Activity of two natural additives in improving the stability of virgin olive oil quality during storage

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Abstract – The activity of natural additives against the deterioration of virgin olive oil (VOO) in relation to storage time and conditions was examined. Thus, carotenoids and phenols previously extracted from carrot and olive mill wastewater, respectively, were added at 200 and 400 ppm to VOO and stored in clear and amber glass bottles at room temperatures during 120 days. The results showed that enriched VOO was largely influenced by the storage time, which resulted in a significant accumulation of hydrolysis and oxidation products. Storage conditions also affected considerably the enriched VOO. Dark glass bottles offered better protection against oxidative deterioration. The impact of both extracts on the oxidation status of examined VOO was significant and concentration-dependent. The natural additives markedly delayed the formation of hydroperoxides and conjugated dienes and trienes. Results from principal component analysis (PCA) showed that most of the variation was captured by the PC1 (89% of the total variance) which grouped samples in four categories according to storage times, each being divided into two clusters linked to storage conditions. Findings from this study revealed that natural additives could minimize VOO deterioration caused by storage time and conditions (light).

Keywords: Carotenoids / phenols / natural additives / storage / VOO

Résumé – Activité de deux additifs naturels pour l’amélioration de la stabilité de la qualité de l’huile d’olive vierge au cours du stockage. L’activité des additifs naturels contre la détérioration de l’huile d’olive vierge en fonction du temps et des conditions de stockage a été examinée dans ce travail. Des caroténoïdes et des phénols préalablement extraits des carottes et des margines, respectivement, ont été ajoutés à des concentrations de 200 et 400 ppm à des échantillons d’huile d’olive et stockés dans des bouteilles en verre clair et foncé à température ambiante pendant 120 jours. Les résultats ont montré que l’huile d’olive enrichie a été largement influencée par le temps de stockage qui a entraîné une accumulation significative des produits d’hydrolyse et d’oxydation. Les conditions de stockage ont également affecté considérablement l’huile enrichie. Les bouteilles en verre foncé ont assuré une meilleure protection contre la détérioration oxydative. L’impact des deux extraits sur l’état d’oxydation de l’huile d’olive a été significatif et concentration-dépendant. Les additifs naturels ont nettement retardé la formation d’hydroperoxydes et des diènes et triènes conjugués. L’analyse en composantes principales (ACP) a révélé que la majeure partie de la variation a été capturée par la première composante (89 % de la variance totale) qui a regroupé les échantillons d’huile d’olive en quatre catégories en fonction du temps de stockage, et chacune d’entre elles a renfermé les deux conditions de stockage. Les résultats de la présente étude ont démontré que les additifs naturels pourraient minimiser la détérioration de l’huile d’olive vierge causée par le temps et les conditions de stockage (lumière).

Mots clés : Caroténoïdes / phénols / additifs naturels / stockage / huile d’olive vierge

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1 Introduction

Olive oil happens to be considered as “the magic elixir of the Mediterranean diet” known for its worldwide appreciation due to its healthy and nutritional aspects that can lead to prevent and reduce the risk rates of many chronic, neurodegenerative and cardiovascular diseases (Huang and Sumpio, 2008; Cicerale et al., 2010; Rizzo et al., 2014). The nutritional value of olive oil is associated to its composition of fatty acids especially the oleic acid and to its richness of natural antioxidants such as phenolic compounds, tocopherols, flavonoids and carotenoids (Covas, 2007; Stefanoudaki et al., 2010). The high content of unsaturated (polyunsaturated) fatty acids in olive oil makes it very susceptible to oxidation. The oxidative reactions that could occur in oil, especially during storage, impact negatively its nutritional quality and flavor, due to the formation of undesirable volatile compounds potentially toxic for human health (Duh et al., 1992; Kiokias and Gordon, 2004; Pan et al., 2007; Ghanbari Shendi et al., 2018, 2020).

Auto-oxidation, photo-oxidation, enzymatic oxidation and ketone oxidation are the various oxidation reactions that can take place in olive oils leading to their deterioration (Taghvaei and Jafari, 2015). However, auto-oxidation is the most frequent phenomena that proceeds via three cyclical stages, namely induction, propagation and termination (Brand-Williams et al., 1995). During the induction period, alkyl radicals are formed and undergo a reaction with oxygen molecules to form hydroperoxides and peroxy radicals during the propagation phase. These unstable compounds generate secondary oxidation products (i.e. alkanes, alcohols, aldehydes and acids). The termination of chain reactions occurs via combination of free radicals to form stable adducts (Velasco and Dobarganes, 2002). In addition, many studies have shown that deterioration reactions of olive oil are more pronounced at longer storage periods (Rodrigues et al., 2016; Di Serio et al., 2018), and are stimulated by oil exposure to light and air (Pristouri et al., 2010; Li et al., 2014; Houshia et al., 2019).

Addition of some synthetic antioxidants into food formulations, especially olive oil, has been used for long time to overcome the lipid oxidation problems and to reduce their damage (Halliwell et al., 1995; Fki et al., 2005; Bouaziz et al., 2008; Venturi et al., 2017). However, recently the use of natural additives has received a great attention because of safety issues of synthetic forms (Lee et al., 2009, Taghvaei and Jafari, 2015).

Carotenoids, whose consumption is linked to the protection against cardiovascular diseases due to its antioxidant functions (Kiokias and Gordon, 2004), are tested as natural additives to improve oil stability in many investigations. Diverse carotenoid extracts (tomato, marigold, paprika and annatto extracts) were added to sunflower oil and showed a significant delay in the formation of secondary oxidation products (Kiokias et al., 2009). The antioxidant potential of carotenoids extracts were confirmed in other studies (Armando et al., 1998; Alavi and Golmakani, 2017).

Similarly, the use of natural phenols from olives and their by-products for the stabilization of olive oils and functional foods has recently been considered. Fki et al. (2005) reported that the addition of phenolic extracts from olive mill wastewater (OMW) had a protective effect against oil oxidation. Sánchez de Medina et al. (2012) have found that enriched edible oils (i.e. maize, soya, high-oleic sunflower, sunflower, olive and rapeseed oils) with phenolic extracts isolated from olive pomace and leaves showed a similar composition to extra virgin olive oil concerning biophenols content. In view of the above mentioned facts, this work investigated the influence of natural antioxidants on the stability of the virgin olive oil (VOO) stored under room temperatures.

2 Materials and methods

2.1 Materials

Olive oils (cv. Moroccan Picholine) used in this study were collected from an industrial mill unit (super-pressure system) located in Taza province (34°19’48"N, 4°12’0"W, ~534 m asl) (northern Morocco) during 2017 extraction period. Samples of OMW were simultaneously drawn from the same industrial unit to obtain phenol extracts. Carrot (Daucus Carota) samples used for the extraction of carotenoids were purchased from a local supermarket in the same area.

The collected olive oil and OMW samples were put in dark glass and plastic bottles, respectively and stored with carrot samples in a deep freezer at 4°C until analysis.

2.2 Determination of initial VOO quality indices

Determination of free fatty acids (FFA), peroxide value (PV), and absorbance at specific wavelengths, namely 232 nm and 270 nm (K232 and K270), were carried out following the analytical methods described in the European Union Commission Regulations (EEC, 1991, 2003). FFA (expressed as % of oleic acid [OA]), being the conventional criterion for olive oil quality and their classification, measures hydrolysis breakdown of fatty acid chains. PV (meq O2/kg oil) estimates the active oxygen content; it is a crude indicator of the amount of primary oxidation products (i.e. peroxide compounds), K232 and K270 (calculated from absorption at 232 nm and 270 nm) are related to the presence in olive oil of conjugated dienes and trienes systems, respectively.

Chlorophylls and carotenoids were assessed at 670 nm and 470 nm, respectively, in cyclohexane, using specific extinction values, following the method described by Minguéz-Mosquera et al. (1991). The extinction coefficients used were E0 = 613 for pheophytin and E0 = 2000 for lutein, as the major components in the chlorophyll and carotenoid fractions, respectively. The concentrations of both pigments were expressed as mg per kg of oil and calculated using the following equations:

\[
\text{Chlorophylls (mg/kg)} = \frac{A_{670}}{613 \times 10^6 \times L}.
\]

\[
\text{Chlorophylls (mg/kg)} = \frac{A_{470}}{2000 \times 10^6 \times L}.
\]
Where A is the absorbance and L is the spectrophotometer cell thickness (1 cm).

Total phenols were isolated by triple extraction of a solution of olive oil (10 g) in n-hexane (10 mL) with 10 mL of a methanol-water mixture (60:40, v/v) (Zumin et al., 1995), then their concentration was determined spectrophotometrically (SPECUVIS1; UV-Visible) following the method proposed by Folin and Ciocalteu (1927). Folin Ciocalteu reagent was added to a suitable dilution of the extract, and the absorbance was measured at 750 nm using as standard the caffeic acid (Sigma-Aldrich, St. Louis, MO, USA). Values for total phenols content are given as mg caffeic acid/kg oil.

### 2.3 Extraction of carotenoids and total phenols

The extraction of carotenoids was carried out using the Soxhlet method (Harwood et al., 1989). The setup consists of a distillation flask, extraction chamber, siphon and condenser. First, a weighed amount of dried carrot was placed in a porous cellulose thimble and put in the extraction chamber. Next, the solvent (acetone) was heated to reflux and vapors passed through the thimble containing the material to be extracted and are liquefied in the condenser. When the liquid reaches the overflow level in the thimble, a siphon aspirates the solution, and the liquid carrying the extracted solutes falls back into the flask. The process is repeated for a number of cycles until complete extraction is achieved and the liquid extract is accumulated in the distillation flask. Finally, carotenoid extract was separated in a rotary evaporator under vacuum at 50 °C; the boiling point of acetone.

Total phenols (TP) were extracted using the analytical methodology described by De Marco et al. (2007). OMW samples were first acidified and washed with hexane in order to remove the lipid fraction, and then a TP extraction was carried out with ethyl acetate which was evaporated to recover the dry phenolic residue.

### 2.4 Enrichment of VOO and experimental design

Weighed quantities of carotenoid (CE) and phenol (PE) extracts (from carrot and OMW, respectively) were dissolved in an appropriate volume of ethanol/water (50/50; v/v) and added at two concentrations (200 ppm and 400 ppm) to VOO. Carotenoid and phenol extracts were mixed with VOO by stirring for 30 min and ethanol traces were evaporated at 37 °C (Farag et al., 2003). The same procedure was applied to L-Ascorbic Acid (AA) as standard. The used AA (purity of 99%) was obtained from Sigma Chemical Co. (Sigma–Aldrich Company Ltd, Great Britain). VOO enriched with CE, PE and AA and the non-enriched VOO (control sample) were stored in clear and dark glass bottles at room temperatures for 120 days during which the oxidation state was evaluated initially at bottling and then each 30 days (0, 30, 60, 90 and 120 days). In fact, a total of 42 glass bottles, divided into 21 for VOO stored in clear glass bottles and 21 for VOO stored in dark ones, were considered. Each category consisted of: (1) control sample + 2 VOO enriched with CE at 200 and 400 ppm + 2 VOO enriched with PE at 200 and 400 ppm + 2 VOO enriched with AA at 200 and 400 ppm) × 3 replicates.

| Table 1. Mean values of analytical characteristics for fresh samples of “Moroccan Picholine” olive oil collected from an industrial mill unit (super-pressure system) located in Taza province (northern Morocco) during 2017 extraction period, and extraction yields of carotenoids and phenols from carrots and olive mill wastewater, respectively. |
|---------------------------------|-----------------|-----------------|
| **Fresh olive oil characteristics** | Free fatty acid (% OA) | 1.003 ± 0.064 |
| | Peroxide value (meq O₂/kg) | 5.643 ± 0.168 |
| | K₂₃₂ | 1.620 ± 0.003 |
| | K₂₇₀ | 0.156 ± 0.002 |
| | Carotenoids (mg/kg) | 3.091 ± 0.052 |
| | Chlorophylls (mg/kg) | 1.395 ± 0.017 |
| **Total phenols (mg/kg caffeic)** | 410.984 ± 2.095 |
| **Extraction yield** | Carotenoids from carrot (mg/100 g FM) | 16 ± 0.66 |
| | Phenols from olive mill wastewaters (g/L caffeic) | 1.26 ± 0.01 |

The experimental design considered to perform statistical analyses was is a Completely Randomized Design with 5 storages times, two storage conditions, seven treatments (1 control + 3 additives at 2 concentrations [6]), and three replicates.

### 2.5 Oxidation stability measurements

Oxidation stability of VOO enriched with carotenoids and phenols extracts was evaluated during 120 days of storage (at 30 days intervals) by measuring the free fatty acids, the peroxide value, and the extinction coefficients at 232 nm and 270 nm (K₂₃₂ and K₂₇₀), according to the European Union Commission Regulation (EEC, 1991, 2003).

### 2.6 Statistical analyses

All determinations were performed in three replicates. Analyses of variance (ANOVA) were carried out over natural additives and storage time and conditions. Least significant difference (LSD) values were calculated at the 5% probability level. Principal component analyses (PCA) and the relationships between parameters were performed on the basis of a correlation matrix calculated on the mean data of all the replicates. The Statgraphics Centurion XVII package (Statpoint Technologies, Inc., Virginia, USA) was used for all the calculations.

### 3 Results and discussion

#### 3.1 Fresh VOO characteristics and amounts of natural extracts

Olive oils from cv “Moroccan Picholine” were analysed for their initial characteristics before being enriched with natural additives. The results for the legal parameters (FFA, PV, K₂₃₂ and K₂₇₀) are listed in Table 1, and indicated that the examined oils belong to the VOO category (EEC, 2003).
Indeed, the mean value recorded for FFA was of 1% OA, while the PV, K232 and K270 that measured the hydroperoxides, the conjugated dienes and trienes, were found around 5.643 meq O2/kg, 1.620 and 0.156, respectively. The average contents of carotenoids and chlorophylls were of 1.395 mg/kg and 3.091 mg/kg, respectively, which corroborates those previous findings of other investigations (Méndez and Falqué, 2007; Ben Youssef et al., 2010; Bedbabis et al., 2015; Mele et al., 2018; El Yamani et al., 2018). Olive oil composition is generally influenced by cultivar, fruit quality, harvesting time, and storage conditions, as well as processing, production site, harvesting practices, water regime and climatic conditions (Salvador et al., 2001; Torres and Maestri, 2006; Maier et al., 2010; Bedbabis et al., 2015; Mele et al., 2018; Piscopo et al., 2018; El Yamani et al., 2020).

Carotenoid and phenol extracts derived from carrot and OMW, respectively, were quantified (Tab. 1). The carotenoids content obtained from 100 g of fresh material was around 16 mg, within the range of the results reported by Rebecca et al. (2014). OMW was found rich in phenols (1.26 g/L caffeic) in agreement with the observations of several authors (Belaqziz et al., 2016; El Yamani et al., 2017, 2019b; Arabi et al., 2018).

### 3.2 VOO enrichment with natural additives

#### 3.2.1 Data variability

Results from the analysis of the variance for the VOO enriched with carotenoid and phenol extracts and stored in clean and dark glass bottles during 120 days are presented in Table 2. They revealed the predominance of storage time influence on all considered parameters, and explained more than 80% of total variance. Storage conditions effect accounted for about 17% of total variance for FFA and less that 8% for PV, K232 and K270. The effect of natural additives was of minor extent for oxidation indices but statistically significant and negligible for FFA.

#### 3.2.2 Influence of storage time

The evolution of mean values recorded for the analysed parameters of VOO enriched with carotenoid and phenol extracts during storage is shown in Table 3. Regardless the effect of natural additives and storage conditions, all measured parameters increased to statistically the highest values \( p < 0.05 \) in enriched VOO after 120 days indicating that they were subjected to substantial reactions of hydrolysis and oxidation.

The FFA accumulation was duplicated after 60 days of storage, and increased by approximately 2.5 times after 120 days, which is consistent with many previous reports (Méndez and Falqué, 2007; Bubola et al., 2014; Gargouri et al., 2015; Di Serio et al., 2018). The lipolytic enzymes present in the oil mainly the lipase, often derived by microorganisms, decomposed triglycerides into glycerol and free fatty acids (Pereira et al., 2002; Abbadi et al., 2014). The degradation of hydroperoxides and oxidation of aldehydes could also increase the formation of free fatty acids (Abramović et al., 2007).

The highest increment was observed for PV that quasi-triplicated at the end of the experiment. The lowest rise (38%) was shown for both K232 and K270. The great increment rate for PV was observed during the first 60 days of storage, then it slowed down and those of K232 and K270 became important. Our results are in good harmony with several published studies (Vekari et al., 2007; Rababah et al., 2011; Rodrigues et al., 2016; Di Serio et al., 2018). The oxidation process could explain the observed behavior. In fact, there was firstly a formation of hydroperoxides which are then decomposed into secondary oxidation products (Baiano et al., 2005; Del Caro et al., 2006; Escudero et al., 2016).

#### 3.2.3 Influence of storage conditions

In relation to storage conditions, significant differences were highlighted between the storage of enriched VOO with carotenoid and phenol extracts at room temperatures in clear conditions.
and dark glass bottles (Table 3). In fact, we detected a delay in the hydrolysis of triglycerides and the oxidation process in enriched VOO stored in dark bottles, which was revealed by the lowest levels of FFA, PV, K232 and K270. In contrast, clear bottles resulted in more advanced deterioration of enriched VOO during storage. Similar results for FFA were displayed by several authors who announced that decomposition of triglycerides, which is responsible of acidity increase in oils, was enhanced by light exposure (Vacca et al., 2006; Pristouri et al., 2010). The formation of hydroperoxides, conjugated dienes and trienes (measured by PV, K232 and K270) was also found to be promoted by light (Caponio et al., 2005; Fadda et al., 2012; Afaneh et al., 2013; Rizzo et al., 2014). The storage in darkness could slow down oxidation rate in olive oil (Del Caro et al., 2006; Pristouri et al., 2010). Moreover, Interesse et al. (1971) indicated that the natural pigments in olive oil act synergistically with the phenols as antioxidants in the dark. However, under light, chlorophylls (especially phytochrome A) have a photosensitizing effect and promoted rapid photooxidation (Sanelli, 1981; Raham and Csallany, 1998).

### Table 3. Mean values of free fatty acids (FFA), peroxide value (PV) and extinction coefficients at 232 and 270 nm (K232 and K270) in “Moroccan Picholine” virgin olive oil enriched with natural additives with carotenoid (CE) and phenol (PE) extracts, and ascorbic acid (AA) at different concentrations (0, 200 and 400 ppm) and stored during 120 days at room temperatures in clear and dark glass bottles.

| Additive   | FFA     | PV       | K232     | K270     |
|------------|---------|----------|----------|----------|
| AA-200     | 1.754 ± 0.64 abc | 10.253 ± 4.49 c | 1.772 ± 0.257 c | 0.175 ± 0.028 bcd |
| AA-400     | 1.730 ± 0.72 abc | 8.877 ± 4.06 e | 1.760 ± 0.238 d | 0.174 ± 0.025 d |
| CE-200     | 1.794 ± 0.63 ab  | 10.842 ± 3.65  b | 1.801 ± 0.222 a  | 0.177 ± 0.021 b  |
| CE-400     | 1.692 ± 0.74 c   | 10.514 ± 4.12 bc | 1.788 ± 0.245 b  | 0.175 ± 0.026 bcd |
| PE-200     | 1.813 ± 0.57 a   | 10.653 ± 3.92 bc | 1.792 ± 0.231 b  | 0.177 ± 0.022 b  |
| PE-400     | 1.707 ± 0.65 bc  | 9.614 ± 3.74 d  | 1.777 ± 0.221 c  | 0.174 ± 0.023 d  |
| Control    | 1.764 ± 0.67 ab  | 11.382 ± 2.81 a  | 1.802 ± 0.214 a  | 0.179 ± 0.022 a  |

| Storage condition | FFA     | PV       | K232     | K270     |
|-------------------|---------|----------|----------|----------|
| Clear glass bottles | 1.868 ± 0.71 a | 10.678 ± 4.00 a | 1.798 ± 0.237 a | 0.179 ± 0.025 a |
| Dark glass bottles | 1.609 ± 0.48 b  | 9.932 ± 3.10 b  | 1.771 ± 0.173 b  | 0.173 ± 0.016 b  |

| Storage time | FFA     | PV       | K232     | K270     |
|--------------|---------|----------|----------|----------|
| 0 day        | 1.003 ± 0.09 e | 5.643 ± 0.24 e | 1.620 ± 0.004 e | 0.156 ± 0.003 e |
| 30 days      | 1.068 ± 0.07 d  | 6.967 ± 0.73 d  | 1.624 ± 0.003 d  | 0.160 ± 0.001 d  |
| 60 days      | 2.014 ± 1.17 c  | 10.599 ± 0.27 c  | 1.651 ± 0.014 c  | 0.165 ± 0.003 c  |
| 90 days      | 2.221 ± 1.51 b  | 13.221 ± 0.26 b  | 1.832 ± 0.040 b  | 0.182 ± 0.006 b  |
| 120 days     | 2.433 ± 1.37 a  | 15.094 ± 0.25 a  | 2.194 ± 0.058 a  | 0.216 ± 0.010 a  |

Means for each character followed by the same letter are not significantly different according to LSD test at P < 0.05.

#### 3.2.4 Influence of natural additives

The changes in enriched-VOO with natural additives (carotenoid and phenol extracts) and acid ascorbic (standard) along with the control sample are given in Table 3. Results showed no clear differences among additives and their concentrations on FFA. Similarly, Limón et al. (2015) reported that addition of carotenoids rich extracts from *Scenedesmus almeriensis* didn’t trigger hydrolytic reactions on olive oils. Non-significant differences were observed on FFA when phenolic compounds and carotenoids were added to refined hazelnut oil (Yalcin, 2011). However, the antioxidant effect of natural additives has been proven in our work and found to be very significant even with lower extent compared to the impact of storage time and conditions. In fact, the VOO “control” displayed a higher accumulation of primary and secondary oxidation products than the enriched VOO. Natural additives had an effect in the retardation of oxidation process, which was greater for phenol extract than that of carotenoids. Moreover, PV, K232 and K270 decreased with increasing the additive concentration from 200 to 400 ppm. However, the effectiveness of ascorbic acid was better despite the effect of both natural additives was very promising. Our findings confirmed those of many researchers that focused their investigation on improving the quality of edible oils and their shelf-life with natural additives from different plant sources and their effectiveness as antioxidants by using different methods (Gordon et al., 2001; Briante et al., 2003; Ranalli et al., 2003; Salta et al., 2007; Abd-El Ghany et al., 2010; Yalcin, 2011; Rafiee et al., 2011).

OMW phenol extracts decreased greatly the oxidation rate (determined by peroxide value) in VOO and sunflower oil (Lafla et al., 2011). Similarly, Fki et al. (2005) reported that OMW extract had an effect in stabilizing husk and refined oils. Farag et al. (2003) indicated that phenols extracted from olive fruits and leaves exerted an antioxidant activity on sunflower oil. Bouaziz et al. (2008) evaluated the antioxidant effect of phenolic extracts recovered from olive leaves on refined and husk olive oils by measuring peroxide values, K232, K270 and Rancimat induction time. They disclosed that the oxidative resistance of oils was significantly improved by the used extracts, which were able to increase the induction time and reduce both lipid hydroperoxides and conjugated dienes and trienes formation. Yahyaoui et al. (2017) noticed that phenolic extracts from aromatic plants and olive leaves could be used to protect extra VOO from oxidation, as well as good substitutes.
for synthetic antioxidants in the industry. Natural antioxidants extracted from OMW were found highly efficient for the oxidative stabilization of lard (De Leonardi et al., 2007).

Carotenoids, which are considered interesting components in several mechanisms that occur in the human organism (Granado et al., 2003), were recovered from different plant sources and tested to improve the stability of foods and oils. Benakmoum et al. (2008) announced that carotenoids from tomato and its by-products enhanced the thermal stability of the refined olive oil, extra virgin oil and sunflower oil. The industrial tomato by-product (skin and seeds) were also used in another investigation to recover carotenoids in order to produce an olive oil naturally enriched with antioxidants (Bendini et al., 2015). Kiokias et al. (2009) inspected the antioxidant activity of natural carotenoid extracts from tomato, marigold and paprika in the emulsions of sunflower oil. They found that the formation of volatile aldehydes was significantly retarded by the addition of the examined carotenoids. The peroxidation was declined and the oxidative stability of olive oils was significantly improved by the addition of carotenoids rich extracts from S. almeriensis, while fatty acids composition and tocopherols content were not influenced (Limón et al., 2015).

In addition, our results indicated that the antioxidant effect of natural carotenoids and phenols was concentration-dependent, and generally augmented with increasing extracts concentration, which was in line with previous researches (Farag et al., 2003; Fki et al., 2005; Kiokias et al., 2009; Lafka et al., 2011; Limón et al., 2015).

The mechanism by which each natural additive acted as an antioxidant could explain the difference in efficiency against oil oxidation between carotenoid and phenol extracts. The antioxidant activity of carotenoids is associated to their highly reactive chemical structure, to the oxygen concentration and to the presence of other antioxidants (Krinsky, 1993; Mordi, 1993). Kiokias and Gordon (2004) recapitulated the action of carotenoids in radical scavenging under low oxygen conditions or in combination with other natural antioxidants, and in the deactivation of singlet oxygen formed by ultraviolet light. β-carotene, the most relevant carotenoids, assured protection against the oxidation of free radicals in lipids by reacting with peroxyl radicals, hence hindering propagation step and favoring termination of the oxidation chain reaction (Burton and Ingold, 1984; Britton, 1995). Niki et al. (1995) reported that antioxidant efficiency of β-carotene was greater in the absence of oxygen. The β-carotene has proved to be an effective quencher of singlet oxygen in the inhibition of photodegradation (Fakourelis et al., 1987). Concerning the antioxidant effect of phenol extract, Lafka et al. (2011) referred its strong protection in the oily system to the amphiphilic properties of the phenolic constituents and suggested that the number of hydroxy groups improves the hydrogen donor capacity, and therefore enhances the inhibition oxidation. The phenolic compounds act as hydrogen donors and suppress the hydrogen atom abstraction from a fatty acid during the first phase of lipid oxidation, which delay oxidation thereby decreasing hydroperoxides formation (Visioli et al., 2002; Farag et al., 2003). The antioxidant potency of OMW extracts was mostly related to their content of hydroxytyrosol and 3,4-dihydroxyphenyl acetic acid, according to several authors (Bouaziz et al., 2005; Fki et al., 2005; Artajo et al., 2006).

### Table 4. Correlations between free fatty acids (FFA), peroxide value (PV) and percent extinction coefficients at 232 and 270 nm (K<sub>232</sub> and K<sub>270</sub>) in “Moroccan Picholine” virgin olive oil enriched with natural additives (carotenoid and phenol extracts) and stored during 120 days at room temperatures in clear and dark glass bottles.

|        | FFA      | PV       | K<sub>232</sub> | K<sub>270</sub> |
|--------|----------|----------|----------------|----------------|
| FFA    | 0.933*** | 0.738*** | 0.789***       | 0.873***       |
| PV     | 0.839*** | 0.988*** | 0.928***       | 0.933***       |
| K<sub>232</sub> | 0.789*** | 0.873*** | 0.873***       | 0.928***       |
| K<sub>270</sub> | 0.873*** | 0.928*** | 0.928***       | 0.933***       |

*Significant at 0.05 probability level; **Significant at 0.01 probability level; ***Significant at 0.001 probability level.

![Fig. 1. PCA projections on axes 1 and 2 accounting for 98% of total variance. Eigenvalues of the correlation matrix are symbolized as vectors representing traits that most influence each axis. The 70 points representing means of olive oil samples for each storage time (0, 30, 60, 90 and 120 days) and storage condition (dark and clear glass bottles) are plotted on the plane determined by axes 1 and 2.](image)

#### 3.2.5 Relationships among parameters and factors

Correlations coefficients among the considered parameters of all VOO samples were calculated and given in Table 4. Positive and significant associations were found among all parameters, with the strongest one was highlighted between K<sub>232</sub> and K<sub>270</sub> (∝ = 0.988), both were highly related to peroxide value (∝ = 0.839 and ∝ = 0.873, respectively). Important relationship was also detected between PV and FFA (∝ = 0.933). Such findings were observed previously by several researchers who have examined the quality of oils and their oxidation status during storage (Caponio et al., 2005; Marmeset et al., 2009). The FFA were also found positively associated in our work with peroxide value. Frega et al. (1999) disclosed that the concentration of FFA had a prooxidant effect on vegetable oils. Besides, the formation of FFA was related to hydroperoxides content and oxidation of aldehydes, according to Abramović et al. (2007).

In addition to the correlation studies, experimental data were processed by principal component analysis (PCA) to select significant parameters reflecting the oxidation stability of enriched VOO during storage and the factors most affecting these parameters (Fig. 1). Results showed that most of the...
variation was captured by the first principal component (PC1) that accounted 89% of the total variance, while PC2 explained about 9%. The obtained PCA also highlighted the presence of four main groups along PC1 arranged according to storage times: The first group consisted of fresh VOO and enriched VOO stored for 30 days, was positioned in the left side of PC1 and characterized by low values of all parameters. The second and third groups were formed by enriched VOO stored for 60 and 90 days, respectively, and interacted with high levels of FFA and PV. The fourth one was located on the right direction of PC1 reacting with high values of K$_{232}$ and K$_{270}$, and corresponded to samples stored for 120 days. In addition, the observation of Figure 1 showed that each group was divided into two clusters consisting of enriched VOO stored in clear glass bottles which appeared to be more sensitive to oxidation than those stored in the dark glass bottles.

We have also plotted the evolution of the considered parameters against the additives concentrations, in order to elucidate and compare their effects on the VOO stability (Fig. 2). Results showed that the addition of all extracts in VOO reduced the FFA and the formation or primary and secondary oxidation products. Indeed, this effect was concentration-dependent and the application of additives at 400 ppm was more efficient than enriched VOO with 200 ppm and the control. Moreover, the comparison between natural additives illustrated that phenol extracts were more active in delaying the formation of hydroperoxides and conjugated dienes than carotenoids extracts. However, the ascorbic acid exhibited the most significant antioxidant effect on VOO stability.

### 4 Conclusions

Olive oil is notable Mediterranean diet for its nutritional and antioxidant effect. However, its high content of unsaturated (polyunsaturated) fatty acids makes it very susceptible to oxidation. To this end, application of synthetic antioxidants has cost implications to processors and producers and therefore, use of natural antioxidants such as carotenoids and phenols extend the stability VOO in storage. Therefore, carotenoids and phenols extracted from carrot and olive mill wastewater, respectively, were added at different amounts to VOO and stored in clear and amber glass bottles at room temperatures during 120 days. The efficiency of the natural antioxidants was assessed by measuring the changes reported in the legal quality indices (free fatty acids and oxidations indices) of the VOO.
According to the obtained results, oxidative stability parameters of enriched VOO increased during storage time. In addition, the used natural additives showed an appropriate oxidation prevention activity on VOO, which was concentration-dependent and could be optimized by storage in darkness. This fact could sustain the use of natural antioxidants in fatty foods as safe substitutes of synthetic ones. This topic is, therefore, of great interest and requires in-depth investigation.

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