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ScienceDirect
Food Chemistry 107 (2008) 607–612
Food Chemistry
www.elsevier.com/locate/foodchem

Phenolics from walnut (*Juglans regia* L.) kernels: Antioxidant activity and interactions with proteins

Diana O. Labuckas, Damián M. Maestri, Milton Perelló, Marcela L. Martínez, Alicia L. Lamarque *

Instituto Multidisciplinario de Biología Vegetal (IMBIV, CONICET-UNC), Instituto de Ciencia y Tecnología de los Alimentos (ICTA), Facultad de Ciencias Exactas, Físicas y Naturales (FCEFyN-UNC), Av. Vélez Sarsfield 1611, X5016GCA Córdoba, Argentina

Received 9 May 2007; received in revised form 4 July 2007; accepted 17 August 2007

Abstract

Walnut (*Juglans regia* L.) kernels have important amounts of phenolic compounds. The objectives of the work were twofold: (a) to extract the phenolic fraction from hulls and walnut flour, and to examine its antioxidant capacity and (b) to evaluate the effect of hull removal on solubility of protein fractions from walnut flour. In accordance with their higher total phenolic content, hull extracts had stronger antioxidant activity than had flour extracts. The presence of phenolic compounds decreased protein solubility in walnut flour obtained from whole kernels. Dehulling of kernels significantly improved protein recovery but this result was strongly affected by the solvent system employed. Proteins from whole kernels, especially those extracted with water and NaCl solution, had a reduced solubility, indicating that phenolics bind to proteins when they are dispersed in aqueous media at neutral pH. The results are discussed in the light of the different complex-forming mechanisms that bind phenolics to proteins.

Keywords: Walnut; Phenolics; Antioxidant activity; Protein–phenolic interactions; Protein solubility

1. Introduction

The walnut tree (*Juglans regia* L.) is cultivated commercially throughout southern Europe, northern Africa, eastern Asia, the USA and western South America. In this region, Argentina is the main producer, with about 8500 metric tons per year.

The walnut seed (kernel) represents from 40 to 60% of the nut weight, depending mainly on the variety. The seed has high levels of oil (52–70%) in which polyunsaturated fatty acids predominate (Greve et al., 1992; Martínez, Mattea, & Maestri, 2006; Prasad, 2003; Savage, Dutta, & McNeil, 1999). In addition to oil, walnuts provide appreciable amounts of proteins (up to 24% of the walnut seed weight), carbohydrates (12–16%), fibre (1.5–2%) and minerals (1.7–2%) (Lavedrine, Ravel, Villet, Ducros, & Alary, 2000; Prasad, 2003; Savage, 2001; Sze-Tao & Sathe, 2000; Wardlaw, 1999).

The slightly astringent flavour of walnut fruit has been associated with the presence of phenolic compounds (Colaric, Veberic, Solar, Hudina, & Stampar, 2005; Prasad, 2003). Most phenolic compounds commonly identified in walnut are phenolic acids and condensed tannins. Walnut phenolics are found in the highest concentration in the hull (the pellicle surrounds the kernel), and they are reported to have favourable effects on human health owing to their apparent antiatherogenic and antioxidant properties (Anderson et al., 2001; Fukuda, Ito, & Yoshida, 2003; Gunduc & El, 2003; Horton et al., 1999; Lavedrine, Zmirou, Ravel, Balducci, & Alary, 1999; Zambon et al.,

* Corresponding author. Tel.: +54 0351 4334141x151; fax: +54 0351 5334439.
E-mail addresses: allamarq@efn.uncor.edu, alamarque@gmail.com (A.L. Lamarque).

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doi:10.1016/j.foodchem.2007.08.051

2000). In spite of these beneficial effects, walnut phenolics may adversely influence the protein solubility (Sze-Tao et al., 2000).

A recent work (Martínez et al., 2006) carried out a complete characterization of walnut oils from the most common walnut varieties grown in Argentina. Results demonstrated that the Franquette variety had a good potential for oil production due to its high oil and oleic acid contents. Now, we are analyzing the oil extraction residue to produce walnut flour suitable for food applications.

The objectives of the present work were twofold: (a) to extract the phenolic fraction from hulls and walnut flour, and to examine its antioxidant capacity and (b) to evaluate the effect of hull removal on solubility of protein fractions from walnut flour.

2. Materials and methods

2.1. Plant material

Walnut fruits, from the varieties Franquette, Chandler and Criolla, were collected from commercial plantations at Belén location, Catamarca Province, Argentina. From each variety, three samples (5 kg each) of fruits at full maturity were picked by hand from the trees. After cleaning, the fruits were dried at 30 ± 2 °C during 24 h, and then were shelled manually. The kernels (from each fruit sample) were divided into two portions. One of them was used to obtain walnut flour from whole kernels (WFWK). The other one was carefully dehulled to obtain the hulls and walnut flour from dehulled kernels (WFDK). To prepare both, WFWK and WFDK, the following experimental procedures were carried out.

The oil was removed with *n*-hexane by cross flow extraction at room temperature using a continuous lixiviation apparatus. For each run, 300 g of crushed kernels were charged into the lixiviation vessel and 5.6 l of organic solvent passed through the packed bed. The process took about 30 min. The resulting meal, containing 4.7% oil, was desolventized under reduced pressure at 30 °C. Walnut flour was obtained by grinding the walnut meal to very fine particles, so that 90% of the product passed through a 100-mesh screen.

2.2. Proximate composition

WFWK and WKDK were evaluated for protein ($N \times 5.3$), oil (Soxhlet, *n*-hexane, 12 h) and ash (furnace, 550 °C, 5 h) contents according to AOCS (1992) methods.

2.3. Extraction of phenolic fraction

Samples of WFWK and hulls were subjected to extraction with the following solvents: methanol:water (6:4 v/v) and ethanol:water (7:3 v/v). The solid/liquid ratio was 1:5 w/v for WFWK and 1:50 w/v for hulls. The extraction was carried out in two successive washings at room temperature, in the dark. At every solvent change, the mixture was filtered through Whatman No. 1 filter paper. For each material (WFWK and hulls), the filtrates were combined and concentrated under vacuum below 40 °C on a rotary evaporator to a final volume of 1 ml. Finally, WFWK and hull extracts were analyzed for their total phenolic content (TPC) and antioxidant activities.

2.4. Total phenolic content (TPC)

The TPC values of walnut extracts were determined using the Folin–Ciocalteu reagent (Siddhuraju, Mohan, & Becker, 2002). The reaction mixture contained 100 μl of walnut extracts, 500 μl of the Folin–Ciocalteu reagent and 2.5 ml of sodium carbonate (20% w/v). The final volume was made up to 10 ml with distilled water. After 1 h of reaction, the absorbance at 725 nm was measured. A reference curve was constructed, using gallic acid as standard. The TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of the extract.

2.5. Radical-scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical

The antioxidant activities of fenolic extracts were determined by a modification of the DPPH radical-scavenging method of Gálvez, Martín-Cordero, Houghton, and Ayuso (2005). A 20 ppm methanolic solution of each extract (1610 μl) was mixed with a 0.022% (w/v) solution of DPPH · (final volume, 1960 μl). A control sample was prepared using methanol instead of phenolic extract. Butylated hydroxy-toluene (BHT) was employed as a standard antioxidant to examine the radical-scavenging activities. The reaction mixture was shaken vigorously and left to stand for 30 min at room temperature in the dark. The remaining DPPH was determined spectrophotometrically at 515 nm and the radical-scavenging activity of each extract was expressed as ppm BHT equivalents. The mean values were obtained from triplicate experiments.

On the basis of the results of the above experiments, the Franquette variety was selected for further studies.

2.6. Oil storage test

Refined soybean oil, without additives, was used to check the antioxidant capacity of walnut extracts from the Franquette variety. WFWK and hull phenolic extracts were mixed with 60 g of soybean oil and sorbitol monooleate as emulsifier. The final concentration of the extracts in soybean oil was 30 ppm. Additional treatments included BHT (0.02% w/w) and a control (soybean oil containing sorbitol monooleate without phenolic extracts). All treatments were stored at 60 °C in open beakers (100 ml) and they were sampled every 24 h to measure peroxide and con-

jugated diene (K_{232}) values according to AOCS (1992) methods.

2.7. Protein solubility

Proteins from WFWK and WFDK (0.25 g) were extracted with the following solvents: distilled deionised water, 1.0 M NaCl solution (pH 7), 0.1 M NaOH solution (pH 13), 0.1 M $Na_2B_4O_7$ solution (pH 10), and aqueous ethanol (70% v/v). In all cases, the defatted flour/solvent ratio was 1:20 w/v. The extraction time was 2 h (25 °C); every 10 min the samples were shaken. Then, they were centrifuged (13,000 × *g*, 25 °C, 10 min) and the supernatants were analysed for protein content by the method of Bradford (1976). Solubility was expressed as mg of protein per gram of total protein.

2.8. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Soluble protein samples (4 ml), from each solvent system tested, were mixed with 20 ml of cold acetone, incubated at −20 °C for 2 h, and pelleted by centrifugation at 14,000 g for 20 min. The pellet (0.004 g) was resuspended in Tris–HCl buffer (100 μl), pH 6.8, 80 mM, containing 2% SDS (w/v) and 0.1 M β-mercaptoethanol, boiled for 2 min, and analyzed by SDS-PAGE in 10% (w/v) acrylamide gels, as described by Leammli (1970). Aliquots (40 μl) of protein samples were loaded into each lane. The polypeptides in gels were fixed with 10% (w/v) trichloroacetic acid and stained with Coomassie Brillant Blue G-250.

2.9. Statistical analysis

Analyses were carried out in triplicate. The data were statistically evaluated by variance analysis (ANOVA). The comparison of means was done by the least significant difference (LSD) test at a significance level of 0.05.

3. Results

3.1. General

The three walnut varieties differed in their TPC (Table 1). As expected, there were significant differences among hulls and WFWK. Minor differences were observed among solvent systems. The hulls from the Criolla variety had the highest TPC in both the MeOH:H_2O and the EtOH:H_2O solvent systems. Among flours, WFWK from the Franquette variety presented the major TPC. Chandler variety was the poorest in TPC in both hulls and WFWK. These results show that the hulls are a much better source of phenolic compounds than are whole kernels. Although the hull contributes only 5% to the fruit weight, its TPC is at least 93–97% higher than that of whole kernels.

The determination of phenols in walnut hulls has gained increasing interest with the recognition that these compounds have antioxidant properties (Anderson et al., 2001; Fukuda et al., 2003). The data from Table 1 shows that hull extracts had stronger antioxidant activity than had flour extracts.

In accordance with the major TPC, the highest antioxidant activity was observed in hulls and WFWK from Criolla and Franquette varieties, respectively. Phenols extracted from flour of the Franquette variety showed an antioxidant capacity as high as that observed in extracts from the hull.

3.2. Oil storage test

Peroxide value (PV) and conjugated dienes (K_{232}) were used to measure the effectiveness of phenolic extracts from the Franquette variety on oxidative stability of soybean oil (SBO) during storage at 60 °C (Torres, Lloret, Sosa, & Maestri, 2006). There were minor differences among treatments: in general, ethanolic extracts from hulls were something lower in peroxide and K_{232} values than were methanolic extracts or pure SBO (Figs. 1 and 2).

Table 1
Total phenol content (TPC, mg of GAE/g of the extract) and antioxidant activity (AA, ppm BHT equivalent) of hulls and walnut flour from whole kernel (WFWK) extracted with two solvent systems

Material	Variety	Solvent system			
		MeOH:H_2O (60:40 v/v)		EtOH:H_2O (70:30 v/v)	
		TPC	AA	TPC	AA
Hull	Chandler	$230^{c,2} \pm 21.1$	$47.7^{b,1} \pm 3.62$	$267^{c,1} \pm 2.13$	$63.0^{b,1} \pm 3.37$
	Criolla	$490^{a,1} \pm 27.3$	$186^{a,2} \pm 14.3$	$479^{a,1} \pm 42.1$	$286^{a,1} \pm 2.72$
	Franquette	$403^{b,1} \pm 37.1$	$63.0^{b,1} \pm 20.1$	$397^{b,1} \pm 6.01$	$95.0^{b,1} \pm 25.7$
WFWK	Chandler	$16.3^{b,1} \pm 0.71$	$14.0^{b,1} \pm 3.95$	$16.3^{b,1} \pm 1.17$	$13.5^{b,1} \pm 5.97$
	Criolla	$17.3^{b,1} \pm 0.4$	$16.1^{b,1} \pm 5.16$	$14.9^{b,1} \pm 3.25$	$15.1^{b,1} \pm 2.92$
	Franquette	$23.7^{a,1} \pm 7.31$	$44.3^{a,2} \pm 4.59$	$25.6^{a,1} \pm 4.73$	$92.6^{a,1} \pm 39.5$

Values are expressed as the means and standard deviation of three replicates.
Different superscript letters indicate significant differences ($p \leqslant 0.05$) among varieties in either TPC or AA from hulls or WFWK for each extraction system.
Average values in the same row followed by different superscripts present significant differences ($p \leqslant 0.05$) in either TPC or AA.

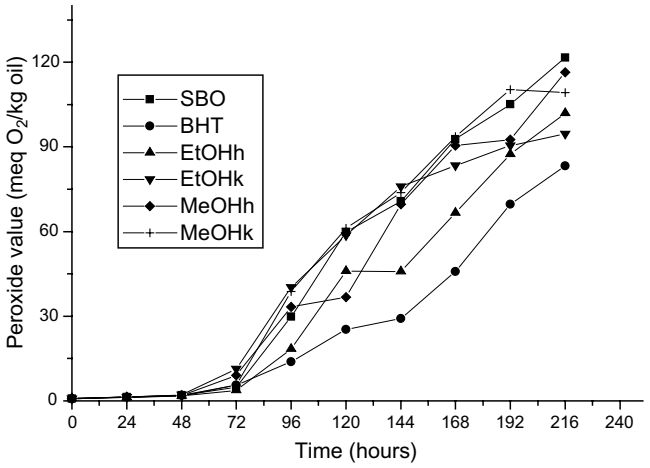

Fig. 1. Kinetic curves of peroxide accumulation during oxidation of soybean oil (SBO), SBO + walnut phenolic extracts and SBO + BHT. All kinetic curves were the averages result of three independent experiments. Abbreviations: EtOHh (ethanolic) and MeOHh (methanolic) extracts from walnut hulls, EtOHk (ethanolic) and MeOHk (methanolic) extracts from walnut whole kernels.

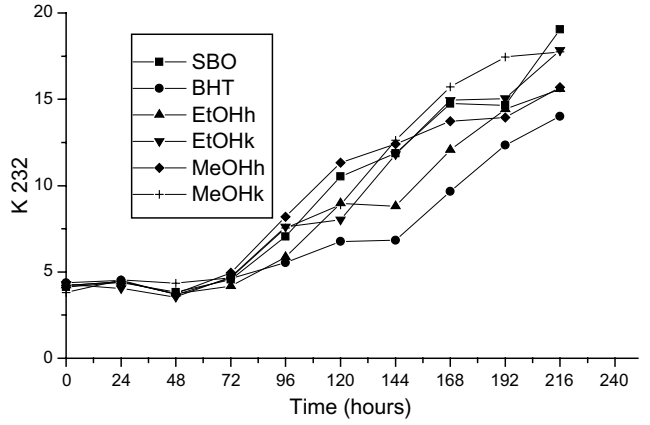

Fig. 2. Kinetic curves of K_{232} values during oxidation of soybean oil (SBO), SBO + walnut phenolic extracts and SBO + BHT. All kinetic curves were the average results of three independent experiments. Abbreviations: EtOHh (ethanolic) and MeOHh (methanolic) extracts from walnut hulls, EtOHk (ethanolic) and MeOHk (methanolic) extracts from walnut whole kernels.

With these results in mind, we analyzed the WFWK and WFDK of the Franquette variety to evaluate the influence of phenols on protein solubility. The choice of this variety was based on the total phenolic content of flours. There were no significant variations among WFWK and WFDK in their proximate composition. They contained 39.8% protein, 4.7% oil, 6.0% ash and the corresponding carbohydrates.

3.3. Protein solubility

With the exception of the $Na_2B_4O_7$ solvent system, the highest protein solubility was observed in WFDK (Table 2).

Proteins from WFWK, especially those extracted with water and NaCl solution, had a reduced solubility, indicating that walnut phenolic compounds may effectively bind to proteins when they are dispersed in aqueous media at neutral pH. This fact may also indicate that an appreciable phenolic fraction is ionically bound to proteins and the NaCl treatment is not sufficient to release proteins from phenolics.

Table 2
Protein solubility data (mg protein/g of total protein) obtained with different solvent systems

Solvent system	WFWK		WFDK	
H_2O	0.19[a]	±0.02	26.1[b]	±0.25
1 M NaCl	0.45[a]	±0.05	44.0[b]	±5.90
Ethanol (70%)	0.09[a]	±0.02	0.14[b]	±0.02
0.1 M $Na_2B_4O_7$	114[a]	±8.2	132[a]	±11.0
0.1 M NaOH	143[a]	±8.1	194[b]	±7.2

Values are expressed as the means and standard deviation of three replicates.
Different superscripts indicate significant differences ($p \leqslant 0.05$) among WFWK and WFDK for each solvent system.

At alkaline pH using $Na_2B_4O_7$ solution, the differences in protein solubility among WFWK and WFDK were less marked. This pH-dependent effect is probably due to the different mechanisms that bind phenolics to proteins. Xu and Diosady (2000, 2002) have observed that both a large free fraction and a significant hydrophobic fraction of condensed tannins are present in alkaline medium, indicating that hydrophobic bonds are involved in tannin–protein interactions at alkaline pH. This means that, under this condition, condensed tannins are free or weakly bound to proteins and, therefore, they do not significantly affect the protein solubility. At alkaline pH, using NaOH solution, denaturation and dissociation of protein molecules occur (Abugoch, Martínez, & Añón, 2003). Under these conditions, the interactions between proteins and phenolics become stronger, owing to exposure of hydrophobic protein residues that may bind to phenolics.

Protein solubility in 70% ethanol was at very low values in both WFWK and WFDK. The reason for this finding is the small proportion (about 5–6%) of prolamins present in

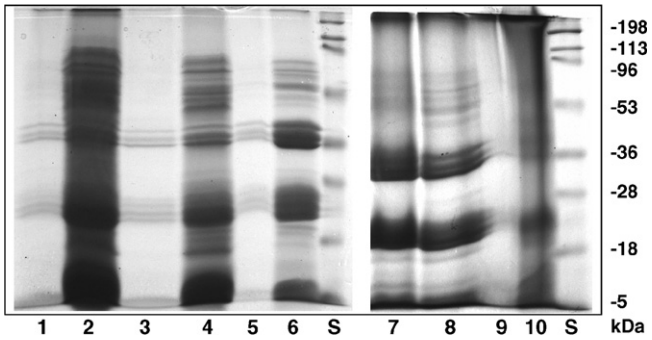

Fig. 3. Electrophoretic patterns of proteins extracted from walnut flour. Lines 1, 3, 5, 7 and 9 were obtained from WFWK using water, 1 M NaCl, 70% EtOH, 0.1 M $Na_2B_4O_7$ and 0.1 M NaOH, respectively, as extracting agents. Lines 2, 4, 6, 8, and 10 were obtained from WFDK using the same extracting agents as stated above. S: Sigma MW standards.

walnut flour (Sze-Tao et al., 2000; Labuckas, Maestri, Martínez, & Lamarque, 2005).

3.4. SDS–PAGE

Electrophoretic analysis (Fig. 3) confirms the effects of phenolics on protein solubility when they are extracted with water, NaCl solution or aqueous ethanol: the patterns obtained from WFWK show lighter and diffuse bands, especially at molecular weights higher than 50 kDa. No quantitative differences were observed among protein profiles from WFWK and WFDK using $Na_2B_4O_7$ as extracting agent. The protein profiles obtained with NaOH as extracting agent show a diffuse band pattern. At variance with $Na_2B_4O_7$, NaOH can produce denaturation and dissociation of protein molecules, favouring their extraction (Abugoch et al., 2003).

4. Discussion and conclusions

Walnut kernels have an important amount of phenolic compounds, which are mostly present in the hull. When kernels are whole-ground and the oil is extracted, most phenolics remain in the flour where they can precipitate proteins through different mechanisms, such as hydrophobic and ionic interactions, hydrogen and covalent bondings (Rubino, Arntfield, Nadon, & Bernatsky, 1996; Xu et al., 2000, 2002). Another consequence of the interaction resulting in protein-phenolic complexes might be a decreased concentration of free phenolics which may reduce their antioxidant efficacy.

Removing hull from walnut kernel improves protein solubility. Although this procedure may be effective for raising the protein bioavailability, it is not practical for industrial applications. Proteins from walnut are mainly composed of glutelins (about 70% of the total seed proteins) and globulins (about 18%) (Sze-Tao et al., 2000; Labuckas et al., 2005). These globular proteins have a very low solubility in aqueous ethanol but, interestingly, this solvent system is suitable for removing phenolics from walnut flour. To sum up: aqueous ethanol solution may be used to extract walnut phenolics without affecting protein recovery. An important utility of the ethanol-extracted phenolic fraction is related to the antioxidant capacity as was demonstrated by its radical-scavenging effect on DPPH radical and less formation of primary oxidation products when it was added to soybean oil.

Proteins are readily extracted from walnut flour at alkaline pH, confirming the trends reported previously (Sze-Tao et al., 2000; Labuckas et al., 2005). Using aqueous $Na_2B_4O_7$ solution at pH 10, the protein extractability is not significantly influenced by the presence of the hulls, suggesting that binding of proteins to phenolics became much weaker than at neutral pH.

In summary, walnut flour is an important source of proteins and a potentially rich source of phenolic compounds. Removal of phenolics by solvent extraction improves protein availability, yielding walnut flour with potential applications as a food ingredient.

Acknowledgements

This research was supported by grants from SECyT-UNC and CONICET

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