FRACTIONATED HIGH-PRESSURE EXTRACTION OF POLYPHENOLS FROM ELDERBERRY BY-PRODUCT

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Abstract
The main focus of this study was to propose new streams to use plant waste, specifically to put on the demonstration of the possibility to implement emerging, green and safe technology into food and pharmaceutical industries in order to enable more economical waste management. For the isolation of valuable compounds from elderberry press cake (by-product left after pressing of juice), solvents and techniques considered as "acceptable" and "generally regarded as safe" in the food and pharmaceutical industries have been applied. Polyphenols rich elderberry press cake extracts were obtained employing a fractioned high-pressure methodology, supercritical fluid (SFE) and enhanced solvent extractions (ESE). SFE-CO_2+ESE comprising a first SFE-CO_2 step in order to remove the low polarity CO_2-soluble compounds, and a second ESE step in order to extract polar compounds like anthocyanins have been carried out, as well as ESE without any pretreatment. The process kinetics of the extraction and increase of extraction yields (EY) were monitored at time intervals from 10 to 90 min, while EYs of ESE were measured after 30-220 min of extraction. Higher extract yield occurred in extract obtained by SFE-CO_2+ESE (28.70%) showing its advantage over ESE. According to the kinetics results for the two-step extraction approach, extraction time of 100 min might be sufficient to achieve the similar final EY obtained after 220 min. HPLC of obtained extracts was utilized for quantification of polyphenolic compounds, showing cyanidin-3-O-sambubioside as a main compound, followed by rutin and cyanidin-3-O-glucoside. This work is the first to produce anthocyanin-rich fractions from black elderberry press cake using such high-performance technique.

Introduction
The importance of green chemistry application and its technologies in improving sustainability is growing, which is reflected in the reduction of water and energy consumption, and the use of hazardous materials, as well as in achieving sustainability in social, economic and environmental performance. Starting from the last decade of the 20th century until today, the efforts have been directed to development of new technologies that comply with strict environmental requirements, so most of the recent research focuses on the development of green technologies and their potential application in industry [1]. Since these are new technologies, it is necessary to constantly and rapidly work on the acquiring new knowledge and comprehensively expand it in all directions and in terms of all green technologies.
Therefore, this study was focused on agro-food waste which is attractive in its composition and in that sense, its processing using green techniques can give a new product with added value and functionality. Black elderberry press cake (*Sambucus nigra* L.) is a byproduct from the

production of black elderberry juice. It is mostly composed of hemicellulose and cellulose, but it also represents an important source of polyphenolic compounds [2]. Namely, the recent studies reported that the main part of the black elderberry polyphenolics - anthocyanins remain in the press cake after juice pressing [3]. Plant-based products rich in anthocyanins have a pharmacological relevance and therapeutic application due to their antioxidant properties. Anthocyanins can reduce neurological diseases and heart diseases risk, and exert an anti-inflammatory role related to obesity and diabetes [4,5]. However, despite the high nutrition and pharmacological value and economical interest in bioactive substances this by-product is underutilized.

In the recent years, thanks to new scientific cognitions related to the mechanisms of separation of compounds from complex matrices, significant progress was made in the technology of pharmacologically active compounds of natural origin and their isolation. Special attention was paid to the development of chemical processes which make use of solvents that do not pollute the environment. Beside of these solvent characteristics, focus of the scientific community was directed to the solvents which are at the same time non-toxic and safer, but efficient enough for bioactive compounds extraction from various natural resources. Therefore, for the isolation of valuable compounds from elderberry press cake, with consideration to safety, low price, selectivity and solvating properties of solvents, this study investigated the possibilities of supercritical fluid extraction (SFE) and enhanced solvent extraction (ESE). These techniques will be used in order to improve polyphenols', specifically anthocyanin's thermal stability without the need of employing high temperatures during isolation, limiting inactivation of the anthocyanins and generally achieving high extraction efficiency. The obtained extracts were analyzed by high performance liquid chromatography with diode array detection (HPLC-DAD) to identify and quantify the main phenolic compounds.

Experimental

Fresh black elderberry press cake (*Sambucus nigra* L.) was provided by a factory focusing on harvesting and processing forest fruits (NISHA d.o.o., Belgrade, Serbia). The raw material was dried using a vacuum dryer and ground in a blender. The average particle size (1.05 ± 0.16 mm) and moisture content (6.69 ± 0.13%) were determined.

The fractionated high pressure extraction assays were performed using the extraction unit described by Seabra et al. [6]. Experiments were carried out with and without a previous defatting step with SFE-CO_2 followed by ESE with CO_2/EtOH-H_2O mixture, to obtain polyphenols rich fractions in the second step. The influence of SFE-CO_2 pretreatment was studied concerning the total extraction mass yield (EY). The EY was expressed as the mass of obtained dry extract (g) per g of dry plant material, i.e., percentage (%). The process kinetics of the extraction and increase of EYs were monitored at time intervals of 10, 20, 30, 45, 60, and 90 min, while EYs of ESE were measured after 30, 40, 70, 100, 130, 160, 190, and 220 min of extraction. The ESE process conditions, pressure (20 MPa), temperature (40°C), and CO_2/EtOH/H_2O mixture proportion (80:1:19, %, v/v/v) were held constant. The same pressure and temperature were applied for SFE-CO_2 pretreatment. Extraction conditions were selected taking into consideration the results obtained by Seabra [7] and the literature information concerning the anthocyanin stability Jackman et al. [8].

Analyses of individual polyphenolic compounds were carried out on an Agilent 1200 RR system (Agilent, Waldbronn, Germany) with a diode array detector. A reverse-phase Lichrospher RP-18 (Agilent) column (250 mm × 4 mm, 5 μm) was used, and the column temperature was maintained at 25°C. The mobile phase consisted of solvent A (10%, v/v solution of formic acid in water) and solvent B (acetonitrile), using gradient elution as follows: 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. The injection volume was 10 µL, the flow rate was 1 mL/min and the detection wavelengths were set at 290, 350 and 520 nm. The contents of the compounds were calculated using calibration curves. The results are presented as micrograms per gram of dried extract (µg/g DE).

Results and discussion

Two different extraction approaches, namely fractionated high-pressure extraction (SFE-CO_2+ESE comprising a first SFE-CO_2 step in order to remove the low polarity CO_2-soluble compounds, and a second ESE step in order to extract polar compounds like anthocyanins) and only ESE, were exploited to isolate polyphenols from elderberry press cake. The elderberry press cake SFE (first step) and ESE (second step) in both extraction approaches exhibited a typical overall extraction curve profiles. For all SFE and ESE experiments, total extraction time was set to 90 and 220 min, respectively, to guarantee the diffusional period. Both experimental extraction kinetics curves regarding ESE and polar fraction isolation are clearly divided in two distinct zones (Figure 1). In the first zone, the yield increases with increasing extraction time, indicating a faster solubility of active compounds into the extraction media. In the second zone, the yield was maximized into the steady-state yield, indicating that mobility of active compounds from elderberry press cake into the extraction media approaches zero in the remaining time. In conclusion, extraction time of 100 minutes might be sufficient to achieve the similar final extraction yield obtained after 220 minutes.

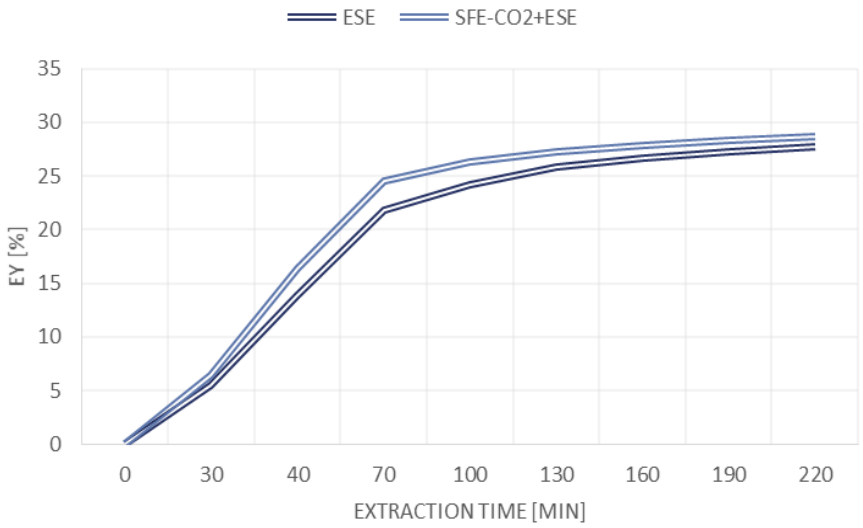

Figure 1. Kinetic curves regarding ESE and polar fractions in both extraction approaches.

During the first SFE-CO_2 step, essentially lipophilic compounds were extracted (Table 1). An extract fraction presenting a yellow color, without anthocyanins, was obtained during the first few minutes of the ESE continuous process (second step), for both extraction approaches. These fractions should contain the low polarity compounds. Higher yields of polar fractions were obtained for fractioned high-pressure extractions with ethanolic mixture in the second step (ESE) when compared to those obtained only by ESE. The main contribution to this difference was essentially due to the first step, wherein the less polar substances were extracted. Since anthocyanins are mainly located in outermost layer of elderberry skin cell walls wherein the fatty acids and waxes exist, an effective lipophilic substances removal, in a first SFE-CO_2 step,

can make polar material more available for extraction. Higher yields of polar and non-polar compounds obtained by SFE-CO_2+ESE show its advantage over ESE.

Table 1. EYs of elderberry press cake extracts obtained by ESE and SFE-CO_2+ESE.

			ESE	**SFE-CO_2+ESE**
		Extraction time [min]	EY [%]	
SFE-CO_2	non-polar fraction	10	-	1.02
		20	-	2.71
		30	-	3.80
		45	-	6.78
		60	-	8.59
		90	-	9.08
ESE	non-polar fraction	30	6.09	0.38
	polar fraction	30	5.53	6.38
		40	13.91	16.32
		70	21.80	24.48
		100	24.22	26.27
		130	25.79	27.23
		160	26.70	27.88
		190	27.25	28.31
		220	27.72	28.70

[1] The phenolic compounds in black elderberry press cake extracts were identified and quantified by HPLC-DAD analysis. The polyphenolic profile of elderberry press-cake extracts is presented in Table 2. A significant difference was observed in the concentrations of quantified compounds in ESE with and without pre-treatment, being the SFE-CO_2+ESE more effienct in terms of phenolic compounds contet. The highest concentration had cyanidin-3-O-sambubioside (2559.37 μg/g DE), followed by rutin and cyanidin-3-O-glucoside.

[2]

[3] Table 2. Characterized compounds in elderberry press cake extracts using HPLC-DAD.

[4]

Sample	C3Gal [μg/g DE]*	C3Sam [μg/g DE]*	C3Glu [μg/g DE]*	RUT [μg/g DE]*	IQ [μg/g DE]*	p-KUM [μg/g DE]*	PC [μg/g DE]*
SFE-CO_2 + ESE	60.66 ± 3.50^a	2559.37 ± 270.48^a	1187.17 ± 81.38^a	1794.70 ± 264.33^a	683.08 ± 43.90^a	21.85 ± 2.80^b	501.59 ± 50.53^a
ESE	tr	tr	tr	tr	tr	97.68 ± 5.02^a	tr

*Different letters within a column indicate a significant difference between samples at $p < 0.05$; SFE - supercritical fluid extraction; ESE - enhanced solvent extraction; DE - dried extract; C3Gal - cyanidin-3-O-galactoside; C3Sam - cyanidin-3-O-sambubioside; C3Glu - cyanidin-3-O-glucoside; RUT - rutin; IQ - isoquercitrin; p-KUM - p-kumaric acid; PC - protocatechuic acid. Tr – trace.

Conclusion

In this work, green-based extraction approaches were applied to extract mainly phenolic compounds from black elderberry press cake being a part of the zero-waste valorization concept. SFE-CO_2+ESE has proven to be highly efficient for the extraction of polyphenolic compounds from black elderberry press cake. The highest concentration was reported for cyanidin-3-O-sambubioside, followed by rutin and cyanidin-3-O-glucoside. These results showed that two step extraction approach enabled the production of natural fractions from industrial elderberry press cake with acceptable characteristics to be used for the development of nutraceuticals and different food formulations.

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