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ORIGINAL PAPER

Analysis of Wild Raspberries (*Rubus idaeus* L.): Optimization of the Ultrasonic-Assisted Extraction of Phenolics and a New Insight in Phenolics Bioaccessibility

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Abstract

A simple and efficient ultrasonic-assisted extraction (UAE) technique was developed in order to find optimal conditions for the extraction of total phenolic compounds, flavonoids and anthocyanins in wild raspberry (*Rubus idaeus* L.) fruits. Several extraction variables, including methanol composition (*v*/v, %), solid-solvent ratio (g/mL), time (min) and extraction temperature (°C) were optimized using response surface methodology (RSM). Under optimal conditions for extraction, the total phenolics were found in the concentration of 383 mg GAE/100 g of fresh fruit weight, while HPLC-PDA analysis of the optimized extract showed the presence of cyanidin-3-glucoside, cyanidin-3-sophoroside, catechin, gallic and ellagic acid. The experimental values of DPPH and ABTS radical scavenging activities were 29.0 and 39.5 μmol Trolox/g of fresh fruit weight, respectively. *In vitro* simulated gastrointestinal digestion showed great raspberry phenolics stability. Our study assessed the bioaccessible phenolics in wild raspberry fruits and showed optimal conditions for the effective extraction of bioactive compounds for their analysis.

Keywords *Rubus idaeus* L. · Extraction · HPLC-PDA · Cyanidin-3-glucoside · Antioxidant activity · *In vitro* digestion

Introduction

The interest in natural products as potential antioxidants is constantly increasing during recent years. Wild raspberries (*Rubus ideaus* L.) are a member of the Rosaceae family and have long been collected and consumed worldwide [1], especially because they are recognized for their possible health benefits [2]. There is an increasing interest in raspberries as a valuable source of bioactive compounds, such as anthocyanins, flavonoids and phenolic acids [3]. Biological activities of the raspberries, such as antioxidant [1], antimicrobial [4] and anticancer [5] are linked to present phenolic compounds, such as hidroxycinnamic acid, gallic acid, galloyl esters, ellagic acid conjugates, flavonols, and anthocyanins [6]. Many studies confirmed that raspberries belong to a group of the fruit of high antioxidant properties and consumption of this fruit is also recommended in the prevention of cardiovascular diseases and type II diabetes [2, 7]. The bioavailability of phenolics in raspberries depends on genetic differences, the cultivar type, growing location etc. [8]. There are a number of studies dealing with the phenolic composition of domesticated raspberry cultivars, but very scarce data about wild ecotypes were reported, especially growing in Serbia. Hence, the aim of this study was the quantification of the biologically important phenolic compounds and determination of the antioxidant activity level of this wild fruit cultivar growing in Serbia.

The extraction procedure is a very important step in the separation and identification of compounds in examined samples. In comparison with conventional solvent extraction techniques, the use of ultrasound for extraction of phenolic compounds has been reported as a faster, solvent saving and more effective method. Ultrasonic waves are capable to cause a cavitation effect, leading to an accelerated release of the target compounds and the extraction rate increase [9]. The efficiency of the UAE can be affected by several variables, such as

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temperature, solvent composition, extraction time, and solid-solvent ratio [10]. Consequently, the aim of this study was to optimize these variables in order to develop the most efficient method for extraction of bioactive compounds in wild raspberries fruits (WRF), to identify and quantify these compounds and to evaluate the antioxidant capacity of this fruit species. Taking into account that after consumption of the fruit, its bioactive compounds must be released from the fruit matrix during digestion to allow their bioaccessibility and provide health benefits, the changes in concentrations of the identified phenolic compounds in WRF during *in vitro* gastrointestinal digestion were also determined.

Material and Methods

Chemicals and Plant Material

Gallic acid, ellagic acid, catechin, cyanidin-3-glucoside and cyanidin-3-sophorozide, DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) and ABTS$^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) were purchased from Sigma Aldrich (Deisenhofen, Germany). HPLC grade acetonitrile and formic acid were purchased from Fisher scientific UK (Leics, UK). *Rubus idaeus* L. (wild raspberries) fruits (approximately 500 g) were collected in South-western Serbia at village Jabuka (43°21′N 19°31′E) at an altitude of 1196 m in August 2017. The voucher specimen (no. 132/019) was prepared and deposited in the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Kragujevac, Serbia, after the identification of species.

Preparation of the Wild Raspberry Extracts and Experimental Design for Extraction Optimization

Wild raspberry samples were frozen at −20 °C after harvesting and used within a week. The defrosted material was homogenized using a laboratory mill (IKA homogenizer, IKA – Werke GmbH & Co. KG, Staufen, Germany). Samples (1 g) were extracted using methanol:water solvent with different methanol composition (20, 60, 100, *v*/v%) at different temperatures (40, 60, 80 °C) and for different extraction times (1, 8, 15 min) in an ultrasonic bath (Bandelin Sonorex RK 52 H, Bandelin electronic GmbH & Co. KG, Berlin, Germany) which was coupled with circular thermostat (Lauda Alpha A6, LAUDA-Brinkmann, Delran, NJ, USA) in order to keep the temperature constant to prevent overheating. The ultrasonic power in the vessel was determined by calorimetric test [11, 12] (Suppl. Data S1). The volume of the solvent was 10, 20 or 30 mL for 1 g of the sample. Thirty extracts were prepared with different combinations of these four variables obtained in Design-Expert® 7.0 software (Stat-Ease, Inc., Minneapolis), as presented in Suppl. Table S3 (Central composite design (CCD) with the responses of the dependent variables). Liquid extracts were separated using filtration (paper filter, Whatman No. 1) and kept at 4 °C until further analysis of total phenolics, flavonoids and anthocyanins content (TPC, TFC and TAC, respectively) in the period of two days. Optimization experiment was carried out using response surface methodology (RSM) for extraction of total phenolics, flavonoids and anthocyanins in the wild raspberry fruits (WRF). More detailed information is given in Suppl. Data S2.

RP-HPLC-PDA Analysis of Phenolic Compounds

Phenolic compounds analysis was performed on an HPLC system (Shimadzu Prominence, Kyoto, Japan) with a PDA detector (SPD-M20A). The separation was carried out using a Luna (Phenomenex, Torrance, CA, USA) C18 column. This method was performed according the method adopted from Tavčar-Benković et al. [13] with slight modifications and detailed information about the instrument and the method is given in the Supplementary Material S3 (RP-HPLC-PDA Analysis of Phenolic Compounds).

Spectrophotometric Measurements

Determination of Phenolics Content Total phenolic content (TPC) in WRF extracts was determined using Agilent UV/Vis spectrophotometer (Santa Clara, CA, USA), by the Folin–Ciocalteu reagent method described by Singleton et al. [14]. The results were calculated from a standard calibration curve based on gallic acid and expressed as mg of gallic acid equivalents (GAE) *per* 100 g of fresh weight (mg GAE/100 g FW). The total flavonoid concentration (TFC) was assessed using the aluminum chloride spectrophotometric method [15]. The results were expressed as mg of rutin equivalents (RUE) *per* 100 g of fresh weight (mg RUE/100 g FW). For the quantification of anthocyanins content (TAC) in the tested extracts, the pH differential method [16] was applied and the results were expressed as cyanidin-3-glucoside equivalents (mg CGE/100 g FW).

DPPH and ABTS Radical Scavenging Activities The DPPH radical scavenging activity method was performed according to the procedure by Kumarasamy et al. [17], whereas the total ABTS$^{\bullet+}$ scavenging activity of the samples was determined according to Re et al. [18]. The results were expressed as Trolox equivalents *per* gram of fresh fruit weight (μmol TE/g FW).

In Vitro Gastrointestinal Digestion Procedures

Simulation of *in vitro* gastrointestinal digestion of the fresh samples was performed according to the methods of Minekus

et al. [19], as previously described by Mihailović et al. [20]. There were two phases for *in vitro* digestion simulation, including the gastric phase and the intestinal phase. Digested raspberry samples for HPLC analysis of phenolic compounds were taken 0, 1, 2, 3 and 4 h after the experiment started. Individual sample tubes were prepared for each time point and after the digestion completed, frozen for further HPLC analyses.

Statistical Analysis

All samples were analyzed in triplicate. Data are reported as the mean ± SD. The results were statistically analyzed with a one-way analysis of variance (ANOVA) using the SPSS statistical software package, version 13.0 (IBM Analytics, Armonk, NY, USA). The results were considered to be statistically significant at $p < 0.05$.

Results and Discussion

Optimization of the Extraction Conditions In order to obtain the highest yields of total phenolics, flavonoids and anthocyanins, four extraction variables (solvent volume, methanol composition, sonication time and extraction temperature) were optimized by statistical experimental design. To understand the effect of methanol concentrations on the extraction of phenolics, the solvent concentration was fixed at different levels because the aqueous solvents extracts are considered to be more efficient than its undiluted forms. The effect of extraction temperature, time and ratio of sample to solvent influences the recovery of phenolics, showing conflicting results of solubilization and degradation of phytochemicals [21]. In our experimental design, all variables were fixed at three levels (−1, 0, 1), *i.e.*, methanol concentration (20, 60, 100%), sample to solvent ratio (1:10, 1:20, 1:30), sonication time (1, 8, 15 min), and extraction temperature (40, 60, 80 ° C). The detailed report of these variable combinations is presented in Suppl. Table S2 (independent variables and their coded and actual values used for optimization). Optimization of the extraction process was carried out by applying a quadratic polynomial equation. The experimental design is shown in Suppl. Table S3. The statistical analysis (ANOVA) and more detailed information about individual statistical parameters of the calculated models are given in Suppl. data S4 (Optimization of Extraction Conditions) and Suppl. Table S4 (The fitted quadratic model in terms of coded variables for Y_1, Y_2 and Y_3 responses). The positive linear effect of extraction time (X_3) and extraction temperature (X_4) were found to be significant for all response variables. However, the quadratic effect of temperature (X_4^2) was found to produce the most significant positive and negative effect on total phenolic content. The most significant interaction between variables is X_2X_4 (sample to solvent ratio/extraction temperature). For the simultaneous optimization of the four responses, a modification of the method developed by Derringer and Suich [22] was used and described in Suppl. Data S5 (Optimization desirability function). The relationship between the most influential factors and responses (combined effects of the methanol concentration and solid to solvent ratio on the extraction yield of total phenolic content) is illustrated by the 3D representation of the response surfaces and Suppl. Fig. S1 shows the 3D response surface obtained for the desirability function of the WRF extract. The partial desirability functions (d_i) of each response and the calculated geometric mean as the maximum global desirability function ($D = 0.996$) are presented in Suppl. Fig. S2 (Bar graph for an individual (d_i) and combined (D) desirability values). Using these conditions, the maximum achieved phenolics content efficiency was 99.6%. This result indicates the suitability and accuracy of the model. Design-Expert® software was used for determining the optimal extraction conditions by maximizing the desirability of the responses. The optimal conditions for the extraction of TPC, TFA and TAC in a single experiment were – methanol concentration (20%), a sample to solvent ratio (10.04 mL/g), extraction time (15 min) and extraction temperature (80 ° C).

Phenolic Compounds of Wild Raspberry Fruit The extraction under obtained optimal conditions showed that the total phenolics (Table 1) concentration in WRF was 383 mg GAE/100 g FW. The applied extraction procedure was also optimized for extraction of total flavonoids and anthocyanins, which concentrations in WRF were found to be 37.6 and 15.9 mg CGE/100 g FW, respectively. Compared to available literature data, result obtained for total phenolic content (TPC) in WRF is significantly higher than TPC found in wild raspberries from Western Serbia (approximately 1.5 mg GAE/kg FW), which may indicate that extraction conditions in our research are optimized for more efficient extraction of these compounds [23]. TPC determined in our extract was also significantly higher than TPC found in species of wild raspberries native to East Asia (Korean), which was 921.84 mg GAE/kg FW, but content of anthocyanins (TAC) found in Korean wild raspberries was slightly higher (179.79 mg CGE/kg FW) than TAC determined in our WRF

Table 1 The contents of total phenols (TPC), total flavonoids (TFC), total anthocyanins (TAC) and free radical scavenging activities of WRF extract

TPC	TFC	TAC	DPPH$^{\bullet}$	ABTS$^{\bullet+}$
383.0 ± 12	37.6 ± 1.2	15.9 ± 0.6	29.0 ± 1.1	39.5 ± 1.3

TPC is expressed as mg GAE/100 g fresh weight (FW); TFC is expressed as mg RUE/100 g FW; TAC is expressed as mg CGE/100 g FW; DPPH and ABTS are expressed as μmol TE/g FW; WRF-wild raspberry fruit

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

extract [24], which may be ascribed to botanical differences of these two species and different growing conditions [8]. TPC obtained in our study is in agreement with the phenolic ranges in wild raspberries (approximately 400 mg GAE100g FW) from Romania [25]. According to Mikulic-Petkovsek et al. [26], TPC in wild raspberries growing in Central Slovenia was 2232 mg GAE/kg FW, which is significantly lower than TPC found in our sample. No data was found in the literature about the total flavonoid content in raspberry fruit expressed in the same units as was in our study. Compared to cultivated raspberry cultivars [8, 27], wild raspberries showed a significantly higher or similar content of total phenolics. Sariburun et al. [28] assessed the anthocyanins content in cultivated ranging from 12.4 to 69.5 mg CGE/100 g FW and our result fits in this range.

Chemical Composition RP-HPLC-PDA analysis of extracts obtained under the optimal conditions demonstrated that the WRF extract contained gallic acid, flavanol catechin, anthocyanins cyanidin-3-glucoside and cyanidin-3-sophoroside, and ellagic acid (Suppl. Fig. S3). Among identified compounds (Table 2), cyanidin-3-glucoside (43.6 mg/100 g FW) and ellagic acid (28.6 mg/100 g FW) were present in the WRF in the highest concentrations. Anthocyanin cyanidin-3-sophoroside was detected in a significantly ($p < 0.05$) lower amount (11.5 mg/100 g FW) compared to cyanidin-3-glucoside. Similar phenolic profile of raspberry fruits was demonstrated by Dantas et al. [1], where cyanidin-3-glucoside was reported as the dominant compound in raspberry fruits frozen pulp, while they did not report any evidence of cyanidin-3-sophoroside and ellagic acid. Anthocyanins are well-known antioxidants and there are literature data that confirm that anthocyanins operate as protective agents against fluidization of cancer membrane [29]. Moreover, the same authors confirmed the anticancer activity of cyanidin-3-glucoside on a human breast cancer cell line. Ellagic acid was determined in Korean wild raspberry in the concentration of 108 mg/kg FW, which is significantly lower than ellagic acid content found in our WRF extract [24]. Milivojevic et al. [23] determined ellagic acid in the wild raspberries from Western Serbia, and its concentration was 12.71 μg/g FW, which is about 20-fold lower than ellagic acid content found in our WRF extract. Considering the results of HPLC analysis obtained in our study, the high content of cyanidin-3-glucoside in WRF indicates that this fruit could be a good natural source of antioxidants with significant biological properties. Ellagic acid, which was detected as the second dominant compound in our analyzed extract, was also reported to exhibit a wide range of biological activities, such as antiviral, antibacterial, cancer preventive and antioxidant [27].

Table 2 Phenolic composition of WRF extract obtained under optimal conditions (mg/100 g FW ± SD)

Compound	R_t (min)	Concentration (mg/100 g FW)
Gallic acid	8.32	0.986 ± 0.009^{a}
Cyanidin-3-glucoside	10.45	43.61 ± 0.16^{b}
Cyanidin-3-sophoroside	10.83	11.46 ± 0.08^{c}
Catechin	13.23	16.36 ± 0.11^{d}
Ellagic acid	15.08	28.67 ± 0.11^{e}

WRF-wild raspbberry fruit; FW-fresh weight; results are presented as mean ± SD

($n = 3$); means with superscripts with different letters are significantly different at $p < 0.05$

Free Radical Scavenging Activities The antioxidant activities of the WRF extract obtained under optimal extraction conditions were evaluated and expressed as Trolox equivalents per gram of fresh fruit weight using DPPH and ABTS radical scavenging activity assays (Table 1). The DPPH radical scavenging method, based on the reduction of DPPH radical in the presence of the hydrogen and electron-donating antioxidants and the tested extract, showed the activity of 29.0 μmol TE/g FW. Also, the extract we tested exhibited effective radical cation scavenging activity, with the value of 39.5 μmol TE/g FW. ABTS radical scavenging activity of this WRF extract proved to be approximately 6-fold higher than activity obtained for Brazilian red raspberry which was 6.27 μmol TE/g FW [30]. Results from the present study revealed that raspberry DPPH radical scavenging activity was slightly higher than antioxidant activity of Brazilian raspberry frozen pulp (1071 μM TEAC/100 g dry weight). All phenolic compounds detected by HPLC analysis are well-known antioxidants, which contributes to the antioxidant activity of the tested WRF extract.

Effect of *In Vitro* Gastrointestinal Digestion on Phenolic Compounds The health benefits of the plant food are closely related to its chemical composition and bioactivity of these molecules depends on their release from the food matrix, their stability during gastrointestinal digestion, and their absorption from the intestine [1]. In this study, we have incorporated *in vitro* simulated gastrointestinal digestion to analyze the stability of WRF phenolic compounds. Changes in the concentration of identified phenolic compounds, analyzed by RP-HPLC, in samples obtained at different times of *in vitro* simulated digestion of fresh raspberries are presented in Table 3. Results showed that the gastric level (after 1 and 2 h) of gallic acid decreased, while its concentration significantly ($p < 0.05$) increased in the intestinal simulated fluid after 3 and 4 h of digestion compared to the first hour of the digestion. Anthocyanins cyanidin-3-glucoside and cyanidin-3-sophoroside, as well as catechin, demonstrated a high degree of release after 1 h of digestion (gastric phase), while their concentrations were slightly affected during the intestinal

Table 3 Phenolic compounds content in the wild raspberries (mg/100 g FW) during simulated *in vitro* gastrointestinal digestion

Time	Gallic acid	Cyanidin-3-glucoside	Cyanidin-3-sophoroside	Catechin	Ellagic acid
0 h	2.61 ± 0.05^{a}	25.5 ± 0.30^{a}	4.16 ± 0.06^{a}	1.01 ± 0.03^{a}	–
1 h	0.556 ± 0.014^{b}	31.2 ± 0.53^{b}	4.40 ± 0.08^{b}	6.03 ± 0.10^{b}	1.50 ± 0.03^{a}
2 h	0.418 ± 0.009^{c}	21.3 ± 0.45^{c}	3.35 ± 0.05^{c}	5.12 ± 0.08^{c}	1.10 ± 0.07^{b}
3 h	0.908 ± 0.015^{d}	24.4 ± 0.48^{d}	4.52 ± 0.06^{b}	4.38 ± 0.06^{d}	0.906 ± 0.05^{c}
4 h	1.82 ± 0.02^{e}	27.1 ± 0.49^{e}	4.32 ± 0.07^{b}	4.98 ± 0.07^{e}	0.768 ± 0.04^{d}

FW-fresh weight; results are mean values ± SD from three measurements ($n = 3$); means in the same column with superscripts with different letters are significantly different at $p < 0.05$ (− not detected)

phase of digestion. Ellagic acid also seemed to be resistant against gastric conditions, while its intestinal level was found to be declined.

Overall, other studies that analyzed the bioaccessibility of phenolic compounds from different raspberries products (extracts, juices or pulps) reported that flavonoids and phenolic acids show higher bioaccessibility than anthocyanins [1]. According to McDougall et al. [31], all the total phenolics and anthocyanins of the raspberry extract survived gastric digestion and all eight anthocyanins identified in raspberry were detected in the extract and the postgastric samples at similar yields, which is in agreement with the results obtained in our study. Our results describe the release of phenolic compounds from raspberry fruit during digestion suggesting that anthocyanins, flavonoids, and phenolic acids, may remain in relatively high concentrations to exert beneficial effects on the organism. The results of *in vitro* digestion together with results of chemical extraction confirmed that although chemical extraction showed certain quantities of phenolic compounds in WRF, not all of these quantities are fully bioavailable for absorption.

Conclusions

The results of this study may serve to expand the knowledge about the present bioactive compounds in the analyzed WRF and indicate that they are a valuable source of phenolics, flavonoids, and anthocyanins as potentially biologically active compounds. RSM was successfully applied to obtain the optimized variables for extraction of the phenolic compounds from WRF. To the best of our knowledge, this is the first study in which UAE has been optimized for the simultaneous extraction of total phenolics, flavonoids, and anthocyanins. This efficient and simple UAE technique together with the HPLC method could be used in further studies based on the examination of phenolic compounds from similar fruit and food sources. The results of chemical composition, antioxidant capacities and simulated *in vitro* digestion show that WRF can be used as an easily accessible source for dietary intake of natural antioxidants and the development of new food supplements and pharmaceutical products.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

Human and Animal Studies This article does not contain any studies with human or animal subjects.

Informed Consent Informed consent was obtained from all individual participants included in the study.

Abbreviations *ABTS,* 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); *CCD,* Central composite design; *CGE,* Cyanidin-3-glucoside equivalents; *DPPH,* 2,2-diphenyl-1-picrylhydrazyl; *FW,* Fresh weight; *GAE,* Gallic acid equivalents; *HPLC,* High performance liquid chromatography; *PDA,* Photo diode array; R_t, Retention time; *RSM,* Response surface methodology; *RUE,* Rutin equivalents; *TAC,* Total anthocyanins content; *TFC,* Total flavonoids content; *TPC,* Total phenolics content; *UAE,* Ultrasonic-assisted extraction; *UV,* Ultra violet; *Vis,* Visible; *WRF,* Wild raspberry fruit

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