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Extraction of natural antioxidants from hazelnut (*Corylus avellana* L.) shell and skin wastes by long maceration at room temperature

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Abstract

The feasibility of obtaining antioxidant phenolic extracts from hazelnut by-products was investigated by long maceration at room temperature. The hard shells and defatted skins of both whole and chopped roasted hazelnut kernels were studied. Three solvent systems were employed and these included aqueous methanol, ethanol and acetone. Extraction yields as well as phenolic contents varied according to the by-product and the solvent used. Among the studied samples, the skin of whole roasted hazelnuts gave remarkably high extraction yields (about 30%) and extracts with the richest phenolic content (up to 502 mg/g, expressed as gallic acid equivalents).

Extracts were screened for antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidation of linoleic acid *in vitro* model systems. The extracts from the skin of whole roasted hazelnuts manifested the strongest antioxidant activity, similar or superior to butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and α-tocopherol, at equivalent concentrations. The presence of hazelnut fragments in the skin residue lowered the yield and the antioxidant activity of the extract. All the extracts were found to be very rich in tannins.

Keywords: Hazelnut by-products; Phenolic extracts; Natural antioxidants; Antioxidant activity

1. Introduction

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are largely employed as preservatives by pharmaceutical, cosmetic and food industries, even if they are suspected of being responsible for liver damage and carcinogenesis in laboratory animals (Madhavi & Salunkhe, 1995). The need to replace synthetic antioxidants with natural and probably safe ones, together with the interest of the food industry and preventive medicine in the development of bioactive naturally-occurring antioxidants, has fostered research on the screening of plant sources, especially the inexpensive residue sources from agricultural industries (Moure et al., 2001).

The world production of hazelnuts (*Corylus avellana* L.) averages nearly one million tonnes per year. Hazelnut fruits have a hard, smooth shell. The seed is covered by a dark brown pellicular pericarp (skin or testa), which is typically removed before consumption after roasting of kernel. It was noted that hazelnut native phenolics are almost exclusively located in the perisperm of the seed (Bignami, Cristofori, & Troso, 2005; Senter, Horvat, & Forbus, 1983), and that they are provided with antioxidant activity (Yurttas, Schafer, & Warthesen, 2000). These phenolic compounds could potentially play a major role in human health promotion and disease risk reduction (Alasalvar, Karamac, Amarowicz, & Shahidi, 2006). A recent research carried out on crude extracts obtained from hazelnut by-products by Shahidi, Alasalvar, and Liyana-Pathirana (2007) supports the hypothesis that hazelnut wastes, especially skin and hard shell, could be a reliable source of new and efficient natural antioxidants.

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Extraction protocols for solvent extraction of plant phenolic antioxidants are very diverse (Moure et al., 2001; Naczk & Shahidi, 2006), due to the variation and complexity of phenolic compounds and to the variety of vegetable matrices. The extraction yield, phenolic content and antioxidant activity of the extracts are strongly dependent not only on the solvent and extraction method, but also on the specific plant materials and their bioactive components (Marinova & Yanishlieva, 1997). Aqueous ethanol, methanol and acetone are the most frequently tested solvents (Moure et al., 2001; Naczk & Shahidi, 2006). The available literature relative to the effect of the extraction temperature reports conflicting results (Moure et al., 2001). Therefore, comparative studies for selecting the extraction technique and the optimal solvent system providing the maximum phenolic content and the greatest antioxidant activity are required for each specific substrate.

The employment of hot-reflux condition (80 °C) to obtain hazelnut by-product phenolic extracts was investigated (Alasalvar et al., 2006; Shahidi et al., 2007). Stévigni, Rolle, Valentini, and Zeppa (2007) utilized cold-extraction (20–22 °C) for short time (30–150 min) to extract phenols from hazelnut shells. The feasibility and opportunity of employing other protocols and temperature conditions (i.e., long cold-maceration) on hazelnut by-product phenolic extraction should be investigated. Furthermore, the antioxidant activity of hazelnut shell and skin phenolic extracts in comparison with the widely used synthetic (BHA, BHT) and natural (α-tocopherol) antioxidants should be evaluated.

This study was aimed at assessing the feasibility of employing a long maceration at room temperature in the presence of three different aqueous solvents (methanol, ethanol and acetone) for extracting antioxidants from hazelnut shell and pellicular wastes coming from the industrial pericarp removal during the processing of whole or chopped hazelnut kernels. The two kinds of skin by-products were studied in order to verify if the high content of kernel fragments, which characterize the residue obtained from the chopping of incompletely blanched hazelnuts, could influence the content and the antioxidant activity of the crude extracts. The antioxidant activity of the extracts in comparison with BHA, BHT, 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox) and α-tocopherol was investigated. Finally, the hypothesis that phenolic extracts obtained from hazelnut shell and skin wastes are rich in tannin compounds was verified.

2. Materials and methods

2.1. Chemicals

Gallic acid, catechin, ferulic acid, protocatechuic acid, *p*-coumaric acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), polyvinylpyrrolidone (PVP), butylated hydroxyanisole (BHA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), linoleic acid and 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma–Aldrich (St. Louis, MO). Folin–Ciocalteau reagent, butylated hydroxytoluene (BHT), α-tocopherol, tannic acid and sinapic acid were purchased from Fluka Co. (Buchs, Switzerland). Quercetin was purchased from Extrasynthése (Genay, France). All the other chemicals and solvents were of the highest commercial level and were used without further purification.

2.2. Samples

The waste shells (SH) constituted the residues of hazelnut (*Corylus avellana* L.) hulling process. The hazelnut skin wastes were the residues of industrial pericarp removal from whole (PW) and chopped (PC) roasted kernel. These by-products were kindly supplied by an Italian hazelnut processing industry (Stelliferi and Itavex spa, Caprarola, VT) and represented the waste of daily industrial processing, carried out on different varieties (Italian Tonda Gentile Romana, Tonda di Giffoni, Tonda Gentile delle Langhe; Turkish Tombul) at different roasting conditions (temperature: varying between 135 and 170 °C; time: varying between 15 and 40 min).

2.3. Extract preparation

All samples were ground in a coffee mill. The skin materials were defatted for 8 h with hexane in a Soxhlet apparatus. Phenolic compounds were extracted overnight at room temperature (20–22 °C), under constant stirring, using 80% (v/v) of aqueous methanol, ethanol or acetone at solid to solvent ratio of 1:10 (w/v). Extraction was carried out in closed bottles, in the dark. The next day, after 20 h of extraction, the suspension was centrifuged (1800*g*, 15 min). The supernatant was removed and stored at 4 °C. The extraction was repeated for additional 20 h. The supernatants were combined and filtered through a Whatman GF/F glass microfibre filter (0.7 μm). The solvent was evaporated in a vacuum at 40 °C in a rotary evaporator, the remaining water was removed by freeze-drying. The crude phenolic extract was weighed to determine the extraction yield, then it was dissolved in methanol (10 mg ml^{-1}) and stored at −20 °C for further analysis.

2.4. Total phenols and total tannins

Total phenols were determined using Folin–Ciocalteau method (Singleton & Rossi, 1965), that involves reduction of reagent by phenolic compounds, with concomitant formation of a blue complex. Briefly, 100 μl of a properly diluted sample were mixed with 1 ml of Folin–Ciocalteau reagent (diluted 10-fold with distilled water) and allowed to stand at room temperature for 3–7 min; 900 μl of Na_2CO_3 (7.5%) were then added to the mixture. After 90 min at room temperature, the absorbance of the blue complex was measured at 725 nm. Methanol was used as a blank. Results were expressed as gallic acid equivalents

(GAE), tannic acid equivalents (TAE) or catechin equivalents (CE), on the basis of curves drawn using each phenol as a standard.

Tannins were estimated indirectly after being adsorbed on insoluble polyvinylpyrrolidone (PVP) and measuring the remaining total phenols in the supernatant, as described by Makkar (2000).

2.5. Antioxidant activity

2.5.1. Radical scavenging ability (DPPH assay)

The radical scavenging ability or hydrogen donating of the extracts was monitored using the stable free radical DPPH, following the method described by Brand-Williams, Cuvelier, and Berset (1995), lightly modified as follows. Different dilutions of crude extract or antioxidant (in 0.4 ml of methanol) were mixed in a 1 cm disposable cuvette with 3 ml of freshly prepared methanolic solution of DPPH (65 μM). A control was prepared with 0.4 ml of pure methanol; its initial absorbance was between 0.716 and 0.720. The cuvette was capped and left to stand in the dark at room temperature for 180 min (time required to reach the steady state). At this time, the decrease in absorbance was measured at 515 nm against a blank of pure methanol, with a Perkin–Elmer Lambda 3 UV/vis spectrophotometer (Perkin–Elmer Inc., Wiesbaden, Germany). For each extract or pure antioxidant at least five different concentrations were tested. BHA, BHT, Trolox and α-tocopherol were used as reference antioxidants.

The percentage of remaining DPPH (%$DPPD_{rem}$) was calculated as follows:

$$\%DPPH_{rem} = DPPH_s/DPPH_c \times 100$$

where $DPPH_s$ was the DPPH concentration in presence of sample and $DPPH_c$ was the DPPH concentration of the control, at $t = 180$ min. DPPH concentration in the reaction medium was calculated using a molar absorption coefficient (ε) of 12,500 L cm^{-1} mol^{-1} (Arnao, 2000). BHA, BHT, Trolox and α-tocopherol were used as reference compounds.

The radical scavenging activity was expressed in terms of EC_{50} (efficient concentration), which is the amount of extract or pure antioxidant necessary to decrease the initial DPPH concentration by 50%. The EC_{50} value was obtained by plotting the %$DPPH_{rem}$ as a function of sample concentration. The dose–response curve was linear in the range tested (30–70% of DPPH remaining; straight line resulting from the fit by linear regression (r^2 above 0.98)). In the case of the extract, EC_{50} was expressed as both μg of crude extract/μg DPPH and as μg of GAE/μg DPPH. In the case of the standard, EC_{50} was expressed in terms of μg/μg DPPH. Antiradical efficiency (AE) was calculated as the inverse of EC_{50}. Reference equivalent antioxidant capacity (REAC) was defined as the amount (μg) of reference antioxidant (BHA, BHT, Trolox, and α-tocopherol) giving the same antioxidant capacity (50% DPPH radical scavenging) as one microgram of sample. REAC was calculated as follows:

$$REAC = EC_{50(ref)}/EC_{50(extr)}$$

where $EC_{50(ref)}$ was the EC_{50} value of the reference antioxidant, and $EC_{50(extr)}$ was the EC_{50} value of the extract, expressed as both μg of dry crude extract/mg DPPH and as μg of GAE/mg DPPH.

2.5.2. Antiperoxyl radical efficiency (AAPH-linoleic acid assay)

The antiperoxyl radical efficiency of extracts was determined measuring the AAPH-induced oxidation of linoleic acid. The method described by Liegeois, Lermusieau, and Collin (2000) was employed. According to these authors, aqueous dispersion of linoleic acid (~16 mM) was prepared in 0.05 M borate buffer solution (pH 9), with Tween20® as emulsifier and sodium hydroxide as a clarifying agent. This solution was distributed in 0.5 ml-aliquots and stored at −20 °C until needed. Before use, the linoleic acid solution was checked for autoxidation, and solutions exhibiting >3% autoxidation were discarded. AAPH solution (40 mM) was prepared in 0.05 M phosphate buffer, pH 7.4; aliquots were stored at −20 °C until required.

The test was carried out as follows. In a quartz cuvette containing 2810 μl of phosphate buffer (0.05 M, pH 7.4), 30 μl of linoleic acid dispersion were mixed with 10 μl of crude extract in methanol, at various concentrations. In the assay carried out with standard antioxidant, 10 μl of BHA, BHT, Trolox and α-tocopherol, at various concentrations in methanolic solution, were employed. The oxidation of linoleic acid was started by adding 150 μl of AAPH solution. In the assay without antioxidant (control), 10 μl of methanol were employed instead of sample. A blank of AAPH was prepared with 2840 μl of phosphate buffer, 10 μl of methanol and 150 μl of AAPH solution. All cuvettes were incubated at 37 °C. The rate of oxidation was monitored at regular intervals, by recording the increase in absorption at 234 nm caused by diene hydroperoxides. The AAPH absorbance at 234 nm changes as the compound decomposes. Therefore, the AAPH-blank measures were subtracted from each experimental point. Absorbance at 234 nm was plotted as a function of time, and the inhibition time (T_{inh}) estimated as the point of intersection between the tangents to the inhibition and propagation phase. The antioxidant power was expressed as rate of inhibition (R_{inh}, min $conc^{-1}$), which represents the slope of the linear regression (r^2 above 0.98) calculated by plotting T_{inh} versus sample concentration. In the case of crude extract, R_{inh} was expressed in terms of both min per mg L^{-1} of dry extract and min per mg L^{-1} of GAE. In the case of standard, R_{inh} was expressed in terms of min per mg L^{-1} of pure antioxidant.

2.6. UV spectra

The UV spectra of the extracts and standard phenols in methanol solution were recorded in the range of 220–

400 nm using a Perkin–Elmer Lambda 25 UV/vis spectrophotometer.

2.7. Statistical analysis

All measurements were done at least in triplicate, and results reported as mean ± SD. The experimental data were elaborated by one-way analysis of variance (ANOVA); significance between means ($p < 0.05$) was determined by the Tukey test, using the SPSS 13.0 for windows package.

3. Results and discussion

It is known that the amount of extractable substances depends on both the plant material and the solvent employed. Many solvent systems have been utilized, but from a careful examination of the bibliographic sources it emerged that 70–80% aqueous methanol, ethanol and acetone were the most utilized (Moure et al., 2001; Naczk & Shahidi, 2006) and promising for phenolic recovery from seed coating (Kähkönen et al., 1999; Yu, Ahmedna, & Goktepe, 2005). This was the reason why we chose to compare the efficiency of methanolic, ethanolic and acetonic aqueous solution (80%, v/v) to obtain phenolic crude extracts from hazelnut skin and shell by-products. As an alternative to the hot-reflux extraction (Shahidi et al., 2007) and to the short cold-extraction (Stévigni et al., 2007), we studied the feasibility of obtaining antioxidants from hazelnut by-products by a long maceration at room temperature.

Hazelnut wastes employed in this work were hard shell and skin residues, because their crude extracts were distinguished by a higher phenolic content and superior antioxidative efficacy as compared with other hazelnut by-products (Shahidi et al., 2007). During industrial blanching, the skin is taken off the whole roasted seeds by abrasion and vacuum suction. The ease of pericarp removal is cultivar-dependent. So, when whole nuts that blanch well are processed, the skin waste is almost totally constituted by pellicular material. However, in some cultivars (such as "Tonda Gentile Romana" or "Barcellona") skin removal is never complete. When such incompletely blanched kernels are subjected to crushing in order to obtain chopped hazelnut formulations, other pellicular materials are removed by vacuum suction, which inevitably sucks off small pieces of broken kernels. Consequently, one obtains skin waste mingled with high amounts of fine fragmented endosperm. In order to investigate the effect of the presence of kernel fragments on phenolic antioxidant extraction, the skin waste samples examined were of two kinds: the by-product obtained from the roasting of whole hazelnuts and the by-product coming from the chopping of incompletely blanched kernels.

3.1. Extraction yield and total soluble phenols

The extraction yields in dry matter, obtained employing the three selected solvent systems, are shown in Table 1. It was evident that the woody shell (SH), the defatted skin waste from whole roasted kernel (PW), and the defatted skin waste from chopped hazelnut (PC) contained different amounts of extractable compounds. SH gave a low extraction yield (2.7–2.8%) which was independent from the solvent used; it was about 7- to 8-fold lower than PC and 10-fold lower than PW.

Noticeable amounts of extractable substances were contained in skin residues, especially PW (up to 32.6%, obtained with acetonic mixture). No significant differences ($p < 0.05$) were detected employing methanol or ethanol, while the acetonic solvent was able to produce the highest

Table 1
Total soluble compounds (TSC), total soluble phenols (TSP); total phenols (TP) and total tannins (TT) of the extracts, with relation to the solvent system

Sample[a]	Solvent[b]	TSC (g/100 g)	TSP (mg GAE[c]/g)	TP			TT (mg TAE[e]/g)
				(mg GAE[c]/g)	(mg CE[d]/g)	(mg TAE[e]/g)	
SH	M	2.7 ± 0.05 a	1.5 ± 0.04 a	56.6 ± 1.7 a	66.2 ± 2.0 a	63.6 ± 2.0 a	40.4 ± 1.5 a
SH	E	2.7 ± 0.07 a	1.6 ± 0.05 a	59.6 ± 1.0 a	69.8 ± 1.1 a	67.1 ± 1.1 a	42.0 ± 1.4 a
SH	A	2.8 ± 0.10 a	2.1 ± 0.03 b	72.2 ± 2.0 b	84.5 ± 2.4 b	81.2 ± 2.3 b	48.6 ± 1.3 b
PW	M	28.9 ± 0.32 a	123.4 ± 2.1 a	426.7 ± 4.6 a	499.7 ± 5.4 a	480.1 ± 5.2 a	283.5 ± 4.6 a
PW	E	27.8 ± 0.48 a	139.6 ± 2.9 b	502.3 ± 9.9 b	588.2 ± 11.6 b	565.3 ± 11.1 b	357.8 ± 9.7 b
PW	A	32.6 ± 0.32 b	152.2 ± 3.0 c	466.8 ± 7.8 c	546.6 ± 9.1 c	525.3 ± 8.7 c	304.3 ± 7.9 c
PC	M	20.8 ± 0.30 a	20.3 ± 0.26 a	97.7 ± 1.7 a	114.5 ± 2.0 a	110.0 ± 2.0 a	71.5 ± 2.3 a
PC	E	20.0 ± 0.41 a	34.9 ± 0.64 b	174.5 ± 2.2 b	204.3 ± 2.5 b	196.4 ± 2.4 b	127.5 ± 2.7 b
PC	A	23.5 ± 0.29 b	48.5 ± 0.86 c	206.1 ± 3.7 c	241.4 ± 4.3 c	232.0 ± 4.2 c	141.3 ± 2.4 c

Mean ± SD ($n = 3$ for TSC, TSP and TP; $n = 4$ for TT). Within the same sample, means in a column with different letters are statistically different ($p < 0.05$).

[a] SH, hazelnut shell waste; PW, skin waste of whole roasted hazelnuts; PC, skin waste of chopped hazelnuts.

[b] M = 80% methanol, E = 80% ethanol, A = 80% acetone.

[c] Gallic acid equivalents.

[d] Catechin equivalents.

[e] Tannic acid equivalents.

amount of crude extract from both hazelnut skin waste samples (PC and PW).

Shahidi et al. (2007) obtained crude phenolic extract from hazelnut by-products employing 80:20 (v/v) ethanol/water mixture under reflux conditions at 80 °C. With regard to hard shell waste, the yield reported by these researchers (2.53%) was similar to that obtained in the present study. In contrast, we obtained a remarkably higher quantity of dry crude extract from hazelnut skin residue (32.6% yield, against the 10.35% reported by the above-mentioned authors), by employing the same solvent (80% ethanol).

Total soluble phenols (Table 1) demonstrated dependence on the sort of residue, as well as the solvent used for the extraction. In the adopted conditions, acetonic mixture revealed the best extracting capacity towards the studied hazelnut by-products. Aqueous acetone is considered a good solvent for phenolics, especially tannin compounds (Makkar, 2000).

Few phenols were obtained from shell (up to 2.1 mg GAE/g of waste, obtained with the acetonic mixture), while PW afforded an excellent source of phenolic extractable compounds (123–152 mg GAE/g of original defatted sample, depending on the solvent). Regarding the two skin waste samples treated with the identical solvent mixture, extractable phenols of PC resulted 3- to 6-fold lower than PW. As shown in Table 1, for both skin samples solvent power towards phenols was in the following order: acetone > ethanol > methanol.

The optimal solvent for phenolic extraction should satisfy the following criteria: the capacity to extract the highest quantity of phenols and also the lowest quantity of foreign substances. Therefore, an optimal solvent should give the highest ratio between total extractable phenols and total extractable compounds. These conditions were satisfied by acetonic solvent, for both SH and PC samples, and by ethanolic solvent, for PW sample. Aqueous acetone extracted more phenolics from PW, but still more extraneous substances; in other terms, there was no relationship between the amount of extractable compounds and extractable phenols. In the case of SH, extraction efficiency of methanol and ethanol were similar, whereas for PC and PW the methanolic solvent was undoubtedly the less suitable.

3.2. Total phenols of the extracts

The amount of total phenolic compounds in the extracts obtained with different solvents is shown in Table 1, where data were expressed as both as gallic acid and catechin equivalents. Total phenolic concentration of the extracts was in the following order: ethanol > acetone > methanol (PW); acetone > ethanol > methanol (PC); acetone > ethanol = methanol (SH). These values confirmed the results discussed above.

Conclusively, the results showed that different solvents used for the extraction had different capacities in extracting substances and phenols from hazelnut by-products. The most suitable phenolic solvent for hazelnut waste shell was 80% aqueous acetone. It provided the highest yield in dry extract, solubilized more phenols and less foreign substances, thus giving the richest phenolic extract (72 mg GAE/g). For this waste, 80% aqueous methanol or ethanol were ineffective. On the contrary, in skin waste from hazelnut roasting, the mixture acetone/water (80:20, v/v) gave a maximum yield and extracted more phenols, but the solvent which provided the most concentrated phenolic extract was 80% ethanol (502.3 mg GAE/g, equal to 588.2 mg CE/g); the methanol/water (80:20, v/v) was ineffective. The presence of hazelnut kernel fragments in the skin waste (sample PC) modified the results. The most effective solvent was 80% aqueous acetone (total phenols in the extract, 206.1 mg/g GAE; maximal value of the other parameters). Eighty percent-ethanolic mixture solubilized less phenols, and gave slightly lower concentration of phenolic compounds in the extract (174.5 mg/g GAE); again, 80% methanol resulted ineffective.

The different results obtained from PC and PW samples indicated that the presence of kernel endosperm in hazelnut skin waste is detrimental to the obtaining of an efficient antioxidant crude extract, because it did not contribute to increasing the phenolic content of raw material (as might be expected on the basis of previous studies, which reported that phenolic content of hazelnut endosperm is insignificant, compared to the perisperm (Bignami et al., 2005), but it also enriched the raw material of non-phenolic soluble solids. Thus, on the assumption of the industrial exploitation of hazelnut skin by-products to obtain natural antioxidants, the results suggest that it is better to use skin material without hazelnut kernel fragments as far as possible.

Considering the inevitable experimental variations, the phenolic content of PW-ethanolic extract obtained in this work was remarkably similar to that reported by Shahidi et al. (2007). By utilizing hot aqueous ethanol (80%), they found 577.7 mg/g of total phenols (expressed as catechin equivalent) in defatted hazelnut skin extract. Therefore, our procedure (long maceration at room temperature) would appear to be as efficient as hot-extraction with regard to phenolic concentration of the extract. On the other hand, yield in dry extract obtained with cold-maceration was much higher (about 3-fold) than that obtained with hot-extraction. Concerning the SH sample, despite the yield in dry extract achieved in this work was in agreement with the value reported by Shahidi et al. (2007), the maximum phenolic content of the extract we obtained (84.5 mg/g CE) resulted about 2.5-fold lower.

Stévigni et al. (2007) utilized cold-maceration (20–22 °C) over a short time (30–150 min) to extract phenols from hazelnut shells. They evaluated the efficiency of different ethanolic and methanolic aqueous mixtures only determining total soluble phenols, which varied from a minimum of 1.37 to a maximum of 6.70 mg GAE/g of shells. The values we obtained (1.5–2.1 mg GAE/g) were positioned in the lower bracket of this range.

Hazelnut crude extracts, and especially PW-ethanolic extracts, were characterized as having a much higher phenolic content compared with those of extracts obtained from similar vegetable matter. In brown almond skin extract, Siriwardhana and Shahidi (2002) found 87.8 mg CE/g; total phenols of peanut and cashew skin extracts were reported to be about 150 and 240 mg GAE/g, respectively (Kamath & Rajini, 2007; Nepote, Grosso, & Guzman, 2002). Therefore, hazelnut skin by-products may represent a very effective source of natural phenolic compounds.

3.3. Antioxidant activity

Numerous analytical methods have been developed to assess the *in vitro* antioxidant activity of plants and their derivates, which diverge from each other in terms of probes, substrate, reaction conditions, kinetic and quantitation method. Consequently, it is difficult to compare the results from different analytical procedures. Further, relationships between assays were found to depend not only on the method, but also on the nature of antioxidants analyzed. These are the reasons why the use of at least two different analytical approaches to test the antioxidant activity of specific substrates is recommended (Schlesier, Harwat, Böhm, & Bitsch, 2002).

In this study, the DPPH assay was selected because it is one of the most accurate, sensitive and widely used in characterizing the antioxidant capacity of vegetable materials or their extracts (Buenger et al., 2006). It evaluates the ability of a sample to scavenge the chromogen long-lived DPPH free radical. The second method we employed evaluates the ability of an antioxidant to protect against peroxyl radicals generated by AAPH-induced oxidation of linoleic acid in aqueous dispersion. This is a convenient and reliable method for determining the efficiency of an antioxidant (Liegeois et al., 2000), since peroxyl radical is the predominant free radical found in lipid oxidation in foods and biological systems.

3.3.1. Radical scavenging ability (DPPH assay)

EC_{50} values (amount of sample required for 50% scavenging of DPPH radicals) are reported in Table 2. The EC_{50} parameter was expressed on a dry weight basis in two measurement units: μg of extract/mg DPPH (indicative of the antiradical ability of the mass of crude extract), and μg of GAE/mg DPPH (indicative of antiradical ability shown by the mass of phenolic compounds present in the extract). The higher the EC_{50}, the lower the antioxidant activity.

All hazelnut by-product extracts showed a scavenging activity against DPPH radical. When EC_{50} value was expressed as μg of extract/mg DPPH, the best free scavenging ability was shown by the richest phenolic extracts (acetonic extracts, for SH and PC; ethanolic extract, for PW). When EC_{50} value was expressed as μg of GAE/mg DPPH, the difference between PC samples was not statistically significant ($p < 0.05$), suggesting similar DPPH-active phenolic compounds were extracted using the three solvents. With regard to SH and PW samples, the results showed the solvent yielded both the highest phenolic concentration and the best DPPH-scavenging activity of crude extract (acetonic and ethanolic mixture, respectively), was able to extract phenolic compounds with a slightly higher antiradical ability. In fact, the phenolic mass (expressed as GAE) required by SH-acetonic and PW-ethanolic extract for 50% scavenging of DPPH radicals was less than those required by the corresponding extracts obtained with the other solvents.

In Fig. 1 the results obtained on the extracts can be visibly compared with those obtained when using pure antioxidants. In the figure, antiradical efficiency ($AE = 1/EC_{50}$)

Table 2
Antioxidant activity of the extracts with relation to the solvent system

Extract[a]	Solvent[b]	EC_{50}[c]		R_{inh}[d]	
		(μg Extract mg^{-1} DPPH)	(μg GAE[e] mg^{-1} DPPH)	(min L mg^{-1} extract)	(min L mg^{-1} GAE[e])
SH	M	2319 ± 51 a	131 ± 3.2 a	6.9 ± 0.1 a	115.9 ± 1.7 a
SH	E	2288 ± 47 a	136 ± 3.6 a	7.0 ± 0.1 a	115.1 ± 1.5 a
SH	A	1634 ± 36 b	118 ± 2.6 b	7.9 ± 0.2 b	113.1 ± 2.3 a
PW	M	141 ± 2.5 a	68 ± 1.2 a	33.3 ± 0.9 a	78.1 ± 1.7 a
PW	E	123 ± 2.0 b	62 ± 1.0 b	37.7 ± 0.6 b	77.9 ± 1.2 a
PW	A	143 ± 3.1 a	67 ± 1.4 a	36.8 ± 0.7 b	80.9 ± 1.6 a
PC	M	674 ± 12.9 a	66 ± 1.3 a	8.0 ± 0.1 a	80.1 ± 1.1 a
PC	E	370 ± 4.7 b	65 ± 0.8 a	13.8 ± 0.2 b	78.2 ± 0.9 a
PC	A	310 ± 4.9 c	64 ± 1.0 a	16.5 ± 0.2 c	80.0 ± 1.1 a

Mean ± SD ($n \geqslant 3$). Within the same sample, means in a column with different letters are statistically different ($p < 0.05$).

[a] SH, obtained from hazelnut shells; PW, obtained from skin of whole roasted hazelnuts; PC, obtained from skin of chopped hazelnuts.

[b] M = 80% methanol, E = 80% ethanol, A = 80% acetone.

[c] DPPH-radical scavenging activity; the lower the EC_{50}, the higher the antioxidant activity.

[d] Antiperoxyl radical efficiency; the higher the R_{inh}, the higher the antioxidant activity.

[e] Gallic acid equivalents.

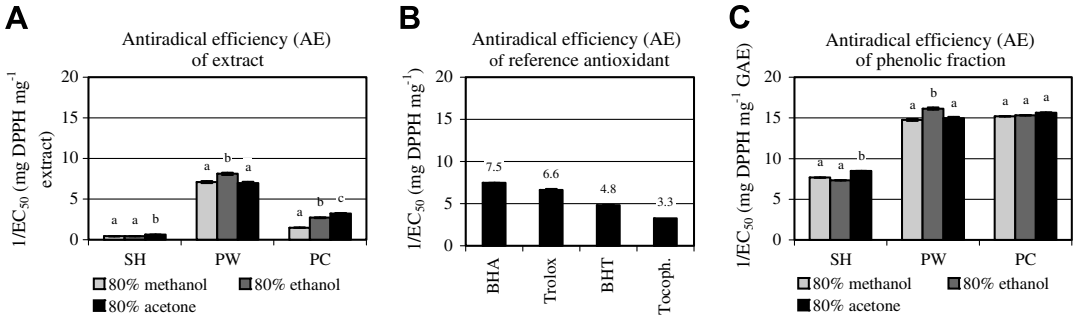

Fig. 1. Antiradical efficiency (AE) of extracts ((A), AE expressed as mg DPPH mg^{-1} of extract; (C), AE expressed as mg DPPH mg^{-1} of GAE), and reference antioxidants ((B), AE expressed as mg DPPH mg^{-1} of pure compound).

was reported, expressed as both mg DPPH/mg of extract (or antioxidant) and mg DPPH/mg of GAE. The AE value expresses antioxidant potency; the higher the AE, the higher the antioxidant power.

The extracts possessing the most powerful DPPH-scavenging activity were decidedly the PW samples; their AE value (ranging from 7.1 to 8.1 mg DPPH/mg of crude extract) was at least 2-fold higher than the best PC (AE = 3.2) and about 12- to 13-fold greater than the best SH (AE = 0.6) extract. When antiradical efficiency was measured in terms of mass phenolic unit, the very high antioxidant power of phenolic compounds contained in PW and PC emerges. Although the phenolic fraction extracted from hazelnut shell showed a much lower antioxidant power than the fractions obtained from skins, it appeared close to or better than that of all the standards tested.

The comparison with the antiradical power of samples and reference antioxidants can be effectively evaluated by calculating reference equivalent antioxidant capacity (REAC), the results are shown in Table 3. This parameter represents the amount (μg) of antioxidant standard (BHA, BHT, α-tocopherol and Trolox) that causes the same (50%) DPPH-scavenging effect as 1 μg of analyzed sample. It is evident that none of the SH crude extracts showed a significant antioxidant power with respect to the standards tested. PC crude extract (namely that obtained with acetonic mixture) exhibited the same antiradical capacity as α-tocopherol (which, of all the standards, gave the lowest AE value), and about half that of BHA, BHT or Trolox. All PW crude extracts, especially those obtained with aqueous ethanol, resulted having an antioxidant capacity equal to BHA or Trolox and higher than BHT or α-tocopherol. These findings suggest that the crude extract of hazelnut skin might be usefully employed without further enrichment/purification if its safety, bioavailability and applicability to food, cosmetic or nutraceuticals products is proved.

Comparison with standard antioxidants evaluated on a weight basis of total phenols (expressed as GAE) rather than on a weight basis of crude extract, allowed us to quantify the DPPH-radical scavenging capacity of reference antioxidants with respect to the mass of phenolic fraction. All SH samples manifested REAC values (expressed as μg of antioxidant/μg GAE) $\geqslant 1$, while 1 μg of phenols contained in PW extract exhibited radical scavenging ability

Table 3
BHA, BHT, Trolox and α-Tocopherol equivalent antioxidant capacity (EAC) of the extracts

Extract[a]	Solvent[b]	BHA[c]		Trolox[c]		BHT[c]		α-Tocopherol[c]	
		μg μg^{-1} of extract	μg μg^{-1} of GAE[d]	μg μg^{-1} of extract	μg μg^{-1} of GAE[d]	μg μg^{-1} of extract	μg μg^{-1} of GAE[d]	μg μg^{-1} of extract	μg μg^{-1} of GAE[d]
SH	M	0.06	1.03	0.06	1.15	0.08	1.50	0.13	2.35
SH	E	0.06	0.99	0.07	1.10	0.09	1.43	0.13	2.25
SH	A	0.08	1.14	0.09	1.28	0.12	1.66	0.19	2.61
PW	M	0.95	1.98	1.07	2.22	1.38	2.88	2.18	4.54
PW	E	1.09	2.17	1.22	2.43	1.58	3.15	2.49	4.96
PW	A	0.94	2.01	1.05	2.26	1.36	2.92	2.15	4.60
PC	M	0.20	2.04	0.22	2.29	0.29	2.97	0.46	4.67
PC	E	0.36	2.06	0.41	2.31	0.53	2.99	0.83	4.71
PC	A	0.43	2.10	0.49	2.36	0.63	3.05	0.99	4.81

[a] SH, obtained from hazelnut shells; PW, obtained from skin of whole roasted hazelnuts; PC, obtained from skin of chopped hazelnuts.
[b] M = 80% methanol, E = 80% ethanol, A = 80% acetone.
[c] EC_{50} (μg mg^{-1}DPPH): BHA, 134.3 ± 2.7; Trolox, 150.6 ± 2.4; BHT, 195.2 ± 3.5; α-Tocopherol, 307.3 ± 5.8.
[d] Gallic acid equivalents.

equivalent to 2 μg of BHA (or Trolox), about 3 μg of BHT and nearly 5 μg of α-tocopherol. REAC values of PC phenolic fractions resulted close to that of PW samples. The findings indicated that the DPPH-scavenging activity of phenolic fractions contained in hazelnut by-products is excellent, when compared with the most largely employed commercial synthetic (BHA, BHT) or natural (α-tocopherol) antioxidative compounds. These considerations enable us to hypothesize that an eventual purification of hazelnut by-product extracts could yield extremely potent natural biophenolic antioxidants.

Our data concerning DPPH-radical scavenging ability (EC_{50}) of the extracts were not directly comparable with the results reported by Shahidi et al. (2007), which investigated hazelnut shell and skin phenolic extracts obtained with aqueous hot ethanolic solvent. These authors evaluated DPPH scavenging activity of the extracts and a reference antioxidant (catechin) by testing only 50 and 100 ppm solutions, obtaining nearly 100% of DPPH scavenging for both the hazelnut by-product extracts; even catechin gave 100% radical scavenging activity at both the concentrations. We think that these concentrations were too high for an effective comparison, because they were out of the linear range when plotted against the percentage of DPPH inhibition (Villaño, Fernández-Pachón, Troncoso, & García-Parrilla, 2005). In fact, it was advisable that the maximal concentration of sample in the reaction vessel does not exceed 70% of DPPH radical scavenging (Buenger et al., 2006).

3.3.2. Antiperoxyl radical efficiency (AAPH-linoleic acid assay)

The AAPH-induced linoleic oxidation assay evaluates the chain breaking activity of an antioxidant. This method allows dynamic quantification of conjugated dienes as a result of initial linoleic oxidation, by measuring UV absorbance at 232 nm. The results of data elaboration are reported in Table 2. Antiperoxyl radical efficiency was expressed as rate of inhibition (R_{inh}, min L mg^{-1} of extract or GAE). The higher the R_{inh}, the higher the antioxidant activity.

A significant difference ($p < 0.05$) was detected among hazelnut by-product extracts. Antioxidant activity (expressed on weight basis of extract) was minimal for SH, intermediate for PC, and maximal for PW. These results were in accordance with those reported by Shahidi et al. (2007), which noted that the antioxidant power of hazelnut skin crude extract was better than the extract obtained from hard shell, employing the β-carotene–linoleate model system assays.

Regarding the solvent mixtures, the highest R_{inh} value was measured in acetonic SH and PC extracts, while both ethanolic and acetonic PW extracts showed the same lower antiperoxyl radical efficiency. Methanolic mixture resulted in affording a slightly lower (but significant different) antioxidant power. Except for acetonic and ethanolic PC extracts, these results were in accordance with those obtained using DPPH assays. However, it can be noted that the difference between extracts of differing sources (shell, and skin of whole or chopped hazelnuts) detected with AAPH-linoleic assay was definitely less pronounced than that highlighted with DPPH-assay. Furthermore, there were no significant differences ($p < 0.05$) between samples of the same source when the ANOVA test was applied to the data expressed in terms of min L mg^{-1} GAE. In other words, from a given hazelnut by-product the three adopted solvent mixtures were able to extract phenolic compounds with analogous protective properties against lipidic peroxidation. Another noteworthy result was that the phenolic mass of PC and PW samples (which manifested the same antiperoxyl radical efficiency) was much less efficient (about 1.5-fold lower) than the phenolic mass of SH extract. Hence, the low antioxidant activity exhibited by SH crude extracts was essentially due to their scarce pureness. Opposite results were obtained when the extracts were tested for their DPPH scavenging ability, the PC and PW phenolic fractions resulting much more efficient than the SH ones.

Differences between the antiperoxyl radical efficiency of samples as well as that between standard antioxidants (BHA, BHT, α-tocopherol and Trolox) are graphically reported in Fig. 2A–C. It is visually noticeable that all PC, and above all SH crude extracts, manifested (on weight basis) very little antiperoxyl radical efficiency in comparison with pure antioxidants. When compared with the standard, the most powerful PW crude extracts exhibited R_{inh} value 1.3 and 1.4-fold smaller than α-tocopherol and, Trolox, respectively, and above 2-fold smaller than BHA.

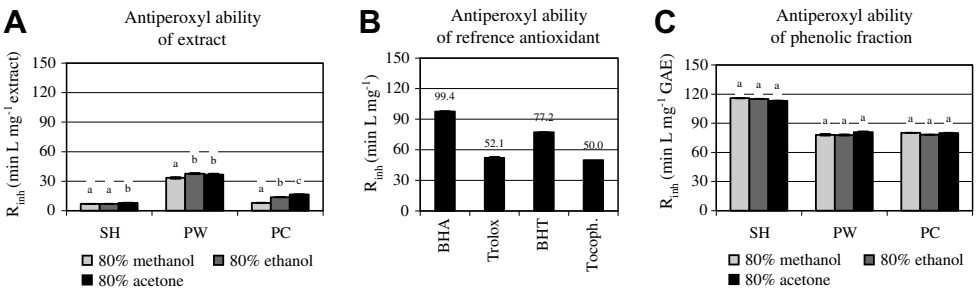

Fig. 2. Antiperoxyl radical efficiency of hazelnut by-product extracts ((A) and (C)) and reference antioxidants (B). (A), R_{inh} expressed as min L mg^{-1} of extract; (B), R_{inh} expressed as min L mg^{-1} of pure compound; (C), R_{inh} expressed as min L mg^{-1} of GAE.

The different results obtained when R_{inh} values were expressed on weight or GAE basis are well noticeable by comparing the graphics reported in Fig. 2A and C. Taking into account the graphics shown in Fig. 2C (referred to R_{inh} value of phenolic fractions) and (B) (referred to R_{inh} value of standards), it is evident that phenolic compounds contained in SH extracts manifested a much greater protective activity against linoleic acid oxidation than all pure antioxidant tested. Even if the phenolic fractions of PW and PC showed a lower antioperoxyl efficiency than SH, they appeared very efficient when compared with standards, being more active than, Trolox and α-tocopherol, and as efficient as BHT under experimental conditions.

3.4. Correlation between total phenols and antioxidant activity

Antioxidant activity of the crude extracts was related to the phenolic content; in fact, a highly significant positive correlation between studied antioxidant parameters and total phenols was found (antiradical ability (y) as function of total phenols (x): $y = 0.0168x - 0.4091$, $r^2 = 0.993$; antiperoxyl radical efficiency (y) as function of total phenols (x): $y = 0.0718x + 2.1033$, $r^2 = 0.995$). The direct proportional correlations found indicated that measured antioxidant activity is primarily attributable to the phenolic compounds present in the extract.

It should be noted that in the correlation between antiradical ability and total phenols the intercept with the abscissa axis is greater than zero; this means that there was a threshold-value of phenolic content (about 24 mg GAE/g) under which the measured DPPH-radical scavenging ability was ineffective. On the contrary, the positive intercept with the ordinate axis found in the correlation between antiperoxyl radical efficiency and total phenols meant that a portion of measured antioxidant activity was due to other active non-phenolic substances. Soluble fibre and/or melanoidin compounds formed during hazelnut roasting could be responsible for such activity. Similar results were found by Llorach, Espin, Tomás-Barberán, and Ferreres (2003), by analyzing the dependence of radical scavenging and antiperoxyl radical efficiency on phenolic content of cauliflower by-product extracts.

When AE (y) and R_{inh} (x) values were plotted, a significant positive correlation was found ($y = 0.2321x - 0.8675$, $r^2 = 0.982$), indicating that there was a relationship between the two analytical methods employed to assess the antioxidant activity in this study.

3.5. Ultraviolet spectra and tannin quantification

After proper dilution with methanol, UV spectra of the obtained phenolic extracts were recorded. As expected, a comparison carried out with the same dilution factor gave a low UV absorption for SH, medium for PC and high for PW (spectra not shown). Samples were then diluted at the same phenolic concentration (26 μg GAE/ml) and scanned again at wavelengths from 220 to 400 nm. The obtained spectra are shown in Fig. 3. All extracts exhibited a single peak in the UV range with maximum absorbance at 280–281 nm. Very similar spectra, characterized by the same UV-max (280 nm), were observed in the phenolic extract

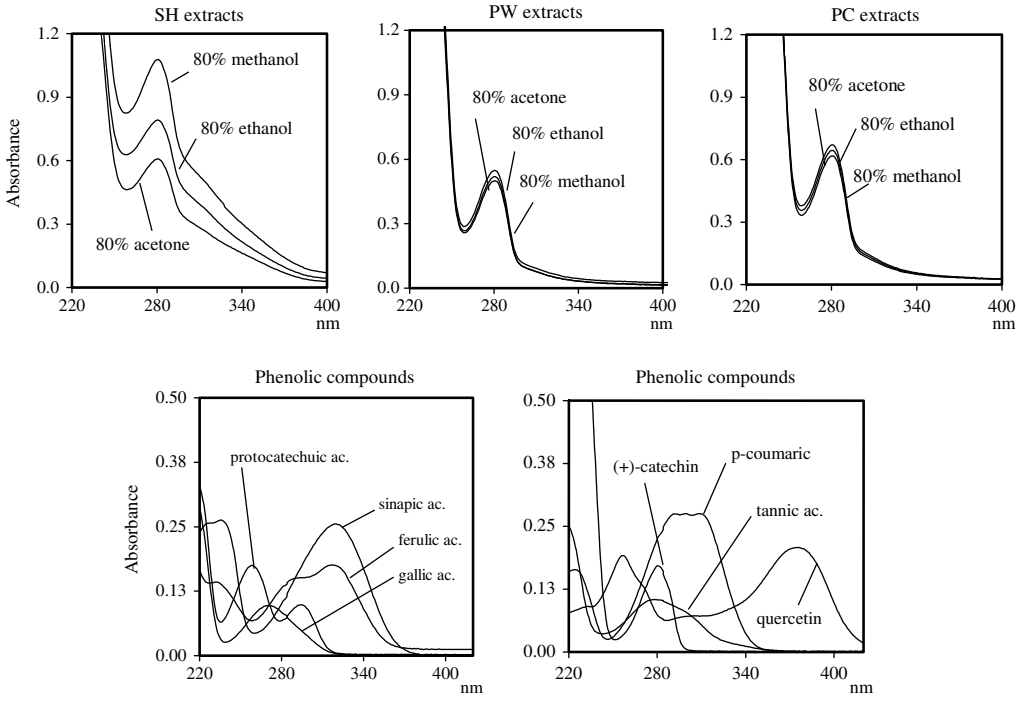

Fig. 3. UV spectra of hazelnut by-product extracts (26 μg GAE/ml) and standard phenolic compounds (15 μg/ml).

obtained from hazelnut kernels (with skin) (Alasalvar et al., 2006) and in the polymeric tannin fraction (obtained by column separation with Sephadex LH-20) of crude almond phenolic extract (Amarowicz, Troszyńska, & Shahidi, 2005).

At the same phenolic concentration, the three spectra of PW extracts, and likewise the three spectra of PC extracts, were almost completely superimposable, independently from the solvent extractor. On the contrary, an increase of background absorption was found for SH extracts obtained with aqueous acetone, ethanol and methanol, respectively. This is probably due to the presence of growing amounts of other UV-absorbers, such as amino acids or peptides containing aromatic ring, lipid oxidation products. An increase of background absorption was observed when comparing the extracts of different sources (PW < PC < SH).

At present very little is known about the nature of phenolic fraction of roasted hazelnut skins and shells. UV spectra of the main monomeric flavonoids (catechin and quercetin) and phenolic acids (protocatechuic, sinapic, gallic, *p*-coumaric, ferulic) detected in the hazelnut kernel and its by-products (Alasalvar et al., 2006; Senter et al., 1983; Shahidi et al., 2007) are shown in Fig. 3. Of these, only gallic acid and catechin exhibited a single peak with maximum absorptivity close to 280 nm (namely, 280 and 277 nm, respectively). Gallic acid and cathechin are components of hydrolyzable and condensed tannins, respectively. The first, was found as the main phenolic acid in hydrolyzed extracts of hazelnut skin and hard shell (Shahidi et al., 2007). UV spectra of commercial tannic acid exhibited a single peak with maximum absorbance at 280 nm (Fig. 3). All these considerations show that the phenolic fraction of hazelnut extracts was characterized by a high presence of catechins and/or polymerized (tannin) polyphenols. Antioxidant activity and health beneficial properties of catechins have been reported widely in literature (Yilmaz, 2006). Historically, tannins were considered antinutrients, but recently the recognition of very effective antioxidative properties has led to second thoughts towards their effect on human health. Tannins resulted much more powerful antioxidants than simple monomeric phenols, and may have unique roles in the human digestive metabolism as both savers of other biological antioxidants and protectors of nutrients (lipids, proteins, and carbohydrates) from oxidative damages (Hagerman et al., 1998).

High tannin content in hazelnut by-product extracts was confirmed by quantitative analyses. As supposed by Shahidi et al. (2007), tannins represented the principal fraction of the phenolic substances of the extracts, ranging nearly 60 to 65% of the total phenols (Table 1). As a general rule, the highest amount of tannins was detected in the extracts with the highest amount of total phenols (namely, ethanolic extract for PW samples, and acetonic extract for both PC and SH samples). Hence, the high antioxidant activity we detected may be due to these polyphenolic compounds, since they are expected to have powerful antioxidant activity.

A positive correlation was found between antioxidant activity and total tannins. The determination coefficient of the correlation between total tannins and AE values resulted very high ($r^2 = 0.99$). When total tannins and R_{inh} were plotted, the resultant determination coefficient was elevated ($r^2 = 0.98$) too. Hence, tannin fraction played a leading role in determining the DPPH-radical scavenging and antiperoxyl radical properties of the hazelnut by-product phenolic extracts.

In conclusion, hazelnut residues represent a rich and inexpensive source of natural and effective phenolic antioxidants. Long maceration at room temperature with selected solvent resulted in a suitable system for obtaining crude phenolic extracts from hazelnut skin and shell by-products. Overall antioxidant activity of extracts was noticeable, but in considering a possible industrial application, roasted hazelnut skins (without kernel fragments as far as possible) represent the most promising raw material. In fact, skin samples coming from whole roasted hazelnuts provided high yield in crude extract (nearly 30% on weigh basis of the defatted source), high phenolic content of the extract (up to 50% of the mass), high antioxidant activity of the crude extract, and high antioxidant efficiency of the phenolic fraction contained in the extract. More research is needed to identify and quantify the antioxidant active compounds in hazelnut by-product extracts, to verify the presence of potentially allergenic residues, and to investigate the relationship between antioxidant properties and health benefits.

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