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Effect of natural and synthetic antioxidants on the oxidative stability of walnut oil under different storage conditions

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ABSTRACT

The aim of the work was to evaluate the combined effects of the storage condition (six months under fluorescent light – 800 Lux – or darkness condition, both at room temperature) with the addition of natural (rosemary extract, RE) and synthetic (ascorbyl palmitate – AP -, and Tert-butylhidroquinone – TBHQ) antioxidants on quality indices related to walnut oil (WO) oxidative stability. Neither RE nor synthetic antioxidants contributed markedly on inhibiting photo-oxidative degradation, resulting in significantly increased amounts of primary and secondary oxidation products in oils exposed to light. Under darkness-storage condition, the addition of the mentioned antioxidants significantly reduced lipid oxidation and improved oil shelf life. Oils added with RE – alone or in combination with TBHQ or TBHQ plus AP – maintain an acceptable quality at least up to six months of storage. Results from this work stressed the influence of the illumination condition on WO oxidative stability, suggesting that this oil should be stored in containers with light-barrier properties, and may be added with the antioxidants examined in the current study.

1. Introduction

Walnut (*Juglans regia* L.) is a crop of high economic interest to the food industry. The edible part of the fruit (the seed or kernel) is globally popular and valued for its nutritional, health and sensory attributes. The high oil and essential fatty acid contents of the walnut kernel make it a good source for commercial production of edible oil. Oil contents as high as 740 g/kg kernel (Soxhlet extraction, *n*-hexane) have been reported for some commercial walnut varieties (Martínez, Mattea, & Maestri, 2006; Martínez & Maestri, 2008).

Walnut oil (WO) can be extracted easily by screw pressing (Martínez, Mattea, & Maestri, 2008). Employing a pilot plant screw-press, the highest oil recovery (660 g/kg kernel) was achieved at 7.5 g/100 g kernel moisture and 50 °C pressing temperature. Fresh WO is very low in free fatty acid concentration, peroxides and phosphatides (Martínez, Labuckas, Lamarque, & Maestri, 2010) because of which it may be consumed directly, without refining.

Walnut oil is composed mainly of triglycerides, in which monounsaturated (oleic acid mainly) and polyunsaturated fatty acids (PUFAs, linoleic and α-linolenic acids) are present in high amounts (Amaral, Casal, Pereira, Seabra, & Oliveira, 2003; Crews et al., 2005; Martínez et al., 2006). According to Simopoulos (2002) WO has a perfect balance of *n*-6:*n*-3 PUFAs, a ratio of 4:1, which was showed to decrease the incidence of cardiovascular risk (Bucher, Hengstler, Schindler, & Meier, 2002). Although it seems clear that such fatty acid composition is favorable from a nutritional point of view, higher contents of linoleic and linolenic acids result in poorer oxidative stability and shorter shelf life of the oil. When PUFAs are exposed to environmental factors such as air, light and temperature, oxidation reactions produce undesirable flavors, rancid odors, discoloration and other forms of spoilage. The primary autoxidation products are hydroperoxides, that have no

Abbreviations: AP, ascorbyl palmitate; CD, conjugated dienes; CT, conjugated trienes; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FAMEs, fatty acid methyl esters; OSI, oxidative stability index; p-AV, *p*-anisidine value; PF, protection factor; PUFAs, polyunsaturated fatty acids; PV, peroxide value; RE, rosemary extract; RSC, radical scavenging capacity; TBHQ, tert-butylhidroquinone; WO, walnut oil.

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taste and flavor, but their degradation products (aldehydes, ketones) are very potent taste and flavor modifiers (Frankel, 2005).

Beyond their chemical composition, the susceptibility of vegetable oils to oxidation also depends on the processing, packing and storage conditions. In a recent work (Martínez, Barrionuevo, Nepote, Grosso, & Maestri, 2011) we determined that WO is highly susceptible to photo-oxidative degradation. When it was stored in transparent glass bottles, exposed to fluorescent light (1100 Lux) and room temperature conditions, without addition of any antioxidant, it could maintain an acceptable quality until two months of storage. This time represents a very short shelf life. Protection against light and addition of appropriate natural or synthetic antioxidants are necessary to preserve WO from oxidation.

Antioxidants can increase shelf life of food products by retarding lipid oxidation. Although synthetic antioxidants are extensively used as food additives, their safety has been questioned (Shahidi & Zhong, 2005) stimulating the search and evaluation of natural compounds with antioxidant properties. As a result, there is a great interest for obtaining and utilizing the antioxidants from natural sources because they are presumed to be safe (Shahidi & Zhong, 2005). Many naturally-occurring compounds from herbs and spices have been extensively studied for their antioxidant activity (Frankel, 2005; Maestri, Nepote, Lamarque, & Zygadlo, 2006; Yanishlieva & Marinova, 2001; Zhang et al., 2010). Among these, rosemary extracts (RE) have been widely used in food systems, and some of them are today available in the market.

Ascorbic acid (AA) is an organic acid occurring widely in the vegetable world. It has a number of antioxidants actions including reaction with free-radical species, as singlet oxygen quencher (Frankel, 2005). Unfortunately, it has very low solubility in pure oils. Ascorbyl palmitate (AP) is a synthetically-derived oil-soluble ester of AA. Although the mechanism of action is not well known, Lee, Jung, and Kim (1997) have reported that AP can act as an effective oxygen scavenger in photosensitized oxidation reactions of vegetable oils.

A number of works have reported on the effect of storage and packing materials on shelf life of shelled walnuts (Bakkalbaşi, Yilmaz, Javidipour, & Artik, 2012; Mexis, Badeka, Riganakos, Karakostas, & Kontominas, 2009): However, up to the moment, there are no reports regarding the effect of added antioxidants and storage conditions on WO oxidative stability. Packing materials with barrier properties against light have been successfully used to protect oils from photo-oxidation (Frankel, 2005; Torres & Maestri, 2006). Ideal containers for oils should be opaque to light and impermeable to air and moisture. Plastic containers are generally used mainly because they allow saving on cost. However, the storage stability of oils containing added antioxidants seems to be lower in plastic containers than in glass bottles (Frankel, 2005).

Torres and Maestri (2006) showed that glass bottles wrapped with aluminum foil were the most appropriate containers to protect olive oils against both photo-oxidative degradation and hydrolytic rancidity. On the other hand, aluminum-coated packages were successfully used to preserve partially defatted walnut flour, mainly because of their light-barrier properties (Labuckas, Maestri, & Lamarque, 2011).

Walnut oil is produced at a small scale in many countries such as France, Spain, Chile and Argentina. Increasing demand for WO consumption encourages finding appropriate methods to enhance its shelf life keeping oil oxidation at the lowest possible level. This work was aimed to evaluate the combined effects of the storage condition (six months under fluorescent light – 800 Lux – or darkness condition, both at room temperature) with the addition of natural (RE) and synthetic (AP and TBHQ) antioxidants on quality indices related to WO oxidative stability.

2. Materials and methods

2.1. Materials

Walnut fruits (*J. regia* L. var. Franquette) were obtained from commercial plantations at Belén location, Catamarca Province, Argentina. At full maturity, fruits were hand-picked directly from the trees. Immediately after harvest, fruits were hulled manually and nuts were dried in an oven at 30 ± 2 °C, in the dark, for one day. Then, nuts were shelled manually, and kernels containing about 740 g oil/kg (dry basis) and 40 g water/kg were immediately processed for oil extraction. Kernels were ground using a home-made stainless steel roller crusher and particles between 2.4 and 4.8 mm (mesh 8-4, Tyler standard screen scale) were selected using an automated screen. This material was sprinkled with distilled water according to Singh and Bargale (2000). Sprinkling was achieved by using a predetermined quantity of water so that the final moisture content of the sample to be pressed was about 75 g/kg. This moisture level was found to give the highest oil recovery when kernels where screw-pressed (Martínez et al., 2008). The water sprinkled sample was then packed in an air-tight, cylindrical stainless steel container, and stored about 48 h for equilibration. The container was shaken at regular time intervals to distribute moisture uniformly throughout the sample. Walnut oil extraction was carried out essentially following the procedure of Martínez et al. (2008). Oil expression was carried out at 50 °C using a Komet screw press (Model CA 59 G, IBG Monforts, Mönchengladbach, Germany), with a 5 mm restriction die and a screw speed of 20 rpm. The screw press was first run for 15 min without seed material but with heating *via* an electrical resistance-heating ring attached around the press barrel, to raise the screw-press barrel temperature to the desired temperature. Running temperature was checked with a digital thermometer inserted into the restriction die. The oil obtained was filtered through a series of cartridge filters from 100 to 2 μm pore size. This procedure allowed eliminating very fine solid particles, so that a filtered oil sample subjected to centrifugation (11,000 × g for 30 min) did not show precipitated solids.

2.2. Experimental design for storage stability test

The antioxidants (RE, AP, TBHQ) or their mixtures were added to oil samples (filtered WO) according to quantities stated in Table 1. Briefly, the additives were dissolved in 50 mL-oil aliquots by using a shaker (approximately 5 min) until a homogeneous oil appearance was achieved. The mixtures (oil plus additive) were transferred separately into transparent glass bottles (250 mL) each containing 200 mL fresh WO. The bottled oils (final volume 250 mL) were mixed thoroughly and then placed inside a thermostated chamber at 25 ± 1 °C. Two sets of bottled oils were prepared: one set was stored under illumination (800 Lux); the other one was kept in the darkness by wrapping each bottle with an aluminum foil. Each treatment (consisting of a combination from oil plus additive/illumination condition) was prepared in duplicate. For the control treatments, oil samples without added antioxidants were used. Oils were stored for six months. Every fifteen days each individual oil sample was withdrawn from the chamber for scheduled analyses.

2.3. Chemical analyses

Acid, peroxide (PV), K_{232} (conjugated dienes, CD), K_{270} (conjugated trienes, CT) and *p*-anisidine (p-AV) values were evaluated using standard AOCS (2009) methods.

Table 1
Treatments used for walnut oil (WO) storage stability test. Each treatment was stored in light (L) and in darkness (D) condition. TBHQ (tert-butylhidroquinone), AP (ascorbyl palmitate), RE (rosemary extract).

Code	Treatment
1	Control (WO without additives)
2	TBHQ 100 μg/g oil
3	TBHQ 200 μg/g oil
4	AP 100 μg/g oil
5	AP 200 μg/g oil
6	RE 800 μg/g oil
7	TBHQ 100 μg/g oil + AP 100 μg/g oil
8	TBHQ 100 μg/g oil + RE 800 μg/g oil
9	AP 100 μg/g oil + RE 800 μg/g oil
10	AP 200 μg/g oil + RE 800 μg/g oil
11	TBHQ 100 μg/g oil + AP 100 μg/g oil + RE 800 μg/g oil

Fatty acid composition was analyzed according to the procedure employed by Martínez et al. (2006) with minor modifications. Briefly, oil samples (0.5 mL) were subjected to alkaline saponification (reflux, 1 mol equi/L KOH in methanol, 10 mL). Unsaponifiable matter was extracted with *n*-hexane (3 × 30 mL). The fatty acid methyl esters (FAMEs) were obtained by reflux (1 mol equi/L H_2SO_4 in methanol, 20 mL) and analyzed by gas chromatography (Perkin Elmer, Shelton, USA) using a fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) CP Wax 52 CB (Varian, Walnut Creek, CA); carrier gas N_2 at 1 mL/min; column temperature programmed from 180 °C (5 min) to 240 °C at 4 °C/min; injector and detector temperatures 250 °C; detector FID. The identification of FAMEs was carried out by comparing their retention times with those of reference compounds (Sigma–Aldrich, St. Louis, USA). Quantification of each fatty acid analyzed was made by using an internal standard (methyl nonadecanoate).

Total tocopherol concentration of WO was quantified by spectrophotometric analysis (Perkin Elmer, Shelton, USA) at 520 nm following the method proposed by Wong, Timms, and Goh (1988). Chlorophyll and carotenoid compounds were also determined spectrophotometrically at 670 and 470 nm, respectively, in cyclohexane, *via* specific extinction values using the method of Minguez-Mosquera, Rejano, Gandul, Sánchez, and Garrido (1991).

Carnosic acid (CA) content from RE was determined by HPLC and UV spectrophotometric analyses according to the procedures employed by Visentin, Cismondi, and Maestri (2011). HPLC (Perkin Elmer, Shelton, USA) analyses were performed on a reverse phase C18 column (250 × 4.6 mm, 5 μm pore size). The mobile phase was programmed with a linear gradient from 90% A (840 mL of water with 8.5 mL of acetic acid and 150 mL of acetonitrile), 10% B (methanol), to 100% B at 30 min, with a flow rate of 1.5 mL/min. Detection was accomplished by using a UV detector at a wavelength of 284 nm. Identification of CA was carried out by comparison of its retention time with that of the pure standard (Sigma–Aldrich, St. Louis, USA). A Perkin–Elmer (Shelton, USA) UV–Vis spectrophotometer was used to determine the amount of CA in the extract, after calibration with gravimetrically prepared standard solutions.

The oxidative stability indices (OSI) were determined using the Rancimat (Metrohm, Switzerland) analysis, and corresponded to the break points in the plotted curves. Air flow rate was set at 20 L/h and temperature of the heating block was maintained at 110 °C.

To evaluate the radical scavenging capacity (RSC) of the oil samples, 100 mg (in 1 mL toluene) of each oil sample was vortexed (20 s, ambient temperature) with 3.9 mL toluene solution (10^{-4} mol/L) of the free stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radical (DPPH•). Against a blank of pure toluene, the absorption at 515 nm was measured in 1 cm quartz cells after 30 min of mixing using a UV–visible spectrophotometer (Perkin–Elmer, Shelton, USA). The RSC was estimated as $DPPH\bullet_r$ which expresses the amount of the radical that remains in the medium after antioxidants depletion (Espín, Soler-Rivas, & Wichers, 2000).

2.4. Statistical analyses

Statistical differences among treatments were estimated from ANOVA test at the 5% level ($P < 0.05$) of significance, for all parameters evaluated. Whenever ANOVA indicated a significant difference, a pair-wise comparison of mean by least significant difference (LSD) was carried out. The multivariate statistical analysis of the data set from the storage stability test was performed using principal component analysis (PCA). Statistical analyses were accomplished by using the software Infostat version 1.1 (FCA-UNC, Argentina).

3. Results and discussion

Table 2 shows some quality and compositional parameters of fresh WO used for the storage stability test. Acid value, PV, CD and CT values were similar to those observed in cold-pressed WO (Martínez et al., 2008) and much lower than the maximum values established by the Codex Alimentarius (2001) for non-refined oils, indicating that the oil extraction method employed in this work did not affect adversely those indicators of hydrolytic and oxidative rancidity.

Linoleic acid was the major fatty acid (52.4 g/100 g oil) followed by oleic (22.9 g/100 g oil) and linolenic (15.2 g/100 g oil) acids. Such composition leads to high unsaturation degree (iodine value = 157). This fact, together with a relatively low tocopherol concentration and the absence of other phenolic compounds with potential antioxidant capacity, results in very low oxidative stability (OSI < 3 h).

According to Wong (1995), vegetable oils are susceptible to photo-oxidation during storage under light, especially when photosensitizers, such as chlorophylls, are present. Although WO analyzed here had very low chlorophyll content (0.52 μg/g oil), such concentration may be sufficient to induce photochemical oxidation (Suzuki, Suzuki, Endo, & Kaneda, 1984).

Table 2
Quality and compositional parameters of walnut oil used for the storage stability test.

Parameter	Value[a]
Acid value (mg KOH/g oil)	0.08 ± 0.006
Peroxide value (mol equi O_2/kg oil)	0.55 ± 0.06
Conjugate dienes (K_{232})	1.18 ± 0.01
Conjugate trienes (K_{270})	0.06 ± 0.001
Oxidative stability index (hours)	2.88 ± 0.22
Iodine value	157 ± 0.09
Total tocopherols (μg/g oil)	289 ± 14.6
Carotenoids (μg/g oil)	0.93 ± 0.05
Chlorophylls (μg/g oil)	0.52 ± 0.02
Fatty acid composition (g/100 g oil)	
Palmitic acid	7.20 ± 0.04
Palmitoleic acid	0.08 ± 0.01
Stearic acid	2.14 ± 0.01
Oleic acid	22.92 ± 0.02
Linoleic acid	52.42 ± 0.02
Linolenic acid	15.24 ± 0.03

[a] Mean values ± standard deviation ($n = 3$).

Regarding carotenoid pigments, Warner and Frankel (1987) have shown that, in soybean oil, the presence of β-carotene – at concentrations between 5 and 20 μg/g oil – has a protective effect against oxidative damage induced by light. Considering the similarities in fatty acid composition of soybean and walnut oils, it can be assumed that the carotenoid content of WO used in this work (0.93 μg/g oil) is not enough to provide some protection against photo-oxidative degradation.

World-wide food regulations recognize the rosemary extracts as GRAS ("generally recognized as safe") additives. There are no regulations that limit their use. Several researches have indicated that carnosic acid (CA) is the most active antioxidant component in rosemary extracts (Terpinc, Bezjak, & Abramovič, 2009; Visentin et al., 2011). According to HPLC analysis, RE used in this work had 1.22 g CA/100 g extract. A preliminary assessment of the RE antioxidant capacity, using the Rancimat method, showed a concentration-dependent effect ($R^2 = 0.96$), which reached a maximum at 800–1200 μg/g oil (Table 3). Nevertheless, the addition of 800 μg/g of RE to light-stored WO had not a noticeable effect on oxidation inhibition: PV, CD and CT differed slightly from those of the control treatment along the storage period (Table 4).

Under light-storage condition, the addition of AP (100 or 200 μg/g oil) to WO caused small decreases in PV and CD with respect to the control treatment (Table 4). At this condition, AP presented a negligible RSC (Table 5). These results agree partially with data from Lee et al. (1997) who reported that AP, at concentrations between 500 and 1500 μg/g oil, was highly effective at minimizing chlorophyll-sensitized photo-oxidation of soybean oil. Beyond variations in composition of the substrate oils, differences between the aforementioned findings may be also due to differences in the AP concentrations employed. It appears that AP can reduce photo-oxidation in a concentration-depending manner, but the effective concentration could be much higher than those usually allowed for synthetic antioxidants in oils (200 μg/g oil). Thus, McMullen, Hawrysh, Lin, and Tokarska (1991) reported that AP (200 μg/g oil) showed a limited effect on canola oil photo-oxidative degradation, but results from Lee et al. (1997) indicated that the addition of 500, 1000 and 1500 μg AP/g oil resulted in 69.3, 83.6 and 94.6% inhibition of linoleic acid photo-oxidation, respectively, after 5-h storage under fluorescent light (3300 Lux).

The AP (at 100 and 200 μg/g oil) and RE treatments stored under light, together with AP (100 μg/g oil) treatment stored in darkness, showed increasing *p*-anisidine values at the end of the storage period (Fig. 1).

Table 3
Preliminary assessment of the antioxidant performance of different rosemary extract (RE) concentrations on walnut oil stability.

RE (μg/g oil)	Protection factor*
200	1.29 ± 0.02^{a}
400	1.54 ± 0.03^{b}
600	1.73 ± 0.01^{c}
800	2.18 ± 0.02^{d}
1000	2.22 ± 0.06^{d}
1200	2.30 ± 0.03^{d}

Mean values ± standard deviation ($n = 4$). Different superscript letters indicate significant differences between treatments ($P < 0.05$). * $PF = IP_{Ant}/IP_o$, where IP_{Ant} is the induction period (the time needed to reach the break point in the plotted curve from Rancimat analysis) of WO in the presence of the added antioxidant, and IP_o is the induction period of WO alone. Regression equation: $y = 0.87 + 2.1.\ 10^{-3}\ x + -6.9.\ 10^{-7}\ x^2$, where '*y*' is the dependent variable (protection factor); '*x*' is the independent variable (RE concentration). Determination coefficient: $R^2 = 0.96$.

Tert-butylhidroquinone (TBHQ), which is a potent radical scavenging in lipid auto-oxidation reactions, seems to have a slight effect on photo-oxidation inhibition. When it was added to light-stored WO, minor but significant reductions in PV, CD and CT were observed in relation to the control treatment at the end of the storage period (Table 4).

The various combinations of the antioxidants tested did not reduce appreciably the WO oxidation rate during storage under light. Nevertheless, the best antioxidant performance was achieved by using a combination of RE (800 μg/g oil), AP (100 μg/g oil) and TBHQ (100 μg/g oil) which showed PV significantly lower than those from the other treatments at the end of the storage period.

Considering PV, CD, CT and OSI values, WO stored in darkness, without addition of any antioxidant, oxidized at similar rate than that stored under light (Tables 4 and 5).

With the exception of AP at 100 μg/g oil, the combination of the antioxidants employed with the darkness-storage condition had a marked influence on WO oxidative stability. OSI value from each sample (oil plus additive) stored in darkness was significantly higher than that obtained from its light-stored counterpart (Table 5). In addition, oil samples stored in darkness did not reach the end point for rancidity or acceptability limit for virgin and cold-pressed vegetable oils (15 mol equi O_2/kg oil) (Codex Alimentarius, 2001), and did not develop secondary oxidation products as indicated by the *p*-anisidine value.

At the end of the storage period, there were minor differences in PV and CD among treatments 2D, 3D (100 and 200 μg TBHQ/g oil, respectively), 7D (TBHQ 100 μg/g oil + AP 100 μg/g oil), 8D (TBHQ 100 μg/g oil + RE 800 μg/g oil), 10 D (AP 200 μg/g oil + RE 800 μg/g oil), and 11D (TBHQ 100 μg/g oil + AP 100 μg/g oil + RE 800 μg/g oil). These findings indicate that – under darkness-storage condition – : a) RE could be used – alone or in combination with TBHQ, AP or TBHQ plus AP – for preserving WO from oxidation at least during a 6-month period; b) the antioxidant effectiveness of TBHQ may be achieved by using the 50% of the maximum concentration (200 μg/g oil) allowed by world-wide food regulations for synthetic antioxidants in oils (Frankel, 2005; Shahidi & Zhong, 2005).

The whole data set from PV was used to perform polynomial regression equations with storage time as the independent variable (Table 6). For light-stored WO, all treatments evaluated could reach the end point for rancidity (15 mol equi O_2/kg oil, Codex Alimentarius, 2001), at 60–66 days of storage. For oils stored in darkness, a PV of 3 mol equi O_2/kg was taken as a reference. In treatments 2D, 3D, 5D, 7D, 8D and 10D, the time needed to reach that value was estimated to be higher than 5 months. In connection with these data, Bakkalbaşi et al. (2012) have found that shelled walnuts stored in polyamide/polyethylene film pouches at darkness and 10–30 °C temperature conditions develop rancid taste after 6 months of storage.

The RSC of the antioxidants examined was slightly influenced by the storage condition (Table 5). In general, oils stored in darkness presented higher RSC (minor amounts of $DPPH^{\bullet}_{r}$) than oils stored under light. Considering the individual antioxidants, a strong effect of the concentration was observed for TBHQ but not for AP. At the concentrations tested neither AP nor RE had a noticeable influence on scavenging DPPH radicals. Regarding antioxidants blends, the RSC from the mixtures of AP and/or RE with TBHQ (treatments 7, 8 and 11) did not differ markedly from that registered for 100 μg TBHQ alone (treatment 2). This fact indicates that AP and RE did not promote a synergistic or even an additive effect on radical quenching activity of TBHQ. There are no reports evaluating the combined effects of AP, RE and TBHQ on vegetable oils photo-oxidation. Nevertheless, a work by Hraš, Hadolin, Knez, and Bauman (2000) has demonstrated that AP (200 μg/g oil) had an

Table 4
Peroxide values, conjugated dienes (K_{232}) and conjugated trienes (K_{270}) from walnut oil during the storage stability test. For clarity, only values at 0, 90 and 180 days are presented. See Table 1 for treatment codes.

Treatment	Peroxide value (mol equi O_2/kg oil)			K_{232}			K_{270}		
	Time (days)			Time (days)			Time (days)		
	0	90	180	0	90	180	0	90	180
Light									
1	0.55 ± 0.06	22.25 ± 0.06^{bcd}	70.92 ± 0.01^{gh}	1.18 ± 0.01	2.62 ± 0.03^{d}	7.36 ± 0.32^{f}	0.06 ± 0.001	0.08 ± 0.001^{a}	0.16 ± 0.001^{g}
2		20.69 ± 0.49^{abc}	58.53 ± 1.04^{cd}		2.61 ± 0.07^{cd}	4.33 ± 0.01^{a}		0.08 ± 0.001^{a}	0.10 ± 0.001^{a}
3		20.86 ± 0.66^{abc}	53.86 ± 1.03^{b}		2.57 ± 0.03^{bcd}	4.39 ± 0.01^{a}		0.09 ± 0.001^{b}	0.10 ± 0.001^{a}
4		23.86 ± 0.71^{de}	59.11 ± 0.90^{cd}		2.50 ± 0.03^{b}	6.36 ± 0.06^{e}		0.08 ± 0.001^{a}	0.13 ± 0.001^{f}
5		20.37 ± 0.04^{ab}	55.52 ± 1.77^{b}		2.32 ± 0.02^{a}	5.65 ± 0.01^{d}		0.08 ± 0.001^{a}	0.12 ± 0.001^{cd}
6		19.31 ± 1.41^{a}	64.37 ± 1.44^{e}		2.63 ± 0.01^{d}	6.19 ± 0.05^{e}		0.09 ± 0.001^{b}	0.16 ± 0.001^{g}
7		22.43 ± 1.81^{cd}	59.50 ± 0.17^{d}		2.58 ± 0.06^{bcd}	4.15 ± 0.26^{a}		0.09 ± 0.001^{b}	0.10 ± 0.001^{a}
8		25.36 ± 1.46^{ef}	69.33 ± 2.11^{fg}		2.54 ± 0.04^{bc}	4.35 ± 0.11^{a}		0.10 ± 0.001^{c}	0.11 ± 0.001^{bc}
9		22.33 ± 0.01^{cd}	72.26 ± 1.04^{h}		2.58 ± 0.06^{bcd}	5.26 ± 0.19^{c}		0.10 ± 0.001^{c}	0.13 ± 0.001^{f}
10		22.29 ± 0.01^{bcd}	56.54 ± 1.92^{bc}		2.52 ± 0.04^{b}	4.89 ± 0.11^{b}		0.10 ± 0.001^{c}	0.13 ± 0.001^{ef}
11		19.21 ± 0.06^{a}	49.10 ± 0.21^{a}		2.61 ± 0.01^{cd}	4.12 ± 0.16^{a}		0.10 ± 0.001^{c}	0.11 ± 0.001^{b}
Darkness									
1	0.55 ± 0.06	17.69 ± 0.66^{e}	68.87 ± 1.53^{h}	1.18 ± 0.01	3.12 ± 0.18^{d}	7.87 ± 0.54^{d}	0.06 ± 0.001	0.09 ± 0.001^{c}	0.17 ± 0.001^{e}
2		1.47 ± 0.07^{abc}	2.31 ± 0.06^{a}		1.27 ± 0.01^{a}	1.32 ± 0.09^{a}		0.07 ± 0.001^{a}	0.07 ± 0.001^{a}
3		1.31 ± 0.03^{ab}	2.55 ± 0.03^{a}		1.23 ± 0.06^{a}	1.32 ± 0.12^{a}		0.07 ± 0.001^{a}	0.07 ± 0.001^{a}
4		1.77 ± 0.18^{bc}	54.11 ± 1.52^{g}		1.56 ± 0.16^{c}	6.63 ± 0.84^{c}		0.07 ± 0.001^{a}	0.13 ± 0.001^{d}
5		1.16 ± 0.07^{ab}	7.20 ± 0.33^{e}		1.25 ± 0.01^{a}	1.81 ± 0.05^{b}		0.07 ± 0.001^{a}	0.07 ± 0.001^{a}
6		2.53 ± 0.39^{d}	8.97 ± 1.13^{f}		1.52 ± 0.02^{bc}	2.09 ± 0.13^{b}		0.08 ± 0.001^{b}	0.09 ± 0.001^{c}
7		1.01 ± 0.16^{a}	2.86 ± 0.01^{ab}		1.29 ± 0.03^{a}	1.37 ± 0.03^{a}		0.08 ± 0.001^{b}	0.07 ± 0.001^{a}
8		1.95 ± 0.11^{cd}	3.19 ± 0.16^{abc}		1.31 ± 0.01^{a}	1.54 ± 0.06^{ab}		0.08 ± 0.001^{b}	0.08 ± 0.001^{b}
9		1.62 ± 0.43^{bcd}	5.75 ± 0.54^{de}		1.32 ± 0.04^{a}	1.86 ± 0.32^{b}		0.08 ± 0.001^{b}	0.08 ± 0.001^{b}
10		1.43 ± 0.01^{abc}	4.50 ± 0.56^{bc}		1.30 ± 0.02^{a}	1.64 ± 0.25^{ab}		0.08 ± 0.001^{b}	0.08 ± 0.001^{b}
11		1.71 ± 0.13^{bc}	4.75 ± 0.47^{bc}		1.35 ± 0.08^{ab}	1.62 ± 0.30^{ab}		0.09 ± 0.001^{c}	0.08 ± 0.001^{b}

Mean values ± standard deviation ($n = 2$). For each storage condition (light and darkness), mean values in each column followed by different superscript letters present significant differences ($P < 0.05$) among treatments.

additive antioxidant effect on RE, when they were used to protect sunflower oil against thermal oxidation (60 °C).

Experimental data from the storage stability test were processed by multivariate analysis. A multidimensional diagram of all treatments (oil samples × illumination condition) in relation to PV, CD and OSI values was obtained by principal component analysis (PCA). The resulting score plot (Fig. 2) provided an overview of all treatments and showed a total of 98.1% of the variance. The analysis of the score plot emphasized a separation on the first principal component (PC1) since it explained 82% of the data variability. Oils

Table 5
Radical scavenging capacity ($DPPH^{\bullet}_{r}$) and oxidative stability index from walnut oil during the storage stability test. For clarity, only values at 0, 90 and 180 days are presented. See Table 1 for treatment codes.

Treatment	$DPPH^{\bullet}_{r}$ (%)			Oxidative stability index (h)		
	0	90	180	0	90	180
Light						
1	56.95 ± 0.41^{h}	57.10 ± 0.60^{e}	61.49 ± 0.40^{j}	2.75 ± 0.07^{a}	1.85 ± 0.07^{a}	0.60 ± 0.14^{a}
2	34.18 ± 0.61^{d}	34.11 ± 0.59^{b}	32.86 ± 0.08^{e}	10.90 ± 2.26^{d}	5.20 ± 0.28^{e}	3.10 ± 0.42^{d}
3	13.04 ± 0.27^{b}	11.60 ± 0.37^{a}	10.35 ± 0.40^{a}	16.45 ± 2.62^{e}	8.45 ± 0.07^{g}	4.75 ± 0.07^{e}
4	49.51 ± 1.39^{g}	61.16 ± 0.21^{f}	57.99 ± 0.48^{i}	4.00 ± 0.01^{abc}	2.35 ± 0.07^{ab}	1.25 ± 0.07^{b}
5	47.05 ± 0.07^{f}	59.74 ± 0.25^{f}	56.46 ± 0.01^{h}	3.50 ± 0.01^{ab}	2.50 ± 0.42^{bc}	1.55 ± 0.07^{bc}
6	47.88 ± 1.23^{fg}	57.47 ± 0.89^{e}	52.97 ± 0.16^{g}	5.35 ± 0.07^{bc}	2.90 ± 0.14^{cd}	1.30 ± 0.14^{b}
7	32.4 ± 1.08^{d}	39.43 ± 1.03^{c}	32.13 ± 0.32^{e}	11.90 ± 0.42^{d}	5.30 ± 0.14^{e}	3.35 ± 0.35^{d}
8	25.79 ± 0.65^{c}	37.79 ± 1.75^{c}	29.50 ± 0.16^{c}	12.10 ± 0.57^{d}	5.45 ± 0.07^{e}	3.40 ± 0.01^{d}
9	46.57 ± 0.62^{f}	57.0 ± 0.37^{e}	50.80 ± 0.65^{f}	6.15 ± 0.21^{c}	3.15 ± 0.21^{d}	1.75 ± 0.07^{bc}
10	43.03 ± 0.14^{e}	52.21 ± 0.35^{d}	50.83 ± 0.47^{f}	6.05 ± 0.21^{c}	3.15 ± 0.21^{d}	1.95 ± 0.21^{c}
11	24.87 ± 1.94^{c}	37.97 ± 1.63^{c}	30.61 ± 0.16^{d}	12.90 ± 0.57^{d}	6.65 ± 0.49^{f}	3.15 ± 0.64^{d}
Darkness						
1	57.09 ± 0.47^{g}	57.68 ± 0.07^{d}	63.64 ± 1.04^{i}	3.00 ± 0.28^{a}	1.80 ± 0.28^{a}	0.90 ± 0.14^{a}
2	34.37 ± 0.61^{c}	35.67 ± 2.65^{b}	29.81 ± 0.24^{d}	11.0 ± 0.85^{d}	12.20 ± 1.13^{d}	10.80 ± 0.57^{f}
3	13.23 ± 1.08^{a}	11.49 ± 0.52^{a}	8.54 ± 0.40^{a}	18.25 ± 0.78^{f}	17.90 ± 0.71^{e}	16.75 ± 1.34^{g}
4	49.56 ± 1.00^{f}	60.92 ± 1.10^{e}	58.16 ± 1.70^{h}	3.95 ± 0.49^{a}	3.05 ± 0.35^{b}	1.35 ± 0.49^{ab}
5	46.69 ± 1.58^{e}	58.0 ± 2.71^{de}	52.33 ± 0.23^{f}	3.75 ± 0.07^{a}	3.10 ± 0.28^{b}	2.70 ± 0.42^{bc}
6	49.02 ± 0.08^{f}	56.36 ± 1.10^{d}	47.38 ± 0.16^{e}	5.15 ± 0.07^{b}	4.55 ± 0.07^{c}	3.45 ± 0.35^{cd}
7	26.28 ± 0.69^{b}	38.27 ± 0.62^{b}	28.88 ± 0.81^{cd}	11.85 ± 0.6^{de} 4	11.45 ± 0.49^{d}	8.95 ± 1.91^{e}
8	24.87 ± 1.51^{b}	36.07 ± 1.56^{b}	26.36 ± 0.23^{b}	11.70 ± 0.14^{de}	11.95 ± 1.06^{d}	10.35 ± 0.07^{ef}
9	46.74 ± 0.54^{e}	56.46 ± 0.75^{d}	46.52 ± 0.08^{e}	6.50 ± 0.28^{c}	5.20 ± 0.01^{c}	4.55 ± 0.07^{d}
10	42.41 ± 0.86^{d}	51.26 ± 0.01^{c}	54.41 ± 0.01^{g}	6.25 ± 0.07^{bc}	5.60 ± 0.01^{c}	4.60 ± 0.28^{d}
11	24.26 ± 0.65^{b}	37.21 ± 0.57^{b}	27.90 ± 0.71^{bc}	12.60 ± 0.85^{e}	11.65 ± 0.2^{d} 1	10.0 ± 0.28^{ef}

Mean values ± standard deviation ($n = 2$). For each storage condition (light and darkness), mean values in each column followed by different superscript letters present significant differences ($P < 0.05$) among treatments.

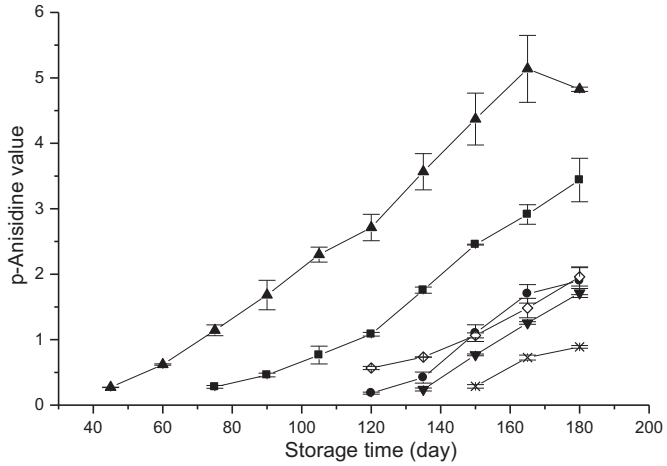

Fig. 1. Kinetic curves of *p*-anisidine values from walnut oil during the storage stability test. Plotted values are means of two independent determinations. Treatment labels: ▲ L1 (control, light); ■ D1 (control, darkness); ◇ L6 (RE 800 μg/g oil, light); ● D4 (AP 100 μg/g oil, darkness); ▼ L4 (AP 100 μg/g oil, light); X L5 (AP 200 μg/g oil, light).

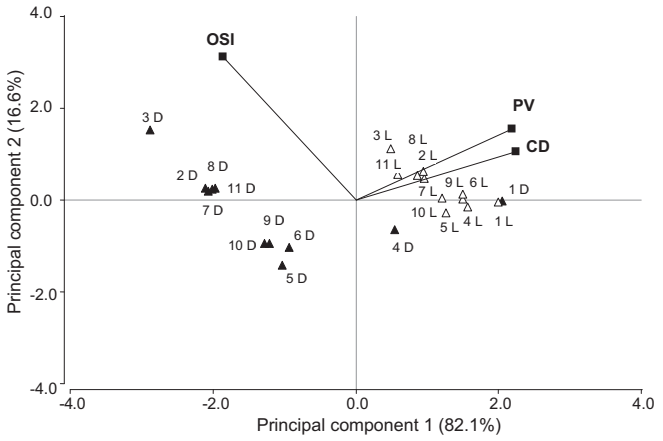

Fig. 2. Score plot of principal components 1 and 2 for chemical data (■) from walnut oil stored in light (△) or in darkness (▲) condition. See Table 1 for treatment codes. OSI, oxidative stability index; PV, peroxide value; CD, conjugated dienes.

stored under light were clustered on the right side of the graph, and were strongly associated with primary oxidation products. Oils kept in the darkness-storage condition were related to higher OSI values. These results strongly stressed the influence of the storage condition on WO oxidative stability, indicating that variations among illumination conditions were greater than differences among antioxidants used.

Table 6
Regression coefficients and determination coefficients (R^2) for peroxide values (PV) from walnut oil during the storage stability test. See Table 1 for treatment codes.

Treatment	Regression coefficients			R^2	*Estimated time (days) for PV = 15 mol equi O_2/kg
	β_0	β_1	β_2		
Light					
1	2.5585	0.0703	0.0018	0.99	60.36^{c}
2	0.0947	0.1853	0.0009	0.99	61.71^{e}
3	0.5184	0.1828	0.0007	0.99	62.54^{f}
4	0.5194	0.1688	0.0009	0.99	61.79^{e}
5	0.3627	0.1491	0.0010	0.99	66.55^{g}
6	3.1484	0.0358	0.0017	0.98	66.50^{g}
7	1.2745	0.1468	0.0011	0.99	61.52^{e}
8	4.1607	0.0306	0.0018	0.96	62.16^{e}
9	1.1173	0.1200	0.0016	0.99	59.01^{b}
10	0.5271	0.1819	0.0008	0.99	61.21^{d}
11	1.4784	0.1767	0.0005	0.99	63.43^{f}
	β_0	β_1	β_2	R^2	Estimated time (days) for PV = 3 mol equi O_2/kg
Darkness					
1	1.1823	−0.0028	0.0022	0.98	25.57^{a}
2	0.4568	0.0084	–	0.90	218.75^{k}
3	0.6620	0.0012	0.0001	0.93	180.60^{j}
4	4.4195	−0.2891	0.0032	0.97	58.85^{b}
5	1.2753	−0.0304	0.0003	0.85	163.89^{h}
6	0.7607	0.0007	0.0002	0.97	92.07^{c}
7	0.6475	0.0017	0.0001	0.96	177.86^{i}
8	0.6693	0.0136	–	0.94	159.00^{g}
9	0.6899	−0.0027	0.0002	0.98	127.48^{d}
10	1.0729	−0.0097	0.0002	0.92	151.51^{f}
11	0.9367	−0.0026	0.0001	0.94	138.74^{e}

Regression equations: $y = \beta_0 + \beta_1 x + \beta_2 x^2$, where '$y$' is the dependent variable (peroxide value); β_0 is a constant that it is equal the value of 'y' when the value of 'x' = 0; β_1 is the coefficient of 'x'; β_2 is the coefficient of 'x^2'; 'x' is the independent variable (time).
*For each storage condition (light and darkness), the values followed by different superscript letters present significant differences ($P < 0.05$) among treatments.

4. Conclusions

This work was primarily aimed to prove the effectiveness of some antioxidants on WO oxidative stability under conditions (fluorescent light, room temperature) normally used for sale in retail markets. At the concentrations used neither RE nor synthetic antioxidants (AP, TBHQ) contributed markedly on inhibiting photo-oxidative degradation, resulting in significantly increased amounts of primary and secondary oxidation products in oils exposed to light.

Under darkness-storage condition, the addition of the mentioned antioxidants significantly reduced lipid oxidation, improving oil shelf life considerably. At the end of the storage period, oils added with RE – alone or in combination with TBHQ or TBHQ plus AP – did not reach the end point for rancidity or acceptability limit for virgin and cold-pressed vegetable oils, and did not develop secondary oxidation products.

The results mentioned previously claim data published elsewhere showing the extremely low stability of WO against photo-oxidation. On the other hand, it was also showed that WO stored in darkness, without addition of any antioxidant, oxidized at similar rate than that stored under light. This means that WO is also highly susceptible to free-radical oxidation (autoxidation) even under relatively mild temperature conditions. The RE (800 μg/g oil) combined with AP (200 μg/g oil) or AP plus TBHQ (at a minimal dose, 100 μg/g oil), seems to be effective to protect the oil from autoxidation. However, this fact may be dependent on the darkness-storage condition. Aluminum-coated glass bottles are suitable containers to reach such condition.

In summary, the oil storage in containers with light–barrier properties, together with the addition of the evaluated antioxidants, could maintain WO quality at least up to six months under room temperature conditions. In order to extend shelf life, future studies may be focused on evaluating the effect of inhibitors of photosensitized oxidation, such as α-tocopherol and carotenoids, as well as their combination with the antioxidants examined in the present study.

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