

Phenolic compounds and antioxidant activity of Pecan [*Carya illinoensis* (Wangenh.) C. Koch] kernel cake extracts

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RESUMEN

Actividad antioxidante y compuestos fenólicos de extractos de tortas de almendra de pecana [*Carya illinoensis* (Wangenh.) C. Koch].

El contenido de taninos condensado y fenoles totales de diferentes extractos de tortas de almendra de pecana (éter, acetona, alcohol y agua destilada) fueron estimados y sus actividades antioxidantes fueron evaluadas mediante los métodos con ABTS, DPPH y el sistema β -caroteno/ácido linoleico. Las variaciones de color de la torta de almendra de pecana fueron determinadas mediante análisis instrumental usando el sistema CIE Lab. Los contenidos de fenoles totales, taninos condensados y actividad antioxidante, medida mediante los métodos con ABTS Y DPPH (30 min y 24 h), fueron significativamente más altos ($p < 0.05$) con el extracto de acetona (16.4 mg GAE/g; 31.2 mg CE/g; 235.3 μ mol TEAC/g and 68.6 and 100.3 mg TEAC/g, respectivamente). El porcentaje de inhibición de la oxidación en el sistema β -caroteno/ácido linoleico vario desde 37.9 a 93.1% con el extracto de acetona a 300 ppm, mostrando resultados significativamente superiores. Las muestras con una mayor tendencia a tonos rojos presento los niveles más altos de taninos condensados.

PALABRAS CLAVE: Actividad antioxidante – *Carya illinoensis* – Compuestos fenólicos – Oxidación – Torta de almendra de pecana.

SUMMARY

Phenolic compounds and antioxidant activity of Pecan [*Carya illinoensis* (Wangenh.) C. Koch] kernel cake extracts obtained by sequential extraction.

The total phenolic and condensed tannin contents of different Pecan kernel cake extracts (ether, acetone, alcohol and distilled water) were estimated and their antioxidant activities were evaluated through ABTS, DPPH and β -carotene/linoleic acid systems. Color variations of the Pecan kernel cake were determined through an instrumental analysis using the CIE Lab system. Significantly higher levels ($p < 0.05$) of total phenolics, condensed tannins and antioxidant activity measured through the ABTS and DPPH (30 min and 24 h) systems were observed for the acetone extract (16.4 mg GAE/g; 31.2 mg CE/g; 235.3 μ mol TEAC/g and 68.6 and 100.3 mg TEAC/g, respectively). The oxidation inhibition percentage in the β -carotene/linoleic acid system varied from 37.9 to 93.1%, with the acetone extract at 300 ppm showing significantly superior results. The samples with the greatest tendency to show red tones presented the highest levels of condensed tannins.

KEY-WORDS: Antioxidant activity – *Carya illinoensis* – Oxidation – Pecan kernel cake – Phenolic compounds.

1. INTRODUCTION

The Pecan nut [*Carya illinoensis* (Wangenh.) C. Koch], which belongs to the *Junglandaceae* family, was originally cultivated by North-American Indians, and can now be found from the regions of Nebraska and Iowa in the USA all the way to the South of Mexico. Its commercial production has been extended to Australia, South Africa, Israel, Argentina, Chile and the South of Brazil. The pecan nut nutrient, micronutrient and phytochemical compound composition, as well as the Pecan nut color, varies depending on growth conditions, water availability, location, weather, horticultural practice, cultivar and maturity level (Shahidi and Naczka, 2004; Venkatachalam, 2004; Wakeling *et al.*, 2001; Thompson *et al.*, 1996).

Pecan nut oil, obtained by mechanical pressing of the nuts, is rich in monounsaturated and polyunsaturated fatty acids and produces, as a residue, the kernel cake. The Pecan kernel cake still contains high levels of lipids (36.2%) and significant quantities of proteins (22.1%), carbohydrates (16.3%), total fiber (14.3%), moisture (7.9%) and minerals (3.2%), when compared to the whole nut (Oro *et al.*, 2008). Also, the Pecan nut presents bioactive molecules, such as sterols, tocopherols and phenolic compounds in its composition. These compounds present antioxidant activity through the stabilization of free radicals (Kornsteiner *et al.*, 2006; Kris-Etherton *et al.*, 1999).

Free radicals are molecules that contain an isolated electron, making them unstable and highly reactive species that trigger oxidation reactions in the fatty acids present in biological membranes and foods, leading to rancidity and the development of unpleasant odors and flavors and loss of nutritional value. Also, the oxidation of compounds related to food pigmentation may lead to discoloration or formation of other compounds with different colors than those of the fresh food. This process may be delayed through changes in environmental conditions or the use of antioxidant substances

(Soares, 2002; Scalbert and Williamson, 2000; Rice-Evans *et al.*, 1997; Senter and Forbus, 1978). Among the antioxidant compounds that exist naturally in plants, tocopherols, phenolic compounds and tannins can be mentioned (Santos-Buelga and Scalbert, 2000).

Tocopherols are natural compounds with antioxidant activity that are widely found in several tissues and are known as vitamin E (Wanasundara and Shahidi, 2005). They are structures derived from the chromanol ring with hydroxyl and methyl groups on the phenolic ring, containing a long terpenic side-chain (Pokorný and Parkányiová, 2005). The antioxidant activity of tocopherols depends on their concentration, temperature, light, substrate and solvent type and on the presence of synergists and chemical species that can act as pro-oxidants. The inhibition of the production of peroxides and the efficiency of the antioxidant activity of the homologous tocopherols also depend on the lipid system tested (Nogala-Kalucka *et al.*, 2005; Bramley *et al.*, 2000).

Tannins are also known as proanthocyanidins of varied degrees of polymerization and can be found in hydrolyzed or condensed forms (Santos-Buelga and Scalbert, 2000). Some of the tannins present in Pecan nut shells can be leached into the interior of the nuts during pre-conditioning, before they are broken in the shelling process. These substances are in part responsible for the stability of color and flavor of the nuts (Heaton and Worthington, 1975). Among the tannins, phlobaphenes, also called red tannins, are a group of colored polymers, derived from condensed tannins, formed through the polymerization of leucoanthocyanidins (Robinson, 1975).

The color of shelled Pecan seed coats is the major criterion in their quality determination. Based on their color, Pecans are graded into light (golden color), light amber (light brown), amber (medium brown) and dark amber (dark brown). A light color is indicative of fully mature Pecans that have been properly harvested, processed and stored. A darker color is caused by exposure of the kernels to adverse conditions, which in turn can lead the plant metabolism to synthesize enzymes, phenolic compounds and condensed tannins, which act in oxidative processes (Balasundram *et al.*, 2006; Shahidi and Naczka, 2004).

The Pecan is a versatile nut with a wide spectrum of possible uses. It can be commercialized shelled, as an ingredient for confectionery, bakery and dairy industries, as a snack in its natural form, roasted, salted, coated with sugar or honey and improving and finishing industrialized products (Swink, 1996). Also, the consumption of nuts is part of the Mediterranean diet, being reported as providing cardioprotective effects and playing an important role in the protection of biological membranes, causing a slowing-down of the effects of free radicals (Kris-Etherton *et al.*, 2002). However, data of the phenolic composition and antioxidant activity of Pecan nut kernel cake from nuts produced in the South of Brazil are still scarce.

Therefore, the objective of the present work was to determine the contents of total phenolic compounds and condensed tannins and the antioxidant activity of different Pecan kernel cake extracts (ether, acetone, alcohol and distilled water), from nuts produced in the South of Brazil, using ABTS [2,2'-azino-bis-(3-ethylbenzotiazoline-6-sulphonic acid)], DPPH (2,2-diphenyl-1-picrylhydrazyl) and β -carotene/linoleic acid systems. Also, the antioxidant activity of the extracts determined through the β -carotene/linoleic acid system was compared to a commercial antioxidant (composed of a mixture of natural tocopherols recommended for use in fats and oils) and to mixtures of extracts and tocopherols in equal proportions. Color variations of Pecan kernel cake were determined through an instrumental analysis using the CIE Lab system.

2. MATERIALS AND METHODS

2.1. Materials

Pecan kernel cake from 3 different batches (2 kg of each batch, harvested on 3 different dates between May and July of 2006) of a mixture of the following varieties: Barton (approximately 50%), Shoshone, Shawnee, Choctaw and Cape Fear was used.

A commercial antioxidant, indicated for use in animal and vegetable fats and oils (from 100 to 500 ppm), composed of a mixture of natural tocopherols (MT), containing 0.64 g tocopherols/g product (of which 58.6% was γ -tocopherol, 25.1% δ -tocopherol, 14.7% α -tocopherol and 1.6% β -tocopherol) was used for the comparison of antioxidant activity.

2.2. Methods

Preparation of Pecan kernel cake extracts

After grinding in a food processor, the Pecan kernel cake was defatted with hexane (1:20 m/v) using a mechanical shaker (model TE-139, Tecnal[®]), dried in a renewed air oven (model 400/D 200 °C, Nova Ética[®]) at 40 °C until complete evaporation of the solvent, milled in an analytical laboratory mill (model A-11, IKA Works[®]), passed through a 60 mesh sieve and submitted to sequential extraction with ether, acetone, alcohol and distilled water at room temperature (Figure 1).

Filtration was then carried out under vacuum, using Whatman 541 (125 mm) filter paper. Volumes were completed to 100 mL and the extracts obtained were stored in triplicate in amber flasks, in a nitrogen atmosphere, and frozen until further analysis (Wilkinson, 2000). Dry weight was determined gravimetrically, placing a 5 mL aliquot in a previously weighed porcelain crucible, followed by drying in an oven at 105.0 \pm 0.5 °C, until constant weight (AOAC, 2005).

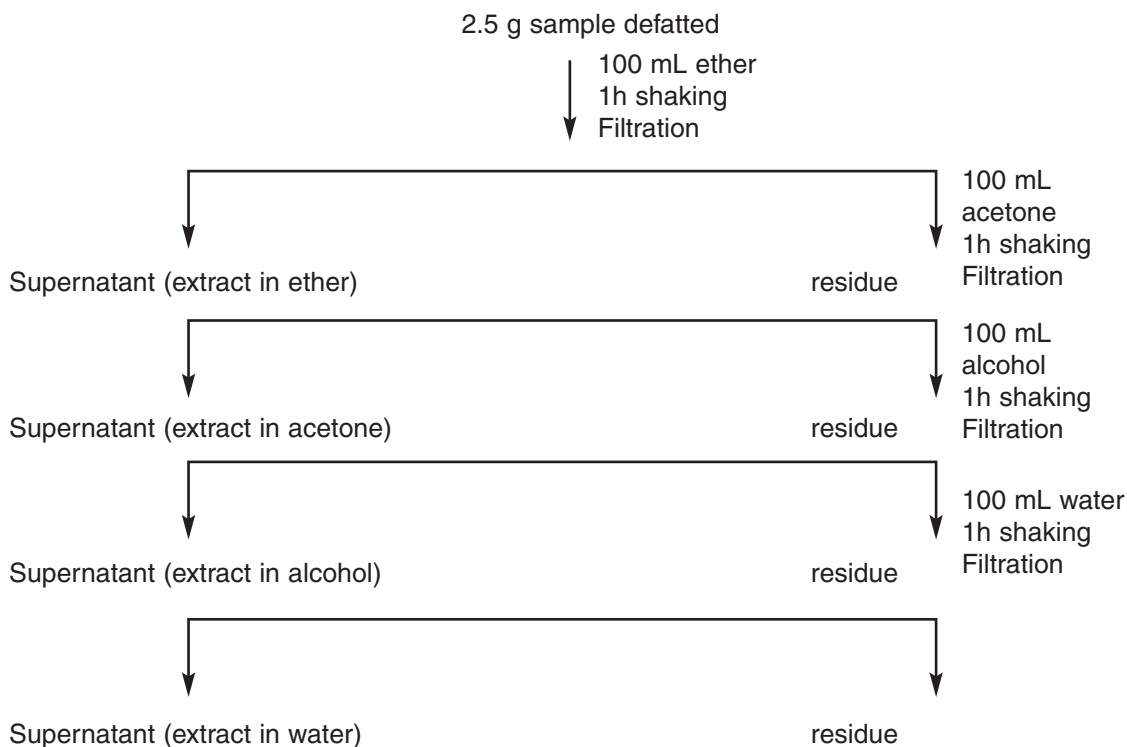


Figure 1
Diagram of the extraction procedure (Moreira, 2003).

Determination of total phenolic compounds

The total phenolic compound content was estimated using the Folin-Ciocalteu colorimetric method with some modifications (Beal, 2006; Budini *et al.*, 1980). Aliquots (100 μ L) of appropriate dilutions of the extracts were oxidized with 500 μ L Folin-Ciocalteu reagent. The reaction was neutralized with saturated sodium carbonate (75 g/L; 1.5 mL) and the volumes were completed to 10 mL by adding deionized water. After incubation for 2 hours at room temperature, absorbance of the resulting blue solution was measured at 764 nm. Analyses were carried out in triplicate. Quantification was done using a gallic acid standard curve and the results were expressed as mg GAE/g of dry weight (mg gallic acid equivalents/g of defatted sample).

Determination of condensed tannin content

To determine the condensed tannin content, the procedure described by Price *et al.* (1978), and adapted by Villarreal-Lozoya *et al.* (2007), was used. Aliquots of 1 mL of appropriate dilutions of the extracts were collected and placed in 2 separate test tubes (1 for the sample and the other for the blank). Then, 5 mL of vanillin reagent (0.5 g reagent and 200 mL 4% HCl/methanol) were added to the samples, and 4% HCl/methanol to the blank. The test tubes were kept in a dark place for 20 minutes and absorbance was measured in a spectrophotometer at 500 nm. The analyses were

carried out in triplicate and the results were expressed as mg CE/g of dry weight (mg catechin equivalents/g of defatted sample).

Determination of antioxidant capacity

ABTS assay: the ABTS [2,2'-azino-bis-(3-ethylbenzotiazoline-6-sulphonic acid)] assay was carried out according to Re *et al.* (1999), with some modifications. After preparing the ABTS radical (7 mM – 0.03836 g ABTS dissolved in 10 mL deionized water), a potassium persulfate solution (2.45 mM – 10 mL ABTS and 10 mL persulfate mixed, homogenized and kept in an amber flask for at least 16 hours, protected from light) was prepared. For the sample, an aliquot of 200 μ L of the radical formed was pipetted and diluted in 10 mL 96° ethanol, analysis grade. Absorbance readings at 734 nm, in 10 mm cuvettes, were made to verify that optical density was around 0.700 ± 0.05 . An aliquot of 980 μ L of the diluted radical was pipetted and transferred to a 10 mm (1 mL) cuvette, measuring absorbance ($A_{734} = A_0$), adding 20 μ L of the sample immediately, homogenizing and agitating for a few seconds. Trolox was used as standard (0.13209 g/500 mL H₂O). The calculation of the radical inhibition percentage was made using the following formula: per cent radical inhibition = $(1 - A_f/A_0) \times 100$, where, “A₀” is initial absorbance and “A_f” is final absorbance. The calculations were made for each concentration of the samples analyzed, so a dose-response curve could be obtained with the results. The results were

expressed as $\mu\text{mol TEAC/g}$ of dry weight ($\mu\text{mol Trolox equivalent antioxidant capacity/g}$ of defatted sample). Analyses were carried out in triplicate.

DPPH assay: the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was carried out according to Brand-William *et al.* (1995), followed by modifications, as described by Kim *et al.* (2002) and Villarreal-Lozoya *et al.* (2007). After preparing the radical (0.1 mM – 0.03943 g DPPH dissolved in 10 mL 80% methanol), an aliquot of 2.9 mL was pipetted and placed in test tubes with 0.1 mL extract. The samples were kept in a dark place, and absorbance was measured in a spectrophotometer at 515 nm, after 30 minutes and after 24 hours. Trolox was used as standard (150 mg/L). The calculation of the radical inhibition percentage was made using the following formula: per cent radical inhibition = $(1 - A_t/A_0) \times 100$, where, “ A_0 ” is initial absorbance and “ A_t ” is final absorbance. The calculations were made for each concentration of the samples analyzed, so a dose-response curve could be obtained with the results. The analyses were carried out in triplicate and the results expressed as mg TEAC/g of dry weight (mg Trolox equivalent antioxidant capacity/g of defatted sample).

β -carotene/linoleic acid assay: the *in vitro* antioxidant activity of the β -carotene and linoleic acid model system was determined following the methodology described by Marco (1968) and modified by Miller (1971). Beta-carotene was dissolved in chloroform (20 mg/mL). An aliquot of 20 μL of this solution was put in an Erlenmeyer flask with 40 μL linoleic acid, 1 mL chloroform and 500 mg Tween 40. Chloroform was completely evaporated with nitrogen vapor. Then, 150 mL distilled deionized water saturated with oxygen (30 min.) were added to the β -carotene emulsion, agitating the mixture. Two hundred, 300 and 500 ppm of the extracts were added, and, in parallel, equal concentrations of a commercial antioxidant, recommended for use in fats and oils in general, composed of a mixture of natural tocopherols (MT) and used as a standard antioxidant. Also, the synergistic effect was evaluated through the mixture of extracts (E) and tocopherols (MT) in concentrations of 100, 150 and 250 ppm each (E + MT, 1:1). Absorbance readings at 470 nm were carried out at 15 minute intervals during a period of 2 hours, keeping the cuvettes in a water bath at 50 °C. Antioxidant activity was calculated as inhibition percentage, relative to the control, using the following equation: $AA = [(A_s - A_c)/(A_0 - A_c)] \times 100$, where A_s and A_c are, respectively, the absorbance of the sample and of the control at the end of the analysis and A_0 is the absorbance of the sample (470 nm) at the beginning of the assay. Analyses were carried out in triplicate.

Instrumental color analysis of Pecan nut kernel cake

For the instrumental color analysis, a Minolta Chromo Meter CR 400 (Minolta, Osaka, Japan)

colorimeter, coupled to a DP-100 processor, with illuminant D65 and angle of 10°, was used. The CIE Lab (Commission Internationale de l'Éclairage) evaluation system was used, with the color scale L^* , a^* and b^* , where L^* is the luminosity (zero for black and 100 for white); a^* is the color variation from green to red [from –80 to zero for green (– a), from zero to +100 for red (+ a)]; b^* is the color variation from blue to yellow [from –100 to zero for blue (– b), from zero to +70 for yellow (+ b)]. Chroma (C^*) –that indicates color saturation, i.e. the proportion in which the color is mixed with white, black or gray– and color hue (H - expressed in angles: 0° is red, 90° is yellow, 180° is green and 270° is blue, corresponding to + a , + b , – a , and – b , respectively), were also determined.

Statistical analysis

The statistical analysis of the data was carried out using the SAS for Windows software. Results were submitted to analysis of variance (ANOVA) and the Tukey test ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Phytochemical analysis and antioxidant activity (ABTS and DPPH)

The contents of Dry Extract (DE), Total Phenolic Compounds (TP), Condensed Tannins (CT) and the Antioxidant Capacity (AC) of Pecan kernel cake extracts can be observed in Table 1.

According to the results, the highest yields (dry extract) were observed for the extracts in water (20.2%) and ether (8.6%), followed by the extracts in alcohol and acetone, which presented lower yields (5.7 and 3.5%, respectively). The total phenolic content obtained for these fractions was higher for the acetone extracts, followed by water, ether and alcohol extracts (16.4 ± 4.2 ; 5.6 ± 0.4 ; 2.4 ± 0.3 and 2.3 ± 1.3 mg GAE/g, respectively). The results found for the yield show that the extraction of other compounds beyond phenolics affected the value.

The relationship between the extraction yield and total phenolic content can be observed in the analysis of the different batches, where those that had higher yields also had higher levels of total phenolic compounds. Batch 1 presented an extraction yield in the acetone fraction of 4.4% and a phenolic compound content significantly higher than the other batches analyzed (20.1 ± 2.5 mg GAE/g). Batches 2 and 3 stand out for presenting higher extraction yields in the fractions obtained in water (22.7%) and ether (9.1%) and higher levels of phenolic compounds (5.9 ± 0.2 and 2.8 ± 0.1 mg GAE/g, respectively) in these fractions. Batches 1 and 2 presented the highest yields in the alcoholic fraction (7.4 and 6.6%). Higher levels of total phenolics for Batch 2 in this fraction (3.7 ± 0.8 mg GAE/g) were also observed.

Table 1
Dry extract (DE), total phenolic compounds (TP), condensed tannins (CT) and antioxidant capacity (AC)
of Pecan kernel cake extracts determined through ABTS and DPPH systems.

	Extract	Batch 1	Batch 2	Batch 3	Average
DE (g/100g)*	Ether	8.7 ^{ab} ± 0.4	7.9 ^b ± 0.3	9.1 ^a ± 0.3	8.6 ± 0.6
	Acetone	4.4 ^a ± 0.4	3.1 ^b ± 0.1	2.9 ^b ± 0.3	3.5 ± 0.8
	Alcohol	7.4 ^a ± 0.9	6.6 ^a ± 1.2	3.1 ^b ± 0.5	5.7 ± 2.3
	Water	16.8 ^b ± 0.4	22.7 ^a ± 0.9	21 ^a ± 1.4	20.2 ± 3
TP (mg GAE/g)*	Ether	2.2 ^a ± 0.5	2.3 ^a ± 0.7	2.8 ^a ± 0.1	2.4 ± 0.3
	Acetone	20.1 ^a ± 2.5	11.8 ^b ± 0.6	17.4 ^a ± 2.6	16.4 ± 4.2
	Alcohol	1.3 ^b ± 0.4	3.7 ^a ± 0.8	1.8 ^b ± 0.1	2.3 ± 1.3
	Water	5.8 ^a ± 1.4	5.9 ^a ± 0.2	5.1 ^a ± 0.6	5.6 ± 0.4
CT (mg CE/g)*	Ether	nd	nd	nd	—
	Acetone	42.9 ^a ± 4.4	21.4 ^b ± 0.1	29.4 ^b ± 3.2	31.0 ± 10.0
	Alcohol	3.6 ^a ± 2.0	3.7 ^a ± 2.4	1.0 ^a ± 1	2.8 ± 1.5
	Water	nd	nd	nd	—
AC _{ABTS} (μmol TEAC/g)*	Ether	7.2 ^a ± 0.1	1.7 ^b ± 1.0	4.3 ^b ± 0.1	4.4 ± 2.7
	Acetone	295.5 ^a ± 0.1	204.7 ^b ± 0.1	205.8 ^b ± 0.03	235.0 ± 52.0
	Alcohol	36.2 ^a ± 0.3	32.5 ^a ± 0.2	19.6 ^a ± 0.1	29.0 ± 8.0
	Water	42.0 ^a ± 0.3	34.3 ^a ± 0.1	49.0 ^a ± 0.04	41.0 ± 7.0
AC _{DPPH} 30 min (mg TEAC/g)*	Ether	ne	ne	ne	—
	Acetone	92.6 ^a ± 0.1	49.9 ^b ± 0.08	63.2 ^b ± 0.1	68.0 ± 21.0
	Alcohol	ne	ne	ne	—
	Water	13.4 ^a ± 0.4	8.8 ^a ± 0.1	15.7 ^a ± 0.2	12.6 ± 3.5
AC _{DPPH} 24 min (mg TEAC/g)*	Ether	ne	ne	ne	—
	Acetone	125.3 ^a ± 0.06	80.4 ^b ± 0.04	95.1 ^b ± 0.1	100.0 ± 22.0
	Alcohol	ne	ne	ne	—
	Water	16.8 ^a ± 0.05	16.1 ^a ± 0.04	19.6 ^a ± 0.1	17.5 ± 1.8

*DE = Dry Extract (gravimetric assay), TP = Total Phenolics - mg GAE/g (gallic acid equivalent) of dry weight (Folin-Ciocalteu assay), CT = Condensed Tannins - mg CE/g (mg catechin equivalent/g of defatted sample) of dry weight (vanillin assay), AC = Antioxidant Capacity - μmol TEAC/g (Trolox equivalent antioxidant activity) of dry weight (ABTS), Antioxidant Capacity (DPPH – 30 minutes), Antioxidant Capacity - mg TEAC/g (Trolox equivalent antioxidant activity) of dry weight (DPPH – 24 hours); ^{a, b} means with the same superscript letter in the same line are not significantly different (Tukey test, $p < 0.05$); overall mean values ± SD (n=3); (nd) not detected; (ne) not evaluated.

Wu *et al.* (2004) reported a level of 20.16 mg GAE/g total phenolic compounds present in extracts of nuts in a mixture of acetone, water and acetic acid (70:29.5:0.5, v/v). Villarreal-Lozoya *et al.* (2007) reported a total phenolic content of 76 mg CAE/g (Chlorogenic Acid Equivalent) in Pecan nut extracts in a mixture of acetone and water (70:30, v/v). According to Kyoung Chun and Kim (2004), it is possible to convert from CAE to GAE, assuming a conversion factor of 0.6, in such a way that the result would be equivalent to 13.68 mg GAE/g. Kornsteiner *et al.* (2006) obtained a content of 12.84 mg GAE/g for Pecan nut extracts, with extraction in hexane of a solution of 75% acetone and 25% 526 μmol/L sodium metabisulfite.

The means obtained in the present work for the fractions in alcohol, ether and water, were much lower than those reported by Wu *et al.* (2004), Villarreal-Lozoya *et al.* (2007) and Kornsteiner *et al.* (2006) (2.3 ± 1.3, 2.4 ± 0.3 and 5.6 ± 0.4 mg GAE/g, respectively), which were close to the mean obtained for the fraction in acetone (16.4 ± 4.2 mg GAE/g).

The content of condensed tannins was higher in the fraction in acetone (mean of 31.0 ± 10.0 mg CE/g), when compared to the fraction in alcohol,

where low concentrations were observed (mean of 2.8 ± 1.5 mg CE/g). For the fraction in acetone, a significant difference ($p < 0.05$) between the contents obtained for Batch 1 (42.9 ± 4.4 mg CE/g) and Batches 2 (21.4 ± 0.1 mg CE/g) and 3 (29.4 ± 3.2 mg CE/g) was observed. There were no significant differences observed ($p < 0.05$) among the batches analyzed for the fraction in alcohol. The extracts in ether and water did not present condensed tannin contents within the levels detected in the assay.

Villarreal-Lozoya *et al.* (2007) reported values of condensed tannins very close to the means of the fraction in acetone obtained in the present work, in extracts composed by a mixture of acetone and water (70:30, v/v), of the Pecan nut kernel of different cultivars from Texas, United States (mean of 34.0 ± 1.3 mg CE/g).

As the results obtained for total phenolic and condensed tannin contents in the fractions in ether and alcohol were relatively low, showing reduced antioxidant activity in the ABTS system compared to the other fractions, the assays of antioxidant activity through the DPPH and the β-carotene and linoleic acid systems were carried out only for the fractions in acetone and water.

The antioxidant activity determined through the ABTS and DPPH systems after 30 minutes and 24 hours of reaction was higher for the extract in acetone (mean of $235.0 \pm 52.0 \mu\text{mol TEAC/g}$; 68.0 ± 21.0 and $100.0 \pm 22.0 \text{ mg TEAC/g}$, respectively). These results are in accordance with the results of TP and CT obtained for this fraction. For the fraction in acetone, the results were significantly higher ($p < 0.05$) for Batch 1 in the ABTS and DPPH systems after 30 minutes of reaction and, in the DPPH system after 24 hours of reaction ($295.5 \pm 0.1 \mu\text{mol TEAC/g}$; 92.6 ± 0.1 and $125.3 \pm 0.06 \text{ mg TEAC/g}$, respectively).

The extract in water showed mean antioxidant activity in the ABTS system of $41.0 \pm 7.0 \mu\text{mol TEAC/g}$ and, in the DPPH system after 30 minutes and 24 hours of reaction, of 12.6 ± 3.5 and $17.5 \pm 1.8 \text{ mg TEAC/g}$, respectively. There were no significant differences observed ($p < 0.05$) for the extract in water in the ABTS and DPPH systems after 30 minutes and 24 hours of reaction among the different batches analyzed.

Villarreal-Lozoya *et al.* (2007) reported an antioxidant activity of Pecan nut in extracts of acetone and water (70:30, v/v) of $97.0 \pm 6.7 \text{ mg TEAC/g}$ in the DPPH system after 24 hours of reaction. This value was higher than the value obtained for the extract in acetone in the present work after 30 minutes of reaction ($68.6 \pm 21.8 \text{ mg TEAC/g}$), but lower than the value obtained for the same fraction after 24 hours of reaction with the DPPH radical (mean of $100.3 \pm 22.9 \text{ mg TEAC/g}$).

The different levels of phenolic compounds in the different solvents observed in this study can be related, among several factors, with the chemical nature of the phenolic substances (like phenolic acid, anthocyanins and tannins), the interaction with other nutrients and micronutrients present in the sample and the polarity of the solvent used in the extraction procedure (Shahidi and Naczki, 1995). These factors explain the difficulties of

comparing the results of different studies due to the high number of variables involved and, particularly, the solvent selected for extraction.

The correlation between the total phenolic compound content (TP) and the antioxidant activity determined in the ABTS and DPPH systems for the extracts can be observed in Figure 2.

The results obtained show a strong correlation between the total phenolic content present in Pecan nut kernel cake extracts and the antioxidant activity of the extracts obtained through the procedure of sequential extraction, in the ABTS ($r = 0.9655$) and DPPH ($r = 0.9821$) systems. The extracts that showed the highest total phenolic compound content (acetone), also showed significantly higher ($p < 0.05$) antioxidant activity in the two systems studied.

3.2. Antioxidant activity in the β -carotene and linoleic acid model system

In Table 2, the oxidation inhibition percentages, measured through the β -carotene and linoleic acid model system, for the mixture of natural tocopherols used as standard (MT), the Pecan nut kernel extracts in acetone and water and the mixtures of extracts and natural tocopherols in the same proportions (100, 150 and 250 ppm each) can be observed.

As can be seen in Table 2, in the analysis of antioxidant activity through the β -carotene and linoleic acid model system, the mixture of natural tocopherols used as standard (MT) showed an oxidation inhibition percentage between 25.2 and 62.4%, being significantly more effective ($p < 0.05$) at the concentration of 500 ppm. These results were inferior to those of the acetone and water extracts and to those of the mixtures of extracts and natural tocopherols tested, which showed significantly superior values ($p < 0.05$).

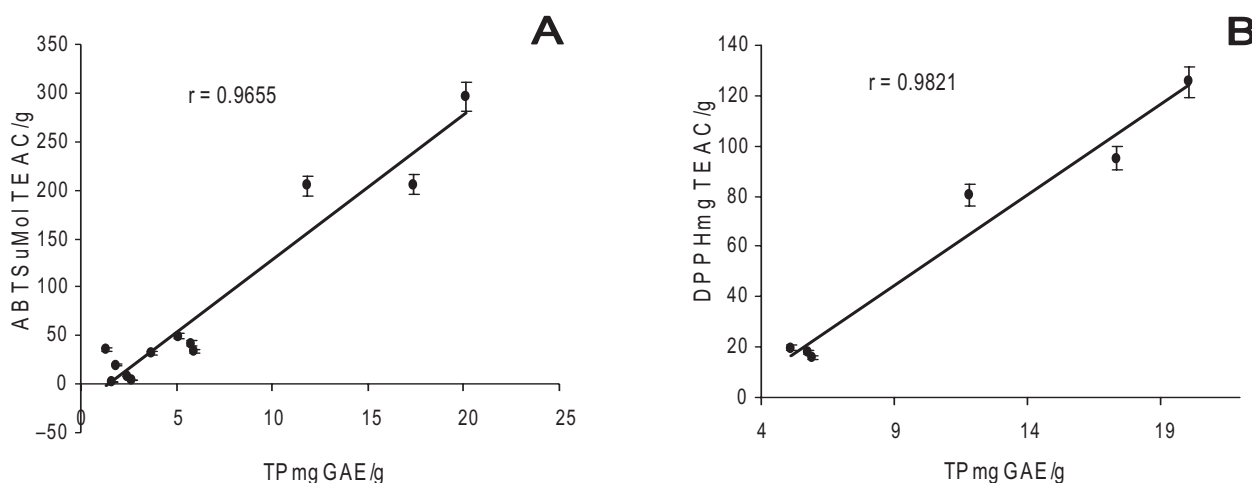


Figure 2

(A) Correlation between antioxidant capacity measured in the ABTS assay and total phenolic compound content (TP). (B) Correlation between antioxidant capacity measured in the DPPH assay (24 hours) and total phenolic compound content (TP).

Table 2
Oxidation inhibition percentage of tocopherols (MT), Pecan kernel cake extracts (E) and mixtures of extracts and tocopherols in the same proportions (E+MT 1:1), measured in *vitro* through the β -carotene and linoleic acid model system.

Extract	% oxidation inhibition at different concentrations		
	200 ppm	300 ppm	500 ppm
MT (standard)	25.2 ^C ± 0.1	42.9 ^B ± 3.2	62.4 ^A ± 1.9
Acetone – Batch 01	85.3 ^{Ac} ± 0.1	87.4 ^{Ab} ± 3.0	85.9 ^{Ab} ± 0.1
Acetone – Batch 02	91.1 ^{Ba} ± 0.4	95.0 ^{Aa} ± 0.1	89.8 ^{Ca} ± 0.1
Acetone – Batch 03	89.0 ^{Bb} ± 0.5	93.1 ^{Ab} ± 0.5	86.2 ^{Bb} ± 0.1
Water – Batch 01	80.4 ^{Ba} ± 0.5	80.6 ^{Ba} ± 0.1	86.5 ^{Aa} ± 0.6
Water – Batch 02	62.2 ^{Bc} ± 2.1	65.8 ^{Ac} ± 0.1	76.0 ^{Aa} ± 3.7
Water – Batch 03	70.9 ^{Bb} ± 0.8	72.8 ^{Bb} ± 0.2	81.0 ^{Aa} ± 2.2
Acetone + MT - Batch 01	84.0 ^{Bab} ± 1.5	89.3 ^{Aa} ± 0.4	90.7 ^{Aa} ± 0.1
Acetone + MT - Batch 02	88.3 ^{Aa} ± 1.5	92.0 ^{Aa} ± 2.2	92.1 ^{Aa} ± 1.8
Acetone + MT - Batch 03	76.0 ^{Bb} ± 3.5	88.1 ^{Aa} ± 0.2	90.0 ^{Aa} ± 1.3
Water + MT - Batch 01	79.7 ^{Ba} ± 2.8	84.0 ^{Aa} ± 1.4	88.9 ^{Aa} ± 0.5
Water + MT - Batch 02	37.9 ^{Cc} ± 2.3	63.7 ^{Bc} ± 3.0	75.9 ^{Ab} ± 3.2
Water + MT - Batch 03	57.9 ^{Cb} ± 0.1	73.4 ^{Bb} ± 0.9	82.1 ^{Ab} ± 1.9

E+MT 1:1 (100, 150 and 250 ppm each); ^{A,B,C} values with the same superscript uppercase letter in the same line are not significantly different (Tukey $p < 0.05$); ^{a, b, c} values with the same superscript lowercase letter in the same column are not significantly different (Tukey $p < 0.05$) among the batches, for the same kind of sample (Extract or E + MT); results expressed as percentage (per cent); overall mean values \pm SD (n=3).

The acetone extract presented an oxidation inhibition percentage between 85.3 and 95%. Batches 2 and 3 had significantly higher ($p < 0.05$) oxidation inhibition percentages and were more effective at the concentration of 300 ppm.

For the extract in water, an oxidation inhibition percentage between 62.2 and 86.5% was observed and the 500 ppm concentration was significantly more effective ($p < 0.05$). Significant differences in the treatments among the batches were observed only at the concentrations of 200 and 300 ppm, when Batch 1 presented significantly higher percentages than the other batches analyzed (80.4 and 80.6%).

The oxidation inhibition percentages of the acetone and water extracts added to the mixture of tocopherols were between 76.0 and 92.1% and 37.9 and 88.9%, respectively. For the mixture of extracts in acetone and natural tocopherols, Batches 1 and 2 showed significantly higher percentages ($p < 0.05$) in the lowest concentration tested (84.0 and 88.3%) and the 500 ppm concentration was more effective for Batches 1 and 3 (90.7 and 90.0%). No significant differences ($p < 0.05$) were observed among the concentrations tested for Batch 2 in the extract with acetone added to tocopherols. For the mixtures of the extract in water and natural tocopherols, Batch 1 presented significantly higher ($p < 0.05$) oxidation inhibition percentages than the other batches analyzed in all the concentrations tested with the most effective concentrations being 300 and 500 ppm (84 and 88.9%). A slight increase in oxidation inhibition occurred at the concentration of 500 ppm for both the fractions, acetone and water, and at the concentration of 300 ppm for Batches 1 and 3 in the fraction in water, when added to natural tocopherols.

It is known that the different concentrations of the substances used as antioxidants and the chemical nature of the structures of those compounds exert a strong influence on the percentage of oxidation inhibition, depending on the environment where they are added (Frankel *et al.*, 1994; Porter, 1993).

Duarte-Almeida *et al.* (2006) evaluated the antioxidant activity of phenolic compounds through the β -carotene and linoleic acid system. They determined the antioxidant activity of several compounds used as standards in a concentration range of 700 to 1000 μ M. Chlorogenic (25.0%), ellagic (34.0%) and gallic (41.0%) acids and catechin (34.0%) had oxidation inhibition percentages lower than those observed for the mixtures of natural tocopherols and Pecan kernel cake extracts.

Tepe (2008) determined the antioxidant activity through the β -carotene and linoleic acid system for BHT, α -tocopherol and rosmarinic acid, using 350 μ L of the extracts in a concentration of 2 g/L. The oxidation inhibition percentages were of 96, 96.6 and 100% for BHT, α -tocopherol and rosmarinic acid, respectively. The results found at the concentration of 500 ppm for the Pecan nut kernel acetone extracts are close to those reported in the studies of these raw materials considered references for antioxidant activity comparison.

3.3. Instrumental color analysis of Pecan nut kernel cake

Table 3 presents the data of the instrumental color analysis of the cake of the three batches of Pecan nut kernel produced in the South of Brazil.

Table 3
**Instrumental evaluation of color through the CIE Lab system
 (Commission Internationale de l'Eclairage) of the three batches of Pecan kernel cake produced
 in the south of Brazil.**

Parameters	Batch 1	Batch 2	Batch 3	Average
L*	35.7 ^b ± 0.4	39.2 ^a ± 0.3	36.7 ^b ± 1.4	37.2 ± 1.8
C*	9.1 ^b ± 0.2	9.9 ^a ± 0.1	9.3 ^{ab} ± 0.4	9.4 ± 0.42
a*	3.2 ^a ± 0.1	2.8 ^b ± 0.01	3.0 ^{ab} ± 0.1	3.0 ± 0.2
b*	8.5 ^b ± 0.1	9.4 ^a ± 0.2	8.8 ^b ± 0.3	8.9 ± 0.46
H	69.4 ^c ± 0.3	73.4 ^a ± 0.2	71.1 ^b ± 0.3	71.3 ± 2.01

* L* = luminosity, C* = saturation of color, a* = Δ green to red, b* = Δ yellow to blue, H = hue ** means with the same superscript letter in the same line are not significantly different (Tukey test, p < 0.05) *** overall mean values ± SD (n=3).

According to the results obtained, Pecan kernel cake presented mean values for luminosity (L*) of 37.2 ± 1.8 . Batch 2 had a significantly ($p < 0.05$) higher value (39.2 ± 0.3) for luminosity compared to Batches 1 and 3 (35.7 ± 0.4 and 36.7 ± 1.4 , respectively).

Regarding color saturation (C*), the mean obtained was of 9.4 ± 0.42 , with Batches 2 and 3 presenting significantly higher ($p < 0.05$) values (9.9 ± 0.1 and 9.3 ± 0.4), when compared to Batch 1 (9.1 ± 0.2).

For the variation from green to red (a*), the mean obtained was of 3.0 ± 0.2 . Batches 1 and 3 presented significantly higher ($p < 0.05$) values (3.2 ± 0.1 and 3.0 ± 0.1 , respectively), representing more red tones than Batch 2 (2.8 ± 0.01). For the variation from blue to yellow (b*), Batch 2 showed results with a greater tendency to yellow (9.4 ± 0.2), when compared to Batches 1 and 3 (8.5 ± 0.1 and 8.8 ± 0.3). The same tendency of Batch 2 to more yellow tones can be confirmed through the analysis of the parameter H, indicator of the hue, which showed a significantly superior ($p < 0.05$) angle for Batch 2, compared to Batches 1 and 3 (73.4 ± 0.2 , 69.4 ± 0.3 and 71.1 ± 0.3 , respectively). Batches 1 and 3 presented cakes with a darker brown coloration, while Batch 2 presented a cake with a lighter coloration.

Senter and Forbus (1978) reported that the alteration in the red-brown coloration of nuts can be due to the oxidation of leucoanthocyanidin and leukodelphinidin present in the nuts, to its respective phlobaphenes of varied levels of polymerization and, to a lesser extent, to the reaction with the formation of cyanidin and delphinidin. The relationship between the accumulation of phlobaphenes (red pigment) and the coloration changes reported by Senter and Forbus (1978), together with the results obtained in the present work, suggest that the variations in the hue represented by the angle H and parameter a* of the coloration of the nut kernel can be directly related to the concentration of phlobaphenes, since Batches 1 and 3, which presented hue values with a greater tendency to red, also showed higher contents of condensed tannins compared to the other batches analyzed.

4. CONCLUSIONS

According to the results obtained, the fraction of the sequential extraction in acetone presented the highest total phenolic and condensed tannin contents extracted from the kernel cake along with a high antioxidant activity determined through different methods. Considering the fact that the sequential extraction method depletes the sample as it is submitted to extraction with different solvents, depending on the solubilization of phenolics during the extraction process, the nut kernel cake presented a higher total phenolic content and antioxidant activity than the results reported in previous works by Wu *et al.* (2004), Kornsteiner *et al.* (2006) and Villarreal-Lozoya *et al.* (2007).

The extracts presented the highest oxidation inhibition percentages when compared to the tocopherols and the infusion plus tocopherol mixtures, indicating that there was no synergistic effect between the components. These results do not agree with the "polar paradox theory" according to which hydrophilic antioxidants are more effective in non-polar media, while lipophilic compounds are better antioxidants in polar media (Frankel *et al.*, 1994). On the other hand, some authors have reported that some compounds do not follow the polar paradox rule or that the partitioning of antioxidants between the oil and water phases depends on the different factors. This suggests that antioxidant activity is affected by complex phenomena and that polarity is not the only parameter to be taken into account (Cuvelier *et al.*, 2000; Gordon *et al.*, 2001). According to Prat and Hudson (1990), structural characteristics of an antioxidant, like the localization of hydroxyl groups, seem to be more important than extract polarity. Besides, the complex composition of extracts influenced by factors like different production locations, soil composition, harvest time, climatic variations and the number of cultivar varieties in the different batches can lead to some interactions between its components like a synergistic, additive or antagonistic effect.

New research is necessary to identify and quantify the phenolic compounds present in Pecan

nut kernel cake, investigating the individual contribution of each compound to its total antioxidant activity. This way, it will be possible to elucidate the action and solubilization of these phenolic substances in the solvent systems used in this work and also to test extracts that can have antioxidant activity in biological systems, through *in vivo* studies.

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