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# Molecular Characterization, Antioxidant and Protein Solubility-Related Properties of Polyphenolic Compounds from Walnut (*Juglans regia*)

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Aqueous ethanol extraction of partially defatted walnut flours provides a simple and reliable method to obtain extracts with high content of polyphenolic compounds. These were characterized by means of HPLC-ESI-MS/MS analytical techniques and molecular parameters. Considering the whole set of polyphenolic compounds identified, a high average number of phenolic-OH groups was found. Although these represent potential hydrogen-atom transfer sites, which are associated with high free-radical scavenging capacity, results show that such a property could be strongly limited by the low lipophilicity of polyphenols affecting the accessibility of these molecules to lipid substrates. Variations in pH values were found to change the ionization behavior of phenolic compounds. These changes, however, had minor effects on walnut protein solubility-related properties. The results obtained in this study highlight the importance of molecular characterization of walnut phenolic compounds in order to assess better their bioactive properties.

**Keywords:** Walnut, Polyphenols, Molecular parameters, Antioxidant activity, Protein solubility.

Walnuts (*Juglans regia* L.) are an excellent source of many nutrients and have been proposed as a promising natural functional food. The edible part of the nut (the seed or kernel) is a nutrient-dense food mainly due to its high oil content (up to 740 g/kg of the kernel weight in some commercial cultivars) [1]. In addition to oil, walnuts provide appreciable amounts of proteins (up to 24% of the kernel weight), carbohydrates (12 – 16%), fibre (1.5 – 2%) and minerals (1.7 – 2%) [1, 2]. Walnut kernels are also an important source of phenolic acids and related polyphenols. They are found in higher amounts in the seed coat, i.e. the skin surrounding the kernel. Although walnut phenolics have no known nutritional function, several studies have shown health-promoting effects arising from their antioxidant, antiatherogenic, antiinflammatory and antimutagenic properties [3, 4].

On the other hand, many *in vitro* studies have shown that interactions of polyphenolic compounds with proteins may lead to changes in structural, functional and nutritional properties of proteins [5]. The structure and molecular weight of polyphenols play an important role in protein-polyphenol interactions. High molecular weight polyphenols, such as the hydrolyzable and non-hydrolyzable tannins which may be present in walnuts, are able to bond more strongly to proteins [6]. In addition, the number of hydrogen donor sites appears to be important as well; it has been reported that the strength of binding to proteins increases as the number of OH groups on the polyphenol molecule increases [7]. Owing to the nature of the complexes formed, interactions between phenolics and proteins are pH sensitive. In general, the lowest solubility of polyphenol-protein complexes occurs at pH values below the isoelectric point. However, this supposition depends on the structure of both the phenolic and the protein molecules. This study was primarily aimed to characterize the phenolic composition of ethanol-soluble walnut extracts. In addition, the antioxidant and protein solubility-related properties are discussed in relation to molecular parameters of the phenolic compounds identified.

Seventeen phenolic compounds were identified in the aqueous ethanol extract obtained from the screw-pressed walnut cake. The major components were ellagic acid (EA, 8.39 mg/L), galloyl hexoside (7.4 mg/L), two digalloyl hexoside isomers (3.15 and 4.32 mg/L) and gallic acid (3.78 mg/L) (Table 1). Ellagic acid (**18**) identity was confirmed by a $[M-H]^-$ signal at *m/z* 301. The $[M-H]^-$ signals at *m/z* 331 and 483 indicated the presence of galloyl hexoside (**3**) and digalloyl-hexosides (**5** and **8**), respectively. The fragmentation pattern of monogalloyl-hexose showed a predominant ion at *m/z* 169 after the loss of the glycosyl, and at *m/z* 271 after cross-ring fragmentation of hexose. Two isomeric forms of the digalloyl esters of hexose with different retention times were found (**5** and **8**). The different *R*t of these compounds in the HPLC-DAD profile may be possibly attributed to isomeric forms due to differences in the points of attachment of the galloyl structures to the hexose. In agreement with data reported previously [8], gallic acid (**9**) presented a $[M-H]^-$ signal at *m/z* 169, with fragmentation at *m/z* 125. Minor amounts of hexahydroxydiphenyl (HHDP) derivatives, ellagic acid bound to hexoses or pentoses, catechin, procyanidin dimer and two non-phenolic structures were also identified. Three peaks corresponding to galloyl-HHDP-hexoside isomers (**2**, **6** and **11**) showed $[M-H]^-$ signals at *m/z* 633 with a fragment ion at *m/z* 301. Two isomeric compounds with $[M-H]^-$ at *m/z* 783 (**4** and **7**), yielding main fragment ions at *m/z* 301 and 481, were identified as bis-HHDP-hexoside structures, presumably pedunculagin or casuariin isomers, in accordance with previous studies [9]. Based on molecular weight and mass spectra data ($[M-H]^-$ signal at *m/z* 481, presence of an intense ion at *m/z* 301), compound **1** was assigned as HHDP-hexoside. This compound was earlier reported in walnut extracts [8]. Monoglycosylated EA derivatives were also observed, such as EA pentoside (*m/z* 433, **15**), two isomers of EA hexoside (*m/z* 463, **13** and **17**), and EA rhamnoside or deoxyhexoside (*m/z* 447, **19**), showing the typical fragment at *m/z* 301 in the MS/MS analysis. Two flavonoid-type structures were recognized. Catechin was identified with a $[M-H]^-$

signal at *m/z* 289 and MS/MS fragment at *m/z* 254, and procyanidin dimer, with a [M-H]$^-$ signal at *m/z* 577 and a fragment at *m/z* 289 (**12** and **10**, respectively). Two non-phenolic compounds (glansreginin A and B; **16** and **14**) were also found with [M-H]$^-$ signals at *m/z* 592 and 565, respectively, yielding fragments ions (403, 343, 241) as described earlier [8].

By relating the structure of the identified compounds (Figure 1) with the calculated molecular parameters (Table 2), it can be seen that most of them have low or negative log D values (indicating that partitioned in water is better than in octanol). Furthermore, these compounds have a high number of both hydrogen donor (HDS) and hydrogen acceptor sites (HAS). These characteristics show that they may establish intra- or intermolecular interactions with similar compounds or with other flour components. Another feature of the compounds is the ionization behavior with pH variation. At acidic pH, all compounds, except glansreginin A, are uncharged, but ionization is accentuated as the pH increases. At alkaline pH (10-13), a high percentage of each compound has a negative net charge. This increase in ionization enhances the hydrophilicity of the compounds and may affect the interaction with other components.

The mean total phenolic content (TPC) from aqueous ethanol extracts (28.8 mg GAE/g) is comparable with those obtained by using other polar organic solvents [8, 10]. This high extraction yield can be attributed mainly to hydrophilic interactions (ionic interactions and hydrogen bonding) between phenolics and solvent molecules. Such a hypothesis is supported by the high number of both hydrogen donor sites (HDS) and hydrogen acceptor sites (HAS) that are present in all compounds identified (Table 2). The antioxidant efficacy of phenolic compounds (measured as the free-radical scavenging capacity) primarily depends on the number of hydrogen-atom donor sites (typically hydroxyl groups attached to aromatic rings), but the position of these active groups is important as well. When simple phenolic molecules are considered, *ortho*- and *para*-dihydric phenols have high antioxidant activity because of their major ability to donate hydrogen atoms and to form quinone-type structures that are stabilized by resonance [11].

Under the assay conditions employed in this study, the mean DPPH radical-scavenging activity, expressed as $IC_{50}$ (10.4 µg/mL), of the aqueous ethanol extracts is similar to that of the BHT (10.1 µg/mL), but lower than that of ascorbic acid (4.14 µg/mL), used as references. Considering the whole set of analyzed compounds, an average number of 6 phenolic-OH groups was found; the isomers of the compound identified as di-HHDP-hexoside had the highest ones (Table 2, Figure 1). These values represent a very high number of hydrogen-atom transfer sites. In addition, all phenolic compounds had at least one aromatic ring with two or more -OH groups as *ortho* substituents. Polyhydric phenols with a high number of OH-groups, such as many compounds identified in the obtained aqueous ethanol extracts, could have strong antioxidant activity in lipid peroxidation reactions owing to their capacity to hydrogen-atom transfer to lipid alkyl radicals. However, such effect must be interpreted with caution because phenolic molecules with more hydroxyl groups tend to have lower lipophilicity. The aqueous ethanol extracts from defatted walnuts contain complex polyphenols, mainly as glycosides. These represent rather polar and hydrophilic forms which are poorly soluble in lipids. This fact could affect the accessibility of the polyphenol molecules to lipid substrates. When walnut flour samples (WF and D-WF) were added to walnut oil, they did not show significant differences in oxidation rates; the presence of polyphenolic compounds in WF samples did not enhance the OSI values (4.62 hours) with respect to samples without ethanol soluble compounds (D-WF, 4.17 hours).

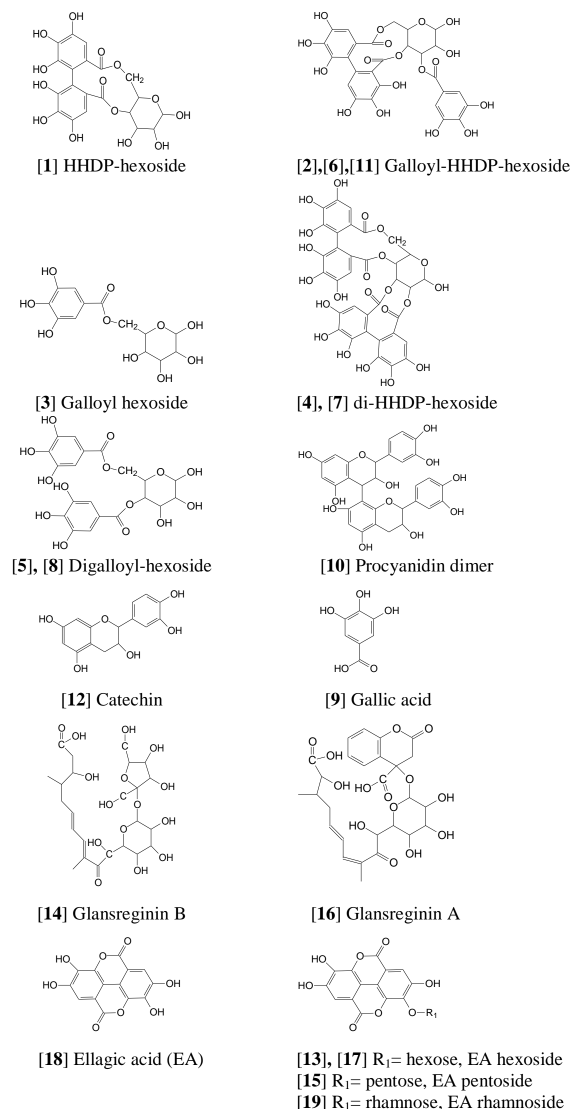


**Figure 1:** 2D molecular structures of the compounds identified by HPLC-ESI-MS/MS. HHDP: hexahydroxydiphenyl.

**Table 1:** Compounds identified by HPLC-ESI-MS/MS analysis in the aqueous ethanol extract obtained from the screw-pressed walnut cake. Compounds are listed on the basis of increasing retention times (*R*t). HHDP: hexahydroxydiphenyl.

| Nº | Compound (Identification, quantification procedure) | *R*t (min) | [M-H]$^-$ | MS$^2$ [M-H]$^-$ | (mg/L) |
|---|---|---|---|---|---|
| 1 | HHDP-hexoside (b, c) | 7.5 | 481 | 301 | 2.7±0.01 |
| 2 | Galloyl-HHDP-hexoside (b, c) | 8.2 | 633 | 301 | 0.4±0.002 |
| 3 | Galloyl-hexoside (b, d) | 8.5 | 331 | 169, 271 | 7.4±0.5 |
| 4 | di-HHDP-hexoside (b, c) | 8.9 | 783 | 481, 301 | 0.4±0.05 |
| 5 | Digalloyl-hexoside (b, d) | 9 | 483 | 169 | 3.1±0.2 |
| 6 | Galloyl-HHDP-hexoside (b, c) | 9.9 | 633 | 301 | 0.3±0.01 |
| 7 | di-HHDP-hexoside (b, c) | 10.1 | 783 | 481, 301 | 0.5±0.05 |
| 8 | Digalloyl-hexoside (b, d) | 10.2 | 483 | 169 | 4.3±0.5 |
| 9 | Gallic acid (a) | 10.9 | 169 | 125 | 3.8±0.51 |
| 10 | Procyanidin dimer (b, e) | 11.1 | 577 | 289 | 0.5±0.005 |
| 11 | Galloyl-HHDP-hexoside (b, c) | 11.1 | 633 | 301 | 0.4±0.01 |
| 12 | Catechin (a) | 12 | 289 | 245 | 2.8±0.01 |
| 13 | Ellagic acid hexoside (b, c) | 14.5 | 463 | 301 | 0.6±0.01 |
| 14 | Glansreginin B (b, c) | 15.1 | 565 | 403, 343, 241 | 2.8±0.2 |
| 15 | Ellagic acid pentoside (b, c) | 18 | 433 | 301 | 2.5±0.2 |
| 16 | Glansreginin A (b, c) | 18.3 | 592 | 403, 343, 241 | 3.4±0.02 |
| 17 | Ellagic acid hexoside (b, c) | 18.9 | 463 | 301 | 0.4±0.01 |
| 18 | Ellagic acid (a) | 20.5 | 301 | | 8.4±1.5 |
| 19 | Ellagic acid rhamnoside (b, c) | 20.9 | 447 | 301 | 0.5±0.01 |

Procedures employed for identification: a, co-analysis relative to a pure compound showing identical retention and mass spectral data; b, comparison with literature MS, MS/MS and UV data. Quantification was made using a calibration curve of the corresponding standard, except where indicated: c, quantified as ellagic acid; d, quantified as gallic acid; e, quantified as catechin.

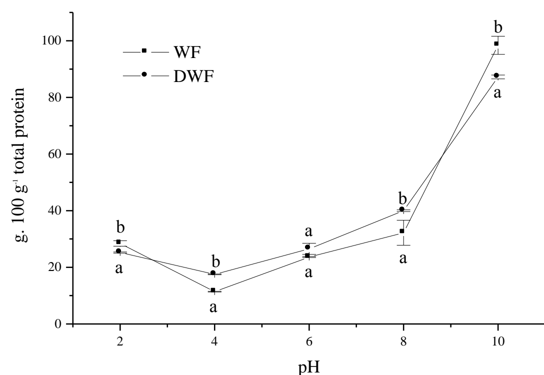


**Figure 2:** Effect of pH on protein solubility of whole walnut flour (WF) and walnut flour without ethanol soluble compounds (D-WF). Each point represents the mean protein solubility value (n = 3) ± standard deviation. For each pH value, statistical differences ($P < 0.05$) in protein solubility between WF and D-WF are indicated by different letters.

Figure 2 shows the effect of pH on protein solubility of flours with and without ethanol-soluble phenolic compounds (WF and D-WF). The lowest protein solubility values were observed at pH 4 (11.4 and 17.5 g/100 g for WF and D-WF, respectively); solubility increased at both increasing and decreasing pH. In general, the dependence of walnut protein solubility on pH follows the tendency observed for many vegetable proteins [12, 13]. WF and D-WF samples showed minor but significant differences in their protein solubility profiles. At both the lowest and the highest pH measured (2 and 10, respectively) proteins from D-WF samples presented lower solubility values than WF samples. This behavior can be explained as a combined effect of two factors. Firstly, the change of the protein conformation that increases the exposure of hydrophobic residues which were previously buried, and secondly the reduction of the hydration layer around the protein which causes aggregation by attractive interactions, reducing the protein percentage to be capable of solubilization. On the other hand, at alkaline pH the phenolic compounds in WF are highly ionized (Table 2) and the electrostatic repulsions with negatively charged proteins reduce the interactions between these components, thus allowing the solubility of higher amount of proteins. At both pH 4 and pH 8, protein solubility from WF was lower than D-WF. At these pH values, protein-polyphenol non-covalent interactions may result in the formation of aggregates that precipitate from the solution and reduce protein solubility. At neutral pH, phenolic compounds are less ionized (Table 2) and the interactions that could be established with the basic amino acids of the proteins, not fully deprotonated, are basically ionic interactions and hydrogen bonding. Similar results have been reported earlier [14].

In conclusion, aqueous ethanol extraction of partially defatted walnut flours provides a simple and reliable method to obtain phenolic extracts with high content of polyphenolic compounds. All of them were characterized by HPLC-ESI-MS/MS analytical techniques, and have been previously identified in walnut extracts obtained from whole nuts or the integument of different walnut cultivars by using other polar organic solvents. The use of ethanol instead of methanol as extraction solvent affects neither the selectivity nor the efficiency (measured as total phenolic content) of the extractions.

Considering the whole set of polyphenolic compounds identified, a high average number of phenolic-OH groups were found. These represent potential hydrogen-atom transfer sites which are related to free-radical scavenging capacity. However, in lipid oxidation reactions, such a property could be strongly limited by the low lipophilicity of polyphenols which may affect the accessibility of the molecules to lipid substrates.

Variations in pH conditions affect the ionization behavior of phenolic compounds identified in the ethanol-soluble walnut extracts. This is mainly associated with the large number of both hydrogen donor sites and hydrogen acceptor sites on the polyphenol molecules. Although these features are known to influence interactions with proteins, data from the present work suggest that walnut flour polyphenols have minor effects on protein solubility-related properties.

Overall the results obtained in this study highlight the importance of molecular characterization of walnut phenolic compounds in order to assess better their bioactive properties.

## Experimental

***Materials:*** Walnut kernels (*Juglans regia* L. cv. Franquette) were partially deoiled by screw-pressing (50ºC, 20 rpm) according to procedures described earlier [15]. A portion (380 g) of the pressed material was homogenized with 1000 mL ethanol (70% v/v). The homogenate was kept under refrigeration (4 ºC) for 72 h. Then, it was filtered (Whatman Nº 1), the solvent reduced (rotary evaporator at 40ºC, in the dark) and partitioned with *n*-hexane (2 x 40 mL each time). The polar phase was evaporated to dryness and the extract re-suspended in 50 mL ethanol (70% v/v) for TPC and HPLC-ESI-MS/MS analyses. Both the whole pressed and pressed material without ethanol soluble compounds were defatted by continuous solid-liquid extraction (*n*-hexane) to obtain the corresponding walnut flours (WF and D-WF, respectively).

**Table 2:** Molecular parameters of compounds identified by HPLC-ESI-MS/MS. HHDP: hexahydroxydiphenyl.

| Nº | Compound | Log D | | | | HDS | HAS | Net charge | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pH | | | | | | pH 3 | | pH 7 | | pH 10 | | pH 13 | |
| | | 3 | 7 | 10 | 13 | | | pKa / (%) | | pKa / (%) | | pKa / (%) | | pKa / (%) | |
| **1** | HHDP-hexoside | -0.5 | -0.6 | -4.6 | -10.7 | 9 | 14 | 0 | (100) | 0 | (72.2) | -2 | (77.4) | -7 | (49.4) |
| **2,6,11** | Galloyl-HHDP-hexoside | 1.5 | 1.3 | -4.2 | -10.1 | 11 | 18 | 0 | (100) | 0 | (64.8) | -5 | (56.1) | -10 | (42.4) |
| **3** | Galloyl-hexoside | -1.3 | -1.4 | -3.1 | -7.3 | 7 | 10 | 0 | (100) | 0 | (92.8) | -1 | (92.6) | -5 | (54.3) |
| **4,7** | di-HHDP-hexoside | 2.7 | 2.6 | -2.6 | -9.2 | 13 | 22 | 0 | (100) | 0 | (65.9) | -7 | (84.8) | -13 | (52.6) |
| **5,8** | Digalloyl-hexoside | 0.2 | 0.2 | -3.2 | -8.1 | 9 | 14 | 0 | (100) | 0 | (85.2) | -2 | (95.2) | -6 | (45.2) |
| **9** | Gallic acid | 0.7 | -2.2 | -3.8 | -6.6 | 4 | 5 | 0 | (89.7) | -1 | (99.0) | -2 | (49.7) | -3 | (91.6) |
| **10** | Procyanidin dimer | 3.1 | 3.1 | 0.0 | -10.4 | 10 | 12 | 0 | (100) | 0 | (98.0) | -5 | (39.6) | -8 | (66.3) |
| **12** | Catechin | 1.8 | 1.8 | 0.2 | -0.5 | 5 | 6 | 0 | (100) | 0 | (99.0) | -2 | (20.0) | -4 | (62.8) |
| **13,17** | Ellagic acid hexoside | -0.6 | -2.6 | -4.8 | -7.9 | 5 | 15 | 0 | (99.8) | -2 | (84.5) | -2 | (94.2) | -5 | (48.1) |
| **14** | Glansreginin B | -2.9 | -5.3 | -6.4 | -9.3 | 9 | 18 | 0 | (96.9) | -1 | (99.7) | -1 | (98.0) | -5 | (41.2) |
| **15** | Ellagic acid pentoside | -0.1 | -0.3 | -3.4 | -8.6 | 6 | 12 | 0 | (100) | 0 | (73.3) | -2 | (85.2) | -5 | (59.4) |
| **16** | Glansreginin A | 0.0 | -6.0 | -6.7 | -8.5 | 5 | 19 | -1 | (49.1) | -2 | (99.9) | -2 | (98.8) | -4 | (47.4) |
| **18** | Ellagic acid | 2.3 | 0.1 | -1.9 | -3.7 | 4 | 8 | 0 | (99.7) | -2 | (85.2) | -2 | (98.3) | -4 | (79.0) |
| **19** | Ellagic acid rhamnoside | -0.3 | -2.4 | -4.5 | -8.1 | 5 | 15 | 0 | (99.8) | -2 | (87.9) | -2 | (93.3) | -5 | (55.4) |

HDS: Hydrogen donor sites; HAS: Hydrogen acceptor sites.

***Identification and quantification of phenolics:*** An Agilent Series 1200 LC System (Agilent, USA) coupled to a MicrOTOF Q II (Bruker Daltonics, USA) was used for HPLC-ESI-MS/MS analysis. A mass spectrometer equipped with electrospray ion source and qTOF analyzer was used in MS and MS/MS mode for the structural analysis. HPLC analyses were performed on a thermostated (40°C) Phenomenex Luna C18 250 × 4.6 mm (5 µm) column at a 0.4 mL/min flow rate using 0.5% (v/v) formic acid (solvent A) and methanol (solvent B) with the following gradient composition: starting with 20%, v/v, and changing to 50%, v/v, B during 3 min, kept for 5 min, followed by a second ramp to 80%, v/v, B in 5 min, maintained for 17 min, a third ramp to 20%, v/v, B in 1 min, remaining at this last condition for 10 min before the next run. ESI-MS detection was performed in negative and positive ion mode with mass acquisition between 100 and 1500 Da. Nitrogen was used as drying and nebulizer gas (7 L/min, 3.5 bar, 180°C). For MS/MS, fragmentation was achieved by using Auto $MS^2$ option and DAD analyses between 200 and 700 nm. The identification of phenolics was carried out by comparison of the spectral properties (UV, ESI-MS and MS/MS) of the compounds with those of reference samples when available or literature data. MS analysis was used for quantification of the compounds with specific calibration curves or with structurally related substances. Compound concentrations were measured in triplicate.

***Molecular parameters:*** The molecular structures of the compounds identified by HPLC-ESI-MS/MS were constructed using the ChemWindow program, transferred to ChemAxon program and the conformer with lower energy was determined. The following structural parameters were calculated: log D at pH 3, 7, 10 and 13; hydrogen donor (HD) and hydrogen acceptor (HA) sites, and pKa (expressed as net charge and percentage of predominant species at each pH). The log D was expressed as the logarithm of the ratio of the concentrations of all forms of the compound (ionized and non-ionized) in a nonpolar solvent (octanol), and the concentrations of all forms in a polar solvent (water).

***Total phenolic content (TPC) and antioxidant activity:*** Analysis of TPC of the aqueous ethanol extract was carried out by the Folin-Ciocalteu method using gallic acid as standard [15]. The radical-scavenging activity was determined using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) [13] and expressed as $IC_{50}$ (extract concentration, which causes 50% decrease of the initial DPPH• concentration). The effect of phenolic extract on the oxidative stability of walnut flours was determined by means of the oxidative stability indices (OSI) with a Rancimat instrument (Metrohm, Switzerland). For these assays, WF and D-WF samples (1 g each) were homogenized with walnut oil (3 g) for 30 s at 15000 rpm. Analyses were carried out at 110ºC with air flow rates of 20 L/h. Results were expressed as induction time (IT) in h and corresponded to the break points in the plotted curves.

***Walnut flour analyses:*** Protein solubility at different pH was determined according to Lawal [13] with some modifications. Suspensions (1%, w/v) of flour samples in distilled water were stirred for 1 h at room temperature. The pH of the mixtures (10 mL) was adjusted to appropriate pH (between 2 and 10) with 0.1 mol/L sodium hydroxide or 0.1 mol/L hydrochloric acid. Each mixture was kept, with constant agitation, for a further 1 h and then was centrifuged (5500 *g*, 30 min at 15 ºC). The soluble protein content in each supernatant was determined by the Kjeldahl method (N x 5.3) and the solubility was expressed as g/100 g of total protein.

***Statistical analyses:*** Analytical determinations were made in triplicate from independent samples. Differences between treatments were estimated by ANOVA and LSD tests ($P < 0.05$) using InfoStat software, version 1.1 (Universidad Nacional de Córdoba).

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