**Modulating oxidoreductase activities modifies the phenolic content of virgin
olive oil**

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Running title: Oxidoreductases alter olive oil phenolic content

Abstract

The effect of modifying polyphenol oxidase (PPO) and peroxidase (POX) activity during the extraction of virgin olive oil has been assessed in terms of its influence on the phenolic profile of the oil produced. These enzymes were modified by adding exogenous enzyme or specific inhibitors during the milling and subsequent kneading step, studying the effect on specific phenolic compounds in the oils. PPO is the main enzyme involved in phenolic oxidation at the milling step whereas POX activity seems to be the main influence during the kneading step. The data obtained suggest it is possible to increase the nutritional and organoleptic quality of virgin olive oil by inhibiting these enzymes during olive fruit processing. Treatment with the PPO inhibitor tropolone produced a two-fold increase in the phenolic fraction, which would therefore seem to be an interesting strategy to improve the nutritional and organoleptic properties of virgin olive oil.

Key words: polyphenol oxidase, peroxidase, virgin olive oil, extraction, phenolic compounds, quality.

Chemical compounds studied in this article

Tyrosol (PubChem CID: 10393); Hydroxytyrosol (PubChem CID: 82755);

Hydroxytyrosol acetate (PubChem CID: 115240); Luteolin (PubChem CID:5280445);

Apigenin (PubChem CID: 5280443); Pinoresinol (PubChem CID: 73399); 1-

Acetoxypinoresinol (PubChem CID: 442831); Vanillic acid (PubChem CID: 8468);

Ferulic acid (PubChem CID: 445858); Cinnamic acid (PubChem CID: 444539).

Introduction

Virgin olive oil (VOO) is the primary source of lipids in the Mediterranean diet and it has been linked with positive health benefits (Konstantinedou et al., 2010; Visioli,

2012). It is well established that phenolic compounds are directly related to the nutritional benefits and organoleptic properties of VOO. The long term dietary consumption of VOO represents the delivery of phenolic compounds over time, which may attenuate the inflammatory responses associated with eating and reduce the associated risk of chronic inflammatory diseases (Lucas, Rusell & Keats 2011). Phenolic compounds are also responsible for the bitter and pungent sensory tones of VOO (Andrewes, Busch, de Joode, Gronewegen & Alexandre, 2003; Mateos, Cert, Perez-Camino & García, 2004; Inarejos-García, Fregapane & Salvador, 2009), common and desirable attributes when present at low to moderate intensity.

In most oils, the main phenolic components found are the dialdehyde forms of decarboxymethyl oleuropein and ligstroside aglycones (3,4-DHPEA-EDA and *p*-HPEA-EDA, respectively), as well as the aldehyde forms of oleuropein and ligstroside aglycones (3,4-DHPEA-EA and *p*-HPEA-EA, respectively). The accumulation of hydrophilic phenols in VOO is related to the initial phenolic glycoside content of the olive fruit, and to the activity of hydrolytic and oxidative enzymes on these glycosides during the oil extraction process (Romero-Segura, Sanz & Pérez, 2009; García-Rodríguez, Romero-Segura, Sanz, Sánchez-Ortiz & Pérez, 2011). However, there is evidence that the phenolic glycoside content should not be regarded as a limiting factor for the phenolic content of VOO, given that the total secoiridoid compounds in VOOs represent an average of 1-4% of the secoiridoid glycosides present in the fruit (Gómez-Rico, Fregapane & Salvador, 2008). Experimental evidence suggests that endogenous enzymes such as β -glucosidase, which hydrolyzes phenolic glycosides, and oxidoreductases like polyphenol oxidase (PPO) and peroxidase (POX), which oxidase phenolic compounds, may be the main biochemical factors affecting the phenolic content of VOO. Indeed, olive β -glucosidase has been shown to play a critical role in

shaping the phenolic profile of VOO (Romero-Segura, García-Rodríguez, Sanz & Pérez, 2011; Romero-Segura, García-Rodríguez, Sánchez-Ortiz, Sanz & Pérez, 2012). However, most kinetic studies into the formation and degradation of phenolic compounds during the extraction of VOO suggest that olive oxidoreductase enzymes that catalyze the oxidative degradation of these compounds during fruit processing are the key factors limiting the phenolic content of the oils (Fregapane & Salvador, 2013; Taticchi, Esposto, Venziani, Urbani, Selvaggini & Servili, 2013). PPO activity is involved in the oxidation of phenols associated with olive fruit ripening and processing (Ortega-García, Balnco, Peinado & Peragón, 2008; Segovia-Bravo, Jarén-Galán, García-García & Garrido-Fernández, 2009). On the other hand, the influence of the olive seed on the phenolic composition of VOO has been related to the high levels of POX activity found in this tissue (Luaces, Romero, Gutiérrez, Sanz & Pérez, 2007). The oxidation of phenolic compounds by POX requires H_2O_2 which may be produced as a consequence of secondary PPO coupled reactions (Takahama & Oniki, 2000). Thus, PPO and POX might act synergistically on the oxidative degradation of olive phenolic compounds during the oil extraction process (El-Riachy, Priego-Capote, León, Rallo & Luque de Castro, 2011).

In recent decades, a number of studies have focused on improving the phenolic composition of VOO by means of technological innovations that substantially modify the operational parameters of the oil extraction process (Inarejos-García et al., 2011; Servili, Taticchi, Esposto, Urbani, Selvaggini & Montedoro, 2008; Frankel, Bakhouch, Lozano-Sánchez, Segura-Carretero & Fernández-Gutiérrez, 2013). Although it is clear that changes in the phenolic profile of the oil induced by any such innovations will be mostly based on the effect of these parameters on the activity of oxidative enzymes, no specific studies have been carried out on the selective modulation of their activities. In

this sense, a few studies have been carried out into the use of enzyme complexes to increase the oil extraction yield, yet very little is known about the specific role of the various constituents present in these formulations, or their implications in the organoleptic and nutritional quality of VOO (Clodoveo, 2012).

We previously showed that partially purified olive PPO and POX can oxidize the main phenolic glycosides present in olive fruit *in vitro*, as well as their secoiridoid derivatives present in the oil (García-Rodríguez et al., 2011). However, there is little information about the specific roles play by PPO and POX during the disruption of fruit tissue in the milling step and the subsequent kneading of the olive paste. Better understanding these biochemical processes could be of interest to help design new biotechnological approaches to improve VOO quality. Therefore, the aim of this study was to evaluate how olive PPO and POX activities shape the phenolic profile of VOO, and to assess the possibility of tailoring this phenolic profile by modulating PPO and POX activities during the industrial extraction of VOO.

Materials and Methods

2.1 Plant material

Olive fruit (*Olea europea*, cv. Picual and cv. Arbequina) was cultivated at the experimental fields of the Instituto de la Grasa and collected during the 2010-2011 and 2011-2012 olive fruit seasons (October-December) with a maturity index (MI) of 1 (fruit with green skin) and 5 (fruit with black skin and <50% purple flesh), according to García and Yousfi (2005).

2.2 Chemicals

Reagents for enzymatic extraction and activity measurements were supplied by Sigma-Aldrich (St. Louis, MO), except for some phenolic compounds, such as oleuropein, hydroxytyrosol, luteolin and apigenin, which were purchased from Extrasynthese (Genay, France). The diol-bonded phase cartridges used to isolate the VOO phenolic compounds were supplied by Supelco (Bellefonte, PA).

2.3 Olive oil extraction

Olive oil extraction was performed using an Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain) that simulates the industrial process of VOO production on a laboratory scale (Martínez, Muñoz, Alba & Lanzón, 1975). The milling of olive fruit (1 kg) was performed using a stainless steel hammer mill operating at 3,000 rpm and equipped with a 5mm sieve. The resulting olive paste was immediately kneaded in a mixer at 50 rpm for 30 min at 30 °C. The kneaded olive paste was then centrifuged in a basket centrifuge at 3,500 rpm for 1 min, after which the oil was decanted and paper-filtered. Samples were stored under nitrogen at -18 °C prior to analysis.

To modulate the PPO and POX activity during the process to obtain the oil, different amounts of enzymes were added to the olive fruit during the milling or kneading steps, such as horseradish peroxidase (HRP, 400 mg enzyme/kg of olive fruit: Sigma-Aldrich, St. Louis, MO), olive seed POX (in the range of 650-850 units/kg of olive fruit) and olive PPO (in the range of 60,000-200,000 units/kg of olive fruit), as well as co-substrates (H_2O_2 , 100-500 μL /kg of olive fruit) or specific inhibitors like sodium azide (66-264 mg/kg of olive fruit) and tropolone (20-480 mg/kg of olive fruit). Olive seeds were used as a source of olive POX (35-45 g seeds/kg of olive fruit), added at the milling step, or the corresponding amount of olive seed acetone powder was added during the kneading step. Olive pulp acetone powder was used as a source of olive-

PPO. All the substances added were previously dissolved in 20 mL of water and the same volume of pure water was added to the control samples. Duplicate experiments were carried out for each cultivar and MI.

2.4 Extraction and analysis of phenolic compounds from virgin olive oil

VOO phenolic compounds were isolated by Solid Phase Extraction (SPE) in a diol-bonded phase cartridge following a previously described protocol (Mateos et al., 2001). A solution of *p*-hydroxyphenyl acetic acid (46.4 µg/mL) and *o*-coumaric acid (9.6 µg/mL) in methanol was used as the internal standard in this extraction procedure. An aliquot (0.5 mL) of standard solution was added to each oil sample (2.5 g) prior to phenolic extraction. VOO and fruit phenolic extracts were further analyzed by HPLC in a Beckman Coulter liquid chromatography on a system equipped with a System Gold 168 detector, a solvent module 126 and a Mediterranea Sea 18 column (4.0 mm i.d. x 250 mm, particle size 5 µm) (Teknokroma, Barcelona, Spain), following a previously described method (Luaces et al., 2007). Phenols (except ferulic acid), cinnamic acid and lignans were quantified at 280 nm using *p*-hydroxyphenyl acetic acid as the internal standard, while flavones and ferulic acid were quantified at 335 nm using *o*-coumaric acid as the internal standard. The nature of the compounds was confirmed by HPLC-MS using the same chromatography system connected on-line with an MAT95 magnetic sector mass spectrometer (Finnigan Mat, Bremen, Germany) and equipped with an ESI-II electrospray ionization (ESI) interface, using the same column and gradient conditions. Duplicate experiments were carried out for each cultivar and MI.

2.5 Enzymatic activities

Acetone powders were prepared from the mesocarp and seed tissues of fresh harvested olive fruit. Typically, 10 g of tissue was ground in 150 mL of cold acetone (-20 °C) in a Waring blender and the residue obtained after filtration was re-extracted twice with 20 mL of cold acetone (-20 °C). The whitish powder obtained was finally rinsed with diethyl ether, dried and stored at -20 °C.

POX enzyme extracts were prepared in an Ultraturrax homogenizer with 0.25 g of olive seed acetone powder and 2.5 mL of a buffer containing: 100 mM sodium phosphate buffer [pH 6.7], 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.1 mM benzamidine, 5 mM α -aminocaproic acid and 0.1% Triton X-100. The resulting homogenate was centrifuged at 10,000 g for 20 min at 4 °C and the cleared supernatant was used as the crude extract. POX activity was monitored through the increase in absorbance at 420 nm due to the formation of tetraguaiacol ($\epsilon = 26600 \text{ M}^{-1}\text{cm}^{-1}$) by the peroxidation of guaiacol (Luaces et al., 2007). One unit of POX activity was defined as the enzyme forming 1 μmol of tetraguaiacol per min.

PPO enzyme extracts were prepared in an Ultraturrax homogenizer with 0.25 g of olive pulp acetone powder in 10 mL of a buffer containing: 100 mM sodium phosphate buffer [pH 6.7] 1 mM EDTA, 0.1 mM PMSF, 0.1 mM benzamidine, 5 mM α -aminocaproic acid and 1 mM sodium dodecyl sulphate (SDS). The resulting homogenate was centrifuged at 27,000 g for 20 min at 4 °C and the cleared supernatant was used as the crude extract. PPO activity was determined by continuously monitoring the increase in absorbance at 400 nm due to the oxidation of tert-butylcatechol (TBC) and the formation of the corresponding quinone. The oxidative reaction was quantified considering an extinction coefficient of $1200 \text{ M}^{-1}\text{cm}^{-1}$ according to García-Molina, Muñoz, Varón, Rodríguez-López & García-Cánovas (2007). One unit of PPO activity was defined as the enzyme forming 1 μmol of TBC-quinone per min.

2.6 Statistical analysis

The data were evaluated using Statgraphics Plus 5.1 (Manugistic Inc., Rockville, MD), applying an analysis of variance (ANOVA) and the means were compared with the Student-Newman-Keuls/Duncan test at a significance level of 0.05.

Results and Discussions

We previously showed that olive PPO and POX catalyze the oxidation of the main phenolic glycosides found in olive fruit *in vitro*, as well as the secoiridoid derivatives arising during the industrial process to obtain the oil, especially those containing hydroxytyrosol such as 3,4-DHPEA-EDA and 3,4-DHPEA-EA (García-Rodríguez et al., 2011). We found that most olive POX activity is located in the seed, accounting for more than 90% of the total fruit POX activity in most olive cultivars (Luaces et al., 2007; Cardoso, Mafra, Reis, Nunes, Saraiva & Coimbra, 2010). Moreover, the POX activity found in the Picual and Arbequina cultivars used in this study are quite similar at the turning stage, 14.5 and 10.9 U/g of seed, respectively. This olive POX activity is partially inactivated during the oil extraction process, presumably by oxidized phenolic compounds that have a well-established inactivating influence on enzymatic activity (Loomis & Battaile, 1966), which explains the weak activity of other enzymes in olive pastes (Sánchez-Ortiz, Romero-Segura, Sanz & Pérez, 2012). Despite this partial

inactivation, significant POX activity (20% of the activity in fresh fruit) was still detected during and at the end of the kneading step (30 min.), consistent with the significant POX activity after even longer kneading times found previously (Taticchi et al., 2013).

As a first approach to determine whether POX activity is involved in the degradation of oil phenolics during extraction, this activity was increased during the milling and kneading steps. HRP was selected for this purpose because it is commercially available and capable of oxidizing olive phenolics *in vitro* (data not shown). The addition of HRP promoted a clear decrease in the oil phenolic content and more specifically, in the content of *o*-diphenolic compounds, which ranged from 14% when added at the milling step to 22% when added during the kneading step (Table 1). Accordingly, increasing POX activity appears to be slightly more effective during the kneading process than in the milling step. This difference might indicate that the dose of HRP added at the milling step is not as significant as it is during the kneading process when the endogenous POX activity has been already partially inactivated. The weak effect obtained with the commercial enzyme, whose activity on guaiacol was 1500 U/mg, suggests that either HRP is not very effective in oxidizing olive phenolics or olive paste is not a suitable reaction medium for HRP.

Given that olive seeds are a natural source of POX activity (Luaces et al., 2007), a second set of experiments was performed to increase POX activity during the oil extraction process by increasing the proportion of olive seeds in the milling step. Olive seeds were added (35-45 g seeds per kg of olive fruit) in order to provide an additional 100% POX activity during this process. The experiments were carried out using green Picual and Arbequina fruit (MI 1) and the addition of olive seeds during milling caused a significant decrease in the phenolics content, particularly in the *o*-diphenolic

compounds (Table 2). This decrease reached 56% of the *o*-diphenolics in Picual and 59% in Arbequina oils. In both cultivars, 3,4-DHPEA-EDA was the compound that experienced the most significant depletion as a consequence of increasing olive seed POX (a decrease of 52% in Picual and 41% in Arbequina). These results agree well with previous *in vitro* oxidation experiments in which 3,4-DHPEA-EDA was the preferred natural substrate for olive seed POX and the oxidative degradation of this compound was selectively catalyzed within a mixture of different VOO phenolic compounds (García-Rodríguez et al., 2011).

Plant peroxidases are known to preferentially oxidase phenolics as substrates at the expense of peroxide species, in most cases H_2O_2 . To explore the possible limitation of the oxidative degradation catalyzed by POX due to the limited availability of this co-substrate, H_2O_2 was added in the range of 100-500 μ L per kg of olive fruit during the milling step. A clear dose/response relationship was observed, whereby the greater availability of H_2O_2 was correlated with a lower phenolic content in the oils. For example, while processing Picual fruit in the presence of 100 μ L H_2O_2 /kg of fruit produced a 10% decrease in the total phenolic compounds, this decrease rose to 13% in the presence of 200 μ L H_2O_2 /kg and to 24% with 500 μ L H_2O_2 /kg (data not shown). Moreover, changes were observed in the phenolic profile of the oils obtained from Picual fruits at two different stages of maturity (MI 1 and MI 5) after the addition of the highest dose of H_2O_2 assessed (500 μ L H_2O_2 /kg: Table 3). Taking into account that H_2O_2 does not promote VOO phenolic oxidation *in vitro per se*, the experimental data suggest that the oxidation of phenolics by POX during VOO extraction is limited by the availability of H_2O_2 in the olive pastes. Hydroxytyrosol derived secoiridoids were the compounds most severely affected by exposure to H_2O_2 (Table 3), their oxidative degradation increasing by 36% and 50% in the oils obtained from unripe and ripe fruits,

respectively. The higher oxidation rates observed when processing ripe compared to unripe fruit after the addition H_2O_2 seem to be related with the higher POX activity found in the former (García-Rodríguez et al., 2011).

Having shown that increasing POX activity or its co-substrate during oil extraction decreases the VOO phenolic content, the effect of reducing POX activity during oil extraction was also studied. Accordingly, different inhibitors of POX activity were tested *in vitro*. Sodium azide, a strong specific non-competitive POX inhibitor and a scavenger of H_2O_2 was selected. A clear dose response effect of this inhibitor was observed in the range 66-264 mg per kg of fruit, with the highest dose (264 mg/kg of fruit) causing a significant increase in the phenolic content of the oils obtained from both Picual MI 1 (34%) and Picual MI 5 (23%) fruit (Table 4). The 3,4-DHPEA-EDA, 3,4-DHPEA-EA phenolic compounds and their immediate precursor, OA isomers (Romero-Segura et al., 2012), were those that were most strongly affected by the inhibition of POX during oil extraction, while little or no effect was found in terms of the main tyrosol derivative, p-HPEA-EDA. These results are consistent with those found when increasing POX activity during oil extraction (Table 1 and 2), yet in the opposite sense. Although it is clear that the toxicity of sodium azide means it could never be used as an additive, these results might indicate the potential benefits of POX inhibitors to increase the phenolic content of olive oils, increasing their nutritional and organoleptic quality.

Unlike POX activity, olive PPO activity is very strong in the mesocarp tissue while almost trace levels are found in the olive seed (García-Rodríguez et al., 2011). Moreover, PPO activity was quite similar in the Picual and Arbequina cultivars used in this study (200 U/g fresh pulp tissues). This PPO activity decreased dramatically during the milling step and only very weak PPO activity was detected in olive pastes during

kneading, indicating that olive PPO is more labile than olive POX (Taticchi et al., 2013).

To determine whether PPO activity is involved in the phenolic degradation that occurs during VOO extraction, the effect of increasing PPO activity on the oil phenolic profile was studied. PPO activity was enhanced by adding olive pulp acetone powders that contain high levels of PPO activity given that the extremely high PPO activity present in olive fruit made it impossible to increase the PPO activity during oil extraction by adding commercial PPO enzymes. To limit the interference caused by β -glucosidase activity present in the olive pulp that may affect the phenolic content, the acetone powders studied were obtained from Picual fruit at different stages of ripening, each with different PPO activity but with very low and similar β -glucosidase activity (Romero-Segura et al., 2011). The increase in PPO activity during milling of Picual and Arbequina fruit caused a decrease in the phenolic content in the VOO produced (Table 5), degradation of phenolics reaching 70% in the Picual and Arbequina oils. The *o*-diphenolic compounds like 3,4-DHPEA-EDA and OA-isomers were almost totally depleted, indicating that PPO activity is involved in the degradation of these compounds. The significant decrease of the monophenol compound *p*-HPEA-EDA could not be attributed to the direct oxidation by olive PPO, which has mostly *o*-diphenolase activity (Goupy, Fleuriet, Amiot & Macheix, 1991), yet its degradation could be associated to the quinones produced through secondary coupled reactions (Falguera, Pagan & Ibarz, 2010).

As described previously for POX, the inhibition of PPO activity during the milling and kneading steps of VOO extraction was explored by studying how different inhibitors affected the phenolic profile. Tropolone is a natural biological active substance used to control pathogens in fruits (Saniewski, Saniewska, Kanlayanarat, 2007) and it that acts

as a specific PPO inhibitor while displaying no effect on POX activity (Cantos, Tudela, Gil & Espín, 2002). At doses in the range of 18-480 mg/kg of fruit, tropolone provoked a clear dose-response effect on the phenolic profiles of the oils, particularly when added at the highest dose (480 mg/kg of fruit) during the milling and kneading of Picual fruit (MI 1: Table 6). Tropolone has a stronger effect when added during the milling step, producing a 95% increase in *o*-diphenolic compounds as opposed to the 53% increase in *o*-diphenolic compounds when it is added during kneading. As indicated above, PPO activity is stronger during milling and it decreases rapidly during kneading. Similarly the effect of the inhibitor is stronger at the milling step than at the kneading step, when the initial PPO activity has been partially inactivated.

Conclusions

The effects of modulating olive PPO and POX activities proves that both enzymatic activities seem to be involved in the degradation of phenolic compounds during oil extraction and consequently, in the conformation of the nutritional and organoleptic quality of VOO. Experimental evidence suggests that the oxidative degradation of phenolic compounds catalyzed by these enzymatic activities occurs immediately after tissue disruption in the milling step, with a substantial reduction in the oxidation rate during kneading. Moreover, PPO seems to be the most relevant enzyme involved in phenolic oxidation during olive fruit milling, whereas POX activity seems to be the main contributor in the kneading step. Moreover, it appears to be possible to improve the nutritional and organoleptic quality of VOO by reducing the degradation of these activities during olive fruit processing by using suitable inhibitors. In this sense, the inhibition of PPO activity with tropolone highlights the potential value of this compound during oil extraction in order to improve the nutritional and organoleptic quality of VOO.

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Table 1. Phenolic contents ($\mu\text{mol/ kg}$ olive oil) of the oils obtained after the addition of HRP (400 mg/ kg fruit) during the processing of olive fruits (Picual IM 1)

Phenolics ($\mu\text{mol/ kg}$ olive oil)	Control	+ HRP	
		at milling	at kneading
hydroxytyrosol	10.5b	6.5a	6.7a
tyrosol	11.2b	7.1a	7.1a
vanillic acid	5.5b	3.5a	3.4a
<i>p</i> -coumaric acid	2.9b	2.2a	2.1a
hydroxytyrosol acetate	17.7b	21.7c	11.4a
3,4-DHPEA-EDA	404.5b	423.1b	334.4a
OA-isomers	575.3b	416.6a	405.2a
<i>p</i> -HPEA-EDA	236.9b	237.3b	219.2a
pinoresinol	0.0	0.0	0.0
cinnamic acid	23.6	22.8	23.4
acetoxypinoresinol	0.0	0.0	0.0
3,4-DHPEA-EA	107.5b	92.4a	85.8a
<i>p</i> -HPEA-EA	23.4b	17.3a	21.3b
luteolin	15.2b	13.1a	13.4a
apigenin	7.2	6.5	6.5
Total phenolics	1441.7c	1269b	1160.3a
<i>o</i> -diphenolics	1130.9c	973.3b	877.1a
Secoiridoids.	1347.7c	1186.6b	1066.1a

*Mean values from three determinations in two different experiments. Values with different letters in the same row are significantly different ($p \leq 0.05$).

Table 2. Phenolic contents ($\mu\text{mol/ kg}$ olive oil) of the oils obtained after the addition of olive seed POX during the processing of olive fruits from cultivars Arbequina and Picual (IM 1)

Phenolics ($\mu\text{mol/ kg}$ olive oil)	Picual		Arbequina			
	Control	+100% POX at milling	Control	+ 100% POX at milling	Control	+ 100% POX at kneading
hydroxytyrosol	10.3b	7.6a	9.5	7.4	10.4b	3.2a
tyrosol	10.5	11.6	11.8	13.0	8.2	6.3
vanillic acid	5.4b	3.6a	2.6	1.8	1.1	1.0
<i>p</i> -coumaric acid	2.7b	2.1a	0.0	0.0	0.0	0.0
hydroxytyrosol acetate	17.5b	13.5a	77.4b	30.8a	66.1b	48.2a
3,4-DHPEA-EDA	400.2b	197.5a	498.7b	297.7a	336.0b	192.6a
OA-isomers	559.1b	313.8a	0.0	0.0	10.9	5.8
<i>p</i> -HPEA-EDA	233.1b	156.6a	124.7	103.7	184.0	143.9a
pinoresinol	0.0	0.0	2.1	2.0	1.1	1.0
cinnamic acid	15.9	19.6	5.7	4.0	5.6b	4.5a
acetoxypinoresinol	0.0	0.0	48.9	39.4	47.2b	37.3a
3,4-DHPEA-EA	97.5b	81.2a	45.0b	34.3a	56.1	46.0
<i>p</i> -HPEA-EA	23.1b	17.6a	17.1	14.4	11.4	9.1
ferulic acid	0.0	0.0	0.0	0.0	0.0	0.0
luteolin	14.8b	11.8a	17.8	13.9	15.4b	7.4a
apigenin	7.0b	6.1a	8.8	8.0	4.9	4.4
Total phenolics	1398.5b	825.7a	870.8b	570.4a	754b	489.5a
<i>o</i> -diphenolics	1099.9b	625.5a	649.1b	384.2a	490.9b	281.9a
Secoiridoids	1313.1b	749.7a	686.2b	450.1a	598.3b	376.1a

*Mean values from three determinations in two different experiments. Values with different letters in the same row within each variety are significantly different ($p \leq 0.05$)

Table 3. Phenolic contents ($\mu\text{mol/ kg}$ olive oil) of the oils obtained after the addition of H_2O_2 ($500\mu\text{L/ kg}$ fruit) during the processing of olive fruits from the Picual cultivar at different maturity stages (MI 1 and 5)

Phenolics ($\mu\text{mol/ kg}$ olive oil)	Picual IM 1		Picual IM 5	
	Control	+ H_2O_2	Control	+ H_2O_2
hydroxytyrosol	4,5	4,3	6,8	4,1
tyrosol	11,8	12,6	20,0	17,7
vanillic acid	3,4	2,1	1,0	0,5
<i>p</i> -coumaric acid	5,0	1,5	4,6	2,6
hydroxytyrosol acetate	19,0	22,3	17,2b	6,7a
3,4-DHPEA-EDA	172,8b	80,5a	52,0b	29,1a
OA-isomers	105,0b	59,4a	116,4b	48,7a
<i>p</i> -HPEA-EDA	140,1	141,8	50,2	41,8
pinoresinol	1,5	1,4	0,9	0,7
cinnamic acid	6,6	0,7	1,4	1,0
acetoxypinoresinol	19,9	14,5	19,3	17,7
3,4-DHPEA-EA	59,3b	31,8a	13,8b	6,6a
<i>p</i> -HPEA-EA	54,5	59,5	8,8	5,5
luteolin	8,8	9,8	13,1	11,5
apigenin	2,1	2,5	4,0	4,0
Total phenolics	614,5b	466,9a	329,5b	198,3a
<i>o</i> -diphenolics	369,6b	230,4a	219,4b	106,8a
Secoiridoids	531,9b	372,9a	241,3b	131,8a

*Mean values from three determinations in two different experiments. Values with different letters in the same row within each ripening index are significantly different ($p \leq 0.05$)

Table 4. Phenolic contents ($\mu\text{mol/ kg}$ olive oil) of the oils obtained after the addition of the POX specific inhibitor sodium azide (264 mg/ kg fruit) during the processing of olive fruits from Picual cultivar at different maturity stages.

Phenolics ($\mu\text{mol/ kg}$ olive oil)	Picual IM 1		Picual IM 5	
	Control	+ Sodium azide	Control	+ Sodium azide
hydroxytyrosol	6,0	5,9	5,6	5,4
tyrosol	18,0	17,1	17,7	20,3
vanillic acid	3,7	2,7	2,0	1,9
<i>p</i> -coumaric acid	9,0	9,2	3,2	3,0
hydroxytyrosol acetate	0,0	0,0	0,0	0,0
3,4-DHPEA-EDA	174.3a	235.4b	84,8a	127,4b
OA-isomers	141,6	134,6	148,7a	165,7b
<i>p</i> -HPEA-EDA	147.1	156.1	37,3	40,7
pinoresinol	6,9	8,7	2,6	1,7
cinnamic acid	19,3a	28,9b	4,6	5,8
acetoxypinoresinol	0,0	0,0	11,9	16,1
3,4-DHPEA-EA	97.6a	155.9b	10,2	14,3
<i>p</i> -HPEA-EA	59,9	88.7	3,4	13,7
ferulic acid	0,0	0,0	0,0	0,0
luteolin	15,5	18,5	12,5	9,8
apigenin	4,4	5,0	3,6	3,5
Total phenolics	703,3a	876,7b	348,0a	429,2b
<i>o</i> -diphenolics	435,0a	550.9b	261,7a	322,5b
Secoiridoids	642,3a	780.7b	284,3a	361,7b

*Mean values from three determinations in two different experiments. Values with different letters in the same row within each ripening index are significantly different ($p \leq 0.05$)

Table 5. Phenolic contents ($\mu\text{mol/ kg}$ olive oil) of the oils obtained after the addition of olive PPO during the processing of olive fruits (Picual IM 5 and Arbequina IM 1).

Phenolics ($\mu\text{mol/ kg}$ olive oil)	Picual		Arbequina	
	Control	+100% PPO	Control	+100% PPO
hydroxytyrosol	6,2	4,2	10.4b	0.9a
tyrosol	12,3	10,4	8.2	8.0
vanillic acid	1,2	0,8	1.1	0.9
<i>p</i> -coumaric acid	0,0	0,0	0.0	0.0
hydroxytyrosol acetate	0,0	0,0	66.1b	3.0a
3,4-DHPEA-EDA	173,7c	15,4a	336.0b	19.4
OA-isomers	79,2c	5,4b	10.9b	0.0a
<i>p</i> -HPEA-EDA	224,8c	92,9a	184.0b	50.2a
pinoresinol	5,4	1,7	1.1b	0.5a
cinnamic acid	3,7b	0,4a	5.6b	3.5a
acetoxypinoresinol	50,3c	24,8a	47.2b	32.9a
3,4-DHPEA-EA	0,0	0,0	56.1b	25.7a
<i>p</i> -HPEA-EA	0,0	0,0	11.4	11.7
ferulic acid	0,0	0,0	0.0	0.0
luteolin	9.9	10,3	15.4b	10.7a
apigenin	4.4	3,4	4.9b	3.4a
Total phenolics	571,2c	169,7a	754b	163.6a
<i>o</i> -diphenolics	269,1c	35,3a	490.9b	59.8a
Secoiridoids	477,8c	113,7a	598.3b	107.1a

*Mean values from three determinations in two different experiments. Values with different letters in the same row within each variety are significantly different ($p \leq 0.05$)

Table 6. Phenolic contents ($\mu\text{mol/ kg olive oil}$) of the oils obtained after the addition of tropolone (480mg/ kg fruit) during the processing of olive fruits (Picual IM 1)

Phenolics ($\mu\text{mol/ kg olive oil}$)	+ tropolone		
	Control	at milling	at kneading
hydroxytyrosol	9,0ab	8,0a	11,0b
tyrosol	10,8a	15,8b	10,5a
vanillic acid	3,6a	6,4b	7,5b
<i>p</i> -coumaric acid	2,7	3,1	3,1
hydroxytyrosol acetate	11,2a	19,0b	16,7b
3,4-DHPEA-EDA	223,6a	420,8c	321,8b
OA-isomers	263,6a	608,7c	478,6b
<i>p</i> -HPEA-EDA	162,1a	203,5b	176,7a
pinoresinol	0,0	0,0	0,0
cinnamic acid	13,2a	25,2c	22,5b
Acetoxypinoresinol	0,0	0,0	0,0
3,4-DHPEA-EA	69,3a	87,6b	63,7a
<i>p</i> -HPEA-EA	15,9a	21,3b	19,5ab
ferulic acid	0,0	0,0	0,0
luteolin	13,9a	16,1b	14,9ab
apigenin	7,0	6,6	6,6
Total phenolics	805,9a	1442,0c	1153,0b
<i>o</i> -diphenolics	590,6a	1160,1c	906,6b
Secoiridoids	734,5a	1341,8c	1060,2b

*Mean values from three determinations in two different experiments. Values with different letters in the same row are significantly different ($p \leq 0.05$)