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Chemical and Cellular Antioxidant Activity of Phytochemicals Purified from Olive Mill Waste Waters

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ABSTRACT: The isolation and identification of a phytocomplex from olive mill waste waters (OMWW) was achieved. The isolated phytocomplex is made up of the following three phenolic compounds: hydroxytyrosol (3,4-DHPEA), tyrosol (*p*-HPEA) and the dialdehydic form of decarboxymethyl elenolic acid, linked with (3,4-dihydroxyphenyl)ethanol (3,4-DHPEA-EDA). The purification of this phytocomplex was reached by partial dehydration of the OMWW, followed by liquid—liquid extraction with ethyl acetate and middle pressure liquid chromatography (MPLC) on a Sephadex LH-20 column. The phytocomplex accounted for 6% of the total phenolic content of the OMWW.

The phytocomplex and individual compounds were tested for antioxidant capacity by the oxygen radical absorbance capacity (ORAC) method. The ORAC phytocomplex produced 10,000 ORAC units/g dry weight, whereas the cellular antioxidant activity, measured by the cellular antioxidant activity in red blood cell (CAA-RBC) method, demonstrated that the phytocomplex and all of the components are able to permeate the cell membrane thus exhibiting antioxidant activity inside the red blood cells.

Our phytocomplex could be employed in the formulation of fortified foods and nutraceuticals, with the goal to obtain substantial health protective effects due to the suitable combination of the component molecules.

KEYWORDS: olive mill wastewater (OMWW), purification, HPLC, liquid chromatography, polyphenols, 3, 4-DHPEA, *p*-HPEA, 3, 4-DHPEA-EDA, antioxidant capacity, cellular antioxidant activity

INTRODUCTION

Olive mill waste waters (OMWW) are emulsions of oils, mucilage, pectins, sugars, nitrogen containing compounds and polyphenols of variable composition, on depending the olives cultivar, ripening stage and technological extraction systems. The concentration of phenolic compounds in OMWW ranges from 0.5 to 2.4% of the raw material;[1] many of these phenolic compounds are formed by the action of hydrolytic enzymes on olive secoiridoids, oleuropein and ligstroside, during oil extraction (Figure 1).

The most interesting products of these enzymes are the phenolic alcohols, hydroxytyrosol (3,4-DHPEA), tyrosol (*p*-HPEA) and a secoiridoid, the dialdehydic form of decarboxymethylelenolic acid, linked to (3,4-dihydroxyphenyl)ethanol (3,4-DHPEA-EDA).[2] The concentration of the latter compound overcomes those of the other two compounds both in oil and in OMWW.[3]

OMWW are a source of pollution due to their influence on biochemical oxygen demand (BOD) and chemical oxygen demand (COD).[2] Therefore, the recovery of phenolic compounds represents a potential use of such waste waters, contributing to the protection of the environment. Moreover, the health benefits of the pure molecules and their applications in industrial products can justify the necessary work required to extract the phenolic compounds. Isolation of high molecular weight phenolics, together with tyrosol and hydroxytyrosol, has been achieved by Cardinali et al.[4] Obied et al.[5] recovered 6 compounds from freeze-dried OMWW by liquid—liquid extraction. Hamden et al.[6] were able to purify hydroxytyrosol in high yields using a three-stage continuous countercurrent liquid—liquid extraction unit. Another extraction process, based on membrane technology, which provided an extract particularly rich in 3,4-DHPEA-EDA, has been developed by Servili et al.[2] The works of the cited groups and many others[7,8] demonstrate the interest in the beneficial health properties of these phenolic compounds, which are mainly recognized as antioxidant,[9] antiatherogenic,[10] anti-inflammatory[11] and antimicrobic.[12]

The aim of the present work was to develop an innovative and effective approach in the recovery of secoiridoid derived phenolics from dehydrated OMWW and to compare these phenolics for the antioxidant capacity, evaluated by both chemical and cellular methods.

MATERIALS AND METHODS

Chemicals. Ethanol and ethyl acetate (analytical grade) were purchased from VWR International Inc. (West Chester, PA). Folin—Ciocalteau's reagent, fluorescein sodium salt, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) and quercetin dehydrate were obtained from Sigma-Aldrich Inc. (St. Louis, MO). 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) was obtained from Polysciences Inc. (Warrington, PA). Acetic acid (HPLC-DAD grade) was purchased from Carlo Erba (Milan, Italy). The 3,4-DHPEA was obtained from Cabru S.p.A.; *p*-HPEA and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were

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Figure 1. Hydrolysis of oleuropein and ligstroside aglycons with the formation of the three main antioxidant compounds: the secoiridoid 3,4-DHPEA-EDA and the two phenolic alcohols 3,4-DHPEA and *p*-HPEA.

from Fluka (Milan, Italy). The 3,4-DHPEA-EDA was extracted from virgin olive oil according to Montedoro et al.[13] with the following modifications: the column used was a Pursuit XRs C18 (250 mm × 10 mm i.d.) with a particle size of 5 μm (Varian Inc., Walnut Creek, CA). The mobile phase was 0.2% acetic acid (pH 3.1) in water (A) and methanol (B) at a flow rate of 3 mL/min. The total running time was 155 min and the gradient changed as follows: 70% A/30% B at time 0 min, 65% A/35% B at 8 min and this composition was maintained for 92 min, 50% A/50% B in 5 min and this maintained for 30 min, 0% A/100% B in 3 min that was maintained for 12 min, before returning to the initial conditions in 5 min.

OMWW Samples. Fresh olive mill waste waters and the related oils were supplied from two different mills located in Cartoceto (Pesaro and Urbino, Marches, Italy). The samples of the OMWW were obtained by a traditional technological system from olives at the early (OMWW-A) at the advanced (OMWW-B) ripening stage; they were collected between October and November 2008 and stored at 4 °C.

One liter of each sample of the OMWW was dried in a ventilated oven at 55 °C for 24 h; the dried waste waters, containing (35 ± 5)% of residue water, were freeze-dried and stored at −20 °C. These OMWW pastes were used for further purification steps.

Samples of OMWW were processed in a muffle oven at 120 °C to determine the percentages of dry matter, which were 16% in OMWW-A and 13% in OMWW-B.

Olive Oil Analysis. Phenols were extracted following the procedure of Montedoro et al.[14] and assayed with the Folin−Ciocalteau method according to Singleton et al.[15]

Maturity Index of Olives. The maturity index was calculated according to Gutierrez et al.[16]

Extraction of Phenolic Compounds from OMWW. Liquid−liquid extraction with ethyl acetate was carried out on OMWW pastes in a separating funnel, at room temperature. The mixture (OMWW:solvent, 1:10 v/v) was vigorously shaken for 10 min to achieve equilibrium and then allowed to settle for 20 min. The phases were separated, and the extraction was repeated twice. The ethyl acetate combined fractions were evaporated under vacuum at 40 °C in a rotary evaporator (Buchi, Milan, Italy).

OMWW Extract Fractionation. OMWW ethyl acetate extracts were subjected to MPLC, conducted with an ÄKTA purifier 10 (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.) equipped with a 901 quaternary pump, on a Sephadex LH-20 column (250 mm × 55 mm i.d.) (Sigma-Aldrich Inc., St. Louis, MO); the mobile phase was 30% ethanol in isocratic mode, at a flow rate of 1 mL/min. Absorbance was read at 320 nm. Five peaks (P1−P5) were obtained from the column, and each one was analyzed by HPLC-DAD. Elution times of peaks were the following: P1 from 120 to 180 min, P2 from 300 to 340 min, P3 from 540 to 580 min, P4 from 700 to 740 min and P5 from 940 to 1000 min.

Reversed Phase HPLC-DAD Analysis. The fractions P1−P5, obtained from the Sephadex LH-20 chromatography column, were concentrated, solubilized in methanol and filtered through PVDF syringe filters (0.2 μm). HPLC analysis of samples was conducted with an Agilent Technologies system model 1100 (Palo Alto, CA) composed of a vacuum degasser, a quaternary pump, an autosampler, a thermostated column compartment, a diode array detector (DAD) and a fluorescence detector (FLD). The column used was a Spherisorb ODS-1 (250 mm × 4.6 mm i.d.) column with a particle size of 5 μm (Phase Separation Ltd., Deeside, U.K.), the injected sample volume was 20 μL. The mobile phase was composed of 0.2% acetic acid (pH 3.1) in water (solvent A)/methanol (solvent B) at a flow rate of 1 mL/min. The gradient changed as follows: 95% A/5% B for 2 min, 75% A/25% B in 8 min, 60% A/40% B in 10 min, 50% A/50% B in 16 min and 0% A/100% B in 14 min and this composition was maintained for 10 min, then was returned to the initial conditions with the equilibration in 13 min; the total running time was 73 min.[17]

Phenolics Assay. Phenolic compounds were assayed with the Folin−Ciocalteau method according to Singleton et al.;[15] values are expressed as mg of caffeic acid equivalents.

Chemical Antioxidant Assay (ORAC). Antioxidant activity of single compounds and P3 was determined by the ORAC assay[18] using a Fluostar Optima plate reader fluorimeter (BMG Labtech, Offenburgh, Germany) equipped with a temperature-controlled incubation chamber and an automatic injection pump. Incubator temperature was set at 37 °C. The reaction mixture for the assay was the following: 200 μL of 0.096 μM fluorescein sodium salt in 0.075 M Na-phosphate buffer (pH 7.0), and 20 μL of sample or Trolox. An initial calibration curve was made each morning using six concentration points of the standard Trolox in the range of 25−150 μM. Subsequent sample concentrations for each plate were verified against this calibration curve, by repeating the

Table 1. Concentration of Phenols and ORAC Values in Olive Mill Waste Waters and Oils[a]

parameters	system A, RI[b] = 3.5		system B, RI = 6	
	OMWW-A	OIL-A	OMWW-B	OIL-B
polyphenols (mg/kg)	6590 ± 577[c]	126 ± 16[c]	5955 ± 198[c]	108 ± 19[c]
ORAC (μmol TE/g)	1080 ± 43[d]	4.27 ± 0.10[d]	699 ± 37[d]	3.83 ± 0.09[d]
ORAC/polyphenols ratio	0.16	0.03	0.11	0.03
polyphenols partition coefficient between OMWW/oil	51.4 ± 3.1		54.8 ± 2.1	

[a] The olive cultivar was Leccino, from trees located in the same area. Values are expressed on a dry weight base after dehydration at 120 °C for 24 h. Results are the mean ± SD of four different samples. Oils and OMWW were obtained in the same day during the processing of 2500 kg of olives both for systems A and B. [b] RI = ripeness index.[16] [c] The phenolic content is the mean value of four independent experiments; waste water and oil polyphenols values of samples A and B are significantly ($P < 0.05$) different among them. [d] The ORAC content is the mean value of seven replicates; waste water and oil ORAC values of samples A and B are significantly ($P < 0.05$) different among them.

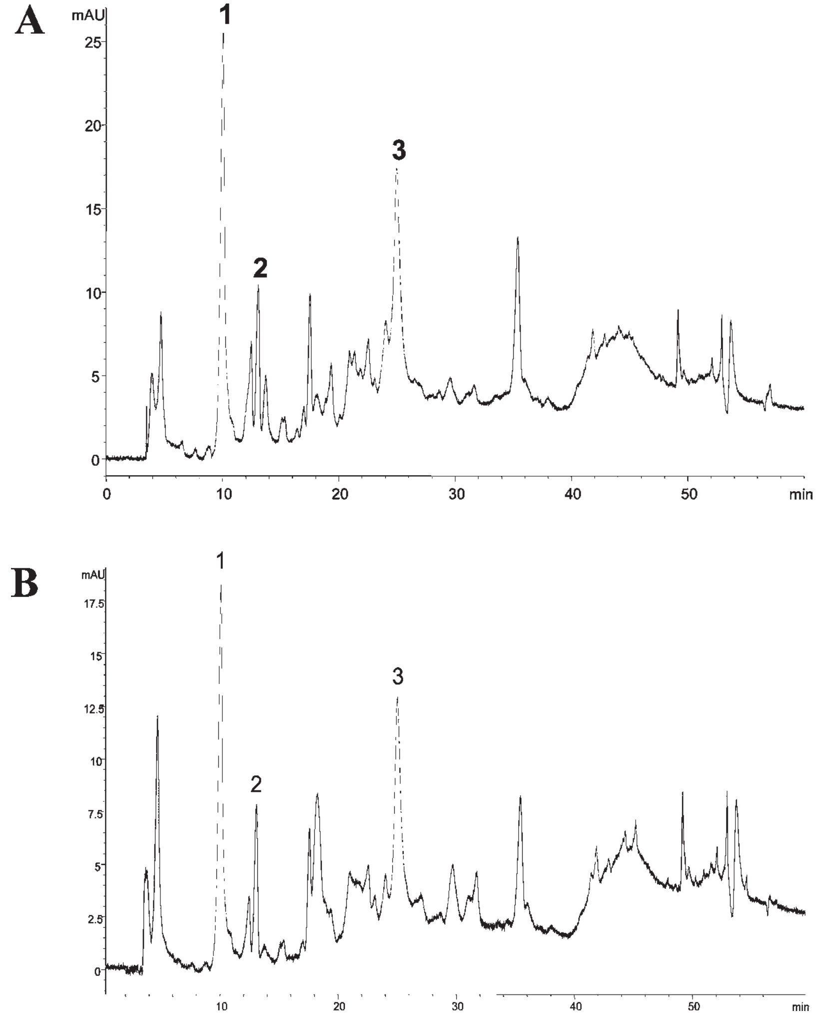

Figure 2. HPLC profile of OMWW-A (A) and OMWW-B (B) extracts. The numbers indicate the peaks of the three target antioxidant molecules identified as 3,4-DHPEA (1), *p*-HPEA (2) and 3,4-DHPEA-EDA (3).

calibration with three concentration points of the standard Trolox (25, 50, 100 μM). The blank was 0.075 M Na-phosphate buffer (pH 7.0). The reaction was initiated with 40 μL of 0.33 M AAPH. Fluorescence was read at 485 nm ex. and 520 nm em. until complete extinction. ORAC values are expressed as μmol Trolox equivalents (TE)/g of standard or vegetable extract and are the means ± SD of eight analyses.

Cellular Antioxidant Activity (CAA-RBC). Human blood from healthy volunteers was kindly provided by the "Blood Transfusion Centre" from the local hospital. RBCs were obtained by consecutive centrifugations and washings in cold phosphate-buffered saline (PBS: 125 mM NaCl, 10 mM sodium phosphate, pH 7.4) to remove plasma, platelets and buffy coat. The packed RBCs were diluted 1:100 in PBS before incubation with DCFH-DA and antioxidants. A stock solution, with a concentration of 20 mM DCFH-DA, was prepared in methanol and diluted in PBS to obtain a 75 μM working solution.

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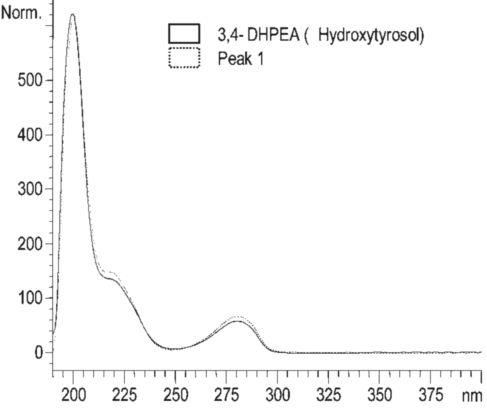
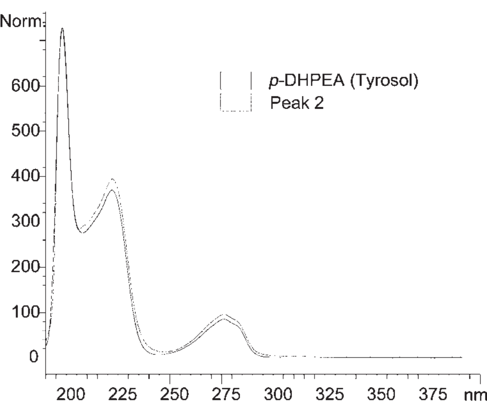




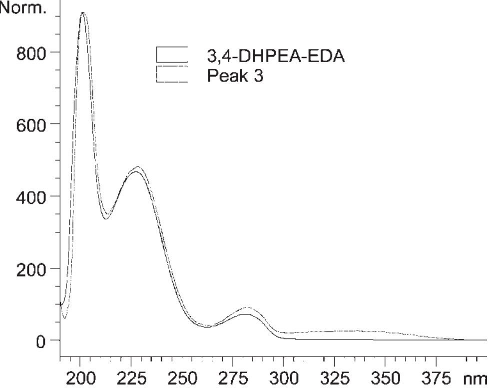


Figure 3. HPLC-DAD spectra of the three target compounds identified in the OMWW column: 3,4-DHPEA, *p*-HPEA and 3,4-DHPEA-EDA in comparison to the relative standard molecules.

The RBC suspension was incubated a 37 °C with 25 μM DCFH-DA, the target compounds and the P3 fraction at the indicated concentrations.

At the end of the incubation, the RBCs were washed twice in PBS to remove the remaining antioxidants in the extracellular medium, resuspended in cold PBS and transferred to a 96-well microplate, which was placed in the Fluostar Optima plate reader fluorimeter. The cell count was evaluated by the use of both a Coulter Counter ZM and hemoglobin assay: a number of $(1.5 \pm 0.2) \times 10^6$ cells per well have been used.

Fluorescence was read at 485 nm ex. and 520 nm em. every min. The cellular antioxidant activities for target compounds and P3 were expressed as micromoles of quercetin equivalent (QE)/100 μmol of total phenols, considering the molecular weight of gallic acid.[19]

Statistical Analysis. Statistical analysis was performed using Microsoft Excel. Statistical significance was tested using Student's *t* test with a *p* value of less than 0.05 indicating a significant difference between data sets.

RESULTS

Total Phenols and Antioxidant Activity of OMWW and Oils. Although this work focuses on the OMWW, we began the research with the comparison of phenolics and antioxidant capacity of both OMWW and correspondent oil samples, in order to have a complete idea of the levels of antioxidants in the two phases.

Table 1 shows the concentrations of phenols and antioxidant capacities, measured with the ORAC method, of OMWW from Leccino olives at two maturity indexes. OMWW and oil from olives at the early ripening stage (system A) showed higher polyphenols and antioxidant activity values with respect to samples produced with olives at an advanced ripening stage (system B). It is worth noting that the concentration of polyphenols of OMWW-A was more than 10% higher than that of OMWW-B; whereas the ORAC value of OMWW-A was 35% higher than that of OMWW-B. The repartition coefficients of phenols between OMWW and oil were in the range of 51−55, with no significant difference between systems A and B (Table 1).

OMWW Extracts and HPLC Analysis. The characterization of the phenolic compounds of OMWW has been carried out with a liquid−liquid extraction with ethyl acetate, followed by HPLC analysis of the concentrated extracts. Figure 2 shows the HPLC chromatograms of the OMWW-A and -B extracts. The two profiles showed a similar pattern, and the only difference was in the concentration of the molecules. The peak numbers in Figure 2 indicate the principal compounds, identified by means of the retention times and the HPLC-DAD spectra with comparison to standard compounds, as shown in Figure 3. The chemical structures of the compounds, together with their chromatographic retention times, physicochemical characteristics and concentrations, are shown in Table 2. It is worth noting that the concentration of 3,4-DHPEA-EDA of OMWW-A is 1.05 ± 0.07 mg/g, a value significantly higher than that of OMWW-B, while the concentrations of 3,4-DHPEA and *p*-HPEA are very similar between OMWW-A and -B. In both systems A and B, the concentration of the secoiridoid 3,4-DHPEA-EDA was higher than that the two phenolic alcohols.

Phenols Purification from OMWW-A. Once we had demonstrated the similarity of the chemical composition of OMWW-A and -B, and that OMWW-A was more rich in phenolic compounds than OMWW-B, we proceeded to the purification of the above-reported molecules from OMWW-A only. For this reason, we have carried out a MPLC on a Sephadex LH-20 column, obtaining the resulting five fractions, labeled P1−P5 (data not shown). After HPLC analysis of the five fractions, we found that P3 only contained significant amounts of the three compounds of interest for our research. Figure 4 shows the chromatographic profile of the P3 fraction, demonstrating the predominant presence of the three antioxidant molecules. From the comparison of the retention times and the HPLC-DAD spectra of the three major peaks with the corresponding molecular standard, we confirmed that the three numbered compounds were actually 3,4-DHPEA, *p*-HPEA and 3,4-DHPEA-EDA respectively.

Figure 5 shows a flowchart which summarizes the working steps and the percentage of polyphenols in each key step. Fraction P3 represents 6% of the total phenols of the starting material, while the relative ratio of concentrations of *p*-HPEA, 3,4-DHPEA and 3,4-DHPEA-EDA, determined by HPLC analysis, was 1:2.5:3.1 respectively .

Table 2. HPLC Data, Structure and Quantification of Compounds Purified in the Two Categories of OMWW

PEAK	COMPOUND	RETENTION TIME (min)	MOLECULAR WEIGHT (Da)	COMPOUND STRUCTURE	SYSTEM-A (mg/g)	SYSTEM-B (mg/g)
1	(3,4-DHPEA)	11.49	154		0.25 ± 0.001	0.18 ± 0.0001
2	(*p*-HPEA)	14.53	138		0.19 ± 0.002	0.11 ± 0.0018
3	(3,4-DHPEA-EDA)	26.12	320		1.05 ± 0.07	0.79 ± 0.002
TOTAL PHENOLICS					1,49 ± 0.07	1,08 ± 0.003

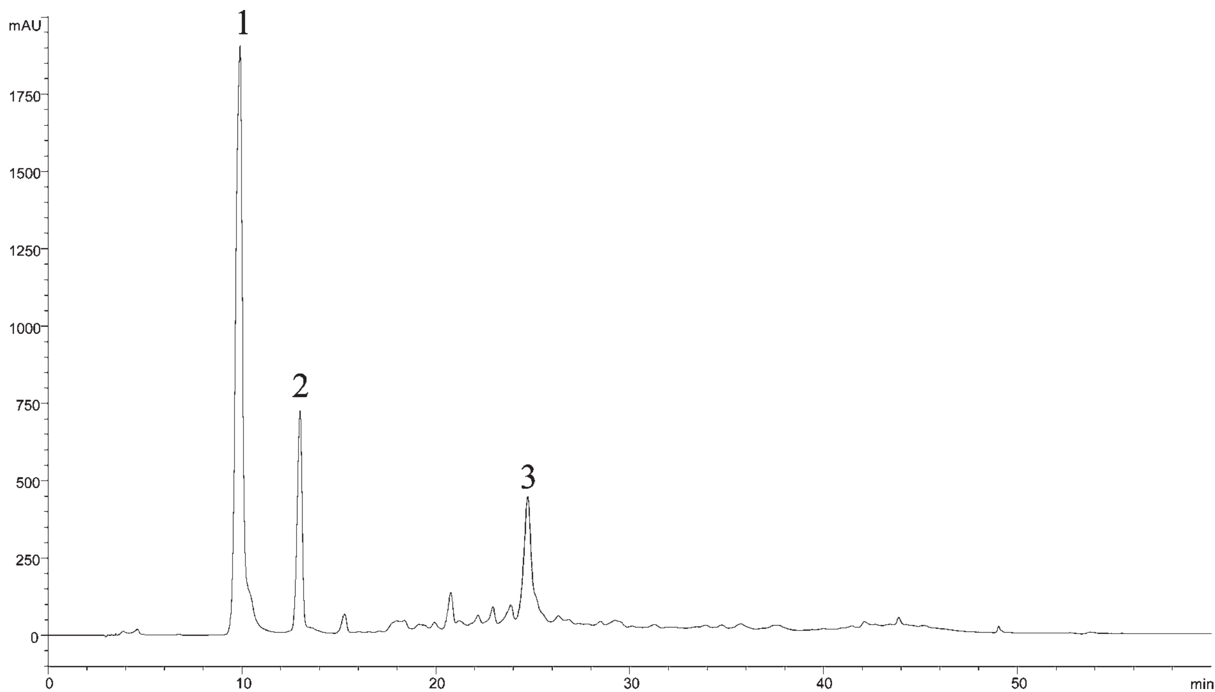


Figure 4. HPLC-DAD profile of P3 fraction eluted from the Sephadex LH-20 column. The numbers indicate the peaks of the three target antioxidant molecules identified as 3,4-DHPEA (1), *p*-HPEA (2) and 3,4-DHPEA-EDA (3).

Chemical and Cellular Antioxidant Activity. The next step in this work was the characterization of the antioxidant activity of the standard phenolic compounds individually and present in the P3 fraction. Figure 6 shows the antioxidant activity values, measured by the chemical (ORAC) and the cellular (CAA-RBC)[19] methods, of the P3 phytocomplex and each pure phenolic compound. Figure 6A shows that the ORAC value of 3,4-DHPEA is the highest, while the ORAC of P3 is not significantly different from that of 3,4-DHPEA-EDA.

Figure 6B shows the CAA-RBC values determined on P3 and on the three compounds. Results provide an indication of the

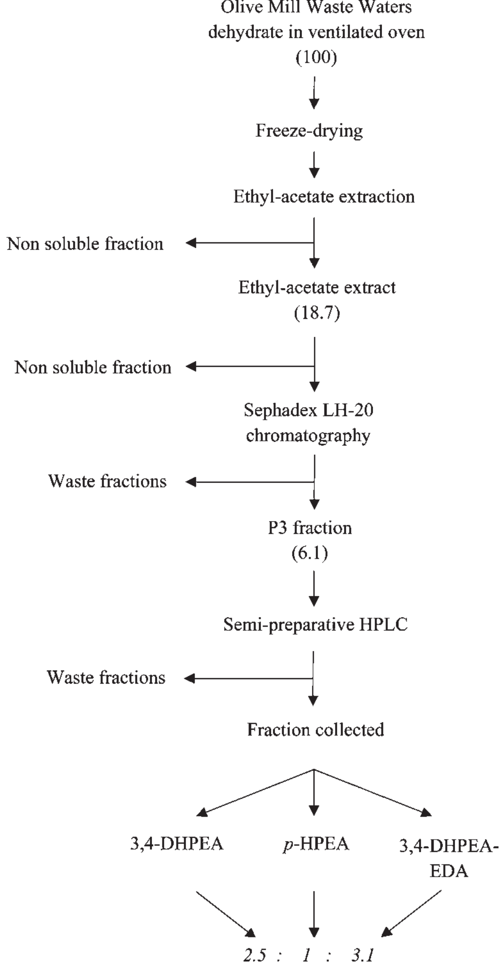


Figure 5. Flowchart of extraction, purification and quantification of the main antioxidants from olive mill waste waters. Numbers in parentheses are the percentages of the phenols found in waste waters at the early maturation stage. Numbers in italics are the relative ratios among the three compounds of the phytocomplex P3.

permeability across the cell membrane of the compounds,[20,21] and data confirm the trend values of the ORAC analysis, with a marked cellular antioxidant protection exerted by 3,4-DHPEA and to a lesser extent by *p*-HPEA; a lower value of P3, not significantly different from that of 3,4-DHPEA-EDA, was detected.

■ DISCUSSION

In this paper, we characterized the OMWW of two oils from the Leccino olive cultivar, collected at two ripening stages. We obtained evidence that the waste waters from olives with a ripeness index of 3.5 showed higher phenolic concentration than those obtained from more mature olives. This result agrees with earlier reports[3,22] indicating that the increase of olive maturation provides lower phenolic content both in oils and waste waters. The repartition coefficient value of phenols between water and oil is also of interest. This value, which amounted to about 50, indicates the high amount of phenols lost in the OMWW and the importance of waste waters as a source of bioactive molecules.

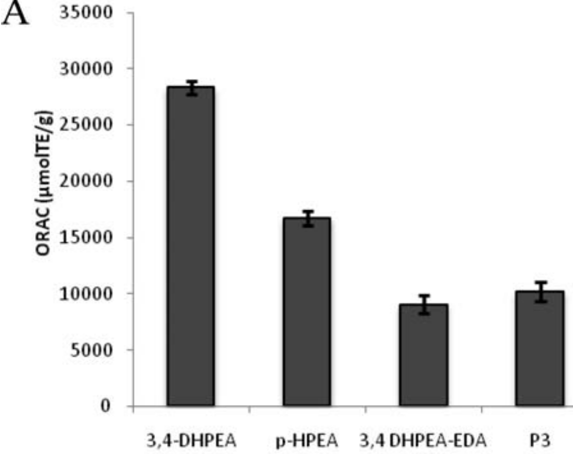
A new protocol for the purification of 3,4-DHPEA, *p*-HPEA and 3,4-DHPEA-EDA in one fraction is described in this work. The separation from many other phenols present in OMWW has been possible by hydrophobic interaction chromatography, adopting an elution in isocratic mode with 30% ethanol. This strategy allowed us to obtain the three compounds of interest in one step, a result which permits the establishment of a cheap large scale separation system. In fact, the scale-up requires neither procedures for the mobile phase pump programming nor very high fluxes.

The quantitative ratio among the antioxidant compounds present in the purified fraction P3 proved to be very easy and reproducible. A similar approach has been adopted by Cardinali et al.,[4] which provided a sample containing also phenolics with high molecular weights.

The phytocomplex P3 purified by us contains the three most interesting phenolic compounds at high purity grade. P3 showed a good potential biological activity as demonstrated by the test performed with the CAA-RBC assay.[19] This test indicates a notable permeability of P3 and single compounds across the RBC membrane. The molecular weight of 3,4-DHPEA, its hydroxyl groups and ethanol chain appears to provide this molecule with the best characteristics for penetrating the RBC membrane. On the contrary, the 3,4-DHPEA-EDA seems to be too big to suitably permeate the cell membrane. The chemical antioxidant capacity (ORAC assay) appears to be linearly correlated with the cellular antioxidant activity (CAA-RBC). The P3 fraction showed a moderate antioxidant activity and permeability, probably due to the large amount of the 3,4-DHPEA-EDA (almost the 50%); however we consider P3 a phytocomplex sufficient for industrial applications. The antioxidant capacity of 3,4-DHPEA-EDA was lower than that of *p*-HPEA and 3,4-DHPEA. This result was unexpected, since it is well-known that the antioxidant activity is related to the number of hydroxylic groups and in this sense it is expected that 3,4-HPEA-EDA and 3,4-DHPEA have the same antioxidant capacity. With the ABTS radical in the TEAC assay, the antioxidant capacity values of 3,4-DHPEA-EDA and 3,4-DHPEA are similar.[23] Our ORAC results, produced with respect to the peroxyl radical, indicate a superior reducing capacity of 3,4-DHPEA vs 3,4-DHPEA-EDA. The antioxidant capacity of the olive oil phenolics as scavengers of the different oxygen radicals is a long debated question in the literature.[24,25] We are not able at present to explain this result, however we believe that the lower reducing capacity of 3,4-DHPEA-EDA toward the peroxyl radical is due to steric hindrance of the elenolic acid of the 3,4-DHPEA-EDA, which could exert a barrier in the reaction with the peroxyl radical.

Different attempts have been made to use olive antioxidants as functional ingredients in foods. Some researchers studied the possibility to use these compounds to fortify fruit juices, vegetable soups, yogurts and cheeses.[26] These compounds may be also useful in nutraceutical preparations for chemoprevention of chronic diseases,[10,27] as well as in cosmetology.[28]

The recovery of olive phenols from waste waters can also provide a significant contribution to the protection of the environment. In fact, the dispersion of the OMWW in the soil represents an environmental hazard and it is limited by the law during only two months following the olive milling. However the period of the milling occurs during the winter, when there is no need of water by the soil. Moreover, the dispersion in cultivated areas is hampered by the discovery that waste waters allow the proliferation a particular strain of fungi, that subtract energy from plant roots.[29] For this motive, the National Agency for New Technologies (ENEA) research group has prepared a technical system which integrates the production of compost with extraction of the biomolecules from the OMWW.[30] These reasons make it very attractive to use OMWW



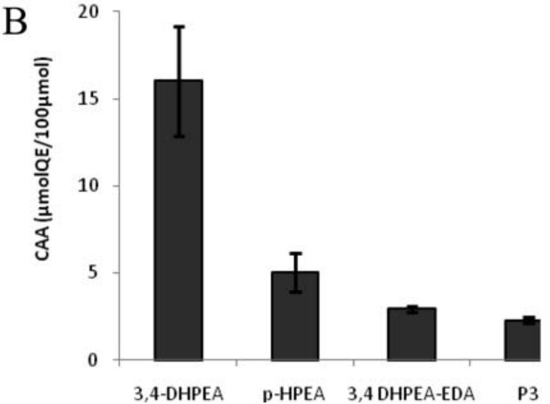


Figure 6. Chemical antioxidant assay (A) and cellular antioxidant activity (B) of the P3 fraction and of the three standard phenolic compounds.

as a starting material for the preparation of various products in industrial or agronomic applications.

In conclusion, OMWW are an interesting source of phenolic compounds, together with carbohydrates and pectins.[1] This study has demonstrated a new and simple method for the extraction of three phenolic compounds of biological interest from OMWW in one fraction.

The P3 phytocomplex has elevated antioxidant activity compared to other botanical extracts,[31] which justifies its employment in the production of functional foods, nutraceuticals or cosmetics; otherwise the P3 phytocomplex can be further purified yielding three single compounds 3,4-DHPEA, *p*-HPEA and 3,4-DHPEA-EDA, which have strong antioxidant capacities as well as cellular antioxidant activities, a parameter highly expressive of their membrane permeability and their biological activity.

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