JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

AUGUST 2001
VOLUME 49, NUMBER 8

Polyphenolic Content in Olive Oil Waste Waters and Related Olive Samples

N. Mulinacci,* A. Romani, C. Galardi, P. Pinelli, C. Giaccherini, and F. F. Vincieri

Dipartimento di Scienze Farmaceutiche, via G. Capponi 9, 50121 Firenze, Italy

The production of olive oil yields a considerable amount of waste water, which is a powerful pollutant and is currently discarded. Polyphenols and other natural antioxidants, extracted from olives during oil extraction process, partially end up in the waste waters. Experimental and commercial olive oil waste waters from four Mediterranean countries were analyzed for a possible recovering of these biologically interesting constituents. Identification and quantitation of the main polyphenols were carried out by applying HPLC-DAD and HPLC-MS methods. Representative samples of ripe olives were also analyzed at the same time to correlate, if possible, their polyphenolic profiles with those of the corresponding olive oil waste waters. The results demonstrate that Italian commercial olive oil waste waters were the richest in total polyphenolic compounds with amounts between 150 and 400 mg/100 mL of waste waters. These raw, as yet unused, matrices could represent an interesting and alternative source of biologically active polyphenols.

Keywords: *Olive oil waste waters; polyphenols; HPLC-DAD*

INTRODUCTION

One of the main problems faced by processing companies involved in the extraction of olive oil is the elimination and/or treatment of olive oil waste waters (OOWW) because they contain powerful pollutants (*1*). This waste is an acidic (pH 5−5.5), malodorous liquid that contains potassium and phosphatic salts and organic substances such as fats, protein, sugars, organic acids, and polyphenols. Moreover, suspended matter consisting of olive pulp, mucilage, pectin, and oil in a relatively stable emulsion is also present (*2*).

The amount of OOWW produced, and therefore the environmental impact, varies depending on the method of olive oil extraction used. In fact, the traditional press method produces ∼50% of OOWW relative to the initial weight of the olives, whereas the continuous process produces 80−110% of OOWW due to the continuous washing of the olive paste with warm water before oil separation from the paste.

* Corresponding author (e-mail nadia.mulinacci@.unifi.it; telephone 0039-55-2757288; fax 0039-55-240776).

The treatment and disposal of waste waters are critical problems, especially in the Mediterranean area, where olive cultivation is widespread and a large volume of the effluent (800000 m^3/year in Italy alone) is produced and concentrated within a period of only a few months (from November to February). For these reasons, increasing attention has been given to finding the best methods to distribute OOWW on agricultural lands and to recycle both organic matter and nutritive elements in the soil−crop system (*3*) or to recover products of biological interest, such as polyphenols. Olive tissue and olive oil polar compounds, according to their partition coefficients, end up in the OOWW during the oil extraction process, rendering this byproduct a potential source of polyphenols.

In fact, several compounds from *Olea europaea* L., such as hydroxytyrosol and oleuropein, have shown antimicrobial, hypoglycemic, hypolipidemic, and hypocholesterolemic properties that can be very interesting for investigations of their role in human health (*4*, *5*). In particular, olives and virgin olive oil contain phenolic

10.1021/jf000972q CCC: $20.00
Published on Web 06/30/2001

Table 1. Percent Yields of Lyophilized Experimental (a) and Commercial (b) Olive Oil Waste Waters

(a) experimental		(b) commercial	
cultivar	% yield (w/v)	sample source	% yield (w/v)
Picual	16.3	Spain	0.98
Frantoio	10.1	Italy B	11.30
Salonenque	7.8	Italy C	10.71
Galega	4.5	Italy D	12.32
		Italy E	9.34
		France	0.50
		Portugal	5.62

Table 2. Polyphenolic Compounds in Ripe Olive Samples

	g/kg of olives from			
compound	Spain	Italy	Portugal	France
hydroxytyrosol	0.29	0.25	0.24	0.13
hydroxytyrosol glucoside	0.19	0.00	0.16	0.93
rutin	0.00	0.53	0.74	0.37
verbascoside	0.24	1.44	0.11	0.00
luteolin 7-*O*-glucoside	0.08	0.48	traces	0.34
cinnamic acid derivative	0.00	0.00	traces	0.29
oleuropein	traces	2.01	0.00	0.98
oleuropein derivative	0.54	1.65	0.43	0.00
elenolic acid derivative	0.57	1.64	0.60	0.64
luteolin	0.00	0.09	0.08	traces
total polyphenols	1.91	8.10	1.69	2.86

compounds (*6*, *7*) that, in vitro, exert potent biological activities including antioxidant and free radical scavenging actions (*8*, *9*).

The growing evidence that free radical-mediated events are involved in several pathological processes has stimulated interest in natural antioxidants. Free radical formations have been shown to be involved in a series of physiopathologies, including neoplastic and coronary heart diseases and aging processes, because free radicals markedly alter the functioning of biomolecules such as lipids, protein, DNA, and biomembranes (*10*, *11*).

The present research is part of an EU project entitled "Natural antioxidants from olive oil processing waste waters" (FAIR PL 973039) with the aim of evaluating the polyphenolic contents in different samples of OOWW and the possibility of recovering them. For this purpose a preliminary qualitative screening was performed working on waste waters obtained from an experimental mill in Tuscany (*12*). Some data from this European project related to antioxidant and other biological activities of extracts obtained from this matrix have recently been published (*13*). Other authors have studied samples of OOWW and found only catecol, 4-methylcatecol, tyrosol, and hydroxytyrosol as the main phenols (*14*). This investigation was carried out by considering only one specific source of raw material coming from a mill of Avellins province (Italy).

The present paper reports investigations performed on several experimental and commercial samples of OOWW obtained from Italy, Spain, France, and Portugal to compare the content and to identify and quantify the main polyphenols present in these matrices. For each country, representative ripe olive samples were also analyzed. For both OOWW and olives HPLC-DAD and HPLC-MS methods were applied.

EXPERIMENTAL PROCEDURES

Sample Preparation. *Olives.* The following cultivars were analyzed: Galega from Portugal, Salonenque from France, Picual from Spain, and Frantoio from Italy. The olive pulp, obtained from 30 olives, was frozen with liquid nitrogen, ground, and extracted with 4 × 400 mL of EtOH/H_2O at pH 2.2 by HCOOH (80:20) as previously described (*15*).

Table 3. Polyphenolic Compounds in Experimental (a) and Commercial (b) Olive Oil Waste Waters

(a) Experimental Olive Oil Waste Water

	mg/100 mL of waste water from			
compound	Spain	Italy	France	Portugal
hydroxytyrosol	5.2	2.1	4.0	3.2
tyrosol	3.8	1.5	1.8	2.6
caffeic acid	0.4	nd	0.2	nd
elenolic acid	21.2	16.7	18.9	nd
elenolic acid derivative	19.3	17.5	1.6	22.3
luteolin-7-O-glucoside	nd	0.5	2.0	0.9
cinnamic acid derivative	6.9	1.0	5.8	nd
luteolin	3.2	3.5	2.4	1.6
total polyphenols	60.1	42.7	71.2	30.5

(b) Commercial Olive Oil Waste Water

	mg/100 mL of waste water from			
compound	Spain	Italy (A)	France	Portugal
hydroxytyrosol	3.6	13.1	nd	nd
tyrosol	4.1	2.9	0.5	9.9
caffeic acid	0.4	nd	nd	nd
elenolic acid	nd	142.6	1.6	nd
elenolic acid derivative	3.1	0.3	0.1	30.1
dAcOlAg	nd	132.4	nd	nd
luteolin 7-*O*-glucoside	0.2	36.6	0.1	1.2
cinnamic acid derivative	0.4	11.8	0.1	nd
luteolin	0.5	62.3	0.1	2.7
total polyphenols	12.4	401.7	2.5	44.0

Table 4. Comparison among Lyophilized Italian Olive Oil Waste Water Samples Collected during 3 Years (See Experimental Procedures)

	mg/100 mL of waste water from sample			
compound	B	C	D	E
hydroxytyrosol	26.2	16.4	10.8	7.9
caffeic acid	1.0	1.0	0.66	0.9
elenolic acid	268.4	274.8	59.4	28.5
elenolic acid derivative	26.6	16.4	30.8	47.7
dAcOlAg	34.2	39.8	59.6	52.6
verbascoside	14.7	2.4	16.5	7.7
rutin	5.8	10.4	2.8	1.0
luteolin 7-*O*-glucoside	4.8	10.6	0.7	0.8
cinnamic acid derivative	0	3.8	1.3	1.2
luteolin	6.0	4.6	9.1	traces
total polyphenols	387.7	380.2	191.7	148.3

Experimental Waste Waters (ExpOOWW). All of the samples, except for the Portuguese one, were obtained from olives at standard degree using a continuous centrifugation process. The OOWW were obtained from the olive residues which had previously had the same weight of water. The solvent was added because the benchtop laboratory mill was a biphasic type. The ExpOOWW were then lyophilized, and the corresponding yields are reported in Table 1a.

Commercial Waste Waters (ComOOWW). One liter of Spanish, French, and Portuguese commercial waste waters was purchased from Tecnoalimenti S.C.p.A. (Italy), responsible for the collection and shipping procedures within the EU project.

Italian commercial waste waters were obtained using a triphasic mill near Florence (Italy). Five samples from different harvest years were compared: three collected in November 1997 (A−C), one in November 1998 (D), and the last in November 1999 (E). All of the commercial samples, except sample A, were lyophilized, and the relative yields are shown in Table 1b.

Treatment of Waste Waters. Twelve milliliters of fresh OOWW were diluted to 15 mL with acid water (pH 2.2 by HCOOH) and then fractionated by applying a liquid−solid

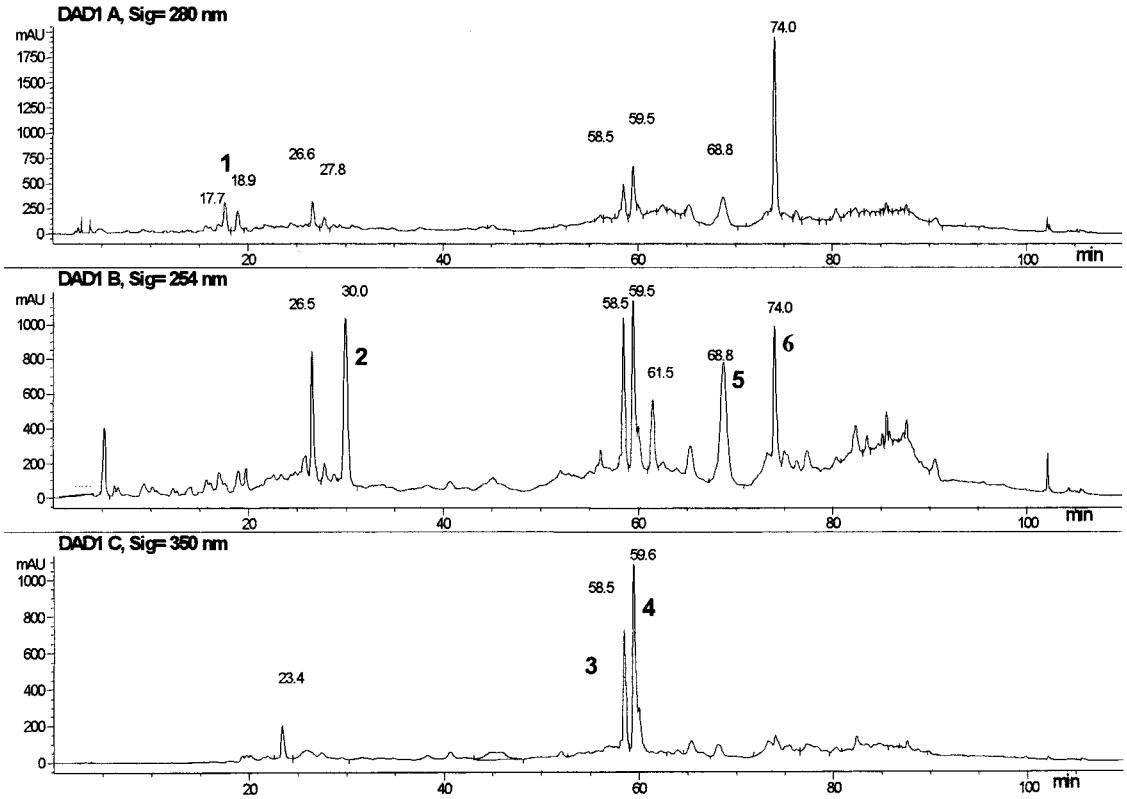

Figure 1. Chromatographic profiles of a French olive sample at 280, 254, and 350 nm. Peaks: 1, hydroxytyrosol; 2, elenolic acid derivative; 3, rutin; 4, verbascoside; 5, oleuropein; 6, cinnamic acid derivative.

extraction (LSE). For the lyophilized samples the weight equivalent to 12 mL of OOWW was dissolved in 15 mL of acid water as described above. Each sample was placed in the Extrelut 20 mL cartridge (Merck, Darmstadt, Germany). Twenty minutes after deposition, the following elution steps were performed: (1) *n*-hexane; (2) ethyl acetate (EtOAc); (3) acid methanol (MeOH), pH 2.2 by HCOOH. The elution steps were carried out up to colorless eluate using 200−300 mL of solvents 1 and 2 and 150−200 mL for solvent 3. The absence of spots in TLC was indicative for stopping the elution due to exhaustive extraction. A TLC plate of silica gel and EtOAc/MeOH/H_2O 77:13:10 as mobile phase were used.

The EtOAc and MeOH fractions were concentrated to dryness under reduced pressure, dissolved in MeOH/CH_3CN/H_2O, pH 3.2 (60/20/20), solution (1−2 mL) and then analyzed by HPLC-DAD and HPLC-MS.

Analytical Techniques and Equipment. HPLC-DAD analyses were performed on an HP 1090L liquid chromatograph equipped with a DAD detector (all from Hewlett-Packard, Palo Alto, CA). The analytical column was a 4.6 × 250 mm LiChrosorb RP18, 5 μm (Merck), maintained at 26 °C. The HPLC-DAD analyses were performed with solvents of analytical grade purchased from Carlo Erba (Milano, Italy).

A seven-step linear solvent gradient was used, starting from 100% H_2O (adjusted to pH 3.2 by H_3PO_4) up to 100% CH_3CN, during a 106-min period, at a flow rate of 1 mL min^{-1}, as previously described (*16*) and already applied for olive samples (*15*). UV−vis spectra were recorded in the 190−450 nm range, and the chromatograms were acquired at 240, 254, 280, 330, and 350 nm.

The HPLC-MS analyses were performed using an HP 1090L liquid chromatograph equipped with a DAD detector, and the interface was an HP 1100 MSD API electrospray (Hewlett-Packard). The mass spectrometer operating conditions were described in a previous work (*17*).

On the whole, the identity of polyphenols was ascertained using data from HPLC-DAD and HPLC-MS analyses, by comparison and combination of their retention time and UV−vis and mass spectra.

Quantitative Analyses. The evaluation of each compound was performed using a four-point regression curve ($r^2 \cong 0.9998$) obtained using the available standards. Tyrosol, caffeic acid, oleuropein, rutin, and luteolin 7-*O*-glucoside standards were purchased from Extrasynthese S.A. (Lyon Nord, Genay, France).

Tyrosol and hydroxytyrosol amounts were calculated at 280 nm with tyrosol as reference compound; oleuropein, deacetoxy oleuropein aglycon, and other oleuropein derivatives were evaluated at 280 nm; elenolic acid and its derivatives were evaluated at 240 nm using oleuropein as reference. For the deacetoxy oleuropein aglycon (dAcOlAg), a correction of molecular weight with a multiplication factor of 320/540 was applied, whereas for elenolic acid and its derivatives a multiplication factor of 242/540 was used.

Flavonoid compounds were calibrated using the corresponding standard evaluated at 350 nm. Verbascoside and cinnamic acid derivatives were quantified using a caffeic acid calibration curve at 330 nm. For verbascoside the correction of molecular weight corresponding to 624/180 as multiplicative factor was calculated.

All of the data reported in Tables 2−4 represent an average of two analysis with the percentage standard deviation ranging within 3−4%.

RESULTS AND DISCUSSION

Olives. Among the considered cultivars, Frantoio (Italy) contained the highest amounts of polyphenolic compounds (>8 g/kg); for the others the concentrations ranged between 1.68 and 2.86 g/kg (Table 2). The quantities of oleuropein, its derivatives, and elenolic acid derivatives were similar within each cultivar and ranged from 57 to 65% of the total amount. Hydroxytyrosol, which is mainly derived from hydrolyzed oleuropein, was present in similar amounts among the cultivars (14−15%), with the exception of the French sample (4.7%). Luteolin 7-*O*-glucoside content varied from 4.1%

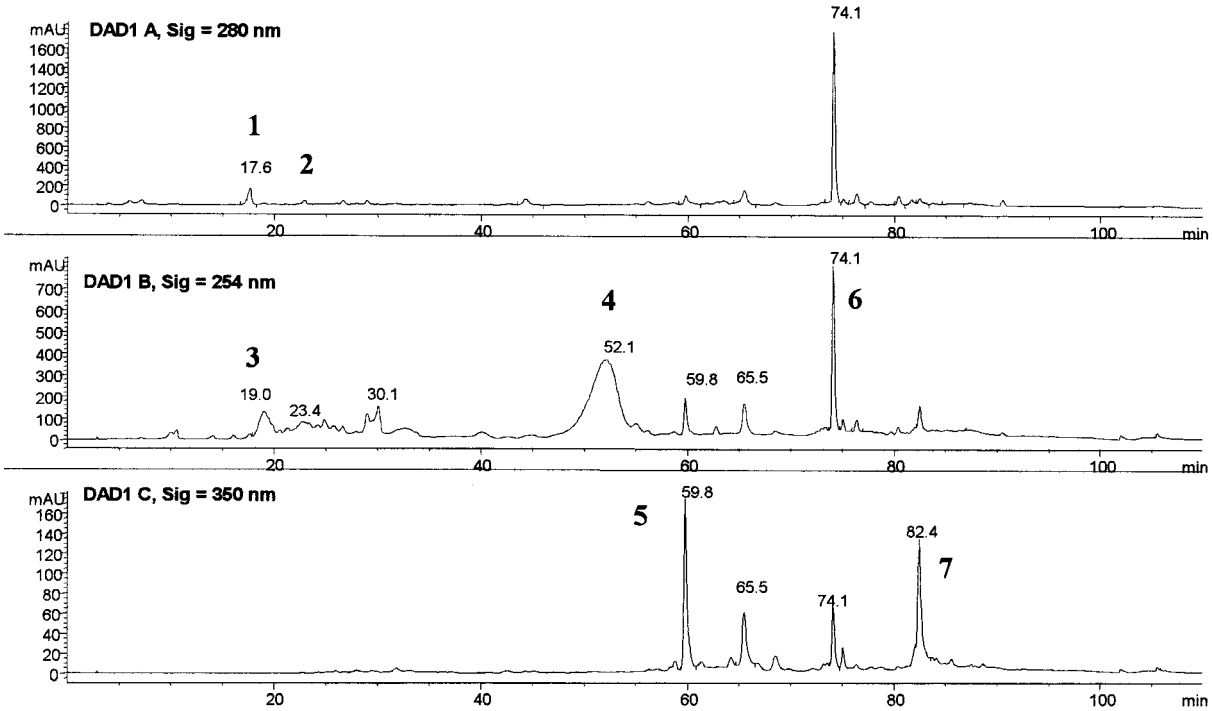

Figure 2. Chromatographic profiles of French ExpOOWW at 280, 254, and 360 nm. Peaks: 1, hydroxytyrosol; 2, tyrosol; 3, elenolic acid derivative; 4, elenolic acid; 5, luteolin 7-*O*-glucoside; 6, cinnamic acid derivative; 7, luteolin.

in cv. Picual (Spain) to 24.8% in cv. Salonenque (France) relative to total polyphenols.

The greatest differences were observed for verbascoside, which ranged between 1.4 g/kg in the Italian olive sample and 0.002 g/kg in the French one. This compound could be an important marker for differentiating specific cultivars harvested at the same time, as previously suggested (*15*). Moreover, its chemical structure suggests that it may be a potential antioxidant. As an example, the HPLC profiles of the French olive sample at 280, 254, and 350 nm are reported in Figure 1.

Olive Oil Waste Waters. Different aliquots of OOWW (from 100 mL to 1 L) were freeze-dried in order to store the samples before analysis and to guarantee the stability of this complex matrix over time. Dry material yields are summarized in Table 1. Comparable values were obtained for the ExpOOWW (ranging from 4.5 to 16.3%), whereas very different values were registered for the ComOOWW. All samples were submitted to an LSE extraction (see Experimental Procedures), and two fractions were obtained: EtOAc and acid MeOH eluates. The EtOAc fraction usually contained 90−100% of the total polyphenolic amounts. Both eluates were analyzed by HPLC-DAD, and quantitative results, reported in Table 3, represent the sum of each compound only when the molecule was also present in the methanolic eluate.

Generally, the same compounds were identified whether fresh commercial or experimental OOWW were considered (Table 3).

The ExpOOWW showed, in every sample, a similar polyphenolic pattern with (EA) and its derivatives as main compounds at values ranging from 28.8 to 80.0% of the total phenolics (Table 3a). Figure 2 shows, as an example, the HPLC-DAD profiles of the French ExpOOWW with the list of identified compounds. The total polyphenolic amounts were comparable among the samples from the various countries: the French sample showed the highest value (71.2 mg/100 mL) and the Portuguese the lowest content (30.5 mg/100 mL) essentially constituted by elenolic acid derivatives (73%). This difference may be due to the fact that the processed Portuguese olives were in a late stage of ripening and not adequately stored. From a comparison of the polyphenolic amounts in the standard degree olives (Table 2) and the corresponding experimental waste waters (Table 3a), it is clear that identical crushing methods strongly reduce the differences observed among the fruits. This fact was particularly evident for the Italian sample, after crushing, the total phenol concentration is strongly reduced.

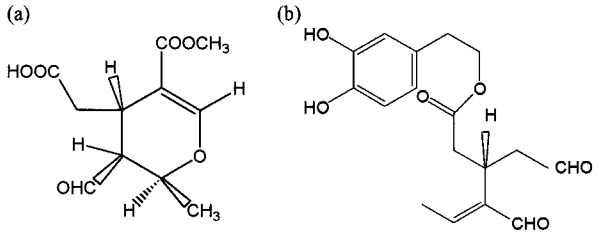

Figure 3. Molecular structures of elenolic acid (a) and deacetoxy oleuropein aglycon (b).

ComOOWW showed a wider variability in the amount of polyphenols and secoiridoids with respect to the corresponding experimental waste waters (Table 3b). Among these, Italian OOWW appeared to be the richest in total polyphenolic compounds with values up to 401.7 mg/100 mL including consistent amounts of secoiridoid derivatives (EA and dAcOlAg). In particular, this latter compound (deacetoxy oleuropein aglycon), which contains the hydroxytyrosol moiety and two dialdeidic groups that are responsible for its high reactivity, was very abundant, whereas it was absent in the other commercial samples. These compounds were identified by HPLC-MS and NMR analyses carried out on purified fractions obtained from semipreparative HPLC, and their structures are shown in Figure 3. NMR data concerning elenolic acid are consistent with those reported by Gariboldi et al. (*18*), and the structure of

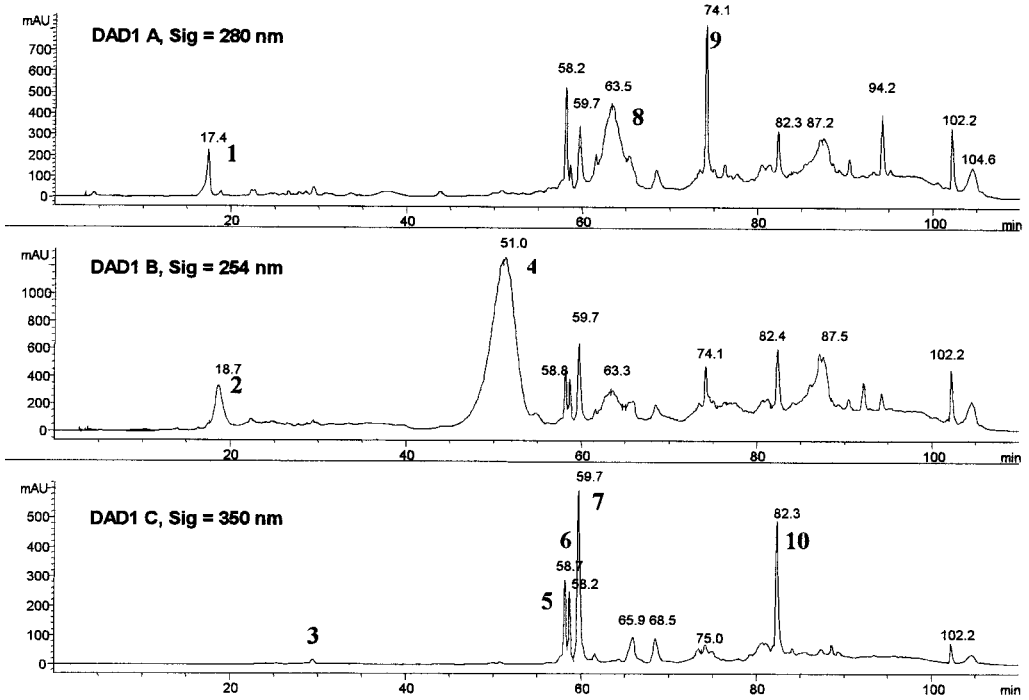

Figure 4. Chromatographic profile of Italian OOWW (sample C) at 280, 254, and 350 nm. Peaks: 1, hydroxytyrosol; 2, elenolic acid derivative; 3, caffeic acid; 4, elenolic acid; 5, verbascoside; 6, luteolin 7-*O*-glucoside; 7, rutin; 8, deacetoxy oleuropein aglycon; 9, cinnamic acid derivative; 10, luteolin.

deacetoxy oleuropein aglycon agrees with that described by Lo Scalzo and Scarpati (*19*).

The peculiarity of the Italian sample (sample A, Table 3b) was the presence of large quantities of suspended material compared with all of the other ComOOWW. Obviously the higher yields in dried material observed for lyophilized Italian waste waters agree with this result as well (Table 1b).

The Italian commercial waste waters seemed to be an interesting source of polyphenols, and therefore it was decided to verify this hypothesis by comparing several commercial samples from the same Italian mill to check the quali-quantitative variability of the raw material over time (Table 4). The HPLC-DAD profiles registered at 240, 280, and 350 nm are reported in Figure 4 for sample C.

In Table 4, it is possible to note similar total amounts for samples B and C, collected in the same year, whereas for the other ones a minor content was observed. However, for all of the waste waters, the amount varies from about 150 to 400 mg/100 mL OOWW. Several compounds, such as verbascoside, flavonoids, and deacetoxy oleuropein aglycon, were present in traces or absent in the clarified waste waters (French, Portuguese, and Spanish, Table 3b), whereas in all Italian commercial waste waters these compounds are present in larger amounts. Two reasons can explain this result: good storage of material, immediately lyophilized after collection, and the presence of a high quantity of solid residue that can represent a source of minor hydrophilic compounds such as dAcOlAg and EA. The richest commercial OOWW samples were also treated by our group to prepare some extracts to use in biological tests, and the results have recently been published (*13*).

It is difficult to find a correlation between raw material (olives) and final waste product (ExpOOWW) indicating that technological processes for oil extraction play a more important role with respect to the cultivar and the ripenings. It is worth noting that the total amount of minor polar compounds in several OOWW samples, collected from the same geographical area and processed in the same mill, show comparable values over time (Table 4).

Work is in progress to test and optimize purified procedures able to recover higher quantities of polyphenols by working on larger quantities of samples in view of possible future industrial applications.

ABBREVIATIONS USED

OOWW, olive oil waste waters; ExpOOWW, experimental olive oil waste waters; ComOOWW, commercial olive oil waste waters; EA, elenolic acid; dAcOlAg, deacetoxy oleuropein aglycon.

ACKNOWLEDGMENT

We thank Tecnoalimenti S.C.p.A. for their help in collecting raw material and Dr. Miari Fulcis, head of the commercial Italian mill in Maiano (Firenze), for having kindly supplied a part of the raw material.

LITERATURE CITED

(1) Saviozzi, A.; Riffaldi, R.; Levi-Minzi, R.; Scagnozzi, A.; Vanni, G. Decomposition of vegetation−water sludge in soil. *Bioresour. Technol.* **1993**, *44*, 223−228.

(2) Amicarelli, V.; Notarnicola, L. L'utilizzazzione delle acque di vegetazione delle olive. Problemi e prospettive. *Atti del XVII Congresso Nazionale di Merceologia.* "*Merci e cicli produttivi nel settore agroindustriale alle soglie del 21° secolo*"; 1996; Vol. 1, pp 741−746.

(3) Sciancalepore, V.; De Stefano, G.; Piacquadio, P.; Sciancalepore, R.; Compostaggio in ambiente protetto del residuo della lavorazione delle olive con impianti ad estrazione bifasica. *Ing. Ambientale* **1995**, *24 (11/12)*, 627−631.

(4) Bisignano, A.; Tomaino, A.; Lo Cascio, R.; Crisafi, G.; Uccella, N.; Saja, A. On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. *J. Pharm. Pharmacol.* **1999**, *51* (*8*), 971−974.

(5) Driss, F.; Duranthon, V.; Viard, V. Effects biologique des composés polyphenoliques de l'olivier. *Corps Gras* **1996**, *3* (*6*), 448−451.
(6) Tsimidou, M.; Papadopolous, G.; Boskou, D. Phenolic compounds and stability of virgin oil—part 1. *Food Chem*. **1992**, *45*, 141−144.
(7) Tsimidou, M. Polyphenols and quality of virgin oil in retrospect. *Ital. J. Food Sci*. **1998**, *10*, 99−116.
(8) Visioli, F.; Galli, C. Olive oil phenols and their potential effect on human health. *J. Agric. Food Chem*. **1998**, *46*, 4292−4296.
(9) Visioli, F.; Galli, C. The effect of minor constituents of olive oil on cardiovascular disease: new findings. *Nutr. Rev*. **1998**, *56*, 142−147.
(10) Chen, J. H.; Ho, C.-T. Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. *J. Agric. Food Chem*. **1997**, *45*, 2374−2378.
(11) Caragay, A. B. Cancer-preventive foods and ingredients. *Food Technol.* **1992**, *46* (*4*), 65−68.
(12) Romani, A.; Baldi, A.; Mulinacci, N.; Vincieri, F. F.; Mancini, P. Acque reflue di frantoio: studio dei composti polifenolici per un eventuale uso alternativo. Problemi e prospettive. *Atti del XVII Congresso Nazionale di Merceologia. Merci e cicli produttivi nel settore agroindustriale alle soglie del 21° secolo*; 1996; Vol. 1, pp 834−841.
(13) Visioli, F.; Romani, A.; Mulinacci, N.; Zarini, S.; Conte, D.; Vincieri, F. F.; Galli C. Antioxidant and other biological activities of olive mill waste waters. *J. Agric. Food Chem*. **1999**, *47*, 3397−3401.
(14) Capasso, R.; Cristinzio, G.; Evidente, A.; Scognamiglio, F.; Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetables waste waters. *Phytochemistry* **1992**, *31* (*12*), 4125−4128.
(15) Romani, A.; Mulinacci, N.; Pinelli, P.; Vincieri, F. F.; Cimato, A. Polyphenolic content in five Tuscany cultivars of *Olea europaea* L. *J. Agric. Food Chem*. **1999**, *47*, 964−967.
(16) Romani, A.; Baldi, A.; Mulinacci, N.; Vincieri, F. F.; Tattini, M. Extraction and identification procedures of polyphenolic compounds and carbohydrates in phyllirea (*Phyllirea angustifolia* L.) leaves. **1996**, *42 (9/10)*, 571−577.
(17) Romani, A.; Pinelli, P.; Mulinacci, N.; Vincieri, F. F.; Tattini, M. Identification and quantitation of polyphenols in leaves of *Myrtus communis* L. *Chromatographia* **1999**, *49*, 17−20.
(18) Gariboldi, P.; Jommi, G.; Verotta, L. Secoiridoids from *Olea europaea*. *Phytochemistry* **1986**, *25*, 865−869.
(19) Lo Scalzo, R.; Scarpati, M. L. A new secoiridoids from olive oil waste waters. *J. Nat. Prod*. **1993**, *56*, 621−623.

Received for review August 2, 2000. Revised manuscript received December 22, 2000. Accepted April 27, 2001.

JF000972Q