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Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters

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Abstract

Liquid–liquid extraction was used in order to recover phenolic compounds from olive mill wastewater (OMWW), a polluting by-product of olive oil production process, and the extraction parameters have been optimized. HPLC analysis showed that hydroxytyrosol is the most abundant biophenol in ethyl acetate extracts from an acidified OMWW. Many other biophenols (tyrosol, caffeic acid, vanillic acid, verbascoside, luteolin-7-glucoside, dialdehydic form of decarboxymethyl oleuropein aglycon, ligstroside, luteolin) were identified.

Fractionation of OMWW phenolic extracts by reversed phase solid phase extraction (RP-SPE) allowed the separation of eight different phenolic fractions, whose radical scavenging activity was measured by the 2,2′-azinobis(3-ethylenbenzothiazoline-6-sulfonic acid) (ABTS) assay. Hydroxytyrosol, in particular, was purified from all the other biophenols, giving a fraction with a very high antioxidant activity.

With the procedure followed in the present study, from one litre of OMWW it seems possible to obtain 1 g of purified hydroxytyrosol.

Keywords: Olive mill wastewater; Phenolic compounds; Antioxidant capacity; Hydroxytyrosol; Solid phase extraction

1. Introduction

In the olive oil production process, the disposal of olive oil mill waste waters (OMWW) represents the main environmental problem. Continuous three phases extraction systems are still widely used in olive oil mills, especially in Italy, where in most cases they have not yet been replaced by more recent two-phases systems, which involve a reduction of OMWW volumes but an increased concentration in organic matter (Roig, Cayuela, & Sánchez-Monedero, 2006). Three phases extraction systems involve the addition of large amounts of water (up to 50 L/100 kg olive paste), resulting in the worldwide production of more than 30 millions m^3 per year of OMWW (Borja, Alba, & Banks, 1997). This represents a great environmental problem, since this by-product is characterized by a high organic load; among the different organic substances found in OMWW, including sugars, tannins, phenolic compounds, polyalcohols, pectins and lipids (D'Annibale, Crestini, Vinciguerra, & Giovannozzi Sermanni, 1998), the toxicity, the antimicrobial activity and the consequent difficult biological degradation of OMWW are mainly due to the phenolic fraction (Bisignano et al., 1999; Borja et al., 1997). The partition coefficients (oil/water) of most olive biophenols, ranging from 6×10^{-4} to 1.5 (Rodis, Karathanos, & Mantzavinou, 2002), are in fact in favour of the water phase: the olive fruit is very rich in phenolic compounds, but only 2% of the total phenolic content of

Abbreviations: OMWW, olive mill wastewater; RP-SPE, reversed phase solid phase extraction; ABTS, 2,2′-azinobis(3-ethylenbenzothiazoline-6-sulfonic acid); LLE, liquid–liquid extraction; OHTy-DEDA, dialdehydic form of decarboxymethyl oleuropein aglycon; TEAC, Trolox equivalent antioxidant capacity; TAA, total antioxidant activity.

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the olive fruit passes in the oil phase, while the remaining amount is lost in the OMWW (approximately 53%) and in the pomace (approximately 45%) (Rodis et al., 2002).

On the other hand, the phenolic compounds, which are very abundant in the OMWW and are the major responsible of their polluting load, are characterized by a strong antioxidant activity (Obied et al., 2005a; Visioli et al., 1999). Extraction of biologically active compounds (namely biophenols) from OMWW may turn a polluting residue into a source of natural antioxidants, object of growing interest in pharmaceutical and food industries since reactive oxygen species are involved in the onset of several human diseases and in the oxidative degradation of food.

The phenolic composition of OMWW has been studied by several recent works (Della Greca, Previtera, Temessi, & Carrelli, 2004; Obied, Allen, Bedgood, Prenzler, & Robards, 2005b). Servili et al. (1999) found an OMWW phenolic composition very different from that of olive fruit: while olives are very rich in secoiridoid glucosides, OMWW shows a high concentration of secoiridoid derivates, such as hydroxytyrosol and the dialdehydic form of decarboxymethyl oleuropein aglycon. The OMWW phenolic fraction is characterized by a great complexity, as demonstrated by Bianco et al. (2003), who identified 20 biophenols in OMWW using HPLC-MS-MS.

Among the biophenols present in OMWW, one of the most abundant and very interesting from a nutritional point of view is hydroxytyrosol, which has been widely studied demonstrating its antioxidant and health-beneficial properties as well as its good bioavailability: hydroxytyrosol scavenges free radicals (Visioli et al., 1999), inhibits human low-density lipoprotein (LDL) oxidation (Aruoma et al., 1998), inhibits platelet aggregation (Petroni et al., 1995) and the production of leucotriene for human neutrophyls (De La Puerta, Gutierrez, & Hoult, 1999) and shows in-vitro antimicrobial activity (Bisignano et al., 1999).

However, hydroxytyrosol is not commercially available in high amount as food additive. Several methods have been proposed for the production of hydroxytyrosol by means of chemical (Tuck, Tan, & Hayball, 2000) or enzymatic synthesis (Espin, Soler-Rivas, Cantos, Tomas-Barberan, & Wichers, 2001), but the protocols are usually slow and expensive, resulting in a little number of commercially available products containing pure hydroxytyrosol and in a high cost of them. By-products from processing materials of biological origin, such as wastewaters from olive oil mills, may then become important sources of high added value compounds, such as hydroxytyrosol or other antioxidant biophenols.

Several protocols have been developed for the purification of hydroxytyrosol and other phenolic compounds from OMWW, also with membrane technologies, but they are under patent protection (Crea, 2002; Fernandez-Bolanos et al., 2002; Pizzichini & Russo, 2005; Villanova, Villanova, Fasiello, & Merendino, 2006).

Recent studies have also dealt with the fractionation of phenolic extracts and with the evaluation of the antioxidant activity of purified compounds (Carrasco-Pancorbo et al., 2005; Fki, Allouche, & Sayadi, 2005; Morello, Vuorela, Romero, Motiva, & Heinonen, 2005).

The present study was aimed to verify the efficiency of the liquid–liquid extraction (LLE) for the recovery of biophenols from OMWW; a second purpose was to fractionate the phenolic extracts by Solid Phase Extraction (SPE), in order to obtain purified compounds, and to assess the antioxidant activity of each fraction, in order to determine the relative contribution of the various components to the antioxidant power of the whole phenolic extracts.

2. Materials and methods

2.1. Materials

Fresh olive oil mill wastewaters were supplied by a continuous three phases olive processing plant located in province of Benevento (Italy). These samples, obtained from olives collected in November 2003 and immediately processed, were stored at −20 °C.

Ethyl acetate, hexane, methanol, acetonitrile, trifluoroacetic acid (TFA), ethanol and formic acid were HPLC-grade solvents purchased from Carlo Erba (Milan, Italy).

Tyrosol, caffeic acid, vanillic acid and luteolin were purchased from Fluka Co. (Buchs, Switzerland); verbascoside and luteolin-7-glucoside were obtained from Extrasynthese (Genay, France). 2,2′-azinobis(3-ethylenbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Trolox) and potassium persulfate were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Extraction of phenolic compounds from OMWW

Liquid–liquid extraction with ethyl acetate was carried out on olive mill wastewater samples obtained from a continuous olive oil processing plant. OMWW was acidified to pH 2 with HCl and washed with hexane in order to remove the lipid fraction: 10 mL of OMWW were mixed with 15 mL of hexane, the mixture was vigorously shaken and centrifuged for 5 min at 3000 rpm. The phases were separated and the washing was repeated successively two times. Extraction of phenolic compounds was then carried out with ethyl acetate: the OMWW samples preventively washed were mixed with 10 mL of ethyl acetate, the mixture was vigorously shaken and centrifuged for 5 min at 3000 rpm. The phases were separated and the extraction was repeated successively four times. The ethyl acetate was evaporated under vacuum, the dry residue was dissolved in 3 mL of methanol and this solution was used for characterization, quantification and fractionation of phenolic compounds.

2.3. Total phenol content determination

The total phenol content of OMWWs and of their extracts was determined colorimetrically at 765 nm, using the Folin–Ciocalteau reagent (Folin & Ciocalteau, 1927) and expressed as tyrosol equivalents (mg tyrosol/mL OMWW).

2.4. HPLC separation and identification of phenolic compounds

The presence and amount of phenolic compounds in the OMWW extracts were studied by reversed phase HPLC analysis using a binary gradient elution. The analysis was performed by reversed phase HPLC on a LC-10AD Shimadzu (Milan, Italy) liquid chromatograph equipped with a SPD M10A VP diode array detector (Shimadzu). The chromatographic separation was achieved on a 5 μm ODS-3 Prodigy (250 mm × 4.6 mm i.d.) reversed-phase column (Phenomenex, Macclesfield, UK). The solvent system used was a gradient of solvent A (water:trifluoroacetic acid, 97:3, v/v), and solvent B (acetonitrile:methanol, 80:20, v/v). A step gradient from 5% to 98% B (45 min) was applied at a flow rate of 1 mL min^{-1}. Peak quantification was carried out at 279 nm. Elenolic acid and its esters were monitored at 239 nm. The main phenolic compounds were identified by comparison with relative retention times of pure compounds, when available, or by comparing the relative elution order and UV spectra with those reported in the literature (Brenes et al., 2000; Rovellini & Cortesi, 2002).

The identity of each peak was confirmed by LC-MS, performed on a LC-10AD VP Shimadzu (Milan, Italy) liquid chromatograph on-line with a LCMS-2010EV Shimadzu (Milan, Italy) mass spectrometer, equipped with an electrospray ionization (ESI) interface. A Discovery HS C18 column (5 μm, 150 mm × 2.1 mm i.d., Supelco, St. Louis, MO, USA), at a flow rate of 0.35 mL min^{-1}, was used. The solvent system used was a gradient of solvent A (water + formic acid 0.25%), and solvent B (methanol + formic acid 0.25%), with a step gradient from 5% to 55% B (45 min). The ESI mass spectra (*m/z* 60–900) in the negative ion mode were obtained under the following conditions: interface voltage 4 kV; nebulizer gas flow 1.5 L min^{-1}; block heater temperature 250 °C; curved desolvation line temperature and voltage 300 °C and −5 V, respectively; Q-Array voltage 0 V DC and 150 V RF; detector voltage 1.5 kV.

Some operating parameters (interface voltage and Q-Array voltage) were then modified in order to obtain a moderate fragmentation of the deprotonated molecular ions: an interface voltage of 5 kV and a Q-Array voltage of −50 V DC and 150 V RF were used.

Quantification of phenolic compounds was achieved by HPLC comparing peak areas with those of tyrosol as an external standard, according to the procedure described by Tsimidou, Papadopoulos, and Boskou (1992). Data were expressed as mg of tyrosol/kg of OMWW for both simple and hydrolizable phenolic compounds.

2.5. Solid phase extraction (SPE)

Fractionation of OMWW phenolic extracts was carried out by SPE using a reversed-phase C18 cartridge (Chromabond C18 ec 1000 mg/6 mL, Macherey-Nagel, Düren, Germany). Cartridges were conditioned with 5 mL of methanol/water 5:95 v/v. An aliquot (1 mL) of OMWW phenolic extract was reduced in a purified nitrogen stream to 300 μL and reported to the original volume with water, before loading on the column. The fractionation was achieved under atmospheric pressure using a binary gradient elution: the solvent system used was a gradient of solvent A (methanol) and solvent B (water). The following elution steps were used: 7 mL A:B 5:95 v/v; 5 mL A:B 10:90 v/v; 5 mL A:B 15:85 v/v; 5 mL A:B 20:80 v/v; 5 mL A:B 25:75 v/v; 5 mL A:B 30:70 v/v; 5 mL A:B 35:65 v/v; 5 mL A:B 50:50 v/v; 5 mL A:B 0:100 v/v. The flow rate was adjusted to about 0.15 mL/min and 1-mL sub-fractions were collected. The absorbance of each sub-fraction was measured spectrophotometrically at 280 nm.

2.6. Antioxidant activity determination

The antioxidant activity of phenolic extracts and their fractions was evaluated according to Re et al. (1999). This assay is based on the ability to scavenge the ABTS radical cation, a chromophore with characteristic absorption at 734 nm, converting it into a colourless product. The degree of decolorization induced by a compound is compared with that induced by Trolox, a polar α-tocopherol analogue.

The ABTS radical cation was produced through the reaction between 7 mM aqueous solution of ABTS and 2.45 mM potassium persulfate (final concentration). This solution was stored in the dark at 4 °C for 12–16 h before use. The concentrated ABTS solution was diluted with ethanol to a final absorbance of 0.70 ± 0.10 at 734 nm. Stock solutions of Trolox were prepared in methanol. 100 μL of a solution containing the reference antioxidant compound (Trolox) or a sample, represented by whole phenolic extracts or each phenolic SPE sub-fraction, were added to 1000 μL of the ABTS solution and the absorbance at 734 nm was measured exactly 2.5 min after initial mixing. This was compared to a blank where 100 μL of the solvent was added to 1000 μL of the ABTS solution.

The antioxidant activity of each phenolic extract or fraction was calculated by relating the decrease in absorbance induced by the sample to that of Trolox and was expressed both as total antioxidant activity (TAA, expressed as mmoles of Trolox L^{-1} OMWW) and as Trolox equivalent antioxidant capacity (TEAC), which was calculated as ratio between the TAA and the total phenol content and represented the concentration of a Trolox solution (mmol L^{-1}) with an equivalent antioxidant potential to 1.0 mmol L^{-1} solution of the substance under investigation.

Analysis of variance (ANOVA) was used to determine if significant differences existed at a level of confidence $p < 0.05$. Statistical analyses were performed with the software XLSTAT 2006, version 2006.6 (Addinsoft, Paris, France).

3. Results and discussion

3.1. Biophenols extraction from OMWW

Among different extraction methods, each one with different efficiency and complexity, the liquid–liquid extraction was preferred for its simplicity and convenience (Obied et al., 2005a). In order to develop an effective (both qualitatively and quantitatively) extraction, different parameters were optimized: solvent nature, pH of OMWW, volumetric ratio between solvent and OMWW, number of extraction stages.

As solvent for the extraction, ethyl acetate was chosen which is frequently used to extract biophenols from aqueous matrices such OMWW (Della Greca et al., 2004; Lesage-Meessen et al., 2001). Allouche, Fki, and Sayadi (2004) demonstrated that ethyl acetate exhibits a higher extraction power respect to other solvents, such as methyl isobutyl ketone, methyl ethyl ketone, diethyl ether, even though it is somewhat selective towards low (about 180 Da) and medium (about 13 kDa) molecular mass phenolic compounds (Visioli et al., 1999).

The effect of OMWW acidification on biophenol extraction was tested. Extracts from crude and acidified OMWW were qualitatively and quantitatively compared. In Fig. 1 the HPLC chromatograms of ethyl acetate extracts from crude and acidified (pH 2 with HCl) OMWW are shown. Acidification determines the protein precipitation, the release of biophenols bounded to cell wall components and the increase of solubility of phenolic compounds in organic solvents (Obied et al., 2005a); these effects are confirmed by the higher total phenol content (determined spectrophotometrically by the Folin–Ciocalteau method) we found in the extract from an acidified OMWW: 2.5 g tyrosol/L OMWW, versus 1.1 g tyrosol/L OMWW in the extract from a crude OMWW. In addition, acidification causes the hydrolysis of complex phenolic compounds with consequent release of phenolic monomers, as confirmed by the chromatograms of extracts from crude and acidified OMWW shown in Fig. 1, where it can be observed an increase of hydroxytyrosol (peak 1), tyrosol (peak 2), caffeic acid (peak 3) and verbascoside (peak 5) and a decrease of dialdehydic form of decarboxymethyl oleuropein aglycon (OHTy-DEDA; peak 8) in the extract from acidified OMWW.

A solvent/OMWW ratio of 1:1 v/v was used (Della Greca et al., 2004; Lesage-Meessen et al., 2001), and the number of extraction stages was optimized, measuring the total phenol content in the sequential extraction washing steps. A number of extraction stages equal to five proved to be adequate to obtain the maximum recovery of biophenols from OMWW in these extraction conditions.

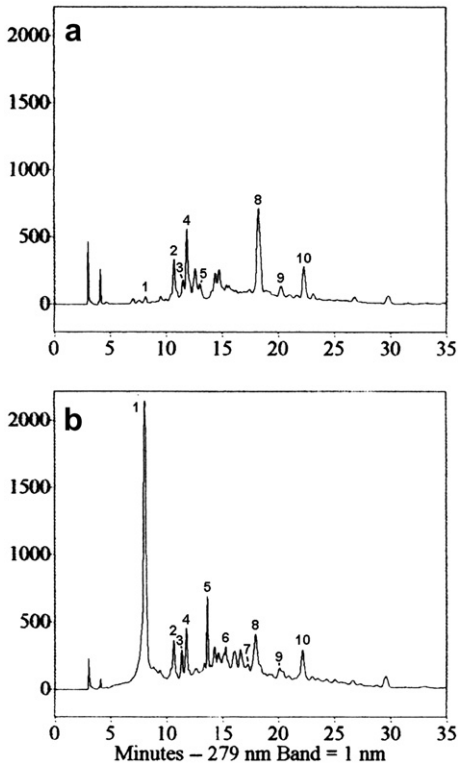

Fig. 1. HPLC chromatograms of phenolic extracts from crude (a) and acidified (pH 2 with HCl) (b) OMWW. 1, hydroxytyrosol; 2, tyrosol; 3, caffeic acid; 4, vanillic acid; 5, verbascoside; 6, luteolin-7-glucoside; 7, elenolic acid; 8, dialdehydic form of decarboxymethyl oleuropein aglycon; 9, ligstroside; 10, luteolin.

3.2. Identification, quantification and fractionation of biophenols extracted from OMWW

Identification of phenolic compounds in the OMWW extracts was preliminary performed by HPLC-UV, comparing the relative retention times and UV spectra with those of standard solutions. A representative chromatogram of an OMWW phenolic extract is reported in Fig. 1b. The HPLC profile showed several peaks corresponding to different biophenols, among which nine compounds were identified: phenyl acids (vanillic acid, caffeic acid), phenyl alcohols (hydroxytyrosol, tyrosol), secoiridoids derivatives (dialdehydic form of decarboxymethyl oleuropein aglycon, ligstroside, verbascoside), flavonoids (luteolin, luteolin-7-glucoside).

Identification was confirmed by LC-MS, in particular for those compounds (hydroxytyrosol and dialdehydic form of decarboxymethyl oleuropein aglycon) which lacked of pure standards. Fig. 2a shows the mass spectrum obtained for hydroxytyrosol in *full scan mode*, dominated by the deprotonated molecule $[M-H]^-$; the presence of the ion fragment at m/z 123 is due to the loss of the CH_2OH group (De la Torre-Carbot et al., 2005). Mass

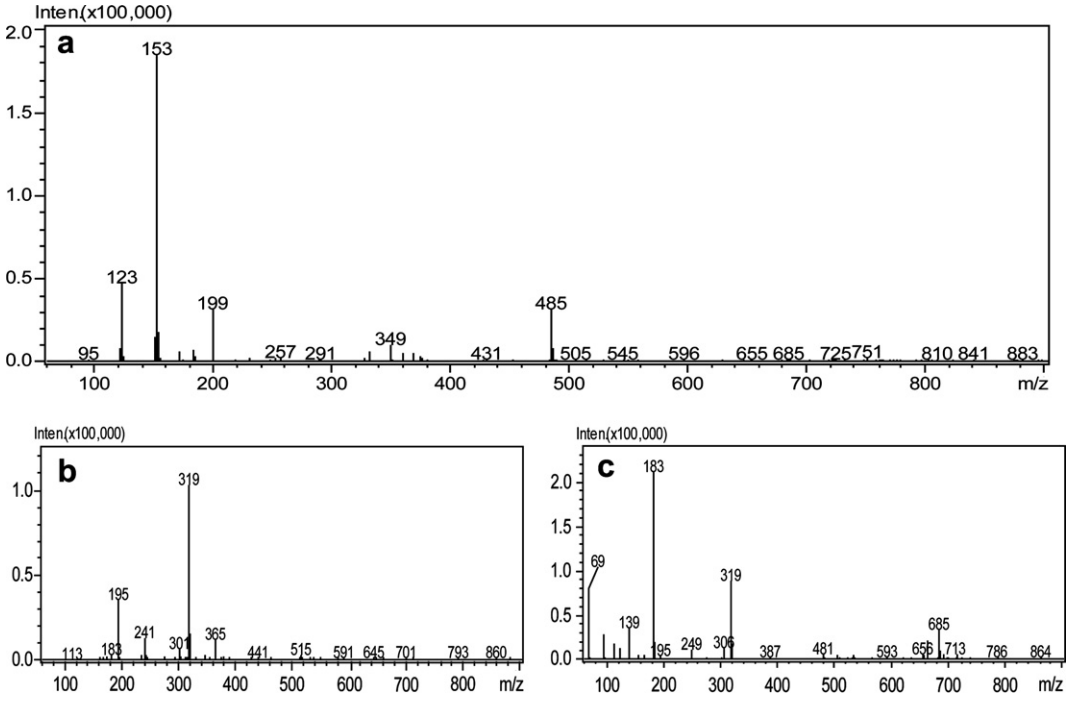

Fig. 2. Mass spectra obtained in *full scan mode* acquisition for hydroxytyrosol (a) and for dialdehydic form of decarboxymethyl oleuropein aglycon (OHTy-DEDA) with initial MS conditions (b) and with modified MS conditions in order to obtain better fragmentation (c) (see Section 2).

spectra obtained for the dialdehydic form of decarboxymethyl oleuropein aglycon are shown in Fig. 2: in the initial MS conditions (see Section 2) a spectrum was obtained dominated by the pseudomolecular ion $[M-H]^-$ (Fig. 2b); changes in the MS conditions (see Section 2) gave a better fragmentation (Fig. 2c), with the appearance of a fragment at m/z 183, which can be explained by the loss of hydroxytyrosol, and of a fragment at m/z 139, originated by the elimination of a CO_2 molecule from the previous fragment.

Concentration of phenolic compounds in the extracts was calculated comparing HPLC peak areas with those of tyrosol as an external standard (values obtained for the main phenolic compounds in the extracts from a crude and from an acidified OMWW are reported in Table 1).

According to previous investigations (Servili et al., 1999), the most abundant phenolic compound present in the OMWW extracts resulted to be hydroxytyrosol, which is formed as a result both of hydrolysis of oleuropein during oil extraction (Capasso, Evidente, & Visca, 1994) and of acid hydrolysis of secoiridoid derivatives caused by the addition of HCl to the OMWW. This biophenol is object of great attention and of numerous studies as it shows antioxidant, cardioprotective and antiatherogenic (Visioli

Table 1
Concentration of identified phenolic compounds (determined by peak integration using tyrosol as external standard) in the extracts from an acidified and from a crude OMWW and their relative abundance (%) in each of the eight fractions obtained from a phenolic extract by SPE

Peak number	Phenolic compound	Concentration (ppm tyrosol)		Relative abundance (%)							
		Extract from acidified OMWW	Extract from crude OMWW	Fractions							
				A	B	C	D	E	F	G	H
1	Hydroxytyrosol	1224	20		87.3	7.9	2.2	1.4	1.2		
2	Tyrosol	208	145			70.8	29.2				
3	Caffeic acid	97	72				79.4	20.6			
4	Vanillic acid	174	198				87.1	12.9			
5	Verbascoside	155	75					40.4		59.6	
6	Luteolin-7-glucoside	214	0				4.0	13.4	11.7	49.4	21.5
8	OHTy-DEDA	333	402							83.5	16.5
9	Ligstroside	87	92								100
10	Luteolin	209	145								100
	Total	2702	1150								

et al., 1999), chemopreventive (Fabiani et al., 2002), antimicrobial (Bisignano et al., 1999) and anti-inflammatory (De La Puerta et al., 1999; Maiuri et al., 2005) activities.

Many other phenolic compounds were found in the phenolic extracts, though not all were identified, due to the complex nature of the OMWW phenolic fraction (Bianco et al., 2003), which has not been completely elucidated yet. This complexity is due to the high number of factors that influence the occurrence of specific biophenols in OMWW, such as olive cultivar, ripeness degree of the fruit, climatic and agronomic conditions, storage conditions prior to extraction, processing technique (Lesage-Meessen et al., 2001; Obied et al., 2005a). Further investigation is required to better understand the phenolic composition of OMWW, as regards in particular the complex compounds, which are reported to be aglycons, glycosides or other derivatives of simple phenols (Obied et al., 2005a; Servili et al., 1999).

Other biophenols quantified in our OMWW extracts were tyrosol, caffeic and vanillic acids, for which antioxidant, antiatherogenic and anti-inflammatory (De La Puerta et al., 1999; Giovannini et al., 1999) activities have been reported. A quite abundant quantity has been found also for verbascoside, a complex biophenol characterized by health beneficial properties (Liu et al., 2003), while lower amounts have been revealed for flavonoids, a group of natural substances possessing antioxidant, anti-inflammatory, antiallergic, antiviral and anticarcinogenic properties (Pietta, 2000). The only flavonoids identified in our OMWW extracts were luteolin and luteolin-7-glucoside. Among secoiridoid derivatives, dialdehydic form of decarboxymethyl oleuropein aglycon (OHTy-DEDA) and ligstroside have been identified and quantified; the first compound derives from hydrolysis and successive decarboxylation (Rovellini & Cortesi, 2002) of oleuropein, one of the two major biophenols in olives (together with ligstroside), and in its turn undergoes an acid hydrolysis with release of monomers, such as hydroxytyrosol and elenolic acid (which was detected at 239 nm).

The biological activities shown by OMWW biophenols induce researchers to make any effort in order to obtain their quantitative extraction and their isolation from OMWW, with the consequent purification of compounds characterized by different properties. Fractionation of OMWW phenolic extracts was performed by means of an atmospheric-pressure reversed phase chromatographic system. The chromatogram shown in Fig. 3 reports the absorbance at 280 nm versus the sub-fraction number. Eight peaks have been identified and the corresponding sub-fractions have been collected in eight different fractions (A–H), which have been characterized by HPLC analysis (Fig. 4). The relative abundance (determined by HPLC data) of identified phenolic compounds in each of the eight fractions is reported in Table 1. A satisfactory separation of the major phenolic compounds between the eight different fractions can be observed. Hydroxytyrosol, purified from all the other phenolic compounds, was present almost

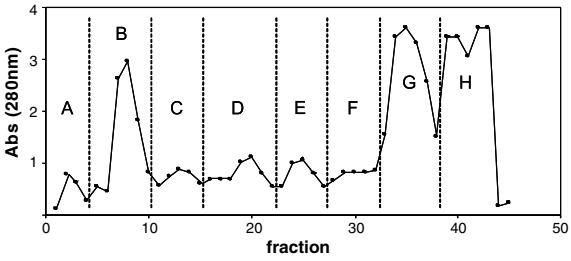

Fig. 3. Fractionation of OMWW phenolic extract: optical density at 280 nm versus sub-fraction number; identification of 8 different fractions (A–H).

exclusively (87%) in fraction B, while tyrosol was found mainly (71%) in the successive one; fraction D was dominated by caffeic and vanillic acid and fraction E by verbascoside and luteolin-7-glucoside; most of verbascoside (60%) and of OHTy-DEDA (84%) was found in fraction G; the total amount of ligstroside and luteolin present in the extract eluted in the last fraction. Fraction F, which subfractions were characterized by a very low absorbance at 280 nm, turned out to be very poor in phenolic compounds, while in the first fraction two very polar compounds were observed which have not been identified jet.

3.3. Antioxidant activity of phenolic extracts and of their fractions

Antioxidants have become a topic of increasing interest recently not only to health and food science researchers or medical experts, but also to the general public. Unfortunately, a validated method for the quantification of the antioxidant activity of foods and biological samples is yet to be developed and widely accepted. There is a great variety of assays which differ from each other in terms of substrates, system compositions, reaction conditions and quantification methods and on which there is no consensus of opinions (Huang, Ou, & Prior, 2005). In the present work, an improved version (Re et al., 1999) of the Trolox equivalent antioxidant capacity (TEAC) assay, first reported by Miller, Rice-Evans, Davies, Gopinathan, and Milner (1993), was selected to evaluate the antioxidant activity of OMWW phenolic extracts and of their fractions. The assay is rapid, easy and is capable of testing the antioxidant activity of both hydrophilic and lipophilic compounds.

Values of total antioxidant activity (TAA, expressed as mmoles of Trolox L^{-1} OMWW) of an extract and of its eight fractions, whose HPLC chromatograms were shown in Fig. 4, are reported in Table 2. It can be noted that the major contribution to the antioxidant activity of an OMWW phenolic extract was given by fractions G and H, in which the principal phenolic compounds were OHTy-DEDA, Luteolin-7-glucoside, Luteolin and Verbascoside. A significant contribution was given also by fraction B, constituted almost exclusively by hydroxytyrosol, while the other fractions contributed to a less extent to

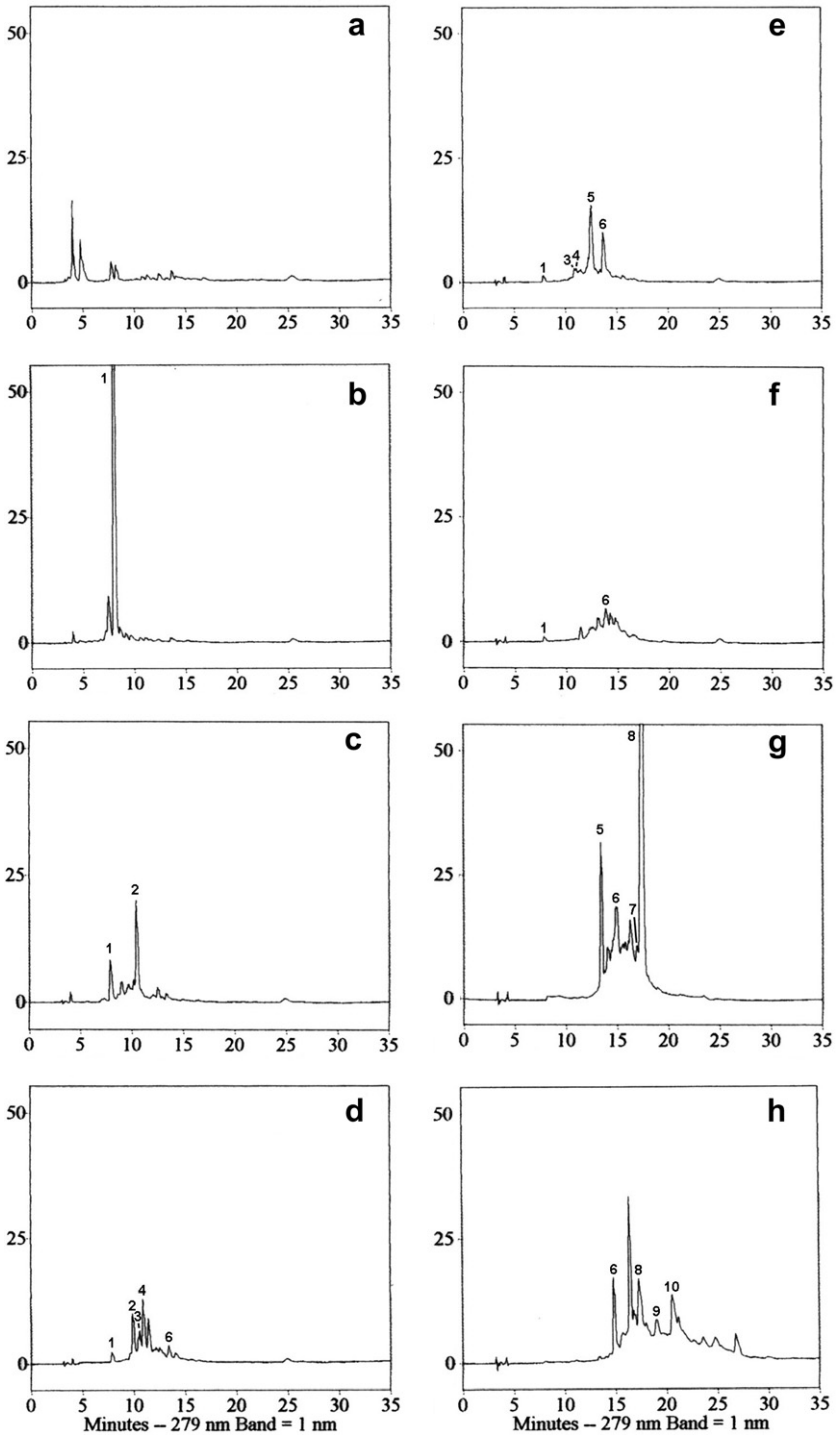

Fig. 4. HPLC chromatograms of the different fractions obtained from an OMWW phenolic extract by means of reversed-phase SPE. 1, hydroxytyrosol; 2, tyrosol; 3, caffeic acid; 4, vanillic acid; 5, verbascoside; 6, luteolin-7-glucoside; 7, elenolic acid; 8, dialdehydic form of decarboxymethyl oleuropein aglycon; 9, ligstroside; 10, luteolin.

the radical scavenging activity of an OMWW phenolic extract.

Values of total phenol content, as determined by peak integration using tyrosol as external standard, and of Trolox equivalent antioxidant capacity (TEAC), calculated as ratio between the TAA and the total phenol content, are also reported in Table 2 for each fraction as well as for the whole extract. The highest value of TEAC (3.2 mmol L^{-1}) can be observed for fraction B, containing the 87% of the total hydroxytyrosol present in the extract, so this compound proved to be the most effective in radical scavenging among the biophenols identified in OMWW. This result is in accordance with previous investigations, that report for this compound high antioxidant properties,

Table 2
Total antioxidant activity (TAA, mmol of Trolox equivalents L^{-1} OMWW), total phenol content (determined by peak integration using tyrosol as external standard) and Trolox equivalent antioxidant capacity (TEAC, mmol L^{-1}) of an OMWW phenolic extract and of its fractions obtained by SPE

Sample	TAA (mmol Trolox L^{-1} OMWW)	Total phenol content (mmol tyrosol L^{-1} OMWW)	TEAC (mmol L^{-1})
OMWW phenolic extract	55.8	25.2	2.2
Fraction A	0.55	0.5	1.1
Fraction B	8.07	2.5	3.2
Fraction C	3.32	1.1	3.0
Fraction D	4.89	2.3	2.1
Fraction E	3.43	1.6	2.2
Fraction F	3.75	1.3	2.9
Fraction G	19.4	8.4	2.3
Fraction H	14.0	9.4	1.5

free radical scavenging activity and a fundamental role in preserving olive oil from oxidative damage (Visioli et al., 1999). Bouaziz, Grayer, Simmonds, Damak, and Sayadi (2005) measured the radical scavenging capacity of pure flavonoids and simple phenols, finding the highest activity for hydroxytyrosol, followed by caffeic acid and quercetin, which showed an antioxidant power comparable of that of BHT, a synthetic compound used as antioxidant in many foods. The hydroxylation pattern of the aromatic ring proved to exert a fundamental influence on the antioxidant power. The presence of two hydroxyl groups at the *ortho* position (such as in hydroxytyrosol and in caffeic acid) confers a high antioxidant activity, while the phenolic compounds with a single hydroxyl group, such as tyrosol, *p*-coumaric acid and *p*-hydroxyphenylacetic acid, show a lower antioxidant activity (Fki et al., 2005). Among flavonoids, the highest antioxidant activity was shown by two *o*-diphenols (quercetin and luteolin), while the lowest by a mono-hydroxylated compound (apigenin), so confirming the importance of the *ortho* hydroxyl group. The stronger antioxidant activity shown by quercetin respect to luteolin suggests the importance of the hydroxylation of the C-3 of the non-phenolic C ring, while the higher power of quercetin respect to rutin and of luteolin respect to luteolin-7-glucoside suggests that glycosilation reduces the radical scavenging activity, as confirmed by other studies (Pietta, 2000; Rice-Evans, Miller, & Paganga, 1996).

Fractions D, E and G showed a TEAC value similar to that measured for the whole OMWW extract (2.2 mmol L^{-1}), while the phenolic compounds of the first and of the last fraction were characterized by a lower antioxidant power than those contained in the other fractions. This result demonstrates that the high contribution to the total antioxidant activity of the extract shown by the fractions G and H is due to their high total phenol content rather than to a high antioxidant power of their phenolic compounds.

Fractions C and F showed TEAC values (3.0 and 2.9 mmol L^{-1}, respectively) not significantly different ($p < 0.05$) from that found for hydroxytyrosol (fraction B) and significantly higher ($p < 0.05$) than those found for the other fractions. This is in contrast with previous studies, which report for tyrosol (the most abundant compound in fraction C) an antioxidant activity significantly lower than that of hydroxytyrosol, in agreement with the assumption that an *ortho*-hydroxyl group confers a strong radical scavenging activity (Bouaziz et al., 2005; Rice-Evans et al., 1996). The second unexpected value is that found for fraction F, which showed a low total phenol content and which major phenolic compound was represented by luteolin-7-glucoside, that is reported to have lower radical scavenging activity than many other flavonoids, such as quercetin and luteolin, and simple phenols, such as hydroxytyrosol and caffeic acid.

These unexpected results may be attributed to the presence of unidentified biophenols characterized by strong antioxidant activity or to a synergistic effect between phenolic compounds. Identification of unknown compounds is currently in progress by a thorough analysis of MS spectra and by comparison of retention times and UV spectra with those of standards.

4. Conclusion

In this study, in addition to the extraction and identification of OMWW biophenols, fractionation of phenolic extracts gave promising results. Eight fractions were obtained by RP-SPE which differed as regards the phenolic composition and consequently the radical scavenging activity. Separation of OMWW biophenols with different polarity, structural complexity and antioxidant activity was achieved quite satisfactorily. Hydroxytyrosol, in particular, was easily purified from all the other biophenols, giving a fraction with a very high antioxidant activity. With the procedure followed in the present study, from one liter of OMWW it is possible to obtain an extract containing 1.2 g of hydroxytyrosol and about 0.4 g of flavonoids, which in its turn can be fractionated with the consequent production of 1 g of purified hydroxytyrosol (87% of the total amount in the extract) from one liter of OMWW. The extraction and purification of hydroxytyrosol from OMWW may represent, then, a rapid, cheap and simple alternative to chemical or enzymatic synthesis. This procedure may be transferred to industrial scale and OMWW may become a valuable source of natural antioxidants, object of great interest for pharmaceutical, food and cosmetic industries.

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