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Research Article

# Stabilization of refined olive oil by enrichment with chlorophyll pigments extracted from Chemlali olive leaves

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This paper presents the first investigation on the effect of enrichment refined olive oil by chlorophyll pigment extracted from Chemlali olive leaves during storage (6 months). The changes that occurred in the quality indices, fatty acids, sterol, and phenolic content were investigated during the storage of refined olive oil under RT (20°C) and accelerated conditions (50°C) in the dark. Additionally, the pigments (chlorophyll and carotene) changes during 6 months of oil storage were evaluated. At the end of the storage, more than 90% of chlorophyll pigments decomposed in all samples, while, carotene pigment loss was lower showing up to 60 and 85% loss for oil stored at 20 and 50°C, respectively, at the end of storage. The reduction of total phenolic compounds exhibited similar degradation profiles, being reduced by 5% and up to 60% for the enriched refined olive oil stored at 20 and 50°C in 6 months, respectively. In the fatty acid composition, an increase in oleic acid and a decrease in linoleic and linolenic acids were less significant in enriched than non-enriched refined olive oil. On the other hand, sterol composition was less affected by storage in enriched oil samples. However, the sterol concentration of the oil samples showed an increase in β-sitosterol, 24-methylene cholesterol, stigmasterol, and a decrease in cholesterol, Δ5, 24-stigmastadienol percentage at the end of storage. Based on the Rancimat method, the oils with added leaf pigment extract had the lowest peroxide value and the highest stability. After 6 months of storage, the oxidative resistance of refined olive oil fell to 0.2 and to zero for enriched refined olive oil stored at 20 and 50°C, respectively.

**Keywords:** Chlorophyll pigments / Oil storage / Olive leaves / Refined olive oil

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## 1 Introduction

*Olea europea* L. (Oleace) cultivation and olive oil production represents one of the most important and traditional agricultural activities in the Mediterranean countries. Olives and olive oil have been reported to contain phenolic compounds, which possess beneficial properties for human health. A great proportion of this oil must be refined to be edible. These oils are susceptible to rapid oxidation during storage due to its low content in polyphenols and losses of oil pigment, mainly chlorophylls, during the extraction process of olive oil [1–3].

Lipid oxidation is one of the most important quality deterioration processes in lipid containing foods and leads to great economic losses in the food industry. In order to overcome the stability problems of oils, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ter-butyl hydroquinone (TBHQ) have been used as food additives. But recent reports reveal that these compounds may be implicated in many health risks, including cancer, and carcinogenesis. 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 list of compounds [4, 5].

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**Abbreviations:** **BHA,** butylated hydroxyanisole; **BHT,** butylated hydroxytoluene; **DPPH,** 2, 2-diphenyl-1-picrylhydrazyl; **ECN,** equivalent carbon number; **FRAP,** ferric reducing antioxidant power; **GA,** gallic acid; **PV,** peroxide value; **TBHQ,** ter-butyl hydroquinone; **VOO,** virgin olive oil

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Therefore, the importance of replacing synthetic antioxidants with natural ingredients has increased greatly. The research on vegetable sources and the screening of raw materials for identifying new antioxidants has recently intensified. The large-scale availability of agricultural and industrial plant waste materials and their low-cost makes them attractive sources of natural antioxidants [6]. In particular, among the natural antioxidants, phenolic compounds, α-tocopherol and β-carotene, are reported to play a key role in preventing oxidation and have been already correlated to the storage stability of virgin olive oils (VOO) [7].

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 and they accumulate during pruning of the olive trees [2, 3, 8]. Olive leaves (*Oleaceae*) have been widely used in folk medicine for several thousand years in European Mediterranean islands and countries [2, 3, 9].

Therefore, the total worldwide consumption of olive oil and the limited VOO production capacity indicate the potential for increasing the range of oils offered to the consumer. The possibility of developing higher quantities of olive oil, with the same stability characteristics found in VOO, by the enrichment of refined olive oil with olive leaves pigments.

This study was set up to investigate the change in non- and enriched refined olive oil quality, as a function of storage time, based on the analysis of parameters such as peroxide index, coefficients $K_{270}$ and $K_{232}$, minor components, such as chlorophylls, carotenoids, and especially in the phenolic fraction, fatty acids, sterols, and triglycerides (equivalent carbon number (ECN) %).

## 2 Materials and methods

### 2.1 Chemical and reagents

Methanol, *n*-hexane, cyclohexane, and acetic acid high-performance liquid chromatography (HPLC)-grade solvents were purchased from Riedel-deHaen (Switzerland). Ethanol was obtained from Carlo Erba (Milan, Italy). The solvents were of appropriate purity. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 6-di-tert-butyl-4-hydroxy-boxylic acid (BHT), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) and 2, 4, 6-tri(2-pyridyl)-*s*-triazine (TPTZ) were purchased from Sigma–Aldrich (Chemie Gmbh, Steinhein, Germany). Iron (III) chloride (FeCl3.6H2O) and sodium acetate (CH3COONa.3H2O) were purchased from Merck (Darmstadt, Germany). FAMEs standards and 2, 6, 10, 15, 19, 23-hexamethyl-2, 6, 10, 14, 18, 22-tetracosahexaene (squalene) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The double distilled water was used in the HPLC mobile phase. The standards used were trilinolein (LLL), triolein (OOO), tripalmitin (PPP), tristearin (SSS), trilinolenin (LnLnLn), and tripalmitolein (PoPoPo) of purity >98% and purchased from Sigma (St. Louis, MO). The abbreviations used for the fatty acids were P for palmitic; S for stearic; O for oleic; L for linoleic, Po for palmitoleic and Ln for linolenic acids.

### 2.2 Plant material

Refined olive oil sample was 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, during 2009/2010 crop from Sfax.

### 2.3 Extraction of chlorophyll pigment from olive leaves

Experiments were carried out on leaves (*Olea europaea L.*) of Chemlali cultivar commonly cultivated in Sfax (South of Tunisia). Ethanol was added to the olive leaves (100 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 35°C and the residue obtained was redissolved in 5 mL of ethanol and stored in glass vials at 0°C in darkness until its use.

### 2.4 Determination of antioxidant activity of chlorophyll rich extract of Chemlali olive leaves

#### 2.4.1 DPPH assay

The total free radical scavenging capacity was determined and compared to that of BHT according to the method described by Rigane et al. [10]. Various concentrations of ethanolic chlorophyll rich extract of Chemlali olive leaves (50, 100, 150, 200, and 250 μg/mL) or BHT (5, 10, 15, 20, and 25 μg/mL) (1 mL), was mixed with 2 mL of $6 \times 10^{-6}$ M DPPH methanolic solution. The mixture was shaken vigorously and left to stand for 30 min at RT, and the absorbance was then measured at 517 nm against a blank. The percentage scavenging effect was calculated as radical Scavenging activity (%RSA) = $[(A_{\mathrm{DPPH}} - A_{\mathrm{E}})/A_{\mathrm{DPPH}}] \times 100$, where $A_{\mathrm{DPPH}}$ was the absorbance of the control (without extract) and $A_{\mathrm{E}}$ was the absorbance in the presence of the extract. The free radical scavenging activity ($IC_{50}$, μg/mL) of extracts was calculated from the graph of RSA percentage against extract concentration. BHT was used as reference compound. All of the samples were analyzed in triplicate.

#### 2.4.2 FRAP assay

The FRAP assay was based on the method of Pulido et al. [11]. Reagents included 300 mM acetate buffer (pH 3.6); 10 mM TPTZ; 20 mM $FeCl_3 \cdot 6H_2O$ in 1 M hydrochloric acid. The FRAP reagent was prepared by mixing 10 mL acetate buffer, 1 mL TPTZ solution and 1 mL $FeCl_3 \cdot 6H_2O$ solution. 100 μL sample was added into the 3 mL FRAP solution, 900 μL of 96% EtOH, and 300 μL $H_2O$was

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added. The reaction was maintained for 6 min, and the absorbance reading was constant at 593 nm. Trolox was introduced as standard compound with the final concentration ranging from 0.25 to 1 mM, and the calibration curve was y (absorbance) = 0.2595 × (mM TE/g of extract) ($r^2 = 0.9965$). The results were expressed as Trolox equivalent (Trolox equivalent mM per 1 g of extract). All of the samples were analyzed in triplicate.

## 2.5 Description of samples

The test material for the study was two refined olive oils, which one was considered as control sample (non-enriched refined olive oil) and the second one was enriched with 400 ppm of chlorophyll pigment extracted from Chemlali olive leaves. Weighed quantities of leaves extracts were dissolved in 1 mL of ethanol, to obtain the desired final molar concentration (400 ppm). Then, these were added to refined olive oil. The chlorophyll extracts were mixed with oil by stirring for 2 h in the darkness and at RT. The samples were stored, in full filled dark bottles, for 6 months at temperatures of 20 and 50°C, and analyzed every 10 days of storage.

## 2.6 Analytical methods

### 2.6.1 Quality indices determinations

Determination of peroxide value (PV), $K_{232}$ and $K_{270}$ were carried out following the analytical methods described by Ayadi et al. [12]

### 2.6.2 Pigment content

Carotenoids and chlorophylls (mg/kg of oil) were determined at 470 and 670 nm, respectively, in cyclohexane using the specific extinction values according to the method of Haddada et al. [13].

$$\text{Chlorophyll (mg/kg)} = \frac{(A_{670} \times 10^6)}{(613 \times 100 \times d)}$$

$$\text{Carotenoid (mg/kg)} = \frac{(A_{470} \times 10^6)}{(2000 \times 100 \times d)}$$

where $A$ is the absorbance and $d$ is the spectrophotometer cell thickness (1 cm). The chlorophyll and carotenoid concentrations are expressed as mg of pheophytin α and lutein per kg of oil, respectively.

### 2.6.3 Fatty acid methyl ester analysis

The fatty acid composition of the oils was determined by GC as FAMEs. FAMEs were prepared by saponification/methylation with sodium methylate according to the EEC 2568/91 [14]. A chromatographic analysis was performed in a SHIMADZU set 17 A Series II GC using a capillary column (Stabilwax, Restek, length 50 m, internal diameter 0.32 mm and film thickness 0.25 μm). The column temperature was isothermal at 180°C and the injector 230°C and detector temperatures were 250°C. Results were expressed as relative percent of total area [2, 3].

### 2.6.4 Sterol determination

The olive oil, with added α-cholestanol as an internal standard, was saponified with potassium hydroxide in ethanolic solution and the unsaponifiables were then extracted with ethyl ether. The bands corresponding to the sterol and triterpene alcohol fractions were separated from the extract by thin-layer chromatography on a basic silica gel plate (Kieselgel 60 $F_{254}$, Merck). 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 chloroform and filtered through a paper filter and then transformed into trimethylsilyl ethers and the mixture was analyzed by GC using a chromatograph SHIMADZU set 17A equipped with capillary column (Stabilwax, Restek, length 50 m, internal diameter 0.32 mm and film thickness 0.25 μm) coated with stationary phase formed by 5% of biphenyl and 95% of dimethyl polysiloxane (0.25 mL thickness). The analytical conditions were: vector gas: Nitrogen; flow rate: 1 mL/min; column temperature: 260°C; injector temperature: 280°C; detector temperature: 290°C; quantity injected: 5 μL [2, 3].

### 2.6.5 Chromatographic analysis of triglycerides (TGAs)

TAG molecular species of olive oil were separated by HPLC equipped with a RP C-18 column (4.6 × 250 mm, particle size 5 μm, Shimadzu, Kyoto, Japan). The eluent was monitored by refractive index detector (model RID-10Avp, Schimadzu). The mobile phase was acetone/acetonitrile (60:40, v/v) with a flow rate of 1.50 mL/min. All solvents were of HPLC grade. Samples (5 mL) were prepared by dissolving the oil in acetone (9:91, v/v). Injection volume was 10 μL. It was assumed that the sum of the areas of the peaks corresponding to the various TGs was equal to 100%, and the relative percentage of each TG was calculated [15].

## 2.7 Gas chromatography analysis of squalene

Olive oils were analyzed in a gas-chromatograph with a mass selective detector (GC/MS 5975B inert MSD Agilent), using a capillary column OV-5 (30m × 0.25mm × 0.25μm); at a flow rate of 1 mL/min; in electronic impact mode (70 eV); split mode injection (1:20), GC/MS interface at 280°C temperature, column temperature program was as follows: 100 (1 min)–300°C to 4°C/min (30 min). Identification of

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the squalene was tentatively identified comparing only the mass spectra obtained with the Wiley library of the equipment [16].

The squalene content of samples was determined by diluting approximately 0.5 mL of olive oil in 1 mL *n*-hexane and analyzing the sample solution by GC/MS. Concentrations of initial squalene contents were then calculated from integrated peak areas of the samples and the calibration curve of squalene standard. Good linearity was achieved in the range 45–354 mg/kg ($y = 62747x$, $r^2 = 0.978$).

### 2.8 Determination of oxidative stability by Rancimat method

Oxidative stability was evaluated by the Rancimat method. Stability was expressed as the oxidation induction time (h), measured with Rancimat equipment (Metrohm Ltd., Herisau, Swiss), using an oil sample of 5 g warmed to 120°C and an air flow of 10 L/h. The conductivity cells were filled with 60 mL of deionized water (2 μS/cm). The time needed for the appearance of a sudden water conductivity rise, caused by the adsorption of volatiles derived from oil oxidation, was registered as the induction time in hours [3].

### 2.9 Analysis of phenolic compounds

#### 2.9.1 Extraction of phenolic fraction from olive oil

The phenolic extracts were obtained following the procedure of Ocakoglu et al. [17]. Briefly, the oil sample (4 g) was added to 2 mL of *n*-hexane, and 4 mL of a methanol/water (60:40, v/v) solution in a 20 mL centrifuge tube. After vigorous mixing, they were centrifuged for 3 min. The hydroalcoholic phase was collected, and the hexanic phase was re-extracted twice with 4 mL of methanol/water (60:40, v/v) solution each time. Finally, the hydroalcoholic fractions were combined, washed with 4 mL of *n*-hexane to remove the residual oil, then concentrated and dried by evaporative centrifuge in vacuum at 35°C.

#### 2.9.2 Determination of total phenolics

The determination of total phenolic compounds included the use of the phenol reagent and the method was adapted from Rigane et al. [10]. In a test tube, 50 μL of phenolic extract was mixed with the Folin–Ciocalteau reagent (250 μL) and, with an aqueous solution of $Na_2CO_3$ (500 μL, 20%). The mixture was vortexed and diluted with water to final volume of 5 mL. The total phenol content was determined colorimetrically at 765 nm. The standard curve was prepared using diluted solutions of gallic acid (GA) in a methanol: water solution (70: 30, v/v). The total phenolic content was expressed as milligrams of GA equivalents per kilogram of oil. For GA, the curve absorbance versus concentration was described by the equation $y = 0.0017 \times$ ($r^2 = 0.9023$).

### 2.10 Statistical analysis

Results were expressed as mean ± SD of three measurements for the analytical determination. Statistical differences were calculated using a one-way ANOVA (ANOVA), followed by Student's *t*-test. Differences were considered significant at $p < 0.05$.

## 3 Results and discussion

### 3.1 Initial composition

One hundred grams of fresh Chemlali olive leaves yielded 10.8 g of extract containing 1.59 g/kg of chlorophyll pigments.

Table 1 presents the results obtained from analysis of the initial compositions of the refined oil sample used for this study. PV, conjugated dienes and secondary oxidation products (carbonylic compounds, aldehydes, and ketones) 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 18.26, 56.50, and 19.1%, respectively (Table 5). The sterol profile showed that the highest sterol levels found in refined olive oil corresponded to β-sitosterol (83.86%) followed by Δ-5-avenosterol (7.17%) (Table 6). Among the substances with antioxidant properties, the total phenols content was 99.41 mg/kg for refined olive oil expressed as GA equivalent. This low content in phenolic compounds had an effect on the oxidative stability as determined by the Rancimat method (4 h 30 min).

### 3.2 Total phenolic contents of chlorophyll rich extract of Chemlali olive leaves

Total phenolic contents of chlorophyll rich extract of Chemlali olive leaves was 80.75 ± 0.30 mg GA/g of ethanolic extract. This result is very low when compared with other study by Lee et al. [9] and Skrget et al. [18], in which total phenolic content of olive leaf extracted by ethanol (80%)

**Table 1.** Initial composition of refined olive oil sample

| | Refined olive oil |
|---|---|
| Free acidity (% oleic acid) | 0.06 ± 0.00 |
| PV (meq $O_2$/kg) | 16.2 ± 0.23 |
| $K_{232}$ | 0.26 ± 0.02 |
| $K_{270}$ | 0.05 ± 0.00 |
| Oxidative stability (h) | 4.30 ± 0.02 |
| Total phenols (mg GA/kg of oil) | 99.41 ± 0.3 |
| Squalene content (mg/kg of oil) | 200 ± 5.10 |

Results are expressed as mean ± SD of three determinations.

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[9] and pure methanol (100%) for 2 h using an ultrasonic bath [18] were 148 and 144 mg/g, respectively.

## 3.3 Determination of antioxidant activity of chlorophyll rich extract of Chemlali olive leaves

To assess the antioxidant potential of bioactive compounds, the application of at least two different assays varying in their mechanisms of antioxidant action has been recommended [19]. The antioxidant capacity of chlorophyll rich extract of Chemlali olive leaves was determined applying the DPPH and ferric reducing antioxidant power (FRAP) assays.

According to a research group in Japan [20, 21], chlorophylls provide protection by preventing autoxidation of vegetable edible oils stored in the dark and suggested a hydrogen donating mechanism breaking the radical chain reactions. Natural chlorophyll pigments had low antioxidant activity when compared to BHT according to results obtained by the DPPH and FRAP assay (Table 2). Therefore, it was reported that the mechanism of antioxidant activity displayed by the natural chlorophyll pigment does not seem to be based on the ability to donate hydrogen but may be, on the protection of polyunsatured fatty acids (mainly linoleic and linolenic acids) against oxidation and/or preventing decomposition of hydroperoxides [22].

## 3.4 Quality indices

To determine the effect of the leaf pigments (chlorophylls) on the oxidation of refined oil, the chlorophyll rich extract of Chemlali olive leaves was added to the refined olive oil at 400 ppm. Lipid oxidation was monitored by measuring PV, conjugated diene and secondary oxidation products formation. Both parameters (conjugated diene and PV) measure the primary product of lipid oxidation [2].

Fig. 1a and b shows the formation of conjugated dienes and secondary oxidation products formation, respectively, in control and stabilized refined oil samples as a function of storage time. The determination of conjugated dienes and secondary oxidation products formation is a good measure of the oxidative state of oils and thus a good indicator of effectiveness of antioxidants [4]. In all samples, $K_{232}$ and $K_{270}$ index went on increasing with the increase in storage time (Fig. 1). A regular pattern of rise was observed for all samples.

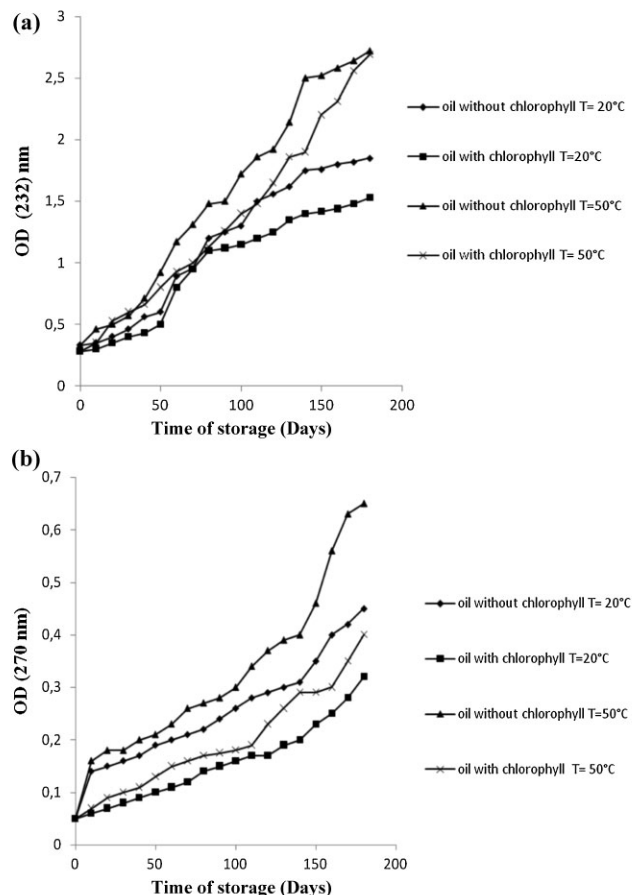


**Figure 1.** Changes in the content of conjugated fatty acids measured by the specific extinction coefficient at 232 nm ($K_{232}$) (a) and in the content on carbonylic compounds measured by the specific extinction coefficient at 270 nm ($K_{270}$) (b). The SD values were not more than 5%.

However, rate of increase in enriched samples was very slow compared to the control samples. Formation of high contents of conjugated dienes may be related to the presence of higher contents of PUFA in refined oils [4].

At the beginning of the assay, the PV was 16.2 meq $O_2$/kg in all samples. As it can be observed in Fig. 2, the PV of enriched refined olive oil with chlorophyll pigment, stored at 20 and 50°C, increased, respectively, to 187.3 and 616.4 meq $O_2$/kg at the end of the assay. However, corresponding values of non-enriched refined olive oils after 6 months of incubation at 50°C, increased dramatically from 16.2 to 775.1 meq $O_2$/kg. After 4 months of storage, it can be seen that oxidation, of control (non-enriched) refined olive oil stored at 50°C, is in advanced phases (489.9 meq $O_2$/kg), due to the sensibility of olive oil to the notable less in the natural pigments of olive oil (chlorophylls) which in the absence of light act as antioxidants, acting synergistically with the phenol

**Table 2.** Determination of antioxidant activity of chlorophyll rich extract of Chemlali olive leaves

| | Chlorophyll pigments | BHT |
|---|---|---|
| DPPH ($IC_{50}$ μg/mL) | 215.53 ± 0.02$^a$ | 8.32 ± 0.09$^b$ |
| FRAP (mM TE/g of extract) | 0.40 ± 0.00$^a$ | 1.32 ± 0.07$^b$ |

Results are expressed as mean ± SD of three determinations. Different letters indicate significant differences ($p < 0.05$).

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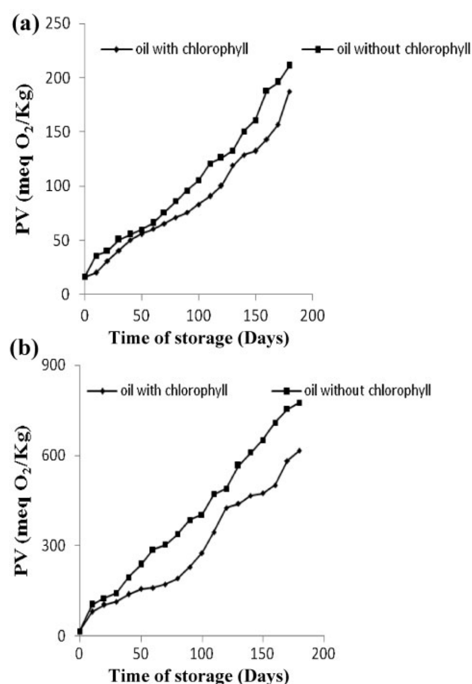


**Figure 2.** Change in peroxide value (PV) of refined olive oils during storage at 20 (a) and 50°C (b). The SD values were not more than 5%.

antioxidants [7], inhibiting the initiation stage of auto-oxidation.

An increase in the PV (propagation phase) is produced in all the samples at 10 days, as, once auto-oxidation is started, it does not stop until all the free radicals that are formed are inactivated [23].

Data presented in this study showed that chlorophyll pigments recovered from Chemlali olive leaves were able to decrease the formation of lipid hydroperoxides, conjugated dienes, and secondary oxidation products formation compared to the control (Figs. 1 and 2). These results were in agreement with those founded by Bouaziz et al. [2, 3], who reported that the enrichment of refined olive oil with phenolic compounds extracted from Chemlali olive leaves resulted in an appreciable resistance to oxidative deterioration due to its phenolic antioxidants content.

### 3.5 Changes in chlorophyll and carotene composition of refined olive oils during storage

Chlorophylls are responsible for the greenish coloration of certain olive oils. Those pigments are also important in olive oil stability [14]. Table 3 presents the change of chlorophyll contents in refined olive oils during storage at 20 and 50°C. In the present work, chlorophyll amount varied between 1.46 and 4.13 mg/kg in non- and enriched refined olive oil, at the beginning of the assay. The oil pigment content, mainly the chlorophyll fraction concentration, decreased gradually with the storage time. Chlorophylls decreased up to 90% of the initial value at the end of storage at 20 and 50°C for non- and enriched refined olive oil (Table 3).

Carotenes are present too in olive oil and are responsible of its yellow coloration [14]. Carotenoid content followed a similar trend to that of chlorophylls but the percentage loss was lower showing up to 60 and 85% loss for oil stored at 20 and 50°C, respectively, at the end of storage (Table 4).

### 3.6 Changes in fatty acid composition during refined oil storage

The main fatty acids and their initial levels of refined olive oils are shown in Table 5. After 6 months of storage, there was a slight increased content in oleic acid in all refined olive oil samples. The changes observed in the PUFAs, linoleic, and linolenic acids, in each sample, are shown in Table 5. A decrease in PUFA percentages was produced, which is assumed to the consequence of the degree of oxidation in the samples [3]. As expected, linolenic acid decreased more, the reductions ranging between 12.67 and 16.90% for linolenic acid and between 9.37 and 10.78% for linoleic acid for enriched refined olive oil with olive leaves extract stored at 20 and 50°C, respectively. The rate of decrease in PUFA percentage was less affected in enriched samples with chlorophyll

**Table 3.** Chlorophyll content of refined olive oil stored at 20 and 50°C

| Time of storage (months) | Refined olive oil storage at 20°C | | Refined olive oil storage at 50°C | |
|---|---|---|---|---|
| | Enriched refined oil | Control refined oil | Enriched refined oil | Control refined oil |
| 0 | $4.13 \pm 0.02^{a,1}$ | $1.46 \pm 0.08^{b,1}$ | $4.13 \pm 0.02^{a,1}$ | $1.42 \pm 0.08^{b,1}$ |
| 2 | $1.7 \pm 0.02^{a,2}$ | $0.63 \pm 0.08^{b,2}$ | $0.9 \pm 0.01^{c,2}$ | $0.3 \pm 0.03^{d,2}$ |
| 4 | $0.8 \pm 0.01^{a,3}$ | $0.27 \pm 0.007^{b,3}$ | $0.4 \pm 0.09^{c,3}$ | $0.18 \pm 0.009^{d,3}$ |
| 6 | $0.4 \pm 0.03^{a,4}$ | $0.13 \pm 0.009^{b,4}$ | $0.2 \pm 0.03^{c,4}$ | $0.07 \pm 0.006^{d,4}$ |

Results are expressed as mean ± SD of three determinations. Significant differences in the same row are shown by different letters ($p < 0.05$). Contents with numbers within time of storage (months) are statistically different at $p < 0.05$.

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**Table 4.** Carotene content of refined olive oil stored at 20 and 50°C

| Time of storage (months) | Refined olive oil storage at 20°C | | Refined olive oil storage at 50°C | |
|---|---|---|---|---|
| | Enriched refined olive oil | Control refined olive oil | Enriched refined olive oil | Control refined olive oil |
| 0 | $0.45 \pm 0.005^{a,1}$ | $0.45 \pm 0.005^{a,1}$ | $0.45 \pm 0.005^{a,1}$ | $0.45 \pm 0.005^{a,1}$ |
| 2 | $0.36 \pm 0.01^{a,2}$ | $0.27 \pm 0.02^{b,2}$ | $0.24 \pm 0.01^{b,2}$ | $0.20 \pm 0.005^{c,2}$ |
| 4 | $0.18 \pm 0.00^{a,3}$ | $0.12 \pm 0.01^{b,3}$ | $0.10 \pm 0.003^{c,3}$ | $0.04 \pm 0.005^{d,3}$ |
| 6 | $0.16 \pm 0.008^{a,4}$ | $0.09 \pm 0.006^{b,4}$ | $0.06 \pm 0.003^{c,4}$ | $0.03 \pm 0.00^{d,4}$ |

Results are expressed as mean ± SD of three determinations. Significant differences in the same row are shown by different letters ($p < 0.05$). Contents with numbers within time of storage (months) are statistically different at $p < 0.05$.

**Table 5.** Refined olive oil fatty acid composition (%) of enriched with chlorophyll pigment

| Fatty acid | Non-enriched refined olive oil time of storage = 0 day | Non-enriched refined olive oil storage at 20°C after 6 months | Non-enriched refined olive oil storage at 50°C after 6 months | Enriched refined olive oil storage at 20°C after 6 months | Enriched refined olive oil storage at 50°C after 6 months |
|---|---|---|---|---|---|
| C16 :0 | $18.26 \pm 0.20^{a}$ | $18.10 \pm 0.08^{a}$ | $18.83 \pm 0.1^{a}$ | $18.14 \pm 0.14^{a}$ | $18.87 \pm 0.16^{a}$ |
| C16 :1 | $2.50 \pm 0.02^{a}$ | $2.37 \pm 0.01^{b}$ | $2.32 \pm 0.02^{c}$ | $2.09 \pm 0.02^{d}$ | $2.04 \pm 0.03^{d}$ |
| C18 :0 | $2.40 \pm 0.06^{a}$ | $3.08 \pm 0.04^{b}$ | $3.39 \pm 0.06^{c}$ | $2.59 \pm 0.04^{d}$ | $2.85 \pm 0.06^{e}$ |
| C18 :1 | $56.50 \pm 0.80^{a}$ | $58.28 \pm 0.68^{b,c}$ | $57.61 \pm 0.80^{a,b,c}$ | $58.58 \pm 0.17^{b}$ | $57.90 \pm 0.20^{c}$ |
| C18 :2 | $19.1 \pm 0.10^{a}$ | $16.95 \pm 0.09^{b}$ | $16.69 \pm 0.10^{c}$ | $17.31 \pm 0.09^{d}$ | $17.04 \pm 0.10^{e}$ |
| C18 :3 | $0.75 \pm 0.02^{a}$ | $0.33 \pm 0.01^{b}$ | $0.32 \pm 0.02^{b}$ | $0.62 \pm 0.03^{c}$ | $0.59 \pm 0.05^{c}$ |
| C20 :0 | $0.38 \pm 0.01^{a}$ | $0.65 \pm 0.01^{b}$ | $0.67 \pm 0.01^{b}$ | $0.38 \pm 0.03^{a}$ | $0.39 \pm 0.03^{a}$ |
| C20 :1 | $0.17 \pm 0.02^{a}$ | $0.14 \pm 0.04^{a}$ | $0.16 \pm 0.02^{a}$ | $0.17 \pm 0.02^{a}$ | $0.19 \pm 0.01^{a}$ |

Results are expressed as mean ± SD of three determinations. Significant differences in the same row are shown by different letters ($p < 0.05$).

pigments when compared to those in non-enriched refined olive oil stored at the same conditions (Table 5). On the other hand, the C20:0 was not affected by the storage when refined olive oil was enriched with chlorophyll pigment extracted from Chemlali olive leaves (Table 5).

After 6 months of storage, our results showed that samples enriched with chlorophyll pigments could minimize the loss of PUFAs. These results are in accordance with those reported by Lanfer-Marquez et al. [22] who declared that chlorophyll pigments protect PUFA against oxidation and/or preventing decomposition of hydroperoxides.

### 3.7 Changes in sterol composition during refined oil storage

Sterols are important non-acylglycerol constituents of olive oil because they relate to the quality of the oil and are widely used to check genuineness [2].

The unsaponifiable matter showed some transformations affected by the storage phase of olive oil. Δ5-avenasterol, Δ7-avenasterol and campesterol were not affected in the presence of chlorophyll pigment extract and its concentration remained constant at the end of storage, while, Δ-5-avenasterol and Δ-7-avenasterol increased however campesterol decreased in non-enriched samples (Table 6). Δ-5, 24-Stigmastadienol showed a decrease in all samples at the end of storage (Table 6). However, the concentration of β-sitosterol (the major sterol of the studied oils) increased from 83.86 to 86% and 85.64% for oil storage at 20 and 50°C, respectively, after enrichment with chlorophyll pigments. The stigmasterol content remained lower than that of campesterol, their behavior was similar, and their concentration increased during storage after enrichment of refined olive oil (Table 6). Storage time influenced the percentage composition of the sterol fraction for non-enriched refined olive oil (Table 6).

The results obtained for sterol composition suggest that enriched refined olive oil could minimize the degradation process in its composition.

### 3.8 Changes in equivalent carbon number (ECN) composition during refined oil storage

The most proportion of ECN in refined olive oils is $ECN_{48}$, followed by $ECN_{46}$ (23.8%), and $ECN_{44}$ (Table 7). Changes

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**Table 6.** Content of the sterols in the refined olive oil after 6 months storage enriched with chlorophyll pigment

| Sterol content (%) | Non-enriched refined olive oil Time of storage = 0 day | Non-enriched refined olive oil storage at 20°C after 6 months | Non-enriched refined olive oil storage at 50°C after6 months | Enriched refined olive oil storage at 20°C after 6 months | Enriched refined olive oil storage at 50°C after 6 months |
|---|---|---|---|---|---|
| Cholesterol | $0.22 \pm 0.01^{a}$ | $0.11 \pm 0.02^{b}$ | $0.12 \pm 0.06^{b}$ | $0.13 \pm 0.02^{b}$ | $0.15 \pm 0.02^{b}$ |
| Brassicasterol | $0.00 \pm 0.00^{a}$ | $0.00 \pm 0.00^{a}$ | $0.00 \pm 0.00^{a}$ | $0.00 \pm 0.00^{a}$ | $0.00 \pm 0.00^{a}$ |
| 24 methylene-Cholesterol | $0.09 \pm 0.001^{a}$ | $0.13 \pm 0.03^{a,b}$ | $0.14 \pm 0.08^{a,b}$ | $0.15 \pm 0.03^{b}$ | $0.17 \pm 0.08^{a,b}$ |
| Campesterol | $3.13 \pm 0.02^{a}$ | $2.77 \pm 0.01^{b}$ | $2.84 \pm 0.29^{b}$ | $3.17 \pm 0.09^{a}$ | $3.24 \pm 0.05^{a}$ |
| Campestanol | $0.06 \pm 0.00^{a}$ | $0.05 \pm 0.01^{a,b}$ | $0.05 \pm 0.00^{b}$ | $0.06 \pm 0.00^{a}$ | $0.07 \pm 0.00^{c}$ |
| Stigmasterol | $0.65 \pm 0.08^{a}$ | $1.88 \pm 0.09^{b}$ | $1.84 \pm 0.08^{b}$ | $0.81 \pm 0.06^{c}$ | $0.79 \pm 0.05^{c}$ |
| Chlerosterol | $1.16 \pm 0.07^{a}$ | $0.95 \pm 0.04^{b}$ | $1.08 \pm 0.00^{c}$ | $1.09 \pm 0.06^{a}$ | $1.25 \pm 0.01^{c}$ |
| β-Sitosterol | $83.86 \pm 0.12^{a}$ | $84.24 \pm 0.13^{b}$ | $83.89 \pm 0.06^{c}$ | $86.00 \pm 0.09^{d}$ | $85.64 \pm 0.04^{e}$ |
| Δ5-Avenasterol | $7.17 \pm 0.07^{a}$ | $8.13 \pm 0.02^{b}$ | $8.26 \pm 0.06^{c}$ | $7.13 \pm 0.02^{a}$ | $7.24 \pm 0.05^{a}$ |
| Δ5,24-Stigmastadienol | $1.40 \pm 0.00^{a}$ | $0.55 \pm 0.03^{b}$ | $0.50 \pm 0.00^{c}$ | $0.51 \pm 0.06^{b,c,d}$ | $0.47 \pm 0.01^{d}$ |
| Δ7-Stigmastenol | $0.38 \pm 0.00^{a}$ | $0.29 \pm 0.05^{b}$ | $0.32 \pm 0.00^{c}$ | $0.33 \pm 0.04^{a}$ | $0.37 \pm 0.03^{a}$ |
| Δ7-Avénasterol | $0.67 \pm 0.001^{a}$ | $1.83 \pm 0.03^{b}$ | $1.81 \pm 0.01^{b}$ | $0.62 \pm 0.07^{c}$ | $0.61 \pm 0.02^{c}$ |

Results are expressed as mean ± SD of three determinations. Significant differences in the same row are shown by different letters ($p < 0.05$).

**Table 7.** ECN (%) of enriched refined olive oil after 6 months of storage

| Triacylglycerols, (%) ECN | Refined olive oil time of storage = 0 day | Non-enriched refined olive oil storage at 20°C after 6 months | Non-enriched refined olive oil storage at 50°C after 6 months | Refined olive oil storage at 20°C after 6 months | Refined olive oil storage at 50°C after 6 months |
|---|---|---|---|---|---|
| 44 | $14.3 \pm 0.19^{a}$ | $26.19 \pm 0.11^{b}$ | $21.66 \pm 0.18^{c}$ | $21.4 \pm 0.1^{c}$ | $17.7 \pm 0.09^{d}$ |
| 46 | $23.8 \pm 0.20^{a}$ | $20.81 \pm 0.25^{b}$ | $17.88 \pm 0.19^{c}$ | $17.8 \pm 0.30^{c}$ | $15.3 \pm 0.24^{d}$ |
| 48 | $48.5 \pm 0.52^{a}$ | $44.19 \pm 0.45^{b}$ | $48.98 \pm 0.42^{a}$ | $48.8 \pm 0.35^{a}$ | $54.1 \pm 0.50^{c}$ |
| 50 | $13.3 \pm 0.09^{a}$ | $4.05 \pm 0.19^{b}$ | $4.5 \pm 0.21^{c}$ | $4.5 \pm 0.25^{c}$ | $5.0 \pm 0.17^{d}$ |

ECN (equivalent carbon number) = CN (carbon number) – 2DB (double bond number). Results are expressed as mean ± SD of three determinations. Significant differences in the same row are shown by different letters ($p < 0.05$).

in ECN composition due to refined olive oil oxidation are scarcely studied. The same trend in all refined olive oil samples were observed after 6 months of storage. Mateos et al. [24] established that olive oil stability mainly depended on the ECN composition. In the storage phase of olive oil, the ECN composition of non-enriched and enriched refined olive oil showed significant changes during storage at 20 and 50°C (Table 7). The proportion of $ECN_{46}$ and $ECN_{50}$ decreased while in $ECN_{48}$ and $ECN_{44}$ an increase was observed. Apparently, the more sensitive ECN were those containing three, two, or one linoleic acid in their structure. It could be possible that $ECN_{48}$ could also have decreased but, due to the marked decrease of the $ECN_{46}$ and $ECN_{50}$, its overall balance was positive. Thus, in general, the sensitivity of the ECN to degradation decreased as the unsaturation degree decreased and none of those with high proportions of saturated fatty acids were affected.

In all studied samples, enrichment of refined olive oil with chlorophyll pigment extracted from Chemlali olive leaves had no effect on ECN composition (Table 7).

### 3.9 Changes in squalene content during refined oil storage

Squalene, one of the more potent, oil soluble, edible antioxidants, is well absorbed by mammals, protects against various oxidative stress-related diseases, and acts as an anti-carcinogenic and anti-inflammatory agent [25].

The squalene content of refined olive oil was 200 mg/kg, this low content of squalene content was due to the removal of squalene in the deodorizer distillate during refining. During refining isomerization products of squalene are also produced by the effect of temperature and bleaching earth during the bleaching process. Examination of the squalene content in enriched refined olive oil showed a further

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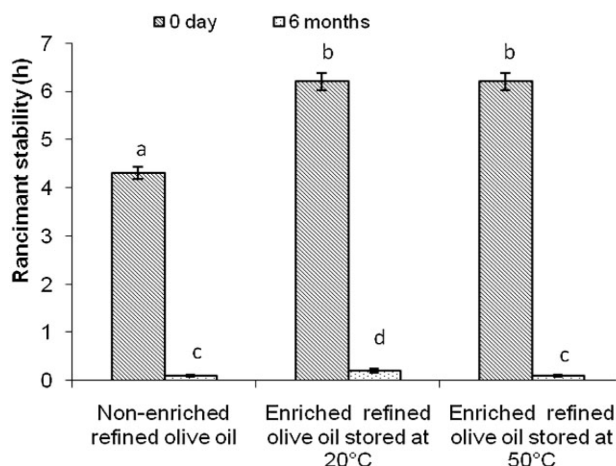


**Figure 3.** Induction time measured by Rancimat for refined olive oil. Different letters indicate significant differences ($p < 0.05$).

reduction in the squalene content so that it was considered practically negligible for the requirements of the study. No squalene was detected in the refined olive oil at the end of storage (Table 1).

### 3.10 Oxidative stability of refined oils added by chlorophyll olive leaves rich extracts

The accelerated oxidation test is useful for evaluating the effects of antioxidants on olive oil resistance to oxidation, and to compare the storage stability of different oils [26].The oxidative stability of oils, measured as the induction time determined using the Rancimat method, is showed in Fig. 3. Indeed, the induction time of refined olive oil increased from 4.3 to 6.2 h by enrichment with chlorophyll extract (Fig. 3).

Moreover, the oxidative resistance at 120°C, fell to 0.2 and zero for all enriched oil samples stored at 20 and 50°C, respectively. The low stability of oils enriched with chlorophyll extract was attributed to the latter's low antioxidant capacity as reported by Lanfer-Marquez et al. [22].

### 3.11 Phenolic content changes during refined olive oil storage

Among the minor compounds characterized by antioxidant activity, the amounts of phenolic compounds were measured with the Folin–Ciocalteu reagent. The reduction in the total phenol content in the oils during storage is a result of their degradation as described previously [2, 3, 27, 28].

Table 8 shows the effects of storage on total phenols in non- and enriched refined olive oil samples during 6 months of storage. After an incubation during 2 months, total phenol contents decreased considerably with more than 2% for samples stored at 20°C. After 6 months, the phenolic content decrease of about 5% of its initial phenolic content. However, storage at 50°C accelerated the degradation of total phenols in all samples, which decreased by more than 60% at the end of storage (Table 8). These results were in accordance with those reported by Bouaziz et al. [3] and Morello et al. [29]

In conclusion, this is the first study to use refined olive oil enriched with chlorophyll pigment from olive leaves and to report on the changes that occurred with regard to chlorophyll pigment during storage time. It is concluded that olive leave pigment extract could stabilize refined olive oil. It inhibits thermal deterioration of oil by improving its hydrolytic stability, inhibiting double bond conjugation and reducing the losses of PUFAs. Chlorophyll pigments had low antioxidant activity when compared to BHT according to results obtained by the DPPH and FRAP assays. Hence, the mechanism of action involved might not be related to the capacity of hydrogen donation but, to the protection of polyunsatured acids against oxidation and/or prevention of the decomposition of hydroperoxides.

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**Table 8.** Total phenol content (mg Gallic acid/kg) of refined olive oil stored at 20 and 50°C

| Time of storage (months) | Refined olive oil storage at 20°C | | Refined olive oil storage at 50°C | |
|---|---|---|---|---|
| | Enriched refined olive oil | Control refined olive oil | Enriched refined olive oil | Control refined olive oil |
| 0 | $131.7 \pm 0.20^{a,1}$ | $99.41 \pm 0.08^{b,1}$ | $131.7 \pm 0.20^{a,1}$ | $99.41 \pm 0.08^{b,1}$ |
| 2 | $129.06 \pm 0.06^{a,2}$ | $97.42 \pm 0.02^{b,2}$ | $98.77 \pm 0.10^{c,2}$ | $74.55 \pm 0.05^{d,2}$ |
| 4 | $127.74 \pm 0.08^{a,3}$ | $96.43 \pm 0.07^{b,3}$ | $77.43 \pm 0.09^{c,3}$ | $58.45 \pm 0.03^{d,3}$ |
| 6 | $125.16 \pm 0.04^{a,4}$ | $94.44 \pm 0.06^{b,4}$ | $49.38 \pm 0.07^{c,4}$ | $37.27 \pm 0.01^{d,4}$ |

Results are expressed as mean ± SD of three determinations. Significant differences in the same row are shown by different letters ($p < 0.05$). Contents with numbers within time of storage (months) are statistically different at $p < 0.05$.

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