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Antioxidant activity of phenolic extracts from olive mill wastewater and their influence on virgin olive oil stability

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Abstract

Fresh olive oil mill wastewaters (OMW) were collected in northern Morocco from industrial mills operating with three extraction systems (dual [C2] and triple phase [C3] centrifugation decanters and super-pressure system [SP]) during the 2017 crop season. Physicochemical and microbiological characteristics of these effluents were examined. OMW phenolic extracts were prepared and their antioxidant activities were evaluated. Moreover, the improvement of virgin olive oil stability by the addition of OMW phenolic extracts (at 100, 300 and 500 ppm) was investigated. Results from the present work confirmed the pollutant load of these effluents characterized by an acidic pH, strong organic content, and considerable amounts of mineral matter. Concerning the microbiological counts, yeasts and molds were the most abundant. Among extraction system, C2 displayed OMW with the high levels for all measured parameters, except for total phenols that were more abundant in C3. Findings from DPPH and FRAP tests showed a good antiradical potential and an important reducing power capacity and were both concentration-dependent. For the two tests, antioxidant activity of OMW phenolic extracts from C2 was the most important. The addition of phenolic extracts to virgin olive oil, stocked at 60°C, resulted in lower values of PV, K232 and K270 compared to the control, indicating a good protective effect against oil oxidation. The great antioxidant effect of OMW extracts was at 500 ppm, similar to that of ascorbic acid.

Keywords: OMW; Phenolic extract; Antioxidant potential; Olive oil stability.

1. Introduction

The increase in demand for olive oil has made its production more expansive because of its beneficial health properties including anti-oxidant, anti-atherogenic, anti-inflammatory, anti-aging, anti-tumor, anti-viral, anti-cancer and immune modulator activities [1-4]. About 97% of worldwide production is provided by Mediterranean countries [5]. In spite of the socio-economic contribution of olive oil industry for these countries, huge amounts of olive oil mills wastewaters (OMW) are produced annually, estimated to reach 30 million per m^3 [6]. Uncontrolled disposal of these effluents creates a substantial environmental problem. In addition, dispersed distribution of the olive mills and high financial requirement of OMW treatments complicate their management and therefore hinder the sustainable development of the olive oil industry. Therefore, OMW management is becoming nowadays a big challenge for producers. OMW contains 83-92% of water as a major component and large amounts of organic molecules, especially polyphenols, nitrogen compounds, sugars, organic acids and pectins [7-11]. The studies carried out on the OMW effect on soil properties, ecosystems and plant growth have all confirmed that their toxicity is mainly due to the phenolic content [12-15]. OMW, known by their highly polluting and complexing organic load, are also a promising source of bioactive compounds and substances of high value and great interest. As olive oils, they could have some health benefits to be exploiting. Phenolic compounds are one of the most valuable substances that OMW might provide as an interesting alternative to attenuate the negative impact of these effluents [16]. Recently, several studies have focused on the functional properties of OMW phenolic compounds and their possible uses. The antioxidant activity of OMW phenolic extracts was widely studied and the results were encouraging compared to synthetic antioxidants [17-20]. Visioli et al. [21] have evaluated and confirmed the antioxidant and anti-inflammatory activities of OMW phenols extracts. Other excellent biological properties such as antimicrobial and anti-carcinogenic activities of the OMW biophenols are documented [16]. El-Abbassi et al. [20] reported that natural phenols from olives and its by-products are recognized as potential targets for the food, cosmetic and pharmaceutical industries. The production of functional foods, especially olive oils, enriched by phenolic compounds extracted from OMW has also been developed [22-24]. Indeed, phenolic compounds are known to function as preventive agents against oxidative damages (formation of flavour and toxic compounds), because of their ability to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation [19, 25-26]. The objectives of the present study were: i) to investigate physicochemical and microbiological characteristics of fresh OMW as affected by extraction systems, ii) to evaluate antioxidant potentials of OMW phenolic extracts, iii) to try improving the stability of virgin olive oil by adding OMW phenolic extracts.

2.Materials and methods

2.1. Sampling

Fresh olive mill wastewaters (OMW) for this study were collected from three industrial units of olive oil extraction, using two phase [C2] and three phase [C3] decanter centrifugation and super-pressure system [SP]), located in Taza province (34 ° 12'36 "N, 3 ° 52'0" W) (northern Morocco). OMW samples were taken at the end of December 2017, in a closed plastic container, and stored at 4 °C until analysis.

2.2. Physicochemical and microbiological characterization

Hydrogen potential (pH) and electrical conductivity of OMW samples were measured in situ using a multi-parameter type (CONSORT C535, Turnhout, Belgium). Suspended solids were assessed by centrifuging a volume of crude OMW samples during 20 min at 4000 rpm, recovering the pellet and drying it for 24 h at a temperature of 105°C.

Total solids were determined by weighing a sample before and after drying at 105°C. The additional treatment of the dry sludge by calcination for 4 h at 550°C allowed to obtain the organic matter content. The difference between total solids and organic matter was defined as mineral matter. Chemical Oxygen Demand was performed using the open reflux method based on a boiling oxidation (150 °C for 2 hours) of the reducing matter with an excess of potassium dichromate in an acidic medium. The remaining unreduced $K_2Cr_2O_7$ was titrated with ferrous ammonium sulfate to determine the consumed potassium dichromate and the oxidizable matter were calculated in terms of oxygen equivalent. Chlorides content was determined according to the standard using the Mohr method. Total kjeldahl nitrogen was evaluated using the Kjeldahl method: A warm mineralization of OMW samples was carried out in an acidic medium using a catalyst. Ammonia nitrogen (mineralization product) was then moved to ammonia after addition of sodium hydroxide. The ammonia is driven by steam and trapped in boric acid and titrated with hydrochloric acid. All traits mentioned above were analyzed according to Rodier et al. [27]. Total phenols were extracted using the analytical methodology described by De Marco et al. [28]. OMW samples were acidified and washed with hexane in order to remove the lipid fraction. Extraction was then carried out on washed phase with ethyl acetate. The dry residue, obtained after ethyl acetate evaporation, was dissolved in methanol and total phenols content was determined by spectrophotometry (Spectrophotometer JENWAY 6100, Dunmow, Essex, UK) using Folin-Ciocalteau reagent [29] using caffeic acid (Sigma-Aldrich, St. Louis, MO, USA) as standard. Total sugars content was determined spectrophotometrically following the method described by Dubois et al. [30]. Total organic carbon was calculated by dividing organic matter by 1.724 [31]. Protein content was calculated by multiplying the total nitrogen by 6.25 [32]. For microbial count, 1 ml of each OMW sample was diluted tenfold in 10 ml of sterile buffered peptone water (0.1% w/v, pH 7.4) followed by preparation of serial decimal dilutions, using the peptone solution, in order to obtain CFU counts in the range of 30-300 per plate. 100 µl of the corresponding decimal dilutions were inoculated in four different media:

- PCA (Plate Count Agar, Difco), at 30°C for count of Total Aerobic and Mesophilic Flora.
- DPA (Potato Dextrose Agar, Difco), at 30°C for count of Yeasts and Molds.
- MRS agar (Man Rogosa Sharpe, Difco,), at 30°C for count of Lactic Acid Bacteria.
- Macconkey agar (Difco), at 37°C for count of Total Coliform Bacteria.

2.3. Antioxidant activity of OMW phenolic extracts

2.3.1. DPPH radical scavenging activity

The antioxidant activity of the OMW phenol extracts was evaluated by using the stable 2,2-diphenyl-2-picrylhydrazyl radical (DPPH*) according to the method described by El-Abbassi et al. [20]. The hydrogen atom donating ability of OMW phenolic extracts was determined by the decolorization of methanolic solution of DPPH. DPPH produces violet/purple color in methanolic solution and fades to shades of yellow color in the presence of antioxidants [33]. The DPPH* solution was prepared by dissolving 4 mg of DPPH in 100 ml of methanol. A 100 µl of each methanolic solution of OMW phenol extracts at different concentrations (0-400 mg/ml), was added to 3 ml of DPPH working solution in a cuvette. All cuvettes were covered, well shaken, and kept in the dark and the decrease in absorbance was measured at 517 nm after 60 min, using a spectrophotometer (SPECUVIS, UV-Visible) against methanol as a blank.
The radical scavenging activities were calculated according to the following formula:

% Radical scavenging activity = {1 - (Asample⁄Acontrol} × 100

Where A_{sample} is the absorbance of the test sample and $A_{control}$ is the absorbance of DPPH in methanol (3 ml) plus methanol (100 µl) instead of samples.

Synthetic antioxidant reagents (ascorbic and caffeic acids) were used as positive controls for comparison. Measurements were repeated three times. Test sample (or control) concentration providing 50% inhibition (IC50, expressed in µg/ml) was calculated from the graph plotted radical scavenging activities (%) against concentration.

2.3.2. Ferric reducing antioxidant power (FRAP) Assay

FRAP assay is based on the ability of the tested antioxidants (OMW phenols extracts) to reduce ferric iron (Fe^{+3}) present in the [$K_3Fe(CN)_6$] complex to the ferrous form (Fe^{+2}). The reducing power of OMW phenols extract was evaluated according to the method reported by Yen and Chen [34]. A 1 ml of sample solutions at different concentrations (0-100 mg/ml) was mixed with 2.5 ml of phosphate buffer solution (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$] at 1%. The mixtures were incubated at 50 °C for 30 min, then, 2.5 ml of trichloroacetic acid (10%) were added. The whole was centrifuged at 3000 rpm for 10 min. The upper layer of each solution (2.5 mL) was then mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm using a spectrophotometer (SPECUVIS1, UV-Visible) against blank. A typical blank solution contained the same solution mixture without OMW phenols extracts (or controls) was incubated under the identical conditions. Higher absorbance of the reaction mixture indicated higher reducing power. Ascorbic and caffeic acids were used as positive controls. Measurements were repeated three times.

2.4. Olive oil enrichment with OMW phenolic extracts

2.4.1. Virgin olive oils (VOO) characterization

VOO were extracted from olives belonging to 'Moroccan Picholine', and collected in an industrial mill unit using continuous process (three phase decanter) located in Taza province (northern Morocco) during 2017 extraction period. Quality characteristics (free fatty acids, peroxide value and extinction coefficients at 232 and 270 nm) of these oils were determined according to the European Union Commission Regulation EEC/2568/91 [35]. Total phenols were extracted according to the method described by Zunin et al. [36], and the content was determined by spectrophotometry (Spectrophotometer JENWAY 6100, Dunmow, Essex, UK) following the Folin-Cicalteu method [29] using as standard the caffeic acid (Sigma-Aldrich, St. Louis, MO, USA). Content of chlorophyll and carotenoid compounds were determined from absorption of the olive oil dissolved in cyclohexane, at 670 and 470 nm respectively following the method of Minguez-Mosquera et al. [37].

2.4.2. Total phenols extraction from OMW

OMW were drawn from the same three phase decanter unit simultaneously with VOO sampling. Total phenols (TP) were extracted using the analytical methodology described by De Marco et al. [28]. OMW samples were acidified and washed with hexane [1:1, (v/v)] in order to remove the lipid fraction: 10 ml of OMW were mixed with 15 ml of hexane; the mixture was shaken and then centrifuged during 5 min at 3000 rpm. The phases were separated and the washing was repeated successively two times. TP extraction was then carried out with ethyl acetate: OMW samples, preventively washed, were mixed with an equal volume of ethyl acetate; the mixture was vigorously shaken and centrifuged for 10 min at 3000 rpm. The phases were separated and the extraction was repeated successively four times. The ethyl acetate was evaporated and the dry residue was recovered.

2.4.3. Enriched virgin olive oil (VOO) preparation and oxidation system

Weighed quantities of OMW phenolic extracts were dissolved in an appropriate volume of ethanol/water (50/50; v/v) and added at different concentrations (100 ppm, 300 ppm and 500 ppm) to VOO to obtain an enriched-virgin olive oil. OMW phenolic extracts were mixed with virgin olive oil by stirring for 30 min and ethanol traces were evaporated at 37 °C [38]. The same procedure was applied to ascorbic acid (AA) as standard. VOO enriched with OMW phenolic extract and ascorbic acid and VOO without enrichment (control) were stored in the dark at 60 °C for 22 days, during which the stability of oils was evaluated at the initial time and every 7 days by the measurement of peroxide value and conjugated dienes and trienes formation [22]. Peroxide value (PV), K232 and K272 were determined according to the European Union Commission Regulation EEC/2568/91 [35].

2.5. Statistical Analysis

All analytical determinations were performed in triplicate. For OMW characterization, least significant differences (LSD) test was applied at the 5% probability level using the STATGRAPHICS Centurion XVII package. For antioxidant activity and enrichment of virgin olive oil with OMW phenolic extracts, data are expressed as means ± standard deviation and curves were fitted using Microsoft Excel software.

2. Results

2.1. OMW characterization

The microbiological and physicochemical characteristics of the fresh OMW are reported in Table 1. Results confirmed the pollutant load of these effluents characterized by an acidic pH (4.89) related to high content of total phenols (1.75 g/l acid caffeic). High values of chlorides content (0.91 g/l) and consequently an important electrical conductivity (10.18 mS/cm) were highlighted. The investigated OMW also showed a strong organic load, expressed by the high values of chemical oxygen demand (164.09 g O2/l), sugar amount (5.19 g/l) and organic matter (73.13 g/l). Total solids and suspended solids were of 91.25 g/l and 42.97 g/l, respectively. Considerable amounts of mineral matter (18.12 g/l), total kjeldahl nitrogen (0.49 g/l) and proteins (3.04 g/l) are recorded. For microbial counts, total aerobic and mesophilic flora was around 21.89 10^4 CFU/ml, including yeasts and molds were the most abundant (17.76 10^4 CFU/ml), while the counted lactic acid bacteria were approximately 5.04 10^4 CFU/ml. No total coliform bacteria were observed. When comparing among extraction systems for physicochemical characteristics, significant statistically differences were found between C2 in one hand and both C3 & SP in the other hand. C2 displayed OMW with the high levels of all traits, except for total phenols which the great amount was for C3. Between C3 and SP, no very important variation was observed, with the exception for pH, sugars and total kjeldahl nitrogen that were low in C3. For microbiological characteristics, wide variations were observed between the three systems, especially for total aerobic and mesophilic flora, and lactic acid bacteria, with C2 exhibiting the highest counts and C3 the lowest ones. Yeasts and molds were more abundant in OMW from C2, whereas, no significant difference was found between C3 and SP.

Table 1. Microbiological and physicochemical characteristics of fresh OMW collected in northern Morocco from industrial mills operating using three extraction systems (dual [C2] and triple [C3] phase centrifugation decanters and super-pressure [SP] system) during the 2017 crop season.

	C2	C3	SP
pH	5.07 a	4.77 c	4.84 b
Electrical conductivity *(mS/cm)*	11.09 a	9.74 b	9.71 b
Suspended solids *(g/l)*	64.89 a	31.52 b	32.50 b
Total solids *(g/l)*	129.69 a	71.86 b	72.21 b
Mineral matter *(g/l)*	29.65 a	9.48 c	15.24 b
Organic matter *(g/l)*	100.04 a	56.63 c	62.73 b
Chemical oxygen demand *(g O_2/l)*	226.74 a	116.00 b	149.53 b
Total phenols *(g/l caffeic acid)*	1.52 b	1.91 a	1.82 a
Chlorides *(g/l)*	1.05 a	0.89 ab	0.79 b
Sugar *(g/l)*	7.99 a	2.65 b	4.93 c
Total kjeldahl nitrogen *(g/l)*	0.53 a	0.44 c	0.49 b
Total organic carbon *(g/l)*	58.03 a	32.85 c	36.39 b
C/N ratio	109.27 a	75.31 b	74.51 b
Proteins *(g/l)*	3.33 a	2.73 c	3.07 b
Total Aerobic Mesophilic Flora *(10^4 CFU/ml)*	23.92 a	19.92 c	21.82 b
Lactic Acid Bacteria *(10^4 CFU/ml)*	6.28 a	4.13 c	4.71 b
Total Coliform Bacteria *(10^4 CFU/ml)*	-	-	-
Yeasts & Molds *(10^4 CFU/ml)*	19.36 a	16.78 b	17.15 b

3.2. Antioxidant activity of OMW phenolic extracts

3.2.1. DPPH radical scavenging activity

The antioxidant capacity of the OMW phenol extracts was studied using two different assays involving different antioxidant mechanisms. The DPPH radical-scavenging activity by hydrogen donation is a well-known antioxidation mechanism. It was evaluated by the monitoring of DPPH radical reduction resulting in a gradually decrease of deep purple color in the presence of OMW phenolic extracts from C2, C3, and SP, as well as the standards caffeic acid (CA) and ascorbic acid (AA). Results represented in Figure 1 showed an antiradical potential of all OMW phenolic extracts and standards that is related to antioxidant concentration (concentration-dependent). The percentage of DPPH scavenging activity increase with increasing concentration until the stationary phase is reached, corresponding to the almost complete DPPH scavenging. OMW phenolic extracts exhibited an antiradical activity greater than 90% at concentrations around 320 µg/ml for C2 and 390 µg/ml for both C3 and SP, while the same score is reached at a relatively low concentration (about 156 µg/ml) for CA and AA. IC 50, the concentration providing 50% of inhibition, was calculated to further compare among phenolic extracts as well as to standards. Results are shown in Figure 2. The comparison of IC50 values showed significant differences between all tests. Among phenolic extracts, C2 displayed the highest antiradical activity (the lowest IC50 equal to 32.5 mg/ml), contrariwise, the lowest one was observed for C3 extract (42.5 mg/ml). Although low concentrations of phenolic extracts allowed significant antiradical activity,

those recorded for both standards remained stronger. IC50 values for CA and AA were 7.5 and 15 mg/ml, respectively.

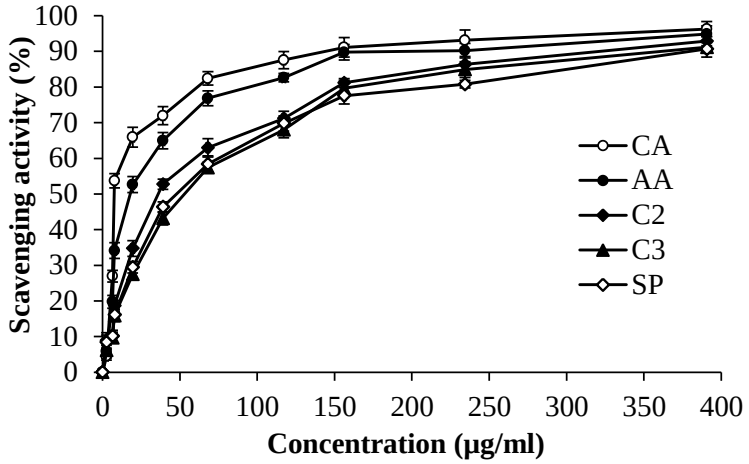

Figure 1. DPPH radical scavenging activities (%) of OMW phenolic extracts from three extraction systems (C2: dual phase decanter, C3: triple phase decanter and SP: super-pressure) compared to ascorbic acid [AA] and caffeic acid [AC]) as standards. Data presented as means ± SD.

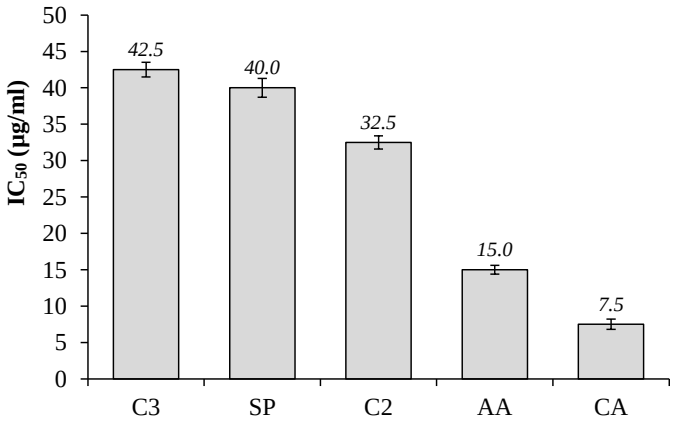

Figure 2. Concentration providing 50% DPPH radical-scavenging (IC50) of OMW phenolic extracts from three extraction systems (C2: dual phase decanter, C3: triple phase decanter and SP: super-pressure) compared to ascorbic acid [AA] and caffeic acid [AC]) as standards. Data presented as means ± SD.

3.2.2. Ferric reducing antioxidant power (FRAP) assay

The antioxidant potential of OMW phenolic extracts from C2, C3 and SP systems and standards (CA and AC) was also evaluated in their ability to reduce ferric iron (Fe^{+3}) present in the $[K_3Fe(CN)_6]$ complex to the ferrous form (Fe^{+2}) (FRAP Test). Results for this test, expressed as absorbance evolution following the formation of Perl's Prussian blue, are displayed graphically in Figure 3. OMW phenolic extracts exhibited a good reducing power capacity, increasing in a concentration-dependent way that was higher for C2 phenolic extract. Nonetheless, the reducing powers of CA and AA (standards) were more pronounced. Indeed, the order of FRAP activity of all samples and standards were as follows: CA > AA > C2 > SP >C3. At a concentration of 100µg/ml, the reducing power (absorbance at 700 nm) were found to be 1.393 ± 0.025, 1.234 ± 0.026, 0.957 ± 0.027, 0.815 ± 0.023 and 0.931 ± 0.021 for CA, AA, C2, C3 and SP, respectively.

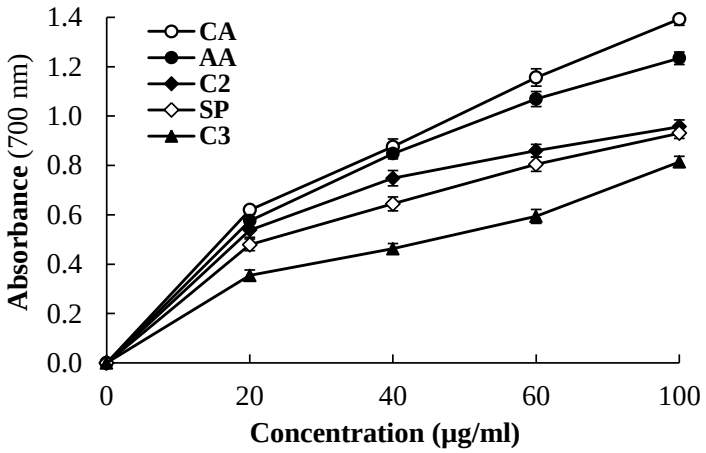

Figure 3. Reducing power of OMW phenolic extracts from three extraction systems (C2: dual phase decanter, C3: triple phase decanter and SP: super-pressure) compared to ascorbic acid [AA] and caffeic acid [AC]) as standards by using the FRAP assay: increase in absorbance indicates increasing reducing power. Data presented as means ± SD.

3.3. VOO enrichment with OMW phenolic extracts

Initial virgin olive oil quality was determined before enrichment with phenolic extracts and results were presented in Table 2. The same concentrations (100, 300 and 500 ppm) of C2 phenolic extracts (PE) and ascorbic acid (AA) were added to virgin olive oil (VOO) samples. Enriched VOO along with the control (VOO without additives) were stored in the dark at 60 °C during 22 days. Olive oil stability was monitored by measuring PV, K232 and K270 and the results are displayed in Figures 4 and 5. Figure 4 show significant increases of PV, K232 and K270 values in relation to the storage time, indicating a significant rise in both lipid hydroperoxides and conjugated dienes and trienes formation, for all samples. However, the increase was more important in the control compared to samples enriched with OMW phenolic extracts (PE) and ascorbic acid (AA). Indeed, PE-500 and AA-500 gave slower increase for the three traits, with more antioxidant effectiveness of AA. Moreover, the effect of PE and AA against lipid oxidation increased with increasing concentration as shown in Figure 5. In fact, we noted a decrease in PV, K232 and K270 values with increasing the additive concentration in VOO (Figure 5A, B, C). In addition, we noticed the good efficiency of AA, while the effect of PE was also promising

Table 2. Analytical parameters (initial quality) of virgin olive oil.

Free fatty acids *(% Oleic acid)*	**Peroxide value** *(meqO_2/kg)*	**K232**	**K270**	**Carotenoids** *(mg/kg)*	**Chlorophylls** *(mg/kg)*	**Total phenols** *(mg /kg caffeic acid)*
0.78	4.13	1.87	0.17	0.85	1.52	398.43

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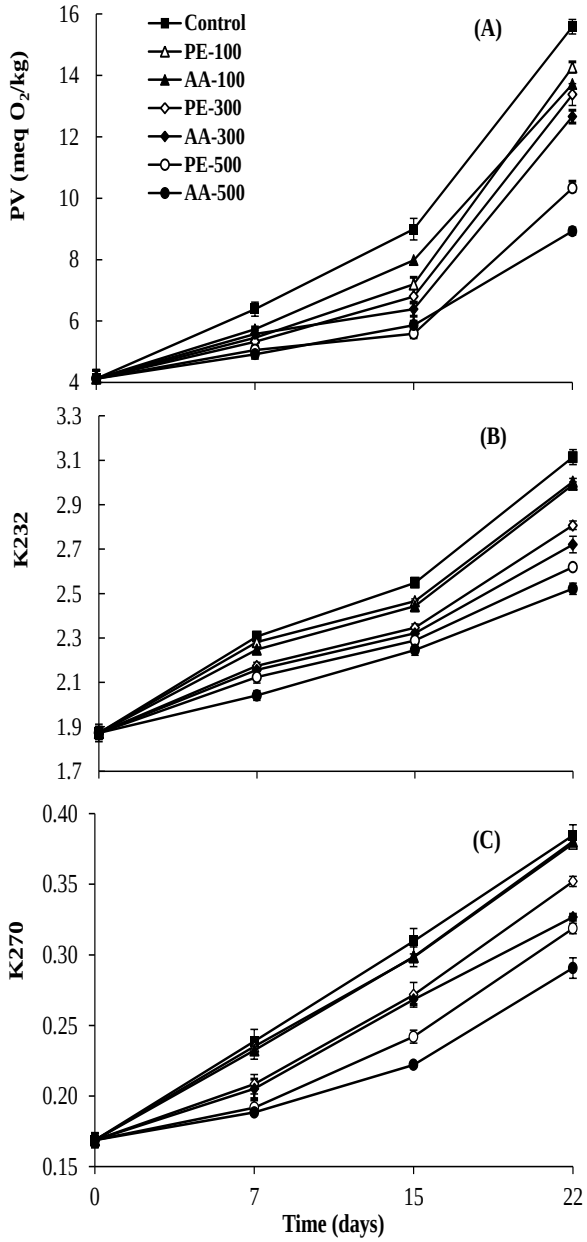

Figure 4. Evolution of peroxide value [PV] (A), K232 (B) and K270 (C) of virgin olive oils (VOO) enriched with different concentrations of OMW phenolic extracts [PE] and ascorbic acid [AA] as standards during the storage period at 60°C. Data presented as means ± SD. *AA-100, AA-300, AA-500 = VOO enriched with 100, 300 and 500 ppm of acid ascorbic, respectively. PE-100, PE-300, PE-500 = VOO enriched with 100, 300 and 500 ppm of OMW phenolic extract, respectively.*

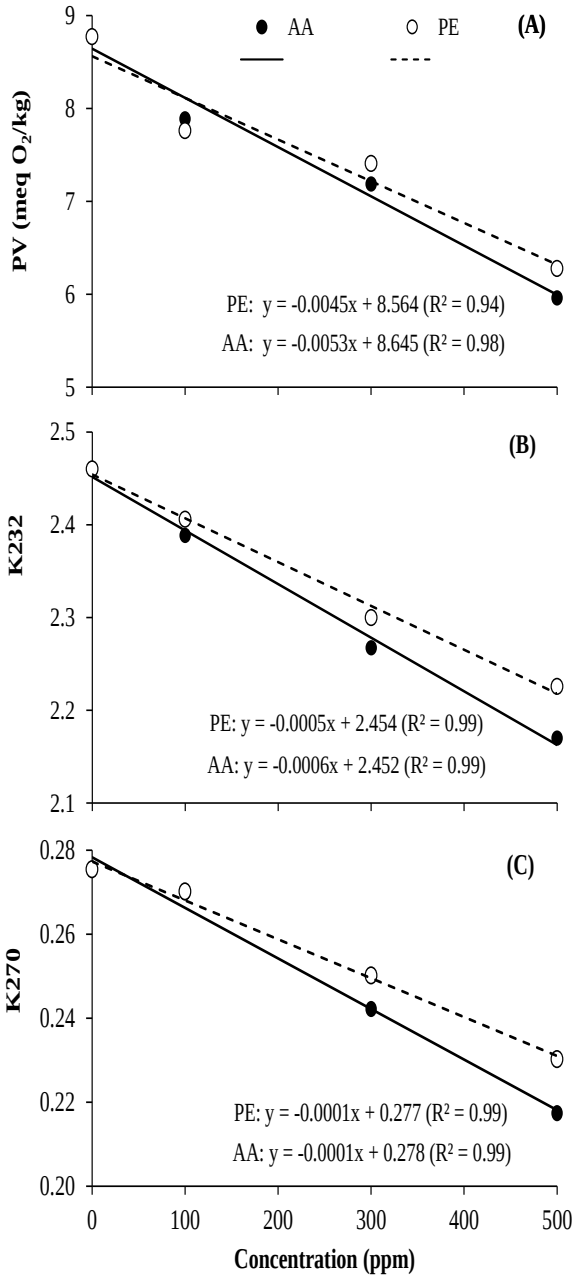

Figure 5. Regressions of peroxide value [PV] (A), K232 (B) and K270 (C) of enriched virgin olive oils against different concentrations of additives (OMW phenol extract [PE] and ascorbic acid [AA]).

4. Discussions

The composition of the OMW used in this study was similar to several previous researches, although some differences were observed for certain characteristics, due to many factors such as variety, maturity of olives, period of production,

climatic conditions, farming methods, region of origin, and the oil extraction technology [15, 39-40]. PH was acidic as a result of high amount recorded for total phenols that was consistent with previous findings [9, 11, 41-42]. High values of chlorides content and consequently electrical conductivity were found, mainly related to salt adding for olive conservation before oil extraction [43]. The investigated OMW also showed a strong organic load, expressed by high values of chemical oxygen demand, sugar amount and organic matter. These finding are usually associated to cultivation practices, irrigation management, olive cultivar and fruit maturity [11, 44]. OMW in our case were heavily loaded by total solids and suspended solids compared to other studies [45-47]. The separation efficiency of the used centrifuge or decanter could explain differences in solid contents. Amounts of mineral matter, total kjeldahl nitrogen and proteins were in line with those detected by Ayoub et al. [48] and De Leonardis et al. [32]. Calculated C/N ratios were similar to values reported by Amaral et al. [7]. Our results for microbiological counts were in good agreement with those documented by El Yamani et al. [49] and Esmail et al. [50], but different to other investigations [7, 39]. Comparisons among extraction systems revealed that OMW from C2 displayed the higher values for all traits, with the exception of total phenols that were higher in C3. The same findings were documented previously [40]. C3 and SP presented relatively similar characteristics. In fact, the large quantities of added water during olive oil extraction using C3 and SP allowed a dilution of OMW produced by these two systems. Moreover, C3 requires addition of warm water that increases the loss of phenols by solubilization through the OMW [11]. The richness of OMW from C3 in total phenols makes them more acidic which could explain the low microbiological counts in these effluents [51]. Although the environmental problem created by OMW because of their polluting load, these effluents seem to be a promising source for valuable compounds such as phenols. In fact, Bouaziz et al. [52] has previously reported that olive oil and olive by-products provide a rich source of natural antioxidants with phenols as one of the most important groups [53]. In the present work, we focused on the antioxidant activity of OMW phenolic extracts from three extraction systems (C2, C3 and SP), using two test: DPPH and FRAP. The results were very encouraging for all extracts and were similar to several previously published researches [19-20, 22, 40, 54]. For both tests, the antioxidant activity of the phenolic extracts was found to be concentration-dependent. This observation is in agreement with that reported in various studies [19, 22, 55]. The correlation of antioxidant activity with phenolic extract content was also reported for olive leaves [56-57] and tea extracts [34]. At equal concentrations, phenolic extract from C2 exhibited the strong antioxidant activity, probably due to the phenolic compound composition characterizing each extract. In similar way, Lesage-Meessen et al. [40] announced that the C2 extract displayed the highest antiradical activity, with a value two-fold greater than that of the C3 extract, and has attributed the difference to the highest concentrations of antioxidant phenolic compounds in the C2 extract such as hydroxytyrosol. Farag et al. [38] had also noticed a close relationship between the antioxidant effectiveness and the chemical composition of phenolic compounds. In another study conducted on olive leaf extracts, it was suggested that the effect of the phenolic profile on the antioxidant activity was greater rather than the total phenolic content [57]. Rice-Evans et al. [58] attributed the antioxidant activity of an extract to both content and structure of phenolic compounds, especially, the number of hydroxyl groups in the phenolic ring. The improvement of virgin olive oil with OMW phenolic extracts was also investigated here in the light of the importance of antioxidants in protecting the quality of foods, and considering the abundant source of phenols in olive oils and OMW as main antioxidants. Our results were encouraging and showed a significant oxidation delay in PE-enriched virgin olive oil as demonstrated by low increases in PV, K232 and K270 values, compared to the control. Moreover, this oxidation delay was concentration-dependent. Similarly, Lafka et al. [20] evaluated the oxidative stability of commercial virgin olive oil and sunflower oil, enriched with OMW phenol extracts, by the measurement of peroxide value and induction time, and they reported a reduction of peroxides formation and an increase in induction

time in samples enriched at a concentration of 150 ppm compared to control samples. Fki et al. [22] reported that the addition of OMW extract, 3,4-dihydroxyphenylacetic acid and hydroxytyrosol in refined oils displayed a significant delay in the oxidation rate, which was dose-dependent. The same authors also indicated that PV values were lower in samples enriched with OMW extracts at 500 ppm than those enriched with butylated hydroxyanisole (BHA), p-hydroxyphenylacetic acid or tyrosol. The effect of total and free phenolic compounds from Picual and Koronaki leaves and fruits on sunflower oil stability was also demonstrated by Farag et al. [38] indicating that total and free phenolic compounds produced a significant antioxidant activity and protected the stability of sunflower oil. Bouaziz et al. [52] have disclosed a decrease in the formation of both lipid hydroperoxides and conjugated dienes and trienes formation as well as an improvement of the oxidative resistance (assessed by Rancimat test) in refined olive oil and refined olive husk oil enriched with phenolics recovered from olives. De Leonardis et al. [59] have examined the influence of natural antioxidants extracted from OMW on the oxidative stabilization of lard and reported that the oxidative resistance, measured using PV and Rancimat, was greatly enhanced by the addition of OMW extracts. Similarly, olive phenols recovered from OMW were added to raw and cooked fresh pork sausages during aerobic storage and resulted in a decrease in pH and peroxide value [60-61]. The qualitative and quantitative analyses of the composition of OMW extracts have revealed the dominance of hydroxytyrosol (66.8%) followed by tyrosol (16.6%), with the presence of caffeic, ferulic and vanilic acids at lesser magnitudes and oleuropein only in traces [40, 59]. Visioli et al. [62-63] attributed the antioxidant activity of hydroxytyrosol to hydrogen donation and its ability to scavenge free radicals by forming an intramolecular hydrogen bond between the free hydrogen of its hydroxyl group and their phenoxyl radicals. Chimi et al. [64] observed the strong inhibition of hydroxide formation by the addition of phenolic compounds, which was related to the hydrogen atom donating ability of the antioxidant to the peroxyl radical, thus acting as chain radical terminator. The same authors reported that the natural phenolic compounds were able to quench OH* radical and peroxyl radical involved in the initiation and propagation steps of lipid peroxidation, respectively.

4. Conclusion

This study confirmed the pollution load of OMW. Their direct disposal into the environment might be a serious problematic. However, phenols, which are the main harmful elements in these effluents, could constitute a promising source of natural antioxidants. OMW phenolic extracts showed great antioxidant potentials either by the in vitro tests or by their effect on oxidation delay of the olive oil. The low cost of this source of bioactive compounds encourages their use as alternatives to synthetic products for other purposes.

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