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Optimization of the Natural Debittering of Table Olives

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16 ABSTRACT

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

29

30 **Keywords:** oleuropein; β -glucosidase; hydrolysis; brine; bitterness

31

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, Papadoulou, & 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 °C, 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 °C in the Gordal cultivar, whereas it is found at 40-45 °C 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 decarboxymethylelenolic acid linked to hydroxytyrosol (HyEDA) were obtained by HPLC preparative system as described elsewhere (Romero, Brenes, García, & Garrido, 2002; Brenes et al., 2000).

107 2.3. Determination of the hydrolase activity

108

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

112 The β -glucosidase activity analysis was based on the methodology proposed
113 elsewhere (Ramírez et al., 2014). Briefly, 0.14 g of acetone powder were suspended in
114 10 mL of a 0.01 mol/L sodium carbonate buffer, containing 0.005 mol/L EDTA, 0.001
115 mol/L PMSF and 10 g/L of 2-mercaptoethanol. The pH was adjusted to 9.0 and the
116 suspension was stirred at 4 °C for 1 h. After 20 min of centrifugation (15550 g) at low
117 temperature, the supernatant was used as the active crude enzyme extract. The β -
118 glucosidase activity analysis was determined by monitoring the increase in absorbance
119 at 405 nm for 30 min related to the increasing amount of p-NP liberated from the
120 synthetic glucoside p-NPG. The evaluation was performed using a regression curve
121 with p-NP. The enzymatic activity is expressed as nano katal/mL enzyme extract. All
122 reactions were carried out in duplicate.

123 The esterase activity analysis was carried out on the enzyme extract obtained
124 from the acetone powder as proposed elsewhere (Ramírez et al., 2014). 0.25 g of
125 acetone powder were suspended in 10 mL of a 0.01 mol/L sodium borate buffer
126 containing 0.005 mol/L EDTA and 0.001 mol/L PMSF. The pH was adjusted to 9.0 and
127 the suspension was stirred at 4 °C for 1 h, after 20 min of centrifugation (15550 g) at
128 low temperature, the supernatant was ready to use as enzyme extract. The esterase
129 activity was determined by continuously monitoring the increase in absorbance at 405
130 nm for 10 min at 40 °C related to the increasing amount of p-NP liberated from the
131 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.

134

2.4. Analysis of phenolic compounds

136

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 μ m pore size nylon filter and an aliquot (20 μ L) 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 μ m, 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.

157

158 *2.5. Chemical analyses*

159

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

163 The concentration of sodium chloride was analyzed in olive juice and brine as
164 described elsewhere (Ramírez, Gandul-Rojas, Romero, Brenes, & Gallardo-Guerrero,
165 2015).

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

176

177 *2.6. Effect of temperature on the oleuropein hydrolysis*

178

179 Crude enzymatic extracts from Manzanilla and Gordal olives were used to study
180 the thermal stability of β -glucosidase and esterase activities, respectively. The pH of the
181 extract solutions was adjusted to 5 with a solution of acetic acid. The extract solutions

182 were stored at three different temperatures (10, 30 and 40 °C). The storage time was 88
183 and 180 days for β -glucosidase and esterase extracts, respectively.

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

189 In order to know the influence of the freezing temperature on the phenolic
190 composition of the fruits, Manzanilla and Hojiblanca olives were frozen and kept at -30
191 °C and -80 °C for 3 months.

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

197

198 *2.7. Effect of the chemical conditions of the brine on the oleuropein hydrolysis*

199

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

205 To study the diffusion rate of sodium chloride and acetic acid from the
206 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.

215

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

217

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.

223

2.9. *Statistical analysis*

225

Data were expressed as mean values $\pm$ 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 $p < 0.05$.

230

3. Results and discussion

231

232

233 *3.1. Effect of temperature on the oleuropein hydrolysis*

234

235 The enzymatic extracts obtained from the Manzanilla and Gordal cultivars were
236 employed to evaluate the thermal stability of β -glucosidase and esterase activities,
237 respectively. The β -glucosidase activity remained stable at the lower temperature tested
238 (10 °C) throughout the storage time and no change was observed in this activity after 3
239 months (Table 1). In contrast, a rapid decrease was monitored at a higher temperature,
240 the residual enzymatic activity was only of 22% after 2 months of storage at 30 °C.
241 Particularly, β -glucosidase activity dropped 10-fold in only five days at 40 °C, and was
242 not detected thereafter. With regard to esterase activity, a statistically similar behavior
243 was observed for all temperatures studied.

244 An experiment with olives of the Manzanilla cultivar was carried out to study
245 the influence of the storage temperature on the hydrolysis reaction of oleuropein in
246 natural green olives preserved under aseptic conditions to eliminate the interference of
247 microorganisms. The oleuropein hydrolysis rate was temperature dependent, with
248 statically significant differences between the four temperatures studied (Table 2). After
249 fifteen days, the oleuropein disappeared almost completely from olive pulp at 10 °C,
250 and it diminished to around 85% at 20 °C, while this hydrolysis was less intensive at
251 higher temperatures. This is very important evidence as the oleuropein hydrolysis was
252 rapid in the early days of brining and then, the decrease in oleuropein concentration in
253 pulp was slow at any temperature tested. A decline in oleuropein of 88% was registered
254 from 15 to 151 days at 40 °C while a decrease in oleuropein of only 50% was recorded
255 at the same time with other temperatures tested.

256 These data were not expected because β -glucosidase and esterase activities
 257 increase with temperature (Ramírez et al., 2014) but their stability also depends on
 258 temperature, as shown in Table 1. Therefore, it seems that a rapid inactivation of these
 259 enzymes occurred at a high temperature during the first days of brining whereas they
 260 stayed active at low temperature such as 10-20 °C.

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

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

276 A drastic reduction of oleuropein in olive pulp was again observed at 10 °C with
 277 time (Table 4). After 30 days of preservation, the content of this glucoside diminished
 278 from 30 g/kg to 1.3 g/kg. In contrast, a residual 9.5 g/kg concentration of oleuropein
 279 was found in olives preserved at 40 °C. These differences between the two temperatures
 280 tested were statistically significant.

281 Contents of other phenolic compounds such as ligustroside, rutin,
 282 hydroxytyrosol 4-glucoside, hydroxytyrosol 1-glucoside, salidroside and comselogoside
 283 also diminished during preservation.

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

290

291 *3.2. Effect of the chemical conditions of the brine on the oleuropein hydrolysis*

292

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

304 Apparently, there is a contradiction between the data presented in Tables 3 and 5
 305 because β -glucosidase was inactivated after 3 days in a model system while it was found

306 in olives even after 10 days of preservation. In order to clarify this point, the diffusion
307 rate of sodium chloride and acetic acid concentration from the surrounding brine to the
308 olives was studied.

309 Previous studies indicate that the diffusion of sodium chloride into the olive pulp
310 is a fast phenomenon during the first days but the equilibrium between the pulp and the
311 surrounding brine is reached after about 30 days (Drusas & Vagenas, 1988; Medina,
312 2008). Experiments carried out at 10 °C with the Manzanilla cultivar showed that
313 equilibrium was not reached after one month of storage (Figure 1). Only 10 g/L of
314 sodium chloride was detected in the olive juice during the first ten days, which would
315 not cause a drastic enzymatic inhibition as seen in Table 5.

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

322 A new experiment was planned to test the effect of chemical conditions on the
323 debittering rate of Manzanilla olives. Two extreme values of salinity (60 and 140 g/L)
324 and acidity (2 and 16 g/L) were evaluated for months under aseptic conditions. A rapid
325 hydrolysis of oleuropein occurred during the first 15 days regardless of the chemical
326 conditions (Table 6) although a slightly higher rate was observed for the milder levels
327 of salt and acid (brine A). Other researchers have indicated that polyphenolic
328 composition decreases in a brine of 70 g/L of sodium chloride to a greater extent than in
329 a brine of 40 g/L (Fadda et al., 2014). In our experiments, the degradation of oleuropein
330 was very fast and showed a statistically different behavior with the storage temperature.

331 It was hydrolyzed up to 76% and 62% of the initial concentration in fresh olives in just
332 fifteen days of storage when the fruits were covered with brines A and B, respectively.

333 The formation of hydroxytyrosol was not statistically different in both brines
334 after fifteen days of storage, but mild conditions allowed for a higher formation of this
335 phenol, increasing by 409% and 404% from the initial concentration at the end of the
336 preservation in brines A and B, respectively. In addition, HyEDA were formed rapidly
337 and a maximal concentration was reached during the first days of storage.

338 No activity was detected for any of the hydrolase enzymes after fifteen days of
339 storage. Moreover, hydroxytyrosol was formed throughout the experiment and this
340 increase may be due to the chemical conditions of the brines (Gikas et al., 2006).

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

347

348 *3.3. Study of the debittering of natural olives at pilot plant scale under optimal* 349 *conditions*

350

351 Olives of the Manzanilla and Hojiblanca cultivars were brined under standard
352 chemical conditions (60 g/L sodium chloride, 6 g/L acetic acid) and stored at 10 °C
353 during fifteen days to favor enzymatic debittering. Afterwards, the olive vessels were
354 kept at room temperature to accelerate chemical debittering. At the same time, control
355 olive vessels were stored at room temperature throughout the study.

356 A rapid enzymatic hydrolysis of oleuropein occurred in the olives of the two
357 treatments assayed during the first 6-15 days of brining (Table 7), although at a higher
358 rate was observed for fruit preserved at 10 °C. In fact, the concentration of the bitter
359 compound was only 0.7 g/kg in the pulp of the olives preserved under the optimized
360 conditions for 190 days whereas it was 3.3 g/kg in the olives of the control treatment,
361 almost five times higher. A higher formation rate of hydroxytyrosol and HyEDA was
362 also found in the olives of the optimized treatment than in the control.

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

368 **4. Conclusion**

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

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380

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385

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471 **Figure captions**

472 **Fig. 1.** Evolution of the concentration of sodium chloride and acetic acid in Manzanilla
473 olives stored for 1 month at 10 °C. ♦ sodium chloride in brine; ● sodium chloride in
474 olive juice; Δ acetic acid in brine; □ acetic acid in olive juice. Error bars mean the
475 standard deviation of duplicate analyses.

476

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.

Enzyme	Storage time (days)	Storage temperature (°C)		
		10	30	40
β -glucosidase	0	10.5 ± 0.2^a	10.5 ± 0.2	10.5 ± 0.2
	5	$9.0a \pm 0.4$	$8.4a \pm 0.4$	$1.0b \pm 0.3$
	56	$8.5a \pm 0.4$	$2.3b \pm 0.2$	ND ^b
	88	$10.0a \pm 0.2$	ND	ND
Esterase	0	28.8 ± 0.5	28.8 ± 0.5	28.8 ± 0.5
	3	$27.5a \pm 0.6$	$26.5a,b \pm 0.6$	$25.5b \pm 0.5$
	7	$29.0a \pm 1.0$	$27.1a,b \pm 2.0$	$26.8b \pm 0.5$
	36	$27.8a \pm 2.0$	$27.8a \pm 3.0$	$22.5b \pm 0.8$
	59	$26.5a \pm 0.4$	$26.7a \pm 0.6$	$26.5a \pm 2.0$
	120	$9.9a \pm 0.3$	$9.8a \pm 0.4$	$10.1a \pm 0.2$
	180	$6.2a \pm 0.5$	$6.8b \pm 0.0$	$7.1c \pm 0.1$

^a Each value is the mean $\pm$ standard deviation of duplicate analyses for β -glucosidase activity and of triplicate analyses for esterase activity. ^bNot 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.

Storage time (days)	Storage temperature (°C)			
	10	20	30	40
0	27 ± 3^a	27 ± 3	27 ± 3	27 ± 3
15	$0.2a \pm 0.0$	$3.5b \pm 0.2$	$8.3c \pm 0.5$	$16.0d \pm 0.4$
30	$0.5a \pm 0.1$	$3.6b \pm 1.0$	$9.8c \pm 0.7$	$12.0d \pm 0.3$
70	$0.2a \pm 0.1$	$2.3b \pm 1.0$	$6.1c \pm 0.2$	$5.8c \pm 0.4$
151	$0.1a \pm 0.0$	$1.5b \pm 0.2$	$4.7c \pm 0.2$	$1.8d \pm 0.0$

^a Each value is the mean $\pm$ 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.

Cultivar	Treatment	Hydroxytyrosol	HyEDA ^a	Oleuropein
Manzanilla	Fresh fruit	ND ^b	ND	28 ± 2 ^c
	-30 °C	0.2 ± 0.0	0.9 ± 0.0	1.6 ± 0.0
	-80 °C	0.1 ± 0.0	0.7 ± 0.0	4.0 ± 0.2
Hojiblanca	Fresh fruit	ND	ND	14 ± 0
	-30 °C	0.3 ± 0.0	0.2 ± 0.0	3.4 ± 0.1
	-80 °C	0.3 ± 0.0	0.1 ± 0.0	1.9 ± 0.1

^aHyEDA is the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. ^bNot detected. ^cEach 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.

Phenolic compounds	Fresh pulp	Storage time at 10 °C (days)			Storage time at 40 °C (days)		
		4	10	30	4	10	30
hydroxytyrosol	ND ^a	0.4a ± 0.1 ^b	0.6c ± 0.1	0.6e ± 0.1	0.4a ± 0.1	0.4c ± 0.1	0.5e ± 0.0
hydroxytyrosol 1-glucoside	0.6 ± 0.0	0.6a ± 0.1	0.3c ± 0.1	0.2e ± 0.1	0.3b ± 0.0	0.4c ± 0.0	0.2e ± 0.0
hydroxytyrosol 4-glucoside	2.1 ± 0.7	1.3a ± 0.2	1.0c ± 0.1	0.3e ± 0.0	1.2a ± 0.2	1.1c ± 0.0	ND
salidroside	0.1 ± 0.0	0.1a ± 0.0	0.0c ± 0.0	0.0e ± 0.0	0.0a ± 0.0	0.0c ± 0.0	0.0e ± 0.0
HyEDA ^c	ND	2.7a ± 1.4	4.1c ± 0.9	3.4e ± 0.6	0.1b ± 0.0	0.0d ± 0.0	0.0f ± 0.0
oleuropein	30 ± 14	4.1a ± 0.9	1.7c ± 0.3	1.3e ± 0.4	20.8b ± 2.1	15.9d ± 0.1	9.5f ± 0.9
rutin	0.5 ± 0.2	0.2a ± 0.0	0.1c ± 0.0	0.0e ± 0.0	0.0b ± 0.0	ND	ND
comselogside	0.2 ± 0.1	0.2a ± 0.0	0.1c ± 0.0	0.1e ± 0.0	0.1b ± 0.0	0.1d ± 0.0	0.1f ± 0.0
ligustroside	0.7 ± 0.2	0.2a ± 0.1	0.0c ± 0.0	0.0e ± 0.0	0.4b ± 0.0	0.3d ± 0.1	0.2f ± 0.0

^a Not detected. ^bEach value is the mean ± standard deviation of quadruplicate analyses. ^cHyEDA 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 β -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.

	Storage time (days)	β -glucosidase	Esterase
Untreated	0	100	100
Treated	0	14	42
	1	16	36
	3	ND ^a	28
	7	ND	28
	15	ND	24
	49	ND	12

^aNot 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.

Compound	Storage time (days)	Brine A		Brine B	
		g	%	g	%
Oleuropein	0	2.6 ± 0.4^a		2.6 ± 0.4	
	15	0.6 ± 0.1	$-76a \pm 2$	1.0 ± 0.2	$-62b \pm 2$
	250	0.2 ± 0.0	$-92a \pm 1$	0.4 ± 0.1	$-85b \pm 1$
Hydroxytyrosol	15	0.1 ± 0.0		0.1 ± 0.1	
	250	0.4 ± 0.0	$+409a \pm 12$	0.4 ± 0.0	$+404a \pm 36$
HyEDA ^b	15	0.7 ± 0.1		0.6 ± 0.1	
	250	0.1 ± 0.0	$-88a \pm 1$	0.1 ± 0.0	$-91b \pm 0$

^aEach value is the mean $\pm$ standard deviation of quadruplicate analyses. ^bHyEDA 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 °C and afterwards, at room temperature; Control: fruits were stored at room temperature.

	Storage time (days)	Phenolic compounds		
		Hydroxytyrosol	HyEDA ^a	Oleuropein
Fresh pulp	0	ND ^b	ND	30 ± 14 ^c
Control	6	0.7 ± 0.1	2.0 ± 0.6	4.7a ± 1.2
	15	0.9 ± 0.1	1.0 ± 0.6	3.9 ± 0.9
	90	1.3 ± 0.1	0.5 ± 0.1	3.4 ± 0.8
	190	1.4 ± 0.1	0.2 ± 0.0	3.3 ± 0.2
Optimized conditions	6	0.4 ± 0.1	3.7 ± 0.8	5.9a ± 3.0
	15	0.7 ± 0.1	4.5 ± 0.7	1.1 ± 0.6
	90	1.4 ± 0.0	1.6 ± 0.1	0.9 ± 0.1
	190	1.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1

^aHyEDA is the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. ^bNot detected. ^cEach 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