JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Toward a High Yield Recovery of Antioxidants and Purified Hydroxytyrosol from Olive Mill Wastewaters

NOUREDDINE ALLOUCHE, INES FKI, AND SAMI SAYADI*

Laboratoire des Bio-procédés, Centre de Biotechnologie de Sfax, B.P. "K" 3038, Sfax, Tunisia

We investigated to develop effective procedures to recover the potentially high-added-value phenolic compounds contained in the discontinuous three-phase olive processing wastewaters (OMW). Particular emphasis was made to extract and purify hydroxytyrosol, one of the major compounds occurring in OMW. Batch optimization experiments showed that ethyl acetate is the most efficient solvent for the recovery of phenolic monomers from OMW. The latter was used with an optimal pH equal to 2. Furthermore, the percentage of each monomer, and particularly hydroxytyrosol, in the extract was maximum for a solvent ratio and a theoretical extraction stage number equal to 2 and 3, respectively. High yield (85.46%) recovery of hydroxytyrosol was achieved from OMW using a three-staged continuous counter-current liquid−liquid extraction unit. Hydroxytyrosol (1.225 g) were extracted per liter of OMW. One gram of hydroxytyrosol per liter of OMW was then purified by means of a chromatographic system which could be adapted to a large scale production process.

KEYWORDS: Olive mill wastewaters; hydroxytyrosol; polyphenols; continuous-extraction; antioxidants; purification

INTRODUCTION

Usually, olive oil is extracted mechanically by pressure and by a three-phase centrifugation system, which result in the worldwide production of more than 30 millions m^3 per year of black olive mill wastewater (OMW) (*1*). This liquid effluent has a high polluting organic load, due to a high content of organic substances, including sugars, tannins, polyphenols, polyalcohols, pectins, and lipids (*2*, *3*). Centrifugation, despite its high water consumption, is still the most widely employed method for production of virgin olive oil, especially in countries that produce large amounts of olives in a short time (*4*). Most frequently, OMW are pumped and discharged into evaporation ponds or directly dumped in rivers or spread on soil (*5*). This becomes a major environmental problem in the main olive-producing countries of the Mediterranean region. Worldwide, its relevance is expected to increase soon because other countries such as Australia, Argentina, and South Africa are promoting intensive olive-tree cultivation even if two-phase mills are more widely used in these countries.

It is known that phenolic compounds are major contributors to the toxicity and the antibacterial activity of OMW. This limits its microbial degradability (*1*, *6*, *7*). However, these phenolic compounds, which are present in general in olives, olive oil, and olive byproducts (*8*) are endowed with several biological activities such as antioxidant property (*9*−*12*).

Among the phenolic compounds present in OMW, hydroxytyrosol (3,4-dihydroxyphenylethanol) stands out as a compound of high added value due to its interesting antioxidant and potential beneficial-human health properties (*13*−*16*). For example, results in vitro demonstrated that hydroxytyrosol inhibits human low-density lipoprotein (LDL) oxidation (*17*), scavenges free radicals (*14*), inhibits platelet aggregation (*18*) and the production of leucotriene for human neutrophyls, (*19*) and confers cell protection (*20*). It has also been demonstrated that hydroxytyrosol acts in vitro against both Gram-positive and Gram-negative bacteria (*21*). Recently, the good bioavailability of hydroxytyrosol has been reported which encourage its addition in the diet (*22*, *23*).

Several methods have been developed to produce hydroxytyrosol by means of chemical synthesis (*24*−*26*), trough conversion of oleuropein (*27*), enzymatic synthesis using tyrosinase as biocatalyst (*28*), and by bench scale purification from OMW (*10*, *29*, *30*). Recently, Bolanos et al. (*31*) reported the production of hydroxytyrosol from the liquid−solid waste of two phase olive processing "Alperujo" by hydrothermal treatment, but the developed protocol for purification was not described due to patent procedure. Other developed protocols as well as industrial applications of hydroxytyrosol are always patented and therefore rather inaccessible (*32*−*35*).

The purpose of this work was to select optimum operational conditions for the solvent extraction of phenolic antioxidants from OMW. Optimized parameters were then validated on a continuous counter-current extraction unit. Additionally, a simple and scalable system of hydroxytyrosol purification was elaborated.

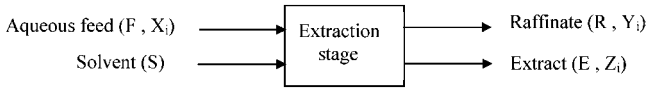
* To whom correspondence should be addressed. Tel.: +216-74-275-373. Fax: +216-74-275-970. E-mail address: sami.sayadi@cbs.rnrt.tn.

10.1021/jf034944u CCC: $27.50
Published on Web 12/24/2003

MATERIALS AND METHODS

Materials. Fresh olive mill wastewaters (50 L) were supplied by discontinuous three-phase olive processing mill from a cooperative in Sfax (Tunisia). This sample, generated from *Chemlali* olive variety, was taken at the end of the olive harvest season and conserved at 4 °C. The phenolic compounds used as standards are hydroxytyrosol, tyrosol, para-hydroxyphenyl acetic acid, 3,4-dihydroxyphenyl acetic acid, caffeic acid, para-coumaric acid and ferulic acid. Hydroxytyrosol was purified in our laboratory from OMW while the other used compounds were purchased from Fluka.

Batch Extraction Procedure. Extraction parameters were optimized in a separatory funnel using 200 mL of OMW. The mixture (solvent-OMW) was vigorously shaken for 10 min to achieve equilibrium and was then allowed to settle for 30 min. All the runs were performed at ambient temperature (20−25 °C). The phases were next separated. Their phenolic compositions were determined by HPLC technique and quantified based on standard curves, in the concentration range 50 ppm to 2000 ppm, according to the method reported by Akasbi et al. (*36*). The used nomenclature for different phases and concentration of each component (i) are given in the following schema:



For each phenolic monomer (i), the distribution coefficient (D_i) and the percentage extracted ($Ex_{(i)}$) were calculated as follows:

$$D_i = Z_i/Y_i$$

$$Ex_{(i)} = (Z_i/X_i) \times 100$$

In the OMW pH optimization experiments, pH was adjusted with HCl (1 N) and measured with a Metrohm pH-meter.

Continuous-Extraction Procedure. Continuous counter-current extractions were conducted at ambient temperature in a polyethylene mixer-settler unit of ROBATEL design (mixer volume, 35 mL; settler volume, 200 mL). The maximum flow rate that could be fed into the battery was 5 L/h. For each run, the steady state was confirmed by phenolic monomers analysis in the organic stream and by verification of the mass flow rates balance. This parameter represents the deviation between the total mass flow fed in the battery and that of the recupered phases (raffinate and extract) (*37*). The obtained counter-current extract was stored at 4 °C.

HPLC Analysis. Phenolic monomers identification and quantification were carried out by HPLC analysis (*36*). It was performed on a Shimadzu apparatus composed of a LC-10ATvp pump and a SPD-10Avp detector. Eluates were detected at 280 nm. The column was (4.6 × 250) mm (Shim-pack VP-ODS) and its temperature was maintained at 40 °C. The flow rate was 0.5 mL/min. The mobile phase used was 0.1% phosphoric acid in water (A) versus 70% acetonitrile in water (B) for a total running time of 40 min, and the gradient changed as follows: solvent B started at 20% and increased immediately to 50% in 30 min. After that, elution was conducted in the isocratic mode with 50% solvent B within 5 min. Finally, solvent B decreased to 20% until the end of running.

GC-MS Analysis. GC-MS was performed with a HP Model 5872A, equipped with a capillary HP5MS column (30-m length; 0.32-mm i.d.; 0.32-μm film thickness). The carrier gas was He used with a 1.7 mL/min flow rate. The oven temperature program was as follows: 1 min at 100 °C, from 100 °C to 260 °C at 4 °C/min, 10 min at 260 °C. BSTFA (100 μL) (bis (trimethylsilyl)-acetamid) was added to 100 μL of the OMW ethyl acetate extract. The obtained solution was incubated within 20 min at 80 °C. Ethyl acetate and BSTFA were evaporated under N_2 current. The residue was redissolved in ethyl acetate (1 mL) and analyzed by GC-MS.

Gel Filtration Analysis. Samples (2 mL) were filtered on a Sephacryl S-200 column 1.6 × 85 cm previously equilibrated with borate buffer pH 9. The flow rate was adjusted to 0.2 mL/min and 2 mL fractions were collected. These fractions were measured spectrophotometrically at 280 nm. The column was calibrated with caffeic acid (180 Da), ribonuclease (13.5 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), catalase (232 kDa), and bleu dextran (2000 kDa).

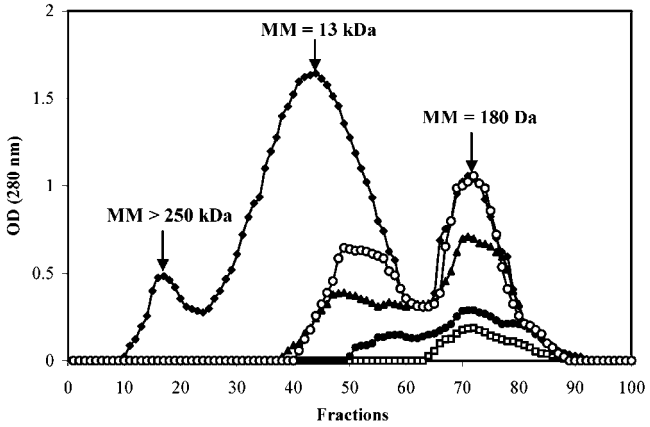


Figure 1. Sephacryl S-200 gel permeation of crude OMW (◆) and its extracts using different organic solvents. Diethyl ether (□); Methyl ethyl ketone (●); Methyl isobutyl ketone (▲); Ethyl acetate (○).

Medium-Pressure Liquid Chromatography. The organic extract obtained from OMW continuous extraction was evaporated under reduced pressure below 45 °C. An aliquot (1 g) of the obtained residue was chromatographed on a C-18 silica gel (LiChroprep RP-18; 25−40 μm) column 2.5 × 70 cm under medium pressure (3 bar). Phenolic compounds elution was carried out with the same gradient solvent used in HPLC. The flow rate was adjusted to 0.3 mL/min, and 4.5-mL fractions were collected. The absorbance of each fraction was measured spectrophotometrically at 280 nm. Compound (**1**) obtained in lot 2 after C-18 silica gel chromatography of the extract was identified to hydroxytyrosol by means of GC-MS analysis and HPLC retention time comparaison with authentic standard.

RESULTS

Polyphenol Extraction from OMW. *Batch Mode Optimization Experiments.* Our objective is to develop effective procedures to recover the potentially high-added-value phenolic compounds contained in OMW. Accordingly, emphasis has been paid to optimize the extraction procedure of phenolic compounds, in batch mode, following four directions: solvent nature, pH of OMW, rate of solvent versus OMW and number of theoretical stages. Results of optimal extraction conditions are summarized as follows:

Solvent Nature. Among several polar solvents such as methyl isobutyl ketone, methyl ethyl ketone, and diethyl ether, ethyl acetate was found to extract broadly the OMW monomeric fraction. Sephacryl S-200 gel filtration analysis (**Figure 1**) shows that OMW is composed by three polyphenolic fractions. Two fractions with high and medium molecular-mass (MM > 250 kDa and MM = 13 kDa respectively) and the third fraction represents the phenolic monomers, flavonoids and other compounds. In addition, it can be noted that all the tested solvents extract polyphenols from OMW but significant difference was found between their extraction power. This latter decreases in the order ethyl acetate > methyl isobutyl ketone > methyl ethyl ketone > diethyl ether.

pH of OMW. The pH effect of the aqueous feed OMW on the polyphenols extraction yield was studied. For each pH value, the corresponding extract was analyzed quantitatively by HPLC and the distribution coefficient (D_i), corresponding for each phenolic monomer (i), was calculated as described in Materials and Methods. **Figure 2** showed that for each phenolic compound, the distribution coefficient increases when the pH

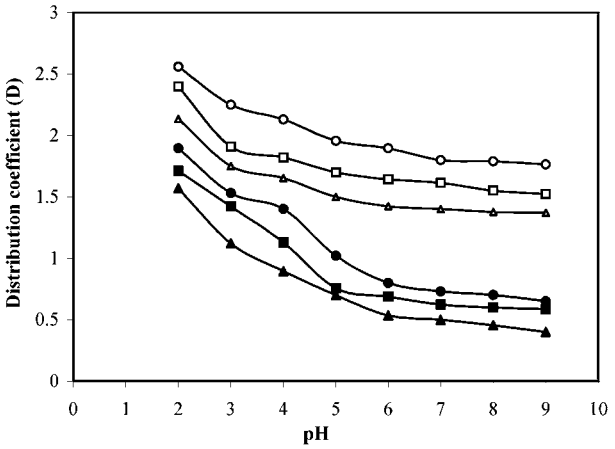


Figure 2. Effect of OMW pH on the distribution coefficient (*D*) of extracted phenolic monomers. Hydroxytyrosol (○); caffeic acid (□); 3,4-dihydroxyphenyl acetic acid (△); para-hydroxyphenyl acetic acid (▲); para-coumaric acid (●); tyrosol (■).

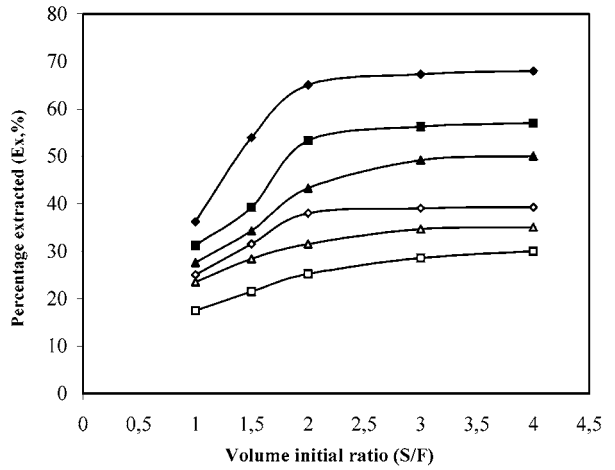


Figure 3. Effect of volume initial ratio (S/F) on the percentage extracted (Ex,%) of extracted phenolic monomers. Hydroxytyrosol (◆); caffeic acid (■); 3,4-dihydroxyphenyl acetic acid (▲); para-hydroxyphenyl acetic acid (□); para-coumaric acid (◇); tyrosol (△).

decreases and reaches its maximum at pH = 2. Furthermore, it was noted that ortho-diphenols (hydroxytyrosol, caffeic acid, and 3,4-dihydroxyphenyl acetic acid) were more extractable than monohydroxylated phenols as a consequence of their high distribution coefficient.

Ratio of Solvent (S/F). To achieve the maximum extraction yield of polyphenols in a single step, equilibrium extractions with ethyl acetate and OMW at pH 2, were carried out using different volume initial ratios (S/F) of 1, 1.5, 2, 3, and 4. To evaluate quantitatively the extraction power of ethyl acetate, the percentage extracted ($Ex_{(i)}$) of each component (i) in the organic phase was calculated as described in Materials and Methods. **Figure 3** shows the percentage of each phenolic monomer extracted by ethyl acetate versus the initial phase ratio (S/F). Accordingly, it is clear that phenolic monomers practically follow the same trend, and as it was observed in the precedent paragraph, ortho-diphenols, and especially hydroxytyrosol have the highest extraction yields with ethyl acetate. In addition, we have observed that the percentage of each monomer, and particularly hydroxytyrosol, in the extract increased rapidly for a ratio ranging from 0 to 2. For higher ratios, the yield of extraction is weakly affected.

Number of Theoretical Stages (N). To improve the recovery of phenolic compounds from OMW, the effects derived from performing sequential extraction stages were studied. For this aim, an extraction cascade was performed using a separatory funnel. Polyphenols in the recovered organic phases were quantified using HPLC analysis (data not shown). Consequently, we found that a number of theoretical extraction stages equal to three is the most adequate for a continuous extraction process.

Continuous Extraction of Polyphenols from OMW. To validate the results obtained in the previous optimization study, continuous counter current extraction was performed, at ambient temperature, on the laboratory-scale mixer-settler unit as described in Materials and Methods. Results described in this paragraph were obtained with three-staged counter current extractions using ethyl acetate at a flow rate = 1.44 L/h and OMW at a flow rate = 0.72 L/h. The global feed flow rate of the mixer−settler battery was 2.16 L/h. The volume initial ratio (S/F) was equal to 2. After 4 h extraction, the steady state was successfully established. Indeed, at this moment, the mass flow rates balance was about 2% (see Materials and Methods), which resulted in a stage efficiency of 98%.

Since hydroxytyrosol is the most abundant compound in OMW, as mentioned in previous investigations (*11*, *25*), continuous extraction yield was determined according to hydroxytyrosol concentration (*C*). Results obtained in steady-state conditions are given in **Figure 4**. In view of that, the recovered quantity of hydroxytyrosol increases with the number of extraction stages. The global continuous extraction yield in term of hydroxytyrosol was equal to 85.48%.

Identification and Quantification of Phenolic Monomers in the Extract. A reversed-phase high-performance liquid chromatographic technique was used to identify and quantify the major phenolic compounds of crude OMW and its ethyl acetate continuous counter-current extract. For this purpose, standards mixture solution of phenolic compounds was analyzed. Sample concentrations were calculated based on peak areas compared to those of each of the external standards as described in the Materials and Methods section. Representative chromatogram of OMW extract obtained after HPLC analysis is given in **Figure 5**.

This chromatogram shows several simple peaks corresponding to different phenolic monomers. From the retention time comparison, it was deduced that the analyzed OMW extract is made mainly of hydroxytyrosol, 3,4-dihydroxyphenyl acetic acid, tyrosol, para-hydroxyphenyl acetic acid, caffeic acid, para-coumaric acid, and ferulic acid. The identification of these phenolic compounds was confirmed by GC-MS analysis (**Table 1**). This identification was achieved according to the comparison of the isolated compounds GC-MS spectra with GC-MS apparatus library. Moreover, the obtained mass fragments agreed with those described by Capasso (*38*). Minor unidentified components were also present in the ethyl acetate extract.

Quantification of the major monomers present in crude OMW and in the ethyl acetate extract is given in **Table 2**. As expected, hydroxytyrosol is the more abundant phenolic monomer in crude OMW (1.433 g/L). Moreover, the distribution coefficient and the extraction yield were maximal for ortho-diphenol compounds and especially for hydroxytyrosol. This result correlates with that previously established during the optimization study carried out in batch experiments.

Hydroxytyrosol Purification from the Extract. To produce large quantities of highly purified hydroxytyrosol, a medium-pressure chromatographic system was performed using C-18 silica gel as stationary phase and the same mobile phase that was used in the HPLC analysis (see Materials and Methods). Chromatogram representing optical density at 280 nm versus

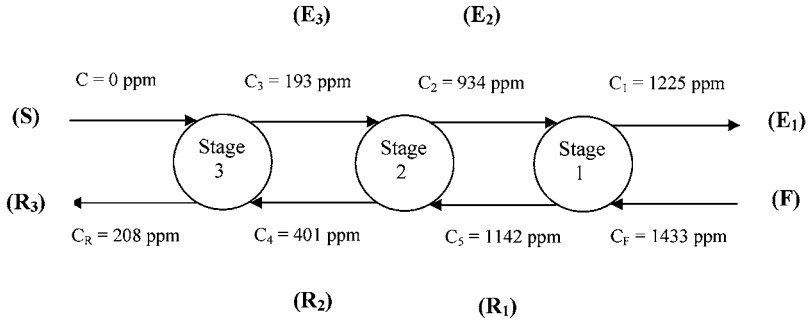


Figure 4. Hydroxytyrosol concentration determination during the continuous counter-current extraction in the three stages. **S**, solvent; **R**, raffinate; **E**, extract; **F**, aqueous feed; *C*, hydroxytyrosol concentration.

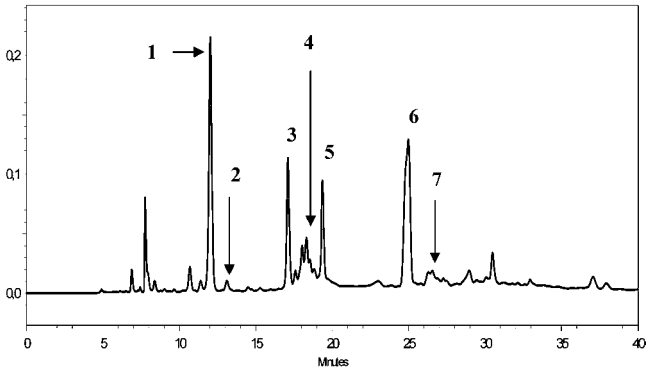


Figure 5. HPLC analysis of the continuous ethyl acetate extract from OMW. **1**, hydroxytyrosol; **2**, 3,4-dihydroxyphenyl acetic acid; **3**, tyrosol; **4**, para-hydroxyphenyl acetic acid; **5**,caffeic acid; **6**, para-coumaric acid; **7**, ferulic acid.

Table 1. Abbreviated Mass Spectra of the Major Phenolic Monomers Identified in the Continuous Counter-Current Ethyl Acetate Extract from OMW

TMS derivatives of	mass spectra (*m/z* and % of the base peak)
hydroxytyrosol	370(M^+, 39); 267(90); 193(25); 179(12); 73(100)
3,4-dihydroxyphenyl acetic acid	384(M^+, 68); 369(66); 311(42); 296(85); 281(67); 252(30); 179(31); 164(28); 147(12); 73(100)
tyrosol	282(M^+, 18); 267(13); 193(15); 179(100); 163(4); 149(5); 103(8); 73(42)
para-hydroxyphenyl acetic acid	296(M^+, 74); 281(57); 252(51); 179(86); 164(40); 147(14); 133(11); 73(100)
caffeic acid	396(M^+, 100); 381(25); 307(12); 293(6); 249(8); 233(4); 219(93); 191(16); 73(55)
para-coumaric acid	308(M^+, 81); 293(100); 249(44); 219(82); 203(7); 179(13); 175(6); 139(5); 115(4); 73(51)
ferulic acid	338(M^+,75); 297(100); 282(30); 267(60); 253(45); 223(60); 207(7); 193(25); 179(6); 165(10); 141(12); 126(28); 89(16); 73(90)

fraction number is given in **Figure 6**. This chromatogram shows five separated peaks. Fractions corresponding to each peak were collected into five different lots. The phenolic monomers constitution of each lot was determined using HPLC analysis. **Figure 7** shows that lot 1, lot 2, and lot 3 are composed of a single major phenolic compound, while lot 4 is made of a

Table 2. Quantification of the Major Phenolic Monomers in Crude OMW and in the Continuous Counter-Current Ethyl Acetate Extract; Distribution Coefficient (*D*) and Extraction Yield Related to Each Phenolic Monomer

	conc (mg/L)		
phenolic monomers	crude OMW	OMW extract (Figure 5)	extraction yield %
hydroxytyrosol	1433	1225	85.48
tyrosol	851	345	40.54
3,4-dihydroxyphenyl acetic acid	87	70	80.45
para-hydroxyphenyl acetic acid	274	198	72.26
caffeic acid	321	256	79.75
para-coumaric acid	298	169	56.71
ferulic acid	94	70	74.46

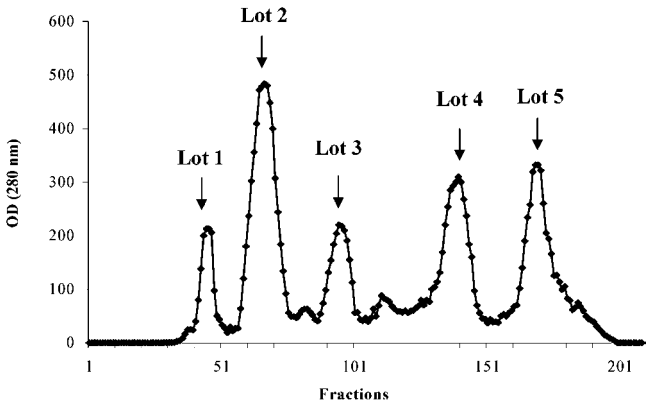


Figure 6. C-18 silica gel chromatography of the continuous ethyl acetate extract from OMW.

mixture of monomers. Lot 5 contains some phenolic polymers, due to the presence of a significant background in the corresponding chromatogram, which has a complex profile. The comparison of the retention times to those of external standards allowed us to identify ferulic acid **(7)** and para-coumaric acid **(6)** in lot 4, caffeic acid **(5)** in lot 3, and hydroxytyrosol **(1)** in lot 2. The other phenolic compounds were not identified. It should be noted that there are some differences between the relative concentrations of the identified compounds in the extract and in the obtained lots after chromatographic purification. This variation could be attributed to the storage conditions of the extract, which can induce polymerization and/or degradation processes. In this way, Ryan et al. (*39*) have noted that the levels of some minor components, present in olive leaves, decreased after 2 h of storage at ambient conditions, and thus, extracts should be generally be examined with minimal delay or stored at low temperature in the dark.

The recovered fractions corresponding to lot 2 were concentrated, and 250 mg of hydroxytyrosol were obtained. Given the

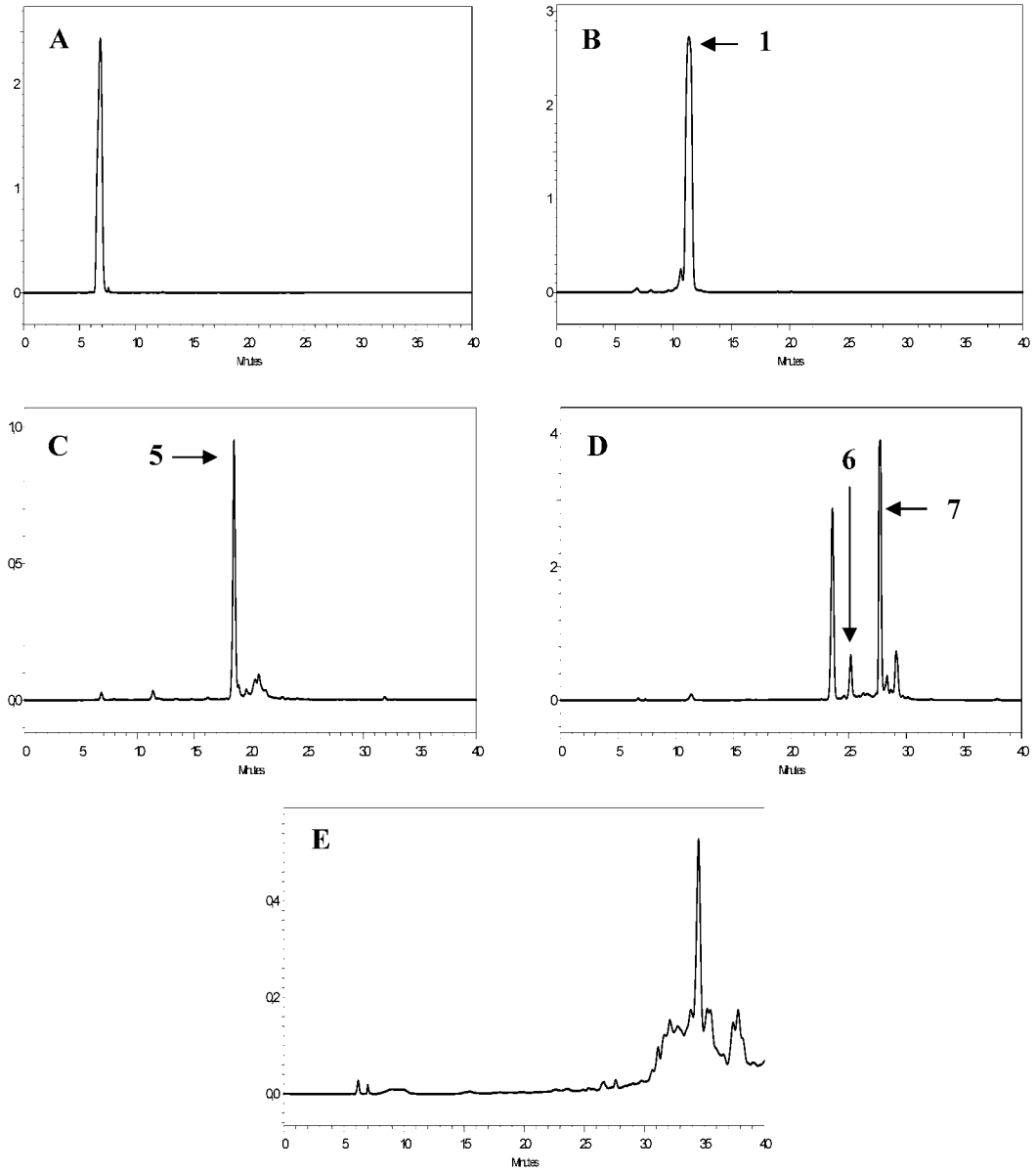


Figure 7. HPLC analysis of the different lots collected from the C-18 silica gel purification of the OMW continuous ethyl acetate extract. Lot 1 (**A**); Lot 2 (**B**); Lot 3 (**C**), Lot 4 (**D**); Lot 5 (**E**).

fact that one liter of crude OMW provides 4 g of washed dry extract and 1 g of the extract was chromatographed, we can conclude that 1 g of hydroxytyrosol can be produced per liter of OMW using the procedure developed in this study.

DISCUSSION

OMW represents a complex medium containing mainly polyphenols of different molecular-mass. Several previous investigations have demonstrated that the monomeric fraction is endowed with interesting biological activities (*9*−*12*). For its recovery from OMW, we have used a liquid−liquid solvent extraction procedure. In previous investigations, it was reported that among all procedures that are employed for natural antioxidants removal, liquid−liquid solvent extraction represents a simple and convenient alternative, and it is widely used in pilot-scale production and in ultimate commercial recovery (*40*). Our optimization study showed that ethyl acetate is the most convenient solvent for phenolic monomers extraction from OMW due to its important polarity comparing with the other tested solvents (diethyl ether, methyl isobutyl ketone, and methyl ethyl ketone). Furthermore, this solvent has a low boiling temperature that facilitates its evaporation below 45 °C. Thus, we focused on the use of ethyl acetate in the continuous extraction procedure. This result is in agreement with previous investigation who reported that polar solvents are among the most employed for removing polyphenols from water (*41*). Moreover, it has been noted that the polyphenols extraction yield increases with increasing polarity of the extractant solvent (*42*). In addition, some other researchers have found that ethers and ketones are among the most employed solvents for removing phenolic compounds from water, whereas ethyl acetate and diethyl ether have been used for extracting low molecular-mass phenolic compounds, and ethyl acetate is the widely used and effective solvent for polyphenols extraction (*43*). The pH of the aqueous feed OMW was optimized. Maximum extraction yield of polyphenols was obtained at pH = 2. This fact agrees with the findings of Sheabar and Neeman (*44*), who reported

that maximum solubility in the organic phase of polyphenols from olive rape is achieved at low pH value.

All the optimized extraction parameters were validated experimentally by performing continuous counter current extraction runs on a laboratory-scale mixer-settler unit. This battery was used for the first time to recover phenolic monomers from OMW and was more effective than batch extractions. Continuous solvent extraction of phenolic monomers from OMW was not reported in the literature. As a result, 1.225 g of hydroxytyrosol were extracted from 1 L of OMW with an extraction yield equal to 85.48%. This process could be used at an industrial scale.

Quantitative study has shown that hydroxytyrosol is the major component in crude OMW and in ethyl acetate extract. In this way, several previous investigations have demonstrated that hydroxytyrosol is the main abundant phenolic compound occurring in OMW (*6*, *45*). However, the identified phenolic compounds in OMW as well as their concentrations vary from a study to another. As an example, Casa et al. (*46*) have identified in an Italian OMW catechol, 4-hydroxybenzoic acid, 4-methylcatechol, 3-hydroxyphenylpropionic acid, 3,4,5-trimethoxybenzoic acid and trans-cinnamic acid, as major compounds. These phenolic compounds have not been identified in the OMW sample used in this investigation. This could be related to olive variety, climatic conditions, period of harvest, and olive oil extraction system as mentioned by Amiot et al. (*47*). In another study, Visioli et al. (*12*) have reported that oleuropein, an ester of elenolic acid and hydroxytyrosol, is a major polyphenol of OMW. Oleuropein was not identified in our extract, probably because our OMW was sampled at late olive harvest (mature olives); hence, this compound is degraded into elenolic acid and hydroxytyrosol (*12*). This is in accordance with the high amount of hydroxytyrosol quantified in the crude studied OMW.

A chromatographic purification of hydroxytyrosol from the OMW extract was performed, which yielded one gram of hydroxytyrosol per liter of OMW. In previous studies, several chromatographic purifications of hydroxytyrosol from OMW were used. The method developed by Capasso et al. (*25*) consisted in silica gel chromatography, C_8-reverse phase chromatography, prep. TLC chromatography and crystallization technique. In this study, researchers have produced only 91 mg of pure hydroxytyrosol per liter of OMW. Chemical synthesis of hydroxytyrosol was performed by the reduction of 3,4-dihydroxyphenyl acetic acid followed by a chromatographic purification. Here, the precursor is very expensive and the method uses toxic reagents, which should be removed (*25*, *26*). Hydroxytyrosol was also synthesized from a commercially available precursor, the tyrosol using a mushroom tyrosinase in the presence of the ascorbic acid reductant (*28*). Although the exact protocol of this process was not described, the main disadvantage of this method is the rapid appearance of quinonic compounds. Hydroxytyrosol was produced from the liquid−solid waste of the two-phase olive processing "Alperujo" by hydrothermal treatment using acid or alkali catalysts.

In conclusion, it is well known that there is an increased preference for natural food ingredients, which are generally believed to be safer, more healthy, and less subject to hazards than foods containing synthetic additives. Because hydroxytyrosol or ethyl acetate extract come from a natural source and has beneficial effects as potent antioxidants, they could be integrated in food as well as cosmetic and pharmaceutical products. Consequently, large scale production of hydroxytyrosol using the low-cost extraction process developed in this study from commercially available OMW constitutes a promising alternative for valorizing this problematic wastewater. Indeed, the effective recovery of these low molecular-mass phenolic compounds, apart from its intrinsic economic validity, might also be beneficial for the ensuing reduction in OMW phenolic content in view of further downstream treatments and/or applications.

ACKNOWLEDGMENT

We wish to thank Dr. Mongi FEKI from the Engineering School of Sfax (ENIS) for his help in the continuous extraction experiments.

LITERATURE CITED

(1) Borja, R.; Alba, J.; Banks, C. J. Impact of the main phenolic compounds of olive mill wastewater (OMW) on the kinetics of acetoclastic methanogenesis. *Process Biochem.* **1997**, *32*, 121−133.

(2) D'Annibale, A.; Crestini, C.; Vinciguerra, V.; Giovannozzi-Sermanni, G. The biodegradation of recalcitrant effluents from an olive mill by a white-rot fungus. *J. Biotechnol.* **1998**, *61*, 209−218.

(3) Sayadi, S.; Ellouz, R. Roles of lignin peroxidase and manganese peroxidase from *Phanerochaete chrysosporium* in the decoloration of olive mill wastewaters. *Appl. Environ. Microb.* **1995**, *61*, 1098−1103.

(4) Benitez, J.; Beltran-Herdia, J.; Torregrosa, J.; Acero, J. L.; Cercas, V. Aerobic degradation of olive mill wastewaters. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 185−188.

(5) Greco, G., Jr.; Toscano, G.; Cioffi, M.; Gianfreda, L.; Sannino, F. Dephenolisation of olive mill wastewaters by olive husk. *Water Res.* **1999**, *33*, 3046−3050.

(6) Capasso, R.; Evidente, A.; Schivo, L.; Orrù, G.; Marcialis, M. A.; Cristinizio, G. Antibacterial polyphenols from olive oil wastewaters. *J. Appl. Bacteriol.* **1995**, *79*, 393−398.

(7) Sayadi, S.; Allouche, N.; Jaoua, M.; Aloui, F. Detrimental effects of high molecular-mass polyphenols on olive mill wastewater biotreatment. *Process Biochem.* **2000**, *35*, 725−735.

(8) Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A.; Montedoro, G. F. HPLC evaluation of phenols in olive fruit, virgin olive oil, vegetation waters and pomace, and 1D- and 2D-NMR characterization. *J. Am. Oil Chem. Soc.* **1999**, *76*, 873−882.

(9) Visioli, F.; Romani, A.; Mulinacci, N.; Zarini, S.; Conte, D.; Vincieri, F. F.; Galli, C. Antioxidant and other biological activities of olive mill wastewaters. *J. Agric. Food Chem.* **1999**, *47*, 3397−3401.

(10) Capasso, R.; Cristinzio, G.; Evidente, A.; Scognamiglio, F. Isolation spectroscopy and selective phytotoxic effects of polyphenols from vegetable wastewaters. *Phytochemistry* **1992**, *31*, 4125−4128.

(11) Visioli, F.; Vinceri, F. F.; Galli, C. Wastewaters from olive production are rich in natural antioxidants. *Experientia* **1995**, *51*, 32−34.

(12) Visioli, F.; Poli, A.; Galli, C. Antioxidant and other biological activities of phenols from olives and olive oil. *Medi. Res. Rev.* **2002**, *22*(1), 65−75.

(13) Badioli, M.; Servili, M.; Peretti, G.; Montedoro, G. F. Antioxidant activity of tocopherols and phenolic compounds in virgin olive oil. *J. Am. Oil Chem. Soc.* **1997**, *79*, 1589−1593.

(14) Visioli, F.; Bellomo, G.; Galli, C. Free radical scavenging properties of olive oil polyphenols. *Biochem. Biophys. Res. Commun.* **1998**, *247*(1), 60−64.

(15) Visioli, F.; Caruso, D.; Galli, C.; Viappiani, S.; Galli, G.; Sala, A. Olive oil rich in natural catecholic phenols decrease isoprostane excretion in humans. *Biochem. Biophys. Res. Commun.* **2000**, *278*, 797−799.

(16) Visioli, F.; Caruso, D.; Galli, C.; Plasmati, E.; Viappiani, S.; Hernandez, A.; Colombo, C.; Sala, A. Olive phenol hydroxytyrosol prevents passive smoking-induced oxidative stress. *Circulation* **2000**, *102*, 2169−2171.
(17) Auroma, O. I.; Deiane, M.; Jenner, A.; Halliwell, B.; Kaur, M.; Bnni, S.; Corongiu, F. P.; Dessi, M. A.; Aesbach, R. Effects of hydroxytyrosol found in extra virgin olive oil on oxidative DNA damage and low-density lipoprotein oxidation. *J. Agric. Food Chem.* **1998**, *46*, 5181−5187.
(18) Petroni, A.; Blasevich, M.; Salami, M.; Papini, N.; Montedoro, G. F.; Galli C. Inhibition of platelet aggregation and eicosanoid production by phenolic component of olive oil. *Thromb. Res.* **1995**, *78*, 151−160.
(19) De la Puerta, R.; Ruiz-Gutiérrez, V.; Hoult, J. R. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem. Pharmacol*. **1999**, *57*, 445−449.
(20) Manna, C.; Galletti, P.; Cucciolla, V.; Moltedo, O.; Leone, A.; Zappia, V. The protective effect of olive oil polyphenols (3,4-dihydroxyphenyl)-ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco- cells. *J. Nutr*. **1997**, *127*, 286−292.
(21) Bisignano, A.; Tomaino, A.; Lo Cascio, R.; Crisafi, G.; Uccella, N.; Saija, A. On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. *J. Pharm. Pharmacol.* **1999**, *51*, 971−974.
(22) Salami, M.; Galli, C.; De Angelis, L.; Visioli, F. Formation of F2-isoprostanes in oxydised low-density lipoprotein: inhibitory effect of hydroxytyrosol. *Pharmacol. Res*. **1995**, *31*, 275−279.
(23) Manna, C.; Galletti, P.; Maisto, G.; Cucciolla, V.; D'Angelo, S.; Zappia, V. Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2-cells. *FEBS Lett.* **2000**, *470*, 341−344.
(24) Tuck, K. L.; Tan, H. W.; Hayball, P. J. Synthesis of tritium-labeled hydroxytyrosol, a phenolic compound found in olive oil. *J. Agric. Food Chem*. **2000**, *48*, 4087−4090.
(25) Capasso, R.; Evidente, A.; Avolio, S.; Solla, F. A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural wastewaters. *J. Agric. Food Chem.* **1999**, *47*, 1745−1748.
(26) Bai, C.; Yan, X.; Takenaka, M.; Sekiya, K.; Nagata, T. Determination of synthetic hydroxytyrosol in rat plasma by GC-MS. *J. Agric. Food Chem*. **1998**, *46*, 3998−4001.
(27) Capasso, R.; Evidente, A.; Visca, C.; Gianfreda, L.; Maremonti, M.; Greco, G. Production of glucose and bioactive aglycone by chemical and enzymatic hydrolysis of purified oleuropein from *Olea europaea. Appl. Biochem. Biotechnol.* **1996**, *60*, 365−377.
(28) Espin, J. C.; Soler-Rivas, C.; Cantos, E.; Tomas-Barberan, F. A.; Wichers, H. J. Synthesis of the antioxidant hydroxytyrosol using tyrosinase as biocatalyst. *J. Agric. Food Chem*. **2001**, *49*, 1187−1193.
(29) Capasso, R. The chemistry, biotechnology, and ecotoxicology of the polyphenols naturally occurring in vegetable wastes. *Curr. Top. Phytochem.* **1997**, *1*, 145−156.
(30) Capasso, R.; Evidente, A.; Visca, C. Production of hydroxytyrosol from olive oil vegetation waters. *Agrochimica* **1994**, *38*, 166−171.
(31) Bolanos, F. J.; Rodriguez, G.; Rodriguez, R.; Heredia, A.; Guillen, R.; Jimenz, A. Production in large quantities of highly purified hydroxytyrosol from liquid−solid waste of two-phase olive oil processing or "Alperujo". *J. Agric. Food Chem*. **2002**, *50*, 6804−6811.
(32) De Tomas, B. F. A.; Ferreres, D. A. F.; Espin, D. G. J. C.; Garcia, V. M.; Wichers, H. J.; Soler, R. C. Enzymatic synthesis of antioxidant hydroxytyrosol. Patent No. EP1310562, 2003.
(33) Crea, R. Hydroxytyrosol-rich composition from vegetation water and method of use thereof. Patent No. US2003108651, 2003.
(34) Crea, R. Method of obtaining a hydroxytyrosol-rich composition from vegetation water. Patent No. WO0218310, 2002.
(35) Fernandez-Bolanos, G. J.; Guillen, B. R.; Rodriguez, A. R.; Rodriguez, G. G.; Heredia, M. A.; Jimenez, A. A. Method for obtaining purified hydroxytyrosol from products and byproducts derived from the olive tree. Patent No. WO02064537, 2002.
(36) Akasbi, M.; Shoeman, D. W.; Csallany, A. S. High-performance liquid chromatography of selected phenolic compounds in olive oils. *J. Am. Oil Chem. Soc.* **1993**, *70*(4), 367−370.
(37) Feki, M.; Stambouli, M.; Pareau, D. H.; Ayedi, F. Study of the multicomponent system wet process phosphoric acid-methyl isobutyl ketone at 40 °C phase equilibria and extraction performances. *Chem. Eng. J.* **2001**, *3932*, 1−10.
(38) Capasso, R. A review on the electron ionization and fast atom bombardment mass spectrometry of polyphenols naturally occurring in olive wastes and some of their synthetic derivatives. *Phytochem. Anal.* **1999**, *10*, 299−306.
(39) Ryan, D.; Lawrence, H.; Prenzler, P. D.; Antolovich, M.; Robards, K. Recovery of phenolic compounds from *Olea europaea*. *Anal. Chim. Acta* **2001**, *445*, 67−77.
(40) Tura, D.; Robards, K. Sample handling strategies for the determination of biophenols in food and plants. *J. Chromatog*. **2002**, *975*, 71−93.
(41) Fernandez de Simon, B.; Cadahía, E.; Conde, E.; García-Vallejo, M. C. Low molecular weight phenolic compounds in Spanish oak woods. *J. Agric. Food Chem.* **1996**, *44*, 1507−1511.
(42) Chien-ya, H.; Gow-Chin, Y. Extraction and identification of antioxidative components of Hsian-Tsao (*Mesona procumbens* Hemsl). *Lebensm-Wiss. U.-Technol.* **2001**, *34*, 306−311.
(43) 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*(3), 964−967.
(44) Sheabar, F. Z.; Neeman, I. Separation and concentration of natural antioxidants from the rape of olives. *J. Am. Oil Chem. Soc.* **1988**, *65*, 990−993.
(45) Capasso, R.; Evidente, A.; Scognamiglio, F. A simple thin layer chromatographic method to detect the main polyphenols occurring in olive oil vegetation waters. *Phytochem. Anal.* **1992**, *3*, 270−275.
(46) Casa, R.; D'Annibale, A.; Pieruccetti, F.; Stazi, S. R.; Giovannozzi, S. G.; Lo Cascio, B. Reduction of the phenolic components in olive-mill wastewater by enzymatic treatment and its impact on durum wheat (*Triticum durum* Desf.) germinability. *Chemosphere* **2003**, *50*, 959−966.
(47) Amiot, M. J.; Fleuriet, A.; Macheix, J. J. Importance and evolution of phenolic compounds in olive during growth and maturation. *J. Agric. Food Chem.* **1986**, *34*, 823−830.

Received for review August 21, 2003. Revised manuscript received October 30, 2003. Accepted October 30, 2003. This research was supported by EEC contract ICA3-CT2002-10033 and "Contrats Programs SERST", Tunisia.

JF034944U