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Walnuts and almonds as model systems of foods constituted by oxidisable, pro-oxidant and antioxidant factors

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ABSTRACT

Walnuts and almonds were selected as complex model systems in order to study possible interactions among oxidisable substances, pro-oxidant species and antioxidants. Lipoxygenase activities in a direct-micelle system were determined for these nuts according their globulin contents in their soluble protein fraction. Walnut lipoxygenase activity was 1.5 times higher than that of almonds. Among antioxidant compounds, hydrophilic contents were analysed in these nuts, hence, extractable, hydrolysable and condensed phenolic compound fractions were determined as well as lipophilic phenolic concentration corresponding to tocopherol fraction. Extracts of brown skins and whole walnuts have significantly higher phenolic contents than those of almonds. Radical scavenging activities of phenolic extracts were measured. Besides, the experiments demonstrated the ability of all nut extracts to inhibit the coupled oxidation of linoleic acid with β-carotene induced by lipoxygenase. The antioxidant substances were mainly found in nut brown skins. Walnut extracts showed higher antioxidant activity than those of almonds. Lipoxygenase pro-oxidant activity was also measured in AOT reverse micelles in conditions that model these nuts.

1. Introduction

Some foods can be studied as models of complex systems constituted by diverse substances distributed in different microphases, according to their lipo or hydrophilicity and physicochemical properties. Walnuts and almonds are interesting cases to investigate possible interactions among oxidisable phytochemicals, oxidant species, antioxidants and other factors that determine the food oxidative status. Oxidation processes of their constituents may result in partial or even total modifications of their nutritional or sensorial properties, due to losses of essential fatty acids or vitamins, off-flavour production, and colour changes by pigment destruction. Walnuts as well as almonds are foods rich in oil especially in unsaturated fatty acids. In the case of almonds, monounsaturated fatty acid content accounts for more than 60% of the total fat content, while, for walnuts, the major constituents are poly-unsaturated compounds with about a 73% (Venkatachalam & Sathe, 2006). These large contents in mono, di and poly-unsaturated fatty acids of these nuts, make them highly susceptible towards oxidation reactions induced by environmental factors such as humidity, temperature and oxygen content in the storage atmosphere (Zacheo, Capello, Perrone, & Gnoni, 1998). These are exogenous factors that promote oxidation processes but there are also endogenous factors such as oxidizing enzymes occurring in these natural foods as the lipoxygenase (LOX). This is a dioxygenase capable of oxidizing fatty acids containing a *cis*, *cis*-1,4-pentadiene system as specific substrates, yielding conjugated diene hydroperoxides (Robinson, Zecai, Claire, & Rod, 1995). It is widely spread in nature and it is present in a great variety of plants as well as in animal kingdom. Its activation normally takes place when a disruption of plant tissue occurs. Then a sequential process starts, lipases catalyze the release of fatty acids, and lipid peroxidation is initiated (López-Nicolás, Pérez-Gilabert, & García Carmona, 2001). Even when LOX is not activated, the oxidation of lipids may occur by an auto-oxidation process forming hydroperoxides as primary products but at a slower rate. Lipid oxidation reactions lead to food organoleptic changes due to the formation of volatile compounds from subsequent degradation of hydroperoxides. Many reports about plant LOXs are found in the literature as those obtained from several legumes (Clemente, Olías, & Olías, 2000; Szymanowska, Jakubczyk, Baraniak, & Kur, 2009; Yoshie-Stark & Wäsche, 2004), tomatoes

Abbreviations: LOX, lipoxygenase; FC, Folin Ciocalteu; CBBG, Comassie blue brillant G-250; PVP, polyvinylpyrrolidone; DPPH·, 1,1-diphenyl-2-picrylhydrazyl radical; GA, gallic acid; LA, linoleic acid; ABTS·+, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; AOA, antioxidant activity; ARA, antiradical activity; AOT, sodium bis(2-ethylhexyl)sulphosuccinate or docusate sodium salt; W, surfactant-water mole ratio; CM, chloroform–methanol; HM, hexane–methanol.

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(Smith, Linforth, & Tucker, 1997), and olives (Ridolfi, Terenziani, & Patumi, 2002). However, their physiological role is still largely unclear. One of the most studied enzymes is that present in soybeans because their LOXs are far more active than those of other plants (Song, Love, & Murphy, 1990). Moreover, isolated soybean LOXs are commercially available. In some cases, such as legumes, LOXs are abundant proteins accumulated in mature seeds and could serve as storage protein. In other cases, as in the case of some nutty species such as almond and hazelnut, the presence of volatile aldehydes produced by the LOX pathway in lipid bodies of mature almonds, could indicate an important role for these enzymes in determining the organoleptic properties of these nuts. LOX activity of walnuts and almond has been investigated after heat treatments and enzyme inactivation produced by heating has been reported (Buranasompob et al., 2007). These enzymes exhibited the greatest activities at pH 7 when the oxygen consumption of the system was monitored. Almond lipoxygenase was identified as a 9-LOX according to the hydroperoxide structure obtained as linoleic acid oxidation products in the enzyme catalyzed reaction (Giovanni et al., 2001).

According to Osborne fractionation of soluble proteins (Venkatachalam, Roux, & Sathe, 2009), LOX belongs by their solubility to the globulin group and, based on their sedimentation constants values at pH 7.6 and 0.5 of ionic strength, to the 7S fraction (Préstamo, Lesmes, Otero, & Arroyo, 2000).

Walnut and almonds have other valuable constituents from the nutritional point of view besides lipids and proteins as dietary fibre, phytoesterols, and tocopherols among other phytochemicals (Amaral, Rui-Alves, Seabra, & Oliveira, 2005). These nuts also possess numerous polyphenolic compounds with potent free radical scavenging ability and therefore, capable to break the propagation chain of lipoperoxidation (Fukuda, Ito, & Yoshida, 2003). The antioxidant activity of extracts from a great variety of nut oils has recently been reported (Arranz, Cert, Pérez-Jiménez, Cert, & Saura-Calixto, 2008; Miraliakbari & Shahidi, 2008).

Nut phenolic compounds include two important fractions, extractable and non-extractable ones. The former fraction includes phenolic acids and flavonoids, while the latter corresponds to condensed tannins-proanthocyanidins and hydrolysable phenolic compounds. These high molecular-weight compounds are usually associated to dietary fibre and indigestible compounds (Saura-Calixto, Serrano, & Goñi, 2007). In the case of walnuts, antioxidant activity of their hydrolysable tannins is reported to be similar to that of superoxide dismutase enzyme (Fukuda et al., 2003).

Unsaturated lipids are prone to oxidation and coexist in these nuts with: (i) pro-oxidant enzymes which use them as specific substrates and (ii) free radical scavengers which could also behave as good inhibitors of such enzymes. As a result, shelf life of these foods will depend on the balance between oxidative damage processes and protective action exert by the antioxidants. The aim of this work was to establish the possibility of interactions between nut active constituents, pro-oxidant, antioxidant and oxidisable substrates, all of them coexisting in these model foods.

2. Materials and methods

2.1. Materials

Walnuts (*Junglans regia*) and almonds (*Prunus dulcis*) were purchased from the local market. Nuts were cracked and shelled. Kernel nut samples were powdered using a mortar or electric mill. Skin of nuts was separated for specific analysis, by soaking the nuts in water overnight and hand removed. Brown skin was dried by air flow and grounded in a coffee grinder for 1 min. Soybeans (*Glycine max*) were purchased from the local market as dried seeds.

Comassie blue brillant G-250 (CBBG) was purchased from Merck. Tween 20 (polyoxyethylene-sorbitan monolaurate), α-tocopherol, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Aldrich. Soybean lipoxidase type I-B, serum bovine albumin, β-carotene, sodium bis(2-ethylhexyl)sulphosuccinate or docusate sodium salt (AOT), and Folin–Ciocalteu's phenol reagent were obtained from Sigma and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was purchased from Fluka. Linoleic acid 99% was obtained from Riedel de Häen. Quercetin and ammonium sulphate were provided by Parafarm. Polyvinylpyrrolidone (PVP) was from Anedra, Buenos Aires, Argentina.

2.2. Methods

All assays were performed by triplicate at room temperature 25 ± 1 °C.

2.2.1. Moisture

The moisture content of fruits was measured using the method of drying to constant weight. Accurately weighed samples of finely chopped nuts were dried in a conventional oven at 105 °C.

2.2.2. Fat content

The total fat content was determined by direct extraction of an accurately weighed sample in a Soxhlet apparatus using as solvent petroleum ether (boiling point range of 35–60 °C) in seven cycles for 6 h. Lipid extract was placed in a weighed round bottomed flask. Solvent was removed in a rotatory evaporator and finally flushed with nitrogen. Fat content was determined gravimetrically.

2.2.3. Soluble protein content

Dyeing solution for protein determination was prepared by diluting 12 mg of CBBG in 10 ml absolute ethanol with 3% perchloric acid solution to 200 ml. Extracts were prepared with 4 g whole nuts with 50 ml ultra-pure water stirred for 45 min, filtered under vacuum and then centrifuged at 8 °C at 10,000 rpm for 10 min. Protein determination was performed by Bradford method (Bradford, 1976), placing 1 ml of CBBG solution and an aliquot of nut extract in a cuvette and completed to 3 ml with buffer pH 7. Absorbance was measured at 596 nm. Serum bovine albumin was used as a reference of a water soluble protein for the calibration curve and expressed in mg of protein/g of nut.

2.2.4. Total phenolic compound content

Extracts were prepared by taking portions of 2 or 0.5 g, of whole nuts and brown skins, respectively, mixed with 50 ml ultra-pure water or methanol and stirred for 45 min, then, filtered under vacuum and centrifuged at 10,000 rpm for 10 min. The supernatant was recovered and total phenolic compound content was determined by the Folin Ciocalteu (FC) method (Singleton & Rossi, 1965). Gallic acid (GA) was used as reference compound for calibration purposes.

2.2.4.1. Extractable phenolic compounds. Approximately 2 g of powdered whole nuts were stirred with 50 ml of 50:50 methanol–water mixtures for 60 min at room temperature and then filtered under vacuum. A second extraction of this residue was carried out with 50 ml of 70:30 acetone–water at room temperature for 60 min. After vacuum filtration, both extracts were combined to determine extractable phenolic compounds of the original sample by FC method.

2.2.4.2. Non-extractable phenolic compounds. The residue obtained after the extractable polyphenol procedure was extracted by

heating with methanol/acetone/water mixture. Condensed tannins-proanthocyanidins and hydrolysable phenolic compounds were determined separately.

- *Condensed tannins/proanthocyanidins:* The residue of the precedent extraction was mixed with 0.25 ml HCl/50 ml of butanol mixture and heated to 100 °C for 3 h. Determinations were performed by measuring the absorbance at 550 nm using cyanidin-3-glucoside as a reference compound whose ε is 26,900 at this wavelength (Kuskoski, Asuero, Troncoso, Mancini-Filho, & Fett, 2005).
- *Hydrolysable phenolic compounds:* This fraction is formed by hydrolysable tannins, phenolic acids and hydroxycinnamic acids released from the food matrix by strong acid hydrolysis. The residue of the precedent extraction was subjected to hydrolysis with 50 ml of 90:10 v/v of methanol/sulphuric acid mixture at 85 °C for 20 h and finally analyzed by the FC method (Hartzfeld, Forkner, Hunter, & Hagerman, 2002).

2.2.4.3. Lipophilic phenolic compounds. Approximately 4 g of powdered whole nuts were stirred with 40 ml of hexane for 20 min at room temperature and then filtered under vacuum. This extraction procedure was repeated three times and the extracts were combined. The lipid extract was treated with anhydrous sodium sulphate, filtrated, evaporated in a rotatory evaporator and dried under nitrogen flush to obtain the oil. To isolate the phenolic minor components from the oil, a 2 g portion of it was placed in a separatory funnel with 60 ml of hexane and extracted three times with 30 ml of methanol. The methanolic fractions were combined and total phenolic content was determined by FC method. An alternative procedure using a (2:1) chloroform–methanol mixture was also assayed to extract the nut oil (Miraliakbari & Shahidi, 2008). An amount of 2 g of oil was mixed with methanol, stirred for 15 min and centrifuged at 10,000 rpm for 10 min. The process was repeated twice and the supernatants were combined. Total phenolic content was determined in this extract by FC method. α-Tocopherol and GA were used as reference compounds for calibration purposes.

2.2.5. Antiradical activity determination

Two methods were used to monitor the antiradical activity (ARA) of nut extracts to scavenge radical species. The disappearance of the coloured radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH·) and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\cdot+}$) was measured by spectrophotometry as the colour loss of their solutions by antioxidant action.

The DPPH· method was carried out according to Brand-Williams, Cuvelier, and Berset (1995) measuring the absorbance reduction at 517 nm after the antioxidant addition. The DPPH· methanolic solution was placed into a cuvette and mixed with aliquots of different extracts or a pure compound solution to a final volume of 3 ml. The initial radical absorbance was 1.00 at 517 nm when a solvent aliquot was added instead of the extract. The absorbance decrease at 517 nm was monitored in 20 cycles in periods of 10 s. The % ARA of the extracts was calculated by the equation proposed by Burda and Oleszek (2001):

$$\%\ \mathrm{ARA} = 100 \times \left[1 - \frac{A_{SS}}{A_0}\right]$$

where A_0 is the absorbance of DPPH· solution before adding the antioxidant and A_{SS} is the absorbance of the solution at the steady state estimated by mathematical fitting of kinetic curves performed with Origin 8.0 software (Chaillou & Nazareno, 2006). Calibration curve was prepared with GA, quercetin and Trolox in order to express ARA in equivalents of these compounds.

The $ABTS^{\cdot+}$ method was carried out according to Re et al. (1999). $ABTS^{\cdot+}$ solution was obtained by reaction of 7 mM ABTS solution with 2.45 mM potassium persulphate in 1:0.5 relation, incubated in darkness for 16 h at room temperature. The radical solution was diluted with ultra-pure water, placed into a cuvette and mixed with aliquots of different extracts or a pure compound solution to a final volume of 3 ml. In the case of the tocopherol fractions obtained from the oil extractions, an aliquot of the methanolic extracts was mixed with an appropriate amount of Tween 20, dried with nitrogen and re-suspended with water in order to assay the antiradical activity of hydrophobic phytochemicals in the aqueous medium. The initial radical absorbance was 1.00 at 734 nm when a solvent aliquot was added instead of the extract. The absorbance measurement at 734 nm was carried out in 20 cycles of 10 s. The data were processed and % ARA of the extracts was calculated by the same previous procedure. Calibration curve was prepared with GA, quercetin, α-tocopherol, and Trolox in order to express ARA values in equivalents of these compounds.

2.2.6. Protein group fractionation

Soluble proteins were fractioned into five main groups according to their solubility (Sugimoto, Tanaka, & Kasai, 1986), namely *albumins* (water soluble), *globulins* (dilute salt soluble), *prolamins* (70% aqueous alcohol soluble), *glutelins*-1 (acid soluble), and *glutelins*-2 (alkali soluble).

Samples of 20 g of chopped walnuts previously defatted were extracted with 100 ml of water using a domestic blender and stirred for 4 h. Liquid phase was centrifuged at 12,000*g* for 30 min and the supernatant corresponds to fraction 1 (*albumins*). Solid residue was extracted by stirring with 100 ml of a 5% NaCl solution at room temperature for 4 h. Extract was centrifuged for 30 min at 12,000*g* and the supernatant corresponds to fraction 2 (*globulins*). The same procedure was used to extract fraction 3 (*prolamins*) with 70% isopropyl alcohol. Subsequently, fraction 4 (*glutelins*-1) and fraction 5 (*glutelins-2)* were extracted from the solid residue with 50% acetic acid and 0.1 M NaOH solution, respectively. Protein contents of these fractions were determined by Bradford method (Bradford, 1976).

2.2.7. Isolation of lipoxygenases

2.2.7.1. Soybean LOX. The enzyme purification procedure was carried out according to Fukushige, Wang, Simpson, Gardner, and Hildebrand (2005) report with minor modifications. A sample of 5 g of soybean seeds were peeled and chopped in an electric coffee grinder and powdered in a mortar. Then, 50 ml of ultra-pure water were added. In order to precipitate polyphenolic compounds, 0.005 g of PVP were added and the mixture was stirred for 15 min. Insoluble solids were filtered under vacuum. Supernatant was centrifuged at 10,000*g* for 10 min. $CaCl_2$ was added to a final concentration of 43 mM and the solution was incubated for 30 min to precipitate storage proteins. The mixture was incubated at 8 °C for 30 min and then centrifuged at 12,000*g* for 10 min. The supernatant was filtered and $(NH_4)_2SO_4$ was added to a final concentration of 1.7 M and then incubated at 8 °C for 60 min. Extract solution was centrifuged at 12,000*g* for 15 min. Supernatant was carefully removed and $(NH_4)_2SO_4$ was added until its concentration increased to 4.08 M. After 60 min of incubation at 8 °C, centrifugation was carried out. Finally, remaining pellet was re-suspended in 5 ml of ultra-pure water and immediately used for activity measurements.

2.2.7.2. Walnut and almond LOX. Some modifications to the methodology previously described for soybeans were needed to isolate the fraction containing the enzymatic activity. Walnuts or almonds

were peeled to remove their brown skins. Nuts were chopped in an electric coffee grinder, and then, powdered in a mortar. An aliquot of 100 ml of ultra-pure water was added to 20 g of powder. The mixture was stirred for 15 min and filtered under vacuum. An amount of 0.2 g of PVP was added to the supernatant. The mixture was stirred for some minutes and the insoluble solids were removed by filtered under vacuum and centrifuged at 10,000*g* for 10 min. $CaCl_2$ was added and taken to a final concentration of 30 mM followed by incubation for 30 min to precipitate storage proteins and to activate LOX (Klein, 1976). The mixture was incubated at 8 °C for 30 min and then centrifuged at 12,000*g* for 15 min. The supernatant was discarded and the pellet was re-suspended in 5 ml of ultra-pure water and immediately used for activity measurements.

2.2.8. Enzymatic activity

Pro-oxidant-enzyme actions in direct and reverse micellar systems were determined by a spectrophotometric analysis at 234 nm based in conjugated diene formation from linoleic acid oxidation enzymatically induced.

Linoleic acid (LA) solution was prepared by mixing 20 μl with 200 μl of Tween 20 and completed with buffer pH 9 up to 5 ml. LA solution was placed in a 3 ml cuvette and an aliquot of LOX solution was added to initiate the reaction, which was measured by monitoring the absorbance at 234 nm for 10 min. LOX activity for soybean, walnut and almond extracts were performed using the same procedure. All the assays were carried out in triplicate at room temperature (25 ± 1 °C). One unit of LOX activity was defined as the amount that causes an increase in absorbance at 234 nm of 0.001 AU/min at pH 9 at 25 °C when LA is the substrate in 3.0 ml volume (1 cm light path).

Enzymatic activity was also evaluated in a reverse-micelle system according to Pérez-Gilabert, Sánchez-Ferrer, and García-Carmona (1992) experiments with some modifications. The experiment was carried out using AOT as surfactant and isooctane as organic solvent. The total amount of water present in the system was expressed as the molar ratio between water and the surfactant ($W = [H_2O]/[AOT]$). A 25 mM stock solution of AOT was prepared in isooctane. A 20 μl aliquot of LA stock solution in isooctane was added with a microsyringe to the AOT solution placed in a quartz cuvette of 0.2 cm optical path. LA concentration in the experiment was 120 μg ml^{-1}. An amount of 4 μl of a 1 mg ml^{-1} LOX solution in 25 mM borate buffer of pH 9 was added to the cuvette. Additional borate buffer of pH 9 was added with a microsyringe to fit *W* to a final volume of 0.600 ml. *W* was varied between 15.4 and 61.8. The effect of LA concentration was evaluated between 60 and 240 μg ml^{-1}. Spectral changes were monitored by spectrophotometry in cycles of 2 min for 20 min and the formation of LA conjugated dienes was determined at 234 nm. Activity values were calculated by correcting the absorbance to a 1 cm optical path.

2.2.9. Antioxidant activity of walnuts and almonds in the β-carotene-linoleic acid co-oxidation enzymatically induced by lipoxygenase

The experiment was carried out according to Chaillou and Nazareno (2006) with minor modifications. Linoleic acid solution was prepared by mixing this compound with Tween 20 and diluting with phosphate buffer pH 7 up to a 330 μg ml^{-1} concentration. An aliquot of 500 μl of a saturated stock solution of β-carotene in chloroform was mixed with the same amount of Tween 20. Chloroform was removed using a stream of nitrogen for 20 min. The final solution was prepared adding phosphate buffer pH 7. β-Carotene and linoleic acid solutions were mixed in a 3 ml cuvette; then, an aliquot of each phenolic extract was added. Finally, 200 μl of 1.0 mg ml^{-1} LOX solution were added to initiate the reaction, which was measured by monitoring the absorbance at 460 nm for 10 min. Carotene initial absorbance was adjusted to 1 AU. The same procedure excluding extract addition was done for control. All the assays were carried out in triplicate at 25 ± 1 °C. Antioxidant activity (AOA) was calculated as suggested by Burda and Oleszek (2001) as the inhibition percentage of the β-carotene bleaching by the samples compared to that of the control using the following equation:

$$\%\ \mathrm{AOA} = 100 \times \left[1 - \frac{(A_s^0 - A_s^t)}{(A_c^0 - A_c^t)}\right]$$

where A_s^0 and A_c^0 were the absorbances at $t = 0$ min of the sample and the control, A_c^t and A_s^t were the absorbances at $t = 10$ min of control and sample, respectively. GA was used as a reference compound.

2.2.10. Antioxidant activity of nut components in a reverse micellar system

The inhibition of LA oxidation induced by LOX was also measured in reverse micelles placing 560 μl of a 25 mM AOT solution in isooctane in a quartz cuvette of 0.2 cm optical path. Aliquots of 20 μl of LA solution in isooctane, 4 μl of 1 mg ml^{-1} LOX solution in a pH 9 borate buffer and additional buffer to fit *W* to 61.8 were added with microsyringes. Different concentrations of the lipophilic antioxidant were assayed, α-tocopherol being the reference compound. Spectral changes were monitored by spectrophotometry at 234 nm for 20 min. AOA was calculated according to Burda and Oleszek (2001) equation.

2.2.11. Statistical analysis

Data were statistically analyzed using Infostat computing program. The average and standard deviation and one-way ANOVA were determined for all the measurements. Significant difference was statistically considered at a level of $P < 0.05$. Correlations among ARA against DPPH· or $ABTS^{\cdot+}$, AOA and phenolic contents were established using analysis of variance models.

3. Results and discussion

3.1. Moisture, total fat and total protein contents

In order to characterise the main constituents of walnuts and almonds, moisture, total fat and total soluble proteins content were determined and the values obtained are shown in Table 1. Nut analyses demonstrated that fat is their main constituent being walnut content higher than that present in almonds. The results obtained in nut major components are quite similar to those reported by Venkatachalam and Sathe (2006). Moreover, Amaral, Casal, Pereira, Seabra, and Oliveira (2003) found similar fat contents in walnuts of six Portuguese cultivars ranging from 62.3% to 66.5%. In the case of soluble protein contents, almonds presented higher amounts than walnuts although both percentages

Table 1
Constituents of walnuts and almonds: moisture, total fat and soluble protein contents.

Constituents	Walnut contents	Almond contents
Moisture%	2.23 ± 0.06	1.9 ± 0.1
Fat%	65 ± 4	56.7 ± 0.5
Soluble protein content%	6.0 ± 0.4	9.9 ± 0.2
Soluble protein fractions (mg g^{-1})		
Albumins	0.5 ± 0.1	11.9 ± 0.3
Globulins	3.1 ± 0.1	12.4 ± 0.5
Prolamins	0.050 ± 0.006	1.82 ± 0.09
Glutelins-1	44.3 ± 0.5	59.7 ± 0.7
Glutelins-2	0.017 ± 0.006	0.041 ± 0.005

were relatively low. Moisture percentages of both, walnuts and almonds, were quite low as expectable for this kind of nuts. This moisture level is related to diverse undesirable chemical and biological reactions and therefore, it is a determining factor that contributes to the long-storage lifetime of nuts.

3.2. Extractable and non-extractable phenolic compounds

Among their minor constituents, walnuts and almonds present a variety of bioactive substances as the phenolic compounds. From the structural and physiological point of view, these are divided in several diverse groups. The content of the different subgroups may be estimated according to the nature of the solvent used for extraction purposes. Table 2 shows values corresponding to phenolic compound contents of aqueous and methanolic extracts prepared with whole walnuts or almonds or their brown skins; lipophilic phenolic compounds from nut oils were also determined.

Extracts of whole walnuts have significantly higher phenolic contents than that of almonds. Phenolic contents of brown skins were remarkably higher than those of whole nuts for both extraction solvents. Nut skins concentrate the major amount of phenolic compounds being 6–9 times higher in brown skins than in whole nuts. This statement agrees with that reported in the literature. In this sense, Wijeratne, Abou-Zaid, and Shahidi (2006) found that ethanolic extracts of almond skin present a total phenolic content 10 times higher than the whole nut. Besides, Monogas, Garrido, Lebrón-Aguilar, Bartolomé, and Gómez-Cordovés (2007) reported that brown skin represents about a 4% of the total almond weight although it contains between 70% and 100% of the total phenolic content.

Methanol was more efficient than water in phenolic extraction from whole walnuts, while, in the case of almonds results were similar. In the case of nut brown skins, water was more efficient as extraction solvent than methanol. According to these results, walnuts phenolic compounds contents are considerably higher than those of almonds and they are concentrated mainly in the brown skins of these nuts.

Table 2
Phenolic fractions in walnuts and almonds in relation to the extraction conditions.

Nuts	Phenolic extracts	Total polyphenol contents (μg gallic acid/mg nut)
Walnuts		
Whole nut	Methanol	15.5 ± 0.5
	Water	11.26 ± 0.04
	Successive extraction[a]	27.5 ± 0.4
	Hydrolysis[b]	3.77 ± 0.09
	Tannins-proanthocyanidins[c]	0.250 ± 0.003
	Oil phenolic fraction[d]	0.49 ± 0.04 (17 ± 1)[f]
	Oil phenolic fraction[e]	0.319 ± 0.002 (9.97 ± 0.04)[f]
Brown skin	Methanol	101 ± 4
	Water	126 ± 5
Almonds		
Whole nut	Methanol	1.44 ± 0.01
	Water	1.42 ± 0.01
	Successive extraction[a]	2.20 ± 0.06
	Hydrolysis[b]	2.65 ± 0.02
	Tannins-proanthocyanidins[c]	0.240 ± 0.001
	Oil phenolic fraction[d]	0.49 ± 0.03 (17.0 ± 0.7)[f]
	Oil phenolic fraction[e]	0.230 ± 0.002 (6.81 ± 0.05)[f]
Brown skin	Methanol	7.9 ± 0.1
	Water	12.57 ± 0.01

[a] Methanol–acetone–water mixture.
[b] Methanol–sulphuric acid mixture.
[c] HCl/butanol.
[d] 1.Hexane–2.Methanol.
[e] Chloroform–methanol mixture.
[f] α-Tocopherol equivalents (μg/100 mg nut).

Moreover, extractable phenols are mentioned in the literature as those that can be removed in successive extractions in methanol–acetone–water mixtures as solvent at room temperature and corresponds to monomeric or non-hydrolysable molecules (Saura-Calixto et al., 2007). Non-extractable fractions include condensed tannins and proanthocyanidins that can be removed in HCl/butanol at 100 °C as well as those hydrolysables that can be dissolved after heating in acidic conditions (MeOH–sulphuric acid mixture). Table 2 also shows those results corresponding to the determination of extractable and non-extractable phenolic compounds. Extractable polyphenol contents obtained by successive extractions were higher than total polyphenol contents from single aqueous or methanolic extracts of both whole nuts. A significant difference was found in walnut extractable fraction in respect to that of almond, whereas the values obtained for non-extractable phenolic compounds did not present significant differences between both types of nuts. In walnuts, the extractable fraction was considerably higher than the non-extractable one corresponding to hydrolysable phenolic compounds, In almonds, the hydrolysable phenolic fraction was larger than the extractable one. The condensed tannin and proanthocyanidin levels were insignificant for both nuts.

Liposoluble phenolic compounds were also extracted as minor constituents from the walnut and almond. Tocopherols and tocotrienols are the active compounds present in this oil fraction (Kornsteiner, Wagner, & Elmadfa, 2006). Total phenolic contents of these extracts by FC method are also shown in Table 2 as α-tocopherol equivalents. Two different procedures were compared as suggested by Miraliakbari and Shahidi (2008) using different extraction conditions. A sequential extraction from the nuts was carried out using hexane in first place and secondly a methanol partition (HM).This was more effective than the oil extraction using a chloroform–methanol mixture (CM) as the oil extraction solvent. Besides, values obtained for the total tocopherol equivalents of these nuts were consistent with literature data (Kornsteiner et al., 2006). Phenolic contents of the oil of walnuts and almonds presented similar values when they were obtained by the first extraction sequence. Although, as Table 2 shows, walnut content was higher than that of almond when the second extraction solvent was used. In order to visualize the relative magnitude of all phenolic groups, tocopherol fractions were also expressed in GA equivalents. Contents of walnuts and almonds were 0.319 and 0.230 μg GA equivalents/mg, respectively, for the CM procedure; although, the HM extracted values were about 0.49 μg GA equivalents/mg of nuts in both cases. These values were remarkably lower than those of the polar phenolic fractions but comparable with tannins contents. These results are in good agreement with Arranz et al. (2008) study of several nut oils. They reported 249 and 250 ppm as the tocopherol levels of walnut and almond oils, respectively. Besides, they found for total phenolic contents by FC, values of 0.32 and 0.27 mg GA/g of oils. The extraction procedure used by the mentioned authors to obtain the polar fraction from the oil gave similar results to the CM extraction of oils and subsequent treatment with methanol, indicated as the alternative procedure in this work.

Several research groups have analysed by GC and HPLC the individual bioactive compounds of the phenolic fractions obtained from nut oils (Amaral et al., 2005; Kornsteiner et al., 2006; Verardo et al., 2009). They identified α-tocopherol as the major constituent of almonds and γ-tocopherol of walnuts, accounting for more than 85% of the tocopherol fractions in both cases.

3.3. Determination of antiradical activity

In order to determine the relative ability of nut antioxidant substances to scavenge free radicals, two spectrophotometric methods

were used. The antiradical capacities against two different reactive species (DPPH· and ABTS·+) were measured by monitoring radical consumptions by the nut extract additions. DPPH· is a free radical that can be obtained ready to be dissolved before using, while ABTS·+ must be previously generated by a chemical reaction.

3.3.1. DPPH· method

Results obtained in the determination of ARA against DPPH· of whole walnuts or almonds or of its brown skins were expressed as GA equivalents and are shown in Table 3. Walnuts have higher % ARA mg^{-1} than almonds, and their brown skin extracts higher than those obtained from whole nuts.

ARA of walnut extracts expressed as GA equivalents presented the following decreasing order: brown skin aqueous extract > brown skin methanolic extract > whole nut methanolic extract > whole nut aqueous extract > tocopherol CM extract > tocopherol HM extract. Fig. 1a shows the kinetic behaviour of DPPH· solution when an aliquot of the extract corresponding to 0.1 mg of whole walnuts or the same amount of its brown skin was added. Since almond ARA was considerably lower than that of walnuts, the addition of 50-fold larger amounts of extracts became necessary to perform these measurements. Fig. 1b shows the kinetic behaviour of DPPH· solution when an aliquot of extract corresponding to 5 mg of whole almonds or its brown skin was added. ARA of almond extracts expressed as GA equivalents presented the following decreasing order: brown skin aqueous extract > brown skin methanolic extracts > whole nut methanolic extract ≅ whole nut aqueous extract > tocopherol CM extract > tocopherol HM extract.

In both cases, the mixture CM resulted more efficient than the HM in extracting antiradical compounds from nuts, although in all cases very low activities were found for these tocopherol extracts when compared to polyphenolic fractions.

3.3.2. ABTS·+ method

This method was employed to measure the ability of antioxidant substances to scavenge free radicals in aqueous solution. Table 3 shows the results obtained in the determination of % ARA mg^{-1} of nuts and the values of ARA equivalents in μg of GA per mg of whole nuts or of its brown skins. In the case of walnut, its brown skin extracts had considerably higher ARA than the whole nut extracts, and skin aqueous extracts had also higher ARA than their respective methanolic extracts. The same results were obtained for almond case where extracts from its brown skin presented higher ARA than those of whole nut. In contrast to this, methanolic and aqueous extracts of almond did not present significant differences.

Table 3
Antiradical activity (ARA) of walnut and almond extracts expressed in gallic acid equivalents (μg GA/mg).

ARA	DPPH· method	ABTS·+ method
Walnuts		
Whole nut		
Methanolic extracts	12.9 ± 0.2	8.9 ± 0.3
Aqueous extracts	4.8 ± 0.4	4.22 ± 0.03
1.Hexane–2.Methanol extracts	0.0088 ± 0.0001 (9.3 ± 0.1)[a]	0.0142 ± 0.0004 (10.2 ± 0.3)[a]
Chloroform–methanol mixture extracts	0.046 ± 0.002 (36 ± 2)[a]	0.057 ± 0.001 (41 ± 1)[a]
Brown skin		
Methanolic extracts	109 ± 9	72 ± 4
Aqueous extracts	137 ± 6	83 ± 4
Almonds		
Whole nut		
Methanolic extracts	0.174 ± 0.002	0.216 ± 0.001
Aqueous extracts	0.16 ± 0.01	0.128 ± 0.003
1.Hexane–2.Methanol extracts	0.0088 ± 0.0001 (9.4 ± 0.2)[a]	0.0143 ± 0.0002 (10.4 ± 0.1)[a]
Chloroform–methanol mixture extracts	0.0120 ± 0.0002 (10.0 ± 0.3)[a]	0.020 ± 0.001 (14.3 ± 0.4)[a]
Brown skin		
Methanolic extracts	2.78 ± 0.01	1.95 ± 0.05
Aqueous extracts	4.0 ± 0.2	3.0 ± 0.2

[a] α-Tocopherol equivalents (μg/100 mg).

The kinetic behaviour of ABTS·+ solution after the addition of an aliquot of extracts corresponding to 0.05 mg of whole walnuts or their brown skins is shown in Fig. 2a. ARA for methanolic and aqueous extracts presented the following decreasing order: brown skin aqueous extract > brown skin methanolic extract > whole nut methanolic extract > whole nut aqueous extract > tocopherol CM extract > tocopherol HM extract. Fig. 2b shows the kinetic behaviour of ABTS·+ solution with 1 mg of whole almond or its brown skin from different extracts. ARA decreases in the following order: brown skin aqueous extract > brown skin methanolic extract > whole nut methanolic extract > whole nut aqueous extract > tocopherol CM extract > tocopherol HM extract.

Lipophilic phenolic fractions presented low ARA compared to polyphenolic fractions. Moreover, ARA values are noticeably lower than those determined by FC. This can be explained by the interference of phospholipids in the FC measurements of oil extracts reported by Arranz et al. (2008) where a overestimation of the phenolic content were observed for oily samples. As it was previously found in DPPH· assays, CM extracts presented higher antiradical activities towards ABTS·+ than HM extracts. These results are consistent with Miraliakbari and Shahidi (2008) report for different nuts. In walnuts, ARA of CM extracts determined for both radicals, ABTS·+ and DPPH·, presented tocopherol equivalent values 4-fold higher than the tocopherol content measured by FC. However in almonds the differences between ARA values and FC contents were smaller. This may be understood if the different composition of the CM fractions obtained from walnuts and almonds is taken into account. The major constituent of walnut fraction is γ-tocopherol; in contrast, almond one is mainly constituted by the α-homologue. Besides, γ-tocopherol is a more active radical scavenger than α-tocopherol (Li, Tsao, Yang, Kramer, & Hernandez, 2007).

From these results it can be summarized that in these nuts the most active radical scavengers were polar phenolic compounds concentrated in their brown skins and walnut extracts presented ARA values markedly higher than almond ones. Good statistical correlations were found between antiradical activity and total phenolic compound contents in GA equivalents for the different nut extracts including tocopherol hexane–methanol extract and all R^2 were higher than 0.96 (P-value < 0.0001). In contrast, when the analysed data included tocopherol CM extracts, the statistical correlation value was lower and R^2 varied between 0.74 and 0.91.

3.4. Protein fractionation

Proteins were classified into five main groups based on their solubility, namely *albumins* (water soluble), *globulins* (dilute salt soluble), *prolamins* (70% aqueous alcohol soluble) *glutelins*-1 (acid soluble), and *glutelins*-2 (alkali soluble). Soluble protein contents of the different fractions are shown in Table 1. For walnuts as well as for almonds the major fraction corresponded to *glutelins*-1 accounting for 92.4% and 69.5% of the total content of soluble proteins, respectively. In the case of almonds, globulin fraction corresponded to a 14.4% of the total soluble protein content. A different situation was found for walnuts where all other fractions different from *glutelins*-1 were remarkably lower and practically insignificant. In particular, globulin fraction of walnuts was a 6.5% of the total soluble protein content. Similar results were found by Venkatachalam et al. (2009) who reported an 18% of globulins in

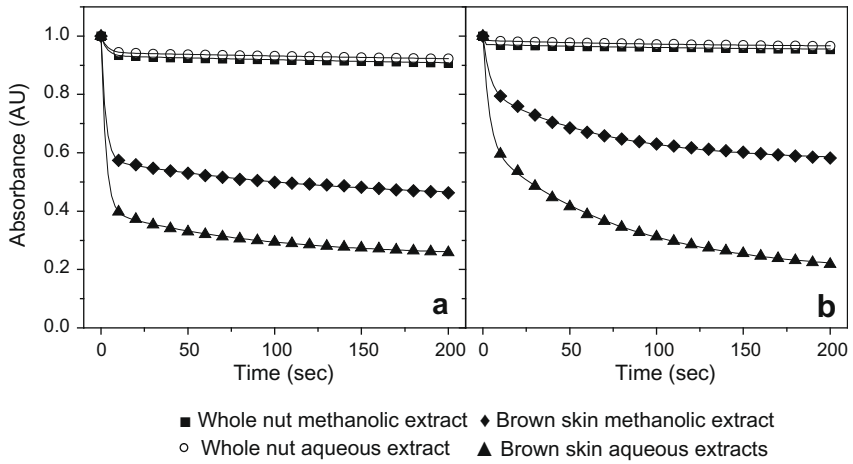

Fig. 1. Kinetic behaviours of DPPH· solutions after addition of aliquots of extracts corresponding to: (a) 0.1 mg of whole walnut or its brown skin and (b) 5 mg of whole almond or its brown skin.

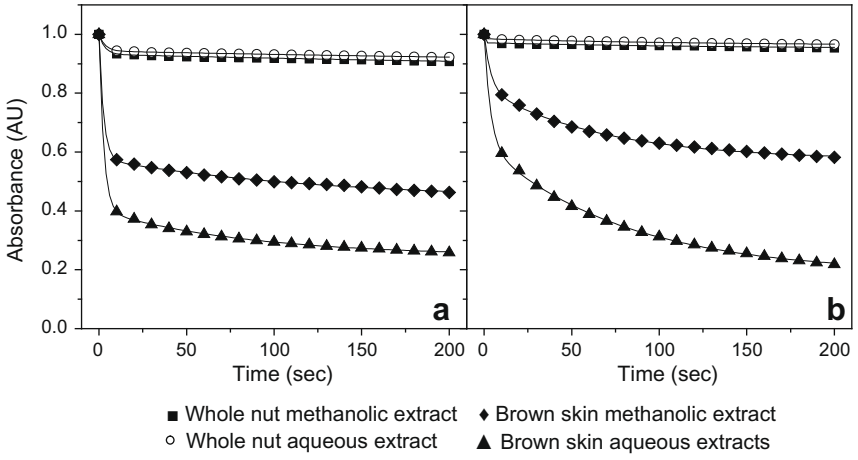

Fig. 2. Kinetic behaviour of $ABTS^{\cdot+}$ solution after addition of aliquots of extracts corresponding to: (a) 0.05 mg of whole walnuts or its brown skin and (b) 1 mg of whole almonds or its brown skin.

the total protein content of almonds. Sze-Tao and Sathe (2000) also reported glutelins as the major constituents of the total walnut proteins with a 70.11% followed by globulin fraction accounting for a 17.57%. In this case certain discrepancy in globulin content between our results and this reported value was observed.

3.5. Enzymatic activity in model systems

In order to determine its enzymatic activity, LOX isolated from nuts were incubated with LA in a micellar medium at pH 7. Diunsaturated substrate oxidation catalyzed by LOX produces hydroperoxides with a conjugated diene system, responsible for the increase of absorbance at 234 nm. Spectral evolutions during LA oxidation reactions induced by different isolated LOX are shown in Fig. 3.

Fig. 4 shows the kinetic behaviours of the lipid system in the oxidation induced by the extracted enzymes at phosphate buffer pH 7. Although the measurements were performed at the pH reported by Buranasompob et al. (2007) for highest activity of both almond and walnut enzymes, the activities of these lipoxygenase extracts were considerably lower than that of soybean LOX used as control.

Results corresponding to the enzymatic activity of soybean, walnut and almond extracts were expressed as unities of enzyme/mg of original sample of grains or nuts. Activity values determined for soybeans, walnuts, and almonds were 78.5, 9.5 and 24.75 U/mg respectively. For the same weight of foods extracted, the higher value was found for soybean, followed by almond and finally by walnut. Globulins are the major proteins in soybean representing the 80% of total proteins (Fukushima, 1991). For a better comparison of the intrinsic activity of these enzymes, their activities were calculated considering the globulin contents of the foods. Results obtained indicate that enzyme activities are 3065, 1996 and 196.25 U/mg globulins for walnuts, almonds and soybean, respectively. Therefore the most active enzyme is that of walnuts although its content in the food is the lowest.

Although nuts are rich in unsaturated lipids and contain enzyme may not be active and do not induce lipid oxidation. Activation of the enzyme seems to be initiated as a response to wounding. In the case of legumes, as soybeans, LOX could serve as storage proteins in mature seeds where they are accumulated. In other cases as in nuts, the LOX are expressed and active only in early developmental stages when the synthesis of storage proteins is not yet initiated. LOX physiological role in nuts can be explained as a defense mechanism against microorganism attack (Santino, Iannaconeb, Hughes, Casey, & Mita, 2005).

3.5.1. Reverse-micelle model

Taking into account that walnuts and almonds are foods with about 65% and 57% corresponding to fat content, respectively,

and a low moisture level (2–3%), appropriate systems to model these nuts are water-in-oil microemulsions or reverse micelles. In these systems, surfactant molecules are dispersed in an apolar solvent. Surfactant polar-head groups are oriented toward the interior of the aggregates where water microdroplets are located and other polar compounds, including enzymes, can be encapsulated. In these microheterogeneous media, the active substances are distributed in different microenvironments according to their affinity. In reverse micelles, amphiphilic compounds as the unsaturated fatty acids are expected to be linked to the micelle interface. The kinetic behaviour and catalytic reactivity of different micelle-entrapped enzymes have a strong dependence on the water pool size or hydration degree represented by W, the water-surfactant molar ratio (Biasutti, Abuin, Silver, Correa, & Lissi, 2008).

LOX activity has been previously studied in reverse micelles made with the surfactant sodium bis(2-ethylhexyl)sulfosuccinate (AOT), isooctane and water (Kurganov, Shkarina, Malakhova, Davydov, & Chebotareva, 1989; Pérez-Gilabert et al., 1992). The highest enzymatic activity has been reported for $W = 30$. However, as far as we know, there are no studies for LOX activity for W values larger than 40. In order to model walnuts and almonds higher W values are needed. Abuin, Lissi, and Jara (2007) reported $W = 62$ for isooctane as the maximum value of water solubility in AOT reverse micelles.

Moreover, as it was previously mentioned, the enzymatic activity was determined spectrophotometrically as the formation of conjugated dienes at 234 nm. This technique has a drawback in AOT reverse micelles due to the AOT absorption band in this spectral region. To overcome this, Pérez-Gilabert et al. (1992) suggested AOT concentration to never exceed 6 mM. To extend this range, in this work we proposed the use of a quartz cuvette of a reduced optical path (0.2 cm). Taking these limitations into account the maximum hydration level that can be modelled in this system is defined by [AOT] below 30 mM and W below 62. Therefore, experimental conditions selected were [AOT] of 25 mM and W of 61.8. On the other hand, it has been reported that the increase in LA concentration produces a decrease in the pH inside the micelles (Rodakiewicz-Novak, Maslakiewicz, & Haber, 1996). In consequence in the present study, the water pool was replaced by a pH 9 borate buffer.

Enzymatic activity variation as a function of W values was evaluated between 15.4 and 61.8. To the best of our knowledge, this is the first study about the pro-oxidant activity of LOX in these extreme W conditions. LOX activity went up when the hydration level increased as shown in Fig. 5, being the highest value that observed for $W = 61.8$ corresponding to system with a 3% water content similar to nut moisture level. The effect of LA concentration

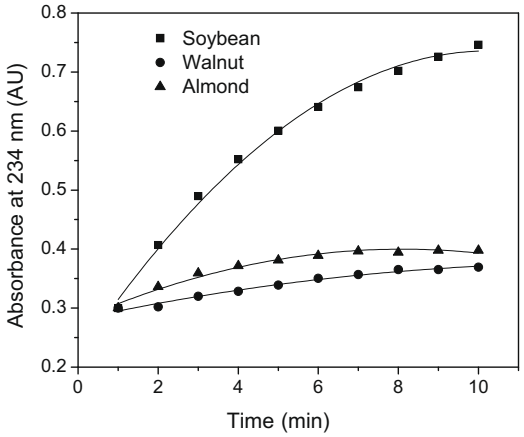

Fig. 4. Kinetic behaviour of linoleic acid oxidation catalyzed by lipoxygenases extracted from soybeans, walnuts and almonds in Tween 20 micelles.

variation on LOX activity was also analyzed in a range of 60–240 μg/ml. As Fig. 5 shows, LOX activity rose when LA concentration increased up to 160 μg/ml, but above this concentration, a slight activity decrease was found. LA must be bound to the AOT interface as Correa, Durantini, and Silber (2001) have reported for carotenoid derivatives bearing a carboxylic acid group. The driving force for the binding to the AOT interface is given by the ability of the polar end groups to interact specifically with the polar heads of AOT by hydrogen bonding.

As a partial conclusion, it can be stated that in a water-in-oil system that models walnuts and almonds, the LOX enzyme is able to interact with its specific substrate, LA, and to induce its oxidation, being the reaction rate dependent on the LA concentration and on the hydration degree. The question to be answered is if the nut phenolic compounds are able to inhibit LA oxidation induced by LOX when they are partitioned in a different microenvironment in respect of the enzyme. Polyphenol compounds are located in the same microphase as LOX and they can indeed inhibit the enzyme as it was previously observed in the direct-micelle system. In contrast to polyphenolic compounds, tocopherols are distributed nearby AOT interface in the lipophilic solvent. Assays were carried out to evaluate the capacity of tocopherol extracts in this water-in-oil model system to prevent LA from oxidizing. Antioxidant activity of α-tocopherol was measured in a reverse-micelle system using a 25 mM AOT solution in isooctane as surfactant. Results indicated that tocopherols are able to retard LA oxidation even they are located in other microphase than LOX. Although

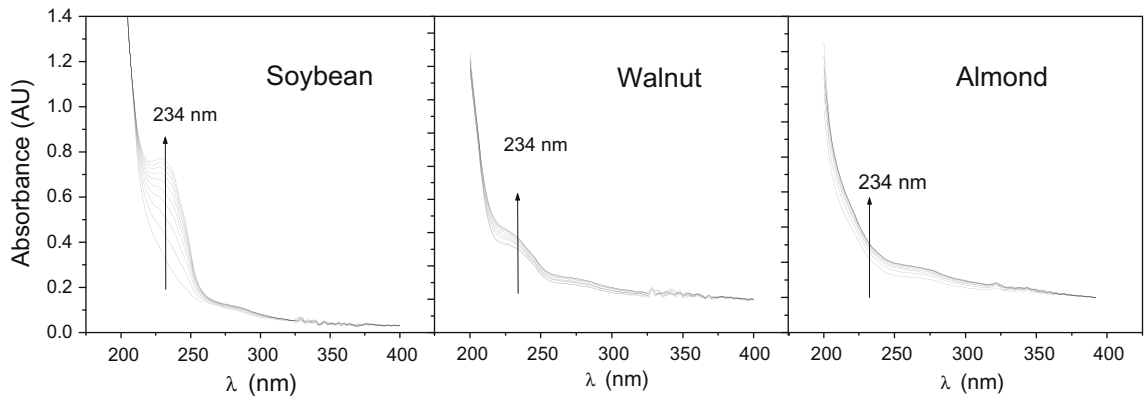

Fig. 3. Spectral changes due to conjugated diene formation during linoleic acid oxidation induced by lipoxygenases extracted from soybeans, walnuts and almonds in a micelle system.

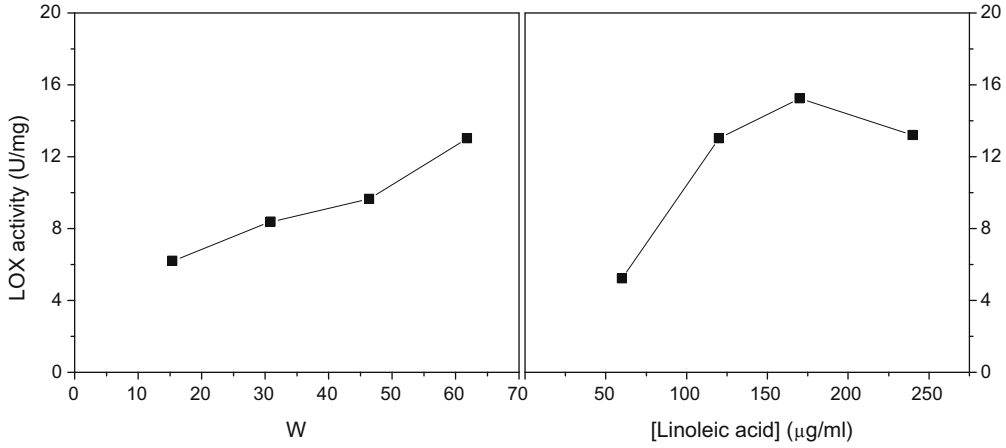

Fig. 5. Effect of micelle size ($W = [H_2O]/[surfactant]$) and substrate concentration on the enzymatic activity in AOT reverse micelles. Experimental conditions: 25 mM AOT/isooctane, LOX = 6.7 μg/ml in borate buffer pH 9.

there is evidence of the LOX inhibition by α-tocopherol in direct micelles (Grossman & Waksman, 1984), the observed anti-oxidative behaviour is better explained as their ability to break the radical propagation reaction leading to peroxidation.

The total and individual contents of the major active compounds in walnuts and almonds have previously been reported by several research groups. Anyway, investigations of the possible interactions among the main constituents as lipids, polyphenols, tocopherols and pro-oxidant enzymes that coexist in these foods are still necessary. Moreover, the inhibitory activity of the lipoxygenase by liposoluble as well as hydrosoluble phenolic compounds has not been compared yet. The direct and reverse micellar systems proposed locate the substrates in different microphases according to their lipo or hydrophilicities and consider not only their intrinsic reactivity but also the consequence of the partition or their distribution in organized systems that mimic a complex matrix as a food.

3.6. Antioxidant activity

In order to determine the possibility of interaction among active species present in nuts, experiments in direct micelles were carried out by mixing oxidisable substrates, antioxidants extracted from walnuts or almonds with the pro-oxidant lipoxygenase. Since the UV absorption due to the conjugated hydroperoxides, primary oxidation products of linoleic acid, takes place at the same spectral region of that due to the phenolic compounds used as antioxidants, the system chosen to evaluate the reaction progress was the β-carotene bleaching in the coupled oxidation with linoleic acid induced by lipoxygenase. Phenolic compounds extracted from nuts were assayed to prevent β-carotene from disappearing. The results obtained in these experiments are shown in Table 4. Most of all the nut extracts presented antioxidant ability especially those of hydrolysable and condensed extracts of whole nuts. Extracts obtained from nut skins were noticeably active in this system.

In all the cases, the AOA of walnut extracts were higher than those of almonds. Nut skins concentrate a remarkable amount of the antioxidants. The hydrolysable and condensed polyphenolic fractions of both whole nuts presented the highest AOA being for almonds remarkably higher than those of the extractable polyphenol extracts. The fractions obtained by successive extractions were more active in inhibiting β-carotene oxidation than the methanolic or aqueous extracts. The AOA of aqueous extracts of whole walnuts accounts for the 44.5% of the activity of the methanolic extracts. In the case of almonds, similar values although lower were obtained for both solvents. For walnuts as well as almonds, CM tocopherol fractions were more active than HM tocopherol extracts. Although α-tocopherol is a stronger antioxidant than GA in this system, the AOA values found for the CM and HM extracts were lower than those of polar phenolic compounds due to their low concentration in these nuts.

Table 4
Antioxidant activity (AOA) of walnuts and almonds expressed in gallic acid equivalents (μg GA/mg nuts) in relation to extraction conditions.

Nuts	Extraction conditions	AOA
Walnuts		
Whole nut	Methanol	119 ± 5
	Water	53 ± 11
	Successive extraction[a]	288 ± 34
	Hydrolysis[b]	103 ± 21
	Tannins-proanthocyanidins[c]	116 ± 20
	Oil phenolic fraction[d]	3.9 ± 0.4 (22 ± 1)[f]
	Oil phenolic fraction[e]	24 ± 3 (150 ± 10)[f]
Brown skin	Methanol	401 ± 23
Almonds		
Whole nut	Methanol	2.2 ± 0.1
	Water	2.0 ± 0.3
	Successive extraction[a]	10 ± 3
	Hydrolysis[b]	82 ± 3
	Tannins-proanthocyanidins[c]	86 ± 2
	Oil phenolic fraction[d]	5.4 ± 0.4 (27 ± 1)[f]
	Oil phenolic fraction[e]	18 ± 2 (90 ± 6)[f]
Brown skin	Methanol	79 ± 11

[a] Methanol–acetone–water.
[b] Methanol–sulphuric acid.
[c] HCl/butanol.
[d] 1.Hexane–2.Methanol.
[e] Chloroform–methanol mixture extracts.
[f] α-Tocopherol equivalents (μg/100 mg).

These experiments demonstrated the ability of phenolic compounds extracted from walnuts and almonds to inhibit the enzymatically-induced coupled oxidation of LA with β-carotene by monitoring the disappearance of the latter. All the extracts showed protective ability against oxidation and possible mechanisms of nut phenolic compounds could be by radical chain breaking as well as enzymatic inhibition. In fact, a flavonoid as quercetin has the ability to inhibit LOX enzymes preventing lipid peroxidation reactions from taking place (Sadik, Sies, & Schewe, 2003). Both nuts have different antioxidant species which can interact with each other, so that synergic or inhibitory effects are able to take place in the food matrix.

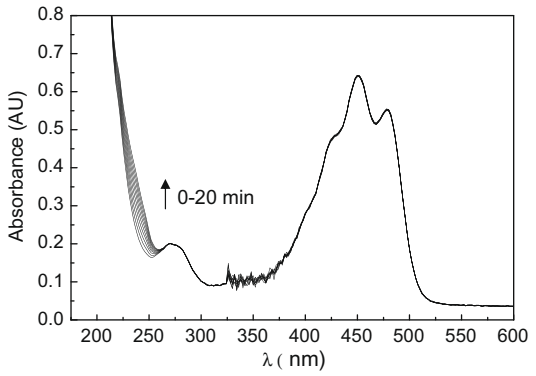

Fig. 6. Spectral changes due to linoleic acid oxidation induced by LOX in a reverse-micelle system containing β-carotene in the organic phase bulk. Experimental conditions: 6 mM AOT/isooctane, $W = 61.8$, LA = 120 μg/ml, LOX = 6.7 μg/ml in borate buffer pH 9.

Antioxidant capacity values of nut extracts expressed in GA equivalents were noticeably high especially when compared with the phenolic compound contents determined by FC method. As was previously reported (Chaillou & Nazareno, 2006), GA as well as ellagic acid present high free radical scavenging capacity in homogeneous solutions but only moderate activity in a micelle medium probably due to their limited distribution in the lipophilic phase having scarce participation in breaking the lipid oxidation chain. Although they are distributed in the same phase as LOX, in this microheterogeneous system their inhibitory activity would be reduced by their structure that limits its access to the enzyme active site. Taking into account that GA response in this system is relatively lower than that of other phenolic compounds, this high AOA values can be ascribed to other constituents of the nut polyphenol extracts.

In contrast to direct micelles, a different behaviour was observed in reverse micelles for the coupled oxidation of LA with β-carotene. The spectral changes were monitored from 200 to 600 nm as Fig. 6 shows. The scans after LOX addition showed LA oxidation progress although β-carotene bleaching did not take place. This result suggested that LA peroxyl radicals formed in the AOT interface by the entrapped enzyme were not able to react with the β-carotene molecule located in the organic solvent bulk. Further experiments using amphiphilic carotenoids as bixin or astaxanthin instead of β-carotene would be valuable to evaluate the antioxidant action of molecules that interfere in the UV region in this reverse-micelle system.

4. Conclusion

Walnuts and almonds are valuable foods from the nutritional point of view according to their chemical composition. They are rich sources of lipids, proteins and antioxidants. Almonds have higher contents of proteins than walnuts while the latter present higher lipid and antioxidant contents than the former. Walnut LOX content was lower although its pro-oxidant activity is higher than those of almonds. The AOA and ARA of polyphenol compounds extracted from nuts were assessed and their protective ability in an enzymatically induced oxidation was corroborated. Walnut phenolic extracts showed higher AOA than those of almonds.

Nuts pro and antioxidants constituents were isolated from the food matrix and their activities were characterized. Walnut and almond lipoxygenases presented catalytic activity towards the oxidation of LA which is the major lipid component of walnuts. In respect to almonds, LA is a minor constituent whereas the major one is the monounsaturated fatty acid. Although oleic acid does not behave as a specific substrate for LOX, it is an oxidisable compound and may take part in the couple oxidation with LA. Phenolic compounds obtained from these nuts were good radical scavengers and were mainly found in nut skins. Besides, they showed their ability to inhibit LA oxidation. This study demonstrated that such active compounds extracted from walnuts and almonds present the capacity to interact with each other. The oxidative status of these nuts will depend on the balance between their interactions and the factor that trigger oxidation initiation by means of enzyme activation as wounding or insect attack. Polyphenolic compound concentration in nut skins, the external layer of the kernels could be linked to nut protection against exogenous oxidative agents. The last result of this confrontation will have direct implications on the final quality of these nuts.

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