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# Changes in the HPLC Phenolic Profile of Virgin Olive Oil from Young Trees (*Olea europaea* L. Cv. Arbequina) Grown under Different Deficit Irrigation Strategies

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The HPLC phenolic profile of virgin olive oils obtained from young olive trees (Arbequina cv.) grown under different deficit irrigation strategies was studied. Deficit irrigation (RDI) did not affect all the phenolic compounds in the same way. Lignans, vanillic acid, vanillin, and the unknown phenolic compound named P24 increased in the oils from the most irrigated treatments. The secoiridoid derivatives and the unknown phenolic compound named P19 increased in the oils from the most stressed irrigation treatments. The period of growth where a water stress significantly affects the phenolic profile of oils was between pit hardening and the first stages of fruit growth and oil accumulation, independently of the water applied during the previous period to harvest. The phenolic profile and those parameters related to phenol content, oxidative stability, and the bitter index were significantly affected only in the most severe RDI strategies. Other strategies produced important savings in irrigation requirements and an increase in the water use efficiency without noticeably affecting the phenolic profile.

**KEYWORDS: Arbequina cultivar; deficit irrigation strategy; olive oil; phenolic compounds**

## INTRODUCTION

The increasing health consciousness of today's society explains the promotion of dietary habits associated with the Mediterranean culture which are believed to represent a healthy and disease-preventive diet. Olive oil is the major lipid component of the Mediterranean diet, and it is characterized by the composition of its triglyceride fraction and by its minor components, including tocopherols and phenolic components. The latter originate from the shikimate pathway and phenylpropanoid metabolism, and most of them are bonded to additional groups from the mevalonic pathway, for example, oleuropein and ligstroside.

The content of phenolic compounds is an important parameter in the evaluation of virgin olive oil quality since phenols largely contribute to oil flavor and aroma (*1*, *2*) and protect it from oxidation owing to their free radical-scavenging and metal-chelating properties (*3*, *4*). The nonvolatile phenolic compounds play an important role in the sensory attributes of virgin olive oil, being responsible for the bitter, pungent, and leafy attributes (*5*, *6*), while volatile phenolic compounds such as vanillin can contribute to flavor and aroma (*7*). Virgin olive oil stability is mainly due to phenolic compounds arising from the glycated precursors present in the olive fruit before extraction. The stability to oxidation has been correlated to the total amount of phenolic compounds (*8*, *9*) and to selected phenol components (*3*, *4*).

It has long been known that the total amount and the composition of the phenolic fraction in a virgin olive oil depend on the olive cultivar (*10*), climatic conditions during ripening (*11*), degree of maturation (*12*), and the industrial processes employed for oil extraction (*13*−*15*). The use of agronomic practices can also affect olive oil phenolic content. Previous studies carried out by our research group proved that irrigation, specifically a linear irrigation strategy where fixed percentages of the total water requirements were applied during the whole growth cycle of the olive tree, not only affected the total amount of phenol compounds present in virgin olive oil but also their HPLC profiles (*16*). These differences were found to be related, on one hand, to the bitter and pungent taste of the oils and therefore to the sensory quality and consumer acceptance, and on the other hand, to virgin olive oil stability to autoxidation. Hence, water availability, considered as a water stress, during certain stages of crop development could influence phenolic metabolism.

As a continuation of those studies, the present investigation was undertaken to confirm water stress influence on the HPLC phenolic profile of virgin olive oil by applying a regulated deficit irrigation (RDI) strategy (*17*). RDI strategy is based on the effect

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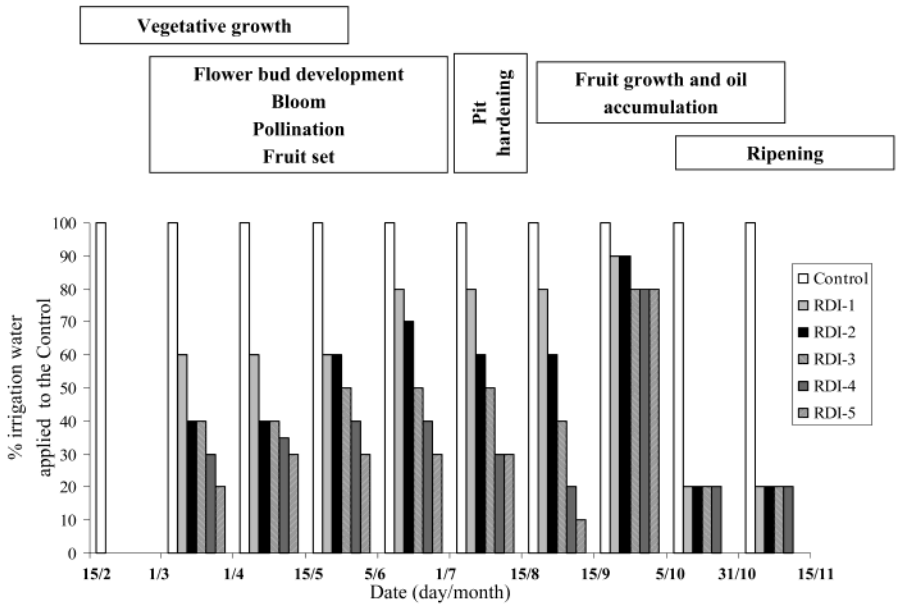


**Figure 1.** Irrigation treatments applied in 2000 during different phenological stages of olive tree.

of water stress on two processes: vegetative growth and photosynthesis. Vegetative growth can be limited by low plant water potentials during particular periods, while fruit growth remains unaffected. This strategy allows a certain degree of water stress during stages of crop development when the tree has a low sensitivity to it. Furthermore, the present paper aims to assess RDI strategy usefulness under limited water irrigation availability on the oil quality in terms of phenolic content in relation to the quality obtained when applying fully irrigation.

## MATERIALS AND METHODS

**Plant Material.** The experiment was conducted in 2000 in a commercial olive (*Olea europaea* L.) orchard of 6-year-old Arbequina trees (6 × 5 m spacing) located in the olive growing region of *Les Garrigues* (Lleida, Catalonia, Spain). The soil was a clay loam with a calcareous layer located at 40−60 cm depth. Annual rainfall for 2000 was 389 mm (mostly in spring and autumn). Daily maximum temperature was about 36 °C.

Six irrigation treatments were applied: control and five regulated deficit irrigation (RDI) strategies. Control was fully irrigated during the whole season following the water budget approach (*18*), with data from a weather station close to the experimental field (*19*), which determine the reference evapotranspiration ($ET_0$) using the FAO modified Penman equation (*20*) and the estimated crop coefficient ($K_c$) initially adapted from Goldhamer et al. (*21*) and recently validated by Girona et al. (*22*). To adjust the potential water consumption to the actual size of the canopy, a $K_r$ factor developed by Fereres et al. (*23*), that relates the percentage of shaded area by trees and the percentage of water consumption, was applied. The $K_r$ values were highly dependent on the irrigation treatments, but for control $K_r = 0.40$. Additionally, five RDI treatments (RDI-1, RDI-2, RDI-3, RDI-4, and RDI-5) were imposed, being irrigated with different percentages of the dose applied to control depending on the phenological stage of the olive tree (**Figure 1**).

Olive trees were daily irrigated with four 6 L/h drippers per olive tree placed in the row and equally distributed through the pipe. A water meter was installed in each experimental unit to verify that the water applied corresponded to each treatment. The system was operated with one general controller that individually manipulates the solenoid valves located in each experimental unit. The irrigation schedule was introduced weekly in the controller. The water applied in 2000, expressed as mm/yr, for each irrigation treatment, control, RDI-1 to RDI-5, was 471, 309, 239, 195, 134, and 114, respectively.

The statistical design was a randomized complete-block with four replicates per treatment. Each one of the 24 experimental unit consisted of three adjacent tree rows with eight olive trees per row. The center six of these 24 trees were monitored, while the others ones served as guard trees. A total 576 trees were used in this experiment, and 144 of them were monitored.

At the harvest period, which started on November 20th, representative samples from each one of the 144 experimental trees were picked and, in 2−3 h, brought to the laboratory for oil extraction.

**Oil Extraction.** An Abencor analyzer (MC2 Ingenierias y Sistemas, Sevilla, Spain) was used to process the olives in a pilot extraction plant. The unit consists of three essential elements: the mill, the thermobeater, and the pulp centrifuge. The oil was separated by decanting, transferred into dark glass bottles, and stored in the dark at 4 °C.

**Analysis of Phenolic Compounds.** *Phenolic Extraction*. Phenols were extracted from virgin olive oil following the procedure of Montedoro et al. (*24*). 2 × 20 mL of methanol/water (80:20 v/v) were added to 45 g of virgin olive oil and homogenized for 2 min with a Polytron. The two phases were separated by centrifugation at 3000 rpm for 10 min. Aqueous alcoholic extracts were then combined and concentrated in a vacuum at < 35 °C until a syrupy consistency was reached. Five milliliters of acetronitrile were added to the extract, and it was washed with 3 × 20 mL of hexane. The nonpolar phases were also purified with 5 mL of acetonitrile. The resulting acetonitrile solution was evaporated under vacuum and dissolved in 5 mL of acetonitrile. Finally, an aliquot of 2 mL was evaporated under a stream of nitrogen.

*HPLC Analysis of Phenolic Compounds*. The extracted phenolic fraction was dissolved in 1 mL of methanol and analyzed by HPLC. The HPLC system consisted of a Waters 717 plus autosampler, a 20 μL loop injector, a Waters 600 pump, a Waters column heater module and a Waters 996 photodiode array detector managed by a Millenium 2000 software (Waters Inc., Milford, MA). The column was a 15 cm × 4.6 mm i.d., 5 μm, Inertsil ODS-3 (GL Sciences Inc.) equipped with a 1 cm × 4.6 mm i.d., 5 μm, Spherisorb S5 ODS-2 (Technokroma, Barcelona, Spain) precolumn. HPLC analysis was performed following the same procedure than Montedoro et al. (*24*). The eluents were 0.2% acetic acid (pH 3.1) and methanol, and the flow rate was 1.5 mL/min. The total run time was 45 min, the initial composition was of 0.2% acetic acid/methanol (95:5) and the gradient was changed as follows: the concentration of methanol was maintained for 2 min, then it was increased to 25% in 8 min, and finally, the methanol percentage was increased to 40, 50, and 100% in 10 min periods. It was maintained at 100% for 5 min. Initial conditions were reached in 10 min. Chromatograms were obtained by monitoring at 240, 280, and 339 nm.

*Reference Compounds*. Tyrosol, *p*-coumaric acid, oleuropein, apigenin, and luteolin were obtained from Extrasynthèse Co. (Genay, France). Vanillic acid, vanillin, and ferulic acid were obtained from Fluka Co. (Buchs, Switzerland). Hydroxytyrosol was kindly donated

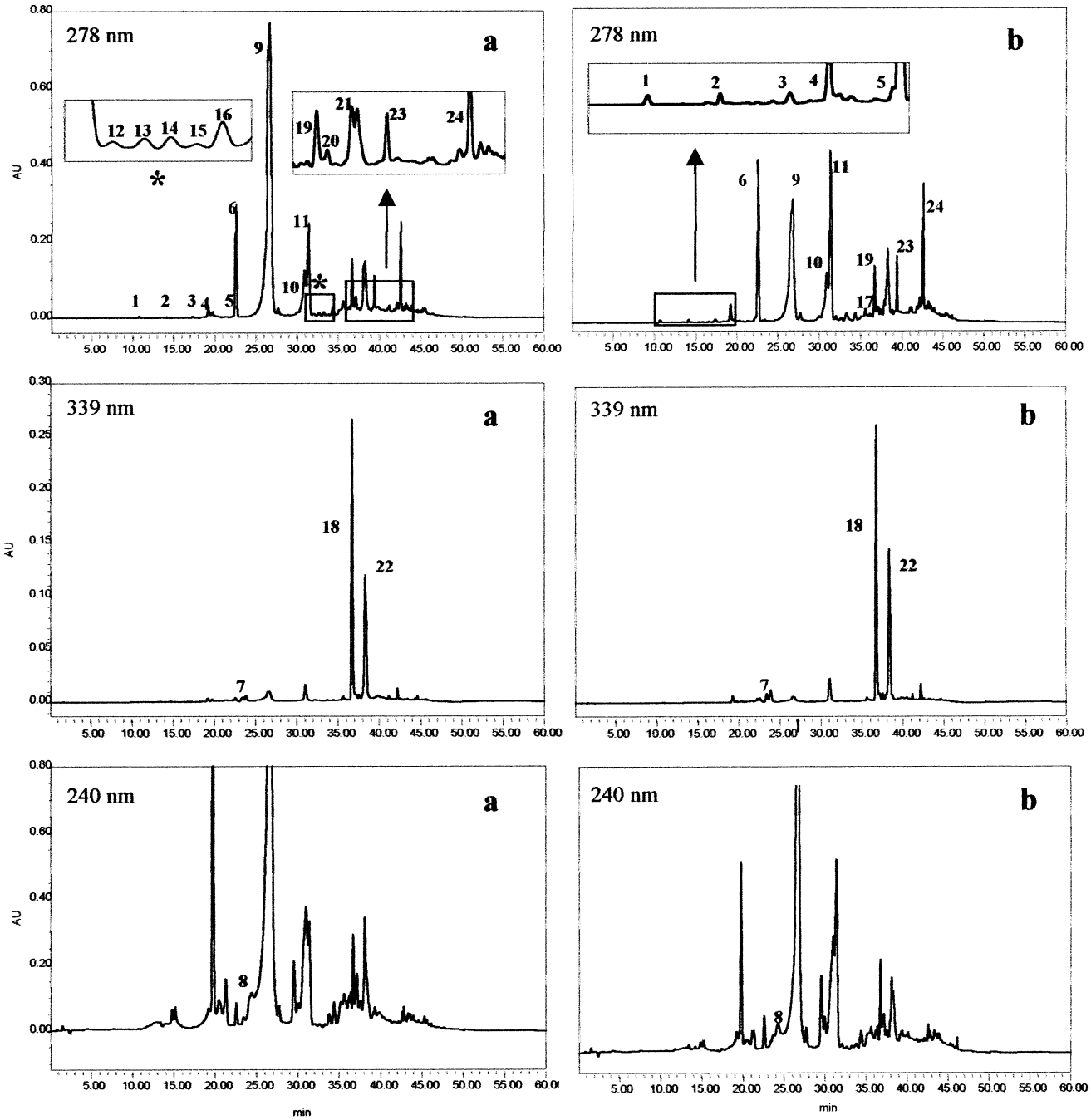


**Figure 2.** HPLC chromatograms (at 278, 339, and 240 nm) of phenolic extracts from virgin olive oil. (a) RDI-5 irrigation treatment; (b) RDI-2 irrigation treatment. See **Table 1** to identify the peaks.

by Professor Montedoro (University of Perugia, Italy). Elenolic acid was obtained from oleuropein by hydrolysis with 1 N sulfuric acid at 100 °C (*25*). The rest of the phenolic compounds were obtained using a semipreparative 25 cm × 10 mm i.d., 5 $\mu$m, Spherisorb ODS-2 (Technokroma, Barcelona, Spain) HPLC column and a flow rate of 4 mL/min. The mobile phases and the gradient have been described previously (*16*).

Individual phenols were quantified by a four-point regression curve on the basis of the standards obtained from commercial suppliers or from preparative HPLC as already described. Quantification of flavones and ferulic acid was carried out at 339 nm, and quantification of elenolic acid was at 240 nm. The quantification of the rest of the phenolic compounds was carried out at 280 nm.

**Olive Oil Analysis.** Stability is expressed as the oxidation induction time (hours) measured with a Rancimat 679 apparatus (Metrohm Co., Switzerland) using an oil sample of 2.5 g warmed to 120 °C, and 20 L/h air flow (*26*).

Bitter index ($K_{225}$) was evaluated by the extraction of the bitter components of a sample of 1.00 ± 0.01 g of oil dissolved in 4 mL of hexane passed over a C18 column (Waters Sep-Pack cartridges), previously activated with methanol (6 mL) and washed with hexane (6 mL). After elution, 10 mL of hexane were passed to eliminate the fat, and then the retained compounds were eluted with methanol/water (1/1) to 25 mL. The absorbance of the extract was measured at 225 nm against methanol/water (1/1) in a 1-cm cuvette (*27*).

**Statistical Analysis.** The data were subjected to an analysis of variance using the version 8.12 SAS system package (SAS Institute Inc. Cary, NC). Separation of the means was obtained using the Lsmeans test, and significant difference was defined at $P \leq 0.05$.

## RESULTS AND DISCUSSION

**Figure 2** shows the chromatographic profile of the phenolic compounds in the methanolic extract of Arbequina virgin olive

**Table 1.** Summary of the Phenolic Components, Retention Time (RT), and Concentration in Virgin Olive Oils from Young Olive Trees (Arbequina Cultivar)

| peak no. | RT (min) | phenolic compd | concn (mg/kg) mean | concn (mg/kg) range |
|---|---|---|---|---|
| 1 | 11.1 | hydroxytyrosol | 0.15 | 0–0.68 |
| 2 | 14.3 | tyrosol | 0.21 | 0.04–0.61 |
| 3 | 17.8 | vanillic acid | 0.27 | 0.08–0.55 |
| 4 | 19.4 | vanillin | 0.41 | 0.24–0.67 |
| 5 | 22.2 | *p*-coumaric acid | 0.06 | 0–0.14 |
| 6 | 22.7 | 4-(acetoxyethyl)-1,2-dihydrobenzene | 158.2 | 54.4–254.7 |
| 7 | 23.6 | ferulic acid | 0.09 | 0.04–0.15 |
| 8 | 24.4 | elenolic acid | 23.3 | 12.2–64.6 |
| 9 | 27.0 | dialdehydic form of elenolic acid linked to hydroxytyrosol | 484.9 | 209.5–1091.9 |
| 10 | 31.1 | dialdehydic form of elenolic acid linked to tyrosol | 36.5 | 26.4–55.1 |
| 11 | 31.6 | lignans | 160.0 | 29.1–239.4 |
| 12 | 32.2 | P12 | 2.79 | 1.49–4.23 |
| 13 | 32.9 | P13 | 2.27 | 1.13–3.29 |
| 14 | 33.5 | P14 | 4.46 | 1.18–8.19 |
| 15 | 34.0 | P15 | 1.70 | 0.48–3.66 |
| 16 | 34.6 | P16 | 8.78 | 4.38–12.1 |
| 17 | 35.8 | oleuropein aglycon | 79.6 | 58.1–127.1 |
| 18 | 36.9 | luteolin | 3.66 | 1.20–5.22 |
| 19 | 37.3 | P19 | 14.4 | 9.02–23.2 |
| 20 | 37.6 | P20 | 7.97 | 6.16–11.4 |
| 21 | 38.2 | P21 | 32.6 | 11.6–62.1 |
| 22 | 38.4 | apigenin | 1.77 | 0.34–2.64 |
| 23 | 39.5 | P23 | 26.9 | 17.1–35.7 |
| 24 | 42.7 | P24 | 40.3 | 17.3–59.3 |

oils from two of the six irrigation treatments applied in the trial (RDI-5 and RDI-2). **Table 1** shows the quantified phenolic components, retention times (RT), and the average concentration in the oils examined in this study.

Peaks 1−6, 9−11, and 17 were identified according to a previous study (*16*). Additionally, we found ferulic and elenolic acids in the oils analyzed, which were identified by comparison of their retention time and spectra with those obtained from the corresponding standards. Elenolic acid was reported for the first time in a virgin olive oil by Montedoro et al. (*24*) and subsequently Mateos et al. (*28*) proved that the presence of this compound could not be attributed to hydrolysis during the analysis of secoiridoids derivatives containing it.

There were a significantly larger number of components in the phenolic fraction as compared to those already defined. Peaks named P12 to P16, P19 to P21, P23, and P24 represent unknown complex phenolic compounds found in all olive oils analyzed, with UV spectra similar to that of secoiridoid derivatives, showing two maxima at approximately 225 and 275 nm. P24, however, shows a UV spectrum similar to that of cinnamic acid, with a maximum at 275 nm. Studies are in progress to isolate, identify, and evaluate their antioxidant potential.

With photodiode array detection at 339 nm, it was possible to establish the presence of the flavones luteolin and apigenin (peaks 18 and 22, respectively), which were identified by comparison of their retention time and spectra with those of the standards. Predominant among naturally occurring flavonoid derivatives are the glycosidic forms that are located primarily in cell vacuoles throughout the plant. Rutin, apigenin-7-glucoside, luteolin-7-glucoside, and luteolin-5-glucoside have been found in the flesh of olive fruits (*29*−*31*). During the oil extraction, the possibility arises of an enzymatic reaction leading to the hydrolysis of the glycosides and to the obtention of the aglycones, a more lipophilic form.

The prevalent phenolic compound found in the Arbequina olive oils analyzed in this trial was the dialdehydic form of elenolic acid linked to hydroxytyrosol followed in order of quantitative importance by 4-(acetoxyethyl)-1,2-dihydrobenzene, lignans, and oleuropein aglycon (**Table 1**).

The HPLC analysis of phenolic extracts obtained from virgin olive oils under different deficit irrigation strategies yielded HPLC profiles containing the same chromatographic peaks but in variable proportions. The concentrations of the phenolic compounds that were affected by irrigation regime are reported in **Table 2**. The content of vanillic acid, vanillin, and lignans increased as the amount of water applied to the tree increased. The main significant differences were observed between the most deficitary treatments (RDI-5 and RDI-4) and the most irrigated ones (RDI-1 and control), while the other irrigation treatments occupied an intermediate position. The content of P24 was only significantly lower in oils from the most stressed irrigation treatment, with no observable differences between the other treatments.

The rest of phenolic compounds affected by the water regime, which correspond to secoiridoid derivatives and the unknown phenolic compound P19, showed a different response to those above, with their concentration in the oil decreasing as the water applied to the tree increased. The concentration of the dialdehydic forms of elenolic acid linked to hydroxytyrosol and to tyrosol and oleuropein aglycon in oils from RDI-5 was significantly higher than in the oils from the other irrigation treatments. In relation to P19, there was only significant differences between RDI-5 and the most irrigated treatments (RDI-2, RDI-1, and control).

As observed in previous work by our research group with linear irrigation strategies (*16*), deficit irrigation did not affect all the phenolic compounds in the same way. On one hand, lignans, which are a major component of the phenolic fraction of olive pits (*32*), vanillic acid, and vanillin, which may be present in soluble conjugated forms as well as bound to cell wall fractions (*33*), and an unknown phenolic compound, P24, with a UV spectrum similar to that of cinnamic acid, increased in the oils from the most irrigated treatments. On the other hand, the dialdehydic forms of elenolic acid linked to hydroxytyrosol and to tyrosol and oleuropein aglycon, secoiridoid derivatives which originate from oleuropein and ligstroside during the oil mechanical extraction process, and the unknown compound P19, increased in oils from the most stressed irrigation treatments.

Different hypothesis have been developed to explain the differences found in the phenol content of oils under irrigation: the different water content of the pastes that could imply a different solubilization of phenols which are more soluble in water than in oil (*34*) and a different effectiveness in the release of phenolic compounds during crushing and malaxation linked to polysaccharides of the cell wall (*15*), and the water stress suffered by the trees that could imply a greater synthesis of phenolic compounds in the fruit and so in the oil obtained from them (*35*, *36*).

From the results obtained in this trial, we could not say that differences in oil phenol content are a consequence of the water content of the pastes, since phenol content in oils from control treatment were not significantly different from that in oils from RDI-3, RDI-2, or RDI-1 treatments, although the water applied to olive trees under those regulated deficit irrigation treatments before harvest time was only 20% of the water applied to control

**Table 2.** Phenolic Compounds of Arbequina Cultivar Virgin Olive Oil Affected by Regulated Deficit Irrigation, Stability, and Bitter Index ($K_{225}$)[a]

| | conc (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|
| phenolic compd | RDI-5 | RDI-4 | RDI-3 | RDI-2 | RDI-1 | control | |
| vanillic acid | 0.12 (0.05)a | 0.23 (0.04)b | 0.28 (0.05)b,c | 0.35 (0.02)c,d | 0.38 (0.01)d | 0.38 (0.02)d | *** |
| vanillin | 0.35 (0.05)a | 0.34 (0.03)a | 0.38 (0.04)a,b | 0.47 (0.06)b,c | 0.50 (0.03)c | 0.56 (0.05)c | ** |
| 3,4-DHPEA-EDA | 784.3 (156.9)a | 482.8 (92.0)b | 404.9 (49.5)b | 333.0 (45.6)b | 344.5 (22.0)b | 310.3 (34.7)b | *** |
| *p*-HPEA-EDA | 49.1 (4.03)a | 38.5 (1.46)b | 35.4 (1.69)b,c | 31.2 (1.91)c | 31.1 (0.47)c | 30.9 (1.28)c | *** |
| lignans | 105.3 (40.8)a | 134.3 (22.3)a,b | 177.3 (7.04)b,c | 181.8 (11.6)b,c | 203.6 (13.7)c | 193.0 (8.78)c | ** |
| 3,4-DHPEA-EA | 101.1 (13.1)a | 80.7 (8.59)b | 71.8 (2.89)b,c | 68.3 (5.63)b,c | 72.3 (2.36)b,c | 64.0 (2.17)c | ** |
| P19 | 17.7 (2.84)a | 15.4 (1.57)a,b | 14.3 (1.76)a,b | 12.0 (1.34)b | 12.0 (0.27)b | 11.1 (1.00)b | * |
| P24 | 32.3 (2.58)a | 45.3 (2.39)b | 44.0 (3.01)b | 45.2 (3.14)b | 44.3 (3.14)b | 42.9 (2.65)b | ** |
| stability (h) | 23.4 (1.65)a | 20.1 (1.77)a,b | 19.2 (0.70)b | 17.7 (1.61)b | 17.3 (0.46)b | 17.1 (1.37)b | * |
| bitter index ($K_{225}$) | 0.345 (0.048)a | 0.294 (0.029)a,b | 0.262 (0.019)b,c | 0.219 (0.024)b,c | 0.219 (0.004)c | 0.210 (0.020)c | ** |

[a] 3,4-DHPEA-EDA: dialdehydic form of elenolic acid linked to hydroxytyrosol, *p*-HPEA-EDA: dialdehydic form of elenolic acid linked to tyrosol, 3,4-DHPEA-EA: oleuropein aglycon. Values are the mean of four independent values and the standard error. Significance level by row. NS, not significant ($p > 0.05$); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

treatment (**Figure 1**). Differences in phenol content should be related to the water stress. In peach trees, it has been reported that soil moisture stress may be related to the increase in phenolics in fruits (*37*, *38*). In fact, in the previous work by our research group (*16*), where a linear irrigation strategy was applied to study its effect on the olive oil phenolic content, a change in the HPLC phenolic profile under water stress conditions reached by applying fixed percentages of the total water requirements during the whole cycle of the olive tree was observed. In the present work, we have delimited the period of the olive cycle where a water stress affects the phenolic profile of oils in a significant way, independently of the water applied during the previous period to harvest. If we consider the irrigation strategies applied in the trial (**Figure 1**), the main differences in the amount of water applied are found in the period comprising pit hardening, the first stages of fruit growth, and oil accumulation (from July to mid-September). Hence, not only is the volume of water applied important but also the stage of crop development when it is applied.

**Table 2** shows the oxidative stability and the bitter index ($K_{225}$) of Arbequina virgin olive oils in relation to the irrigation treatment applied to olive tree. Oils from RDI-5 treatment show the highest values of oxidative stability, while there are no significant differences between the rest of irrigation treatments. Oxidative stability seems to be defined by the content of the dialdehydic form of elenolic acid linked to hydroxytyrosol. It is a compound of major interest because of its presence as the major secoiridoid antioxidant compound in virgin olive oils. Its antioxidant activity has already been evaluated, and it has been shown to extend the shelf life of olive oil (*3*, *4*).

The bitter index ($K_{225}$), an analytical determination to evaluate the bitter taste, is an important index to take into account since sensory quality plays a principal role in directing the preference of consumers. The bitter attribute is one of the characteristic attributes of Arbequina virgin olive oils; however, RDI-5 treatment showed values of bitter index close to the value 0.360 corresponding to quite bitter oils (*27*), which may negatively affect consumer acceptance. Bitter index progressively decreased as the irrigation treatments applied were less severe (**Table 2**) following the same trend that the concentration of the secoiridoid derivatives in the oils. They have been reported to be related to the bitter attribute of oils (*5*), and strong positive correlations between them and the bitter index have also been found (*16*).

Water stress during a determined period of the olive cycle (pit hardening and fruit growth) could influence not only the total amount of phenolic compounds in the oil but also their profile. Only in the most severe RDI strategies applied in the present trial (RDI-5 and RDI-4) were HPLC phenolic profile and those parameters related to phenol content significantly affected by water regime. The rest of the RDI strategies indicated important savings in irrigation requirements and an increase in the water use efficiency in relation to the control treatment without affecting in a noticeable way the phenolic profile, which clearly plays a role in the organoleptic properties and the antioxidant capacity of the virgin olive oil.

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