Journal of Food and Nutrition Research (ISSN 1336-8672) Vol. *56*, 2017, No. 3, pp. 299–303

SHORT COMMUNICATION

Pressurized hot water extraction followed by high-performance liquid chromatography for determination of polyphenols in *Sambucus nigra* L. branches in dependence on vegetative period of the plant

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Summary
Pressurized hot water extraction (PHWE) was optimized for the determination of selected polyphenols (catechin, epicatechin, rutin, quercetin and chlorogenic acid) in *Sambucus nigra* L. branches. Pressure 15 MPa, temperature 100 °C and extraction time 1 × 5 min were found to be sufficient for the extraction of target polyphenols from the matrix. Chlorogenic acid and rutin were the most abundant substances, the highest contents of them were observed in December (922 mg·kg^{-1} for rutin and 108 mg·kg^{-1} for quercetin).

Keywords
Sambucus nigra L. branches; pressurized hot water extraction; high performance liquid chromatography; polyphenols

Sambucus nigra L., commonly known as black elder, is a wide-branched shrubby tree growing up to 10 m. It is native in Europe and it also grows in Asia, North Africa, and the United States. All parts of the plant have been used for generations in traditional medicine because of their positive effects on the human health (e. g. reduction of coronary heart disease, antioxidant properties), which are mainly ascribed to the presence of numerous taste- and health-related compounds (e.g. sugars, vitamins, and polyphenols) [1]. These compounds can be used as food additives in a class of foods with declared health-promoting effects.

The elderberry-processing industry produces a large amount of by-products, with the branches as the most abundant waste, which could be used as alternative sources of valuable bioactive compounds for pharmaceutical or cosmeceutical industries (e.g. polyphenols). Nowadays, the branches of the plant are mainly used as a valuable source of ribosome inactivating proteins (RIPs) and lectins [2]. To our best knowledge, only one study about polyphenols content in *S. nigra* branches has been recently reported [3]. The authors compared the polyphenolic profile, total phenols, anthocyanin contents and antioxidant activity of the extracts from the branches with those of elderberries.

Pressurized hot water extraction (PHWE) is recognized as a fast, effective, less labour intensive and environmentally friendly extraction technique for extraction of biologically active compounds from plant matrices [4, 5]. The method utilizes water as an extraction solvent at a temperature above the atmospheric boiling point of water (100 °C/373 K) but below the critical point of water (374 °C/647 K) at a sufficiently high pressure to keep the water in the liquid state. It brings several benefits including higher solubility of analytes, enhanced mass transfer processes and easier penetration of the solvent into the sample matrix micropores. Moreover, the relative permittivity of water in the liquid state decreases noticeably as the temperature increases, which makes it possible to employ water as an extraction solvent for moderately polar or non-polar compounds. On

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the other side, degradation of the compounds and a variety of reactions, such as hydrolysis or oxidation, can occur with the increased temperature. Theory and principle of the extraction was reviewed in several papers [6–9].

The aim of this study was to investigate cultivated *S. nigra* branches, variety Bohatka, as a potential source of polyphenols. For this purpose, PHWE was optimized as a sample treatment method for the determination of polyphenols in elderberry branches by high-performance liquid chromatography with diode-array detection (HPLC-DAD). Moreover, the influence of vegetative stage of the plant on the abundance of individual polyphenols was examined.

MATERIALS AND METHODS

Chemicals and standards

Acetonitrile, methanol and formic acid, all HPLC-grade, were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Water was purified by a reverse osmosis system Ultra Clear UV (SG Wasseraufbereitung und Regenerierstation, Barsbüttel, Germany). Standards of (+)-catechin hydrate, (–)-epicatechin, rutin, quercetin and chlorogenic acid were obtained from Sigma-Aldrich. Stock solutions of all target compounds (1 $g \cdot l^{-1}$ of each) were prepared in methanol and stored at 5 °C. Anthracene, used as internal standard, was supplied by Sigma-Aldrich.

Plant material

Branches of cultivated *Sambucus nigra* L. variety Bohatka, grown in sub-region Hustopeče (South Moravia region, Czech Republic), were used in all experiments. The whole branches were collected during autumn 2015 (September – December), dried, ground to 4 mm size and stored in brown glass vials at room temperature.

Pressurized hot water extraction of *Sambucus nigra* branches

Pressurized hot water extraction (PHWE) was performed an extraction apparatus of in-house design, described previously by Pol et al. [10] at a static arrangement. A portion (2 g) of the sample mixed at 1:1 (v/v) with glass beads (570–700 μm) was put into 11 ml stainless steel extraction cell, flushed with nitrogen to prevent oxidation of the analytes and extracted at a pressure of 15 MPa, temperature 100 °C, during 5 min in a single extraction step. A 20 s rinsing time and a 90 s nitrogen purge time were programmed for each extraction cycle. The extracts were passed through a Strata SDB-L SPE cartridge (bed weight 500 mg, cartridge volume 6 ml; Phenomenex, Torrance, California, USA). The SPE cartridge was conditioned with 2 ml of methanol and then with 2 ml of water. The crude extract was applied and target compounds were eluted by 2 ml of methanol. The internal standard for HPLC analysis was added to the SPE eluate and the solution was injected to HPLC apparatus. The recovery of extraction process, including SPE clean-up step, was determined by addition of a defined volume of a standard solution of known concentration to real sample before performing the extraction procedure mentioned above. The recovery of whole extraction process was ≥ 93 %.

HPLC-DAD analysis

The analysis of the extracts was carried out on Agilent 1200 series HPLC-DAD system (Agilent Technologies, Santa Clara, California, USA) using a Synergi Hydro-RP column (4.6 mm × 250 mm, 4 μm particle size; Phenomenex) that was kept at 30 °C throughout the run. Mobile phase was composed of acetonitrile (A) and water containing 0.3 % formic acid (B). The following gradient program was employed: linearly from 5% A to 60% A for 15 min, then linearly from 60% A to 100% A for 5 min and isocratically for 15 min 100% A, at a constant flow rate 0.5 $ml \cdot min^{-1}$. The column was re-equilibrated at initial conditions (5% A) for 10 min. The injection volume was 20 μl and the analytes were detected at 280 nm and 360 nm. Identification of target compounds was based on comparison of their retention times with those of authentic standards and on the method of standard addition. Quantitative analysis was performed by the internal standard method using anthracene as the internal standard. The content of the analytes was expressed in milligrams per kilogram of dry weight (DW).

RESULTS AND DISCUSSION

HPLC-DAD method validation

Standard calibration solutions were prepared in a concentration range of 5–50 $\mu g \cdot ml^{-1}$, 30 μl aliquot of internal standard solution (500 $\mu g \cdot ml^{-1}$) was added to 1 ml of each calibration level and the solutions were analysed by HPLC-DAD system. The calibration curves were constructed by linear regression of the peak-area ratio of individual standard to the internal standard versus the concentration. Triplicate injections were made for each calibration level. The determined limit of detection (*LOD*, $s/n = 3$), limit of quantification

Tab. 1. Parameters of calibration curves constructed for the analysed compounds.

Compound	t_R [min]	*a*	*b*	r^2	*LOD* [ng·ml^{-1}]	*LOQ* [ng·ml^{-1}]	*RSD* [%]
Chlorogenic acid	13.1	11.54	–0.138	0.9995	15.6	52.0	2.2
Catechin	13.4	1.139	–0.020	0.9997	30.2	101.0	2.7
Epicatechin	13.9	1.386	–0.029	0.9997	31.4	105.0	2.6
Rutin	14.4	9.035	–0.127	0.9991	19.5	65.0	1.1
Quercetin	19.2	18.26	–0.073	0.9994	25.4	84.7	2.3

t_R – retention time, *a* – slope, *b* – intercept, r^2 – coefficient of determination, *LOD* – limit of detection, *LOQ* – limit of quantification, *RSD* – relative standard deviation of triplicate injection.

(*LOQ*, $s/n = 10$), repeatability (*RSD*) and linearity (r^2) are summarized in Tab. 1, together with retention times of individual standards.

Optimization of pressurized hot water extraction

Optimization of the extraction parameters was performed with branches of *S. nigra* collected in November. The main parameters that influence the extraction efficiency of PHWE include particle size, temperature, pressure, extraction time and number of extraction steps. Based on our previous experience, the extraction pressure was set to 15 MPa without any optimization. The pressure of 15 MPa was adequate not only for keeping the solvent in the liquid state but also, in our particular experimental arrangement, for improving the tightness of the system. The impact of particle size on extraction efficiency was evaluated as a parameter often overlooked in the literature. The branches were gradually ground by grinder and passed through the sieve with a defined mesh size. Three different particle size fractions were collected (4 mm, 6 mm and 10 mm) and the fractions were extracted separately at identical experimental conditions (15 MPa, 60 °C, 1 × 5 min). Approximately 15% increase of extraction yields of target analytes with the decrease of particle size fraction was observed. This difference was theoretically caused by the larger surface area per unit mass, which resulted in greater accessibility of the analyte from the matrix pores by the extraction solvent. Studying the number of extraction steps, the branches were extracted by pressurized water at 60 °C for 1 × 5 min, 2 × 5 min and 3 × 5 min using fresh water for each extraction cycle. No relevant improvement in the extraction yields of target analytes was observed after the period of 1 × 5 min. The extraction process was subsequently repeated at time periods 1 × 5 min, 1 × 10 min and 1 × 15 min. The time period 1 × 5 min was found to be sufficient for the extraction of target compounds from the plant material. Temperature is the main factor that affects the extraction efficiency and selectivity in PHWE. The impact of temperature on the extraction efficiency and stability of target compounds from *S. nigra* branches was evaluated in the range of 40–140 °C with 20 °C steps. As shown in Fig. 1, a gradual increase in extraction yields of target polyphenols with temperature up to 100 °C was noticed. At higher temperatures, a decrease in extraction yields of the analytes was observed. This decrease in the extraction yields could be due to degradation of compounds caused by hydrolysis, oxidation or various other reactions [11].

Comparison of polyphenols content in *Sambucus nigra* branches in different vegetative stages

The influence of the plant vegetative stage on the abundance of individual polyphenols in the branches was examined during plant pruning for the next vegetative season. The *S. nigra* branches were collected in September, October, November and December, extracted by PHWE at optimized

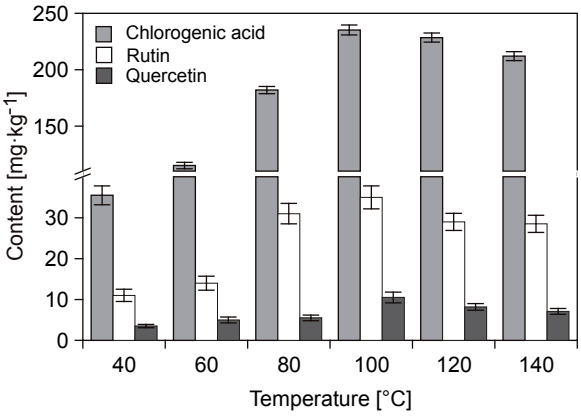

Fig. 1. Influence of temperature on extraction yields of target compounds.

The results are expressed in milligrams per kilogram of dry weight ± standard deviation ($n = 3$).

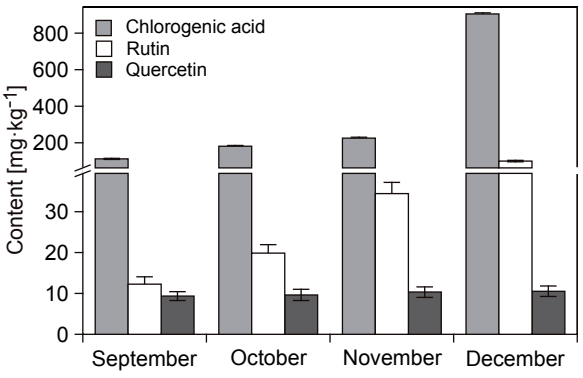

Fig. 2. Influence of picking period of *Sambucus nigra* L. branches on extraction yields of target compounds.

The results are expressed in milligrams per kilogram of dry weight ± standard deviation (n = 3).

conditions (P = 15 MPa, T = 100 °C, 1 × 5 min, particle size 4 mm) and analysed by HPLC-DAD. The results are summarized in Fig. 2. A HPLC-DAD chromatogram of PHWE extract of *S. nigra* branches is presented in Fig. 3. Catechin, rutin, quercetin and chlorogenic acid were identified in the extracts, chlorogenic acid being the most abundant substance. Catechin was found at concentrations under the limit of quantification and epicatechin was not detected. Identification of other significant peaks (e.g. at 7.9 min, 11.5 min and 18.8 min) was not feasible because the HPLC system was equipped only with DAD array detector. The calculation at least of relative extraction yields of these compounds would provide different results in dependence on wavelength of the measurement and would not match the compounds representation obtained by calibration with pure standards. The content of each analysed compound was highest in December and the lowest content of individual polyphenols was found in September. Significant differences in the abundance of chlorogenic acid and rutin along the season period were noticed. The content of chlorogenic acid ranged from 120 mg·kg^{-1} DW in September to 922 mg·kg^{-1} DW in December. Similar trend was also observed for rutin, its content ranged from 12.5 mg·kg^{-1} DW in September to 108 mg·kg^{-1} DW in December. The content of quercetin along the season was almost constant, 9.53 mg·kg^{-1} DW to 10.7 mg·kg^{-1} DW. Silva et al. [3] quantified chlorogenic acid (0.15 mg·kg^{-1}), quercetin (0.05 mg·kg^{-1}) and rutin (1.21 mg·kg^{-1})

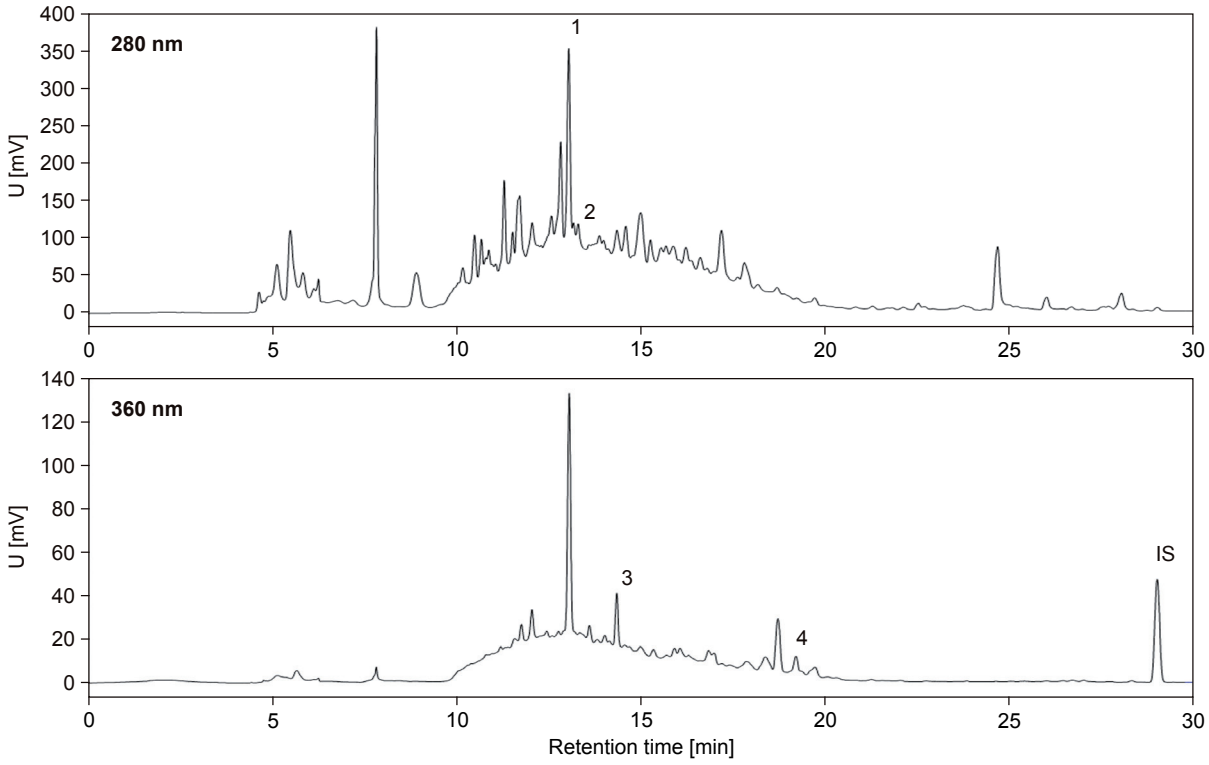

Fig. 3. HPLC-DAD chromatogram of the pressurized hot water extract of *Sambucus nigra* L. branches obtained at 100 °C with detection at 280 nm and 360 nm.

Peak identification: 1 – chlorogenic acid, 2 – catechin, 3 – rutin, 4 – quercetin, IS – internal standard.

in water extracts of elderberry branches collected in Portugal during August. Determination of catechin and epicatechin was not the object of their study. The variations were probably caused not only by different physiological stages of the plant, climatic and environmental conditions, but also by different extraction techniques (PHWE versus maceration).

CONCLUSIONS

PHWE was introduced and optimized for the extraction of polyphenols from *Sambucus nigra* branches. The extraction yields of the target compounds were mainly influenced by extraction temperature and particle size of the branches. Additionally, an interesting variance in abundance of individual polyphenols along the season period was noticed. The highest extraction yields of polyphenols were observed in December. The results may be useful to producers of nutraceuticals. Identification of other valuable compounds (e.g. amino acid, proteins) will be the subject of our next research.

Acknowledgements

This contribution has been supported by the Czech Science Foundation (Project No. 16-03749S) and the Czech Academy of Science (Institutional Support RVO:68081715).

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Received 27 February 2017; 1st revised 19 April 2017; 2nd revised 31 May 2017; accepted 7 June 2017; published online 3 September 2017.