



Walnut paste: oxidative stability and effect of grape skin extract addition

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ABSTRACT

Walnut paste, obtained by roasting and grinding of kernels, was characterized and supplemented with encapsulated grape skin extract aiming to evaluate its potential effect on oxidative stability and/or antioxidant capacity. Based on the oxidation induction period in screening trials 5000 ppm (w/w) extract addition was selected as effective in inhibiting oxidation processes. Walnut paste with and without 5000 ppm grape skin extract were maintained for 15 days at 60 °C, simulating 2 year storage at 20 °C, based on an estimated activation energy of 80,327 kJ/mol for walnut lipid oxidation. Monitoring of data from peroxides, conjugated dienes and trienes, total phenolics, ABTS, ORAC, FRAP, and tocopherols values showed the deterioration of walnut paste started at the end of the observed period, even remaining below the threshold of unacceptability. Moreover, 5000 ppm extract addition did not prove to enhance oxidative stability nor antioxidant properties of the walnut paste. In the future, specific parameters of oxidation kinetics and antioxidant activity in the advanced phase of storage could be investigated.

1. Introduction

Widely cultivated and consumed all around the world, walnuts have recently aroused great interest as a promising natural functional food. In fact, several studies demonstrated walnut role in preventing neurodegenerative disorders and the inversely correlation between their consumption and the occurrence of cardiovascular diseases. Health properties of walnuts are related to their chemical composition: since, among nuts, they are the richest in polyunsaturated fatty acids (PUFA), with a favourable $\omega 6$: $\omega 3$ ratio, and show the highest antioxidant capacity due to their content of polyphenols. They also include other important bioactive compounds such as melatonin, serotonin, minerals and vitamins (especially tocopherols) (Tapia et al., 2013; Martínez et al., 2010).

Walnuts are commonly commercialized as shelled or unshelled (as whole or ground kernels), but they also can be processed to obtain ingredients such as walnut oil, flour and paste. The major constituents of kernels are lipids (62–74%), with relatively high contents of linoleic acid (~63%) and linolenic acid (~13%), notably susceptible to oxidative degradation resulting in rancidity development and shorter shelf life. Walnut oil has a low oleic to linoleic acid ratio, ranging between 0.08 and 0.57, being therefore highly unstable compared with other plant oils (Gama et al., 2018; Martínez et al., 2010).

As known, oxidation rate is correlated to temperature according to Arrhenius law (Abedi Gonabad et al., 2015). Ling et al. (2014) performed

an accelerated shelf life test on walnut paste, showing that a storage at 35 °C for 20 days simulated approximately two years at +4 °C or two months at room temperature (25 °C). Furthermore, several researches were carried out to study the effect of packaging and storage conditions on shelf life of walnut kernels (Bakkalbaşı et al., 2012) and oil, stating that oxidation also depends on both endogenous (walnut variety, activity of lipolytic enzymes) and environmental factors (lightning conditions, temperature, humidity, oxygen concentration, presence of metals).

Oxidative stability of food products can also be strongly affected by technological processes. Roasting is extensively applied on nut fruits with the aim of improving both sensory properties and shelf life. After roasting, walnuts acquire characteristic colour, favourable taste and flavour, as well as delicate and crispy texture. Moreover, roasted walnuts show a longer shelf life because of water evaporation, lipoxygenase inactivation (Buranasompob et al., 2007), greater retention of tocopherols (Vaidya and Eun, 2013) and higher antioxidant activity (Vinson and Cai, 2012).

According to Arranz et al. (2008), the major contributors to antioxidant capacity of walnuts are polyphenols, mainly hydrolysable ellagittannins located in their pellicle. The phenolic content of kernels has been investigated by several authors, revealing that it is affected by extraction methodologies (solvents, temperatures and times), cultivar, harvest year, orchard location, processing steps and storage (Tapia et al., 2013; Arranz et al., 2008). Walnuts also contain tocopherols in the lipid fraction, with γ -tocopherol as the prevalent form, followed by δ - and α -, and trace

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amounts of β -isomer (Amaral et al., 2005). Despite their radical scavenging activity, tocopherols seem to contribute to total antioxidant capacity of walnut with less than 5% (Arranz et al., 2008).

In order to extend the shelf life of walnut products, antioxidants could be added to delay rancidity development. Nowadays there is a great interest for obtaining and exploiting antioxidants from natural sources, avoiding the use of synthetic additives, scarcely accepted by consumers. However, there are few studies related to their use for the preservation of nuts and/or derivatives: rosemary extract proved an efficient additive in delaying oxidation of stored walnut oil (Martínez et al., 2013), or even the addition of powdery encapsulated extract from red-grape marc was successfully tested to delay lipid oxidation in hazelnut paste (Spigno et al., 2013). Grape marc is a wine-making by-product of wine industry, rich in phenolic compounds (mainly anthocyanins, flavanols and flavonols) that can be recovered to obtain extracts useful in the development of new food products as functional ingredients or as natural antioxidant additives (Lavelli et al., 2016).

Based on these premises, the objective of this work was to determine chemical composition and oxidative stability of walnut paste. In addition, accelerated storage tests on walnut paste, with or without added antioxidant, were performed in order to investigate the potential protective effect exerted by the addition of a red grape skin phenolic extract on the oxidative status during storage.

2. Materials and methods

2.1. Materials

Walnuts in shell (var. *Howard*, California, USA) were purchased from a local market in Piacenza (Italy). Grape skin extract, encapsulated into maltodextrin, was obtained from grape pomace of the Barbera variety according to the procedure reported in Dordoni et al. (2019). Briefly, grape skin powder was extracted with 60% aqueous ethanol under continuous stirring for 2h at 60 °C. The mixture was then centrifuged and the supernatant recovered. The extract was concentrated 15 times with a rotary evaporator (Büchi Rotavapor R-144), diluted with an aqueous solution of maltodextrins, and spray-dried. Maltodextrins (Glucidex IT 12 DE, Roquette Italia S.P.A.) were used at a dosing level of 0.6 M ratio DE/gallic acid equivalents (GAE). Spray-drying operation conditions were described in Lavelli et al. (2016). The spray-dried extract was characterized for moisture content (7.19 ± 0.39 g/100g), total phenolics (98.24 ± 3.50 mg_{GAE}/g), ABTS value (790.91 ± 50.51 $\mu\text{mol}_{\text{Trolox}}$ /g), FRAP value (2.25 ± 0.15 mmol_{Fe(II)}/g), and ORAC value (985.78 ± 59.51 $\mu\text{mol}_{\text{Trolox}}$ /g).

All standards and chemicals used in the analytical determinations were purchased from Sigma Aldrich (Milan, Italy).

2.2. Walnut paste preparation

After manual shell removal, kernels were roasted in a forced convection oven (Tecnoeka, Italy) at 160 °C for 15 min, as reported by Vaidya and Eun (2013). Roasted kernels were firstly ground by an electric domestic grinder (La Moulinette, Moulinex) and then reduced to paste using a planetary micro mill (Pulverisette, Fritsch, Germany) at 700 rpm for 2 min. The paste was collected in plastic and opaque containers to limit its exposure to light. Before closing, the head space was saturated with nitrogen to avoid contact with air. The samples were then stored at -20 °C until use.

2.3. Experimental plan

The obtained walnut paste was analysed for moisture, fat, protein, reducing sugar, ash, fibre, pH, and total acidity.

Preliminary tests were carried out to select the extract concentration to be used in the next accelerated storage test: three different doses of the spray-dried grape skin extract were handily and thoroughly mixed with

the walnut paste. The obtained samples were subjected to accelerated oxidative stability tests by using an Oxidation Test Reactor (Oxitest) device (see section 2.3.1).

Therefore, an accelerated storage test (see section 2.3.2) was designed and performed to compare samples with and without the addition of the grape extract at the best concentration selected from preliminary tests. For this purpose oxidative quality indexes were monitored during storage: peroxide values, conjugated dienes and trienes, and tocopherols (determined in the lipid fraction); total polyphenols content and antioxidant capacity (ABTS and ORAC tests) (evaluated on the defatted matter). Antioxidant capacity by FRAP tests was assessed on defatted samples, at the beginning and end of the test.

2.3.1. Accelerated oxidative stability test (Oxitest)

Walnut paste was enriched with the spray-dried grape skin extract at three different concentrations: 2500, 5000 and 10000 ppm (w/w). To assess the extract concentration with the greatest antioxidant efficacy, the Oxidation Test Reactor (Oxitest, VELP Scientifica, Milan, Italy) was employed. Walnut pastes (10 g), with and without extract addition, were distributed homogeneously on the instrument sample holders and test conditions were set at 90 °C and 6 bar oxygen pressure. The Induction Period (IP in min) was calculated by the instrument software (OXISoft™).

2.3.2. Accelerated storage test

In order to evaluate the oxidative stability of walnut paste and the protective effect exerted by grape skin extract, an accelerated storage study was performed on both the walnut paste and the walnut paste added with the extract concentration selected from the accelerated oxidative stability test.

According to Abedi Gonabad et al. (2015) an activation energy of 80.327 kJ/mol was assumed for walnut oil oxidation. In order to simulate storage at room temperature (20 °C), the Arrhenius law was applied, finding the acceleration factor of oxidation:

$$\frac{k_{60^{\circ}\text{C}}}{k_{20^{\circ}\text{C}}} = 52.3$$

A day storage at 60 °C can then be considered equivalent to a minimum of 52 days at 20 °C. Therefore, in the present study a period of 15 days at 60 °C was chosen for the accelerated storage tests, aiming to simulate a 2 year shelf life at 20 °C and notice quality deterioration.

Thirty grams of both samples (paste with and without grape extract) were placed in open ceramic vessels and stored at 60 °C for 15 days (Vaidya and Eun, 2013). Samples were analysed after 2, 4, 7, 9, 11, and 15 days. For each type of walnut paste (with and without extract) one vessel was used at each sampling time. Walnut paste quality was assessed on triplicate at each sampling time by determination of oxidative indexes.

2.4. Chemical composition

Moisture content was determined as stated by the AOAC official method 931.04 (AOAC, 2005). The fat content was assessed according to the method 948.22 (AOAC, 2005) suitably modified. In detail, the samples were mixed with distilled water and hydrochloric acid (25% w/w), and subjected to 30 min-boiling for hydrolysis. The resulting solutions were then filtered through a folded filter (Whatman 595 ½). The filters containing the samples were washed with distilled water and subsequently dried for 6 h at 100 °C. The dried filters were then transferred into thimbles and extracted for 6 h with 250 mL n-hexane through a Soxhlet apparatus. The solvent was finally evaporated (Rotavapor Buchi R-3) and the residual fat weighed.

Official methods of analysis were used to determine ash and total protein content (method 950.48) (AOAC, 2005). For the evaluation of free reducing and non-reducing sugar content (glucose, fructose, maltose,

lactose and sucrose) the volumetric method of Luff-Shoorl was applied (Egan et al., 1981). Finally, neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) contents were determined on the defatted samples according to the Van Soest et al. (1991) procedure adapted to the ANKOM Fiber Analyzer (ANKOM Technology Corporation, Macedon, NY).

Total acidity and pH were evaluated on walnut paste samples according to the methods recommended by the Office International du Cacao, du Chocolat et de la Confiserie (OICCC, 1972, 9. In particular, 10 g of walnut paste were dissolved in 90 mL of boiling distilled water. The pH was measured after cooling to 25 °C. Total acidity was measured on the same dispersion added with 20 mL of 0.1 N sodium hydroxide, then titrated with 0.1 N hydrochloric acid until pH of 7.00, and expressed as goic acid/100 g of dry sample.

2.5. Oxidative quality indexes

2.5.1. Defatting of walnut paste

Oil fraction and defatted powder were separated by a cold extraction procedure. Samples were added with hexane at a 1:5 ratio (Belščak et al., 2009), stirred at 180 rpm for 1 h at 20 °C (orbital shaker Infors HT), then centrifuged at 3000 rpm for 15 min at 15 °C (Varifuge 20 RS, Heraeus Sepatech). The supernatant mixture was filtered throughout a filter paper (Whatman n° 595 ½) and the free oil was collected after hexane evaporation (Rotavapor Buchi R-3). The solid residue was kept for about 8 h under nitrogen flow, in order to evaporate all the remaining solvent and obtain the defatted powder.

Peroxide value, conjugated dienes and trienes, and tocopherols were measured on the lipid fraction, whereas total phenolic content and antioxidant capacity (through ABTS, ORAC, and FRAP assays) were evaluated on defatted matter.

2.5.2. Peroxide value, conjugated dienes and trienes

The peroxide values (expressed as meq O₂/kg_{oil}) and conjugated diene and triene systems (expressed as extinction coefficients at 232 and 270 nm, K₂₃₂ and K₂₇₀) were determined on the oil fraction as described in the European Union Commission Regulations (1991).

2.5.3. Tocopherols

The chromatographic determination of tocopherols was performed on a HPLC system including a Perkin Elmer (Norwalk, CT, USA) 200 Series pump equipped with a Perkin-Elmer 650-10S fluorescence detector, Jasco LC-Net II/ADC (Oklahoma City, OK, USA) communication module and ChromNAV Control Center software. The analysis was carried out according to Calvo et al. (2011): a LiChrosorb Si60-5 C18 column 250 mm × 4.6 mm, 5 μm (Supelco, Bellefonte, PA, USA) was used, the injection volume was 20 μL, and the mobile phase was hexane:isopropanol:ethanol (98.5:1:0.5). Fluorescence detector was set at 290 nm excitation and 330 nm emission wavelengths.

α-, γ- and δ-tocopherols were identified by comparing retention times with those of commercial standards. Results were expressed as mg/100g of dry sample for each tocopherol.

2.5.4. Total phenolic content

Two grams of defatted sample were added with 5 mL methanol:water/70:30 and kept in ultrasonic bath for 30 min, as reported by Belščak et al. (2009). Then, the mixture was centrifuged at 3000 rpm for 10 min at 15 °C (Varifuge 20 RS, Heraeus Sepatech): the supernatant was recovered and the solid residue was re-extracted with 5 mL of the same solvent. Filtered supernatants (Whatman n° 595 ½) were collected into 10 mL flasks and made up to the mark with methanol:water/70:30. The extracts were stored in the dark, at -20 °C, until further analysis.

Total phenolic content was determined through the Folin-Ciocalteu assay (Moncalvo et al., 2016). Briefly, an aliquot (50 μL) of extract (diluted as necessary with water) was mixed with 250 μL of Folin-Ciocalteu reagent; then 4.7 mL of Na₂CO₃ 2.2% were added and

the mixture was brought to 50 mL with distilled water. The samples were kept in a thermostatic bath at 40 °C for 30 min, then the absorbance of the samples was measured at 750 nm (Shimadzu UV-1601), against blank with no extract addition. Results were expressed as equivalents of gallic acid (GAE) per 100 g of paste (dry weight) through a calibration curve made with a standard of gallic acid (≥98%) in the range 100–800 mg/L.

2.5.5. ABTS assay

The test assesses the ability of a sample to reduce the ABTS radical (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt) by measuring its absorbance decrease at 734 nm. A radical solution (7 mM ABTS and 2.45 mM potassium persulfate) was prepared and kept in the dark at room-temperature for 16 h before use. The solution was then diluted with ethanol:water/50:50 to an absorbance of 0.700 (±0.020) at 734 nm against ethanol:water/50:50 and equilibrated at 30 °C. For the analysis, 2 mL of the final radical solution were mixed with 20 μL of different dilutions (in ethanol:water/50:50) of the phenolic extract. The samples, along with a control (2 mL of the radical solution) and a blank (2 mL of the radical solution mixed with 20 μL of the sample solvent) were kept in the dark for 6 min at 30 °C and then their absorbance was read at 734 nm (Vadivel et al., 2016). Antioxidant capacity was calculated as percentage of absorbance decrease and expressed as mmol Trolox® equivalents/100g or mg Trolox® equivalents/100g (based on paste dry weight) by a calibration curve obtained with standard Trolox® (0.1–3.2 mM final concentration in the cuvette).

2.5.6. ORAC assay

The ORAC (Oxygen Radical Absorbance Capacity) assay was performed to measure the peroxy radical-scavenging activity of phenolic extracts based on the method described by Huang et al. (2002). A 4 μM fluorescein solution was daily prepared in 75 mM phosphate buffer (pH 7.4). Two hundred microliters of fluorescein solution and 33 μL of 0.153 M AAPH solution (2,2'-azobis-2-amidinopropane dihydrochloride) were added to all working wells. In addition, blank wells received 33 μL of 75 mM phosphate buffer (pH 7.4), while standards received 33 μL of Trolox® dilution, and samples received 33 μL of diluted extracts. The fluorescence decay of each well was then measured and recorded by a fluorescence reader (Bio Tek Synergy HT - Bio-Tek Instruments, Inc., Winooski, VT): the fluorescence was monitored kinetically with data taken every minute, with a 485 nm excitation filter and a 528 nm emission filter. Results were expressed as μmol Trolox® equivalents/100 g of dry paste.

2.5.7. FRAP assay

The FRAP assay (Ferric Reducing Antioxidant Power) was carried out as described by Vadivel et al. (2016). The FRAP reagent was daily prepared with 2.5 mL of 20 mM TPTZ solution (2,4,6-Tris (2-pyridyl)-s-triazine) in 40 mM hydrochloric acid, plus 2.5 mL of 20 mM FeCl₃·6H₂O solution, and 25 mL of 0.3 M acetate buffer (pH 3.6). An aliquot (3.7 mL) of this reagent, kept for 15 min at 37 °C, was mixed with 360 μL of distilled water and 120 μL of the phenolic extract opportunely diluted. The samples and the blank were incubated at 37 °C for 30 min; then, the absorbance of the sample was read at 593 nm against the blank. The absorbance was converted into mMFe(II) through a calibration curve prepared with different concentrations of FeSO₄·7H₂O solutions, then expressed as mmoles of Fe(II) per 100g of dry paste.

2.6. Statistical analysis

Chemical analysis were carried out on triplicate; mean values and standard deviations were calculated for each case. Peroxide values were analysed by Student's t-test at p ≤ 0.05. Analysis of variance (ANOVA) followed by Tukey's post-hoc test was performed at the p ≤ 0.05 level using statistical software SPSS® (version 23.0, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Characterization of initial walnut paste

Walnut composition can vary considerably by genotype, pre- and post-harvest condition, as well as processing and storage conditions. However, there are few studies (Tapia et al., 2013) that evaluated the nutritional value of the *Howard* cultivar. The proximate characterization of the walnut paste obtained in this study by *Howard* cv is shown in Table 1. More than a half of the macro-components was represented by lipids, followed by the protein content. These data partially agreed with results obtained by Tapia et al. (2013) on not-roasted walnut kernels, reporting a higher fat content (61.3%) and a lower protein content (15.1%). As expected, the negligible moisture value (0.66%) was a consequence of the kernel roasting process for walnut paste production. Fibre fractions were higher than those reported in studies on different varieties (Tapia et al., 2013; Savage, 2001): to the neutral detergent fibre contributed 5.42% of hemicellulose, 3.37% of cellulose, and 3.89% of lignin and cutin, mainly located in the walnut pellicle (Dordoni et al., 2017).

Walnut paste exhibited pH slightly lower than the neutrality (6.24 ± 0.03) and acidity value of 1.87 ± 0.02 (g_{oleic acid}/100g) that can be correlated to the degree of oil hydrolytic rancidity induced by the roasting process (Ziaolhagh et al., 2017). As a matter of fact, roasting and subsequent grinding of the kernels can adversely affect indicators of hydrolytic and oxidative alterations (Martínez et al., 2013). With regard to the lipid fraction, peroxide values, conjugated dienes (K_{232}) and conjugated trienes (K_{270}) agreed with studies on walnut oils dark stored and/or heat-treated (Martínez et al., 2013; Vaidya and Eun, 2013) (Table 2, Fig. 1). In particular, peroxides are the primary products of lipid oxidation and their formation is promoted by mechanical and thermal stress on the product. By analysing the characteristics of a cold-pressed walnut oil, Martínez et al. (2010) reported peroxide numbers between 0.05 and 0.50 meq_{O₂}/kg oil, lower than the amount detected in the present study (Table 2). On the contrary, by applying the same roasting conditions used in this work (160 °C for 15 min) Vaidya and Eun (2013) scored a similar peroxide value (2.34 meq_{O₂}/kg oil) and showed that, despite the immediate increase of oxidative parameters, roasted nuts were more stable over time, due to enzyme inactivation.

Furthermore, the walnut high susceptibility to oxidation may be counterbalanced by its richness in antioxidant compounds, mainly represented by tocopherols and phenolic compounds.

Thermal treatment can also induce the degradation of phenolic compounds and/or increase the total phenolic content and the antioxidant activity, due to release of bound phenolic compounds and formation of Maillard reaction products (Chang et al., 2016). Data related to the content of polyphenols and the antioxidant capacity of walnut paste are reported in Fig. 2.

Walnut phenolic compounds are located especially in the brown pellicle surrounding the nut and include hydrolysable and condensed

Table 1

Characterization of initial walnut paste (NDF: Neutral Detergent Fibre; ADF: Acid Detergent Fibre; ADL: Acid Detergent Lignin). Values are means \pm standard deviations (n = 3).

Parameter	Value
Moisture (g/100g)	0.66 \pm 0.28
Fat (g/100g)	56.82 \pm 9.23
Protein (g/100g)	20.49 \pm 1.04
Reducing sugars (g/100g)	0.012 \pm 0.000
Ash (g/100g)	1.94 \pm 0.01
Fibre fractions	
NDF (g/100g)	12.68 \pm 1.07
ADF (g/100g)	7.26 \pm 0.48
ADL (g/100g)	3.89 \pm 0.56
pH	6.24 \pm 0.03
Acidity (g _{OLEIC ACID} /100g)	1.87 \pm 0.02

Table 2

Peroxide values of walnut paste (WP) and of walnut paste added with 5000 ppm (w/w) of extract (WP + E) determined at different storage times at 60 °C. Values are means \pm standard deviations (n = 3). Within each column, different superscript letters indicate statistically different values according to post-hoc comparison (Tukey's test) at $p \leq 0.05$. Within each row, different subscript letters indicate statistically different values according to Student's t-test at $p \leq 0.05$.

Storage time (days)	Peroxide value (meq _{O₂} /kg _{oil})	
	WP	WP + E
0	2.31 \pm 0.60 ^{ab} _a	2.32 \pm 0.57 ^a _a
2	5.82 \pm 0.76 ^c _a	6.82 \pm 1.76 ^b _a
4	3.94 \pm 0.03 ^b _a	6.45 \pm 2.09 ^b _a
7	2.11 \pm 0.71 ^a _a	1.78 \pm 0.30 ^a _a
9	0.66 \pm 0.29 ^a _a	3.30 \pm 0.76 ^a _b
11	1.79 \pm 0.28 ^a _a	1.98 \pm 0.01 ^a _a
15	5.81 \pm 0.74 ^c _a	7.77 \pm 0.57 ^b _b

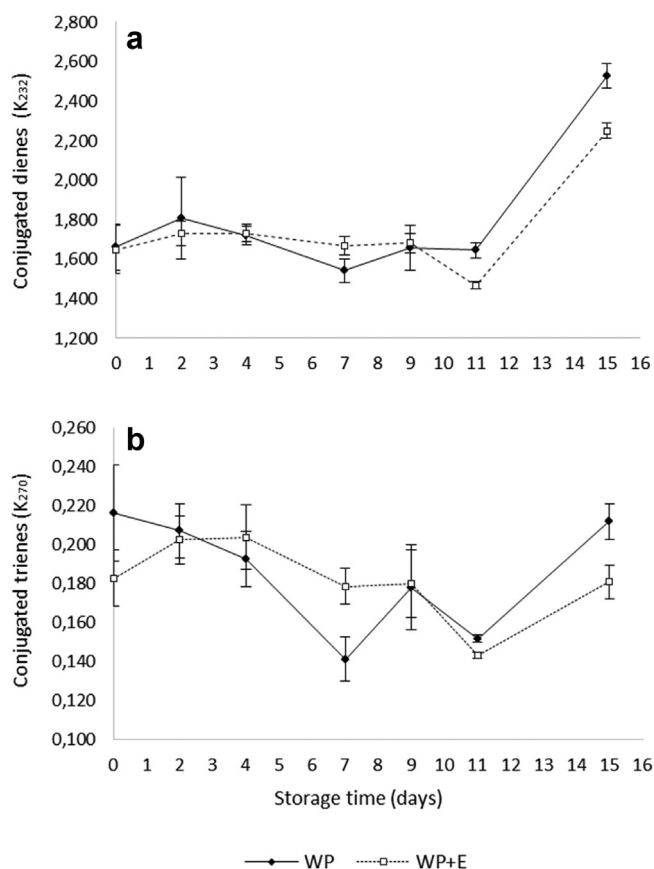


Fig. 1. (a) Conjugated diene values (K_{232}), and (b) conjugated triene values (K_{270}) of walnut paste (WP) and of walnut paste added with 5000 ppm (w/w) of extract (WP + E) determined at different storage times. Error bars indicate \pm standard deviations (n = 3).

tannins, flavonoids and phenolic acids. Ellagitannins, ellagic acid and its derivatives were revealed as the most abundant (Dordoni et al., 2017).

The content of phenolic compounds in 100g of dry paste was 406.9 ± 19.0 mg_{GAE}. A similar concentration (414 ± 18 mg_{GAE}/100g of fresh weight) was reported by Arcan and Yemencioğlu (2009) who evaluated it through aqueous extraction of whole walnuts, while higher values were obtained by aqueous-organic extraction: Salcedo et al. (2010) extracted 15.5 mg_{GAE}/g of walnut through 50:50 methanol:water (v/v) extraction of whole fruit, Arranz et al. (2008) found 1071 \pm 35 mg_{GAE}/g by extraction of defatted walnuts with acidic methanol:water (50:50 v/v), and Tapia et al. (2013) 58.2 mg_{GAE}/g of fresh weight by acidic ethanol:water (80:20 v/v) extraction of whole *Howard* walnuts.

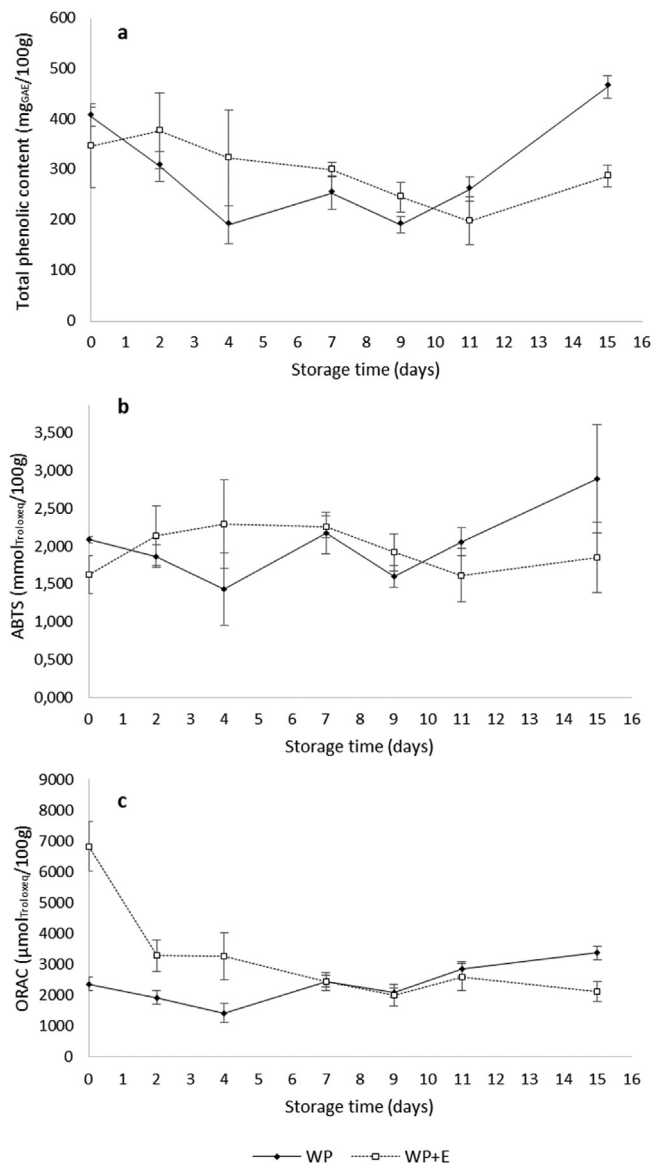


Fig. 2. (a) Total phenolic content (mg_{GAE}/100g), (b) antioxidant capacity (mmol_{Trolox}/100g) based on the ABTS assay, and (c) antioxidant capacity (μmol_{Trolox}/100g) based on the ORAC assay of walnut paste (WP) and of walnut paste added with 5000 ppm (w/w) of extract (WP + E) determined at different storage times. Error bars indicate ± standard deviations (n = 3).

In addition to environmental factors, differences observed on phenolic contents may be partly explained by the different extraction methods and interference from other reducing non-phenolic constituents. Therefore, the values obtained based on Folin's assay are often overestimated in comparison with the sum of individual phenolics identified using high-performance liquid chromatography (Chang et al., 2016).

The phenolic content of walnuts is in general correlated to their antioxidant capacity which resulted equal to 2.088 ± 0.046 mmol_{Trolox}/100g, 7.036 ± 0.456 mmol_{Fe(II)}/100g and 2363.763 ± 216.880 μmol_{Trolox}/100g according to the ABTS, FRAP and ORAC test, respectively. In literature there are various studies related to the antioxidant capacity of walnuts, but it is difficult to compare the obtained results since they employed different extraction methods and reported antioxidant activities as equivalents of different compounds (Arcan and Yemencioğlu, 2009). Schlörmann et al. (2015) evaluated the effect of roasting on the hydrophilic antioxidant activity of walnuts, showing a significant decrease from 11.0 mmol_{Trolox}/100g in natural nuts to 3.8–10.5 mmol_{Trolox}/100g in kernels roasted at 123 °C/25 min to 185.5

°C/25 min.

Although no specific data for the *Howard* variety have been found in the literature, the relative abundance of tocopherol vitamers agree with reported values (Amaral et al., 2005) for other varieties: in the examined paste γ-tocopherol was the predominant form, followed by α-tocopherol and δ-tocopherol, while the presence of β-tocopherol was not detectable (Fig. 3). The observed values were high, despite the roasting process that generally causes a tocopherol decrease (Vaidya and Eun, 2013).

3.2. Preliminary test to select the extract addition level

The antioxidant efficacy of different levels of encapsulated grape skin extract (2500, 5000, and 10000 ppm (w/w)) was preliminarily measured by comparing the induction periods (IP) of the different samples. The IP corresponds to either a level of detectable rancidity or a sudden change in the rate of oxidation, with higher IP value corresponding to higher oxidative stability. Walnut paste without additions showed an IP of 726 ± 40 min. Addition of grape extract at 2500 ppm did not significantly influence the IP (769 ± 53 min), while a 5000 ppm enrichment gave a

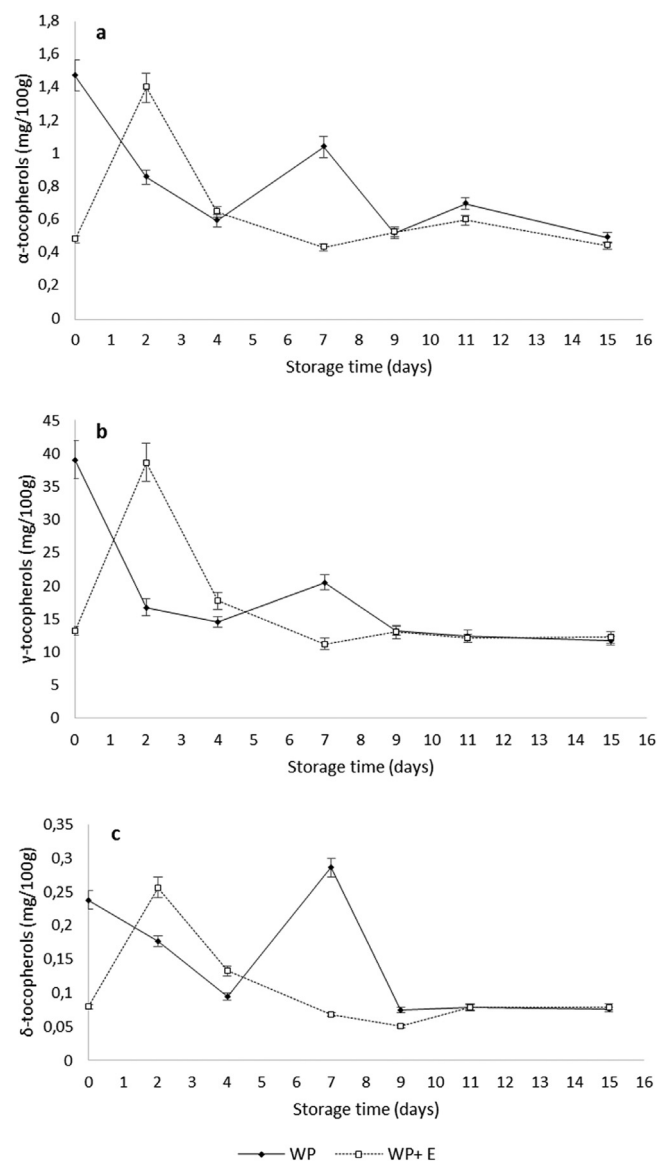


Fig. 3. (a) α-tocopherol content (mg/100g), (b) γ-tocopherol content (mg/100g), and (c) δ-tocopherol content (mg/100g) of walnut paste (WP) and of walnut paste added with 5000 ppm (w/w) of extract (WP + E) determined at different storage times. Error bars indicate ± standard deviations (n = 3).

longer IP (831 ± 29 min), which did not further increase with 10000 ppm addition (830 ± 27 min). Taking into account these results and considering that 5000 ppm dosing level also represents the common maximum allowed additives incorporation into foods (Spigno et al., 2013), this concentration was selected for the accelerated shelf life study.

3.3. Accelerated storage study

Literature reported data on shelf life of walnut stored at temperature ranging between -18 °C and 38 °C do not exceed 18 months (Gama et al., 2018) and information on the expiration date of processed walnut derived products such as walnut paste is not available. In the present study, accelerated storage conditions were set in order to simulate a 24-month shelf-life (at 20 °C), to largely exceed the common commercial expiration period of 1 year generally assigned to the semi-finished nut-based products (Spigno et al., 2013).

Results showed some variations in the measured peroxide values (Table 2) explainable by the heterogeneity nature of the matrix (Ling et al., 2014). Contrary to expectations, after 15 days of high temperature storage, walnut paste with extract reached a slightly higher value than the one without addition, suggesting that grape antioxidant molecules do not exert any protective effect on the lipid fraction. Furthermore, these maximum levels were still lower than the value (10 meqO₂/kg of fat) recommended for avoiding rancidity flavour (Kong and Singh, 2011). Our results are in general in agreement with those reported by Stark et al. (2000) and Ling et al. (2014) who reported good stability of walnut oil and walnut paste during 115 days at 60 °C, and 20 days at 35 °C storage, respectively.

Hydroperoxides accumulation during the initial oxidation period proved to be correlated with increase of the conjugated dienes. Since walnut paste production involved a roasting process, initial K₂₃₂ of both natural and enriched samples (1.662 ± 0.117 and 1.648 ± 0.122 , respectively) (Fig. 1a) can be considered index of a slight oxidative status. The conjugated dienes showed a trend similar to that obtained for peroxide values: large increase of the diene conjugation rate occurred at the end of the storage showing, nevertheless, a slightly higher value in the sample without extract (Fig. 1a). The production of secondary oxidation products (conjugated trienes) over time is presented in Figure 1b. Their formation is due to reactions of decomposition and light degradation of primary oxidation compounds resulting in the generation of a wide variety of different molecules (aldehydes, ketones, alcohols, etc.) (Ferreira et al., 2018). In both samples, the content of trienes generally tended to fluctuate over time. Anyway, final values (after 15 day storage) remained low even in comparison to reported data for walnut oil during different storage conditions (Ferreira et al., 2018). Hence, results observed on lipid oxidation indicate that both the tested walnut pastes have a reasonably long shelf life. As a matter of fact, no substantial differences on oxidative parameters were revealed between samples with or without grape extract.

Total phenolic content of walnut paste samples had a very high variability during storage test (Fig. 2a). Although a decrease in the content of these molecules was observed during storage especially in the walnut paste with extract, both the samples values were not significantly different between initial and final time. This behaviour might be ascribed to the ability of polyphenols to oxidize and further polymerise to form oligomers with a higher antioxidant activity, since the Folin's assay actually measures the reducing power of samples (Spigno et al., 2013).

The same trend was observed in the FRAP and ABTS test (Fig. 2b). In fact, the FRAP assay on walnut paste gave 7.036 ± 0.456 and 8.803 ± 1.555 mmol_{Fe(II)}/100g, at day 0 and 15, respectively, and 5.978 ± 2.596 and 5.558 ± 0.970 mmol_{Fe(II)}/100g for the grape extract enriched sample.

Similarly ABTS based antioxidant capacity (Fig. 2b) did not vary significantly during storage in both the samples. On the opposite, ORAC test revealed significantly higher values at time zero (Fig. 2c). These data confirm the correlation between the Folin's assay and antioxidant activity

measured by FRAP, ABTS and other assays based on electron transfer (Huang et al., 2005).

Comparing the total polyphenols content in walnut paste with and without extract, no significant differences appeared during the studied period, with the exception of the 9th and, particularly, of the last day, when the sample of walnut paste showed a greater and significantly different phenolic content (465.79 ± 22.05 mg_{GAE}/100g) than the enriched paste (288.98 ± 21.97 mg_{GAE}/100g) (Fig. 2a). This difference was noticeably significant also in the ORAC (Fig. 2c) and FRAP results. ABTS capacity appeared slightly higher in the initial walnut paste; after that, samples with and without extract could not be considered as significantly different (Fig. 2b).

The presence of grape marc extract had the effect of considerably increasing the initial antioxidant capacity of walnut paste only based on the ORAC assay, but this difference attenuated during the storage, up to annulment at day 7. As already mentioned, at the end of the study, samples showed a slightly higher antioxidant activity in the walnut paste without extract (Fig. 2c).

Considering added amount (5000 ppm w/w) and antioxidant capacity of the grape extract (section 2.1), the measured values in the initial supplemented walnut paste (day 0) did not correspond to the theoretical ones based on extract addition. In particular, ABTS and FRAP were lower than expected by 34% and 28%, respectively, while total phenolic content was 24% lower. The ORAC assay, on the other hand, provided much higher results than expected (+139 %). It must be considered that evaluation of total phenolic content and antioxidant capacity were carried out on the defatted walnut paste (as recommended by Arranz et al., 2008). However, it was demonstrated high fat content of walnut can interfere in the determination of antioxidant capacity and total polyphenols in the extracts: in some cases high fat content can prevent quantification of hydrolysable tannins and their antioxidant capacity in extraction residue (if emulsions are formed), in other cases actual values may be overestimated (Arranz et al., 2008). Nevertheless, on the basis of results obtained over time, the grape extract addition did not exert any significant and decisive role in terms of enhancing antioxidant capacity of walnut paste.

Walnuts are a good source of antioxidants in their lipid fraction and, in fact, α -, γ -, and δ -tocopherols, the main lipid soluble compounds with antioxidant activity, were identified both in fresh and roasted walnut oil. Among them, γ -tocopherol was reported to be the major homologous (Vaidya and Eun, 2013). Tocopherols can transfer a hydrogen atom at the 6-hydroxy group on their chroman ring to lipid peroxy radical and scavenge the peroxy radicals. Lipid peroxy radicals react with tocopherols much faster than with lipids: one tocopherol molecule can protect about 10^3 to 10^8 polyunsaturated fatty acid molecules at low peroxide value. The effectiveness of tocopherols as antioxidants depends on the isomers and on concentration. Free radical scavenging activity of tocopherols is the highest in δ -tocopherol followed by γ -, β -, and α -tocopherol (Choe and Min, 2006).

In the present study, γ -tocopherol resulted the predominant homologous in all samples, followed by α - and δ -tocopherol (Table 2). The ratio of the isomers remained constant over the observation period (Fig. 3a, b, and c). By analysing trend data on the walnut paste, it can be noted that all the tocopherols underwent a similar and drastic reduction (-60.8%) in the first 4 days. These results are in disagreement with those reported by Vaidya and Eun (2013) who observed a greater retention of γ - and δ -tocopherols, and a significantly lower stability of the α -isomer in oil from roasted walnuts stored at 60 °C: the average decreases of γ -, δ -, and α -tocopherols were 0.7%, 2.8%, and 16.4%, respectively, at 6th day of storage. In the walnut paste all the tocopherol homologues increased at day 7, then decreased again and stabilized from the 9th to the last day. Although a negative correlation between storage time and tocopherol content is known, the tocopherol contents can increase during heating due to the release of tocopherols from ruptured membranes and broken bond between tocopherol and phospholipids or proteins (Vaidya and Eun, 2013).

Since the added grape extract supposedly does not contain fatty fraction, nor even tocopherols, no substantial differences were expected in the initial content of the enriched walnut paste. Conversely, the samples with extract showed on average a tocopherol content equal to one third of the value of the walnut paste without extract (Figs. 3a, 3b, and 3c). Again, the trends were like those observed in not enriched paste. Tocopherols increased at day 2 (reaching values close to those measured in the initial sample without extract), then decreased until 9th day, and subsequently stabilized to values comparable to the initial ones. The procedure of handily mixing walnut paste with grape extract (with consequent greater exposure of the sample to light and air) could be responsible of the initial depletion of tocopherols. They might have exerted their antioxidant action already at this stage, supporting the behaviour of enriched samples that seemed to anticipate the trend of samples without extract. At the end of the storage, the samples were similar for the δ - and γ -tocopherol residual content. Only α -isomer was slightly higher in walnut paste without extract (Fig. 3a).

This seems in contrast with the results obtained by the Oxitest in the preliminary tests. However, forced conditions kept during Oxitest (6 bar oxygen pressure and 90 °C temperature) could provide information on oxidative stability of samples not comparable with those obtained during accelerated shelf life test at 60 °C, open air.

Overall, data obtained from the 15 days storage at 60 °C show that the grape extract addition to the walnut paste does not improve its oxidative stability. Indeed, natural and/or exogenous antioxidants at high levels (most of 0.01% or less) can behave as pro-oxidants, because involved in the initiation step of lipid oxidation, and in important phenomena such as synergisms and degradations (Madhavi et al., 1996).

The polyphenols contained in the grape extract should to be able to directly reduce peroxyradicals, but their hydrophilic nature and remoteness from lipophilic radicals could hinder all direct contact reactions (Spigno et al., 2013). Moreover, edible oil and fat matrices often contains multicomponent antioxidants, and interactions among them were widely demonstrated (Vaidya and Eun, 2013). Alpha tocopherol and phenolic compounds proved to be more effective in preserving olive oil when used together than used separately (Velasco and Dobarganes, 2002); however, α -tocopherol can also exert an adverse effect on the antioxidation of olive oil by phenolic compounds, such as tyrosol or oleuropein, during the period of low hydroperoxide accumulation (Blekas et al., 1995). Hence, the joint action of antioxidant compounds, deriving from roasted walnuts and grape pomace extract, may have influenced the antioxidant capacity and/or its evaluation in the examined samples.

Over time, foods generally undergo changes that depends on chemical composition and environmental factors (Abedi Gonabad et al., 2015). These aspects interactively affect the oxidation of the lipid fraction and it is not easy to differentiate their individual effect (Choe and Min, 2006)). As temperature is one of the most important external factor affecting the quality and the shelf life of food products, in this paper the accelerated storage test was performed by keeping the samples at 60 °C to simulate an abuse (Solco, 2007).

Fifteen-day storage at 60 °C overall resulted in an increase in peroxides and only a slight raise in conjugated dienes (both primary oxidation indicators). Tocopherols significantly decreased only in the walnut paste without extract. However, in sample with extract, the initial values were lower, hence the obtained final levels were comparable. It can be therefore deduced that walnut paste was in itself a stable product, as the set storage conditions still provided final sample of acceptable quality.

Since parameters indicating oxidative process progression (peroxides and conjugated dienes) started increasing on the 15th day, it is conceivable that the quality deterioration of the product started at the end of the observation period, even though remaining below the threshold of unacceptability.

The activation energy (E_a) of 80,327 kJ/mol (Abedi Gonabad et al., 2015) considered in setting accelerated shelf life test conditions referred to the oxidative kinetics of a fresh walnut oil, while in the present work a walnut paste obtained from whole roasted kernels was studied. Preliminary thermal treatment and more complex matrix (richer in antioxidants and dietary fibre than oil) may have accordingly affected the activation energy of the oxidative process, increasing it (Solco, 2007). The adoption of a different E_a value than the real one could have led to overestimating the acceleration factor of oxidation and, as a consequence, the corresponding storage at ambient conditions (20 °C). The results described above are in good agreement with those reported by Ling et al. (2014) who showed that the walnut paste from thermal treated kernels was more stable against oxidation than walnut kernels and cold pressed walnut oils (Ling et al., 2014). In a previous study Vaidya and Eun (2013) already supported roasting of walnut kernels as an appropriate method for extending the oxidative stability of oil during storage in the dark.

4. Conclusions

Walnut paste is usually obtained by roasting and grinding of kernels; it is used in confectionery and traditional products, and may find application in innovative foods (e.g. sausages and meat preparations) as fat replacer and functional component. In the present study, oxidative stability of walnut paste was evaluated during 15-day storage at 60 °C. Moreover, the potential efficiency of the incorporation of a grape skin extract at 5000 ppm in improving the shelf life of the walnut paste was assessed.

The experimental results showed that deterioration in quality of walnut paste itself started only at the end of the observation period, even though remaining below the threshold of unacceptability. The extract addition did not prove to enhance oxidative stability and/or antioxidant properties of the walnut paste.

Further investigations are needed to identify the specific parameters of oxidation kinetics (i.e. activation energy) and, eventually, to detect any antioxidant activity exerted by the supplemented extracts in the most advanced phase of storage, extending the monitoring time.

Declarations

Author contribution statement

Roberta Dordoni: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Silvia Cantaboni: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Giorgia Spigno: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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