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The use of polyphenolic extract, purified hydroxytyrosol and 3,4-dihydroxyphenyl acetic acid from olive mill wastewater for the stabilization of refined oils: a potential alternative to synthetic antioxidants

Ines Fki, Noureddine Allouche, Sami Sayadi *

Laboratoire des Bio-procédés, Centre de Biotechnologie de Sfax, B.P. "K", 3038 Sfax, Tunisia

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Abstract

Ethyl acetate extracts of olive mill waste water (OMWW) were prepared, under in optimal conditions, using a continuous counter-current extraction unit. The antiradical and antioxidant activities of the OMWW extract as well as pure phenolic compounds identified in this extract were evaluated. Results showed that pure hydroxytyrosol and 3,4-dihydroxyphenyl acetic acid had the highest radical-scavenging effects on 1,1-diphenyl-2-picrylhydrazyl radical and the highest antioxidant activities using the β-carotene linoleate model system.

The effect of addition of individual phenolic compounds and OMWW extract to refined olive and husk oils was compared with that of control, BHA and BHT at 50 °C. Unexpectedly, 3,4-dihydroxyphenyl acetic acid had the highest protective effect against oil oxidation. Oils with added 3,4-dihydroxyphenyl acetic acid had lower PV than oils with added hydroxytyrosol, the most studied powerful antioxidant. Moreover, the addition of OMWW extract, at 500 ppm, resulted in lower PV values than BHA, *p*-hydroxyphenylacetic acid, tyrosol on the control.

The results suggested that 3,4-dihydroxyphenyl acetic acid, hydroxytyrosol and OMWW extract possess useful antioxidant properties and may be used as alternatives in the search for natural replacement of synthetic antioxidant food additives.
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Keywords: Antioxidants; Olive mill waste water; Phenolic compounds; Refined oils

1. Introduction

The olive oil market has recently developed, since the "Mediterranean diet" is widely appreciated throughout the world by consumers more attentive to both health and nutritional aspects of food (Key, 1995). The increasing popularity of olive oil has mainly been attributed to its high content of oleic acid, which may affect the plasma lipid/lipoprotein profiles (Delplanque, Jusselin, Le Roy, & Motta, 1999) and its richness in phenolic compounds, acting as natural antioxidants, which may contribute to the prevention of diseases in humans (Fito et al., 2000; Saija et al., 1998; Visioli, Bellomo, & Galli, 1998; Visioli & Galli, 1998).

Usually, olive oil is extracted mechanically by pressure and by a three-phase centrifugation system, which results in the production of more than 30 million m^3 of black olive mill wastewater (Borja, Alba, & Banks, 1997). This liquid effluent has a high polluting organic load, due to a high content of organic substances, including sugars, tannins, polyphenols, polyalcohols, pectins, and lipids (D'Annibale, Crestini, Vinciguerra,

* Corresponding author. Tel.: +216 74 440 452; fax: +216 74 440 818.
E-mail address: sami.sayadi@cbs.rnrt.tn (S. Sayadi).

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& Giovannozzi Sermanni, 1998; Sayadi & Ellouz, 1995). Centrifugation, despite its high water consumption, is still the most widely employed method for production of virgin olive oil, especially in countries that produce large amounts of olives in a short time (Benitez, Beltran-Herdia, Torregrosa, Acero, & Cercas, 1997). Most frequently, OMWW are pumped and discharged into evaporation ponds or directly dumped in rivers or spread on soil (Greco, Toscanoa, Cioffi, Gianfreda, & Sannino, 1999). This becomes a major environmental problem in the main olive-producing countries of the Mediterranean region. It is known that phenolic compounds are major contributors to the toxicity and the antibacterial activity of OMWW. This limits its microbial degradability (Borja et al., 1997; Capasso et al., 1995). However, these phenolic compounds possess strong antioxidant properties (Galli & Visioli, 1999), which may turn the olive oil residues into a cheap source of natural antioxidants. An antioxidant may be defined as a substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or prevents the oxidation of that substrate (Halliwell, 1990). Oxidizable substrates include carbohydrates, DNA, proteins and lipids.

Olive and husk refined oils are characteristic by-products of olive oil production and are gaining importance in the food industry. Olive and husk refined oils are susceptible to oxidation, especially in warm countries such as Tunisia. Lipid oxidation, not only produces undesirable off-flavour, but also decreases the nutritional quality of oils due to the loss of essential fatty acids.

Oil manufacturers aim at producing foods that maintain their shelf life and nutritional quality over a defined period. Thus, the use of antioxidants to minimize the oxidation of lipids in food materials is extensively practised (Loliger, 1991; St Angelo, 1992).

Antioxidants, such as BHT and BHA and propyl gallate are widely used in many foods. These compounds are added at concentrations ranging from 50 to 200 ppm to fat and oils to suppress the development of peroxides during food storage (Loliger, 1991).There has been some recent discussion about the undesirable use of synthetic antioxidants. For example, dietary administration of BHT to rats caused fatal haemorrhages in the pleural and peritoneal cavities and in organs such as epididymis testes and pancreas (Hirose, Masuda, Imaida, Kagawa, & Ito, 1987). Also, BHT caused changes in rat livers, stimulation of DNA synthesis and induction of enzymes (Thamavit et al., 1985). BHA has toxic and carcinogenic effects (Ito et al., 1986). However, these antioxidants are approved for food use within limits. Consequently, there is an urgent need for other types of compounds to use as antioxidants. Olives, olive leaves and OMWW represent a potential source of such natural antioxidants.

This present work was realized in the frame of an INCO project "contract ICA3-CT2002-10033" entitled: "New technologies for olive mill wastewater detoxification and product recovery". We have attempted to develop effective procedures to recover the potentially high-added-value phenolic compounds contained in OMWW, to produce antioxidant additives extracts; and to evaluate their antioxidant activity and potential application for stabilisation of refined oils (husk and olive).

2. Materials and methods

2.1. Chemicals

Refined olive oil and refined olive husk oil samples were obtained from a local commercial refining plant. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), linoleic acid, ethylene diamine tetra acetic acid (EDTA), *p*-coumaric acid, ferulic acid, *p*-hydroxyphenylacetic acid and Tween 20 were purchased from Sigma Aldrich Chemical (Germany). Tyrosol,3,4-dihydroxyphenyl acetic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and β-carotene were purchased from Fluka (Switzerland). Ethyl acetate, ethanol, methanol, acetonitrile and orthophosphoric acid were HPLC-grade solvents and purchased from Prolabo (France).

2.2. Extraction of phenolic compounds from OMWW

Olive mill wastewaters (OMWW) were obtained from a discontinuous olive oil processing plant located in Sfax. Continuous counter-current extractions were conducted at ambient temperature in a polyethylene mixer-settler unit of ROBATEL design (mixer volume: 35 ml; settler volume: 200 ml). The total feed flow rate ranged from 2 to 5 l/h. For each run, the steady state was confirmed by phenolic monomer analysis in the organic stream and by verification of the mass flow rate balance. The maximum deviation of the latter was 2% (Feki, Stambouli, Pareau, & Ayedi., 2001).

The organic extract was evaporated under vacuum at 40 °C in rotary evaporator. The residue was re-dissolved in a minimum volume of solvent and analysed by HPLC.

2.3. HPLC analysis of OMWW extract

A reversed-phase high-performance liquid chromatographic technique was developed to identify and quantify the major phenolic compounds contained in the crude OMWW and in the ethyl acetate continuous counter-current extract. For this purpose, a standards mixture solution of phenolic compounds was analysed. Sample concentrations were calculated, based on peak

areas compared to those of each of the external standards. The HPLC chromatograph was a Schimadzu apparatus equipped with a (LC-10ATvp) pump and a (SPD-10Avp) detector. The column was (4.6 × 250) mm (Shim-pack, VP-ODS) and the temperature was maintained at 40 °C. The flow rate was 0.5 ml/min. The mobile phase used was 0.1% phosphoric acid in water (A) versus 70% acetonitrile in water (B) for a total running time of 40 min, and the gradient changed as follows: solvent B started at 20% and increased immediately to 50% in 30 min. After that, elution was conducted in the isocratic mode with 50% solvent B within 5 min. Finally, solvent B decreased to 20% until the end of running time.

2.4. Chromatographic purification of hydroxytyrosol

OMWW extract (1 g) was chromatographed on a C-18 silica gel (liChroprep RP-18; 25–40 μm) column (2.5 × 70 mm) under medium pressure. Phenolic compound elution was carried out with the same gradient solvent as used in the HPLC. The flow rate was adjusted to 0.3 ml/min and 4.5 ml fractions were collected. These fractions were measured by optical density at 280 nm and the chromatogram (optical density versus fraction number) was represented (data not shown). The first separated peak corresponds to pure hydroxytyrosol.

2.5. DPPH radical-scavenging effect

The DPPH radical-scavenging effect was evaluated according to Kim, Kim, Chung, and Choi (2000). A volume of four millilitres of methanolic solution of varying sample concentrations was added to a 10 ml of DPPH methanol solution (1.5×10^{-4} M). After mixing the two solutions gently and leaving for 30 min at room temperature, the optical density was measured at 520 nm, using a spectrophotometer. The antioxidant activity of each sample was expressed in terms of IC_{50} (microgrammes per ml required to inhibit DPPH radical formation by 50 per cent) and calculated from the log-dose inhibition curve.

2.6. Antioxidant activity in aqueous media

In this experiment, scrupulous care was taken to avoid contamination by heavy metals. All experimental work was carried out in all-glass equipment to minimize metal contamination. All glassware was immersed for at least 24 h in EDTA (0.1%), rinsed several times with deionised water and dried at 150 °C before use. The antioxidant activity in aqueous media was evaluated according to Farag, Badei, Hewedi, and El-Baroty (1989). An aliquot of β-carotene dissolved in chloroform (10 ml, 0.05%) was pipetted into a flask containing linoleic acid and Tween 20. The solvent was evaporated, deionised water was then added and emulsification was achieved in an ultrasonic bath for 15 min. The phenolic compounds and OMWW extract were added to emulsified linoleic acid β-carotene at 200 ppm. The oxidation of linoleic acid was followed by coupled oxidation with β-carotene, monitored by optical density reading at 462 nm.

2.7. Antioxidant activity in refined oils

Weighed quantities of OMWW extract and pure phenolic compounds were dissolved in 1ml of ethanol, to obtain the desired final molar concentration (200 ppm). Then, these were added to refined olive oil and refined husk oil. The phenolic compounds were mixed with oil by stirring for 30 min. Oil samples were stored in the dark at 50 °C. The stability of oils was evaluated by the measurement of peroxide value (PV; AOCS, 1989) and conjugated dienes formation (IUPAC, 1987).

3. Results

3.1. Extraction and analysis of olive mill wastewater extract

Results of optimal extraction conditions are summarized as follows: among several polar solvents such as methyl isobutyl ketone, methyl ethyl ketone and diethyl ether, ethyl acetate was found to extract broadly the whole OMWW monomeric fraction. The acidification of OMWW to pH 2 increased the solubility of the phenolic compounds in the organic phase, which allowed the extraction of the most extractable polyphenols. Furthermore, we have observed that the percentage of each monomer, and particularly hydroxytyrosol, in the extract was maximum for a solvent ratio and a theoretical extraction stage number equal to 2 and 3, respectively. The extraction yield, calculated on the basis of hydroxytyrosol, was 85.5 per cent. One litre of OMWW yielded 4 g of extract containing 1225 mg/l of hydroxytyrosol (Table 1).

The HPLC chromatogram of the final extract, obtained from OMWW using the continuous counter-current extractor (described in Material and methods) under the optimal conditions fixed above, is shown in Fig. 1. Hydroxytyrosol and tyrosol were the major compounds detected (Table 1). Their concentrations in the extract were 1225 and 345 mg/l, respectively. *para*-Hydroxyphenyl acetic acid, caffeic acid and *p*-coumaric acid were present at lower concentrations. 3,4-Dihydroxyphenylacetic acid and ferulic acid were also detected at the same concentration, 70 mg/l (Table 1). Protocatechuic acid, vanillic acid and synergic acids and other compounds were detected but not quantified.

Table 1
HPLC evaluation of major phenolic compounds identified in OMWW extract (milligrammes per litre)

Phenolic monomers	Concentration in OMWW (mg/l)	Concentration in the extract (mg/l)
Hydroxytyrosol	1433.4	1225.6
Tyrosol	851	345
3,4-Dihydroxyphenyl acetic acid	87.9	70.2
p-Hydroxyphenyl acetic acid	274	198.9
Caffeic acid	321	256.7
p-Coumaric acid	298	169
Ferulic acid	95.0	70.2

Table 2
Radical-scavenging effects of phenolic compounds on DPPH radical

Samples	IC_{50}(μg/ml)
OMWW extract	1.20
Hydroxytyrosol	0.57
3,4-Dihydroxyphenylacetic acid	0.64
Ferulic acid	1.21
Caffeic acid	0.87
Tyrosol	10.9
p-Coumaric acid	9.50
p-Hydroxyphenylacetic acid	12.2
BHA	0.91
BHT	0.89

3.2. DPPH radical-scavenging effect of OMWW extract and some phenolic compounds

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging effect of OMWW extract and its major phenolic compounds is shown in Table 2. Among compounds obtained from OMWW, *ortho*-dihydroxylated aromatic components exhibited higher scavenging activity on DPPH, particularly hydroxytyrosol and 3,4-dihydroxyphenyl acetic acid, which exhibited IC_{50} values of 0.57 and 0.64 μg/ ml, respectively. However, tyrosol and *p*-hydroxyphenylacetic acid, possessing only one hydroxyl function, showed no activity (IC_{50} = 10.9 and 12 μg/ml). In particular, the antioxidant activity of hydroxytyrosol and 3,4-dihydroxyphenyl acetic acid, as well as, caffeic acid were higher than those of BHA and BHT. These results suggest that the radical scavenging effect of the OMWW extract (1.2 μg/ml), which is partially related to its content of hydroxytyrosol, caffeic acid and 3,4-dihydroxyphenyl acetic acid, is comparable to those of synthetic antioxidants BHA and BHT.

3.3. Antioxidant activity of OMWW extract and its phenolic compounds tested using the β-carotene bleaching method

β-Carotene was added to the model systems as monitor for linoleate oxidation. The results of individual experiments showed considerable variation in the rates of oxidation in comparison with the control experiments. The rate of β-carotene bleaching for linoleate systems is shown in Fig. 2 and the time required for the complete disappearance of β-carotene in the model system is shown in Table 3. The curve slopes of β-carotene loss per time unit were broadly the same for hydroxytyrosol and BHT. The comparison of the effect of phenolic compounds in preventing the oxidation of β-carotene makes it possible to establish a classification of

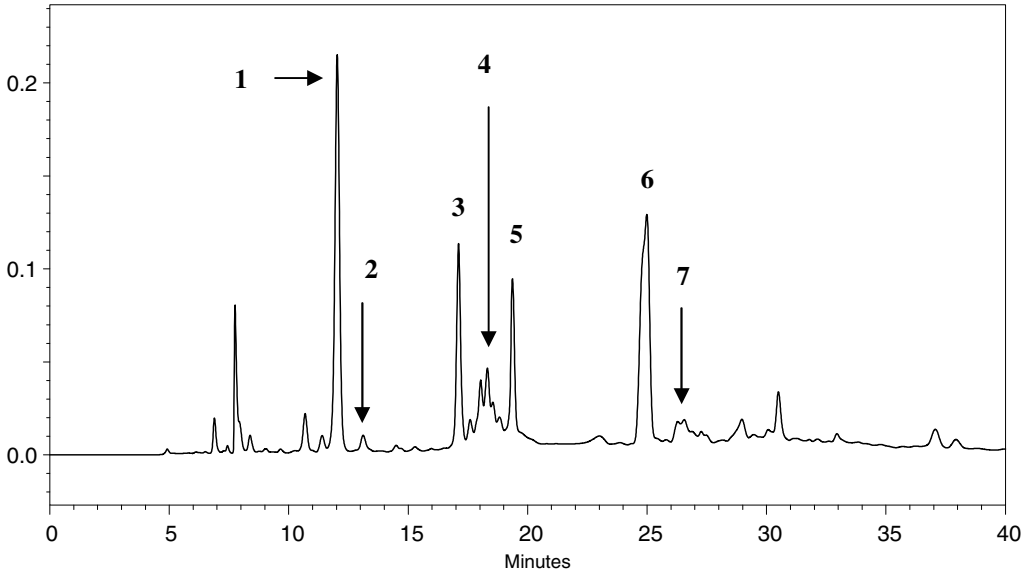

Fig. 1. HPLC-chromatogram of the liquid-liquid continuous ethyl acetate extract of olive mill wastewater. **1**, Hydroxytyrosol; **2**, 3,4-dihydroxyphenyl acetic acid; **3**, tyrosol; **4**, *para*-hydroxyphenyl acetic acid; **5**, caffeic acid; **6**, *para*-coumaric acid; **7**, ferulic acid.

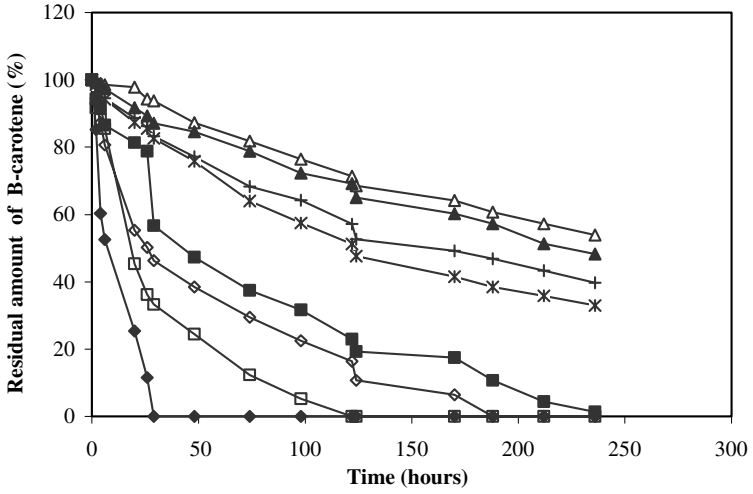

Fig. 2. Oxidation of emulsified linoleic acid–β-carotene catalysed by individual phenolic compounds. (◆) control, (×) BHA, (△) BHT, (□) tyrosol, (▲) hydroxytyrosol, (■) OMWW extract, (◇) *para*-hydroxyphenyl acetic acid, (+) 3,4-dihydroxyphenyl acetic acid.

Table 3
Coupled oxidation of β-carotene and linoleic acid catalysed by some phenolic compounds in aqueous media

Compounds	Time required for complete disappearance of β-carotene (h)
Hydroxytyrosol	>236
OMWW extract	>236
3,4-Dihyroxyphenyl acetic acid	>236
BHT	>236
BHA	>236
Caffeic acid	>236
Ferulic acid	>236
Tyrosol	122
p-Coumaric acid	148
p-Hydroxyphenyl acetic acid	188
Control	29

these compounds according to their antioxidant potencies. In decreasing order, we have: hydroxytyrosol = BHT > 3,4-dihydroxyphenyl acetic acid > caffeic acid > BHA > OMWW extract > *p*-hydroxyphenyl acetic acid > tyrosol (Fig. 2).

3.4. Antioxidant effects of OMWW extract and its major components on refined oils

The present work show the application of OMWW extract and some phenolic compounds on the oxidative stability of husk and olive refined oils. The oxidation of refined husk and olive oils, stored at 50° after the addition of OMWW extract, was measured by peroxide values (PV) and conjugated diene formation CD. Both parameters (CD and PV) measure the primary product of lipid oxidation. OMWW extract had an effect in stabilizing refined oils (Fig. 3). Indeed, the rate of peroxide formation decreased with added OMWW extract. This effect increased as OMWW extract concentration increased. The PVs of olive and husk oils treated with 200 and 500 ppm of OMWW extract were similar up to day 14. After this period, PVs of samples containing 500 ppm of OMWW extract were different. For up to 110 days, PV of the controls of olive and husk oils increased from 16 to 644 and 640 meq/kg, respectively. However, corresponding values of husk and olive oils after 110 days of incubation in the presence of OMWW at 500 ppm increased from 16 to 191 meq/ kg (Fig. 3) and 344 meq/kg (data not shown), respectively. OMWW extract at 500 ppm was most effective and gave a much lower PV than the control or the sample treated at 200 ppm.

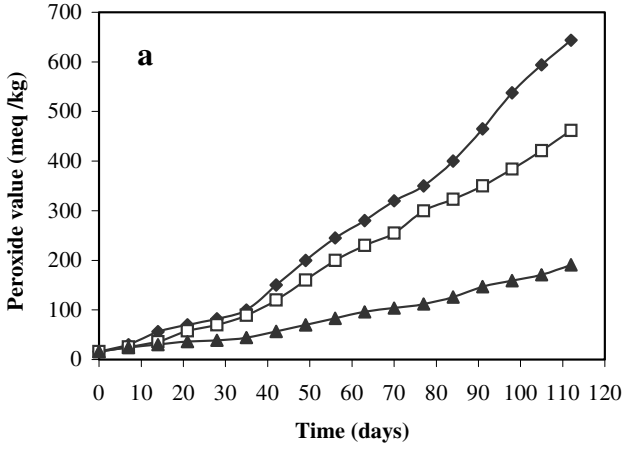

Fig. 3. Peroxide values (PV) of refined husk oil with added olive mill wastewater (OMWW) extract during storage at 50 °C. (◆) Control, (□) 200 ppm, (▲) 500 ppm.

The effects of some phenolic compounds, contained in the extracted OMWW at 200 ppm, on olive and husk refined oil oxidation at 50° were compared to those of BHA and BHT (Figs. 4 and 5). Oils treated with 3,4-

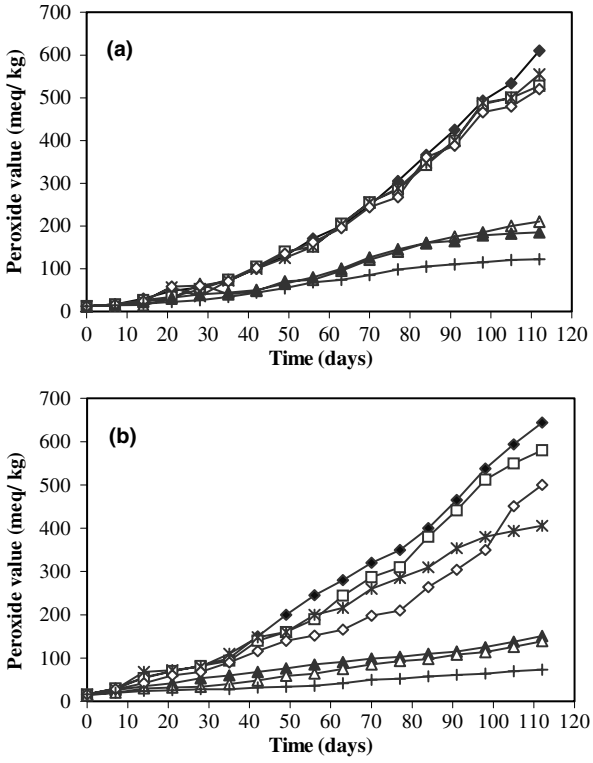

Fig. 4. Peroxide values (PV) of refined husk oils (a) and refined olive oils (b) with added phenolic compounds at 200 ppm during storage at 50 °C. (◆) Control, (×) BHA, (△) BHT, (□) tyrosol, (▲) hydroxytyrosol, (◇) *para*-hydroxyphenyl acetic acid, (+)3,4-dihydroxyphenyl acetic acid.

dihydroxyphenyl acetic acid exhibited the lowest PV value (<20%), up to 110 days as compared with the control sample. 3,4-Dihydroxyphenyl acetic acid delayed the oxidation of either husk (Fig. 4a) or olive (Fig. 4b) refined oils significantly more than did hydroxytyrosol and BHT. 3,4-dihydroxyphenyl acetic acid was superior to all other compounds and gave, after 110 days of oxidation, PVs of olive and husk oils, respectively, of 73 and 122 meq/kg, whereas corresponding values of the control samples were 644 and 640 meq/kg, respectively. The rises in PV of the control and of the oils treated with tyrosol and *p*-hydroxyphenylacetic were not significantly different from each other.

Among phenolic compounds extracted from OMWW and tested, 3,4-dihydroxyphenyl acetic acid and hydroxytyrosol appeared as real alternatives for lowering the formation of peroxides in stored oil samples.

As an example, the appearance of conjugated diene (CD) profiles, shown in Fig. 5 confirmed the high protective effect of 3,4-dihydroxyphenyl acetic acid and hydroxytyrosol against oxidation of husk oils. Indeed, samples treated with these latter compounds exhibited the lowest absorbance at 232 nm than the other compounds BHT, BHA, tyrosol and *p*-hydroxyphenyl acetic acid. Results of this study indicate that 3,4-dihydroxyphenyl acetic acid and hydroxytyrosol, compounds of OMWW, had a marked effect on the prevention of refined oil autoxidation. Moreover, the anti-oxidative efficacy of OMWW extracts was directly related to their contents of *ortho*-diphenols, such as hydroxytyrosol and 3,4-dihydroxyphenyl acetic acid. Indeed, the addition of the extract of OMWW at 500 ppm and hydroxytyrosol at 200 ppm had similar effects in retarding oxidation of husk refined oils (Fig. 6).

4. Discussion

Antioxidants are major ingredients which protect the quality of foods by retarding oxidation. In the edible oil industry, synthetic antioxidants are often used because they are effective and inexpensive. However, increased

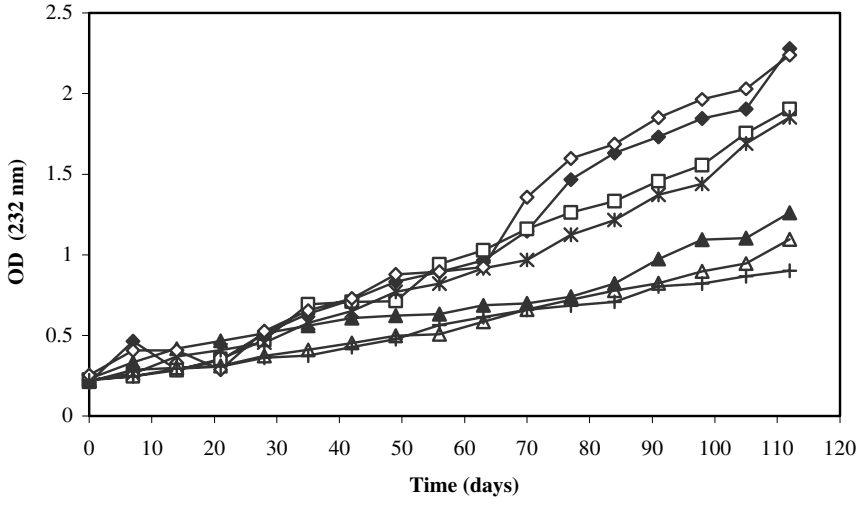

Fig. 5. Conjugated diene values (CD) of refined husk oils with added phenolic compounds at 200 ppm during storage at 50 °C. (◆) Ccontrol, (×) BHA, (△) BHT, (□) tyrosol, (▲) hydroxytyrosol, (◇) *para*-hydroxyphenyl acetic acid, (+) 3,4-dihydroxyphenyl acetic acid.

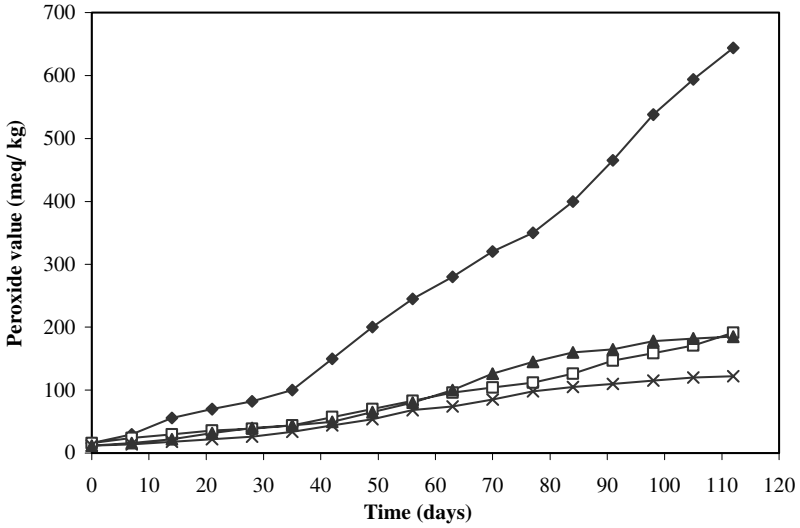

Fig. 6. Peroxide values of refined husk oils with added phenolic compounds during storage at 50 °C. (◆) Control, (▲) hydroxytyrosol at 200 ppm, (×) 3,4-dihydroxyphenyl acetic acid at 200 ppm, (□) OMWW extract at 500 ppm.

popularity of natural food additives may prompt more food manufacturers to replace synthetic antioxidants with ingredients containing natural antioxidative compounds. Therefore, research on natural ingredients has gained momentum as they are generally considered to pose no health risk to consumers (Halliwell, 1990).

OMWW might be a natural source of useful substances. Indeed, some of the polar phenolic compounds present in extra virgin olive oil, particularly hydroxytyrosol and oleuropein, have potent scavenging action on superoxide radicals. Since the concentration of these antioxidants in OMWW is 100- to 500-fold higher than that in olive oil, their recovery from OMWW could be addressed with high priority. A simple, relatively low-cost continuous extraction technique is adopted in this study in order to recover these high-value products. Optimal operational conditions were developed to extract the phenolic compounds present in OMWW using a continuous counter-current extraction unit. The HPLC analysis showed a predominant presence of hydroxytyrosol (1.43 g/l).

The effective recovery of these low-molecular-mass phenolics, apart from the intrinsic economic validity, might also be beneficial for the ensuing reduction in OMWW phenolic content in view of further downstream treatments and/or applications (Sayadi, Allouche, Jaoua, & Aloui, 2000). Most of the published research has dealt with hydroxytyrosol as the most interesting *ortho*-diphenol that occurs in olives and their derivatives, due to its antioxidative, antibacterial and beneficial effects on human health (Capasso, 1997; Capasso, Cristinzio, Evidente, & Scognamiglio, 1992; Capasso, Evidente, & Visca, 1994; Visioli et al., 1999). However, 3,4-dihydroxyphenyl acetic acid was not reported previously as a potent antioxidant. Product recovery, particularly of hydroxytyrosol from OMWW, is a fairly well studied technology at European level. Since this polyphenolic compound is not commercially available, several methods for chemical synthesis of hydroxytyrosol, have been previously reported in the literature, particularly those performed by Capasso, Evidente, Avolio, and Solla (1999) Bai, Yan, Takenaka, Sekiya, and Nagata (1998) and Verhe, Papadopoulos, and Boskou (1992).

We have applied relevant assays to screen the antioxidant properties of some phenolic compounds of OMWW that may be exploited as food antioxidants. The data reported in this paper indicate that ethyl acetate OMWW extract contains potent antioxidants. Hydroxytyrosol and 3,4-dihydroxyphenylacetic acid are the most active components isolated from OMWW. They showed a strong DPPH radical scavenging activity and a strong inhibition of linoleic acid in aqueous media. The addition of 3,4-dihydroxyphenylacetic acid and hydroxytyrosol, at 200 ppm, to refined oils showed a significant retardation in the oxidation rate comparable and even superior to that of BHT. These results suggest also that the antioxidant activity of OMWW extract could be a synergistic effect of hydroxytyrosol, 3,4-dihydroxyphenylacetic and other *o*-diphenols.

In conclusion, our data show that low-cost natural polyphenolic extracts could be produced from commercially available OMWW for use as alternatives to BHA and BHT. Moreover, the commercially available compound 3,4-dihydroxyphenyl acetic acid, which has the highest antioxidant power, and the OMWW-derived hydroxytyrosol, may potentially be used as alternative natural antioxidants to stabilize edible oils, while at the same time appeasing a major concern of consumers over the use of synthetic antioxidants in food products.

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