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Time Course of Pentacyclic Triterpenoids from Fruits and Leaves of Olive Tree (*Olea europaea* L.) cv. Picual and cv. Cornezuelo during Ripening

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ABSTRACT: Pentacyclic triterpenoids are plant secondary metabolites of great interest for health and disease prevention. HPLC-UV/vis was used to determine the concentration of the pentacyclic triterpenoids present in fruits and leaves of Picual and Cornezuelo olive tree cultivars. Maslinic acid (MA) and oleanolic acid (OA) are the only two compounds present in fruits, MA being the more abundant. In leaves, in addition to MA and OA, uvaol (UO), and erythrodiol (EO) are found, with OA being the most abundant. In this work, the changes in the concentrations of these compounds during ripening as well as the effect of Jaén-style table-olive processing are reported. The amount of MA and OA found in Picual and Cornezuelo olives after processing was 1.26 ± 0.06, 1.30 ± 0.06, 0.31 ± 0.02, and 0.23 ± 0.01 mg per fruit, respectively. These results enable us to calculate the average intake of pentacyclic triterpenoids and reinforce the importance of table olives as a source of healthy compounds.

KEYWORDS: *maslinic acid, oleanolic acid, ursolic acid, uvaol, erythrodiol, Olea europaea, ripening, pentacyclic triterpenoids*

INTRODUCTION

Pentacyclic triterpenes comprise a group of plant secondary metabolites that have important biological properties related to human health and disease prevention. Among them, the main acids are maslinic [(2α,3β)-2,3-dihydroxyolean-12-en-28-oic acid, $C_{30}H_{48}O_4$; molecular mass, 472.7, MA], oleanolic (3β-hydroxy-olean-12-en-28-oic acid, $C_{30}H_{48}O_3$; molecular mass, 456.7, OA), and ursolic (3β-hydroxy-ursan-12-en-28-oic acid, $C_{30}H_{48}O_3$; molecular mass, 456.7, UA), while the main alcohols are erythrodiol (olean-12-ene-3b,28-diol, $C_{30}H_{50}O_2$; molecular mass, 442.7, EO), and uvaol (12-ursen-3-beta,28-diol, $C_{30}H_{50}O_2$; molecular mass, 442.7, UO). These molecules are constituted by 30 carbons that are grouped in five cycles of six carbons with different substituents (Figure 1). These molecules are synthesized via the cytoplasmic acetate/mevalonate pathway from acetyl-CoA, which is converted into units of active isoprene. Triterpenes are synthesized from the condensation of six molecules of active isoprene, and (3*S*)-2,3-oxidosqualene (OS) is a common precursor of all of them.[1] OS is a substrate for various OS cyclases or triterpene cyclases, among them β-amyrin synthase. This enzyme catalyzes the production of β-amyrin, the first pentacyclic triterpenoid of this pathway. Subsequently, EO, OA, and MA are produced from β-amyrin. α-Amyrin is another pentacyclic triterpenoid produced by OS cyclation from which UO and UA are successively synthesized.[1]

Although the biological function of pentacyclic triterpenes in plant metabolism is not clearly understood, they are present in a wide range of plants used in traditional medicine.[2,3] Recently, more of them have been found to exert important effects as antioxidant,[2,4,5] anti-inflammatory,[6] antimicrobial and antiviral,[7−9] and even antitumor agents.[10,11] With respect to this latter aspect, it is of great interest that MA selectively induces apoptosis in human colon-cancer cells[10,11] and that UA inhibits tumorigenesis and tumor promotion while suppressing angiogenesis in human breast-cancer cells.[12] In addition, OA inhibits proliferation and induces apoptosis in osteosarcoma cells,[13] while EO and UO show antioxidant, antiproliferative, and pro-apoptotic effects on human breast-cancer cells.[14] Thus, at present, synthetic triterpenoids and their derivatives are an extremely potent class of new anticancer therapeutic agents, either alone or in combination with conventional chemotherapy in cancers that resist standard agents.[15] For all of these, the knowledge of the presence and concentration of these molecules in food is of great interest.

In previous works, the composition of pentacyclic triterpenoids in olive fruit and leaf of different cultivars has been studied.[16−19] Human intake of olive fruits is traditional in Spain, and Picual and Cornezuelo (also named Cornicabra) are two olive cultivars used in Jaén (S. Spain) for table olives, which constitute a source of pentacyclic triterpenes in the Mediterranean diet. In line with the above studies, the aim of the present work is to identify the pentacyclic triterpenes found in fruits and leaves of the Picual and Cornezuelo cultivars and to determine how the concentration of each changes during ripening. The study of olive-fruit metabolism during ripening is one of our research objectives. In previous studies,[20−22] the metabolism of phenolic compounds was investigated by a HPLC-UV/vis and HPLC-MS analysis. In this work, the time course of triterpenic compounds is analyzed using a similar procedure that was previously used.

Moreover, the effects of the Jaén-style processing of table olive fruits on the concentration of pentacyclic triterpenic acids are studied. The aim is to ascertain the final content of these compounds present in table olives of both cultivars, so as to determine the average human intake of triterpenic compounds. Olive oil and olive fruits are two foods valued for their good properties for human health. The high contents in triterpenic

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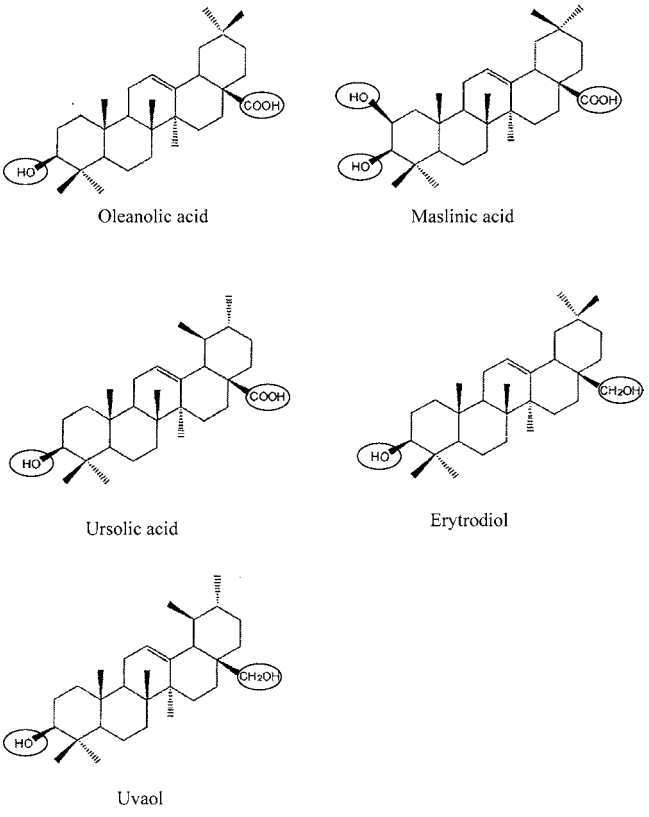

Figure 1. Chemical structure of oleanolic acid, maslinic acid, ursolic acid, erythrodiol, and uvaol. The main functional groups of each compound are marked.

compounds constitute one element that contributes to the health benefits and to stimulate the consumption of this food.

MATERIALS AND METHODS

Chemicals. The chemical compounds used for making the reagents came from Sigma Chemical Co. (St. Louis, USA) and Fluka Chemie GmbH (Buchs, Switzerland). OA, UA, EO, and UO used as standards were purchased from Extrasynthèse (Z.I. Lyon-Nord, Genay, France) and Sigma Chemical Co. (St. Louis, USA). MA was kindly donated by Dr. A. García-Granados, Department of Organic Chemistry, University of Granada, Spain. Specific reagents were of analytical or HPLC grade as required.

Plant Material and Experimental Design. Olive trees cv. Picual and cv. Cornezuelo, 35 years old, were studied in an orchard at Torredonjimeno (Jaén, Spain; 37°45′61″N, 3°57′12″W, 655 m a.s.l.). Cornezuelo trees were located at random between Picual trees. These trees were under traditional rain-fed cultivation. This study was conducted in 3 trees of each cultivar taken at random in the orchard. From each position in each tree, five 25-cm segments of branches with fruits near the apical end were collected. The leaves and fruits were immediately removed and frozen in liquid nitrogen until analyzed. Nine different samples were collected throughout the ripening period of olive fruit, from July to November. Specifically, the samples were picked on days 2 and 29 in July (samples 1,2), 2 and 17 in September (samples 3,4), 1 and 21 in October (samples 5,6), 4 and 18 in November (samples 7,8), and 2 in December of 2010 (sample 9). On each date, the ripeness index (RI) of fruits was calculated using a color evaluation of the skin and flesh proposed by Uceda and Frías.[23] The procedure consisted of distributing 100 olives among 8 groups, according to the following characteristics: group 0, bright green skin; group 1, yellowish green skin; group 2, green skin with reddish spots; group 3, reddish brown skin; group 4, black skin with white flesh; group 5, black skin with lower than 50% of purple flesh; group 6, black skin with more than 50% purple flesh; and group 7, black skin with 100% purple flesh. The RI was determined by the following equation:

$$\mathrm{RI} = \frac{\Sigma(i n_i)}{100}$$

where i is the number of the group and n_i the number of olives in it.

Table 1 shows the time course of the RI over the experiment.

Processing Jaén-Style Table Olives cv. Picual and cv. Cornezuelo. Olive fruits of Cornezuelo (picked on 17 September, sample 4) and Picual (picked on 21 October, sample 6) were processed in the traditional Jaén style of table olives. These stages were selected because it is the date on which the fruits of both cultivars are traditionally processed in Jaén (Spain). Fruits of each cultivar were crushed with a wooden mallet and put into a hermetically sealed 4-L plastic container. Fruits (2.5 kg) were covered with 2 L of tap water. A total of six containers were used, three for each cultivar. For 10 days, the tap water was changed daily until no green pigmentation appeared in the rinsewater. Subsequently, olives were covered with 6% NaCl solution, and then, 5 g of fennel (*Foeniculum vulgare*) and 5 g of thyme (*Thymus zygis*) were added to each container. In this mixture, olives were kept for 3 days before being used for the experiment.

Extraction and Analysis of Triterpenic Compounds of Fruits and Leaves of cv. Picual and cv. Cornezuelo. *Determination of Water Content.* For each ripening stage, samples of leaves and pulp of fruits previously weighed and destoned were finely diced and then pulverized in mortar with liquid nitrogen. The water content of fruits and leaves was determined by weighing 3 g of the organ and then oven drying at 100 °C to constant weight. The samples were cooled for 30 min in a dryer and reweighed.

Extraction of Triterpenic Compounds. For each sample, 0.125 g of dry tissue was mixed with 1.5 mL of methanol/ethanol (1:1, v/v). After vigorous shaking in a Vortex for 1 min and sedimentation, the sample was centrifuged at 7700*g* for 5 min at 4 °C. The supernatant was removed and collected in another tube. The residue was again re-extracted 5 times with the same volume of methanol/ethanol. All supernatants were pooled and evaporated with a SpeedVac concentrator (Thermo Scientific, USA). The residue was dissolved in 1 mL of methanol. This is the phase used for HPLC analysis of triterpenic compounds.

HPLC Analysis. HPLC analysis of triterpenic compounds was made using two chromatographic systems.

Chromatographic System 1: HPLC-UV–Vis. A reverse-phase Spherisorb ODS-2 (Waters Corporation, Milford, USA) (25 cm−4.6 mm, 5 μm) column was used. The Shimadzu HPLC system consisted of two pumps, a column-heater module and a UV−vis detector operated with LC-Solutions software (Shimadzu Corporation, Kyoto, Japan). Separation was achieved by an isocratic elution for 20 min. The solvent used for separation was methanol/water with acetic acid (pH 3.1) at a proportion of 92:8 (v/v). A flow rate of 0.8 mL min^{-1} was used. Absorbance at 210 nm was recorded. Typical chromatograms for fruits and leaves of Picual and Cornezuelo are shown in Figures 2 and 3. MA, OA, EO, and UO were identified and quantified at 210 nm by the external standard method. For this, methanolic extracts of fruits or leaves plus the corresponding standard compounds were used. The total injection volume was 20 μL: 10 μL of methanolic extracts plus 10 μL of the corresponding standard. In all cases, the concentration of the standard compound used for identification was 0.5 mg mL^{-1}. By comparing the chromatograms obtained with and without the standard, each compound (MA, OA, EO, and UO) was identified. The concentration of MA, OA, EO, and UO was determined on the basis of peak area. Standard calibration curves were previously constructed with 10 concentrations of MA (from 0.001 to 1 mg mL^{-1}), OA (from 0.005 to 1 mg mL^{-1}), EO (0.1 to 5 mg mL^{-1}), and UO (from 0.005 to 1 mg mL^{-1}) standards. The equations formulated relating peak areas (y in arbitrary units) and concentrations (x in mg mL^{-1}) were y = 10790536.98x + 38259.17744 for MA; y = 11679048.51x + 92813.66 for OA, y = 9583746.07x + 22392.95953 for EO, and y = 16133897.81x + 72415.1863 for UO. Moreover, a standard calibration curve was constructed with 10 concentrations of ursolic acid (UA, from 0.01 to 1 mg mL^{-1}) standard. The equation

Table 1. Time Courses of the Ripeness Index, Fruit Mass, and Fruit and Leaf Water Content in *Olea europaea* cv. Picual and cv. Cornezuelo during Ripening[a]

sample[b]	July		September		October		November		December
	1	2	3	4	5	6	7	8	9
day	0	27	62	77	92	112	126	141	162
Picual									
ripeness index	0 ± 0.00 a	0 ± 0.00 a	0.25 ± 0.02 b	0.50 ± 0.03 c	0.75 ± 0.05 d	1.72 ± 0.09 e	2.84 ± 0.14 f	3.66 ± 0.18 g	4.86 ± 0.24 h
fruit mass (g)	0.67 ± 0.02 a	2.29 ± 0.13 b	3.00 ± 0.06 c	3.19 ± 0.09 c	3.27 ± 0.30 c	4.37 ± 0.08 d	4.35 ± 0.14 d	4.09 ± 0.24 d	4.58 ± 0.12 d
pulp mass (g)	0.57 ± 0.02 a	1.48 ± 0.08 b	2.21 ± 0.05 c	2.52 ± 0.07 c	2.63 ± 0.13 c	3.63 ± 0.06 de	3.54 ± 0.12 de	3.23 ± 0.16 d	3.87 ± 0.19 e
water content (%)	71.89 ± 3.60 a	73.61 ± 3.70 a	66.41 ± 3.30 a	65.59 ± 3.30 a	60.87 ± 3.00 b	60.74 ± 3.00 b	61.97 ± 3.10 ab	54.74 ± 2.70 b	52.02 ± 2.60 c
leaf water content (%)	53.13 ± 2.60 a	49.28 ± 1.90 a	48.50 ± 1.50 a	48.05 ± 2.00 a	43.02 ± 2.10 b	48.44 ± 2.50 a	51.86 ± 2.40 a	44.15 ± 2.20 ab	45.38 ± 2.30 ab
Cornezuelo									
ripeness index	0 ± 0.00 a	0 ± 0.00 a	0.25 ± 0.01 b	0.50 ± 0.03 c	0.75 ± 0.05 d	1.00 ± 0.04 e	1.15 ± 0.06 e	1.75 ± 0.09 f	2.73 ± 0.14 g
fruit mass (g)	0.97 ± 0.04 a	2.27 ± 0.06 b	2.92 ± 0.09 c	2.75 ± 0.18 c	3.00 ± 0.21 c	3.57 ± 0.07 d	2.83 ± 0.04 c	2.88 ± 0.16 c	2.37 ± 0.10 b
pulp mass (g)	0.65 ± 0.03 a	1.58 ± 0.04 b	2.23 ± 0.07 c	2.21 ± 0.14 c	2.36 ± 0.17 c	2.79 ± 0.11 d	2.22 ± 0.03 c	2.25 ± 0.11 c	1.59 ± 0.08 b
water content (%)	73.31 ± 3.70 ab	80.64 ± 4.00 a	76.48 ± 3.80 ab	72.20 ± 3.60 ab	70.99 ± 3.50 ab	68.04 ± 3.40 b	69.87 ± 3.50 ab	61.14 ± 3.00 bc	53.36 ± 2.70 c
leaf water content (%)	53.58 ± 2.50 a	52.01 ± 2.00 a	52.21 ± 1.80 a	48.86 ± 2.40 ab	44.74 ± 2.20 b	50.45 ± 2.50 a	50.36 ± 1.80 a	44.46 ± 2.00 b	43.89 ± 1.80 b

[a]Values are the means ± standard error of the mean. In each row, values followed by different letters are significantly different ($P < 0.05$). [b]Samples were harvested on nine dates, corresponding to the different ripening states.

formulated was $y = 9309745.471x - 5213.776667$. In all cases, the correlation coefficient (r^2) was 0.99 or above.

Chromatographic System 2: HPLC-MS/MS. This system was used to corroborate the identification of MA and OA provided by chromatographic system 1. The method was developed using an Agilent Series 1100 (Agilent Technologies, Santa Clara, USA) system consisting of a vacuum degasser, an autosampler, a quaternary pump, a diode-array detector, and an ion-trap mass spectrometer Esquire 6000 (Bruker Daltonics, Billerica, USA) equipped with an electrospray ionization source (ESI) operating in negative ion mode. Separation was achieved by isocratic elution using the same column described in chromatographic system 1. The solvent used for separation was methanol/water with 0.1% formic acid (pH 3.1) at a proportion of 92:8 (v/v). A flux of 0.8 mL min^{-1} and a temperature of 35 °C were also used. Ions were detected in an ion-charged control (ICC) (target: 2500 ions) with an accumulation time of 170 ms, using the following operation parameters: capillary exit voltage (fragmentor), −300.0 V; capillary voltage, 4000 V; nebulizer pressure, 60 psig; drying gas, 11 l min^{-1}; and gas temperature, 350 °C. This chromatographic system operates with Bruker Daltonics Data Analysis Software (Bruker Daltonics, Billerica, USA). The fragmentation options used for the MS/MS analyses were energy of 0.8 V, width of 4 m/z, and time of 40 ms. Figure 2 shows the results of these analyses.

Statistical Analysis. The results are expressed as the mean ± SEM. Data were analyzed by one-way or two-way analysis of variance. Differences between means were analyzed by an unpaired Student's t-test. Linear correlations were determined by least-squares regression analysis. The criterion of significance was taken as $P < 0.05$.

RESULTS

In a typical chromatogram of a triterpenic extract of fruit, the only two triterpenic compounds detected and identified were MA and OA (Figure 2). The mass spectra of these compounds (Figure 2) confirm the identification made by HPLC-UV/vis. The mass spectrum of peaks eluted with a retention time of 8.23 and 8.18 min displayed a major signal at m/z 471 in the negative-ion mode. This molecule was also fragmented in the MS/MS mode and showed a major signal at m/z 423, 393, and 405. The 423 m/z fragment may have been the result of the fragmentation and removing of the carboxylic group of MA, while the 393 m/z fragment could have resulted from the fragmentation and removal of the carboxylic and hydroxylic group of MA. This mass spectrum coincided with that found for the MA standard. The mass spectrum of peaks eluted with a retention time of 12.16 and 12.17 min displayed a major signal at m/z 455 in the negative-ion mode. This molecule was also fragmented in the MS/MS mode and showed major signals at m/z 407.0. The 407 m/z fragment could have been the result of the fragmentation and removal of the carboxylic group of OA. This mass spectrum coincided with that found for the OA standard. MA was the main triterpenic compound found in the chromatographic profile of methanolic extract from fruits. The percentage that represented MA with respect to total triterpenic acids (MA + OA) in fruit samples of Picual and Cornezuelo was 70.62 ± 2.46% and 79.79 ± 0.80%, respectively.

In a typical chromatogram of a triterpenic extract of leaves, the main triterpenic compounds detected and identified were MA, OA, EO, and UO (Figure 3). In Cornezuelo leaves, UA and other unidentified compounds were also detected in some samples. OA was the main triterpenic compound found in the chromatographic profile of methanolic extract from leaves. The percentage that represents OA with respect to total triterpenic compounds (MA + OA + EO + UO + UA) in leaf samples of

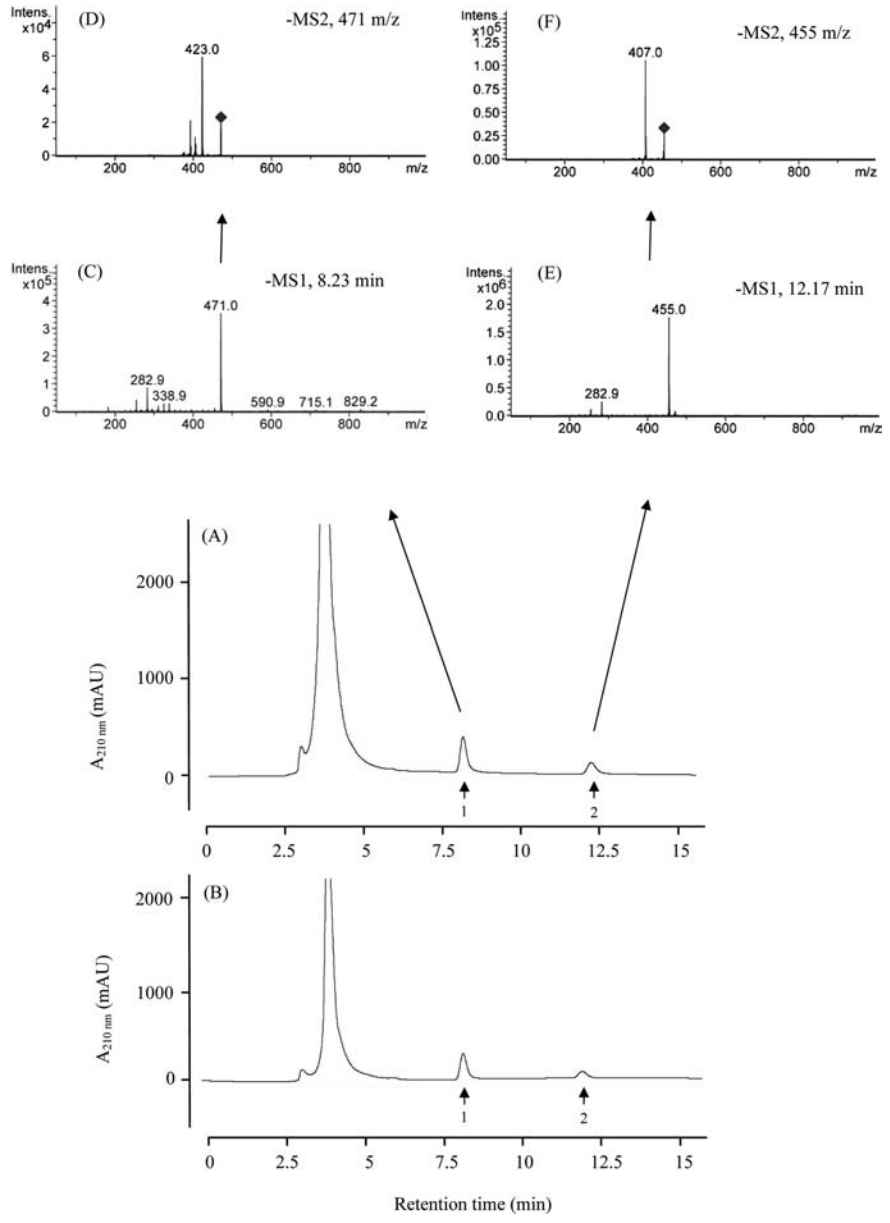

Figure 2. HPLC chromatogram at 210 nm of methanolic extracts of cv. Picual (A) and cv. Cornezuelo (B) fruit pulp and HPLC tandem mass spectra of maslinic acid (C,D) and oleanolic acid (E,F). The peaks corresponding to maslinic acid (MA) (1) and oleanolic acid (OA) (2) are marked on the chromatogram. The chromatogram shown in the figure corresponds to the samples collected in July, when the ripeness index was 0.00. In the HPLC mass mode, the ionization process was in ion-negative mode. The peaks observed in the spectra indicated the pseudomolecular ion $[M - 1]^-$ of the analyte.

Picual and Cornezuelo was 81.39 ± 1.15% and 61.92 ± 1.50%, respectively.

Effects of Ripening on the Concentration of Triterpenic Acids Present in Olive Fruits of cv. Picual and cv. Cornezuelo. Figure 4 shows the time course of the concentration of triterpenic compounds found in fruit pulp of the olive tree Picual and Cornezuelo during ripening. In Picual, from sample 2 to sample 9, a significant and progressive decrease occurred in MA concentration expressed as mg (g dry weight)$^{-1}$ (Figure 4, panel A). The content (mg) of MA per fruit followed a biphasic trend during ripening (Figure 4, panel B). In the first phase, MA content increased from 1.54 ± 0.06 (sample 1) to 9.06 ± 0.49 mg (sample 5). In the second phase, MA content decreased until reaching 5.07 ± 0.25 mg (sample 9). In the case of Cornezuelo, a similar trend was noted, although the absolute values were lower than those in Picual (Figure 4, panels C and D). In the case of mg MA per fruit in Cornezuelo, the level was significantly lower than that of Picual. The values changed from 1.08 ± 0.04 mg (sample 1) to 3.80 ± 0.16 (sample 2) without showing important variations until the end of ripening (sample 9).

In Picual, the OA concentration changed with a trend similar to that described for MA, with the exception of samples 7 and 8, in which an increment was detected (Figure 4, panels A and B). In Cornezuelo, the OA concentration showed a trend similar to that in Picual, although with low absolute values (Figure 4, panels C and D).

Effects of Ripening on the Concentration of Triterpenic Compounds Present in Olive Leaves of cv. Picual and cv. Cornezuelo. Figure 5 shows the time course

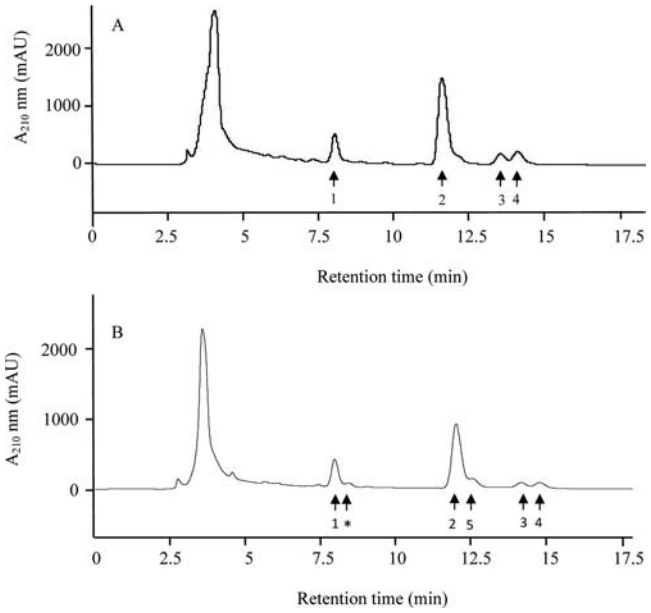

Figure 3. HPLC chromatogram at 210 nm of methanolic extracts of cv. Picual (A) and cv. Cornezuelo (B) leaves. The peaks corresponding to maslinic acid (MA) (1), oleanolic acid (OA) (2), erythrodiol (EO) (3), and uvaol (UO) (4) are marked on the chromatogram. In the chromatogram of Cornezuelo leaves, the peaks corresponding to ursolic acid (UA) and other unidentified compounds are marked as 5 and *. The chromatogram shown in the figure corresponds to the samples collected in July when the ripeness index was 0.00.

of the concentration of triterpenic compounds found in leaves of Picual and Cornezuelo during ripening. Moreover, of MA and OA, alcohols, EO and UO, are also present in all of the samples analyzed. In Picual (Figure 5, panel A), OA is the main triterpenic compound found in the leaf. The values of the OA concentration ranged from 16.03 ± 0.33 (sample 7) to 25.09 ± 0.72 mg (g dry weight)$^{-1}$. The value range of the MA concentration was from 2.05 ± 0.18 (sample 9) to 5.05 ± 0.21 (sample 1) mg (g dry weight)$^{-1}$. The values of the EO concentration ranged from 0.56 ± 0.20 (samples 1 and 9) to 1.05 ± 0.05 (sample 8) mg (g dry weight)$^{-1}$. The values of UO concentration ranged from 0.42 ± 0.03 (sample 9) to 0.96 ± 0.06 (sample 8) mg (g dry weight)$^{-1}$.

In Cornezuelo (Figure 5, panel B), the value range of the OA concentration was from 10.94 ± 0.26 mg (sample 7) and 18.83 ± 0.66 (sample 9) mg (g dry weight)$^{-1}$, and that of the MA concentration was from 2.58 ± 0.13 (sample 7) to 6.30 ± 0.15 (sample 4) mg (g dry weight)$^{-1}$. The EO concentration ranged from 0.54 ± 0.04 (sample 6) and 1.78 ± 0.07 (sample 3) mg (g dry weight)$^{-1}$ and the UO concentration from 0.46 ± 0.02 (sample 4) to 1.24 ± 0.04 (sample 7). UA was detected in samples 1 to 7 with a concentration range from 1.94 ± 0.49 (sample 6) to 4.25 ± 0.33 mg (sample 4) mg (g dry weight)$^{-1}$.

Effects of Jaén-Style Processing of Table Olives on the Concentration of Triterpenic Acids of Fruits of cv. Picual and cv. Cornezuelo. Table 2 shows the values of MA and OA concentration in olive fruits of Picual and Cornezuelo after Jaén-style processing for table consumption in comparison with nonprocessed olives. Picual and Cornezuelo fruits were harvested on the traditional dates at which each cultivar is harvested for table-olive processing in Jaén.

The results showed a significant decrease in the concentration and content of MA and OA after fruit processing in both cultivars. The content of MA per fruit was, in processed

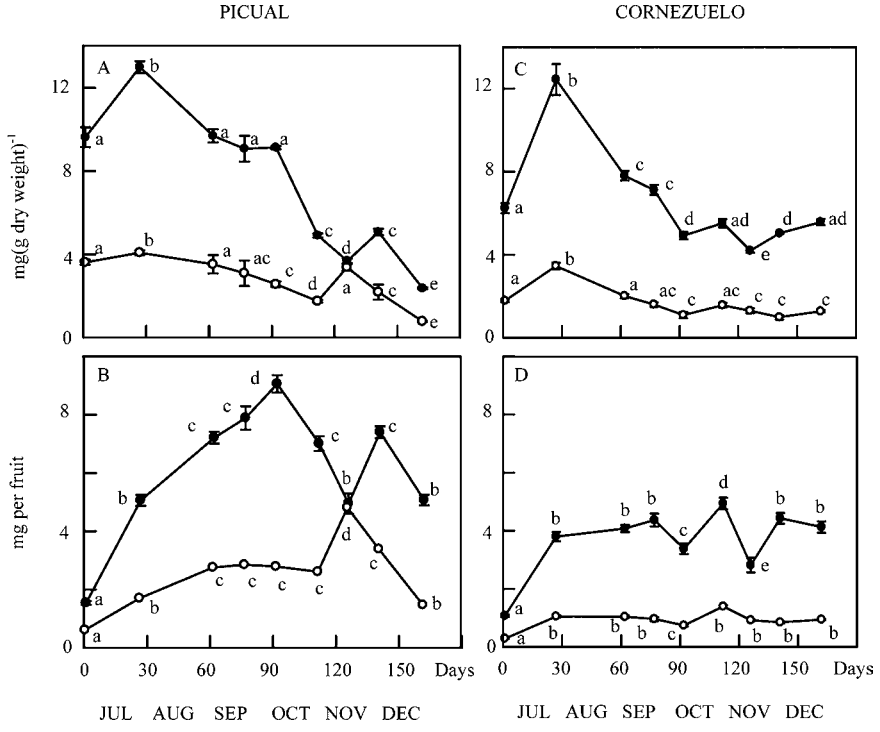

Figure 4. Concentrations of maslinic (●) and oleanolic (○) acid in the pulp of the fruits of olive tree *Olea europaea* L. cv. Picual (panels A and B) and cv. Cornezuelo (panels C and D) during ripening. Time 0 was considered the day on which the first sample was picked: 2 July, 2010. Samples were collected on nine dates, corresponding to the different ripening stages. Results are expressed as the means ± standard error of the mean. Points followed by different letters are statistically different ($P < 0.05$).

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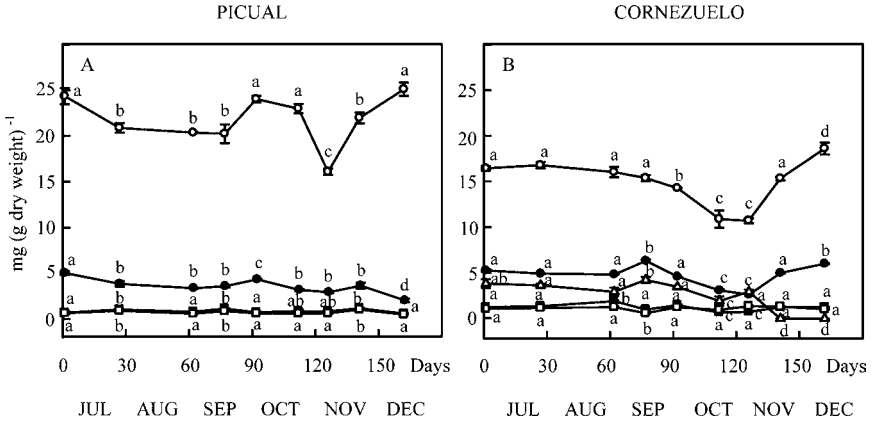

Figure 5. Concentration of maslinic acid (●), oleanolic acid (○), ursolic acid (△), erythrodiol (■), and uvaol (□) in leaves of olive tree *Olea europaea* L. cv. Picual (panel A) and cv. Cornezuelo (panel B) during ripening. Time 0 was considered the day on which the first sample was picked: 2 July, 2010. Samples were collected on nine dates, corresponding to the different ripening stages of the fruits. Results are expressed as the means ± standard error of the mean. Points followed by different letters are statistically different ($P < 0.05$).

Table 2. Effects of Jaén-Style Processing of Table Olives on the Concentration of Triterpenic Acids of Fruits of *Olea europaea* cv. Picual and cv. Cornezuelo[a]

	Picual fruits		Cornezuelo fruits	
	not processed	processed	not processed	processed
mg MA[b]/g dry weight	4.91 ± 0.10 ax	1.11 ± 0.02 bx	7.12 ± 0.24 ay	2.64 ± 0.15 by
mg MA/g wet weight	1.93 ± 0.04 ax	0.34 ± 0.02 bx	1.98 ± 0.07 ax	0.59 ± 0.03 by
mg MA per fruit	7.01 ± 0.35 ax	1.26 ± 0.06 bx	4.37 ± 0.22 ay	1.30 ± 0.06 bx
mg OA[c]/g dry weight	1.84 ± 0.06 ax	0.27 ± 0.03 bx	1.60 ± 0.11 ax	0.47 ± 0.04 by
mg OA/g wet weight	0.72 ± 0.02 ax	0.08 ± 0.01 bx	0.44 ± 0.03 ay	0.10 ± 0.01 bx
mg OA per fruit	2.61 ± 0.13 ax	0.31 ± 0.02 bx	0.97 ± 0.07 ay	0.23 ± 0.01 by

[a]Values are means ± standard error of the mean. In each row, for comparisons between Picual and Cornezuelo fruits (not processed vs not processed; processed vs processed), values followed by different letters (x,y) are significantly different ($P < 0.05$). In each variety, for comparisons between not-processed and processed, values followed by different letters (a,b) are significantly different ($P < 0.05$). [b]MA, maslinic acid. [c]OA, oleanolic acid.

olives, 82.02% and 70.25% lower than that in nonprocessed fruits, respectively. In both cultivars, the OA content per fruit was, in processed olives, 88.12% and 76.29% lower, respectively, than that in nonprocessed fruits. The total OA content in Picual was 25.81% higher than that in Cornezuelo.

■ DISCUSSION

In this work a method of HPLC analysis was applied to detect and quantify triterpenic compounds present in olive fruits and leaves of cv. Picual and cv. Cornezuelo. The procedure used for drying and extracting triterpenoids is the most appropriate for these previous steps.[24] Triterpenic acids (MA, UA, and OA) and dialcohols (EO and UO) were clearly identified on the chromatograms. When the effect of the standard concentration on the peak area was studied, an appropriate linearity was found for all the triterpenic compounds studied. The concentration of triterpenic compounds in fruit and leaf samples was found to be in the range defined by the calibration line. Specifically, the results of this study demonstrate that this is an appropriate method to quantify these compounds in these samples. The values found for MA and OA were of the same magnitude as previously reported in a study made with table olives of other cultivars under a similar procedure.[25] In leaves, the results of our work are also of the same magnitude as previously reported using gas chromatography.[18] Nevertheless, our results for OA and MA in fruits are significantly higher than those reported by Guinda et al.[18] and Romero et al.[25] We detected a change in MA from 9.63 ± 0.48 to 2.37 ± 0.05 mg (g dry weight)$^{-1}$, the highest value being 13.00 ± 0.28 mg (g dry weight)$^{-1}$. Guinda et al.[18] showed that MA values ranged between 1.5 ± 0.1 to 1.2 ± 0.1 mg (g dry weight)$^{-1}$, and Romero et al.[25] reported values around 1.5−3.0 mg (g dry weight)$^{-1}$ for green−yellow olives. A similar trend was found for OA. The differences detected in fruit samples but not in leaf samples versus those by Guinda et al.[18] may be due to the different processing and analysis methods used. The differences detected in fruit samples but not in processed table olives versus those by Romero et al.[25] may be due to the effects of specific climatic, land, and growth conditions. The concordance found in the results reported in olive leaves and processed olives fruits confirm that the method used is appropriated. The results of the present work demonstrate that the concentrations of triterpenic acids in fruits samples from Picual and Cornezuelo, in our experimental conditions, are significantly higher than those reported by Guinda et al.[18] and Romero et al.[25] These data reinforce the dietary value of olive fruits as a source of triterpenoids presented in olive fruits of Picual and Cornezuelo.

The only presence of MA and OA in fruits indicated that the biosynthesis pathway of pentacyclic triterpenoids in the fruit of olives involves the route of β-amyrin synthase. The failure to detect UO and UA indicated that the α-amyrin synthase pathway does not significantly deviate in the fruit. MA is the main triterpenic acid, representing about 1.3% and 1.2% of the dry weight of the fruit at the point of the highest concentration.

This implies that MA must have an important function in fruit physiology and metabolism. The presence of UO and EO in the leaf demonstrated the specific metabolism of the triterpenic compounds in this organ, and the presence of UA in Cornezuelo also distinguished this cultivar from Picual.

Moreover, the present work shows the time course of the concentration of triterpenic compounds during fruit ripening in both cultivars. This is of great interest for clarifying the function of these compounds in the overall metabolism of the fruits and leaves. The MA concentration significantly increases in the first stages of ripening studied, coinciding with the higher growth in the mass of the fruit pulp produced during the so-called "green ripening". Afterward, coinciding with "black ripening", the MA concentration and MA content significantly decreased. This indicates that at the same time as the fruit grows in mass, the synthesis of MA and OA increases.

Whereas the biological roles of other secondary metabolites are better known, the functions of pentacyclic triterpenoids in plants in general and in the olive tree in particular remain poorly understood. Other works have reported that these metabolites contribute to plant defense, as attested by the production of triterpenic phytoalexins[26] or saponins[27] in response to biotic and abiotic stress. Triterpenoids are constituents of waxes on the surfaces of the leaves and fruits of various species and consequently form part of the cuticle and are involved in the maintenance of its structure, provide water permeability, and appear to play a role in plant−insect interactions.[28−30] Currently, very few studies have studied the health properties of olive fruit. Aside from the content in polyunsaturated fatty acids, olive fruit contains a high concentration of phenols and pentacyclic triterpenes, two bioactive compounds which have important biological functions, such as hepatoprotection, cardioprotection, vaso-protection, and anti-inflammatory, antimicrobial, and anticancer effects. [31]

Moreover, the present work shows how the concentration of pentacyclic triterpenes changes in table olives after Jaén-style processing. This processing method is traditional in the Jaén province. Only natural products such as sodium chloride, fennel, and thyme are used in a simple process that does not require fermentation and NaOH treatment.[25] After olive pressing for oil, the processing of fruits for table olives is the second most important economic application of olive products in Jaén. A comparison was made between Picual and Cornezuelo, two main olive cultivars in Jaén (Spain). As has been reported with other processing methods,[25] the one used for table olives in Jaén significantly lowered the concentration of triterpenic compounds in both cultivars. At the end of the processing, the content of MA and OA in both fruits was similar, although the initial concentration differed. The total content of triterpenic compounds in a Picual or Cornezuelo fruit is around 1.5 mg per fruit. This data demonstrates that the inclusion of olive fruit in the daily diet is highly recommended because this product is a major source of pentacyclic triterpenes, compounds that have important beneficial effects on health and disease prevention, and that are practically absent in olive oil,[32] the other olive-tree product typical of the Mediterranean diet.

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Notes

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ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; UV/vis, ultraviolet−visible; MS/MS, mass/mass spectrometry; MA, maslinic acid; OA, oleanolic acid; UA, ursolic acid; UO, uvaol; EO, erythrodiol

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