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Quantification of bioactive substances in Picual and Arbequina olive leaves and fruit

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Running title: Bioactive substances in olive leaves and fruit

Abstract

BACKGROUND: Olive leaves and fruit possess bioactive substances such as phenolic compounds and triterpenic acids that can be obtained from olive by-products generated during olive oil extraction. The aim of this work was the characterization and quantification of them in Picual and Arbequina cultivars from different locations and for several seasons in both olive leaves and fruit.

RESULTS: The major phenolic compound identified in the leaves was oleuropein, and the total content of phenolic compounds in this material reached 70 g kg^{-1} fresh weight. The leaves were also rich in triterpenic acids (20 g kg^{-1} fresh weight), with oleanolic being the most concentrated in them. With regard to olives, oleuropein and demethyloleuropein were the main phenolic compounds in the pulp of Picual and Arbequina cultivars, and the total concentration of these phenolic compounds reached 3.5% fresh weight. Olives can also be an important source of triterpenic acids although mainly the skin part, where maslinic and oleanolic acids are concentrated.

CONCLUSIONS: Olive leaves can contain up to 70 g kg^{-1} phenolic compounds and 20 g kg^{-1} triterpenic acids, and olive fruit up to 35 g kg^{-1} of the former and 3 g kg^{-1} of the latter. It must also be noted that this level was constant between seasons and orchard location.

Keywords: olive; phenolic; triterpene; oleuropein; maslinic; oleanolic

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2 **INTRODUCTION**

3 The worldwide production of olive oil accounts for ca. 3 million tons per year, and
4 around half of them are produced in Spain, Picual as the most important cultivar grown
5 in this country. Moreover, the number of olive trees of the Arbequina cultivar is highly
6 increasing both in Spain and worldwide because of the appreciated organoleptic
7 characteristics of its oil and the good adaptation of the tree to super-high-density
8 hedgerow cultivation.

9 Both olive cultivars are mainly intended for olive oil production that gives rise to
10 olive pomace (alperujo) and leaves as the main by-products, which are rich in bioactive
11 substances such as phenolic compounds and triterpenic acids. They have been found to
12 exert important effects such as antioxidant, anti-inflammatory, antimicrobial and
13 antitumor agents, among others.¹⁻⁴ In addition, the resistance of these olive trees to
14 insect and microbe attack has also been associated to the presence of phenolic and
15 triterpene compounds.⁵⁻⁷ Consequently, there have been many studies and patents with
16 the aim of obtaining extracts rich in these substances from olive by-products.

17 Although the chemical characterization of phenolic and triterpene substances in
18 olive products is well-documented,⁸⁻¹¹ many of these studies were carried out with
19 olives and leaves picked from experimental orchards; few samples were used or they
20 were analyzed during one unique crop season.¹²⁻¹⁵ In addition, contradictory data on the
21 concentration of these substances in olive material can be found in the literature, and
22 they are scarce on the phenolic and triterpenic composition of the different parts of the
23 olives (skin, seed and pulp).¹⁶⁻¹⁸ Hence, a deep knowledge of the amount of them in
24 olive leaves and fruit cultivated under common agronomic conditions for several
25 seasons is required, mainly if these bioactive substances are to be extracted from olive
26 by-products.

27 The main objective of this work was to quantify the phenolic and triterpenic
28 substances in olive leaves and fruit of the Picual and Arbequina cultivars grown in
29 different areas of the province of Jaen, which is the main Spanish producer of olive oil.
30 The effect of the degree of olive maturation on the content in these bioactive substances
31 was also assessed.

1

2 MATERIALS AND METHODS

3 Samples

4 Samples from olive leaves and fruit (*Olea europaea* L.) of the Picual and Arbequina
5 varieties were used in this study. Leaves from 7 orchards located in the north of the
6 province of Jaen (south of Spain) were obtained from trees of the Picual variety in
7 November, January and April during the 2012/2013 season. Leaves of trees of the
8 Arbequina variety were also picked on the same dates from only one orchard. All
9 samples were collected in triplicate from all sides and orientations of 9 olive trees.
10 During the 2013/2014 season, the olive leaves were obtained from 3 orchards of the
11 Picual variety and one of Arbequina on the same dates as the former season.

12 Olive fruits of the Picual variety were picked from the same 7 and 3 orchards
13 used for leaf characterization during the 2012/2013 and 2013/2014 seasons,
14 respectively. The same orchard of Arbequina variety was used for olive samples of the
15 two seasons. The fruits were harvested in December with three different degrees of
16 maturation: (i) green-yellowish skin (green), (ii) reddish-brown skin (purple), and (iii)
17 black skin with white flesh (black). As was done with leaves, each sample of olive fruit
18 was collected in triplicate from all sides and orientation of 9 olive trees.

19 Olives of the Picual variety were also picked from 4 orchards in December
20 (2012/2013 season) at the two extreme degrees of maturation (green and black), and
21 phenolic and triterpenic compounds were analyzed in the pulp, skin and seed.

22 It must be noted that all samples (olive and leaf) were immediately transferred to
23 the laboratory and analyzed on the same day without any storage period.

24 The olive orchards were cultivated under standard cultural practices and trees
25 were irrigated ad libitum to avoid water stress.

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27 Analysis of phenolic and oleosidic compounds

28 The extraction of phenolic and oleosidic compounds from the olive pulp was based on
29 the methodology proposed elsewhere.¹⁸ Around 10 g of fresh olive pulp was mixed in

1 an Ultra-turrax homogenizer with 30 mL of dimethyl sulfoxide (DMSO). After 30 min
2 of resting contact, the mixture was centrifuged at 6000 g for 5 min (22 °C), and 0.25 mL
3 of the supernatant were diluted with 0.5 mL of DMSO plus 0.25 mL of 0.2 mM of
4 syringic acid in DMSO (internal standard). The extraction of these substances from the
5 skin and the seed was made similarly to the olive pulp as described above, but this time
6 the mixing ratio was 0.1 g of olive skin or seed and 0.5 mL of DMSO.

7 Samples were filtered through a 0.22 µm pore size nylon filter, and an aliquot
8 (20 µL) was injected into the chromatograph. The chromatographic system consisted of
9 a Waters 717 plus autosampler, a Waters 600 pump, a Waters column heater module,
10 and a Waters 996 photodiode array detector operated with Empower.2 software
11 (Waters, Inc.). A 25 cm x 4.6 mm i. d., 5 µm, Spherisob ODS-2 (Waters, Inc.) column
12 at a flow rate of 1 mL min⁻¹ and a temperature of 35 °C, was used in all experiments.
13 Separation was achieved by gradient elution using (A) water (pH 2.5 adjusted with
14 0.15% phosphoric acid) and (B) methanol. The initial composition was 90% A and 10%
15 B. The concentration of B was increased to 30% over 10 min and was maintained for 20
16 min. Subsequently, B was raised to 40% over 10 min, maintained for 5 min, and then
17 increased to 50%. Finally, B was increased to 60, 70, and 100% in 5 min periods. The
18 initial conditions were reached in 10 min. Chromatograms were recorded at 280 nm and
19 240 nm for phenolic and oleosidic compounds, respectively.

20 The evaluation of each compound was performed using a regression curve with
21 the corresponding standard. Hydroxytyrosol, oleuropein, verbascoside, luteolin 7-
22 glucoside, rutin, and apigenin were purchased from Extrasynthese S. A. (Genay, France)
23 and tyrosol, caffeic acid and *p*-coumaric acid from Sigma Chemical Co. (St Louis, MO,
24 USA). Hydroxytyrosol-1-glucoside, caffeoyl ester of secologanoside, and
25 comselogoside were quantified using the response factors of hydroxytyrosol, caffeic
26 acid, and *p*-coumaric acid, respectively. Salidroside and ligustroside were quantified
27 using the response factor of tyrosol. Hydroxytyrosol-4-glucoside was obtained using an
28 HPLC preparative system. All oleosidic compounds were quantified using the response
29 of oleoside 11-methyl ester.

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31 **Moisture**

1 The water content of fruits and leaves was determined by weighing 3 g of the organ and
2 then oven drying at 105 °C to constant weight.

4 **Analysis of sugars**

5 Fresh olive pulp (25 g) or olive leaf (10 g) was mixed with 5 mL of 7.5% sorbitol
6 (internal standard) and 50 mL of distilled water at 100 °C. The mixture was
7 homogenized in Ultra-turrax equipment, and centrifuged at 6000 g for 5 min. After that,
8 the supernatant was filtered through filter paper and the paste was washed twice with 30
9 mL of distilled water at 100 °C. The supernatant was collected in a 125 mL volumetric
10 flask and distilled water was added to the mark. The solution was left at 5 °C for 24 h
11 and 5 mL were filtered through a 0.22 µm pore size nylon filter. Subsequently, 2 mL of
12 the extract were de-salted by adding 1g of cationic resin (Amberlite IR-120,
13 FlukaChemieAG , Switzerland) and 1 g of anionic resin (Amberlite IR-96, Fluka).
14 Samples were shaken occasionally during a 30 min de-salting period. An aliquot (≈
15 1mL) of the solution was centrifuged at 6000 g for 5 min, and 20 µL were injected into
16 the chromatograph. The HPLC system was the same used for the phenolic analysis, and
17 the detection was carried out with a Waters 410 differential refractometer detector. A
18 Rezex RCM-monosaccharide Ca⁺ (8%) column (300 mm x 7.8 mm i.d., Phenomenex)
19 held at 85 °C with deionized water as the eluent at 0.6 mL/min was used for sugar
20 analyses. Sucrose, fructose, glucose, mannitol and sorbitol were purchased from Sigma
21 Co. (St. Louis, USA).

23 **Analysis of triterpenic acids**

24 Ten grams of de-stoned and cut olive fruits or cut leaves were desiccated at 105 °C until
25 weight stabilization. Subsequently, 1 g of dry and triturated olive pulp or leaf were
26 mixed in a 10 mL centrifuge tube with 4 mL of methanol/ethanol (1:1, v/v) and
27 vortexed for 1 min, centrifuged at 6000 g for 5 min at 20 °C, and the solvent was
28 separated from the solid phase. This step was repeated six times, and the pooled solvent
29 extract was vacuum evaporated. The residue was dissolved in 4 mL of methanol, which
30 was filtered through a 0.22 µm pore size nylon filter and an aliquot (20 µL) was injected
31 into the liquid chromatograph. The chromatographic system and column were the same

1 as those used for the phenolic compound analysis. The mobile phase
2 (methanol/acidified water with phosphoric acid at pH 3.0, 92:8, v/v) was delivered to
3 the column at a flow rate of 0.8 mL min⁻¹ and the eluate was monitored at 210 nm.
4 Oleonic and maslinic acids were quantified using external standards (Sigma, USA).

5
6 **Statistical analysis**

7 Statistical comparisons of the mean values for each experiment were performed by one-
8 way analysis of variance (ANOVA), followed by the Duncan's multiple range test ($p <$
9 0.05) using Statistica software version 8.0 (Stat-Soft, 2001).

10
11 **RESULTS AND DISCUSSION**

12 The sampling of olive leaves were taken in November and January because this is the
13 harvesting period of olives and when leaves can be obtained at the olive oil mills as a
14 by-product. They were also analyzed in April when olive trees are pruned. The data in
15 Table 1 on the concentration of phenolic compounds in leaves of the Picual and
16 Arbequina cultivars show great differences with respect to the reported values. Many
17 previous studies have been carried out during the time course of fruit ripening from
18 May to December but no clear conclusions have been obtained on the phenolic
19 compounds in the leaves.^{15,19-20} However, our data indicate that the concentration of
20 oleuropein was much higher in November than in the following months, whereas the
21 rest of the phenolic compounds increased their concentrations from November onwards,
22 which was observed for both Picual and Arbequina cultivars. These differences cannot
23 be attributed to changes in the water content of the leaves harvested from November to
24 April (Table S1), although a much higher percentage of water was always found in
25 leaves of the Arbequina (~520 g kg⁻¹) than Picual (~ 430 g kg⁻¹) cultivar.

26 First, these substances were found in a much higher concentration than recorded
27 in the literature. Moreover, the four main phenolic compounds detected were
28 oleuropein, verbascoside and the hydroxytyrosol glucosides. By contrast, the presence
29 of metabolites from the hydrolysis of the secoiridoid glucosides such as hydroxytyrosol
30 and tyrosol was not observed. The concentration of oleuropein in the seven samples of

Picual leaves collected from seven different growing areas of the province of Jaen ranged from 47 g kg⁻¹ for those picked in April up to 72 g kg⁻¹ for those in November. Up to now, the concentration of oleuropein and total phenolic compounds in the leaves of Spanish and Italian cultivars did not exceed 1-3% fresh weight.^{8,15,19-20,21} In this study, samples were directly analyzed without any drying or storage period which may explain the high amount of phenolic compounds measured. It must be noted that during the drying and storage of leaves enzymatic reactions occur that can reduce the level of these substances as well as originate metabolites from their hydrolysis. The content of the hydroxytyrosol glucosides was also higher than reported in the literature;⁴ their concentration increased from November to April, while that of oleuropein decreasing in both Picual and Arbequina olive leaves (Table 1). The concentration of verbascoside and other minor compounds also increased from November to April.

Concerning oleosides, a similar behavior to oleuropein was found for all of them (Table S2), and the maximum concentration was reached in November. Besides, the concentration of oleoside and oleoside 11-methyl ester was always higher in Picual than Arbequina leaves, and the opposite was observed for secoxyloganin and secologanoside.

As was found in Picual leaves, the main phenolic compound in the fruit was oleuropein, its concentration slightly decreasing with maturation degree (Table 2). By contrast, this secoiridoid glucoside was detected only in the pulp of green Arbequina olives, whereas demethyloleuropein was the main phenolic compound in this cultivar, whose concentration slightly decreased as ripening increased. The presence of demethyloleuropein in Arbequina olives has previous been reported,¹³ but not at a high concentration of 1 % fresh weight.⁹ Similarly, the concentration of oleuropein in Picual olives was found to be around 18 g kg⁻¹ fresh weight, which is much higher than previous data.⁹ The high content of hydroxytyrosol glucosides in the pulp of both cultivars must also be noted, as has been documented in olives intended for table olives.¹⁸

Many studies have shown a decrease in total polyphenols, particularly oleuropein, with the time course of olive ripening,²²⁻²³ which has also been observed in this work. By contrast, many other phenolic compounds such as hydroxytyrosol glucosides, verbascoside and rutin increased with olive maturation (Table 2). In

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1 particular, a decrease in hydroxytyrosol glucosides during olive maturation has been
2 previously reported,¹³ but the opposite has also been found.²⁴ Indeed, it has been
3 observed in this work that while the concentration of oleuropein decreases with time in
4 both the fruit and leaves of Picual and Arbequina cultivars, many others phenolic
5 compounds increase (Tables 1 and 2). The moisture of the fruit did not significantly
6 change with olive maturation (Table S3), although, as happened with the leaves, this
7 parameter was higher for the Arbequina than Picual cultivar, with these differences
8 registering higher during the 2012/2013 than the 2013/2014 season.

9 There is scarce information on the presence of oleosidic substances in olives.²⁵
10 Talhaoui et al (2015c) did not observe any changes or only a slightly decrease in these
11 compounds in olives harvested from October to December, which is in agreement with
12 the data reported in Table S4. In this study, the concentrations of oleoside 11-methyl
13 ester and secoxyloganin were higher in olives of the Arbequina than Picual cultivar, and
14 the opposite trend was detected for oleoside and secologanoside.

15 The phenolic compounds were analyzed in different parts of olives of the Picual
16 cultivar harvested with green/yellow and black color on the surface (Table 3). The most
17 significant finding was the much higher concentration of these substances in the skin
18 than in the pulp regardless of the degree of maturation. Surprisingly, there is little data
19 revealing the enrichment of the skin of olives in these substances,^{18,26} which was found
20 not only for oleuropein but for most of the phenolic compounds analyzed. As
21 previously reported, the main phenolic compound in the seed was nuzhenide with a
22 rather similar level for the both degrees of olive maturation studied.²⁷ With regard to
23 oleosides, none of them were detected in the seeds (Table S5), and secoxyloganin was
24 not found in the skin of the fruit. Moreover, oleoside and secologanoside were more
25 concentrated in the skin than in the pulp, whereas the opposite for oleoside 11-methyl
26 ester was detected. The later substance is a precursor of oleuropein biosynthesis, and the
27 lower content detected in the skin could be related to the higher concentration of
28 oleuropein in this part of the olive. A trend to lower oleoside concentration in black
29 than green olives was also observed, regardless of the part of the fruit.

30 The profile and concentration of phenolic compounds in olive leaves have been
31 proposed to discriminate among cultivars and cultivation zones.^{8,19,28} The data depicted
32 in Figure 1 indicate that the content of phenolic compounds in Picual leaves was not

1 useful to discriminate among three cultivation zones in the province of Jaen, regardless
2 of the sampling time and season year. Seasonal changes have been reported in the
3 phenolic content of olive leaves,²⁹⁻³⁰ but this effect was not detected in this study
4 (Figure 1). Among the three cultivation zones analyzed, the phenolic content in olives
5 from Santisteban del Puerto (ST) presented the highest concentration for the two
6 seasons studied. A higher concentration of these substances was found in the olives of
7 the 2012/2013 season than the 2013/2014 season when they were harvested from
8 orchards located in Chilluevar (CH) and Santisteban del Puerto (ST) but not
9 Villacarrillo (VI) regardless of the degree of maturation.

10 Triterpenic acids are other important bioactive substances analyzed in olives and
11 leaves. The skin of the fruit is very rich in triterpenic acids,¹⁶ which was confirmed in
12 this study for olives collected with two degrees of ripeness (Table 3). The concentration
13 of maslinic acid was higher than oleanolic in both skin and pulp although the
14 differences were higher in the pulp. There are few and contradictory data on the content
15 of triterpenic acids in olive pulp. Guinda et al (2010) reported lower concentrations than
16 1 g kg^{-1} in the olives of the Picual and Arbequina cultivars, whereas Fernández-
17 Hernández et al (2015) found a concentration higher than 25.5 g kg^{-1} in olive oil mill
18 paste (alperujo). Figure 2 shows a rather similar concentration of triterpenic acids in
19 both cultivars ranging from 2400 to 3100 mg kg^{-1} fresh weight that coincides with our
20 previous data.¹¹ Peragón (2013) followed the evolution of these substances in the olives
21 of the Picual and Cornezuelo cultivars during maturation and observed a drastic
22 decrease in their concentration at the first step of ripening but from October to
23 December the evolution was contradictory. In this study, the concentration of triterpenic
24 acids decreased from green to black olives of the Picual cultivar (Figure 2), and it also
25 occurred from green to purple olives of the Arbequina cultivar but not significantly.
26 These findings cannot be attributed to changes in the water content of the fruit (Table
27 S3). Besides, the concentration of maslinic was always much higher than that of
28 oleanolic acid.

29 In contrast, the concentration of oleanolic acid was much higher than that of
30 maslinic acid in the leaves (Figure 3), as previously reported.¹⁰ The total concentration
31 of triterpenic acids ranged between 11 and 21 g kg^{-1} fresh weight, values similar to
32 those found by other researchers.^{15,32} Similarly, the content of these substances was
33 always higher in leaves of the Picual than Arbequina cultivar, and a slight decrease was

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1 detected from sampling in November to April (Figure 3). In fact, the concentration of
2 these substances in the Picual leaves obtained from three different orchards during two
3 consecutive seasons was very similar (Figure 4) regardless of the location of the trees,
4 although a slightly lower value was observed in the olives picked in April. It must be
5 noted that the orchards were situated at different altitudes in the province of Jaen.
6 Moreover, differences in the concentration of triterpenic acids in the olives between
7 seasons were minimal, and the content in fruit harvested with black color tended to be
8 lower than with the green color (Figure 4).

9 Sugars are the carbon source for microorganisms during table olive
10 fermentation;³³ the precursors in the biosynthesis of lipids thereby a good correlation
11 between the content in olives of lipids and sugars has been found,²³ but they can also be
12 a good source of bioactive substances such as mannitol.¹⁵ Figure 5 shows the
13 concentration of sugars in the olives and leaves of the Picual and Arbequina cultivars.
14 As expected, glucose, sucrose, fructose and mannitol were the main sugars identified in
15 these materials. Glucose was the main sugar in both the fruit and leaves although the
16 concentration of mannitol was similar to glucose in the leaves of the two cultivars
17 studied, which is in disagreement with results obtained by other,³⁴ who found a rather
18 similar concentration of mannitol and glucose in the pulp of the olives. The
19 concentration of mannitol in the leaves was similar in both cultivars analyzed and both
20 sampling times, which reached 20 g kg⁻¹ fresh weight. This substance is useful for
21 pharmaceutical purposes and has been attributed to beneficial properties for health. In
22 fact, it can be selectively extracted from olive leaves by using ethanol as solvent.¹⁵

23
24 **CONCLUSIONS**

25 Picual is the olive cultivar most employed worldwide for the extraction of the healthy
26 fat, olive oil. However, it can also be a good source of bioactive substances obtained
27 from the by-products generated during oil extraction, particularly phenolic compounds
28 and triterpenic acids. The leaves of this cultivar but also those of the Arbequina cultivar
29 possess a very high concentration in both phenolic and triterpene substances when
30 obtained from orchards cultivated under common agronomic conditions. The contents in
31 phenolic compounds and triterpenic acids in leaves can reach up to 70 g kg⁻¹ and 20 g
32 kg⁻¹ fresh weight, respectively. It must be noted that this level was constant between

seasons and orchard location, which is very important for a continuous supply of raw material to a future factory of bioactive substances. Olives can also have an important concentration in triterpenic acids, mainly in the skin, although they are particularly rich in phenolic compounds that can reach 35 g kg⁻¹ fresh weight, with oleuropein and demethyloleuropein being the main phenolics in the pulp of Picual and Arbequina cultivars, respectively. These results will contribute to the knowledge of the presence of phenolic and triterpene compounds in olive material suitable for the extraction of bioactive substances in the future.

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Figure legends

Figure. 1. Concentration of total phenolic compounds (mg kg⁻¹) in Picual olives harvested in December with green and black surface color, and Picual leaves collected in November and April. Samples were obtained from three different orchards during the 2012/2013 and 2013/2014 seasons. CH, Chillluevar, ST, Santistebandel Puerto, VI, Villacarrillo. Vertical bars with different letters indicate significant differences between orchards according to Duncan’s multiple-range test ($p<0.05$).

Figure.2. Concentration (mg kg⁻¹) of triterpenic acids in the olives of the Picual (P) and Arbequina (A) cultivars collected with different surface color in December during the 2012/2013 season. Bars mean the data of 7 samples of Picual olives analyzed in triplicate, and the mean of one sample of Arbequina olives analyzed in triplicate. Triplicate samples were obtained from three different olive trees. Vertical bars with different letters indicate significant differences between cultivars according to Duncan’s multiple-range test ($p<0.05$).

Figure. 3. Concentration (mg kg⁻¹) of triterpenic acids in the leaves of the Picual (P) and Arbequina(A) cultivars collected from November to April during the 2012/2013 season. Bars mean the data of 7 samples of Picual leaves analyzed in triplicate, and the mean of one sample of Arbequina leaves analyzed in triplicate. Triplicate samples were obtained from three different olive trees. Vertical bars with different letters indicate significant differences between cultivars according to Duncan’s multiple-range test ($p<0.05$).

Figure. 4. Concentration of total triperpenic acids (mg kg⁻¹) in Picual olives harvested in December with green and black surface color, and Picual leaves collected in November and April. Samples were obtained from three different orchards during the 2012/2013 and 2013/2014 seasons. CH, Chillluevar, ST, Santistebandel Puerto, VI, Villacarrillo. Vertical bars with different letters indicate significant differences between orchards according to Duncan’s multiple-range test ($p<0.05$).

Figure. 5. Concentration of sugars (mg kg⁻¹) in Picual and Arbequina olives harvested in December with green and black surface color, and Picual and Arbequina leaves collected in November and April. Data are the mean of 4 samples from 4 different orchards. Vertical bars with different letters indicate significant differences between

cultivars according to Duncan's multiple-range test ($p < 0.05$). S, sucrose, G, glucose, F, fructose, and M, mannitol.

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Table 1
Phenolic compounds (mg kg⁻¹) in olive leaves of the Picual and Arbequina cultivars collected from November to April. They were analyzed during the 2012/2013 season.

Month	Oleuropein		Hy 4-glucoside ^y		Hy 1-glucoside		Verbascoside		Others	
	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina
November	72117 (10963)a ^x	55577 (1567)a	772 (452)a	779 (16)a	243 (219)a	241 (16)a	533 (431)a	474 (102)a	206 (126)a	349 (17)a
January	54018 (5688)b	32539 (5978)b	1124 (592)a	1853 (430)b	337 (219)a	381 (55)b	805 (445)a	856 (322)b	218 (164)a	338 (79)a
April	47468 (6501)b	30456 (5955)b	2550 (575)b	3332 (712)c	424 (101)a	954 (67)c	1263 (708)a	1026 (283)b	262 (222)a	594 (80)b

^xData are the mean of 7 samples of Picual leaves and one of Arbequina leaves analyzed in triplicate. Standard deviation is shown in parentheses. ^yHy 4-glucoside, hydroxytyrosol 4-glucoside; Hy 1-glucoside, hydroxytyrosol 1-glucoside; others is the sum of salidroside, rutin, luteolin-7-glucoside, comselogoside and caffeoyl-6-secologanoside. Column values followed by the same letter do not differ at the 5% level of significance according to Duncan's multiple-range test.

Table 2

Phenolic compounds (mg kg⁻¹) in the pulp of olives of the Picual and Arbequina cultivars harvested with different degree of maturation. They were analyzed during the 2012/2013 season.

	Oleuropein		Hy 4-glucoside ^y		Hy 1-glucoside		Verbascoside		Demethyloleuropein	
	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina
Green	18608 (7156)a ^x	1781 (295)	976 (350)a	898 (166)a	171 (110)a	10 (4)a	298 (141)a	498 (208)a	nd	10337 (2763)a
Purple	17229 (6096)a	nd	1402 (479)b	971 (153)a	261 (144)a	16 (6)a	604 (301)a	999 (287)b	nd	8836 (563)a
Black	17117 (7073)a	nd	1866 (648)b	1230 (89)b	476 (165)b	30 (8)b	1236 (322)b	970 (273)b	nd	9192 (295)a

(continued)

	Ligustroside		Comselogoside		Rutin		Salidroside		Others	
	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina
Green	315 (117)a	79 (24)a	104 (46)a	33 (7)a	266 (68)a	206 (35)a	32 (9)a	12 (3)a	73 (40)a	99 (34)a
Purple	317 (124)a	92 (21)a	117 (50)a	33 (6)a	363 (55)a	266 (63)a	76 (14)b	26 (2)b	67 (31)a	295 (85)b
Black	306 (128)a	87 (20)a	107 (46)a	27 (1)a	493 (113)b	196 (8)a	165 (73)c	41 (4)c	55 (35)a	181 (34)c

^xData are the mean of 7 samples of Picual olives and one of Arbequina leaves analyzed in triplicate. Standard deviation is shown in parentheses. ^yHy 4-glucoside, Hydroxytyrosol 4-glucoside; Hy-1-glucoside, Hydroxytyrosol 1-glucoside; others: vanillic, caffeic and *p*-coumaric acids, luteolin-7-glucoside and caffeoyl-6-secologanoside. Column values followed by the same letter do not differ at the 5% level of significance according to Duncan's multiple-range test.

Table 3

Concentration (mg kg⁻¹) of phenolic and triterpenic acids in different parts of Picual olives harvested with green and black surface color. Data are the mean of 4 samples collected from 4 different growers.

Compound	Green/yellow olives			Black olives		
	Skin	Pulp	Seed	Skin	Pulp	Seed
Oleuropein	27937 (5655)	16760 (3960)	183 (82)	25714 (7418)	11080 (4744)	325 (167)
Nuzhenide	nd ^b	nd	4042 (335)	nd	nd	3759 (1205)
Total phenolics ^a	31788 (5601)	19438 (3292)	5128 (261)	310500 (61097)	14787 (4481)	5138 (1090)
Oleanolic acid	4356 (2120)	59 (23)	nd	3094 (1116)	27 (22)	nd
Maslinic acid	7209 (3527)	393 (210)	nd	5556 (1935)	147 (109)	nd

^aTotal phenolics is the sum of oleuropein, nuzhenide, hydroxytyrosol 1-glucoside, hydroxytyrosol 4-glucoside, salidroside, vanillic, caffeic and *p*-coumaric acids, verbascoside, comselogoside, caffeoyl-6-secologanoside, luteolin 7-glucoside, rutin and ligustroside. ^bnd, not detected. Standard deviation is shown in parentheses.

Table S1

Moisture (g kg^{-1}) of leaves of the Picual and Arbequina varieties collected from November to April. They were analyzed during the 2012/2013 and 2013/2014 seasons.

Month	Season 2012/2013		Season 2013/2014	
	Picual	Arbequina	Picual	Arbequina
November	460 (22) ^a	513 (13) ^b	449 (9)	535 (1)
January	455 (18)	524 (23)	457 (2)	517 (2)
April	409 (15)	543 (30)	427 (16)	474 (2)

^aData are the mean of 7 and 3 samples of leaves of the Picual variety collected during the seasons 2012/2013 and 2013/2014 respectively. ^bData are the mean of 3 samples of leaves of the Arbequina variety. Standard deviation is shown in parentheses.

Table S2

Oleosidic compounds (mg kg⁻¹) in olive leaves of the Picual and Arbequina cultivars collected from November to April. They were analyzed during the 2012/2013 season.

Month	Oleoside		Secoxyloganin		Secologanoside		Oleoside-11-methyl ester	
	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina
November	2792 (453)a ^x	2275 (335)a	1169 (896)a	1268 (217)a	1724 (596)a	2188 (536)a	1368 (256)a	824 (41)a
January	2660 (369)a	1730 (532)b	660 (323)a	852 (506)a	721 (233)b	1079 (328)b	865 (170)b	494 (78)b
April	2398 (325)a	1372 (485)b	281 (182)b	299 (129)b	461 (207)b	1068 (248)b	607 (180)b	425 (88)c

^xData are the mean of 7 samples of Picual leaves analyzed in triplicate, and the mean of Arbequina leaves analyzed in triplicate. Standard deviation is shown in parentheses. Column values followed by the same letter do not differ at the 5% level of significance according to Duncan's multiple-range test.

Table S3

Moisture (g kg⁻¹) of the pulp of olives of the Picual and Arbequina cultivars harvested with different surface color. They were analyzed during the 2012/2013 and 2013/2014 seasons.

Colour	Season 2012/2013		Season 2013/2014	
	Picual	Arbequina	Picual	Arbequina
Green	673(28) ^a	725 (5) ^b	620 (23)	625 (3)
Purple	666 (35)	718 (14)	594 (22)	613 (1)
Black	653 (38)	704 (17)	583 (39)	630 (1)

^aData are the mean of 7 and 3 samples of leaves of the Picual variety collected during the seasons 2012/2013 and 2013/2014 respectively. ^bData are the mean of triplicates.

Table S4

Oleosidic compounds (mg kg⁻¹) in the pulp of olives of the Picual and Arbequina cultivars harvested with different color on their surface. They were analyzed during the 2012/2013 season.

Compound	Green/yellow		Purple		Black	
	Picual	Arbequina	Picual	Arbequina	Picual	Arbequina
Oleoside	431 (181)a ^x	298 (102.2)a	432 (160.8)a	313 (20.6)a	417 (162.0)a	458 (13.2)a
Secoxyloganin	716 (413)a	1714 (412)b	398 (383)a	502 (127)a	264 (194)a	292 (18)a
Secologanoside	1789 (669)a	1345 (375)a	1599 (666)a	1072 (110)a	1447 (549)a	1110 (84)a
Oleoside 11-methyl ester	3089 (856)a	5192 (1929)b	2948 (1054)a	3819 (268)b	3117 (1437)a	5889 (292)b

^xData are the mean of 7 samples of Picual olives and one of Arbequina leaves analyzed in triplicate. Standard deviation is shown in parenthesis. Column values followed by the same letter do not differ at the 5% level of significance according to Duncan's multiple-range test.

Table S5

Concentration (mg kg⁻¹) of oleosidic compounds in different parts of Picual olives harvested with green and black surface color. Data are the mean of 4 samples collected from 4 different growers. Standard deviation is shown in parentheses.

Compound	Green olives			Black olives		
	Skin	Pulp	Seed	Skin	Pulp	Seed
Oleoside	697 (146)	453 (141)	nd	560 (172)	337 (141)	nd
Secoxyloganin	nd ^a	881 (271)	nd	nd	565 (169)	nd
Secologanoside	2475 (341)	1832 (449)	nd	1825 (684)	1363 (540)	nd
Oleoside 11-methyl ester	1150 (442)	5296 (3391)	nd	1385 (896)	4188 (3426)	nd

^aNot detected.

Figure 1
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Figure 2
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