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Optimization of the Natural Debittering of Table Olives

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ABSTRACT

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 ºC) 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 ºC) 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.

Keywords: oleuropein; β-glucosidase; hydrolysis; brine; bitterness
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 (Romero, Brenes, García, García, & Garrido, 2004; Arroyo-López et al., 2005). 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; Servili et al., 2006; Medina et al., 2008). 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). 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 ºC, respectively in Spain and many other countries worldwide. Maximum β-glucosidase activity is reached at a pH close to 5 units (Ramírez et al., 2014; Romero-Segura, Sanz, & Pérez, 2009; Kara, Sinan, & Turan, 2011), 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 activity is reached at 10 and 30 ºC in the Gordal cultivar, whereas it is found at 40-45 ºC for Picual and Hojiblanca cultivars (Ramírez et al., 2014; Kara et al., 2011). 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

Ethylenediaminetetraacetic 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 decarboxymethylenelelenolic acid linked to hydroxytyrosol (HyEDA) were obtained by HPLC preparative system as described elsewhere (Romero, Brenes, García, & Garrido, 2002; Brenes et al., 2000).
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 ºC) 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 ºC 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 nanokatal/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 ºC 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 ºC 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 x 4.6 mm i.d., 5 µm, Spherisorb ODS-2 column, at a flow rate of 1 mL/min and a temperature of 35 ºC 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 x 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 esterase activities, respectively. The pH of the extract solutions was adjusted to 5 with a solution of acetic acid. The extract solutions
were stored at three different temperatures (10, 30 and 40 °C). The storage time was 88 and 180 days for β-glucosidase and esterase extracts, respectively.

Olives of the Manzanilla cultivar (season 2011/2012) were stored under aseptic conditions (Medina, et al., 2007). The fruits were placed in autoclaved bottles and covered with a brine of 60 g/L sodium chloride and 2 g/L acetic acid. The bottles were sealed and stored at four temperatures (10, 20, 30 and 40 °C) for 5 months. Two bottles were analyzed from each batch every 15 days.

In order to know the influence of the freezing temperature on the phenolic composition of the fruits, Manzanilla and Hojiblanca olives were frozen and kept at -30 °C and -80 °C for 3 months.

A new experiment with the olives of the Manzanilla cultivar treated in aseptic conditions (season 2011/2012) was carried out. The bottles were sealed and stored at two temperatures (10 and 40 °C) for one month. The chemical characteristics of the brine were initially 60 g/L sodium chloride and 6 g/L acetic acid. Two bottles were analyzed from each batch every 5 days.

2.7. Effect of the chemical conditions of the brine on the oleuropein hydrolysis

A crude enzymatic extract from Gordal olives was used as model system solution to study the stability of hydrolases against the salinity and acidity conditions of the brines. The enzymatic extracts were supplemented with 30 g/L sodium chloride, 3 g/L acetic acid and the pH was adjusted to 4.2 with HCl. The model solutions were stored at 10 °C for 5 and 50 days from the β-glucosidase and esterase tests, respectively.

To study the diffusion rate of sodium chloride and acetic acid from the surrounding brine into the olive pulp, fruits of the Manzanilla cultivar were placed in
bottles of 100 mL capacity and covered with a brine of 60 g/L sodium chloride and 6 g/L acetic acid. The bottles were stored at 10 °C and periodically analyzed for 1 month. A new experiment was carried out with olives of the Gordal, Manzanilla and Hojiblanca cultivars under aseptic conditions (season 2012/2013). Fruits were covered with two different brines; brine A, 60 g/L sodium chloride and 2 g/L acetic acid and brine B, 140 g/L sodium chloride and 16 g/L acetic acid. The bottles were sealed and stored at room temperature (22-25 °C) for 8 months. Two bottles were analyzed from each batch every 15 days.

2.8. Study of debittering of natural olives at pilot scale under optimal conditions

Fruits of Manzanilla and Hojiblanca cultivars (season 2013/2014) were placed in 5.6 kg PVC vessels and covered with a brine of 60 g/L sodium chloride and 6 g/L acetic acid. The vessels were stored for 15 days at 10 °C and afterward at room temperature (optimized conditions) or from the beginning at room temperature (control conditions). The experiments were run in duplicate.

2.9. Statistical analysis

Data were expressed as mean values ± standard deviation. Statistical software version 7.0 was used for data processing (Statistical for Windows, Tulsa, USA). A comparison among mean variables was made by Duncan’s multiple-range tests (one way ANOVA), and the differences were considered significant when p < 0.05.

3. Results and discussion
3.1. Effect of temperature on the oleuropein hydrolysis

The enzymatic extracts obtained from the Manzanilla and Gordal cultivars were employed to evaluate the thermal stability of $\beta$-glucosidase and esterase activities, respectively. The $\beta$-glucosidase activity remained stable at the lower temperature tested (10 °C) throughout the storage time and no change was observed in this activity after 3 months (Table 1). In contrast, a rapid decrease was monitored at a higher temperature, the residual enzymatic activity was only of 22% after 2 months of storage at 30 °C. Particularly, $\beta$-glucosidase activity dropped 10-fold in only five days at 40 °C, and was not detected thereafter. With regard to esterase activity, a statistically similar behavior was observed for all temperatures studied.

An experiment with olives of the Manzanilla cultivar was carried out to study the influence of the storage temperature on the hydrolysis reaction of oleuropein in natural green olives preserved under aseptic conditions to eliminate the interference of microorganisms. The oleuropein hydrolysis rate was temperature dependent, with statically significant differences between the four temperatures studied (Table 2). After fifteen days, the oleuropein disappeared almost completely from olive pulp at 10 °C, and it diminished to around 85% at 20 °C, while this hydrolysis was less intensive at higher temperatures. This is very important evidence as the oleuropein hydrolysis was rapid in the early days of brining and then, the decrease in oleuropein concentration in pulp was slow at any temperature tested. A decline in oleuropein of 88% was registered from 15 to 151 days at 40 °C while a decrease in oleuropein of only 50% was recorded at the same time with other temperatures tested.
These data were not expected because β-glucosidase and esterase activities increase with temperature (Ramírez et al., 2014) but their stability also depends on temperature, as shown in Table 1. Therefore, it seems that a rapid inactivation of these enzymes occurred at a high temperature during the first days of brining whereas they stayed active at low temperature such as 10-20 °C.

Many researchers preserve fruits at -30 °C for months in the freezer before polyphenol analysis. Considering the above results, the polyphenol content in frozen fruits was analyzed after three months of storage in the freezer at -30 °C and -80 °C. It was confirmed that degradative enzymes worked not only at -30 °C but also at -80 °C (Table 3). When the analysis was carried out with fresh fruits, oleuropein was the main polyphenol and its derivatives such as hydroxytyrosol and HyEDA were not detected. Nevertheless, the concentration of oleuropein diminished to a large extent in olives preserved at -30 and -80 °C, whereas the concentration of metabolites from its degradation increased significantly. Therefore, all our polyphenol analyses were carried out on olives non-preserved in the freezer.

These data confirmed the importance of the enzymatic action on the oleuropein transformation and its dependency with temperature. A new experiment with Manzanilla olives was carried out at 10 and 40 °C and a higher acidity was employed, 6 g/L acetic acid, to be closer to common chemical characteristics during the processing of natural green olives.

A drastic reduction of oleuropein in olive pulp was again observed at 10 °C with time (Table 4). After 30 days of preservation, the content of this glucoside diminished from 30 g/kg to 1.3 g/kg. In contrast, a residual 9.5 g/kg concentration of oleuropein was found in olives preserved at 40 °C. These differences between the two temperatures tested were statistically significant.
Contents of other phenolic compounds such as ligustroside, rutin, hydroxytyrosol 4-glucoside, hydroxytyrosol 1-glucoside, salidroside and comselogoside also diminished during preservation.

Additionally, HyEDA was formed in a high concentration in those olives preserved at 10 ºC but not at 40 ºC, which is in agreement with the data reported in Table 1. There were no statistically significant differences in the hydroxytyrosol formed from the hydrolysis of oleuropein and HyEDA at both temperatures, which confirmed that esterase activity was less sensitive than β-glucosidase to storage temperature in a real system.

3.2. Effect of the chemical conditions of the brine on the oleuropein hydrolysis

It has been reported that sodium chloride inhibits β-glucosidase activity in almonds (Bowers, Ragland, & Byers, 2007) but there is no knowledge about the combined influence of the salinity and acidity of olive brine on the activity of endogenous enzymes during table olive processing. We have carried out a new experiment at 10 ºC with a mild chemical condition, 30 g/L sodium chloride and 3 g/L acetic acid. The β-glucosidase enzyme was very sensitive to the brine components (Table 5). The simple addition of sodium chloride and acetic acid caused a rapid decrease in enzymatic activity; the residual activity being 14% at time zero, and it was even not detectable after three days of storage. In contrast, the esterase activity was less affected, the esterase activity was 42% at the beginning of incubation and it slowly decreased and still remained at 12% after 49 days of storage.

Apparently, there is a contradiction between the data presented in Tables 3 and 5 because β-glucosidase was inactivated after 3 days in a model system while it was found
In olives even after 10 days of preservation. In order to clarify this point, the diffusion rate of sodium chloride and acetic acid concentration from the surrounding brine to the olives was studied.

Previous studies indicate that the diffusion of sodium chloride into the olive pulp is a fast phenomenon during the first days but the equilibrium between the pulp and the surrounding brine is reached after about 30 days (Drusas & Vagenas, 1988; Medina, 2008). Experiments carried out at 10 ºC with the Manzanilla cultivar showed that equilibrium was not reached after one month of storage (Figure 1). Only 10 g/L of sodium chloride was detected in the olive juice during the first ten days, which would not cause a drastic enzymatic inhibition as seen in Table 5.

The diffusion of acetic acid was also slow, only 1 g/L acid was detected in olive juice after 10 days of storage. Medina (2008) reported a rapid diffusion of this acid from the surrounding brine into the pulp of the Manzanilla cultivar, the equilibrium was reached in only ten days but assays were run at room temperature. Therefore, a low temperature during the brining of olives retards the diffusion of chemicals into the pulp and favors the action of endogenous enzymes.

A new experiment was planned to test the effect of chemical conditions on the debittering rate of Manzanilla olives. Two extreme values of salinity (60 and 140 g/L) and acidity (2 and 16 g/L) were evaluated for months under aseptic conditions. A rapid hydrolysis of oleuropein occurred during the first 15 days regardless of the chemical conditions (Table 6) although a slightly higher rate was observed for the milder levels of salt and acid (brine A). Other researchers have indicated that polyphenolic composition decreases in a brine of 70 g/L of sodium chloride to a greater extent than in a brine of 40 g/L (Fadda et al., 2014). In our experiments, the degradation of oleuropein was very fast and showed a statistically different behavior with the storage temperature.
It was hydrolyzed up to 76% and 62% of the initial concentration in fresh olives in just fifteen days of storage when the fruits were covered with brines A and B, respectively. The formation of hydroxytyrosol was not statistically different in both brines after fifteen days of storage, but mild conditions allowed for a higher formation of this phenol, increasing by 409% and 404% from the initial concentration at the end of the preservation in brines A and B, respectively. In addition, HyEDA were formed rapidly and a maximal concentration was reached during the first days of storage. No activity was detected for any of the hydrolase enzymes after fifteen days of storage. Moreover, hydroxytyrosol was formed throughout the experiment and this increase may be due to the chemical conditions of the brines (Gikas et al., 2006).

The last experiment was repeated with Hojiblanca and Gordal cultivars and the results were similar to those found with Manzanilla. Again, a rapid hydrolysis of oleuropein was observed during the first 15 days and 96% and 65% of this compound were degraded in olives of the Hojiblanca cultivar preserved under brines A and B, respectively. The hydrolysis of this substance in the Gordal cultivar was almost complete in fifteen days.

3.3. Study of the debittering of natural olives at pilot plant scale under optimal conditions

Olives of the Manzanilla and Hojiblanca cultivars were brined under standard chemical conditions (60 g/L sodium chloride, 6 g/L acetic acid) and stored at 10 °C during fifteen days to favor enzymatic debittering. Afterwards, the olive vessels were kept at room temperature to accelerate chemical debittering. At the same time, control olive vessels were stored at room temperature throughout the study.
A rapid enzymatic hydrolysis of oleuropein occurred in the olives of the two treatments assayed during the first 6-15 days of brining (Table 7), although at a higher rate was observed for fruit preserved at 10 ºC. In fact, the concentration of the bitter compound was only 0.7 g/kg in the pulp of the olives preserved under the optimized conditions for 190 days whereas it was 3.3 g/kg in the olives of the control treatment, almost five times higher. A higher formation rate of hydroxytyrosol and HyEDA was also found in the olives of the optimized treatment than in the control.

With regard to fruit of Hojiblanca cultivar, similar results were obtained. A large oleuropein concentration was hydrolyzed in the first 15 days, especially at a low temperature. Also, an elevated concentration of HyEDA was observed at 10 ºC and hydroxytyrosol formation was independent of the storage temperature (data not shown).

4. Conclusion

The studies at pilot plant scale confirmed that low storage temperature favors the enzymatic hydrolysis of oleuropein in green natural olives during the first months of storage. Also, high acidity and salinity conditions can favor subsequent chemical hydrolysis at room temperature. A new process is proposed: a first month of brining at low temperature under soft chemical conditions, followed by increasing the salt and acid concentration for the storage of olives at ambient temperature. These results will contribute to improving the preparations of natural table olives by reducing the time of the debittering process.

Acknowledgements
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References


Figure captions

Fig. 1. Evolution of the concentration of sodium chloride and acetic acid in Manzanilla olives stored for 1 month at 10 °C. ♦ sodium chloride in brine; ● sodium chloride in olive juice; ∆ acetic acid in brine; □ acetic acid in olive juice. Error bars mean the standard deviation of duplicate analyses.
Table 1
Thermal stability of hydrolases. An enzymatic extract from Manzanilla and Gordal olives were used to check β-glucosidase and esterase activities, respectively. The enzymatic activity is expressed as nano katal/mL enzyme extract.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Storage time (days)</th>
<th>Storage temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>0</td>
<td>10.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>9.0a ± 0.4</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>8.5a ± 0.4</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>10.0a ± 0.2</td>
</tr>
<tr>
<td>Esterase</td>
<td>0</td>
<td>28.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>27.5a ± 0.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>29.0a ± 1.0</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>27.8a ± 2.0</td>
</tr>
<tr>
<td></td>
<td>59</td>
<td>26.5a ± 0.4</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>9.9a ± 0.3</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>6.2a ± 0.5</td>
</tr>
</tbody>
</table>

a Each value is the mean ± standard deviation of duplicate analyses for β-glucosidase activity and of triplicate analyses for esterase activity. b Not detected. Different letters in the same mean value row indicate significant differences according to a Duncan’s multiple-range test (p< 0.05).
Table 2

Evolution of oleuropein in the pulp (g/kg) of Manzanilla olives stored under aseptic conditions at four different temperatures. The chemical characteristics were 60 g/L sodium chloride and 2 g/L acetic acid.

<table>
<thead>
<tr>
<th>Storage time (days)</th>
<th>Storage temperature (ºC)</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>27 ± 3a</td>
<td>27 ± 3</td>
<td>27 ± 3</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>0.2a ± 0.0</td>
<td>3.5b ± 0.2</td>
<td>8.3c ± 0.5</td>
<td>16.0d ± 0.4</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>0.5a ± 0.1</td>
<td>3.6b ± 1.0</td>
<td>9.8c ± 0.7</td>
<td>12.0d ± 0.3</td>
</tr>
<tr>
<td>70</td>
<td></td>
<td>0.2a ± 0.1</td>
<td>2.3b ± 1.0</td>
<td>6.1c ± 0.2</td>
<td>5.8c ± 0.4</td>
</tr>
<tr>
<td>151</td>
<td></td>
<td>0.1a ± 0.0</td>
<td>1.5b ± 0.2</td>
<td>4.7c ± 0.2</td>
<td>1.8d ± 0.0</td>
</tr>
</tbody>
</table>

* Each value is the mean ± standard deviation of quadruplicate analyses. Different letters in the same mean value row indicate significant differences according to a Duncan’s multiple-range test (p< 0.05).
Table 3
Concentration of phenolic compounds (g/kg) in the pulp of Manzanilla and Hojiblanca olives stored for 3 months at different freezing temperatures.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Hydroxytyrosol</th>
<th>HyEDA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Oleuropein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manzanilla</td>
<td>Fresh fruit</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>28 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>-30 °C</td>
<td>0.2 ± 0.0</td>
<td>0.9 ± 0.0</td>
<td>1.6 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>-80 °C</td>
<td>0.1 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>Hojiblanca</td>
<td>Fresh fruit</td>
<td>ND</td>
<td>ND</td>
<td>14 ± 0</td>
</tr>
<tr>
<td></td>
<td>-30 °C</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>-80 °C</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>HyEDA is the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. <sup>b</sup>Not detected. <sup>c</sup>Each value is the mean ± standard deviation of duplicate analyses.
### Table 4

Evolution of the concentration of phenolic compounds (g/kg) in the pulp of Manzanilla olives stored under aseptic conditions in brine at two different temperatures. The chemical characteristics were initially 60 g/L sodium chloride and 6 g/L acetic acid.

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Fresh pulp</th>
<th>Storage time at 10 ºC (days)</th>
<th>Storage time at 40 ºC (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>hydroxytyrosol</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>hydroxytyrosol 1-glucoside</td>
<td>0.6 ± 0.0</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>hydroxytyrosol 4-glucoside</td>
<td>2.1 ± 0.7</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>salidroside</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>HyEDA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>2.7 ± 1.4</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>oleuropein</td>
<td>30 ± 14</td>
<td>4.1 ± 0.9</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>rutin</td>
<td>0.5 ± 0.2</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>comselogoside</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>ligustroside</td>
<td>0.7 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Not detected.  
<sup>b</sup> Each value is the mean ± standard deviation of quadruplicate analyses.  
<sup>c</sup> HyEDA is the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. For each compound, different letters in the same mean value row at the same storage time indicate significant differences according to a Duncan’s multiple-range test (p< 0.05) between both treatments.
Table 5
Residual $\beta$-glucosidase and esterase activity (%) in a model system stored for 2 months at 10 °C. Enzymatic extracts from the Gordal cultivar were used as a model system. The treated enzymatic extract content 30 g/L sodium chloride, 3 g/L acetic acid and pH 4.2.

<table>
<thead>
<tr>
<th>Storage time (days)</th>
<th>$\beta$-glucosidase</th>
<th>Esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14</td>
<td>42</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>ND$^a$</td>
<td>28</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
<td>28</td>
</tr>
<tr>
<td>15</td>
<td>ND</td>
<td>24</td>
</tr>
<tr>
<td>49</td>
<td>ND</td>
<td>12</td>
</tr>
</tbody>
</table>

$^a$Not detected.
Table 6

Evolution of phenolic compounds in olives of the Manzanilla cultivar stored under aseptic conditions at room temperature (22 ± 2 °C). Brine A, 60 g/L sodium chloride and 2 g/L acetic acid; Brine B, 140 g/L sodium chloride and 16 g/L acetic acid.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Storage time (days)</th>
<th>Brine A</th>
<th>Brine B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>g</td>
<td>g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Oleuropein</td>
<td>0</td>
<td>2.6 ± 0.4(^a)</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.6 ± 0.1</td>
<td>-76(^a) ± 2</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.2 ± 0.0</td>
<td>-92(^a) ± 1</td>
</tr>
<tr>
<td>Hydroxytyrosol</td>
<td>15</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.4 ± 0.0</td>
<td>+409(^a) ± 12</td>
</tr>
<tr>
<td>HyEDA(^b)</td>
<td>15</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.1 ± 0.0</td>
<td>-88(^a) ± 1</td>
</tr>
</tbody>
</table>

\(^a\)Each value is the mean ± standard deviation of quadruplicate analyses. \(^b\)HyEDA is the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. Different letters in the same mean value row indicate significant differences according to a Duncan’s multiple-range test (p< 0.05).
Table 7

Evolution of the concentration of phenolic compounds (g/kg) in the pulp of Manzanilla olives stored in acidified brine (60 g/L sodium chloride and 6 g/L acetic acid). Optimized conditions: fruits stored during 15 days at 10 ºC and afterwards, at room temperature; Control: fruits were stored at room temperature.

<table>
<thead>
<tr>
<th>Storage time (days)</th>
<th>Phenolic compounds</th>
<th>Hydroxytyrosol</th>
<th>HyEDA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Oleuropein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh pulp</td>
<td></td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>30 ± 14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control 6</td>
<td></td>
<td>0.7 ± 0.1</td>
<td>2.0 ± 0.6</td>
<td>4.7a ± 1.2</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.6</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td>90</td>
<td></td>
<td>1.3 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>190</td>
<td></td>
<td>1.4 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Optimized conditions</td>
<td>6</td>
<td>0.4 ± 0.1</td>
<td>3.7 ± 0.8</td>
<td>5.9a ± 3.0</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>0.7 ± 0.1</td>
<td>4.5 ± 0.7</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>90</td>
<td></td>
<td>1.4 ± 0.0</td>
<td>1.6 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>190</td>
<td></td>
<td>1.7 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>HyEDA is the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. <sup>b</sup>Not detected. <sup>c</sup>Each value is the mean ± standard deviation of quadruplicate analyses. For each compound, same letter in the same mean value indicate not significant differences according to a Duncan’s multiple-range test (p< 0.05) between control and optimized conditions.
Highlights

- Low storage temperature favors enzymatic hydrolysis of oleuropein
- The chemical hydrolysis of oleuropein is a slow process
- High salinity and acidity enhances chemical hydrolysis of oleuropein
- A new natural debittering process of table olives has been proposed