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AKADÉMIAI KIADÓ

Phenolic profile, color parameters and antioxidant activity of walnut kernel extracts as influenced by different time and temperature during extraction

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ABSTRACT

The aim of the present study was to find the best extraction parameters to obtain the highest amounts of polyphenols and antioxidants from the walnut. Walnut kernels from 'Alsószentiváni 117' cultivar were used for extraction. The extraction methods were as the follows:

Method 1: shaking water-bath at 50 °C for 30 min.
Method 2: shaking water-bath at 50 °C for 30 min, then storing at 5 °C for 20 h.
Method 3: shaking water-bath at 40 °C for 30 min.
Method 4: shaking water-bath at 40 °C for 30 min, then storing at 5 °C for 20 h.

According to our results Method 1 showed the highest FRAP value (34.43 mg AAE g^{-1}), the DPPH value (52,94%) and the highest HPLC peaks for chlorogenic acid, epicatechin and rutin were also seen in extracts obtained using Method 1. TPC values of Method 3 were 26.06 mg GAE g^{-1} for Method 1 it was 25.65 mg GAE g^{-1}. The results of color values, L^* and ΔE^* were similar in all extracts as well. In our experiments extraction Method 1 proved to be better than others.

KEYWORDS

'Alsószentiváni 117' walnut cultivar, TPC, FRAP, DPPH, HPLC analysis

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INTRODUCTION

Persian or common walnut (*Juglans regia* L.) growing goes back for many years in Hungary. Walnut breeding started unsuccessfully with the domestication of seed-propagated French cultivars in the early 1900s. After that different selections were used during the Hungarian breeding researches for the breeding processes (Bujdosó and Cseke, 2021). Hungary produced 5,950 tons of walnuts in 2021 ("FAOSTAT," 2023). Its production in Hungary is increasing steadily which can attributed to the increased interest and demand for walnuts due to their beneficial effects on human health.

Walnuts have been consumed for centuries as a highly nutritious food in many diets and societies around the world. Recent research has shown that they are helpful to tackle life-style diseases like arteriosclerosis, cardiovascular diseases, and diabetes mellitus (Bullo et al., 2011; Pan et al., 2013; Ros et al., 2004). The health benefits of walnuts are more or less attributed to ω-3 fatty acids and vitamin E (Maguire et al., 2004; Ros et al., 2004). Walnuts are rich in bioactive components such as polyphenols that have positive effects on health (Vinson and Cai, 2012). Recent work suggest that higher phenolics compounds found in nuts including walnuts reduce inflammation and increase antioxidant defenses (Sánchez-González et al., 2017). Different studies revealed that the polyphenol-rich foods have influence on the blood lipids by lowering low-density lipoprotein (LDL) and increasing high-density lipoprotein (HDL) (Potì et al., 2019). Amen et al. (2023) reported that nuts can increase the total polyphenol content in the human diet, so walnut alone added to a regular diet can multiply the polyphenol intake. In addition, the concentration of polyphenolic compounds in walnuts is significantly higher than in other nuts (peanuts, pistachios, hazelnuts, almonds, etc.) (Abe et al., 2010; Vinson and Cai, 2012). In PREDIMED (Prevención con Dieta Mediterránea) clinical trials, Mediterranean diet of healthy adults was supplemented with 30 g different nuts/day (15 g walnuts, 7.5 g hazelnuts, and 7.5 g almonds). In the trials results, better cognitive ability and memory functions were observed, against the control group on a low-fat diet (Valls-Pedret et al., 2015). Results from studies of (Park et al., 2020) indicate that walnut polyphenol extracts possess anticancer properties. Different polyphenols present in walnuts are responsible for all their all their health promoting properties. Catechin, chlorogenic acid, epicatechin, elagic acid, juglone, rutin, tannic acid are some of the polyphenols present in good amount in walnuts (Nguyen and Vu, 2023; Ni et al., 2022).

Our experiments were focused on maximum extraction of polyphenols from the walnuts. A lot of research has been done on the extraction of fatty acids from walnuts, but polyphenols are often not preferred. We wanted to develop an accurate and comprehensive process to maximize the extraction of polyphenols and antioxidants.

MATERIALS AND METHODS

Hungarian walnut cultivar 'Alsószentiváni 117' was obtained from Pálháza (Hungary, (48°28′18.88″ N, 21°30′34.85″ E) for the research. The walnuts were first deshelled, and the kernels were collected. The kernels were ground for 30 s by multifunctional grinder (Princess 22104001), and a fine powder was prepared. This kernel powder was used to extract polyphenols using 100% methanol in 1:5 (w/v) sample:solvent ratio. 100% methanol for extraction was used

based on the previous experiments done in our laboratory. The extraction was done using four different methods to find the one which gives the maximum amount of polyphenols. The four different methods are shown as follows:

Method 1: Shaking walnut sample and solvent in a shaking water-bath at 50 °C for 30 min.
Method 2: Shaking walnut sample and solvent in a shaking water-bath at 50 °C for 30 min, then storing at 5 °C for 20 h.
Method 3: Shaking walnut sample and solvent in a shaking water-bath at 40 °C for 30 min
Method 4: Shaking walnut sample and solvent in a shaking water-bath at 40 °C for 30 min, then storing at 5 °C for 20 h.

After completing the extraction, the extracted solution was transferred into centrifuge tubes. Then the tubes were centrifuged at 4500 RPM for 10 min and the supernatant solution was filtered using Whatman filter paper of 1 μm pore size and 55 mm diameter. The samples were kept at −18 °C until further analysis.

Total phenolic concentration was measured using the Folin–Ciocalteu method based on Singleton and Rossi (1965). 1,250 μL of Folin reagent (1:10 v/v Folin; distilled water) was added in the test tube followed by 200 μL of methanol (4:1 v/v methanol; distilled water). After that, 50 μL of the sample was added, followed by the addition of 1,000 μL of sodium carbonate after 1 min. After that samples were kept in a water bath at 50 °C for 5 min. Finally, the absorbance was detected at 760 nm. The results were given in gallic acid equivalents (mg GAE g^{-1} walnut kernel). The range of calibration curve was from 0.099 to 0.599 with $R^2 = 0.9836$.

Antioxidant capacity was measured by ferric reducing ability of plasma (FRAP) method (Benzie and Strain, 1996). FRAP reagent was prepared by using acetate buffer (pH 3.6), 2, 4, 6-tripyridyl-s-triazine (TPTZ), and FeCl3 × 6H2O. The absorbance was detected at 593 nm after 5 min. Results were given in ascorbic acid equivalent (mg AAE g^{-1} walnut extract) using an ascorbic acid standard calibration curve. The range of calibration curve was from 0.309 to 1.543 with $R^2 = 0.999$.

Free radical scavenging activity (DPPH) was performed based on the method of Blois (1958). 1000 μL DPPH solution (prepared using 9 mg 2,2-diphenyl-1-picrylhydrazyl per 100 mL MeOH) was added to the test tube before adding 990 μL distilled water and 10 μL sample. Test tubes were closed and kept in the dark for 30 min and then the analysis was done at 517 nm. The results were expressed in percentage of radical scavenging power. The range of calibration curves was from 0.197 to 0.714 with $R^2 = 0.9994$.

The spectrometric measurements were carried out with Hitachi U-2900 equipment (Hitachi High Technologies Europe GmbH, Krefeld, Germany). All the reagents were purchased in analytical grade from Sigma-Aldrich Chemical Co. (3,050 Spruce Street, St. Louis, MO 63103, USA).

Color coordinates were determined according to C.I.E.LAB system using a digital colorimeter (Konica Minolta CR 410, Minolta Canada Inc.). ΔE^* was calculated using (Lukács, 1982) the following equation:

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

The color differences are presented in Table 1.

Table 1. Summary of color difference (Lukács, 1982)

ΔE^*	Color change
0–0.5	Not noticeable
0.5–1.5	Hardly noticeable
1.5–3.0	Visible
3.0–6.0	Clearly visible

For the HPLC analysis the sample was filtered on a 0.45 µm MILLEX®-HV Syringe Driven Filter Unit (SLHV 013 NL, PVDF Durapore), obtained from Millipore Co. (Bedford, MA, USA), and injected into the HPLC system. The Shimadzu High Performance Liquid Chromatograph (was equipped with an absorbance detector (2,487 Dual λ), a binary HPLC pump (1,525), and in-line degasser, a column thermostat (set at 40 °C) and an 717plus auto sampler (set at 5 °C) and was controlled using Labsolution software. A KINETEX C18 2.6 µm 150 × 4.6 mm column (Phenomenex 411 Madrid Avenue Torrance, CA 90501-1430 USA) was used, and the gradient mobile phase was A: 1% formic acid with HPLC grade water and B: 1% formic acid with acetonitrile (0–30 min: B 5%–100%, 30–35 min: B 100%, 35.5–45 min: B 5%) with a flow rate 1.5 mL min^{-1}. The compounds were detected at 280 and 310 nm wavelength. HPLC standards (Sigma Aldrich) and four points external calibration were used for the qualification and quantification of the individual components. The unit of the individual phenolic compounds are given in mg g^{-1}.

Statistical analysis was performed using one factor complete randomized ANOVA using IBM SPSS version 27.

RESULTS AND DISCUSSION

Insignificant differences in amount of TPC in walnuts ($P = 0.343$) were found between all the four methods. TPC using extraction Method (1) was 25.65 mg GAE g^{-1} which was insignificantly less than Method (3) (26.06 mg GAE g^{-1}) and for Method (2) and (4) it was 25.21 and 23.68 mg GAE g^{-1} respectively. TPC of deoiled walnut cake reported by (Garcia-Mendoza et al., 2021) was 8.9 mg GAE g^{-1} meal. Ojeda-Amador et al. (2018) reported 19 mg GAE g^{-1} meal TPC for Lara variety of walnuts (Pop et al., 2021). reported TPC of 2.86 mg GAE g^{-1} of walnut kernels (Kumar et al., 2022), reported similar TPC results.

The highest values of FRAP (34.43 mg AAE g^{-1}) were observed in case of Method 1. According to ANOVA this was significantly different ($P < 0.05$) from other methods. Romano et al. (2021) reported different FRAP values using different extraction solvents with methanol they reported 0.083 (mmol TE g^{-1}), and with Supercritical CO_2 + Ethanol, 0.139 (mmol TE g^{-1}).

The results of TPC and FRAP are given in Figs 1 and 2.

The values of DPPH for all extracts were not significantly different ($P = 0.487$) from each other for all extraction methods. DPPH values are given in Fig. 3. Pop et al. (2021) reported 77 µmol Trolox Equiv. g^{-1} meal DPPH for walnut kernels and Ojeda-Amador et al. (2018) reported 149 µmol g $meal^{-1}$.

The extraction methods had no significant effect on L^* color values of the extracts ($P = 0.107$). The b^* values were highest for Method 1. There was a significant difference in

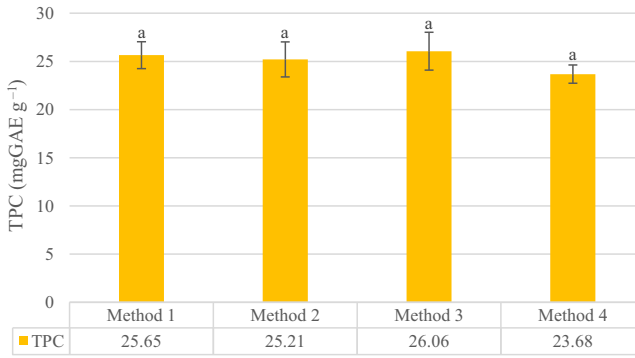

Fig. 1. Results of total polyphenol content (TPC) of the walnuts; Superscript letters above error bars, a: no different groups based on the statistical analysis by extraction methods

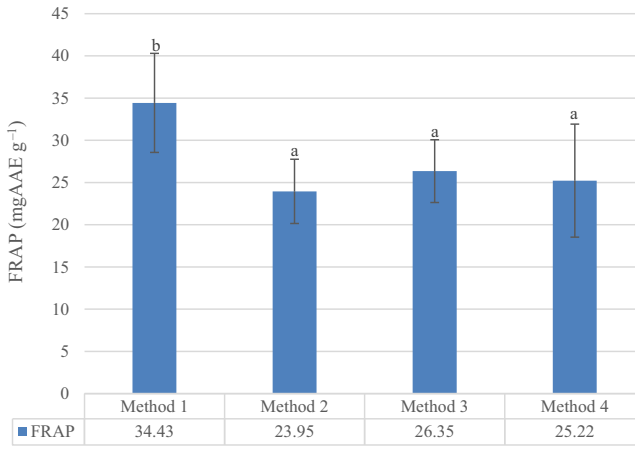

Fig. 2. Results of antioxidant capacity (FRAP) of the walnuts; Superscript letters above error bars, a, b: indicate significance difference by extraction methods

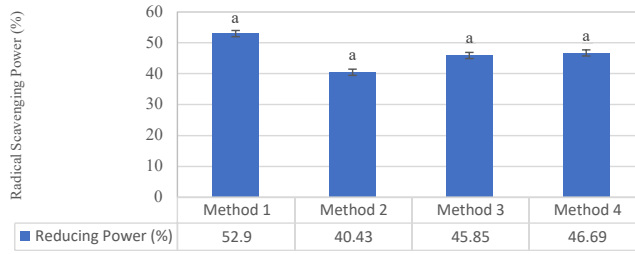

Fig. 3. Results of DPPH values of the extracts; Superscript letters above error bars, a: no different groups based on the statistical analysis by extraction methods

the results of a^* ($P < 0.005$) and b^* ($P < 0.0001$) color values. The results for L^*, a^* and b^* are presented in Table 2.

Based on Table 3, the difference between Method 1–3 and Method 2–4 is "hardly noticeable". However, the difference between Method 1–2, 1–4 and 2–3 is "Visible" by human eye.

During the quantitative determination of individual polyphenols, chlorogenic acid was present in a significantly higher amount ($P < 0.005$) in Method 1 compared to others. We found 2.54 mg g^{-1} chlorogenic acid in walnut kernels using Method (1). Romano et al. (2021) found 23.27 (mg 100 g^{-1} extract) chlorogenic acid using methanol and 16.16 (mg 100 g^{-1} extract) using Supercritical CO_2 + Ethanol as extraction solvents in walnuts. The amounts of other polyphenols like rutin ($P = 0.111$), catechin ($P = 0.233$), epicatechin ($P = 0.195$) were also higher in samples extracted using Method (1) but they were not significantly higher than in samples extracted by other methods. The results for individual polyphenols are expressed in mg g^{-1} and are presented in Table 4. Trandafir et al. (2017) found 4.1 mg 100 g^{-1} catechin; 13.3 mg 100 g^{-1} epicatechin and 35.5 mg 100 g^{-1} rutin in the walnut kernels.

According to Table 5, a strong correlation can be shown between TPC and DPPH ($R^2 = 0.802$), so the radical scavenging capacity generally caused by polyphenolic compounds

Table 2. L^*, a^* and b^* values of extracts

Color values of extracts			
	L^*	a^*	b^*
Method 1	25.98 ± 0.31[a]	−0.35 ± 0.10[b,c]	6.96 ± 0.32[b]
Method 2	28.28 ± 1.83[a]	−0.73 ± 0.09[a]	5.35 ± 0.39[a]
Method 3	26.63 ± 0.57[a]	−0.15 ± 0.05[c]	5.93 ± 0.11[a]
Method 4	27.59 ± 0.81[a]	−0.48 ± 0.23[ab]	5.68 ± 0.15[a]

[a-c]: significance difference by extraction methods.

Table 3. ΔE^* color values of extracts

ΔE^* color values				
Method 1–2	Method 1–3	Method 1–4	Method 2–3	Method 2–4
2.83	1.23	2.06	1.84	1.04

Table 4. Results of analysis of individual polyphenols

Amount of individual polyphenols in walnuts (mg g^{-1})				
Extraction Methods	Chlorogenic acid	Catechin	Epicatechin	Rutin
Method 1	2.54 ± 0.15[b]	2.80 ± 0.93[a]	3.22 ± 0.82[a]	3.61 ± 0.11[a]
Method 2	2.18 ± 0.13[a]	3.59 ± 0.07[a]	2.00 ± 0.03[a]	3.25 ± 0.05[a]
Method 3	1.95 ± 0.08[a]	3.53 ± 0.13[a]	2.32 ± 0.68[a]	3.11 ± 0.04[a]
Method 4	2.06 ± 0.10[a]	3.38 ± 0.06[a]	2.58 ± 0.79[a]	3.35 ± 0.42[a]

[a-b]: significance difference by extraction methods

Table 5. Correlation between the variables

	TPC	FRAP	DPPH	L*	a*	b*	Chlorogenic acid	Cathecin	Epicathecin	Rutin
TPC		0.376	0.802	–0.515	0.460	0.390	0.185	–0.125	0.029	–0.143
FRAP			0.758	–0.867	0.409	0.990	0.834	–0.964	0.925	0.812
DPPH				–0.925	0.782	0.808	0.374	–0.566	0.565	0.235
L*					–0.808	–0.926	–0.453	0.754	–0.807	–0.458
a*						0.529	–0.159	–0.254	0.405	–0.105
b*							0.749	–0.941	0.932	0.749
Chlorigenic acid								0.301	0.731	0.919
Cathecin									–0.969	–0.929
Epicathecin										0.863
Rutin										

can be easily measured in walnut extracts using the DPPH method. FRAP was strongly correlated with chlorogenic acid ($R^2 = 0.834$), epicatechin ($R^2 = 0.925$), and rutin ($R^2 = 0.812$), so primarily these phenolic compounds cause the antioxidant activity based on iron-reducing ability. This relationship also appears in the color analysis of the extracts, since most of the phenolic compounds tested show a strong correlation with the b* values, which represent the yellowish shade. A correlation can be seen between several of the tested phenolic compounds, e.g. chlorogenic acid was associated with epicatechin and rutin, and epicatechin was correlated to rutin.

CONCLUSION

Bioactive compounds such as polyphenols have been proven to be beneficial in a number of diseases and in healthy living. The modern lifestyle requires more and more bioactive compounds in the diet. Thus, in addition to fatty acids, more emphasis should also be placed on walnuts polyphenols. Walnuts are one of the best sources of polyphenols. With the advent of technology and new research more and more information about walnut polyphenols is coming into light. Fundamental scientific research is essential for expanding our understanding of nature and the compounds present in walnuts. This knowledge can pave the way for further discoveries in nutrition and medicine. In summary, research on walnut polyphenols is crucial for advancing our understanding of their potential health benefits and their broader impact on human well-being, as well as for promoting sustainable agriculture and innovation in the food industry.

We chose to find out more about walnut polyphenols extraction. Our results showed that shaking walnut sample and solvent in a shaking water-bath at 50 °C for 30 min could be more suitable for extracting higher amount of polyphenols with high antioxidant power. However, there is a requirement of more research on stability of polyphenols and further experiments should be done to evaluate their efficacy on human health.

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