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ARTICLE

Impact of Sampling Parameters on the Radical Scavenging Potential of Olive (*Olea europaea* L.) Leaves

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The impact of sampling parameters, that is, cultivar, leaf age, and sampling date, on the radical scavenging potential of olive leaf extracts was examined via the DPPH• and other assays. Total phenol content was estimated colorimetrically and by fluorometry, whereas phenol composition was assessed by RP-HPLC coupled with diode array, fluorometric, and MS detection systems. Oleuropein was not always the major leaf constituent. Considerable differences noted in individual phenol levels (hydroxytyrosol, oleuropein and other secoiridoids, verbascoside, and flavonoids) among samples were not reflected either in the total phenol content or in the radical scavenging potential of the extracts. It can be suggested that olive leaf is a robust source of radical scavengers throughout the year and that differentiation in the levels of individual components depends rather on sampling period than on cultivar or age. The latter does not present predictable regularity. Exploitation of all types of leaves expected in an olive tree shoot for the extraction of bioactive compounds is feasible.

KEYWORDS: Olive (*Olea europaea* L.) leaves; oleuropein; secoiridoids; flavonoids; total phenol content; radical scavenging activity; sampling impact

INTRODUCTION

Olive leaf is considered to be a source rich in oleuropein and related compounds known for multifunctional bioactive properties related to radical scavenging activity (*1*−*3*). The generic term "olive leaf" covers an entity holding a vital role in primary and secondary plant metabolism and is, as is any other natural source, subject to the influence of factors, such as cultivar, environment (production zone, agronomic practices), leaf age, and phenological stage during sampling (*4*). Trees of various cultivars, found all over the world, bear leaves with different characteristics and chemical composition, whereas leaves of different age (i.e., "new", "mature", "old-season", and "yellow" ones) can be found concurrently all over the tree canopy throughout the year. The above factors may notably affect the composition and antioxidant potential of the final raw plant material collected from the tree to be further utilized in the preparation of dry or liquid formulations. Currently, a whole range of olive leaf based products, with health or function−structure claims, are advertised on the international market. Such preparations should be standardized with regard to the compounds responsible for the claimed properties directly (on a mass basis) or indirectly on the basis of a certain attribute (e.g., ORAC value).

Although studies on olive leaf antioxidants are gaining rising interest, to our knowledge, studies on the effect of sampling parameters on the antioxidant potential of olive leaf are not available. The latter is possibly due to the fact that this plant material attracted the interest of researchers more as a farming or industrial byproduct (see, e.g., refs (*5*−*7*)) than as an olive tree product. Limited and conflicting is the information about the influence of cultivar, leaf age, and sampling period on the content of one or more phenolic compounds (*8*−*13*), whereas only one study (*8*) is, to our knowledge, about the effect of such parameters on the antioxidant potential of leaves.

The present work is part of a larger project on the establishment of olive leaf as a natural source of bioactive ingredients, suitable for commercialization throughout the year. Examination of the impact of sampling parameters such as cultivar, leaf age, and sampling date on the radical scavenging potential of extracts prepared from a great number of systematically collected samples over a two year period was based on the determination of total and individual phenolic compounds as well as the antioxidant activity assessment using complementary techniques and methods.

MATERIALS AND METHODS

Chemicals. Oleuropein (98%) was purchased from Extrasynthèse (Genay, France); tyrosol (98%), caffeic acid, and luteolin (99%) were from Sigma-Aldrich (Steinheim, Germany); morin was from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azobis(2-aminopropane) dihydrochloride (AAPH, > 98%) and fluorescein sodium were from Fluka Chemie (Buchs, Switzerland). DPPH• radical (1,1-diphenyl-2-picrylhydrazyl, 90%), $ABTS^{\bullet+}$ diammonium salt, and potassium persulfate were from Sigma Chemical Co. Folin−Ciocalteu (F−C) reagent, $AlCl_3$, and diethyl ether, stabilized with ethanol, were purchased from Panreac Quimica (Barcelona, Spain). Saffron red stigmas were donated by Saffron Cooperative of Kozani (Greece). HPLC grade methanol (MeOH) and acetonitrile (ACN) were from Merck KgaA (Darmstadt, Germany). Ultrahigh-purity

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water was produced using a Millipore Milli-Q system. All other common reagents and solvents were of the appropriate purity from various suppliers.

Leaf Sampling. *(a)* Leaves from various cultivars (cv.) were sampled from trees of an olive orchard (Agricultural Research Station, Agios Mamas, Chalkidiki, Greece) as indicated in the plan of Figure S1 (see Supporting Information). The trees of each cultivar were of the same age (24 years old), grown under the same climatic conditions and cultivation treatments. Variability was investigated for one type of leaves (mature leaves, obtained from the middle to inner end of 1-year-old shoots) from trees that belonged to 10 different Greek cultivars [Adramatiani, Amfissis, Chondrolia Chalkidikis (Ch.), Kalamon, Kolovi, Koroneiki, Kothreiki, Megaritiki, Tsounati, Vassilikada], one Spanish cultivar (Picual), and one Italian cultivar (Frantoio). For each cultivar, samples were collected from the four trees of one parallelogram placed in block B of the orchard on the same date (December 22, 2006). Within cultivar, variability was investigated for two of the cultivars (Chondrolia Ch. and Koroneiki). Leaves were, therefore, collected from all blocks (A, B, and C). On February 15, 2008, sampling was repeated for selected cultivars (Adramatiani, Amfissis, Chondrolia Ch., Koroneiki, and Vassilikada) in the way already described. For the study of sampling date effect, new-season leaves (from the extreme tip of 1-year-old shoots) were obtained from Chondrolia Ch. and Koroneiki trees of the same orchard. Sampling for each cultivar was from two of the four trees of the same parallelogram (Figure S1, sixth row, north orientation) during the period between July 15 and November 29, 2006.

(b) Leaves from one randomly selected tree (Chondrolia Ch.) grown in another orchard (Aristotle University of Thessaloniki, Greece), placed ~80 km northwest from the one described in the preceding paragraph, were collected for the study of leaf age effect. New, mature, old-season leaves from 2-year-old shoots, and yellow ones were collected on the same day (October 24, 2007). On April 1, 2008, sampling was repeated, and new, mature, old-season, and yellow leaves were collected from the same tree.

In all cases, sampling was performed between 10 a.m. and 1 p.m. and the leaves were selected from branches within arm's reach, from the whole perimeter of trees, to minimize environmental and orientation variability. After sampling, leaves were immediately cleaned of dust and subsequently freeze-dried. Finally, dried samples were placed under nitrogen in airtight opaque glass jars and stored in a dry, dark, and cool place until analysis. Analysis was accomplished as close as possible to sampling date.

Leaf Characteristics. Characteristics such as leaf length, width, and weight were given as a mean value of randomly selected leaves ($n = 15$). Moisture content (percent loss of weight) was determined for samples before and after lyophilization.

Leaf Extract Preparation. Prior to further analysis leaves were cut into small pieces with the aid of a laboratory blade cutter. Extraction solvent was either methanol or 50% aqueous methanol. Lyophilized plant material (0.25 g in 10 mL of solvent) was treated in an ultrasonic bath at room temperature for 5 min. Suitable filtered aliquots were then used for spectrometric and HPLC analyses.

Determination of Total Polar Phenol (TPP) Content. TPP content of methanol extracts was estimated using two assays, the F−C and a fluorometric one, recently developed in our laboratory (*14*). The F−C assay was applied as described elsewhere (*15*). Oleuropein was used as an external standard. Measurement and extraction repeatabilities were found to be satisfactory (CV % = 1 and 8, respectively, $n = 5$). For the fluorometric assay an aliquot (~100 μL) of the extract was transferred into a 10 mL volumetric flask, and the volume was made up to 10 mL with MeOH (stock solution). Then, working solutions were prepared in duplicate by diluting an appropriate aliquot (~100−400 μL) from stock solution in 5 mL of the solvent, so that the reading was within the acceptable range. Other analytical details are given in ref *14*. Oleuropein was used as the external standard. Measurement repeatabilities for oleuropein and an extract were satisfactory (CV % = 1 and 3, respectively, $n = 5$).

Determination of Flavonoid Content (FL). Flavonoid content was estimated according to the validated protocol of Cvek and collaborators (*16*). The latter is based on the formation of a flavonoid−aluminum chloride complex. Results were expressed as micrograms of FL per gram of dry leaf through a morin calibration curve. The repeatabilities of measurement calculated for a morin standard solution and an extract were found to be satisfactory (CV % = 1 for both, $n = 5$). The same procedure in the absence of acid was also carried out to codetermine all of the *o*-dihydroxy groups present (*17*).

Antioxidant Activity Studies. Radical scavenging activity of methanol extracts was determined via the DPPH$^{\bullet}$ assay. In certain cases ABTS$^{\bullet+}$, ORAC, and CBA assays were also applied for further investigation of the differences in the antioxidant potential of the examined extracts. Oleuropein was the reference compound in all assays, which were accomplished using protocols repeatedly applied to our laboratory with appropriate adjustments (*18*−*22*).

HPLC Analysis of Phenolic Compounds. The HPLC system consisted of a pump, model P4000 (Thermo Separation Products, San Jose, CA), a Midas autosampler (Spark, Emmen, The Netherlands), and a UV 6000 LP diode array detector (DAD; Thermo Separation Products) in series with an SSI 502 fluorescence detector (FLD; Scientific Systems Inc., State College, PA). Phenolic compounds in the tested extracts were monitored at 245, 280, and 335 nm using DAD and at 280 nm excitation and 320 nm emission using FLD. The data were processed with the aid of ChromQuest software (version 3.0, Thermo Separation Products). Peak identification was based on literature data, relative retention times, spectra matching, and standards available. Quantitification was achieved using calibration curves of tyrosol (280 nm), caffeic acid (335 nm), and oleuropein (245 nm). Two chromatographic columns were used in the analysis. Comparisons are made only for data produced under the same experimental conditions. The following columns and elution protocols were used. Protocol A: Chromolith RP-18e (100 × 4.6 mm) column (Merck); aqueous acetic acid solution (3%, v/v) (A) and ACN (B); 0−1 min, 4% B; 1−26 min, 4−30% B; 26−36 min, 30−60% B; 36−46 min, 60−98% B; 46−50 min, 98% B; 50−60 min, 98−4% B; flow rate 0.9 mL/min. Protocol B: Nucleosil C18, (125 × 4 mm, 5 μm) column (Macherey-Nagel), aqueous acetic acid solution (3%, v/v) (A) and ACN/MeOH (50:50, v/v) (B); 0−50 min, 5−66% B; 50−52 min, 66−95% B; 52−65 min, 95% B; 65−70 min, 95−5% B; 70−85 min, 5% B; flow rate, 1.0 mL/min.

LC-MS Analysis. LC-MS analysis was used for verification of certain identified major compounds using the above-mentioned procedures. Instrumentation and detection mode were as described in ref *23*. Elution was achieved using aqueous acetic acid solution (1%, v/v) (A) and ACN (B); 0−20 min, 5−25% B; 20−40 min, 25−50% B; 40−50 min, 50−80% B; 50−60 min, 80−5% B, on an Alltech 250 mm × 4.6 mm, 5 μm, Altima C18 column (Breda, The Netherlands), 0.6 mL/min flow rate, and 20 μL injection volume.

Statistical Analysis. Statistical comparisons of the mean values for each experiment were performed by one-way analysis of variance (ANOVA), followed by the multiple Duncan test ($p < 0.05$ confidence level) using SPSS 14.0 software (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Results of the impact of sampling parameters such as cultivar, leaf age, and sampling date on the radical scavenging potential of olive leaves demanded a series of experiments covering antioxidant activity assessment, evaluation of TPP content of tested extracts, and characterization of bioactive ingredients. Assays based on different principles were conducted to verify findings concerning differences in the TPP content and antioxidant activity among extracts. The results are presented in **Tables 1**−**4** and **Figures 1** and **2**. Morphological data within each leaf category given as Supporting Information (Tables S1−S3) point out that sampling was carefully accomplished. Discussion is organized per factor effect as follows.

Table 1. Differences in the TPP Contents and Radical Scavenging Activities of Extracts Obtained from Leaves of the Studied Cultivars[a]

	TPP content		DPPH• assay,[b] antioxidant activity on the same	
cultivar (block B)	F−C assay[c]	fluorometric assay[c]	TPP basis[d]	dry extract wt basis[e]
		December 2006 Sampling		
Adramatiani	42 de	21 b	91 ± 1 gh	59 ± 3 e
Amfissis	35 bc	22 b	84 ± 1 e	45 ± 1 c
Chondrolia Ch.	34 ab	24 c	69 ± 1 a	40 ± 1 b
Kalamon	29 a	16 a	77 ± 1 c	40 ± 2 b
Kolovi	40 cd	17 a	89 ± 1 fg	34 ± 2 a
Koroneiki	31 ab	24 cd	73 ± 1 b	32 ± 3 a
Kothreiki	60 g	33 e	84 ± 1 e	57 ± 4 e
Megaritiki	45 e	26 d	90 ± 2 fgh	56 ± 1 e
Tsounati	54 f	33 e	92 ± 1 h	58 ± 1 e
Vassilikada	33 ab	21 b	79 ± 1 d	52 ± 2 d
Frantoio	30 a	21 b	80 ± 2 d	33 ± 2 a
Picual	61 g	25 cd	88 ± 1 f	40 ± 1 b
		February 2008 Sampling		
Adramatiani	41 b	29 b	75 ± 3 b	40 ± 3 b
Amfissis	43 b	33 c	77 ± 4 b	39 ± 1 b
Chondrolia Ch.	29 a	34 c	83 ± 1 c	39 ± 3 b
Koroneiki	41 b	28 b	68 ± 1 a	44 ± 1 b
Vassilikada	53 c	17 a	76 ± 2 b	28 ± 2 a

[a] Values within the same column bearing different letters are significantly different ($p < 0.05$). [b] Results are expressed as % DPPH• inhibition ± standard deviation ($n = 3$); measurement and extraction repeatability CV % = 5 and 8, $n = 5$. [c] Results are expressed as mg of oleuropein/g of dry leaf; mean value of two measurements. [d] Final assay concentration ~23 mg/L TPP (F−C) expressed as oleuropein. [e] Final assay concentration ~33 mg of dry extract/L.

Table 2. "Within Cultivar" Variability in TPP Content and Radical Scavenging Activity of Leaf Extracts, December 2006 Sampling[a]

	TPP content		DPPH• assay,[b] antioxidant activity on the same		individual phenol content[c] (μg of tyrosol/g of dry leaf)				
cultivar/block	F−C assay[d]	fluorometric assay[d]	TPP basis[e]	dry extract wt basis[f]	1	2	3	4	6
Chondrolia Ch./A	35 a	27 b	73 ± 1 b	41 ± 1 a	2172	2598	1249	621	<LOD
Chondrolia Ch./B	34 a	24 a	69 ± 1 a	40 ± 1 a	235	471	1278	693	124
Chondrolia Ch./C	64 b	36 c	94 ± 1 c	56 ± 3 b	398	369	62	337	45
Koroneiki/A	36 a	29 b	73 ± 2 a	33 ± 2 a	927	367	<LOD	326	132
Koroneiki/B	31 a	24 a	73 ± 1 a	32 ± 3 a	306	339	<LOD	346	<LOD
Koroneiki/C	44 b	32 c	81 ± 3 b	41 ± 1 b	520	202	<LOD	213	122

[a] Values within the same column bearing different letters are significantly different ($p < 0.05$). [b] Results are expressed as % DPPH• inhibition ± standard deviation ($n = 3$), measurement and extraction repeatability CV % = 5 and 8, $n = 5$. [c] Peak numbering according to **Figure 1** corresponding to compounds **1**−**4** and **6**. [d] Results are expressed as mg of oleuropein/g of dry leaf; mean value of two measurements. [e] Final assay concentration ~23 mg/L TPP (F−C) expressed as oleuropein. [f] Final assay concentration ~33 mg of dry extract/L.

Table 3. TPP Content and Radical Scavenging Activity of Extracts Obtained from New, Mature, Old, and Yellow Leaves in October 2007; Data in Parentheses Correspond to April 2008 Sampling[a]

	TPP content		antioxidant activity on the same TPP basis			
leaf age	F−C assay[b]	fluorometric assay[b]	DPPH• assay[c]	$ABTS^{\bullet+}$ assay[d]	ORAC assay[e]	CBA assay[f]
new	73 b (55 b)	49 b (40 a)	82 ± 1 a (78 ± 2 a)	57 ± 3 d	1.0 ± 0.1 a	56 ± 1 b
mature	69 b (46 ab)	42 a (44 ab)	84 ± 2 a (83 ± 3 b)	52 ± 1 cd	0.9 ± 0.1 a	55 ± 1 b
old	70 b (39 a)	42 a (47 b)	82 ± 2 a (80 ± 1 ab)	46 ± 3 ab	0.9 ± 0.1 a	58 ± 4 bc
yellow	53 a (37 a)	43 a (48 b)	83 ± 3 a (80 ± 1 a,b)	56 ± 4 d	1.0 ± 0.2 a	61 ± 1 c
oleuropein[g]			96 ± 1	42 ± 3	1.0 ± 0.1	49 ± 2

[a] Values within the same column bearing different letters are significantly different ($p < 0.05$). [b] Results are expressed as mg of oleuropein/g of dry leaf, mean value of two measurements. [c] Results are expressed as % DPPH• inhibition ± standard deviation ($n = 3$), final assay concentration ~23 mg/L TPP (F−C) expressed as oleuropein. [d] Results are expressed as % inhibition ± standard deviation ($n = 3$), final assay concentration ~11 mg/L TPP (F−C) expressed as oleuropein. [e] Results are expressed as oleuropein equivalents, mean value ± standard deviation ($n = 3$), final assay concentration ~0.54 mg/L TPP (F−C) expressed as oleuropein, measurement repeatability CV% = 14, $n = 5$ for oleuropein and extract, between day repeatability CV % = 17, $n = 15$ for oleuropein and CV % = 18, $n = 9$ for extract. [f] Results are expressed as % inhibition ± standard deviation ($n = 3$), final assay concentration ~16 mg/L TPP (F−C) expressed as oleuropein. [g] Antiradical ability of equimolar oleuropein standard solution.

Table 4. TPP Content, FL Content, and Radical Scavenging Activity of Extracts Obtained from New Leaves; Sampling Period: June 2006 to November 2006[a]

	TPP content		FL content	DPPH• assay[b], antioxidant activity on the same		
sampling date	F−C assay[c]	fluorometric assay[c]	flavonoid−Al(III) complexation[d]	TPP basis[e]	FL basis[f]	dry extract wt basis[g]
			Cv. Chondrolia Ch.			
June 15	39 b	31 ab	2996 b (3507 bc)	95 ± 1 d	37 ± 2 a	68 ± 2 b
July 31	29 a	33 bc	2382 a (3183 b)	94 ± 1 d	47 ± 4 b	52 ± 2 a
Aug 21	32 a	26 a	2678 ab (3714 c)	95 ± 1 d	44 ± 1 b	52 ± 1a
Sept 11	42 b	26 a	2127 a (2749 a)	88 ± 1 c	44 ± 1 b	90 ± 1d
Oct 17	53 c	40 d	4741 c (5939 d)	86 ± 1 b	37 ± 2 a	74 ± 1 c
Nov 29	49 c	38 cd	2656 ab (3417 bc)	80 ± 1 a	36 ± 1 a	66 ± 2 b
			Cv. Koroneiki			
June 15	47 bc	30 b	3098 d (4936 d)	77 ± 1 b	64 ± 2 a	58 ± 3 c
July 31	32 a	23 a	2523 b (3907 b)	86 ± 1 c	63 ± 5 a	45 ± 1 a
Aug 21	34 a	21 a	2225 a (3611 a)	94 ± 1 e	76 ± 1 b	55 ± 2 b
Sept 11	59 c	24 a	3108 d (5195 d)	65 ± 1 a	78 ± 1 b	62 ± 1 d
Oct 17	50 bc	37 c	2726 c (4220 c)	91 ± 1 d	83 ± 2 c	77 ± 1 e
Nov 29	41 ab	47 d	2308 a (3511 a)	94 ± 1 e	66 ± 3 a	76 ± 1e

[a] Values within the same column bearing different letters are significantly different ($p < 0.05$). [b] Results are expressed as % DPPH• inhibition ± standard deviation ($n = 3$). [c] Results are expressed as mg of oleuropein/g of dry leaf, mean value of two measurements. [d] Results are expressed as μg of morin/g of dry leaf, mean value of two measurements, final assay concentration ~400 mg dry extract/L; data in parentheses were obtained without acid treatment. [e] Final assay concentration ~23 mg/L TPP (F−C) expressed as oleuropein. [f] Final assay concentration ~ 1 mg/L FL expressed as morin. [g] Final assay concentration ~ 33 mg of dry extract/L.

Cultivar Effect. Variability due to cultivar effect was examined for 10 Greek cultivars, an Italian cultivar, and a Spanish cultivar grown under the same environment. Mature leaves, commonly used in leaf diagnostic agricultural studies, were chosen as the test material (*24*). "Within cultivar" effect was examined for two of the above cultivars, Chondrolia Ch. and Koroneiki. The former, a large-fruited cultivar, presenting a distinct 2-year fruit-bearing cycle, is famous in table olive production, whereas the latter, a small-fruited one, bearing a regular crop every year, is considered to be the major cultivar in Greek olive oil production.

Data on the TPP content and radical scavenging potential for the studied cultivars are presented in **Table 1**. Values were of the same magnitude even when statistically significant different. Variability within the same cultivar is not expected to differentiate the above findings (**Table 2**). Leaf extracts, irrespective of cultivar, were found to be good sources of radical scavengers. Variability in relative inhibition values (~69−94%) of extracts—on the same TPP content basis—indicated that individual phenol levels may influence overall activity. Repetition of sampling for characteristic cases a year later led to similar observations (**Table 1**), which indicated that cultivar impact with regard to radical scavenging potential of olive leaves is rather limited.

Composition of individual constituents was then investigated by RP-HPLC analysis. Due to differences in absorption maxima except for the general use wavelength, 280 nm, recording at 245 nm aimed at selective detection of oleuropein, whereas flavonoid and cinnamic type phenolics were recorded at 335 nm. Fluorescence detection (280 nm excitation/320 nm emission) was found useful to identify oleuropein and hydroxytyrosol. Representative chromatographic phenolic profiles of olive leaf extracts are illustrated in **Figure 1**. Quantification data for compounds identified as hydroxytyrosol (**1**), luteolin 7-*O*-glucoside (**2**), verbascoside (**3**), luteolin 4′-*O*-glucoside (**4**), oleuropein (**5**), and luteolin (**6**) using various means showed that the levels of these most frequently reported olive leaf bioactive ingredients may vary significantly among cultivars. Despite the alleged view, neither oleuropein nor other secoiridoid compounds were the major ingredient in the majority of the examined extracts. Flavonoids and verbascoside shared with them total peak area recorded. Most of the extracts (~3/4) contained oleuropein in trace levels (280 nm), whereas, when present, its content varied widely (40−2159 μg as tyrosol/g of dry leaf). Two of the cultivars had extremely high oleuropein content the date of sampling (December 2006). Repetition of sampling a year later for the same cultivars did not show a similar trend. Nevertheless, other cultivars that showed trace to moderate oleuropein content in the first sampling appeared to have moderate to high levels, respectively. Such results point out that oleuropein level depends on sampling period irrespective of cultivar. Quantitative differences in oleuropein content reported in the literature among cultivars may be, consequently, misleading for the appreciation of the antioxidant potential of leaf extracts, especially in cases when sampling is from trees grown in different geographic regions (see, e.g., ref *25*). Our observation was strengthened by data obtained for the content of other constituents such as hydroxytyrosol (trace−2172 μg), luteolin 7-*O*-glucoside (trace−2598 μg), verbascoside (trace−1278 μg), luteolin 4′-*O*-glucoside (trace−1398 μg), and luteolin (trace−1054 μg); values are expressed as micrograms of tyrosol per gram of dry leaf. Nevertheless, these considerable differences in individual phenol contents were not reflective of either the TPP content or the antioxidant activity values, as shown in **Table 1**. This fact supports our objective to investigate which sampling parameters are possibly the most influential, with regard to the antioxidant activity of an olive leaf extract.

Variability in the composition of extracts may be partially assigned to the asynchrony of developmental stages of trees from various cultivars rather than to cultivar impact itself. Moreover, parameters such as canopy density and shading of leaves that are reported to affect leaf dry matter and

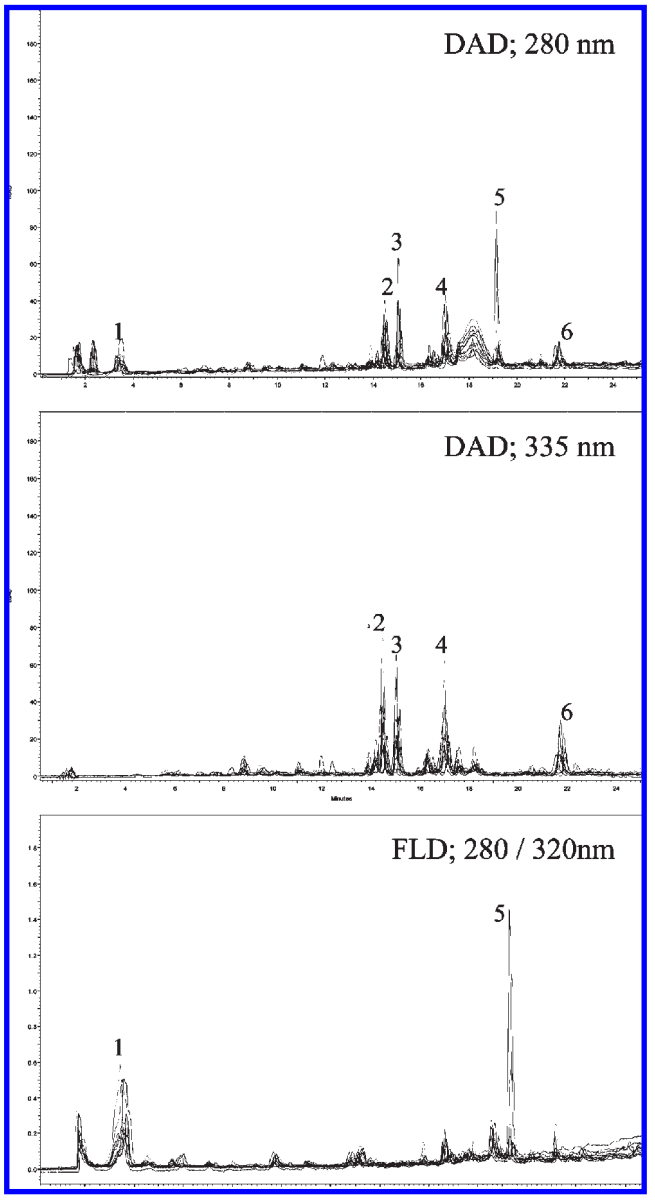

Figure 1. Overlay of RP-HPLC phenolic profiles of 50% aqueous methanol leaf extracts from the 12 cultivars examined; chromatographic protocol A; detection using DAD (280 and 335 nm) and FLD (280 nm excitation/320 nm emission). Peaks: 1, hydroxytyrosol; 2, luteolin 7-*O*-glucoside; 3, verbascoside; 4, luteolin 4′-*O*-glucoside; 5, oleuropein; 6, luteolin.

carbohydrate content (*26*) may also influence the phenolic composition of leaves and consequently the respective antiradical potential. Nonetheless, the above factors, although numerous and interrelated, were not proven to affect dramatically the radical scavenging activity of the tested extracts. This was also confirmed by "within cultivar" study data (**Table 2**), where the notable differences observed in the levels of some individual phenolics are not reflective of either the TPP content or the percent DPPH$^{\bullet}$ inhibition values.

Sampling and analysis of mature leaves from Chondrolia Ch. and Koroneiki trees, grown in another region during the same period (December 2006), confirmed the above remarks (data available not shown). Regardless of the slightly lower TPP content and radical inhibition values, these plant materials were good antioxidant sources, too. Undoubtedly, olive leaves are a rather robust source of antioxidants highly appreciated in the food, pharmaceutical, and cosmetic industries.

"Leaf Type Effect". So far, leaves used in the study were mature ones. Considering "olive leaves" as an olive tree product, collectable directly from it and not as an olive mill waste material, the question of whether leaf type influences apparently or not the level and composition of phenolic compounds and/or the antioxidant potential of the corresponding extracts was to answer next. The olive tree is an evergreen plant, and thus leaves of different ages can be found concomitantly throughout its canopy. For an adult tree, leaf life span is up to three years, even if the majority of leaves fall during the second year. Olive leaves can be distinguished to new and mature, located in current year shoots, whereas old and yellow leaves are also found in older season shoots. On the other hand, throughout the growing season leaves present a developmental stage (March to November), during which new leaves appear continually (*27*). To study "leaf age" factor, we collected new, mature, old-season, and yellow leaves. Our sampling aimed at investigating the possibility of pooling together all types of leaves expected in the shoots of an olive tree branch for extraction of bioactive ingredients.

Observations similar to those made during the study of cultivar effect were also driven from the data presented in **Table 3**. TPP content and percent DPPH$^{\bullet}$ inhibition values of the methanol extracts prepared from leaves of different age were comparable. The DPPH$^{\bullet}$ scavenging activity of all studied extracts was of the same magnitude as that of an oleuropein equimolar standard solution, also presented in **Table 3**. Small were also the differences found among extracts using other characteristic antioxidant assays (ABTS$^{\bullet+}$, ORAC, CBA) to verify DPPH findings as suggested by (*28*).

All leaf extracts, in accordance with our previous findings, presented qualitatively similar HPLC phenolic profiles. Monitoring showed remarkable differences in the content of certain phenolics. Marked was the variability in oleuropein content (5732−26330 μg of oleuropein/g of dry leaf), although within the range reported by various investigators (*8*, *10*). Extracts prepared from mature leaves had 1.5-fold higher oleuropein content than those of yellow ones. New and old leaves presented similar oleuropein levels, almost 3.5 times lower than those found for mature ones. Total flavonoid content (expressed as total peak area at 335 nm) varied noticeably among extracts. Luteolin 7-*O*-glucoside, luteolin 4′-*O*-glucoside, and luteolin accounted for ∼60−70% of the total flavonoid content. The contents of luteolin 7-*O*-glucoside and luteolin 4′-*O*-glucoside were found to vary with leaf age. The absence of luteolin was observed in yellow leaves, which were found to contain higher amounts of luteolin glucosides and verbascoside, in comparison with the rest of the studied leaves. Comparison of chromatographic profiles of extracts from the two sampling periods (October 2007 and April 2008) showed only qualitative similarities. Mature leaves from the second sampling were found to contain oleuropein in traces, whereas yellow leaves exhibited the highest oleuropein content.

"Sampling date" effect examined for new leaves from Chondrolia Ch. and Koroneiki cultivars confirmed our view that olive leaf is suitable for commercialization throughout the year, as far as radical scavenging potential is concerned. New leaves were collected once a month, over a period coinciding with that from growth to maturation of olive fruits. During that period changes in phenol content and composition of fruits are expected to be large (*29*). To our knowledge, evidence for the content and composition

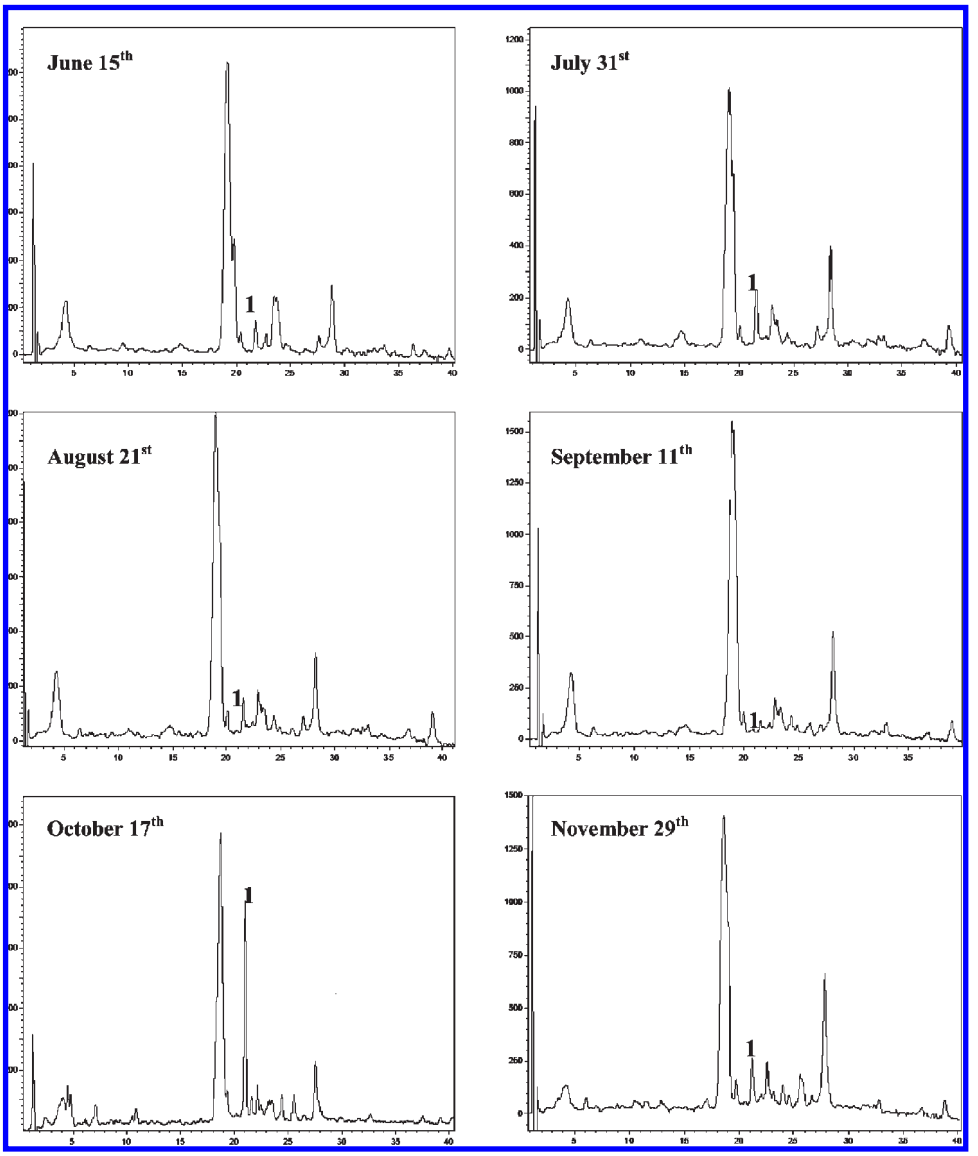

Figure 2. RP-HPLC phenolic profiles of methanol leaf (cv. Koroneiki) extracts from the six different sampling dates examined; chromatographic protocol B; detection using DAD at 245 nm. Peak: 1, oleuropein.

of new leaf phenolics over the same time span is limited to the work of Ryan and collaborators (*10*) for an Australian cultivar (Hardy's Mammoth) grown in the southern hemisphere. However, no information relevant to the impact of sampling date factor on the antioxidant potential of leaf extracts is available. Data presented in **Table 4** indicate that new leaves were a robust source of radical scavengers, in terms of total phenol or flavonoid content, over the 6 month period sampling. Differences in the percent $DPPH^{\bullet}$ inhibition values of extracts containing the same TPP or FL content, or assessed on the same dry weight basis, were once more trivial despite observed discrepancies in the level of individual compounds, as illustrated in **Figure 2** for oleuropein.

General Remarks. The above findings reinforce our view that, apart from oleuropein, other secoiridoids and flavonoids contribute notably to the final antioxidant potential of olive leaf, which is a rather robust entity, despite discrepancies in the level or activity of individual compounds. Indeed, the predominance of certain secoiridoids eluting just before oleuropein (see **Figures 1** and **2**) was seen in all of the samples. Our chromatographic data coupled with similar published ones suggest the presence of these derivatives due to possible transformation pathways of oleuropein (*10*, *30*, *31*). These secoiridoids are expected to exhibit antioxidant activity (*3*). In addition, flavonoids and verbascoside contribute to the overall antioxidant activity of olive leaf extracts (*2*), whereas synergistic behavior of the various leaf phenolics has been suggested to affect its overall antioxidant potency, too (*2*).

Conclusion. Our systematic study gave us the opportunity to be more critical about the impact of leaf cultivar, type, and sampling date on the levels of its bioactive compounds. Total phenol content and radical scavenging potential values seem to be safe criteria for the selection and appreciation of olive leaf batches for further usage. Our findings were the outcome of careful sampling and postharvest treatment of leaves, which then were analyzed within a short period of time to allow comparisons among quantitative data. Establishment of olive leaf as a robust source for radical scavengers throughout the year seems to be promising for its commercialization. Investigation of the contribution of individual compounds to the overall antioxidant activity is expected to add to the knowledge needed

for further fractionation of the polar extracts. This task is currently underway.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amino propane) dihydrochloride; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); ACN, acetonitrile; CBA, crocin bleaching assay; Chondrolia Ch., Chondrolia Chalkidikis; cv., cultivar; DAD, diode array; DPPH, 1,1-diphenyl-2-picrylhydrazyl; F−C, Folin−Ciocalteu; FLD, fluorescence detector; MeOH, methanol; ORAC, oxygen radical absorbance capacity; FL, flavonoids; TPP, total polar phenols.

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Supporting Information Available: Figure S1, experimental orchard; Tables S1, S2, and S3, morphological characteristics and mean moisture content of leaf samples examined. This material is available free of charge via the Internet at http://pubs.acs.org.

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