**Changes in glutathione, ascorbate and antioxidant enzymes during
olive fruit ripening**

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ABSTRACT

The content of glutathione, ascorbate (ASC) and the enzymatic antioxidants superoxide dismutase, catalase and components of the ascorbate-glutathione cycle were investigated in olive fruit (cv. Picual) selected at the green, turning and mature ripening stages. The changes observed in total and reduced glutathione (GSH), oxidised glutathione (GSSG), the ratio GSH/GSSG, ASC and antioxidant enzymes (mainly superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase) indicate a shift to a moderate cellular oxidative status during ripening and suggest a role for antioxidants in the process. The antioxidant composition of olive oils obtained from the olive fruits of the study was investigated. A model is proposed for the recycling of antioxidant polyphenols mediated by endogenous molecular antioxidants in olive fruit.

Keywords: Antioxidant, olive (*Olea europaea*), ripening.

INTRODUCTION

The generation of reactive oxygen species (ROS) is a consequence of aerobic metabolism in plant and animal cells. Some ROS are free radicals like superoxide ($O_2^{\cdot-}$), hydroxyl ($\cdot OH$), peroxy ($RO_2^{\cdot-}$) and hydroperoxyl ($HO_2^{\cdot-}$), whilst others are non-radicals like hydrogen peroxide (H_2O_2), ozone (O_3) and singlet oxygen ($^1O_2$). Although increased production of ROS originates oxidative stress and damage to molecules such as lipids, proteins and DNA, ROS can also act as signalling molecules to induce synthesis or degradation of genes and proteins related to biotic and abiotic stress and are also involved in plant development and ripening.^{1,2} Indeed, fruit ripening has been described as an oxidative process genetically regulated in which production and scavenging of ROS take place. The production of ROS is controlled by antioxidants, so they play an important role in the ripening process.³⁻⁵

There are two categories of antioxidants: enzymatic and low-molecular weight-antioxidants. Among the enzymatic antioxidants, superoxide dismutase (SOD), catalase and the enzymes of the ascorbate-glutathione cycle (AGC), including ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR), constitute a first line of defence against ROS.^{1,6} Dehydrogenases like glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), isocitrate dehydrogenase (ICDH) and malic enzyme (ME) generate the necessary supply of NADPH to maintain the AGC.⁷ Low-molecular weight antioxidants, including ascorbate (ASC), glutathione (both key molecules of the AGC), carotenoids, tocopherols and polyphenols, are also important for the control of ROS. ASC is one of the strongest antioxidants, prominently present in fruit and vegetables which content varies considerably between species.^{8,9} Besides participating as the first electron donor in the AGC, it is involved in the direct

scavenging of ROS, the regeneration of the lipophilic molecular antioxidant α -tocopherol and is also involved in many plant metabolic reactions.¹ Reduced glutathione (GSH), a component of the AGC, is a main cellular antioxidant. GSH is oxidised to GSSG by ROS as part of the antioxidant barrier that prevents excessive oxidation of key cellular components. GSSG is rapidly recycled to GSH by GR with the use of NADPH. GSH is also the principal cellular thiol and is involved in synthesis, redox turnover, metabolism and cell signalling.⁶ Tocopherols detoxify lipid peroxides preventing lipid peroxidation and oxidation of fatty acids. Apart from the very efficient ROS scavenging activities, the water-soluble antioxidants ASC and glutathione and the lipophilic antioxidant α -tocopherol act in conjunction and are interconnected.¹

Olive trees (*Olea europea*) and the commercialisation of olive oil and olives has a great impact on the economy of Mediterranean countries like Spain, Italy and Greece. The study of olive ripening is of great interest, because the ripening stage of the fruit influences olive oil quality and extraction yield and therefore the production of olive oil and table olives. Olive maturation is a very complex process that involves mesocarp development, fruit softening, change of texture, decrease of carbohydrates and increase in oil synthesis.¹⁰ Olives are very rich in phenolic compounds which possess strong antioxidant activity and metal quelating properties.¹¹ Oleuropein and hydroxytyrosol (HT) are the main phenolic compounds in olive pulp.¹²⁻¹⁴ The phenolic content and composition of olive fruit can be strongly affected by several agronomic parameters including the variety of olive and the stage of ripening.^{12,15}

The response of glutathione, ASC and of the antioxidant enzymes during the process of olive fruit ripening has been poorly studied, if at all. In our previous work, we reported for the first time the presence of glutathione in mature olives. We also characterised the enzymatic antioxidants SOD, CAT, enzymes of the AGC and

NADPH-generating dehydrogenases.¹⁶ In this study we investigated how glutathione, ASC and antioxidant enzymes were influenced by ripening in the Picual variety of olives, one of the most widely used in Spain for the production of olive oil.¹⁷ The profile of polyphenols in oils extracted from olive fruits at the three ripening stages was also followed and an interactive model among the low-molecular weight antioxidants is proposed.

MATERIALS AND METHODS

Plant material.

“Olive fruits (*Olea europea* L.) from the Picual variety were obtained from the Experimental Orchard of the “Instituto de Investigación y Formación Agraria y Pesquera (IFAPA)”, Centro “Venta de Llano” located in Mengibar (province of Jaén), Spain, 37° 56' 27" N, 03° 47' 15" W, 293 m above sea level. The 25-year old olive trees were grown using traditional techniques and under irrigation in silty clay soil, well-aerated, well-drained, with no evidence of soil erosion. The study was carried out during the 2014/2015 crop season. The general climate of this area is Mediterranean-subcontinental with cold winters and hot summers. The average temperature and humidity values (and ranges) registered at the weather station located on the site [<https://www.juntadeandalucia.es/agriculturaypesca/ifapa/riaweb/web/estacion/23/104>] were 17.6°C (range 31.6- 0.3°C) and 62.4% (range 99.9-23.1%) for the year 2014, 19.0°C (range 23.5- 13.8 °C) and 64.84% (range 90.4-46.1%) for October 2014 and 12.8°C (range 16.6- 8.8°C) and 82.6% (range 96.9-59.9%) for November 2014, respectively.

About 2 Kg of healthy olive fruit samples were hand-picked from three different trees of the above variety at different stages of ripening according to the scale and method

described in ¹⁸. The ripening index (RI) scale used classifies olives with regards to fruit colour in both skin and pulp and goes from 0 to 7, with 0 being the green stage and 7 the end of olive maturation (black epidermis, dark purple mesocarp and endocarp). Olive samples were harvested from the trees at three stages: the yellow-green phase of maturation (green olives, RI 1, date of harvest 1/10), at the end of turning phase (red or purple skin in more than half of the olive surface, RI 3, date of harvest 20/10) and at the beginning of the maturation stage (black skin and purple mesocarp, RI 6, date of harvest 10/11). The olives were thoroughly washed with distilled water and dried before use.

Extraction and determination of glutathione and ascorbate from olives.

Olive skin and pulp were obtained with a scalpel and the fragments were immediately frozen in a mortar containing liquid nitrogen. The tissues were ground in the mortar with a pestle until a very fine powder was obtained. 0.4 g of olive powder was transferred to an ice cold tube then 7 mL of cold 0.1 M of HCl were added. The tube was shaken vigorously for 1 minute and then spun at 5500 x g for 20 min at 4°C. The supernatant was then transferred to fresh tubes and spun again. The supernatant was loaded into Oasis MAX 3 cc/60 mg solid phase extraction cartridges (Waters, Milford, USA) as indicated by the manufacturer's instructions. Samples were eluted with 2 mL of 5% (v/v) NH₄OH and filtered through 0.45 µm nylon filters before analysis. To avoid degradation of the analytes, all procedures were carried out in darkness and at 4°C.

Analysis and quantification of GSH, GSSG, and ASC was performed by liquid chromatography-electrospray/mass spectrometry (LC-ES/MS), using an Allience 2695 Separation module connected to a Quattro Micro triple quadrupole mass spectrometer detector from Waters, as described in ¹⁹. GSH, GSSG and ASC standards were purchased from Fluka Chemical Corp. Stock solutions of each standard were prepared at a concentration of 500 mg/L from which serial dilutions were obtained for analysis.

The injection volume in the LC-ES/MS was 10 μ L of the sample. All the samples were analysed in triplicate, and the concentration of the molecules present in them was established by calculating the area of each individual compound and by interpolation on its corresponding calibration curve. Solutions of pure standards in a concentration range of 0.25-200 mg/Kg were used for quantification. The analysis data are expressed as means $\pm$ standard deviation (SD).

Protein extracts and enzymatic activities from olive fruits.

Proteins from olive fruits were extracted as described in ¹⁶. Briefly, seedless olive fruits (10 g) were ground to a very fine powder in a mortar with liquid nitrogen. The powder was added to an ice-cold beaker containing 80 mL (1/8; w/v) of extraction buffer consisting of 100 mM Tris-HCl buffer pH 8.0, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 7% (w/v) PVPP, 15 mM DTT and 0.8 mL of protease inhibitor cocktail (P9599, Sigma, USA). The mixture was left on ice for 1 minute and then a manual blender was applied for 30 seconds to improve protein extraction. In the cold room, the mix was filtered through two layers of cloth and centrifuged at 20,000 g for 30 min at 2°C. The supernatant contained a thick upper layer of lipids (solid at this temperature) that was carefully removed. The remaining supernatant was collected with a plastic pipette, transferred to fresh tubes and centrifuged again. The supernatants were then purified by gel-filtration through Sephadex G-25 PD-10 prepacked columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) pre-equilibrated with 10 mM sodium-phosphate buffer, pH 6.8, at 4°C. Two gel-filtration steps were made. Samples were finally eluted with 10 mM potassium-phosphate buffer, pH 7.8, for enzyme activity determinations or with 10 mM Tris-HCl buffer, pH 7.8, for electrophoresis and protein gel blot analysis. The total superoxide dismutase activity (SOD; EC 1.15.1.1) was assayed according to the ferricytochrome *c* method of McCord and Fridovich.²⁰ Catalase activity (EC

1.11.1.6) was determined by measuring the disappearance of H₂O₂, as described by Aebi.²¹ Ascorbate peroxidase (APX; EC 1.11.1.11) was determined from the decrease in A290 due to the ascorbate oxidation by H₂O₂ according to the method of Hossain and Asada.²² Glutathione reductase (GR; EC 1.6.4.2) activity was assayed by recording the NADPH oxidation, as described by Jiménez et al.²³ The NADPH-generating enzymes glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44), NADP-isocitrate dehydrogenase (ICDH; EC 1.1.1.42) and NADP-malic enzyme (ME; EC 1.1.1.40) were assayed according to Corpas et al.⁷ The protein concentration in samples was determined by the Bradford method²⁴, using bovine serum albumin as standard.

Electrophoresis and protein gel blot analysis.

Olive fruit protein extracts were separated by standard SDS-PAGE on 4–20% gradient precast Mini-Protean TGX gels and transferred to PVDF membranes using specific PVDF- transfer packs for Trans-Blot Turbo Transfer System, all supplied by Biorad®. After protein transfer, membranes were used for cross-reactivity assays with polyclonal antibodies against the following proteins: catalase obtained from *Arabidopsis thaliana* (Agrisera, ref AS09 501), Mn-SOD from pea (*Pisum sativum* L.) leaves, Cu,Zn-SOD from watermelon (*Citrullus vulgaris* Schrad.) cotyledons and Fe-SOD from synthetic peptides were obtained as described in¹⁶. GR was detected by using an antibody raised against GR from maize (*Zea mays*) purchased from Agrisera (ref. No. AS 06181). Ascorbate peroxidase protein was detected using antibodies raised against *Arabidopsis thaliana* APX obtained from Agrisera (ref. No. AS06 181) and dehydroascorbate reductase (DHAR) was detected using against *Arabidopsis thaliana* DHAR1 also obtained from Agrisera (ref. No. AS11 1746).

Extraction of olive oil and determination of analytical indices.

The extraction of olive oil was carried out within 24 hours after the harvest of the olives with a laboratory scale olive oil extraction system (Abencor, MC2 Ingeniería y Sistemas SL, Spain). The extracted oils were stored at -20° C for further analysis. Free fatty acids, peroxide value and ultraviolet spectrophotometric parameters were determined as indicated in Commission Regulation No. 2568/91 of the European Union. Oxidative stability index was evaluated using a Rancimat device (Metrohm CH 9100) by measuring the time required to induce oxidation of the oil as described in ²⁵. All parameters were analysed in triplicate for each olive oil sample by the olive oil producing company Deoleo SA (Spain), with certified quality which follows UNE-EN-ISO 9001 directives.

Quantification of olive oil polyphenols and tocopherols

Polyphenols were extracted from olive oils following the method of Montedoro et al. ¹¹ Briefly, 5 g of virgin olive oil were dissolved in 5 ml of hexane. The mixture was extracted with 4 x 10 mL of methanol:water (80:20%, v/v). The methanol/water extracts were concentrated in vacuum under nitrogen and the residue was suspended with 10 mL of acetonitrile. The mixture was then washed three times with 20 mL of hexane and the final acetonitrile solution was evaporated. The remaining residue was dissolved with 1 mL of 100% methanol and filtered through a 0.2 µm filter before use. Analysis of phenolic compounds in olive oils was carried out by high performance liquid chromatography with diode array detector (HPLC-DAD) with a C₁₈ column (5 µm particle size) obtained from Phenomenex (UK) and HPLC-MS/MS, using the method described in ²⁶. All the samples were analysed in triplicate, and the concentration of phenols was determined by calculating the area of each individual compound and by interpolation on its corresponding calibration curve. Solutions of pure standards in a concentration range of 0.1-300 mg/Kg were used for quantification.

Tocopherol content was measured in olive oils by HPLC following the IUPAC Standard Method 2432.²⁷ Tocopherols β and γ were quantified together. The results were expressed as mg of tocopherol per kg of oil. Polyphenols and tocopherols in olive oils were measured by Deoleo SA (Spain). The analysis data were expressed as means $\pm$ standard deviation

Chemicals

All solvents were of HPLC-grade (Panreac Quimica, Spain). The water used was of 18 M Ω ·cm quality produced by a Milli-Q integral Water Purification System (Millipore). GSH, GSSG and ascorbic acid were purchased from Fluka Chemical Corp. Phenolic standards, HT, tyrosol, oleuropein, apigenin and luteolin were purchased from Extrasynthese (Genay, France). *p*-coumaric acid was acquired from Sigma-Aldrich.

Statistical analysis

Data treatment was carried out with Microsoft Excel 2010 (Microsoft Corp, USA). All the data is expressed as means $\pm$ standard deviation. Normality was assessed by the Kolmogorov-Smirnov test. The effect of maturation was assessed by one-way repeated-measures ANOVA followed by Tamhane's T2 posthoc test for not assumed equal variances. *P* values < 0.05 were considered significant. SPSS statistical software version 23.0 was used to analyse the data (SPSS, Chicago, USA).

RESULTS

Analysis of GSH/GSSG in olive fruits showed that GSH was the main form of glutathione in the three maturation stages of our study. The concentration of GSH significantly increased by 5 times from the green stage until the mature stage of ripening and the concentration of GSSG also increased significantly by about 10 times. The glutathione redox state defined as the ratio [GSH]/[GSSG] was high in the green

phase (>20) but decreased sharply at the turning and mature stages (Table 1). Analysis of ascorbate in olives showed that the concentration remained constant at the green and turning phases but the levels increased significantly at the mature phase by 2.5 fold.

Activities of CAT, SOD, GR, APX and four NADPH-generating dehydrogenases were determined in olive extracts at the three ripening stages of the study (Table 2). The CAT activity decreased gradually from the green to the mature stage. Total SOD and GR showed a modest increase at the turning stage of ripening compared with the green and mature stages. No APX activity was detected in the samples using the method described in ²². The APX enzyme was further investigated by protein gel blot analysis as it seemed that APX activity could be very sensitive to protein degradation. Regarding NADPH producing dehydrogenases, G6PDH did not modify its activity during ripening, 6PGDH increased only at the turning stage and ICDH and ME almost doubled their activity from the green to the mature phase. Overall, the dehydrogenase activity increased significantly from the green to the mature phase. The changes in antioxidant enzymes during ripening were also investigated by protein gel blot analysis using specific antibodies and by densitometry of the cross-reactive bands.

Characterization of the antibodies used was carried out in our previous work.¹⁶ The levels of CAT showed a remarkable reduction from the green to the mature ripening stage, also in agreement with the CAT activity measured in the protein extracts. The three SOD isozymes Fe-SOD, Mn-SOD and Cu,Zn-SOD investigated during ripening in olive protein extracts exhibited different patterns. Fe-SOD showed the highest protein levels in the turning phase of ripening and then it was reduced at the mature phase. Mn-SOD increased at the turning and mature phases compared with the green phase and Cu,Zn-SOD showed its highest levels at the green phase and then it

was reduced in the two other consecutive phases. Regarding enzymes of the AGC, the levels of GR increased progressively from the green to the mature phase whilst APX and DHAR reduced their levels during the same ripening phases.

We investigated whether the changes in glutathione, ASC and antioxidant enzymes during ripening have an influence in the composition of olive oil antioxidants. The content of polyphenols, tocopherols and the oxidative stability was investigated in the olive oils extracted from the same batches of olives at the three different ripening stages used in the study. The main phenolic compounds found in the olive oils of our study were oleuropein and ligstroside aglycons, in agreement with previous reports.²⁸ The low concentrations of simple phenols like HT or tyrosol detected have also been reported in oils from the same cultivar.²⁹ The concentration of total polyphenols showed a modest but gradual increase from the green to the mature stage of ripening (Table 3), whilst α -tocopherol and total tocopherols showed the opposite response during the ripening phases (Table 4). The oxidative stability of the extracted oil did not show any significant changes during ripening.

DISCUSSION

The concentrations of glutathione found in olives were similar to those reported in tomato, peach or cherry.^{3,5,30} During maturation, olives increased the concentrations of GSH and GSSG by five-fold and ten-fold, respectively, as fruit developed from the green to the turning and mature phases. Indeed, GSSG increased from 4.5% of total glutathione at the green phase to 13.5% of total glutathione at the mature stage, indicating an increase in cellular redox status during ripening. In the absence of stress, tissues such as leaves usually maintain GSH/GSSG ratios of at least 20:1.³¹ Ripening decreased the GSH/GSSG ratio, which suggests a displacement towards a higher oxidative status, and this could also have acted on the synthesis of total glutathione and

ASC. The response of glutathione during the ripening of olives was very similar to that reported in other crops. Tomatoes progressively increased their GSH concentration from 2.2 mg/100g FW at the stage of mature green to 4.8 mg/100 g in the over-ripe stage of ripening.³ Other studies have also reported increases of GSH during the maturation of sweet pepper³², saskatoon fruit³³ and white grapes.³⁴

The content of ASC detected in olives was similar to that reported in other nutritionally relevant fruits and vegetables such as peaches⁵, peas, carrots, spinach or green beans.⁸ We only found one study reporting ASC content in olives (only in the green phase) that showed values much lower than those quantified by us.³⁵ In our study, the ASC content in olives increased by more than two-fold at the mature phase of ripening. In agreement with this, two other studies showed that the ASC content of tomatoes (cv. Ailsa Craig) and peppers (cv. California) increased by about 2-fold at the end of the red ripening phase compared with the green phase.^{3,4}

The reduction of catalase activity during ripening observed in our study has been shown in other studies performed in tomato and pepper.^{3,4} In the case of pepper fruit, it has been reported that the decrease of the catalase activity is because the enzyme undergoes tyrosine nitration and S-nitrosylation during ripening, two posttranslational modifications promoted by reactive nitrogen species.³⁶ Regarding SOD activity, previous studies in different plant species have shown different patterns of activity during ripening.³ In our study, the SOD activity showed a small at the turning stage of ripening and then returned to the same activity values of the green phase. In previous research we showed that the main SOD isozyme in olives was due to an Fe-SOD¹⁶, which explains why the SOD total activity detected during the ripening process resembles the protein Fe-isozyme levels detected by gel blot analysis. This pattern has also been reported in pepper fruit when SOD has been shown to increase from the green

to the yellow/green stage but to decline at the end of ripening³⁷, also reported in tomatoes³⁸ and in Saskatoon fruits.³³ A previous study showed that the turning phase of olive ripening coincides with a peak of respiration rate which is then reduced to a minimum at the end of the maturation.³⁹ Production of ROS is a consequence of respiration so the respiratory rate increase potentially feeds the signalling network that controls ripening and senescence.² Overall, the reduction of catalase activity and increase of SOD suggests an increase in intracellular levels of H₂O₂ during the ripening of olives. In plants, ROS are produced (and regulated) in different subcellular organelles, including chloroplasts, mitochondria and peroxisomes.² This suggests a role for catalase and peroxisomes in the control of olive ripening. Likewise, the different responses of SOD isozymes during ripening could be associated with a different type of ROS control and metabolism in subcellular organelles, as described earlier.⁹ Regarding the lower levels of APX and DHAR during the ripening of olives, other studies have shown a similar response during tomato and pepper fruit ripening.^{4,38} As mentioned before, ASC content was higher in black mature olives however the DHAR levels reduced by about 80% at the mature phase which suggests that the synthesis of ASC was perhaps more active than its enzymatic reduction from oxidised ASC. The lower levels of APX detected at the end of ripening may have also contributed to the accumulation of ASC.

When we analysed the polyphenol composition of the oils of our study, we observed an increase in the content of polyphenols during ripening, particularly oleuropein and hydroxytyrosol. However, in the mature phase of ripening the oxidative status of olive fruit increased, as indicated by the elevated levels of GSSG and the reduced ratio of GSH/GSSG. We expected that this increased oxidative status should

have had effects on the content of key molecular antioxidants such as polyphenols. However, we only detected a significant reduction in the levels of α -tocopherol, which is a common effect of ripening in the α -tocopherol content of olive oils.²⁵

We hypothesised that the antioxidant enzymes and the molecular antioxidants ASC, glutathione and α -tocopherols could act in the olive fruit as a first defence barrier to control the production of ROS, so during increased production of ROS, they may oxidise first, or contribute to the recycling of oxidised polyphenols directly, or act in cooperation with polyphenols, thereby protecting or saving polyphenols from oxidation. When for example HT quenches free radicals, semiquinone radicals and quinones are formed.⁴⁰ One possibility is the direct reduction of oxidised HT (or other oxidised polyphenols) back to their reduced forms mediated by ASC or GSH. The reduction potentials (which determines the feasibility that a molecule can chemically reduce another) of α -tocopherols (500 mV), ASC (330), GSH (310 mV) and HT (in vicinity of 400-500 mV^{41,42 43,44}), would allow this possibility. For example, ASC is able to reduce oxidised gallic acid, which is structurally related to HT.^{43 46} Another example is quercetin and its phenoxyl radicals, which can be reduced back to quercetin by ASC.¹ Likewise, phenoxyl radicals can be recycled back to their reduced form via oxidation of intracellular GSH.⁴⁴

Apart from their additive antioxidant effects against ROS, polyphenols, tocopherols and ASC could act in cooperation to control lipid peroxides ($\text{LOO} \cdot$) or molecular peroxides in general (Fig 2). Several lines of evidence support this possibility, including the antioxidant activity of green tea polyphenols involved in the recycling of tocopherols⁴⁵

and the antioxidant synergy activities between caffeic, coumaric acid or HT with
ASC.^{46, 47}

In conclusion, the changes observed during ripening in the levels of glutathione,
ascorbate and the antioxidant enzymes investigated in the study suggest a role for these
antioxidants in the process of olive maturation. In addition, they are likely to influence
the composition and concentration of olive oil molecular antioxidants such as
polyphenols and tocopherols, which are essential to maintaining the stability and
organoleptic properties of olive oil

ABBREVIATIONS: 6PGDH, 6-phosphogluconate dehydrogenase; AGC, ascorbate-
glutathione cycle; APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; DHAR,
dehydroascorbate reductase; E^0 , reduction potential; G6PDH, glucose-6-phosphate
dehydrogenase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidised
glutathione; H_2O_2 , hydrogen peroxide; $HO_2\cdot$, hydroperoxyl radical; HT,
hydroxytyrosol; ICDH, isocitrate dehydrogenase; $LOO\cdot$, lipid hydroperoxide radicals;
MDHAR, monodehydroascorbate reductase; ME, malic enzyme; $O_2^{\cdot-}$, superoxide
radical; $\cdot OH$, hydroxyl radical; PPOH, reduced polyphenols; ROS, reactive oxygen
species; $RO_2\cdot$, peroxy radical; SOD, superoxide dismutase; α -Toc, α -Tocopherol.

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Table 1. Reduced glutathione (GSH), oxidised glutathione (GSSG), redox state defined as [GSH]/[GSSG] and ascorbate (ASC) content in green, turning and mature olives, expressed as mg/100g of fresh weight (FW). Values are means $\pm$ SD of three different analyses. *, differences are significantly different ($P < 0.05$) vs. green phase.

	Green	Turning	Mature
GSH mg/100g FW	1.296 $\pm$ 0.016	1.918 $\pm$ 0.233*	5.208 $\pm$ 0.781*
GSSG mg/100g FW	0.061 $\pm$ 0.021	0.331 $\pm$ 0.046*	0.702 $\pm$ 0.201*
[GSH]/[GSSG]	21.3	5.8	7.4
ASC mg/100g FW	5.467 $\pm$ 0.058	5.471 $\pm$ 0.190	12.77 $\pm$ 4.580*

Table 2. Changes in antioxidant enzyme activities of olive fruit during ripening. Data are expressed as means $\pm$ SEM of at least three different experiments. ¹ in $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1}$; ² in U mg prot^{-1} ; ³ in $\text{nmol NADPH min}^{-1} \text{ mg}^{-1}$; ⁴ in $\text{mmol NADPH min}^{-1} \text{ mg}^{-1}$. ND, not detected. SD. *, differences are significantly different ($P < 0.05$) vs. green phase. Values are means $\pm$ SD of five different analyses.

	Green	Turning	Mature
Catalase ¹	30.07 ± 5.53	$15.56 \pm 4.49^*$	$4.78 \pm 5.11^*$
Superoxide dismutase ²	19.54 ± 3.20	$28.01 \pm 4.46^*$	22.11 ± 5.99
Glutathione reductase ³	45.15 ± 6.0	$72.57 \pm 8.0^*$	40.27 ± 7.6
Glucose-6-phosphate dehydrogenase ⁴	17.62 ± 6.21	23.20 ± 8.89	19.80 ± 2.03
6-Phospho-gluconate dehydrogenase ⁴	25.22 ± 2.51	$35.11 \pm 3.90^*$	20.57 ± 3.43
NADP-isocitrate dehydrogenase ⁴	27.65 ± 2.80	$43.01 \pm 1.32^*$	$68.71 \pm 13.01^*$
NADP-malic enzyme ⁴	34.40 ± 3.18	$57.65 \pm 5.01^*$	$51.66 \pm 7.15^*$

Table 3. Concentrations of phenols detected in the olive oil extracted from olives (cv Picual) at the green, turning and mature ripening phases. Data expressed as mg of phenols/Kg olive oil. HT, hydroxytyrosol; Ole Agl der. (oleuropein aglycon derivative); 3,4-DHPEA-EDA, dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol; p-HPEA-EDA, dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol; concentrations obtained from the different varieties expressed in mg/Kg of olive oil. Values are expressed as means $\pm$ SD of three different sample analyses. *, differences are significantly different ($P < 0.05$) vs. green phase.

Phenolic compounds	Green	Turning	Mature
HT	0,130 $\pm$ 0,020	1,962 $\pm$ 0,150	2,387 $\pm$ 0,031
HT–Acetate	0,046 $\pm$ 0,020	0,159 $\pm$ 0,060	0,348 $\pm$ 0,031
Tyrosol	1,386 $\pm$ 0,060	1,260 $\pm$ 0,015	1,488 $\pm$ 0,078
Oleuropein Aglycon	92,10 $\pm$ 4,532	126,22 $\pm$ 4,596	149,05 $\pm$ 3,121
Ole Agl der.	0,857 $\pm$ 0,040	0,368 $\pm$ 0,030	1,128 $\pm$ 0,030
Ole Agl der.	1,792 $\pm$ 0,020	1,036 $\pm$ 0,015	1,782 $\pm$ 0,016
3,4- DHPEA-EDA	1,274 $\pm$ 0,030	0,448 $\pm$ 0,030	1,596 $\pm$ 0,031
Ligstroside Aglycon	3,057 $\pm$ 0,004	1,684 $\pm$ 0,150	1,953 $\pm$ 0,016
p-HPEA-EDA (oleocanthal)	0,258 $\pm$ 0,005	0,127 $\pm$ 0,009	0,096 $\pm$ 0,005
Pinoresinol	1,05 $\pm$ 0,040	0,532 $\pm$ 0,015	0,986 $\pm$ 0,055
Luteolin	2,968 $\pm$ 0,046	3,221 $\pm$ 0,150	4,758 $\pm$ 0,062
Apigenin	0,137 $\pm$ 0,003	0,166 $\pm$ 0,003	0,223 $\pm$ 0,062
<i>p</i> -coumaric acid	1,064 $\pm$ 0,022	0,563 $\pm$ 0,030	0,445 $\pm$ 0,016
Total Phenols	106,3$\pm$4,8	137,1$\pm$5,3*	165,5$\pm$3,6*

Table 4. Chemical parameters determined in oils extracted from olives (cv. Picual) at different ripening indices. Values are expressed as means $\pm$ SD of three different analyses of olive oil samples. *, differences are significantly different ($P < 0.05$) vs. green phase.

Chemical parameters	Green	Turning	Mature
Free fatty acids (as % of oleic acid)	0,12 $\pm$ 0.02	0,16 $\pm$ 0.02	0,12 $\pm$ 0.02
Peroxide value (mequiv O ₂ /Kg)	2,085 $\pm$ 0.039	1,655 $\pm$ 0.028	2,455 $\pm$ 0.037
K_{270}	0,127 $\pm$ 0.017	0,131 $\pm$ 0.012	0,103 $\pm$ 0.019
K_{232}	1,548 $\pm$ 0.11	1,594 $\pm$ 0.14	1,430 $\pm$ 0.18
ΔK	-0,0050	-0,0030	-0,0030
Oxidative stability index (Rancimat, h)	23,45 $\pm$ 1,67	27,3 $\pm$ 2,37	26,36 $\pm$ 3,20
α -tocopherol	339,12 $\pm$ 4,2	273,13 $\pm$ 3,8*	248,51 $\pm$ 9.1*
β + γ -tocopherol	16,20 $\pm$ 0.6	33,45 $\pm$ 0.9*	32,35 $\pm$ 1.3*
δ -tocopherol	0,15 $\pm$ 0,1	2,14 $\pm$ 0,1*	1,14 $\pm$ 0,2*
Total tocopherols	355,5 $\pm$ 4,9	308,7 $\pm$ 4,8*	281,9 $\pm$ 10,6*

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653 **Figure 1.** Western blot and densitometry analysis of antioxidant enzymes present in
654 olive fruits during ripening. 1, green; 2, turning; 3, mature. Densitometry data
655 expressed in arbitrary units; numbers over the bars indicate relative density expressed as
656 % from the highest density value. Representative of three experiments

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660 **Figure 2.** A model for possible collaboration mechanisms between α -tocopherol (α -
661 Toc), polyphenols (PPOH) such as hydroxytyrosol and ascorbate (ASC) in olive fruit:
662 1. Lipid hydroperoxide radicals (LOO•) are reduced to non-toxic hydroperoxides by α -
663 Toc which is oxidised to tocopheryl radicals (α -Toc •). These are recycled back to α -
664 Toc by ASC with the production of dehydroascorbate (DHA), which is reduced back to
665 ASC in the ASC-Glutathione cycle (AGC). 2. LOO• could be directly reduced by PPOH
666 with the production of semiquinone radicals (PPO•, or quinones) which are reduced to
667 their original forms by ASC, which is then recycled in the AGC, as before. 3. LOO• are
668 reduced by α -Toc with the production of α -Toc •, as in 1. These are reduced to α -Toc by
669 PPOH with the production of PPO• which are reduced to PPOH by ASC, originating
670 DHA, which is reduced to ASC in the AGC.

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