JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Factors Affecting the Contents of Iridoid Oleuropein in Olive Leaves (*Olea europaea* L.)

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In this study, for the first time, the impact of the genetic factor on the contents of oleuropein in olive leaves was not only evaluated but the influence exerted by the color/age of leaves (green, green-yellowish, and yellow) and the collecting period (spring or autumn) was also evaluated. A repetitive high-resolution gas chromatographic quantitation method and an accurate high-performance liquid chromatographic method were developed. These analytical methods gave results showing a highly linear relationship. Samples of olive leaves were taken from seven major Italian olive cultivars, such as Dritta, Leccino, Caroleo, Coratina, Castiglionese, Nebbio, and Grossa di Cassano. Such a vegetal raw material could actually be exploited for recovering oleuropein, considered to be a high-added value molecule. This could be converted into hydrxytyrosol, a compound known to possess strong bioactive properties. Olive leaves showed considerable contents of oleuropein, which with some cultivars were even higher with respect to those present in the corresponding olive fruits (reported in the literature). The amounts of oleuropein in the collected leaves were markedly modified by the color/age and genetic factors, whereas meaningless variations were ascribable to the quantitation method and the collecting period factors. Various chemometrics, applied to the obtained analytical data, appeared to be effective in discriminating the samples on the basis of the above-examined experimental factors, thus confirming how these should be taken into account in future industrial recovery of oleuropein from olive leaves.

KEYWORDS: Olive leaves; oleuropein; extraction; quantitation; impacting factors; chemometrics

INTRODUCTION

Olive tree (*Olea europaea* L., Oleaceae) is known for its capacity of making an organic matter containing a high proportion of biophenol compounds. These amphiphilic molecules accumulate essentially into the fruit (and leaf), especially during growth and the first stages of maturation. Several investigations involved the phenol composition of olive fruit and its derivatives. Recently, some studies considered the phenol fraction present in olive leaves (*1*, *2*). This research seemed to indicate that olive leaf has a qualitative phenol composition similar to that of olive fruit. The iridoid oleuropein (a bitter compound) is the main phenol component followed by other phenol glycosides (or esters) (*3*).

Such a functional compound belongs to the group of phenol secoiridoids (including also demethyloleuropein, ligstroside, and nüzenide), which form through opening of the five-membered ring of iridoids. The parent of this group of plant metabolites, which occur only in the Olearaceae family (including *O. europaea* L.), is the secologanin compound. Iridoids are monoterpenes (C_{10}) arising from the folding of geraniol, characterized by a bicyclic fused ring system (six-membered heterocyclic ring fused to a cyclopentane ring) (*4*, *5*).

Oleuropein was detected for the first time in the fruit of *O. europaea* L. by Bourquelot and Ventilesco (*6*), but its chemical structure was assigned by Panizzi et al. (*7*). It is the heterosidic ester of 3,4-dihydroxyphenilethanol (hydroxytyrosol) with β-glucosylated elenolic acid. Hydroxytyrosol in turn consists of two bounded molecular structures, such as catechol and ethanol (*1*).

Oleuropein is easily transformed by the endogenous or exogenous β-glycosidase enzyme into glucose and oleuropein aglycon. The product obtained by subsequent esterolysis of the latter compound is hydroxytyrosol, a high-added value phenol molecule claimed to be highly bioactive. The intermediate aglycon structure can also undergo the opening of the elenolic ring moiety giving rise to chemical rearrangements (decarboxilation, methylation, and oxidation) with formation of new phenol aglycon structures, which also can exist as unstable ketoenolic tautomer forms (e.g., mono- and dialdehydic forms) (*7*−*11*). **Figure 1** shows the chemical structure of oleuropein and related compounds.

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10.1021/jf051647b CCC: $33.50
Published on Web 12/17/2005

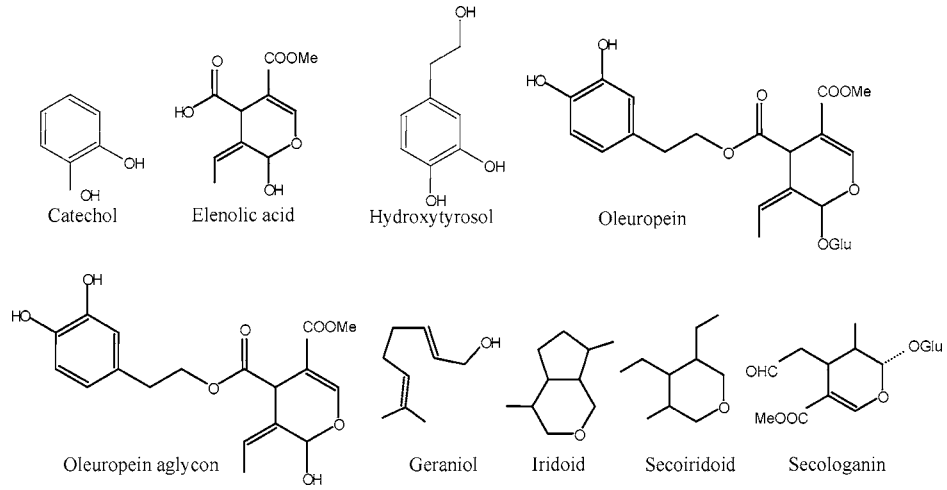

Figure 1. Chemical structures of oleuropein and related compounds.

The antimicrobial, antioxidative, and healthy effects of oleuropein and hydroxytyrosol have been documented (*3*). This latter is claimed to be the main functional biophenol molecule and the most potent antioxidant because of the presence of the catechol moiety in its molecule (*12*, *13*). The oleuropein molecule is known to be antiatherogenic and cardioprotective (inhibition of low-density lipoprotein oxidation and platelet aggregation), hypoglycemic, antihypertensive (vasodilatator), antiviral (even against the HIV virus), antiinflammatory (by inhibiting the 5-lipoxygenase enzyme), cytostatic (against the McCoy cells), molluscicidal, endocrinal, and an enzyme modulator (*14*−*17*). Hydroxytyrosol, in addition to exerting more markedly most of the biolological activities ascribed to oleuropein, protects the human erythrocytes against the oxidative damage, reduces superoxide anion production in the human promonocyte cells, inhibits peroxynitrite-dependent damage, induces cytochrome C-dependent apopostis, inhibits tumor cell proliferation, and finally inhibits leukocytes leukotriene B_4 (*18*, *19*).

Because of their richness in antioxidant phenols (compounds also claimed to be basic natural flavorings), olive leaves have recently been successfully mixed with overripe olives (2−3%) before processing to produce oils with a more marked flavor and a higher resistance to oxidation (*20*, *21*). Also, tablets of olive leaf extracts have recently been marketed as a dietetic product or a food integrator (*13*). Finally, studies targeted to recover the significant amounts (∼3% dry weight) of oleanolic acid from olive leaves have been carried out. The oils supplemented with this acid have been found to be useful in preparing functional foods (*22*).

However, olive leaves (resulting from pruning or defoliation of olive fruits before processing) have not yet been exploited industrially. They are still regarded as a byproduct/residue to use as a fuel after drying or as an animal feed. This work was designed to study the content of oleuropein in leaves from seven major Italian olive cultivars in order to elucidate its range of variation with respect not only to the genetic factor but also to the collecting period and the color/age of leaves, as well as to the quantitation method used. A general knowledge including such important aspects is in fact not yet available in the literature. This study could have both scientific and economic consequences for the olive-growing sector.

MATERIALS AND METHODS

Chemicals. The reagents used were methanol, water plus, ethyl acetate, ethanol anhydrous, pyridine, hexamethyldisilazane, trimethylchlorosilane, *o*-phosphoric acid, hydrochloric acid, and acetonitrile. They were of analytical grade and were all provided by Carlo Erba SpA (Milan, Italy). Pure oleuropein standard was from Extrasynthése (Genay, France).

Preparation of Olive Leaf Samples. The Italian olive cultivars Dritta, Leccino, Caroleo, Coratina, Castiglionese, Nebbio, and Grossa di Cassano (eight trees for each cultivar having the same age and uniform vegetative features), grown in the same orchard, in Middle Italy, were selected. They were submitted to the same cultural conditions (pruning, watering, and harvesting) and the same geographical, geological, and climatic conditions. From each of these cultivars, a 2 kg sample of leaves was taken uniformly from the canopy of the trees. Sampling was carried out in March and October of 2003 because olive tree is an evergreen plant characterized by two periods of foliation (which in our climate fall in spring and autumn, respectively). From each homogenized sample, three 200 g subsamples of leaves were taken, one consisting of young/developing leaves with an intense green color, one of leaves with a green-yellowish color (average age), and one of semihardened leaves with a faded yellow color and near to the shedding stage (old leaves).

Preparation of Leaf Extracts. Each of the above subsamples of leaves was immediately blended into a fine powder. This (1 g) was extracted with a 3 × 50 mL methanol/water mixture (2.3:1, v/v) that was vortexed for 6 min. The extracts (150 mL in total) were transferred to a 250 mL volumetric flask and evaporated by a rotary evaporator at a temperature of 30 °C. The residual aqueous phase (∼30 mL) was transferred to a 100 mL separator funnel, acidified with hydrochloric acid up to pH 3.1, and extracted with 3 × 50 mL ethyl acetate. After vigorous stirring of the mixture for 1 min, the phases separated thoroughly after an interval of 10 min. The surnatants (ethyl acetate phases) were transferred to a volumetric flask (200 mL) and evaporated to dryness in a rotary evaporator at a temperature of 30 °C. The residual aqueous phase was subjected to solid−liquid extraction using columns SPE coated with strata-X 33 μm polymeric sorbent (Phenomenex, Torrance, CA). Elution was done with methanol (4 × 5 mL). The eluates were transferred to the above 200 mL volumetric flask and evaporated as above-described. The end residue was redissolved in 20 mL of methanol.

High-Resolution Gas Chromatographic (HRGC) Analysis of Oleuropein. A reliable HRGC method (repetitiveness expressed as RSD% = 5.1%; $n = 10$) was set up. One milliliter of the above final methanol solution was dried under a nitrogen stream, silanized with a pyridine/hexamethyldisilazane/trimethylchlorosilane mixture (2:1:1, v/v/v), and kept in a cold dark environment for ∼2 h. Next, injection (0.5 μL) in the gas chromatograph was done. This was equipped with a 30 m × 0.32 mm i.d., 0.10 μm HP-1 capillary column coated with dimethylpolysiloxane (Agilent Technologies, Wilmington, DE). The oven temperature was programmed as follows: started at 70 °C, increased to 90 °C at a rate of 10 °C/ min, increased to 300 °C at a rate of 4 °C/min, increased to 315 °C at a rate of 5 °C/min, and held

Table 1. Contents (as g/kg Fresh Weight) of Oleuropein (as Determined by HRGC or HPLC Methods) in Green (GR), Green-Yellowish (GY), and Yellow (YL) Leaves Collected from Seven Italian Olive Cultivars in March of 2003[a]

	GR		GY		YL	
olive cultivars	HRGC	HPLC	HRGC	HPLC	HRGC	HPLC
Dritta	3.21 ± 0.23 Aa	2.99 ± 0.20 Aa	2.22 ± 0.12 Ab	2.36 ± 0.18 Ab	1.10 ± 0.06 Ac	1.23 ± 0.06 Ac
Leccino	2.33 ± 0.15 Ba	2.44 ± 0.20 Ba	1.38 ± 0.10 Bb	1.36 ± 0.09 Bb	0.98 ± 0.06 Ac	1.01 ± 0.07 ABc
Caroleo	2.36 ± 0.20 Ba	2.24 ± 0.76 Ba	1.43 ± 0.11 Bb	1.51 ± 0.10 Bb	0.92 ± 0.05 Ac	0.90 ± 0.07 Bc
Coratina	4.28 ± 0.29 Ca	4.43 ± 0.13 Ca	3.10 ± 0.25 Cb	2.99 ± 0.25 Cb	1.77 ± 0.12 Bc	1.84 ± 0.14 Cc
Castiglionese	7.29 ± 0.50 Da	7.11 ± 0.53 Da	5.38 ± 0.36 DEb	5.05 ± 0.37 Dc	3.01 ± 0.23 Cd	2.89 ± 0.16 Dd
Nebbio	8.61 ± 0.68 Ea	8.55 ± 0.71 Ea	5.08 ± 0.46 Db	5.16 ± 0.40 DEb	3.61 ± 0.25 Dc	3.50 ± 0.24 Cc
Grossa di Cassano	7.98 ± 0.45 Fa	8.10 ± 0.59 Fa	5.63 ± 0.21 Eb	5.55 ± 0.38 Eb	3.73 ± 0.34 Dc	3.62 ± 0.18 Cc

[a] Each reported value is the mean of three independent samples ± standard deviation (SD). Means within the same row with different lower case letters or within the same column with different capital letters are significantly different (Tukey's HSD range test, $p \leq 0.05$).

Table 2. Contents (as g/kg Fresh Weight) of Oleuropein (as Determined by HRGC or HPLC Methods) in GR, GY, and YL Leaves Collected from Seven Italian Olive Cultivars in October of 2003[a]

	GR		GY		YL	
olive cultivars	HRGC	HPLC	HRGC	HPLC	HRGC	HPLC
Dritta	2.88 ± 0.21 Aa	2.95 ± 0.19 Aa	1.87 ± 0.11 Ab	1.78 ± 0.12 Ab	1.00 ± 0.05 Ac	0.95 ± 0.06 Ac
Leccino	2.20 ± 0.16 Ba	2.09 ± 0.17 Ba	1.56 ± 0.12 Bb	1.63 ± 0.08 Ab	0.83 ± 0.06 ABc	0.80 ± 0.06 ABc
Caroleo	2.11 ± 0.14 Ba	2.05 ± 0.14 Ba	1.23 ± 0.09 Cb	1.30 ± 0.07 Bb	0.80 ± 0.06 Bc	0.78 ± 0.06 Bc
Coratina	4.01 ± 0.20 Ca	3.96 ± 0.30 Ca	2.87 ± 0.23 Db	2.79 ± 0.18 Cb	1.42 ± 0.10 Cc	1.50 ± 0.12 Cc
Castiglionese	6.95 ± 0.47 Da	6.89 ± 0.45 Da	5.01 ± 0.37 Eb	5.08 ± 0.40 Db	2.81 ± 0.19 Dc	2.88 ± 0.20 Dc
Nebbio	7.93 ± 0.67 Ea	8.04 ± 0.60 Ea	4.93 ± 0.34 Eb	4.86 ± 0.36 Db	3.31 ± 0.16 Ec	3.27 ± 0.18 Ec
Grossa di Cassano	7.62 ± 0.36 Ea	7.70 ± 0.53 Fa	5.01 ± 0.29 Eb	5.11 ± 0.35 Db	3.18 ± 0.25 Ec	3.08 ± 0.21 EDc

[a] Each reported value is the mean of three independent samples ± SD. Means within the same row with different lower case letters or within the same column with different capital letters are significantly different (Tukey's HSD range test, $p \leq 0.05$).

at this temperature for 15 min. Both injector and detector temperatures were 320 °C. Hydrogen was the carrier gas (column pressure = 40 kPa). The amounts of oleuropein in the extracts were calculated using a calibration curve ($r = 0.9896^{+++}$) obtained from four standard solutions containing increasing concentrations of this pure glycoside. The experimental findings were the averages of four different measurements.

High-Performance Liquid Chromatography (HPLC) Analysis of Oleuropein. A repetitive HPLC method was also developed (repetitiveness expressed as RSD% = 4.4%; $n = 10$). Separation of oleuropein from the leaf extract, prepared according to the above-described procedure, was carried out by a reversed silica phase adsorboshere XL C18 90 Å column (250 mm × 4.6 mm, 5 μm) (Altech, Deerfield, IL). The mobile phase consisted of an acetonitrile/water (21:79, v/v) mixture acidified with *o*-phosphoric acid (up to pH 3). This mixture was pumped at 1 mL/min at room temperature. The pressure and flux were 0.75 mL/min and 1100 psi, respectively. The detector was a spectrophotometer operated at 280 nm wavelength. Samples were prepared by diluting 1 mL of olive leaf extract with 1 mL of the above mobile phase mixture. A volume of ~5 μL was injected. To calculate the contents of oleuropein in the olive leaf extracts, a four-point calibration curve ($r = 0.9936^{+++}$) prepared using the standard solutions (with increasing concentrations) was used.

Rancimat Test. The antioxidative power of the olive leaf extracts was evaluated by the established and repetitive Rancimat test. A Rancimat apparatus (model 679) operated at 120 °C with an air flow rate of 20 L/h was from Metrohom Co. (Basel, Switzerland). Refined olive oil purified according to a previously described procedure was the medium submitted to forced oxidation (*23*). The volatile oxidation products (small molecules) were stripped from the lipid medium by dissolving in cold distilled water with an increase of the electrical conductivity parameter value. The time (h) taken to reach a determined level of conductivity, corresponding to the flex point of the peroxidation curve (recorded at the paper speed of 1 cm/h), was considered as an oxidation mark (induction time). The higher the induction time value, the higher the antioxidizing potency of the olive leaf extract. All tests were run in triplicate and averaged. Each extract was tested in comparison with a blank (purified refined olive oil alone).

Statistics. A 7 × 3 × 2 × 2 factorial design (seven olive cultivars × three kinds of olive leaves × two analytical methods × two collecting periods) was adopted. The analytical data were processed by analysis of variance (ANOVA). For each test, three replications were planned. When a significant *F* value was found, means were separated by Tukey's posthoc pairwise test (*24*). Multivariate techniques (*24*−*26*), such as principal component analysis (PCA), hierarchical cluster analysis (HCA), and canonical discriminant analysis (CDA), were also used. The statistical method used depended on the characteristics of the data matrix, the chosen one being that leading to lower error rate and lower risk. A nonparametric multivariate method, such as classification and regression tree (CART), was also used (*24*). The statistical software packages Statistica release 6.0 (Statsoft Inc., OK), Minitab release 13.0 (Minitab Inc., State College, PA), and SPSS release 12.0 (SPSS Inc., Chicago, IL) were used (*24*−*26*).

RESULTS AND DISCUSSION

The obtained analytical data concerning oleuropein contents in *O. europaea* L. leaves are given in **Tables 1** and **2** [means ± standard deviations (SD) along with the statistical significances]. These data confirm how olive leaves are a source of oleuropein (*8*, *9*). Another work in progress in our laboratory suggests how the contents of oleuropein in young olive leaves are comparable or even higher (with some cultivars) than those detected in unripe olive fruits.

Impact of Cultivar and Collecting Season factors on Contents of Oleuropein in Olive Leaves. In general, the genetic factor significantly impacted the content of oleuropein in the collected olive leaves (**Tables 1** and **2**). In order, Nebbio, Grossa di Cassano, and Castiglionese leaves showed higher contents of oleuropein (whatever the collecting period considered, the quantification procedure used, and the color/age of leaves), whereas Caroleo, Leccino, and Dritta leaves had the lower concentrations. Finally, Coratina leaves showed average concentrations of oleuropein. The differences seemed to be more marked when the comparisons among cultivars involved the

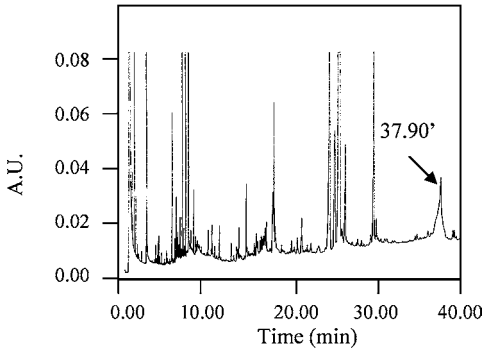

Figure 2. HRGC chromatogram of an extract of *O. europaea* L. leaves picked from the Coratina cultivar. The arrow indicates the peak corresponding to oleuropein.

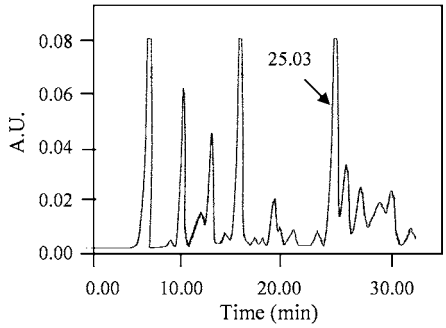

Figure 3. HPLC chromatogram of an extract of *O. europaea* L. leaves picked from the Leccino cultivar. The arrow indicates the peak corresponding to oleuropein.

green leaves (because these were very rich in oleuropein). Such a genetic feature could be exploited for chemotaxnomic purposes as well as for evaluation of the homogeneity of the olive cultivars.

The contents of oleuropein in olive leaves were in general barely influenced by the collecting period factor. In fact, no very substantial differences were observed between the homologous kinds of leaves (characterized by the same color/age) collected in March or October (independently of the olive cultivar considered and the analytical determination method used) (**Tables 1** and **2**). The samples of leaves collected in October seemed to contain slightly lower amounts of oleuropein than those collected in March, likely due to a higher degradation rate of this glycoside in autumn and in addition to lower production of green young leaves (leaf renovation) during this season with respect to the spring period.

Effects of Quantitation Method and Color/Age of Leaves on Their Contents of Oleuropein. No statistically significant differences were observed between the two compared developed methods (HRGC vs HPLC) of determination of oleuropein in olive leaves (regardless of the olive cultivar examined, the collecting period considered, and the color/age of leaves) (**Tables 1** and **2**). These analytical methods were both very effective, but with the HPLC method, the time of analysis was significantly shorter, since the prepared sample could be injected in the HPLC system without prior silanization (one step that took ~1 h). In the obtained HRGC and HPLC chromatograms (the first being very complex), the major peak, corresponding to oleuropein (the only compound to be quantified in this work), has been identified. This identification was made by comparison with a pure standard of oleuropein. The other peaks, which will be identified by combined instrumental techniques within other research projects, could mainly include other secoiridoids as well as flavonoids and verbascoside. A highly positive linear relationship ($r = 0.9498^{+++}$; $p \leq 0.001$) between the two series of analytical data ($n = 21$) obtained with the HRGC and HPLC methods was found. An example of HRGC (**Figure 2**) and one of HPLC (**Figure 3**) chromatograms have been reported.

We also observed that the contents of oleuropein, irrespective of the olive cultivar, the quantification method, and the collecting period factors, were markedly affected by the color/age factor, which was quite higher in the leaves with an intense green color (including mainly those at the developing stage). The green-yellowish leaves and noticeably the yellow ones (near to the falling stage) displayed markedly lower contents of oleuropein. This suggests that oleuropein in olive leaves is gradually degraded with their progressive aging, due most probably to biochemical pathways in which the endogenous β-glucosidase enzyme could play a key role (*9*−*12*).

Antioxidant Potency of Olive Leaves Extracts. Results of the Rancimat test (data not shown) proved that the olive leaf extracts are in general endowed with marked antioxidizing properties, thus confirming previous results (*2*, *13*). However, our research showed how these properties are quite affected by the studied leaf color/age variable. The green leaves, in fact, exhibited the highest antioxidizing potency. The genetic factor (cultivar) also induced a significant effect. The induction time values of the olive leaf extracts ranged from 12 to 21 h and appeared to be significantly higher than those reported in the literature (*23*) concerning reference antioxidants, such as trolox, L-ascorbic acid, BHA (2-3-di-*tert*-butyl-4-hydroxyanisole), BHT (3,5-di-*tert*-butyl-4-hidroxytoluene), TBHA (*tert*-butyl-hidroquinone), NDGA (nord-hydroguaiaretic acid), and gallates. A quite good linear relationship between oleuropein content and antioxidant potency of the extracts was found ($r = 0.9018^{++}$; $p \leq 0.01$; $n = 21$). The oleuropein extracts from Nebbio, Grossa di Cassano, and Castiglionese leaves displayed the higher antioxidizing potency values. Those from Dritta, Caroleo, and Leccino leaves showed the lower values. A medium value was displayed in Coratina leaves in agreement with its average levels of oleuropein.

The antioxidizing potency of *O. europaea* L. leaf extract can further be enhanced by hydrolyzing the contained oleuropein amounts in hydroxytyrosol, an *o*-diphenol compound (making up the catechol moiety of this glycoside) endowed with strong radical hydrogen donor ability and capable of scavenging the superoxide anion. These properties are strictly related to the *o*-dihydroxy (catecholic) substitution possibility, whereas single hydroxyl substitution, as in the case of tyrosol, provides no antioxidative activity (*12*, *13*). It is noteworthy to point out that BHT and vitamin E do not scavenge the superoxide anion, so the olive leaf extract could actually add stability to food industry products exposed to high $O_2^{\bullet -}$ levels (*2*, *3*). The β-glycosidase enzyme needed for conversion of oleuropein into hydroxytyrosol can be immobilized on the chitosan matrix (*8*). It is obtainable from either the almond fruits or the hyperthermophilic microorganisms, like *Sulfolobulus solfataricus* (*9*, *10*).

Results of Multivariate Analyses. Figure 4 shows how the PCA method, applied to the olive leaf oleuropein content data of **Table 2** and based on cases including the factors color/age of leaves and oleuropein quantitation method (HRGC or HPLC method), is capable of characterizing by the dimensions 1 and 2 (accounting for 67.8 and 32.2% of variance, respectively) the color/age variable. However, within each leaf color, the quantitation method variable is not or is poorly discriminated. These

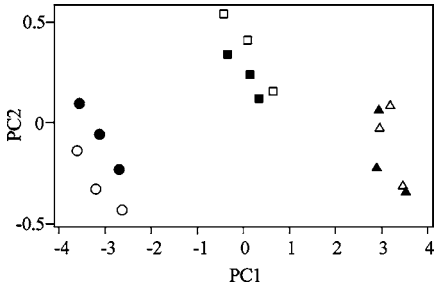

Figure 4. Score plot by dimensions 1 and 2 from PCA, based on the analytical data of **Table 2** and on cases including the factors color/age of leaves and oleuropein quantitation method (HRGC or HPLC method). Symbols: green–HRGC, ●; green–HPLC, ○; green-yellowish–HRGC, ■; green-yellowish–HPLC, □; yellow–HRGC, ♦; and yellow–HPLC, ◇.

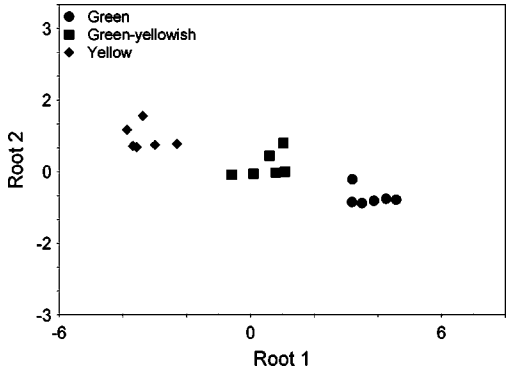

Figure 5. Classification of olive leaves by color, using CDA, based on the analytical data of **Table 2** and on cases including the factors color/age of leaves and oleuropein quantitation method (HRGC or HPLC method). Abbreviations are as in **Table 2**.

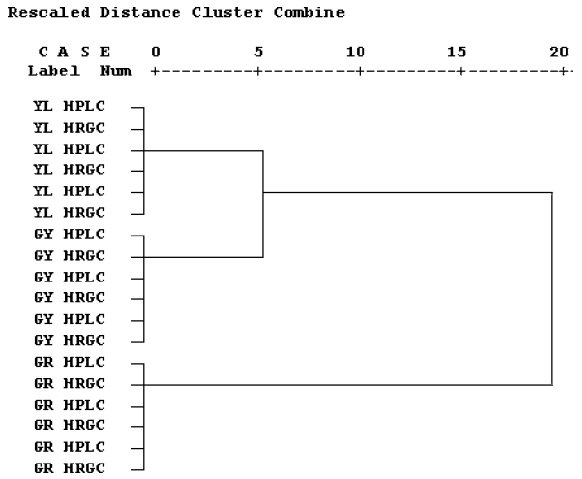

Figure 6. Dendrogram showing the clustering of olive leaf samples by color, based on the analytical data of **Table 2** and on cases including the factors color/age of leaves and oleuropein quantitation method (HRGC or HPLC method). Abbreviations concerning color: GR, green; GY, green-yellowish; and YL, yellow.

results are confirmed by the plot (**Figure 5**) generated by CDA analysis, applied to the same data set and based on the roots (canonical functions) 1 and 2, as well as by the dendrogram from HCA analysis (**Figure 6**).

Figure 7 shows how the CART's nonparametric method, applied to the data of **Table 1** and based on cases including the factors olive cultivar and oleuropein quantitation method (HRGC or HPLC method), is able to discriminate the cultivar variable,

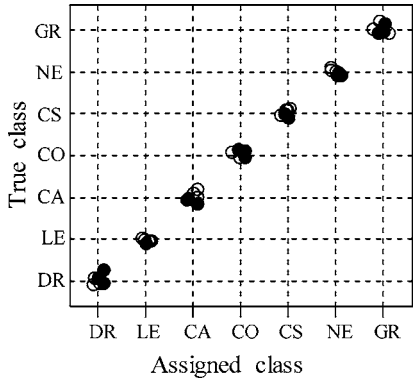

Figure 7. CART's classification of olive leaf samples by cultivar, based on the analytical data of **Table 1** and on cases including the factors oleuropein contents of leaves, cultivar, and quantification method (HRGC or HPLC method). Cultivar abbreviations: DR, Dritta; LE, Leccino; CA, Caroleo; CO, Coratina; CS, Castiglionese; NE, Nebbio; and GR, Grossa di Cassano.

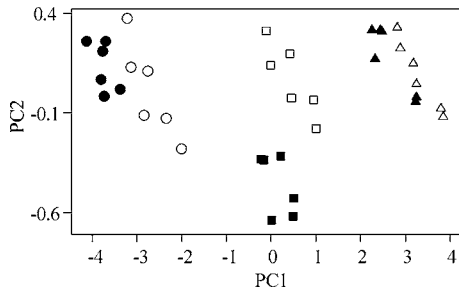

Figure 8. Score plot by dimensions 1 and 2 from PCA, based on the combined analytical data of **Tables 1** and **2** and on cases including the factors color/age of olive leaves, oleuropein quantitation method (HRGC or HPLC method), and leaf collecting period. Symbols: green–first period, ●; green–second period, ○; green-yellowish–first period, ■; green-yellowish–second period, □; yellow–first period, ♦; and yellow–second period, ◇.

whereas within this no discrimination seems to involve the quantitation method variable. **Figure 8**, obtained from the combined data of **Tables 1** and **2** and based on cases including the factors color/age of leaves, collecting season, and oleuropein quantitation method (HRGC or HPLC method), shows how the PCA dimensions 1 and 2 (accounting for 69.6 and 31.4% of variance, respectively) are capable of discriminating the color/age variable. Within this, two subgroupings corresponding to the two collecting periods seem to be relatively separated, while no discrimination seems to involve the quantitation method variable. The dendrogram in **Figure 9** from HCA analysis, applied to the same combined data of **Tables 1** and **2**, confirms that the leaf samples differentiate by their color/age.

The concentrations of oleuropein in olive leaves referred to in this work are in general consistent with those given by other authors (*1*, *4*), even though these do not report the effects related to the various impacting factors, which as this study has proven clearly can substantially modify the oleuropein levels. The confirmed (although quite variable) high levels of secoiridoid oleuropein in olive leaves suggest how this vegetal material could actually be exploited at an industrial level (especially the green leaves) to recovery such an organic high-added value molecule (one transformable into the much more bioactive hydroxytyrosol molecule). Such an industrial activity could result in a significant additional income for the olive growing sector, thus solving at least in part the lasting problems related to its high production costs.

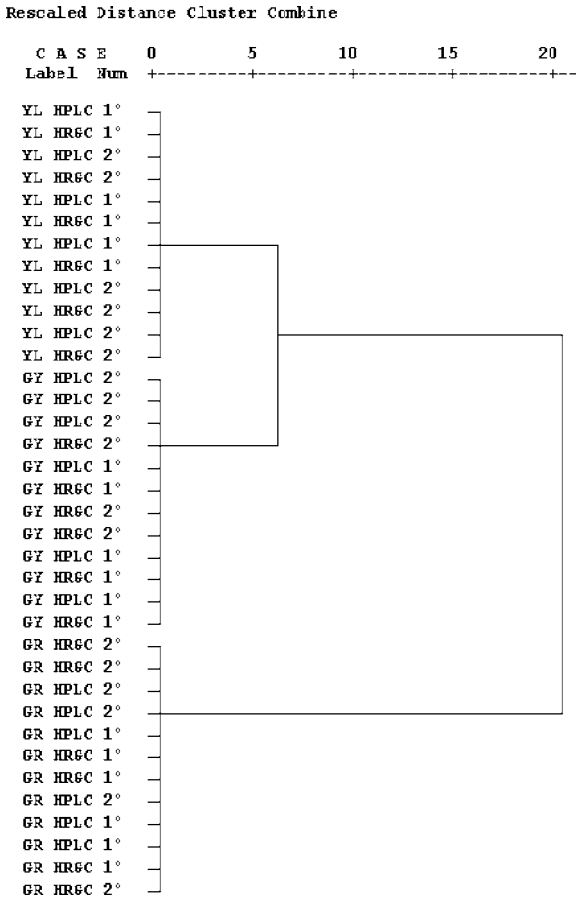

Figure 9. Dendrogram showing the clustering of olive leaves by color, based on the combined data of **Tables 1** and **2** and on cases including the factors oleuropein quantitation method (HRGC or HPLC method), color/age, and collecting period of leaves. Color abbreviations are as in the **Figure 4** legend.

ABBREVIATIONS USED

PCA, principal component analysis; HCA, hierarchical cluster analysis; CDA, canonical discriminant analysis; CART, classification and regression tree; ANOVA, analysis of variance; BHA, 2-3-di-*tert*-butyl-4-hydroxyanisole; BHT, 3,5-di-*tert*-butyl-4-hidroxytoluene; TBHA, *tert*-butyl-hidroquinone; NDGA, nord-hydroguaiaretic acid.

ACKNOWLEDGMENT

We gratefully acknowledge the skilled technical assistance of M. Benzi, G. Pavone, and M. Ottaviano.

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Received for review July 11, 2005. Accepted November 21, 2005. This work was part of a special project "Agricultural and Industrial Exploitation of Byproducts and Residues from Olive Growing Sector" funded by the Ministero per le Politiche Agricole e Forestali (MiPAF). D.M. and M.B. are the recipients of a fellowship from MiPAF.

JF051647B