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ORIGINAL ARTICLE

Antioxidant activity, and phenolic and mineral contents of the walnut kernel (*Juglans regia* L.) as a function of the pellicle color

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Abstract – Introduction. The objective of this study was to investigate some nutritional and functional components of the walnut fruit kernel and pellicle across twelve genotypes of *Juglans regia* L., six with a red pellicle and six with a light yellow pellicle. **Materials and methods.** Antioxidant activity, and contents of total phenolics, flavonoids, minerals and trace elements were determined as well as anthocyanins in red pellicles. **Results and discussion.** Total phenolic content ranged from 1,131 to 2,892 mg GAE 100 g^{-1} in kernels and from 11,525 to 33,833 mg GAE 100 g^{-1} in pellicles. The ratio between the average content in pellicles and that in kernels was found to be 12.7 for total phenolics and 12.9 for the antioxidant activity. Significantly higher average levels of total phenolic content and flavonoid content were found in yellow pellicles than in red pellicles, while no significant differences between the two groups of genotypes were found in kernels. High concentrations of anthocyanins were found in the red pellicles (1,636−2,956 mg CGE 100 g^{-1}). The contents of calcium, magnesium, iron and manganese were significantly higher in genotypes with a red pellicle compared with genotypes with a yellow pellicle both in kernels and pellicles, while the opposite is true for potassium and sodium. **Conclusion.** The data confirm that walnuts are a good dietary source of total phenolics with high antioxidant potential, minerals (Ca, Mg and K) and essential elements (Fe, Mn, Cu and Zn), most of which are concentrated in the walnut pellicle. Despite the large amounts of anthocyanins found in the red pellicles, yellow pellicles had a stronger antioxidant activity than red pellicles.

Keywords: Romania / walnut / *Juglans regia* / phenolics / antioxidant activity / mineral composition / nutritional value

Résumé – Activité antioxydante, teneurs en composés phénolique et minéraux de la noix (*Juglans regia* L.) en fonction de la couleur de la pellicule de l'amande. Introduction. L'objectif de cette étude était d'étudier certains composants nutritionnels et fonctionnels de l'amande du fruit du noyer et de sa pellicule à travers douze génotypes de *Juglans regia* L., dont six à pellicule rouge et six à pellicule jaune clair. **Matériels et méthodes.** L'activité antioxydante, les teneurs en composés phénoliques totaux, en flavonoïdes, en minéraux et oligo-éléments ont été déterminées, ainsi que les teneurs en anthocyanines des pellicules rouges. **Résultats et discussion**. La teneur totale en composés phénoliques a varié entre 1 131 et 2 892 mg GAE 100 g^{-1} dans les noix et entre 11 525 et 33 833 mg GAE 100 g^{-1} dans les pellicules. Le rapport entre la teneur moyenne des pellicules et celle des noix a été de 12,7 pour les composés phénoliques totaux et de 12,9 pour l'activité anti-oxydante. Des niveaux moyens significativement plus élevés de teneur en composés phénoliques totaux et en flavonoïdes ont été trouvés dans les pellicules jaunes que dans les pellicules rouges, tandis que pour les noix aucune différence significative n'a été observée entre les deux groupes génotypiques. De fortes concentrations en anthocyanes ont été mesurées dans les pellicules rouges (1 636−2 956 mg CGE 100 g^{-1}). Les teneurs en calcium, magnésium, fer et manganèse ont été significativement plus élevées chez les génotypes à pellicule rouge que chez ceux à pellicule jaune, à la fois dans les noix et les pellicules, alors c'est l'inverse pour les teneurs en potassium et sodium. **Conclusion.** Ces données confirment que les noix sont une bonne source alimentaire en composés phénoliques totaux avec un potentiel anti-oxydant élevé, en éléments minéraux (Ca, Mg et K) et microéléments essentiels (Fe, Mn, Cu et Zn) dont la plupart sont concentrés dans la pellicule de la noix. En dépit de la grande quantité d'anthocyanes présents dans les pellicules rouges, les pellicules jaunes ont présenté une activité anti-oxydante plus forte que les pellicules rouges.

Mots clés : Roumanie / noyer / *Juglans regia* / composés phénoliques / activité anti-oxydante / composition minérale / valeur nutritionnelle

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1 Introduction

The walnut (*Juglans regia* L.) is an extremely valuable nut species, not only due to the high nutritional value of the seeds and high-quality wood, but also for its non-edible parts such as leaves, green walnut husks (epicarp), shells and barks, which are of considerable interest for applications in the cosmetic and pharmaceutical industries [1–3].

Walnuts, the seeds of *Juglans regia* L., have generated great interest in recent years and have been hailed as a natural functional food, being one of the best dietary sources of compounds with health benefits [4, 5].

The health benefits of walnut consumption include reducing the risk of cardiovascular disease [6], type 2 diabetes [7] and some types of cancer [8]; mitigating postprandial oxidative stress [9]; reducing adiposity and low-grade systemic inflammation [10] and improving human serum lipid profiles, by decreasing total and LDL cholesterol, as well as triglycerides, and increasing HDL cholesterol and apolipoprotein A1 [4, 11].

These beneficial effects are attributed to their chemical composition. Walnut kernels generally contain about 60% oil, the most important fatty acids found in walnut oil being oleic (18:1 n−9), linoleic (18:2 n−6) and α-linolenic (18:3 n−3) acids [2, 12]. Therefore, walnuts contain a high amount of n−6 and n−3 polyunsaturated fatty acids (PUFAs) and have a high n−6 to n−3 ratio, which can affect different biochemical and physiological processes, including glucose and lipid metabolism, blood pressure regulation, erythrocyte deformability and platelet aggregation [5, 13, 14].

They also contain other beneficial components such as plant protein, and are unusually rich in essential amino acids (*e.g.* arginine, leucine), carbohydrates (*e.g.* dietary fiber), vitamins (tocopherols and tocotrienols, phytosterols, folate), biogenic amines (melatonin and serotonin), pectic substances, tannins and other polyphenols (phenolic acids, flavonoids) [15, 16].

Walnut phenolics have favorable effects on human health owing to their apparent antiatherogenic and antioxidant properties [17]. Phenolic compounds give walnuts their slightly astringent flavor and, together with tocopherols, they play an important role in protecting unsaturated fatty acids from oxidation [4]. Furthermore, previous studies indicated that walnut polyphenols have a stronger antioxidant action than non-polar antioxidants present in the lipid fraction, such as tocopherols and tocotrienols, making them the main compounds responsible for the antiradical protection provided by walnuts [8].

Walnuts contain significant amounts of essential micronutrients that are associated with an improved health status. Micronutrients constitute a small fraction of the overall nutritional intake of our diet, but they are structural and functional components of many important enzymes and play vital roles as catalysts and antioxidants in different metabolic processes. Walnuts are considered a good source of potassium (K), phosphorus (P), magnesium (Mg), sulphur (S), copper (Cu), zinc (Zn), manganese (Mn) and iron (Fe), and have low sodium (Na) content [12, 18–20].

Walnuts have the highest contents of antioxidants, most of them being concentrated in the walnut pellicle, the thin coating that surrounds the kernel [21]. Together with tocopherols, found in high concentration in the kernel itself, antioxidant polyphenols from the pellicle are probably responsible for the protection of the unsaturated fatty acids in the kernel against oxidation [22].

Previous studies confirm that the hydrolysable tannins, and specifically ellagitannins, are the most abundant phenolic constituents in the walnut pellicle [17]. These tannins consist of high-molecular-weight phenolic compounds, possessing, as a common structural feature, a glucose core to which gallic acid or ellagic acid are ester-linked. More complex ellagitannins are derived from pentagalloylglucose by oxidative reactions [23]. The hydrolysable tannins in the walnut pellicle and their hydrolysis products, gallic and ellagic acids, have been proven to be responsible for the extraordinary resistance of walnuts to aflatoxigenesis relative to other nuts [24].

The present study was carried out to investigate the total phenolics, total flavonoids, mineral (Na, K, Ca, Mg) and trace element (Fe, Mn, Cu, Cr, Zn) contents, and the antioxidant activity of kernels and pellicles from twelve walnut genotypes grown under the same environmental conditions and collected during the same period. Since six of the walnut cultivars had a red pellicle, these were also evaluated in terms of total anthocyanin content. The final aim was to identify differences in the chemical content of the kernels and pellicles depending on the genotype and pellicle color.

2 Materials and methods

2.1 Walnut samples

Twelve walnut genotypes, six of them with a red pellicle (H1R19, H1R9, H2R21, H3R3, H21R23 and H27R23) and another six with a light yellow pellicle (H1R7, H1R3, H1R13, H1R22, H2R18 and H1R10) were analysed. Two of the genotypes (H1R3 and H3R3) are open-pollinated seedlings of cv. Valcor (VL 4) and the rest are open-pollinated seedlings of walnut selections (VL 20, VL 51, VL 38, etc.) from local populations [25]. The walnut fruit of these genotypes was harvested in October 2014 from a selection plot belonging to the fruit growing research extension station (SCDP) Valcea (45° 08′26″ N, 24°22′ 00″ E, elevation 279 m above sea level), located in Oltenia, the central-south area of Romania, at the foothills of the Southern Carpathians. This region, well known as highly suitable for cultivating walnut trees, has a particularly mild temperate climate. The mean annual precipitation and air temperature are 715 mm and 10.2 °C, respectively. The trees examined in this study were 17 to 20 years old. No phytosanitary treatments were applied within the selection plot, in order to evaluate the walnut genotypes' susceptibility to diseases and pests.

From each genotype, three samples (1 kg each) of fruits at full maturity were picked by hand from the trees. After harvest, walnut fruits were immediately transported to the laboratory, cleaned and dried in an oven (UM200, Memmert, Germany) at 30 ± 2 °C for 24 h. They were stored in the shell, closed in plastic bags, and frozen at −20 °C, until the analyses were performed. Before each chemical analysis, the walnuts were manually cracked and shelled and their kernels and pellicle (yellow or red skin) were separated.

2.2 Extraction of the phenolic fraction

Walnut kernels and pellicles were dried and ground to very fine particles in a mechanical grinder. The oil was removed from the kernel samples using n-hexane in a Soxhlet apparatus. The pellicle and defatted kernel samples (1.5 g) were extracted with 10 mL methanol in a Bandelin Sonorex Digital 10P ultrasonic bath (Bandelin Electronic GmbH, Germany) for 60 min at ambient temperature. After extraction, the samples were centrifuged for 5 min at 4,200 rpm. Supernatants were filtered through polyamide membranes with pore diameter of 0.45 μm and stored at −20 °C.

2.3 Chemicals and reagents

Folin-Ciocalteu reagent (2 N), hydrochloric acid (37%), potassium chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and methanol were purchased from Merck (Germany). Gallic acid (99% purity), anhydrous sodium carbonate (99% purity), anhydrous sodium acetate (98% purity), aluminium nitrate, potassium acetate, quercetin and 2, 2-diphenyl-1-picrylhydrazyl (DPPH, 90% purity) were procured from Sigma-Aldrich (Germany). The water used for analysis was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.4 Total phenolic content

The total phenolic content was assessed colorimetrically by using the Folin-Ciocalteu phenol reagent method [26] with minor modifications [27]. One mL of each methanolic extract (diluted 1:20 with methanol) was mixed with 1 mL distilled water and 500 μL Folin-Ciocalteau reagent and stirred for one minute. After 2 min, 4 mL of 7.5% sodium carbonate aqueous solution were added and the mixture was incubated for 2 h at 25 °C. The same procedure was also applied to the standard solutions of gallic acid. Finally, the absorbance of the mixture was measured at 765 nm using an Evolution 600 UV/VIS spectrophotometer (Thermo Scientific, USA). Gallic acid was used for preparing the standard curve (0−250 mg L^{-1}) and the results were expressed as mg gallic acid equivalents (GAE) 100 g^{-1} dry weight (DW).

2.5 Total flavonoid content

The determination of flavonoids was done following the aluminium nitrate colorimetric method [28]. Briefly, 0.5 mL of each methanolic extract was diluted with methanol (1:10) and mixed in a test tube with 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M aqueous potassium acetate and 4.3 mL methanol. After keeping it for 40 min at room temperature, the absorbance of the reaction mixture was measured at 415 nm using an Evolution 600 UV/VIS spectrophotometer (Thermo Scientific, USA). Quercetin was used for preparing the standard curve (0−100 mg L^{-1}). Results were expressed as mg quercetin equivalents (QE) 100 g^{-1} DW.

2.6 Total anthocyanin content

The anthocyanins were extracted from red pellicles with 0.1% HCl (v/v) in methanol for 20 h at room temperature, in complete darkness. The quantification of total anthocyanins was performed using the pH differential spectrophotometric method [29]. Briefly, the extracts were separately diluted in a potassium chloride buffer (0.025 M, pH 1.0) and a sodium acetate buffer (0.4 M, pH 4.5). After incubation for 15 min at 23°C the absorbance was read against a blank at both 510 and 700 nm in a Varian Cary 50 UV spectrophotometer (Varian Co., USA). The total anthocyanin content of red pellicles was calculated using the equation below and was expressed in mg cyanidin 3-O-glucoside equivalents (CGE) 100 g^{-1} considering the amount of pellicles subjected to anthocyanin analysis.

$$Total\ anthocyanins\ (mg\ CGE\ 100\ g^{-1}) = (A \times MW \times DF \times 1,000)/(\varepsilon \times l)$$

where $A = (A_{510\ nm} - A_{700\ nm})_{pH\ 1.0} - (A_{510\ nm} - A_{700\ nm})_{pH\ 4.5}$; MW (molecular weight) = 449.2 g mol^{-1} for cyanidin-3-O-glucoside; DF (dilution factor of the samples); ε (molar extinction coefficient) = 29,600 L mol^{-1} cm^{-1}; l = cuvette path length in cm; and 1,000 = factor for conversion from g to mg.

2.7 DPPH radical-scavenging activity

The capacity to scavenge the DPPH free radical of pellicle and kernel extracts was determined according to a previously reported method [30]. Briefly, each methanol-diluted extract (50 μL) was mixed with 3 mL of a 0.004% (v/v) DPPH methanolic solution. The mixture was shaken vigorously and left to stand in the dark for 30 min at room temperature, and the absorbance was measured at 517 nm using an Evolution 600 UV/VIS spectrophotometer (Thermo Scientific, USA).

The inhibition of the DPPH radical by the samples was calculated according to the following formula:

$$DPPH\ scavenging\ activity\ (\%) = [1 - Abs.sample/Abs.blank] \times 100.$$

The DPPH scavenging activity was subsequently calculated with respect to the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), which was used as a standard reference. Six calibration solutions of Trolox were also tested to establish a standard curve. A blank control of methanol/water mixture was run in each trial. Results were expressed as mmol Trolox equivalents 100 g^{-1} DW.

2.8 Mineral composition

Calcium (Ca), magnesium (Mg), sodium (Na), iron (Fe), manganese (Mn), copper (Cu), chromium (Cr) and zinc (Zn) were determined by inductively coupled plasma mass spectrometry (ICP-MS), while potassium (K) was determined by flame atomic absorption spectrometry (FAAS) according to the methods previously described [18]. Mineralisation of samples

Table I. Antioxidant activity (AA, mmol Trolox 100 g^{-1} DW), and contents of total phenolics (TPC, mg GAE 100 g^{-1} DW) and total flavonoids (TFC, mg QE 100 g^{-1} DW) of the kernels of 12 walnut (*Juglans regia* L.) genotypes. Values are means ± standard deviation (n = 3).

Genotypes	AA	TPC	TFC
with red pellicle			
H1R19	14.97 ± 0.78^{a}	1,588.64 ± 77.23^{cd}	117.52 ± 6.22^{c}
H1R9	23.29 ± 1.33^{f}	1,131.05 ± 50.08^{a}	89.12 ± 5.41^{ab}
H2R21	19.01 ± 0.93^{de}	1,625.21 ± 79.91^{d}	93.44 ± 4.18^{b}
H3R3	19.32 ± 1.04^{e}	1,593.71 ± 56.88^{d}	137.55 ± 7.31^{e}
H21R23	19.44 ± 0.67^{e}	1,780.12 ± 68.34^{e}	97.80 ± 5.56^{b}
H27R23	26.49 ± 1.45^{g}	2,538.56 ± 110.98^{g}	158.30 ± 8.81^{f}
Mean	20.42 ± 3.84$_{A}$	1,709.52 ± 437.67$_{A}$	115.62 ± 26.51$_{A}$
with yellow pellicle			
H1R7	26.62 ± 1.28^{g}	2,052.49 ± 98.77^{f}	130.79 ± 7.11^{de}
H1R3	16.19 ± 0.83^{abc}	1,618.79 ± 72.76^{d}	96.17 ± 3.33^{b}
H1R13	17.29 ± 0.94^{bc}	2,892.12 ± 130.56^{h}	164.78 ± 7.92^{f}
H1R22	15.68 ± 0.62^{ab}	1,349.69 ± 61.55^{b}	81.03 ± 4.18^{a}
H2R18	17.29 ± 0.95^{bc}	1,803.44 ± 88.81^{e}	120.70 ± 5.88^{cd}
H1R10	17.54 ± 0.92^{cd}	1,450.15 ± 74.67^{bc}	125.31 ± 6.68^{cd}
Mean	18.44 ± 3.91$_{A}$	1,861.11 ± 535.50$_{A}$	119.80 ± 27.82$_{A}$

*Values in the same column followed by different superscript lower-case letters are significantly different at $P < 0.05$; **Means in the same column followed by different upper-case subscript letters are significantly different at $P < 0.05$.

was carried out by performing a wet acid digestion at high pressure in a microwave digester set at 180 °C for 20 min (Milestone Ethos EZ, Shelton, CT, USA). Briefly, samples of approximately 1.0 g ground kernel or pellicle were weighed in TFM containers, over which 5 mL nitric acid 65% and 2 mL hydrogen peroxide 30% were added. The containers were then closed and heated in the microwave digestion system. Reagent blanks were included in each series of digestions. After digestion was completed, the clear, colorless solution was transferred into a 50-mL volumetric flask and made up to the mark with ultrapure water. The samples were analysed by using an Elan 9000 inductively coupled plasma mass spectrometer (Perkin Elmer Sciex, Canada) equipped with a Meinhard nebuliser and Scott-type double-pass spray chamber, and an Avanta PM atomic absorption spectrometer in flame mode (GBC, Australia). The mineral contents of the samples were quantified against standard solutions of known concentrations and the results were expressed in mg 100 g^{-1} DW.

2.9 Statistical analysis

Values (mean ± SD) are the average of three samples from each genotype, analysed individually in triplicate. Data were subjected to analysis of variance (ANOVA), and the significance of differences between means was assessed by Duncan's multiple range test at $P < 0.05$. Correlation between total phenolic content and antioxidant activity was established by linear regression analysis at $P < 0.05$. All statistical analyses were performed using Statgraphics Centurion XVI software (StatPoint Technologies, VA, USA).

3 Results and discussion

3.1 Cultivar differences in phenolics

The total phenolic content, as determined by the Folin-Ciocalteu assay, varied widely in walnut genotypes' kernels and ranged from 1,131 (genotype H1R9) to 2,892 (genotype H1R13) mg GAE 100 g^{-1} (*table I*). There were significant differences among phenolic contents in kernels of various genotypes, but no significant differences were identified between the average content of phenolic compounds of the genotypes with a yellow pellicle and those with a red one.

Our results are in agreement with those of Anderson *et al.* [4], who reported a total phenolic content of 1,604 mg GAE 100 g^{-1}, and Labuckas *et al.* [17], who reported between 1,630 and 2,370 mg GAE 100 g^{-1} in walnut flour from the whole kernel extracted with methanol:water (6:4 v/v). Tosun *et al.* [16] also found total phenolic contents between 954 and 2,106 mg GAE 100 g^{-1} in commercial national cultivars and regional genotypes from Turkey. However, other authors registered much higher levels (*i.e.* between 2,820 and 5,820 mg GAE 100 g^{-1}) in four walnut cultivars grown in the southwest region of Spain [5], or even between 5,890 and 9,510 mg GAE 100 g^{-1} [15]. These differences could be explained by the influence of the genotype and environmental conditions on the biosynthesis and accumulation of these phenolic compounds, or by the different extraction methodologies.

The content of phenolic compounds was notably higher in pellicles than in kernels, ranging between 11,525 and 21,927 mg GAE 100 g^{-1} in genotypes with a red pellicle and between 17,473 and 33,833 mg GAE 100 g^{-1} in genotypes with a yellow pellicle (*table II*). Higher total phenolic contents were reported by Labuckas *et al.* [17] in walnut pellicles, *i.e.* between 23,000 and 40,300 mg 100 g^{-1}.

The average total phenolic content of pellicles was 12.7 times higher than that of the kernel. Rong *et al.* [31] also found that the content of polyphenols of the walnut pellicle was 12.51 times higher than that of the walnut kernel, while Colaric *et al.* [32] reported that the ratio between the phenolic contents in the pellicle and kernel varied by at least 14.8 times for caffeic acid and by up to 752.0 times for *p*-coumaric acid. Labuckas *et al.* [17] noted that although the hull contributes

Table II. Antioxidant activity (AA, mmol Trolox 100 g^{-1}), and contents of total phenolics (TPC, mg GAE 100 g^{-1}), total flavonoids (TFC, mg QE 100 g^{-1} DW) and total anthocyanins (TAC, mg CGE 100 g^{-1} DW) of the kernel pellicles of 12 walnut (*Juglans regia* L.) genotypes. Values are means ± standard deviation (n = 3).

Genotypes	AA	TPC	TFC	TAC
with red pellicle				
H1R19	143.86 ± 7.89^{a}	11,525.74 ± 677.25^{a}	18.30 ± 0.88^{a}	1,655.22 ± 69.61^{a}
H1R9	213.21 ± 12.13^{bc}	19,572.03 ± 966.15^{c}	45.74 ± 1.98^{ef}	2,931.11 ± 126.18^{c}
H2R21	200.20 ± 11.01^{bc}	17,203.30 ± 875.37^{b}	38.86 ± 1.72^{d}	2,956.23 ± 124.42^{c}
H3R3	197.48 ± 9.84^{b}	17,567.32 ± 830.16^{b}	43.81 ± 2.11^{e}	1,636.71 ± 71.50^{a}
H21R23	192.23 ± 10.92^{b}	18,148.39 ± 866.23^{bc}	27.06 ± 1.55^{b}	1,737.17 ± 62.53^{a}
H27R23	243.31 ± 14.30^{de}	21,927.07 ± 1233.21^{d}	31.32 ± 1.66^{c}	2,239.33 ± 122.21^{b}
Mean	198.38 ± 31.86$_{A}$	17,657.31 ± 3343.85$_{A}$	34.18 ± 10.04$_{A}$	2,192.24 ± 591.29
with yellow pellicle				
H1R7	334.08 ± 18.14^{g}	31,639.60 ± 1544.55^{f}	68.89 ± 3.33^{h}	nd
H1R3	312.56 ± 15.57^{fg}	28,272.01 ± 1246.46^{e}	48.74 ± 2.71^{f}	nd
H1R13	306.12 ± 16.43^{f}	32,519.60 ± 1335.09^{fg}	45.83 ± 2.44^{ef}	nd
H1R22	394.23 ± 18.24^{h}	33,833.80 ± 1100.66^{g}	55.41 ± 1.89^{g}	nd
H2R18	220.70 ± 11.64^{cd}	17,473.80 ± 573.78^{b}	54.68 ± 2.34^{g}	nd
H1R10	250.82 ± 13.09^{e}	23,684.40 ± 992.15^{d}	38.10 ± 2.09^{d}	nd
Mean	303.01 ± 59.15$_{B}$	27,903.87 ± 5,980.09$_{B}$	51.94 ± 7.95$_{B}$	

*Values in the same column followed by different superscript lower-case letters are significantly different at $P < 0.05$; **Means in the same column followed by different upper-case subscript letters are significantly different at $P < 0.05$; ***nd – not determined.

only 5% to the walnut fruit weight, its total phenolic content is at least 93–97% higher than that of the whole kernel.

It is noteworthy that the average total phenolic content in pellicles was significantly higher in the genotypes with a yellow pellicle then in those with a red pellicle.

The results obtained from the estimation of the total flavonoid content also indicated great variations among genotypes (*tables I, II*). The content of flavonoid compounds as quercetin equivalents was higher in kernels (89.12 to 130.79 mg QE 100 g^{-1}) than in pellicles (18.30 to 68.89 mg QE 100 g^{-1}). As for the total phenolic content, the average total flavonoid content in pellicles was significantly higher in the genotypes with a yellow pellicle then in those with a red pellicle, while no significant differences were found in kernels between the two groups.

The contrast between the low flavonoid content and the high phenolic content, in kernels but especially in pellicles, should also be noted. These findings are consistent with previous reports showing that walnut phenolics are mostly of the nonflavonoid type, belonging to the phenolic acid and hydolysable tannin categories [4, 9]. Colaric *et al.* [32] identified chlorogenic, caffeic, *p*-coumaric, ferulic, sinapic, ellagic and syringic acids, as well as syringaldehyde and juglone in ripe walnut fruit, while Slatnar *et al.* [33] detected twenty-seven phenolic compounds in walnut kernels using high-performance liquid chromatography-tandem mass spectrometry. The main polyphenolic subclass comprising hydrolysable tannins accounted for approximately 60.8%, the most abundant compound being glansreginin A.

3.2 Cultivar differences in antioxidant activities

The DPPH antioxidant activity was in the range of 14.97–26.62 mmol Trolox 100 g^{-1} for walnut kernels and 143.86–394.23 mmol Trolox 100 g^{-1} for walnut pellicles (*tables I, II*). As with total phenolic content, the extracts from pellicles presented higher scavenging capacity than those obtained from walnut kernels, the average antioxidant activity of pellicles being 12.9 times higher. These results support the fact that walnut pellicles are a rich source of phenolics, and they could be responsible for effective scavenging of free radicals.

A strong positive correlation was found between the total phenolic content and antioxidant activity of walnut pellicles (R^2 = 0.92), but a low correlation coefficient was observed in the case of kernels (R^2 = 0.10). Akbari *et al.* [1] found similar results and argued that the chemical nature and content of phenolics present in each walnut fraction may have a profound effect on their antiradical activity.

Besides phenolic acids, flavonoids and hydrolysable tannins, anthocyanins can contribute to the protective effect against oxidative damage to the cells. Large amounts of anthocyanins were found in the red pellicles, *i.e.* between 1,636 and 2,956 mg CGE 100 g^{-1}. Despite this, the average antioxidant activity of pellicles was significantly higher in walnut genotypes with a yellow pellicle than in those with a red pellicle.

3.3 Cultivar differences in minerals

Tables III and *IV* illustrate the mineral contents of the walnut kernels and pellicles of the twelve walnut genotypes, expressed as mg 100 g^{-1} DW. The data indicated that the walnut genotypes studied contain high quantities of many dietary minerals, which are an essential part of many important enzymes and play significant roles as catalysts and antioxidants. In fact, some authors consider that nuts, especially walnuts, have a mineral profile that is one of the most beneficial for health out of all other common food groups [20].

There were significant differences in mineral contents ($P <$ 0.05) both in kernels and pellicles, reflecting the specific plant

Table III. Mineral and trace element contents (mg 100 g^{-1} DW) of the kernels of 12 walnut (*Juglans regia* L.) genotypes. Values are means ± standard deviation (n = 3).

Genotypes	la	Mg	K	Na	Fe	Mn	Cu	Cr	Zn
with red pellicle									
H1R19	110.42 ± 6.21^{f}	258.81 ± 14.36^{c}	384.85 ± 18.98^{bc}	0.43 ± 0.03^{b}	4.24 ± 0.28^{ef}	14.09 ± 0.82^{h}	2.52 ± 0.16^{f}	0.69 ± 0.04^{de}	3.45 ± 0.24^{ef}
H1R9	74.60 ± 4.11^{d}	217.19 ± 11.55^{b}	337.80 ± 15.78^{ab}	0.32 ± 0.02^{a}	3.49 ± 0.21^{bc}	8.94 ± 0.38^{ef}	1.83 ± 0.1^{b}	0.41 ± 0.02^{b}	3.07 ± 0.19^{d}
H2R21	74.19 ± 3.37^{d}	253.61 ± 16.21^{c}	341.11 ± 16.66^{ab}	0.90 ± 0.03^{d}	3.87 ± 0.26^{cde}	6.30 ± 0.41^{c}	2.04 ± 0.14^{bcd}	0.54 ± 0.03^{c}	2.30 ± 0.15^{b}
H3R3	75.54 ± 3.87^{d}	212.36 ± 12.66^{b}	410.10 ± 22.34^{c}	0.38 ± 0.02^{ab}	4.24 ± 0.31^{ef}	8.30 ± 0.36^{e}	2.21 ± 0.12^{de}	0.35 ± 0.02^{a}	2.69 ± 0.18^{c}
H21R23	53.60 ± 2.26^{b}	213.34 ± 15.78^{b}	341.91 ± 15.56^{ab}	1.03 ± 0.06^{e}	3.57 ± 0.19^{bcd}	10.57 ± 0.66^{g}	1.88 ± 0.08^{bc}	0.59 ± 0.03^{c}	2.10 ± 0.11^{ab}
H27R23	60.59 ± 3.24^{c}	215.36 ± 9.88^{b}	308.90 ± 14.44^{a}	0.63 ± 0.03^{c}	3.94 ± 0.25^{de}	18.79 ± 0.89^{i}	2.38 ± 0.18^{def}	0.39 ± 0.02^{ab}	3.76 ± 0.28^{f}
Mean	$74.82 \pm 18.72_{B}$	$228.45 \pm 23.31_{A}$	$354.11 \pm 37.41_{A}$	$0.62 \pm 0.28_{A}$	$3.89 \pm 0.37_{B}$	$11.16 \pm 4.32_{B}$	$2.14 \pm 0.28_{A}$	$0.50 \pm 0.13_{A}$	$2.89 \pm 0.63_{A}$
with yellow pellicle									
H1R7	55.45 ± 2.28^{bc}	252.91 ± 13.34^{c}	811.40 ± 38.87^{fg}	0.68 ± 0.04^{c}	3.55 ± 0.18^{bc}	7.20 ± 0.29^{d}	2.10 ± 0.15^{cd}	0.73 ± 0.04^{ef}	3.60 ± 0.22^{f}
H1R3	51.47 ± 1.98^{b}	197.21 ± 10.44^{b}	791.22 ± 36.65^{ef}	0.46 ± 0.02^{b}	3.30 ± 0.16^{b}	10.87 ± 0.55^{g}	2.19 ± 0.18^{de}	0.65 ± 0.04^{d}	2.86 ± 0.16^{cd}
H1R13	77.91 ± 4.41^{d}	217.94 ± 14.45^{b}	856.18 ± 43.33^{g}	0.87 ± 0.04^{d}	3.63 ± 0.23^{bcd}	7.27 ± 0.37^{d}	1.95 ± 0.16^{bcd}	0.71 ± 0.03^{e}	4.52 ± 0.31^{g}
H1R22	35.45 ± 1.67^{a}	104.01 ± 7.71^{a}	561.92 ± 30.08^{d}	0.61 ± 0.03^{c}	2.23 ± 0.13^{a}	1.42 ± 0.10^{a}	1.04 ± 0.07^{a}	$0.40 \pm 0.\ 01^{ab}$	1.80 ± 0.12^{a}
H2R18	54.03 ± 2.52^{b}	248.33 ± 15.89^{c}	752.58 ± 35.55^{e}	1.02 ± 0.04^{e}	4.27 ± 0.25^{f}	9.62 ± 0.46^{f}	2.82 ± 0.21^{g}	0.78 ± 0.04^{fg}	3.19 ± 0.19^{de}
H1R10	90.63 ± 4.66^{e}	197.32 ± 9.67^{b}	816.65 ± 39.11^{fg}	2.19 ± 0.12^{f}	4.03 ± 0.26^{ef}	4.61 ± 0.25^{b}	2.06 ± 0.23^{bcd}	0.79 ± 0.03^{g}	1.81 ± 0.09^{a}
Mean	$60.82 \pm 18.91_{A}$	$202.95 \pm 51.85_{A}$	$764.99 \pm 103.62_{B}$	$0.97 \pm 0.59_{B}$	$3.50 \pm 0.69_{A}$	$6.83 \pm 3.23_{A}$	$2.03 \pm 0.56_{A}$	$0.68 \pm 0.14_{B}$	$2.96 \pm 1.01_{A}$

*Values in the same column followed by different superscript lower-case letters are significantly different at $P < 0.05$; **Means in the same column followed by different upper-case subscript letters are significantly different at $P < 0.05$.

Table IV. Mineral and trace element contents (mg 100 g^{-1} DW) of the pellicles of 12 walnut (*Juglans regia* L.) genotypes. Values are means ± standard deviation (n = 3).

Genotypes	Ca	Mg	K	Na	Fe	Mn	Cu	Cr	Zn
with red pellicle									
H1R19	198.51 ± 11.52^{d}	180.14 ± 11.55^{g}	287.55 ± 17.78^{a}	4.21 ± 0.29^{a}	8.95 ± 0.53^{f}	18.69 ± 0.78^{g}	1.59 ± 0.09^{e}	0.58 ± 0.04^{d}	2.10 ± 0.13^{fg}
H1R9	222.02 ± 13.66^{e}	143.25 ± 8.22^{e}	298.98 ± 21.17^{a}	3.21 ± 0.22^{a}	10.17 ± 0.66^{g}	16.24 ± 0.88^{f}	1.92 ± 0.11^{f}	0.67 ± 0.05^{e}	2.31 ± 0.18^{g}
H2R21	204.58 ± 9.98^{d}	163.30 ± 8.84^{f}	245.84 ± 15.55^{a}	7.21 ± 0.38^{c}	10.91 ± 0.48^{g}	7.64 ± 0.41^{d}	1.64 ± 0.08^{e}	0.78 ± 0.04^{f}	1.89 ± 0.10^{f}
H3R3	132.83 ± 7.72^{c}	146.43 ± 6.98^{e}	265.34 ± 16.61^{a}	6.73 ± 0.30^{bc}	10.75 ± 0.69^{g}	9.26 ± 0.55^{e}	1.53 ± 0.07^{e}	0.69 ± 0.04^{e}	1.61 ± 0.09^{de}
H21R23	136.65 ± 8.21^{c}	142.02 ± 6.77^{e}	199.69 ± 11.79^{a}	3.12 ± 0.17^{a}	7.75 ± 0.41^{e}	8.09 ± 0.39^{d}	3.25 ± 0.17^{h}	0.66 ± 0.05^{e}	1.02 ± 0.07^{a}
H27R23	213.46 ± 11.96^{de}	98.31 ± 5.35^{d}	226.54 ± 12.28^{a}	5.58 ± 0.29^{b}	10.45 ± 0.59^{g}	21.59 ± 1.22^{h}	2.10 ± 0.11^{g}	0.55 ± 0.03^{d}	1.52 ± 0.11^{cde}
Mean	$184.68 \pm 38.19_{B}$	$145.57 \pm 26.67_{B}$	$253.99 \pm 37.80_{A}$	$5.01 \pm 1.67_{A}$	$9.83 \pm 1.25_{B}$	$13.58 \pm 5.69_{B}$	$2.00 \pm 0.62_{B}$	$0.65 \pm 0.08_{B}$	$1.74 \pm 0.45_{B}$
with yellow pellicle									
H1R7	81.36 ± 5.21^{a}	88.59 ± 5.11^{bcd}	$1,773.55 \pm 96.65^{b}$	16.28 ± 0.89^{d}	4.96 ± 0.38^{bc}	4.90 ± 0.27^{bc}	0.99 ± 0.06^{d}	0.30 ± 0.02^{a}	1.66 ± 0.09^{e}
H1R3	91.92 ± 6.13^{ab}	92.25 ± 6.61^{cd}	$2,023.99 \pm 115.77^{c}$	19.92 ± 1.09^{f}	5.72 ± 0.32^{c}	8.21 ± 0.47^{d}	0.93 ± 0.07^{cd}	0.42 ± 0.03^{c}	1.33 ± 0.18^{bc}
H1R13	103.96 ± 8.85^{b}	79.09 ± 4.89^{ab}	$1,963.40 \pm 102.26^{c}$	18.02 ± 0.96^{e}	3.62 ± 0.28^{a}	4.17 ± 0.26^{b}	0.74 ± 0.04^{ab}	0.33 ± 0.02^{a}	1.41 ± 0.15^{bcd}
H1R22	91.43 ± 6.66^{ab}	74.62 ± 4.33^{a}	$2,219.67 \pm 120.08^{d}$	20.31 ± 1.23^{f}	5.09 ± 0.41^{bc}	1.82 ± 0.11^{a}	0.84 ± 0.05^{bc}	0.34 ± 0.02^{a}	1.07 ± 0.11^{a}
H2R18	95.29 ± 7.11^{ab}	92.12 ± 5.66^{cd}	$2,192.64 \pm 116.68^{d}$	19.61 ± 1.27^{f}	4.68 ± 0.27^{b}	5.79 ± 0.33^{c}	0.71 ± 0.04^{ab}	0.35 ± 0.03^{ab}	1.10 ± 0.13^{a}
H1R10	133.52 ± 8.44^{c}	83.06 ± 5.03^{abc}	$1,921.77 \pm 104.46^{c}$	19.14 ± 0.89^{ef}	6.53 ± 0.40^{d}	7.28 ± 0.41^{d}	0.62 ± 0.04^{a}	0.40 ± 0.03^{bc}	1.22 ± 0.12^{ab}
Mean	$74.82 \pm 18.72_{A}$	$84.95 \pm 8.14_{A}$	$2,015.84 \pm 183.82_{B}$	$18.88 \pm 1.76_{B}$	$5.10 \pm 0.97_{A}$	$5.36 \pm 2.17_{A}$	$0.80 \pm 0.14_{A}$	$0.36 \pm 0.05_{A}$	$1.30 \pm 0.23_{A}$

*Values in the same column followed by different superscript lower-case letters are significantly different at $P < 0.05$; **Means in the same column followed by different upper-case subscript letters are significantly different at $P < 0.05$.

genetic capacity to accumulate minerals. Nevertheless, in the kernel, the relative order of macroelements by content is the same in all genotypes, namely: K > Mg > Ca > Na. Among macroelements, K (potassium) presented the highest concentration, ranging from 308.9 to 856.18 mg 100 g^{-1} in the kernel, values similar to those reported by many authors [2, 5, 12, 14, 18].

Potassium content also ranked first in pellicles but varied within a wider range, the average potassium content being significantly higher in yellow pellicles (2,015.84 mg 100 g^{-1}) than in red pellicles (253.99 mg 100 g^{-1}). In the kernel as well, the potassium content was significantly higher in genotypes with a yellow pellicle than in those with a red pellicle. It is well known that the elemental composition and pH of the soil greatly influence mineral absorption by plants, which may explain, along with the genotype, the observed differences.

Walnuts are among the most concentrated sources of magnesium, making them a very suitable food to compensate for deficiencies in this mineral. The levels of magnesium in our kernel samples ranged between 104.01 and 258.81 mg 100 g^{-1} while the USDA National Nutrient Database for Standard Reference listed 158 mg 100 g^{-1} [34]. Our results were higher compared with values reported by Çağlarirmak [35] and Gharibzahedi *et al.* [13], but lower than the results reported by Tapia *et al.* [5] and Aryapak and Ziarati [12].

There were no significant differences in the average magnesium content of the kernel between the two groups of genotypes. Magnesium contents in the pellicle were consistently

lower than in the kernel, ranging from 74.62 to 92.25 mg 100 g^{-1} in yellow pellicle genotypes and from 98.31 to 180.14 mg 100 g^{-1} in red pellicle genotypes, significantly higher in the latter.

High calcium contents were found in walnut samples, ranging from 35.45 to 110.42 mg 100 g^{-1} in the kernel and from 91.43 to 222 mg 100 g^{-1} in the pellicle. Calcium contents were significantly higher in genotypes with a red pellicle, both in the kernel and in the pellicle. The values obtained in this study were similar to those reported in walnuts by other authors [2, 5, 18]. The calcium content was high in pellicles; therefore, in some genotypes, the calcium content exceeded the magnesium content.

Sodium content ranged from 0.32–2.19 mg 100 g^{-1} in the kernel and 12.00–20.31 mg 100 g^{-1} in the pellicle, significantly higher in genotypes with a yellow pellicle, both in the kernel and in the pellicle. The mineral content of walnuts – low in sodium but high in potassium, calcium and magnesium – represents one of the main reasons why they would make an ideal component of the well-known Dietary Approaches to Stop Hypertension (DASH) diet, recommended for the prevention and treatment of elevated blood pressure levels [36].

The interest in the nutritional value of walnuts is largely due to their trace element contents. The amounts of trace elements found in the walnut kernels ranged from 2.23–4.27 mg 100 g^{-1} for iron, 1.80–4.52 mg 100 g^{-1} for zinc, 1.04–2.82 mg 100 g^{-1} for copper and 0.35–0.79 mg 100 g^{-1} for chrome. These results were in line with data from previous studies [5, 13, 14], except for manganese, for which we recorded higher levels (1.82–21.59 mg 100 g^{-1}). These observed differences may be due to genetic factors, harvesting time, soil and water properties, geographical conditions and analytical procedures.

There were no significant differences between the two groups of genotypes in so far as the copper and zinc content in the kernel is concerned, but the iron and manganese contents were significantly higher in kernels of the genotypes with a red pellicle. Walnut pellicles were found to have higher iron content (3.62–10.91 mg 100 g^{-1}) compared with the kernels, but lower zinc content (1.02–2.31 mg 100 g^{-1}). As for calcium and magnesium, the contents of all trace elements in the pellicle were significantly higher in genotypes with a red pellicle compared with genotypes with a yellow pellicle.

4 Conclusion

The selected genotypes have nutritionally valuable levels of phenolics and antioxidant activity, but significant differences were found among them. The average total phenolic content of pellicles was 12.7 times higher than that of kernels, while antioxidant activity was 12.9 times higher. Thus, the results of this investigation confirm previous studies showing that most of the antioxidants are located in the walnut pellicle. A strong positive correlation was found between total phenolic content and antioxidant activity in walnut pellicles, but a low correlation coefficient was found for the kernels.

Low flavonoid contents were found in both kernels and pellicles (particularly low in pellicles), findings which are consistent with previous reports showing that walnut phenolics are mostly of the non-flavonoid type, belonging to the phenolic acid and hydolysable tannin categories.

Despite the large amounts of anthocyanins found in the red pellicles, the average antioxidant activity of pellicles was significantly higher in walnut genotypes with a yellow pellicle than in those with a red pellicle. Both kernels and pellicles contain significant amounts of minerals needed in a healthy diet, such as calcium, magnesium and potassium, and essential micronutrients that are associated with an improved health status. Macroelements were found to be more abundant in pellicles, with the exception of magnesium. The high content of essential elements such as iron, manganese, copper and zinc allows some Romanian walnut genotypes to be considered as an excellent source of bioactive compounds with recognised health benefits.

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