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Investigation of Australian Olive Mill Waste for Recovery of Biophenols

HASSAN K. OBIED,† MALCOLM S. ALLEN,‡ DANNY R. BEDGOOD, JR.,† PAUL D. PRENZLER,† AND KEVIN ROBARDS*,†

E.H. Graham Centre for Agricultural Innovation, School of Science and Technology, and School of Wine and Food Sciences, Charles Sturt University, Wagga Wagga, NSW 2678, Australia

Olive mill waste is a potential source for the recovery of phytochemicals with a wide array of biological activities. Phytochemical screening of hexane, methanol, and water extracts revealed a diversity of compounds, perhaps overlooked in previous studies through intensive cleanup procedures. Methanol and water extracts contained large amounts of biophenols, and further testing of polar extraction solvents, including ethyl acetate, ethanol, propanol, acetone, acetonitrile, and water/methanol mixtures, highlighted the latter as the solvent of choice for extraction of the widest array of phenolic compounds. Stabilization of the resulting extract was best achieved by addition of 2% (w/w) sodium metabisulfite. Quantitative data are reported for nine biophenols extracted using 60% (v/v) methanol in water with 2% (w/w) sodium metabisulfite. Six compounds had recoveries of greater than 1 g/kg of freeze-dried waste: hydroxytyrosol glucoside, hydroxytyrosol, tyrosol, verbascoside, and a derivative of oleuropein.

KEYWORDS: Phenols; phytochemical screening; sample pretreatment; hydroxytyrosol; oleuropein; verbascoside; HPLC

INTRODUCTION

Olive oil production is no longer restricted to the traditional production area in the Mediterranean Basin. New producers include Australia, where olive oil production is the fastest growing horticultural industry (*1*). Australians are the largest consumers of olive oil per capita outside the Mediterranean (*1*). For these new producers of olive oil to be able to occupy a position on the global market and support this growing industry, sustainability will be a major issue. Like most other agroindustrial processes, the large volume of processing byproducts is a concern. Olive mill waste (OMW) has been regarded as a hazardous waste with negative impact on the environment and an economic burden on the olive oil industry. This view has changed to one that recognizes the waste as a valuable starting material for the production of bioactive compounds, particularly biophenols (**Figure 1**) for food and pharmaceutical industries. The bioactivity and analysis of OMW biophenols has been reviewed (*2*). OMW is a general term that may be misleading. According to the type of the mill used to process olives, two different byproducts are produced. The three-phase mill uses large volumes of water to aid the separation of oil, and in this case, the waste formed is mainly wastewater, which is known as olive mill wastewater (OMWW), black water, vegetable water, vegetation water, or alpechin. Modern, two-phase mills consume little or no water and in this case the waste is mainly solid in nature and is called alperujo or pomace. This study focuses on two-phase mill waste, as this is the main wastestream from Australian mills. For the sake of consistency, OMW will be used exclusively to denote the waste generated from a two-phase processing system.

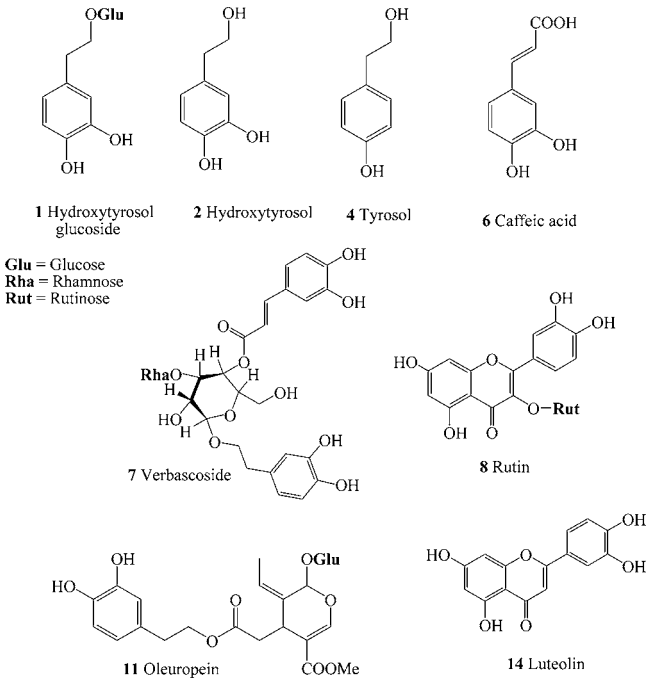

Figure 1. Structure of selected biophenols.

* Corresponding author. Phone: +61-2-6933-2547. Fax +61-2-6933-2737. krobards@csu.edu.au.
† School of Science and Technology.
‡ School of Wine and Food Sciences.

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Variability in the type and amount of biophenols in OMW (*3*, *4*) may be due to geographical, varietal, seasonal, or methodological factors (*5*). Recovery of the biophenols from lyophilized material is a prerequisite to analysis and represents a source of variability (*2*). The choice of the extraction method, extraction solvent, extraction time, and extraction temperature is critical (*6*). The use of different techniques to retard degradation in OMW samples during extraction has been reported. Servili et al. (*7*) used sodium diethyldithiocarbamate to inhibit polyphenol oxidase (PPO) and lipoxygenase. They studied the use of antioxidants and extraction under inert atmosphere on the recovery of phenolic compounds from olive fruit and pomace. The most commonly applied technique to stop enzyme activity is precipitation at pH 3 or less (*3*, *4*, *8*).

This study undertook the phytochemical screening of OMW extracts, followed by a systematic investigation of the recovery, identification, and quantitation of the biophenolic content. Despite general agreement that endogenous enzymes cause a loss of the recovered phenols, there has been no systematic study of factors affecting this process and to find the best practice to thwart their activity. We were interested in finding the most suitable method to inhibit or minimize the degradation of biophenols during extraction. We have examined the effect of air, enzymes, and light on phenol degradation, both during extraction and storage, to develop a strategy to maximize recovery of biophenols.

MATERIALS AND METHODS

Reagents and Standards. Reagents used without further purification were Folin-Ciocalteu reagent, mercuric chloride, and ferric chloride hexahydrate from Sigma-Aldrich (Steinheim, Germany); HPLC-grade methanol and *n*-hexane from Mallinckrodt (Paris, KY); anhydrous acetonitrile from Unichrome (Sydney, Australia); glacial acetic acid, formic acid, lead acetate, hydrochloric acid (32%), sodium carbonate, sodium metabisulfite, sodium chloride, potassium sodium tartarate, and sodium molybdate dihydrate from Univar (Sydney, Australia); aluminum chloride from Aldrich (Milwaukee, WI); chloroform and copper sulfate pentahydrate from Chem-Supply (Adelaide, Australia); acetic anhydride from BDH (Poole, England); and sodium hydroxide, sulfuric acid, formaldehyde, potassium iodide, and absolute ethanol from Biolab (Melbourne, Australia).

Water used in all analytical work was purified by a Modulab Analytical model (Continental Water Systems Corp., Melbourne, Australia) water system.

Phenolic standards used without further purification were catechol, gallic acid, caffeic acid, *p*-coumaric acid, rutin, luteolin, and quercetin dihydrate from Sigma-Aldrich (Steinheim, Germany); tyrosol from Aldrich (Milwaukee, WI); hydroxytyrosol from Cayman (Ann Arbor, MI); oleuropein and cyanidin chloride from Extrasynthese (Genay, France); *p*-hydroxybenzoic acid from Fluka (Switzerland); and verbascoside, generously provided by Dr. Emi Okuyama of Chiba University, Chiba, Japan.

Most standards were dissolved in 80% methanol to prepare stock solutions of 1 mg/mL. The exceptions were quercetin dihydrate and luteolin, which were dissolved in absolute methanol; rutin, which was dissolved in hot 80% methanol; and cyanidin chloride, which required 80% methanol containing 1% HCl for stabilization.

Sampling and Sample Pretreatment. OMW sample from a commercial two-phase olive oil mill (Pieralisi, Italy) was collected from Riverina Olive Grove (Wagga Wagga, NSW, Australia) on May 26, 2003, and was stored under liquid nitrogen without delay. The OMW sample was obtained from Frantoio cultivar, 90% black skin coloration, using a malaxation time of 1 h and temperature of 20 ± 1 °C. Freeze-drying was performed in a Dynavac FD12 (Sydney, Australia) freeze-dryer. Freeze-dried powder was stored in screw capped amber-colored glass containers at −20 °C.

Gross Characterization of OMW. Water content was determined according to the gravimetric method for "articles of botanical origin" using USP 26 procedures (*9*) with 10 g of fresh OMW. Extractable matter (dry matter) was also determined according to USP 26 (*9*) using 2 mL of the extracts. Fat content was determined on freeze-dried powder (10 g) extracted with hexane in a Soxhlet extractor according to the AOAC method (*10*). The pH of an aqueous suspension (25 mL) of the freeze-dried powder (10 g) was determined using a pH meter calibrated at pH 4, 7, and 11.

Phytochemical Screening. Freeze-dried powder (5 g) was extracted sequentially with solvents of increasing polarity, hexane, methanol, and water (50 mL), at ambient temperature for 1 h. Sequential extraction was repeated with fresh freeze-dried sample using a Soxhlet extractor for 30 min. The freeze-dried powder (5 g) was also extracted with aqueous methanol (80% v/v; pH 2, HCl; 50 mL) at room temperature. Extractable matter was determined for each extract following evaporation in vacuo to a constant mass. Similarly, total phenol content of each extract was determined using Folin−Ciocalteu (FC) reagent.

The dried extracts were reconstituted in the same solvent while, for hexane extract, reconstitution was performed in ethanol to overcome the immiscibility of hexane. For both methanol and ethanol, 5 mL was enough to dissolve the dry extracts, but for the residue from the aqueous extract, 10 mL water was necessary. Reconstituted extracts were subjected to phytochemical screening according to the methods of Evans (*11*) and Karumi et al. (*12*).

UV/vis absorption spectra of the extracts were obtained following suitable dilution. Spectra of the material obtained by aqueous methanolic extraction were obtained in neutral, acidic, and alkaline solution by dilution of an aliquot (0.2 mL) of the reconstituted extract to 10 mL with water, hydrochloric acid (2%), or 2 M sodium hydroxide, respectively.

Extraction of Biophenols. Various solvents and extraction procedures were evaluated for recovery of biophenols. Aqueous mixtures of methanol, ethanol, *n*-propanol, acetonitrile, and acetone were investigated for extraction of biophenols using simple extraction, blending with Ultra Turrax (20 s at 11 000 rpm twice), and stirring with a magnetic stirrer. Mixtures with methanol were examined in the range 30−80% v/v in water while the study of other solvents was restricted to 50% v/v in water. Various combinations of extraction time (15 min, 15 min × 2, 15 min × 3, 30 min + 15 min, 45 min) and temperature [5 °C, ambient (i.e. 20 ± 2 °C), 30 °C] were investigated.

For ethyl acetate extraction, the method reported by Lesage-Meessen et al. (*4*) was used with some modification to suit the freeze-dried sample. The freeze-dried sample (5 g) was reconstituted in aqueous ethanol (30% v/v; 50 mL) and the pH of the solution was adjusted to pH 3 using hydrochloric acid (5%). The adjusted extract was filtered using a Buchner filtration apparatus, and a 10-mL aliquot from the red-colored filtrate was extracted with ethyl acetate (15 mL × 3). The ethyl acetate combined fraction (yellow) was dried over anhydrous sodium sulfate and the ethyl acetate was evaporated in a rotary evaporator at 35 °C. The residue was dissolved in methanol (10 mL) and filtered through a nonsterile 0.45 μm plastic syringe filter (Advantec MFS, Japan). The residual aqueous ethanolic fraction (still red in color) was filtered through a GF/F filter paper using a Buchner filtration apparatus and then refiltered through a nonsterile 0.45 μm plastic syringe filter.

Extracts were compared quantitatively by measuring absorbance of total phenols (FC) at 280, 320, 360, and 520 nm and qualitatively by HPLC chromatograms at 280 and 335 nm. Methanol (60% v/v in water) was used as control for comparison purposes.

Optimized Extraction of Biophenols. Freeze-dried OMW (1 g) was extracted for 30 min with methanol:water (60:40 v/v; 5 mL) containing sodium metabisulfite (2% w/w) in a 50-mL stoppered-conical flask. The mixture was stirred continuously using a magnetic stirrer at low speed. The extract was filtered through Whatman No. 1 filter paper. The residue was re-extracted as before with fresh solvent but for only 15 min. The combined extract was defatted with hexane (10 mL × 3) and filtered through GF/F filter paper. The filtrate was refiltered using 0.45 μm plastic nonsterile filters. All extractions were performed at 20 ± 2 °C. The biophenol extract was stored at −20 °C until analyzed.

Stability Tests. All extracts and treatments described under stability studies were prepared according to the optimized extraction procedure.

Spectrophotometric Measurements. Spectrophotometric measurements were performed with a Cary 50 UV/vis spectrophotometer, using Cary WinUV "version 3" software (Varian), on crude extracts after dilution (1:10) with water.

Determination of Total Phenols Using FC Reagent. A modified version of Singleton and Rossi's method (*13*) was developed. The diluted extract (100 μL) was added to a 10-mL volumetric flask containing 6−7 mL water. FC reagent (500 μL) was added and after 1 min, aqueous sodium carbonate solution (20% w/v; 1.5 mL) was added. The flask was shaken and the volume was made up to 10 mL with water. The flask was kept for 1 h at ambient temperature after mixing the contents thoroughly. The absorbance was read at 760 nm. Results are expressed by reference to a six-point regression curve as milligrams of gallic acid equivalents per gram dry weight of freeze-dried material (mg GAE/g).

Determination of o-Diphenols. Determination of *o*-diphenols was performed according to Mateos et al. (*14*). Sodium molybdate dihydrate solution (5% w/v in ethanol 50%; 1.0 mL) was added to 1 mL of the diluted extract in a 10-mL volumetric flask. After mixing, the volume was made up to 10 mL with ethanol 50%. After 15 min the absorbance was measured at 370 nm. Results are expressed by reference to a six-point regression curve as milligrams of caffeic acid equivalents per gram dry weight of freeze-dried material (mg CAE/g).

*Determination of Different Biophenolic Classes***.** The determination of different biophenolic classes was performed according to the method used by Mazza et al. (*15*) originally for wine biophenols. Aqueous ethanol (95% v/v; 1 mL) containing 0.1% hydrochloric acid was added to the dilute extract (1 mL) in a 10-mL volumetric flask, and the volume was made up to 10 mL with 2% hydrochloric acid. The absorbance was measured at 280 nm to determine total biophenols using gallic acid as standard, at 320 nm to determine hydroxycinnamic acid derivatives using caffeic acid as standard, at 360 nm to estimate flavonols using quercetin as standard, and at 520 nm for anthocyanins using cyanidin chloride as standard.

High-Performance Liquid Chromatography. *System 1*. HPLC was performed routinely on a binary LC pump 250 (Perkin-Elmer, Norwalk, CT) equipped with a Perkin-Elmer LC-235 array detector monitoring at 280 and 335 nm. An LC 290 UV/vis spectrophotometric detector (Perkin-Elmer) was connected in series when monitoring the absorbance in the visible range. The system was maintained in a controlled temperature room 21 ± 1 °C. A flow rate of 1 mL/min and an injection volume of 20 μL were used. Separation was performed by gradient elution on a 150 mm × 4.6 mm i.d., 5 μm, Luna C-18(2) column (Phenomenex, Pennant Hills, Australia) attached to a SecurityGuard guard cartridge (Phenomenex). The HPLC system was controlled using a Varian Star chromatography workstation (version 6.2). The mobile phases were freshly prepared and degassed under vacuum using Phenomenex nylon 45 μm membranes and sonicated in a Sanophon ultrasonic bath (Ultrasonic Industries Pty. Ltd., Sydney, Australia) for 15 min prior to HPLC analysis.

Solvent A was a mixture of 100:1 water/acetic acid (v/v), and solvent B was a mixture of 90:10:1 methanol/acetonitrile/acetic acid (v/v/v). A six-step linear gradient analysis for a total run time of 60 min was used as follows: Starting from 90% solvent A and 10% solvent B, increase to 30% solvent B over 10 min and then isocratic for 5 min, increase to 40% solvent B over 10 min, to 50% over 15 min and to 100% solvent B over 10 min, and finally isocratic for 10 min. The system was equilibrated between runs for 20 min using the starting mobile phase composition.

System 2. HPLC was performed with a Varian 9021 solvent delivery system equipped with Varian a 9065 Polychrom UV diode array detector (190−367 nm). Software used for data processing was Star Polychrom version 5.2. Other conditions were the same as for system 1 except the between-run equilibration time, which was 10 min.

Data Treatment. All experiments were performed in triplicate. Data are expressed as means ± standard deviations. Data analysis was performed by Microsoft Excel. One-way ANOVA was carried out to test for significant differences using SPSS 11.5 (SPSS Inc., Chicago, IL). Results were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Frantoio was chosen for investigation as it is one of the most popular olive cultivars in Australia (*1*). Its OMW samples were collected in the middle of the olive oil processing season. The sample comprised the solid byproducts (pomace) from a two-phase continuous system. The high water content, 74.8%, is consistent with literature values for Spanish OMW (*16*, *17*). The pH of the OMW was 5.2 in aqueous solution (10 g/25 mL), similar to the values reported for Spanish OMW (pH 4.86−6.45) (*17*) and French OMW (pH 5.7) (*4*). The fat content at 16% (based on freeze-dried weight) is double the values reported by Fernandez-Bolanos et al. (*16*) and toward the upper end of the fat content range reported by Alburquerque et al. (7.8−19.5%) (*17*). This may reflect varietal or processing differences, but analytical effects cannot be eliminated as hexane was used for the determination instead of petroleum ether due to its superior defatting efficiency (*18*).

Flash-freezing and freeze-drying of OMW were used to minimize production of artifacts, avoid degradation of biophenols, and ensure long-term availability of a stable reproducible sample. Prolonged freeze-drying of the waste (1 kg, 2 weeks) was required by its high water content and the occurrence of a water−oil emulsion (*2*); it provided a fluffy, hygroscopic, brownish-yellow colored powder. Lyophilized plant powder should be stored under strictly dry conditions due to its hygroscopic nature. Airtight screw capped glass containers stored at −20 °C demonstrated good stability of the dried powder for more than 1 year. Exposure of the sample to the surroundings during processing was minimized to avoid absorption of atmospheric moisture, which gave reduced recovery of biophenols and a darker colored powder. Storage of an opened container in a desiccator at −20 °C was found to be suitable, provided that the sample was used within 1 week.

Phytochemical Screening of OMW Extracts. Phytochemical screening, when combined with sequential extraction with solvents of different polarities, provides an overview of major classes of phytochemicals present and likely interferences in analytical and biological assays. Results of screening tests were identical for extracts obtained at room temperature and by Soxhlet extraction. Phytochemical screening provided no indication of the occurrence of alkaloids, catechins, anthraquinones, cardiac glycosides, or saponins in the extracts.

Screening of hexane extracts showed no positives except for sterols/triterpenes. The presence of chlorophyll and carotenes accounts for the yellowish green color of the hexane extracts, and spectra exhibited a broad band with two small sharp peaks at 430 and 665 nm typical of chlorophyll *a* (*19*), with a further maximum at 470 nm indicative of carotenes. The presence of biophenols in aqueous, methanolic, and aqueous methanolic extracts is supported by a positive reaction with iron(III) chloride and a positive Shinoda's test for the methanolic extract, indicating flavonoid aglycons. Carbohydrates were detected in the aqueous extracts by Molisch's test, in agreement with the identification and quantification of water-soluble carbohydrates in OMW (*16*, *17*). Polyuronides (mucilage) were also detected.

Methanolic extracts were green due to chlorophyll content, with a characteristic absorption maximum at 665 nm. The browning of the aqueous extract during extraction is suggestive of oxidation and/or polymerization reactions. Acidification of the aqueous extracts produced a bright red color (525 nm maximum), which due to the extraction conditions and the stability of the red color (>4 months) is not consistent with anthocyanins. Further evidence that the pigment is not anthocyanin-based is that addition of aluminum chloride did not

Table 1. Effect of Solvent and Extraction Conditions on Extractable Matter and Total Phenols in OMW

	extract						
fraction	methanol/ H_2O/HCl	hexane (Soxhlet)	methanol (Soxhlet)	water (Soxhlet)	hexane (ambient temp)	methanol (ambient temp)	water (ambient temp)
extractable matter %[a]	26.2 ± 1.3	13.0 ± 0.6	8.6 ± 0.1	5.0 ± 0.6	15.7 ± 0.6	13.1 ± 1.3	10.4 ± 0.9
total phenols (% GAE w/w)	1.47 ± 0.01	*b*	0.33 ± 0.01	0.25 ± 0.06	*c*	0.37 ± 0.06	0.45 ± 0.02

[a] Weight per dry weight. [b] Blue color (positive reaction) was detected but could not be measured due to the turbidity caused by water-insoluble constituents. [c] Colorless (negative reaction) and turbid solution caused by water-insoluble constituents.

produce the expected bathochromic shift of cyanidins, gradual addition of sodium hydroxide did not give a blue color, and addition of sodium metabisulfite to the acidified solution caused minimal bleaching (*20*). Ryan et al. (*21*) suggested that the red/brown color of black and purple olive fruit extract developed upon acidification may be due to phlobaphenes, which result from acid-catalyzed condensation of leucoanthocyanins. Phlobaphenes are essentially high molecular weight water-insoluble reddish-brown plant pigments. As the red color was only detected in aqueous extracts and not extractable into organic solvents (hexane or ethyl acetate), this suggestion is unlikely. Pigments from OMW have been identified as catechol−melanin macromolecules that result from polymerization of phenolics linked to sugars, proteins, and fatty acids with molecular masses between 50 and 200 kDa (*22*, *23*). These pigments are believed to be one of the principal reasons for the high recalcitrance of OMW toward biodegradation (*23*).

The 280-nm absorption peak common to all biophenols was detected in both methanolic and aqueous extracts. This peak underwent a hypsochromic shift to 270 nm on alkalinization with a doubling of absorbance while, on acidification, absorbance increased by only 30%. The change in absorbance with pH indicates that care must be taken when using absorbance at 280 nm to quantify biophenols. A peak at 335 nm was also present in these extracts and may be due to flavonoids (flavones) and/or hydroxycinnamic acid derivatives (caffeoyl esters). However, a bathochromic shift of +15 nm in alkaline solution for this peak suggests that it was mainly due to hydroxycinnamic esters, not flavones, as the latter show a large bathochromic shift of +45 to +65 nm in alkaline solutions. A small peak at 520−525 nm was only detected under acidic conditions, suggesting the possible presence of cyanic compounds.

Recovery of Biophenols. Several different extraction solvents are useful for the recovery of biophenols from OMW and olive fruits (*2*). Our goal was to achieve the highest possible recovery of phenols (qualitative/quantitative) as measured by total phenols and absorbance at 280, 320, 360, and 520 nm with the optimum response from the HPLC detector using 280 and 335 nm for detection. These wavelengths represent a suitable compromise for detection of biophenols.

In our preliminary study of biophenolic recovery, hydroalcoholic extraction had the highest recovery of extractable matter at 26.2% w/w (**Table 1**). The hexane extract comprised mainly residual olive oil, according to our findings in fat determination. The recovery by hydroalcoholic extraction was equal to or more than the sum of the extractable matter from methanolic or aqueous extracts alone. Total phenols measured by the FC method stress the superiority of hydroalcoholic extraction, with a recovery of 1.47% gallic acid equivalents (GAE) w/w, which is significantly higher than that achieved with methanolic or aqueous extraction. The recovery of biophenols (FC method) from Australian OMW was comparable to that reported for Spanish OMW (*17*, *24*). Hexane extracts obtained at ambient temperature were phenol-free, confirming the suitability of defatting OMW with hexane as a cleanup procedure.

Poor recoveries of some phenols have been encountered with ethyl acetate (*8*), but its use is reported frequently in the extraction of biophenols from aqueous matrixes such as OMWW (*4*, *22*, *25*). Ethyl acetate is selective for small and medium molecular weight phenols (*26*), allowing extraction and cleanup simultaneously, facilitating extraction of simple phenols (*4*) and sequential extraction of phenolic fractions (*22*, *26*). In the present study, ethyl acetate recovery of biophenols from an ethanolic extract of the freeze-dried powder was not encouraging. From **Table 2**, the ethyl acetate fraction contained only 43% or 45% of the total phenols (280 nm or FC method, respectively) in the aqueous ethanol extract. However, ethyl acetate demonstrated high selectivity for phenols in that 50% of the extractable matter comprised phenols, while the original ethanol extract contained only 10% phenols. Recovery of specific classes of biophenols from the ethanolic extract was correspondingly low at 49% of the hydroxycinnamic acids (absorbance at 320 nm) and 45% of flavonoids (absorbance at 360 nm), and pigment compounds (absorbance at 520 nm) were below quantitation limit. Generally, spectroscopic measurements showed that the recovery of biophenols was always less than 50% and almost null for olive pigments. HPLC chromatograms generated at 280 nm gave additional insight to the composition of the ethyl acetate and the residual aqueous ethanolic (30%) fractions compared with the original aqueous ethanolic (30%) extract. Ethyl acetate successfully removed the less polar compounds eluting after 30 min and 60−80% of compounds such as hydroxytyrosol (80%), verbascoside (75%), and oleuropein derivative (70%). On the other hand, ethyl acetate recovered no hydroxytyrosol glucoside and none of the highly polar compounds eluting before hydroxytyrosol glucoside. The cleanup effect of ethyl acetate was evident as the broad peaks typically observed in reversed-phase separations of biophenols (P1 and P2 in **Figure 2**) were much reduced in the ethyl acetate fraction (results not shown).

A more detailed investigation of solvents (**Table 2**) confirmed the suitability of aqueous methanolic extractants. Although aqueous acetonitrile, acetone, ethanol, and propanol recovered more phenols (FC), their selectivity was much lower than aqueous methanol, as indicated by higher recovery of extractable matter. Nevertheless, other factors must be considered in solvent choice. For the hydroalcoholic extractants, increasing the molecular weight of the alcohol increased the lipophilicity of the extraction solvent and hence the recovery of the more lipophilic biophenols, as seen from an increased size of late-eluting peaks in HPLC chromatograms of aqueous ethanolic and aqueous propanolic extracts (results not shown). Moreover, ethanol and propanol tended to dissolve more oil, necessitating more intense defatting of the extract. Defatting of ethanol extracts was troublesome due to emulsion formation. The use of propanol has been previously reported (*22*). Acetonitrile has not been reported as an extraction solvent for OMW or OMWW,

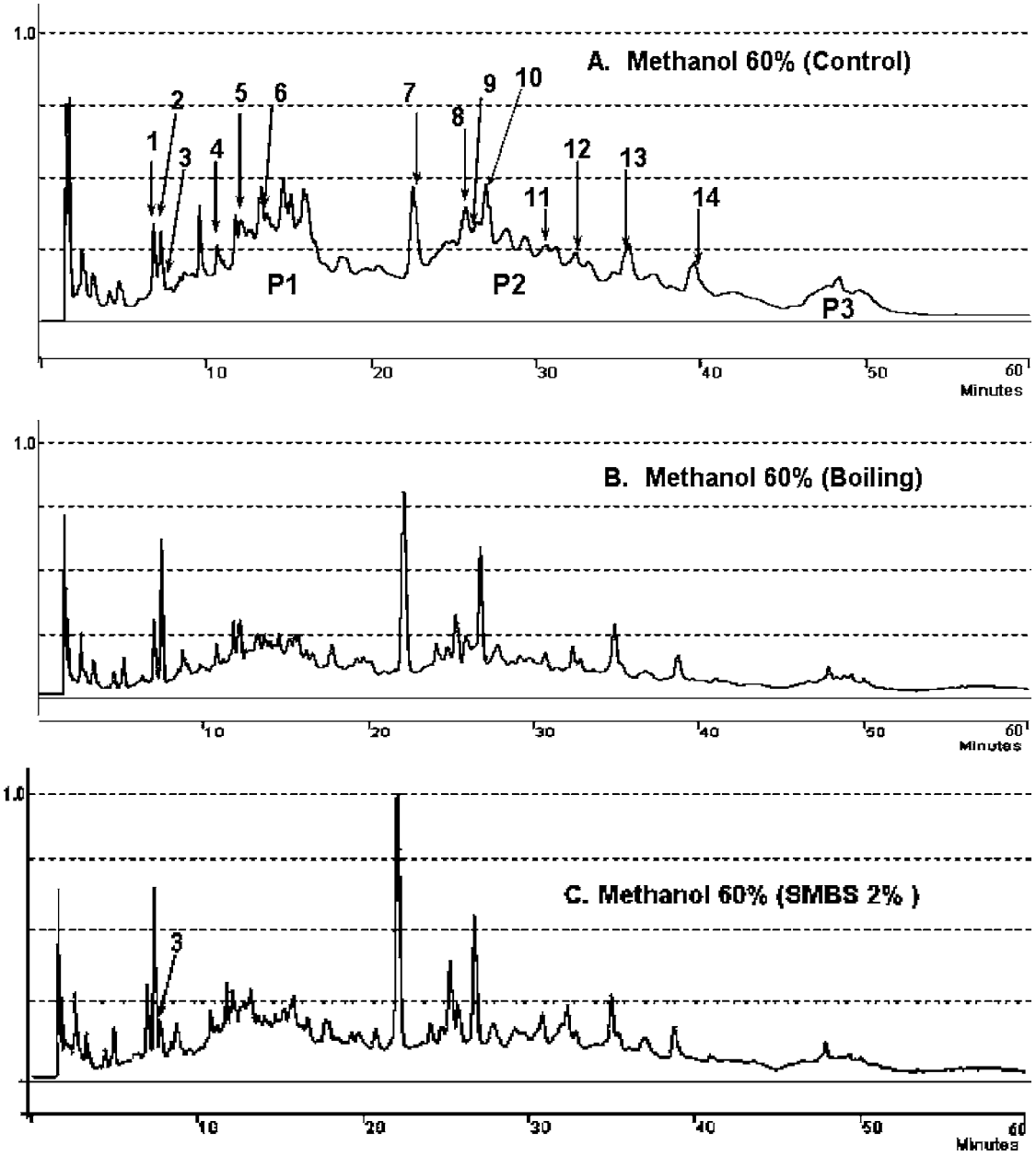

Figure 2. Representative RP-HPLC chromatograms of OMW biophenolic extracts at 280 nm showing effect of extraction conditions on the recovery. **1**, hydroxytyrosol glucoside; **2**, hydroxytyrosol; **3**, unknown; **4**, tyrosol; **5**, flavonoidal glycoside; **6**, caffeic acid; **7**, verbascoside; **8**, rutin; **9**, verbascoside isomer; **10**, oleuropein derivative; **11**, oleuropein; **12**, oleuropein isomer; **13**, unknown; **14**, luteolin; and P1, P2, P3, polymeric substances. Chromatograms were generated using HPLC system 1.

though it is very frequently used as a constituent of HPLC mobile phases for biophenols. Although the highest recoveries were reported for aqueous propanol and acetonitrile, their low selectivity and low volatility were a disincentive for their use in screening and other experiments. The high UV cutoff of acetone at 330 nm interfered with spectrophotometric measurement at 280 nm. Furthermore, as aqueous acetone provided lower total phenols (FC) than the optimized solvent, and the other spectroscopic measures apart from absorbance at 280 nm were not exceptionally high, aqueous acetone was not favored. Although Ceccon et al. (*8*) utilized acetone in a final concentration of 50% to precipitate colloids, they evaporated the solvent under vacuum and the final measurements were performed on a methanolic solution. On the basis of these considerations, the most suitable solvent for recovery of biophenols was aqueous methanol.

Different combinations of methanol and water were examined, ranging from 30% to 80% methanol (v/v). With methanol concentrations of 50% or less, the extract was colloidal and the filtration process was long and incomplete. The highest total recoveries were achieved with 60%−80% methanol. However, 60% methanol was better than 80% methanol at maintaining a balance between recovery of the polar, early-eluting compounds and the less polar, later-eluting compounds, in the HPLC chromatograms.

In optimizing extraction conditions, it was observed that faster extraction improved the recovery of biophenols. Extraction time and temperature are important variables in any extraction procedure, but in the case of reactive labile compounds such as biophenols, they become critical. There is a compromise between rapid extraction to minimize possible reactions such as oxidation and allowing sufficient time for complete wetting

Table 2. Comparison of the Extraction Efficiency of Various Solvents for the Recovery of OMW Biophenols[a]

extraction solvent	extractable matter (mg/g)	total phenols (mg GAE/g)	*o*-diphenols (mg CAE/g)	TP (280 nm) mg GAE/g	hydroxycinnamic acids (320 nm) mg CAE/g	flavonols (360 nm) mg QE/g	pigments (520 nm) mg CCE/g
control[b]	259.5 ± 17.5a	28.1 ± 0.6a	14.1 ± 0.2a	15.39 ± 0.36a	5.02 ± 0.10a	6.47 ± 0.03a	0.85 ± 0.01a
aqueous ethanol (30% v/v)	205.8 ± 40.6b	20.3 ± 1.0b	9.2 ± 0.1b	10.39 ± 0.10b	3.26 ± 0.05b	3.59 ± 0.04b	0.46 ± 0.02b
ethanol 30% residual fraction	187.0 ± 7.5b	14.7 ± 1.5c	7.8 ± 0.1c	9.17 ± 0.22c	2.4 5 ± 0.02c	2.98 ± 0.09c	0.56 ± 0.02c
ethyl acetate fraction	20.0 ± 3.5c	9.1 ± 1.2d	4.1 ± 0.1d	4.44 ± 0.07d	1.59 ± 0.15d	1.62 ± 0.03d	–[e]
OES*c*	275.7 ± 0.7a[d]	37.0 ± 0.3e	16.0 ± 0.3e	15.57 ± 0.09a	5.98 ± 0.02e	6.47 ± 0.03e	0.75 ± 0.02d
aqueous ethanol (50% v/v)	335.0 ± 2.1d	32.9 ± 0.9f	16.2 ± 0.2e	18.76 ± 0.51e	6.03 ± 0.15e	6.58 ± 0.16e	1.00 ± 0.02e
aqueous propanol (50% v/v)	447.6 ± 12.6e	42.6 ± 1.1g	22.2 ± 0.3f	26.50 ± 0.53f	8.22 ± 0.18f	8.85 ± 0.19f	1.32 ± 0.04f
aqueous acetonitrile (50% v/v)	410.4 ± 15.5e	41.4 ± 1.3g	22.8 ± 0.3g	26.42 ± 0.62f	7.88 ± 0.18g	8.58 ± 0.17g	1.14 ± 0.03g
aqueous acetone (50% v/v)	351.2 ± 6.2d	35.2 ± 0.8h	19.4 ± 0.2h	28.23 ± 0.32g	6.86 ± 0.07h	7.61 ± 0.12e	0.96 ± 0.01h

[a] Results are mean ± standard deviation from triplicate analyses. Values in each column having different letters (a–h) are significantly different from one another at $p < 0.05$. [b] Aqueous methanol (60% v/v). [c] OES optimized extracting solvent (60% methanol, sodium metabisulfite 2% w/w). [d] Corrected for the weight of sodium metabisulfite. [e] Below limit of quantitation.

of the powder. Investigation of time and temperature produced the optimum extraction conditions of 20 ± 2 °C with sequential extraction (30 min + 15 min).

The effect of vortexing and magnetic stirring on recovery of biophenols was investigated. Although neither method improved recovery, stirring did improve reproducibility. However, stirring at high rates oxidized caffeic acid standard, as demonstrated by a decrease in the peak area in chromatograms at 280 nm. Lowering the extraction temperature to 5 °C decreased the recovery of total phenols (FC) and absorptions at all measured wavelengths. Increasing the temperature to 30 °C did not increase the total phenols (FC) significantly, but it did increase absorptions at all measured wavelengths. It may be that increasing the temperature enhanced the solubility of biophenols; on the other hand, it also enhanced the rate of the oxidative reactions of the recovered biophenols. Extraction at ambient temperature offered a compromise between recovery of total phenols and marked degradation.

Characterization of the Biophenolic Extract. Further characterization of the extracts was performed by reversed-phase (RP)-HPLC. Various mobile phase gradients were examined. The gradient devised by Lesage-Meessen (*4*), which provided good resolution of simple phenols extractable from OMW with ethyl acetate, was unsuitable for the OMW hydroalcoholic extract in this study. The most suitable gradient for these extracts involved aqueous methanol and acetonitrile with 1% acetic acid. Decreasing the ratio of acetic acid in the mobile phase from 1:100 to 0.1:100 increased the solvent pH from 3.0 to 3.3, but peak resolution deteriorated and the retention time for most peaks increased by up to 1 min (data not shown). The effect of mobile phase acid concentration on retention time can be demonstrated with data for gallic acid with retention times of 4.67, 5.36, and 18.32 min for mobile phase gradients in which the acetic acid content was 1:100, 0.1:100 and 0.0, respectively. When formic acid replaced acetic acid, the retention time for gallic acid increased to 5.71 min in the mobile phase at pH 3. This indicates that the polarity of the acid (*27*) is important in determining the retention time, not just the mobile phase pH. Compounds 1−3 (**Figure 2C**) were only resolved using acetic acid at 1:100.

Retention times for selected standards (gallic acid, hydroxytyrosol, caffeic acid, rutin, and oleuropein) and principal peaks in the sample chromatograms showed excellent reproducibility with a coefficient of variation (CV) of less than 3.3% over several months. Peak area data demonstrated good reproducibility (CV = 2.8%) for the standards, and for sample peaks CV was 20% over the short term of days to weeks, decreasing to 30% over months (CV = 30%).

Three-dimensional plotting of the diode array data (not shown) plus the chromatograms generated at single wavelengths stressed the complexity of the OMW extracts and the difficulty of representing the huge number of compounds at a single wavelength (**Figure 3**). Nevertheless, detection at 280 nm represented a good compromise at which most biophenols could be detected with reasonable intensity and with an acceptable background level. Detection at 234 nm showed high sensitivity compared with 254 nm, but interference from the matrix was strong. Detection at 334 nm was promising for quantitation of compounds absorbing at this wavelength, such as verbascoside, as the background interference was minimal. Two small peaks coelute with hydroxytyrosol and all three compounds absorb at 280 nm, and may result in overestimation of hydroxytyrosol concentration. However, these compounds can be measured independently at 334 nm, where hydroxytyrosol does not absorb. In accord with our phytochemical screening, no major peaks were detected at 520 nm. A very wide band of low intensity extended over the whole chromatogram, suggesting the polymeric nature of OMW pigment.

Identification was performed by comparing retention times and UV spectra of standards by HPLC-DAD using chromatographic system 2 (**Figure 2A**). The common olive biophenols, hydroxytyrosol (**2**), tyrosol (**4**), caffeic acid (**6**), verbascoside (**7**), rutin (**8**), oleuropein (**11**), and luteolin (**14**), were identified using available standards (**Table 3**). When there was no available standard, as with hydroxytyrosol glucoside (**1**), verbascoside isomer (**9**), and oleuropein isomer (**12**), identification was based on their UV spectra and comparison of the relative retention time with literature data (*5*, *28*). A number of frequently reported biophenols in OMWW were not detected in the current sample, e.g. gallic acid, catechol, *p*-coumaric acid, and *p*-hydroxybenzoic acid. However, there is a good agreement between our chromatograms and those produced by Romero et al. (*5*) for OMWW and pomace. Hydroxytyrosol glucoside was identified as a discrete peak eluting immediately before hydroxytyrosol. Romero et al. (*5*) found that resolution of this peak from hydroxytyrosol depended on the analytical column used. In the current study, hydroxytyrosol glucoside was not extracted in the ethyl acetate fraction, in accord with the findings of Romero et al.

The quantitation of such complicated chromatograms is a challenge. In addition to the biological variability of the OMW sample, integration of some peaks could only be achieved manually. The results shown in **Table 3** reflect the large variability of some peaks. Verbascoside was the major peak at 280 nm and the second most abundant biophenol, while the unidentified oleuropein derivative (**10**) was the most abundant

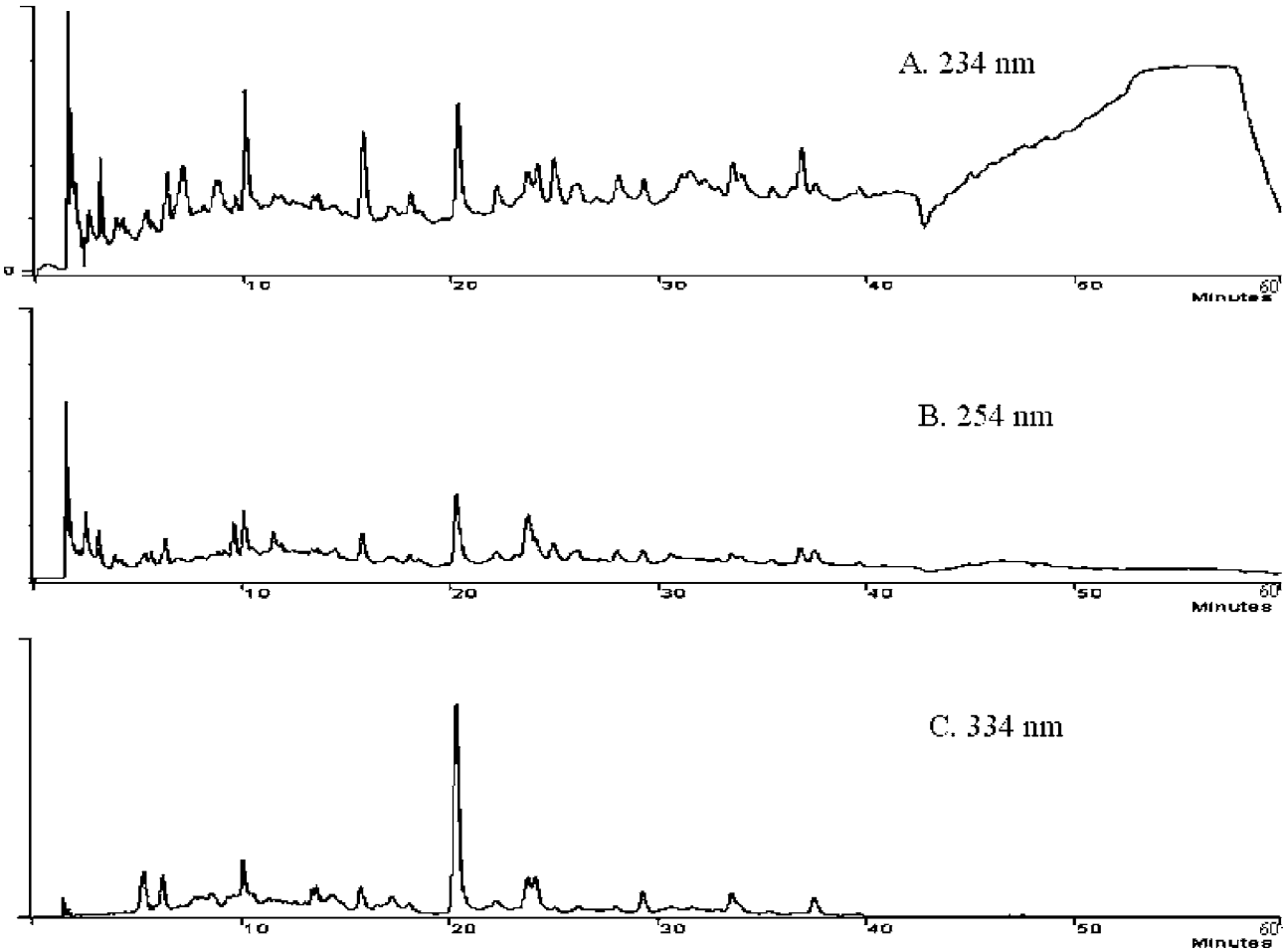

Figure 3. Representative RP-HPLC chromatograms of OMW biophenolic extracts generated at different wavelengths: **A**, 234 nm; **B**, 254 nm; and **C**, 334 nm. Chromatograms were generated using HPLC system 2.

Table 3. Identification and Amounts of the Principal Compounds in OMW Extract

peak	identity	retention time (min)	λ_{max} (nm) standard	λ_{max} (nm) sample	amount (mg/kg of freeze-dried waste)[a]
1	hydroxytyrosol glucoside	5.71		**217.4**,[b] 273.0	1522 ± 246[c]
2	hydroxytyrosol	6.36	**220**, 276.8	**218.3**, 277.2	1258 ± 29
3	unknown	6.78		230.9, **289.8**	not quantified
4	tyrosol	9.18	**218.5**, 272.7	**218.4**, 272.5	1373 ± 191
5	flavonoidal glycoside	10.06		**228.0**, 353.1	not quantified
6	caffeic acid	12.02	216.4, 235.2, **321.2**, 290 nm (s)[d]	**322.9**[e]	not quantified
7	verbascoside	20.27	217.9, **328.5**, 293 (s)	217.5, **328.3**, 290 (s)	1689 ± 73
8	rutin	23.72	216.8, **253.3**, 352.8	220.9, **251.7**, 352.6	537 ± 100
9	verbascoside isomer	24.11		**228.3**, 331.3, 290 (s)	not quantified
10	oleuropein derivative	24.89		**219.0**, 277.7	6526 ± 1093[f]
11	oleuropein	28.62	**228.6**, 277.7	**226.0**, 276.8	489 ± 73
12	oleuropein isomer (oleuroside)	30.57		219.1, 273.1	330 ± 95
13	unknown	33.48		226.1, **309.2**	not quantified
14	luteolin	37.45	250.3, **347.2**	252.9, **348.2**	393 ± 120

[a] Results are mean ± standard deviation from triplicate analyses; standards used are hydroxytyrosol, tyrosol, verbascoside, oleuropein, rutin and luteolin. [b] Peaks in bold are the major ones in the spectrum. [c] Quantified using hydroxytyrosol with correction based on molecular weight. [d] (s), shoulder. [e] Coeluting with a compound with maxima at 215, 282. [f] Calculated as oleuropein equivalent.

phenolic compound in the OMW extract. Hydroxytyrosol was an abundant constituent of our sample, but it was not the major component, as previously reported for Tunisian OMWW (*29*) and for French OMW (*4*). Furthermore, oleuropein and caffeic acid were not among the principal peaks in the current study. The amount of oleuropein was relatively small and caffeic acid was not quantified, as it coeluted with an unidentified compound.

Stability of Biophenol Extract. During the extraction process, the extract underwent rapid darkening from dark red to blackish brown, and the solid raffinate was also dark brown. OMWW was also reported to change color from dark violet to brown (black water) due to the complete disappearance of the anthocyanins and the formation of the brown catechol−melanin polymer through a process of oxidation and polymerization of the phenolics, specially *o*-diphenols (*22*). A direct correlation has been reported between PPO activity and the browning of olive fruit crude homogenate (*30*). Nevertheless, browning (oxidative polymerization) can be an enzymatic or nonenzymatic

process. Fernández-Bolaños et al. (*31*) suggested a condensation reaction between phenolic and nonphenolic constituents as the main cause of the reduction in the recovery of biophenols from OMW instead of the widely accepted oxidative degradation. Thus, OMW represents a complex matrix in which oxidation (enzymatic and/or nonenzymatic), condensation, polymerization, and enzymatic processes (e.g. hydrolysis) can take place simultaneously.

Sample pretreatment ideally halts the action of the different degrading enzymes through flash freezing in liquid nitrogen and subsequent freeze-drying. However, lyophilization of plant materials preserves the enzymes, which resume their activity upon wetting. The enzymatic reaction may be more favored under these conditions, where the enzymes and the substrates are in close contact. Freeze-drying and storage at −20 °C were suitable for the storage of OMW for 12 months. However, this did not stop the degradation of biophenols, since after 2 years of storage, dark brownish black extracts with complete loss of some labile biophenols and reduced recovery of others were noticed. The browning (degradation) reaction was observed in our 60% methanolic extract.

Seven sample treatments were devised to assess the effect of various extraction conditions on the recovery of OMW biophenols and the inhibition of degradation during extraction. The efficacy of these treatments was assessed against a control comprising extraction with aqueous methanol (60% v/v) at ambient conditions (**Figure 2A**). Both gross spectrophotometric measurements and visual inspection of the chromatograms generated at 280 nm were used to compare different treatments and the control.

Gross spectrophotometric measures of biophenol recovery showed good reproducibility with CVs less than 10% for the same container within three successive days and less than 20% from different containers on different occasions. Although the chromatographic results gave a more accurate and detailed view, some restrictions were still encountered. Secoiridoids did not absorb at 280 nm and hence they were not included in the study. As a result of the intentionally limited cleanup in the method applied here, the chromatograms acquired at 280 nm are complex, showing more than 65 peaks. With this very large number of compounds, resolution was seriously affected and some adjacent peaks may appear as one broad peak or a number of separated peaks in different runs. Many of these peaks were minor constituents (ca. 50% of the total peak area was produced by only 15 peaks). Polymeric compounds could be observed as the three very broad humps (P1, P2, and P3, **Figure 2A**) that caused a shift in the chromatogram baseline with subsequent low resolution and overestimation of the coeluting constituents. Manual integration eliminated background interference by these polymeric species.

Increasing or decreasing extraction temperature should decrease the enzymatic degradation of biophenols, if the temperatures are outside the range for maximal enzyme activity. Of the seven treatments, the lowest recoveries were obtained with extraction at subzero temperature (−10 °C). A marked decrease (>20% relative to control) in the recoveries of total and all subclasses of biophenols as measured by spectrophotometric parameters was observed (data not shown). The decrease in temperature should slow most degradation reactions, but the solubility was evidently reduced as well. Alternatively, Harborne (*19*) recommended plunging samples into boiling alcohol to stop enzymatic oxidation or hydrolysis of fresh plant material. Extraction with boiling aqueous methanol (60%) had slightly reduced total phenols, pigments, and *o*-diphenols content, possibly due to the thermal instability of some biophenols. In contrast, HPLC chromatograms (280 nm) of the two extracts showed an improved response for most major phenols relative to the control (e.g. **Figure 2B**).

Oxygen is required for both enzymatic and nonenzymatic oxidation; it is a cosubstrate for PPO (*32*). Extraction under nitrogen using prepurged (15 min) solvents improved the recovery of the flavone content (absorbance at 360 nm) with some increases in peak response in HPLC chromatograms. Extraction in the dark markedly improved the recovery of the OMW pigment, suggesting that these compounds are photosensitive. On the other hand, total phenol content (FC) was decreased, and this is also seen in chromatograms. This suggests that the OMW pigment is not a potent reducing agent to FC reagent and the products of photodegradation may be more reducing to FC than their precursors.

The addition of sodium diethyldithiocarbamate at 100 and 1000 ppm to aqueous methanol showed concentration-dependent changes in total phenols and *o*-diphenols with some increases in peak response in HPLC chromatograms. The data are consistent with the action of sodium diethyldithiocarbamate as a noncompetitive PPO inhibitor (*7*). Nevertheless, the most effective way to control PPO activity is the use of antioxidants or reducing agents (*32*). Their antioxidant activity relies on their ability to reduce benzoquinones to *o*-dihydroxyphenols. Ascorbates, bisulfites, and thiols have a direct inhibitory activity against PPO (*32*). Sodium metabisulfite has a wide array of activities, including potent competitive PPO inhibition and antimicrobial activity that support its use in the extraction of biophenols. Its addition to the extracting solvent increased the FC total phenols markedly with a significant increase in other spectrophotometric measurements apart from the absorbance at 520 nm (OMW pigment). It seems that the pigment substances may still have some cyanic constituents that were bleached by sodium metabisulfite. Different concentrations of sodium metabisulfite were tested (results not shown) and the optimum concentration was 2% (w/w) (**Figure 2C**). Higher concentrations of sodium metabisulfite showed pro-oxidant activity (reduced recovery of biophenols) and interfered with the FC assay. Similar enhancements in recoveries were observed with the addition of formic acid to bring the extract to pH 2.0. This is understandable in terms of the known oxidation pathways of biophenols, in which the anionic form of phenolate is more vulnerable to oxidation than the neutral form, and the precipitation of enzymes that occurs at low pH values. The reduced recovery of pigment compounds may be attributed to conversion of the salt form to the less water-soluble acidic form or the decreased conjugation of the phenolic chromophore.

Extraction with inclusion of sodium diethyldithiocarbamate in the dark and under nitrogen reduced P1, P2, and P3 but to a lesser extent than cooling, heating, and addition of sodium metabisulfite. Addition of formic acid was not very effective in reducing the humps. A similar pattern (P1, P2, and P3) was reported by Romero et al. (*5*) for OMWW that was directly injected into the chromatograph after 0.45 μm filtration. However, addition of sodium diethyldithiocarbamate, cooling, and cleanup by SPE reduced the recovery of such polymeric compounds from pomace (*5*), while these humps were almost absent in the olive pulp chromatograms.

Both the control and extraction in dark showed a greater peak area for hydroxytyrosol glucoside (**1**) relative to hydroxytyrosol (**2**), contrary to all other treatments. It appears that an enzymatic degradation mechanism that consumes hydroxytyrosol more readily than its glucoside and does not depend on light is

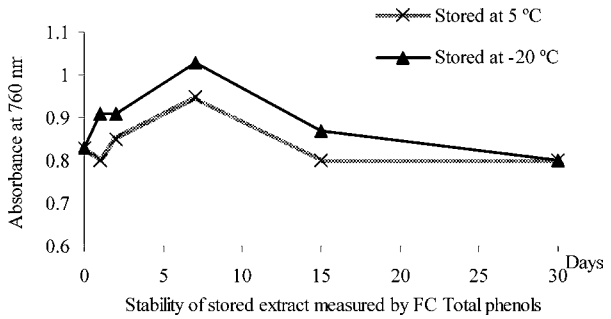

Figure 4. Stability of the stored extract measured by FC Total phenols.

operative in which case glycosidation of hydroxytyrosol acts as a protection mechanism. This is consistent with the lower antioxidant activity of flavonoid glycosides relative to the corresponding aglycons (*33*). The high amount of hydroxytyrosol in the pH 2 extract may be due to acid hydrolysis of the glucoside.

The recovery of verbascoside (**7**) was enhanced markedly by all treatments compared to the control. The higher recovery with boiling alcohol (190%) than cooling (150%) suggests thermal stability. The recovery of verbascoside under nitrogen was comparable to the results with boiling alcohol. The presence of oxygen seems to be essential in the degradation of verbascoside. The highest recovery for verbascoside was achieved with addition of formic acid, suggesting good stability of verbascoside toward acid hydrolysis. The absence of light and the use of sodium diethyldithiocarbamate did not prove to be effective in protecting verbascoside from degradation.

The oleuropein derivative (**10**) was the major biophenol in the OMW extracts, and its recovery showed a pattern similar to that of verbascoside. The highest recoveries were achieved with addition of sodium metabisulfite (240%) and formic acid (340%). The highest recovery for luteolin (**14**) was under nitrogen, and this supports the high readings in the spectrophotometric test at 360 nm. In general, the recoveries of biophenols (peak area) with boiling methanol were higher than subzero and ambient extraction (control), and this suggests the reasonable thermal stability of OMW biophenols. The highest recoveries were observed with formic acid and sodium metabisulfite, in line with the findings from spectrophotometric measurements. Unknown compound **3** was recovered with the addition of sodium metabisulfite. Thus, where profiling and qualitative analysis are more critical than absolute yield, sodium metabisulfite is probably the preferred treatment to stabilize the OMW during extraction. Caution is however needed as the strong negative charge of sodium metabisulfite makes it reactive toward nucleophilic addition reactions such as bleaching of anthocyanins and precipitation of aldehydic compounds as bisulfite addition adducts, e.g. oleuropeindial (*7*). Sodium metabisulfite can react with *o*- or *p*-hydroxybenzyl alcohol derivatives to form sulfuric acid derivatives. Ester hydrolysis may also be assisted by the sulfite ion.

Storage of the sodium metabisulfite stabilized aqueous methanolic extract at −20 and 5 °C showed an initial increase in total phenols by FC followed by a gradual decline over 3 weeks (**Figure 4**). The most notable changes in the HPLC chromatograms were a decrease in hydroxytyrosol glucoside (**1**) and hydroxytyrosol (**2**) in the first week followed by a gradual increase by the end of the study and a significant increase in the amounts of the two peaks that coeluted with hydroxytyrosol. The change in these coeluting peaks was more easily monitored at 335 nm. Compound 3 showed an initial increase but then decreased gradually after 1 week.

Short-term storage of the extract up to 5 h at ambient temperature showed compound **3** to be particularly sensitive. Of the principal peaks, the peak area of compound **3** declined by over 90% during the 5 h. While peaks due to compounds **5** and **9** had increased marginally after 5 h, that of compound **13** had decreased markedly and the other major peaks had not changed. On the other hand, compound **3** was still detectable in samples stored at subambient temperatures after 30 days. It appears that the loss of compound **3** is probably due to simple volatilization. This was consistent with total loss of the peak for this compound following preconcentration of the extract by solvent evaporation under a stream of nitrogen at room temperature.

In summary, OMW constitutes a distinct matrix compared to those found when biophenols are extracted from whole fruit or plant material, since the cells are no longer intact and released enzymes have been intimately mixed with their substrates during malaxation, prior to sample collection. Such a reactive matrix requires careful attention to sample handling: stabilization of the extract was best achieved by addition of sodium metabisulfite 2% (w/w), a well-known antioxidant and potent PPO inhibitor, while prompt analysis of the extract is suggested, and storage at −20 °C is recommended if delayed analysis is inevitable. Aqueous methanol showed superior extraction of olive biophenols and Australian Frantoio OMW demonstrated a distinct biophenol profile compared to Italian Frantoio OMWW (*7*, *34*). The unidentified oleuropein derivative (**10**) was the major phenolic compound recovered, followed by verbascoside.

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LITERATURE CITED

(1) Kailis, S. G.; Considine, J. A. The olive *Olea europea* L. in Australia: 2000 onwards. *Adv. Hortic. Sci.* **2002**, *16*, 299−306.

(2) Obied, H. K.; Allen, M. S.; Bedgood, D. R.; Prenzler, P. D.; Robards, K.; Stockmann, R. Bioactivity and analysis of biophenols recovered from olive mill waste. *J. Agric. Food Chem.* **2005**, *53*, 823−837.

(3) Mulinacci, N.; Romani, A.; Galardi, C.; Pinelli, P.; Giaccherini, C.; Vincieri, F. F. Polyphenolic content in olive oil wastewaters and related olive samples. *J. Agric. Food Chem.* **2001**, *49*, 3509−3514.

(4) Lesage-Meessen, L.; Navarro, D.; Maunier, S.; Sigoillot, J. C.; Lorquin, J.; Delattre, M.; Simon, J. L.; Asther, M.; Labat, M. Simple phenolic content in olive oil residues as a function of extraction systems. *Food Chem.* **2001**, *75*, 501−507.

(5) Romero, C.; Brenes, M.; Garcia, P.; Garrido, A. Hydroxytyrosol 4-β-D-glucoside, an important phenolic compound in olive fruits and derived products. *J. Agric. Food Chem.* **2002**, *50*, 3835−3839.

(6) Robards, K. Strategies for the determination of bioactive phenols in plants, fruit and vegetables. *J. Chromatogr. A.* **2003**, *1000*, 657−691.

(7) Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A.; Montedoro, G. High-performance liquid chromatography evaluation of phenols in olive fruit, virgin olive oil, vegetation waters, and pomace and 1D-and 2D-nuclear magnetic resonance characterization. *J. Am. Oil Chem. Soc.* **1999**, *76*, 873−882.

(8) Ceccon, L.; Saccu, D.; Procida, G.; Cardinali, S. Liquid chromatographic determination of simple phenolic compounds in waste waters from olive oil production plants. *J. AOAC Int.* **2001**, *84*, 1739−1744.

(9) *United States Pharmacopeia XXVI (USP 26), The National Formulary 21 (NF21)*; United States Pharmacopeia Convention: Rockville, MD, 2002.

(10) *Official methods of analysis of the Association of Official Analytical Chemists;* Kennith H., Ed.; Association of Official Analytical Chemists: Gaithersburg, MD, 1990.
(11) Evans, C. W. Pharmacopoeial and related drugs of biological origin. In *Trease and Evans Pharmacognosy;* Evans, C. W., Ed.; 15th ed.; W. B. Saunders: London, UK, 2002.
(12) Karumi, Y.; Onyeyili P. A.; Ogugbuaja V. O. Identification of active principles of *M. balsamina* (Balsam apple) leaf extract. *J. Med. Sci.* **2004**, *4*, 179−182.
(13) Singleton, V. L.; Rossi Jr., J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticult.* **1965**, *16*, 144−158.
(14) Mateos, R.; Espartero, J. L.; Trujillo, M.; Rios, J. J.; Leon-Camacho, M.; Alcudia, F.; Cert, A. Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction and high-performance liquid chromatography with diode array ultraviolet detection. *J. Agric. Food Chem.* **2001**, *49*, 2185−2192.
(15) Mazza, G.; Fukumoto, L.; Delaquis, P.; Girard, B.; Ewert, B. Anthocyanins, phenolics, and color of Cabernet Franc, Merlot, and Pinot Noir wines from British Columbia. *J. Agric. Food Chem.* **1999**, *47*, 4009−4017.
(16) Fernández-Bolaños, J.; Rodriguez, G.; Gomez, E.; Guillen, R.; Jimenez, A.; Heredia, A.; Rodriguez, R. Total recovery of the waste of two-phase olive oil processing: Isolation of added-value compounds. *J. Agric. Food Chem.* **2004**, *52*, 5849−5855.
(17) Alburquerque, J. A.; Gonzalvez, J.; Garcia, D.; Cegarra, J. Agrochemical characterization of "alperujo", a solid byproduct of the two-phase centrifugation method for olive oil extraction. *Bioresource Technol.* **2004**, *91*, 195−200.
(18) Almazan, A. M.; Adeyeye, S. O. Fat and fatty acid concentrations in some green vegetables. *J. Food Compos. Anal.* **1998**, *11*, 375−380.
(19) Harborne, J. B. *Phytochemical methods: A guide to modern techniques of plant analysis*, 3rd ed.; Chapman and Hall: London, UK, 1998.
(20) Berke, B.; Cheze, C.; Vercauteren, J.; and Deffieux, G. Bisulfite addition to anthocyanins: Revisited structures of colourless adducts. *Tetrahedron Lett.* **1998**, *39*, 5771−5774.
(21) Ryan, D.; Robards, K.; Lavee, S. Determination of phenolic compounds in olives by reversed-phase chromatography and mass spectrometry. *J. Chromatogr. A* **1999**, *832*, 87−96.
(22) Vázquez Roncero, A.; Maestro Durán, R.; Graciani Constante, E. Componentes fenólicos de la aceituna. ii. Polifenols del alpechin. *Grasas Aceites* **1974**, *25*, 341−345.
(23) Moreno, E.; Quevedo-Sarmiento, J.; Ramos-Cormenzana, A. Antibacterial activity of wastewaters from olive oil mills. In *Encyclopedia of environmental control technology;* Cheremisoff, P. N., Ed.; Gulf Publishing Co.: Houston, TX, 1989.
(24) Gimeno, E.; Castellote, A. I.; Lamuela-Raventos, R. M.; De La Torre, M. C.; Lopez-Sabater, M. C. The effects of harvest and extraction methods on the antioxidant content (phenolics, alpha-tocopherol, and beta-carotene) in virgin olive oil. *Food Chem.* **2002**, *78*, 207−211.
(25) Capasso, R.; Cristinzio, G.; Evidente, A.; Scognamiglio F. Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. *Phytochemistry* **1992**, *31*, 4125−4128.
(26) 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.
(27) Cuyckens, F.; Claeys, M. Optimization of a liquid chromatography method based on simultaneous electrospray ionization mass spectrometric and ultraviolet photodiode array detection for analysis of flavonoid glycosides. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 2341−2348.
(28) Ryan, D.; Antolovich, M.; Herlt, T.; Prenzler, P. D.; Lavee, S.; Robards, K. Identification of phenolic compounds in tissues of the novel olive cultivar Hardy's Mammoth. *J. Agric. Food Chem.* **2002**, *50*, 6716−6724.
(29) Allouche, N.; Fki, I.; Sayadi, S. Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters. *J. Agric. Food Chem.* **2004**, *52*, 267−273.
(30) Sciancalepore, V.; Longone, V. Polyphenol oxidase activity and browning in green olives. *J. Agric. Food Chem.* **1984**, *32*, 320−321.
(31) Fernández-Bolaños, J.; Rodriguez, G.; Rodriguez, R.; Heredia, A.; Guillen, R.; Jimenez, 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) Martinez, M. V.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195−200.
(33) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure−antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.
(34) Visioli, F.; Vinceri, F. F.; Galli, C. Waste waters from olive oil production are rich in natural antioxidants. *Experientia* **1995**, *51*, 32−34.

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