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Increasing espresso coffee brew antioxidant capacity using phenolic extract recovered from hazelnut skin waste

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ABSTRACT

The simultaneous consumption of different classes of phytochemical antioxidants in the diet can result in more beneficial effects than when consumed alone. In the present study, the *in vitro* and *in vivo* antioxidative effects of espresso coffee brew (EC) (rich in chlorogenic acids) with added crude hazelnut skin phenolic extract (HSPE) from hazelnut skin waste (rich in flavonoids) were studied. Both post-brewing and pre-brewing phenolic-enriched espresso coffees (PE-ECs) were analysed for total phenols and screened for their *in vitro* antiradical ability. Moreover, the *in vivo* biological effect on the antioxidant potential of plasma in rats was evaluated. The PE-ECs showed increased both *in vitro* and *in vivo* antiradical activity proportional to the added HSPE. The *in vivo* experiments suggested that HSPE was much more antioxidant active than the phenolic fraction naturally contained in EC. Moreover, evidence of possible synergic effects of EC and HSPE phenolics was observed *in vivo*.

1. Introduction

Food antioxidants contribute to the strengthening of endogenous defences in the body against oxidative stress, which is responsible for many chronic degenerative alterations, such as cardiovascular, rheumatoid and neurodegenerative diseases as well as osteoporosis, type 2 diabetes, hypertension, and cancer (Valko et al., 2007). Numerous experimental studies have shown the beneficial effects of plant phenolics in maintaining health and preventing diseases (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). For these reasons, medicine experts and food manufactures are showing increasing interest towards developing functional foods enriched with natural phenolic antioxidants which could improve both nutritional status and humans health.

Several thousands of natural phenolic compounds have been identified, although a more limited number are at significant levels in most human diets. The antioxidant properties of these phytochemicals are strongly dependent on their chemical structures (Tucker & Robards, 2008), according to which they are subdivided into several main categories: phenolic acids, flavonoids, stilbenes, and lignans. The chemical

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Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DPPH*, 2,2-diphenyl-1-picrylhydrazyl radical; EC, espresso coffee; IC_{50}, inhibitory concentration; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; HSPE, hazelnut skin phenolic extract; PE-EC, phenolic-enriched espresso coffee; TPTZ, 2,4,6-tripyridyl-S-triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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structure of phenolics also affects their biological properties, such as bioavailability, antioxidant bioactivity and interactions with basic cellular activities.

Recent studies have shown that hazelnut and its wastes are a rich source of phenolic compounds (Alasalvar, Karamać, Amarowicz, & Shahidi, 2006). In particular, it was demonstrated that the hazelnut skin by-product is exceptionally concentrated in phenolics, which are characterized by multiple and efficient *in vitro* antioxidant properties as follows: 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), hydrogen peroxide and superoxide radical scavenging activity; reducing and chelating power; the ability to inhibit oxidation of human low-density lipoprotein (LDL) cholesterol; and the capacity to reduce DNA damage induced by hydroxyl radical (Contini, Baccelloni, Massantini, & Anelli, 2008; Shahidi, Alasalvar, & Liyana-Pathirana, 2007; Alasalvar et al., 2009; Contini et al., 2009). Moreover, *in vivo* tests have demonstrated that crude hazelnut skin phenolic extract (HSPE) is biologically active in rats (Contini et al., 2009). Hazelnut phenolics have recently been characterized and constituted mainly by flavonoids, especially flavanols, potent antioxidants whose beneficial action on human health promotion and disease risk reduction, especially the risk of cardiovascular disease, is supported by numerous studies (Erdman et al., 2007; Hackman et al., 2008; Wang, Melnyk, Tsao, & Marcone, 2011). High amounts of monomeric (mainly (+)catechin), dimeric and polymeric flavanols (proanthocyanidins) have been found in roasted hazelnut skins (Monagas et al., 2009). Moreover, minor flavonoids, such as myricetin, kaempferol and quercetin (Coisson et al., 2002), in addition to some phenolic acids (mainly gallic acid) have also been found in roasted hazelnut skins (Shahidi et al., 2007). Therefore, HSPE may be a valuable and convenient source of natural dietetic flavonoids. To date, however, no scientific study has been developed with the aim of evaluating the use of HSPE as a food antioxidant.

Coffee is the third most widely consumed beverage in the world, after water and tea (Villanueva et al., 2006). Its consumption has been frequently associated with an increase in serum cholesterol, blood pressure and cardiac rhythm, with adverse effects on cardiovascular outcome. However, data on the detrimental effects of coffee are controversial and it has been suggested that a moderate consumption (4–5 cups/day) of espresso coffee (EC) is not related with increased risks of cardiovascular diseases or hypertension (Ranheim & Halvorsen, 2005; Viola, 2005). In contrast, recent reports have demonstrated that coffee intake has beneficial effects, which are mediated by the antioxidant fraction in the beverage, on cardiovascular and type 2 diabetes mellitus risks (Ranheim & Halvorsen, 2005).

Coffee has been reported to be a better contributor towards human antioxidant defense than fruits, vegetables and wine (Svilaas et al., 2004) and the major source of dietetic phenolic antioxidants in several countries, such as Denmark, USA, Brazil and Japan (Farah, 2009). As a result, coffee could represent an excellent food-base for the preparation of functional beverages rich in phenolic antioxidants.

The antioxidant fraction of coffee brews is mainly composed of chlorogenic acids (with 5-caffeoylquinic acid being by far the most abundant) (Wang & Ho, 2009), powerful antioxidants (Daglia et al., 2004) to which several positive physiological actions have been attributed both *in vitro* and *in vivo*, such as chemopreventive anticancer properties (Belkaid, Currie, Desgagnés, & Annabi, 2006). Other antioxidants are Maillard reaction products formed during coffee roasting, whose relative contribution to the overall radical scavenging activity of the brew was much lower (Sacchetti, Di Mattia, Pittia, & Mastrocola, 2009). In contrast, coffee brews lack flavonoids. No detectable flavonols, flavones (Lakenbrink, Lapczynski, Maiwald, & Engelhardt, 2000) or proanthocyanidins (Hellström, Törrönen, & Mattila, 2009) have been found in coffee brews.

Chocolate rich in proanthocyanidins, tea integrated with catechins or coffee brew enriched with chlorogenic acids, are examples of traditional foods/beverages available on the market which serve as a good source of phenolic antioxidants. However, ideally it would be more opportune to develop functional foods having structurally different classes of phenolics, rather than enriching a food with a surplus of its own. In fact, several studies have suggested that a network of chemically different antioxidants may be convenient for offering wide-ranging protection against oxidative stress (Schafer & Buettner, 2001) or cancer development because cooperative actions within a pool of antioxidants can be more efficient than single components (Onyeneho & Hettiarachchy, 1992).

The interest of nutritionists on the potential synergistic actions or the complementary physiological activities exercised by the combination of strategically selected specific foods or food-derived phytochemicals is expanding (Kelloff et al., 2000; Wang et al., 2011). The present research was aimed at giving a suggestion to the food industry for developing a novel coffee-based functional beverage, provided with very high antioxidant potential. In this context, the effects of EC, which is rich in chlorogenic acids, when added with HSPE, which is rich in flavonoids, were evaluated. Two alternative methods for HSPE integration were investigated as follows: direct HSPE addition to a conventionally prepared EC (post-brewing integration) or mixing HSPE with the ground coffee used for preparing EC (pre-brewing integration). Total phenolic content of the phenolic-enriched espresso coffee brews (PE-ECs) was evaluated. Moreover, the antioxidant potential of the brews was screened both *in vitro* (scavenging ability against the DPPH radical) and *in vivo* (capability for improving the antioxidant potential of plasma in rats after oral intake).

2. Materials and methods

2.1. Chemicals

Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, butylated hydroxyanisole (BHA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and ferric-2,4,6-tripyridyl-S-triazine complex ($TPTZ\text{-}Fe^{3+}$), were purchased from Sigma–Aldrich (St. Louis, MO). Folin–Ciocalteau reagent, butylated hydroxytoluene (BHT) and α-tocopherol were purchased from Fluka Co. (Buchs, Switzerland). All the other chemicals and solvents were of the highest commercial level and were used without further purification.

2.2. Samples

Commercial ready-to-use coffee pods that were singularly sealed into a protective wrapper under nitrogen for use in an automatic espresso machine (8.0 ± 0.1 g of ground coffee) were supplied by AromEspresso (Roma, Italy). The coffee blend consisted in a mixture of *Coffea arabica* (60%) and *Coffea canefora* var. *robusta* (40%).

The hazelnut skin waste employed as raw material for obtaining HSPE consisted of the residue resulting from industrial pericarp removal from roasted kernels. This residue was kindly supplied by an Italian hazelnut processing industry (Stelliferi & 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. HSPE preparation

After manual removal of all kernel residue fragments, the hazelnut skin waste was milled and defatted for 8 h with hexane in a Soxhlet apparatus. HSPE was obtained by maceration at room temperature using 80% (v/v) aqueous ethanol at a solid/solvent ratio of 1:10 (w/v). After 20 h of extraction, the suspension was filtered through Whatman No. 1 filter paper, and the residual cake was repeatedly washed with 80% ethanol. The solvent was removed under vacuum at 40 °C and subsequent freeze-drying. The resulting crude HSPE appeared as a brownish fine powder that was easily soluble in alcohol but only partially soluble in water. When mixed with ground coffee, it results undistinguishable.

2.4. Assessment of HSPE heat stability

To assess the heat stability of HSPE, boiling water was added to the HSPE, and the temperature was maintained for 150 s in a thermostatic bath. The sample was then cooled on ice to 20 °C prior to analysis. The heating time of 150 s was considered as the summation of brewing time (30 s) and a brief standing time (2 min) before drinking, even though EC is ideally consumed immediately after preparation.

2.5. EC and PE-EC samples

The EC and PE-EC samples (serving size of 25 ml) were prepared using an automatic espresso machine (model Carezza, Gaggia SpA, Italy). The water pump pressure was 15 bars, and the extraction temperature was approximately 90 °C.

In the HSPE post-brewing integration studies (Trial 1), increasing amounts of HSPE were mixed with freshly prepared EC samples. In the HSPE pre-brewing integration studies (Trial 2), a series of PE-ECs were prepared using ground coffee (removed from a pod) mixed with a known amount of HSPE. Natural mineral water was used for brewing. The brews were cooled to room temperature in an ice bath and then transferred into a 50-ml volumetric flask where distilled water was added up to volume. For the *in vivo* tests in rats, no further dilutions were performed. To evaluate the total phenols and *in vitro* antiradical activity (DPPH assay), further dilutions in methanol were performed.

2.6. Total phenols

The HSPE and EC total phenol contents were determined by the Folin–Ciocalteau method as previously described (Contini et al., 2008). Gallic acid was used as a reference, and a five-point calibration curve was constructed with 3–25 mg of the standard (R^2 = 0.9997). The results were expressed as mg gallic acid equivalents (GAE) per HSPE weight (1 g or mg) or one serving (25 ml) of EC.

2.7. Antioxidant activity (DPPH assay)

The radical scavenging ability of the extracts was monitored using the stable DPPH free radical following the method described by Brand-Williams, Cuvelier, and Berset (1995) with slight modifications (Contini et al., 2008). At least five different concentrations were tested for each sample. BHA, BHT, Trolox and α-tocopherol were used as reference antioxidants. The inhibitory concentration (IC_{50}) value is the amount of sample (HSPE, EC, PE-EC or reference antioxidant) necessary to decrease the initial DPPH concentration by 50%, and it was obtained by plotting the percentage of remaining DPPH as a function of sample concentration. The results were expressed as Trolox equivalents. A Trolox equivalent is defined as the amount of Trolox (g or mg) giving the same antioxidant capacity (50% DPPH radical scavenging) as 1 g (or mg) of dry sample (HSPE or reference antioxidant) or one serving (25 ml) of brew (EC or PE-EC). The Trolox equivalents were calculated as the ratio between the IC_{50} of Trolox and the IC_{50} of the sample.

2.8. Groups of rats

A total of 21 male Sprague–Dawley rats (Charles River, Milano, Italy) were used (body weights of 140 ± 10 g) in this study. The rats were individually housed in wire bottom stainless steel cages in a temperature- and light-controlled room (22 ± 1 °C; 12 h light/dark cycle), and they were fed with a commercial laboratory diet and allowed food and water ad libitum.

Rats were randomly divided into seven equal groups with three rats per group. In group 1 (control group), each animal received only basic food as a vehicle. In group 2, the rats received EC (tq). In groups 3, 4 and 5, the rats received PE-EC fortified with the pre-brewing addition of HSPE (180, 270 and 360 mg, respectively). In groups 6 and 7, the rats received PE-EC fortified with the post-brewing addition of HSPE (120 and 180 mg, respectively). In all groups, 1 ml of 1:2 diluted EC or PEC-EC samples were mixed with basic food. Three hours after consumption, the rats were anesthetised and sacrificed, and blood was drawn from the heart. Plasma was obtained by centrifugation at 1000*g* for 10 min at 4 °C.

The care and use of the rats were approved by the Animal Care and Ethics Committee of Tuscia University (Viterbo, Italy).

2.9. Antioxidant potential of rat plasma (FRAP assay)

The ferric reducing antioxidant power (FRAP) assay was performed by measuring the ability of plasma samples to reduce the colourless TPTZ-Fe^{3+} to its ferrous coloured form (TPTZ-Fe^{2+}) (Benzie & Strain, 1999). The results were expressed as μmol Fe^{2+}/l.

2.10. Statistical analysis

All measurements were carried out at least in triplicate ($n = 3$) and the results were reported as the means ± SD. The significance between means was determined by one-way variance analysis (ANOVA) followed by Duncan post-hoc test, using the SPSS 15.0 for windows package. Microsoft Excel was used to calculate the correlation curves.

3. Results and discussion

3.1. HSPE and EC characteristics

The extraction yield of HSPE was nearly 27% defatted hazelnut skin, and the phenolic content of the extract was 680.3 mg GAE/g. This phenolic value was in accordance with previous studies that reported HSPE phenolic concentration varying between 577.7 mg/g catechin equivalents (Shahidi et al., 2007) and 743.5 mg/g GAE (Contini et al., 2009).

Two batches of ground coffee were employed for the post-brewing (Trial 1) and pre-brewing (Trial 2) HSPE addition studies with EC (control) phenolic contents of 290.2 and 288.6 mg GAE/serving, respectively (differences were not statistically significant; $p \leqslant 0.01$). These contents were higher than those observed by Sánchez-González, Jimenéz-Escrig, and Saura-Calixto (2005), which in EC reported values varying between 150 and 220 mg GAE/serving. Table 1 summarises the HSPE and EC characteristics employed in the present study.

The antioxidant efficiency of HSPE and EC with respect to selected reference antioxidants is shown in Fig. 1. The HSPE had a better antiradical power against the DPPH radical compared to most common natural (α-tocopherol) and synthetic (BHA, BHT) antioxidants. In particular, the HSPE antiradical efficiency was approximately twofold higher than the antiradical efficiency of BHT and over three times more than the antiradical efficiency of α-tocopherol for the same weight. These results agreed with previously reported results (Contini et al., 2008). Regarding EC, one serving of the brew obtained under these experimental conditions had antiradical power close to that of 1 g of pure α-tocopherol (Fig. 1). The high phenolic content and the elevated antioxidant (antiradical) activity we measured in EC confirmed our conviction that this brew is an optimal base for preparing functional beverages very rich in phenolic antioxidants.

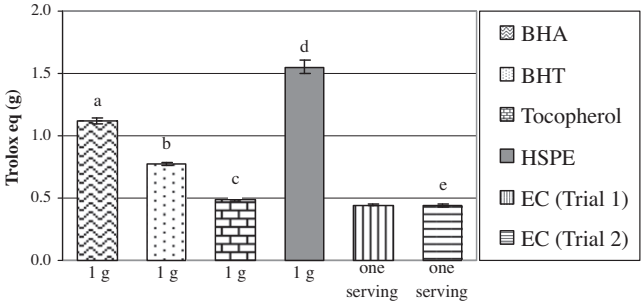

Fig. 1 – Antiradical efficiency of HSPE and EC with respect to reference antioxidants. HSPE, hazelnut skin phenolic extract; EC, espresso coffee. Samples with different letters are significantly different ($p \leqslant 0.01$).

EC is typically brewed with water at an approximate temperature of 90–95 °C, and it is consumed extremely hot immediately after the brewing. Consequently, an important prerequisite to evaluate was the low lability of the HSPE phenolic fraction to heat. No statistically significant difference ($p \leqslant 0.01$) was found in the HSPE phenolic content and antiradical activity (DPPH test) after maintaining the extract in boiling water for 150 min (data not shown). This result was not predictable, because if it is true that the waste for the HSPE production already underwent a long heat treatment (135–170 °C for 15–40 min) in rather dry conditions (approximate initial hazelnut humidity of 4–5%), the effects of heat in the presence of water may have induced new hydrolytic and/or oxidative chemical reactions, which may have modified the phenolic profiles and/or properties. The results in this study suggested that no apparent chemical changes in phenolic compounds or their antioxidant characteristics occurred during brewing, when the HSPE was in the hot water for the time necessary to prepare and drink a cup of coffee. This outcome strengthened our confidence that HPSE phenolics might be suitably used in combination with coffee phenolics for obtaining brews rich in phenolic antioxidants.

3.2. Effects of HSPE addition to EC

The addition of HSPE (that is a crude phenolic concentrate constituted prevalently by flavonoids) to a complex matrix such as EC (whose phenolic fraction is essentially constituted by chlorogenic acids), could lead to different matrix effects. If

Table 1 – Total phenols and antiradical activity of HSPE and EC samples.

	Total phenols (mg GAE)	Antiradical activity (mg Trolox eq)	mg Trolox eq/mg GAE
HSPE (1 g)	680.3 ± 11.6	1549.9 ± 54.7	2.28
EC1[a] (1 serving)	290.2 ± 2.3	445.0 ± 8.0	1.53
EC2[a] (1 serving)	288.6 ± 4.3	442.2 ± 11.6	1.53

HSPE, hazelnut skin phenolic extract; EC, espresso coffee; GAE, gallic acid equivalents.

[a] EC1 and EC2 represent the espresso coffees employed in Trial 1 and Trial 2, respectively (the differences between the two samples were not statistically significant; $p \leqslant 0.05$).

no interactions between components occur, simply additive results should be obtained, both in the assessment of total phenol concentration and in the evaluation of their antioxidant activity. Alternatively, we could observe diminishment or enhancement with respect to the theoretical additive results, if inhibitive or cooperative/synergic effects occur, respectively.

3.2.1. *Post-brewing HSPE addition to EC*

To verify the feasibility of obtaining PE-EC by simply adding HSPE to a conventionally prepared EC, we tried to dissolve known amounts of HSPE by depositing it on the bottom or on the top of a freshly prepared EC. However, an incomplete and quantitatively variable amount of the HSPE was transferred into the liquid portion of the brew after mixing. A part of the undissolved HSPE adhered to the spoon or to the walls of the cups, and a portion was entrapped within the characteristic creamy foam on the surface of the brew. Therefore, the post-brewing experiments were continued by employing water-dispersed HSPE, which was added in a known amount to freshly prepared EC. The aim of this experiment was to show eventual interactions and effects amongst water solubilised/dispersed components of EC and HSPE, in particular so as to exclude eventual pro-oxidative properties of the mixtures, which were concentrated in phenolics. It is known, in fact, that some antioxidants such as β-carotene can exhibit a concentration-dependent inversion of antioxidant and pro-oxidant effects (Lankin, Tikhaze, Konovalova, & Kozachenko, 1999).

The amount of HSPE added to the EC varied from 48.2 to 481.6 mg/serving, corresponding to a range of 32.8–327.6 mg of hazelnut phenolics (expressed in GAE). The total phenols in the PE-EC samples increased (Table 2), and a high correlation ($R^2 = 0.9986$) existed between the added hazelnut phenolics and the increase in measured phenolics in the brews (Fig. 2A). The correlation curve was close to the theoretical additive curve. Moreover, the ratio of the detected phenolic increase to added HSPE phenolics was close to one (Table 2). These results demonstrated that the addition of hazelnut phenolics to EC yielded simple additive effects.

The increase in total phenolics was accompanied by improved antiradical activity (Table 2). Moreover, a good correlation ($R^2 = 0.9988$) was observed between the added hazelnut Trolox eq and PE-EC Trolox eq increase (Fig. 2B). These results showed that hazelnut phenolics improved the antiradical activity of the beverage when they were added to the EC. However, a reduction in the antiradical efficiency of the added hazelnut phenolics was found. In fact: (a) the correlation curve in Fig. 2B was not parallel to the putative curve; (b) the mean value of the ratio of Trolox eq increase to added Trolox eq was not close to one, but 0.75 (Table 2); and (c) each milligram of GAE of the HSPE phenolics resulted in an antiradical efficiency value equal to 2.28 mg Trolox eq (Table 1), whereas the mean antiradical efficiency for each unit of phenolic increase in the PE-EC samples was significantly lower ($p \leqslant 0.01$), with a value equal to 1.72 mg Trolox eq (Table 2). These findings indicated that HSPE phenolics, when added to EC, did not fully realise their antioxidant action against DPPH radicals.

There was no evidence of pro-oxidant effects in the PE-EC samples with HSPE phenolic additions of up to 327.6 mg.

3.2.2. *Pre-brewing HSPE addition to EC*

During classical EC preparation, only part of the compounds present in the ground coffee is transferred into the brew. We can consider the results of the post-brewing HSPE integration as representative of the theoretical maximal (i.e., total) transfer of hazelnut phenolics in the coffee brew if no loss of active components remaining in the exhausted coffee cake took place. Clearly, this condition does occur in reality.

To evaluate the proportion of hazelnut phenolics transferred into the cup during the brewing process and to further assess the antiradical property of the PE-EC brews, we analysed a series of fortified samples prepared by charging the

Table 2 – Effect of increasing amounts of HSPE added to EC (post-brewing addition) on the phenolic content and antiradical activity of the brews.

HSPE added to EC	Phenolics/serving				Antiradical activity/serving				mg Trolox eq /mg GAE[e]
	Added (Pa)[a]	Found (Pf)	Increase (Pi)[b]	Pi/Pa	Added (Ta)[c]	Found (Tf)	Increase (Ti)[d]	Ti/Ta	
mg	mg GAE	mg GAE	mg GAE		mg Trolox eq	mg Trolox eq	mg Trolox eq		
48.2	32.8	322.2 ± 2.3	32.1	0.98	74.6	502.1 ± 2.5	57.1	0.76	1.78
79.0	53.7	342.1 ± 4.8	51.9	0.97	122.4	537.5 ± 2.4	92.5	0.76	1.78
111.7	76.0	366.5 ± 3.0	76.3	1.00	173.2	569.7 ± 3.3	124.7	0.72	1.63
149.3	101.6	395.7 ± 9.9	105.5	1.04	231.4	617.9 ± 0.8	172.9	0.75	1.64
192.6	131.1	422.4 ± 8.0	132.2	1.01	298.6	662.0 ± 12.9	217.0	0.73	1.64
240.8	163.8	449.5 ± 11.7	159.3	0.97	373.2	733.9 ± 4.8	288.9	0.77	1.81
481.6	327.6	600.7 ± 12.5	310.5	0.95	746.4	994.6 ± 9.6	549.6	0.74	1.77
Mean				0.99 ± 0.03				0.75 ± 0.02	1.72 ± 0.08

HSPE, hazelnut skin phenolic extract; EC, espresso coffee; GAE, gallic acid equivalents.

[a] Pa = HSPE phenolics (mg GAE/mg) × added HSPE (mg).

[b] Pi = Pf – espresso coffee (tq) phenolics.

[c] Ta = HSPE Trolox eq (mg Trolox eq/mg) × added HSPE (mg).

[d] Ti = Tf – espresso coffee (tq) Trolox eq.

[e] Ratio of Ti to Pi.

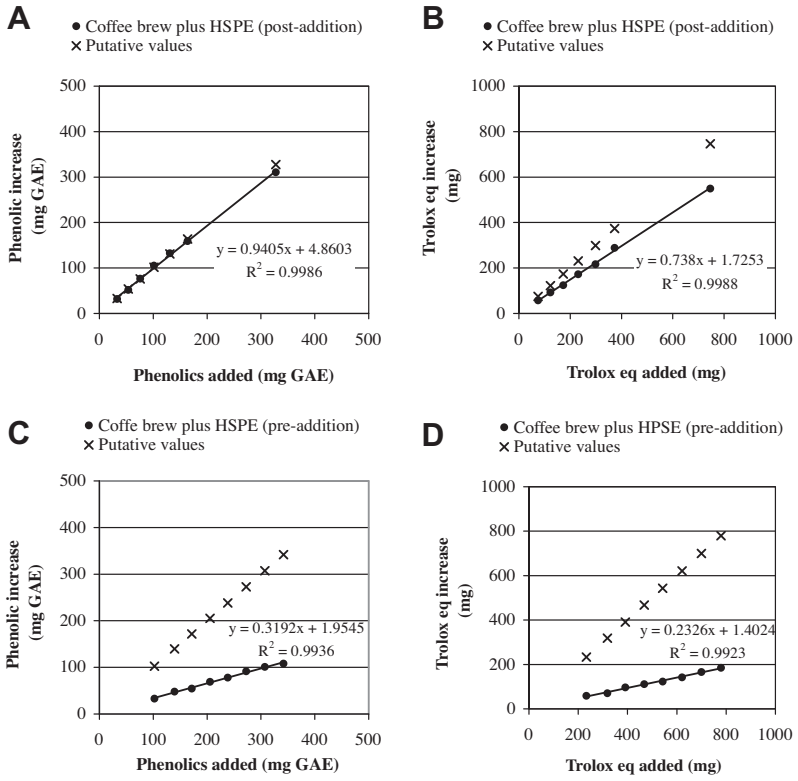

Fig. 2 – Effect of increasing amounts of HSPE added to EC. (A and B) post-brewing addition: regression curve between added hazelnut phenolics and the increase in measured phenolics (A); regression curve between added Trolox eq and the increase in measured Trolox eq. (B). (C and D) pre-brewing addition: regression curve between added hazelnut phenolics and the increase in measured phenolics (C); regression curve between added Trolox eq and the increase in measured Trolox eq. (D). HSPE, hazelnut skin phenolic extract; EC, espresso coffee; GAE, gallic acid equivalents. The crosses indicate the theoretical values if all added phenolics/Trolox eq were to be found in the brews.

espresso machine filter with 8 g of ground coffee accurately mixed with increasing amounts (from 150.6 to 502.8 mg) of HSPE. The results are shown in Table 3 and Fig. 2C and D. On the basis of preliminary experiments, the amounts of added HSPE were selected to yield phenolic increases in the PE-EC up to approximately 40% with respect to EC.

Table 3 – Effect of increasing amounts of HSPE added to EC (pre-brewing addition) on the phenolic content and antiradical activity of the brews.

HSPE added to EC	Phenolics/serving				Antiradical activity/serving				mg Trolox eq /mg GAE[e]
	Added (Pa)[a]	Found (Pf)	Increase (Pi)[b]	Pi/Pa	Added (Ta)[c]	Found (Tf)	Increase (Ti)[d]	Ti/Ta	
mg	mg GAE	mg GAE	mg GAE		mg Trolox eq	mg Trolox eq	mg Trolox eq		
150.6	102.4	321.5 ± 7.5	32.9	0.32	233.3	501.1 ± 12.9	58.9	0.25	1.79
205.2	139.6	336.9 ± 7.5	48.3	0.35	318.0	512.5 ± 11.9	70.2	0.22	1.46
252.2	171.5	343.1 ± 11.0	54.5	0.32	390.8	539.2 ± 23.1	97.0	0.25	1.78
301.6	205.2	357.7 ± 8.4	69.1	0.34	467.5	553.1 ± 24.8	110.9	0.24	1.60
350.2	238.2	366.6 ± 10.1	78.0	0.33	542.7	565.6 ± 20.7	123.4	0.23	1.58
400.7	272.6	380.4 ± 4.6	91.8	0.34	621.0	583.9 ± 14.6	141.7	0.23	1.54
451.6	307.2	389.5 ± 7.4	100.9	0.33	699.9	608.6 ± 20.6	166.4	0.24	1.65
502.8	342.1	396.7 ± 4.5	108.1	0.32	779.3	627.4 ± 12.6	185.2	0.24	1.71
Mean				0.33 ± 0.01				0.24 ± 0.01	1.64 ± 0.12

HSPE, hazelnut skin phenolic extract; EC, espresso coffee; GAE, gallic acid equivalents.

[a] Pa = HSPE phenolics (mg GAE/mg) × added HSPE (mg).

[b] Pi = Pf − espresso coffee (tq) phenolics.

[c] Ta = HSPE Trolox eq (mg Trolox eq/mg) × added HSPE (mg).

[d] Ti = Tf − espresso coffee (tq) Trolox eq.

[e] ratio of Ti to Pi.

In this case also, the total phenols in the PE-EC samples increased with regularity (Table 3), and a high correlation ($R^2 = 0.9936$) existed between the added hazelnut phenolics and the increase in measured phenolics in the brews (Fig. 2C). As expected, however, the experimental curve was different from the additive theoretical curve. The mean ratio of measured phenolic increase to added phenolics was equal to 0.33 (Table 3); this value represent the phenolic yield. Thirty-three percent is a good phenolic solubilisation percentage, considering that it was found to be around 21% in EC (Sánchez-González et al., 2005). The higher value obtained in this study may be due to the fact that the HSPE was a fine powder. Thus, it was much more easily solubilised/dragged by the pressurised hot water during brewing with respect to the coffee phenolic compounds entrapped in a coarse grounding matrix.

The mean antiradical power per unit of GAE for the HSPE pre-brewing trials (1.64) (Table 3) was not significantly different ($p \leqslant 0.01$) from the value found in the HSPE post-addition trials (1.72) (Table 2), indicating that no further loss in the hazelnut phenolic antiradical activity occurred when the phenolics were subjected to the brewing process. Considering that the initial antiradical efficiency per unit of HSPE phenolics was 2.28 mg Trolox eq/mg GAE, the antiradical efficiency of hazelnut phenolics diminished by approximately 28% after brewing.

Thus, HSPE addition to ground coffee induced proportional increases both in total phenols and in antiradical efficiency of the PE-EC. A phenolic yield of 33% was found. Evidence of a partial inhibition in hazelnut phenolic antiradical activity was found in the PE-ECs. No pro-oxidant effects of the HSPE phenolics were observed.

3.3. *Correlation between total phenols and antioxidant activity*

Some researchers have found high correlation between EC phenolic content and antioxidant power as measured by ferric reducing power (FRAP) and antiradical activity against 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt cation ($ABTS^{\cdot+}$) (Sánchez-González et al., 2005). Similarly, a high positive correlation between total phenolic content (mg GAE) and antiradical activity against DPPH (mg Trolox eq) both in pre-brewed and post-brewed PE-EC samples ($R^2 = 0.9976$ and 0.9874, respectively) was obtained in this study. These findings indicated that the measured antiradical activity is primarily attributable to the phenolic compounds present in the brews.

3.4. *Effect of HSPE-fortified EC in rats*

The effects of EC and PE-EC on the antioxidant potential of plasma in rats are reported in Table 4. The amount of HSPE employed was selected to have a maximum phenolic increase in the brews similar to that previously tested in the *in vitro* evaluation of antioxidant activity (approximately 40%). In particular, the rats consumed PE-EC samples having the following estimated phenolic increases with respect to the EC: 14.0, 20.9 and 27.8% GAE eq (pre-brewing addition of 180, 270 and 360 mg of HSPE, respectively); and 28.5% and 41.9% (post-brewing addition of 120 and 180 mg of HSPE, respectively). Both pre-brewing and post-brewing HSPE-integrated PE-EC samples were employed to assess whether the response on rats depended on the fortification method. In particular, two PE-EC samples having similar estimated phenolic increases (approximately 28%) but obtained with pre-brewing or post-brewing fortification methods (employing 360 and 120 mg of HSPE, respectively) were used in this study. Moreover, two PE-EC samples in which the same HSPE amount (180 mg) was pre-brewing or post-brewing added, were also analysed.

Three hours after intake, the rats that consumed the EC had a slight but significant ($p \leqslant 0.05$) improvement (+2.9%) in plasma antioxidant potential compared to the control (Fig. 3A). When HSPE was added to the brews, additional significant ($p \leqslant 0.05$) improvements were found, showing a good linear correlation ($R^2 = 0.9522$) with the phenolic increase measured in the brews. Importantly, the rats consumed 5.8 mg GAE of phenolics from the EC, which induced a slight increase (2.9%) in plasma antioxidant potential. When the rats were fed PE-ECs, they consumed an extra amount of phenolics varying from 0.8 up to 2.4 mg GAE. These small quantities of hazelnut skin phenolics produced high increases in plasma antioxidant potential (from 7.1% to 19.8%) (Fig. 3B). In addition to confirming the previous reports showing the ability of coffee brew to improve plasma antioxidant capacity (Natella, Nardini, Giannetti, Dattilo, & Saccini, 2002), the present study demonstrated that the PE-EC phenolic fraction had more activity in rats than the fraction naturally contained in EC.

Table 4 – Effects of EC and PE-EC on the antioxidant potential of plasma in rats.

Sample no.	Sample	HSPE addition	Amount of HSPE added (mg)	Amount of HSPE phenolics added (mg GAE)	Rat plasma antioxidant potential[a] (Fe^{+2} eq, μmol/l)
1	Control	–	–	–	390.7 ± 4.5[a]
2	EC	–	–	–	402.0 ± 5.7[b]
3	PE-EC	Pre-brewing	180	122.5	418.5 ± 8.1[c]
4	PE-EC	Pre-brewing	270	183.7	440.5 ± 6.1[d]
5	PE-EC	Pre-brewing	360	244.9	452.0 ± 5.9[e]
6	PE-EC	Post-brewing	120	81.6	458.1 ± 5.2[e]
7	PE-EC	Post-brewing	180	122.5	468.2 ± 6.6[f]

HSPE, hazelnut skin phenolic extract; EC, espresso coffee; PE-EC, phenolic-enriched espresso coffee; GAE, gallic acid equivalents.
[a] Means with different letters are statistically different ($p \leqslant 0.05$).

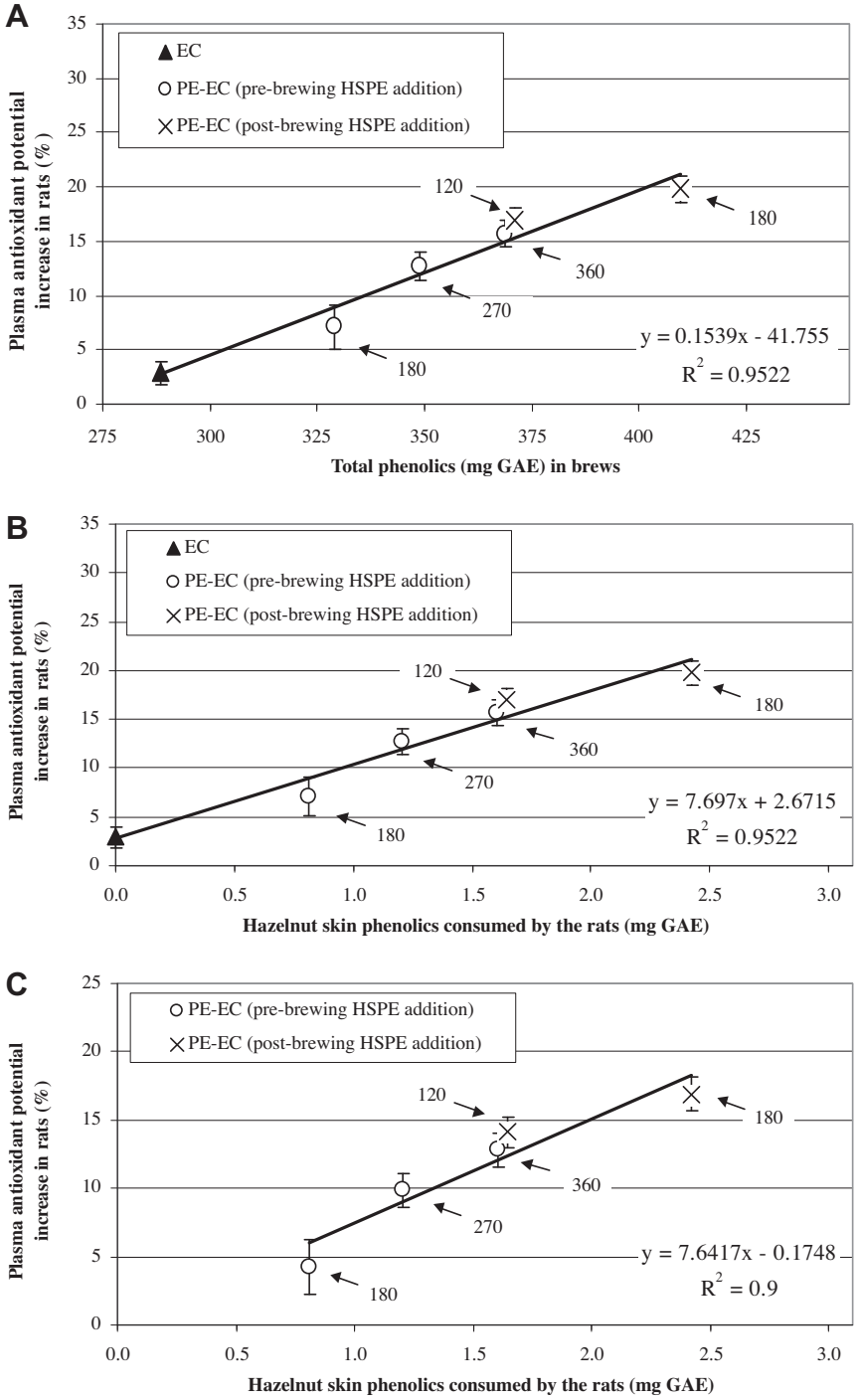

Fig. 3 – Increase (%) in plasma antioxidant potential of rats after intake of EC or PE-EC with added HSPE (pre-brewing and post brewing addition of HSPE). (A) Phenolic content in brews versus increase in plasma antioxidant potential, with respect to control. (B) Hazelnut skin phenolics consumed by the rats versus increase in plasma antioxidant potential, with respect to control; (C) Hazelnut skin phenolics consumed by the rats versus increase in plasma antioxidant potential, excluding the contribution of coffee. HSPE, hazelnut skin phenolic extract; EC, espresso coffee; PE-EC, phenolic-enriched espresso coffee; GAE, gallic acid equivalents. The numbers by the arrows represent the amounts (mg) of HSPE employed for integration.

The two differently fortified PE-EC samples inducing similar phenolic increases (80.1 and 82.4 mg GAE/serving with 360 mg HSPE pre-addition or 120 mg HSPE post-addition, respectively) resulted in no statistically significant differences ($p \leqslant 0.01$) in rat plasma antioxidant potential increases (15.7% and 17.0%, respectively) compared to the control. This finding verified that no apparent chemical changes in the antioxidant properties occurred during brewing.

This study demonstrated that the hazelnut phenolics present in PE-EC acted as bioactive antioxidants in rats. In a

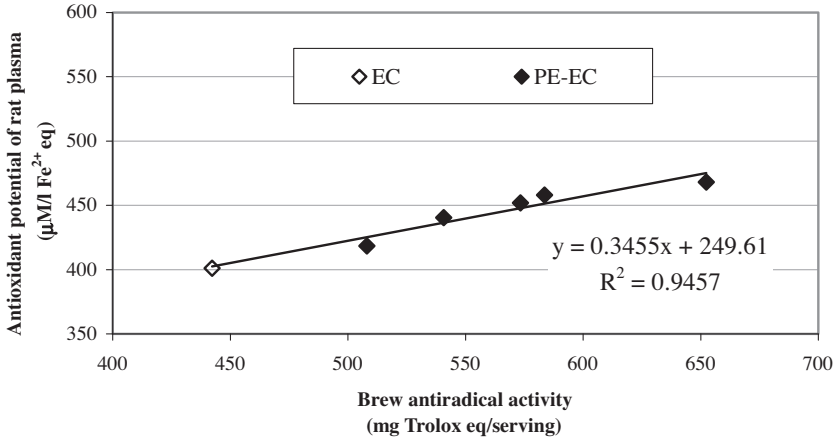

Fig. 4 – Relationship between *in vitro* (DPPH test) and *in vivo* (FRAP assay on rat plasma) antioxidant activity evaluation of EC or EC with added HSPE (PE-EC). EC, espresso coffee; PE-EC, phenolic-enriched espresso coffee.

previous study using Sprague–Dawley rats following an analogous experimental design, we observed that the rat plasma antioxidant potential increases by 25% with respect to the control 3 h after oral administration of 7.4 mg GAE of hazelnut skin phenolics (Contini et al., 2009). However, the results of the present study showed that a 25% increment in rat plasma antioxidant potential was obtainable with only 3 mg GAE of hazelnut skin phenolics consumed by the rats (value estimated by extrapolation based on the regression curve shown in Fig. 3C). These findings indicated that HSPE phenolics were much more active in rats when consumed together with EC rather than alone, suggesting that synergic effects enhancing the global plasma antioxidant power occurred *in vivo*. We remember that no synergisms were apparent in the *in vitro* DPPH tests, which demonstrated a contradictory result with a reduction in antioxidant activity power of the HSPE phenolics when they were added to EC. The *in vivo* affect may be attributed to the ability of some phenolic antioxidants to exercise their physiological action not only as free radical scavengers but also as agents able to interact with many basic cellular activities (Wang et al., 2011).

Furthermore, a good correlation ($R^2 = 0.9457$) existed between the *in vitro* (DPPH test) and *in vivo* (test on rats) methods used to evaluate antioxidant activity (Fig. 4). This correlation suggested that the relatively simple antiradical activity DPPH test under the adopted experimental conditions may be a good predictor of PE-EC biological activity in rats as measured by the FRAP assay.

In conclusion, HSPE potentially represent an inexpensive source of natural and very effective flavonoids, useful to upgrade the phenolic heritage of EC both quantitatively and qualitatively. Post-brewing or pre-brewing addition of HSPE to EC allowed enhancement of the antioxidant activity of the brew both *in vitro* and *in vivo*. The *in vivo* studies suggested that the HSPE phenolics had more antioxidant activity in rats with respect to the phenolics naturally present in coffee; moreover, synergic effects between HSPE and EC phenolics may have occurred.

The combination of coffee and HSPE phenolic antioxidants might strengthen and extend the spectrum of the physiologically positive activities presently attributed to coffee brew phytochemicals. Furthermore, the generally recognised cardioprotective properties of flavonoids might help to overcome the possible negative effects of the coffee brews on cardiovascular health. Obviously, further studies are necessary to evaluate the efficiency and the biological effects of HSPE fortified EC on humans, as well as assessing the eventual presence of hazelnut allergens in the product.

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