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Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique

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ABSTRACT

Traditional maceration method was used for the extraction of polyphenols from chokeberry (*Aronia melanocarpa*) dried fruit, and the effects of several extraction parameters on the total phenolics and anthocyanins contents were studied. Various solvents, particle size, solid–solvent ratio and extraction time have been investigated as independent variables in two level factorial design. Among examined variables, time was not statistically important factor for the extraction of polyphenols. The optimal extraction conditions were maceration of 0.75 mm size berries by 50% ethanol, with solid–solvent ratio of 1:20, and predicted values were 27.7 mg GAE/g for total phenolics and 0.27% for total anthocyanins. Under selected conditions, the experimental total phenolics were 27.8 mg GAE/g, and total anthocyanins were 0.27%, which is in agreement with the predicted values. In addition, a complementary quantitative analysis of individual phenolic compounds was performed using HPLC method. The study indicated that maceration was effective and simple technique for the extraction of bioactive compounds from chokeberry fruit.

1. Introduction

Chokeberry (*Aronia melanocarpa* [Michx] Elliot, Rosaceae) is a perennial shrub native to North America, and it was introduced to Eastern Europe, Scandinavia, and Russia in early 20th century (Kokotkiewicz, Jaremicz, & Luczkiewicz, 2010; Kulling & Rawel, 2008). Fully ripened chokeberry fruits contain different phenolic compounds such as proanthocyanidins, flavan-3-ol and flavonol glycosides, and phenolic acids (Rugina et al., 2012; Suiero et al., 2006). Moreover, chokeberry represents one of the richest plant sources of anthocyanins exhibiting strong antioxidant activity (Braunlich et al., 2013). Anthocyanins and proanthocyanidins play important role in human nutrition and the growing interest for their utilization is mainly due to their antioxidant potential and the association between their consumption and the prevention of cancer, coronary heart disease, diabetes and other degenerative disorders (Kokotkiewicz et al., 2010; Ovando, Hernandez, Hernandez, Rodriguez, & Galan-Vidal, 2009; Sainova et al., 2012).

The main source of chokeberry fruit on the market originate from plantations, and beside fresh fruits various processed products like juices, jams, jellies as well as extracts and dietary supplements are available (Gonzales-Molina, Moreno, & Garcia-Viguera, 2008; Kokotkiewicz et al., 2010; Kulling & Rawel, 2008). For the products of pharmaceutical, cosmetic and food industry, quality of the extracts as component of the products is critical and increment of the amount of biologically active phenolics in extracts is a challenging task. Extraction is the first and important step in isolation and purification of bioactive components from plant material. Various extraction techniques can be applied for polyphenol recovery from plants, and generally these techniques can be divided into traditional and modern ones. The traditional extraction methods include maceration, maceration assisted with stirring, and Soxhlet extraction. In recent years a new techniques have been used for the extraction of bioactive compounds including ultrasound-assisted extraction, microwave-assisted extraction, sub- and supercritical fluid extraction and accelerated solvent extraction (Khoddami, Wilkes, & Roberts, 2013). Each technique has its own advantages and disadvantages, but the main goal of the chosen method is the achievement of complete extraction of the compounds of interest and avoidance of their chemical modification. Extraction efficiency is influenced by several factors such as type and concentration of solvent, solid–solvent ratio, time, temperature, pH, etc. However, in the most studies the influence of a single factor has been explained while the interactions between the factors have not been examined thoroughly. Therefore, in order

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to optimize the extraction conditions it would be useful to investigate the influence of different factors on extraction of chokeberry polyphenols using experimental design strategy. Factorial design is a good and simple statistical tool that allows the simultaneous study of the effects that several factors may have on an optimization of a particular process. It also allows measuring the interaction between each factor in order to achieve the best overall optimization of the process.

Many studies have reported extraction of polyphenols from different plant material, but literature data concerning optimization of polyphenol extraction from chokeberry is very scarce. Only optimization of ultrasound-assisted extraction of polyphenols from chokeberry and chokeberry by-products has been studied previously (Galvan D'Alessandro, Dimitrov, Vauchel, & Nikov, 2014; Galvan D'Alessandro, Kriaa, Nikov, & Dimitrov, 2012; Ramić et al., 2015). Considering that phenolic molecules possesses beneficial effects on human health, and the industry set up demands for reduction of production costs, it is worthwhile to investigate the optimal conditions for the efficient extraction of chokeberry polyphenols.

In the present study, the effects of four factors (time, solid–solvent ratio, solvent type and particle size) on the extraction efficiency of polyphenols from chokeberry dried fruit were analyzed using factorial design in order to optimize experimental procedure using maceration as traditional technique for the extraction. Moreover, quantitative analysis of individual phenolic compounds, i.e. flavonoids and anthocyanins by HPLC was also performed.

2. Materials and methods

2.1. Plant material

Berries were collected in August 2013, at fully ripened stage from plantation located at mountain Suvobor, Serbia (44°08′16.04″N, 20°10′56.28″E, 779 m a.s.l.). Soil type was Calcocambisol, weakly skeleted with rock (<25%), well drained, with slightly acidic reaction (pH = 6.2). Climate was continental and characterized with average rainfall precipitation of 50 mm and average minimal–maximal temperature range of 3.3–27.3 °C during vegetation period (March–September). Beside pruning and regular weeding no other agro-technical measurements were applied. Collected berries were dried in a tunnel dryer at 40 °C and moisture content was 10.65 ± 1.39%.

Dried berries were grounded by industry mill and obtained particles were separated using sieves into 5 particle sizes according to Yugoslav Pharmacopoeia (Ph Yug V, 2000). The samples were stored at room temperature before the extraction.

2.2. Reagents and standards

Folin–Ciocalteu phenol reagent (Sigma–Aldrich, Steinheim, Germany), sodium carbonate, methanol, formic acid, and orthophosphoric acid were purchased from (Sigma–Aldrich Chemie GmbH, Munich, Germany). Ethanol was of analytical grade, acetonitrile (Merck, Germany) was of HPLC grade, and ultra pure water was prepared using a Milli-Q purification system (Millipore, France). The anthocyanin standards cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-arabinoside, and flavonoids quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-galactoside (hyperoside) and quercetin-3-*O*-glucoside (isoquercetin) were purchased from Extrasynthese (Cedex, France). Gallic acid was obtained from Sigma–Aldrich (Steinheim, Germany).

2.3. Extraction procedures

Maceration in an Erlenmeyer flask (100 mL) was performed on a shaker (Unimax 1010, Heidolph, Germany) with agitation fixed on 170 rpm, at ambient temperature. An initial step was carried out in order to determine the optimum and rational duration of extraction, because maceration usually requires performance over a longer period of time. The experiments were performed under selected conditions for polyphenols extraction based on the literature data. Duration of extraction was studied with 50% ethanol (Galvan D'Alessandro et al., 2012; Ramić et al., 2015), 1:20 solid–solvent ratio (Cacace & Mazza, 2003; Galvan D'Alessandro et al., 2012), and 2 mm particle size (as a medium sized particle). The samples were collected at 15 min, 30 min, 60 min, 90 min, 2 h, 2.5 h, 5 h, 10 h and 18 h. As the extraction time of more then 90 min had no significant influence on the amount of phenolics (data not shown), the optimization of the extraction was further monitored at 15, 30, 60 and 90 min.

In the next set of experiments, the influence of each factor on the extraction process has been studied and the results are shown in the preliminary screening. Each factor in all levels was separately tested in combinations with other investigated factors. To find out the suitability of the solvent for extraction of polyphenols, four different solvents levels, 25 mL of distilled water, 50%, 70% and 96% mixture ethanol–water were used. Three levels of the solid–solvent ratio (1:10, 1:20 and 1:30) were studied in order to select out suitable ratio for extraction, and five levels of particle classes (6, 3, 2, 1, and 0.75 mm) were extracted to find out the optimal particle size.

Ultrasound-assisted extraction was performed in an ultrasonic bath (bath power 35 W, continuous mode at frequency of 40 kHz, Maget, Bela Palanka, Serbia) for 30 and 60 min. The generator of ultrasounds was placed on the lateral sides of the bath, and an Erlenmeyer flask (100 mL) was positioned in the same distance from three sides. The berry extract in the Erlenmeyer was 5 cm below the surface of water in the bath and sonicated without agitation. The solvent volume (50% ethanol) was 25 mL, solid–solvent ratio was 1:20, and 0.75 mm sieve was used.

2.4. Design of experiments

2.4.1. Preliminary screening of process levels

The selection of the process levels of each factor that have significant influence on the extraction of total phenolics (TP) and total anthocyanins (TA) has been performed. Statistical significance among factor levels has been estimated on triplicate samples through one-way ANOVA followed by Duncan's multiple range test at $p < 0.05$ level. Data in charts were presented as means coupled with vertical bars which denote 0.95 confidence intervals of triplicate measurements. Means followed by different letters in charts and tables differ significantly, based on Duncan's test at $p < 0.05$ level. Selected two levels with the highest yields of both observed quality parameters (TP and TA) were subjected to further factorial designs.

2.4.2. Factorial design

Two experimental design methods were used for the screening and optimization of process factors. In the first factorial design (Plackett–Burman design), four independent variables time, solid–solvent ratio, solvent type and particle size, each at two levels, were screened forming the 2^4 full factorial design (Table 1). The purpose of this step is to identify which variable have significant effect on the total phenolics and total anthocyanins contents.

Based on the results obtained in the first factorial design, a new 2^3 full factorial design was employed to investigate the effect and

Table 1
The factor levels used in 2^4 full factorial design.

Factor	Notation	Factor levels	
		Low (−)	High (+)
Solid–solvent ratio	1	1:20	1:30
Ethanol concentration (%)	2	50	70
Sieve (mm)	3	1.00	0.75
Time (min)	4	60	90

choose the optimum values of solid–solvent ratio (1), solvent type (2) and particle size (3) on the total phenolic content and total anthocyanins content (dependent variables). Each factor was tested at two most promising levels using the upper and lower limits chosen on the basis of preliminary screening.

For statistical analysis of factorial design multivariate ANOVA has performed using STATISTICA 7.0 software, where factor influence on total phenolic content and total anthocyanin content were observed through absolute values of standardized estimated effects, and presented on Pareto charts with level of significance set at $p \leqslant 0.05$ for the first factorial design. For the second factorial design, observed and predicted means for each dependent variable are presented in Table 2. The effects and corresponding regression coefficients of factors and factor interactions are listed in Table 3. For each analysis of factorial design residuals were normally distributed.

2.5. Total phenolics

The total phenolic content in the extracts was determined by a modified Folin–Ciocalteu method (Waterman & Mole, 1994). Briefly, 200 μL of extracts were added to 1 mL of 1:10 diluted Folin–Ciocalteu reagent. After 4 min, 800 μL of sodium carbonate (75 g/L) were added. After 2 h of incubation at room temperature in the dark, the absorbance at 765 nm was measured by a spectrophotometer. Gallic acid (0–100 mg/L) was used for calibration of a standard curve. The results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW). Triplicate measurements were taken and mean values were calculated.

2.6. Total anthocyanins

The total anthocyanin content was investigated according to the procedure described in European Pharmacopoeia 6.0. (2008) with slight modifications. The absorbance of the solution was measured at 528 nm, using a 0.1% (v/v) solution of hydrochloric acid in methanol as the compensation liquid. The percentage content of anthocyanins extracts, expressed as cyanidin-3-*O*-glucoside chloride, was calculated from expression: $A \times 5000/718 \times m$ (A = absorbance at 528 nm; 718 = specific absorbance of cyanidin-3-glucoside chloride at 528 nm; m = mass of dried chokeberry to be examined in grams). Triplicate measurements were taken and mean values were calculated.

Table 2
Experimental design for screening of factor influence on total phenolics and total anthocyanins content with the observed and predicted values.

S–S ratio	Solvent EtOH	Sieve (mm)	Total phenolics content (mg GAE/g DW)		Total anthocyanins content (%DW)	
			Observed	Predicted	Observed	Predicted
1:20 (−1)	50% (−1)	1.00 (−1)	21.31	21.41	0.208	0.208
1:20 (−1)	50% (−1)	0.75 (+1)	27.82	27.72	0.273	0.272
1:20 (−1)	70% (+1)	1.00 (−1)	21.85	21.74	0.210	0.210
1:20 (−1)	70% (+1)	0.75 (+1)	26.30	26.41	0.258	0.259
1:30 (+1)	50% (−1)	1.00 (−1)	20.37	20.27	0.203	0.202
1:30 (+1)	50% (−1)	0.75 (+1)	24.79	24.89	0.244	0.245
1:30 (+1)	70% (+1)	1.00 (−1)	19.64	19.75	0.205	0.206
1:30 (+1)	70% (+1)	0.75 (+1)	22.83	22.73	0.235	0.234

Table 3
Statistical analysis of extraction optimization using 2^3 factorial design.

	Effect	Std. Err.	Effect estimates	Coeff.	Std. Err. Coeff.	*P*
Total phenolics						
Constant				23.115	0.259	0.000
Main factors						
S–S ratio (1)	−2.797	0.517	−5.404	−1.398	0.259	0.000
Solvent (2)	−0.988	0.517	−1.909	−0.494	0.259	0.073
Sieve (3)	4.320	0.517	8.346	2.160	0.259	0.000
Interaction of two factors						
1 by 2	0.308	0.517	0.595	0.154	0.259	0.560
1 by 3	−0.792	0.517	−1.531	−0.396	0.259	0.144
2 by 3	−0.268	0.517	−0.517	−0.134	0.259	0.611
Total anthocyanins						
Constant				0.230	0.001	0.000
Main factors						
S–S ratio (1)	−0.015	0.003	−5.125	−0.008	0.001	0.000
Solvent (2)	−0.005	0.003	−1.555	−0.002	0.001	0.128
Sieve (3)	−0.046	0.003	−15.408	−0.023	0.001	0.000
Interaction of two factors						
1 by 2	0.001	0.003	0.462	0.001	0.001	0.646
1 by 3	0.011	0.003	3.532	0.005	0.001	0.001
2 by 3	0.007	0.003	2.393	0.004	0.001	0.021

2.7. HPLC analysis

2.7.1. Quantitative analysis of anthocyanins

Analyses were carried out on Agilent series 1200 RR HPLC instrument (Agilent, Waldbronn, Germany), with DAD detector, on a reverse phase Lichrospher RP-18 (Agilent) analytical column (250 × 4 mm i.d., 5 μm particle size). The mobile phase consisted of solvent A (10% of formic acid in water) and solvent B (acetonitrile). Samples were separated by gradient elution according to the following scheme: 1% B 0–0.5 min; 1–7% B 0.5–1 min; 7% B 1–4 min; 7–10% B 4–7.5 min; 10–14% B 7.5–11.5 min; 14–25% B 11.5–15.5 min; 25–40% B 15.5–18.5 min; 40–75% B 18.5–22 min; 75% B 22–25 min. Flow was adjusted to 1 mL/min, and detection wavelengths were set at 290, 350 and 520 nm. Quantification was done using calibration curves of anthocyanin standards cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-arabinoside. All experiments were repeated at least three times. The results are expressed as mean value ± standard deviation in milligrams per grams of dry weight (mg/g DW).

2.7.2. Quantitative analysis of flavonoids

Analyses were carried out on Agilent series 1200 RR HPLC instrument (Agilent, Waldbronn, Germany), with DAD detector, on a reverse phase Lichrospher RP-18 (Agilent) analytical column (250 × 4 mm i.d., 5 μm particle size). The mobile phase consisted of solvent A (1% solution of orthophosphoric acid in water) and mobile phase B (acetonitrile), using the gradient elution as follows: 90–80% A 0–5 min, 80% A 5–20 min, 80–40% A 20–30 min, 40–0% A 30–35 min. Detection wavelengths were set at 260 and 350 nm, and the flow rate was 1 mL/min. The amounts of the quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside were calculated using calibration curve. All experiments were repeated three times. The results are presented as milligrams per grams of dry weight (mg/g DW).

3. Results and discussion

3.1. Preliminary screening

To determine the optimal levels of each factor for the extraction of total phenolics (TP) and total anthocyanins (TA), a preliminary screening was performed. The purpose of this screening was to find out two most promising levels of each factor which exhibit significant influence on the yield of TP and TA which will be further included in full factorial design.

3.1.1. Effect of extraction time

The influence of extraction time on the TP and TA contents is shown in Fig. 1A and B, respectively. The extraction efficiency of both observed quality parameters was improved as the duration of the extraction increased. The maximum yields of both TP and TA were achieved at 90 min (13.3 mg GAE/g DW and 0.15%, respectively), but no significant difference was observed between 60 min and 90 min. Two stages of extraction could be observed, an initial increase of the concentration of polyphenols in the beginning of the process followed by slow extraction (after 60 min) characterized by a low enhancement of polyphenol content with the progress of extraction. Galvan D'Alessandro et al. (2012) have also reported that under sonication TP and TA extraction yields increased rapidly with the extraction time for the first 15 min and slowly in the next 4 h.

Since the both observed parameters (TP and TA) obtained maximal yields at longer extraction periods (60 min and 90 min), these factor levels have been chosen to be included in full factorial design.

3.1.2. Effect of solid–solvent ratio

The effect of solvent volume on the extraction yield of TP and TA was evaluated (Fig. 1C and D, respectively). The results indicated that yield of TP increased gradually from 1:10 solid–solvent ratio and achieved the highest value at 1:30 ratio (13.4 mg GAE/g DW). On the other hand, different trend was observed for TA, their extraction yield increased significantly from 1:10 to 1:20 followed by the slight decrease at 1:30. For both observed parameters, solid–solvent ratio of 1:10 showed significantly lower yields than it was achieved at 1:30 and 1:20 ratios. The results are in accordance with the previous findings that higher solid–solvent ratio leads to the higher yield of polyphenols (Galvan D'Alessandro et al., 2012). It should be noted that higher solid–solvent ratio generate a decrease in the consumption of plant material and decrease in the cost of extraction.

Since the solid–solvent ratio 1:10 produced lower yields of both observed parameters, ratios 1:20 and 1:30 have been chosen to be included in full factorial design.

3.1.3. Effect of solvent type and concentration

In order to investigate the effect of different solvents on the extraction efficiency, ethanol (at concentrations of 50%, 70% and 96%) and water were used. The results revealed that the yield of TP was maximized at 50% ethanol (18.2 mg GAE/g DW) and significantly decrease with further increase in ethanol concentration (Fig. 1E). Similar pattern was observed regarding TA where the highest yield was also achieved with 50% ethanol (0.21%), followed by non-significant decrease when extracted with 70% ethanol (Fig. 1F). Significantly lower yields of TA were obtained with other two solvents, 96% ethanol and water. Galvan D'Alessandro et al. (2012) also reported that the use of medium concentration for ethanol/water mixture (50%) resulted in higher TP yield compared with water or other ratios of the ethanol/water mixture. Our results are in accordance with previous studies which reported that binary solvent system containing hydro-organic solvents was superior than mono-component solvent system (pure water or ethanol) in the extraction of phenolic compounds (Cacace & Mazza, 2003; Zhang et al., 2007). Water plays an important role in swelling of plant material, whereas ethanol is responsible for disrupting the bonding between the solutes and plant matrix thus enabling better mass transfer of the compounds. Therefore, the mixture of water and ethanol as solvent agent shows synergistic effect which facilitates phenolic extraction from chokeberry fruit.

Taking into account that the yields of TP and TA obtained with 50% and 70% ethanol have been much higher than those obtained with 96% ethanol and water, these factor levels were chosen to be included in further factorial design.

3.1.4. Effect of particle size

The extraction efficiency regarding TP content showed improvement as the particle size decreased (Fig. 1G). Yields obtained with particle sizes 1 and 0.75 mm were significantly higher (14.9 and 15.4 mg GAE/g DW, respectively) than those obtained with larger particle sizes of 2, 3 and 6 mm (11.8, 11.4 and 10.5 mg GAE/g DW, respectively). Similarly, the smallest particle size (0.75 mm) had the highest yield of TA (0.178%), while other classes of particles (1, 2 and 3 mm) achieved lower yields with no statistical differences between them (Fig. 1H). Significantly lower yield of total anthocyanins was obtained from 6 mm sieve fraction (0.125%). Our results are in accordance with previous report where the extraction from grounded chokeberries was more efficient than the extraction from berries cut in half (Galvan D'Alessandro et al., 2012). Smaller particle size had higher contact surface which allows the increase of mass transfer. Since both TP and TA exhibited maximal yields with a smaller particle size, sieves 1 and 0.75 were chosen to be included in full factorial design.

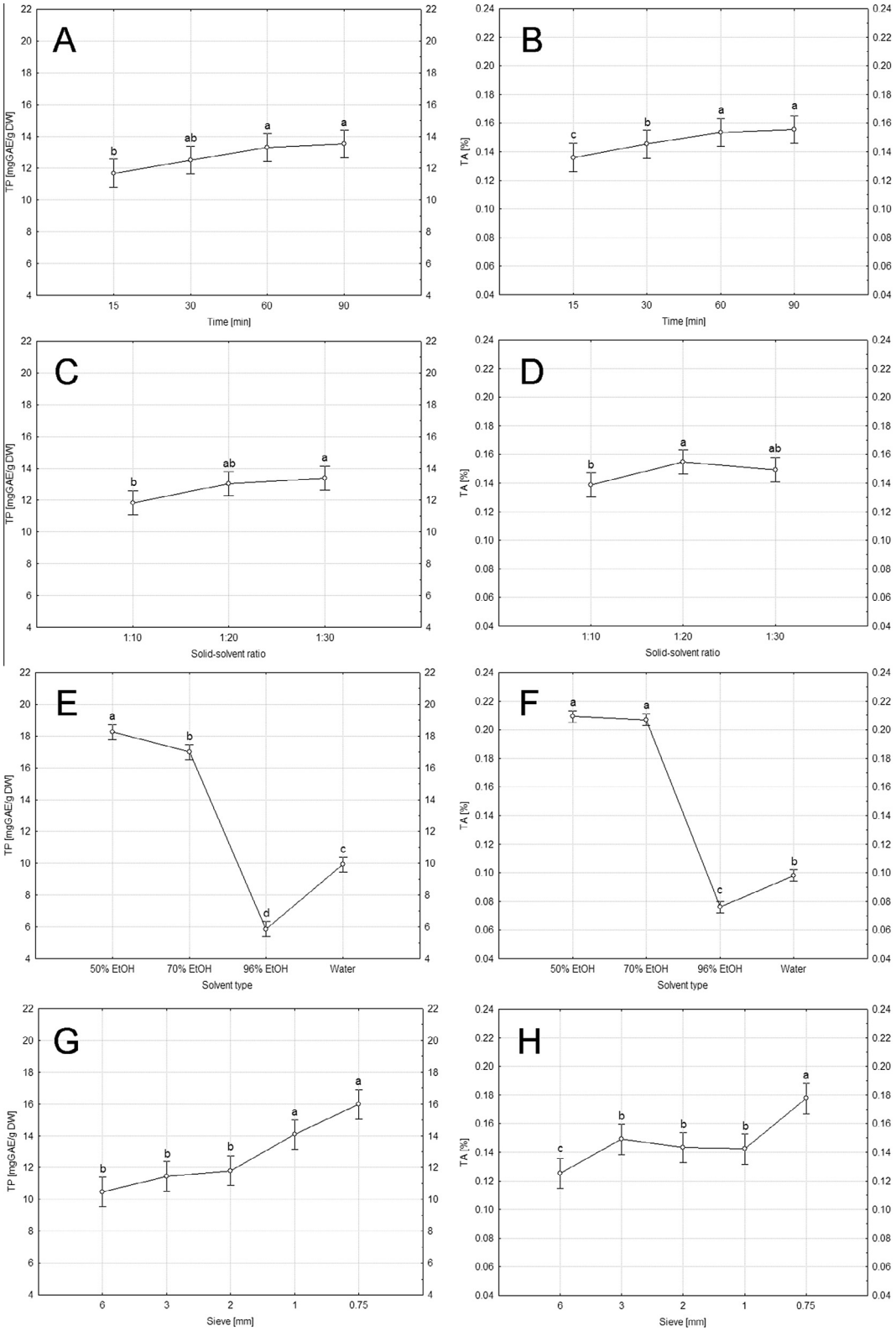

Fig. 1. Preliminary screening of each factor level's influence on total phenolics (TP) and total anthocyanins content (TA) in dried chokeberry fruits; (A) (TP) and (B) (TA) – extraction time; (C) (TP) and (D) (TA) – solid–solvent ratio; (E) (TP) and (F) (TA) – solvent type; (G) (TP) and (H) (TA) – sieve diameter. Vertical bars on graphs denote 0.95 confidence intervals.

3.2. Optimization of extraction conditions

Optimization procedures involve simultaneous alteration of all experimental factors studied according to an experimental design. The levels of independent factors for the extraction of polyphenols were selected based on the results obtained from our preliminary experiments, and they are included in the 2^4 full factorial design (Table 1). The Pareto chart (Fig. 2) presents the factors that have

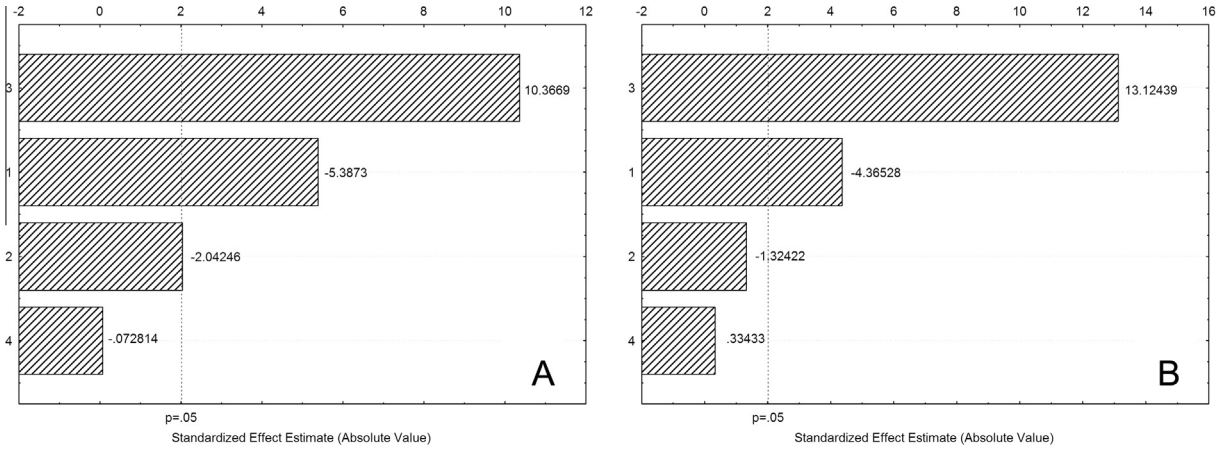

Fig. 2. Pareto diagrams of screened factor influence on the yields of TP (A) and TA (B) in the extraction process; factor codes: 1, solid–solvent ratio; 2, solvent type; 3, particle size; 4, time.

a significant effect on the response obtained at $p < 0.05$. The lengths of the bars are proportional to the absolute value of the estimated effects and the values which are located on the right of the dashed line are significant. It could be observed that particle size (factor 3) and solid–solvent ratio (factor 1) were the most significant factors for the yields of TP and TA (Fig. 2A and B). Similar observation has been also reported by Cacace and Mazza (2003) for black currants. In their study, ethanol concentration and solid–solvent ratio play a significant role in the extraction of total phenolics, while the major factor that influenced extraction of anthocyanins was solid–solvent ratio. Extraction time (factor 4) was the least important single factor for obtaining high yields of TP and TA at 5% probability level. Therefore, this factor was excluded from the optimization step and the optimum extraction time was fixed at 60 min.

A 2^3 full factorial design was performed to further investigate the influence of solid–solvent ratio, solvent type and particle size on the yields of TP and TA (Table 2), as well as to determine the optimum values. This method optimization also allows the evaluation of the interactions between the factors. The main effects, interaction effects, coefficients of the model, and probability for the full 2^3 factorial design are presented in Table 3. It can be seen that the particle size was the most relevant factor concerning the content of total phenolics. The second important factor for obtaining high yield of TP was the solid–solvent ratio whose absolute value was lower than particle size factor. The main factor solvent concentration as well as all interactions between factors were not significant since their probability level was higher than 0.05. For the total anthocyanin content, the results indicated that particle size was the most significant factor for their extraction. Solid–solvent ratio was also significant factor for obtaining high yield of TA, with lower absolute value compared with particle size. The third important factor was the interaction of solid–solvent ratio and particle size, followed by interaction of solvent concentration and particle size. The two non-significant factors at 95% of confidence level were solvent concentration and interaction between solid–solvent ratio and solvent concentration.

The observed results and predicted values of extraction of total phenolics and total anthocyanins according to the factorial design are presented in Table 2. The highest yield of both TP and TA (27.82 mg GAE/g DW and 0.273%, respectively) was observed under experimental parameters of 50% ethanol, solid–solvent ratio of 1:20 and particle size of 0.75 mm. The lowest content of TP (19.64 mg GAE/g DW) was recorded with 70% ethanol, 1:30 solid–solvent ratio and 1 mm particle size, whereas lowest yield

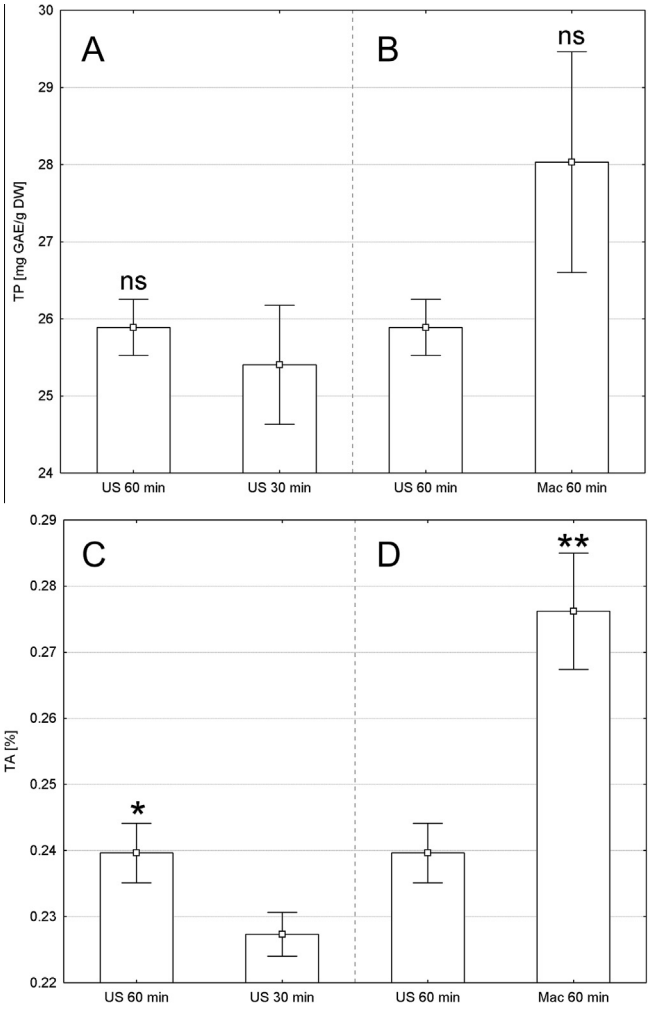

Fig. 3. Effects of extraction procedures on the TP (A and B) and TA (C and D) content in dried chokeberry fruits; (A and C) different duration of ultrasound-assisted extraction; (B and D) comparison of ultrasound-assisted extraction with maceration process; labels on bars are given according to the Student's *t*-test: ns, not significant; * at $p < 0.05$; ** at $p < 0.01$.

of TA was obtained at 50% ethanol, 1:30 solid–solvent ratio and particle size of 1 mm (0.203%). There is a close agreement between the observed values of TP and TA amounts and theoretical values predicted by factorial design. The model predicted a maximum

Table 4
Content of anthocyanins and flavonoids (mg/g DW) in chokeberry extracts determined by HPLC analysis.

S–S ratio	Solvent (EtOH) (%)	Sieve (mm)	Cyanidin-3-galactoside	Cyanidin-3-glucoside	Cyanidin-3-arabinoside	Quercetin-3-rutinoside	Quercetin-3-galactoside	Quercetin-3-glucoside
1:20	50	1.00	0.40 ± 0.04 d	0.08 ± 0.01 c	0.14 ± 0.01 d	0.38 ± 0.01 c	0.27 ± 0.01 a	0.14 ± 0.01 a
1:20	50	0.75	0.81 ± 0.06 a	0.14 ± 0.02 a	0.30 ± 0.01 a	0.42 ± 0.01 a	0.27 ± 0.03 a	0.15 ± 0.07 a
1:20	70	1.00	0.48 ± 0.01 c	0.08 ± 0.01 c	0.17 ± 0.01 c	0.31 ± 0.01 e	0.22 ± 0.01 c	0.12 ± 0.01 b
1:20	70	0.75	0.85 ± 0.06 a	0.13 ± 0.01 a	0.32 ± 0.03 a	0.36 ± 0.05 d	0.25 ± 0.02 b	0.13 ± 0.05 b
1:30	50	1.00	0.42 ± 0.01 d	0.07 ± 0.01 c	0.15 ± 0.01 d	0.32 ± 0.02 e	0.17 ± 0.01 d	0.10 ± 0.01 c
1:30	50	0.75	0.74 ± 0.08 b	0.13 ± 0.01 a	0.29 ± 0.01 b	0.41 ± 0.05 b	0.23 ± 0.01 c	0.12 ± 0.02 b
1:30	70	1.00	0.40 ± 0.01 d	0.07 ± 0.01 c	0.14 ± 0.01 d	0.36 ± 0.01 d	0.22 ± 0.01 c	0.12 ± 0.01 b
1:30	70	0.75	0.71 ± 0.12 b	0.11 ± 0.01 b	0.27 ± 0.03 b	0.40 ± 0.01 b	0.22 ± 0.01 c	0.12 ± 0.01 b

Means followed by different letters in the column differ significantly based on Duncan's test at $p < 0.05$.

extraction of TP and TA (27.72 mg GAE/g DW and 0.272%, respectively) under the same factor levels combination (50% ethanol, 1:20 solid–solvent ratio and 0.75 mm particle size) as obtained from the real experiments. The good correlation between these results confirmed that full factorial design was adequate to reflect the expected optimization.

3.3. Ultrasonic-assisted extraction

In order to evaluate the effect of ultrasonic on the yields of TP and TA during extraction under selected optimal conditions determined by factorial design, extraction was performed in an ultrasonic bath for 30 and 60 min and the results are presented in Fig. 3. Extract obtained by maceration contained higher yield of TP (27.6 mg GAE/g, Fig. 3A and B) than both extracts obtained by ultrasonic-assisted extraction for 30 and 60 min (25.4 and 25.9 mg GAE/g, respectively). TA content (Fig. 3C and D) was significantly higher ($p < 0.01$) in maceration extract (0.27%) than in ultrasonic extracts obtained after 30 and 60 min (0.23% and 0.24%, respectively). These preliminary results indicate that maceration could be preferable method for the extraction of polyphenols from chokeberry fruit under tested conditions.

3.4. HPLC analysis

The quantification of individual anthocyanins and flavonoids in chokeberry extracts afforded under different extraction conditions was carried out using an HPLC method, and the results are shown in Table 4. The main anthocyanin compound was cyanidin-3-galactoside, followed by cyanidin-3-arabinoside and cyanidin-3-glucoside. Among flavonoids, the dominant were quercetin derivatives, such as quercetin-3-glucoside, quercetin-3-galactoside and quercetin-3-rutinoside. The highest content of the majority of all individual compounds was achieved with extraction parameters of 50% ethanol, 1:20 solid–solvent ratio and 0.75 mm particle size, which was also shown for the TP and TA contents. Only cyanidin-3-galactoside and cyanidin-3-arabinoside were obtained in highest amount under experimental conditions of 70% ethanol, 1:20 solid–solvent ratio and 0.75 mm particle size. Extracts obtained when 0.75 mm particles were used contained higher amounts of flavonoids and anthocyanins compared with those obtained with 1 mm particle size. This result confirms previous statistical finding that particle size was the most relevant factor concerning the contents of total phenolics and total anthocyanins.

4. Conclusion

An effective maceration technique for extracting polyphenols from chokeberry was optimized using two experimental designs. The first 2^4 factorial design showed that solvent, solid–solvent ratio and particle size were significant factors, whereas time was not relevant in affecting the total phenolic and total anthocyanins contents. The second 2^3 design enabled us to determine the optimal values for the significant factors. This optimization showed that the best conditions for obtaining high yields of total phenolics and total anthocyanins were 50% of ethanol, solid–solvent ratio of 1:20 and particle size of 0.75 mm. HPLC analysis showed that the same extraction conditions provided the highest yield of the majority of individual anthocyanins and flavonoids. The study indicated that applied factorial design was adequate model for optimization of extraction process and there was a good agreement between predicted and observed values. Our results suggest that maceration was effective and simple method for the extraction of phenolic compounds from chokeberry fruit.

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