Postprint of J. Agric. Food Chem., 2014, 62 (39), pp 9569–9575

DOI: 10.1021/jf5027982

**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 35°C. The β -glucosidase and
11 esterase activities were at their maximum at 30 °C and 55 °C 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.

INTRODUCTION

The olive is a popular fruit in Mediterranean countries, especially the foodstuff that derives from it, such as olive oil and table olives. The raw fruit is unpalatable due to the presence of the bitter compound named oleuropein. This substance is formed by glucose, elenolic acid and the *o*-diphenol hydroxytyrosol (Figure 1). When the fruits are processed, the oleuropein is transformed by chemical or enzymatic actions to obtain a non-bitter final product.¹

There is an increased demand to mechanically harvest the fruit intended for table olives but this technology is not applicable to some varieties due to the formation of brown spots. Mechanical harvesting gives rise to a rupture of the cellular tissue that releases phenolic compounds and oxidases. The oleuropein is oxidized by the action of polyphenoloxidase (PPO) and peroxidase (POD) to give its quinone that polymerizes to brown pigments.²⁻⁴ Oleuropein can also be oxidized by the action of PPO in natural olives under an overpressure of oxygen.⁵

A correlation between PPO activity and the rate of browning of a crude homogenate of five Italian olive varieties has been reported,⁶ and browning has also been associated with the oleuropein content in the olive varieties.⁷ Additionally, a correlation between PPO activity and total phenol concentration during the fruit maturation of Picual, Verdial, Arbequina and Frantoio varieties has been shown.^{8,9} Although there are some studies on the evolution of PPO activity during olive maturation^{7,10} there is only one report on Spanish table olive varieties,¹¹ in which a decrease in PPO activity with fruit ripening was found.

Most commercial table olives, Spanish-style green and California-style black olives are debittered by treating the fruits with a dilute NaOH solution. Nevertheless, there are other trade preparations that involve the direct brining of olives without any

alkaline treatment, which are known as Natural olives, and the bitterness disappears slowly during brining.¹² Moreover, the hydrolysis of oleuropein is mainly carried out during the extraction of olive oil by the action of endogenous β -glucosidase.¹³ Precisely, there are many phenolic glucosides in olives and β -glucosidase is especially active with oleuropein, its activity being lower with ligustroside, and demethyloleuropein.¹⁴ The oleuropein is transformed to its aglycone which can lead to the formation of the decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA), an important antimicrobial compound present in virgin olive oil and natural table olives.¹⁵

There is a lack of studies on the evolution of endogenous β -glucosidase and esterase activity during the maturation of olives intended for table olives. Gutiérrez-Rosales et al.,¹⁶ found a high content of oleuropein aglycon during the olive ripening of Arbequina and Hojiblanca varieties although it depended to a large extent on the tree location.¹⁷ Other researchers have also observed an increase in β -glucosidase and esterase activity with olive ripening of the Tunisian¹⁸ and Italian varieties.¹⁰

Most of these studies were focused on the participation of these enzymes in the elaboration process of olive oil but not table olives. Hence, the aim of this work was to evaluate both the phenolic composition and the endogenous enzymes in the raw fruits of the main Spanish table olive varieties.

MATERIALS AND METHODS

Raw material. Fruits of the Gordal, Manzanilla and Hojiblanca varieties (*Olea europaea* L.) in the ripening stage corresponding to a green-yellow surface color were harvested from mid-September to mid-October and supplied by different olive Cooperatives located in the Seville province (Spain). Fruit with this pigmentation was only considered in this study because this is the optimum degree of maturation for fruit

processed as green Spanish-style and black California-style. Twelve and six samples for each variety during the 2010/2011 and 2011/2012 seasons, respectively, were assessed. The fruits were processed on the same day without any storage period.

Analysis of phenolic compounds. The extraction of phenolic compounds from the olive pulp was based on the methodology proposed elsewhere.¹⁹ Around 10 g of fresh olive pulp were mixed in an Ultra-Turrax homogenizer with 30 mL of dimethyl sulfoxide (DMSO). After 30 min resting contact, the mixture was centrifuged at 6000 g for 5 min (22 °C), and 0.25 mL of the supernatant were diluted with 0.5 mL of DMSO plus 0.25 mL of 0.2 mM syringic acid in DMSO (internal standard). The extraction of phenolic compounds from the fresh olive skin was made similarly to the olive pulp as described above, but this time the mixing ratio was 0.1g of olive skin and 0.5 mL of DMSO.

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 consisted of a Waters 717 plus autosampler, a Waters 600 E pump, a Waters column heater module, and a Waters 996 photodiode array detector operated with Empower software (Waters Inc.). A 25 cm x 4.6 mm i.d., 5 μ m, Spherisorb ODS-2 (Waters Inc.) 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) water (pH 2.5 adjusted with 0.15% phosphoric acid) and (B) methanol. The initial composition was 90% A and 10% B. The concentration of B was increased to 30% over 10 min and was maintained for 20 min. Subsequently, B was raised to 40% over 10 min, maintained for 5 min, and then increased to 50%. Finally, B was increased to 60, 70 and 100% in 5-min periods. The initial conditions were reached in 10 min. Chromatograms were recorded at 280 nm.¹⁵

The evaluation of each compound was performed using a regression curve with the corresponding standard. Hydroxytyrosol, oleuropein, verbascoside, luteolin 7-glucoside, luteolin, rutin and apigenin were purchased from Extrasynthese S.A. (Lyon Nord, Genay, France), and tyrosol, caffeic and *p*-coumaric acid from Sigma Chemical Co. (St. Louis, MO). Hydroxytyrosol-1-glucoside, caffeoyl ester of secologanoside and comselogoside were quantified using the response factors of hydroxytyrosol, caffeic and *p*-coumaric acid respectively. Salidroside and ligustroside were quantified using the response factors of tyrosol. Hydroxytyrosol-4-glucoside was obtained using an HPLC preparative system.²⁰

Preparation of acetone powder. Acetone powders were obtained from 50 g of olive pulp or skin homogenized with 100 mL of cold acetone (-30 °C) containing 2.5 g of polyethylene glycol.⁶ The mixture was then vacuum-filtered in a Büchner funnel through paper, and the residue was re-extracted three times with 100 mL of cold acetone, obtaining a white solid that was dried overnight at room temperature to remove residual acetone. The acetone powder was stored at -40 °C until use.

PPO and POD activity. A crude enzyme extract was obtained from the above acetone powder as described elsewhere.¹¹ 0.5 g of acetone powder was suspended in 20 mL of 0.1 M sodium phosphate buffer, containing 1 M NaCl, and the pH was adjusted to 6.2 units with NaOH. The suspension was stirred at 4 °C for 30 min and then centrifuged at 15,550 x *g* for 20 min at 4 °C. The pellet was discarded and the supernatant divided into two aliquots; one was used as the active crude enzyme extract, and the other was boiled for 30 min to get the denatured enzyme extract.

The PPO activity was determined spectrophotometrically using a Shimadzu UV-vis 1800 spectrophotometer as described elsewhere.²¹ All measurements of PPO activity 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 °C 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 °C 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 °C 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 ethylenediaminetetraacetic 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 °C for 1 h and then centrifuged at 15,550 x g for 20 min at 4 °C. 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 μ L of crude enzyme extract and 100 μ L 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 °C. 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 μ mol 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 °C for 1 h and
163 then centrifuged at 15,550 x g for 20 min at 4 °C. 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

absorbance at 405 nm for 10 min at 40 °C related to the increasing amount of p-NP liberated from the synthetic p-nitrophenyl acetate (p-NPA) (Sigma Chemical Co., St. Louis, MO). The incubation mixture contained 50 µL of crude enzyme extract, 50 µL of 0.15 M p-NPA in ethanol as substrate and 2.9 mL of a 0.0092 M Tris-HCl buffer at pH 7.5. The reaction was initiated by the addition of the enzyme extract. The assay mixture with 50 µL of extraction buffer served as the control.

The evaluation was performed using a regression curve with p-NP in a range of 0-0.00003 M. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of p-NP/min. All reactions were carried out in duplicate.

Effect of pH. The enzyme assays at different pH was performed by measuring the activity of each enzyme using different buffer solutions under standard conditions. In the case of PPO and POD, 0.1 M sodium citrate and a 0.1 M sodium phosphate buffer in the pH range of 4-5 and 6-8 units were used, respectively. The effect of pH on olive β-glucosidase and esterase activity was assessed with a 0.05 M acetic acid-sodium acetate trihydrate solution, 0.05 M sodium phosphate and a 0.0092 M Tris-HCl buffer in the pH range of 4-5, 6-7 and 7.5-8 units, respectively. In all cases, the pH was finally adjusted with suitable amounts of 1.0 M NaOH and 1.0 M HCl.

Effect of temperature. The optimal temperature for PPO, POD, β-glucosidase and esterase enzymes was determined by measuring the activity of each enzyme in the temperature range of 10-70 ° C under standard conditions.

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

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 olives²⁰ 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.

Enzyme activity. Figure 3 shows the PPO activity in fruits of the Gordal, Hojiblanca and Manzanilla varieties picked with a green-yellow color for two seasons and from several Cooperatives.

The first finding that must be noted is the high variability found among the different samples analyzed within the same variety, especially for those of the Gordal variety. The standard error of the mean PPO activity of Gordal was of 1.28 and 2.61 U/mL enzyme extract for the two seasons studied whereas these data were recorded as 0.63 and 0.06 U/mL enzyme extract for the Hojiblanca variety. This variability is common in nature and it has been observed for Italian olive varieties²⁸ and many other fruits such as peaches.²⁹

Regardless of the season, the Gordal variety showed a high PPO activity, followed by Manzanilla and Hojiblanca. In Spain, the Hojiblanca variety destined to table olives is mechanically harvested whereas the other varieties (Manzanilla and Gordal), which are very prone to bruising, are picked by hand. Indeed, Jiménez-Jiménez et al.,³⁰ found the Hojiblanca variety to have a lower susceptibility to bruising than the others but no explanation for this phenomenon was reported. Both phenolic compounds and PPO participate in the appearance of bruising after harvesting but from the data reflected in Figures 2 and 3, it seems that the PPO activity could better explain the behavior of the Hojiblanca variety. The low PPO activity in this variety probably contributes to the slow formation of brown spots in harvested olives while the high activity in Manzanilla and Gordal favors their appearance.

The phenolic composition and the PPO activity in both the pulp and the skin of the Manzanilla variety were also characterized (Table 1), which is the more prone variety to bruising. The concentration of the phenolic compounds, except verbascoside, was much higher in the skin than in the pulp but, in contrast, the PPO activity was much

lower in the skin than in the pulp. This data are in agreement with those reported by Chang et al.,²⁹ who found that in five peach varieties out of nine, the PPO activity was higher in the pulp than in the skin.

Regarding the peroxidase activity (Figure 3), the variability of results within a single variety was much higher than that of PPO activity. Particularly, 33% of Hojiblanca samples did not present POD activity for the two seasons studied. In contrast, 50% of Manzanilla samples had a very high POD activity, higher than 170 U/mL enzyme extract, but 15% of the samples had less than 20 U/mL enzyme extract. The mean values reflect that the Manzanilla variety had the highest POD activity, followed by Hojiblanca and Gordal. The participation of POD in the browning of olives is unknown but some researchers have found a much higher concentration of this enzyme in the olive seed than in the rest of the fruit.^{31,32} This has been associated with phenolic oxidation of the olive paste during the malaxation step of the olive oil extraction process.

In relation to the β -glucosidase activity, again a high variability among orchards was found within the same variety for the two seasons (Figure 3). The Gordal variety showed the highest activity, followed by the Manzanilla and Hojiblanca varieties. There is scarce data about endogenous β -glucosidase activity in olive varieties, and they were focused mainly on Spanish,^{14,17} Tunisian¹⁸ and Italian¹⁰ olive oil varieties. Furthermore, these studies were carried out with samples from one orchard, one season and few varieties, which did not find the great variability detected in this study.

The cleavage of the ester bond in the oleuropein molecule either chemically with the use of NaOH or enzymatically by esterases gives rise to non-bitter compounds (Figure 1). The activity of this enzyme for the three olive varieties and the two seasons studied is reflected in Figure 3. The two varieties with a high concentration in phenolic

compounds and bitterness, Hojiblanca and Manzanilla, had a very low esterase activity whereas the sweet variety Gordal with low concentrations in phenolic compounds showed a very high activity of this enzyme, particularly during the second season. Similar behavior has been reported for the sweet Tunisian variety Dhokar, a low concentration in oleuropein was correlated with a high β -glucosidase and esterase activity.¹⁸

Influence of external parameters: pH and temperature. The influence of pH on enzyme activity in the fruits of Gordal, Manzanilla and Hojiblanca varieties is shown in Figure 4. Oxidase enzymes showed a maximum activity in a pH range between 5 and 6 units, which is in agreement with previous data reported for the PPO in Manzanilla olives.³³ However, there are many contradictory findings in the literature on the effect of pH on oxidases activity. Researchers have reported a maximum activity of PPO at pH 4.5³⁴ and 7.5³⁵ for the Turkish Domat variety. A maximum POD activity has also been reported at pH 7 and in the range of 4-6 units for Portuguese and Greek olive varieties, respectively.^{36,37} These data agree with the result shown in Figure 4, where an optimal pH value of 6 units for POD activity is evident for all varieties.

It must be noted that these oxidase enzymes primarily act in olives during the postharvest period when the pH of the fruit is around 5.

The β -glucosidase activity was at its maximum at pH 5, which is in accordance with data reported by other researchers.^{38,14} In contrast, the esterase activity decreased from pH 7 to 4, particularly for the Gordal variety. To our knowledge, there is no data available about the influence of pH on the olive esterase but our findings mean that the low pH in olives during brining is not favorable for the action of this enzyme.

The effect of temperature on enzyme activity is reflected in Figure 5. It can be observed that the PPO activity increased with increasing temperature up to 70 °C for the

three olive varieties studied, which is in contrast to previous data that indicated a maximum activity in the range of 30-50 °C.^{34,39} We have no explanation for these results. It could be thought that some non-enzymatic reactions took place during the analyses at high temperature but the PPO activity was calculated with the difference in absorbance originated from the enzyme extract and the denatured control. It must be noted that PPO stability is high at elevated temperature^{35,39} and the analysis was carried out in a few minutes.

The POD presented maximum activity over a temperature range of 30-40 °C which is in agreement with the range of 30-35 °C reported by other researchers.^{36,37}

The β -glucosidase activity in the Gordal variety presented two maximum activity levels at temperatures of 10 °C and 30 °C, the former temperature was not expected, and the second is similar to previous data found for other olive varieties,³⁸ even for the Manzanilla variety. Moreover, Hojiblanca showed a maximum activity at 40-45 °C, which agrees with the results found for the Picual variety.¹⁴

Regarding the esterase activity, the behavior was similar in all varieties, reaching an optimal temperature at around 50-60 °C, particularly for the Gordal variety. This is a very high temperature and not common during the processing of Spanish table olives. Also, there is no information in the literature on the influence of temperature on olive esterase activity.

In conclusion, a great variability in the phenolic content and enzyme activity of olives harvested with the same degree of maturation but from different orchards was found. Variety and season also had a great influence on the contents of polyphenols and enzyme activity in olive fruits. The Manzanilla variety showed the highest concentration in phenolic compounds, followed by Hojiblanca and Gordal with the lowest. With respect to the enzyme activity, it is relevant to say that the lowest PPO activity was

found in the Hojiblanca variety which could explain why this variety is less prone to bruising than the others. Therefore, the results obtained in this study could contribute to the explanation of the great variability found in the quality of processed table olives. Besides, the enzyme knowledge can help processors to optimize the elaboration methods of table olives due to the enzymatic transformation of the bitter substance oleuropein instead of the current alkaline hydrolysis.

ACKNOWLEDGEMENTS

Eva Ramirez wishes to thank MINECO for her FPI fellowship.

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441 NOTE OF ACKNOWLEDGEMENT

442 This work was supported by project AGL 2009-07512 from the Spanish Government
443 and European Union (European Regional Development Funds).

444

FIGURE CAPTIONS

Figure 1. Oleuropein and products of its enzymatic transformations.

Figure 2. Concentration of phenolic composition (mmol/kg) in fruit of Gordal (G), Hojiblanca (H) and Manzanilla (M) varieties. The phenol compounds analyzed were hydroxytyrosol-1-glucoside, hydroxytyrosol-4-glucoside, salidroside, verbascoside, oleuropein and the sum of tyrosol, luteolin-7-glucoside, rutin, caffeic and *p*-coumaric acid, caffeoyl ester of secologanoside, comselogoside, luteolin, apigenin and ligustroside (Others). Bars mean the standard error of twelve and six samples from the 2010/2011 (white bars) and 2011/2012 (black bars) seasons, respectively. Columns with the same letter are not significantly different by Duncan's multiple range test at the 5% level for each phenol.

Figure 3. Enzymatic activity (U/mL enzyme extract) in fruit of Gordal (G), Hojiblanca (H) and Manzanilla (M) varieties. Bars mean the standard error of twelve and six samples from the 2010/2011 (white bars) and 2011/2012 (black bars) seasons, respectively. Columns with the same letter are not significantly different by Duncan's multiple range test at the 5% level for each enzyme.

Figure 4. Effect of pH on enzymatic activity (U/mL enzyme extract) in the fruits of Gordal (♦), Hojiblanca (Δ) and Manzanilla (□) varieties. Bars mean the standard deviation of two samples.

Figure 5. Effect of temperature on enzymatic activity (U/mL enzyme extract) in the fruit of Gordal (♦), Hojiblanca (Δ) and Manzanilla (◆) varieties. Bars mean the standard 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
<i>Enzyme activity</i>		
PPO	4.3 ± 0.1 ^a	19.5 ± 0.4
<i>Polyphenol compounds</i>		
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. ^bSum of rutin, caffeoyl ester of secologanoside, comselogoside and ligustroside.

Figure 1

Hydroxytyrosol **Oleoside 11-methyl ester**

The diagram illustrates the chemical structures and enzymatic conversion pathways of Oleuropein. Oleuropein is shown as a central molecule with a hydroxytyrosol moiety linked to a glucose moiety via an ester bond. The pathways are as follows:

- Oleuropein** is converted to **Oleuropein aglycon** (a glucose-free structure) via β -glucosidase activity, releasing **Glucose**.
- Oleuropein** is converted to **Hydroxytyrosol** (a dihydroxybenzyl alcohol structure) and **Oleoside 11-methyl ester** (a glucose-linked structure) via **Esterase activity**.
- Oleuropein** is converted to **Oleuropein quinone** (a quinone structure) via **PFO and POD activity**.

Oleuropein **Oleuropein aglycon** **Oleuropein quinone**

Figure 2

Figure 3

Figure 4

Figure 5

Table of Contents