Phenolic acid profile and antioxidant capacity of hazelnut (*Corylus avellana* L.) kernels in different solvent systems

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Summary

Limited information is available relating to the qualitative and quantitative composition of phenolic acids in hazelnut kernels. Many authors have proposed different techniques to extract phenolic compounds from nuts, therefore, the objective of this study was to investigate how the experimental conditions commonly used to detect soluble phenolics affected the yield of phenolic acids and the antioxidant capacity of hazelnut kernel extracts. Phenolic compounds were extracted using three different solvent mixtures (80% v/v ethanol, methanol and acetone), at different temperatures. The extracts were examined for their total phenolic contents (TPC) and phenolic acid profiles. In addition, antioxidant capacities were tested using four different spectrophotometric assays. The results showed that acetone/water mixture applied at 50 °C exhibited the best extracting capacity, and 80% acetone extracts had the highest TPC and antioxidant capacity. HPLC analysis of the extracts highlighted the presence of eight phenolic acids (gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, *o*-coumaric acid and sinapic acid). In all extracts, benzoic acid derivatives were the most abundant. The acetone solution (80% v/v) at 50 °C was the most effective solvent for the quantitative extraction of both benzoic and cinnamic acid derivatives.

Keywords

hazelnut kernel; polyphenols extraction; phenolic acids; antioxidant capacity

Hazelnuts (*Corylus avellana* L.), also known as filberts, play a fundamental role in the tree nut industry. This worldwide tree nut is grown in many countries including Turkey, Italy, Spain and the United States. The annual crop production varies by country, but generally Turkey supplies 70% and Italy 15% of the world's hazelnuts. About 90% of the global production of shelled hazelnuts is used by the food industry as an ingredient of confectionery and chocolate. The remaining 10% supplies the in-shell consumption. The edible seed is best known for its mild, sweet, exotic flavour and distinctive texture, but it is also one of the most nutritious nuts. It contains high and valuable quantities of nutrients such as lipids, saccharides and proteins, but also significant micronutrients, minerals and other minor components, non-nutrient phytochemicals, such as polyphenols. Phenolic compounds have an array of health-promoting benefits; they possess substantial antioxidant and antiradical activities, anticarcinogenic and antimutagenic effects [1], and antiproliferative potential [2]. In recent literature, it has been reported that nut consumption is inversely associated with the incidence of cardiovascular disease, diabetes and some forms of cancer [3–8].

Typically, when discussing phenolics in plant foods, flavonoids are the predominant class described, because they account for approximately two thirds of the dietary phenols [9]. However, phenolic acids account for almost all of the remaining third. There is an increasing awareness of and interest in the antioxidant behaviour, and potential health benefits associated with this class of phenolic compounds. It is their role as dietary antioxidants that have received the most attention in recent literature [10–12]. Phenolics are structurally various, and are generally part of a complex mixture isolated from matrices of plant and other biological origin. Thus, the rapid and systematic

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measurement of phenolic acids and flavonoids is a serious challenge for analytical chemists, phytochemists and biochemists because of their inherent structural diversity and dietary impact. The first problem is exhaustive extraction of selected compounds from the plant material – usually performed by liquid-solid extraction (LSE). The goal of every extraction process is rapid and effective isolation of compounds from the matrix by use of a minimum amount of solvent [13]. Numerous extraction procedures have been described in literature. A common feature of these extraction methods is their suitability to only one plant material type for certain phenolic acid(s) or certain form(s) of phenolic acid(s) [14]. Hydroxybenzoic and hydroxycinnamic acids are contained in all plants and plant derivatives (e.g. fruits, vegetables, grains) [15], in both free and bound forms. Only a minor fraction exists in the free form, the remainder is linked through ester, ether or acetal bonds to various plant components, creating a very heterogeneous group of compounds. Hence their analysis is very complex and problematic. As for the phenolic acid extraction from vegetable matrices, the location of phenolic acids in the plant needs to be taken into account. Most phenolic acid derivatives present in the plant matrix are stored in vacuoles and are commonly extracted in alcoholic or organic solvents. The exceptions are those bound to insoluble saccharides and proteins within the plant matrix. Considering the variety of food matrices involved, there is surprisingly a great deal of coherency in the choice of solvents for extracting the phenolic acids and their conjugates [16]. Commonly used extraction solvents are hot water, methanol, ethanol, acetone and ethyl acetate, the latter being the most common. However, very polar phenolic acids could not be extracted completely with pure organic solvents, and mixtures of alcohol-water or acetone-water are recommended [11].

The difficulty of determination of phenolic acid originates from the extraction of these compounds from food matrices. In order to investigate the effect of different solvent systems on phenolic acid extraction from hazelnut kernels, we chose and compared the most common solvents reported in the literature [16, 17], applying them at different experimental conditions: under hot-reflux extraction conditions [18, 19] and by a long maceration at room temperature [20]. Therefore, the completeness of extraction and the consequent effect on antioxidant activities were investigated. The final objective of this work was to identify suitable conditions for extracting phenolic acids from hazelnut kernels, as a base for future research.

MATERIALS AND METHODS

Samples

Sample of Italian "Nocciola Piemonte PGI" hazelnut kernels, namely Tonda Gentile delle Langhe cultivar, harvested in 2007, was purchased from a Piedmont market. Dried, shelled, and calibrated (12–13 mm diameter) kernels were received in vacuum plastic bags, and stored in a dark refrigerated room at 4 °C until analysis.

Chemicals

The standards of phenolic acids (gallic acid, caffeic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, *o*-coumaric acid, *m*-coumaric acid, ferulic acid, sinapic acid, vanillic acid, protocatechuic acid, and syringic acid) were from Fluka Chemicals (Sigma-Aldrich Division, Milan, Italy). 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical, Trolox and Neocuproine (2,9-dimethyl-1,10-phenenthroline) were purchased from Sigma-Aldrich; HPLC-grade trifluoroacetic acid (TFA), 2,2'-azino-bis-(3-ethylbenzothiazolin-6-sulfonate) diammonium salt (ABTS), Folin-Ciocalteu reagent and 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) were from Fluka Chemicals. All organic solvents (analytical grade methanol, ethanol, acetone, *n*-hexane, diethyl ether) were obtained from Sigma-Aldrich.

HPLC instrumentation

The analytical HPLC system consisted of a Thermo Scientific SpectraSYSTEM LC (Thermo Fisher Scientific, San Jose, California, USA) with a SpectraSYSTEM SCM1000 degasser, a binary gradient pump system (SpectraSYSTEM P2000), an autosampling injector (SpectraSYSTEM AS100) and a UV-DAD detector (SpectraSYSTEM UV6000LP) with a cell of 5 cm length, operating in full-scan modality. UV-Vis spectra were recorded in the 220–360 nm range. ChromQuest 4.2 software (Thermo Fisher Scientific) was used for data processing. A 4.6 mm × 250 mm, 5 μm C18 RP Lichrosphere column (Merck, Darmstadt, Germany), with a guard column, was used for separations at room temperature (maintained at 22 °C). Elution was performed according to the procedure adopted from Xu and Chang [21], using mobile phase A (0.1% TFA aqueous solution) and mobile phase B (methanol); the flow rate was set to 0.7 ml·min^{-1} and the injected volume was 20 μl. The solvent gradient in volumetric ratios was as follows: 5–30% B over 50 min. The solvent gradient was held at 30% B for additional 15 min, and the gradient was increased to 100% B at 66 min. The sol-

vent gradient was held at 100% B for additional 10 min to clean up the column.

Preparation of defatted samples

Hazelnuts (30 g portions) were ground for 30 s in a domestic electric mixer (Imetec 7334 180W Dolcevita CH5, Tenaca Group, Azzano S. Paolo, Bergamo, Italy) to the smallest obtained particle size without creating a puree (10–35 mesh), and then defatted for 6 h with *n*-hexane in a Soxhlet apparatus. Defatted samples were subsequently dried under vacuum at 35 °C in a rotary evaporator (Büchi Rotavapor R-210, Büchi Labortechnick, Flawil, Switzerland) for 15 min, and then stored in vacuum-packaging polyethylene pouches at –20 °C until further analysis.

Extraction of crude phenolics

Phenolic compounds were extracted applying three different solvent systems (80 : 20 v/v ethanol/water, methanol/water, and acetone/water mixture) at a solid solvent ratio of 1 : 10 (w/v) and at three different temperatures (80 °C for ethanol/water and methanol/water mixtures, 50 °C, and room temperature, 20–22 °C, for each solvent). Extractions at high temperatures (80 °C and 50 °C) were carried out in the dark, under reflux conditions in a thermostated water bath, for 30 min. The resulting slurries were filtered with a Büchner funnel through a Whatman qualitative filter paper grade 113 (Whatman International, Maidstone, England). The residues were re-extracted twice under the same conditions, and finally, after filtration, the three extracts were combined. For extraction at room temperature (20–22 °C), the mixture was shaken in the dark on a magnetic stirrer (2 × 90 min), the suspensions were filtered, and the filtrates were collected and stored at 4 °C. The next day, after 18 h of additional extraction under the same conditions, the suspension was filtered and the three extracts were combined. The solvents were removed by a rotary evaporator under vacuum at 35 °C, and the remaining water solution was frozen and lyophilized for 72 h at –50 °C (LIO-5P, Cinquepascal, Trezzano sul Naviglio, Milan, Italy). The dry crude phenolic extracts were weighed to determine the extraction yield, and were then stored at –20 °C, in amber vials in vacuum-sealed pouches, for further analyses. Three replications for each extraction protocol were done. During this step, and the subsequent extraction and hydrolysis of phenolic acids, particular care was kept to limit the exposure of the samples to intense light, in order to avoid structural modifications of the analyte.

Determination of total phenolic content (TPC)

The hazelnut crude phenolic extracts were dissolved in methanol to obtain a concentration of 1 mg·ml^{-1}. The total phenolic content of the extracts was assayed spectrophotometrically by means of the Folin–Ciocalteu method, as modified by SINGLETON and ROSSI [22]. The absorbance was measured at 765 nm with a UV-1700 PharmaSpek UV-Vis spectrophotometer (Shimadzu, Osaka, Japan), after 15 min heating at 45 °C [23]. Gallic acid was used for the preparation of a standard curve (0–250 mg·l^{-1}). The average of triplicate measurements was used to calculate the phenolic content as gallic acid equivalent (GAE) in mg·g^{-1} of dry extract.

Determination of total antioxidant capacity (TAC)

In order to determine the antioxidant capacities of the hazelnut crude extracts, four different spectrophotometric assays were applied: the ABTS or TEAC (Trolox equivalent antioxidant capacity) assay, the DPPH radical-scavenging assay, the ferric reducing antioxidant power (FRAP) assay, and the cupric reducing antioxidant capacity (CUPRAC) assay. All determinations were carried out in triplicate and averaged.

TEAC assay

The Trolox equivalent antioxidant capacity (TEAC) was estimated following the original analytical procedure described by RE et al. [24] with some modifications. A stable stock solution of ABTS radical cation (ABTS$^{\bullet+}$) was produced by reacting 7 mmol·l^{-1} ABTS aqueous solution with 2.45 mmol·l^{-1} potassium persulphate (final concentration), and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Just before the analysis, an ABTS$^{\bullet+}$ working solution was obtained by dilution of the stock solution with ethanol, to an absorbance of 0.70 (± 0.02) at 734 nm. ABTS$^{\bullet+}$ working solution was equilibrated at 30 °C. A volume of 30 μl of the sample (or standard) solutions were mixed with 3 ml of ABTS$^{\bullet+}$ solution. Absorbance readings were taken at 30 °C exactly 6 min after the initial mixing. Appropriate solvent blank was obtained by mixing 30 μl of absolute ethanol with 3 ml of ABTS$^{\bullet+}$ solution. Pure ethanol was used as a control. A calibration curve was prepared with Trolox as a standard (final concentration 0–350 μmol·l^{-1}). The ABTS$^{\bullet+}$ scavenging effect (% inhibition) was calculated as in Eq. (1):

$$\text{Inhibition } [\%] = \frac{(A_{734\text{blank}} - A_{734\text{sample}})}{A_{734\text{blank}}} \times 100 \quad (1)$$

where $A_{734blank}$ and $A_{734sample}$ are the absorbances of ABTS$^{\bullet+}$ solution at 734 nm before and after the samples addition. Results were expressed as μmol Trolox equivalent (TE) per mg of dry extract by means of a dose-response curve for Trolox.

DPPH assay

The radical-scavenging activity (RSA) of the extracts was estimated according to the procedure reported by von GADOW et al. [25], being slightly modified by increasing the volume of the working solution to 3 ml. The working solution was prepared daily by dilution of DPPH$^{\bullet}$ (6.1×10^{-5} mol·l^{-1}, absorbance at 515 nm equal to or greater than 0.70) in methanol. A 75 μl aliquot of the sample was added to 3 ml of DPPH$^{\bullet}$ methanol solution and incubated for 1 h at room temperature in the dark. After this time, the absorbance was measured at 515 nm against methanol as a control, and methanol solution of DPPH$^{\bullet}$ as a blank. To minimize the underestimated values due to sample interferences at wavelengths near the visible region, methanol control was added of a 75 μl aliquot of each sample. The inhibition percentage (*IP*) of the DPPH$^{\bullet}$ by the extracts was calculated according to Eq. (2):

$$IP\,[\%] = \frac{(A_{0\,min} - A_{60\,min})}{A_{0\,min}} \times 100 \qquad (2)$$

where A_{0min} is the absorbance of the blank at $t = 0$ min, and A_{60min} is the absorbance of the samples at 60 min.

FRAP assay

The FRAP assay was conducted according to BENZIE and STRAIN [26] with slight modifications. Briefly, to 3 ml of the FRAP reagent, prepared freshly and warmed at 37 °C, 300 μl of distilled water and 100 μl of test sample, or water for the reagent blank, were mixed. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 ml of a 10 mmol·l^{-1} TPTZ solution in 40 mmol·l^{-1} HCl plus 2.5 ml of 20 mmol·l^{-1} $FeCl_3 \cdot 6H_2O$ and 25 ml of 300 mmol·l^{-1} acetate buffer (pH 3.6). Readings at the absorption maximum (593 nm) were taken after 4 min and 30 min [27]. Temperature of the test sample was maintained at 37 °C. The change in absorbance (ΔA_{593nm}) between the final reading and the reagent blank was calculated for each sample and related to ΔA_{593nm} of a Fe(II) standard solution tested in parallel. The sample interferences were minimized by subtracting the water control with 100 μl of sample from the sample value. Aqueous solutions of known Fe(II) concentration in the range 0–1 000 μmol·l^{-1} ($FeSO_4 \cdot 7H_2O$) were used for calibration. The resulting regression equation was used to calculate the FRAP value of the samples. Data were expressed as mmol·l^{-1} of $FeSO_4$ per g of dry extract.

CUPRAC assay

The cupric reducing antioxidant capacity assay was performed according to the method of APAK and co-workers [28].

Normal Sample Measurement

To a test tube 1 ml each of 10 mmol·l^{-1} copper(II) chloride, 7.5 mmol·l^{-1} neocuproine and NH_4Ac buffer (1 mol·l^{-1}, pH 7) solutions were added. Antioxidant sample (or standard) solution (100 μl) and 1 ml of H_2O were added to the initial mixture so as to make the final volume of 4.1 ml. The tubes were stoppered and, after 30 min, the absorbance at 450 nm (A_{450}) was recorded spectrophotometrically against a reagent blank. Also in this case, the sample interferences were minimized by subtracting the solvent control with 100 μl of sample from the sample value.

Incubated Sample Measurement

The mixture solutions containing sample and reagents were prepared as described under Normal Measurement; the tubes were stoppered and incubated for 20 min in a thermostatically controlled cell at a temperature of 50 °C. The tubes were cooled to room temperature under running water, and their A_{450} values were measured. Standard curve was prepared using different concentration of Trolox (0–350 μmol·l^{-1}). CUPRAC levels were expressed as μmol Trolox equivalent (TE) per mg of dry extract.

Extraction and hydrolysis of phenolic acids

Phenolic acids in crude extracts were fractioned in free and esterified forms according to the procedure described by SHAHIDI et al. [19] with slight modifications. The final procedure adopted is outlined in Fig. 1. An amount of 100 mg of dried crude phenolic extract was suspended in 10 ml of distilled water. The aqueous suspension was acidified to pH 2 using 6 mol·l^{-1} HCl, and then free phenolic acids were extracted five times with diethyl ether (1:1, v/v) at room temperature, using a separation funnel. The combined ether extracts of phenolic acids (referred to as free phenolic acids) were evaporated to dryness under vacuum at room temperature. The water phase was neutralized and then evaporated to almost dryness under vacuum at ≤ 40 °C. The residue was dissolved in 10 ml of 2 mol·l^{-1} NaOH and hydrolysed under nitrogen

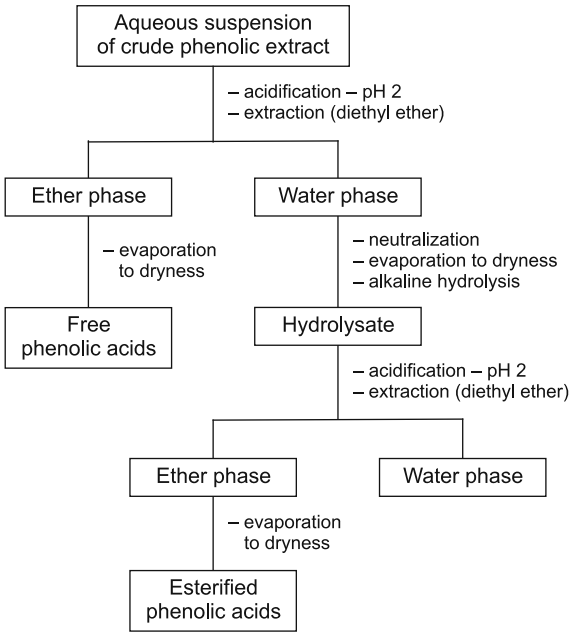

Fig. 1. A simplified flow diagram for extraction and fractionation of free and esterified phenolic acids.

for 4 h at room temperature in the dark. The reaction mixture was then acidified with 6 mol·l^{-1} HCl to pH 2, and phenolic acids from soluble esters (referred to as esterified phenolic acids) were extracted, five times, each into 15 ml of diethyl ether. Ether from the combined extracts was then evaporated to dryness. The dry residues of free and esterified phenolic acids were dissolved separately in 2 ml of methanol/formic acid 0.05% (1 : 1 v/v) and finally filtered through a Incofar PTFE single use syringe filter for HPLC analysis (Incofar, Modena, Italy) with 0.20 μm pore size. Each fraction was transferred into amber vial and stored at –20 °C until HPLC analytical assessments.

Identification and quantification of phenolic acids

The identification of phenolic acids, separated by HPLC, was achieved by comparing retention times (*RT*) and spectra with those of pure standards (gallic acid, caffeic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, *o*-coumaric acid, *m*-coumaric acid, ferulic acid, sinapic acid, vanillic acid, protocatechuic acid and syringic acid). Calibration curves were made from phenolic acid standard solutions in methanol/TFA/H_2O solvent mixture (5 : 0.1 : 94.9, v/v) at 5, 3, 1, 0.5, and 0.1 μg·ml^{-1}. A five point calibration curve was calculated for each phenolic acid. The regression coefficient of each standard curve was greater than 0.99.

Statistical analysis

Statistical analysis of data (one-way ANOVA) was performed using SPSS software (version 12.0 for Windows; SPSS, Chicago, Illinois, USA). Duncan's test was carried out to test any significant differences between extraction protocols applied, while Pearson correlation test was conducted to determine the correlation between variables.

RESULTS AND DISCUSSION

Extraction yield and total soluble phenols

The extraction yields after lyophilization of hazelnut crude extracts are shown in Tab. 1. The extraction conducted with 80% ethanol under reflux conditions at 80 °C produced the highest amount of crude extract (TSC). Under the same extraction conditions, we obtained a considerably higher

Tab. 1. Total soluble compounds (TSC), total soluble phenols (TSP) and extractable efficiency (TSP/TSC) of the extracts.

Extraction protocol	TSC [g·g^{-1}]	TSP [mg·g^{-1}]	TSP/TSC
80% ethanol, 80 °C	0.165 ± 0.012^{c}	1.90 ± 0.5^{a}	11.56 ± 3.5
80% ethanol, 50 °C	0.132 ± 0.015^{b}	3.67 ± 0.9^{bc}	27.63 ± 3.7
80% ethanol, room temperature	0.111 ± 0.002^{a}	4.20 ± 0.7^{cd}	38.02 ± 6.5
80% methanol, 80 °C	0.136 ± 0.009^{b}	2.27 ± 0.1^{a}	16.71 ± 2.0
80% methanol, 50 °C	0.131 ± 0.006^{b}	3.01 ± 0.2^{ab}	23.02 ± 0.6
80% methanol, room temperature	0.124 ± 0.005^{ab}	2.03 ± 0.1^{a}	16.36 ± 0.7
80% acetone, 50 °C	0.110 ± 0.013^{a}	7.22 ± 0.6^{e}	66.20 ± 9.2
80% acetone, room temperature	0.119 ± 0.001^{ab}	5.05 ± 1.0^{d}	42.51 ± 7.6

Data are expressed as mean ± standard deviation (n = 3). Means ± standard deviation in a column with different letters are statistically different ($p < 0.05$). TSP values are expressed as gallic acid equivalents.

Tab. 2. Total phenolic content (TPC) and total antioxidant capacity (TAC) of crude extracts.

Extraction protocol	TPC [mg·g^{-1}]	TEAC – ABTS$^{\bullet+}$ [μmol·mg^{-1}]	FRAP [mmol·g^{-1}]	CUPRAC normal [μmol·mg^{-1}]	CUPRAC incubated [μmol·mg^{-1}]	RSA – DPPH$^{\bullet}$ [%]
E, 80 °C	11.56 ± 3.5^{a}	0.06 ± 0.01^{a}	0.14 ± 0.01^{a}	0.13 ± 0.01^{a}	0.17 ± 0.01^{a}	3.00 ± 0.5^{a}
E, 50 °C	27.63 ± 3.7^{abc}	0.20 ± 0.01^{b}	0.33 ± 0.04^{ab}	0.26 ± 0.03^{abc}	0.38 ± 0.06^{abc}	10.63 ± 1.5^{a}
E, room *T*	38.02 ± 6.5^{bc}	0.31 ± 0.05^{c}	0.50 ± 0.09^{bc}	0.36 ± 0.05^{bc}	0.54 ± 0.07^{bc}	18.10 ± 2.7^{b}
M, 80 °C	16.71 ± 2.1^{a}	0.13 ± 0.01^{ab}	0.22 ± 0.02^{a}	0.17 ± 0.02^{a}	0.24 ± 0.03^{a}	6.87 ± 1.5^{a}
M, 50 °C	23.02 ± 0.6^{ab}	0.20 ± 0.01^{b}	0.29 ± 0.02^{ab}	0.23 ± 0.00^{ab}	0.31 ± 0.00^{ab}	9.49 ± 0.5^{a}
M, room T	16.36 ± 0.0^{a}	0.13 ± 0.01^{ab}	0.22 ± 0.02^{a}	0.19 ± 0.00^{a}	0.29 ± 0.01^{a}	9.02 ± 0.6^{a}
A, 50 °C	66.20 ± 9.2^{d}	0.55 ± 0.06^{d}	0.94 ± 0.13^{d}	0.59 ± 0.08^{d}	0.87 ± 0.13^{d}	30.97 ± 4.0^{c}
A, room *T*	42.51 ± 7.6^{c}	0.32 ± 0.02^{c}	0.58 ± 0.01^{c}	0.38 ± 0.04^{c}	0.55 ± 0.04^{c}	18.46 ± 1.9^{b}

Data are expressed as mean ± standard deviation (n = 3). Means ± standard deviation in a column with different letters are statistically different ($p \leq 0.001$).
TPC values are expressed as gallic acid equivalents. TEAC and CUPRAC values are expressed as Trolox equivalents. FRAP values are expressed as $FeSO_4$ equivalents. RSA values are expressed as inhibition percentage of DPPH$^{\bullet}$ radical.
E – 80% ethanol, M – 80% methanol, A – 80% acetone, *T* – temperature.

quantity of dry crude extract from hazelnut kernel (16.49%) than that reported by SHAHIDI et al. [19] (2.26%).

Total soluble phenols (TSP) showed dependence both on the solvent used and the temperature applied. In accordance with results reported by CONTINI et al. [20], we noticed that acetone/water mixture showed the best extracting capacity, in particular when it was applied at 50 °C. Extraction is the main step for the recovery and isolation of bioactive phytochemicals from plant material before analysis. It is influenced by their nature, the extraction method employed, sample particle size, as well as the presence of interfering substances [11]. Solubility of phenolic compounds is governed by the type of solvent (polarity) used, degree of polymerization of phenolics, interaction with other food constituents and formation of insoluble complexes. Methanol, ethanol, acetone, water, ethyl acetate and their combinations are frequently used for phenolics extraction [15]. 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 (TSP) and total extractable compounds (TSC) [20]. Our results indicated that these conditions were satisfied by acetone solvent, both at 50 °C and at room temperature. The same results were reported by CONTINI et al. [20] for hazelnut shell waste and skin waste of chopped hazelnuts, in these cases acetone solvent showed also the highest amount of crude extracts. The extraction rate of polyphenols from plant matrices using organic solvents can be improved with an increase of the concentration gradient, a larger diffusion coefficient or a smaller particle size. Increasing the temperature and decreasing the viscosity coefficient also increases the diffusivities [29]. Acetone solvent is characterized by the lowest viscosity (0.306 cP at 25 °C), followed by methanol, water and ethanol (0.544, 0.894, and 1.074 cP respectively) [30]. This physical characteristic could partially explain the better performance of acetone/water mixture. The data reported in Tab. 1 suggested that at the temperature of 80 °C and 50 °C, ethanol and methanol solvents had similar extractable efficiencies (TSP/TSC). Extraction at room temperature gave similar responses for ethanol and acetone aqueous solvents. Aqueous ethanol at 80 °C extracted more compounds, but probably the greater quantity of extraneous substances. Thus, in accordance with other authors [20], we confirmed that it was not possible to establish a correspondence between total extractable compounds and total extractable phenols.

Total phenolic content of crude extracts

The total phenolic content (TPC), expressed as milligram of GAE per gram of crude extracts, is shown in Tab. 2. It ranged from 11.56 mg·g^{-1} to 66.20 mg·g^{-1}. Significant differences ($p < 0.001$) exist among extracts obtained with different extraction protocols. The highest amounts of phenolics were assessed in 80% acetone extracts, in particular when the extraction was conducted at 50 °C but, in contrast with data reported by other authors [20], they did not provide the highest yield in dry extract. Nevertheless, they had

the best extracting capacity. Minor differences were observed among other solvent systems. The extract obtained using 80% ethanol at 80 °C had a lower content of total phenolics. SHAHIDI et al. [19], operating at the same extraction conditions, reported for kernel extract a comparable phenolic content (13.7 mg catechin equivalents (CE) per g of extract). In a previous work, ALASALVAR et al. [18] found that 80% ethanol extracts had a significantly lower content of total phenolics, compared to those obtained using 80% acetone. The same results were published by AMAROWICZ et al. [31] about almond seed. CONTINI et al. [20] reported that the most suitable phenolic solvent for hazelnut skin waste in presence of kernel fragments was 80% aqueous acetone (total phenol in extract 206.1 $mg \cdot g^{-1}$; maximal value of other parameters). The 80% ethanol mixture gave a slightly lower content of phenolic compounds (174.5 $mg \cdot g^{-1}$), while 80% methanol was found ineffective. Under long maceration at room temperature, our data showed the same trend, and the phenolic content of extracts was in the following order: acetone > ethanol > methanol (42.51 $mg \cdot g^{-1}$, 38.02 $mg \cdot g^{-1}$, and 16.36 $mg \cdot g^{-1}$ respectively). In any case, the assessed TPC corresponded with data published in the literature. OLIVEIRA et al. [32] reported values from 11.17 $mg \cdot g^{-1}$ to 14.77 $mg \cdot g^{-1}$ in hot water extracts of different hazelnut cultivars, while WU et al. [33], applying an ASE 200 accelerated solvent extractor (Dionex Corporation, Sunnyvale, California, USA), found that hazelnut had a TPC of 8.35 $mg \cdot g^{-1}$ fresh mass.

Total antioxidant capacity of crude extracts

The total antioxidant capacities (TAC) determined by different antioxidant assays are listed in Tab. 2. The data indicate that, among the extraction protocols, the total phenolic content and the total antioxidant capacity indices of crude extracts followed the same trend, in the following order: 80% acetone, 50 °C > 80% acetone, room temperature > 80% ethanol, room temperature > 80% ethanol, 50 °C > 80% methanol, 50 °C >

Tab. 3. Pearson correlation coefficient (*r*) between antioxidant capacity assays and total phenolic content (TPC) in crude phenolic extracts.

r	TPC	TEAC – ABTS$^{\bullet+}$	FRAP	CUPRAC normal	CUPRAC incubated	RSA – DPPH$^{\bullet}$
TPC	1					
TEAC – ABTS$^{\bullet+}$	0.982**	1				
FRAP	0.986**	0.990**	1			
CUPRAC normal	0.989**	0.993**	0.990**	1		
CUPRAC incubated	0.984**	0.990**	0.991**	0.996**	1	
RSA – DPPH$^{\bullet}$	0.979**	0.993**	0.989**	0.992**	0.996**	1

** Correlation is significant at the 0.01 level (2-tailed).

Tab. 4. Total phenolic acid amount (TPA) and their fractions in extracts obtained by different precedures.

Extraction protocol	TPA [$\mu g \cdot g^{-1}$]	Free acids [$\mu g \cdot g^{-1}$]	Esterified acids [$\mu g \cdot g^{-1}$]	Benzoic acid [$\mu g \cdot g^{-1}$]	Cinnamic acid [$\mu g \cdot g^{-1}$]
E, 80 °C	60.76 ± 1.4^{b}	34.34 ± 3.1^{b}	26.42 ± 2.0^{b}	54.27 ± 1.5^{b}	6.49 ± 0.4^{bc}
E, 50 °C	101.28 ± 5.4^{c}	48.64 ± 2.8^{c}	52.64 ± 4.8^{c}	91.81 ± 3.6^{c}	9.46 ± 2.0^{cd}
E, room *T*	22.60 ± 2.3^{a}	18.32 ± 2.9^{a}	4.28 ± 1.9^{a}	21.60 ± 2.2^{a}	1.00 ± 0.3^{ab}
M, 80 °C	101.95 ± 1.7^{c}	37.40 ± 2.2^{bc}	64.55 ± 0.9^{c}	85.98 ± 2.2^{c}	15.97 ± 0.9^{de}
M, 50 °C	122.81 ± 14.7^{d}	61.95 ± 5.7^{d}	60.86 ± 9.6^{c}	105.79 ± 16.4^{c}	17.03 ± 1.7^{e}
M, room *T*	5.95 ± 1.6^{a}	5.95 ± 1.6^{a}	nd	5.95 ± 1.6^{a}	nd
A, 50 °C	161.01 ± 13.1^{d}	47.55 ± 5.0^{bc}	113.46 ± 8.8^{d}	141.55 ± 9.8^{d}	19.46 ± 4.0^{e}
A, room *T*	97.51 ± 12.7^{c}	38.46 ± 5.5^{bc}	59.05 ± 7.2^{c}	82.48 ± 12.7^{c}	15.03 ± 2.0^{e}

Data are expressed as mean ± standard deviation ($n = 3$). Means ± standard deviation in a column with different letters are statistically different ($p \leq 0.001$).
E – 80% ethanol, M – 80% methanol, A – 80% acetone, *T* – temperature, nd – not detected.

Tab. 5. Free and esterified phenolic acids in extracts obtained by different precedures.

Extraction protocol	Gallic acid [$\mu g \cdot g^{-1}$]	Protocatechuic acid [$\mu g \cdot g^{-1}$]	4-Hydroxybenzoic acid [$\mu g \cdot g^{-1}$]	Vanillic acid [$\mu g \cdot g^{-1}$]	Syringic acid [$\mu g \cdot g^{-1}$]	*p*-Coumaric acid [$\mu g \cdot g^{-1}$]	*o*-Coumaric acid [$\mu g \cdot g^{-1}$]	Sinapic acid [$\mu g \cdot g^{-1}$]
Free phenolic acids								
E, 80 °C	11.19 ± 1.1 ^{c}	2.68 ± 0.3 ^{a}	6.21 ± 1.3 ^{abcd}	5.86 ± 0.9 ^{ab}	5.23 ± 0.5 ^{bc}	1.85 ± 0.4 ^{b}	1.31 ± 0.3 ^{abc}	nd
E, 50 °C	8.87 ± 1.6 ^{abc}	3.21 ± 0.8 ^{a}	9.51 ± 4.0 ^{cd}	12.13 ± 1.4 ^{cd}	10.09 ± 1.5 ^{d}	3.28 ± 0.4 ^{cd}	1.55 ± 0.8 ^{bc}	nd
E, room *T*	3.77 ± 0.3 ^{ab}	2.32 ± 0.2 ^{a}	3.03 ± 0.9 ^{ab}	4.45 ± 1.0 ^{a}	3.74 ± 1.1 ^{ab}	0.50 ± 0.2 ^{a}	0.50 ± 0.2 ^{ab}	nd
M, 80 °C	10.66 ± 0.6 ^{bc}	3.31 ± 0.3 ^{a}	4.01 ± 0.4 ^{abc}	7.96 ± 1.1 ^{abc}	7.37 ± 0.3 ^{bcd}	1.84 ± 0.2 ^{b}	2.25 ± 0.4 ^{cd}	nd
M, 50 °C	10.28 ± 4.8 ^{bc}	8.07 ± 1.2 ^{c}	12.27 ± 2.1 ^{d}	15.12 ± 1.9 ^{d}	9.30 ± 2.9 ^{cd}	4.25 ± 0.7 ^{d}	2.67 ± 0.4 ^{cd}	nd
M, room *T*	3.33 ± 0.4 ^{a}	nd	2.62 ± 1.3 ^{ab}	nd	nd	nd	nd	nd
A, 50 °C	9.33 ± 0.9 ^{abc}	4.65 ± 0.6 ^{ab}	7.19 ± 1.1 ^{bcd}	9.85 ± 1.3 ^{bc}	10.33 ± 0.3 ^{d}	2.79 ± 0.5 ^{bc}	3.41 ± 0.3 ^{e}	nd
A, room *T*	10.61 ± 1.5 ^{bc}	6.19 ± 1.0 ^{bc}	nd	11.05 ± 1.8 ^{cd}	8.52 ± 0.9 ^{cd}	nd	2.09 ± 0.3 ^{cd}	nd
Esterified phenolic acids								
E, 80 °C	5.52 ± 1.8 ^{ab}	8.44 ± 0.8 ^{b}	nd	9.13 ± 0.6 ^{ab}	nd	3.33 ± 0.4 ^{a}	nd	nd
E, 50 °C	13.83 ± 2.3 ^{ab}	9.38 ± 1.7 ^{bc}	15.36 ± 3.4 ^{a}	9.44 ± 5.1 ^{ab}	nd	2.97 ± 0.8 ^{a}	1.66 ± 0.8 ^{a}	nd
E, room *T*	3.00 ± 1.8 ^{ab}	1.29 ± 0.2 ^{a}	nd	nd	nd	nd	nd	nd
M, 80 °C	18.65 ± 1.1 ^{bc}	7.64 ± 0.4 ^{b}	18.53 ± 1.1 ^{a}	nd	7.85 ± 0.2 ^{b}	6.53 ± 0.1 ^{b}	nd	5.35 ± 0.9 ^{a}
M, 50 °C	12.76 ± 8.8 ^{ab}	9.32 ± 1.9 ^{bc}	14.42 ± 2.8 ^{a}	10.45 ± 0.5 ^{b}	3.80 ± 1.0 ^{a}	3.72 ± 0.6 ^{a}	nd	6.39 ± 0.9 ^{a}
M, room *T*	nd	nd	nd	nd	nd	nd	nd	nd
A, 50 °C	16.42 ± 4.5 ^{abc}	12.66 ± 0.7 ^{bc}	25.90 ± 2.7 ^{b}	45.22 ± 5.7 ^{c}	nd	6.20 ± 1.2 ^{b}	3.68 ± 0.8 ^{b}	3.38 ± 1.4 ^{a}
A, room *T*	31.21 ± 7.8 ^{c}	14.90 ± 3.9 ^{c}	nd	nd	nd	2.31 ± 1.2 ^{a}	nd	10.63 ± 1.9 ^{b}

Data are expressed as mean ± standard deviation ($n = 3$). Means ± standard deviation in a column with different letters are statistically different ($p \leq 0.001$).
E – 80% ethanol, M – 80% methanol, A – 80% acetone, *T* – temperature, nd – not detected.

80% methanol, room temperature > 80% methanol, 80 °C > 80% ethanol, 80 °C. All applied antioxidant assays gave the same trends.

The TEAC of crude hazelnut extracts ranged from 0.06 μmol·mg^{-1} to 0.55 μmol·mg^{-1}, being the lowest in 80% ethanol, 80 °C extract, and the highest in 80% acetone, 50 °C extract. The TAC values of 80% acetone, 50 °C extract were about three times greater than those of other solvents at the same operating temperatures. Similar results were obtained by ALASALVAR et al. [18]. They observed that operating at 50 °C, extracts obtained from 80% (v/v) ethanol had significantly lower ($p < 0.05$) TEAC compared to extracts obtained using 80% (v/v) acetone (0.20 mmol·g^{-1} and 0.62 mmol·g^{-1}, respectively), absolutely in accordance with our data (0.20 μmol·mg^{-1} and 0.55 μmol·mg^{-1}). SHAHIDI et al. [19], operating with 80% ethanol solvent at 80 °C, reported a hazelnut kernel TEAC value of 0.03 μmol·mg^{-1}. At the same extraction conditions, we obtained a double TEAC value of 0.06 μmol·mg^{-1}. Moreover, data reported in Tab. 2 suggest that acetone and ethanol solvents produce the same results if referred to a long maceration extraction at room temperature.

The content of total phenolics and the antioxidant capacities were closer and very well correlated (Tab. 3). Pearson correlation coefficient (r) ranged from 0.979 to 0.989.

Phenolic acids

The HPLC analysis of the extracts highlighted the quantifiable presence of eight phenolic acids, belonging to benzoic and cinnamic acid derivatives. The amounts of free and esterified phenolic acids in the extracts are reported in Tab. 4. Unlike the data previously reported [18], free phenolic acids were identified. The benzoic acid derivatives were gallic acid, protocatechuic acid, 4-hydroxybenzoic acids, vanillic acid and syringic acids. Among

the cinnamic acids derivatives, *p*-coumaric acid, *o*-coumaric acid and sinapic acid were identified (Tab. 5). Sinapic acid was found only in esterified fractions, which is in concordance with the report by ALASALVAR and co-workers [18]. This is probably due to the fact that sinapic acid is involved in lignin synthesis [34]. In all extracts, benzoic acid derivatives were the most abundant, in both free and esterified forms. The order of total phenolic acid content was as follows: 80% acetone, 50 °C > 80% methanol, 50 °C > 80% methanol, 80 °C ≥ 80% ethanol, 50 °C > 80% acetone, room temperature > 80% ethanol, 80 °C > 80% ethanol, room temperature > 80% methanol, room temperature. However, the dominance of each acid depended upon the extraction procedure adopted. Generally, all solvent systems, at the same temperature of extraction, extracted the same type of phenolic acids, and the extractions at 50 °C were qualitatively and quantitatively more effective.

SENTER et al. [35] compared phenolic acids of nine edible tree nuts produced in the United States. A total of eight phenolic acids were isolated and identified. Both qualitative and quantitative differences were observed among nut samples in the phenolic acids present, with gallic acid being predominant except for pine nut, almond and hazelnut. YURTTAS et al. [36] isolated and tentatively identified six phenolic aglycones in Turkish and American hazelnut extracts; these were gallic acid, *p*-hydroxybenzoic acid, epicatechin and/or caffeic acid, sinapic acid, and quercetin. However, the hazelnut variety and extraction solvents used in this study were different from those used by YURTTAS et al. In another work, TROSZYŃSKA et al. [37] extracted phenolic compounds from hazelnut, walnut and almond seeds with 80% aqueous acetone solvent. They identified a total of five phenolic acids (vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid), but only *p*-coumaric, ferulic and sinapic acids were detected in hazelnut. In a preliminary investigation conducted on the same cultivar harvested in 2006 [38], we compared the effect of two solvents (ethanol and acetone). We tentatively identified twelve phenolic acids (some only in trace amounts) and quantified five phenolic acids (gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid). In this study, we did not detect caffeic and ferulic acids, contrarily with data reported by SHAHIDI and co-workers [19], but in accordance with results previously reported by ALASALVAR et al. [18], which applied the same extraction protocols. They identified five and three phenolic acids respectively. Gallic, caffeic, *p*-coumaric, ferulic and sinapic acids were reported by SHAHIDI et al. [19], while gallic, *p*-coumaric and sinapic acids were mentioned by ALASALVAR and co-workers [18]. Similar results were obtained by WIJERATNE et al. [39], who identified caffeic, *p*-coumaric, ferulic and sinapic acids in almond seed extract, predominantly in the esterified form, the free form being present only in trace amounts. Loss of phenolic acids during alkaline hydrolysis has been described. For a range of hydroxycinnamic acids, the loss has been estimated not to exceed 10% of the initial values (*o*-coumaric, *p*-coumaric, isoferulic, ferulic acids; 4 mol·l^{-1} NaOH, 4 h under nitrogen) [40]. In the same experimental conditions, the loss of caffeic acid and sinapic acid was 67% and 36.5%, respectively. When exposed to acidic hydrolysis, the loss for hydroxycinnamic acid derivatives has been described to be even more dramatic [40]. Caffeic acid has already been reported to undergo spontaneous oxidation, particularly at alkaline hydrolysis pH [41, 42]. Moreover, a somewhat incomplete recovery after alkaline hydrolysis of ferulic and sinapic acids has been reported [43]. The partial discrepancy in the data between our previous and present study is probably based on the intrinsic differences of the samples, which were harvested in two different years, in addition to the likely different amount of the hydroxycinnamic acids loss due to the alkaline hydrolysis.

CONCLUSIONS

Numerous investigators have extracted phenolic compounds with aqueous ethanol, methanol and acetone. Extracting conditions varied from room temperature to reflux or boiling conditions. In this work, different assays used for examining antioxidant efficacies of hazelnut extracts revealed that acetone extracts exhibited superior antioxidant capacity compared to that of other solvents. When we consider the contents of total soluble phenolic acids (free and esterified), all the suggested methods showed the best extraction capacity at 50 °C, but acetone solution (80% v/v) at 50 °C was the most effective solvent for the quantitative extraction of both benzoic and cinnamic acid derivatives, particularly when referring to the esterified fractions. Therefore, aqueous acetone can be considered an effective solvent for phenolic acids extraction. Nevertheless, more research is still needed to establish the effect of cultivars, pre-harvest environmental conditions, post-harvest storage conditions and processing on phenolic acid profiles of hazelnut kernels.

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