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Phenylalanine Ammonia-Lyase, Polyphenol Oxidase, and Phenol Concentration in Fruits of *Olea europaea* L. cv. Picual, Verdial, Arbequina, and Frantoio during Ripening

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The kinetics and protein-expression level of phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) in fruits of olive trees (*Olea europaea*) cv. Picual, Verdial, Arbequina, and Frantoio have been studied in relation to the concentration of total phenolic compounds, oleuropein, hydroxytyrosol, and tyrosol during fruit ripening. Frantoio was the variety that showed the highest total phenol concentration, the highest PAL activity, the lowest PPO activity, and the lowest protein levels. In contrast, Verdial was the variety that showed the lowest total phenol concentration, the least PAL activity, the greatest PPO activity, and the highest protein levels. Arbequina and Picual showed intermediate levels. These results suggest the existence of a coordinated response between PAL, PPO, and the concentration of total phenols over ripening in the four varieties. The concentration of total and specific phenols differed between varieties and specifically changed over ripening.

KEYWORDS: Phenylalanine ammonia-lyase; polyphenol oxidase; oleuropein; hydroxytyrosol; *Olea europaea*; Picual; Verdial; Arbequina; Frantoio; ripening

INTRODUCTION

Phenols are secondary plant metabolites that have important functions in the physiology of the plant, and they also affect appearance, flavor, and health-promoting properties of olive (*Olea europaea* L.) products (*1*, *2*). Oleuropein is the most abundant phenolic compound in olive fruits (*3*); it is a heterosidic ester of β-glucosylated elenolic acid and hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol). Oleuropein and hydroxytyrosol are the two most important antioxidants present in olive fruits (*4*, *5*). An oleuropein derivative is also the prime phenolic compound responsible for the oil bitterness, one important property that determines the taste of the olive oil and thus its price. Tyrosol (*p*-hydroxyphenylethanol) is the second most abundant phenolic alcohol in olives (*6*, *7*). The antioxidant effects of polyphenolic compounds are correlated to the high stability of virgin olive oil against thermoxidation and autoxidation processes over time (*4*).

The level of phenolic compounds in a tissue is a result of the balance between biosynthesis and catabolism. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is the regulatory enzyme of the biosynthesis of phenylpropanoids in plants (*8*, *9*). PAL catalyzes the nonoxidative deamination of L-phenylalanine (L-Phe) to form *trans*-cinnamic acid and free ammonium. This reaction is the first step in the biosynthesis of a large group of phenylpropanoid-derived secondary products in plants, such as flavonoids, isoflavonoids, coumarins, lignins, wound-protective hydroxycinnamic acid esters, and other phenolic compounds such as tyrosol together with derivatives such as salidroside (*8*). In the oxidative degradation of phenolic compounds, the enzyme polyphenol oxidase (PPO, EC 1.10.3.1) has a major function (*10*). PPO, an enzyme that contains copper, catalyzes the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-diquinones (*11*). This oxidative reaction occurs during fruit browning and in response to injury by biotic as well as abiotic factors (*10*). During ripening, hydroxytyrosol can be formed by hydrolysis of tyrosol glucoside and oleuropein. During fruit browning, hydroxytyrosol and other *o*-diphenols are oxidized by fruit PPO.

In our study, we have selected four olive varieties used in oil production. Picual, cultivated on approximately 900 000 ha in Spain, is the prime variety from Jaén, Andalusia, Spain (*12*). It is considered well adapted to several climate and soil conditions, being especially tolerant to cold, salinity, and excess soil water. Nevertheless, it is sensitive to drought and limy soil. The oil produced by this variety is very stable, with great resistance to becoming bitter (*13*) and a high content of polyphenols (*12*). Verdial is cultivated on approximately 30 000 ha in Andalusia and Extremadura, Spain. This variety is adapted to wet and dry soil and is frost resistant. Arbequina, cultivated on approximately 91 000 ha in Spain, is the most important variety in Catalonia and Aragón, Spain. It is considered to be cold and frost resistant and tolerant to salinity but is sensitive to ferric chlorosis in limy soil (*12*). The oil from this variety has a high amount of lignans (*14*) and is highly appreciated in the foreign trade, probably for its fruity taste (*15*). Frantoio is cultivated in Tuscany on around 48% of the growing area in Italy (*16*). This variety has

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a high capacity of adaptation to different environmental conditions, although it is sensitive to cold stress. Its oil is highly appreciated for its organoleptic properties and its stability (*12*, *17*).

Previous studies have described the differences between the phenolic profiles of different Italian and Portuguese olive cultivars (*18*−*20*) and their changes over the ripening process (*5*, *21*, *22*). We have previously reported the molecular and kinetic characterization of PAL and PPO in fruits and leaves of olive tree of the Picual variety (*23*, *24*). In this work, we study and compare the evolution of kinetics and protein expression of PAL and PPO and their relationship to the phenol concentrations in the fruits of four varieties of olive (Picual, Verdial, Arbequina, and Frantoio) during ripening. The hypothesis of our work is that a direct relationship exists between PAL, PPO, and the phenol concentration that explains the differences between varieties. The characteristics and quality of the mill olives are key factors in the quality of the final product. A knowledge of the factors that regulate the phenol composition of the fruits of different varieties can be useful to determine how to control the phenol composition of olive oil and thus the nutritional quality and flavor of it; both are essential elements for a healthy, profitable product.

MATERIALS AND METHODS

Chemicals. Substrates, coenzymes, and other reagents such as β-mercaptoethanol, polyvinylpolypyrrolidone, soybean trypsin inhibitor type II, tropolone, and 1,4-aminobenzotriazole were purchased from Sigma Chemical Co. (St. Louis, MO) and Fluka Chemie GmbH (Buchs, Switzerland). The chemicals used for SDS-PAGE and immunoblot were from BioRad Laboratories (Hercules, CA) and Pierce Biotechnology Inc. (Rockford, IL). Oleuropein, hydroxytyrosol, and tyrosol were purchased from Extrasynthèse (Z.I. Lyon-Nord, Genay, France). The reagents used in HPLC were of HPLC grade.

Plant Material and Experimental Design. Olive trees (*Olea europaea*) of cv. Picual, Verdial, Arbequina, and Frantoio, 20 years old, located in the Agricultural Research and Training Centre "Estación de Olivicultura y Elaiotecnia Venta del Llano" (Mengíbar, Jaén, Junta de Andalucía, Spain) were used for this study. The one-trunk trees were grown at 7×7 m apart in the experimental farm located at 280 m above sea level under traditional rain-fed cultivation. Four different samples (called samples 1−4) were collected throughout the ripening period of olive fruit, from July to December. Samples 1−4 were picked on July 31, Oct 10, Nov 1, and Dec 3 of 2002, respectively. In each orchard, five trees periodically distributed were chosen and fruits were sampled from all orientations of the tree. From each orientation in each tree, five 25 cm segments of branch with fruits near the apical end were collected. The fruits were separated from the branches, and all the fruits from all five trees were pooled. The index of ripeness (IR) was determined, and immediately afterward the samples were divided into five replicates and frozen in liquid nitrogen until analyzed. IR was determined using a color evaluation of the skin and flesh proposed by Uceda and Frías (*25*). The procedure consists of distributing approximately 100 olive fruits into eight groups, according to the following characteristics: group 0, skin bright green; group 1, skin green-yellowish; group 2, skin green with reddish spots; group 3, skin reddish brown; group 4, skin black with white flesh; group 5, skin black with less than 50% of purple flesh; group 6, skin black with more than 50% purple flesh; and group 7, skin black with 100% purple flesh. Afterward, the number of fruits was counted in each group and the IR was determined by the equation $IR = \sum(in_i)/100$, where i is the number of the group and n_i the number of olives in it.

The water content of the fruits was determined by weighing 5 g of fruit pulp and then drying at 55 °C in an oven to constant weight. The samples were cooled for 30 min in a drier and reweighed.

Extraction and Assay of Fruit PAL and PPO. The procedure used to extract and assay PAL followed the method of Ortega-García et al. (*24*). This procedure was carried out with fruit samples of all different ripening states of the four olive-tree varieties studied. Five replicates were made of each sample corresponding to each ripening state of each variety. After being defrosted, olives were pitted and 5 g of pulp and skin was pulverized in a mortar with liquid nitrogen. A homogenate was made at a proportion of 1:5 w/v with an extraction buffer that contained 0.1 mol L^{-1} sodium phosphate buffer pH 8.0, 50 g L^{-1} polyvinylpolypyrrolidone, 3 g L^{-1} soybean trypsin inhibitor type II, and 2 mmol L^{-1} β-mercaptoethanol. The homogenate was filtered through glass wool. and the filtrate was centrifuged at 20 000*g* for 20 min at 4 °C. The proteins of the supernatant were precipitated by adding ammonium sulfate to 80% saturation. The pH in the samples was maintained between 7 and 7.5. The precipitated proteins were collected by centrifugation at 20 000*g* at 4 °C for 20 min. The pellet was dissolved and dialyzed overnight at 4 °C. The dialyzed samples were used to assay the PAL activity, protein determination, and immunoblot analysis.

PAL was assayed in a medium containing 37.5 mmol L^{-1} borate buffer pH 8.8, 2 mmol L^{-1} mercaptoethanol, 6.67 μmol L^{-1} tropolone (an inhibitor of polyphenol oxidase), 66.67 μmol L^{-1} 1,4-aminobenzotriazole (an inhibitor of cinnamate-4-hydroxylase), L-Phe at variable concentration, from 0.05 to 10 mmol L^{-1}, and 0.12 mL of enzyme extract. These media were incubated at 40 °C for 0, 10, 20, 30, 40, and 60 min. The reaction was stopped by adding 0.05 mL of 4.5 mol L^{-1} H_2SO_4. In the samples at time 0, first the acid was added and then the enzyme extract. The acidified samples were incubated in a boiling water bath for 10 min and afterward centrifuged at 1500*g* for 10 min. An aliquot of 2.5 mL of diethyl ether was added to each supernatant, and the mixture was vigorously shaken in a vortex for 2 min to extract the cinnamate. One milliliter of the ether phase was collected and evaporated. The resulting residue was dissolved in 0.05 mol L^{-1} NaOH, and the absorbance at 268 nm was recorded. The initial velocity of the reaction was determined considering the reaction-progress curve drawn on plotting the results found at the different assay times. One unit of PAL activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of cinnamic acid min^{-1} in a cuvette of 1 cm under optimum assay conditions.

The protein concentration was determined by the Bradford method (*26*) using bovine serum albumin as the standard.

The procedure used to extract and assay the PPO followed the method of Ortega-García et al. (*23*). Acetone powders of the pulverized fruit samples were prepared using cold acetone and polyethylene glycol. Immediately before each PPO assay, acetone powder was resuspended in 0.1 mol L^{-1} sodium phosphate buffer, pH 6.2 with 0.3 g L^{-1} of type-II trypsin inhibitor. After filtration with glass wool, the filtrate was centrifuged at 10 000*g* for 20 min at 4 °C. The supernatants were used for PPO assays, protein quantification, PAGE, and Western blotting.

Polyphenol oxidase activity was determined by spectrophotometry at 420 nm using catechol as the substrate. Assays were performed at 30 °C in a medium containing 0.1 mol L^{-1} sodium phosphate buffer, pH 6.2, 1.25−500 mmol L^{-1} catechol, and 10 μL of fruit enzyme extract in a total volume of 1 mL. The unspecific photooxidation of catechol was evaluated and corrected for. Specific activity was expressed as units mg^{-1} protein; 1 unit is defined as the amount of enzyme needed to prompt an increase of 0.1 optical density unit min^{-1} in a 1 cm cuvette at 30 °C under our standard assay conditions.

The PAL and PPO kinetic parameters (maximum velocity V_{max} and Michaelis constant K_m) and kinetic behavior were determined by the nonlinear-regression analysis program GraFit (Erithacus Software Ltd., Surrey, U.K.).

SDS-PAGE and in-Gel PPO Assay. Partially denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Ortega-García et al. (*23*). SDS-PAGE was carried out without the addition of 2-mercaptoethanol and without heating to preserve PPO activity. Samples of supernatants of fruit acetone powders were mixed with 0.5 mol L^{-1} Tris-HCl, pH 6.8 containing 100 mL L^{-1} glycerol and 0.02 g L^{-1} bromophenol blue and subjected to 10% SDS-PAGE in a minigel Bio-Rad system (Bio-Rad Laboratories, Hercules, CA), following the method of Laemmli (*27*). Electrophoresis was performed for 1.5 h at constant voltage (100 V) in a buffer containing 15 g L^{-1} (w/v) Tris base, 72 g L^{-1} glycine, and 5 g L^{-1} (w/v) SDS. All operations were performed at 4 °C.

After electrophoresis, gels were incubated for 45 min with 5 mmol L^{-1} of DL-DOPA in the presence of 100 000 U L^{-1} catalase dissolved in 0.1 mol L^{-1} sodium phosphate buffer pH 7.3. The reaction was stopped with 0.2 mmol L^{-1} of diethyldithiocarbamic acid. A second gel was stained

Table 1. Time Course of the Weight and Concentration of Water and Total Phenols in Fruits of *Olea europaea* cv. Picual, Verdial, Arbequina, and Frantoio during Fruit Ripening[a]

	sample 1 (July)	sample 2 (Oct)	sample 3 (Nov)	sample 4 (Dec)
time (days)	0	41	62	95
wt (g)				
Picual	$1.63 \pm 0.20_a^x$	$2.17 \pm 0.22_a^x$	$2.34 \pm 0.49_a^x$	$3.86 \pm 0.44_b^x$
Verdial	$1.86 \pm 0.20_a^x$	$3.73 \pm 0.35_b^y$	$4.01 \pm 0.40_b^y$	$5.24 \pm 0.38_c^y$
Arbequina	$0.73 \pm 0.10_a^y$	$1.79 \pm 0.15_b^x$	$1.76 \pm 0.09_b^x$	$1.83 \pm 0.15_b^z$
Frantoio	$1.58 \pm 0.16_a^x$	$2.40 \pm 0.24_b^x$	$2.34 \pm 0.24_b^x$	$2.52 \pm 0.25_b^w$
water (g kg dry weight^{-1})				
Picual	$730.6 \pm 4.1_a^x$	$631.3 \pm 3.6_b^x$	$586.0 \pm 7.8_c^x$	$551.5 \pm 18.3_c^x$
Verdial	$729.7 \pm 6.7_a^x$	$704.4 \pm 7.9_b^y$	$667.0 \pm 3.0_c^y$	$693.2 \pm 3.5_b^y$
Arbequina	$683.7 \pm 3.7_a^y$	$640.1 \pm 15.1_b^x$	$629.8 \pm 27.5_b^{xy}$	$577.1 \pm 27.8_b^x$
Frantoio	$718.7 \pm 4.4_a^x$	$582.2 \pm 3.9_b^z$	$548.6 \pm 27.8_b^x$	$552.2 \pm 49.4_b^x$
total phenols (g kg dry weight^{-1})				
Picual	$35.15 \pm 0.49_a^x$	$21.97 \pm 0.43_b^x$	$15.21 \pm 0.39_c^x$	$13.76 \pm 0.18_c^x$
Verdial	$23.62 \pm 0.87_a^y$	$17.78 \pm 0.68_b^y$	$17.51 \pm 0.21_b^y$	$12.57 \pm 0.49_c^y$
Arbequina	$31.52 \pm 0.33_a^z$	$16.03 \pm 1.12_b^y$	$19.77 \pm 0.71_c^z$	$13.34 \pm 0.43_b^{xy}$
Frantoio	$64.09 \pm 0.17_a^w$	$22.87 \pm 0.23_b^x$	$20.90 \pm 0.19_c^z$	$23.66 \pm 0.38_b^z$

[a] Samples were harvested on four dates corresponding to different ripening status. Values are means ± SEM of 10 data. Results were analyzed by a two-way ANOVA and Student's *t*-test. For each parameter, data in each row followed by different sub-indices (a, b, c, d) are statistically different ($p < 0.05$). For a comparison between varieties, data in each column followed by different super-indices (x, y, z, w) are statistically different ($p < 0.05$).

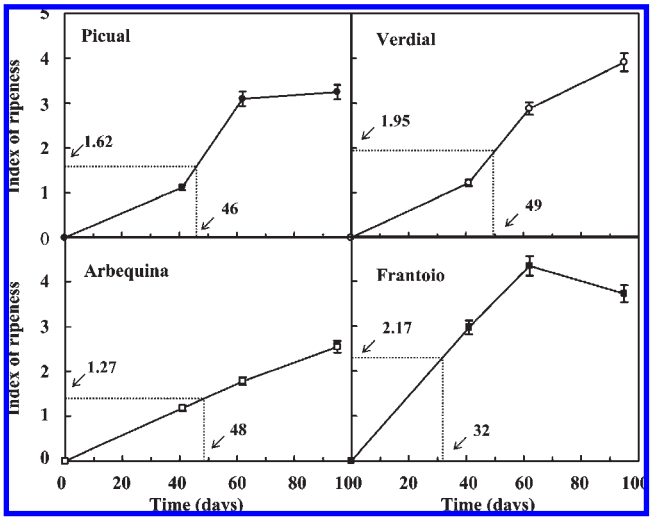

Figure 1. Changes in the index of ripeness (IR) of olive fruit of Picual, Verdial, Arbequina, and Frantoio varieties during maturation. Data are mean ± SEM. The time 0 was considered the day on which the first sample was picked, July 31, 2002. For each variety, the mean value of the IR obtained during maturation is shown. Also, the time at which this value was recorded is indicated.

specifically for peroxidase (POD) activity with 50 mmol L^{-1} guaiacol plus 0.2 mL of 10 mL L^{-1} H_2O_2.

Determination of PAL and PPO Protein Levels by Immunoblot. PAL and PPO specific protein levels were determined by Western blotting as described by Ortega-García et al. (*23*, *24*). Samples prepared as described above were mixed with 62.5 mmol L^{-1} Tris-HCl pH 6.8 buffer containing 20 g L^{-1} SDS, 100 mL L^{-1} glycerol, 25 g L^{-1} β-mercaptoethanol, and 0.045 mmol L^{-1} bromophenol blue and then heated for 3 min at 95 °C. Polypeptides were separated on 7.5% or 10% SDS-PAGE and afterward transferred to polyvinylidene fluoride membranes with a semidry electroblotting system at 1.5 mA cm^{-2} for 45 min in a medium containing 25 mmol L^{-1} Tris-HCl, 192 mmol L^{-1} glycine, 200 mL L^{-1} methanol, and 1 g L^{-1} SDS. Blots were blocked for 4.5 h at room temperature with a buffer containing 25 mmol L^{-1} Tris-HCl, 100 mmol L^{-1} NaCl, 2.5 mmol L^{-1} KCl, pH 7.6 (TBS), 1 mL L^{-1} Tween20, and 15 g L^{-1} bovine serum albumin (BSA) pH 7.6. Membranes were washed with TBS containing 1 mL L^{-1} Tween20 (TBS-T) for 15 min and afterward incubated with a rabbit antiserum antiparsley PAL-1 (1:5000) or with a rabbit antiserum antibroad bean PPO (1:10000). The antiserum anti-PAL-1 was generously donated by Dr. N. Amrheim, Institut für Pflanzenwissenschafte, Eidgenössische Technische Hochschule, Zurich, Switzerland. The antiserum anti-PPO was generously donated by Dr. B. B. Buchanan, Department of Plant and Microbial Biology, University of California, Berkeley, CA. After three washes with TBS-T containing 10 g L^{-1} BSA (TBS-T-BSA) for 10 min, membranes were incubated with a biotinylated goat antibody antirabbit-IgG. After three washes with TBS-T-BSA, immunodetection was performed using a chemiluminescent intensification system. Membranes were scanned with a Hewlett-Packard scanner and densitometer with a Bio-Rad Fluor-S Multimager and quantifier using Bio-Rad Quantity One software.

Extraction, Analysis, and Quantification of Phenolic Compounds. The phenolic compounds in the fruits were extracted as described by Ortega-García et al. (*23*, *24*). Fruit samples were pulverized in a mortar with liquid nitrogen and afterward homogenized in 800 mL L^{-1} methanol (1:4, w:v). The homogenates were vigorously shaken in a vortex for 2 min. After sedimentation, the methanol phase was decanted and the residue re-extracted with the same volume of 800 mL L^{-1} methanol. All the methanol phases were pooled, and an equal volume of hexane was added. The mixture was again vigorously shaken in a vortex for 2 min, and after phase separation, the hexane was removed. This extraction was repeated twice more. Finally, for enhanced separation and removal of contaminating residues, the mixture was centrifuged at 1500*g* for 5 min. The resulting methanolic phase was used to analyze the phenolic compounds by high-performance liquid chromatography (HPLC) and for the measurement of total phenol content by spectrophotometry.

The HPLC analyses of the methanol extracts from the fruits were made using a reverse-phase Spherisorb ODS-2 column (5 μm, 25 cm by 4.6 mm; Waters Corp., Milford, MA) and a Shimadzu HPLC system consisting of two pumps, a column-heater module, and a UV−visible detector that operates with LC-Solutions software (Shimadzu Corporation, Kyoto, Japan). Separation was achieved by an elution gradient using an initial composition of 900 mL L^{-1} water (pH adjusted to 3.1 with 2 mL L^{-1} acetic acid) and 100 mL L^{-1} methanol. The concentration of the latter solvent was increased to 300 mL L^{-1} over 10 min and maintained for 20 min. Subsequently, the methanol concentration was increased to 400 mL L^{-1} over 10 min, maintained for 5 min, increased to 500 mL L^{-1} over 5 min, and maintained for another 5 min. Finally, the methanol percentage was increased to 600, 700, and 1000 mL L^{-1} in 5 min periods. Initial conditions were reached in 15 min. A flux of 1 mL min^{-1} and a temperature of 35 °C were also used. Oleuropein, hydroxytyrosol, and tyrosol were identified and quantified in fruit methanol extracts at 280 nm by using the external standard method.

The concentration of total phenols was determined colorimetrically at 725 nm using the Folin−Ciocalteau reagent and caffeic acid as standard following the method of Vázquez-Roncero et al. (*28*).

Table 2. Changes in Kinetic Parameters of PAL in Fruits of *Olea europaea* cv. Picual, Verdial, Arbequina, and Frantoio during Fruit Ripening[a]

	sample 1 (July)	sample 2 (Oct)	sample 3 (Nov)	sample 4 (Dec)
V_{max}[b]				
Picual	$14.98 \pm 1.03_a^x$	$15.50 \pm 0.55_a^x$	$13.22 \pm 1.23_a^x$	$4.21 \pm 0.35_b^x$
Verdial	$8.06 \pm 0.78_a^y$	$4.85 \pm 0.50_b^y$	$2.57 \pm 0.25_c^y$	$1.05 \pm 0.10_d^y$
Arbequina	$16.94 \pm 1.06_a^x$	$8.39 \pm 0.78_b^z$	$0.90 \pm 0.08_c^z$	$0.82 \pm 0.08_c^{yz}$
Frantoio	$10.68 \pm 0.96_a^y$	$24.48 \pm 1.86_b^w$	$1.08 \pm 0.09_c^z$	$0.73 \pm 0.06_d^z$
K_m[c]				
Picual	$0.26 \pm 0.02_a^x$	$0.30 \pm 0.03_a^x$	$0.26 \pm 0.02_a^x$	$0.23 \pm 0.02_a^x$
Verdial	$0.06 \pm 0.01_a^y$	$0.10 \pm 0.01_b^y$	$0.13 \pm 0.01_{bc}^y$	$0.18 \pm 0.02_c^x$
Arbequina	$0.12 \pm 0.01_a^z$	$0.17 \pm 0.01_b^z$	$0.21 \pm 0.02_{bc}^x$	$0.25 \pm 0.02_c^{xy}$
Frantoio	$0.13 \pm 0.01_a^z$	$0.18 \pm 0.01_b^z$	$0.20 \pm 0.02_b^x$	$0.32 \pm 0.02_c^y$
activity ratio[d]				
Picual	$0.22 \pm 0.02_a^x$	$0.13 \pm 0.01_b^x$	$0.25 \pm 0.02_a^x$	$0.13 \pm 0.01_b^x$
Verdial	$0.50 \pm 0.05_a^y$	$0.41 \pm 0.04_{ab}^y$	$0.37 \pm 0.04_b^y$	$0.24 \pm 0.02_c^y$
Arbequina	$0.24 \pm 0.02_a^x$	$0.12 \pm 0.01_b^x$	$0.22 \pm 0.02_a^x$	$0.40 \pm 0.04_c^z$
Frantoio	$0.43 \pm 0.04_a^y$	$0.32 \pm 0.03_{ab}^y$	$0.28 \pm 0.02_b^{xy}$	$0.27 \pm 0.02_b^y$
catalytic efficiency[e]				
Picual	$57.61 \pm 3.96_a^x$	$51.67 \pm 3.01_a^x$	$50.85 \pm 2.54_a^x$	$18.30 \pm 0.91_b^x$
Verdial	$134.33 \pm 6.71_a^y$	$48.50 \pm 3.91_b^x$	$19.77 \pm 0.99_c^y$	$5.83 \pm 0.29_d^y$
Arbequina	$141.17 \pm 10.73_a^y$	$49.35 \pm 3.56_b^x$	$4.29 \pm 0.21_c^z$	$3.28 \pm 0.16_d^y$
Frantoio	$82.15 \pm 6.12_a^z$	$136.00 \pm 9.91_b^y$	$5.40 \pm 0.27_c^z$	$2.28 \pm 0.11_d^z$

[a] Samples were harvested on four dates corresponding to different ripening status. Results are expressed as mean ± SEM of five values. [b] V_{max} is expressed as mU mg^{-1}. [c] K_m is expressed as mM. [d] Activity ratio is determined as the ratio $V_{subsaturating}/V_{max}$. The subsaturating concentration considered was 0.05 mM. [e] Catalytic efficiency is determined as the V_{max}:K_m ratio and is expressed as nmol min^{-1} mg^{-1} mM^{-1}. For each parameter, data in each row followed by different subindices (a, b, c, d) are statistically different ($p < 0.05$). For the comparison between varieties, data in each column followed by different superindices (x, y, z, w) are statistically different ($p < 0.05$).

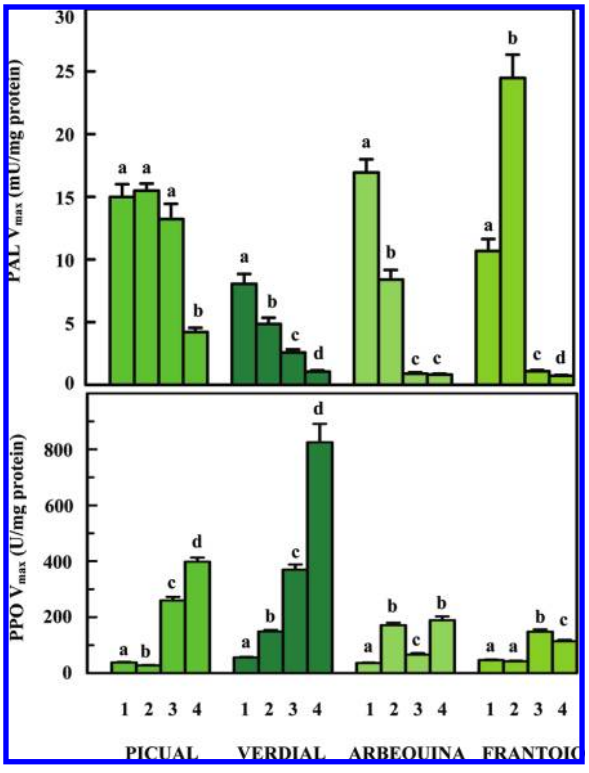

Figure 2. Changes in fruit phenylalanine ammonia-lyase (upper panel) and polyphenol oxidase (lower panel) V_{max} values of Picual, Verdial, Arbequina, and Frantoio varieties during maturation. Data are mean ± SEM of five values. For each variety, four samples, denoted 1−4, were harvested during the ripening process. Sample 1 was picked on July 31, 2002, sample 2 on Oct 10, 2002, sample 3 on Nov 1, 2002, and sample 4 on Dec 3, 2002. For each variety, columns with different letters are statistically different ($p < 0.05$). For PPO during ripening, V_{max} and time of maturation (t, days) are related by the following power equations: $V_{max} = 30.18t^{0.392}$, $r = 0.61$, Picual; $V_{max} = 54.94t^{0.464}$, $r = 0.87$, Verdial; $V_{max} = 36.77t^{0.303}$, $r = 0.80$, Arbequina; $V_{max} = 42.66t^{0.186}$, $r = 0.61$, Frantoio.

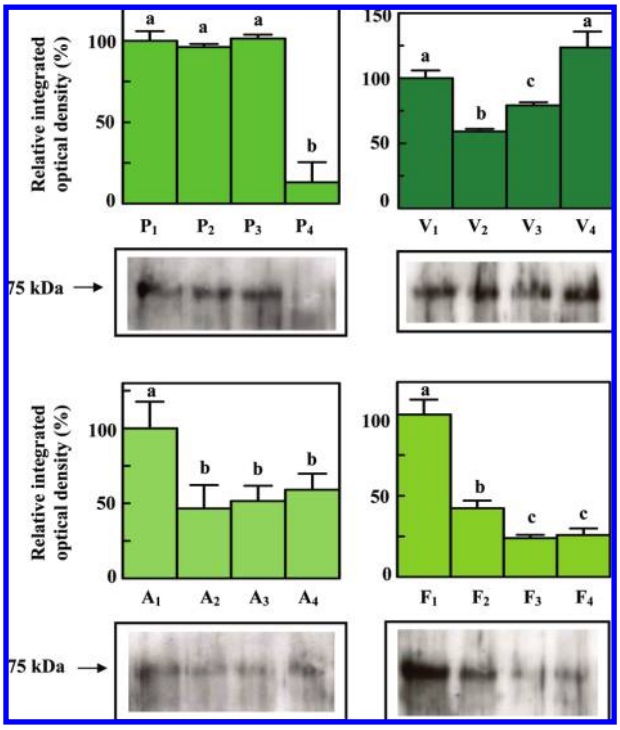

Figure 3. Western-blot analysis of phenylalanine ammonia-lyase (PAL) in fruits of *Olea europaea* cv. Picual (P), Verdial (V), Arbequina (A), and Frantoio (F) during fruit ripening: P_1, V_1, A_1, and F_1 give fruit samples of July; P_2, V_2, A_2, and F_2 give fruit samples of October; P_3, V_3, A_3, and F_3 give fruit samples of November; P_4, V_4, A_4, and F_4 give fruit samples of December. Proteins from extracts of fruits of different indices of ripeness were separated by 7.5% SDS-PAGE (6 μg per lane) and then blotted onto a polyvinylidene difluoride (PVDF) membrane. The 75 kDa polypeptide corresponding to a monomeric form of the enzyme was detected with specific polyclonal anti-PAL rabbit serum. The quantification of PAL levels by densitometric analysis is shown in bar graphics. The results are means ± SEM of five values and are expressed as arbitrary units of integrated optical density compared to sample number 1. Bars with different letters are significantly different ($p < 0.05$).

Table 3. Changes in Kinetic Parameters of PPO in Fruits of *Olea europaea* cv. Picual, Verdial, Arbequina, and Frantoio during Fruit Ripening[a]

	sample 1 (July)	sample 2 (Oct)	sample 3 (Nov)	sample 4 (Dec)
V_{max}[b]				
Picual	$38.31 \pm 1.94_a^x$	$27.19 \pm 1.38_b^x$	$259.91 \pm 12.17_c^x$	$397.94 \pm 15.11_d^x$
Verdial	$62.86 \pm 2.77_a^y$	$148.68 \pm 5.65_b^y$	$369.87 \pm 18.49_c^y$	$825.26 \pm 72.54_d^y$
Arbequina	$36.23 \pm 1.27_a^x$	$171.32 \pm 8.77_b^z$	$66.39 \pm 4.88_c^z$	$189.02 \pm 13.79_b^z$
Frantoio	$46.43 \pm 1.97_a^z$	$42.09 \pm 2.23_a^w$	$148.24 \pm 7.96_b^w$	$114.04 \pm 5.50_c^w$
K_m[c]				
Picual	$7.91 \pm 0.76_a^x$	$5.30 \pm 0.51_b^x$	$23.06 \pm 1.66_c^x$	$20.04 \pm 1.48_c^x$
Verdial	$97.68 \pm 6.59_a^y$	$63.61 \pm 2.74_b^y$	$9.55 \pm 0.48_c^y$	$15.36 \pm 0.76_d^y$
Arbequina	$13.38 \pm 1.33_a^z$	$17.23 \pm 0.86_b^z$	$4.75 \pm 0.43_c^z$	$5.64 \pm 0.67_c^z$
Frantoio	$12.02 \pm 0.74_a^z$	$4.30 \pm 0.40_b^x$	$4.63 \pm 0.36_b^z$	$6.57 \pm 0.39_c^z$
activity ratio[d]				
Picual	$0.28 \pm 0.01_a^x$	$0.31 \pm 0.02_a^x$	$0.08 \pm 0.02_b^x$	$0.20 \pm 0.03_c^x$
Verdial	$0.11 \pm 0.01_a^y$	$0.09 \pm 0.01_a^y$	$0.22 \pm 0.02_b^y$	$0.25 \pm 0.01_b^x$
Arbequina	$0.43 \pm 0.02_a^z$	$0.26 \pm 0.01_b^z$	$0.19 \pm 0.01_c^y$	$0.34 \pm 0.02_d^y$
Frantoio	$0.09 \pm 0.01_a^y$	$0.32 \pm 0.03_b^x$	$0.33 \pm 0.02_b^z$	$0.27 \pm 0.02_b^x$
catalytic efficiency[e]				
Picual	$4.84 \pm 0.24_a^x$	$5.13 \pm 0.31_a^x$	$11.27 \pm 0.45_b^x$	$19.86 \pm 1.00_c^x$
Verdial	$0.64 \pm 0.03_a^y$	$2.33 \pm 0.14_b^y$	$38.73 \pm 1.55_c^y$	$53.73 \pm 2.69_d^y$
Arbequina	$2.71 \pm 0.13_a^z$	$9.94 \pm 0.60_b^z$	$13.98 \pm 0.56_c^x$	$33.51 \pm 2.01_d^z$
Frantoio	$3.86 \pm 0.19a^w$	$9.79 \pm 0.58b^z$	$32.02 \pm 1.28_c^w$	$17.36 \pm 0.87_d^x$

[a] Samples were harvested on four dates corresponding to different ripening status. Results are expressed as mean ± SEM of five values. [b] V_{max} is expressed as units mg^{-1}. [c] K_m is expressed as mM. [d] Activity ratio is determined as the ratio $V_{subsaturating}/V_{max}$. The subsaturating concentration considered was 2.5 mM. [e] Catalytic efficiency is determined as the V_{max}:K_m ratio and is expressed as units mg^{-1} mM^{-1}. For each parameter, data in each row followed by different subindices (a, b, c, d) are statistically different ($p < 0.05$). For the comparison between varieties, data in each column followed by different superindices (x, y, z, w) are statistically different ($p < 0.05$).

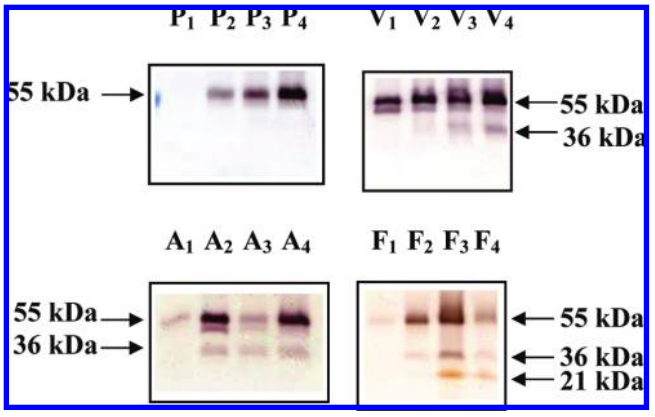

Figure 4. Partially denaturing SDS-PAGE followed by in-gel staining for polyphenol oxidase activity of native extracts of fruits of *Olea europaea* cv. Picual, Verdial, Arbequina, and Frantoio during different stages of ripening. For each variety, four samples, denoted 1−4, were harvested during the ripening process. Fruit extracts were dissolved in native buffer and separated by 10% SDS-PAGE at 4 °C. In each lane, 5.5 μg of protein was loaded. Gels were stained with DL-DOPA and catalase. The molecular mass of each band with PPO activity is indicated.

Statistical Analysis. The results are expressed as the mean ± standard error of the mean (SEM). Initially the data were analyzed by a one-way analysis of variance. The differences between means were analyzed using a Student's *t*-test. Potential correlations were determined by least-squares linear-regression analysis. The criterion of significance was taken as $p < 0.05$.

RESULTS

Fruit Growth and Ripening. The pattern of growth and ripening of the fruit of the Picual, Verdial, Arbequina, and Frantoio cultivars investigated in this study differed between varieties (**Table 1**, **Figure 1**). The weight gain of the fruits also differed between varieties (**Table 1**, **Figure 1**). In Arbequina and Frantoio the main weight gain occurred between stages 1 and 2. In Verdial, weight increased linearly throughout the ripening period. In Picual, the main weight increase occurred between stages 3 and 4. Verdial fruits showed the greatest weight, followed by Picual, Frantoio, and Arbequina. Frantoio was the variety that reached the highest IR (4.34 ± 0.22), followed by Verdial (3.91 ± 0.20), Picual (3.25 ± 0.16), and Arbequina (2.55 ± 0.13). Frantoio was the first variety to ripen (32 days for half IR), followed by Picual (46 days), Arbequina (48 days), and Verdial (49 days) (**Figure 1**). Arbequina fruits registered the lowest IR and mass at the end of the experiment. The water content of the fruits decreased during ripening, at the end of the maturation reaching values of close to 550 g kg dry weight $^{-1}$ for Picual, Arbequina, and Frantoio and 693.2 for Verdial (**Table 1**). In terms of total phenols, all varieties underwent a progressive fall during ripening. Between samples 1 and 2, this fall was more pronounced than during the rest of maturation. Throughout the experiment, Frantoio showed the highest values, followed by Picual, Arbequina, and Verdial (**Table 1**).

Kinetic Behavior and Protein Expression of PAL. PAL-specific activity was assayed in all fruit samples. The effect of substrate concentration on PAL-specific activity was studied, and the kinetic parameters were calculated (**Table 2**). In the fruits of the four varieties, hyperbolic kinetics were observed. In Picual, Verdial, and Arbequina varieties, the highest PAL activity values were recorded during the first stage of ripening. In Frantoio, the highest PAL activity was reached in the second stage of ripening studied. This value was the highest in the entire experiment. All varieties showed a significant decrease with maturation (**Table 2**, **Figure 2**). In the last two stages of ripening, the highest PAL-specific activity was found in the Picual variety.

The K_m values of PAL for L-Phe were different between varieties and during ripening. In Picual the K_m values were close to 0.26 mM in all ripening stages, in Verdial from 0.06 to 0.18 mM, in Arbequina from 0.12 to 0.25 mM, and in Frantoio from 0.13 to 0.32 mM. In the two first stages of ripening the K_m values in Verdial, Arbequina, and Frantoio were significantly lower than in Picual. In these varieties, a significant increment in the K_m values was found over the course of ripening.

Catalytic efficiency showed a trend similar to that of V_{max} (**Table 2**). The highest values were found in the first stages of

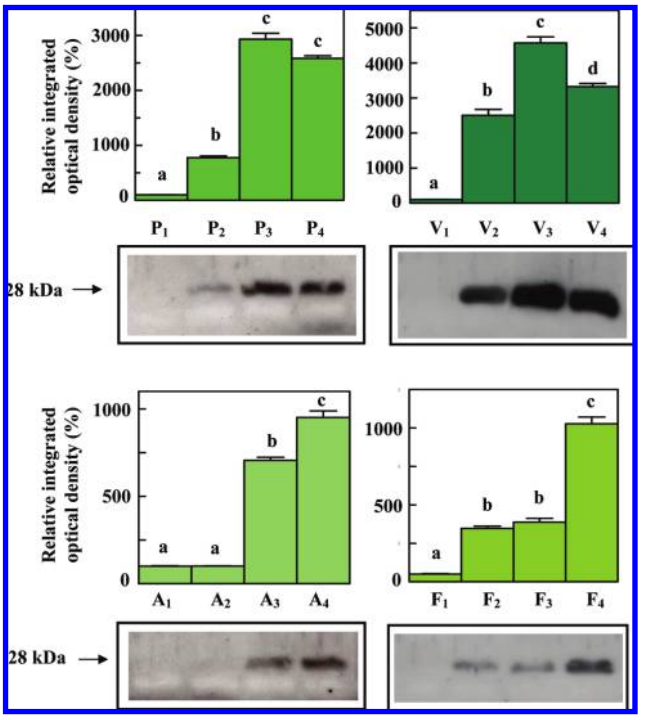

Figure 5. Western-blot analysis of polyphenol oxidase (PPO) in fruits of *Olea europaea* cv. Picual (P), Verdial (V), Arbequina (A), and Frantoio (F) during fruit ripening: P_1, V_1, A_1, and F_1 give fruit samples of July; P_2, V_2, A_2, and F_2 give fruit samples of October; P_3, V_3, A_3, and F_3 give fruit samples of November; P_4, V_4, A_4, and F_4 give fruit samples of December. Proteins from extracts of fruits of different ripeness indices were separated by 10% SDS-PAGE (7.2 μg per lane) and then blotted onto a polyvinylidene difluoride (PVDF) membrane. The 28 kDa polypeptide corresponding to a monomeric form of the enzyme was detected with specific polyclonal anti-PPO rabbit serum. The quantification of PPO levels by densitometry analysis is shown in bar graphics. The results are means ± SEM of five values and are expressed as arbitrary units of integrated optical density compared to sample number 1. Bars with different letters are significantly different ($p < 0.05$).

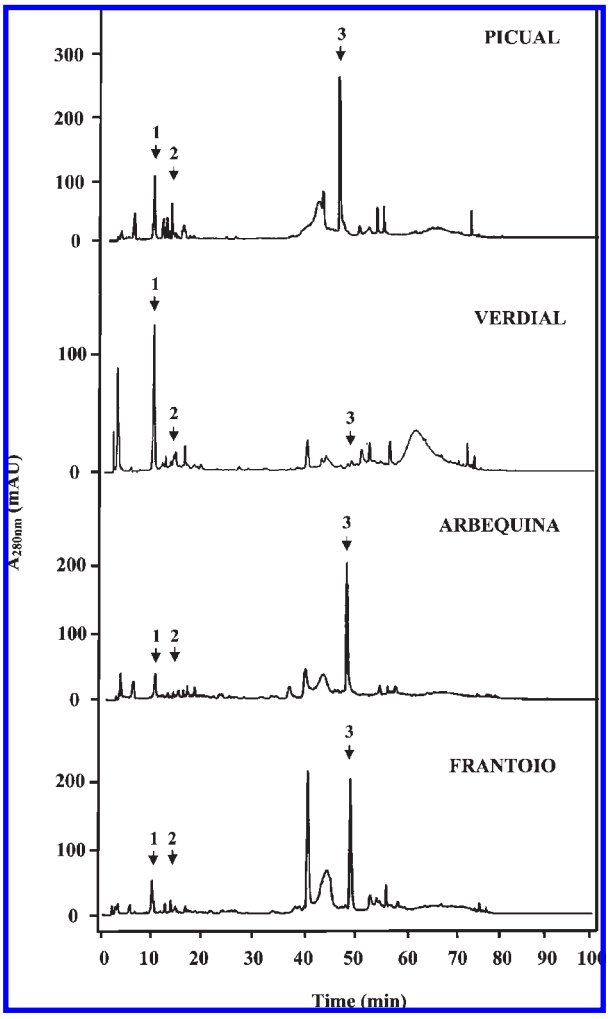

Figure 6. HPLC chromatograms at 280 nm of methanol extracts of olive fruits of *Olea europaea* cv. Picual, Verdial, Arbequina, and Frantoio. Samples of 20 μL methanol extracts of olive fruits were HPLC chromatographed in a Spherisorb ODS-2 column and eluted with a methanol−water gradient for 100 min. The peaks corresponding to hydroxytyrosol (1), tyrosol (2), and oleuropein (3) are marked on the chromatogram made at 280 nm. The chromatograms shown in this figure are representative of five independent determinations. The chromatograms for Picual, Arbequina, and Frantoio correspond with the sample picked on July 31, 2002, and that for Verdial corresponds with the sample picked on Dec 3, 2002.

ripening, and a sharp decrease occurred over ripening. In the last stage, the highest values were observed in Picual, followed by Verdial, Arbequina, and Frantoio.

The PAL protein content was determined by Western blot using a specific antiserum anti-PAL-1 of parsley with samples of fruits of the different varieties at different ripening stages (**Figure 3**). Only one 75 kDa immunoreactive polypeptide was detected in all the samples assayed. As found with the specific activity, as the ripeness progressed, the intensity of the immunoreactive band declined. In Picual, this decline occurred between stages 3 and 4. In Verdial, although a decrease was found between stages 1 and 2, in stages 3 and 4 the intensity of the band rose to reach the values found in stage 1. These results demonstrate that, in general, throughout ripening, the amount of immunoreactive PAL protein decreased.

Kinetic Behavior and Protein Expression of PPO. PPO-specific activity was assayed using catechol as a substrate in all fruit samples. Hyperbolic kinetics appeared in all cases (**Table 3**). In all the ripening states, Verdial fruits showed the highest PPO specific activities, followed by Picual, Arbequina, and Frantoio, respectively. In contrast to PAL-specific activity, throughout the ripening, a significant and potential increase in PPO-specific activity was registered (**Figure 2**). The increment was higher in Verdial, in which the PPO-specific activity at stage 4 was 12-fold higher than at stage 1. In Picual, Arbequina, and Frantoio the PPO activity at stage 4 was 9-, 4-, and 1.5-fold higher than in stage 1, respectively.

Significant differences were shown between the K_m values for catechol in the four varieties during ripening (**Table 3**). Picual showed a value of 7.91 mM at stage 1 that changed to 20.04 mM at stage 4. Verdial showed a value of 97.68 mM at stage 1 that changed to 15.36 at stage 4. Arbequina registered 13.38 mM at stage 1 that decreased to 5.64 mM at stage 4. For Frantoio, a value of 12.02 mM at stage 1 fell to 6.57 mM at stage 4. Picual behaved differently with respect to Verdial, Arbequina, and Frantoio during ripening; in Picual the K_m values significantly rose, while in the rest of the varieties the K_m values dipped. This indicates that in Picual the affinity of PPO for its substrates diminishes during ripening, while in Verdial, Arbequina, and Frantoio this affinity is augmented.

PPO activity, following partially denaturing SDS-PAGE, was assayed in all fruit samples (**Figure 4**). After electrophoretic

Table 4. Time Course of the Concentration of Hydroxytyrosol, Tyrosol and Oleuropein in Fruits of *Olea europaea* cv Picual, Verdial, Arbequina, and Frantoio during Fruit Ripening[a]

	sample 1 (July)	sample 2 (Oct)	sample 3 (Nov)	sample 4 (Dec)
hydroxytyrosol (g kg dry mass^{-1})				
Picual	$3.26 \pm 0.04_a{}^x$	$3.65 \pm 0.03_a{}^x$	$1.04 \pm 0.01_b{}^x$	$0.34 \pm 0.01_c{}^x$
Verdial	$0.63 \pm 0.01_a{}^y$	$1.40 \pm 0.01_b{}^y$	$1.75 \pm 0.04_c{}^y$	$2.03 \pm 0.01_d{}^y$
Arbequina	$1.51 \pm 0.04_a{}^z$	$1.01 \pm 0.04_b{}^z$	$0.62 \pm 0.03_c{}^z$	$0.62 \pm 0.05_c{}^z$
Frantoio	$2.01 \pm 0.01_a{}^w$	$1.88 \pm 0.02_b{}^w$	$2.41 \pm 0.06_c{}^w$	$1.11 \pm 0.05_d{}^w$
tyrosol (g kg dry mass^{-1})				
Picual	$1.61 \pm 0.12_a{}^x$	$0.87 \pm 0.05_b{}^x$	$0.35 \pm 0.02_c{}^x$	$0.59 \pm 0.01_d{}^x$
Verdial	$0.59 \pm 0.04_a{}^y$	$0.08 \pm 0.01_b{}^y$	$0.11 \pm 0.01_b{}^y$	$0.10 \pm 0.01_b{}^y$
Arbequina	$0.25 \pm 0.04_a{}^z$	$0.43 \pm 0.07_b{}^z$	$0.39 \pm 0.01_b{}^x$	$0.15 \pm 0.06_c{}^y$
Frantoio	$0.54 \pm 0.03_a{}^y$	$0.15 \pm 0.02_b{}^w$	$0.17 \pm 0.03_b{}^y$	$0.12 \pm 0.03_b{}^y$
oleuropein (g kg dry mass^{-1})				
Picual	$36.17 \pm 3.03_a{}^x$	$17.49 \pm 0.19_b{}^x$	$6.53 \pm 0.20_c{}^x$	$7.37 \pm 0.12_d{}^x$
Verdial	$1.32 \pm 0.03_a{}^y$	$1.19 \pm 0.03_a{}^y$	$0.46 \pm 0.02_b{}^y$	$0.63 \pm 0.13_b{}^y$
Arbequina	$29.53 \pm 3.35_a{}^x$	$4.22 \pm 1.33_b{}^z$	$0.90 \pm 0.02_c{}^z$	$1.56 \pm 0.19_c{}^z$
Frantoio	$33.47 \pm 0.59_a{}^x$	$2.27 \pm 0.08_b{}^z$	$2.25 \pm 0.18_b{}^w$	$1.42 \pm 0.06_c{}^z$

[a] Samples were harvested on four dates corresponding to different ripening states. Results are expressed as means ± SEM of five values. Results were analysed by a two-way ANOVA and Student's *t*-test. For each compound, data in each row followed by different sub-indices (a, b, c, d) are statistically different ($p < 0.05$). For the comparison between varieties, data in each column followed by different super-indices (x, y, z, w) are statistically different ($p < 0.05$).

separation of polypeptides from fruit samples and in-gel staining with DL-DOPA, one (Picual), two (Verdial and Arbequina), or three (Frantoio) bands with PPO activity appeared. The apparent molecular masses of these proteins were 55, 36, and 21 kDa. In the Picual variety, one 55 kDa PPO band was found, this increasing as ripening progressed. In the Verdial and Arbequina varieties, two bands of 58 and 36 kDa were found, respectively. The 36 kDa band was detected at the stages with higher PPO activity that in most cases coincided with the last stages of ripening. In the Frantoio variety, in addition to the 56 and 36 kDa bands, a new 21 kDa band with PPO activity appeared at the stages with higher PPO activity (stages 3 and 4). The PPO activity level rose throughout ripening, coinciding with the increase previously described in the PPO V_{max}. In parallel with this determination, gels under identical conditions were stained for POD activity. No POD or catalase activity was detected in the 55, 36, and 21 kDa protein bands.

A single band of 28 kDa was detected in the most of samples assayed by SDS-PAGE under denaturing conditions and immunoblotting (**Figure 5**). The intensity of this immunoreactive band significantly increased from stage 1 to stage 4 in all the varieties. In the Verdial and Picual varieties, the highest immunoreactive level was found at stage 3, while in the Arbequina and Frantoio varieties the peak occurred at stage 4. Coinciding with the relative PPO specific-activity level between varieties, the highest PPO protein level was detected in the Verdial variety, followed by Picual, Arbequina, and Frantoio (**Figure 5**).

Concentrations of Oleuropein, Hydroxytyrosol, and Tyrosol. Samples of phenolic extracts of fruits of Picual, Verdial, Arbequina, and Frantoio varieties were analyzed by HPLC to determine the concentrations of oleuropein, hydroxytyrosol, and tyrosol during ripening (**Figure 6**). Oleuropein, hydroxytyrosol, and tyrosol were identified with retention times of 49.37 ± 0.28, 10.01 ± 0.06, and 13.81 ± 0.13 min, respectively. Picual fruits had the highest oleuropein concentration at all stages of ripening (**Table 4**). The oleuropein concentration in Picual during the last stage of ripening was 3.74-, 4.1- and 10.7-fold higher than in Arbequina, Frantoio, and Verdial varieties, respectively. In all cases, the oleuropein concentration significantly decreased throughout ripening.

With respect to the hydroxytyrosol concentration, significant differences appeared between varieties, depending on the ripening stage considered. In samples 1 and 2, the highest concentration was found in Picual, followed by Frantoio, Arbequina, and Verdial. Throughout ripening, the hydroxytyrosol concentration significantly declined in Picual, Arbequina, and Frantoio but increased in Verdial. Thus, at the end of the experiment, Verdial had the highest hydroxytyrosol concentration (2.03 g kg^{-1}), followed by Frantoio (1.11 g kg^{-1}), Arbequina (0.62 g kg^{-1}), and Picual (0.34 g kg^{-1}).

DISCUSSION

As the olive ripens, major changes occur in the fruit, changes that affect the pulp to stone ratio, color, and the chemical composition (including phenolic compounds). In this study, investigating four varieties that produce different types of oil, we found that each olive variety showed a different pattern of fruit growth and ripening in an experiment in which the agronomical and environmental conditions were very similar between cultivars. Also, each variety had different concentrations of total phenols in the fruit. The growth pattern and phenolic concentration appear to be determined by specific genetic or metabolic conditions typical of each variety. The specific pattern of fruit growth and ripening appears to determine the most appropriate date to harvest the crop in each variety because the state of the fruit at harvest influences the chemical composition and sensory properties of the olive oil (*29*). Hence, Frantoio and Picual are two varieties in which the harvest could begin 1 month earlier than in this experiment because the IR and phenol concentration maxima are reached by that time.

In our study, the phenol concentrations significantly declined over the ripening period in all four varieties. This trend is consistent with findings by Tovar et al. (*30*) and Gómez-Rico et al. (*31*) and agrees with a decrease in the importance of these compounds in the metabolism of olive fruit over the ripening period. Also, this decline and the lower PAL activity found in all the varieties suggest the biosynthesis of these compounds diminishes over ripening.

The results found in the present work demonstrate the existence of a protein with PAL activity in the fruit of the Picual, Verdial, Arbequina, and Frantoio varieties that showed hyperbolic kinetics. The V_{max} values differed between varieties and during ripening, although in all cases they presented a generally similar pattern with a significant decrease over the ripening period. This decrease agrees qualitatively with the decline in the concentration

of total phenols previously mentioned and may indicate that the two are related. These results are consistent with our previous findings in Picual (*24*) and with reports by other authors in the varieties Arbequina, Farga, and Morrut (*22*, *30*). In these works, coinciding with the decrease in phenol concentrations, lower PAL activity at saturate−substrate concentration was also reported during ripening. Our results indicate that the high PAL-specific activity was found before veraison, the stage in which the color of olive fruit begins to change from yellow-green to black. At this stage the synthesis of phenylpropanoids is high. One group of phenylpropanoids are anthocyanins, the most important water-soluble pigments in plants. After the accumulation of anthocyanins, the fruit color changes to purple, violet, and black. The different levels of PAL-specific activity found in fruits of different varieties indicate that a different level of phenylpropanoid biosynthesis exists in the four varieties. The immunoreactive PAL protein showed a molecular mass of 75 kDa under denaturing conditions. This agrees with previous findings in olive and in other plants (*24*). Significant differences in the K_m values of the different varieties and over ripening were also found. In the Picual variety the values were close to 0.26 mM, and no significant changes were found over ripening. In Verdial, Arbequina, and Frantoio at stages 1 and 2, the K_m values were lower than in Picual and increased during ripening to reach values similar to those registered by Picual. In Verdial, Arbequina, and Frantoio, the affinity of the enzyme and the catalytic efficiency by the substrate declined with an advance in ripening. This indicates that a different regulation mechanism occurs in the different varieties during ripening. In Picual the changes in activity during ripening appear to be triggered by a decrease in the PAL protein-expression level. In the rest of the varieties, in addition to a change in the number of PAL molecules present, a change in the catalytic efficiency or in isoenzyme expression may also occur.

Previously, we reported the molecular characterization of PPO in the fruit and leaf of Picual (*23*). Segovia-Bravo et al. (*32*) showed a kinetic, thermodynamic, and isokinetic study of the reaction catalyzed by PPO from the Manzanilla cultivar. In this new work, we reported the kinetic behavior and protein expression of PPO in fruits of Verdial, Arbequina, and Frantoio during ripening compared with Picual. PPO catalyzes reactions in which *o*-diphenols are oxidized to *o*-diquinones with the incorporation of oxygen molecules. In the past, PPO activity has been related to the browning that accompanied fruit maturation. A significant increment in the specific activity and in the V_{max} was observed in the four varieties over ripening. This increment was highest in Verdial, followed by Picual, Arbequina, and Frantoio. In parallel with this increase was a significant decrease in the K_m value for catechol in Verdial, Arbequina, and Frantoio, while in Picual K_m increased throughout the ripening period. During the last stages of fruit ripening, new 36 and 21 kDa PPO protein was detected in fruits. Under denaturing conditions, PPO showed a molecular mass of 28 kDa in the four varieties. The PPO protein-expression level significantly increased with ripening. The PPO-specific activity and protein levels in the fruit differed between varieties and during ripening, although in all cases the pattern was in general the same. Significant differences between the K_m values of the varieties were also reported. In Verdial, Arbequina, and Frantoio, the K_m values significantly decrease during ripening, whereas in Picual K_m increased. This indicates that an increment in the affinity for its substrate occurs in Verdial, Arbequina, and Frantoio as ripening progresses, whereas in Picual it decreases. These changes in the affinity are parallel with the changes found in the level of expression of this enzyme, and both in conjunction can explain the increase in the catalytic efficiency also found during ripening.

As the fruit ripens, the rise in PPO activity indicates a significant increment in the oxidative reactions of phenols. In this work, we found that although the PPO protein-expression level rose during ripening, no direct relationship appeared between the PPO-specific activity and protein-expression level and the maximum index of ripening reached by the different varieties of olive tree. Thus, Frantoio was the variety that had the highest ripening index and the lowest PPO-specific activity and protein-expression level, Verdial, a variety that usually has a lower ripening index, showed the highest PPO-specific activity and protein-expression level, Arbequina, with the lowest IR, had a PPO-protein expression level similar to that of Frantoio, and Picual showed a PPO protein-expression level higher than that of Arbequina and Frantoio but lower than that of Verdial. This signifies that the PPO-specific activity is not the only element that determines the browning level reached by the olive fruit. Rather, other elements must be involved in the browning reactions responsible for the ripening index of the olive fruit. In this sense, it has been shown (*33*, *34*) that, under different stress conditions, a browning process occurred as products of the reaction catalyzed by PPO are accumulated. PPO is involved in the browning reaction in the fruits, although it may not be the main cause of the browning. Moreover, the browning reaction in olives due to polyphenol oxidation may have an enzymatic and/or nonenzymatic origin (*35*). At alkaline pH values, it is probable that a percentage of the browning reaction observed on bruised fruits was due to a nonenzymatic reaction (*32*). This may be a second element involved in the browning reaction coupled with ripening.

Our results show a possible relationship between PAL, PPO, and total phenols in the varieties Picual, Arbequina, Verdial, and Frantoio throughout ripening. The levels of PAL and PPO seem to be coordinated in the different varieties during ripening. Also, this is related to the concentration of total phenols in each variety. This affirmation is based on the fact that Frantoio was the variety with the highest concentration of total phenols, the highest PAL-specific activity, and the lowest PPO activity. Verdial registered the lowest concentration of total phenols, the lowest PAL-specific activity, and the highest PPO activity and protein level. Picual and Arbequina showed medium levels of PAL and PPO activity and total phenols. Thus, it appears that the biosynthesis and oxidation of phenolic compounds, regulated by PAL and PPO, were involved in the regulation of the levels of phenolic compounds in the different varieties.

The analyses of the chromatographic profile of phenolic fraction in the fruit during ripening in the varieties Picual, Verdial, Arbequina, and Frantoio indicate major differences. These differences can be used to distinguish one variety from the other, indicating a distinct phenol metabolism in the fruit of the four varieties. Verdial was the only variety in which the concentration of hydroxytyrosol increased over ripening. At harvest, Verdial registered the highest hydroxytyrosol level of the four varieties. Picual, the main variety for olive-oil production in Spain, showed the highest oleuropein concentration. For this compound, our results in Verdial, Arbequina, and Frantoio show levels similar to those described by Esti et al. (*18*) and Romani et al. (*19*) for several Italian varieties, with the exception of the values found in Picual. The values found in our work for Picual are significantly higher than those reported by Esti et al. (*18*) and Romani et al. (*19*) and are consistent with those of Gomez-Rico et al. (*31*) for the last stages of ripening. Tyrosol is present in lower amounts than hydroxytyrosol, as previously described by Romani et al. (*19*), with the exception of Picual in the last stage of ripening. Although the decrease in the oleuropein concentration throughout ripening has previously been described (*21*), our results indicate marked differences between varieties, leading to major dissimilarities in

the last stages of ripening, which afterward are reflected in the oil derived from the fruits of the different varieties. The high oleuropein concentration found in Picual fruits is responsible for the bitter taste of olive oil obtained from this variety and the high stability against oxidation of this type of oil. Also, this oil offers excellent nutritional quality due to its high concentration of antioxidant phenolic compounds.

ABBREVIATIONS USED

PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase; K_m, Michaelis constant; V_{max}, maximum velocity; L-Phe, L-phenylalanine; IR, index of ripeness; SDS-PAGE, polyacrylamide-gel electrophoresis with sodium dodecyl sulfate; PVDF, polyvinylidenedifluoride; TBS, Tris buffer solution; TBS-T, Tris buffer solution-Tween20; BSA, bovine serum albumin; TBS-T-BSA, Tris buffer solution-Tween20-bovine serum albumin; IgG, immunoglobulin G; PBS, phosphate buffer solution; NGS, normal goat serum; HPLC, high-performance liquid chromatography; UV−visible, ultraviolet−visible.

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