Endogenous Enzymes Involved in the Transformation of Oleuropein in Spanish Table Olive Varieties

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1   **ABSTRACT**

2   The main Spanish table olives varieties supplied by different olive Cooperatives were

3   investigated for their polyphenol compositions and the endogenous enzymes involved in

4   their transformations during two growing seasons. Olives of the Manzanilla variety had

5   the highest concentration in total polyphenols, followed by the Hojiblanca and Gordal

6   varieties. The Gordal and Manzanilla cultivar showed the highest polyphenoloxidase

7   activity. The Gordal cultivar presented a greater β-glucosidase and esterase activity than

8   the others. An important influence of pH and temperature on the optimal activity of

9   these enzymes was also observed. The polyphenoloxidase activity increased with

10   temperature and peroxidase activity was optimal at 35ºC. The β-glucosidase and

11   esterase activities were at their maximum at 30 ºC and 55 ºC respectively. The oxidase

12   and β-glucosidase activities were at their maximum at the pH of the raw fruit. These

13   results will contribute to the knowledge of the enzyme transformation of oleuropein in

14   natural table olives.

15   KEYWORDS: table olive varieties; phenolic compounds; β-glucosidase; esterase;

16   polyphenol oxidase; peroxidase.

17

18   INTRODUCTION

19   The olive is a popular fruit in Mediterranean countries, especially the foodstuff

20   that derives from it, such as olive oil and table olives. The raw fruit is unpalatable due to

21   the presence of the bitter compound named oleuropein. This substance is formed by

22   glucose, elenolic acid and the *o*-diphenol hydroxytyrosol (Figure 1). When the fruits are

23   processed, the oleuropein is transformed by chemical or enzymatic actions to obtain a

24   non-bitter final product.[1]

25   There is an increased demand to mechanically harvest the fruit intended for table

26   olives but this technology is not applicable to some varieties due to the formation of

27   brown spots. Mechanical harvesting gives rise to a rupture of the cellular tissue that

28   releases phenolic compounds and oxidases. The oleuropein is oxidized by the action of

29   polyphenoloxidase (PPO) and peroxidase (POD) to give its quinone that polymerizes to

30   brown pigments.[2-4] Oleuropein can also be oxidized by the action of PPO in natural

31   olives under an overpressure of oxygen.[5]

32   A correlation between PPO activity and the rate of browning of a crude

33   homogenate of five Italian olive varieties has been reported,[6] and browning has also

34   been associated with the oleuropein content in the olive varieties.[7] Additionally, a

35   correlation between PPO activity and total phenol concentration during the fruit

36   maturation of Picual, Verdial, Arbequina and Frantoio varieties has been shown.[8,9]

37   Although there are some studies on the evolution of PPO activity during olive

38   maturation[7,10] there is only one report on Spanish table olive varieties,[11] in which a

39   decrease in PPO activity with fruit ripening was found.

40   Most commercial table olives, Spanish-style green and California-style black

41   olives are debittered by treating the fruits with a dilute NaOH solution. Nevertheless,

42   there are other trade preparations that involve the direct brining of olives without any

43 alkaline treatment, which are known as Natural olives, and the bitterness disappears

44 slowly during brining.[12] Moreover, the hydrolysis of oleuropein is mainly carried out

45 during the extraction of olive oil by the action of endogenous β-glucosidase.[13] Precisely,

46 there are many phenolic glucosides in olives and β-glucosidase is especially active with

47 oleuropein, its activity being lower with ligustroside, and demethyloleuropein.[14] The

48 oleuropein is transformed to its aglycone which can lead to the formation of the

49 decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA), an important

50 antimicrobial compound present in virgin olive oil and natural table olives.[15]

51   There is a lack of studies on the evolution of endogenous β-glucosidase and

52 esterase activity during the maturation of olives intended for table olives. Gutiérrez-

53 Rosales et al.,[16] found a high content of oleuropein aglycon during the olive ripening of

54 Arbequina and Hojiblanca varieties although it depended to a large extent on the tree

55 location.[17] Other researchers have also observed an increase in β-glucosidase and

56 esterase activity with olive ripening of the Tunisian[18] and Italian varieties.[10]

57   Most of these studies were focused on the participation of these enzymes in the

58 elaboration process of olive oil but not table olives. Hence, the aim of this work was to

59 evaluate both the phenolic composition and the endogenous enzymes in the raw fruits of

60 the main Spanish table olive varieties.

61

62 MATERIALS AND METHODS

63   **Raw material.** Fruits of the Gordal, Manzanilla and Hojiblanca varieties (*Olea*

64 *europaea* L.) in the ripening stage corresponding to a green-yellow surface color were

65 harvested from mid-September to mid-October and supplied by different olive

66 Cooperatives located in the Seville province (Spain). Fruit with this pigmentation was

67 only considered in this study because this is the optimum degree of maturation for fruit

68 processed as green Spanish-style and black California-style. Twelve and six samples for

69 each variety during the 2010/2011 and 2011/2012 seasons, respectively, were assessed.

70 The fruits were processed on the same day without any storage period.

71 **Analysis of phenolic compounds.** The extraction of phenolic compounds from

72 the olive pulp was based on the methodology proposed elsewhere.[19] Around 10 g of

73 fresh olive pulp were mixed in an Ultra-Turrax homogenizer with 30 mL of dimethyl

74 sulfoxide (DMSO). After 30 min resting contact, the mixture was centrifuged at 6000 g

75 for 5 min (22 ºC), and 0.25 mL of the supernatant were diluted with 0.5 mL of DMSO

76 plus 0.25 mL of 0.2 mM syringic acid in DMSO (internal standard). The extraction of

77 phenolic compounds from the fresh olive skin was made similarly to the olive pulp as

78 described above, but this time the mixing ratio was 0.1g of olive skin and 0.5 mL of

79 DMSO.

80 Samples were filtered through a 0.22 μm pore size nylon filter and an aliquot (20

81 μL) was injected into the chromatograph. The chromatographic system consisted of a

82 Waters 717 plus autosampler, a Waters 600 E pump, a Waters column heater module,

83 and a Waters 996 photodiode array detector operated with Empower software (Waters

84 Inc.). A 25 cm x 4.6 mm i.d., 5 μm, Spherisorb ODS-2 (Waters Inc.) column, at a flow

85 rate of 1 mL/min and a temperature of 35 ºC were used in all experiments. Separation

86 was achieved by gradient elution using (A) water (pH 2.5 adjusted with 0.15%

87 phosphoric acid) and (B) methanol. The initial composition was 90% A and 10% B. The

88 concentration of B was increased to 30% over 10 min and was maintained for 20 min.

89 Subsequently, B was raised to 40% over 10 min, maintained for 5 min, and then

90 increased to 50%. Finally, B was increased to 60, 70 and 100% in 5-min periods. The

91 initial conditions were reached in 10 min. Chromatograms were recorded at 280 nm.[15]

92   The evaluation of each compound was performed using a regression curve with

93   the corresponding standard. Hydroxytyrosol, oleuropein, verbascoside, luteolin 7-

94   glucoside, luteolin, rutin and apigenin were purchased from Extrasynthese S.A. (Lyon

95   Nord, Genay, France), and tyrosol, caffeic and *p*-coumaric acid from Sigma Chemical

96   Co. (St. Louis, MO). Hydroxytyrosol-1-glucoside, caffeoyl ester of secologanoside and

97   comselogoside were quantified using the response factors of hydroxytyrosol, caffeic and

98   *p*-coumaric acid respectively. Salidroside and ligustroside were quantified using the

99   response factors of tyrosol. Hydroxytyrosol-4-glucoside was obtained using an HPLC

100   preparative system.[20]

101   **Preparation of acetone powder.** Acetone powders were obtained from 50 g of

102   olive pulp or skin homogenized with 100 mL of cold acetone (-30 ºC) containing 2.5 g

103   of polyethylene glycol.[6] The mixture was then vacuum-filtered in a Büchner funnel

104   through paper, and the residue was re-extracted three times with 100 mL of cold

105   acetone, obtaining a white solid that was dried overnight at room temperature to remove

106   residual acetone. The acetone powder was stored at -40 ºC until use.

107   **PPO and POD activity.** A crude enzyme extract was obtained from the above

108   acetone powder as described elsewhere.[11] 0.5 g of acetone powder was suspended in 20

109   mL of 0.1 M sodium phosphate buffer, containing 1 M NaCl, and the pH was adjusted

110   to 6.2 units with NaOH. The suspension was stirred at 4 ºC for 30 min and then

111   centrifuged at 15,550 x *g* for 20 min at 4 ºC. The pellet was discarded and the

112   supernatant divided into two aliquots; one was used as the active crude enzyme extract,

113   and the other was boiled for 30 min to get the denatured enzyme extract.

114   The PPO activity was determined spectrophotometrically using a Shimadzu UV-

115   vis 1800 spectrophotometer as described elsewhere.[21] All measurements of PPO activity

116   were carried out with 4-methylcatechol (Sigma Chemical Co., St. Louis, MO) as

substrate by measuring the changes in absorbance at 410 nm at 25 ºC for 10 min at intervals of 5 s. The incubation mixture contained 0.5 mL of crude enzyme preparation and 2.5 mL of 0.1 M sodium citrate buffer at pH 5 with 0.02 M of substrate. The assay mixture with the denatured enzyme extract served as the control. One unit of enzyme activity was defined as the amount of the enzyme giving a change in absorbance of 0.05 units AU/min under the above-mentioned conditions. All assays were carried out in duplicate.

The POD activity was determined spectrophotometrically as described elsewhere.[22] All measurements of POD activity were carried out with guaiacol (Sigma Chemical Co., St. Louis, MO) as substrate by measuring the changes in absorbance at 470 nm at 25 ºC for 10 min at intervals of 1 min. The assay mixture with the denatured enzyme extract served as the control. The incubation mixture contained 0.3 mL of crude enzyme extract and 2.7 mL of phosphate buffer at pH 6 containing 0.04 M of substrate and 0.026 M of hydrogen peroxide. The composition of the phosphate buffer was 0.02 M sodium acid phosphate and 0.08 M sodium monobasic phosphate. Initially, the reaction buffer was incubated for 15 min at 30 ºC and the reaction was started by the addition of crude enzyme extract.

One unit of enzyme activity was defined as the amount of enzyme required to increase 0.001 units AU/min under the above-mentioned conditions. All reactions were carried out in duplicate.

β-glucosidase activity. A crude enzyme extract was obtained from the above mentioned acetone powder. 0.14 g of acetone powder were suspended in 10 mL of a 0.01 M sodium carbonate buffer, containing 0.005 M ethylenediaminotetraacetic acid (EDTA), 0.001 M phenylmethylsulfonyl fluoride (PMSF) and 1% of 2-mercaptoethanol. The pH was adjusted to 9.0 units with NaOH. The suspension was

142   stirred at 4 ºC for 1 h and then centrifuged at 15,550 x g for 20 min at 4 ºC. The pellet

143   was discarded and the supernatant divided into two aliquots; one was used as the active

144   crude enzyme extract, and the other was boiled for 30 min to obtain the denatured

145   enzyme extract.

146   The β-glucosidase activity analysis was based on the methodology proposed

147   elsewhere.[14] This activity was determined by monitoring the increase in absorbance at

148   405 nm for 30 min at intervals of 5 min related to the increasing amount of p-

149   nitrophenol (p-NP) liberated from the synthetic glucoside p-nitrophenyl-β-D-

150   glucopyranoside (p-NPG) (Sigma Chemical Co., St. Louis, MO). The reaction medium

151   consisted of 100 µL of crude enzyme extract and 100 µL of 0.050 M sodium acetate

152   buffer at pH 5.4 units containing 0.015 M p-NPG as substrate. The mixture was

153   incubated for 30 min at 45 ºC. Then, the reaction was stopped by adding 1 mL of 0.5 M

154   sodium carbonate and the spectrophotometrical measurement started at 405 nm. The

155   assay mixture with the denatured enzyme extract served as the control.

156   The evaluation was performed using a regression curve with p-NP in a range of

157   0-0.0003 M. One unit of enzyme activity was defined as the amount of enzyme required

158   to produce 1 µmol of p-NP/min. All reactions were carried out in duplicate.

159   **Esterase activity.** A crude enzyme extract was obtained from the above

160   mentioned acetone powder. 0.25 g of acetone powder were suspended in 10 mL of a

161   0.01 M sodium borate buffer, containing 0.005 M EDTA and 0.001 M PMSF. The pH

162   was adjusted to 9.0 units with NaOH. The suspension was stirred at 4 ºC for 1 h and

163   then centrifuged at 15,550 x g for 20 min at 4 ºC. The pellet was discarded and the

164   supernatant was the enzyme extract.

165   The esterase activity was analyzed according to the methodology proposed

166   elsewhere.[23] This activity was determined by continuously monitoring the increase in

167 absorbance at 405 nm for 10 min at 40 ℃ related to the increasing amount of p-NP

168 liberated from the synthetic p-nitrophenyl acetate (p-NPA) (Sigma Chemical Co., St.

169 Louis, MO). The incubation mixture contained 50 µL of crude enzyme extract, 50 µL of

170 0.15 M p-NPA in ethanol as substrate and 2.9 mL of a 0.0092 M Tris-HCl buffer at pH

171 7.5. The reaction was initiated by the addition of the enzyme extract. The assay mixture

172 with 50 µL of extraction buffer served as the control.

173 The evaluation was performed using a regression curve with p-NP in a range of

174 0-0.00003 M. One unit of enzyme activity was defined as the amount of enzyme

175 required to produce 1 µmol of p-NP/min. All reactions were carried out in duplicate.

176 **Effect of pH.** The enzyme assays at different pH was performed by measuring

177 the activity of each enzyme using different buffer solutions under standard conditions.

178 In the case of PPO and POD, 0.1 M sodium citrate and a 0.1 M sodium phosphate

179 buffer in the pH range of 4-5 and 6-8 units were used, respectively. The effect of pH on

180 olive β-glucosidase and esterase activity was assessed with a 0.05 M acetic acid-sodium

181 acetate trihidrate solution, 0.05 M sodium phosphate and a 0.0092 M Tris-HCl buffer in

182 the pH range of 4-5, 6-7 and 7.5-8 units, respectively. In all cases, the pH was finally

183 adjusted with suitable amounts of 1.0 M NaOH and 1.0 M HCl.

184 **Effect of temperature.** The optimal temperature for PPO, POD, β-glucosidase

185 and esterase enzymes was determined by measuring the activity of each enzyme in the

186 temperature range of 10-70 ° C under standard conditions.

187 **Statistical analysis.** Data were expressed as mean values ± error standard.

188 Statistical software version 7.0 was used for data processing (Statistical for Windows,

189 Tulsa, OK, USA). A comparison among mean variables was made by the Duncan's

190 multiple range tests and the differences were considered significant when $p < 0.05$.

191

RESULTS AND DISCUSSION

Polyphenolic compounds. Hojiblanca, Manzanilla and Gordal are the main olive varieties employed by the Spanish table olive industry. The concentration of the phenolic composition in the fruit of these varieties for two seasons and several orchards is shown in Figure 2. As was expected from previous works,[24-26] the main phenolic compound was oleuropein, followed by hydroxytyrosol 4-glucoside, hydroxytyrosol 1-glucoside, verbascoside and salidroside, with minor concentration of tyrosol, luteolin 7-glucoside, caffeic and *p*-coumaric acids, the ester of caffeic acid with secologanoside, comselogoside, luteolin, apigenin and ligustroside. The hydroxytyrosol 4-glucoside concentration was particularly high in the Hojiblanca variety. This polyphenol increases its concentration in mature olives[20] but all samples, including those of the Hojiblanca variety, were picked at the same maturity index. Salidroside was not detected in the samples of the Gordal variety as other authors have reported.[27]

In summary, the olives of the Manzanilla variety showed the highest concentration in total polyphenols, 25.4±2.6 mM and 59.6±6.7 mM, followed by the Hojiblanca variety, 19.7±1.0 mM and 44.7±1.5 mM, and the Gordal variety, 5.2±0.3 mM and 21.6±1.4 mM, for the 2010/2011 and 2011/2012 seasons, respectively. These differences among varieties were statistically significant according to a Duncan's multiple range test at the 5% level, and it must also be noted that, big differences were found between seasons. A much higher concentration of polyphenols was found in olives from the 2011/2012 season than the 2010/2011 season, except for hydroxytyrosol 1-glucoside and salidroside. These data are of great importance because of the lack of previous studies and the fact that research was only carried out for one season. In addition, the hydrolysis rate of oleuropein and its residual concentration in edible olives depends on the concentration of this substance in the raw material.

217   **Enzyme activity.** Figure 3 shows the PPO activity in fruits of the Gordal,

218   Hojiblanca and Manzanilla varieties picked with a green-yellow color for two seasons

219   and from several Cooperatives.

220   The first finding that must be noted is the high variability found among the

221   different samples analyzed within the same variety, especially for those of the Gordal

222   variety. The standard error of the mean PPO activity of Gordal was of 1.28 and 2.61

223   U/mL enzyme extract for the two seasons studied whereas these data were recorded as

224   0.63 and 0.06 U/mL enzyme extract for the Hojiblanca variety. This variability is

225   common in nature and it has been observed for Italian olive varieties[28] and many other

226   fruits such as peaches.[29]

227   Regardless of the season, the Gordal variety showed a high PPO activity,

228   followed by Manzanilla and Hojiblanca. In Spain, the Hojiblanca variety destined to

229   table olives is mechanically harvested whereas the other varieties (Manzanilla and

230   Gordal), which are very prone to bruising, are picked by hand. Indeed, Jiménez-Jiménez

231   et al.,[30] found the Hojiblanca variety to have a lower susceptibility to bruising than the

232   others but no explanation for this phenomenon was reported. Both phenolic compounds

233   and PPO participate in the appearance of bruising after harvesting but from the data

234   reflected in Figures 2 and 3, it seems that the PPO activity could better explain the

235   behavior of the Hojiblanca variety. The low PPO activity in this variety probably

236   contributes to the slow formation of brown spots in harvested olives while the high

237   activity in Manzanilla and Gordal favors their appearance.

238   The phenolic composition and the PPO activity in both the pulp and the skin of

239   the Manzanilla variety were also characterized (Table 1), which is the more prone

240   variety to bruising. The concentration of the phenolic compounds, except verbascoside,

241   was much higher in the skin than in the pulp but, in contrast, the PPO activity was much

242 lower in the skin than in the pulp. This data are in agreement with those reported by

243 Chang et al.,[29] who found that in five peach varieties out of nine, the PPO activity was

244 higher in the pulp than in the skin.

245   Regarding the peroxidase activity (Figure 3), the variability of results within a

246 single variety was much higher than that of PPO activity. Particularly, 33% of

247 Hojiblanca samples did not present POD activity for the two seasons studied. In

248 contrast, 50% of Manzanilla samples had a very high POD activity, higher than 170

249 U/mL enzyme extract, but 15% of the samples had less than 20 U/mL enzyme extract.

250 The mean values reflect that the Manzanilla variety had the highest POD activity,

251 followed by Hojiblanca and Gordal. The participation of POD in the browning of olives

252 is unknown but some researchers have found a much higher concentration of this

253 enzyme in the olive seed than in the rest of the fruit.[31,32] This has been associated with

254 phenolic oxidation of the olive paste during the malaxation step of the olive oil

255 extraction process.

256   In relation to the β-glucosidase activity, again a high variability among orchards

257 was found within the same variety for the two seasons (Figure 3). The Gordal variety

258 showed the highest activity, followed by the Manzanilla and Hojiblanca varieties. There

259 is scarce data about endogenous β-glucosidase activity in olive varieties, and they were

260 focused mainly on Spanish,[14,17] Tunisian[18] and Italian[10] olive oil varieties. Furthermore,

261 these studies were carried out with samples from one orchard, one season and few

262 varieties, which did not find the great variability detected in this study.

263   The cleavage of the ester bond in the oleuropein molecule either chemically with

264 the use of NaOH or enzymatically by esterases gives rise to non-bitter compounds

265 (Figure 1). The activity of this enzyme for the three olive varieties and the two seasons

266 studied is reflected in Figure 3. The two varieties with a high concentration in phenolic

267 compounds and bitterness, Hojiblanca and Manzanilla, had a very low esterase activity

268 whereas the sweet variety Gordal with low concentrations in phenolic compounds

269 showed a very high activity of this enzyme, particularly during the second season.

270 Similar behavior has been reported for the sweet Tunisian variety Dhokar, a low

271 concentration in oleuropein was correlated with a high β-glucosidase and esterase

272 activity.[18]

273 **Influence of external parameters: pH and temperature.** The influence of pH

274 on enzyme activity in the fruits of Gordal, Manzanilla and Hojiblanca varieties is shown

275 in Figure 4. Oxidase enzymes showed a maximum activity in a pH range between 5 and

276 6 units, which is in agreement with previous data reported for the PPO in Manzanilla

277 olives.[33] However, there are many contradictory findings in the literature on the effect

278 of pH on oxidases activity. Researchers have reported a maximum activity of PPO at pH

279 4.5[34] and 7.5[35] for the Turkish Domat variety. A maximum POD activity has also been

280 reported at pH 7 and in the range of 4-6 units for Portuguese and Greek olive varieties,

281 respectively.[36,37] These data agree with the result shown in Figure 4, where an optimal

282 pH value of 6 units for POD activity is evident for all varieties.

283 It must be noted that these oxidase enzymes primarily act in olives during the

284 postharvest period when the pH of the fruit is around 5.

285 The β-glucosidase activity was at its maximum at pH 5, which is in accordance

286 with data reported by other researchers.[38,14] In contrast, the esterase activity decreased

287 from pH 7 to 4, particularly for the Gordal variety. To our knowledge, there is no data

288 available about the influence of pH on the olive esterase but our findings mean that the

289 low pH in olives during brining is not favorable for the action of this enzyme.

290 The effect of temperature on enzyme activity is reflected in Figure 5. It can be

291 observed that the PPO activity increased with increasing temperature up to 70 ºC for the

292 three olive varieties studied, which is in contrast to previous data that indicated a

293 maximum activity in the range of 30-50 ºC.[34,39] We have no explanation for these

294 results. It could be thought that some non-enzymatic reactions took place during the

295 analyses at high temperature but the PPO activity was calculated with the difference in

296 absorbance originated from the enzyme extract and the denatured control. It must be

297 noted that PPO stability is high at elevated temperature[35,39] and the analysis was carried

298 out in a few minutes.

299   The POD presented maximum activity over a temperature range of 30-40 ºC

300 which is in agreement with the range of 30-35 ºC reported by other researchers.[36,37]

301   The β-glucosidase activity in the Gordal variety presented two maximum

302 activity levels at temperatures of 10 ºC and 30 ºC, the former temperature was not

303 expected, and the second is similar to previous data found for other olive varieties,[38]

304 even for the Manzanilla variety. Moreover, Hojiblanca showed a maximum activity at

305 40-45 ºC, which agrees with the results found for the Picual variey.[14]

306   Regarding the esterase activity, the behavior was similar in all varieties, reaching

307 an optimal temperature at around 50-60 ºC, particularly for the Gordal variety. This is a

308 very high temperature and not common during the processing of Spanish table olives.

309 Also, there is no information in the literature on the influence of temperature on olive

310 esterase activity.

311 In conclusion, a great variability in the phenolic content and enzyme activity of olives

312 harvested with the same degree of maturation but from different orchards was found.

313 Variety and season also had a great influence on the contents of polyphenols and

314 enzyme activity in olive fruits. The Manzanilla variety showed the highest concentration

315 in phenolic compounds, followed by Hojiblanca and Gordal with the lowest. With

316 respect to the enzyme activity, it is relevant to say that the lowest PPO activity was

317   found in the Hojiblanca variety which could explain why this variety is less prone to

318   bruising than the others. Therefore, the results obtained in this study could contribute to

319   the explanation of the great variability found in the quality of processed table olives.

320   Besides, the enzyme knowledge can help processors to optimize the elaboration

321   methods of table olives due to the enzymatic transformation of the bitter substance

322   oleuropein instead of the current alkaline hydrolysis.

323   ACKNOWLEDGEMENTS

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440

444

445 **FIGURE CAPTIONS**

446 **Figure 1.** Oleuropein and products of its enzymatic transformations.

447 **Figure 2.** Concentration of phenolic composition (mmol/kg) in fruit of Gordal (G),

448 Hojiblanca (H) and Manzanilla (M) varieties. The phenol compounds analyzed were

449 hydroxytyrosol-1-glucoside, hydroxytyrosol-4-glucoside, salidroside, verbascoside,

450 oleuropein and the sum of tyrosol, luteolin-7-glucoside, rutin, caffeic and *p*-coumaric

451 acid, caffeoyl ester of secologanoside, comselogoside, luteolin, apigenin and

452 ligustroside (Others). Bars mean the standard error of twelve and six samples from the

453 2010/2011 (white bars) and 2011/2012 (black bars) seasons, respectively. Columns with

454 the same letter are not significantly different by Duncan's multiple range test at the 5%

455 level for each phenol.

456 **Figure 3.** Enzymatic activity (U/mL enzyme extract) in fruit of Gordal (G), Hojiblanca

457 (H) and Manzanilla (M) varieties. Bars mean the standard error of twelve and six

458 samples from the 2010/2011 (white bars) and 2011/2012 (black bars) seasons,

459 respectively. Columns with the same letter are not significantly different by Duncan's

460 multiple range test at the 5% level for each enzyme.

461 **Figure 4.** Effect of pH on enzymatic activity (U/mL enzyme extract) in the fruits of

462 Gordal (♦), Hojiblanca (Δ) and Manzanilla (□) varieties. Bars mean the standard

463 deviation of two samples.

464 **Figure 5.** Effect of temperature on enzymatic activity (U/mL enzyme extract) in the

465 fruit of Gordal (♦), Hojiblanca (Δ) and Manzanilla (◆) varieties. Bars mean the standard

466 deviation of two samples.

Table 1. Enzyme Activity (U/mL enzyme extract) and Polyphenol Compounds (mmol/ kg) in the Skin and Pulp of Manzanilla Olives

	Skin	Pulp
Enzyme activity		
PPO	4.3 ± 0.1^{a}	19.5 ± 0.4
Polyphenol compounds		
Hydroxytyrosol 1-glucoside	2.7 ± 0.1	0.6 ± 0.2
Hydroxytyrosol 4-glucoside	3.8 ± 0.8	2.2 ± 0.7
Salidroside	0.2 ± 0.1	0.2 ± 0.1
Verbascoside	0.3 ± 0.1	2.4 ± 0.4
Oleuropein	129.4 ± 18.4	45.9 ± 8.6
Others[b]	6.8 ± 1.8	2.5 ± 0.2

[a] Each value is the mean ± standard deviation of three samples. [b]Sum of rutin, caffeoyl ester of secologanoside, comselogoside and ligustroside.
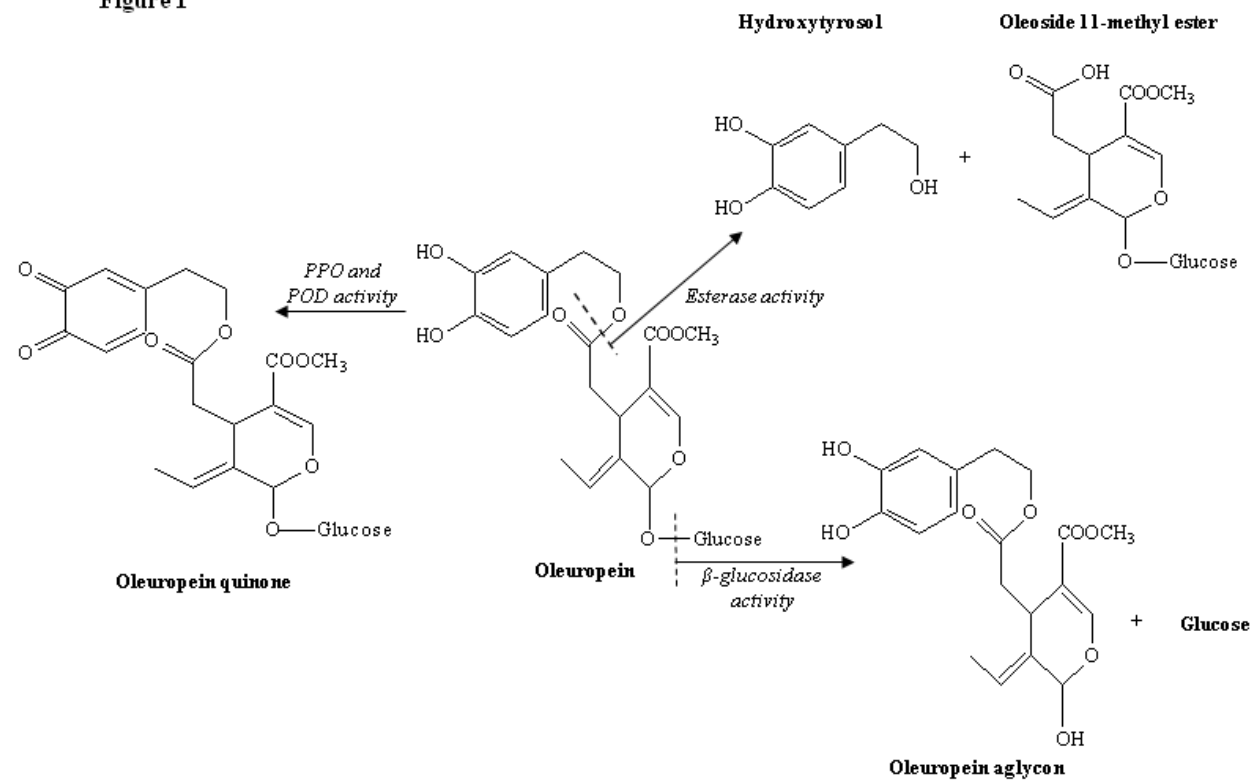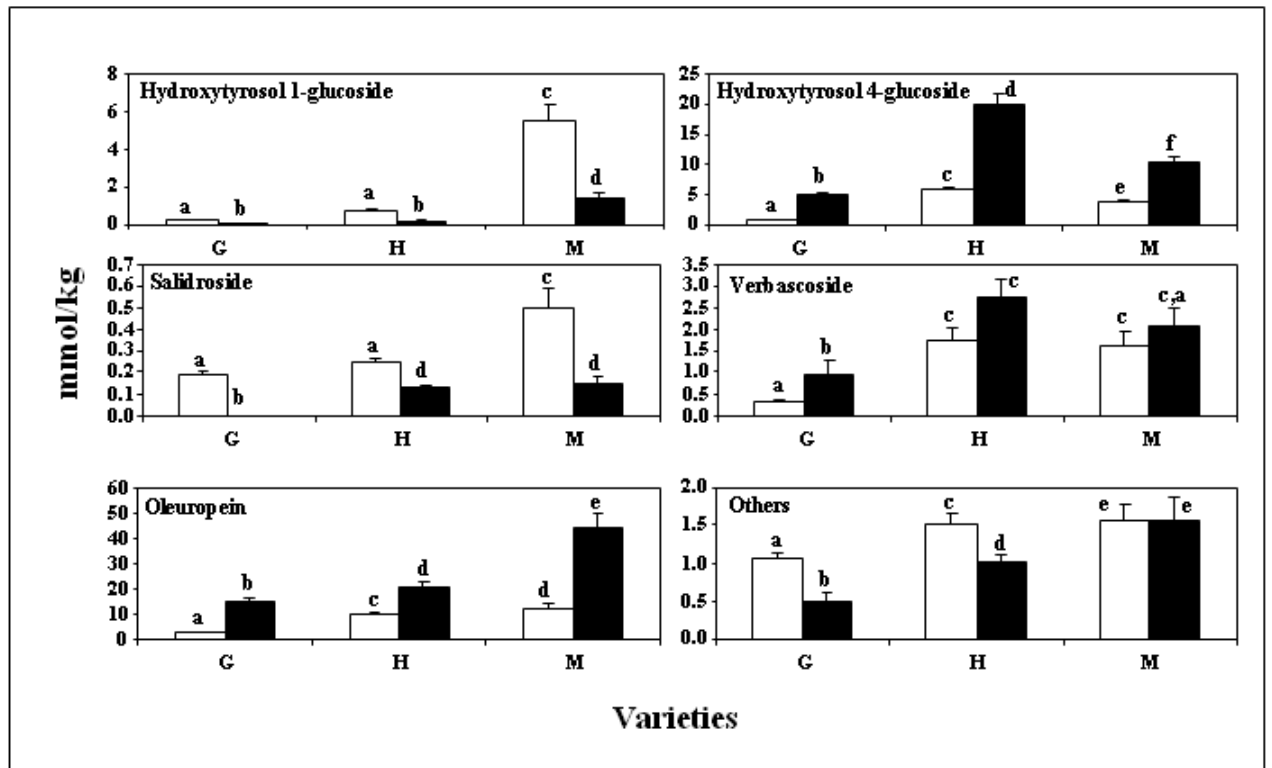
Figure 1

Hydroxytyrosol
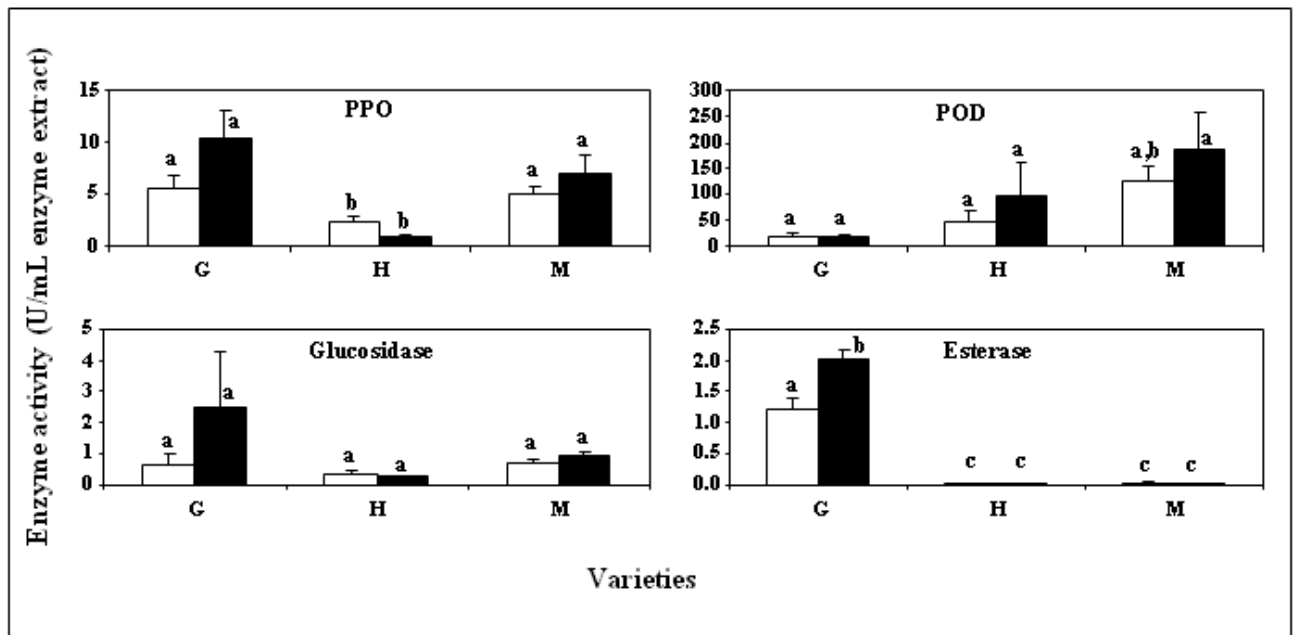
Oleoside 11-methyl ester

Oleuropein quinone

Oleuropein

Oleuropein aglycon

PPO and POD activity

Esterase activity

β-glucosidase activity

Figure 2

Figure 3

Figure 4
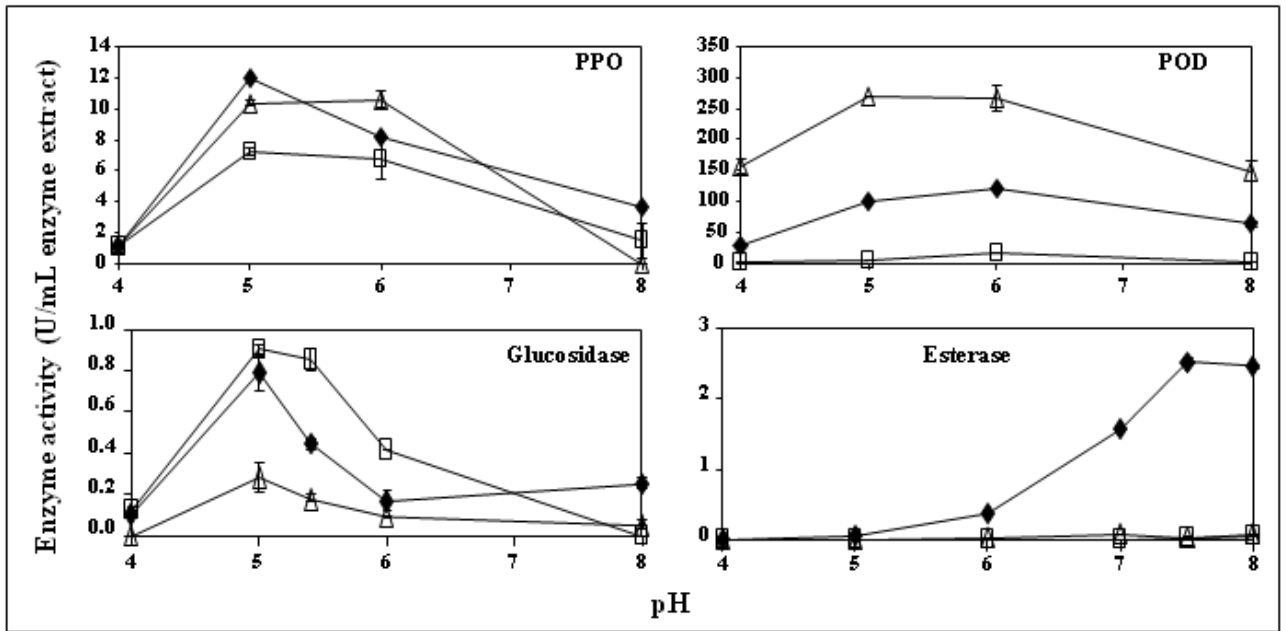
Figure 5

Table of Contents