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Effect of storage on refined and husk olive oils composition: Stabilization by addition of natural antioxidants from Chemlali olive leaves

Mohamed Bouaziz [a], Ines Fki [a], Hedya Jemai [a], Mohamed Ayadi [b], Sami Sayadi [a,*]

[a] *Laboratoire des Bioprocédés, Pôle d'Excellence Régional AUF (PER-LBP), Centre de Biotechnologie de Sfax, BP: K, 3038 Sfax, Tunisia*
[b] *Institut de l'olivier de Sfax, 3029 Sfax, Tunisia*

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Abstract

Two samples of refined olive and husk oils have been analysed in order to evaluate the influence of storage time on their quality. The following parameters were determined: peroxide values, absorption coefficients K270 and K232, Rancimat induction time, sterols and fatty acid contents. Six months storage at 50 °C in the dark revealed a loss in oil stability. This finding was reflected by the greater increase in peroxide value and a decrease of Rancimat induction time and sterol content. The enrichment of refined olive and husk oils with olive leaves and its hydrolysate extract resulted in an appreciable resistance to oxidative deterioration due to its phenolic antioxidants content. Oleuropein and hydroxytyrosol were the major compounds in Chemlali olive leaves extract and hydrolysate solution, respectively. The antiradical activity of leaves extract as well as its hydrolysate solution was evaluated and compared to that of the BHT. The antioxidant activity of the enriched refined olive and husk oils with leaves and hydrolysate extracts at 400 ppm showed that the latter had the highest protective effect against oil oxidation. Oils with added hydrolysate extract had the lower peroxide value and the higher stability measured with a Rancimat method. After six months of storage the induction time increased from 23.3 to 83.5 h for refined olive oil and from 16.6 to 49 h for husk oil. Furthermore, during oil storage, there was no significant variation in fatty acid composition. However, the total sterol concentration of the oils treated with hydrolysate extract increased. The results suggested that hydrolysate and leaves extracts are excellent antioxidants and can serve as substitutes for synthetic antioxidants.

Keywords: Chemlali olive leaves; Refined oils; Stabilization; Natural antioxidants

1. Introduction

Olive and husk refined oils are characteristic by products of olive oil production and are gaining importance in the food industry. These oils have a lower content of polyphenols since these compounds are among the substances eliminated during the refining process (García, Ruiz-Méndez, Romero, & Brenes, 2006). Olive and husk refined oils are therefore unstable and subjected to rapid oxidation during storage. Oxidation of oils modifies their organoleptic properties, affecting the shelf life of this product. It results in the loss of nutritional value of food as well as changes in colour, texture, sensory and other physiological properties (Iqbal & Bhanger, 2007). Due to these changes, consumers do not accept oxidized products and industries suffer from economic losses.

The oil industry has to pay special attention in this context, as oils, fats and fatty foods suffer stability problems (Valenzuela, Sanhueza, & Nieto, 2003). The oils with higher contents of unsaturated fatty acids, especially polyunsaturated fatty acids, are more susceptible to oxidation. In order to overcome the stability problems of oils and fats, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ter-butyl hydroquinone (TBHQ) have been used as food additives.

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

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However, recent reports reveal that these compounds may be implicated in many health risks, including cancer and carcinogenesis (X Hou, 2003; Prior, 2004). Therefore, the most powerful synthetic antioxidant (TBHQ) is not allowed for food application in Japan, Canada and Europe. Similarly, BHA has also been removed from the generally recognized as safe (GRAS) list of compounds (Goli, Barzegar, & Sahari, 2005).

Due to these safety concerns, there is an increasing trend among food scientists to replace these synthetic antioxidants with natural ones, which, in general, are supposed to be safer (Yanishlieva & Marinova, 2001). Phenols are one of the most important groups of natural antioxidants (Artajo, Romero, Morello, & Motilva, 2006). They occur only in material of plant origin and they are known to protect easily-oxidizable constituents of food from oxidation. Olive oil and olive by products provide a rich source of natural antioxidants. These include carotenoids, tocopherols and phenolic compounds which may act, by different mechanisms, to confer an effective defence system against free radical attack (Morello, Motilva, Tovar, & Romero, 2004). The enrichment of processed food with polyphenols protects against oxidation and means better keeping quality because the formation of toxic oxidation products, such as cholesterol oxides, is prevented. Such enrichment also benefits human health (Antolovich et al., 2004).

Olive leaves are one of the by products of farming of the olive grove; they can be found in high amounts in the olive oil industries (10% of the total weight of the olives) and they accumulate during pruning of the olive trees (Tabera et al., 2004). Olive leaves are considered as a cheap raw material which can used as useful source of high-added-value products (Briante et al., 2002). Historically, olive leaves have been used as a folk remedy for combating diseases, such as malaria. Several reports have shown that olive leaf extract has the capacity to lower blood pressure in animals and increase blood flow in the coronary arteries, to relieve arrhythmia and prevent intestinal muscle spasms (Garcia, Castillo, Lorente, Ortuno, & Del Rio, 2000). In a recent study, it was found that phenolic compounds from olive leaves have a strong protective effect against oil oxidation. Indeed, it was reported that the enrichment with extract from 1 kg of leaves is sufficient to fortify 50–320 l of refined olive oil to a similar stability as a virgin olive oil (Paiva-Martins, Correia, Felix, Ferreira, & Gordon, 2007). Similarly, it was reported that phenolic extracts obtained from the olive plant (fruit, leaves, and pomace) showed remarkable antioxidant activity in retarding sunflower oil oxidative rancidity (Farag, El-Baroty, & Basuny, 2003). In previous work, we reported the extraction and isolation of oleuropein and hydroxytyrosol from olive leaves and hyrolysate extracts using a C-18 silica gel column. Isolated hydroxytyrosol and hydrolysate extract showed strong antioxidant activity better than BHT (Bouaziz & Sayadi, 2005).

The aims of the present work were to evaluate the (i) fatty acid as well as sterol compositions of refined and husks oils during storage, and (ii) antioxidant efficacy of leaf extract and its hydrolysate, in the retardation of their oxidation. The oils were examined before and after six months storing. Each sample was examined for P.V., K_{232}, K_{270}, total phenols, fatty acids and sterols. Stability was measured using a Rancimat apparatus at 100 °C.

2. Materials and methods

2.1. Plant material

Refined olive oil and refined olive husk oil samples were obtained from a local commercial refining plant (AGRO–ZITEX, Sfax). Fresh green olive leaves (200 g) were collected from the Chemlali olive tree cultivar commonly cultivated in Tunisia. The Watter were dried on site in a Gold star microwave oven, three times for 2 min at maximum power (1250 W). Dried leaves were powdered and stored in a dry place in the dark for extraction.

2.2. Reagents and standards

Oleuropein was purchased from Extrasynthèse, Genay, France. Hydroxytyrosol was purified from olive leaves as described previously by Bouaziz and Sayadi (2005). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemicals Co. (St. Louis, MO). All phenolic compounds solutions were made in a mixture of methanol/water 80/20 (v/v), the double distilled water was used in the HPLC mobile phase. Pure HPLC solvents were used in all cases.

2.3. Extraction of phenolic compounds from olive leaves

Experiments were carried out on leaves (*Olea europaea* L.) of Chemlali cultivar commonly cultivated in Tunisia. The extraction method adopted was able to achieve the extraction of compounds of interest (Bouaziz & Sayadi, 2005; Bouaziz, Chamkha, & Sayadi, 2004). A mixture of methanol and water (300 ml, 80:20 v/v) was added to the olive leaves powder (60 g) and this was left to stand overnight under agitation in the dark. Subsequently, the solution was filtered using GF/F filter paper. The extract was concentrated in vacuum to dryness at 40 °C and the residue obtained was redissolved in 5 ml of methanol and stored in glass vials at 0 °C in darkness before chromatographic analysis.

2.4. Acid hydrolysis of olive leaves extract

One gram of the olive leaf extract was dissolved in 10 ml of a MeOH/H_2O (4:1) mixture in sealed vial. The solution was hydrolysed at 100 °C for 1 h using 5 ml of a 2 M solution of HCl (Prolabo, France). After 1 h, the sample was cooled and diluted with water (10 ml) and the hydrophobic fraction was extracted in a separating funnel for three times with 15 ml of ethyl acetate (Prolabo, France), which was subsequently removed by evaporation.

2.5. HPLC analysis of phenolic compounds

HPLC analysis used for monomeric phenols and oleuropein was performed on a Shimadzu apparatus composed of a (LC-10ATvp) pump and a (SPD-10Avp) UV detector. The columns used to analyse phenols was a C-18 (4.6 × 250 mm) Shim-pack VP–ODS. Eluates were detected at 280 nm. The temperature was maintained at 40 °C. The mobile phase used was 0.1% phosphoric acid (Prolabo, France) in water (A) vs. 70% acetonitrile (Dharmadrug, GmbH, Germany) in water (B) for a total running time of 50 min. The elution conditions applied for phenolic compounds were: 0–25 min, 10–25% B; 25–35 min, 25–80% B; 35–37 min, 80–100% B; 37–40 min, 100% B, and finally washing and reconditioning steps of the column were included (40–50 min) linear gradient 100–10% B. The flow rate was 0.6 ml/min and the injection volume was 50 μl. The identification and quantification of phenolic compounds in *O. europaea* L. Chemlali leaves variety was based on their spectra, on their retention time in comparison with phenolic standards analysed in the same condition and on the method of standard addition to the samples.

2.6. LC–MS/MS analysis

The LC–MS/MS experiments were carried out with an Agilent 1100 LC system consisting of degasser, binary pump, auto sampler, and column heater. The column outlet was coupled to an Agilent MSD Ion Trap XCT mass spectrometer equipped with an ESI ion source. Data acquisition and mass spectrometric evaluation was carried out on a personal computer with Data Analysis software (Chemstations). For the chromatographic separation a Zorbax 300 Å Extend-C-18 Column (2.1 × 150 mm) was used. The column was held at 95% solvent A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in ACN) for 1 min, followed by an 11 min step gradient from 5% B to 100% B, then it was kept for 4 min with 100% B, finally, the elution was achieved with a linear gradient from 100% B to 5% B in 2 min. The flow rate was 200 μl min^{-1} and the injection volume 5 μl. The following parameters were employed throughout all MS experiments: for electrospray ionisation with positive ion polarity the capillary voltage was set to 3.5 kV, the drying temperature to 350 °C, the nebulizer pressure to 40 psi, and the drying gas flow to 10 l min^{-1}. The maximum accumulation time was 50 ms, the scan speed was 26,000 m z^{-1} s^{-1} (ultra scan mode) and the fragmentation time was 30 ms. Identification of compounds by LC–MS analysis was carried out by comparing retention times and mass spectra of the unknown peaks to those of standards.

2.7. Fatty acid determination (GC)

The fatty acid composition of the oils was determined by gas chromatography (GC) as fatty acid methyl esters (FAMEs). FAMEs were prepared by saponification/methylation with sodium methylate according to the European Union Commission modified Regulation EEC 2568/91. A chromatographic analysis was performed in a SHIMADZU set 17 A Series II gas chromatography using a capillary column (stabilwax, Restek). The column temperature was isothermal at 180 °C and the injector 230 °C and detector temperatures were 250 °C. Fatty acids were identified by comparing retention times with standard compounds. Six fatty acids were considered in this study. These were palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acids expressed as percentages of fatty acid methyl esters.

2.8. Sterol determination

The analysis of sterolic fractions extracted from olive oil was determined according to the method adopted by the international olive council (European Union Commission Regulation, 1991). Briefly, 5 g of olive oil from different treatment were added to α-cholestanol, used as internal standard was saponified with potassium hydroxide in ethanolic solution. After 1 h of boiling, 100 ml of water was added and the extraction of unsaponifiable was carried out by 200 ml of ethyl ether. 20 mg of unsaponifiable were dissolved in 0.5 ml of chloroform then deposited on a basic silica gel plate. The elution was achieved by a mixture of hexane, and ether (65/35 v/v) then the plate was pulverized by a solution of 2,7-dichlorofluorescein (0.2% in the ethanol), we scrape the band corresponding to sterols. Sterols recovered from the plate were dissolved in chloroforme and filtered through a paper filter. The solvent was evaporated under N_2 and the sterols were transformed into trimethylsilyl ethers and the mixture was analysed by gas chromatography using a chromatograph SHIMADZU set 17A equipped with capillary column (30 m length × 0.32 mm i.d.) coated with stationary phase formed by 5% of biphenyl and 95% of dimethyl polysiloxane (0.25 μm thickness). The analytical conditions were: vector gas: Nitrogen; flow rate: 1 ml/mm; column temperature: 260 °C; injector temperature: 280 °C; detector temperature: 290 °C; quantity injected: 5 μl.

2.9. DPPH radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH radical) scavenging effect was evaluated according to the method employed by Bouaziz, Grayer, Simmonds, Damak, and Sayadi (2005). Four millilitres of each sample extract (or a pure compound) at different concentrations (25, 50, 100 and 150 μg/ml) were added to 10 ml 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 Shimadzu UV-160 A spectrophotometer. The test samples were tested and positive control BHT over a range of concentrations. The antioxidant activity of each test sample and BHT was expressed in terms of concentration required to inhibit

50% DPPH radical formation (IC_{50} μg/ml) and calculated from the log-dose inhibition curve.

2.10. Antioxidant activity in refined oils

Weighed quantities of leaves and hydrolysate extracts were dissolved in 1 ml of ethanol, to obtain the desired final molar concentration (400 ppm). Then, these were added to refined olive and husk oils (Farag et al., 2003). The phenolic extracts 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 and conjugated dienes and trienes formation as previously described by Fki, Allouche, and Sayadi (2005).

Oil oxidative stability was also evaluated by measuring the oxidation induction time, on a Rancimat apparatus (Metrohom Series 679). Air (20 l/h) was bubbled through oil (5 g) heated at 100 °C, the volatile compounds were collected in water, and the increasing water conductivity was continually measured. The time taken to reach the conductivity inflection time was recorded.

2.11. Statistical analysis

Results were expressed as mean ± standard deviation (SD). Statistical significance (*t*-test: two-sample equal variance, using two-tailed distribution) was determined using Microsoft Excel statistical software (Microsoft Corporation, Microsoft Office Excel 2003, Redmond, WA). Differences at $P < 0.05$ were considered to be significant.

3. Results and discussion

3.1. Initial composition

Table 1 presents the results obtained from analyses of the initial compositions of the refined and husk oil samples used for this study. Peroxide value (PV), conjugated dienes and trienes were measured by extinction value at 232 and 270 nm, while total sterol and fatty acid were quantified by GC. In refined olive oil, the mean values of the main saturated (palmitic), monounsaturated (oleic) and polyunsaturated (linoleic) acids were 11.99, 68.27 and 16.12%, respectively. Among the substances with antioxidant properties, the total phenols contents shows significant differences between the two samples, with initial concentrations of 158.0 mg kg^{-1} for refined olive oil and 78.1 mg kg^{-1} for refined husk oil expressed as pyrogallol equivalent. Consequently, the oxidative stability, as determined by the Rancimat method, also differed between the two oils samples (34.4 h for refined olive oil and 29.1 h for the husk oil), since samples with higher concentrations of phenolic compounds, especially ortho-diphenols, exhibited greater oxidative stability as reported elsewhere (Gómez-Alonso, Salvador, & Fegapane, 2004; Gutfinger, 1981).

Table 1
Initial composition of refined olive and husk oil samples

	Refined olive oil	Refined husk oil
Peroxide values (meq kg^{-1})	15.91 ± 0.11	14.33 ± 0.26
Conjugated dienes (K_{232})	0.247 ± 0.035	0.22 ± 0.022
K_{270}	0.048 ± 0.002	0.125 ± 0.005
Total sterol (mg kg^{-1})	1286 ± 0.4	3505 ± 1.6
Total phenols (mg kg^{-1})	158 ± 0.1	78.1 ± 0.4
Fatty acid (% peak area)		
$C_{16:0}$	11.99 ± 0.14	14.96 ± 0.21
$C_{16:1}$	1.62 ± 0.02	2.18 ± 0.15
$C_{18:0}$	1.48 ± 0.04	1 ± 0.09
$C_{18:1}$	68.27 ± 0.17	64.08 ± 0.20
$C_{18:2}$	16.12 ± 0.09	16.09 ± 0.29
$C_{18:3}$	0.34 ± 0.03	0.71 ± 0.06

3.2. Phenolic profile and antioxidant activity of olive leaf extract and its hydrolysis

A high amount of oleuropein was obtained using methanol/water (80:20 v/v) as an extraction mixture. HPLC analyses showed that oleuropein was the major compound of the leaf extract (Fig. 1a). The identification of oleuropein was based on comparison of the chromatographic retention time, UV absorbance spectra comparison with the authentic standard and confirmed by using LC–MS apparatus in the positive mode. Oleuropein concentration reached 14% (all data are reported on a dry mass basis) and was far in excess in comparison with the other phenolics.

Acid treatment of aqueous methanolic leaf extract induced hydrolysis or break down of the more complex phenolic molecules. The most notable effect seen was the increase in hydroxytyrosol concentration followed by a complete disappearance of oleuropein. Hydroxytyrosol is the principal product of oleuropein degradation. Indeed, HPLC profile of phenols in the olive leaf extract after acid hydrolysis showed that hydroxytyrosol was the major compound in the hydrolysate extract (Fig. 1b). The identification of purified hydroxytyrosol was confirmed by using LC–MS apparatus in the positive mode. The spectrum exhibited a molecular ion at *m/z* 155 with fragments at *m/z* 137, 119, 99, and 91, which was consistent with the known fragmentation scheme for hydroxytyrosol (Bouaziz & Sayadi, 2005). It has been reported that acid hydrolysis causes the degradation of oleuropein to produce hydroxytyrosol, and other oleuropein derivatives (Bouaziz et al., 2005; Bouaziz & Sayadi, 2003).

The DPPH radical scavenging activity of the leaf extracts and hydrolysate, were measured and compared to that of BHT ($IC_{50} = 0.89$ μg/ml). Olive leaf extract ($IC_{50} = 1.5$ μg/ml) exhibited antioxidant potential comparable to that of pure oleuropein ($IC_{50} = 1.19$ μg/ml). Hydrolysate extract showed strong antioxidant activity and was the most effective with IC_{50} value 0.65 μg/ml. The IC_{50} value of hydrolysate suggests that this extract contains a high concentration of hydroxytyrosol ($IC_{50} = 0.58$ μg/ml) which resulted from oleuropein hydro-

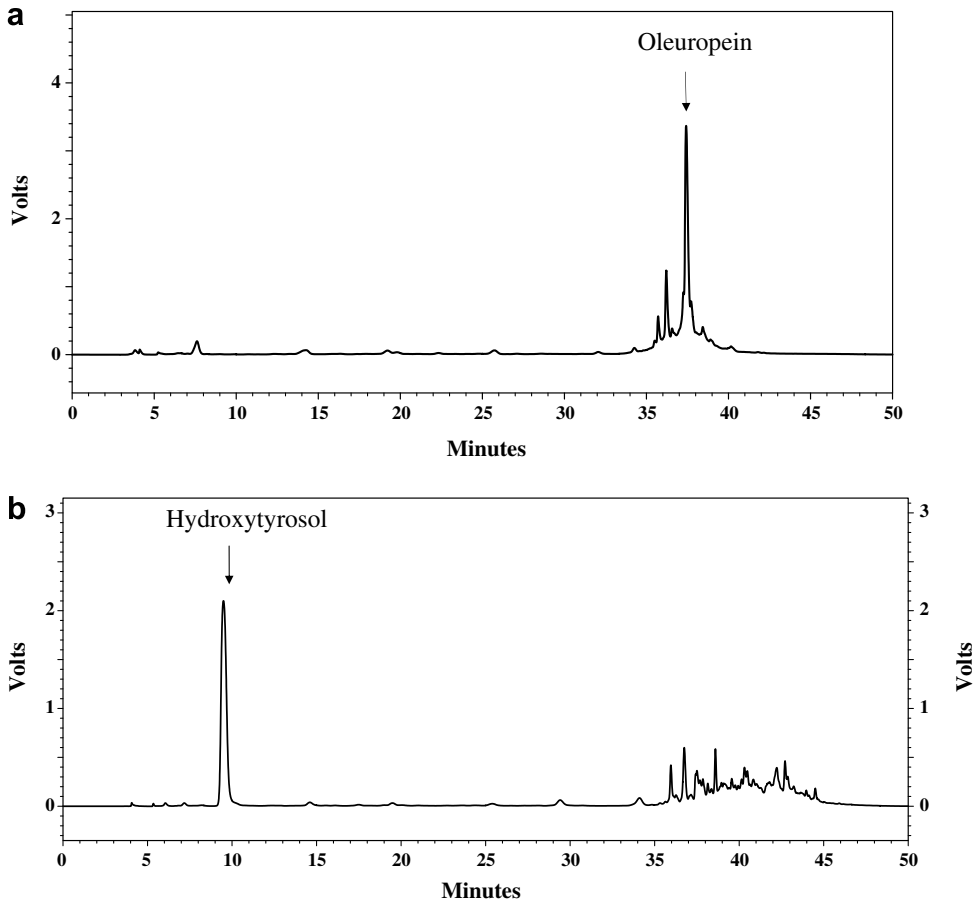

Fig. 1. HPLC chromatogram of Chemlali olive leaf extract (a) and its hydrolysate (b). Detection was at 280 nm.

lysis as it was previously reported (Bouaziz & Sayadi, 2005).

3.3. Oxidative stability of refined oils with and without leaves extracts

To determine the effect of the leave phenolics on the oxidation of refined oil, the leaf extract and hydrolysate were added to the refined husk and olive oils at 400 ppm (Farag et al., 2003). Lipid oxidation was monitored by measuring peroxide value (PV) and conjugated diene CD and triene formation CT. Both parameters (CD and PV) measure the primary product of lipid oxidation.

Data presented in this study showed that phenolics recovered from olive leaves were able to decrease the formation of both lipid hydroperoxides and conjugated dienes and trienes formation compared to the control. However, the hydrolysate was more efficient than olive leaves extract at decreasing the formation of both lipid oxidation markers. As it can be observed in Fig. 2, the peroxide value of the controls of olive and husk oils increased from 16 and 14 to 828 and 875 meq/kg, respectively. However, corresponding values of husk and olive oils after six months of incubation in the presence of hydrolysate increased from 16 to 328 meq/kg and 14 to 331 meq/kg, respectively. Furthermore, a regular increase in CD and CT contents as a function of storage time was observed for all the samples at all intervals. However, the rate of increase in hydrolysate stabilized oil samples was slow compared to the control sample (Figs. 3 and 4).

The refined oils oxidative resistance, measured by Rancimat, was greatly improved in the presence of leaves and hydrolysate extracts (Table 2). Indeed, the induction time of refined olive oil increased significantly from 34.4 to 89.8 h by enrichment with hydrolysate extract and to 64.8 h with the addition of olive leaves extract (Table 2). Equally, the induction period of husk oil augmented from 29.1 to 85.0 and 59.0 h in the presence of hydrolysate and leaves extract, respectively (Table 2).

Moreover, the oxidative oil stability after six months of storage was influenced by the presence of leaves phenolics. In fact, the oxidative resistances at 100 °C of refined olive oil increased significantly from 23.1 to 83.2 h by enrichment with hydrolysate extract and to 53.0 h in samples added with olive leaves extract (Table 2). Similarly, the induction period of husk oil augmented from 16.9 to 75 and 49 h in the presence of hydrolysate and leaf extract, respectively (Table 2).

The highest stability of refined oils added with hydrolysate extract can be explained by its richness in hydroxytyrosol. Indeed, previous reports showed that this phenol is the main antioxidant components in Chemlali olive

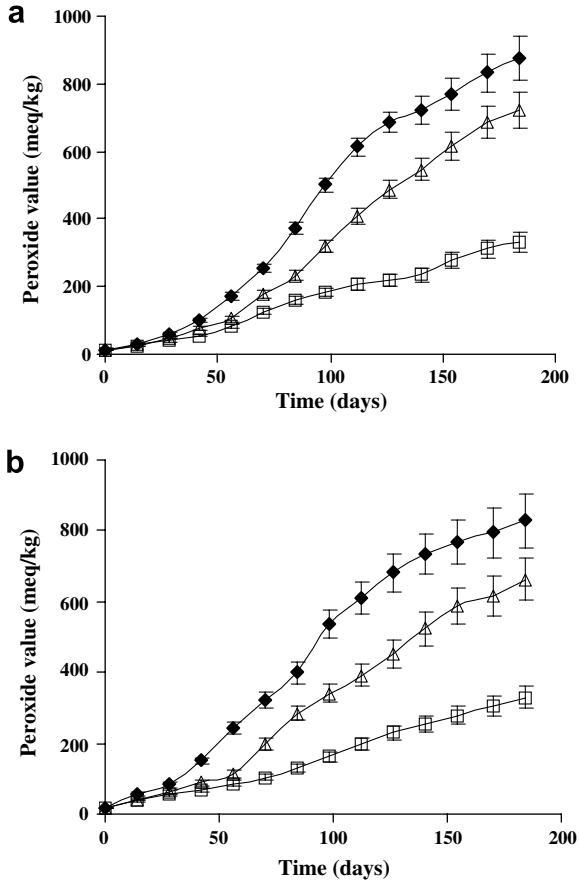

Fig. 2. Peroxide values (PV) of refined husk oils (a) and refined olive oils (b) with added phenolic compounds at 400 ppm during storage at 50 °C. (♦) Control, (Δ) olive leaves extract, (□) hydrolysate extract. Values given are the mean of three replicates ± standard deviation.

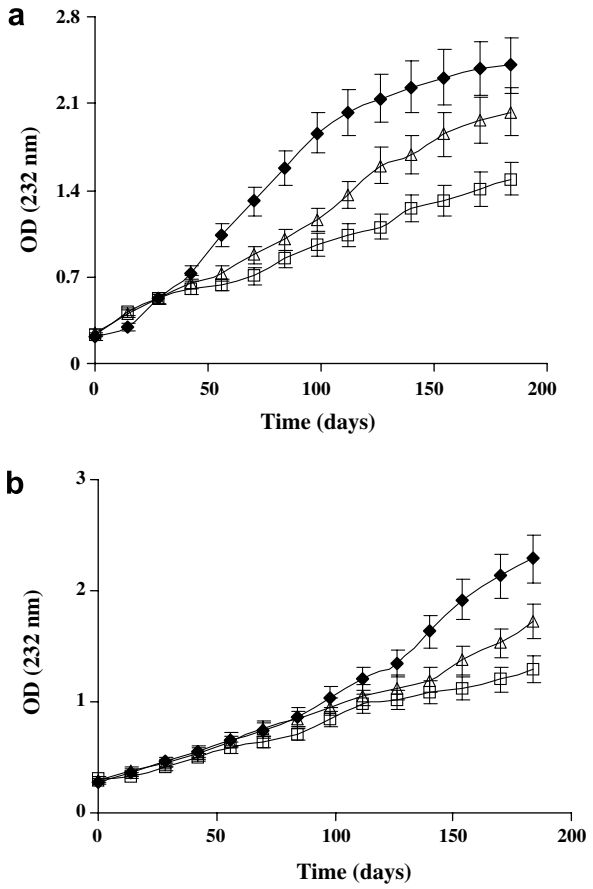

Fig. 3. Conjugated diene values (K_{232}) of refined husk oils (a) and refined olive oils (b) with added phenolic compounds at 400 ppm during storage at 50 °C. (♦) Control, (Δ) olive leaves extract, (□) hydrolysate extract. Values given are the mean of three replicates ±standard deviation.

cultivar: olive oil and olive by products (Bouaziz et al., 2005). Recent work in our laboratory has shown that hydroxytyrosol recovered from olive mill wastewaters increased the oxidative stability of refined oils in rate comparable to that of BHT at 200 ppm. In addition, it was reported that hydroxytyrosol had a highest protective effect against refined olive oil oxidation (Fki et al., 2005).

The antioxidant activity of hydroxytyrosol is ascribed to hydrogen donation and its ability to scavenge free radicals by forming an intra-molecular hydrogen bond between the free hydrogen of its hydroxyl group and their phenoxyl radicals (Visioli, Poli, & Galli, 2002) (Fig. 5). It generates a new radial that is stabilized by the resonance effect of the aromatic structure, the propagation phase is therefore blocked and the development of oxidation is delayed (Chimi, Cillard, Cillard, & Rahamani, 1991).

3.4. Changes in fatty acid composition during oils storage

The major fatty acids present as glycerides in refined husk and olive oil are oleic (C18:1), linoleic (C18:2), palmitic (C16:0), and stearic acid (C18:0) (Tables 3 and 4). Oleic acid is the main monounsaturated fatty acid in both refined and husk oil during storage and was present in higher concentrations than other acids (68.3–66.0%) and (64.1–63.1%), respectively. Palmitic acid, the main saturated fatty acid was not affected by the storage condition and addition of hydrolysate and leaf extracts in refined husk oil. However, the C16:0 content showed a significant increase in control and treated olive oil samples. Linoleic acid was the dominant polyunsaturated fatty acid in both refined olive oil and husk oil. It showed a non-significant variation ($P > 0.05$) during oil storage. Linolenic acid (C l8:3) content increased significantly ($P < 0.05$) after six months of storage from 0.34% to 0.51% in untreated olive oil sample, while it was not affected in the presence of hydrolysate and leaf extract. In addition, its concentration remained constant in refined husk oil.

Our results showed that polyunsaturated fatty acids remained almost constant during six months of storage. This could be a consequence of the impermeability of the containers to oxygen. It has been reported that the unsaturated fatty acids are very important for the stability of oils because of the chemical reactions occurring at the double bonds and their oxidation is accelerated by the exposure to the light, which causes formation of both singlet and/

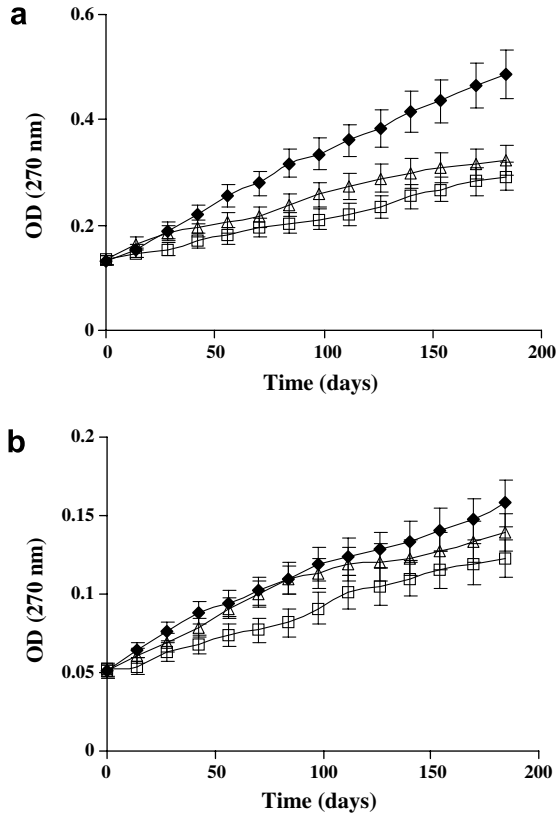

Fig. 4. Conjugated diene values (K_{270}) of refined husk oils (a) and refined olive oils (b) with added phenolic compounds at 400 ppm during storage at 50 °C. (♦) Control, (Δ) olive leaves extract, (□) hydrolysate extract. Values given are the mean of three replicates ± standard deviation.

or superoxide anion radicals (Morello et al., 2004; Rastrelli, Passi, Ippolito, Vacca, & De Simone, 2002).

3.5. Changes in sterol content during oils storage

Sterols are important nonglyceridic constituents of oil and are widely used to check authenticity. Storage time influenced the percentage composition of the sterol fraction. The variation in composition was significant in both refined olive and husk oils compared to the control oil sample without any additive after six months storage. Tables 5 and 6 showed a significant increase of total sterol for the samples added with hydrolysate and leaves extracts. As can be observed, hydrolysate leaves extract exhibited the most effect on total sterol content. It increased from 947 in control refined olive oil sample to 1802 mg/kg. These results agree with others previously reported, where the increase of total sterol content was explained by the thermal hydrolysis reaction in olive oil (Gutierrez & Fernaandez, 2002; Soupas, Juntunen, Lampi, & Piironen, 2004).

The concentration of sitosterol (the major sterol of the two studied oils) behaved differently in the two oils after six months storage. In refined olive oil, it increased from 1043 to 1480 mg/kg and 1263 mg/kg after enrichment with hydrolysate and olive leaves extracts, respectively. The Δ-7-avenasterol also behaved differently in the two oils, decreasing in refined husk oil and increasing in refined olive oil added with olive leaves and hydrolysate extracts. Δ-5-avenasterol content was constant throughout storage in husk oil supplemented by hydrolysate extract and showed a decrease in its concentration by the enrichment with leaves extract, while it decreased significantly in refined

Table 2
Antioxidant effects on refined olive and husk oils measured by Rancimat, under 120 °C temperature and 20 l h

Table 3
Refined olive oil fatty acid composition (%) after six months of storage enriched with olive leaves and hydrolysate extracts

Fatty acid	Refined olive oil $T = 0$ day	Refined olive oil $T = 6$ months	Refined olive oil + leaves extract $T = 6$ month	Refined olive oil + hydrolysate extract $T = 6$ months
C16:0	11.99 ± 0.14^{a}	13.35 ± 0.05^{b}	13.37 ± 0.07^{b}	13.57 ± 0.07^{b}
C16:1	1.62 ± 0.02^{a}	1.79 ± 0.01^{a}	1.75 ± 0.06^{a}	1.81 ± 0.03^{a}
C18:0	1.48 ± 0.04^{a}	1.8 ± 0.03^{b}	1.73 ± 0.03^{b}	1.92 ± 0.02^{c}
C18:1	68.27 ± 0.17^{a}	66.03 ± 0.03^{a}	65.49 ± 0.52^{a}	65.42 ± 0.44^{a}
C18:2	16.12 ± 0.09^{a}	15.72 ± 0.02^{a}	15.79 ± 0.02^{a}	15.43 ± 0.03^{a}
C18:3	0.34 ± 0.03^{a}	0.51 ± 0.02^{b}	0.32 ± 0.01^{a}	0.32 ± 0.02^{a}

Values given are the mean of three replicates ±standard deviation.
Means with the same letter in the same line are not significantly different at $P > 0.05$.

Table 4
Refined husk oil fatty acid composition (%) after six months of storage enriched with olive leaves and hydrolysate extracts

Fatty acid	Refined husk oil $T = 0$ day	Refined husk oil $T = 6$ months	Refined husk oil + leaves extract $T = 6$ months	Refined husk oil + hydrolysate extract $T = 6$ months
C16:0	14.96 ± 0.21^{a}	15.01 ± 0.02^{a}	15.06 ± 0.06^{a}	15.01 ± 0.02^{a}
C16:1	2.18 ± 0.15^{a}	1.92 ± 0.03^{a}	1.89 ± 0.01^{a}	1.91 ± 0.01^{a}
C18:0	1 ± 0.09^{a}	2.21 ± 0.01^{b}	2.14 ± 0.05^{a}	2.23 ± 0.03^{b}
C18:1	64.08 ± 0.20^{a}	63.13 ± 0.04^{a}	63.07 ± 0.08^{a}	63.07 ± 0.03^{a}
C18:2	16.09 ± 0.29^{a}	16.27 ± 0.18^{a}	16.35 ± 0.05^{a}	16.30 ± 0.11^{a}
C18:3	0.71 ± 0.06^{a}	0.72 ± 0.02^{a}	0.65 ± 0.04^{a}	0.62 ± 0.03^{a}

Values given are the mean of three replicates ±standard deviation.
Means with the same letter in the same line are not significantly different at $P > 0.05$

Table 5
Contents of the sterols in the refined olive oil after six months storage enriched with olive leaves and hydrolysate extracts (mg/Kg of oil)

Sterol	Oil			
	Refined olive oil $T = 0$ day	Refined olive oil $T = 6$ months	Refined olive oil + leaves extract $T = 6$ months	Refined olive oil + hydrolysate extract $T = 6$ months
Campesterol	35.67 ± 0.06^{a}	26.91 ± 0.58^{b}	41.02 ± 0.53^{c}	46.49 ± 0.78^{d}
Stigmasterol	22.87 ± 0.42^{a}	14.07 ± 0.08^{b}	33.25 ± 0.13^{c}	38.23 ± 0.12^{d}
β-Sitosterol	1043 ± 0.2^{a}	794.5 ± 0.2^{b}	1263 ± 0.4^{c}	1480 ± 0.2^{d}
Δ-5-Avenasterol	102.9 ± 0.46^{a}	78.28 ± 0.14^{b}	72.4 ± 0.2^{c}	79.69 ± 0.34^{d}
Δ-7-Avenasterol	24.68 ± 0.19^{a}	17.18 ± 0.09^{b}	58.12 ± 0.06^{c}	77.84 ± 0.17^{d}
Erythrodiol + uvaol	57.94 ± 0.05^{a}	15.96 ± 0.12^{b}	67.35 ± 0.07^{c}	79.66 ± 0.34^{d}
Total (mg/kg)	1286 ± 0.4^{a}	947 ± 0.02^{b}	1535 ± 0.06^{c}	1802 ± 0.4^{d}

Values given are the mean of three replicates ±standard deviation.
Means with the same letter in the same line are not significantly different at $P > 0.05$.

Table 6
Contents of the sterols in the refined husk oil after six months storage enriched with olive leaves hydrolysate extract (mg/kg of oil)

Sterol	Oil			
	Refined husk oil $T = 0$ day	Refined husk oil $T = 6$ months	Refined husk oil + l eaves extract $T = 6$ months	Refined husk oil + hydrolysate extract $T = 6$ months
Campesterol	104.5 ± 0.1^{a}	130.3 ± 0.2^{b}	133 ± 0.1^{c}	139.1 ± 0.05^{d}
Stigmasterol	36.69 ± 0.08^{a}	45.73 ± 0.02^{b}	40.71 ± 0.05^{c}	52.27 ± 0.14^{d}
β-Sitosterol	2738 ± 0.5^{a}	2511 ± 0.5^{b}	2622 ± 0.3^{c}	2805 ± 0.1^{d}
Δ-5-Avenasterol	152.5 ± 0.3^{a}	135.1 ± 0.06^{b}	143.5 ± 0.25^{c}	152.6 ± 0.3^{a}
Δ-7-Avenasterol	329.7 ± 0.3^{a}	257.4 ± 0.18^{b}	258 ± 0.1^{c}	259.4 ± 0.2^{d}
Erythrodiol + uvaol	145.1 ± 0.05^{a}	71.00 ± 0.52^{b}	75.53 ± 0.27^{c}	82.41 ± 0.21^{d}
Total (mg/kg)	3505 ± 1.6^{a}	3151 ± 0.2^{b}	3272 ± 0.5^{c}	3490 ± 0.9^{d}

Values given are the mean of three replicates ± standard deviation.
Means with the same letter in the same line are not significantly different at $P > 0.05$.

olive oil (Tables 5 and 6). The stigmasterol content remained lower than that of campesterol, their behaviour was similar, and their concentration increased significantly during storage after enrichment of refined olive oil and husk oil with hydrolysate extract and leaves extract (Tables 5 and 6).

The results suggest that the influence of storage time on sterol composition is more important than its influence on fatty acids. This agrees with other findings previously obtained by Gutierrez, Varona, and Albi (2000) who reported that during olive fruit storage no significant variation in fatty acid composition was observed. However, the sterol composition of the oil varied markedly.

4. Conclusion

From the present study, it is concluded that olive leave hydrolysate extract with high antioxidant activity can stabilize refined olive and husk oils up to a greater extent than commonly employed synthetic antioxidants as previously described by Fki et al. (2005). It inhibits rancidity deterioration of oils by improving their stability and increasing their sterol content. Therefore, olive leaves can be considered as a potential antioxidant source of natural origin. Thus, incorporation of such extracts in food industry may contribute to the health benefit of the consumers significantly and also to prolong the shelf life of food products.

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