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ORIGINAL ARTICLE

Antioxidant Activity Displayed by Phenolic Compounds Obtained from Walnut Oil Cake used for Walnut Oil Preservation

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Abstract The objective of this work was to evaluate the performance of various antioxidants (ethyl acetate-soluble polyphenols: PAE; water-soluble polyphenols: PH; and butylated hydroxytoluene [BHT]) on the preservation of walnut oil (W) during storage. An oven test was conducted on the walnut oil samples: without antioxidants (control: WC) and with antioxidants: PAE (WPAE), PH (WPH), and BHT (WBHT) during a 16-day storage at 60 °C temperature. Chemical parameters related to deterioration were analyzed during storage: peroxide value (PV), conjugated dienes (CD) and conjugated trienes (CT), and carotenoid content (CC). Volatile compounds were also analyzed. PV, CD, CT, and volatile compounds related to oxidation of lipids such as; nonanal, hexanal, butanal, and (E)-2-heptenal increased in all samples during storage. The increase of these variables was greater in WC and lower in WPAE and WPH. WPAE, WPH, and WBHT displayed a better preservation of volatile compounds that influence the walnut-characteristic flavor like D-limonene, and a lower increase of aldehydes and furans. The CC exhibited a decrease in all samples during storage. WPAE presented the greatest CC value along the storage and was followed by WPH. Antioxidants helped to preserve walnut oil against deterioration processes. PAE and PH displayed good antioxidant activity and in some cases showed a better performance for quality preservation of walnut oil compared to the BHT antioxidant.

Keywords Walnut oil · Polyphenol · Antioxidants · Preservation

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Introduction

Walnut kernels typically contain 52% to 72% oil, depending on the cultivar, geographical location, and irrigation rate (Martínez et al., 2006; Slatnar et al., 2015). This oil is extracted on a small scale for use in food as a flavoring agent, mainly as a salad dressing and in bakery products. It also finds use in the cosmetic industry, as a component of dry skin creams, antiwrinkle, and antiaging products (Martínez et al., 2011).

Like most nuts, walnuts are valuable sources of polyunsaturated fatty acids (PUFA; 90% of the oil), predominantly linoleic (47.4%) and α-linolenic acids (15.8%) (Slatnar et al., 2015). Because of its composition, walnut oil consumption reduces serum cholesterol levels in humans, by lowering the low-density lipoprotein, increasing the high-density lipoprotein and decreasing the total triacylglycerol levels (Liang et al., 2013). PUFA have been associated with health-promoting properties, which are thought to improve lifestyle-related diseases, such as decreasing the risk of cardiovascular disease (Estruch et al., 2013). However, these fatty acids limit the shelf life of walnuts and

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walnut-containing products due to their high susceptibility to oxidation. The lipid oxidation of walnut-containing products during storage affects their quality parameters, by decreasing their nutritional, sensory, and chemical properties, in addition to their economic value.

Besides oil, walnuts also contain significant amounts of crude protein (15.17–19.24%), carbohydrates (8.05–13.23%), and other bioactive compounds, such as plant sterols, dietary fiber, and polyphenols that may exert health benefits (Regueiro et al., 2014). For instance, phenolic compounds are commonly associated with antioxidant, antiatherogenic, anti-inflammatory, and antimutagenic properties. High content of walnut phenolics has been measured in the skin that covers the kernel. This polyphenol content is greatly influenced by factors, such as temperature, precipitation, natural causes (infection, damage, and pests), fruit maturity stage, and storage (Slatnar et al., 2015).

The addition of antioxidants to lipid-rich foods is a simple way to reduce lipid-oxidative reactions. Synthetic antioxidants, such as butylated hydroxytoluene (BHT), are widely used in food because of their efficacy and low cost. However, the safety of synthetic antioxidants has been questioned (Hudson, 1990). For this reason, natural antioxidants are more accepted by consumers, because they are perceived as safe products. Many natural antioxidants have been proven to be effective in many lipid-rich foods. Nevertheless, the food industry has been intensely addressing the necessities of food-allergic consumers, not only concerning the proper food labeling but also seeking ways to minimize allergen cross-contamination among foodstuffs (López-Calleja et al., 2015). Consequently, the use of antioxidants that have the same origin as the food in which they are going to be applied is a great advantage for the industry.

Walnut oil is highly susceptible to both thermal and photooxidative degradation. Considering that walnut phenolics could display antioxidant and free radical-scavenging activities, the use of walnut polyphenols could enhance walnut oil stability, increase its shelf life, and help avoid the use of synthetic antioxidants. Therefore, the objectives of this study were to characterize the antioxidant properties of walnut polyphenols and to evaluate their antioxidant effect on walnut oil, considering the changes in chemical and volatile indicators of lipid oxidation during storage.

Materials and Methods

Materials

Unshelled walnuts (variety Chandler) and cold press-extracted walnut oil "VitaNuss," both from a 2016 harvest, were provided by Nogales SRL, Huaco, La Rioja, Argentina.

Methods

Phenolic Fraction Extraction

Walnuts were defatted using n-hexane for 10 h in a Soxhlet apparatus. Then, polyphenols were extracted from the defatted walnuts using a magnetic stirrer with ethanol-water (70:30 v/v) solution as according to (Larrauri et al., 2016b). This action was performed at room temperature (23 ± 1 °C) for 24 h. Then, the extract obtained was filtered through a cellulose filter and the solvent was removed using a rotary evaporator. The concentrated extract was further purified by solvent partitioning using distilled water and ethyl acetate. First, the concentrated extract was dissolved in distilled water. Ethyl acetate was added in this solution and then was poured in a separation funnel to separate both phases. Afterward, the ethyl acetate and water phases were separated in different containers. Ethyl acetate solvent was chosen because phenolic compounds are known to have good solubility in it (Nepote et al., 2004; Larrauri et al., 2016b). Afterward, the solvent was removed under reduced pressure using a rotary evaporator at 40 °C temperature (Larrauri et al., 2016b). The obtained extract (ethyl acetate-soluble polyphenols: PAE) was stored at −18 °C. In addition, the fraction soluble in distilled water was lyophilized to obtain a dried extract. The obtained extracts (water-soluble polyphenols: PH) were also stored at −18 °C.

Total Phenolic and Flavonoid Content

Total phenolic compounds in both extracts were determined using the Folin–Ciocalteu method as Larrauri et al. (2016b). The sample absorbance was measured using a spectrophotometer (Spectrum SP-2100, Zhejiang, China) at 760 nm. Gallic acid (GAE) was used as standard for plotting a calibration curve. The total phenolic content was expressed as mg GAE g^{-1} sample. The total flavonoid content in both extracts (PAE and PH) was determined using the $AlCl_3$ method (Luximon-Ramma et al., 2005). Absorbance was measured at 367 nm. The calibration curve was prepared using quercetin (QE). The results were expressed in mg QE g^{-1} sample.

DPPH Radical-Scavenging Activity

The radical-scavenging activity of both fractions was determined using diphenyl picryl hydrazyl radicals (DPPH). First, DPPH solution was prepared using ethanol (20 ug mL^{-1}). Then, different aliquots of the samples on ethanol were added to 950 uL DPPH solution. The absorbance of

samples was measured at 517 nm. The radical-scavenging activity was calculated using the following formula: % RSA = [1 − (absorbance of DPPH and sample − absorbance of sample)/absorbance of DPPH] × 100. The inhibitory concentration 50% (IC50) was calculated using the equation obtained from the curve formed by plotting inhibition percentage vs. final extracts concentrations (Quiroga et al., 2011).

Chelating Activity on Ferrous Ions (Fe^{2+})

The Fe^{2+}-chelating activity of extracts and fractions was determined according to Yamaguchi et al. (2000). Samples and $FeSO_4$ solutions were prepared and measured at 522 nm. Ethylene diamine tetraacetate (EDTA) (Biopack, Buenos Aires, Argentina) was used as positive control. The ability to chelate the ferrous iron was reported as EDTA equivalents.

ABTS Assay

A modified method was used, according to Asensio et al. (2011). The ABTS reagent (0.0384 g) and potassium persulfate (0.0066 g) were diluted in 10 mL of ethanol (absorbance 0.7 ± 0.02). Then, 990 uL of this solution were added to 10 uL of extracts (in ethanol), and the standard Trolox reagent. The Trolox concentration (mg g^{-1}) of polyphenol extracts (PAE and PH) was calculated using the absorbance inhibition of the radical cation at 734 nm. An equation was obtained using a Trolox calibration curve.

Accelerated Oxidation Test in Walnut Oil

The antioxidant activity of walnut extracts (PH and PAE) and BHT on walnut oil was determined using an accelerated oxidation test. Walnut polyphenols were added to the walnut oil. After that, the walnut oil samples were sonicated at 20 kHz for 10 min using a QSonica Q500 sonicator. The concentration of walnut extracts was 0.1% w/w for PAE (WPAE) and PH (WPH), which was the same as the peanut polyphenol concentration applied by Larrauri et al. (2013) in salami. The BHT was added at 0.02% (w/w) (WBHT). Also, a sample of walnut oil without additives (control sample: WC) was prepared. The samples were stored in test tubes at 60 ± 1 °C (Quiroga et al., 2011) for a 16-day storage period. The experimental design consisted of 4 treatments (WC, WPAE, WPH, and WBHT) × 3 replicates × 5 periods of time. The variables considered for the data analysis were chemical oxidation parameters (peroxide values [PV], carotenoid content [CC], conjugated dienes [CD], and conjugated trienes [CT]) and volatile compounds that changed during storage (hexanal, nonanal, butanal, 2-heptenal (E), 1,2,3-trimethyl benzene, 1,3,5-trimethyl benzene, 2-propyl furan, and D-limonene).

Chemical Analysis: PV, CD, CT, and CC

PV was measured according to AOAC (2010). CD and trienes were measured at 232 and 268 nm, respectively (COI, 2001). CC were analyzed at 470 nm following the procedures reported by Asensio et al. (2011). The concentration of carotenoids was expressed using the following equation: Carotenoids (mg kg^{-1}) = ($Abs_{470} \times 10^6$)/(2000 × 100 × density).

Volatile Analysis

Solid phase microextraction fiber (divinylbenzene/carboxen/polydimethyl-siloxane, DVB/CAR/PDMS 50/30 μm, Stable Flex, 1-cm long, Supelco) was used to capture volatile compounds according Larrauri et al. (2016a). Walnut oil samples were weighed (2 g) and placed in vials at 70 °C for 20 min. The fiber was exposed to the glass flask's (5 mL) headspace for 10 min and then injected into a Gas Chromatograph (Perkin Elmer Clarus 600) coupled with a mass detector. An ELITE 5MS (30 * 0.25 mm i.d., 0.25 mm film thickness; Perkin Elmer) column was used. The column temperature program was: 40 °C initial temperature, 10 °C min^{-1} for rate 1 until 100 °C, 15 °C min^{-1} for rate 2 until 250 °C (2 min hold). The injector temperature was 250 °C. Helium was used as a carrier gas at 0.9 mL min^{-1} flow. Ionization was performed by electron impact at 70 eV. Mass spectral data were obtained in the full scan mode (m/z range 40–550). The identification of compounds was performed via a combination of the National Institute of Standards and Technology (NIST) mass spectral library and gas chromatographic retention times of standard compounds. When standards were not available, volatile compounds were tentatively identified using the gas chromatography-mass spectrometry (CG-MS) spectra only. Chromatographic responses of detected volatile compounds (peak area electronic counts: e.c.) were monitored for comparison of each compound between samples.

Statistical Analysis

Three replications were made for the experiment. The data obtained were analyzed using the InfoStat software, version 2016p (Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba). A two-way analysis of variance (factors: "treatment" and "time") was performed and DGC was used to detect significant differences between mean values (ANOVA, $\alpha = 0.05$). Principal component analysis (PCA) was performed on the correlation matrix of standardized data to explore associations between treatments and variables. Pearson coefficients were calculated to confirm correlations between dependent variables.

Table 1 Means and standard errors of total phenolic and flavonoid contents, ABTS, ferrous ion chelating activity, and IC50 of DPPH from walnut polyphenol extracts

Sample[a]	Total phenolic content (mg GAE g^{-1} sample)[a,b]	Total flavonoids content (mg QE g^{-1} sample)[a,b]	ABTS (mg trolox g^{-1} sample)[a,b]	Chelating activity on ferrous ions (Fe^{2+}) (mg EDTA g^{-1} sample)[a,b]	IC50 (g mL^{-1})[a] DPPH radical scavenging activity[a,b]
PH	41.39 ± 1.89a	20.00 ± 0.12a	1136.96 ± 1.17b	11.59 ± 0.19a	2.13 ± 0.04a
PAE	230.45 ± 2.04b	49.85 ± 1.27b	1105.00 ± 6.93a	70.29 ± 0.56b	2.57 ± 0.05b

[a] PH = walnut water-soluble polyphenols; PAE = walnut ethyl acetate-soluble polyphenols; GAE = gallic acid equivalent g^{-1} extract; QE = quercetin equivalent g^{-1} extract; DPPH = 2,2-diphenyl-1-picrylhydrazyl; and IC50 = scavenging/chelating activity required to inhibit 50% radical/ion.

[b] Different letters in the same column are significantly different. ANOVA, DGC ($\alpha = 0.05$; $n = 3$).

Results and Discussion

Total Phenolic and Flavonoid Determinations

Phenolic compounds are naturally present in walnut oils and are the main compounds responsible for the stability of the oil during storage (Fu et al., 2016). The PAE extract presented a higher phenolic content (230.45 mg GAE g^{-1} sample) compared to the PH extract (41.40 mg GAE g^{-1} sample) (Table 1). Kornsteiner et al. (2006) detected a 1625 mg GAE per 100 g of phenolics in fresh walnuts. In addition, Gharibzahedi et al. (2013) found different values of total phenolics in walnut oil, according to the oil extraction method. The highest values were obtained in oil extracted using modified Bligh–Dyer (MBD method) (0.22 mg GAE g^{-1}), whereas the maceration method (Mac) provided the lowest phenolic content (0.17 mg GAE g^{-1}).

Flavonoids are valuable in the treatment of many diseases owing to their potent antioxidant properties. In addition to their higher phenolic content, PAE samples also displayed a higher flavonoid content (49.85 mg QE g^{-1} sample) than PH (20.00 mg QE g^{-1} sample). Fu et al. (2016) observed that the flavonoid content in walnuts was subjected to intermittent oven drying, direct oven drying, and sun drying, which were 1.07, 0.87, and 0.45 g QE kg^{-1} fresh walnuts, respectively.

Due to their chemical structure, polyphenols are soluble in ethyl acetate. For that reason, the ethyl acetate fractions (PAE) showed a greater proportion of total phenolics and flavonoids than the other extract studied (PH), which could explain a higher antioxidant activity in the ethyl acetate fractions.

DPPH Radical-Scavenging Activity

The DPPH assay measures the ability of the antioxidants present in the oil, kernels, and pellets to scavenge free radicals (Slatnar et al., 2015). The samples were able to reduce the stable violet DPPH radical to the yellow DPPH-H, exhibiting 50% inhibition (IC50) values of 2.57 for PAE and 2.13 for the PH fraction (Table 1). A lower IC50 value indicates a higher antioxidant activity. Therefore, the PH fraction was considered to be the more efficient radical scavenger of the samples. This result contrasts with Larrauri et al. (2016b), who found that the ethyl acetate extract of peanut skins exerted greater activities toward DPPH radicals than the aqueous fraction.

ABTS Assay

Compared to the DPPH assay, the ABTS method can provide a more accurate estimation for hydrophilic and lipophilic compounds, as well as those with high pigmentation. According to the ABTS results, the PH fraction showed the highest antioxidant activity, followed by the PAE fraction (1136.96 and 1105.00 mg Trolox g^{-1} sample, respectively) (Table 1). Among the previous works that have also evaluated the ABTS antioxidant capacity of walnut polyphenols, Regueiro et al. (2014) reported 25.7 mmol Trolox per 100 g for an ethyl acetate extract of walnut polyphenols, measured using a different method to the one used in the current work.

Ferrous Ion (Fe^{2+})-Chelating Activity

Metal-chelating capacity is significant as it reduces the concentration of catalyzing transition metals in lipid peroxidation. The highest Fe^{2+}-chelating activity was observed for the PAE extract (70.29 mg EDTA g^{-1} sample), while the PH activity only represented 11.59 mg EDTA g^{-1} sample (Table 1). Larrauri et al. (2016b) also found that the ethyl acetate fractions of polyphenols from peanut skins showed more effective Fe^{2+}-chelating activities than aqueous fractions. According to the results, PAE extracts are expected to have protective effects against the peroxidation process.

Accelerated Oxidation Test in Walnut Oil

Chemical Analysis: PV, CD, CT, and CC

The PV represents a classical method for quantification of oxidative rancidity in walnuts and other types of nuts.

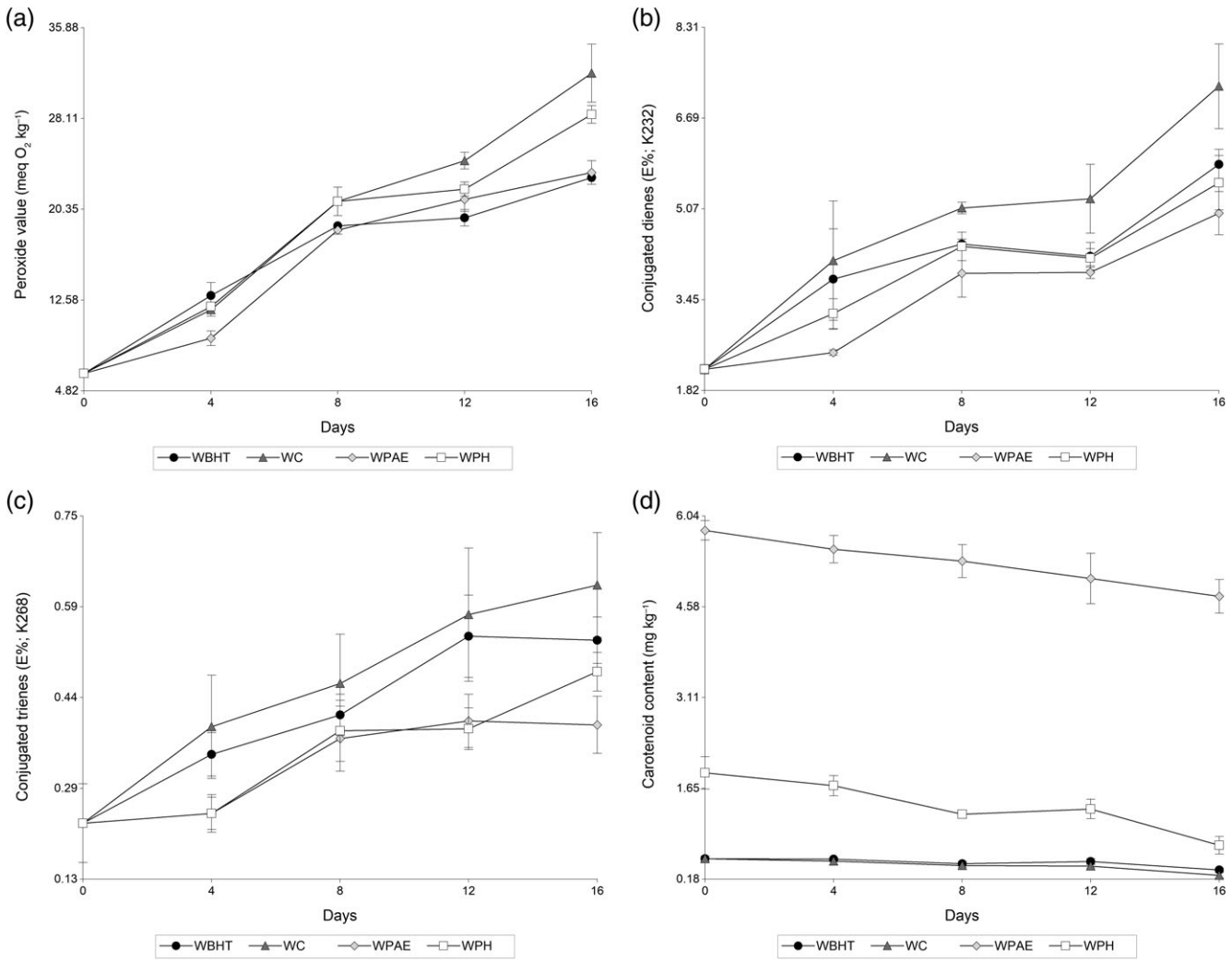

Fig. 1 (a) Peroxide value (meq O_2 kg^{-1}), (b) conjugated dienes (E1%, K232), (c) conjugated trienes (E1%, K268), and (d) carotenoid content (mg kg^{-1}) in walnut oil without antioxidants (WC, control sample), and with the addition of walnut water-soluble polyphenol fraction (WPH), ethyl acetate-soluble polyphenol fraction (WPAE) and, BHT (WBHT); analyzed during a 16-day storage period at 60 °C temperature ($n = 3$)

According to CODEX-STAN 210–1999, the acceptability limit for vegetable cold-pressed oils corresponds to a PV of 15 meqO_2 kg^{-1} oil. In this study, the PV increased for all walnut oil samples during the storage period (Fig. 1).

On day 0, the mean PV (Fig. 1a) of the treatments was 6.30 meqO_2 kg^{-1} walnut oil, with no significant differences between treatments ($P < 0.05$). At the next evaluation (day 4), the PV begun to rise for all treatments. During this period, WPAE presented a lower PV value (9.32 meqO_2 kg^{-1} walnut oil) compared to the rest of the treatments: WC, WBHT, and WPH (11.76, 12.97, and 12.03 meqO_2 kg^{-1} walnut oil, respectively). On the last day of measurement (day 16), the treatments with the lowest PV were WPAE and WBHT (23.49 and 23.07 meqO_2 kg^{-1} walnut oil, respectively) with no significant differences between them ($P < 0.05$) followed by WPH (28.46 meqO_2 kg^{-1} walnut oil). In a study conducted by Martínez et al. (2013), a protective effect among various commercial antioxidants, such as tert-butylhydroquinone, ascorbyl palmitate, and rosemary extract, was also found against the development of PV on walnut oil during storage. Another study performed on walnut oil stated that as the storage time increased a linear increase in PV was registered for walnut oil of Franquette variety, along with an intensified oxidized flavor (Martínez et al., 2011).

The specific extinction coefficients at wavelengths 232 and 268 nm, namely K_{232} and K_{268}, are sensitive markers of lipid oil deterioration. While K_{232} is an indicator of primary oxidation products (CD), K_{268} reflects the concentrations of secondary oxidation products, mainly CT and carbonyl compounds (Ayadi et al., 2009). Regarding CD (Fig. 1b), all treatments presented an initial (day 0) mean K_{232} of 2.20, without significant differences. On day 4, WPAE and WPH (K_{232} 2.50 and 3.20, respectively)

recorded the lowest CD values while WBHT and WC displayed higher values (K_{232} 3.81 and 4.14, respectively). On the last day of storage (day 16), the control sample (WC) contained the most CD (K_{232} 7.26). In comparison, the remaining samples had less CD, with no significant differences among them (WBHT: K_{232} 5.86; WPH: K_{232} 5.53 and WPAE: K_{232} 4.99). The data evidenced that WC was the most oxidized, among the samples, at the end of the storage. Similarly, Martínez et al. (2013) also found that CD increased during storage on walnut oil but walnut oil with antioxidants had a lower increase than the control sample.

In the case of CT (Fig. 1c), on day 0, the treatments exhibited a K_{268} of 0.23, with no significant differences ($P < 0.05$). The WPH and WPAE treatments displayed the lowest values during storage, in contrast to WC that displayed the highest. The WBHT was located between them. At the final measurement (day 16), the WPAE treatment and WC exhibited the lowest value (K_{268} 0.39) and highest value (K_{268} 0.63), respectively. Ayadi, Grati-Kamoun, & Attia (2009) noted that olive oil with natural antioxidants, such as rosemary and thyme, exhibited a significant low K_{268} relative to the control, throughout the storage period.

The antioxidant property of the plant material is due to the presence of many active phytochemicals. Carotenoids are phytochemicals that can play an important role in the oxidative stability of walnuts, considering that the leading cause of carotenoid loss is oxidation (Dias et al., 2014). The CC of the treatments diminished throughout storage (Fig. 1d). WPAE treatment presented the highest initial CC reading (5.81 mg kg^{-1}) on day 0, followed by WPH (1.90 mg kg^{-1}). In both samples, the CC remained superior for the entire storage duration of the experiment, but this content presented a greater decrease until 4.75 and 0.73 mg kg^{-1} on day 16 (end of storage) in WPAE and WPH, respectively. Regarding samples WC and WBHT, the CC through the experiment decreased but without significant differences between them starting in 0.51 and, decreasing until 0.24 and 0.33, respectively. In an earlier work, the presence of β-carotene at concentrations between 5 and 20 mg g^{-1} oil exerted a protective effect against oxidative damage to soybean oil induced by light (Warner and Frankel, 1987). Given this result, it can be assumed that the CC reading of WPAE on day 0 has a sufficient quantity of bioactive compounds to protect against oxidative degradation. The reason for the largest readings of CC found in the WPAE and WPH samples could be due to the presence of other bioactive compound present in the polyphenol extracts. Slatnar et al. (2015) reported that hydrolyzable tannins are the major phenolic compounds determined in extracts of walnut kernels (60.8%), followed by flavanols (24.7%). These dark-colored and yellow compounds, respectively, can influence the color of walnut kernels and the products into which they are incorporated. Gomez-Caravaca et al. (2008) noticed that a higher content of glansreginin A in Fernette variety could be the reason for its darker kernels compared to Franquette. To summarize the observed results in CC, the walnut polyphenols (PH and PAE) added to the walnut oil were read in the 470 nm range, which is the same used for measuring CC. This explains the higher reading detected in WPAE and WPH samples. The greater decrease in both samples during storage can be due to the oxidation process that affects primarily the walnut polyphenols that are acting as natural antioxidants. For that reason, walnut oil samples with walnut polyphenols evidenced a good antioxidant activity during storage.

GC–MS Volatile Compound Analysis

When lipid oxidation occurs, the chemical composition, quality, and safety of a food product change. Oxidation of PUFA generates aldehydes, ketones, alcohols, acids, and other carbonyl compounds. According to Elmore et al. (2005), the volatile compound fraction in walnuts is mainly represented by hydrocarbons, low-molecular-weight alcohols, saturated, and nonsaturated aldehydes and furans. In that investigation, hexanal was the predominant volatile compound in walnut samples, followed by 1-pentanol, pentanal, 1-hexanol, and 1-penten-3-ol. Most of these compounds are mainly formed from the oxidation of linoleic acid. It is also known that the levels of hexanal increase as walnut sensory quality deteriorates.

In the present study, diverse volatile compounds were found initially (day 0) in the walnut oil samples (Table 2). The quantities of many of these volatile compounds changed as storage progressed (Fig. 2), while others remained the same. Nonanal, hexanal, butanal, and (*E*)-2-heptenal evidenced some of the most pronounced changes and all these compounds increased during the storage period, in all treatments.

The presence of hexanal and nonanal is directly related to the development of rancid flavors in lipid-rich foods (Larrauri et al., 2016a). Hexanal contents (Fig. 2a) were comparable among the treatments on day 0, with a mean value of 162,690 e.c. From storage day 4, the hexanal content of WC dominated over the rest of the treatments. At the last measurement (day 16), the hexanal content was the highest in WC (645,554 e.c.) compared to WBHT (228,947 e.c.), WPAE (218,655 e.c), and WPH (204,145 e.c.), which had no significant differences with each other ($P < 0.05$). Based on the mean values for the entire 16-day storage, WC presented the highest nonanal content (Fig. 2b) (32,788 e.c.), and the remaining treatments recorded the lowest amounts (WBHT 19308, WPAE 14864 e.c., and WPH 13352). The distinction among the treatments

Table 2 Volatile compounds (electronic counts, 10^3) per gram of walnut oil without antioxidants (WC) and with BHT (WBHT), walnut water-soluble polyphenols (WPH), and walnut ethyl acetate-soluble polyphenols (PAE), analyzed on day 0 of the storage period

Volatile compound	Day 0				Day 16			
	WC[a]	WBHT[a]	WPH[a]	WPAE[a]	WC[a]	WBHT[a]	WPH[a]	WPAE[a]
Alcohols								
Cyclopropyl carbinol	ND	10.01 ± 1.83b	7.56 ± 0.66b	ND	14.65 ± 1.55a	15.84 ± 0.48a	ND	ND
2-Octen-1-ol, (Z)-	ND	ND	ND	ND	ND	8.68 ± 0.57a	ND	ND
Cyclobutanol	38.05 ± 1.35ab	44.72 ± 3.29ab	34.72 ± 7.62b	47.89 ± 4.83a	ND	ND	ND	ND
Aldehydes								
Butanal	15.32 ± 0.87d	13.84 ± 0.73d	13.65 ± 2.02d	14.65 ± 1.19d	86.97 ± 4.27a	54.67 ± 1.44b	48.04 ± 1.16c	43.69 ± 2.55c
Pentanal	ND	ND	ND	ND	15.54 ± 1.81a	ND	10.78 ± 0.52b	9.61 ± 0.87b
Heptanal	ND	ND	ND	ND	24.64 ± 0.37a	ND	ND	ND
Octanal	ND	ND	ND	ND	17.56 ± 0.78	ND	ND	ND
Hexanal	168.52 ± 33.57b	181.86 ± 17.33b	155.19 ± 23.19b	145.19 ± 18.15b	645.55 ± 64.85a	228.95 ± 15.74b	204.15 ± 32.51b	218.66 ± 10.99b
2,4-Octadienal, (E,E)-	10.89 ± 1.70de	8.85 ± 0.59e	12.22 ± 1.21de	10.72 ± 1.75de	52.88 ± 0.13a	15.77 ± 3.02 cd	41.54 ± 1.66c	48.68 ± 2.65b
2-Heptenal, (E)-	8.61 ± 0.17c	9.28 ± 0.48c	6.96 ± 0.81c	8.30 ± 0.67c	133.41 ± 12.72a	101.30 ± 2.92b	95.03 ± 4.77b	90.71 ± 0.91b
Nonanal	10.07 ± 1.31de	8.41 ± 1.15e	8.68 ± 1.10e	9.07 ± 0.90e	32.79 ± 1.13a	19.31 ± 2.50b	13.35 ± 1.32 cd	14.86 ± 0.72c
Butanal, 3-methyl-	5.67 ± 0.44	ND	ND	ND	50.12 ± 1.09a	ND	40.96 ± 5.16b	30.54 ± 2.39c
Decanal	6.58 ± 2.33	ND	ND	ND	26.66 ± 0.90	ND	ND	ND
Aromatic hydrocarbons								
Benzene, 1,2,3-trimethyl-	ND	ND	ND	6.63 ± 0.81a	ND	ND	ND	6.10 ± 0.24a
Benzene, 1,3,5-trimethyl-	ND	ND	ND	34.70 ± 0.52a	ND	ND	ND	46.85 ± 3.10a
Organic acids								
Oxalic acid	119.22 ± 11.56a	131.23 ± 2.30a	95.88 ± 8.45b	132.42 ± 2.29a	ND	ND	37.26 ± 1.06c	ND
Furans								
Furan, 2-pentyl-	16.33 ± 1.85	ND	ND	ND	26.51 ± 1.26a	14.96 ± 0.78b	ND	14.90 ± 0.21b
Furan, 2-propyl-	1.36 ± 0.27c	1.82 ± 0.13c	1.59 ± 0.21c	1.68 ± 0.13c	47.83 ± 1.75a	45.51 ± 2.14ab	48.25 ± 1.32a	41.64 ± 1.61b
Ketones								
3-Hexanone, 2,5-dimethyl-	14.56 ± 1.00a	15.06 ± 0.99a	13.89 ± 1.54a	12.79 ± 0.60a	8.30 ± 0.26b	ND	ND	ND
3,5-Octadien-2-one	10.63 ± 1.14c	11.63 ± 1.29c	9.97 ± 1.47c	11.63 ± 1.29c	24.42 ± 0.45b	36.49 ± 8.27a	8.12 ± 0.93	ND
Monoterpenes								
D-Limonene	85.76 ± 3.71a	92.43 ± 8.72a	79.09 ± 4.33a	75.76 ± 6.33a	ND	13.73 ± 1.15b	14.73 ± 0.80b	15.57 ± 1.80b
α-Sabinene	1.574 ± 1.83a	14.48 ± 1.03a	15.79 ± 0.42a	16.81 ± 0.84a	ND	ND	ND	ND
Amines and amides								
1-Nonanamine, N,N-dimethyl-	ND	30.12 ± 2.03a	ND	ND	ND	ND	ND	ND
2-Propen-1-amine, 2-bromo-N-methyl-	16.16 ± 1.61a	14.49 ± 0.26a	14.49 ± 0.83a	16.18 ± 1.07a	21.47 ± 10.03a	ND	ND	10.26 ± 0.85a
Others								
Butylated Hydroxytoluene	ND	100.70 ± 4.88a	ND	ND	ND	29.70 ± 0.55b	ND	ND

ND = not detected.

[a] Means ± standard error followed by different letters in each row indicate significant differences between treatments for each volatile compound ($\alpha = 0.05$; $n = 3$).

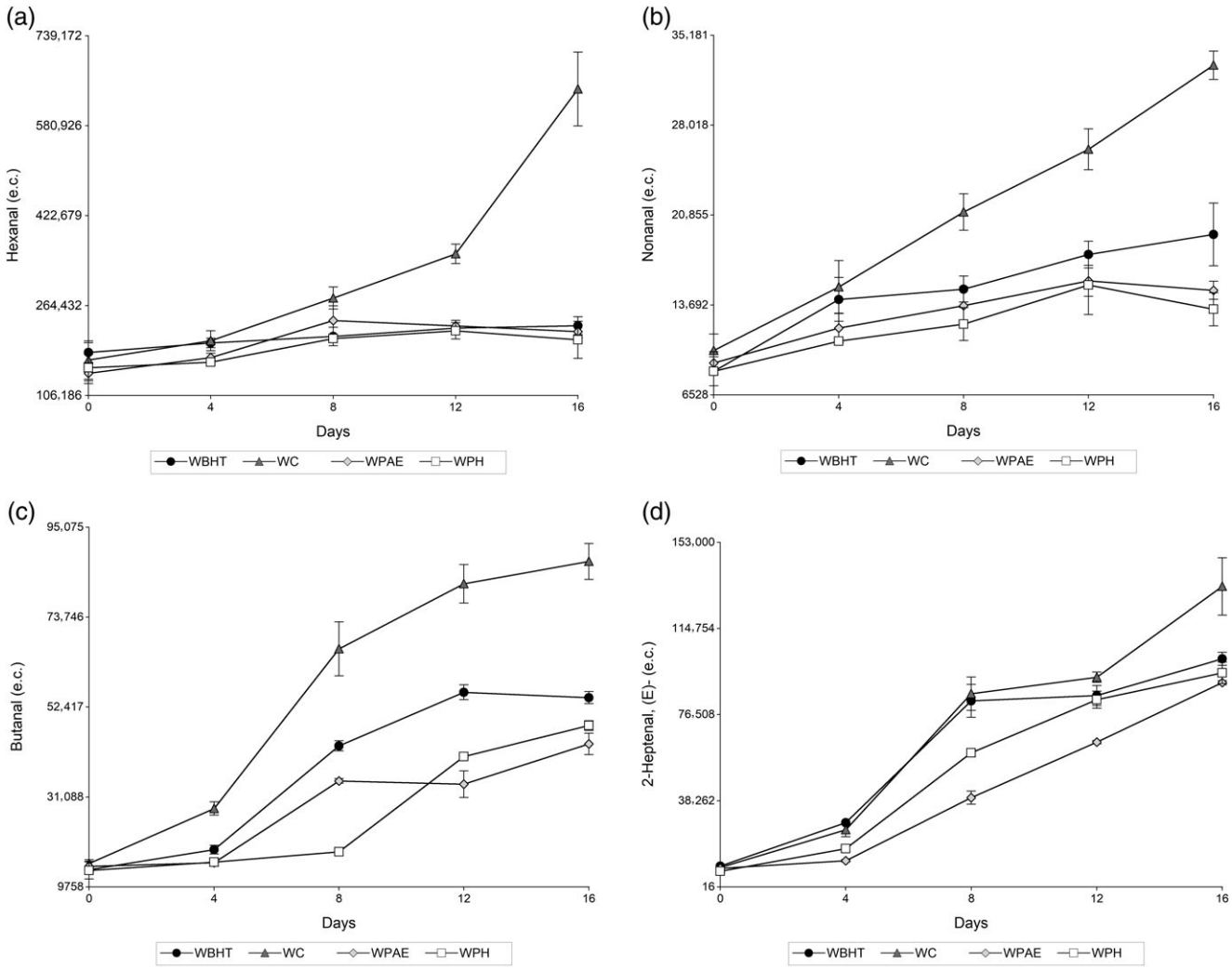

Fig. 2 (a) Hexanal, (b) nonanal, (c) butanal, and (d) 2-heptenal, (E)-measured in electronic counts in walnut oil without antioxidants (WC, control sample), and with the addition of walnut water-soluble polyphenol fraction (WPH) and ethyl acetate-soluble polyphenol fraction (WPAE), and BHT (WBHT) analyzed during a 16-day storage period at 60 °C temperature ($n = 3$)

occurred on day 8, with WC recording the highest value and this tendency continued throughout the storage.

For both lipid oxidation indicators (nonanal and hexanal), the control sample accumulated higher amounts, while the treatments with antioxidants (WBHT, WPAE, and WPH) displayed the lowest. In corroboration with this trend, Larrauri et al. (2016a) obtained a lower increase of nonanal and hexanal content in almonds with a BHT-containing solution compared to the control, considering 126-day storage at 40 °C.

For butanal, the mean values of the measurements that were conducted throughout the entire duration for all treatments decreased in the following order (Fig. 2c): WC (55,713 e.c.) > WBHT (37,254 e.c.) > WPAE (28,576 e.c.) and WPH (27,221 e.c), without significant differences. On day 0, no significant differences were observed among the samples (14,366 e.c.). However, from day 8, the control (WC) showed a higher butanal content (66,232 e.c.). This trend was maintained, even at the end of storage (16 day) showing the following order: WC (86,970 e.c.), followed by WBHT (54,667 e.c.), and finally WPAE (48,043 e.c) and WPH (43,694 e.c.) without significant differences ($P < 0.05$). Butanal is an aldehyde that has been found in walnuts in other studies (Elmore et al., 2005; Martínez et al., 2006). This compound is derived from the oxidation of linoleic acid (Frankel, 2005), which could explain its increasing prevalence in all the samples during the storage, but mainly in WC.

2-Heptenal is considered one of the main volatile compounds formed by oxidation of unsaturated fatty acids. In the present study, the initial (day 0) mean value of (*E*)-2-heptenal (Fig. 2d) was 8284 e.c., which was statistically

similar among the samples. On day 4, WC and WBHT could be distinguished by higher values. Nevertheless, on the last day, only the control treatment, which presented the highest value (133,414 e.c.), was significantly different to the rest of the treatments (WBHT 101301, WPH 95033, and WPAE 90714 e.c.). These data further confirmed that WC was the treatment most affected by oxidation processes. Frankel (2005) describes the importance of 2-heptenal, as one of the main components mainly responsible for the oxidized flavor of soybeans.

2-Propyl-furan was another compound detected in the samples. The amount of this compound increased with storage duration. Based on the mean values obtained for each treatment considering all periods measured, WC displayed the highest 2-propyl-furan content (24,914 e.c), followed by WBHT and WPH (21,197 and 22,355 e.c., respectively), and then WPAE (14,092 e.c.) ($P < 0.05$). Furans are secondary oxidation products and are a by-product of the disruption of hydroperoxides, possibly accounting for the observed increase of this compound in the samples. The lowest accumulation, which occurred in the WPAE sample, could be due to the antioxidant activity of the PAE extract.

The BHT compound was only found in the WBHT treatment but was more prevalent on day 0 (100,703 e.c) than day 16 (29,703 e.c). This synthetic antioxidant decreasing in the oxidation process was advancing in WBHT.

The compounds benzene, 1,2,3-trimethyl- and benzene, 1,3,5-trimethyl-, have been previously detected in black walnut (*Juglans nigra*) skins (Buchbauer and Jirovetz, 1992). These compounds were only found in the WPAE sample and did not change significantly during storage. The presence of these compounds in the treatment could be related to the composition of the PAE extract that was added to the sample.

D-Limonene is an aromatic compound that makes an important contribution to the flavor of various foods. This compound has already been reported in the walnuts' volatile composition (Elmore et al., 2005). The D-limonene content gradually diminished as storage progressed. Initially (day 0), the treatments did not display significant differences (mean value: 83260 e.c.). However, by day 16, WC had lost all its D-limonene content, whereas it was partially retained in WPH (14,728 e.c.), WPAE (15,570 e.c.), and WBHT (13,729 e.c), without significant differences among them. Its presence in these samples could be justified by the protection afforded by the antioxidants compared to the control sample. The samples with antioxidants (WPH, WPAE, and WBHT) had superior ability to preserve the original flavor components of walnuts, like D-limonene. Moreover, these samples prevented the development of compounds related to lipid oxidation and unpleasant flavors, resulting in a lower evolution of compounds, like hexanal, butanal, and nonanal.

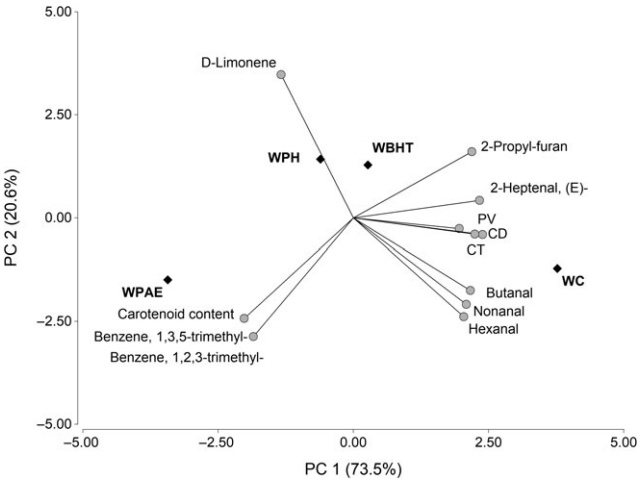

Fig. 3 Biplots of principal component analysis. Independent variables: peroxide value (PV), conjugates dienes (CD), conjugated trienes (CT), carotenoid content (CC) hexanal, nonanal, butanal, 2-heptenal, (E)-, benzene, 1,2,3-trimethyl-, benzene, 1,3,5-trimethyl-, furan, 2-propyl-, D-Limonene, and butylated hydroxytoluene. Treatments: walnut oil without antioxidants (WC, control sample), and with the addition of walnut water-soluble polyphenol fraction (WPH) and ethyl acetate-soluble polyphenol fraction (WPAE), and BHT (WBHT) analyzed during a 16-day storage period at a 60 °C temperature ($n = 3$)

Certain volatile compounds did not appear on day 0 of storage but evolved throughout the storage. These were mainly aldehydes and alcohols: (*Z*)-2-octen-1-ol, pentanal, butanal, 3-methyl-, heptanal, 2,4-Octadienal, (E,E)-, octanal, and decanal. Even though these compounds are related to oxidation processes, they did not change significantly as a function of increasing storage duration or did not display significant differences between treatments analyzed in the same period.

PCA

PCA was conducted to understand the oxidative stability of walnut oil that was endowed by the various treatments, with and without antioxidants during storage. The biplot was obtained from the first two principal components (PC) (Fig. 3), which explained 94.1% variability over the 16-day storage. The chemical parameters and volatile compounds that increase along with lipid oxidation were placed on the right side of the biplot: CD, CT, PV, nonanal, hexanal, butanal, (*E*)-2-heptenal, and 2-propyl-furan. A positive association existed among these variables but they were negatively associated with the variables located on the left side: CC, 1,2,3-trimethy-benzene, 1,3,5-trimethyl-benzene, and D-limonene. These last variables decreased during storage except the benzene derivatives. The oxidation indicators were closely linked with the WC sample and were negatively associated with the sample WPAE, which was

placed on the left side of the biplot and was related to CC, benzene,1,2,3-trimethyl-, D-limonene, and benzene, 1,3,5-trimethyl- variables. In the upper half of the biplot (PC2), the most important variable was D-limonene, which was nearer to the WBHT and WPH samples. The location of the samples in the biplot evidenced that WPAE followed by WPH and WBHT exhibited a minor oxidation process as a result of the application of the antioxidants.

The associations between the samples and oxidation indicators were confirmed by correlation analysis (Pearson's coefficient). 2-Propyl- furan and (*E*)-2-heptenal showed the strongest correlation of all volatile compounds analyzed, represented by a coefficient (r) of 0.92. Butanal ($r = 0.87$) and hexanal ($r = 0.84$) were also highly correlated with nonanal ($P < 0.0001$). Likewise, Larrauri et al. (2016a) also found a positive correlation between hexanal and nonanal in a storage study of coated almonds. Some chemical indicators were also correlated with each other, like, PV and CD ($r = 0.85$) and CD with CT ($r = 0.73$). Riveros et al. (2013) found a high correlation between PV and CD values ($r = 0.95$), in a study aimed at preserving the sensory attributes of peanuts using edible coatings. Some correlations between chemical and volatile variables were also evident. There was a high correlation between CD with (*E*)-2-heptenal ($r = 0.86$). For instance, D-limonene had a convincing negative correlation with PV ($r = -0.90$) and CC was strongly correlated with benzene, 1,2,3-trimethyl- ($r = 0.95$).

The results of the current research showed that the polyphenols extracted from walnut oil cake exhibited good antioxidant properties against walnut oil oxidation. Currently, walnut oil cake is a by-product from walnut oil production, which is only being exploited for animal feed. Therefore, it is possible to use this by-product for developing a new food additive with a high added value. In addition, these natural compounds (walnut polyphenols) are derived from a food product (walnuts), therefore, could be considered a GRAS (generally recognized as safe by FDA) product, and probably, its approval as food additive will not be required.

Conclusions

The current study reveals that the application of antioxidants improves the stability of walnut oil. Both walnut polyphenol extracts efficiently preserve the product walnut oil and, in some lipid oxidation indicators, they show a better performance than the commercial synthetic antioxidant BHT. Consumers are reluctant to food preserved with synthetic antioxidants due to their associated human health concerns. Walnut polyphenols are natural compounds that when applied in walnut oil, walnuts, or walnut by-products can help prolong their shelf life. Additionally, the inclusion of walnut polyphenol extracts in walnut oil or walnut products does not provide the risk of cross-contamination of allergens, which is an important point to consider in a food safety system for the food industry.

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Conflict of interest The authors declare that they have no conflict of interest.

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