*Article*

# Olive Oil Dregs as a Novel Source of Natural Antioxidants: Extraction Optimization towards a Sustainable Process

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**Abstract:** Olive oil dregs (OOD), which are an underutilized by-product from oil mills, were used for the extraction of antioxidant compounds. The residues from three oil mills located in Campania (Southern Italy) were extracted with acidified methanol, and hydroxytyrosol (HT) was the main phenolic compound detected. Total phenolic content (TPC) and HT amount were measured. EVO Campania oil mill provided the residue with the highest TPC and HT quantities: 6.801 ± 0.159 mg Gallic Acid Equivalents (GAE)/g OOD and 519.865 ± 9.082 µg/g OOD, respectively. Eco-friendly extractions at different temperatures and times were performed on EVO Campania OOD, obtaining 9.122 ± 0.104 mg GAE/g OOD and 541.330 ± 64.087 µg/g OOD for TPC and HT, respectively, at 121 °C for 60 min. Radical Scavenging Activity (RSA), Superoxide Scavenging Activity (SSA), and Ferric Reducing Antioxidant Power (FRAP) were measured in OOD aqueous extracts. Extract prepared at 37 °C for 60 min showed the greatest RSA and SSA values (44.12 ± 1.82 and 75.72 ± 1.78, respectively), whereas extract prepared at 121 °C for 60 min exhibited the highest FRAP value (129.10 ± 10.49 µg Ascorbic Acid Equivalents (AAE)/mg). OOD extracts were able to protect sunflower oil from oxidation for 4 weeks at 65 °C. The overall results suggest that this novel residue can be usefully valorized by providing HT-rich extracts to use as antioxidant agents.

**Keywords:** olive oil dregs; hydroxytyrosol; antioxidant; phenolic compounds; Radical Scavenging Activity; Superoxide Scavenging Activity; Ferric Reducing Antioxidant Power; sunflower oil; extraction process; eco-friendly extraction

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## 1. Introduction

Each year, thousands of tons of agro-industrial wastes are generated globally, creating severe disposal problems and environmental contamination. Hence, a current challenge is to identify attractive solutions for their rational and fruitful exploitation. Because agro-industrial wastes are rich in bioactive compounds, they can be regarded as a cheap and easily available source for the production of high added-value compounds, such as polyphenols and polysaccharides, that can be used in the pharmaceutical, food, and cosmetic sectors [1,2].

Olive oil is one of the main components of the Mediterranean diet, which is well known for its beneficial effects on human health. Italy is the third-largest manufacturer of olive oil in the European Union, after Spain and Greece, producing 277,713 tons in 2018 [3]. The extraction of olive oil is performed through processes yielding large amounts of waste that require disposal: olive mill wastewater (OMWW) (0.2–0.3 and 0.3–1.2 $m^3$/ton of processed olives from two-phase and three-phase extraction processes, respectively), and olive pomace (OP) (740 and 580 kg/ton of processed olives from two-phase and three-phase extraction processes, respectively) [4]. Furthermore, additional wastes are created by

pruning and harvesting procedures, such as leaves, and during the storage period before olive oil is sold, such as the semi-solid residue naturally deposited at the bottom of the unfiltered oils (dregs).

Interest in exploiting olive oil wastes is due to the presence of by-products containing bioactive compounds with valuable properties, particularly phenolic compounds, which are widely recognized as powerful antioxidants with significant therapeutic properties [5]. In addition, they have potential applications as antimicrobial drugs, stabilizing agents in food and cosmetic sectors, fertilizers, and plant protection agents [6]. In addition, the utilization of olive oil waste also minimizes the environmental impact associated with their disposal.

Among olive oil residues, OMWW has attracted the greatest attention from producers and researchers, as evidenced by the vast literature on this subject. OMWW is characterized by a strong olive oil smell, acid pH (3–6), organic compounds (25–45 g/L), high content of phenolic compounds (0.5–24 g/L), and high content of solid matter [7]. Due to the presence of these pollutants, particularly phenolic compounds, OMWW cannot be directly discharged into water or onto land. OMWW is not readily biodegradable because phenolic compounds have toxic effects on aquatic organisms and soil microorganisms. Several treatment procedures have been tested to reduce the undesirable properties [8], and to exploit OMWW as a cheap source of high added-value compounds for application in many fields, including bioenergy production [9,10].

OP is the residue obtained after the olive oil extraction. It is made of peels, kernels, and pulp, which are pressed to form a cake rich in valuable bioactive compounds [11]. OP is also considered a renewable cheap energy source as an alternative to fossil fuels [12].

Olive leaves constitute 10% of the total weight of the olives harvested. They are rich in phenolic compounds, including tyrosol, hydroxytyrosol (HT), oleuropein, quercetin, luteolin-7-O-glucoside, and luteolin-5-O-glucoside, which have many biological activities such as anticancer, anti-inflammatory, and antimicrobial properties [13]. One of the main utilizations of this residue is the extraction of oleuropein, because this secoiridoid is the precursor of HT, one of the most powerful natural antioxidants.

Olive oil dregs (OOD) represent a further residue, constituted by residual oil, water, polyphenols, cellulose, and small impurities that precipitate to the bottom of tanks, resulting in a mix of solid and aqueous waste (1 kg from 100 kg of olives) [14]. After a period of olive oil storage, dregs are separated from the oil by decanting in small olive mills or by filtering at an industrial level. Shepherds usually use OOD to grease cheeses and to treat a number of infections of animals living in the wild; moreover, this residue can be used to extract the oily fraction for the preparation of soaps. However, despite the presence of organic compounds acting as antioxidants, this waste is little considered as an exploitable and cheap source of high added-value phenolic compounds, as witnessed by the scarce scientific literature available. Lozano-Sánchez et al. identified several phenolic compounds endowed with significant activities after pressurized liquid extraction, such as decarboxymethylated and hydroxylated forms of oleuropein and ligstroside aglycones, HT, luteolin, and apigenin [15].

It is known that the healthy properties of phenolic compounds are correlated with their antioxidant power; these molecules have a positive effect on cardiovascular diseases, and are able to reduce the oxidation of low-density lipoproteins due to their ability to scavenge superoxide radicals, which are involved in the pathogenesis of atherosclerosis [16]. Phenolic compounds may also reduce blood pressure, inhibit platelet aggregation, and protect against neurodegenerative disorders [17–19].

Furthermore, cellular studies have demonstrated that olive oil phenolic compounds enhance the proliferation of pre-osteoblasts and the differentiation of osteoblasts, and decrease the formation of osteoclast-like cells; thus, these compounds can be regarded as an effective treatment for the prevention of bone loss and in bone tissue regeneration [20]. In particular, HT exhibits high antioxidant power, and acts as a free radical scavenger and metal chelator. Its remarkable antioxidant capacity is due to the "catechol" structure of the

molecule, which has two hydroxyl groups in the ortho position. This chemical feature has the ability to stabilize the phenoxyl free radicals through the formation of intramolecular hydrogen bonds between the radical oxygen and the adjacent hydroxyl group [21]. It is used as an active ingredient in cosmetic formulations and has a high commercial value. In nature, HT can be found in leaves and fruits of the olive tree (*Olea europea* L.) and is a constituent of olive oil and its processing wastes. Therefore, from a circular economy perspective, the identification of new sources not yet considered can open new possibilities for exploiting existing resources, previously considered waste, to obtain high added-value products. In the current study, eco-friendly extraction processes from HT-rich OOD were investigated for the production of aqueous extracts provided with antioxidant activity. Three OOD from local oil mills were analyzed, and total phenol and HT contents were determined in the extracts. The antioxidant power was also evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide anion, and Ferric Reducing Antioxidant Oower (FRAP) assays. Moreover, the protective capacity of the extracts against sunflower oil oxidation during storage was also investigated.

## 2. Materials and Methods

### *2.1. Chemical*

Chemicals needed for the phenolic content determination (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, $FeCl_3 \cdot 6H_2O$, butylatedhydroxytoluene-BHT), and High-Performance Liquid Chromatography (HPLC) standard (hydroxytyrosol-HT) were purchased from Sigma-Aldrich Co. (Milano, Italy). HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany). Glacial acetic acid was purchased from Carlo Erba (Rodano, Milan, Italy). 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*

OOD necessary to carry out the activities were kindly provided by the following oil mills located in the Italian region of Campania: Basso oil mill (San Michele di Serino, Avellino), Dell'Orto oil mill (Oliveto Citra, Salerno), and EVO Campania oil mill (Campagna, Salerno). Once in the laboratory, the samples were aliquoted and frozen at −80 °C to avoid any degradation activity until their use. After thawing, they were treated and analyzed as follows.

### *2.3. Extractions of Antioxidants from OOD*

Three different methods (1, 2, and 3) of extracting antioxidants from OOD were applied. Method 1 [22] with some modifications was used to select the sample with the highest content of phenolic compounds and HT among the three available wastes. It involved the use of organic solvents. The other two methods (2 and 3) represent optimizations designed to avoid the use of organic solvents.

Method 1: OOD was mixed with *n*-hexane (1:4 $w/v$) and stirred at room temperature for 20 min to promote the defatting of the sample. After organic phase removal, the residual waste was mixed with methanol/1% HCl (70:30) or acidified water (pH 1.25–1.27) in a 1:1 ratio (OOD original weight (g): extracting solution (mL)). The mixture was stirred at 37 °C for 30 min and then centrifuged at 13,200 rpm at 4 °C for 60 min. The methanol or aqueous phase was recovered and further clarified by filtering the solution through a 0.45 µm pore size filter. The sample was stored at 4 °C until analysis.

Method 2: The highest polyphenol-rich OOD was mixed with acidified water (pH 1.25–1.27) in a 1:1 ratio (OOD original weight (g): extracting solution (mL)). The mixture was stirred at 37 °C for 30 min and then centrifuged at 13,200 rpm at 4 °C for 60 min. The aqueous phase was recovered and further clarified by filtering the solution using a 0.45 µm pore size filter. The sample was stored at 4 °C until analysis.

Method 3: The highest polyphenol-rich OOD was mixed with acidified water (pH 1.25–1.27) in a 1:2 ratio (OOD original weight (g): extracting solution (mL)). The mixture was stirred at 37, 83, and 121 °C for 30 or 60 min and then centrifuged at 13,200 rpm at 4 °C for 60 min. The aqueous phase was recovered and further clarified by filtering the solution through a 0.45 μm pore size filter. The sample was stored at 4 °C until analysis.

### 2.4. Total Phenolic Content

The Total Phenolic Content (TPC) in the extracts was determined by the Folin–Ciocalteu assay [23]. An appropriate amount of each sample was diluted up to 150 μL with deionized water and placed in 2 mL Eppendorf tubes; then, 750 μL of the Folin–Ciocalteu reagent, diluted 1:10 with deionized $H_2O$, and 600 μL of 7.5% (*w/v*) $Na_2CO_3$ were added in the described order. After adding the reagents, the tubes were quickly shaken and incubated in the dark for 2 h at room temperature. The absorbance was read at 765 nm (Thermo Scientific spectrophotometer, Genesys 180 model, Rodano, Milan, Italy) against a blank prepared using 150 μL of deionized water.

Quantification was obtained from a calibration curve built with increasing quantities of a standard solution of gallic acid (range 1.5–10 μg). The results were expressed as mg of Gallic Acid Equivalents (GAE)/g OOD.

### 2.5. Identification and Quantification of Hydroxytyrosol by Reverse Phase High-Performance Liquid Chromatography

HT, the main phenolic compound present in OOD, was identified and quantified by Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) using a Dionex Ultimate 3000® HPLC system equipped with quaternary pump and an Ultimate 3000® Diode Array Detector. OOD extracts were filtered and pumped through a Luna C18 (2) column (250 × 4.6 mm, 5.0 μm, Phenomenex, Torrance, CA, USA) equipped with a SecurityGuard™ pre-column containing a C18 cartridge. The elution method was as follows: 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 comparing the retention time and the absorption spectrum of a pure commercial standard. The content in the extracts was measured by means of a calibration curve obtained by eluting fixed amounts of the standard compound (range 0.3–5 μg) and expressed as μg HT/g OOD.

### 2.6. Antioxidant Activity

#### 2.6.1. DPPH Assay

The free Radical Scavenging Activity (RSA) of the extracts was measured by the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^\bullet$) assay as described in Squillaci et al. [24]. Briefly, an appropriate volume of sample containing 5 μg GAE was diluted in deionized water to reach a volume of 150 μL. This solution was mixed with 1.35 mL of 60 μM DPPH in methanol. The antioxidant activity was followed at 517 nm for 30 min, against a control solution consisting of 150 μL of deionized water instead of the sample. The blank solution used to zero the instrument was made up of 1.50 mL pure methanol. The RSA was calculated using the formula:

$$\text{RSA (\%)} = (\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Sample}} / \text{Absorbance}_{\text{Control}}) \times 100 \quad (1)$$

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

#### 2.6.2. Superoxide Scavenging Assay

The Superoxide Scavenging Activity (SSA) of the extracts was determined by the pyrogallol autoxidation method [25] with minor changes. An appropriate amount of sample containing 50 μg GAE was mixed in a quartz cuvette for a spectrophotometer with

0.05 M Tris-HCl, pH 7.4, 1 mM EDTA-$Na_2$ buffer solution. Then, 17 µL of 60 mM pyrogallol in 1 mM HCl was added to the solution. The resulting mix was quickly stirred and its absorbance recorded at 325 nm every 30 s, for 300 s, against a blank containing 1 mL of 0.05 M Tris-HCl, pH 7.4, 1 mM EDTA-$Na_2$. As a control, 1 mL buffer solution containing 17 µL of 60 mM pyrogallol in 1 mM HCl was prepared. The control solution was also 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:

$$\text{SSA (\%)} = (\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Sample}} / \text{Absorbance}_{\text{Control}}) \times 100 \quad (2)$$

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

#### 2.6.3. Ferric Reducing Antioxidant Power

Ferric Reducing Antioxidant Power (FRAP) assay was performed according to Fernández-Agulló et al. [26]. Briefly, the assay solution, containing 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 10:1:1 (A:B:C) ratio at the time of use. Extracts containing 0.01 mg were diluted to 50 µL and added to 1.5 mL of FRAP solution. After 4 min incubation, the absorbance was read at 593 nm against a blank made of FRAP solution. FRAP was calculated using a calibration curve built with amounts of ascorbic acid ranging from 0.5 to 6 µg, and compared with FRAP values obtained with 5 µg GAE of BHT used as the reference compound. The results were expressed as µg Ascorbic Acid Equivalents (AAE)/mg extract and µg AAE/mg standard.

### *2.7. Preparation of Sunflower Oil Samples and Determination of $K_{232}$ and $K_{270}$ Values*

The preparation of sunflower oil samples enriched with OOD extracts was carried out as follows: sunflower oil was mixed with an amount of each tested extract corresponding to 800 µg GAE/g oil. A positive control, containing 0.02% BHT, and a blank, without any addition, were also prepared. All samples were stored at 65 °C for four weeks. At the start of the test and for each week, $K_{232}$ and $K_{270}$ values were measured as described below: an amount of sunflower oil sample was diluted to 1% in *n*-hexane and absorbance at 232 and 270 nm was recorded.

### *2.8. Statistical Analysis*

All tests 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 analysis of variance (ANOVA) completed by Bonferroni post-tests. Mean values were considered significantly different at $p \leq 0.05$.

## 3. Results and Discussion

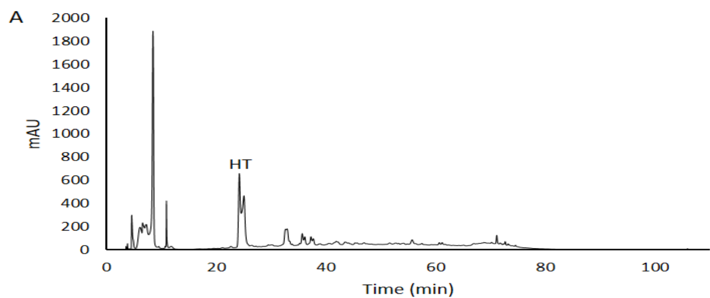
### *3.1. Selection of OOD with the Highest Polyphenol and Hydroxytyrosol Contents*

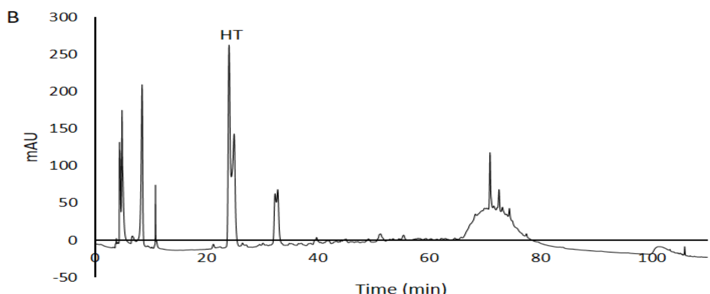
To select the most suitable residue for further studies, the three OOD received in the laboratory were subjected to extraction of the phenolic compounds using Method 1. The residue supplied by EVO Campania oil mill was the best in terms of polyphenol yield, with a TPC released during extraction of 6.801 ± 0.159 mg GAE/g OOD (Table 1). OOD from Basso oil mill provided 6.487 ± 0.249 mg GAE/g OOD, whereas the residue from Dell'Orto oil mill contained the lowest quantity of phenolic compounds (1.915 ± 0.104 mg/g OOD). The three extracts were also analyzed by RP-HPLC to identify and quantify, if present, HT. This was represented by the main peak in the chromatograms (Figure 1), and all extracts showed a notable HT content. As well as for TPC, the extract from EVO Campania was the richest in HT, with 519.865 µg/g OOD. HT content in Basso extract was 482.828 ± 38.539 µg/g OOD, whereas the extract from Dell'Orto contained 206.276 µg HT/g OOD. Based on the results obtained, EVO Campania OOD was chosen for the optimization of the extraction process.

**Table 1.** Extraction yields (Method 1–acidified methanol).

| Extract | TPC (mg GAE/g OOD) | HT (μg/g OOD) |
|---|---|---|
| Basso | 6.487 ± 0.249 $^{a}$ | 482.828 ± 38.539 $^{c}$ |
| Dell'Orto | 1.915 ± 0.104 $^{b}$ | 206.276 ± 7.411 $^{d}$ |
| EVO Campania | 6.801 ± 0.159 $^{a}$ | 519.865 ± 9.082 $^{c}$ |

TPC: Total Phenolic Content; HT: hydroxytyrosol; GAE: Gallic Acid Equivalents; OOD: olive oil dregs. Values in the same column followed by the same superscript letter indicate not significant differences at $p > 0.05$.



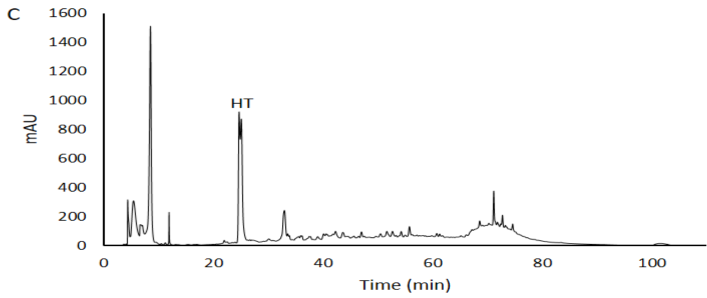




**Figure 1.** Chromatographic profile of OOD extracts from Basso (**A**), Dell'Orto (**B**), and EVO (**C**) oil mills. OOD were extracted according to Method 1–acidified methanol, as described in Section 2.3. HT: hydroxytyrosol.

Comparison with results obtained from other research groups is difficult because the available literature on this topic is scarce. OOD from EVOOs produced in the San Placido production plant (Oleoestepa S.L., Sevilla, Spain) was extracted with MeOH/water in different proportions after defatting with *n*-hexane. The amount of HT ranged from 159.31 ± 14.58 μg/g (MeOH/water 50/50) to 194.67 ± 3.59 μg/g (MeOH/water 75/25) [27]. The OOD analyzed herein showed a higher content of HT, but variability is often linked to environmental factors, which can affect the composition of olives, and to different extraction procedures.

A recent review of the valorization of by-products from the olive oil industry reports several by-products and their possible uses: from olive tree leaves to branches, from OMWW to pomace, from olive stones to skin [28]. No OOD is mentioned among the wastes, thus testifying that this residue is not currently considered to be a source to exploit, even though it contains bioactive molecules of great value.

### 3.2. Optimization of the Extraction Method

As a source of phenolic compounds with high antioxidant power, OOD is scarcely investigated in the literature [27]. In the few works reported, the extraction process of these molecules is conceived from a laboratory perspective, and attempts to maximize yields at the expense of the environmental sustainability of the processes and their industrial feasibility, particularly in agricultural contexts, such as oil mills. The use of solvents such as methanol or hexane in these fields is substantially impractical.

Thus, the present work aimed to eliminate steps involving the use of substances that are dangerous for the environment and humans. EVO Campania OOD, the richest waste in terms of phenolic compounds and HT obtained using Method 1, was taken as a benchmark for the optimization of the extraction process in terms of environmental sustainability.

In the first step of the optimization, a second set of extractions from EVO Campania OOD was established. In this experiment, TPC and HT yields, obtained from OOD extracted by Method 1 with acidified methanol, were compared with the yields from extraction carried out by the Modified Method 1, in which the methanol was replaced with an equal quantity of acidified water (pH 1.25, the same pH value of the methanol phase). TPC and HT yields are shown in Table 2.

**Table 2.** Extraction yields obtained using different methods.

| Extraction Method | TPC (mg GAE/g OOD) | TPC Gap from Method 1 (%) | HT (μg/g OOD) | HT Gap from Method 1 (%) |
|---|---|---|---|---|
| Method 1 (acidified methanol) | $4.818 \pm 0.059$ [a] | - | $507.547 \pm 17.910$ [a] | - |
| Modified Method 1 (acidified water) | $2.895 \pm 0.085$ [b,i] | −39.9 | $352.605 \pm 15.670$ [b,f] | −30.5 |
| Method 2 | $2.855 \pm 0.097$ [c,i] | −40.7 | $321.126 \pm 56.933$ [c,f,g] | −36.7 |
| Method 3 | | | | |
| A (37 °C—30 min) | $3.824 \pm 0.030$ [d,j] | −20.6 | $383.298 \pm 24.796$ [d,f,g,h] | −24.5 |
| B (37 °C—60 min) | $3.444 \pm 0.038$ [e,j] | −28.5 | $363.834 \pm 6.348$ [e,f,g,h,i] | −28.8 |
| C (83 °C—30 min) | $4.576 \pm 0.311$ [a,k] | −5.0 | $409.950 \pm 21.908$ [a,f,g,h,i,j] | −19.2 |
| D (83 °C—60 min) | $4.588 \pm 0.034$ [f,k] | −4.8 | $428.510 \pm 5.829$ [a,f,g,h,i,j,k] | −15.6 |
| E (121 °C—30 min) | $6.954 \pm 0.017$ [g] | +44.3 | $538.137 \pm 10.986$ [a,k,l] | +6.0 |
| F (121 °C—60 min) | $9.122 \pm 0.104$ [h] | +89.3 | $541.330 \pm 64.087$ [a,k,l] | +6.6 |

TPC: Total Phenolic Content; HT: hydroxytyrosol; GAE: Gallic Acid Equivalents; OOD: olive oil dregs. Values in the same column followed by the same superscript letter indicate not significant differences at $p > 0.05$. For explanation of methods, see Section 2.3.

The replacement of the methanol in Method 1 with an equal quantity of acidified water led to a decrease in TPC and HT yields of 39.9% ($p < 0.0001$) and 30.5% ($p < 0.001$), respectively.

To pursue the elimination of organic solvents from the extraction process, the next step was to verify the impact of the removal of the defatting phase (*n*-hexane) on the TPC and HT yields. For this purpose, a third set of extractions was performed by applying Method 2 to the OOD provided by EVO Campania. As shown in Table 2, the elimination of the defatting step had a very limited impact ($p > 0.05$) on TPC and HT yields, with a decrease of 1.4% and 8.9%, respectively, compared to Modified Method 1. These results also indicate that the total removal of organic solvents (Method 2) from the extraction process resulted in a loss of 40.7% for TPC ($p < 0.0001$) and 36.7% for HT ($p < 0.001$) compared to the initial values (Method 1–acidified methanol).

In light of the results obtained, the aim was, therefore, to restore as much as possible the extraction yield values obtained by Method 1, by investigating the effect of three variables—OOD/extraction solvent ratio, temperature, and extraction time—while continuing to avoid the use of organic solvents.

For this purpose, a new set of extractions (Method 3), in which the OOD/extraction solvent (acidified water) ratio was set to 1:2, was established. The EVO Campania OOD was extracted at 37, 83, and 121 °C for 30 and 60 min. As shown in Table 2, the increase in the OOD/extraction solvent ratio at 37 °C for 30 min led to a partial return of the TPC and HT extraction yields to those obtained by Method 1. The gaps from Method 1 were −20.6% for TPC ($p < 0.0001$) and −24.5% for HT ($p < 0.05$). By increasing the extraction

time to 60 min, the extraction yields did not improve. They were 28.5% and 28.8% lower than Method 1 for TPC and HT, respectively ($p > 0.05$).

When the extraction temperature was increased to 83° C, a further significant increase in the TPC yield ($p < 0.01$) was obtained; the differences were 5.0% (30 min) and 4.8% (60 min), respectively, compared to Method 1. However, for HT, the differences were 19.2% (30 min) and 15.6% (60 min), respectively. Even in the case of extractions at 83 °C, the increase in the extraction time from 30 to 60 min did not lead to significant increases in terms of yields ($p > 0.05$).

A final set of Method 3 experiments consisted of extractions at 121 °C for 30 and 60 min. Under these conditions, TPC yield drastically increased. The gaps from Method 1 were +44.3% at 30 min and +89.3% at 60 min ($p < 0.0001$). The HT content was 6.0% and 6.6% higher than that of Method 1 at 30 and 60 min, respectively, and the statistical analysis did not indicate significant differences in terms of HT yield between the two methods ($p > 0.05$).

It has been previously reported that the degradation of phenolic compounds can occur when temperature is increased [29,30]. The overall findings reported herein show that this is not always true.

Similarly, it is not always the case that, as the extraction time increases, the extraction yield will also increase. This is evidenced by the low differences measured at 30 and 60 min at the same extraction temperature. A similar result was previously observed during the extraction of phenolic compounds from grape canes of typical Italian cultivars. TPC content increased from 10 to 40 min, and then slightly decreased after 60 min of extraction. Such behavior can be explained by Fick's second law of diffusion: the equilibrium between solute concentration in solid and liquid phases is reached after some time, and any further increase in time has no effect [31].

Conventional and non-conventional extraction techniques have been applied by several authors for the extraction of antioxidant compounds from by-products of olive oil industries [32,33]. Olive pomace (OP) is one of the most exploited residues for the production of highly valuable compounds, and the recent widespread trend is to apply eco-friendly extraction processes that avoid or limit the use of organic solvents; this is also the purpose of our work. Chanioti and Tzia compared several non-conventional extraction methods of phenolic compounds from OP, obtained from a Greek oil mill, using water as a solvent at 60 °C [34]. HT was the main phenolic compound detected, together with oleuropein, among the identified molecules in the extracts. The amount of HT ranged from $0.23 \pm 0.01$ to $0.32 \pm 0.00$ mg/g using Ultrasound-Assisted Extraction and Microwave-Assisted Extraction, respectively. These quantities were lower than those measured in the OOD extracts under study in the present paper. Even when OP was extracted with hydro-alcoholic mixtures or with alcohols, the quantity of HT was lower than that found in OOD from EVO Campania. OP from olives harvested in olive groves located in the same area as the EVO Campania oil mill were extracted with methanol after defatting. HT was among the minor components of the phenolic extract because methanol allowed the extraction of less polar compounds compared to water. The amount of HT was low: $10.4 \pm 0.24$ µg/g in OP from "La Pepa" and $8.4 \pm 0.56$ µg/g in OP from "Severini" [35]. The results from OP extractions indicate that OOD is an interesting by-product of olive oil processing that can be considered for the production of antioxidants such as HT. Its content is higher than that of the above cited OP, although it must be emphasized that the composition of oil mill by-products is affected by many environmental and technological factors.

### 3.3. Antioxidant Activity of OOD Extracts

The antioxidant power of the aqueous OOD extracts was investigated by means of different assays because the antioxidant capacity of natural compounds can be due to diverse action mechanisms. Thus, three assays were used for determining the antioxidant capacity of aqueous OOD extracts: Radical Scavenging Activity (RSA), Superoxide Scavenging Activity (SSA), and Ferric Reducing Antioxidant Power (FRAP). The deleterious

effects of free radicals in oxidative processes involving biological systems, foods, cosmetics, and pharmaceuticals are well recognized. Thus, the prevention of the initiation step in the radical chain by scavenging reactive species, such as free radicals, is considered to be an important antioxidant mode of action. Considering these factors, the first two methods were used to measure the capacity of the extracts to counteract the deleterious effects of free radicals by using the stable radical $DPPH^{\bullet}$ (RSA assay) and the superoxide radical anion $^{\bullet}O_2^{-}$ (SSA assay).

In detail, the antioxidant power of OOD aqueous extracts was evaluated by the first assay as a function of their RSA through the evaluation of the discoloration of the purple $DPPH^{\bullet}$ radical. This radical accepts an electron or a hydrogen radical to become a stable molecule. This method represents the choice of numerous scientists for measuring RSA of natural compounds due to its simplicity and reproducibility [36,37]. All of the tested extracts exhibited antioxidant activity, with values of RSA ranging from 36.63% ± 0.11 (A) to 44.12% ± 1.82 (B) (Figure 2A). Statistically significant differences were recorded between these two extracts, which differed in terms of their diverse extraction time (Table 2) ($p < 0.001$). RSA measured for a number of extracts prepared at 83 and 121 °C were not statistically significant ($p > 0.05$). BHT, whose safety has been recently discussed [38], is added as a preservative to foods rich in oils and fats to prevent their oxidation. Here, it was chosen as a representative of antioxidant compounds. Its RSA was higher than that of all of the OOD aqueous extracts tested ($p < 0.0001$).

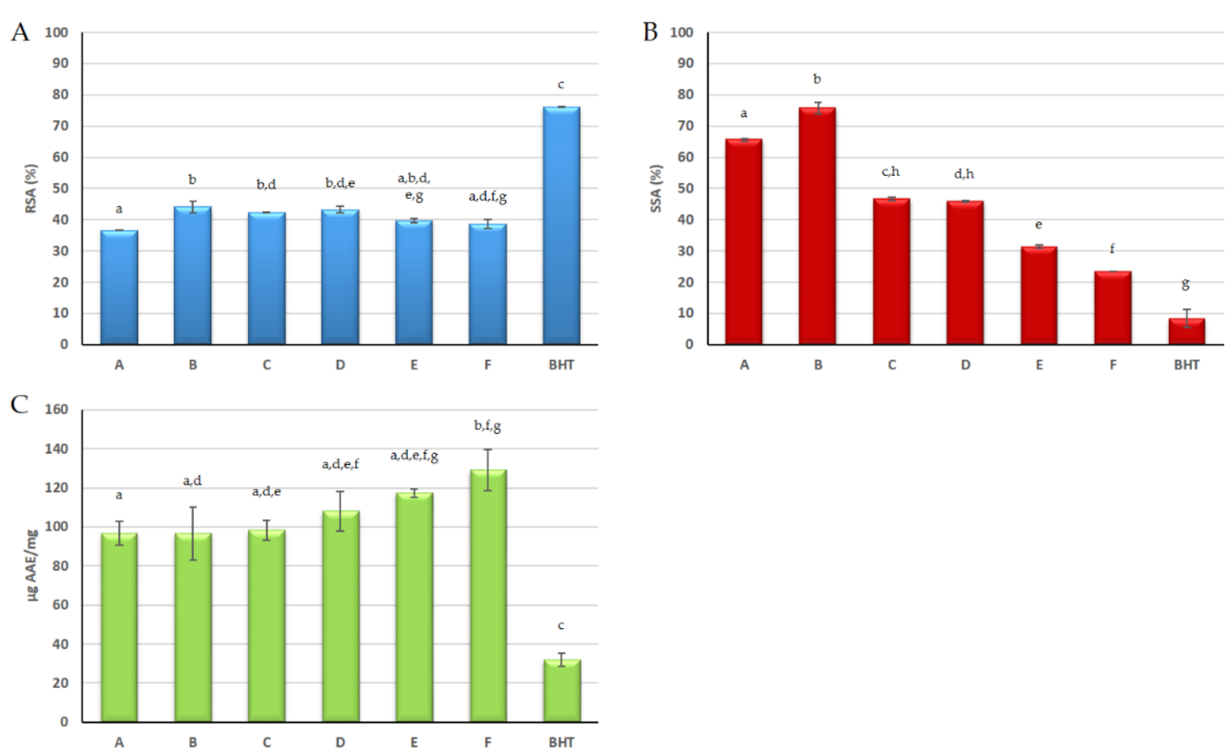



**Figure 2.** Antioxidant power of OOD extracts prepared by Method 3. Radical Scavenging Activity—RSA (**A**), Superoxide Scavenging Activity—SSA (**B**), Ferric Reducing Antioxidant Power (**C**). BHT: butylatedhydroxytoluene; AAE: Ascorbic Acid Equivalents. Bars with the same letter indicate not significant differences at $p > 0.05$.

Although superoxide is a weak oxidant, its combination with molecules such as nitric oxide can generate powerful species that are harmful for human health. The capability of hydrogen donation to the superoxide radical, thus inhibiting the chain propagation reaction, is a measure of the antioxidant activity of the molecules. Several methods are available for the determination of SSA. In this paper, the assay based on the autoxidation of pyrogallol was used because it is relatively easy and cheap; furthermore, this method

has been used for the measurement of the antioxidant power of phenolic compounds by several authors [39]. The consumption of the superoxide radical in the reaction mixture is indicated by a decrease in its absorbance. OOD aqueous extracts exhibited SSA values between 23.33 ± 0.01 (F) and 75.72 ± 1.78 (B) (Figure 2B). The temperature increase led to an SSA decrease from 37 to 121 °C, and results were highly significant ($p < 0.0001$). The derivation of different SSA values from different extraction times is difficult to rationalize because at 37 °C the extract prepared at 60 min showed the highest SSA, whereas extracts prepared at 121 °C showed the opposite result, with SSA of 31.29 ± 0.50 (30 min) and 23.33 ± 0.01 (60 min) ($p < 0.05$). SSA values of C and D were not statistically significant ($p > 0.05$). All OOD extracts had SSA values higher than those of BHT, which was used as the reference antioxidant ($p < 0.0001$).

In both assays, extract B was endowed with the greatest radical scavenging effect, perhaps due to the highest HT/TPC ratio (10.57) among the OOD extracts tested.

The FRAP assay evaluates the antioxidant power of phenolic compounds by measuring the reaction ability with ferric ions that are reduced to ferrous form. OOD extracts exhibited FRAP values higher than that of BHT ($p < 0.0001$) and increased with increasing temperature (Figure 2C). F and B had the highest (129.10 ± 10.49 µg AAE/mg) and the lowest (96.64 ± 13.47 µg AAE/mg) values, respectively ($p < 0.001$), showing the opposite behavior compared to the ability of scavenging free radicals. As the temperature increased, the content of total phenolic compounds and HT in the extracts increased, but the HT/TPC ratio decreased from 10.57 (B) to 5.93 (F). It can be hypothesized that the greater FRAP of OOD extracts prepared at high temperature is due to an enrichment in which phenolic species exert their antioxidant action using a different mechanism than that of HT. HT is known to be a powerful radical scavenger, due to the presence in its chemical structure of two hydroxyl groups in the ortho position [40].

Antioxidant power is related to the phytochemical composition of an extract, and phytochemical composition is affected by pedoclimatic conditions and cultivar variability. RSA values of olive leaf water extracts from 21 cultivars in Turkey were found to be between 52.72% ±1.93 (Uslu) and 82.35% ± 3.18 (Domat) at 100 µg/mL concentration [41], whereas ethanol extracts of olive cake from two oil mills in the Tadla-Azilal region (Morocco) exhibited RSA values of about 35% at 50 µg/mL concentration [42].

OP from Castellar (Jaén, Spain) was subjected to Ultrasound-Assisted Extraction in an ethanol/water mixture. The extract showed a FRAP value of 54.23 ± 3.62 mmol Trolox equivalents/100 g [43]. Although a comparison between the results obtained here and those reported by other groups is difficult, due to differences in expression and the diversity of the investigated by-products, it can be attested that residues from olive plant processing, including OOD, are good sources of antioxidant agents.

### *3.4. Protection Effect of OOD Extracts on Accelerated Oxidation of Sunflower Oil*

The measurement of $K_{232}$ and $K_{270}$ values yields information about oxidative alteration. In detail, absorbance at 232 nm estimates the primary oxidation due to the formation of conjugated dienes, and measurement at 270 nm is related to the presence of secondary oxidation products (conjugated trienes) [44]. Here, the protection effect of OOD extracts from EVO Campania oil mill prepared at different temperatures and times (A–F) on sunflower oil was investigated. To carry out accelerated oxidation tests, samples were stored for 4 weeks at 65 °C, because 24 h of storage at this temperature corresponds to 1 month of storage at usual temperatures [45]. $K_{232}$ and $K_{270}$ values of extract-enriched samples were compared with a BHT-enriched sample (positive control) and sunflower oil without any addition (blank). Extract E showed the highest protection effect after 4 weeks ($K_{232}$ 5.66 ± 0.03, $K_{270}$ 1.43 ± 0.01), followed by C ($K_{232}$ 5.69 ± 0.06, $K_{270}$ 1.62 ± 0.01) (Table 3). However, extracts A–E exhibited statistically significant $K_{232}$ values with respect to the blank ($p < 0.0001$), whereas $K_{270}$ values were significant for all of the extracts ($p < 0.0001$). Furthermore, with the exception of F, the extracts had a protection capacity higher than that of BHT ($K_{232}$ 7.43 ± 0.03, $K_{270}$ 1.74 ± 0.05).

**Table 3.** $K_{232}$ and $K_{270}$ values of sunflower oil samples enriched with OOD extracts obtained by Method 3.

| | $K_{232}$ | | | | $K_{270}$ | | | |
|---|---|---|---|---|---|---|---|---|
| **Extract** | **0** | **2 Weeks** | **3 Weeks** | **4 Weeks** | **0** | **2 Weeks** | **3 Weeks** | **4 Weeks** |
| Blank | 2.87 ± 0.00 | 6.51 ± 0.03 $^{a}$ | 7.72 ± 0.08 $^{a}$ | 7.98 ± 0.06 $^{a}$ | 1.10 ± 0.00 | 1.68 ± 0.02 $^{a}$ | 1.82 ± 0.01 $^{a}$ | 2.15 ± 0.01 $^{a}$ |
| A | | 4.75 ± 0.16 $^{b}$ | 5.35 ± 0.07 $^{b}$ | 6.23 ± 0.01 $^{b}$ | | 1.02 ± 0.01 $^{b}$ | 1.17 ± 0.03 $^{b}$ | 1.62 ± 0.00 $^{b}$ |
| B | | 4.49 ± 0.03 $^{c}$ | 4.94 ± 0.18 $^{c}$ | 5.89 ± 0.03 $^{c}$ | | 0.96 ± 0.01 $^{c}$ | 1.10 ± 0.02 $^{c}$ | 1.83 ± 0.01 $^{c}$ |
| C | | 4.58 ± 0.16 $^{d}$ | 5.07 ± 0.01 $^{d}$ | 5.69 ± 0.06 $^{d}$ | | 1.11 ± 0.01 $^{d}$ | 1.48 ± 0.01 $^{d}$ | 1.62 ± 0.01 $^{d}$ |
| D | | 4.79 ± 0.04 $^{e}$ | 5.11 ± 0.18 $^{e}$ | 6.24 ± 0.03 $^{e}$ | | 1.12 ± 0.01 $^{e}$ | 1.28 ± 0.01 $^{e}$ | 1.84 ± 0.01 $^{e}$ |
| E | | 5.18 ± 0.05 $^{f}$ | 5.28 ± 0.23 $^{f}$ | 5.66 ± 0.03 $^{f}$ | | 1.15 ± 0.13 $^{f}$ | 1.26 ± 0.01 $^{f}$ | 1.43 ± 0.01 $^{f}$ |
| F | | 5.02 ± 0.04 $^{g}$ | 6.2 ± 0.13 $^{g}$ | 7.86 ± 0.08 $^{a}$ | | 1.32 ± 0.01 $^{g}$ | 1.95 ± 0.00 $^{g}$ | 1.90 ± 0.01 $^{g}$ |
| BHT | | 5.96 ± 0.02 $^{h}$ | 6.34 ± 0.04 $^{h}$ | 7.43 ± 0.03 $^{g}$ | | 1.57 ± 0.03 $^{a}$ | 1.72 ± 0.01 $^{h}$ | 1.74 ± 0.05 $^{h}$ |

Blank: sunflower oil without any addition; BHT: butylatedhydroxytoluene. Values in the same column followed by the same superscript letter indicate not significant differences at $p > 0.05$.

Souidi et al. investigated the effect on the oxidative stability of a commercially available lampante olive oil after addition of extracts prepared from olive leaves and pomace obtained from *Olea europea* L., Picholine variety (Morocco) [46]. An accelerated oxidation test was carried out by storing oil samples at 60 °C and $K_{270}$ values were recorded after 2 weeks. Both enriched oils showed a lower $K_{270}$ value in comparison to the control sample, indicating that the added extracts were able to protect from the formation of secondary oxidation products (1.053 ± 0.009 leaves extract-enriched oil, 1.168 ± 0.006 pomace extract-enriched oil, and 1.378 ± 0.008 control oil).

Günal and Turan used OMWW and OP to study the protection power of their extracts against accelerated oxidation conditions in sunflower oil [47]. OMWW and OP methanol extracts at 1 mg/g concentration were able to slow the formation of oxidation products during the storage of sunflower oil at 60 °C for 21 days. $K_{232}$ values of 75.23 ± 0.71 and 81.32 ± 1.28 were measured at day 21 for OMWW and OP, respectively, compared to the 98.25 ± 4.69 value of the blank (sunflower oil), whereas $K_{270}$ values were 4.28 ± 0.21 (OMWW), 3.72 ± 0.19 (OP), and 4.49 ± 0.44 (blank). In contrast to the OOD extracts of the present work, the methanol extracts reported above were less active than BHT in protecting sunflower oil from primary oxidation ($K_{232}$ value of BHT 30.90 ± 2.49).

## 4. Conclusions

In the present study, valorization of OOD, a currently unexploited by-product of olive oil processing, was achieved via the production of HT-rich aqueous extracts that possess antioxidant activities. The diffusion and size of the olive oil industry in the Mediterranean area, and the continuously increasing demand for bio-based antioxidant agents as substitutes for synthetic sources, justify the need to direct this waste to more valuable uses. The extraction method applied here is simple, fast, and easily scalable. Thus, the proposed method could be quickly spread among oil mills, enabling OOD to be used in a more productive application. The extracts prepared at higher temperature contained greater amounts of TPC and HT. Furthermore, a greater quantity of total phenolic compounds was associated with a decrease in HT/TPC ratio, which can increase the time required in the purification of HT. Organic solvents were avoided, thus indicating the improved sustainability of the proposed extraction process.

In conclusion, the extracts produced using the proposed approach could be safely used as antioxidants in several fields, such as the food, pharmaceutical, and cosmetic industries. This proposal represents a response to the growing attention of consumers to their health and the environment, and the increased preference for natural preparations to achieve health benefits.

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