PAPER

EFFECTS OF ARCHAIC OLIVE AND OIL STORAGE METHODS STILL USED IN SOUTHERN TUNISIA ON OLIVE OIL QUALITY

S. EL-GHARBI[1,2], M. TEKAYA[1], A. BENDINI[3,4], E. VALLI*[3,4], R. PALAGANO[3], T. GALLINA TOSCHI[3,4], M. HAMMAMI[1] and B. MECHRI[1]

[1]Laboratoire de Biochimie, USCR Spectrométrie de Masse, LR-NAFS /LR12ES05, Nutrition –Aliments Fonctionnels et Santé Vasculaire, Faculté de Médecine, Université de Monastir, 5019 Monastir, Tunisia

[2]Faculty of Science of Gabes, University of Gabes, 6072 Gabes, Tunisia

[3]Department of Agricultural and Food Sciences, Alma Mater Studiorum, University of Bologna, Italy

[4]Inter-Departmental Centre for Agri-Food Industrial Research, Alma Mater Studiorum, University of Bologna, Italy

*Corresponding author. Tel.: +39 0547 338121; Fax +39 0547382348

*E-mail address: enrico.valli4@unibo.it

ABSTRACT

The present paper investigated how virgin olive oil quality is influenced by two different storage conditions that residents of Gabes (Southern Tunisia) usually apply to fruits of the Zarazi cultivar: long conservation as oil in glass bottles or traditional storage of olives as sun-dried fruits before processing for oil production. Even if both storage conditions are associated with strong losses in the qualitative characteristics of olive oil, the changes observed were more accentuated for oil stored for two years after its production compared to the oil obtained from olives stored by traditional methods.

Keywords: fatty acid alkyl esters, olives, phenols, traditional storage, virgin olive oil

1. INTRODUCTION

Long known to many generations in the Mediterranean basin populations as essential to their diet, virgin olive oil is now widely appreciated around the world for its healthy and sensory properties. The nutritional benefits of virgin olive oil are firstly related to the fatty acid composition, mainly due to both the high content of oleic acid and the balanced ratio of saturated and polyunsaturated fatty acids (GARGOURI et *al*., 2015; RUIZ-CANELA and MARTÍNEZ-GONZÁLEZ, 2011); in addition, virgin olive oil presents considerable amounts of natural antioxidants. Because of these natural characteristics, virgin olive oil is particularly resistant to storage and more suitable for cooking than other vegetable oils (PELLEGRINI et *al*., 2001). It is also considered important in prevention of many diseases, such as cardiovascular disease, obesity, metabolic syndrome, type 2 diabetes and hypertension (NUNEZ-CORDOBA et *al*., 2009).

It is one of the few vegetable oils that can be consumed in the raw state and contains important nutritional elements (fatty acids, vitamins, sterols, etc.). Extra virgin olive oil (EVOO) is considered the highest quality virgin olive oil due to its organoleptic and chemical characteristics (JABEUR et *al*., 2015).

Nevertheless, oxidation is the main cause of deterioration of EVOOs during the storage period. Auto-oxidation follows a free radical mechanism, wherein a first result of the absorption of oxygen is the formation of hydroperoxydes (FRANKEL, 2005). These labile compounds also decompose to produce a complex mixture of volatile molecules, such as aldehydes, ketones, hydrocarbons, alcohols and esters, among which some are directly responsible for perception of the rancid sensory defect (PRISTOURI et *al*., 2010).

The most important factors affecting the quality of olive oil during storage are environmental, especially temperature, light exposure and contact with oxygen (PRISTOURI et *al*., 2010). Much research has been carried out to study the effects of storage conditions and packaging materials on olive oil quality (DABBOU et *al*., 2011; PRISTOURI et *al*., 2010).

Moreover, one of the primary factors responsible for the low quality and stability of virgin olive oils is linked to the mishandling of the olives during the period between harvesting and processing. Indeed, the storage of olives, which leads to degenerative processes in a short period of time, should be carried out by simple heaping in fruit piles, awaiting their transformation (RABIEI et *al*., 2011). Unfortunately, olive processing is often not well synchronized with crop harvests; in particular, for countries in which the production of olives is very high and concentrated in a short period, if the capacity of the local olive oil mills is not adequate to support such enormous amounts, olives are often piled into large heaps or in plastic sacks. Moreover, olives can be stored in big cumuli outside the olive oil mill for periods that may range from weeks to months prior to oil extraction (KIRITSAKIS et *al*., 1998). During this storage period, mechanical, physical, chemical and physiological alterations occur in the fruit, which can cause the rupture of cellular structures and subsequent negative chemical and sensory changes in the oils obtained (BIASONE et *al*., 2012; INAREJOS-GARCÍA et *al*., 2010; VICHI et *al*., 2009). The olive fruit deteriorates rapidly due to the combined action of pathogenic microorganisms and internal processes of senescence. Both processes are accelerated by temperature increases due to fruit fermentation and mechanical damage due to compression. The degradation of fruit causes the loss of the texture of the flesh and tanning of the skin, and finally complete decomposition (GARCÍA et *al*., 1996). Virgin olive oils obtained from damaged olives usually present high acidity, low oxidation stability and high levels of oxidation, due to the increased peroxide value and specific extinction coefficients at 232 and 270 nm (GARCÍA et *al*., 1996; INAREJOS-GARCÍA et *al*., 2010). Moreover, the content in fatty acid

ethyl esters also increases with storage time of olives due to fruit degradation (BIEDERMANN et *al*., 2008).
In this context, herein we investigated the effects on olive oil quality of *i)* the olive oil immediately processed, and *ii)* of the olive oil and olive fruits stored for two-years following the traditional method of Gabes. Although some data have already been published on the effects of olive storage before oil extraction (BIASONE et *al*., 2012; INAREJOS-GARCÍA et *al*., 2010; VICHI et *al*., 2009), in the present investigation a particular interest was given to a traditional conservation procedure that the residents of Gabes (southern Tunisia) still apply to olive fruits of the *Zarazi* cultivar. In fact, after harvesting, olive fruits are traditionally sun-dried, and when completely dried they are stored in plastic bags at room temperature and then processed to obtain olive oil. Therefore, the objective of this investigation was to compare the deteriorative effects on the oil quality of two different conservation methods: conservation as oil or as intact fruits both carried out for a long period of time (two years). For this aim, different quality parameters were investigated, including composition in minor compounds, such as phenolics and fatty acid alkyl esters.

2. MATERIALS AND METHODS

2.1. Olive fruit samples

The present study was carried out on olives picked up from *O. europaea* L. trees (cv. *Zarazi*) grown in an orchard located in the region of Gabes (southern Tunisia: 33° 32′ N, 10° 06′ E). In particular, olive samples were harvested manually and were divided into two unequal lots:
- About 4 kg of olives were processed (see paragraph 2.3) directly after harvest (about 24 hours between harvest and start of processing) and the virgin olive oil obtained was divided into two samples: one used as a control sample (sample code: "TC") and the other stored in dark glass bottles at room temperature for two years (sample code: "T1").
- About 2 kg of olives were cleaned from leaves, washed and stored following a traditional method described later (see the section 2.2) for two years, oil (sample code: "T2") was then extracted from dried fruit using the same lab-scale mill described in paragraph 2.3, in order to evaluate the effects of both conservation methods (as intact fruits or as oil) on virgin olive oil quality.

2.2. Olive fruit conservation

In this investigation, a traditional storage procedure that the residents of *Gabes* still apply to olive fruits of *Zarazi* cultivar was simulated. After harvesting, olive fruits were sun-dried (thrown singly upon a stone-floor, exposed to the sun in the open air for 10 to 15 days) and, once completely dried, they were stored in plastic bags at room temperature for two years, before processing to obtain the oil.

2.3. Oil extraction

Olive oil was extracted under similar industrial extraction conditions using a bench hammer mill. A sample of 2 kg of olive fruits was firstly crushed with a small hammer crusher, and the paste was mixed at about 25°C for 30 min and centrifuged in a two-phase decanter (3500 rpm for 1 min). After extraction, oils were transferred into 250 mL amber glass bottles and stored in darkness at room temperature (25°C) for further analyses.

2.4. Quality indices determination

Free acidity (FA, expressed as % oleic acid), peroxide value (PV, expressed as milliequivalents active oxygen - meq O_2 kg^{-1} oil) and extinction coefficients (K_{232} and K_{270}) were determined by analytical methods described respectively in ISO 660:2009, ISO 3960: 2010 and ISO 3656: 2011.

2.5. Total phenol and *o*-diphenol content

The total phenol and *o*-diphenol content of olive oil samples were quantified colorimetrically using an UV–Vis 1800 spectrophotometer (Shimadzu Co., Kyoto, Japan), according to the procedures previously described (MATEOS et *al*., 2001; SINGLETON et *al*., 1965). Phenolic compounds were extracted in methanol/water (80:20, v/v), according to IOC/T.20/Doc No 29. Folin-Ciocalteu reagent and sodium carbonate (15% p/v) were added to a suitable aliquot of the extract, and the absorption of the solution at 750 nm was measured to evaluate the total phenolic content, by a specific calibration curve built with different concentrations of gallic acid (Sigma-Aldrich, St. Louise, MO, USA) (r^2 =0.995). For *o*-diphenol content determination, 0.5 mL of the extract was dissolved in 5 mL of a methanol/water solution (50:50, v/v). Four mL of the obtained solution were withdrawn, then 1 mL of sodium molybdate (5% in ethanol/water 50:50) was added. After vigorous mixing, they were centrifuged for 5 min at 3000 rpm. The concentration was determined colorimetrically at 370 nm. A specific calibration curve (r^2 = 0.985) was built using gallic acid for the quantification. Data were expressed as mg gallic acid per kg of oil for both total phenolic compounds and *o*-diphenols.

2.6. Determination of phenolic compounds by HPLC-DAD-MS

HPLC analysis was performed using an Agilent Technologies 1100 series system equipped with an automatic injector, a diode array UV-Vis detector (DAD) and a mass spectrometer detector (MSD) (LERMA-GARCIA et *al*., 2009). A C18 column KINETEX (100 cm x 3.00 mm x 2.6 μm; Phenomenex, Torrence CA, USA) was used, maintained at 40°C during the analyses, with an injection volume of 5 μL and a flow rate of 0.7 mL min^{-1}. The wavelength was set at 280 nm for detection of phenolic acids, phenyl ethyl alcohols and secoiridoids. The mobile phase was a mixture of water/formic acid (99.5:0.5%, v/v) (solvent A) and acetonitrile (solvent B). A linear gradient was run from 95% (A) and 5% (B) to 80% (A) and 20% (B) during 3 min, it changed to 60% (A) and 40% (B) in 1 min, after 1 min it changed to 55% (A) and 45% (B), it changed to 40% (A) and 60% (B) after 4 min, and then in 1 min it becomes 100% (B), finally it changed to 95% (A) and 5% (B) in 3 min (13 min total time). Phenolic compounds were identified by comparison of their relative retention times and maximum absorbance according to IOC/T.20/Doc No 29, and by interpretation of their mass spectra (LERMA-GARCIA et *al*., 2009). Syringic acid (Sigma-Aldrich) was used as an internal standard for the quantification of identified phenols and results are expressed as mg of tyrosol per kg of oil (according to the IOC/T.20/Doc No 29).

2.7. Tocopherol determination

Oils were dissolved in a solution of isopropanol and the isomers of α-tocopherol, β-tocopherol and γ-tocopherol were analysed as described (ANWAR et *al*., 2013). An Agilent Technologies 1100 series HPLC apparatus (Paolo Alto, California, USA), comprising a HP pump series 1050 (Darmstadt, Germany) and a DAD detector set at 292 nm was used.

The eluting solvents were methanol/water (90:10, v/v) acidified with 0.2% H_3PO_4 (solvent A), and acetonitrile (solvent B). Samples were eluted through a Cosmosil column (π NAP 4.6 mm × 150 mm x 5 μm; Nacalai-Tesque, Kyoto, Japan) according to the following gradient: 100% A maintained for 22 min; 100% B for 13 min; 100% A for 15 min (total run = 50 min). The injection volume was 20 μL. The flow rate was 1 mL min^{-1}. The concentrations of different tocopherol isomers were expressed on a calibration curve constructed using solutions of *α*-tocopherol at different concentrations (r^2 = 0.998). Results are expressed in mg of *α* tocopherol per kg of oil.

2.8. Oxidative stability measurements

Oxidative stability was measured by a Rancimat 743 apparatus (Metrohm Ω, Schweiz AG, Zofingen, Switzerland) following the method described (TURA et *al*., 2007). Air (20 L h^{-1}) was passed through a sample (3 g) held at constant temperature (120°C). Stability was expressed as the oxidation induction time (h).

2.9. Determination of fatty acid alkyl esters (FAAE) by gas chromatographic analyses

Fatty acid alkyl esters were extracted following the methods described in IOC/T.20/ DOC. NO.28 (2009). Subsequent separation of alkyl esters (methyl and ethyl esters) was performed on a gas chromatograph equipped with an injector port and a FID both set at 325°C. The capillary column was a ZB–5MS (30 m length × 0.25 mm i.d. × 0.25-*μ*m-film thickness; Phenomenex) with a split ratio 1:10. Helium was the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was programmed from 80°C (kept for 1 min) to 140°C at a rate of 15°C min^{-1}, then raised to 325°C at a rate of 4.5°C min^{-1} and kept for 20 min. The amounts of alkyl esters were expressed as mg of methyl heptadecanoate (C17:0 ME) per kg of oil.

2.10. Statistical analysis

The Statistical Package for Social Sciences (SPSS) program, release 16.0 for Windows (SPSS, Chicago, IL, USA) was used for all statistical analyses. The results were expressed as mean±standard deviation (SD) of three measurements for each analytical determination. Significant differences between the values of all parameters were considered at $p< 0.05$ according to the one-way ANOVA Post Hoc Comparisons (Duncan's test).

3. RESULTS AND DISCUSSIONS

3.1. Effect of storage on basic chemical quality parameters

The basic chemical quality parameters of the olive oil samples were investigated and the results are shown in Table 1. The values of FA, PV, K_{232} and K_{270} for the control sample (TC) were below the legal limits established by IOC for extra virgin olive oils (IOC/T.15/NC No. 3/Rev. 10 November 2015). Regarding the effects of storage, both methods of storage (T1 and T2) negatively affected oil quality (p <0.001) by increasing all four above-mentioned parameters, which all exceeded the limits established by IOC for extra virgin olive oils. Comparing the differences between the two storage methods, the increase of these parameters was more accentuated for the oil extracted from dried fruits (T2) compared to the oil stored for two years (T1). In both cases (T1 and T2), the adopted

storage methods led to the classification of the olive oil samples in the category of "lampante oils".
The results obtained for free acidity agree with a previous study (JABEUR et *al*., 2015), which confirmed the increase of this parameter in olive oil over time. A similar result was reported by (MÉNDEZ and FALQUÉ, 2007) after 3 and 6 months of storage. Moreover, the acidity value was also related with the storage temperature and the percentage of damaged olives (NABIL et *al*., 2012). The increase of oil acidity during storage of oils obtained from stored olives is likely the result of fungal lipase activity (KIRITSAKIS et *al*., 1998).
Regarding the PV, in the present study the olive oil quality was affected by an increase of this parameter after both storage conditions. These results are in accordance with the study performed by MÉNDEZ and FALQUÉ (2007) during 3 and 6 months that showed an increase of PV in olive oil during storage. As reported by SERVILI and GIANFRANCESCO (2002), the PV is basically affected by several factors that damage the fruits (e.g. olive fly attacks or improper systems of harvesting, transport and storage) or the oil (processing technology and oil storage conditions).
Finally, concerning the extinction coefficients, the K_{270} of olive oil increased more slowly than K_{232} over two years of storage. However, the highest increase of K_{232} and K_{270} took place in the oil obtained from stored olives (T2). These results are in accordance with those of (GUTIERREZ et *al*., 1992; VICHI et *al*., 2009) which suggested that K_{232} and K_{270} progressively increased during olive storage. The increase in the extinction coefficient values during storage could be due to the presence of conjugated dienes. As reported by FRANKEL (1993), it is important to consider that the storage temperature can affect the formation of certain volatile compounds resulting in the formation and degradation of different hydroperoxydes formed by oxidation processes. In general, an increase in these parameters can affect the conservation of EVOOs, as well as their nutritive and organoleptic characteristics.

Table 1. Quality indices (free acidity, peroxide value, K_{232} and K_{270}) of *Zarazi* olive oil not stored (TC), stored for two years (T1) and obtained from intact sun-dried olive fruits stored for two years before being processed (T2).

Quality indices	TC	T1	T2
FA (% oleic acid)	0.77±0.01^{c}	1.68±0.04^{b}	2.40±0.03^{a}
PV (meq O_2 kg oil^{-1})	10.1±0.1^{c}	21.4±0.5^{b}	23.5±1.7^{a}
K_{232}	1.85±0.34^{c}	2.89±0.48^{b}	3.80±0.52^{a}
K_{270}	0.05±0.01^{c}	0.2±0.01^{b}	0.43±0.05^{a}

FA: free acidity; PV: peroxide value; K_{232} and K_{270}: spectrophotometric indices.
Each value represents the mean of three determinations (n = 3) ± standard deviation.
$^{a-c}$Different letters in the same row indicate significantly different values ($p < 0.05$) according to Duncan test.

3.2. Total phenols and *o*-diphenols as influenced by storage methods

Total phenols and *o*-diphenols were quantified in samples and the results are shown in Fig. 1. Statistical analysis showed a significant decrease in both total phenol and *o*-diphenol contents compared to the control sample under the effect of T1 and T2 (p <0.001). Concerning the *o*-diphenol concentration, it also dramatically decreased from 506.8 mg kg ^{1} (sample TC) to 82.0 mg kg^{-1} in T1 and to 80.1 mg kg^{-1} in T2 (Fig.1 B). No significant differences were found between the two storage methods.

The decrease of total phenols with storage time is in accordance with other studies showing that, during storage, phenols undergo qualitative and quantitative variations due to decomposition and oxidation reactions (DABBOU et *al*., 2011; GARGOURI et *al*., 2015). The decrease of the total phenol content in all samples during storage is due to hydrolysis and oxidation processes (SICARI et *al*., 2010). The losses of total phenols could be also be the result of an increase in the oxidative state of fruit. In fact, the oxidation of polyphenols in olive fruits is the result of enhanced activity of oxidative enzymes such as polyphenoloxidase and peroxidase, which contribute to the impairment of health-related qualities and sensory characteristics of olive oil (CLODOVEO et *al*., 2007; SERVILI et *al*., 2003).

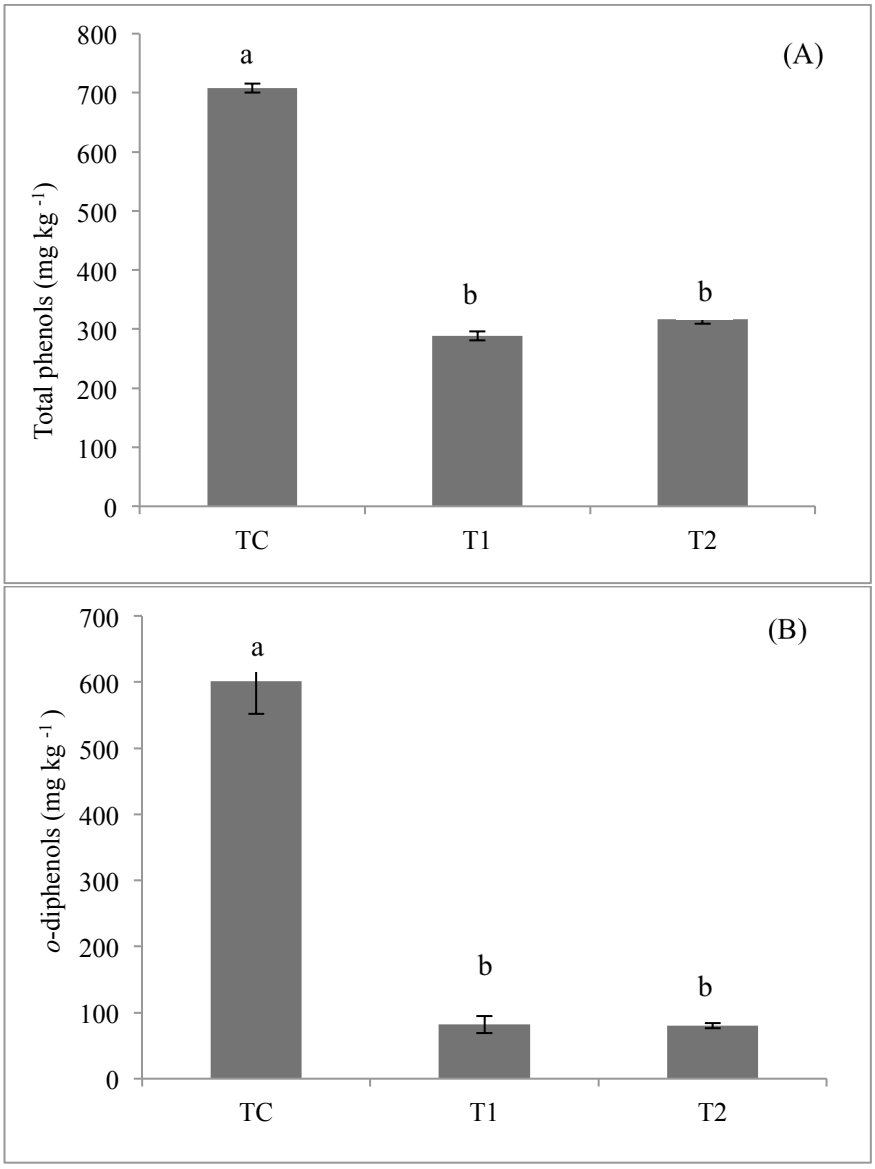

Figure 1. Total phenol (A) and *o*-diphenol (B) content in control olive oil (TC), in olive oil stored for two years (T1) and in olive oil obtained from intact sun-dried olive fruits stored for two years before being processed (T2). Results are shown as mean ± SD (n = 3).

[a-b] Different letters indicate significantly differences at $p < 0.05$ according to Duncan's test.

3.3. Modification of phenolic profiles during different methods of storage

The analysis of phenolic profiles allowed the separation and identification of several phenolic compounds (Table 2). In all olive oil samples, secoiridoid derivatives were the most abundant. The major secoiridoids detected were oleuropein aglycone (3,4-DHPEA-EA) and its decarboxymethylated derivative (3,4-DHPEA-EDA). The results presented in Table 2 showed significant differences in the concentration of a wide number of phenolic compounds among samples. In general, in both the methods tested, it is possible to observe a significant decrease of all phenolic compounds compared to control samples (TC). In fact, 3,4-DHPEA-EA decreased significantly (p<0.001) by 69.87% in T1 and by 58.81% in T2, compared to TC. Another compound (3,4-DHPEA-EDA) also decreased significantly (p<0.001) by 84.34% in T1 and 68.40% in T2 compared to TC.
For the other phenolic compounds, T1 had the lowest content of tyrosol (p-HPEA), decarboxymethyl ligstroside aglycon (p-HPEA-EDA) and luteolin, while T2 oil samples contained the lowest concentration of ligstroside aglycon (p-HPEA-EA) and acetoxypinoresinol. On the other hand, hydroxytyrosol (3,4-DHPEA) was not detected in T1 oil, while its concentration was 19.8 mg kg^{-1} and 0.9 mg kg^{-1}, respectively in samples TC and T2.

Table 2. Phenolic compounds (mg of tyrosol kg^{-1} oil) identified and quantified by HPLC-DAD-MS in the control olive oil (TC), stored for two years (T1) and obtained from intact sun-dried olive fruits stored for two years before being processed (T2).

Phenolic compounds	TC	T1	T2
3,4-DHPEA	19.8±1.0^{a}	ND	0.9±1.5^{b}
p-HPEA	14.9±0.7^{a}	3.4±0.3^{c}	7.8±0.3^{b}
p-HPEA-EA	6.8±0.3^{ab}	8.4±1.4^{a}	5.6±0.7^{b}
3,4-DHPEA-EA	35.3±1.6^{a}	10.6±1.5^{c}	14.5±0.8^{b}
p-HPEA-EDA	15.9±0.9^{b}	9.1±0.5^{c}	19.5±0.4^{a}
3,4-DHPEA-EDA	33.2±1.7^{a}	5.2±1.1^{c}	10.5±0.4^{b}
Acetoxypinoresinol	26.5±1.7^{a}	11.6±0.8^{b}	10.0±0.2^{b}
Luteolin	12.2±0.9 ^{a}	3.2±0.1^{c}	5.3±0.5^{b}

3.4-DHPEA: Hydroxytyrosol; p-HPEA: Tyrosol; p-HPEA-EA: Ligstroside aglycon; 3.4-DHPEA-EA: Oleuropein aglycon; p-HPEA-EDA: Dialdehydic form of elenolic acid linked to tyrosol; 3.4-DHPEA-EDA: Dialdehydic form of elenolic acid linked to hydroxytyrosol; ND: not detected.
Results as expressed as means ± SD (n = 3).
$^{a-c}$ Different letters in the same row show statistically significant differences (p < 0.05) according to Duncan test.

Given the importance of the phenolic fraction, with regards to antioxidant activities (BENDINI et *al*., 2006), sensory properties and health benefits (BENDINI et *al*., 2007) of olive oil, the content of phenolic compounds could be an important quality control parameter of EVOO. The trends of simple phenols, secoiridoids, lignans and flavones during storage are related to the stability and nature of their molecular structure. Indeed, these compounds may undergo alterations due mainly to hydrolysis, oxidation and increase of decarboxymethylated derivatives during storage of EVOO in the industry or after sales (LERMA-GARCIA et *al*., 2009). The reduced contents of different phenolic compounds observed in this study could be related to hydrolytic processes that may occur in parallel with oxidation, which is in concordance with previous studies (PAGLIARINI et *al*., 2000) that suggested aglycone esters undergo hydrolysis during long term storage.

Several studies have focused on possible hydrolytic and oxidative degradation forms of phenolic compounds present in EVOO during storage (LOZANO-SÁNCHEZ et *al*., 2013). In addition, secoiridoids, 3,4-DHPEA-EDA and 3,4- DHPEA-EA, were the most affected phenolic compounds during the storage period. These results are in accordance with those of other authors (HACHICHA et *al*., 2015).
The results of this study show that under oil storage conditions, almost all phenolic compounds (except *p*-HPEA-EA and acetoxypinoresinol) were lost more in samples of stored oil (T1) than in those produced after fruit storage conditions (T2). It is not easy to provide an explanation for this phenomenon, but it is possible that when conserved as fruits, exposure of the oil to external environmental factors is avoided, which may cause alteration of phenolic profiles, such as availability of oxygen, presence of light and temperature (MÉNDEZ and FALQUÉ, 2007; PRISTOURI et *al*., 2010).

3.4. Modification of the concentrations of tocopherol isomers as an effect of storage methods

As reported in Table 3, α-tocopherol was the most predominant isomer in olive oil samples and its content was 180.4 mg kg^{-1} in the control olive oil (TC), while the β and γ isomers were present at relatively minor concentrations (6.0 mg kg^{-1} and 17.7 mg kg^{-1}, respectively). Statistical analysis revealed a significant decrease of total tocopherol content as well as the individual concentrations of each isomer ($p<0.001$) as an effect of the two storage methods. Comparing the latter two methods, we observed that T1 oil samples had the lowest content of total tocopherols compared to TC and T2, especially because of its significantly lower content of α-tocopherol.
Tocopherols play an important role as antioxidants in the oxidative stability of olive oil by helping to maintain its shelf-life and in preserving oils from rancidity by interrupting the chain reactions involved in the formation of hydroperoxydes (MORELLÓ et *al*., 2004). Our study showed that the loss of α-tocopherol content was about 50% in T1, which confirms the data by KRICHENE et *al*., (2010) who observed a strong reduction in α-tocopherol during storage. As described for most phenolic compounds in paragraph 4.3, α-tocopherol also decreased more in samples of stored oils (T1) than in those obtained from olives stored for two years (T2). However, for both storage methods, it appears that polyphenols and *o*-diphenols were much more sensitive to storage time than tocopherols. This is definitely attributable to the fact that polyphenols, particularly *o*-diphenols, are the preferred substrates for oxidation (BLEKAS et *al*., 1995).

Table 3. Tocopherols isomer contents (mg α-tocopherol kg^{-1}) in the control olive oil (TC), in olive oil stored for two years (T1) and in olive oil obtained from intact sun-dried olive fruits stored for two years before being processed (T2).

	Tocopherols (mg kg^{-1})			
	***α*-tocopherol**	***β*-tocopherol**	***γ*-tocopherol**	**Total tocopherols**
TC	180.4±2.8^{a}	6.0±0.4^{a}	17.7±0.6^{a}	204.2±3.0^{a}
T1	109.0±6.8^{c}	5.3±0.6^{ab}	13.1±0.9^{b}	127.5±8.2^{c}
T2	140.4±2.4^{b}	5.1±0.1^{b}	13.3±0.2^{b}	158.9±2.2^{b}

$^{a-c}$ Different letters in the same column indicate significantly different values ($p < 0.05$) according to Duncan test.
Results as expressed as means ± SD (n = 3).

3.5. Effect of the two storage methods on oxidative stability

Oxidative stability was studied by the Rancimat test (Fig. 2). The stability (OS) decreased significantly from 8.47 h (TC) to 1.50 h in T1 and to 2.77 h in T2, reaching a significant lower value in of the latter.
As reported by some studies in the literature (GARCÍA et *al*., 1996; GUTIERREZ et *al*., 1992), oil stability is clearly affected by storage time and conditions. This is in accordance with our results: OSI time decreased more in the stored oil (T1) than in the that obtained from olives stored for two years (T2). This is a quite interesting finding, since it confirms that the traditional storage method of the olives had a lesser negative effect on OSI time than the storage of the oil.

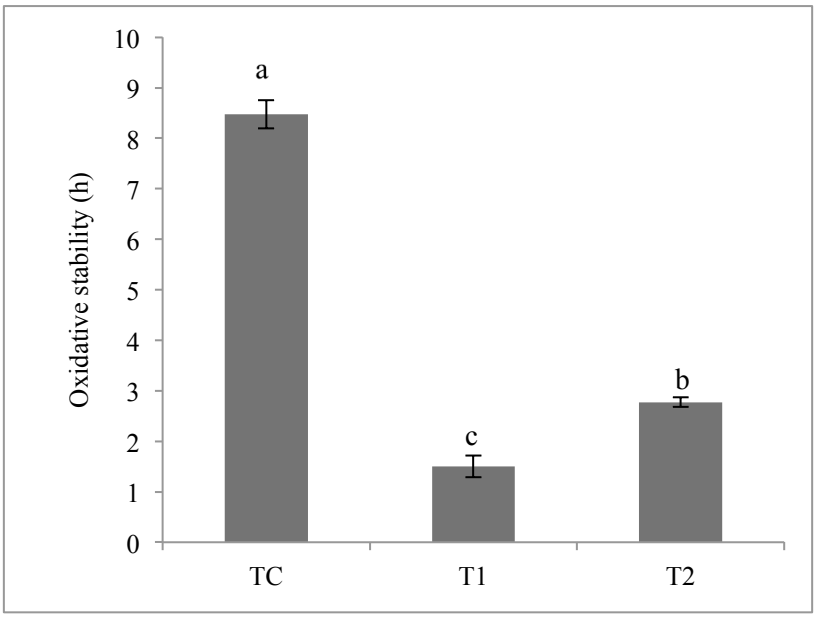

Fig. 2 - Variation of oxidative stability of control olive oil (TC), olive oil stored for two years (T1) and olive oil obtained from intact sun-dried olive fruits stored for two years before being processed (T2). Results are shown as mean ± SD (n = 3).
[a-c] Different letters indicate significantly differences at $p < 0.05$ according to Duncan's test.

3.6. Changes in fatty acid alkyl esters in olive oil samples following the two storage methods

The experimental results of the amounts of FAAEs, FAMEs, FAEEs and FAEEs/FAMEs in oil extracted directly after harvesting (from fresh olives) and in oils stored after two years using the two different methods are summarized in Table 4. For EVOO, the concentration of the FAEEs must be ≤35 mg kg^{-1} (IOC, 2015).
The major fatty acid alkyl esters (FAAEs) present in olive oil are those corresponding to palmitic, oleic and linoleic acids. Indeed, the amount of FAEEs in sample TC did not exceed the quantity of 35 mg kg^{-1}, thus indicating the classification of this olive oil as extra virgin.
Compared with the control oil, the content of FAEEs showed significantly higher values in samples T1 and T2 (p <0.001) with 624.2 mg kg^{-1} and 62.6 mg kg^{-1}, respectively. The two types of storage (T1 and T2) both negatively affected the quality of the oil. This leads to their classification as non-extra virgin olive oils.

Fatty acid alkyl esters (FAAEs), mainly ethyl (FAEEs) and methyl esters (FAMEs), are formed by esterification of free fatty acids (FFAs) with low molecular alcohols, such as methanol and ethanol. In good quality extra virgin olive oils, FAMEs and FAEEs are present in very small amounts (VALLI et *al*., 2013), while they are present in higher amounts in "lampante" olive oils, as confirmed in the current study. The high content of FAAEs in samples T1 and T2 can be due to the presence of specific components, such as free fatty acids (FFAs) and low chain alcohols. These compounds are, respectively, originated through substantial liberation of FFAs from triglycerides by lipolysis and by a parallel consistent formation of ethanol due to the aerobic metabolism of microorganisms and methanol due to the degradation of the pectins linked to the action of endogenous pectinmethyl-esterases (BIEDERMANN et *al*., 2008).
JABEUR et *al*., (2015) found that in lampante oils the amounts of ethyl esters were greater than those of methyl esters: in our study, this was only observed for sample T1. The results also highlighted that the change in the content of FAAEs, and particularly FAEE, that occurred in the stored oil (T1) was significantly higher than that in oils extracted from olives stored for two years (T2). In fact, (CONTE et *al*., 2014; GÓMEZ-COCA et *al*., 2016) reported that the FAEE concentration is a quality parameter that reflects fruit quality at the moment of extraction, and the presence of ethyl esters are markers of fermentation (low quality of olive fruits and extracted oil). It could be assumed that sunlight drying of olives and subsequent long storage could lead to a higher liberation of methanol (from degradation of pectins) respect to a fermentative process, explaining the highest content of FAMEs compared to FAEEs for T2. On the other hand, the high content of FAEEs in sample T1 could be explained by the low quality of olives before processing, as shown by the remarkable content of FAAEs as well as a free acidity value close to the legal limit in sample TC.

Table 4. Fatty acid alkyl esters (mg i.s. kg^{-1}) of olive oil not stored (TC), stored for two years (T1) and obtained from intact sun-dried olive fruits stored for two years before being processed (T2).

	TC	T1	T2
Σ FAMEs	33.7±0.2^{c}	185.2±9.8a	172.4±31.9^{b}
Σ FAEEs	23.6±3.6^{c}	624.2±15.5^{a}	62.6±8.7^{b}
FAEEs/FAMEs	0.7±0.1^{b}	3.4±0.2 ^{a}	0.4±0.1^{c}
Total FAAEs	53.5±8.1^{c}	809.5±20.5 ^{a}	235.0±33.0^{b}

FAMEs: fatty acid methyl esters; FAEEs: fatty acid ethyl esters; FAAEs: fatty acid alkyl esters.
Each value represents the mean of the three determination (n = 3) ± standard deviation.
$^{a-c}$Different letters show statistically significant differences ($p < 0.05$) according to Duncan test.

4. CONCLUSIONS

Overall, from this study it can be concluded that both the storage methods herein investigated had a strong negative influence on the quality of the olive oils. This is a crucial information to be communicated to the local olive oil producers residents in Gabes (Southern Tunisia), in order to make them aware about the strong influence on the quality of the product. In particular, it should be underlined that the basic quality parameters of the stored oils exceeded the limits established by the IOC trade standard for virgin olive oils, thus declassifying the oil as "lampante". A significant degradation ($p<0.05$) in phenolic compounds also occurred, being more accentuated in the oil conserved for two

years than in that obtained from stored and dried olive fruits. A similar trend was also seen in α-tocopherol content and in oxidative stability time (OSI) of the samples. The high FAAEs level observed in both the two storage conditions suggested that the oils were obtained from olives of suboptimal quality, wherein fermentation and other types of degradations (oxidation) have occurred.
However, the results of the current study highlight that some traditional methods, still applied in Gabes for storage of olives and oil, exerted a notable negative effect on all parameters investigated.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Higher Education and Scientific Research in Tunisia. We want to thank the members of LR-NAFS /LR12ES05 «Nutrition-Functional Food and Vascular Health» and the laboratory staff of the Department of Agricultural and Food Sciences, Alma Mater Studiorum – University of Bologna (Italy) for their contribution to this research.

REFERENCES

Anwar P., Bendini A., Gulfraz M., Qureshi R., Valli E., Di Lecce G. and Gallina Toschi T. 2013. Characterization of olive oils obtained from wild olive trees (*Olea ferruginea* Royle) in Pakistan. Food Res. Int. 54:1965-1971.

Bendini A., Cerretani L., Carrasco-Pancorbo A., Gómez-Caravaca A.M., Segura-Carretero A., Fernández-Gutiérrez A. and Lercker G. 2007. Phenolic molecules in virgin olive oils: A survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. Molecules 12:1679-1719.

Bendini A., Cerretani L., Pizzolante L., Gallina Toschi T., Guzzo F., Ceoldo S. and Levi M. 2006. Phenol content related to antioxidant and antimicrobial activities of Passiflora spp. extracts. Eur. Food Res. Technol. 223:102-109.

Biasone A., Di Loreto G., Preziuso S.M., Serraiocco A. and Di Giacinto L. 2012. Changes in the volatile and sensory profile of virgin olive oil as a function of olive fruits mould process. Riv. Ital. delle Sost. Grasse 89:117-125.

Biedermann M., Bongartz A., Mariani C. and Grob K. 2008. Fatty acid methyl and ethyl esters as well as wax esters for evaluating the quality of olive oils. Eur. Food Res. Technol. 228:65-74.

Blekas G., Tsimidou M. and Boskou D. 1995. Contribution of α-tocopherol to olive oil stability. Food Chem. 52:289-294.

Clodoveo M.L., Delcuratolo D., Gomes T. and Colelli G. 2007. Effect of different temperatures and storage atmospheres on Coratina olive oil quality. Food Chem. 102:571-576.

Conte L., Mariani C., Gallina Toschi T. and Tagliabue S. 2014. Alchil esteri e composti correlate in oli d'oliva vergini: loro evoluzione nel tempo. Riv. Ital. delle Sost. Grasse 91:21-29.

Dabbou S., Gharbi I., Dabbou S., Brahmi F. and Nakbi A. 2011. Impact of packaging material and storage time on olive oil quality. African J. Biotechnol. 10:16937-16947.

Frankel EN. 1993. Formation of headspace volatiles by thermal decomposition of oxidised fish oils vs. vegetable oils. J Am. Oil Chem. Soc. 70:767-772.

García J.M., Gutiérrez F., Castellano J.M., Perdiguero S., Morilla A. and Albi M.A. 1996. Influence of Storage Temperature on Fruit Ripening and Olive Oil Quality. J. Agric. Food Chem. 44:264-267.

Gargouri B., Zribi A. and Bouaziz M. 2015. Effect of containers on the quality of Chemlali olive oil during storage. J. Food Sci. Technol. 52:1948-59.

Gómez-Coca R.B., Fernandes G.D., Pérez-Camino M. del C. and Moreda W. 2016. Fatty acid ethyl esters (FAEE) in extra virgin olive oil: A case study of a quality parameter. LWT - Food Sci. Technol. 66:378-383.

Gutierrez F., Perdiguero S., Garcia J.M. and Castellano J.M. 1992. Quality of Oils from Olives Stored Under Controlled Atmosphere. J. Am. Oil Chem. Soc. 12:1215-1218.

Hachicha R., Kotti F., Garcia-Rodriguez R., Gargouri M., Sanz C. and Pirez A. G. 2015. Monitoring endogenous enzymes during olive fruit ripening and storage: Correlation with virgin olive oil phenolic profiles. Food Chem. 174:240-247.

Inarejos-García A.M., Gómez-Rico A., Desamparados Salvador M. and Fregapane G. 2010. Effect of preprocessing olive storage conditions on virgin olive oil quality and composition. J. Agric. Food Chem. 58:4858-65.

International Olive Council. 2009. COI/T.20/Doc No 29, Determination of Biophenols in Olive Oils by HPLC.

International Olive Oil Council. 2015. Trade Standard Applying to Olive Oil and Olive-Pomace Oil. 1-17.

ISO 3656: 2011:"Animal and vegetable fats and oils" - Determination of ultraviolet absorbance expressed as specific uv extinction.

ISO 3960: 2010: "Animal and vegetable fats and oils" - Determination of peroxide value.

ISO 660:2009: "Animal and vegetable fats and oils" - Determination of acid value and acidity.

Jabeur H., Zribi A., Abdelhedi R. and Bouaziz M. 2015. Effect of olive storage conditions on Chemlali olive oil quality and the effective role of fatty acids alkyl esters in checking olive oils authenticity. Food Chem. 169:289-296.

Kiritsakis A., Nanos G.D., Polymenopulos Z., Thomai T. and Sfakiotakis E.M. 1998. Effect of fruit storage conditions on olive oil quality. J. Am. Oil Chem. Soc. 75:721-724.

Krichene D., Allalout A., Mancebo-campos V., Salvador M.D., Zarrouk M. and Fregapane G. 2010. Stability of virgin olive oil and behaviour of its natural antioxidants under medium temperature accelerated storage conditions. Food Chem. 121:171-177.

Lerma-Garcia M.J., Simó-Alfonso E.F., Chiavaro E., Bendini A., Lercker G. and Cerretani L. 2009. Study of chemical changes produced in virgin olive oils with different phenolic contents during an accelerated storage treatment. J. Agric. Food Chem. 57:7834-7840.

Lozano-Sánchez J., Bendini A., Quirantes-Piné R., Cerretani L., Segura-Carretero A. and Fernández-Gutiérrez A. 2013. Monitoring the bioactive compounds status of extra-virgin olive oil and storage by-products over the shelf life. Food Control 30:606-615.

Mateos R., Espartero JL., Trujillo M., Ríos J. J., León-Camacho M., Alcudia F. and Cert A. 2001. Determination of Phenols, Flavones, and Lignans in Virgin Olive Oils by Solid-Phase Extraction and High-Performance Liquid Chromatography with Diode Array Ultraviolet Detection. J. Agric. Food Chem. 49:2185-2192.

Méndez A.I. and Falqué E. 2007. Effect of storage time and container type on the quality of extra-virgin olive oil. Food Control 18:521-529.

Morelló J.R., Motilva M.J., Tovar M.J. and Romero M.P. 2004. Changes in commercial virgin olive oil (cv Arbequina) during storage, with special emphasis on the phenolic fraction. Food Chem. 85:357-364.

Nabil BY., Youssef O., Nizar D., Bechir B. and Chedly A. 2012. Effect of olive storage period at two different temperatures on oil quality of two tunisian cultivars of *Olea europea*, Chemlali and Chétoui. African J. Biotechnol. 11:888-895.

Nunez-Cordoba J.M., Alonso A., Beunza J.J., Palma S., Gomez-Gracia E. and Martinez-Gonzalez M.A. 2009. Role of vegetables and fruits in Mediterranean diets to prevent hypertension. Eur. J. Clin. Nutr. 63:605-612.

Pagliarini E., Zanoni B. and Giovanelli G. 2000. Predictive study on tuscan extra virgin olive oil stability under several commercial conditions. J. Agric. Food Chem. 48:1345-1351.

Pellegrini N., Visioli F., Buratti S. and Brighenti F. 2001. Direct Analysis of Total Antioxidant Activity of Olive Oil and Studies on the Influence of Heating. J. Agric. Food Chem. 49:2532-2538.

Pristouri G., Badeka A. and Kontominas MG. 2010. Effect of packaging material headspace, oxygen and light transmission, temperature and storage time on quality characteristics of extra virgin olive oil. Food Control 21:412-418.

Rabiei V., Ghorbani S. and Hajnajari H. 2011. Effect of temperature and storage period of olive (*Olea europaea* cv. Zard) fruit on olive oil quality. J. Food. Agric. Environ. 9:74-77.

Ruiz-Canela M. and Martínez-González MA. 2011. Olive oil in the primary prevention of cardiovascular disease. Maturitas 68:245-250.

Sicari V., Giuffrè AM. and Louadj MP. 2010. Evolution of phenolic compounds of virgin olive oil during 12 months. J. Am. Oil Chem. Soc. 74:109.

Servili M. and Gian Francesco M. 2002. Contribution of phenolic compouns to virgin olive oil quality. Eur. J. Lipid Sci. Technol. 104:602-613.

Servili M., Selvaggini R., Taticchi A., Esposto S. and Montedoro G. 2003. Volatile compounds and phenolic composition of virgin olive oil: optimization of temperature and time of exposure of olive pastes to air contact during the mechanical extraction process. J. Agric. Food Chem. 51:7980-8.

Singleton V.L. and Rossi J.A.J. 1965. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Am. J. Enol. Vitic. 16:144-158.

Tura D., Gigliotti C., Pedò S., Failla O., Bassi D. and Serraiocco A. 2007. Influence of cultivar and site of cultivation on levels of lipophilic and hydrophilic antioxidants in virgin olive oils (Olea Europea L.) and correlations with oxidative stability. Sci. Hortic. (Amsterdam) 112:108-119.

Valli E., Bendini A., Maggio RM., Cerretani L., Toschi T. G., Casiraghi E. and Lercker G. 2013. Detection of low-quality extra virgin olive oils by fatty acid alkyl esters evaluation: A preliminary and fast mid-infrared spectroscopy discrimination by a chemometric approach. Int. J. Food Sci. Technol. 48:548-555.

Vichi S., Romero A., Gallardo-Chacón J., Tous J., López-Tamames E. and Buxaderas S. 2009. Influence of olives' storage conditions on the formation of volatile phenols and their role in off-odor formation in the oil. J. Agric. Food Chem. 57:1449-1455.

Paper Received July 21, 2017 Accepted September 2, 2017