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Small Branches of Olive Tree: A Source of Biophenols Complementary to Olive Leaves

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The extraction of biophenols (BPs) from small branches (fibrous softwood) of olive tree accelerated by microwave assistance is proposed for the first time. Under optimal working conditions, no further extraction of the target analytes was achieved after 10 min, so complete removal of them within this interval was assumed (amounts ca. to 19000, 1000, 2000, 900, and 700 mg/kg of oleuropein, verbascoside, tyrosol, α-taxifolin, and hydroxytyrosol, respectively; the three last BPs are absent in branch-free olive leaves). The extracts required no cleanup or concentration prior to injection into a chromatograph−photodiode array detector assembly for individual separation−quantification. Extraction from this raw material was also implemented in continuous and discontinuous−continuous extractors using ultrasound assistance and superheated liquids, respectively, as auxiliary energies, and the results were compared with those obtained by microwave-assisted extraction. The simultaneous extraction of small branches and leaves from olive tree provided extracts with a higher variety of BPs, but either extracts richer in oleuropein and verbascoside without tyrosol, α-taxifolin, and hydroxytyrosol or rich in these three BPs can be obtained by separate extraction of leaves and branches, respectively.

KEYWORDS: Olive small branches; olive leaves; oleuropeih; verbascoside; hydroxytyrosol; biophenols; phenols; microwave-assisted extraction

INTRODUCTION

The leaves of *Olea europaea* L. have been studied, among other reasons, for their therapeutic effects. Interest in the potential health benefits of olive leaf extracts dates back to the mid-19th century, when reports were made of the ability of an extract (made from boiling the leaves) to reduce fever, including the ability to prevent or cure malaria symptoms even more effectively than quinine. Since the second half of the 20th century olive leaf extracts have been investigated and demonstrated to possess healthy properties that are a consequence of the function of olive biophenols (OBPs) in the olive tree [namely, reactivity against pathogen attack and response to insect injury (*1*, *2*)]. Oleuropein is the most abundant biophenol in olive leaves, which has been used in a number of medical treatments since its first reference in the literature (*3*); thus, oleuropein prevents cardiac diseases by protecting membrane lipid oxidation acting on coronary dilation (*4*) and by antiarythmic action (*5*), improves lipid metabolism to mitigate obesity problems (*6*), protects enzymes and hypertensive cell death in cancer patients (*7*), and presents antiviral properties (*8*, *9*). A derivative of oleuropain, hydroxytyrosol, is a better scavenger and/or antioxidant than typical antioxidants such as vitamins C and E or 2,6-di-*tert*-butyl-4-methylphenol (BHT) (*10*); as a result, this OBP prevents a number of cardiac [through antiaggregating platelet action (*6*) or antimycoplasmal activity (*11*), among others] and tumoral diseases [e.g., by avoiding the protein damage induced by long-wave ultraviolet radiation in melanoma cells (*12*)]. This phenolic compound can also be used in applications such as the prevention of macrophage activation (*13*), inhibition of bacterial plaque accumulation on teeth, and prevention and treatment of diseases of the dental cavity and periodontium (*14*). The healthy characteristics of oleuropein and hydroxytyrosol are the best studied; nevertheless, other OBPs such as tyrosol (*15*), verbascoside (*16*, *17*), apigenin-7-glucoside (*18*, *19*), and luteolin-7-glucoside, which have traditionally been considered as components of olive leaves, are also endowed with similar properties.

Olive leaves and branches are agricultural residues resulting from the beating of olive trees for fruit removal; however, they may also be considered industrial byproducts as they find their way to the olive mill in substantial amounts [around 10% of the total weight of fruit arriving at mills (*20*)]. The scientifically proven healthy properties of olive leaf extracts are leading to new research (*21*−*25*) and an incipient industrial exploitation (although the majority are transported from the mills and burned).

This research is aimed at checking the potential of olive small branches as a biophenols source. The lack of information in the literature on this material makes advisable its characterization

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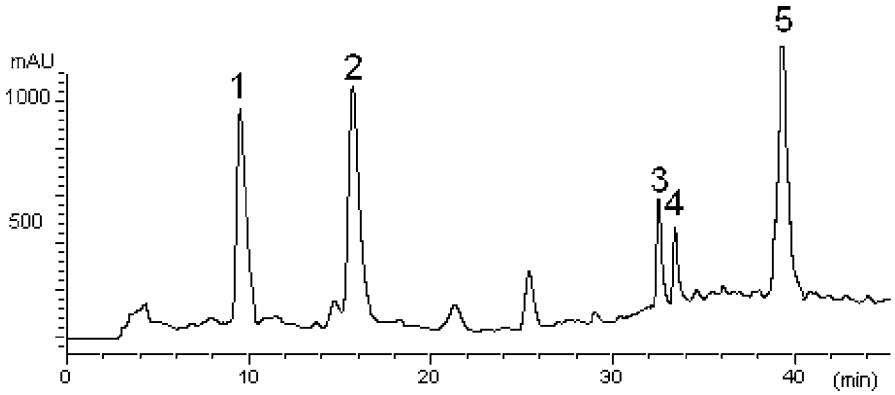

Figure 1. Chromatogram at 280 nm of an extract from olive small branches obtained under the optimal working conditions. Peaks: 1, hydroxytyrosol; 2, tyrosol; 3, α-taxifolin; 4, verbascoside; 5, oleuropein.

for possible value. Microwave assistance as well as ultrasound or superheated liquids have been used to accelerate the extraction process, and HPLC-DAD has been used to carry out individual separation−detection.

MATERIALS AND METHODS

Apparatus. A Microdigest 301 digestor of 200 W maximum power (Prolabo, Paris, France) furnished with a microprocessor programmer (Prolabo) to control the microwave unit was used for favoring extraction. Description of the equipment used for ultrasound-assisted and superheated liquid extractions can be found elsewhere (*26*, *27*).

A Selecta Angular 6 centrifuge was used to remove particles in the extract. An Agilent 1100 liquid chromatograph consisting of a G1322A vacuum degasser, a G1315A diode array detector (DAD), and a Rheodyne 7725 high-pressure manual injection valve (20 μL injection loop) was used for the analysis of the target analytes by HPLC. The analytical column was a Lichrospher 100 RP-18 (250 × 4 mm i.d., 5 μm) from Análisis Vínicos (Ciudad Real, Spain). A Kromasil 5 C-18 column (15 × 4.6 mm i.d., 5 μm) protected with a steel holder, both from Scharlab (Barcelona, Spain), was also used.

Reagents and Working Solutions. Ethanol, acetonitrile, and acetic acid were from Panreac (Barcelona, Spain). Deionized water (18 mΩ) from a Millipore Milli-Q water purification system was used to prepare both water−ethanol extractant mixtures and mobile chromatographic phases.

Oleuropein, hydroxytyrosol, tyrosol, verbascoside, and α-taxifolin from Extrasynthèse (Genay, France) were used for the identification and quantification of these compounds, the most abundant in small branches of the olive tree.

Samples. Small branches (fibrous softwood) and leaves from different varieties of olive trees (viz., Alameño, Arbequina, Azulillo, Chorna, Hojiblanca, Lechín, Manzanillo, Negrillo, Nevadillo, Ocal, Pierra, Sevillano, and Tempranillo), all from Córdoba, were collected in December 2005. In all cases, the samples were obtained from at least five different and healthy trees. All samples were dried, milled, and kept at 4 °C until use.

Extraction of Biophenols from Small Branches of Olive Tree. For microwave-assisted extraction, 1 g of milled small branches and 8 mL of 80:20 ethanol/water were placed into the quartz extraction vessel located in the microwave irradiation zone. After extraction (10 min of microwave irradiation at 200 W), the suspension was centrifuged at 3000 rpm for 5 min and the extract 1:1 diluted prior to injection into the liquid chromatograph to avoid overpressure problems in the chromatographic column.

The continuous ultrasound-assisted extraction and discontinuous−continuous superheated liquid extraction procedures can be found elsewhere (*26*, *27*). In short, ultrasonic irradiation was applied by means of a cylindrical titanium alloy probe, which was immersed into a water bath in which the extraction cell was placed. The latter consisted of a stainless steel cylinder, closed with screws at either end, allowing circulation of a leaching solvent through it. A low-pressure peristaltic pump (programmed for changing the rotation direction a preset intervals) and PTFE tubing of 0.5 mm i.d. were used to build the flow manifold. The discontinuous−continuous superheated extractions were performed using a high-pressure pump to propel the extractant through the system; an extraction chamber similar to that above; a gas chromatograph oven used as heating source into which the chamber and a stainless steel preheater were placed; a cooler system, consisting of a loop made from a 1 m length stainless steel tubing, cooled with water, used to cool the extract from the oven to ca. 25 °C; and two valves, one of the them (an open−close valve) located between the high-pressure pump and the oven and used to prevent the extract from going backward to the extractant reservoir in the discontinuous extraction mode, and the other (a restrictor valve) located after the oven.

Table 1. Optimization of Microwave-Assisted Extraction of OBPs from Small Branches of Olive Tree

variable	tested range	optimum value
microwave power (W)	100–200	200
irradiation time (min)	5–15	10[a]
ethanol (%)	60–80	80

[a] Obtained by a univariate study.

HPLC-DAD Separation−Detection. The elution solvents used were as follows: A (6% acetic acid, 2 mM sodium acetate, in water) and B (acetonitrile). The samples were eluted according to the following gradient: 0−19 min, 100−97% A and 0−3% B, flow rate = 0.8 mL/min; 19−27 min, 97−89.6% A and 3−10.4% B, flow rate = 0.8−1 mL/min; 27−28 min, 89.6−87% A and 10.4−13% B, flow rate = 1.0 mL/min; 28−58 min, 87−47% A and 13−53% B, flow rate = 1.0 mL/min; 58−59 min and 47−0% A and 53−100% B, flow rate = 1.0−0.8 mL/min.

RESULTS AND DISCUSSION

Optimization of the Individual Chromatographic Separation−Detection Step. Oleuropein, tyrosol, hydroxytyrosol, and α-taxifolin were monitored at 280 nm and verbascoside was monitored at 330 nm using a diode array detector.

The experimental variables optimized in previous research (*26*−*28*) were checked in this case.

The best separation is achieved using the mobile phases, flow rates, and gradient program given under HPLC-DAD Separation−Detection. A chromatogram obtained at 280 nm under these working conditions is shown in **Figure 1**.

Optimization of Microwave Assistance for the Extraction of OBPs from Small Branches. The three potentially more influential variables on the extraction from small branches (i.e., irradiation power, irradiation time, and extractant composition) were optimized using as response variable the extraction

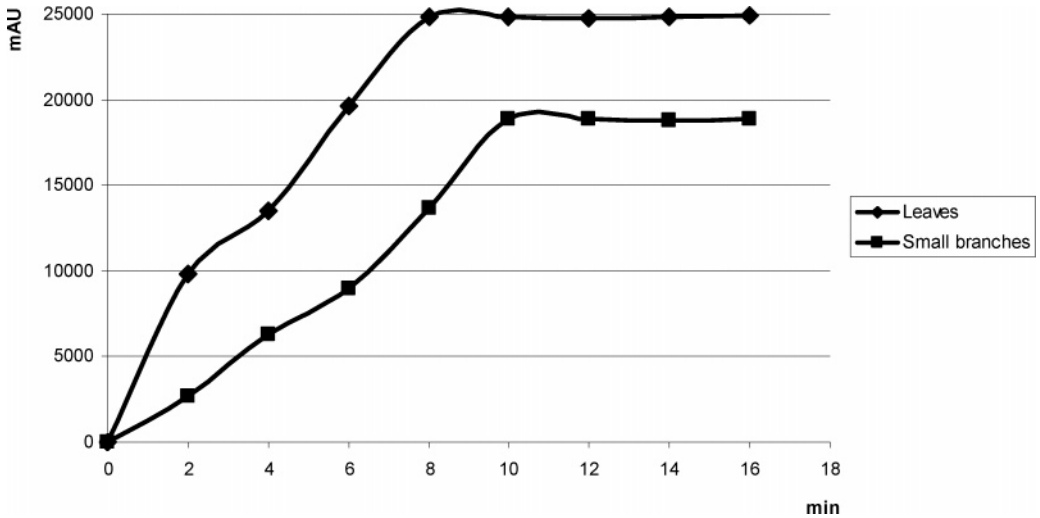

Figure 2. Extraction kinetics of oleuropein for small branches and leaves of olive tree under the optimal working conditions.

efficiency expressed as the peak area for each compound under the optimum chromatographic conditions.

A full two-level factorial design allowing four degrees of freedom and involving 11 randomized runs including three center points was built for a screening study of the behavior of the three variables influencing the extraction process. The upper and lower values given to each variable were selected from the available data and experience gathered in preliminary experiments.

None of the three variables were found to be statistically influential within the ranges under study; therefore, their optimum values are within these intervals. Nevertheless, the best results were obtained with the maximum percentage of ethanol and radiation power. The tested and optimum values obtained for each variable, the latter used in further experiments, are shown in **Table 1**. These values do not differ from those found in the extraction of BPs from olive leaves (*28*).

Biophenols present in olive leaves such as oleuropein and verbascoside (*26*−*28*) were identified in small branches; hydroxytyrosol, tyrosol, and α-taxifolin present in olive oil and alperujo (*7*) were also found in branch extracts.

A kinetics study was made to determine the time necessary for total removal of phenolic compounds from small branches, which was achieved after irradiation for 10 min, so this time was selected and used for further experiments. The extracts obtained with longer times did not increase the concentration of OBPs or involve detectable degradation. Total BP extraction from small branches requires only 2 min more than in the case of olive leaves, as can be seen in **Figure 2** for oleuropein. The rest of the analytes showed a similar behavior to that of oleuropein.

Characterization of the Method. The studies were aimed at calculating the concentration of BPs in the extracts and thus in the raw material (calibration curves) and knowing the variability in concentration caused by the extraction step (precision study).

Calibration curves were obtained by plotting the peak area of each OBP as a function of standard concentration. The regression coefficients ranged between 0.9968 and 0.9998 for all analytes. The limit of detection (LOD) was expressed as the mass of analyte which gives a signal that is 3σ above the mean blank signal (where σ is the standard deviation of the blank signal). The LODs obtained ranged between 1.3 and 3.3 mg/kg. The limits of quantification (LOQs), expressed as the mass of analyte which gives a signal that is 10σ above the mean blank signal, ranged from 3.8 to 9.0 mg/kg. LODs and LOQs were estimated from both branch extracts and standard solutions of these compounds.

Table 2. Precision of the Proposed Method, Expressed as Repeatability Relative Standard Deviation (s_r) and Within-Laboratory Intermediary Precision Relative Standard Deviation (s_{WR}), for Each Biophenol in Olive Small Branches and Limits of Detection and Quantification, Expressed as Concentration

compound	s_r (%)	s_{WR} (%)	LOD (mg/kg)	LOQ (mg/kg)
hydroxytyrosol	3.18	4.95	1.3	3.8
tyrosol	3.40	5.76	2.1	5.2
oleuropein	6.27	9.54	3.3	9.0
α-taxifolin	2.43	4.98	1.8	4.6
verbascoside	6.54	10.01	2.1	6.2

The precision of the proposed method was calculated by within-laboratory reproducibility and repeatability studies. The experiments were carried out using 1 g of milled small branches under the optimal working conditions. Two measurements of each compound per day were performed on 7 days. The repeatability, expressed as relative standard deviation, was from 3.18 to 6.54%; meanwhile, within-laboratory reproducibility ranged from 4.95 to 10.01%. Limits of detection and quantification and the results of precision studies are listed in **Table 2**.

Additional Studies. The efficacy of pure water for the extraction of phenolics from olive small branches was compared with that of the optimum extractant (an 80:20 ethanol/water mixture). With this aim, an experiment was carried out under the optimum working conditions given in **Table 1**, but using water as extractant. Oleuropein, hydroxytyrosol, and α-taxifolin were partially extracted (see **Table 3**), whereas the other two BPs were not detected.

Other experiments involved both dynamic ultrasound-assisted and static−dynamic superheated liquid extractions to test the extraction behavior of small branches in continuous and discontinuous−continuous modes, respectively. Taking into account the similar behaviors, the optimal values of the methods proposed in previous research to extract BPs from olive leaves assisted by ultrasound (*26*) or using superheated extractant (*27*) were also applied to small branches. The results shown in **Table 3** prove that continuous and discontinuous−continuous modes helped by ultrasound or superheated liquids, respectively, are also suitable for carrying out the extraction BPs from olive small branches, to which the methods previously reported for olive leaves (*26*, *27*) can be applied.

Table 3. Efficiency of the Proposed Method As Compared with Those Using Other Auxiliary Energies for the Extraction of BPs from Olive Small Branches of Arbequina Variety

biophenol	proposed method (mg/kg of dry weight)	microwave-assisted extraction (water as extractant)[a] (%)	ultrasound-assisted extraction[a] (%)	superheated liquid extraction[a] (%)
hydroxytyrosol	685	15	100	75
tyrosol	1254	<LOD	63	65
oleuropein	18856	27	42	82
α-taxifolin	852	56	100	23
verbascoside	1044	<LOD	59	31

[a] The amount extracted with the proposed method, expressed as mg/kg (second column), is taken as 100% efficiency of the extraction, to which columns 3–5 are referred.

Table 4. Concentration of BPs in Small Branches from Different Varieties of Olive Tree, Expressed as Milligrams per Kilogram

variety/OBP	oleuropein	verbascoside	hydroxy-tyrosol	tyrosol	α-taxifolin
Alameño	4365 (5310)[a]	52 (371)	369	1254	569
Arbequina	18856 (24800)	1044 (10303)	685	1915	852
Azulillo	2957 (4922)	558 (2240)	524	1741	587
Chorna	1602 (2127)	156 (722)	600	1845	847
Hojiblanca	4213 (5584)	69 (475)	471	1423	695
Lechín	8541 (10554)	362 (1853)	474	1659	753
Manzanillo	2451 (3302)	101 (779)	214	1201	412
Negrillo	8369 (10542)	120 (1173)	469	1833	455
Nevadillo	6897 (8002)	230 (1362)	698	1697	502
Ocal	4993 (5972)	65 (284)	453	1660	554
Pierra	6541 (8089)	164 (1091)	475	1477	741
Sevillano	8741 (10913)	841 (6702)	740	1635	854
Tempranillo	5429 (7000)	83 (427)	580	1507	699

[a] Concentrations of these BPs present in leaves are given in parentheses.

Concentration of BPs in Olive Small Branches versus That in Olive Leaves. Thirteen varieties of olive tree (i.e., Alameño, Arbequina, Azulillo, Chorna, Hojiblanca, Lechín, Manzanillo, Negrillo, Nevadillo, Ocal, Pierra, Sevillano, and Tempranillo) were used to extract BPs both from small branches and leaves. The concentrations of the most abundant biophenols in these raw materials (namely, oleuropein, tyrosol, hydroxytyrosol, verbascoside, and α-taxifolin) in each variety are shown in **Table 4**. Only oleuropein and verbascoside were found in both small branch and leaf extracts (depending on the variety, the ratio of oleuropein and verbascoside extracted from branches to those in leaves is between 0.60−0.86 and 0.10−0.25, respectively); hydroxytyrosol, tyrosol, and α-taxifolin, present in small branch extracts, were below their detection limits in olive leaf extracts, if any. Biophenols in leaves not detected in branches are apigenin-7-glucoside and luteolin-7-glucoside. These results (which could be generalized to any olive tree due to the the wide variety used in the study) prove that in the studies on BPs in olive leaves developed so far, mixtures of both leaves and branches have been used (*29*, *30*).

The extracts obtained with the proposed method have the same composition and concentration of OBPs when separate portions of leaves and branches are extracted and then mixed as when a mixture of them are extracted—providing that both the weight of the raw materials and the volume of the extracts are equal. Therefore, the simultaneous extraction of small branches and leaves provides extracts with a higher variety of OBPs and avoids physical separation of both raw materials, making easier their industrial exploitation. Alternatively, both have to be separated to obtain extracts containing hydroxytyrosol, tyrosol, and α-taxifolin (small branches) or a higher content of oleuropein and verbascoside (leaves).

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