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# Effect of the Treatment of the Olive Tree (*Olea europaea* L.) on the Phenolic Content and Antioxidant Properties in Olive Fruits

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**Abstract** We here investigate the effects of the application of methyl jasmonate to olive trees on antioxidant composition of olive fruits. Two cultivars (ie, *Arbequina* and *Picual*) were evaluated in our study. As a result, the total phenol content increased significantly with the treatment in *Arbequina* (from 155.89 to 434.22 mg gallic acid $kg^{-1}$) whereas decreases were observed in *Picual* (from 338.27 to 127.71 mg gallic acid $kg^{-1}$). Similarly, decreases in phenolic acid content were measured in *Arbequina* whilst no effect was observed in *Picual* olives. However, the contents of oleuropein and hydroxytyrosol did not increase with the pre-harvest methyl jasmonate for both *Arbequina* and *Picual*. Also for both cultivars the treatment of the olive trees increased the free radical scavenging activity of the olive fruits ($IC_{50}$ from 514.36 to 1125.46 µg/mL in *Arbequina* and from 611.98 to 114.55 µg/mL in *Picual*). The results here found are deeply discussed.

***Keywords:*** *olive fruit, olive tree, methyl jasmonate, antioxidant, pre-harvest treatment, phenolics, quality*

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## 1. Introduction

Olive tree (*Olea europaea* L.) is very popular in the Mediterranean area because of its fruits and oil. Both of them are worldwide known by their beneficial health properties [1]. These benefits are in part associated with the high amounts of nutritionally relevant constituents, mainly mono-unsaturated fatty acids, but also certain minor components such as polyphenols. Among polyphenols, oleuropein and related compounds are generally the most predominant phenolic compounds in olive cultivar [2,3]. Oleuropein is a secoiridoid compound present in all olive tree derivatives (olive oil, olive fruit, olive mill wastewater and pomace). The beneficial effects on human health of oleuropein and some related compounds such as hydroxytyrosol have been widely reported. In particular, they have been described to possess antioxidative [4], antimicrobial [5], antiviral [6], anti-inflammatory [7], cardioprotective [8] and neuroprotective [9] properties. Other important phenolics in olives are phenolic acids. Although their occurrence is minor, their therapeutic effect is equally remarkable. Phenolics acids have demonstrated protection against a range of diseases, including cancer, heart diseases and diabetes [10,11]. These positive effects have been mainly attributed to their antioxidant properties.

Nowadays the awareness of the relationship between diet and health has led to a search for functional foods to prevent naturally nutrition-related diseases. In this regard, it has been demonstrated that the chemical elicitation of plant foods is an interesting technique to develop functional foods by means of the enrichment in bioactive compounds.

Various elicitors have been reported to induce bioactive compound production in plant foods; among them methyl jasmonate (MJ) is considered to be particularly effective.

In the past few years we have focused our research on the development of plant foods enriched in phenolics by using the exposition of the food to chemical elicitors. We have mainly studied MJ effects on berries and potato [12,13,14]. More recently we have also investigated MJ effect on fatty acids and phenolic acids in olive fruits [15]. These studies were mostly centered on the postharvest treatment which implies the application of the elicitor over the food immediately after harvest. However, occasional works have also been accomplished on the pre-harvest elicitation effect, in other words application of the elicitor to the plant before harvest instead of to the food. Besides, pre-harvest treatments possess the addition advantage of protecting the plant from chilling injuries and diseases [16].

The goal of this work was to investigate the effect of the pre-harvest MJ application on the phenolic fraction of olive fruits. For that purpose, we sprayed MJ to olive trees

during the ripening process of the olive fruits. Our final intention was to obtain olive fruits enriched in phenolic compounds with antioxidant properties. We evaluated MJ effects on the total phenolic content (TPC), free radical scavenging assay and the contents of some relevant phenolic compounds (ie, oleuropein, hydroxytyrosol and phenolic acids). The results were compared with those obtained from olive fruits picked from untreated olive trees, which were used as controls. Two olive cultivars (*Arbequina* and *Picual*) were included in our study to consider the varietal influence on MJ effect.

# 2. Materials and Method

## 2.1. Samples and Chemicals

HPLC-grade MeOH was supplied by VWR Inc. (Bridgeport, PA, USA). Ultrapure water was collected from a purification system (Millipore Milford, MA, USA). Acetic acid was obtained from Probus (Barcelona, Madrid). MJ was purchased from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate and Folin-Ciocalteu reagent were supplied by Merck (Darmstadt, Germany). Oleuropein, hyrdroxytyrosol, 1,1-diphenyl-2-picrylhydrazil (DPPH) and phenolic acid standards (i.e., gallic, vanillic, *p*-coumaric, caffeic, chlorogenic, and ferulic acids) were acquired by Sigma-Aldrich (Steinheim, Germany). Olive fruits (*Arbequina* and *Picual* cultivars) were hand-picked from the trees in November and December 2016 in the University of Extremadura (Badajoz, Spain). Only undamaged fruits without any kind of infection of physical injury were selected for the experiments. All fruits exhibited the same maturity stage. After harvesting, the olive fruits, from both untreated and treated trees, were immediately frozen at -80℃ up to the analysis, as explained below.

## 2.2. Plan Material and MJ Treatment

Twenty-year-old olive tree (*Olea europaea* L.) of two cultivars, *Arbequina* and *Picual*, grown under trip irrigation and fertirrigation (irrigation with suitable fertilizers in the solution) in the same orchard near Badajoz (Spain) under the same agronomical and environmental condition were studied.

Treatments were performed on olive trees (5 per treatment) of *Arbequina* and *Picual* cultivars. Two branches per tree were selected for experiments for uniform size and fruit load. For each treatment, 5 branches (1 branch for tree) were sprayed with a solution of 500 mg/L of MJ in ethanol and the other 5 branches (1 branch for tree) were not sprayed at all to be used as controls. A 500 mL volume of MJ solution was applied per branch at the time of harvest. To avoid contamination during spraying, at least one guard tree was used to separate each of the test trees, and the trees were sprayed with the solutions only when there was a weak and no wind. For the purposes of this study, olive fruit samples of each cultivar were collected from each tree (300 fruits) on days 3 and 6 after treatments. A total of 1500 fruits were collected for each treatment and time point of each cultivar, and were immediately frozen in liquid nitrogen and stored at -80℃ until analysis.

## 2.3. Analysis of Phenolic Compounds

### 2.3.1. Extraction

Phenolic composition was examined in untreated and MJ treated olive fruits. The extraction was performed on the basis of the method described in the literature [17] with slight modifications. First, a 60 mL-volume of 80:20 (v/v) methanol:water was added to a 5 g-weight of de-stones olive sample. Then, the mixture was homogenized by using and Ultraturrax (IKA, Sigma-Aldrich, Madrid, Spain) and subsequently centrifuged at 1500 rpm for 10 min at room temperature. The supernatant was filtered through filter paper. An additional 60 mL of methanol:water was added to the extract, which was re-extracted. After that, 30 mL of hexane was added to the resulting extract to eliminate the remaining oil. Once discharged the hexane layer, the combined methanolic extracts were collected, filtered through Whatman No. 1 filter paper and analyzed by HPLC as detailed below. Extractions of each single sample including controls and olives treated with MJ were accomplished in duplicate. As detailed below, the extracts obtained were used for the free radical scavenging assay and to determine TPC and contents of oleuropein, hydroxytyrosol and phenolic acids.

### 2.3.2. Determination of TPC

A Beckman Coulter DU-800 spectrophotometer (Barcelona, Spain) was used to perform the measurements. The determination of TPC was carried out by following the method described elsewhere [18]. Basically, the method is based on the oxidation of the hydroxyl groups of phenols in basic media by the Folin-Ciocalteu reagent. A 0.1-mL volume of the extract, 0.5 mL of Folin-Ciocalteu reagent and 10 mL of sodium carbonate solution (75 g/L) were mixed, and the volume was made up to 25 mL with distilled water. After 1 h, the absorbance was measured at 750 nm against a blank prepared in the same way but without adding the reagent. Gallic acid was used as the standard to prepare the calibration curve. The results were expressed as milligrams of gallic acid equivalents per kg of olive fruit. Analyses were performed in triplicate.

### 2.3.3. 1,1-Diphenyl-2-picrylhydrazyl Free Radical (DPPH*) Scavenging Assay

The same equipment as that used for TPC was used for DPPH measurements. The ability of the extracts to scavenge DPPH* radicals was performed according to a slight modification of the method elsewhere developed [19]. Each extract was further diluted to final concentrations of 15.6, 62.5, 125, 250 and 500 µg/mL before being transferred to a 96-well microliter plate. Each extract solution, before adding DPPH, was used as a blank. Each well contained 50 µL aliquot of the sample and 150 µL of DPPH (400 µM). Decrease of absorbance, with respect to DPPH solution measured immediately, was monitored at 517 nm after 30 min of incubation at 37℃. The percentage inhibition of the DPPH by each dilution of samples was calculated considering the percentage of the steady DPPH in solution after reaction. Results were expressed as the concentration of extracts that gives rise to a 50% reduction in the DPPH. The experiments were performed in triplicate.

#### 2.3.4. Determination of Oleuropein and Hydroxytyrosol

A Konik-Tech model 560 (Barcelona, Spain) liquid chromatograph fitted with a manual injection valve (model 7725i, Konik-Tech, Barcelona, Spain) and having a 20 µl sample loop was used for the analyses. The separation was accomplished on a ODS reverse phase (C18) column (250 nm × 4.6 mm i.d., 5 µm particle size, ACE, Madrid, Spain). A mixture of water/acetic acid (95/5, v/v) and methanol were used as solvents A and B, respectively and the flow rate was 1 mL/min. A linear gradient was programmed as follows: initial composition 95/5% A/B, 85/15 A/B at 3 min, 80/20 A/B at 13 min, 75/25 A/B at 25 min, 70/30 A/B at 35 min, 65/35 A/B at 40 min, 60/40 A/B at 45 min, 55/45 A/B at 47 min, 53/47 A/B at 50 min, 52/48 A/B at 60 min, 50/50 A/B at 64 min, 50/50 A/B at 70 min, 95/5 A/B at 75 min. Chromatograms were recorded at 280 nm. Blanks between consecutive runs were performed to assure the washing of the equipment. Three HPLC runs were performed for each single extract. Stock solutions of the standard compounds were prepared in 70% (v/v) methanol to final concentration of 1 mg/mL. Each stock solution was further diluted to obtain six concentrations of the standard. Calibration curves of the standards were established on six data points, and each standard dilution was injected in triplicate. Peak areas for the extracts and standards were integrated by use of Konikrom Plus (KNK-725-240). Analyses were performed in triplicate.

#### 2.3.5. Determination of Phenolic Acids

The equipment and method used were the same as the one described above for oleuropein and hydroxytyrosol. Chromatograms were recorded at two different wavelengths. Gallic acid and vanillic acid were detected at 280 nm whereas caffeic, *p*-coumaric, ferulic and chlorogenic acids were measured at 320 nm. Blanks between consecutive runs were performed to assure the washing of the equipment. Three HPLC runs were performed for each single extract. Stock solutions of the standard compounds were prepared in 70% (v/v) methanol to final concentration of 1 mg/mL. Each stock solution was further diluted to obtain six concentrations of the standard. Calibration curves of the standards were established on six data points, and each standard dilution was injected in triplicate. Peak areas for the extracts and standards were integrated by use of Konikrom Plus (KNK-725-240). Analyses were performed in triplicate

### 2.4. Statistical Analysis

The results are presented as the average of the all values obtained and standard deviation (± SD). The two varieties *Arbequina* and *Picual* are included in the statistical analysis. The data were analyzed using one-way analysis of variance (ANOVA), and differences were considered significant at $p < 0.05$.

## 3. Results and Discussion

### 3.1. TPC and DPPH Activity

Table 1 represents the TPC, expressed as mg gallic acid $kg^{-1}$ in olives fruits picked from olive trees untreated (control) and treated with MJ. Olive samples were collected on two different days, day 3 and day 6 after treatment. Data provided by two different varieties, *Arbequina* and *Picual*, were included in our study to evaluate the varietal influence on the MJ effect. Statistical analysis was carried out between control and treated samples and between *Arbequina* and *Picual* varieties. Data on days 3 and 6 were not statistically compared.

From Table 1, TPCs ranged from 127.71 to 472.23 mg gallic acid $kg^{-1}$, which is in accordance with data published in olives in the literature [20]. By comparing controls from *Arbequina* and *Picual*, differences between cultivars were found. In particular, *Picual* exhibited significantly ($p < 0.05$) higher TPCs than *Arbequina* on both days 3 and 6. This observation supports bibliographic reports which have already described varietal differences in the phenolic content in olives [21].

It is also interesting to note that, although data on days 3 and 6 were not statistically compared, the decline of the composition of TPC overtime in both *Arbequina* and *Picual* varieties was apparent. Particularly, TPC on day 3 were 265.61 mg gallic acid $kg^{-1}$ in *Arbequina vs* 472.23 mg gallic acid $kg^{-1}$ in *Picual*. In the same way 155.89 mg gallic acid $kg^{-1}$ were measured in *Arbequina vs* 338.27 in *Picual* on day 6. Decrease in TPC as ripening progresses has been reported in the literature [22]. These values might indicate that olives on day 6 were picked close to overripe and, therefore, day 3 would be more recommendable as a picking day.

As far as the MJ treatment effect is concerned, different responses to MJ were observed in olives according to the variety. As seen, TPC increased significantly ($p < 0.05$) in *Arbequina* samples after the treatment on both harvesting days (ie, day 3 and 6). By contrast, for *Picual* the MJ treatment resulted in a significant ($p < 0.05$) decrease of TPC, particularly on day 6 (ie, from 338.27 to 127.71 mg gallic acid $kg^{-1}$). MJ is a phytohormone that regulates relevant metabolic processes and may bring about opposite effects on polyphenol metabolism in plant foods. On the one hand, MJ is involved in the ethylene production in such a way that accelerates the ripening process, which, in turn, conducts to the drop of the polyphenol content. On the other hand, it is already known the promoting MJ effect on phenyl-alanine lyase (PAL), which is the first enzyme regulating the bioformation of phenolics through phenylpropanoid pathway. In particular, PAL catalyzes the formation of cinnamic acid from phenylalanine and then cinnamic acid is transformed in naringenin, which is subsequently converted into different flavonoids including phenolic acids [23,24]. In view of our results, the activation of PAL as a result of the preharvest MJ application to olive trees prevailed over ethylene production for *Arbequina* samples whereas the stimulation of ripening process was clearly predominant over the induction of the polyphenol biosynthesis by PAL activation for *Picual* olives. This finding is in accordance with metabolic differences previously observed in *Arbequina* and *Picual*. In particular, both cultivars have shown different ripening behavior. Whereas *Arbequina* does not continue to ripen after harvest, *Picual* olives are capable of ripening during the postharvest period (data submitted for publication). The different response of both cultivars to MJ in terms of TPC suggests higher susceptibility of *Picual* to the ethylene production.

**Table 1. Total phenol content (mg gallic acid $kg^{-1}$) in olive fruits from *Arbequina* and *Picual* olive trees (*Olea europaea L.*) untreated-control and treated with MJ. Olive samples were picked on days 3 and 6 after MJ treatment**

| TOTAL PHENOLS (mg gallic acid $kg^{-1}$) | ARBEQUINA | PICUAL |
|---|---|---|
| **DAY 3** | | |
| *CONTROL* | 265.61 ± 0.23Aa | 472.23 ± 0.65Ba |
| *MJ TREATED* | 430.55 ± 0.35Ab | 380.10 ± 0.54Bb |
| **DAY 6** | | |
| *CONTROL* | 155.89 ± 0.38Aa | 338.27 ± 0.55Ba |
| *MJ TREATED* | 434.22 ± 0.46Ab | 127.71 ± 0.68Bb |

Data are presented as means ($n$=3) ± SD, where $n$ refers to three independent samples.
Different upper-case letters in the same row in control samples between cultivars indicate differences at $p < 0.05$
Different lower-case letters in the same column between control and MJ treated samples within the same cultivar indicate differences at $p < 0.05$.

**Table 2. DPPH scavenging activity expressed as $IC_{50}$ (µg/mL) of olive fruits from *Arbequina* and *Picual* olive trees (*Olea europaea* L.) treated with MJ. Olive samples were picked on days 3 and 6 after MJ application**

| IC50 (µg/mL) | *ARBEQUINA* | *PICUAL* |
|---|---|---|
| **DAY 3** | | |
| *CONTROL* | 514.36 ± 1.02Aa | 539.12 ± 1.13Aa |
| *MJ TREATED* | 1125.46 ± 0.86Ab | 638.57 ± 0.92Bb |
| **DAY 6** | | |
| *CONTROL* | 585.02 ± 0.78Aa | 611.98 ± 0.96Aa |
| *MJ TREATED* | 343.67± 0.89Ab | 1146.55 ± 0.74Bb |

Data are presented as means ($n$=3) ± SD, where $n$ refers to three independent samples.
Different upper-case letters in the same row in control samples between cultivars indicate differences at $p < 0.05$
Different lower-case letters in the same column between control and MJ treated samples within the same cultivar indicate differences at $p < 0.05$.

Table 2 represents the $IC_{50}$ values, expressed as µg/mL, of olive fruits from *Arbequina* and *Picual* olive trees (*Olea europaea* L.) treated with MJ. Data from olives picked on days 3 and 6 after pre-harvest MJ application were included in the table, although they were not in the statistical comparison. As seen in the table, $IC_{50}$ measured in control olives were statistically similar ($p > 0.05$) for both varieties. In particular values of 514.36 and 539.12 µg/mL were obtained on day 3 and 585.02 and 611.98 µg/mL on day 6 for *Arbequina* and *Picual* respectively. Therefore, the radical scavenging activity was not found to depend on the cultivar.

Regarding the MJ effect, differences in $IC_{50}$ values were significant ($p < 0.05$) in MJ treated samples as compared with controls for both varieties. Specifically, *Arbequina* olives exhibited a significant ($p < 0.05$) increase in the radical scavenging activity after pre-harvest MJ application on day 3 (1125.46 *vs* 514.36 µg/mL). By contrast, the radical scavenging activity declined significantly ($p > 0.05$) in MJ treated olives on day 6 (343.67 *vs* 585.02 µg/mL). However, *Picual* olives exerted a significant ($p < 0.05$) increase in the radical scavenging activity on both days 3 and 6 as a result of the exposition of olive trees to MJ, although this increase was more pronounced on day 6 (1146.55 µg/mL in MJ treated olives *vs* 611.98 µg/mL in controls). These results reflect, once more, the different metabolic processes during the ripening process for both *Arbequina* and *Picual* cultivars.

A possible correlation between TPC and the DPPH activity was investigated. As a result, the linear regressions corresponding to TPC and DPPH data obtained were $y = 0.6467x + 434.17$ ($r^2 = 0.0674$) for *Arbequina* and $y = -1.8353x + 1338.9$ ($r^2 = 0.9246$) for *Picual*. As observed, a linear correlation between TPC and DPPH activity could not be established either for *Arbequina* or *Picual*. This lack of linearity was more visible for *Picual* olives since the effectiveness of pre-harvest MJ treatment in terms of DPPH activity (see Table 2) was noteworthy despite the decrease in TPC in MJ treated olives (see Table 1). The discrepancy between TPC and DPPH activity suggests the presence of some potent antioxidants other than those here studied in MJ treated olives whose content is low enough not to affect TPC. It is necessary to bear in mind that the biological activity is not directly related to concentration. Actually, some minor constituents are usually described as particularly active compounds in a number of areas [25]. Structural elucidation studies in combination with free radical scavenging assays are currently scheduled to get an insight of structure-activity relation in olive phenolics other than those here considered.

## 3.2. Oleuropein and Hydroxytyrosol

Table 3 depicts the oleuropein content expressed as mg $kg^{-1}$ weight in olive fruits from *Arbequina* and *Picual* olive trees (*Olea europaea* L.) untreated-control and treated with MJ. Data obtained from fruits picked on days 3 and 6 after the treatment are also represented although, as already mentioned, they were not considered in the statistical study. Oleuropein contents here found are in general lower than those reported in olive fruits by other authors [20]. This can be due to a number of factors such as geographical aspects, agronomic conditions, among others. By comparing controls of *Arbequina* and *Picual*, the oleuropein content was statistically ($p > 0.05$) similar in both varieties on day 3 (129.96 and 122.59 mg $kg^{-1}$ for *Arbequina* and *Picual* respectively). However, measurements on day 6 exhibited higher oleuropein content in *Picual* than in *Arbequina*. Besides, higher contents of oleuropein on day 6 than on day 3 were evident for both cultivars (ie, 220.45 mg $kg^{-1}$ and 348 mg $kg^{-1}$ for *Arbequina* and *Picual*, respectively). Bibliographic reports have already described higher contents of oleuropein during the final stages of olive fruit ripening [26]. This supports the above mentioned observation that the day 6 represents the final of the olive ripening process, close to overripe. It is also interesting that, despite oleuropein is a major phenolic in olive, the increase of the oleuropein content on day 6 does not correlate with the drop in TPC on that same day. This fact can be attributed to relevant contributions of phenolics other than oleuropein to TPC. These authors have also reported no direct relation between the trend of oleuropein and TPC during the fruit ripening process [26]. In particular, these authors have found that during the first stages of olive ripening process, oleuropein content starts increasing whereas no change in TPC is observed. Nevertheless, they always found coincidence between enzymatic activity and oleuropein concentration.

**Table 3. Oleuropein contents (expressed as mg kg$^{-1}$ weight ± SD) in olive fruits from *Arbequina* and *Picual* olive trees (*Olea europaea* L.) treated with MJ. Data from olive samples picked on days 3 and 6 after MJ application are included**

| OLEUROPEIN CONTENT (mg kg$^{-1}$) | *ARBEQUINA* | | *PICUAL* | |
|---|---|---|---|---|
| | *CONTROL* | *MJ TREATED* | *CONTROL* | *MJ TREATED* |
| **DAY 3** | 129.96 ± 1.52Aa | 121.00 ± 1.93Aa | 122.59 ± 0.96Aa | 139.26 ± 1.76Aa |
| **DAY 6** | 220.45 ± 2.05Aa | 159.15 ± 1.35Ab | 348.00 ± 1.83Ba | 576.38 ± 1.45Bb |

Data are presented as means ($n$=3) ± SD, where $n$ refers to three independent samples.
Different upper-case letters in the same row in control samples between cultivars indicate differences at $p < 0.05$
Different lower-case letters in the same row between control and MJ treated samples within the same cultivar indicate differences at $p < 0.05$

**Table 4. Hydroxytyrosol contents (expressed as mg kg$^{-1}$ weight ± SD) in olive fruits from *Arbequina* and *Picual* olive trees (*Olea europaea* L.) treated with MJ. Data from olive samples picked on days 3 and 6 after MJ application are included**

| HYDROXYTYROSOL CONTENT (mg kg$^{-1}$) | *ARBEQUINA* | | *PICUAL* | |
|---|---|---|---|---|
| | *CONTROL* | *MJ TREATED* | *CONTROL* | *MJ TREATED* |
| **DAY 3** | 146.92±1.23Aa | 169.49±1.95Aa | 161.92±1.72Aa | 271.03±1.63Bb |
| **DAY 6** | 229.72±0.98Aa | 150.76 ± 2.01b | 268.24±1.88Aa | n.d. |

Data are presented as means ($n$=3) ± SD, where $n$ refers to three independent samples.
Different upper-case letters in the same row in control samples between cultivars indicate differences at $p < 0.05$
Different lower-case letters in the same row between control and MJ treated samples within the same cultivar indicate differences at $p < 0.05$.

Surprisingly, the exposition of *Arbequina* and *Picual* trees to MJ did not provide in general significantly ($p < 0.05$) higher oleuropein contents. In fact, oleuropein content on day 6 decreased from 220.45 to 159.15 mg kg$^{-1}$ after MJ treatment in *Arbequina*. An exception was *Picual* olives picked on day 6 whose content increased from 348.00 to 576.38 mg kg$^{-1}$ after pre-harvest treatment. Since PAL enzyme has been demonstrated to be activated by MJ [24,26], two possibilities are considered to explain the results on oleuropein content here found. Firstly, MJ might not have any effect on the oleuropein metabolism when pre-harvest applied. Alternatively, MJ might be activating PAL enzyme and, at the same time, inhibiting specific enzymes (ie, polyphenol oxidase, PPO) regulating the formation of oleuropein further in the phenylpropananoid pathway [26].

Table 4 represents the hydroxytyrosol content expressed as mg kg$^{-1}$ weight in olive fruits from *Arbequina* and *Picual* olive trees (*Olea europaea* L.) untreated-control and treated with MJ. Data obtained from samples picked on days 3 and 6 after MJ application are also shown. From Table 4, varietal differences in hydroxytyrosol contents in olive controls were not found. Values of 146.9 and 161.92 mg kg$^{-1}$ were estimated on day 3 and 229.72 and 268.24 mg kg$^{-1}$ on day 6 for *Arbequina* and *Picual*, respectively. Similarly to oleuropein, contents of hydroxytyrosol in controls on day 6 seemed to be higher than those on day 3 for both cultivars (compare Table 3 and 4). Concerning the MJ effect, the hydroxytyrosol content was not in general terms significantly ($p > 0.05$) increased by the pre-harvest MJ treatment. Actually, values on day 6 exhibited significant ($p < 0.05$) decreases of the hydroxytyrosol content as a result of MJ treatment (from 229.72 to 150.76 mg kg$^{-1}$ for *Arbequina* and from 268.24 mg kg$^{-1}$ to even not detected for *Picual*). As an exception, *Picual* olives treated with MJ and picked on day 3 showed an increase of hydroxytyrosol content after MJ application (ie, 271.03 mg kg$^{-1}$ in treated *vs* 161.92 mg kg$^{-1}$ in controls). It is important to point out that hydroxytyrosol is a product derived from hydrolysis of oleuropein [27]. For this reason, similar evolution during the ripening process and similar response to MJ is somehow expected.

## 3.3. Phenolic Acids

Table 5 and Table 6 summarize the phenolic acid contents (expressed as mg kg$^{-1}$ weight ± standard deviation) in olive fruits from MJ treated *Arbequina* and *Picual* olive trees (*Olea europeae* L.), respectively. Data from olive fruits picked on days 3 and 6 after MJ treatment are also represented in both tables.

As seen in Table 5 and Table 6 the contents of phenolic acids varied in a wide range in control olive fruits (ie, from 1.1 to 75.3 mg kg$^{-1}$). These values are in the same range as data reported by other authors [20] as well as values found by ourselves in a previous study carried out in our laboratory [15]. However, it is worthy to note that *Picual* (see Table 6) exhibited lower contents than *Arbequina* (see Table 5). As also commented for TPC, varietal differences in phenolics are usual in plant-derived foods. In addition, differences in phenolic acid contents can also indicate a slightly different maturity stage between *Arbequina* and *Picual*. Interestingly, the phenolic acid contents in controls were similar on days 3 and 6 within each cultivar. For instance, gallic acid exhibited 10.5 mg kg$^{-1}$ on day 3 and 13.2 mg kg$^{1}$ on day 6 in untreated *Arbequina* olives (Table 5). Similarly, its content in untreated *Picual* olives was 2.1 and 3.1mg kg$^{-1}$ on days 3 and 6, respectively.

As far as the MJ effect is concerned, *Arbequina* olives exhibited in general a significantly ($p < 0.05$) drop in the content of phenolic acids in treated olives whereas no MJ effect was determined on phenolic acid content in *Picual* olives. This finding is in disagreement with the increase in phenolic acid contents observed in an earlier study on the postharvest MJ effect [15]. The discrepancy between pre-harvest and post-harvest MJ effect in the phenolic acid content can be explained by differences in phenolic acid metabolism between the olive tree and the olive fruit. In

other words, it is believed that PAL enzyme is affected differently depending on the moment in which the elicitor is applied. This theory explains results obtained for *Picual* cultivar since TPC decreased in pre-harvest MJ treated olives, which would indicate inhibitory effect of MJ on PAL when applied pre-harvest. However, this theory does not justify the increase of TPC in *Arbequina* treated samples, which reflects that PAL enzyme is always activated by exogenous MJ regardless the moment of its application.

In view of these results, varietal differences on pre-harvest MJ effect on olive phenolics are concluded. For *Arbequina*, MJ activates PAL, which in turn increases TPC, and at the same time, MJ inhibits other specific enzymes in the phenylpropanoid pathway responsible for bioformation of phenolic acids. For example, it is likely that MJ inhibits certain enzymes belonging PPOs, such as phenolasa and catechol-O-methyl transferasa, regulating the bioformation of caffeic acid and ferulic acid [28,29]. In contrast to *Arbequina*, MJ inhibits PAL activity in *Picual* olives resulting in a decrease of TPC. The inhibition of PAL leads to a decrease of cinnamic acid, which is a primary precursor in the pathway. This slows down the metabolism of the rest of phenolic compounds further in the pathway. On the other hand, it is interesting to note that phenolic acid content is not statistically changed in *Picual* by pre-harvest MJ. This involves that the specific enzymes regulating the last steps to the bioformation of phenolic acids are not inhibited by MJ. This is not the first time that varietal differences in the MJ treatment effects are observed. Bibliographic reports have proven that exogenous MJ effect can differ according to the variety in pak choi and plums [30,31]. In summary, the exogenous MJ application to olive tree affects differently to the phenolic composition in olive fruits according to the cultivar. In *Arbequina*, pre-harvest MJ results in increase of TPC and decrease of oleuropein, hydroxytyrosol and phenolic acids. From these results, increment of olive phenolics other than those studied in the present work (ie, tyrosol, cinnamic acid, luteolin…) as a consequence of the pre-harvest MJ application must be the reason for the increase of TPC. Contrary to *Arbequina*, the pre-harvest MJ application in *Picual* brought about the decline of TPC, oleuropein and hydroxytyrosol. No effect on phenolic acid content was however observed in *Picual* olives. On the other hand, it is convenient to highlight that the free radical scavenging activity of olive fruits increased with the pre-harvest MJ treatment for both *Arbequina* and *Picual* cultivars.

Concluding, the exposition of olive trees to MJ vapor in the right moment of the fruit ripening process provides olive fruits with better antioxidant properties. The effect of pre-harvest MJ in olives is cultivar dependent. That involves that the MJ influences varies with the olive cultivar and the specific phenolic compound studied. This is the first time that olive trees are elicited with the aim of improving the characteristics of olive fruits. The results found in the present study are promising, particularly, taking into account that the pre-harvest MJ treatment additionally protects the olive tree from diseases and plagues. The purpose now is to extent the study to extra olive phenolics and elicitation conditions to develop an effective and productive agronomic procedure to enhance the antioxidant properties in olive fruits.

**Table 5. Phenolic acid contents (expressed as mg kg$^{-1}$ weight ± SD) in olive fruits from *Arbequina* olive trees (*Olea europaea* L.) treated with MJ. Data from olive samples picked on days 3 and 6 after the MJ application are included**

| SAMPLES | Gallic Acid | Chlorogenic Acid | Vanillic Acid | Caffeic Acid | *p*-Coumaric Acid | Ferulic Acid |
|---|---|---|---|---|---|---|
| DAY 3 | | | | | | |
| *CONTROL* | 10.5±0.2a | 28.6±0.1a | 46.5±0.1a | 6.9±0.1a | 5.6±0.1a | 75.3±0.2a |
| *MJ TREATED* | 23.7±0.1b | n.d. | 27.2±0.1b | n.d. | 1.8±0.2b | 15.2±0.2b |
| DAY 6 | | | | | | |
| *CONTROL* | 13.2±0.1a | 20.4±0.2a | 41.8±0.3a | 4.0±0.2a | 18.1±0.2a | 52.4±0.1a |
| *MJ TREATED* | 18.1±0.01b | 4.6±0.1b | 10.3±0.2b | 7.5±0.1a | 4.3±0.1b | 20.1±0.3b |

Data are presented as means ($n$=3) ± SD, where $n$ refers to three independent samples.
Different lower-case letters in the same column between control and MJ treated samples within the same compound indicate differences at $p < 0.05$

**Table 6. Phenolic acid contents (expressed as mg kg$^{-1}$ weight ± SD) in olive fruits from *Picual* olive trees (*Olea europaea* L.) treated with MJ. Data from olive samples picked on days 3 and 6 after MJ application are included**

| **SAMPLES** | **Gallic Acid** | **Chlorogenic Acid** | **VanillicAcid** | **Caffeic Acid** | ***p*-Coumaric Acid** | **Ferulic Acid** |
|---|---|---|---|---|---|---|
| DAY 3 | | | | | | |
| *CONTROL* | 2.1±0.2a | n.d. | 1.3±0.1a | 1.1±0.1a | 1.8±0.3a | 1.1±0.1a |
| *MJ TREATED* | 5.1±0.3a | 2.2±0.1a | 5.7±0.1a | 3.4±0.2a | 3.8±0.1a | 5.1±0.1a |
| DAY 6 | | | | | | |
| *CONTROL* | 3.1±0.1a | 1.2±0.1a | 4.2±0.2a | 2.8±0.3a | 1.7±0.1a | 1.8±0.2a |
| *MJ TREATED* | 6.2±0.2a | 4.6±0.4a | 7.3±0.1a | 4.5±0.2a | 5.5±0.2a | 4.2±0.2a |

Data are presented as means ($n$=3) ± SD, where $n$ refers to three independent samples.
Different lower-case letters in the same column between control and MJ treated samples within the same compound indicate differences at $p < 0.05$.

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