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Valorization of an Underutilized Waste from Olive Oil Production by Recovery of Hydroxytyrosol

Giuseppe Squillaci [1,*], Ismene Serino [1,2], Sara Errichiello [1], Francesco La Cara [1] and Alessandra Morana [1,*]

[1] Research Institute on Terrestrial Ecosystems, National Research Council of Italy, via Pietro Castellino 111, 80131 Naples, Italy
[2] Department of Experimental Medicine, University of Campania "Luigi Vanvitelli", via Costantinopoli 16, 80138 Naples, Italy
* Correspondence: giuseppe.squillaci@iret.cnr.it (G.S.); alessandra.morana@cnr.it (A.M.)

Abstract: Hydroxytyrosol (HT) is one of the most powerful natural antioxidants, mainly contained in olive oil and its by-products. Here, a procedure for the preparation of an HT-enriched sample is described. An acidic aqueous extract (pH 1.25) from Olive Oil Dregs (OOD), a by-product from oil mills, was prepared by incubation at 37 °C for 1 h. The total phenolic content and HT amount were 6.24 ± 0.10 mg gallic acid equivalent/g OOD and 532.98 ± 5.78 µg/g OOD, respectively. Amberlite XAD16N and XAD7HP resins were used for the recovery of HT from the raw extract. Several elution conditions were tested with both resins, and elution with 25% ethanol provided the highest HT recovery (92.50% from XAD7HP). Antioxidant activities were assessed in the pool containing the highest quantity of HT. The results were compared with those of the raw extract. Ferric reducing antioxidant power values were comparable (95.71 ± 2.50 and 96.64 ± 13.47 µg ascorbic acid equivalent/mg for HT-enriched pool and raw extract, respectively), while the radical scavenging activity was higher for the pool (92.83% ± 0.44 and 44.12% ± 1.82, respectively). The results reported here demonstrate that HT can be recovered with a high yield from OOD, providing a preparation with high radical scavenging power. In addition, it is proved that this by-product, poorly considered up to now, can be usefully exploited.

Keywords: olive oil dregs; hydroxytyrosol; phenolic compounds; Amberlite XAD7HP; Amberlite XAD16N; antioxidant; radical scavenging activity; superoxide scavenging activity; ferric reducing antioxidant power; waste valorization

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1. Introduction

Phenolic compounds, secondary plant metabolites, are widely known for the antioxidant power they exert; moreover, they greatly contribute to the benefits of human wellness being that their healthy properties are strictly correlated to antioxidant activity [1]. These compounds are able to prevent human diseases associated with oxidative stress: they protect against neurodegenerative disorders, counteract the oxidation of low-density lipoproteins with consequent positive effects on cardiovascular diseases, and exhibit anticancer activity [2–5].

Among them, hydroxytyrosol (3,4-dihydroxyphenylethanol, HT) is known as one of the most powerful natural antioxidants [6]. Several studies have ascertained that it has many biological activities such as antidiabetic, anticancer, and anti-inflammatory, and exerts neuroprotective effects on Alzheimer's and Parkinson's diseases [7–11]. Furthermore, HT exhibits: antibacterial effects against *Salmonella typhi*, *Haemophilus influenzae*, *Vibrio parahaemolyticus*, and *Vibrio cholerae*, as reported by Bisignano et al.; antiviral effects against influenza enveloped viruses, as described by Yamada et al. [12,13]. The remarkable antioxidant activity of HT lies in its "catechol" structure, where two hydroxyl groups are in the *ortho* position (Figure 1).

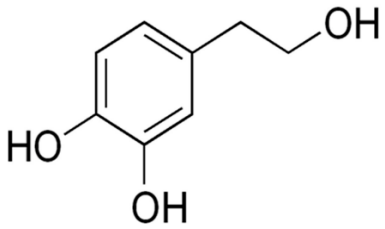

Figure 1. Chemical structure of hydroxytyrosol.

This chemical structure has the ability to stabilize the phenoxyl free radicals by forming intramolecular hydrogen bonds between the radical oxygen and the adjacent hydroxyl group [14].

The main natural sources of HT are table olives and olive oil which, together with other phenolic compounds such as tyrosol and oleuropein, contribute to the organoleptic features of this product besides the benefits to human health [15,16]. However, its content is greatly variable as demonstrated by del Monaco et al. in a set of analyses of Italian extra virgin olive oils (from 0.18 ± 0.005 mg/kg cv Lavagnina, Liguria to 17.00 ± 0.405 mg/kg cv Cerasuola, Sicily) [17].

Additional sources of HT are represented by several by-products of olives generated in oil mills during the production of olive oils such as olive leaves, olive mill wastewater (OMWW), pomace, and olive oil dregs (OOD).

HT is currently used as an active ingredient in cosmetic formulations and has a high commercial value. Several methods have been proposed for HT synthesis, such as chemical, electrochemical, and enzymatic but they are usually time-consuming, costly, or produce low yields [18–20]. To overcome this gap, HT-rich waste can be considered a cheaper and alternative way to obtain this phenolic compound.

A vast scientific literature concerning the recovery of HT from olive oil by-products is available, attesting the great interest in this valuable molecule and its production from inexpensive sources; in particular, from leaves through the bioconversion of oleuropein (its precursor) into HT and from OMWW by the means of solvent extractions or sequential membrane filtration techniques [21–23]. Very recently, it was demonstrated that OOD, an underexploited waste from oil mills not considered for HT production up to now, can be regarded as a low-cost and easily available source for HT recovery [24]. OOD are the semi-solid sediment deposited on the bottom of tanks containing unfiltered extra virgin olive oils. It is made of residual oil, water, polyphenols, cellulose, and small impurities (1 kg OOD from 100 kg of olives). Shepherds use OOD to grease cheeses and to cure the infections of wild animals. However, the utilization of OOD can be directed to more valuable uses because it contains not negligible amounts of HT, even higher than those estimated in olive oils. In our previous study, an OOD extract prepared by a simple and eco-sustainable method contained 363.83 ± 6.35 mg HT/kg OOD [24].

The aim of the present work is to valorize the underutilized OOD by obtaining an HT-enriched sample to use as an active antioxidant ingredient, and to create awareness among olive oil producers about the potential of OOD thanks to the presence of noteworthy bioactive molecules.

Two resins with different features were used for HT purification from the raw extract prepared as previously described [24], and three diverse chromatographic conditions were tested for each resin. The antioxidant power of the HT-enriched fractions was tested by measuring the radical scavenging activity (RSA), the superoxide scavenging activity (SSA), and the ferric reducing antioxidant power (FRAP). To the best of our knowledge, this work is the first to report an HT enrichment procedure from OOD.

2. Materials and Methods

2.1. Chemical

Chemicals needed for the estimation of the total phenolic content (Folin–Ciocalteu reagent, Na_2CO_3, and gallic acid), antioxidant power assays (2,2-diphenyl-1-picrylhydrazyl-DPPH, ascorbic acid, pyrogallol, Tris-HCl, EDTA-Na_2, 2,4,6-tripyridyl-S-triazine-TPTZ, $FeCl_3 \cdot 6H_2O$, and butylatedhydroxytoluene-BHT), High-Performance Liquid Chromatography (HPLC) pure standard (HT), activated charcoal, and Amberlite resins XAD7HP and XAD16N were purchased from Sigma-Aldrich Co. (Milano, Italy). Glacial acetic acid and absolute ethanol (99%) were purchased from Carlo Erba (Rodano, Milan, Italy). HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany). HPLC-grade water (18.2 MΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA, USA).

2.2. Olive Oil Dregs and Extract Preparation

OOD were kindly provided by EVO Campania, an oil mill, located in southern Italy (Campagna, Salerno). They were aliquoted and frozen at −80 °C to avoid any degradation and were thawed before use.

In a previous study that aimed to optimize the extraction of phenolic compounds from OOD by an eco-sustainable method, different extraction temperatures and times were tested using acidified water as the solvent [24]. The best extraction method, which gave the highest HT/TPC ratio and radical scavenging activity, was selected for the preparation of the extract of the present work. OOD were mixed with acidified water (pH 1.25) at 1:2 ratio (OOD (g): extraction solvent (mL)). The resulting mixture was incubated under continuous stirring for 1 h at 37 °C and then centrifuged at 13,200 rpm for 1 h at 4 °C. The aqueous phase was recovered and filtered through a 0.45 µm pore size filter for further clarification. The sample was stored at 4 °C until analysis.

2.3. HT Recovery from OOD

2.3.1. Activated Charcoal

An adsorption/desorption test using activated charcoal (AC) at 5% (*w*/*v*) was carried out by adding AC (500 mg) to the raw extract (10 mL). The mix was incubated at 30 °C for 1 h with continuous stirring and then centrifuged at 13,200 rpm at 4 °C for 1 h. The AC was separated from the supernatant, and the phenolic compounds adsorbed on AC were desorbed by using the following eluents (10 mL): water, ethanol, and ethanol/1% HCl (70:30). Each desorption test was performed at 65 °C for 1 h under continuous stirring. The mixture was centrifuged at 13,200 rpm at 4 °C for 1 h, and the supernatant was recovered. The total phenolic content and HT amount were measured in all supernatants as reported below.

2.3.2. Amberlite Resins

A glass chromatography column (10 mm × 46 cm) was packed with Amberlite XAD7HP or XAD16N. The resins were preconditioned by extensive washing in methanol and equilibrated in acidified water (pH 1.25) before loading the sample.

The raw extract (1 mL) was loaded onto the column and washed with two beds of acidified water to remove the unadsorbed molecules.

Then, the following three different chromatographic conditions were tested with both resins in order to elute the highest amount of HT: Method 1) elution with 50% ethanol in acidified water (3-bed volumes);

Method 2) elution with 25% ethanol in acidified water (3-bed volumes) followed by 50% ethanol in acidified water (3-bed volumes); Method 3) elution with 25% ethanol in acidified water (4-bed volumes). Total phenols and HT were measured in the fractions as reported below.

The fractions containing HT, obtained from the same eluent, were pooled and dried. Ethanol was removed under a nitrogen stream and the water was lyophilized.

2.4. Total Phenolic Content

The total phenolic content (TPC) was estimated by the Folin–Ciocalteu assay [25]. A proper amount of sample was diluted with ultrapure water up to 150 µL in 2 mL Eppendorf tubes; then, Folin–Ciocalteu reagent was diluted 1:10 with ultrapure H_2O (750 µL), and 7.5% (*w*/*v*) Na_2CO_3 (600 µL) were added in the described order. The tubes were rapidly shaken and incubated at room temperature for 2 h. The absorbance was recorded at 765 nm (Thermo Scientific spectrophotometer, Genesys 180 model, Rodano, Milan, Italy) against a blank made of ultrapure water (150 µL). Quantification was obtained by a calibration curve ($y = 0.075x$; $r^2 = 1.0$) built with increasing quantities of a standard solution of gallic acid. The standard compound was dissolved at 1 mg/mL in ultrapure water, and the reference curve was obtained by analyzing the following increasing amounts (1.5, 3.0, 5.0, 6.0, and 8.0 µg). The results were expressed as mg of gallic acid equivalent (GAE).

2.5. Identification and Quantification of HT by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

HT was identified and quantified in the raw extract and the fractions by RP-HPLC using a Dionex Ultimate 3000® HPLC system equipped with a quaternary pump and an Ultimate 3000® Diode Array Detector (ThermoFisher Scientific, Waltham, MA, USA). After filtration by a 0.22 µm pore size filter, the samples were pumped through a Phenomenex Luna C18 (2) column (250 × 4.6 mm, 5.0 µm), equipped with a SecurityGuard™ pre-column containing a C18 cartridge (Phenomenex, Torrance, CA, USA). HT was eluted according to the following method: flow rate fixed at 700 µL/min; solvent A: 0.5% acetic acid in degassed ultrapure water; solvent B: 0.1% acetic acid in degassed ultrapure water/acetonitrile (1:1 *v*/*v*); from min 0 to min 5 stable flow at 5% of B, from min 5 to min 55 a linear gradient reaching 55% of B, from min 55 to min 65 a linear gradient reaching 95% of B followed by 10 min of maintenance. HT was identified by comparison with the retention time and the absorption spectrum of a pure commercial standard. The amount in the analyzed samples, expressed as µg HT, was estimated by means of a calibration curve ($y = 26.862x$; $r^2 = 0.9975$) built by injecting increasing quantities of HT standard solution. The standard compound was dissolved at 1 mg/mL in ultrapure water, and the reference curve was obtained by analyzing four increasing amounts (0.3, 1.0, 2.5, and 5.0 µg). The suitability of the method applied was defined by determining the limit of detection (LOD) and limit of quantitation (LOQ) in accordance with the ICH guidelines (1994) [26].

2.6. Antioxidant Activity

The liquid raw extract was directly used for the antioxidant activity determination, whereas the dried HT-enriched pool obtained from XAD7HP by Method 3 was solubilized in ethanol/acidified water (1:1) at 5.9 mg/mL for the RSA and SSA assays, and at 0.59 mg/mL for the FRAP assay.

2.6.1. Radical Scavenging Activity

The free radical scavenging activity (*RSA*) was measured by the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) assay, as described in Squillaci et al. [24]. Briefly, a proper amount of sample containing 5 µg GAE was diluted with ultrapure water to 150 µL. This solution was mixed with 60 µM DPPH in methanol (1.35 mL). The antioxidant activity was recorded for 30 min at 517 nm against a control made of 60 µM DPPH in methanol (1.35 mL) and ultrapure water (150 µL). The blank solution used to set to zero the instrument was made of pure methanol (1.50 mL). The *RSA* was calculated using the formula:

$$RSA\ (\%) = \left(1 - \frac{Absorbance_{sample}}{Absorbance_{control}}\right) \times 100 \quad (1)$$

and compared to the *RSA* of 5 μg GAE of BHT used as the antioxidant reference.

2.6.2. Superoxide Scavenging Assay

The superoxide scavenging activity (*SSA*) was measured by the pyrogallol autoxidation method with minor modifications [27]. A proper amount of sample containing 50 μg GAE was mixed with 0.05 M Tris-HCl, pH 7.4, and 1 mM EDTA-Na_2 buffer solution in a quartz cuvette. Then, 60 mM pyrogallol in 1 mM HCl (17 μL) was added to the solution. The solution was rapidly stirred, and the absorbance was recorded every 30 s, up to 300 s, at 325 nm against a blank containing 0.05 M Tris-HCl, pH 7.4, and 1 mM EDTA-Na_2 (1 mL). One mL of buffer solution containing 60 mM pyrogallol in 1 mM HCl (17 μL) was used as a control and subjected to absorbance measurement at 325 nm for the same time of assay. The scavenging ability of the superoxide anion $^{\bullet}O_2^-$ was calculated according to the formula:

$$SSA\ (\%) = \left(1 - \frac{Absorbance_{sample}}{Absorbance_{control}}\right) \times 100 \quad (2)$$

and compared to the *SSA* of 5 μg GAE of BHT used as the antioxidant reference.

2.6.3. Ferric Reducing Antioxidant Power

The ferric reducing antioxidant power (FRAP) assay was performed according to Fernández-Agulló et al. [28]. Briefly, FRAP solution, made of 300 mM sodium acetate buffer, pH 3.6 (A), 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) in 40 mM HCl (B), and 20 mM $FeCl_3 \cdot 6H_2O$ (C) was mixed in a 10:1:1 (A:B:C) ratio at the time of use. A proper volume of the samples, containing 0.01 mg, was diluted to 50 μL and added to FRAP solution (1.5 mL). Four minutes after incubation, the absorbance was recorded at 593 nm against a blank prepared with FRAP solution. The FRAP was calculated using a calibration curve built with increasing amounts of ascorbic acid. In detail, the standard compound was dissolved 1 mg/mL in ultrapure water, and the calibration curve (y = 0.265x; r^2 = 0.9989) was obtained by analyzing the following amounts: 0.5, 1.0, 2.0, 3.0, 5.0, and 6.0 μg. FRAP values were compared with the antioxidant power obtained from the same amount of BHT, used as the reference compound. The results were expressed as μg ascorbic acid equivalent (AAE)/mg sample and μg AAE/mg standard.

2.7. Statistical Analysis

Analyses were performed in triplicate and expressed as mean standard deviation (SD) calculated by Microsoft Excel. Experimental data were analyzed using GraphPad Prism (version 5). Significant differences were determined by one-way (antioxidant assays) and two-way (chromatographies) analyses of variance (ANOVA) completed by Bonferroni post-tests. Mean values were considered significantly different at $p \leq 0.05$.

3. Results and Discussion

3.1. Preparation of the OOD Extract and HT Recovery

OOD from EVO Campania oil mill were subjected to extraction in acidified water at 37 °C for 1 h. TPC and HT in the raw extract were 6.24 ± 0.10 mg GAE/g OOD and 532.98 ± 5.78 μg/g OOD, respectively.

Activated charcoal (AC) is widely used for the detoxification of several kinds of samples such as lignocellulosic biomass hydrolysates and wastewaters, but few descriptions are available about the recovery of the adsorbed compounds for following uses [29]. However, as a phenolic compounds recovery of 70.3% was previously obtained from AC used for the detoxification of a chestnut shell hydrolysate [30], an adsorption/desorption process using AC was performed in order to recover HT from the raw extract. The total phenolic compounds contained in the extract were completely adsorbed on charcoal. The desorption

yield was extremely low with all the eluents used (water, ethanol, and acidified ethanol); furthermore, HT was totally absent in the desorbed phenolic mixtures (data not shown).

Therefore, the raw extract was subjected to column chromatography by using two different resins: the non-polar Amberlite XAD16N and the weakly polar Amberlite XAD7HP. Ethanol at 25% and 50% (*v*/*v*) in acidified water was used as the eluent with both resins. This solvent was selected because it is a recognized GRAS solvent.

Fractions eluted with the same solvent were pooled and then analyzed for HT and total phenols quantification. Data obtained by means of Amberlite resins are reported in Table 1. Elution by Method 1 applied to XAD7HP allowed for recovering 44.23% of the total phenols and 57.90% of the HT with an HT/TPC ratio of 11.15%. The same method applied to XAD16N provided 55.45% of total phenols and 82.43% of HT with an HT/TPC ratio of 12.66%.

Table 1. HT purification on Amberlite XAD7HP and XAD16N.

	Chromatographic Conditions (Elution Steps)	TPC (mg GAE)		HT (μg)		HT Yield (%)		HT/TPC (%)	
		XAD7HP	XAD16N	XAD7HP	XAD16N	XAD7HP	XAD16N	XAD7HP	XAD16N
Raw extract (1 mL)	None	3.12 ± 0.14		266.49 ± 2.89		100		8.54	
Method 1	50% ethanol in acidified water	1.38 ± 0.03 a	1.73 ± 0.00 e	154.30 ± 14.80 a	219.66 ± 20.20 e	57.90	82.43	11.15	12.66
Method 2									
Step 1	25% ethanol in acidified water	0.53 ± 0.01 b	0.56 ± 0.01 f	117.17 ± 1.63 b	141.25 ± 3.19 f	43.97	53.00	22.11	25.36
Step 2	50% ethanol in acidified water	0.63 ± 0.01 c	0.39 ± 0.01 g	87.11 ± 2.36 c	54.06 ± 3.13 g	32.69	20.29	13.75	13.69
Method 3	25% ethanol in acidified water	0.76 ± 0.01 d	1.47 ± 0.09 h	246.51 ± 3.34 d	202.74 ± 8.30 e	92.50	76.08	32.44	13.84

TPC: Total phenolic content; GAE: Gallic acid equivalents; HT: hydroxytyrosol. Different letters in the same column denote significant differences at $p < 0.05$ (TPC: XAD7HP a–d; XAD16N e–h. HT: XAD7HP a–d; XAD16N e–g).

Method 2 was designed to foresee two elution steps. The purpose of this method was to verify if the HT elution would have been obtained with a lower recovery of total phenols using two different eluents. Steps 1 and 2 provided lower polyphenols recovery compared to Method 1, but HT was distributed in both eluents reaching yields lower than Method 1.

When the last condition was tested (Method 3), the following percentages of TPC and HT were obtained: 55.07% and 92.50%, respectively, from XAD7HP; 47.12% and 76.08%, respectively, from XAD16N.

A two-way ANOVA analysis allowed us to study the effects of the interactions between the different elution methods and the resins on the yields. For total phenols, the interaction accounts for 13.51% of the total variance. The *p*-value is < 0.0001. Therefore, the interaction is considered extremely significant. The influence of the elution methods on the results obtained accounts for 81.30% of the total variance with a *p*-value < 0.0001. This effect is considered extremely significant. Finally, the influence of the resins on the results accounts for 4.82% of the total variance. The *p*-value is 0.0002, and thus the effect is extremely significant.

In the case of HT, the interaction accounts for 12.38% of the total variance. The *p*-value is 0.0002; thus, the interaction is considered extremely significant. The influence of the elution methods on the results accounts for 86.39% of the total variance. The *p*-value is < 0.0001; this effect is considered extremely significant. The influence of XAD7HP and XAD16N on the results accounts for less than 0.1% of the total variance. The *p*-value is 0.6966, and therefore the effect is considered not significant.

The overall data led us to conclude that the most efficient elution of HT was obtained with 25% ethanol (Method 3). This condition provided 92.50% of HT recovery and 32.44% HT/TPC ratio when Method 3 was applied to XAD7HP resin. Figure 2 shows the chromatograms of the raw extract (A) and the HT-enriched pool (B). In panel B, HT is the main peak detected.

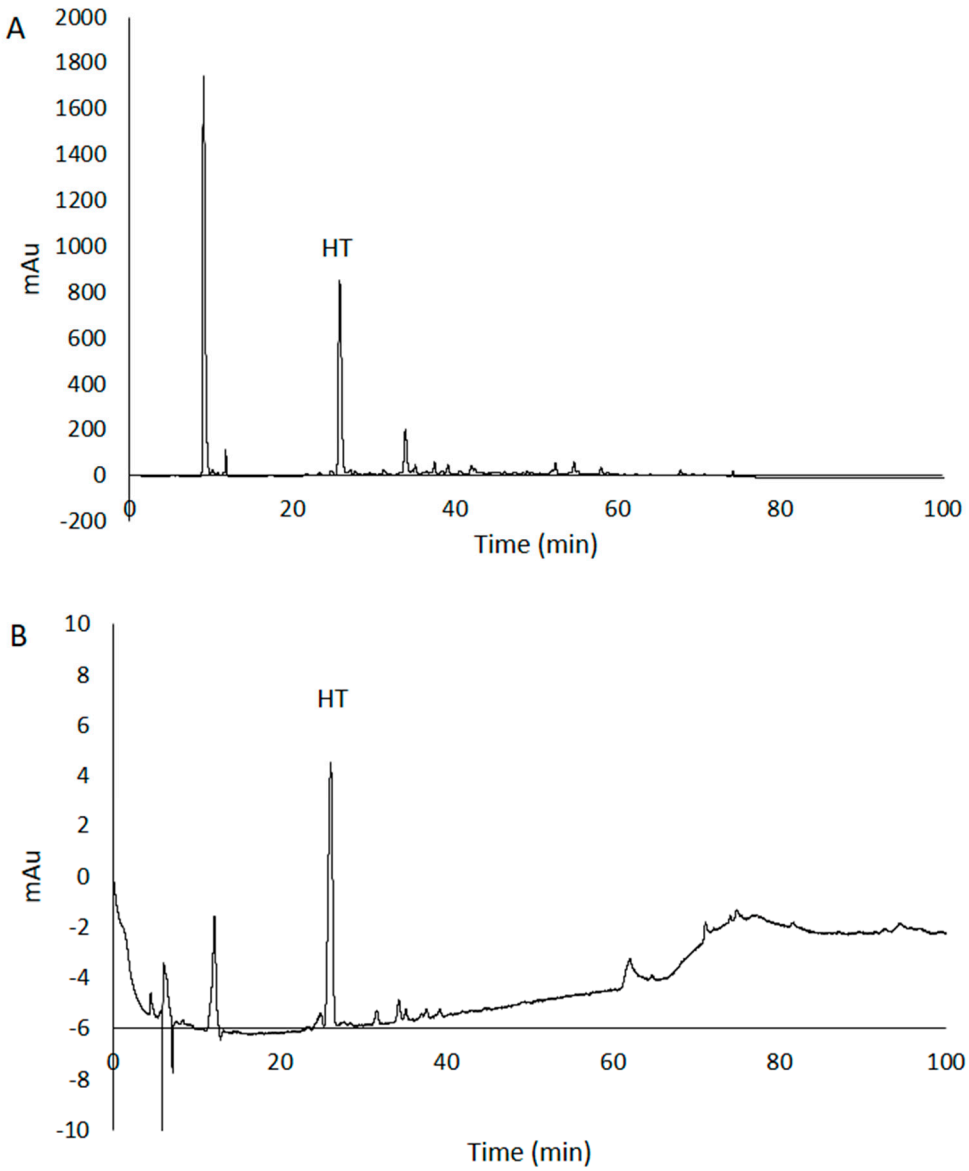

Figure 2. Chromatographic profile of OOD raw extract (**A**) and HT−enriched pool (**B**). HT−enriched pool was prepared by means of XAD7HP resin according to Method 3, as described in Section 2.3. HT: hydroxytyrosol.

Macroporous resins, such as the styrene-divinylbenzene XAD16 and acrylic ester XAD7, are widely used for the recovery and concentration of phenolic compounds from fruit and vegetable raw extracts or phenolic-rich waste such as olive mill wastewater [31,32]. The most diffuse and simple use is the batch mode where a phenolic-rich sample is mixed with the resin under selected operative conditions, and the phenolic compounds are adsorbed on the adsorption matrix. Then, a desorption step through an opportune solvent allows for the recovery of the molecules. Bertin et al. described a solid phase extraction for the removal of phenolic compounds from two olive mill effluents by four different adsorption matrices, including Amberlite XAD16 and XAD7. The resins worked with high efficiency; XAD16 allowed the highest recovery of HT (97%) followed by XAD7 (94%) when ethanol was used as the desorbing solvent. However, almost the total phenols adsorbed onto XAD7 were released with HT, whereas about 60% were desorbed from XAD16 [33].

Several papers describe the use of these resins also as adsorbents for column chromatography. A high-purity anthocyanin mixture from blueberries of *Vaccinium uliginosum* L. was obtained through several chromatographic steps, the first of which was represented

by a chromatographic column containing XAD7 as adsorption resin. Elution with 35% ethanol allowed for the recovery of almost all the anthocyanins from the crude extract loaded [34].

The majority of published papers deal with the use of Amberlite resins for the concentration or recovery of phenolic compounds from complex mixtures, while there is little interest in the recovery of a specific compound or its enrichment by means of XAD resins. In the present paper, we have tested elution with different solvents in order to obtain a pool enriched as much as possible in HT to use as an antioxidant ingredient. Our purpose is justified by the elevated content of HT in this type of by-product (533 mg/kg), comparable to or higher than that contained in other oil mill waste such as OMWW. OMWW from an oil mill located in Isernia (Italy) was extracted with ethyl acetate by continuous (Soxhlet) and discontinuous (separatory funnel) methods. The HT content ranged from 460 to 584 mg/L and from 402 to 538 mg/L in extracts obtained by the first and second procedures, respectively [35]. Furthermore, Abu-Lafi et al. measured an HT content of 371 and 300.22 mg/L in OMWW from Hebron (Southern Palestine) extracted with ethyl acetate and methanol/ethyl acetate (2:1), respectively [36].

3.2. Antioxidant Power of HT-Enriched Pool

With the purpose of investigating the antioxidant power of an HT-enriched preparation, the pool from XAD7HP by Method 3, containing the highest amount of HT and HT/TPC ratio (Table 1), was selected for the estimation of the antioxidant power and the results were compared with those of the raw extract.

HT can exert its antioxidant capacity both as a free radical scavenger and as a metal chelator [37]. Moreover, Visioli et al. demonstrated that HT is also able to scavenge superoxide anions produced by the xanthine/xanthine oxidase system or by the neutrophil-based generation method [38]. Because of this, the following three assays were chosen: radical scavenging activity (RSA), superoxide scavenging activity (SSA), and ferric reducing antioxidant power (FRAP). The RSA and SSA assays measured the capacity to counteract the harmful effects of free radicals by using the stable radical DPPH$^{\bullet}$ and the superoxide radical anion $^{\bullet}O_2^-$, respectively, whereas FRAP evaluated the ability of an antioxidant of reacting with ferric ions.

The antioxidant power of the HT-enriched pool was evaluated as a function of its radical scavenger capacity by measuring the reduction of the stable free radical DPPH$^{\bullet}$ through the disappearance of the purple colour at 517 nm. It showed a high RSA value, reaching 92.83% $\pm$ 0.44 after 30 min of the assay. This value was much higher than the RSA of the raw extract (44.12% $\pm$ 1.82) and higher than BHT chosen as representative of antioxidant compounds (76.07% $\pm$ 0.18) (Figure 3A). Statistically significant differences were measured for the RSA values observed ($p < 0.001$).

The generation of the superoxide anion for the antioxidant power determination can be obtained in several ways. Here, the assay based on the autoxidation of pyrogallol was used for SSA estimation because this method was already used in our previous paper for testing the antioxidant capacity of different OOD extracts [24]; moreover, this assay is fast and cheap. The SSA value measured for the HT-enriched pool after 300 s of the assay was 42.31% $\pm$ 0.86, lower than the SSA value of the raw extract (75.76% $\pm$ 1.78), but higher than BHT (8.10% $\pm$ 2.86) (Figure 3B). Statistically significant differences were measured among all the SSA values observed ($p < 0.001$).

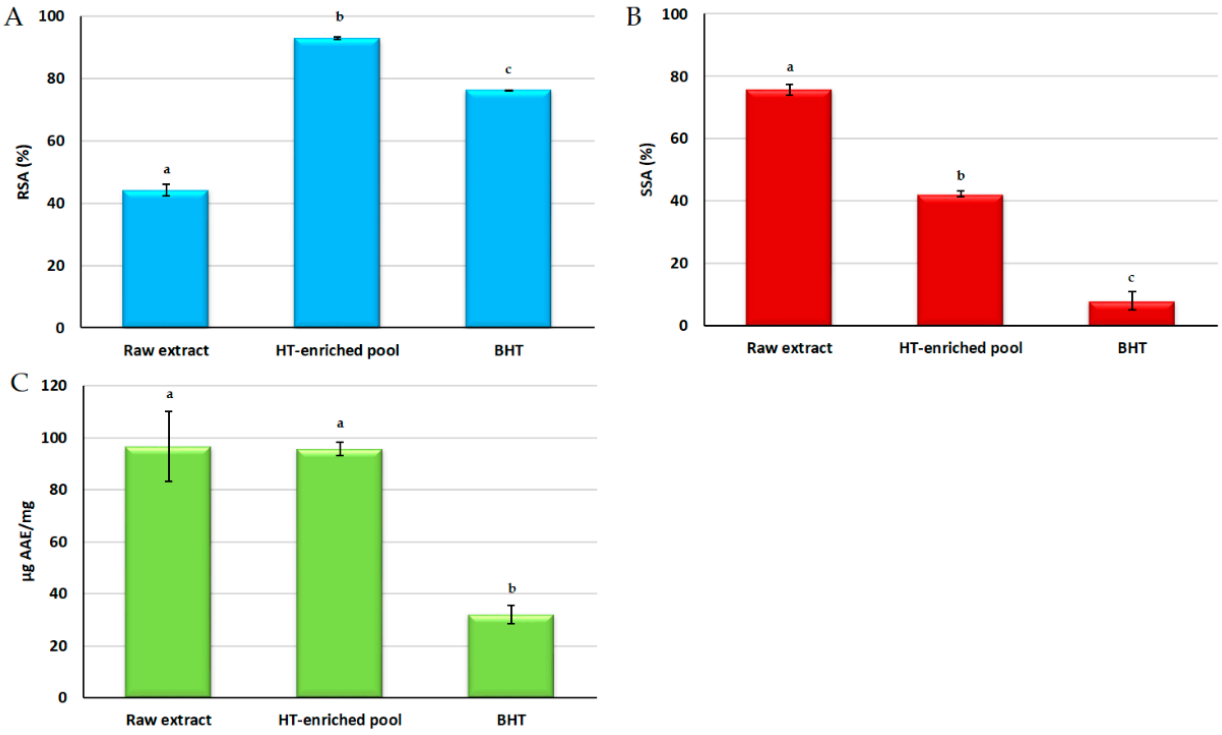

Figure 3. Antioxidant power of OOD raw extract and HT-enriched pool prepared by means of XAD7HP resin, according to Method 3, as described in Section 2.3. RSA: Radical scavenging activity (**A**), SSA: Superoxide scavenging activity (**B**), FRAP: Ferric reducing antioxidant power (**C**). BHT: butylatedhydroxytoluene; AAE: Ascorbic acid equivalent. Bars with different letters indicate significant differences at $p < 0.001$ (RSA and SSA) and $p < 0.0001$ (FRAP).

The HT-enriched pool and the raw extract showed the ability to reduce the ferric ions to the ferrous form to the same extent. In fact, comparable FRAP values were measured (95.71 ± 2.50 and 96.64 ± 13.47 µg AAE/mg for HT-enriched pool and raw extract, respectively) (Figure 3C). No significant statistical differences were observed between the FRAP values of the two samples ($p > 0.05$) but they were more antioxidant than BHT used as a reference compound (32.08 ± 3.46). In fact, highly statistically significant differences were measured between the antioxidant power of the samples and BHT ($p < 0.0001$).

Natural extracts are rich in several kinds of phytochemicals that exert their antioxidant power through diverse mechanisms of action; therefore, it is not easy to find certain explanations for the different behaviors of a blend of molecules towards different antioxidant assays, but hypotheses may be advanced.

The RSA was measured by testing the same amount, expressed as GAE, for the HT-enriched pool and the raw extract. The higher Radical Scavenging Activity displayed by the HT-enriched pool could be probably due to the elevated concentration of HT in the sample. The opposite behavior was observed with the SSA assay. The antioxidant o of the HT-enriched pool was lower than the raw extract. It is evident from the results that the concentration process of HT determines the removal of molecules with high antioxidant power toward the superoxide anion, making HT-enriched pools less active than the raw extract. However, it should be taken in mind that the HT-enriched pool is in any case provided with moderate activity toward the superoxide anion. Concerning the FRAP assay, it is only possible to speculate that HT can be the main responsible factor for the activity of ferric ions for justifying the observed result.

The overall findings demonstrate that the HT-enriched pool is provided with antioxidant activity in all the assays used. Furthermore, under the selected assay conditions its antioxidant power is always higher than BHT (known by the European food additive

number E321). This synthetic molecule was chosen as the reference antioxidant as it is currently used as a preservative in food and cosmetic products rich in oils and fats to prevent their autoxidation, which involves a free radical chain reaction. At present, the permitted concentrations range from 0.01% to 0.04%, depending on the type of food [39]. However, the safety of this antioxidant is discussed [40], and its replacement with natural molecules of equal or superior antioxidant power is of great interest.

BHT is a potent free radical scavenger and many polyphenol-rich extracts show comparable or lower antioxidant activity. Three hydro-alcoholic extracts from leaves of the Chinese plants *Apocynum venetum* L., *Poacynum pictum* (Schrenk) Baill., and *Poacynum hendersonii* (Hook.f.) Woodson were prepared and their RSA was tested by DPPH assay and compared with BHT at the same concentration. The extract from *A. venetum* L. was the only one showing RSA slightly higher than BHT at all concentrations tested (about 94% and 89%, respectively, at the highest concentration), whereas the other extracts had an RSA of about 60% at the maximum concentration used [41]. In addition, the RSA of single compounds was investigated by DPPH assay, in order to find possible substitutes for BHT. The five flavonoids tested (malvin, pelargonin, oenin, callistephin, and silychristin) exhibited RSA comparable to or slightly lower than BHT (96.28%), ranging from 91.07% to 95.57%. Among the three non-flavonoid structures studied, arachidonoyl dopamine and 3,4-dihydroxy-5-methoxybenzoic acid exhibited an RSA higher than BHT (about 98.5%) [42].

In the present paper, the raw extract has an RSA value lower than BHT, but it is shown that the HT enrichment produces a preparation with a free Radical Scavenging Activity superior to both the extract and BHT. This result proves that OOD can be considered for the preparation of an HT-enriched product to be used as an antioxidant in various products.

4. Conclusions

In the present study, the preparation of an HT-enriched pool provided with high radical scavenging activity from OOD was achieved by using a weakly polar resin. The wide distribution of the olive oil industry in the Mediterranean area and the increasing interest of public opinion toward environmental protection and health explain the need for exploiting this waste that is currently scarcely valorized. This fast and simple method applied for the extract preparation, with the high recovery percentage of HT obtained by a GRAS solvent that can be easily distilled and recycled, opens the way for more valuable exploitation of OOD.

In conclusion, the HT-enriched preparation obtained using the approach described herein could be safely used as an antioxidant ingredient in numerous sectors, such as pharmaceuticals, cosmetics, and food. The results obtained confirm that OOD can reasonably be considered as a further low-cost and easily available residue coming from olive mills for the production of high value-added molecules in addition to the most widely utilized OMWW and olive pomace. Furthermore, the olive oil fraction produced during the extract preparation, now discarded, could be used as an ingredient in soaps or other products intended for personal care, thus increasing the interest of olive oil producers in valorizing this by-product.

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