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ARTICLE

# Fruit Quality and Olive Leaf and Stone Addition Affect Picual Virgin Olive Oil Triterpenic Content

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The present research aimed to evaluate whether Picual virgin olive oil triterpenic compounds are affected by the addition of variable quantities of stones and leaves before processing or by fruit resting on the ground during 3 months. Results showed that stone addition did not influence triterpenic dialcohol content (uvaol and erythrodiol), whereas triterpenic acids (oleanolic and maslinic) increased significantly when 20 and 30% stones were added. Leaves added at 2% increased significantly oleanolic acid, maslinic acid, and erythrodiol content by 83, 41, and 36%, respectively. During fruit resting on the ground, olive oils showed no differences in uvaol content, a slight increase in erythrodiol, and a gradual increase in both oleanolic and maslinic acids, obtaining at the end of the experiment contents nearly 10- and 3-fold higher than control oils. These results confirm that olive oil triterpenic composition is modified by the factors analyzed.

**KEYWORDS: Picual olives; olive stone; olive leaf; olive oil; ground-picked olives; quality; triterpenic dialcohols; triterpenic acids**

## INTRODUCTION

Triterpenoids are natural compounds that are widely distributed throughout the plant kingdom, *Olea europaea* L. included. These natural compounds have long been used for medicinal purposes in many Asian countries (*1*) and even now are still showing attractive interests due to their multiple biological properties (*2*−*6*). Oleanolic, ursolic, and maslinic acids as well as uvaol and erythrodiol dialcohols constitute the most abundant triterpenes in *O. europaea* L. These compounds are mainly located in the skin of olive fruit (*7*, *8*), although their presence in olive stone has also been reported (*9*, *10*). Therefore, during the virgin olive oil extraction process, these compounds are partially transferred to the oily phase but in only minor amounts (*11*) compared to olive pomace oil, in which higher levels are found (*12*, *13*). Nevertheless, according to EU regulation (*14*), pomace oil cannot be consumed directly and needs to be refined, thus removing the triterpenoids (*12*, *13*).

Given the important health properties of these compounds and their unique presence in virgin olive oil, research on factors that improve their content is essential. In previous papers, we have reported that virgin olive oil triterpenic concentration is cultivar dependent (*11*) and that, according to the olive cultivar, triterpenic content may be improved by regulating some variables of the extraction process (*15*). The great influence of olive cultivar may be attributed in part to fruit characteristics such as size and flesh/stone ratio because skin and stone constitute the main sources of these compounds. Furthermore, fruits picked from the tree can include variable amounts of leaves and small twigs that are crushed with olives, raising the in virgin olive oil green color and volatile content (*16*). Virgin olive oil triterpenic content may be also changed because of the abundance of these compounds in olive leaf (*17*−*22*).

On the other hand, olive fruits can fall on the ground and remain there for variable periods of time until harvesting, giving poor-quality oils. It has been reported that the triterpenic content increases as the quality of the oil decreases (*12*, *23*); however, there are no available data about changes on triterpenes during olive fruit resting on the ground.

The present study was undertaken (i) to investigate whether the addition of stones and leaves before the crushing step influences virgin olive oil triterpenic content and (ii) to study the effect of fruit resting on the ground on the final olive oil triterpenic content. All experiments were performed on the Picual cultivar, which is most widely cultivated in Spain.

## MATERIALS AND METHODS

**Plant Material.** Three olive trees of the Picual cultivar (*O. europaea*, L.), 25 years old, were selected on the basis of uniformity and yield index (between 3 and 4). The trees were spaced 7 × 7 m and grown in the experimental orchard of Centro IFAPA 'Venta del Llano'- Mengibar, Jaen (Spain), using standard growing techniques. The study was carried out during the 2007/2008 crop year.

For the first part of the study, 25 kg of fruits were harvested from the pool of trees by hand at ripening index 3, according to the fruit classification based on skin and flesh color described in the ripening index method (*24*). Olive leaves were also collected by hand at the same time from the olive trees studied.

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For the second part of the study, the ground under the selected olive trees was previously cleaned of old leaves and fallen olives, and then olive fruits were dropped by mechanical shaker, ensuring that no fruits remained on the tree canopy. For this experiment, each olive tree was considered to be an elemental unit.

**Experimental Procedure.** For the first experiment, olive stones were removed from the flesh manually using a depitting machine. Stones were subsequently washed thoroughly under water, dried between sheets of filter paper, and finally incorporated to olive fruits at 20 and 30% by fresh olive weight to include the variability of pulp/stone ratio described for Picual fruits. Olive leaves were washed with water and dried between sheets of filter paper. Leaves were then added to olive fruits at a proportion of 1 or 2% by fresh olive weight (*16*).

For the study of fruit resting on the ground, olives were collected at biweekly intervals, by hand, and immediately processed for oil extraction. Samples harvested from the trees for the other experiments were used as control. The operation was repeated six times.

**Olive Oil Extraction.** Oil extraction was performed using an Abencor laboratory oil mill (Abengoa, Seville). The fruits were crushed in a hammer mill, and the olive paste was kneaded for 30 min at 28 °C and then centrifuged for 1 min at 3500 rpm. The oily must was left for decantation and then filtered. Oils were stored at −20 °C until analysis.

**Determination of Quality Indices.** Free acidity, peroxide values, absorption at 232 and 270 nm ($K_{232}$ and $K_{270}$), and the sum of uvaol and erythrodiol were measured following the analytical methods described in European Regulation EEC 2568/91 (*14*). Free acidity was expressed as percent of oleic acid, peroxide values were expressed as milliequivalents of active oxygen per kilogram of oil (mequiv of $O_2$/kg); $K_{232}$ and $K_{270}$ extinction coefficients were calculated from absorption at 232 and 270 nm, respectively; and the sum of erythrodiol and uvaol was expressed as percent of total sterols.

**Determination of Triterpenic Dialcohols.** The analysis of uvaol and erythrodiol was performed according to EU Regulation 2568/91 (*14*) for the determination of sterols in olive oil. The oil sample was saponified with ethanolic potassium hydroxide solution. The unsaponifiable fraction was removed with ethyl ether, and the sterol fraction was separated by silica gel plate chromatography. Separation and quantification of silylated sterol were performed on a Helwett-Packard instrument model 6890 gas chromatograph, equipped with a HP-5 capillary column (25 m, 0.25 mm i.d., 0.25 $\mu$m of thickness). The working conditions were as follows: oven temperature, 260 °C; injector temperature, 305 °C; split/splitless; FID detector temperature, 330 °C. The injected volume was 1 $\mu$L at a flow rate 1 mL/min, using helium as carrier gas. For quantification, betulin was used as internal standard. The same response factor was considered for both triterpenic dialcohols uvaol and erythrodiol. For each oil sample, analyses were performed in duplicate and results were expressed as milligrams per kilogram.

**Determination of Triterpenic Acids.** The acidic fraction was isolated by solid phase extraction using bonded aminopropyl cartridges, and betulinic acid was added as internal standard according to the method described by Pérez-Camino and Cert (*12*). Then, the extract was evaporated, silylated, and analyzed by gas chromatography. The chromatographic analysis was performed using a Perkin-Elmer gas chromatograph, Autosystem model, fitted with a flame ionization detector and a split injection system (split ratio 1:0.25). Separation was carried out on a HP-5 capillary column (30 m, 0.32 mm i.d, 0.25 $\mu$m of thickness). The operating conditions were as follows: oven temperature, 260 °C for 5 min and then increased at 4 °C/min to 320 °C; injector and detector temperature, 320 °C. Helium was used as carrier gas at a column head pressure of 25 psi. Triterpenic acids were quantified by assuming the same response factor for all triterpenes. For each oil sample, analyses were performed in duplicate and results were expressed as milligrams per kilogram of betulinic acid.

**Statistical Analysis.** Results are expressed as mean values ± standard error (SE). A general variance analysis (ANOVA) was carried out on all data. A two-sided Dunnett's multiple-comparison test was then performed to establish differences between treatments and their respective control ($p \leq 0.05$). These statistical analyses were performed using the program Statistix, version 8.0.

## RESULTS AND DISCUSSION

Addition of olive stones and leaves to olive fruits prior to the crushing step did not affect the oil quality parameters. All of the oils fit within the "extra virgin" category (acidity ≤ 0.8%; peroxide values ≤ 20 mequiv of $O_2$/kg; $K_{270} \leq 0.22$; $K_{232} \leq 2.5$), as stated by EU Regulation 2568/91 (*14*) (data not shown). Moreover, the sum of erythrodiol and uvaol, expressed as sum of percent of total sterols, was checked, showing values below the upper limit of 4.5% set for the extra virgin olive oil category (*14*).

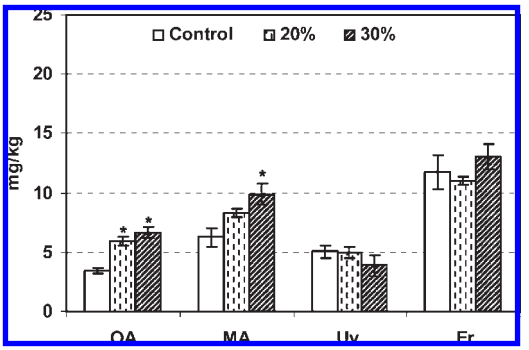


**Figure 1.** Effect of stone addition (%) on the triterpenic compounds of Picual virgin olive oil. OA, oleanolic acid; MA, maslinic acid; Uv, uvaol; Er, erythrodiol. * denotes significant differences versus control for each compound ($p \leq 0.05$).

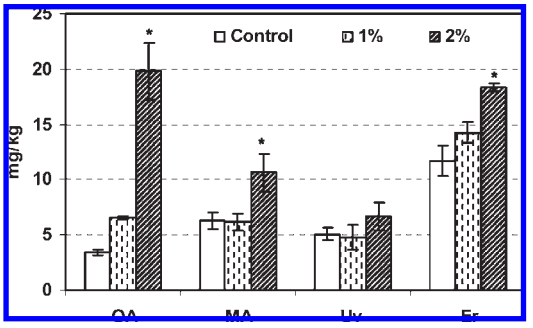


**Figure 2.** Effect of leaf addition (%) on the triterpenic compounds of Picual virgin olive oil. OA, oleanolic acid; MA, maslinic acid; Uv, uvaol; Er, erythrodiol. * denotes significant differences versus control for each compound ($p \leq 0.05$).

However, the addition of olive stones and leaves produced changes in oil triterpenic content. By adding 20 and 30% olive stones, no significant changes were obtained for dialcoholic triterpenes when compared with control (**Figure 1**). Studies about the triterpenic dialcoholic fraction of Greek cultivars have reported that olive stone oil contains lower amounts of erythrodiol and uvaol than those from flesh and skin (*25*). Furthermore, in a study performed in seven Italian olive cultivars, uvaol was not detected in oil seed, whereas erythrodiol was found in smaller quantities in comparison with oil from whole fruits (*10*). All of these data indicate that olive stone contains negligible quantities of uvaol and small amounts of erythrodiol, in agreement with our results. Although not significant, erythrodiol content showed a slight increase, approximately 10% higher than control, when stones were incorporated at 30%.

Stone addition gave an increase in virgin olive oil triterpenic acids (**Figure 1**). When 20 and 30% were included, oleanolic acid rose by 42.5 and 48.3%, respectively. Maslinic acid increased significantly by 36.8% for the highest stone proportion. At 20% a rise was observed, too (24.4%), although it was not significant. In any case, oleanolic acid was transferred more easily than maslinic acid. These findings are in accordance with those reported in the literature as oleanolic acid was found to be the major triterpenic acid in fresh olive husks (*9*, *26*).

**Table 1.** Seasonal Changes in Oil Quality Parameters during Picual Fruit Resting on the Ground[a]

| | acidity (% of oleic acid) | peroxide values (mequiv of $O_2$/kg) | $K_{232}$ (nm) | $K_{270}$ (nm) | % uvaol + erythrodiol |
|---|---|---|---|---|---|
| control | 0.35 ± 0.03 | 6.23 ± 0.32 | 1.40 ± 0.05 | 0.14 ± 0.00 | 1.42 ± 0.14 |
| 15 days | 0.34 ± 0.01 | 7.45 ± 1.07 | 1.51 ± 0.04 | 0.12 ± 0.01 | 1.20 ± 0.06 |
| 30 days | 0.39 ± 0.03 | 8.52 ± 0.35 | 1.55 ± 0.02 | 0.12 ± 0.00 | 1.14 ± 0.07 |
| 45 days | 0.66 ± 0.06 | 8.55 ± 0.78 | 1.49 ± 0.02 | 0.12 ± 0.02 | 1.32 ± 0.06 |
| 60 days | 0.98 ± 0.05 | 9.06 ± 0.96 | 1.50 ± 0.01 | 0.13 ± 0.00 | 1.34 ± 0.08 |
| 75 days | 1.20 ± 0.08 | 10.33 ± 0.57 | 1.50 ± 0.01 | 0.12 ± 0.00 | 1.40 ± 0.04 |
| 90 days | 2.01 ± 0.14 | 10.29 ± 0.38 | 1.43 ± 0.01 | 0.12 ± 0.01 | 1.32 ± 0.06 |

[a] Mean values ± standard error (SE).

With regard to leaf addition, 1% of olive leaves did not produce significant changes in triterpene content compared to reference oil, although there was a trend to increase for both oleanolic acid and erythrodiol. However, when leaves were incorporated at 2%, significant increases ($p \leq 0.05$) in oleanolic acid, maslinic acid, and erythrodiol content were obtained, whereas only a slight rise was observed for uvaol (**Figure 2**). Picual leaf was reported to contain 4.71 mg/kg (dry weight) triterpenic acids (*19*) and 837 mg/kg dialcohols (*20*). Among the triterpenic acids, oleanolic acid was found to be the most abundant (content more than twice that of maslinic acid), whereas for triterpenic dialcohols, uvaol content was greater than that of erythrodiol. In this study, both acids and dialcohols increased their contents for higher leaf percent. Oleanolic acid rise was the most pronounced, being nearly 6-fold higher than control oil, followed by maslinic acid and erythrodiol (respectively, 1.7- and 1.6-fold higher) (**Figure 2**). These results confirm the high concentration of oleanolic acid in Picual leaves (*19*). Moreover, although uvaol content is higher than erythrodiol content in olive leaf, no significant changes were detected in the oils obtained. Ursolic acid was reported to be at trace levels in both Picual virgin olive oil (*11*) and leaf (*19*), which may explain its absence in the oil samples studied.

In conclusion, our experimental results confirm that the addition of stones and leaves to olive fruits before the crushing step enhances Picual virgin olive oil triterpenic content. Addition of olive leaves was more significant than that of olive stones, obtaining total triterpene contents of 55.40 ± 4.18 and 33.39 ± 1.13 mg/kg, respectively, compared to 26.44 ± 2.36 mg/kg in reference oil. Addition of small proportions of leaves can therefore be considered suitable to increase virgin olive oil triterpenic content besides their ability to modify the oils' sensorial characteristics. Screening on leaf composition from different cultivars must be conducted, and studies on the processing of other cultivars should be done.

The second part of the study consisted of studying the effect of olive fruit resting on the ground for 15, 30, 45, 60, 75, and 90 days on oil triterpenic content. Seasonal changes in regulated oil quality parameters are presented in **Table 1**. As can be seen, after 45 days and using only chemical determinations, the oils belonged to the category "extra virgin" as established by European Regulation EEC 2568/91 (*14*). Oils obtained after 60 and 75 days were classified into the category "virgin" (free acidity ≤ 2.0% oleic acid; peroxide values ≤ 20 mequiv of $O_2$/kg; $K_{232} \leq 2.60$; $K_{270} \leq 0.25$). Finally, the oils obtained after 90 days were classified as "lampant" (free acidity > 2.0% oleic acid). **Table 2** shows the contents of oleanolic acid, maslinic acid, uvaol, and erythrodiol for control oils and those obtained at different fruit sampling dates. No changes were observed for uvaol content, whereas a slight increase (22%) was observed for erythrodiol at the end of the experiment. By contrast, both oleanolic and maslinic acids were significantly affected by fruit resting on the ground, showing higher contents as the harvesting date was delayed. After 3 months, oleanolic acid was nearly 10-fold higher than reference, whereas the maslinic acid increase was around 3-fold. Overall, these results indicate that fruit resting on the ground increased Picual olive oil triterpenic fraction, achieving a total content of 70.97 ± 2.01 mg/kg compared to 26.44 ± 2.36 mg/kg in control oil.

**Table 2.** Variation of Oleanolic Acid, Maslinic Acid, Uvaol, and Erythrodiol Contents (Milligrams per Kilogram) of Picual Olive Oils during Fruit Resting on the Ground[a]

| | oleanolic acid | maslinic acid | uvaol | erythrodiol |
|---|---|---|---|---|
| control | 3.41 ± 0.23 | 6.24 ± 0.74 | 5.06 ± 0.57 | 11.73 ± 1.37 |
| 15 days | 10.51 ± 0.61* | 10.33 ± 0.28* | 4.34 ± 0.28 | 12.90 ± 0.31 |
| 30 days | 8.47 ± 0.94* | 6.25 ± 0.41 | 3.87 ± 0.52 | 11.95 ± 0.76 |
| 45 days | 21.16 ± 1.75* | 14.87 ± 0.78* | 5.64 ± 0.30 | 14.31 ± 0.62 |
| 60 days | 25.10 ± 1.27* | 14.28 ± 0.68* | 4.95 ± 0.49 | 14.10 ± 0.72 |
| 75 days | 28.98 ± 0.68* | 14.31 ± 0.39* | 5.56 ± 0.11 | 14.40 ± 0.21 |
| 90 days | 33.59 ± 0.94* | 17.51 ± 0.33* | 4.87 ± 0.29 | 15.00 ± 0.62* |

[a] Mean values ± standard error (SE). * denotes significant differences vs control for each harvesting date ($p \leq 0.05$).

To the authors' knowledge, there have been no studies to date that have investigated the effect of fruit resting on the ground on oil triterpenic content. However, some papers have reported that higher amounts of triterpenes are related to poor-quality oils. Paganuzzi (*23*) found that triterpenic dialcohol (uvaol and erythrodiol) content increases as the oil conservation state worsens, considering quality parameters. Furthermore, Pérez-Camino and Cert (*12*) have reported that triterpenic acid content increases in oils with acidity > 1%, suggesting that hydrolytic processes taking place in the olive fruit facilitate the liberation of these compounds from the olive skin. In our experiment, we found that acidity and triterpenic content increase according to fruit sampling dates (**Tables 1** and **2**). Therefore, we suggest that the rise in triterpenic content may be related to higher acidity values. To confirm this hypothesis and because the oleanolic acid rise was the most pronounced, we studied the relationship between them, obtaining a correlation with an $R^2$ value of 0.9873.

Results obtained in this set of experiments point out that Picual virgin olive oil triterpenic content can be changed by other factors different from that of the extraction process as we recently described (*15*). Addition of small proportions of stones and leaves to olive fruits prior to processing and fruit resting on the ground effectively improved Picual virgin olive oil triterpenic content. Nevertheless, all of these factors can occur naturally. (i) As previously reported, the great influence of olive cultivar on the final virgin olive oil triterpenic content may be attributed in part to fruit characteristics such as pulp/stone ratio that can be changed in part by the growing conditions. Under drought conditions, the pulp/stone ratio increases, thus increasing the oil triterpenic content. (ii) Olive fruits picked either from the tree or from the ground usually include some amount of olive leaves. Noncomplete leaf removal before fruit processing is frequent, giving oils with greater triterpenic content. (iii) Picual olive fruits

are known to ripen early and to have a low resistance to detachment, so a significant amount of the crop is frequently collected from the ground. Consequently, although oil quality decreases slightly, oil triterpenic content increases.

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