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Analytical Methods

Total antioxidant activity of hazelnut skin (Nocciola Piemonte PGI): Impact of different roasting conditions

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ABSTRACT

The thin brown perisperm (skin) that envelops hazelnut kernels is usually removed after roasting process, leading a phenolic-rich by-product. Principal aim of this work was to characterise the total antioxidant activity of phenolic extracts obtained from roasted "Nocciola Piemonte PGI" hazelnuts skin. Different extraction solvents (methanol, acidified methanol, ethanol, acidified ethanol, and acetone/water) and different protocols (cold solvent-assisted extraction and semi-automated Soxhlet extraction) were employed. The influence of different roasting degree (180 °C/10 min and 180 °C/20 min) was also investigated. DPPH· and $ABTS^{\cdot+}$ radical-scavenging methods, ferrous ions chelation activity and inhibition of lipid peroxidation investigated in this study demonstrated significant antioxidant properties for hazelnut skin phenolics. The main mechanism involved appeared the antiradical activity, strictly related to the total phenolic content ($r = -0.8798$ and -0.8285 for DPPH· and $ABTS^{\cdot+}$ assays, respectively). The acidification of extraction solvents led to a significant decrease of antiradical activity, whilst the different roasting conditions significantly influenced the chelation activity and the inhibition of lipid peroxidation, showing higher effectiveness for high-roasted hazelnut skin extracts. Conversely, the direct measure of the antioxidant capacity of defatted hazelnut skins revealed higher $ABTS^{\cdot+}$ scavenging properties for medium-roasted sample.

1. Introduction

In food science, antioxidants are very important in that they act preventing lipid oxidation in food and decreasing the adverse effects of reactive species (ROS: reactive oxygen species; RNS: reactive nitrogen species) on normal physiological functions in humans (Huang, Ou, & Prior, 2005).

Antioxidant synthetically obtained, like BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene), are largely used in food industry and are included in human diet. However, in recent years the use of natural antioxidants has been promoted because of concerns regarding the safety of synthetic ones. Dietary components, including polyphenols, carotenoids and vitamins C and E, are considered effective antioxidants useful in the prevention of oxidative stress and related diseases (Kaur & Kapoor, 2001; Moure et al., 2001).

Widely distributed in the plant kingdom and abundant in our diet, polyphenols are among the most studied about classes of antioxidants. Phenolics are the products of secondary metabolism in plants, providing essential functions in the reproduction and the growth of the plants, acting as defense mechanisms against pathogens, parasites, and predators, as well as contributing to the colour of plants (Liu, 2004). In addition to their roles in plants, several epidemiological and clinical researches demonstrated that phenolic antioxidants occurring in cereals, fruits and vegetables are principal contributing factors for the decreased incidence of several chronical and degenerative diseases (Shahidi, 2000).

For all these reasons, in last few years several studies have been conduced in order to investigate the antioxidant activity of phytoextracts obtained from vegetable sources. Particularly, agricultural and industrial residues are considered as very attractive sources of natural antioxidants (Moure et al., 2001). By-products of grape (*Vitis vinifera* L.) processing, such as seeds and peels, are the most studied and promising antioxidants sources (Shi, Yu, Pohorly, & Kakuda, 2003). The extraction and antioxidant activity of phenolic compounds from other residual materials such as apple peel (Kim et al., 2005), apple pomace (Lu & Foo, 2000), sweet orange peel (Anagnostopoulou, Kefalas, Kokkalou, Assimopoulou, & Papageorgiou, 2005), blanched artichoke and artichoke blanching waters (Llorach, Espín, Tomás-Barberán, & Ferreres, 2002), leaves and stems of cauliflowers (Llorach, Espín, Tomás-Barberán, & Ferreres, 2003), olive mill waste (Mulinacci et al., 2005), cocoa by-products (Arlorio et al., 2008; Azizah, Ruslawati Nik, & Swee Tee, 1999) and nut hulls (peanut, cashew nut, hazelnut, almond, pistachios,

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Chilean hazelnut, etc.) (Goli, Barzegar, & Sahari, 2005; Kamath & Rajini, 2007; Moure et al., 2000; Shahidi, Alasalvar, & Liyana-Pathirana, 2007; Wijeratne, Abou-Zaid, & Shahidi, 2006; Yu, Ahmedna, & Goktepe, 2005) have been also investigated. The presence of polyphenols in outer layers (skins, peels, and hulls) of fruits, vegetables and seeds (nuts) may offer protection against oxidative stress: it is known that hulls play the major role in the defense of the plant seeds and, together with bran fractions, concentrate most phenols and tannins (Shahidi & Naczk, 1995). Moreover, polyphenols play an important role in the astringent taste, causing typical long-lasting puckering, shrinking, rough, and drying sensation in the oral cavity (Stark, Bareuther, & Hofmann, 2005).

The antioxidant activity of nuts and their by-products has been previously studied. These studies have highlighted that nut by-products are rich sources of natural antioxidants and phenolic compounds. Among nuts, hazelnuts (*Corylus avellana* L.) are very interesting in that rich in phenols and, particularly, proanthocyanidins (Gu et al., 2003). Recent studies tentatively identified several phenolic acids in both hazelnut kernels (Alasalvar, Karamać, Amarowicz, & Shahidi, 2006; Yurttas, Schafer, & Warthesen, 2000) and hazelnut by-products (skin, green leafy cover, hard shell and tree leaf) (Contini, Baccelloni, Massantini, & Anelli, 2008; Shahidi et al., 2007). These works demonstrated that hazelnut skin (or perisperm, or testa) is a rich and low-cost source of natural phenolic antioxidants. More recently, Alasalvar et al. (2009) obtained two fractions from crude phenolic extracts of Turkish Tombul hazelnuts skin (low-molecular-weight phenolics and tannins, respectively), showing higher antioxidant/antiradical activity for tannin fraction, followed by the crude extract and low-molecular-weight phenolic compounds. However, the impact of different roasting conditions on both phenols extraction and antioxidant activity should also to be investigated, particularly considering the formation of Maillard products (melanoidins) during roasting.

The aim of this work was to characterise the total antioxidant activity of phenolic extracts obtained from roasted "Nocciola Piemonte PGI" hazelnuts skin, considering different approaches (free radical-scavenging activity, chelation of pro-oxidant ferrous ions, inhibition of lipid peroxidation). Different extraction protocols were employed (cold solvent-assisted extraction and semi-automated Soxhlet extraction) and the influence of different roasting processes (medium- and high-roasting degrees) was investigated. Finally, the total antioxidant capacity of hazelnut skins (defatted powders) was determined using a direct measurement protocol.

2. Materials and methods

2.1. Samples

Samples of hazelnut skins were kindly provided by Dr. Giuseppe Zeppa (University of Turin, Italy). Hazelnut skins were obtained from Italian "Nocciola Piemonte PGI" hazelnut kernels (*C. avellana* L.), namely Tonda Gentile delle Langhe cultivar, cultivated only in specific areas and according to the disciplinary of production of the protected geographical indication (PGI) "Nocciola Piemonte". Dried unshelled hazelnuts were roasted at two different conditions: 180 °C for 10 min (medium roasting, MR) and 180 °C for 20 min (high roasting, HR), and hazelnut skins were recovered after spontaneous separation from the kernels after roasting. All samples were stored under vacuum and kept in the dark at −20 °C until they were analysed.

2.2. Chemicals

All reagents and standard chemicals ((±)-catechin monohydrate, trolox, gallic acid monohydrate, caffeic acid, (−)-epicatechin, quercetin dihydrate, butylated hydroxyanisole (BHA) and disodium ethylenediaminetetraacetate dihydrate (Na_2-EDTA)) used for the determination of total phenol content and antiradical activity were purchased from Sigma–Aldrich (Milano, Italy). All chemicals and solvents were of reagent-grade level and purchased either from Sigma–Aldrich (Milano, Italy).

2.3. Proximate composition analysis

The moisture content of hazelnut skin samples was determined using a thermo-balance Sartorius MA30 (Sartorius AG, Goettingen, Germany). Total nitrogen content and total protein content (conversion factor: 6.25) were obtained according to Kjeldahl method using the Kjeltec system I (FOSS Tecator, Sweden). The ash content was determined in a muffle furnace according to AOAC (1990) procedure. Lipid fraction was extracted from ground hazelnut skins (after grinding and sieving particles size <1 mm) using a semi-automatic Soxhlet Büchi Extraction System B-811 (Büchi Labortechnik AG, Flawil, Switzerland) for 12 h, employing dichloromethane as solvent. All the results have reported as percentage on the basis of dry weight (dw).

2.4. Extraction of phenolic fraction

The extraction of phenolic fraction from high- and medium-roasted defatted hazelnut skins was performed using two different methods: (i) cold-extraction under magnetic stirring and (ii) Soxhlet extraction. For cold-extraction five different solvents were used: methanol, acidified methanol (hydrochloric acid 0.1%, v/v), ethanol, acidified ethanol (hydrochloric acid 0.1%, v/v), acetone/water 80:20, v/v; methanol was chosen as solvent for Soxhlet extraction.

2.4.1. Cold-extraction under stirring

Four grams of defatted hazelnut skins powders were extracted using 100 mL of solvent; extraction was carried out in closed Erlenmeyer flasks and under constant magnetic stirring, in the dark at room temperature (22 °C). After 1 h of stirring/extraction, the suspension was filtered (Buchner funnel) through Perfecte 2 paper filter (Superfiltro, Milan, Italy), and the solid residue was re-extracted with 50 mL of solvent for 30 min. This last step was repeated until the complete decolouration was achieved (exhaustive extraction); then, filtrates were collected. The total time required to obtain the complete extraction varied depending on the solvents employed and on the different roasting conditions (data not shown). Finally, the solvent was evaporated to dryness (vacuum, 40 °C) and dry extract was stored at −20 °C until use.

2.4.2. Soxhlet extraction

Ten grams of the defatted hazelnut skin powders were extracted with Soxhlet apparatus using methanol for 7 h. The solvent was then evaporated to dryness (vacuum, 40 °C) and the dry extract was stored at −20 °C until use.

2.5. Determination of phenolic content

The determination of total phenolic content was obtained using the classic Folin-Ciocalteu assay, as previously described in Arlorio et al. (2008). Results were expressed as catechin equivalents, through the calibration curve of (±)-catechin monohydrate. The calibration curve linearity range was 50–250 μg (r = 0.9987).

2.6. DPPH· scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging assay was performed according to the method reported by Locatelli

et al. (2009). Samples and standard molecules were dissolved in methanol and appropriately diluted in order to obtain a calibration curve (concentration range from 1 to 20 μg mL^{-1}). Antiradical activity was expressed as inhibition percentage (*I*%) and calculated using the following equation:

$$\text{Inhibition percentage } (I\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Results were finally expressed as EC_{50} (antioxidant dose required to obtain a 50% inhibition), calculated by probit regression analysis.

2.7. ABTS$^{\cdot+}$ scavenging activity

The ABTS radical cation (ABTS$^{\cdot+}$) scavenging assay was performed according to the method reported by Re et al. (1999). Samples and standard molecules were dissolved in ethanol and appropriately diluted in order to obtain a calibration curve (concentrations range from 10 to 900 μg mL^{-1}). Antiradical activity was expressed as inhibition percentage (*I*%), as previously described for DPPH$^{\cdot}$ assay. Results were expressed as EC_{50}, calculated by linear regression analysis.

2.8. Chelation activity (ferrozine method)

The determination of ferrous ions chelation activity was performed according to the method reported by Liyana-Pathirana and Shahidi (2005) with some modifications. Briefly, 500 μL of sample or its relative solvent (control: water for Na_2-EDTA, methanol for hazelnut skin extracts and other standard molecules), 10 μL of aqueous 2 mM $FeCl_2$, 35 μL of 5 mM ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt) and 2.5 mL of ethanol were added, adequately mixed, and left to stand for 10 min. Absorbance was immediately read at 562 nm, using a Kontron UVIKON 930 Spectrophotometer (Kontron Instruments, Milan, Italy). Samples and standard molecules were dissolved in methanol (water in the case of Na_2-EDTA) and appropriately diluted to obtain a calibration curve. Because of high extract concentrations employed (ranging from 0.2 to 7 mg mL^{-1}), absorbance of blank solutions (without ferrozine) was measured to correct any influence due to colour of the extracts. The chelation activity, measured as inhibition percentage of ferrozine–Fe^{2+} complex formation, was calculated by using the following equation:

$$\text{Chelation activity } (\text{CA}\%) = \frac{\text{Abs}_{\text{control}} - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}} \times 100$$

Results were finally expressed as EC_{50}, calculated by linear regression analysis.

2.9. Inhibition of lipid peroxidation (ferric-thiocyanate method)

The determination of inhibition of lipid peroxidation was performed according to the ferric-thiocyanate (FTC) method reported by Zin, Hamid, Osman, and Saari (2006), with some modifications. First, 100 μL of linoleic acid were dissolved in 4 mL of EtOH, 8 mL of 0.05 M phosphate buffer (pH 7.0) and 3.9 mL of distilled water. Three-hundred and fifty micro-litres of sample or solvent (5% methanolic ethanol, control) were added to 1.4 mL of the previously described linoleic acid solution. This mixture was kept in a screwed-cap container in the dark and at a temperature of 50 °C; the accelerated oxidation of linoleic acid was measured after 24, 48, 72 and 96 h of thermal treatment. The determination of oxidation degree (as peroxides formation) was performed according to the ferric-thiocyanate method: 30 μL of the reaction mixture were added to 2910 μL of 75% ethanol, 30 μL of 30% ammonium thiocyanate and 30 μL of 0.02 M ferrous chloride in 3.5% hydrochloric acid. Mixtures were shaken and exactly after 3 min the absorbance was measured at 500 nm, using a Kontron UVIKON 930 Spectrophotometer (Kontron Instruments, Milan, Italy). Samples and standard molecules were tested at three different concentrations (10, 100, and 1000 μg mL^{-1}). Results were expressed as inhibition of lipid peroxidation percentage calculated by using the following equation:

$$\text{Inhibition of lipid peroxidation } (\text{IP}\%) = 100 - \frac{\text{Abs}_{\text{sample}}(t) - \text{Abs}_{\text{sample}}(0)}{\text{Abs}_{\text{control}}(t) - \text{Abs}_{\text{control}}(0)} \times 100$$

where (*t*) and (0) indicate that absorbances of the samples and the control were measured at time *t* (*t* = 24, 48, 72 and 96 h of thermal treatment) and at time zero (before the starting of the oxidation reaction), respectively.

2.10. Direct measurement of total antioxidant capacity (QUENCHER approach)

The direct measurement of total antioxidant capacity of hazelnut skin was obtained following the procedure described by Serpen, Gökmen, Pellegrini, and Fogliano (2008). ABTS$^{\cdot+}$ reagent was prepared as described and further diluted in a mixture of ethanol:water (50:50, v/v) to obtain an absorbance of 0.700 ± 0.020 at 734 nm. Hazelnuts skin (defatted powders) were finely ground and sieved (particles size <250 μm); then, samples were tested in the ratio of 0.15 mg per 6 mL of ABTS$^{\cdot+}$ reagent. Absorbance measurements were performed at 734 nm after exactly 6, 15, 30 and 60 min to determine the time required to reach the steady state. Results were expressed as mol of trolox equivalents per kg of sample through a calibration curve (linearity range: 20–160 mmol; $r = 0.9998$).

2.11. Statistical analysis

Results were expressed as mean ± standard deviation (SD) of at least three independent experiments. Differences were estimated by analysis of variance (ANOVA) followed by Tukey's "Honest Significant Difference" test. Differences were considered significant at $p < 0.05$. Pearson's correlation analysis was used to determine correlation coefficients and their statistically significance. All statistical analyses were performed using the free statistical software R 2.8.1 version (http://www.R-project.org) (R Development Core Team, 2008).

3. Results and discussion

3.1. Proximate composition of roasted hazelnut skin

First, the proximate composition of roasted hazelnut skin was determined (Table 1). As expected, prolonged roasting time allowed the decreasing of moisture content. In order to avoid the influence of roasting conditions, lipids, proteins and ashes contents were reported on dry weight (dw) basis. Both MR and HR samples

Table 1
Proximate composition of roasted hazelnut skin samples.

	MR hazelnut skin	HR hazelnut skin
Moisture	11.89 ± 0.27	7.71 ± 0.21
Lipids (dw)	39.48 ± 0.32	37.41 ± 0.76
Proteins ($N \times 6.25$) (dw)	9.02 ± 0.03	10.42 ± 0.20
Ashes (dw)	1.91 ± 0.09	2.52 ± 0.03

Results are expressed as mean ± SD ($n = 3$).

showed a proximate composition similar to that reported for raw hazelnut skin (Anil, 2007).

3.2. Extraction of phenolic fraction

Extraction of phenolic compounds is strongly affected by their chemical nature, the sample particles size, the extraction method employed, and the presence of interfering substances. Moreover, the solubility of phenolic substances is strictly dependent by the polarity of the solvent used, as well as their degree of polymerisation (Naczk & Shahidi, 2004). In this work, the extraction of phenolic fraction from hazelnut skin was first performed using different solvents (methanol, acidified methanol, ethanol, acidified ethanol, and acetone/water). An additional extraction by semi-automatic Soxhlet was also performed using methanol as solvent (MeOHsox). In order to avoid the influence of lipid interfering compounds on phenols extraction and following analyses, matrix was previously defatted by extraction with dichloromethane. Phenolics extraction yield, expressed as grams per 100 g of defatted samples, was in the order MeOH > MeOH/H^+ > Ac_2O/H_2O > EtOH/H^+ > EtOH > MeOHsox for MR hazelnut skin (range from 43.67% to 28.59%) and in the order Ac_2O/H_2O > MeOH/H^+ > EtOH/H^+ > MeOHsox > MeOH > EtOH for HR hazelnut skin (range from 42.65% to 27.95%). Extraction yields obtained for aqueous acetonic extracts (38.54% and 42.65% for MR and HR samples, respectively) were higher than that reported by Contini et al. (2008), which obtained a 32.6% extraction yield employing the same solvent, but using a long-time maceration extraction method. A recent study showed that aqueous acetone (80:20 acetone/water, v/v) was a more effective solvent than aqueous methanol (80:20 methanol/water, v/v) to extract condensed tannins from hazelnut skin (Alasalvar et al., 2009). The other solvents used in this work (MeOH, MeOH/H^+, EtOH and EtOH/H^+) were not previously considered for phenolic extraction from hazelnut skin. It can be observed a different order of solvents depending on medium roasted and high-roasted samples: in fact, different thermal treatments might be induce modifications on the chemical composition and cellular structure of the original matrix (Saklar, Ungan, & Katnas, 2003; Özdemir et al., 2001).

3.3. Determination of total phenolic content

Total phenolic content of hazelnut skin is shown in Table 2; results are expressed as milligrams of catechin equivalent (CE) per gram of extract. The amount of phenolic compounds ranges from 637.65 to 380.24 mg CE g^{-1} and 706.35 to 551.59 mg CE g^{-1} for MR and HR samples, respectively. The very low phenols content obtained for medium-roasted sample using methanol under stirring is presumably due to the unexpected incomplete solubility of this extract in methanol. So, this value is considered underestimated. Concerning both medium and high-roasted skin the most effective extraction solvent resulted ethanol (total phenolic content expressed per gram of extract); however, considering results on a dw basis, the higher phenolic content was obtained using the aqueous acetonic mixture (181.51 and 190.88 mg CE g^{-1} of hazelnut skin, dw for MR and HR samples, respectively). Significant differences were observed among the extracts. Overall, acidified solvents extracted lower amounts of phenolic compounds than not-acidified ones ($p < 0.05$). Instead, different roasting conditions do not significantly influence the quantity of extractable polyphenols; the prolonged thermal treatment seems to not affect the total phenolic content of hazelnut skin ($p > 0.05$). Results obtained in this work are similar to that obtained by other researchers using different solvents and extraction method. Alasalvar et al. (2009) reported 686 and 701 mg CE g^{-1} for crude extracts obtained from three consecutive extractions at 50 °C (each of 30 min), using 80:20 acetone/water (v/v) and 80:20 methanol/water (v/v) as solvent, respectively. Shahidi et al. (2007) reported a 577.7 mg CE g^{-1} phenolic content for hazelnut skin employing 80/20 (v/v) ethanol/water mixture under reflux conditions at 80 °C; Contini et al. (2008) obtained 499.7, 588.2 and 546.6 mg CE g^{-1} for skin waste from whole roasted kernel using aqueous methanol (80/20, v/v), aqueous ethanol (80/20, v/v) and aqueous acetone (80/20, v/v), respectively, after long maceration at room temperature. Differences in the total phenols content might be ascribed to different solvents and extraction methods used, but also to different cultivars, geographic origin and harvest season of the samples (all these parameters are strictly related to biosynthesis of secondary metabolites such as phenolics). In this work, we analysed hazelnut skin obtained exclusively from "Nocciola Piemonte PGI" hazelnut kernels, while authors previously cited used by-products obtained from Turkish Tombul hazelnut (Alasalvar et al., 2009; Shahidi et al., 2007) or from a mixture of different varieties (Italian Tonda Gentile Romana, Tonda di Giffoni, Tonda Gentile delle Langhe; Turkish Tombul) (Contini et al., 2008).

3.4. Determination of antioxidant activity of hazelnut skin extracts

In order to better understand the antioxidant properties of hazelnut skin phenolic extracts, we performed four different chemical *in vitro* assays, based on different antioxidant mechanism. Antiradical properties were analysed using both DPPH· and $ABTS^{\cdot+}$ scavenging assays, in that these methods show several important differences in their response to antioxidants and in their manipulation (Arnao, 2000); then, ferrous ions chelation activity and inhibition of lipid oxidation (autoxidation of linoleic acid system) were determined. Trolox (useful and available as commercial standard compound in the evaluation of antioxidant properties), BHA (a synthetic antioxidant largely used by food industry), some phenolic acids (gallic acid and caffeic acid) and some natural flavonoids (epicatechin and quercetin) (qualitatively identified in hazelnut skin extracts, data not showed) were assayed for their antioxidant properties as reference compounds.

Table 2
Total phenols content and antiradical activity (DPPH· and $ABTS^{\cdot+}$ methods) of hazelnut skin extracts.

Samples	Total phenols (mg CE g^{-1} of extract)		DPPH· (EC_{50}; μg mL^{-1})		$ABTS^{\cdot+}$ (EC_{50}; μg mL^{-1})	
	MR	HR	MR	HR	MR	HR
MeOH	380 ± 24^{a}	$701 \pm 107^{a,c}$	10.11 (8.49–12.09)^{a}	3.67 (3.19–4.21)^{a}	655 ± 6^{a}	350 ± 1^{a}
MeOH/H^+	513 ± 50^{b}	$564 \pm 66^{b,d}$	5.43 (4.55–6.49)^{b}	6.33 (5.53–7.31)^{b}	484 ± 26^{b}	439 ± 27^{b}
EtOH	$638 \pm 35^{c,d}$	706 ± 104^{a}	4.70 (3.75–5.90)^{c}	3.91 (3.38–4.52)^{a}	318 ± 20^{c}	364 ± 6^{c}
EtOH/H^+	$575 \pm 21^{c,b}$	$552 \pm 64^{b,d}$	5.31 (4.29–6.61)^{c,a}	4.71 (4.00–5.54)^{c}	374 ± 19^{d}	379 ± 14^{c}
Ac_2O/H_2O	631 ± 19^{d}	$631 \pm 88^{a,b,c}$	5.02 (3.99–6.31)^{c,b}	5.00 (4.13–6.06)^{c}	$336 \pm 29^{c,d}$	$351 \pm 10^{a,c}$
MeOHsox	562 ± 29^{b}	$594 \pm 8^{c,d}$	5.46 (4.30–6.94)^{c}	4.01 (3.33–4.83)^{a}	317 ± 13^{c}	$361 \pm 10^{a,c}$

Results are expressed as mean ± SD ($n \geqslant 3$). In the case of DPPH· assay, the 95% confidence limits are reported. Mean ± SD followed by the same letter, within a column, are not significantly different ($p > 0.05$).

3.4.1. DPPH· scavenging activity

DPPH· is one of the most used synthetic radicals to evaluate antiradical properties of bioactive compounds and food extracts. It is more stable than common natural radicals (hydroxyl and superoxide radicals) and it is unaffected by certain side reactions, such as metal–ion chelation and enzyme inhibition. In this work, DPPH· scavenging properties were evaluated testing at least six different concentrations for each extract and repeating experiments at least in triplicate. Results were reported as concentration required to obtained a 50% radical inhibition (EC_{50}, expressed as μg of extract per millilitre of solvent; Table 2); higher antiradical activity corresponds to lower EC_{50} values. Because of the restricted linearity range between antioxidant concentration and radical inhibition (*I*%), EC_{50} values were calculated on the basis of probit regression, according to the method reported by Locatelli et al. (2009). As previously observed for total phenolic content, methanolic extract obtained from MR hazelnut skin showed the lowest DPPH antiradical activity (EC_{50}: 10.11 μg mL^{-1}), in accordance with their incomplete solubility in the reaction solvent (methanol); so, antiradical activity of this sample has to considered underestimated. Ethanolic and methanolic extracts were characterised by higher scavenging properties for MR and HR samples, respectively. DPPH antiradical activity ranged from 3.67 to 10.11 μg mL^{-1}; MR and HR samples activity was not significantly different ($p > 0.05$), whilst acidified solvents extracts were less actives than corresponding not-acidified ones ($p < 0.05$, methanolic extract from MR hazelnut skin was not considered because of their incomplete solubility). Antiradical activity of hazelnut skin extracts was compared with that of antioxidant standard compounds (Table 3). Except for MR methanolic extract, all hazelnut skin extracts were at least 1.5-fold more active than BHA; HR methanolic extract was comparable to trolox (no significant difference was observed). Compared to the other standard molecules, hazelnut skin extracts appeared less effective to scavenge DPPH radical. Because of experimental differences among the DPPH methods reported in literature, it is difficult to compare our results with that obtained by other authors.

3.4.2. $ABTS^{\cdot+}$ scavenging activity

The basis of the method is to monitor the decay of the radical-cation $ABTS^{\cdot+}$ produced by the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) caused by the addition of antioxidants. Generally, results are expressed relative to trolox (trolox equivalents); however, on analogy to DPPH· assay, in this work results were expressed as EC_{50} values. For each extract and standard molecule at least nine concentrations were tested and at least three different experiments were performed. EC_{50} values, expressed as μg of extract per millilitre of solvent (Table 2), were calculated by linear regression analysis; linearity range between antioxidant concentration and antiradical activity ($ABTS^{\cdot+}$ inhibition percentage, *I*%) was verified for *I*% values upper than 90%. Hazelnut skin extracts were solved in ethanol; both methanolic and acidified methanolic extracts from medium-roasted sample were partially insoluble in the solvent (solid insoluble residue was higher for methanolic extract than for acidified methanolic one), so values obtained for these extracts should be considered underestimated. $ABTS^{\cdot+}$ scavenging activity (EC_{50} values) ranged from 655.15 to 317.24 μg mL^{-1}. Methanolic extracts obtained by Soxhlet apparatus (317.24 μg mL^{-1}) and cold-extraction under stirring (350.05 μg mL^{-1}) exhibited the highest ABTS antiradical properties for MR and HR hazelnut skin, respectively. As previously reported for DPPH· method, MR and HR samples activity was not significantly different ($p > 0.05$), whilst acidification of solvents led to a significant decrease of antiradical activity ($p < 0.01$, partially insoluble extracts were not considered in this analysis). Compared to standard compounds, MR hazelnut skin extracts showed $ABTS^{\cdot+}$ scavenging properties similar to epicatechin and trolox ($p > 0.05$, partially insoluble extracts not included). The study of Alasalvar et al. (2009) showed for acetonic and methanolic aqueous extracts of hazelnut skin 6.33 and 6.36 mmol of trolox equivalents per gram of crude extract, respectively. Shahidi et al. (2007) obtained for hazelnut skin ethanolic extract an ABTS radical anion ($ABTS^{\cdot-}$) scavenging activity equal to 132.0 mg of trolox equivalent per gram of extract (TEAC); at the same concentration the aqueous ethanolic (80% ethanol) extract of almond brown skin showed a 52.9 TEAC (Siriwardhana & Shahidi, 2002). Kamath and Rajini (2007) reported that ethanolic extract of cashew nut skin was equally potent as BHA in $ABTS^{\cdot+}$ scavenging assay (EC_{50} of cashew nut skin extract: 1.30 μg mL^{-1}).

3.4.3. Chelation activity

Metal-mediated formation of free radicals may cause various modifications to DNA bases, enhanced lipid peroxidation, and changes in calcium and sulphydryl homeostasis. Because of high reactivity, iron is one of most important lipid oxidation pro-oxidants, particularly in its ferrous state. So, the effective Fe^{2+} chelators may afford protection against oxidative damage by inhibiting production of ROS and lipid peroxidation (Liyana-Pathirana & Shahidi, 2007). In this work, the ferrous chelation activity of hazelnut skin extracts was evaluated using the ferrozine method. At least six different concentrations for each extract were tested and experiments were repeated at least in triplicate. EC_{50} values, expressed as mg of extract per millilitre of solvent, were calculated by linear regression analysis; linearity range between antioxidant concentration and chelation activity (expressed as percentage, CA%) was verified for CA% values up to 75–90%, depending on different samples analysed.

Table 4 shows the results obtained measuring the ferrous chelation activity of roasted hazelnut skin extracts. It is important to highlight that it was not possible to evaluate the activity of the samples extracted using acidified solvents (their absorbance values resulted higher than control and so not considered).

If compared to the antiradical activity, iron chelation capacity of hazelnut skin extracts were relatively weaker, ranging from

Table 3
Antioxidant activity of standard compounds.

Samples	Antiradical activity (EC_{50}; μg mL^{-1})		Chelation activity (EC_{50}; mg mL^{-1})	Inhibition lipid peroxidation (IP%; t = 96 h)	
	DPPH·	$ABTS^{\cdot+}$		100 μg mL^{-1}	1000 μg mL^{-1}
Trolox	3.32 (3.05–3.62)[a]	300 ± 9[a]	–	17.2 ± 3.2[a]	100.0 ± 0.7[a]
Gallic acid	1.03 (0.96–1.1)[b]	84 ± 5[b]	–	46.6 ± 0.5[a,b]	82.6 ± 0.4[b]
Caffeic acid	2.93 (2.73–3.14)[c]	214 ± 10[c]	–	29.4 ± 4.7[a,b]	78.7 ± 1.4[b]
BHA	8.18 (7.44–8.99)[d]	213 ± 13[c]	–	97.6 ± 0.8[c]	97.4 ± 0.3[a]
Quercetin	1.99 (1.77–2.23)[e]	206 ± 12[c]	5.07 ± 0.42	56.4 ± 2.5[b]	99.1 ± 1.7[a]
Epicatechin	3.11 (2.88–3.34)[a]	309 ± 21[a]	–	30.8 ± 1.9[a,b]	68.1 ± 4.8[c]

Results are expressed as mean ± SD ($n \geqslant 3$). In the case of DPPH· assay, the 95% confidence limits are reported. Means ± SD followed by the same letter, within a column, are not significantly different ($p > 0.05$).

Table 4
Ferrous ions chelation and inhibition of lipid peroxidation of hazelnut skin extracts.

Samples	Chelation activity (EC_{50}; mg mL^{-1})		Inhibition lipid peroxidation (IP%; 100 μg mL^{-1}, t = 96 h)		Inhibition lipid peroxidation (IP%; 1000 μg mL^{-1}, t = 96 h)	
	MR	HR	MR	HR	MR	HR
MeOH	1.01 ± 0.07[a]	2.51 ± 0.24[a]	43.7 ± 4.3[a]	67.5 ± 1.6[a]	77.9 ± 0.1[a,d]	90.9 ± 1.7[a,b]
MeOH/H^+	–	–	58.4 ± 0.7[b]	65.5 ± 1.3[a,b]	90.7 ± 2.9[b]	93.0 ± 3.8[a]
EtOH	4.45 ± 0.09[b]	3.41 ± 0.29[b]	44.0 ± 2.4[a]	49.6 ± 0.1[c]	87.1 ± 0.8[b,c]	88.6 ± 3.1[b]
EtOH/H^+	–	–	51.3 ± 2.0[a,b]	60.0 ± 1.0[d]	83.6 ± 3.0[a,b,c]	91.4 ± 3.3[a,b]
Ac_2O/H_2O	4.93 ± 0.33[b]	3.30 ± 0.23[b]	55.9 ± 5.1[a,b]	61.7 ± 0.6[b,d]	82.5 ± 3.9[a,c]	89.6 ± 4.5[b]
MeOHsox	3.32 ± 0.22[c]	2.54 ± 0.15[a]	46.0 ± 3.2[a,b]	60.5 ± 1.4[d]	71.1 ± 0.9[d]	81.8 ± 0.9[c]

Results are expressed as mean ± SD ($n \geqslant 3$). Mean ± SD followed by the same letter, within a column, are not significantly different ($p > 0.05$).

4.93 mg mL^{-1} (MR aqueous acetonic extract) to 1.01 mg mL^{-1} (MR methanolic extract) EC_{50} values. For both MR and HR hazelnut skin, methanol appeared the best extraction solvent. Moreover, in contrast to its reduced solubility, methanolic extract of MR hazelnut skin showed the highest iron chelation activity (1.01 mg mL^{-1}); it would seem that insoluble fraction of this extract (presumably polymeric compounds) has a negative influence on the chelation properties of total extract. Altogether, chelation activity obtained for MR and HR samples was significantly different ($p < 0.001$), indicating higher chelation properties for HR extracts. Amongst standard compounds, only quercetin revealed the capacity to bind Fe^{2+} (EC_{50}: 5.07 mg mL^{-1}), showing a chelation activity comparable to MR ethanolic and acetonic extracts ($p > 0.05$); all the other molecules were ineffective at the concentration of 7 mg mL^{-1}. Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) was also tested as positive control, showing the highest chelating power (EC_{50}: 8.36 μg mL^{-1}). Our results are in agreement with literature; Kamath and Rajini (2007) reported for EDTA higher chelation activity than cashew nut skin extract (EC_{50}: 6.00 mg mL^{-1}). Liyana-Pathirana and Shahidi (2005) revealed for 100 ppm EDTA solution a complete chelation activity, whilst observed that trolox did not chelate ferrous ions at all. Using the tetramethylmurexide method, Wijeratne et al. (2006) obtained a 96% chelation of ferrous ions for both quercetin (100 ppm) and almond skin extract (100 ppm quercetin equivalents).

3.4.4. Inhibition of lipid peroxidation

Lipid oxidative damage has been recognised of fundamental importance because of its numerous biological and nutritional implications (deterioration of flavour and aroma of food, decay of nutritional and safety qualities, cellular damage related to carcinogenesis, premature aging and other diseases) (Kanner & Rosenthal, 1992). Measurement of lipid hydroperoxides is an essential part of understanding lipid oxidation processes. A convenient spectrophotometric method to measure lipid hydroperoxides is the ferric-thiocyanate method. Hazelnut skin extracts and standard compounds were assayed at three different concentrations (10, 100 and 1000 μg mL^{-1}) and their protective effect against hydroperoxide generation was monitored during oxidation at 50 °C (24, 48, 72 and 96 h). At concentration of 10 μg mL^{-1} hazelnut skin extracts did not show significant lipid peroxidation inhibition (data not showed). In some cases, pro-oxidant activity was observed; however, other and more specific studies should to be conduced in order to evaluate the real pro-oxidant properties of the extracts. Samples tested at concentrations 100 and 1000 μg mL^{-1} reduced the ferric-thiocyanate complexes formation, showing absorbance values significantly lower than control (Fig. 1). Antioxidant activity, expressed as inhibition of lipid peroxidation percentage (IP%), increased in a dose-dependent manner and depending on the oxidation time. In a general way, we observed that inhibition of lipid peroxidation increased with oxidation time.

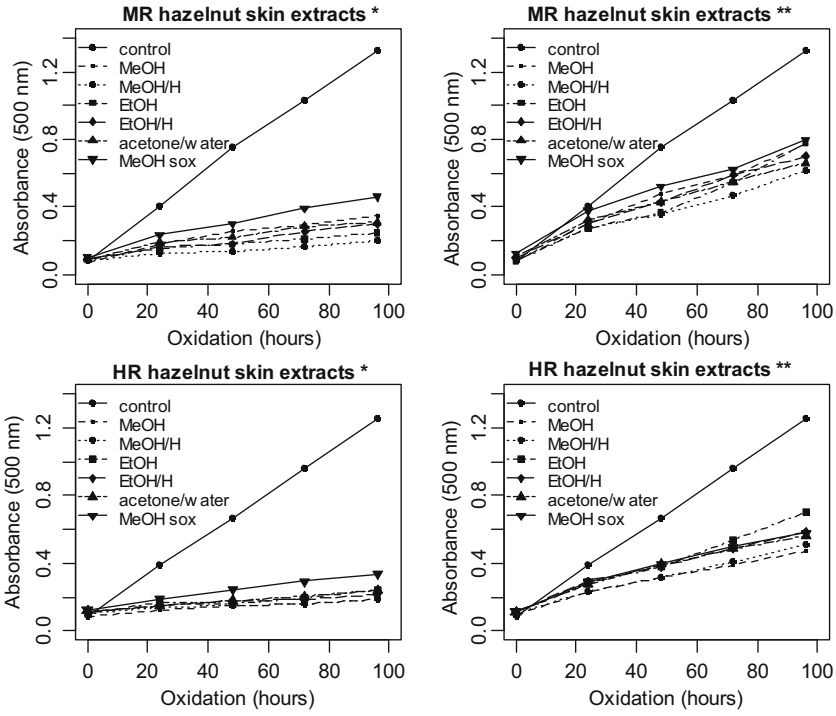

Fig. 1. Inhibition of lipid peroxidation (FTC method) of hazelnut skin extracts during oxidation at 50 °C. * Concentration 1000 μg mL^{-1}. ** Concentration 100 μg mL^{-1}.

The IP% values obtained for MR and HR extracts (at concentrations 100 and 1000 μg mL^{-1}, after 96 h of thermal treatment) are reported in Table 4. Also in this case, antioxidant activity of MR hazelnut skin methanolic extract has to be considered underestimated. As previously observed for chelation activity, HR samples showed significantly higher antioxidant properties than MR ones ($p < 0.0001$, for both 100 and 1000 μg mL^{-1}). Acidification of extraction solvent showed a significant and positive influence on IP% values only considering the concentration 100 μg mL^{-1} ($p < 0.05$); at 1000 μg mL^{-1} no significant differences were observed between acidified solvents and not-acidified ones. These data indicate that the choice of sample concentrations is fundamental in antioxidant activity studies, since different results could be obtained. Antioxidant activity determined for hazelnut skin extracts were not significantly different from that obtained for standard molecules (Table 3).

3.5. Correlation between total phenolic content and antioxidant activity of hazelnut skin extracts and comparison among antioxidant methods

The extent to which the antioxidant potential of hazelnut skin extracts is accounted for by their phenolic content was assessed. The degree of linear association between both radical-scavenging activity (DPPH· and ABTS·$^+$ assays) and inhibition of lipid peroxidation (1000 μg mL^{-1} after 96-h oxidation), and total phenolic content was determined by means of correlation analysis; Pearson's product moment correlation coefficients (r) and significance levels (p) were calculated (Fig. 2). Correlation between chelation activity and polyphenols content was not considered in this section because of the lack of results from chelation activity assay. As depicted in Fig. 2, a significantly strong correlation with polyphenols content was observed for antiradical activity, showing correlation coefficients $r = -0.8798$ and -0.8285 for DPPH· and ABTS·$^+$ assays, respectively. Negative r values were obtained in that higher antiradical activity corresponded to lower EC_{50} values. Many studies have reported a highly correlation between total phenol content and antiradical activity in various food and plant species (Wojdyło, Oszmiański, & Laskowski, 2008). On the contrary, no correlation was observed between total phenolic content and inhibition of lipid peroxidation. This fact may be due to the specific molecular structure of phenolics involved in this specific action. Furthermore, the total phenolic content determined according to the Folin-Ciocalteu method is not an "absolute" measurement of the amount of phenolic materials and various phenolic compounds show different responses in this assay (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1999). Conversely, phenol content is not the unique factor influencing the total antioxidant activity of the samples and synergistic effects of phenolics with other components, for example melanoidinic compounds formed during roasting, should also be taken into account.

Comparing the different antioxidant methods used in this work, DPPH· assay was the most sensitive, because required lower sample concentrations than other methods. Considering ABTS·$^+$ and DPPH· methods, DPPH· is likely more selective than ABTS·$^+$ in the reaction with H-donors; nevertheless, in contrast to DPPH·, ABTS radicals are able to react with flavonoids which contain no OH-groups in B-ring as well as with aromatic acids containing only

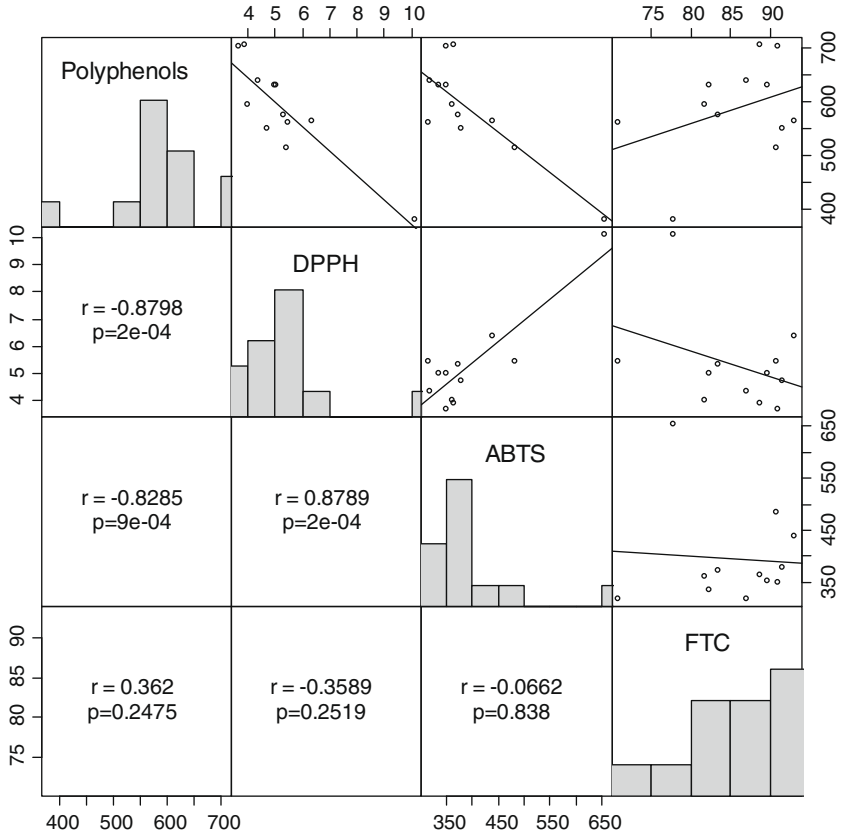

Fig. 2. Correlation of antioxidant activity with total phenolic content and among antioxidant assays (DPPH, ABTS and FTC methods). The upper diagonal part of the figure shows scatterplot and linear regression lines; the diagonal part represents the histogram of frequencies; in the lower diagonal part are reported Pearson's correlation coefficient (r) and the probability (p) values. Correlations were considered statistically significant for $p < 0.05$. Polyphenols content is expressed as CE (mg g^{-1}); antiradical activity against DPPH and ABTS is expressed as EC_{50} (μg mL^{-1}); inhibition of lipid peroxidation is expressed as percentage, determined for concentration 1000 μg mL^{-1}, after 96-h oxidation.

one OH-group (Roginsky & Lissi, 2005). However, it should be considered that sample concentrations are strongly dependent from the specific conditions and the physical composition of test systems.

A significant correlation was observed between radical-scavenging activity methods (DPPH· and $ABTS^{\cdot+}$ assays, $r = 0.8789$), whilst no correlation was observed with FTC method. These results would seem to suggest that inhibition of lipid peroxidation (FTC method) involve a different antioxidant mechanism from radical-scavenging activity. All these observations confirm the necessity to perform more than a single test to determine the total antioxidant activity of complex extracts.

3.6. Direct measurement of total antioxidant capacity (QUENCHER approach)

Finally, the total antioxidant activity of MR and HR hazelnut skin was determined employing a direct procedure, namely the QUENCHER approach (Gökmen, Serpen, & Fogliano, 2009; Serpen et al., 2008). The method was applied to defatted hazelnut skin powders and the results were expressed as mol of trolox equivalents (TE) per kg of sample. $ABTS^{\cdot+}$ scavenging activity was measured during different times (from 6 to 60 min) in order to determine the time needed to reach the steady state. Even if in the case of trolox the reaction was completed within 6 min, the $ABTS^{\cdot+}$ scavenging curves of hazelnut skin reached the plateau in 30 min. So, the antioxidant activity of both hazelnut skin samples and trolox was measured after exactly 30 min of reaction. Results were 1.10 ± 0.01 and 0.94 ± 0.06 mol TE/kg for MR and HR defatted hazelnut skin, respectively.

As previously suggested in literature, many food items have insoluble components that may exert an antioxidant activity. Common procedures to enhance the solubility of insoluble fractions (for example, esterified phenolic acids) and determine their contribute to antioxidant activity is to perform alkali, acid or enzymatic treatments (usually, hydrolysis using NaOH). However, severe chemical hydrolysis can alter the food structure and the resulting extracts are no longer representative of the real antioxidant capacity that the food could have (Gökmen, Serpen, & Fogliano, 2009). In addition, the use of sequential extractions and alkaline hydrolysis procedure to evaluate the total antioxidant capacity in cereal products allowed to results comparable or, in some cases, lower than those obtained using a direct measurement approach (Serpen et al., 2008). Compared to data previously reported in literature, hazelnut skin was more active than several cereal products, for whom were obtained values lower than 120 mmol TE/kg (Serpen et al., 2008). These results confirm very high antioxidant properties for hazelnut skin. Moreover, in contrast to the results previously obtained for hazelnut skin extracts ($ABTS^{\cdot+}$ method), antioxidant activity measured for MR hazelnut skin was significantly higher than that obtained for HR one ($p < 0.05$). This fact could indicate that insoluble fraction of hazelnut skin is more relevant for antioxidant activity of MR sample.

4. Conclusions

All the methods employed in this work demonstrated significant antioxidant properties for hazelnut skin extracts; however, the main mechanism involved appeared the antiradical activity. Particularly, considering the DPPH radical scavenging the concentrations employed were one order of magnitude less than the chelation activity, indicating higher antiradical properties. The acidification of extraction solvents led to a significant decrease of antiradical activity (both DPPH· and $ABTS^{\cdot+}$ assays), whilst the different roasting conditions appeared significantly influence the chelation activity and the inhibition of lipid peroxidation, showing higher effectiveness for high-roasted hazelnut skin extracts. Conversely, results obtained by direct measure of the antioxidant activity of defatted hazelnut skins (QUENCHER method) revealed higher $ABTS^{\cdot+}$ scavenging properties for medium-roasted sample. Concluding, roasted hazelnut skin can be considered a low-cost natural source of antioxidants; however, being the overall activity of the extracts dependent on both extraction solvents and the roasting degree, the identification and quantification of phenolic compounds (flavonoids, phenolic acids, and proanthocyanidins), as well as melanoidins formed during roasting, should have to be performed.

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