Rapid High-Performance Liquid Chromatography Analysis for the Quantitative Determination of Oleuropein in *Olea europaea* Leaves

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Olea europaea (Oleaceae) leaves of 14 different cultivars have been studied by a new isocratic HPLC method. Qualitative and quantitative determinations of principal compounds were established for each cultivar. Oleuropein concentration was determined for each sampled tree, using coumarin as internal standard. Bid el Haman, Chemlali, Meski, Cailletier, Tanche, a Verdale−Picholine hybrid, and Lucques, in particular, had high oleuropein concentrations and could be useful sources for industrial extractions.

Keywords: *Olea europaea; Oleaceae; cultivars; phenolic compounds; oleuropein; HPLC analysis; NMR*

INTRODUCTION

The olive tree (*Olea europaea*, Oleaceae) is one of the most important fruit trees in Mediterranean countries. Many data on the polyphenols of olive fruits (*1*−*13*) and olive oil (*14*−*21*) have been reported, but few studies have been published on olive leaves (*22*−*26*). However, popular medicine and phytotherapy use olive leaves to treat and prevent hypertension. The secoiridoids such as oleuropein are suggested to support hypotensive activity (*27*, *28*). Oleuropein is also known for its antioxidant activity (*26*), and its hydrolysis leads to antimicrobial compounds (*29*, *30*). Leaves could be useful sources for oleuropein extraction. Moreover, polyphenol studies could be helpful in ecophysiology to estimate the response mechanisms to several environmental constraints.

An analytical method was required that permitted rapid and simple characterization of major phenolic compounds while giving oleuropein concentrations. A new isocratic method has been developed to identify and quantify the major compounds extracted from the leaves (secoiridoids, flavonoids, and verbascoside). Quantitation was realized with external standards except for oleuropein, which was quantified using coumarin as internal standard. Because oleuropein, demethyloleuropein, oleuroside, and verbascoside were not of analytical grade or commercially available, isolation of standards has been performed on dried olive leaves and their identification has been achieved using NMR spectra.

MATERIALS AND METHODS

Plant Material. Experiments were carried out on leaves (*O. europaea*) of 11 French varieties (Aglandau, Cailletier, Cayet Rouge, Cayon, Grossanne, Lucques, Picholine, Picholine Noire, Tanche, Verdale de l'Hérault, Verdale−Picholine hybrid) and 3 varieties commonly cultivated in Tunisia (Bid el Haman, Chemlali, and Meski). Leaves were collected from the 7th to the 11th of February 1998, in the National Botanical Conservatory of Porquerolles (France). In this place, different cultivars from different origins are submitted to the same cultural conditions and the same geographical, geological, and climatic conditions. Leaves were dried on site in a Panasonic 1330 microwave oven, three times for 2 min at maximum power (2680 W). Dried leaves were powdered and stored in a dry place in the dark.

Standards. *Extraction and Identification*. Oleuropein, demethyloleuropein, oleuroside, luteolin 7-glucoside, and verbascoside were obtained from microwave-dried leaves of Bid el Haman cultivar. Powdered leaves (500 g) were extracted with 50% aqueous methanol. After evaporation of methanol, the aqueous phase was extracted with chloroform and then saturated with NaCl and filtered. Phenolic compounds were extracted with ethyl acetate. The ethyl acetate phase was totally evaporated to obtain 23 g of dried extract. Five grams of the extract was subjected to low-pressure liquid chromatography on Chromatospac Prep 10 (Jobin-Yvon) with a 40 × 500 mm column filled with Lichroprep RP-18 (25−40 μm, 200 g, Merck). The gradient solvent system was methanol/water [v/v, 35:65 (500 mL); 40:60 (500 mL); 45:55 (500 mL); 70:30 (500 mL); 100:0 (1000 mL)]. Collected fractions (100 mL) were examined by TLC. Five compounds were isolated: demethyloleuropein and luteolin 7-glucoside in the 35% methanol fractions, verbascoside in the 40% methanol fractions, and oleuropein and oleuroside in the 45% methanol fractions. Identification was established using ^{13}C and ^{1}H NMR.

Commercial Standards. Coumarin, rutin, and apigenin 7-glucoside were purchased from Extrasynthèse (Genay, France).

Analytical Techniques and Equipment. HPLC analysis was performed on a Waters apparatus (two solvent delivery systems model 510, automatic sample injector WISP model 717, tunable absorbance detector model 486, and photodiode array detector model 996) using a Symmetry C18, 5 μm, 3.9 × 250 mm column (Waters) with a SentryGuard Symmetry C18, 3.9 × 20 mm insert (Waters). Data acquisition and quantitation were performed with Millenium 32, v 3.0, software (Waters). The mobile phase was 79% distilled water acidified (pH 3) with 0.1 M orthophosphoric acid (v/v, 1000: 2.3) and 21% acetonitrile (Carlo Erba, HPLC grade) acidified

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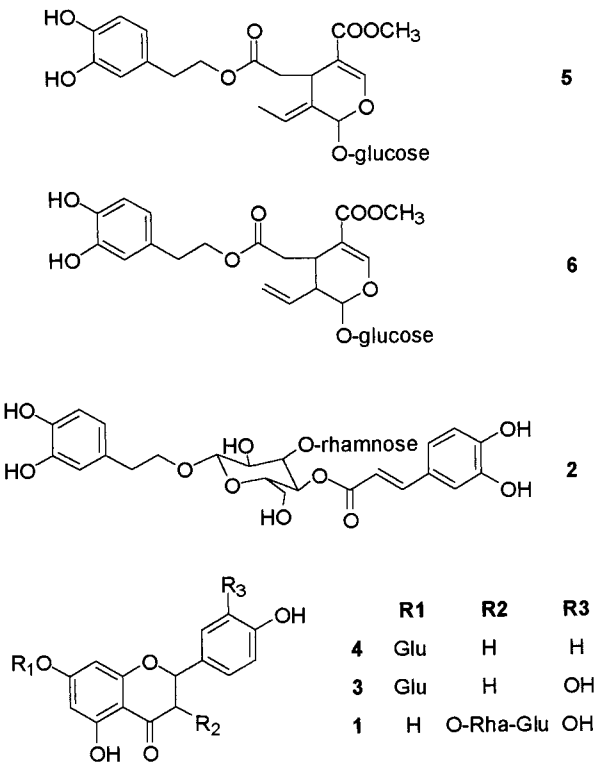

Figure 1. Structures of identified compounds in *O. europaea* L. leaves: **1**, rutin; **2**, verbascoside; **3**, luteolin 7-glucoside; **4**, apigenin 7-glucoside; **5**, oleuropein; **6**, oleuroside.

with 0.1 M orthophosphoric acid (v/v, 1000:2.3). The flow rate was 1 mL/min, and the injection volume was 20 μL. Routine quantitation of oleuropein was assessed at 280 nm. Run time was 35 min.

TLC controls were performed on silica gel 60 F254 (Merck) with chloroform/methanol/acetic acid (70:30:10). Plates were visualized under UV light at 254 nm and after ferric chloride solution (10%) had been sprayed for secoiridoids and aminoethyl diphenylborate solution (10%) had been sprayed for flavonoids.

NMR measurements were performed on a Brücker AMX400 spectrometer, operating at 400.13 MHz for ^{1}H and at 100.61 MHz for ^{13}C. Chemical shifts (δ) were referred to tetramethylsilane (TMS) in CD_3OD for verbascoside and iridoids and in DMSO-d_6 for flavonoids.

Samples for HPLC Analysis. Powdered leaves (5.0 g) were extracted three times for 15 min with 30 mL of 60% aqueous methanol in an ultrasonic bath (Branson 3510). Filtered solutions were mixed and diluted to 100 mL with the same solvent. Extracts were diluted (1:5) with mobile phase and added to an equal volume of internal standard (coumarin, 65.0 μg/mL, prepared with mobile phase).

RESULTS AND DISCUSSION

Isolated Standards. Five phenolic compounds (Figure 1) were isolated from the ethyl acetate extract (5 g). Luteolin 7-glucoside (21.5 mg) and verbascoside (30 mg) were identified by comparison of their ^{1}H and ^{13}C spectra with published data (*31*, *32*). Comparison of ^{13}C NMR spectra of demethyloleuropein (16 mg), oleuropein (1200 mg), and oleuroside (133.6 mg) with those of the literature data (*24*, *25*, *33*) permitted unambiguous confirmation of their structures.

HPLC Chromatogram of Phenolic Compounds. Chromatographic profiles of the different cultivars studied showed no differences in qualitative composition. The main compounds detected by HPLC (Figure 2) were identified as rutin (t_R = 6.8 min), verbascoside (t_R = 7.8 min), luteolin 7-glucoside (t_R = 8.9 min), apigenin 7-glucoside (t_R = 15.3 min), oleuropein (t_R = 23.8 min), and oleuroside (t_R = 33.8 min). The unidentified compound (t_R = 16.4 min) is currently under investigation; its UV spectrum suggests a flavonoid structure. Demethyloleuropein was not detected in leaf extracts of the 14 varieties studied.

HPLC Validation of Oleuropein Quantitation. The HPLC method was validated for its specificity, linearity, and precision. Calibration curves were plotted by correlating the area ratio (oleuropein/internal standard) versus the corresponding concentration ratios. Assays of oleuropein (0.5−1.0 mg; n = 9) and powdered leaves (0.5−10.0 g; n = 9) were linear in the concentration range studied. The least-squares regression lines (Y = oleuropein/internal standard peak area ratio, X = oleuropein/internal standard concentration ratio) were, respectively, $Y = 0.0656X - 0.0089$ for pure oleuropein and $Y = 0.0052X - 0.0132$ for leaf extracts. Coefficients of correlation were >0.999. Repeatability (3 days, n = 6) and intermediate precision (n = 18) were assessed on 5 g of powdered leaves. Their coefficients of variation were <2%. The detection limit for oleuropein was 0.4 μg/mL, and the limit of quantitation was 0.85 μg/mL.

Oleuropein Concentrations. Oleuropein concentration in leaves was calculated in percentage (w/w) for each cultivar (Table 1). Samples were collected from several varieties exposed to the same environmental (geographical, geological, and climate) and cultural

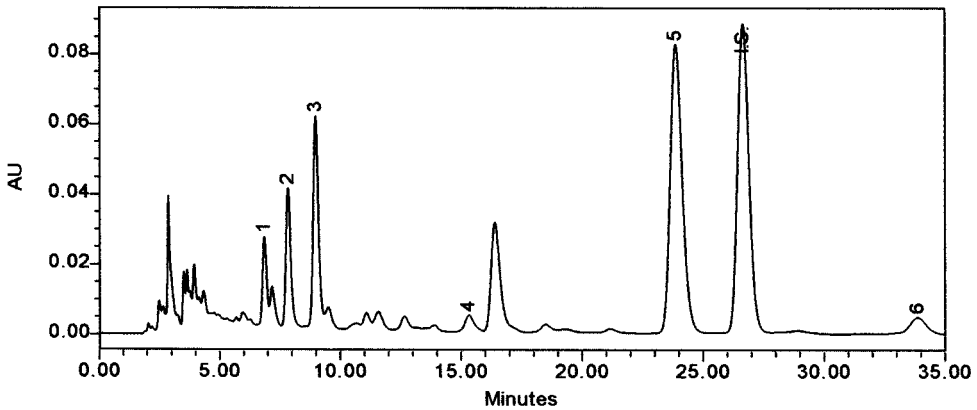

Figure 2. HPLC chromatogram of an extract of *O. europaea* L. leaves picked from Verdale−Picholine hybrid. Peaks: 1, rutin; 2, verbascoside; 3, luteolin 7-glucoside; 4, apigenin 7-glucoside; 5, oleuropein; 6, oleuroside; I.S., coumarin. Detection was at 280 nm.

Table 1. Oleuropein Concentrations in 14 Cultivated Varieties of Olive Tree in Percent (w/w) of Powdered Leaves

variety	n[a]	oleuropein concn	mean value	rel SD (%)
Aglandau	1	9.04	9.27	2.89
	2	9.62		
	3	9.14		
Cailletier	1	12.90	13.05	1.35
	2	13.21		
Cayet Rouge	1	10.05	10.54	4.91
	2	11.02		
Cayon	1	12.02	11.36	6.22
	2	10.70		
Grossanne	1	11.26	11.09	2.03
	2	10.82		
	3	11.18		
Lucques	1	14.16	13.43	5.17
	2	13.58		
	3	12.56		
Picholine	1	11.10	11.66	13.83
	2	13.66		
	3	9.46		
	4	12.43		
Picholine Noire		11.11		
Tanche		12.80		
Verdale de l'Herault	1	10.49	10.35	1.54
	2	10.21		
V × P Hybrid	1	14.19	13.23	7.72
	2	12.28		
Bid el Haman	1	13.22	12.91	10.52
	2	14.32		
	3	11.19		
Chemlali		12.75		
Meski		12.66		

[a] Number of individuals.

(pruning, watering, and harvesting) conditions. Short sampling time (5 days) made seasonal variations irrelevant. Under these conditions, this chemical study showed intrinsic variations among sampled cultivars. The three Tunisian varieties had concentrations of 12 ± 2%. Almost all of the French varieties had concentrations <12% except for Cailletier, Lucques, Tanche, and the Verdale−Picholine hybrid. Aglandau had the lowest concentrations (9.04−9.62%) and Lucques the highest (12.56−14.16%). Values obtained in this study were higher than usually reported. This was probably due to the drying method rather than the extraction method. A previous study (*34*) showed that microwave drying avoided ester hydrolysis of saponins, which occurred with air-drying.

Varieties with more than one sample showed two groups of variations in oleuropein concentrations: a group with a low variation of concentrations (relative standard deviation <5%, Aglandau, Cailletier, Cayet Rouge, Grossanne, and Verdale de l'Hérault); and a group with a high variation of concentrations (relative standard deviation ≥5%, Bid el Haman, Cayon, Lucques, Picholine, and Verdale−Picholine hybrid).

Other Phenolic Compounds. Flavonoids, verbascoside, and oleuroside were quantified by an external standard method to estimate variations among cultivars (Table 2). Cultivars were clustered following their deviation to the mean value of each compound.

Rutin had the highest values (≥0.26%) in Cayon and the Verdale−Picholine hybrid and the lowest (≤0.10%) in Aglandau, Grossanne, and Verdale. Verbascoside was relatively high (≥0.50%) in Aglandau and Lucques but very low in Cailletier and Chemlali (≤0.24%). Inversely, apigenin 7-glucoside was more important (≥0.07%) in these two varieties and very poor in others, especially in Cayon, Picholine, Picholine Noire, Verdale, Bid el Haman, and Meski (≤0.03%). Luteolin 7-glucoside was also low (≤0.31%) in Grossanne, Bid el Haman, and Meski but was high (≥0.53%) in Cayet, Cayon, Tanche, and the Verdale−Picholine hybrid. Oleuroside was especially high in Aglandau, Cayet, Tanche, and Bid el Haman (≥0.69%).

Table 2. Quantitation of Other Phenolic Compounds in Olive Tree Leaves (Values Are in Percent w/w of Powdered Leaves)

variety	rutin	verbascoside	luteolin 7-glucoside	apigenin 7-glucoside	oleuroside
Aglandau	0.07	0.53	0.34	0.06	0.79
Cailletier	0.14	0.15	0.44	0.08	0.51
Cayet	0.19	0.30	0.54	0.04	0.75
Cayon	0.31	0.43	0.56	0.03	0.45
Grossanne	0.09	0.45	0.31	0.04	0.56
Lucques	0.15	0.50	0.44	0.05	0.48
Picholine	0.20	0.48	0.50	0.03	0.51
Picholine Noire	0.21	0.44	0.36	0.02	0.46
Tanche	0.17	0.44	0.55	0.06	0.70
Verdale	0.10	0.29	0.36	0.02	0.43
V × P hybrid	0.35	0.37	0.57	0.05	0.45
Bid el Haman	0.18	0.30	0.27	0.03	0.75
Chemlali	0.22	0.12	0.40	0.11	0.45
Meski	0.18	0.42	0.28	0.03	0.60

Several varieties shared similar features. Chemlali and Cailletier, and Bid el Haman and Meski, were closely related in most compounds. Also, Cayon and Cayet Rouge presented similarities. However, as observed before for oleuropein, Bid el Haman, Cayon, Lucques, Picholine, and the Verdale−Picholine hybrid have a great range in variation of compound concentrations among individuals.

The isocratic HPLC method described in this study allowed rapid separation and identification of the major compounds of olive tree leaves. The quantitation of oleuropein using an internal standard is of particular importance because commercially available oleuropein standards are not of HPLC grade. This method could be used for the routine analysis of leaves, extracts, and industrial products. The chromatographic profiles and the evaluation of compound distribution, especially oleuropein, could lead to characterization of varieties and evaluation of their homogeneity. Additional investigations should increase the number of analyzed samples in order to reach significant statistical results to correlate with chemotaxonomy. In the varieties studied, the Tunisian varieties, Cailletier, Tanche, Verdale−Picholine, and particularly Lucques, had high oleuropein concentrations. Studies of ecological and cultural variations should permit optimization of culture conditions for olive leaf crops.

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