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Extraction and Analytical Characterization of Phenolic Compounds from Brazil Nut (*Bertholletia excelsa*) Skin Industrial by-Product

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Abstract

Pressurized liquid extraction (PLE) and ultrasound assisted extraction (UAE) were applied to extract phenolic antioxidants from defatted Brazil nut skin (DBNS) industrial by-product. Water/alcohol solvents (ethanol and methanol) (0 - 50 % v/v water) were used at temperatures of 50 - 200 °C in PLE and 50 °C was set in UAE. The effect on extraction yield, total phenolic compounds content (TPC), antioxidant capacity and phenolic characterization was determined. The temperature and water/alcohol mixture affected the dielectric constant, and this in turn influenced the yield, TPC, antioxidant activity and phenolic composition. In PLE, at 200 °C and 50 % (v/v) water/alcohol (ethanol and methanol) the highest yields (33.8 and 38.4 %, respectively), TPC (4673 and 5435 mg GAE/100 g DBNS) and antioxidant capacity (397 and 502 µmol TE/g DBNS) were obtained. Furthermore, at 50 °C, PLE had better extraction efficiency than UAE. Characterization of the extracts indicated that at 50 - 100 °C the extraction of protocatechuic acid and ellagic acid derivative were predominant, while at 150 - 200 °C catechin and vanillin derivatives stand out. In general, hydroxybenzoic acids and flavonoids were the most abundant phenolics, and the addition of 25 % v/v water to ethanol resulted in extracts with their highest concentration. Then, Brazil nut skin is an interesting phenolic-rich source with potential use to produce bioactive ingredients.

Keywords: Antioxidant capacity, Bioactive ingredient, Brazil nut skin, Dielectric constant, Phenolic compounds, Pressurized liquid extraction, Ultrasound assisted extraction

Introduction

Brazil nut (*Bertholletia excelsa* HBK) (BN) is a native fruit (tree nut) from the Amazon region with large industrial production, which in turn generates large amounts of by-products (husk, shell and skin) without current outstanding value. For every ton of clean nut (Brazil nut shelled and dried) around 1.4 tons of waste is generated [1]. Although the Brazil nut skin (BNS) by-product represents a small percentage of the fruit, the industrial scale processing generates significant quantities without any particular use and skins are generally discarded as waste, becoming a type of environmental pollutant.

In recent years, several studies have reported that skin of nuts represents an important source of phytochemicals, such as fiber, polyphenols, and other bioactive substances. Skins of almond and peanut are the most investigated, both in *in vitro* and *in vivo* studies, and properties such as antioxidant, antimicrobial, anti-inflammatory, cytoprotective, antiobesity, among others, have been attributed mainly to their phenolic components [2-4]. Nevertheless, scarce studies were reported concerning BNS. John and Shahidi [5] claimed that BNS contains a total phenolic compound (TPC) content of 1588 mg GAE/100g dry weight (dw), about four times higher than BN kernel (410.9 mg GAE/100g dw), mostly in the soluble form (ca. 80 %), with large content of hydroxybenzoic acids and flavanols.

Indeed, the technological approach used for the phenolic compound extractions from certain plant matrix influences yield and selectivity. Conventional solid-liquid extraction at atmospheric pressure, such as maceration, decoction and Soxhlet methods, that have limitations such as long extraction times, large solvent consumption, low yields, risk of degradation of thermolabile phenolic compounds and/or environmental problems [6]. In this sense, novel extraction technologies using high pressure fluids as solvents, or assisted by ultrasounds, microwaves, electric pulse fields, among others, have shown great preference due to their lower environmental impact, less use of organic solvents, shorter extraction times,

increased yield and/or selectivity of target compounds. Among the most recognized innovative techniques in the recovery of phenolic compounds, ultrasound-assisted extraction (UAE) and pressurized liquid extraction (PLE) are the most preferred. UAE is based on the phenomenon of acoustic cavitation, induced through the formation and explosion of microbubbles that generate the rupture of the matrix tissue and facilitate the diffusion of phytochemicals to the solvent [6]. PLE technique use organic solvents under high pressure (500 - 3000 psi) which exerts a high penetrability in the matrix and maintain the solvent in liquid state under a wide range of temperatures (40 - 200 °C), enhancing the solubility of analytes and allowing a fast extraction [7]. There are reports such as from Odabaş and Koca [8] who achieved improved phenolic extraction of hazelnut skin with water/ethanol mixtures using UAE (123 mg GAE/g dw) followed by microwave (111.5 mg GAE/g dw), supercritical CO_2-ethanol (70 mg GAE/g dw) and maceration (59 mg GAE/g dw). This favorable effect of UAE technique was also reported in the case of peanut skins, with a TPC content 4-fold higher than the value obtained by traditional solid-liquid extraction [9,10]. On the other hand, the use of PLE in the extraction of nut skins is scarcely reported, but there are references on other plant sources, such as apple by-product and grape pomace, where high extraction yield and TPC content were obtained at high temperatures (above 100 °C) [11,12].

Considering the potential improvement of phenolic extraction of BNS by non-conventional extraction technologies, this work presents an evaluation of the use of PLE and UAE methods, under different conditions (temperature and water/alcohol solvents), in the phenolic extraction and chemical composition, together with an assessment of the antioxidant capacity of extracts obtained from defatted BNS. The importance of the present study applies to the analytical field and potential use of BNS as a novel source of bioactive ingredients, stimulating the valorization of agri-food industry by-products.

Materials and methods

Sample and conditioning

Brazil nut skin (BNS) industrial byproduct was kindly donated by the Asociacion de Castañeros de la Reserva de Tambopata los Pioneros - ASCART, located in the department of Madre de Dios, Perú. After elimination of fragments (Brazil nut chips), shell and foreign materials, the BNS were ground (GM200, Retsch, Haan, Germany) to a particle size of less than 1000 µm and then were defatted by supercritical CO_2 extraction (SC-CO_2) at 40 °C and 400 bar, following the procedure described by Vasquez *et al.* [13]. Finally, the defatted Brazil nut skin (DBNS) was packed in a bag, stored at room temperature and protected from light until the extraction.

Reagents

CO_2 (99.98 % purity) was purchased from Carburos Metálicos (Madrid, Spain). Ultra-pure water (Mili-Q) was obtained from a Milipak® Express 40 system (Merk-Milipore, Dormstadt, Germany). Methanol and ethanol were purchased from Panreac (Barcelona, Spain). The reagents Sodium carbonate, Gallic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin and Ciocalteau's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For HPLC analysis, catechin, epicatechin, vanillic acid, protocatechuic acid, quercetin, vanillin, gallic acid and hydroxybenzoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction procedures

Pressurized liquid extraction

PLE device used was an Accelerated Solvent Extraction System (ASE 350, Dionex Corporation, Sunnyvale, CA, USA) equipped with a solvent controller unit, in which about 1 g of DBNS plus 4 g of sea sand were loaded in cells (10 mL capacity). Binary mix, water/methanol and water/ethanol ratio (0, 25 and 50 % v/v) were used as solvents, at temperatures of 50, 100, 150 and 200 °C, under pressurization with nitrogen gas to ~1500 psi and static extraction time of 20 min. After extraction, the liquid extract was collected in a vial and the alcohol solvent was evaporated using a rotary evaporator at 40 °C. The water residue in the extracts was removed by lyophilization, stored in vials protected from light, and kept at −20 °C until analysis.

Ultrasound assisted extraction

UAE assays were carried out using a probe type sonicator (Branson SFX250 Digital Sonifier, Branson Ultrasonics, Danbury, CT, USA). About 4 g of DBNS raw material was used with the extraction solvents water/methanol and water/ethanol (0, 25 and 50 % v/v) at the following process conditions: temperature ~50 °C, amplitude 60 %, power 44 W, time 20 min and a ratio 1/10 (w/v) of skin/solvent. The liquid extract

was filtered by Buchner flask with filter paper and then the alcohol solvent was evaporated with a rotary evaporator at 40 °C. The water residue in the extracts was removed by lyophilization, stored in vials protected from light, and kept at −20 °C until analysis.

Extraction with acetone/water mixture

The reference method proposed by John and Shahidi [5] for phenolic analysis of Brazil nut skin was used to compare the extraction efficiency of individual phenols with the assayed PLE and UAE methods under similar temperature conditions (~50 °C). Briefly, 3 g of DBNS was homogenized (T-25 Ultraturrax, IKA-Werke GmbH & Co., KG, Staufen, Germany) with 50 mL of 70 % acetone and then subjected to reflux conditions at 60 °C for 40min. It was then cooled and centrifuged at 3000 rpm for 10 min. The supernatant was reserved and the sediment was extracted again under the same conditions. The extracts were pooled, concentrate under vacuum with rotary evaporator and dried by lyophilization (Lyobeta-15, Azbil Telstar, S.L., Terrasa, Spain) and kept at −20 °C until analysis.

Analysis of total phenolic compounds (TPC)

TPC of the extracts were analyzed spectrophotometrically with the Folin-Ciocalteu reagent, according to the method of Singleton *et al.* [14] with some modifications. In a 96-microwell plate 20 µL of samples (diluted extracts, standard and blank) were placed and reacted with 100 µL Folin-Ciocalteu reagent (10 %) and then alkalized with 80 µL of Na2CO3 7.5 % (w/v). The mixture was stirred and kept one hour in darkness at room temperature. The reading was done at 756 nm spectrophotometrically with a Synergy HT microplate reader (Bio-Tek instruments, Winooski, VT, USA). Gallic acid was used as standard to elaborate the calibration curve, with concentrations in the range of 50 to 400 µg/mL. The results were expressed as mg of gallic acid equivalents (GAE)/100 g DBNS.

Analysis of phenolic individual compounds by HPLC-UV/Vis-MS

PLE and UAE lyophilized extracts were dissolved with water/methanol (1:1, v/v) and filtered with 0.45 µm syringe filters before analysis by HPLC and UV/Vis detection. Chromatographic analyses were performed according to the protocol reported by Vasquez-Rojas *et al.* [13] using a 1200 Series Agilent HPLC System (Agilent Technologies, Santa Clara, CA, USA) with a reverse phase C18 column (Zorbax SB-C18, 250×4.6 mm, 5 µm; Agilent) maintained at 20 °C. Elution solvent A consisted of 1 % formic acid (v/v) in water, while solvent B was a mixture of methanol and formic acid (1 %, v/v). Separation was achieved using an initial solvent composition of 15 % (B) during 15 min, increased to 25 % (B) within 10 min, and subsequently ramped to 50 % (B) within 10 min, increased to 75 % (B) in 15 min, followed by a decreased period of 15 % (B) in 5 min prior to isocratic re-equilibration at 15 % (B) for 10 min. The flow rate was fixed at 0.8 mL/min and the injection volume was 20 µL. The UV/vis photodiode array detector was set at 3 wavelengths (280, 320 and 380 nm) for monitoring simultaneously different phenolic chemical families.

The HPLC was also coupled to a mass spectrometry detector (LCMS SQ 6120, Agilent, CA, USA) with an electrospray ionization (ESI) source operating in positive ion mode. The drying gas was nitrogen at 3 L/min at 137.9 KPa. The nebulizer temperature was 300 °C and the capillary had 3500 V potential. The coliseum gas was helium and the fragmentation amplitude was 70 V. Spectra were recorded m/z from 100 to 1000. Further mass spectrometry analyses were performed in a maXis II LC-QTOF equipment (Bruker Daltonics, Bremen, Germany) with an ESI source and the same chromatographic conditions. The ESI-QTOF detector worked in positive ion mode and recorded spectra m/z from 50 to 3000. Operation conditions were 300 °C, capillary voltage 3500 V, charging voltage 2000 V, nebulizer 2.0 bar and dry gas at 6 L/min. MS/MS analysis used the bbCID (Broad Band Collision Induces Dissociation) method at 30 eV.

Phenolic compounds were identified according to retention time, UV/vis and mass spectral data compared to those of commercial standards or purified standards. Quantification of most phenolic compounds was determined using the calibration curves of standards: catechin, epicatechin, myricetin, gallic acid, 4-hidroxibenzoic acid, protocatechuic acid, vanillic acid, p-coumaric, ellagic acid and vanillin.

Analysis of the antioxidant capacity

The antioxidant capacity was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging method according to the method of Abe *et al.* [15] with some modifications. In a 96-well microplate, the reaction was carried out as follows: A 50 µL aliquot of the sample extract, previously diluted with ethanol or methanol solution (the same used in the extraction), was mixed with 250 µL of DPPH (0.5 mM) and after 25 min in the dark the absorbance was measured at 517 nm using spectrophotometric

equipment (Synergy HT microplate reader (Bio-Tek instruments, Winooski, VT, USA). The reference standard for obtaining the calibration curve consisted of a methanolic solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at different concentrations (100 to 800 µM/ml). The antioxidant capacity was expressed as µmol Trolox equivalent (TE)/g DBNS.

Estimation of solvent dielectric constant

Values of the dielectric constant of the water/ alcohol mixed solvents were calculated using the mathematical relationship provided by Prakongpan and Nagai [16], following the equation:

$$Ɛ1,2\ (T) = Ø1 \times Ɛ1(T) + Ø2 \times Ɛ2(T) \quad (1)$$

Being Øi the volume fraction of solvent i and Ɛi(T) the dielectric constant of pure solvent i at the specific temperature (T). The dielectric constant of water was taken from Uematsu and Frank [17] and for the alcohols (methanol and ethanol) were estimated through interpolation of data reported by Dannhauser and Bahe [18] as shown in **Appendix A**.

Statistical analysis

The resulted data, with duplicate assays and triplicate analyses, were assessed via an analysis of variance (ANOVA) and using Tukey's post hoc test ($p < 0.05$). The correlation coefficient (r) was also calculated. Statistical processing was carried out using the software "IBM SPSS Statistic 20".

Results and discussion

PLE and UAE extraction yield

Tables 1 and **2**, for aqueous alcohol of methanol and ethanol, respectively, show the extraction yields, TPC values and antioxidant capacity of DBNS extracts obtained by PLE and UAE. The solvent dielectric constants, calculated using Eq. (1), are also included in the tables. Regarding yield, in the PLE method, as show in Figure 1a, the extraction temperature had a direct effect on yield. Thus, the highest yields were obtained at 200 °C, being about 3 times higher than the ones obtained at the lowest temperature (50 °C). At 200 °C, with 0 to 50 % of water/methanol and water/ethanol, the yields vary from 24.4 to 38.4 % and 22.8 to 32.9 %, respectively. The water addition to both alcohols, showed a poor influence on yield at the low temperatures (50 and 100 °C), but at high temperatures (150 and 200 °C) a significant increase of the yields was observed. Similar effect of temperature and water/alcohol solvent on yield was observed in other studies with apple peel and olive leaves, evaluated in an extraction range of 50 to 200 ºC [11,19]. In both reports, the highest temperature resulted in the highest yields (between 40 - 60 %) using water or ethanol/water as solvents, effect mainly attributed to the increase of analytes solubility and diffusivity in solvent, decrease of solvent viscosity and assist in breaking down analyte-matrix interactions, factors that encourage improving solvent penetration into the vegetal matrix and mass transfer. Therefore, under these conditions (150 °C and 50 % water/alcohol), the solvents have a higher solvating power and higher extraction rates, but not necessarily with good extraction selectivity of target compounds. When compared both alcohols in the PLE at the same water/alcohol ratio and temperature range, similar yields were obtained ($p > 0.05$), with the exception of 50 % water/alcohol at 200 °C, in which the aqueous methanol produced a significant higher extraction yield (38.4 %) than aqueous ethanol (32.9 %).

Table 1 Yield (%), Total Phenolic Compounds (TPC) and antioxidant capacity (DPPH) of the PLE and UAE extracts obtained with methanol as solvent from DBNS. Ɛ: Solvent dielectric constant.

Nº	Method	T (°C)	Water (%)	Yield* (%)	Ɛ	TPC (mg GAE/100g DBNS)	DPPH (µmol TE/ g DBNS)
1	PLE	50	0	9.5 ± 0^{bc}	28.2	211 ± 3^{a}	11.9 ± 0^{a}
2	PLE	100	0	14.6 ± 1.4^{d}	20.4	324 ± 10^{ab}	87.7 ± 1^{c}
3	PLE	150	0	16.5 ± 1.4^{d}	13.6	603 ± 25^{c}	110.5 ± 4^{c}
4	PLE	200	0	24.4 ± 1.6^{f}	7.8	2106 ± 28^{d}	233.6 ± 13^{e}
5	PLE	50	25	10.5 ± 0.1^{c}	38.7	274 ± 11^{ab}	11.8 ± 1^{a}
6	PLE	100	25	15 ± 0.1^{d}	29.3	513 ± 1^{bc}	34.4 ± 0.3^{ab}

Nº	Method	T (°C)	Water (%)	Yield* (%)	Ɛ	TPC (mg GAE/100g DBNS)	DPPH (µmol TE/ g DBNS)
7	PLE	150	25	21.2 ± 0^{e}	21.3	2185 ± 73^{d}	150.5 ± 2^{d}
8	PLE	200	25	32.2 ± 0.3^{g}	14.6	3977 ± 95^{f}	370.6 ± 5^{f}
9	PLE	50	50	10.4 ± 0.2^{c}	49.3	301 ± 0^{ab}	11.3 ± 0^{a}
10	PLE	100	50	13.7 ± 0.2^{d}	38.1	540 ± 18^{bc}	42.8 ± 0.1^{b}
11	PLE	150	50	25 ± 0.2^{f}	29.0	3098 ± 7^{e}	158.5 ± 1^{d}
12	PLE	200	50	38.4 ± 0.5^{h}	21.5	5435 ± 21^{g}	501.9 ± 17^{g}
13	UAE	50	0	7.1 ± 0.4^{ab}	21.1	110 ± 4^{a}	16.9 ± 0.3^{a}
14	UAE	50	25	6.4 ± 0.4^{a}	33.4	125 ± 6^{a}	14.3 ± 0.2^{a}
15	UAE	50	50	6.2 ± 0.1^{a}	45.7	110 ± 10^{a}	12.6 ± 2^{a}

* g extract/ 100 g DBNS. Superscripts with different letters within column indicate significant differences in data at $p < 0.05$.

Table 2 Yield (%), Total Phenolic Compounds (TPC) and antioxidant capacity (DPPH) of the PLE and UAE extracts obtained with ethanol as solvent from DBNS. Ɛ: solvent dielectric constant.

Nº	Method	T (ºC)	Water (%)	Yield* (%)	Ɛ	TPC (mg GAE/100 g DBNS)	DPPH (µmol TE/ g DBNS)
1	PLE	50	0	7.2 ± 0.4^{a}	21.1	198 ± 37^{a}	62.8 ± 10^{abc}
2	PLE	100	0	11.3 ± 1.4^{b}	15.2	273 ± 33^{a}	79.6 ± 0.4^{bcd}
3	PLE	150	0	15.7 ± 1.4^{c}	10.9	417 ± 25^{a}	94.7 ± 8^{cde}
4	PLE	200	0	22.8 ± 1.4^{d}	8.0	1049 ± 24^{b}	132.5 ± 5^{ef}
5	PLE	50	25	10.8 ± 0.1^{b}	33.4	271 ± 12^{a}	12.9 ± 1^{a}
6	PLE	100	25	14.7 ± 0.1^{c}	25.4	415 ± 20^{a}	30.6 ± 1^{ab}
7	PLE	150	25	20.1 ± 0.5^{d}	19.2	1767 ± 73^{c}	129.8 ± 0.3^{def}
8	PLE	200	25	29.1 ± 0.8^{e}	14.8	3198 ± 17^{d}	340.9 ± 34^{g}
9	PLE	50	50	10.7 ± 0.7^{b}	45.7	310 ± 14^{a}	12.7 ± 1^{a}
10	PLE	100	50	15.7 ± 0.3^{c}	35.5	580 ± 4^{a}	41.1 ± 1^{ab}
11	PLE	150	50	23.4 ± 0.1^{d}	27.6	2758 ± 67^{d}	151.5 ± 1^{f}
12	PLE	200	50	32.9 ± 1.6^{f}	21.5	4673 ± 97^{e}	396.9 ± 35^{h}
13	UAE	50	0	6.8 ± 0.5^{a}	28.2	194 ± 26^{a}	61.1 ± 1^{abc}
14	UAE	50	25	7 ± 0.3^{a}	38.7	136 ± 6^{a}	18.1 ± 0.5^{a}
15	UAE	50	50	9.8 ± 0.6^{ab}	49.3	167 ± 12^{a}	19.5 ± 0.2^{a}

* g extract/ 100 g DBNS. Superscripts with different letters within column indicate significant differences in data at $p < 0.05$.

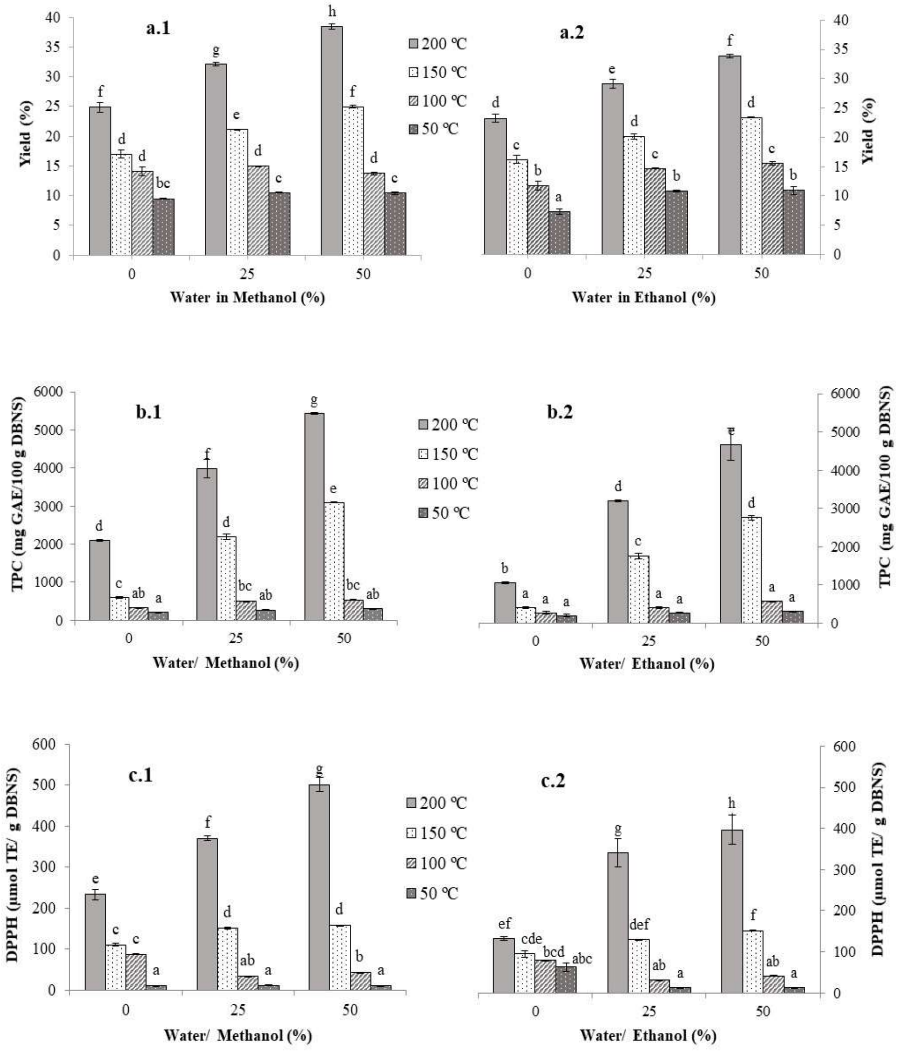

Figure 1 Effect of temperature and water/alcohol ratio on a) yield, b) TPC content and c) DPPH values determined in the PLE extraction of DBNS.

In the case of UAE extractions, which were carried out at 50 °C, no significant differences were observed in the yields obtained with methanol, ethanol or their aqueous mixtures, with the exception of the case using 50 % water/alcohol (v/v), in which ethanol resulted in higher yield than methanol, 9.8 and 6.2 %, respectively. In general, at this low extraction temperature, the addition of water in alcohol solvent had a poor influence on yield, either PLE and UAE. When compared both techniques at the same temperature (50 °C), significant differences ($p < 0.05$) were observed in water/methanol mixtures, where the PLE yields (9.5 to 10.5 %) were higher than UAE yields (6.2 to 7.1 %); but in ethanol/water mixtures were similar between PLE yields (7 to 10.8 %) and UAE yields (6.8 to 9.8 %).

Total phenolic compounds (TPC) in PLE and UAE extracts

Figure 1b shows the influence of temperature and type of solvent on the TPC values of PLE extracts, noticing a strong increase of extraction efficiency with the addition of water on the extraction solvent at temperatures higher than 100 °C, for both methanol and ethanol solvents. Thus, at 200 °C and 50 % v/v water/alcohol, the highest TPC values were obtained at 5435 mg GAE/100 g DBNS with water/methanol and 4673 mg GAE/100 g DBNS with water/ethanol. This improvement of the extraction of phenolic compounds from plant matrix with increasing temperature and water addition is corroborated in the

literature, such as in the case of macadamia peel [20] and apple residue [11]. In addition to improving the solubility and diffusivity properties of the solvent, high temperatures disrupt the vegetal cell wall improving the released of substances and extraction, as in the case of phenolic compounds, which are crosslinked in cellulose and hemicellulose to form the cell wall structure [21].

Comparing the TPC values of extracts obtained with both alcohols (methanol or ethanol), under the same conditions of water concentration and temperature, not significant differences ($p > 0.05$) were observed at low temperatures (50 and 100 °C), but above 150 °C differences are notorious, i.e. with water/methanol mixtures TPC values were higher than those corresponding to water/ethanol mixtures. In the literature, similar TPC extraction capacity between methanol and ethanol were noted at low extraction temperatures (≤ 50 °C), as reported in case of the peanut skin [22] and Brazil nut skin [5].

In order to analyze the relationship between the dielectric constant (Ɛ) and the TPC extraction, Figure 2 showed a good correlation (r > 0.95) of TPC and Ɛ, indicating the relevant influence of the solvent dielectric constant on TPC level, mainly above 100 ºC. Therefore, the highest TPC values were obtained at 200 °C and 50 % (v/v) water/alcohol with dielectric constant around ~21.5 in both water-alcohol solvents.

Despite the recognized effect of increasing temperatures to improve phenolic compounds extraction, degradation of some thermolabile phenolic compounds may occur. Moreover, temperatures higher than 120 °C favor the Maillard reaction, between amino acids and reducing sugars, which give rise to various compounds known as Maillard reaction products (MRPs), such as furfural compounds and melanoidins, with structures and chemical properties that can interfere in spectrophotometric measurements, as Folin-Ciocalteu assay [23]. At high temperature processes, this phenomenon is easily perceived due to a change of coloration and this was the case of our study, in which extracts showed a color modification from pale yellow to dark brown as the extraction temperature was increased.

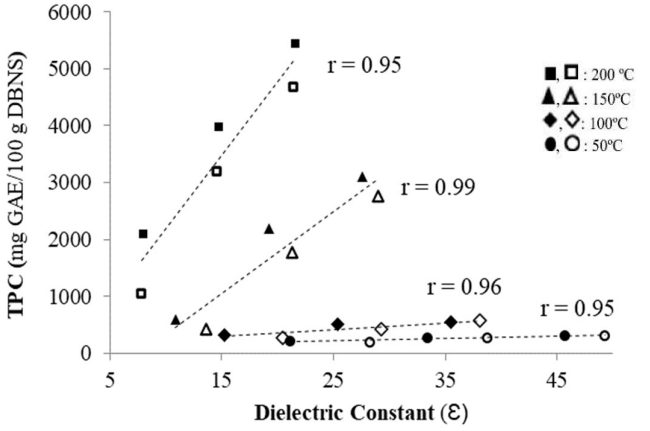

Figure 2 TPC values of DBNS PLE extracts as a function of solvent dielectric constant (Ɛ) at temperatures from 50 to 200 °C. Methanol: Full symbols; Ethanol: Open symbols; r: Pearson correlation coefficient.

With respect to the UAE extraction, according to results given in **Tables 1** and **2**, the variation of water/alcohol mixture (0 to 50 %, v/v) did not have a major influence on the TPC values ($p > 0.05$). In methanolic mixtures values were from 110 to 125 mg GAE/100 g DBNS and in the ethanolic mixtures from 136 to 194 mg GAE/100 g DBNS. This results are consistent with the reports where the maximum TPC values are obtained in a wide range of aqueous alcohol (20 to 80 %, v/v, water/ethanol) depending on the type of nut skin extracted [8,24]. Between UAE and PLE techniques, at the same temperature (50 °C), our study found no significant differences ($p > 0.5$) in the corresponding TPC values.

Antioxidant capacity of PLE and UAE extracts

In this section the results concerning the antioxidant capacity (AC) of the DBNS extracts obtained by PLE and UAE are analyzed. In the PLE extraction, according to **Figure 1c** there is a significant influence on the AC values with water/alcohol mixtures as solvent and high temperatures. Therefore, the highest DPPH values were obtained making the PLE process at 200 °C using 50 % v/v water in methanol (509 µmol TE/g DBNS) and 50 % v/v water in ethanol (397 µmol TE/g DBNS). Furthermore, a strong correlation between DPPH and TPC values was obtained, with Pearson coefficients of 0.95 and 0.94 for

water/methanol and water/ethanol solvents, respectively (**Appendix B**). This correlation are usually reported [11,25], but high temperatures can degraded the phenolic compounds and thus reduce the antioxidant capacity. On the other hand, high temperatures may also enhance the release of phenolics due to hydrolysis of polymeric polyphenols and/or glycosylated flavonoids and enable the formation of MRPs, such as melanoidins, with metal chelating capacity, scavenging of reactive oxygen species, and inhibition of lipid peroxidation [26,27].

In the UAE method, no influence of water/methanol concentration (0 to 50 %, v/v) on the antioxidant activity of the extracts was observed, with values from 12.3 to 17.0 µmol TE/g DBNS. In water/ethanol, at 25 and 50 % water/ethanol, no significant differences were obtained on AC, with 19.5 and 18.1 µmol TE/g DBNS, respectively; but with pure ethanol the AC increased significantly up to 61.1 µmol TE/g DBNS, similar effect was observed in the PLE method at 50 °C (62.8 µmol TE/g DBNS), attributable to the antioxidant power of the phenolic profile specific depending on the extraction method. In the literature, extraction at ~50 °C, the influence of the water/alcohol ratio on antioxidant capacity of other nut skins is variable, but in general is favored with the addition of water to the alcohol solvent, in a range of 40 to 70 % water/alcohol (ethanol o methanol) [8,9]. Regarding the comparison between PLE and UAE (both at 50 °C), no differences in antioxidant power were observed.

Identification of Phenolic Compounds in the PLE and UAE extracts

Figure 3 and Table 3 show the phenolic compounds identified and quantified in the extracts obtained from DBNS using the different extraction techniques and conditions. Detection at a wavelength of 280 nm showed the best resolution and abundance of the peaks, suitable for quantification. Therefore, 19 phenolic compounds were identified, comprising 9 phenolic acids, 6 flavonoids and 2 phenolic aldehydes. The standards employed, their retention time (Rt), UV λmax and MS spectral data allowed the identification of gallic acid (peak 1), protocatechuic acid (peak 3), 4-hydroxybenzoic acid (peak 8), epicatechin (peak 11), vanillin (peak 12), myricetin (peak 18) and ellagic acid (peak 19). Other compounds were identified by comparison of the UV spectra and MS spectral data (**Appendix C**) and previous studies on Brazil nut [5,13], allowing a tentative identification of gallic acid derivative (peak 2), catechin derivative (peak 4 and 5), vanillin derivative (peak 6), p-coumaric (peak 10), catechin gallate (peak 13), epicatechin gallate (peak 14), taxifolin (dihydroquercetin) (peak 15), ellagic acid derivative (peak 16) and a vanillic acid derivative (peak 17). Overall, the phenolic profile obtained from DBNS extracts is consistent with the phenolics identified reported previously [5], even our study detected some new compounds, such as vanillin, epicatechin, epicatechin gallate, hydroxybenzoic acid, p-coumaric and myricetin. Therefore, the DBNS extracts are mainly composed of hydroxybenzoic acids (phenolic acids) and flavan-3-ols (flavonoids), a common feature in the phenolic profile of other nut skins [27].

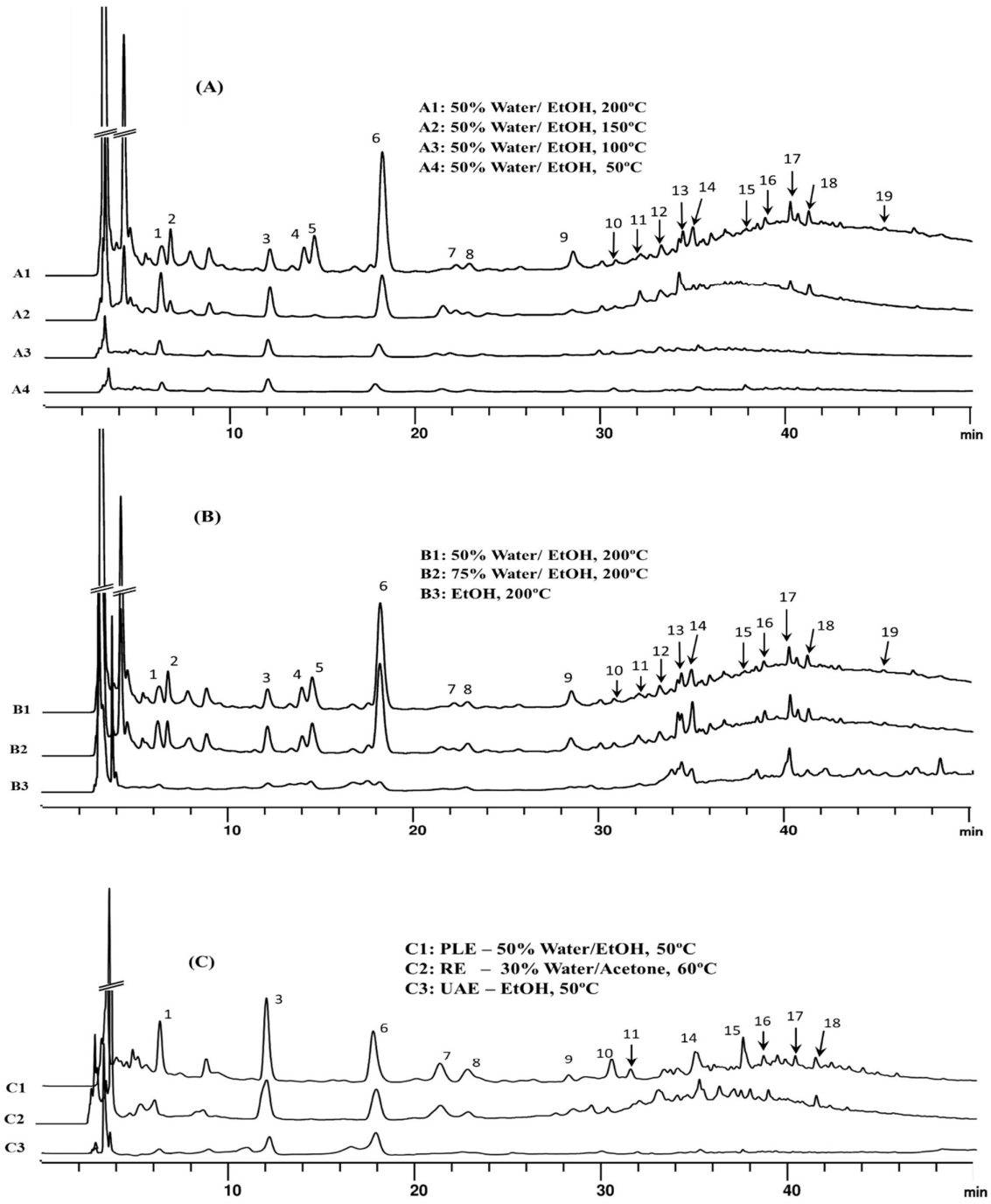

Figure 3 HPLC chromatogram (280 nm) of phenolic compounds from DBNS extracts obtained with water/ethanol solvent: (A) PLE with 50 % (v/v) water at 50, 100, 150 and 200 °C, (B) PLE at 200 °C with 0 - 50 % (v/v) water and (C) PLE with 50 % (v/v) water/ethanol, reference extraction (RE) and UAE with ethanol at 50 °C.

Table 3 Quantification of individual phenolic compounds (µg/g extract) in PLE and UEA extracts from defatted Brazil nut skin (DBNS).

Peak	Compound	Rt	PLE							UAE	RE
			1	2	3	4	5	6	7		
			Ethanol 100 % 200 °C	Ethanol 50 % 50 °C	Ethanol 50 % 100 °C	Ethanol 50 % 150 °C	Ethanol 50 % 200 °C	Methanol 50 % 200 °C	Ethanol 75 % 200 °C	Ethanol 100 % 50 °C	Acetone 70 % 60 °C
1	Gallic acid	6.3	326.0 ± 7^{ab}	756.4 ± 30^{d}	701.7 ± 41^{cd}	1083.4 ± 39^{e}	633.2 ± 1^{c}	739.4 ± 28^{d}	780.9 ± 1^{d}	242.1 ± 1^{a}	346.8 ± 4^{b}
2	Gallic acid derivative	6.8	*	*	*	507.8 ± 37^{a}	686.8 ± 14^{b}	477.2 ± 16^{a}	650.0 ± 19^{b}	*	*
3	Protocatechuic acid	12.2	1084.9 ± 15^{a}	3126.5 ± 106^{e}	2798.3 ± 76^{d}	2641.1 ± 8^{d}	1767.2 ± 79^{b}	1900.3 ± 47^{bc}	2127.5 ± 87^{c}	1234.2 ± 18^{a}	1836.2 ± 1^{b}
4	Catechin derivative	14.1	904.8 ± 9^{a}	*	*	*	2380.8 ± 93^{e}	1364.6 ± 28^{b}	1835.1 ± 17^{c}	*	*
5	Catechin derivative	14.5	909.2 ± 48^{a}	*	*	*	3788.6 ± 37^{e}	2807.0 ± 21^{b}	3577.1 ± 95^{c}	*	*
6	Vanillin derivative	18.3	168.6 ± 3^{a}	362.4 ± 43^{b}	399.8 ± 15^{b}	1055.4 ± 48^{c}	1888.1 ± 17^{d}	2195.3 ± 81^{e}	1931.7 ± 22^{d}	116.2 ± 6^{a}	336.3 ± 8^{b}
7	Catechin	22.3	472.1 ± 13^{b}	1178.7 ± 57^{c}	774.8 ± 43^{b}	1773.7 ± 151^{e}	839.6 ± 33^{b}	2842.6 ± 41^{f}	734.5 ± 58^{b}	149.7 ± 0.5^{a}	1387.1 ± 32^{c}
8	4-hydroxybenzoic acid	22.9	250.8 ± 9^{b}	426.3 ± 19^{d}	333.0 ± 21^{c}	462.1 ± 2^{d}	357.0 ± 6^{c}	845.9 ± 29^{f}	598.8 ± 11^{e}	42.1 ± 3^{a}	266.3 ± 14^{b}
9	Vanillic acid	28.5	172.6 ± 14^{bc}	112.6 ± 11^{ab}	126.1 ± 7^{b}	249.5 ± 31^{c}	662.6 ± 20^{d}	757.3 ± 35^{e}	758.2 ± 26^{e}	39.1 ± 2^{a}	154.6 ± 0.3^{b}
10	p-Coumaric	30.9	*	178.1 ± 7^{e}	75.9 ± 6^{c}	*	69.1 ± 4^{bc}	59.1 ± 2^{b}	103.4 ± 7^{d}	31.5 ± 0.2^{a}	*
11	Epicatechin	32.2	344.9 ± 15^{a}	574.0 ± 47^{b}	718.2 ± 46^{b}	1134.2 ± 112^{c}	567.5 ± 7^{b}	538.6 ± 46^{b}	1114.5 ± 23^{c}	161.2 ± 1^{a}	643.3 ± 26^{b}
12	Vanillin	33.3	233.0 ± 0.3^{e}	29.5 ± 1^{a}	123.3 ± 12^{bc}	154.7 ± 24^{cd}	99.1 ± 4^{bc}	132.9 ± 8^{b}	153.5 ± 1^{cd}	4.3 ± 0.2^{a}	177.7 ± 2^{d}
13	Catechin gallate	34.5	2287.2 ± 133^{d}	352.7 ± 2^{a}	528.3 ± 22^{ab}	2087.1 ± 97^{d}	1367.9 ± 66^{c}	678.3 ± 20^{b}	2954.1 ± 8^{e}	*	*
14	Epicatechin gallate	34.9	1193.8 ± 60^{d}	1209.2 ± 69^{d}	*	462.4 ± 5^{b}	1201.9 ± 37^{d}	1882.1 ± 46^{e}	2612.7 ± 19^{f}	162.2 ± 4^{a}	934.6 ± 26^{c}
15	Taxifolin (dihydroquercetin)	37.3	-	-	-	-	-	-	-	-	-
16	Ellagic acid derivative	38.9	1305.6 ± 1^{c}	3111.0 ± 45^{d}	2101.6 ± 26^{e}	1522.6 ± 23^{f}	1245.6 ± 4^{c}	1100.5 ± 22^{b}	1415.7 ± 17^{g}	*	987.1 ± 4^{a}
17	Vanillic acid derivative	40.3	888.3 ± 6^{f}	180.3 ± 17^{b}	96.3 ± 10^{a}	222.8 ± 20^{c}	296.1 ± 8^{d}	218.3 ± 6^{bc}	515.1 ± 1^{e}	*	*
18	Myricetin	42.0	1209.4 ± 92^{c}	631.3 ± 32^{b}	597.5 ± 27^{b}	1132.8 ± 32^{c}	1140.3 ± 43^{c}	1075.7 ± 14^{c}	1192.0 ± 100^{c}	69.0 ± 2^{a}	589.2 ± 8^{b}
19	Ellagic acid	45.5	1254.4 ± 12^{a}	2949.3 ± 28^{b}	1946.8 ± 37^{c}	1445.0 ± 6^{d}	1090.2 ± 11^{e}	952.9 ± 8^{f}	1237.8 ± 28^{a}	1732.5 ± 8^{g}	*
	Phenolic acids		5283 ± 63^{b}	10841 ± 9^{e}	8180 ± 182^{d}	8134 ± 72^{d}	6808 ± 66^{c}	7051 ± 95^{c}	8187 ± 17^{d}	3322 ± 11^{a}	3591 ± 13^{a}
	Phenolic aldehydes		402 ± 3^{bc}	392 ± 42^{b}	523 ± 27^{c}	1210 ± 24^{d}	1987 ± 21^{e}	2328 ± 73^{f}	2085 ± 23^{e}	120 ± 5^{a}	514 ± 7^{bc}
	Flavonoids		7321 ± 186^{e}	3946 ± 93^{c}	2619 ± 94^{b}	6590 ± 203^{d}	11287 ± 31^{e}	11189 ± 55^{e}	14020 ± 170^{f}	542 ± 7^{a}	3554 ± 39^{c}
	Total Phenolic		13006 ± 253^{a}	15178 ± 42^{b}	11322 ± 302^{c}	15935 ± 107^{d}	20082 ± 118^{e}	20568 ± 76^{e}	24292 ± 176^{f}	3984 ± 10^{g}	7659 ± 33^{h}

* No detected; - Detected but unquantified. Values with different superscript letters in each row are significantly different ($p < 0.05$).

Individual phenolic compounds content in PLE and UEA extracts

Quantification of phenolic compounds was accomplished using the calibration curves of the available standards (catechin, epicatechin, myricetin, gallic acid, 4-hidroxibenzoic acid, protocatechuic acid, vanillic acid, p-coumaric, ellagic acid and vanillin). The content in the individual phenolics is showed in **Table 3** and the data were expressed on extract weight basis. These results were compared with the DBNS extract obtained according to the reference extraction (RE) method (acetone/water 70 %, 60 °C and solvent reflux) [5].

The concentrations of the observed phenolic compounds change with temperature (samples 2 to 5 in **Table 3**). With increasing temperature, a decrease in the content of protocatechuic acid (from 3126 to 1767 µg/g extract), ellagic acid (from 2949 to 1090 µg/g extract) and ellagic acid derivative (from 3111 to 1245 µg/g extract) was observed; conversely, an increase of vanillic acid (from 113 to 663 µg/g extract) and vanillin derivative (from 362 to 1888 µg/g extract) was noted. In case of catechin derivatives, they were only detected in important concentrations (6169 µg/g extract) at 200 °C. These results confirm the degradation with temperature raise of certain phenols, mainly phenolic acids, although flavonoids and phenolic aldehydes increase notoriously, particularly at 200 °C. Reports about extraction from nut skins, at high temperature, are scarce, but PLE reports from vegetal byproduct such apple by-product [11] at temperatures above 150 °C confirm the degradation of thermosensitive phenolic compounds, but also a release of simple and thermostable phenols due to hydrolysis of polymeric polyphenols and/or glycosylated.

The effect of water addition to ethanol solvent on the extraction of individual phenolics is reported in **Table 3** for samples 1 (pure ethanol), 7 (25 %, v/v, water) and 5 (50 %, v/v, water) at constant temperature (200 °C). Phenolic compounds were better extracted using aqueous ethanol, among the most abundant were catechin derivatives (6169.4 µg/g extract), protocatechuic acid (2127 µg/g extract) and vanillin derivative (1931.7 µg/g extract). However, vanillic acid derivative (888 µg/g extract) was better recovered with pure

ethanol, aspects linked to the polarity of the compounds and the extraction solvent. In grouped compounds, the highest extraction was achieved with 25 % water in ethanol: in phenolic acid was 8187.3 µg/g (mainly protocatechuic acid, vanillin derivative, ellagic acid and its derivative); and, in flavonoids was 14020 µg/g of (mainly catechin derivatives, catechin gallate and epicatechin gallate). The influence of water/alcohol mixtures on the specific phenolic profile was also reported in the case of other vegetal by-products. In extraction from grape skin and wine pomace, at ~160 °C, the higher amounts of phenolic acid was to ~45 % water/ethanol and in flavonoids was with ~73 % water/ethanol [12,28]. When comparing the alcoholic solvents (ethanol and methanol) in the PLE at 200 °C with 50 % water (samples 6 and 7 in **Table 3**), there was no greater difference in the content of phenolic compounds.

Finally, comparing PLE, UAE (both at 50 °C) and the referential method (RE) [5], the extraction efficiency of flavonoids was similar between PLE and RE (3945.8 and 3554.2 µg/g extract, respectively), much higher than UAE (542 µg/g extract). In the phenolic acids, PLE (10840 µg/g extract) allowed better extraction than UAE and RE (3321.5 and 3590.9 µg/g extract, respectively). In general, most phenolics presented the following order: PLE > RE > UAE.

Conclusions

PLE is a suitable technique for the extraction of phenolic compounds from Brazil nut skin, with better extraction efficiency in comparison to UAE. The increase of temperature and addition of water to alcohol solvents (methanol and ethanol) enhance extraction yield, content of total phenolic compounds and antioxidant activity of the extracts. At constant temperature, in a range water-alcohol mixture of 0 to 50 % (v/v), the TPC of PLE extracts exhibit linear dependence with the solvent dielectric constant; thus, at a dielectric constant ~21.5 (obtained to 200 °C and 50 % water/alcohol) the highest TPC is obtained. The antioxidant activity of the extracts showed a linear correlation with TPC, demonstrating the influence of this type of compounds. About individual phenolic compounds, most are better extracted in water/alcohol mixture (25 - 50 % v/v), with similar effect between ethanol and methanol. High temperatures (above 50 °C), produce a progressive degradation of certain phenolic acids, but an increase of flavonoids. Further studies are suggested, the color change observed in the extracts with increasing temperature suggest the formation of Maillard reaction products. This study contributes to the knowledge in the framework of bioactive compounds, improvement of extraction techniques and promotes the potential use of Brazil nut skin industrial as a high-value ingredient.

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Appendix A

The methanol and ethanol dielectric constant data reported by Dannhauser [1] were used to develop the quadratic correlations (**Figure S1**):

$\varepsilon = a \times T^2 + b \times T + c$

where **ε** is the dielectric constant and T is the temperature in °C.

The corresponding equation coefficients are given in **Table S1**, and were used to calculate the dielectric constants of methanol and ethanol at the different extraction temperatures (50, 100, 150 and 200 °C) evaluated in this work. The regression coefficients (R^2) were, respectively, 0.9997 for methanol and 0.9999 for ethanol.

Table S1 Quadratic correlation coefficients for the methanol and ethanol dielectric constants as a function of temperature.

	a	**b**	**c**
Methanol	0.0002	-0.1863	37.057
Ethanol	0.0003	-0.1625	28.480

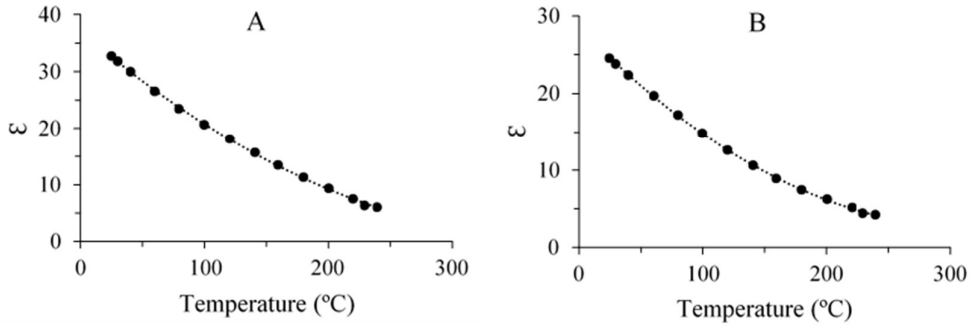

Figure S1 A) Correlation of methanol and B) ethanol dielectric constant (ε) vs temperature (25 to 240 °C) from data of Dannhauser (1964).

Appendix B

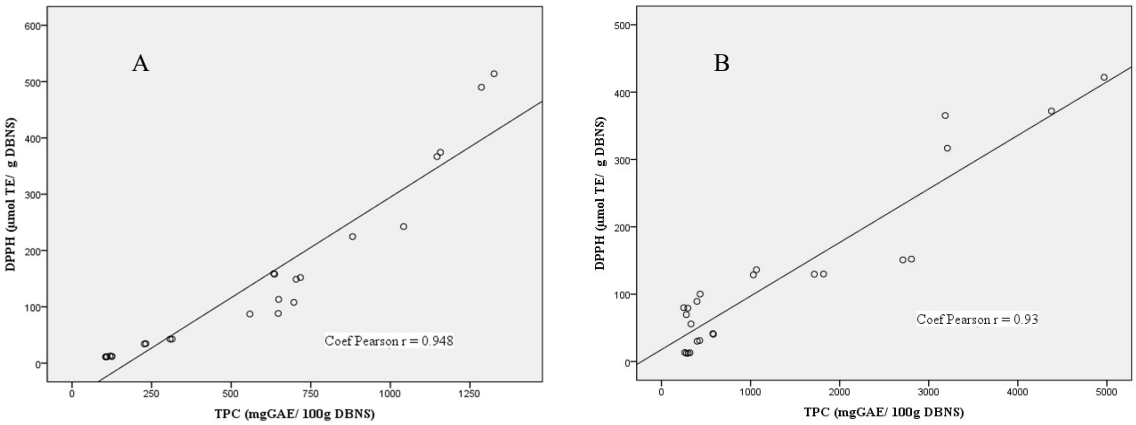

Figure S2 Correlation of TPC and antioxidant capacity (DPPH) of the PLE extracts obtained from DBNS, using water/alcohol solvents: (A) water/methanol and (B) water/ethanol.

Appendix C

Table S2 HPLC retention times (Rt), UV/Vis spectra and MS spectral data of individual phenolics in DBNS extracts.

Peak	Rt (min)	Identification	UV λmax (nm)	[M-H]-	MS/MS (m/z)
1	6.3	Gallic acid	230, 271	169.01	125.02, 107.01, 97.03, 79.02, 69.03, 51.02, 41.04
2	6.8	Gallic acid derivative	232, 282	187	125.02, 169.01
3	12.2	Protocatechuic acid	230, 258, 294	297.11	286.09, 153.04, 86.09
4	14.1	Catechin derivative	232, 288	167.03	289.1
5	14.5	Catechin derivative	232, 278	167.03	289.1
6	18.3	Vanillin derivative	232, 276 (310)	441.19	137.05, 123.05, 109.0, 81.0
7	22.3	Catechin	232 (275)	137.03	136.8, 150.7, 160.8
8	22.9	4-hydroxybenzoic acid	232 (275)	289.1	106.64, 93.03
9	28.5	Vanillic acid	232 (275) (350)	151.05	152.01, 108.02
10	30.9	p-Coumaric	232 (280) (310)	303.05	119.05, 91.05
11	32.2	Epicatechin	232 (278)	441.03	109.01, 121.01, 123.03, 125.01, 137.00
12	33.3	Vanillin	232 (280) (311)	441.19	137.05, 123.05, 109.0, 81.0
13	34.5	Catechin gallate	232 (272)	457.3	109.01, 125.00, 168.98, 289.03
14	34.9	Epicatechin gallate	232 (280)	164.05	109.08, 125.08,137.08, 151.10, 203.14, 245.16, 289.16
15	37.3	Taxifolin (dihydroquercetin)	232 (330)	301	285.05, 179.00, 125.03
16	38.9	Ellagic acid derivative	232 (280)	609.15	301, 257, 229
17	40.3	Vanillic acid derivative	232 (260) (290)	285	167
18	42.0	Myricetin	232 (280) (330)	329	317
19	45.5	Ellagic acid	232, 370	317.03	285, 283, 257, 229, 184.92, 134.92