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ORIGINAL ARTICLE

Extraction methods, LC-ESI-MS/MS analysis of phenolic compounds and antiradical properties of functional food enriched with elderberry flowers or fruits

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Abstract Due to its pharmacological activity, *Sambucus nigra* L. is excellent potential component of functional food. The main aim of presented study was the quantitative and qualitative analysis (LC-ESI-MS/MS) of phenolic acids and flavonoids present in extracts obtained from corn snacks with addition of elderberry fruits or flowers. Additionally, antiradical activity of samples was determined. Moreover, the comparison of various extraction methods of polyphenols from that functional food was made. In corn snacks, without additives, nine phenolic compounds were identified. These were protocatechuic, 4-OH-benzoic, caffeic, *p*-coumaric, salicylic, ferulic acids, rutin, isoquercetin and apigenin-7-glucoside. In the extract from *Sambucus* flowers and extract from snacks containing 20% of *Sambucus* flowers fifteen polyphenols were found. They were additionally gallic, gentisic, vanillic, sinapic acids, kaempferol-3-rutinoside and astragalin. The radical-scavenging activity of the extracts was determined spectrophotometrically against DPPH· (2,2-diphenyl-1-picrylhydrazyl) radical and using thin layer chromatography. The high antiradical potential was observed for crude extracts from *Sambucus* flowers or fruits and for extracts enriched with 20%, 10%, 5% of *Sambucus* flowers. The product with a 20% addition of fruits revealed moderate free radical scavenging properties.

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Snacks enriched with *Sambucus* flowers, prepared by the extrusion process, have great potential to be a good source of natural antioxidants and may be a convenient, health-promoting product useful in the prevention of lifestyle diseases.

1. Introduction

Plants are a source of many substances affecting functioning of the human organism and its well-being (Costa et al., 2013; Sidor and Gramza-Michałowska, 2015). Antioxidant activity is one of the most desirable property of natural compounds (Campillo et al., 2015; Magiera and Zaręba, 2015; Tarola et al., 2013). Polyphenolic compounds are group of phytochemicals demonstrating excellent antiradical properties. They were found to exhibit a wide range of pharmacological activities, e.g.: anti-inflammatory (Mehta et al., 2014), immunomodulatory (Olejnik et al., 2015; Peluso et al., 2015), neuroprotective, cardioprotective, antiviral, anti-cancer (Andriani et al., 2015; Romagnolo and Selmin, 2012) and antimicrobial (Jindal and Kumar, 2012). Moreover, polyphenols are effective natural food preservatives, preventing oxidative deterioration and microbial contamination.

Sambucus nigra L. (European elderberry or black elderberry, Familia *Adoxaceae*) is a good source of biologically active components, primarily flavonols, phenolic acids and antocyanins. Polyphenols are found in its leaves, fruits and flowers (Veberic et al., 2009; Mandore et al., 2014; Sidor and Gramza-Michałowska, 2015).

S. nigra can modulate reactive oxygen species (ROS) production and concentration in the organism, thus may be useful for the prevention and mitigation of oxidative stress-related diseases (Olejnik et al., 2016).

Elderberry fruits or flowers are traditionally used because of their diuretic and diaphoretic properties, and in the treatment of viral infections, for example influenza. Many studies confirmed the benefits of *S. nigra* as an antiviral drug (Barak et al., 2001; Chen et al., 2014; Zakay-Rones et al., 1995) but also in the treatment and prevention of diabetes, cardiovascular diseases and cancers (Ciocoiu et al., 2009, 2012; Jing et al., 2008). Therefore, elderberry is one of the medicinal plants of particular interest, suitable for pharmaceutical and food purposes.

Recently consumers are looking for food, in which natural substances replaced synthetic components. A healthy lifestyle and attempt to prevent civilization-diseases contributes to diet trends with health-promoting properties. Functional foods are products, which have a potentially positive effect on health beyond basic nutrition. They contain well-known biologically active natural compounds, that promote optimal health and reduce the risk of chronic disease (Krüger et al., 2015). Plants are inexhaustible source of natural substances. Studies toward determination of plant extracts' composition are one of the most desirable part of scientific work. Numerous scientists focus attention on remarkable activities of polyphenols. These substances are often responsible for high antioxidant activity of functional food.

Due to its pharmacological activity, *S. nigra* fruits and flowers are potentially excellent components of functional food. Therefore, snacks enriched with elderberry produced for first time in the Department of Food Process Engineering, University of Life Science in Lublin were the basis of the presented study. Detailed qualitative and quantitative analysis (LC-ESI-MS/MS) of polyphenols composition in snacks without and with addition of elderberry fruits or flowers (5%, 10% and 20% addition) was performed. Influence of aforementioned additives on snacks' free radical scavenging ability was analyzed. Since the results of polyphenolic content and antiradical activity analyses are closely connected with extraction method, various extraction techniques of food product were compared.

2. Materials and methods

2.1. Chemicals

Analytical grade standards of flavonoids (astragalin, rutin, hyperoside, isoquercetin, kaempferol-3-rutinoside, quercitrin, and apigenin-7-O-glucoside) and phenolic acids (4-OH-benzoic, caffeic, *p*-coumaric, salicylic, ferulic, gallic, gentisic, vanillic, sinapic acids, protocatechuic, syringic), 2,2-diphenyl-1-picrylhydrazyl, as well as liquid chromatography grade (LC) acetonitrile were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). LC grade and analytical grade solvents were obtained from J.T. Baker (Phillipsburg, USA).

2.2. Plant materials

S. nigra L. fruits and flowers were purchased from *Herbapol* herbal industrial plant (Lublin, Poland). Plant material was dried in the air, in the shade, at average temperature 29.0 ± 0.5 °C and relative humidity 57.9 ± 6.9%. The average value of air speed was 0.003 m s^{-1}. The plant material was stored in a dry room and at 20 °C. Before proceeding dry plant material was milled and sieved.

Corn grit (Dasca Poland, Radomin, Poland) was used as basic raw material (composition: protein 9.2%, fat 1.7%, ash 0.5%, dietary fiber 7.2%).

2.3. Extrusion-cooking procedure

Blends of corn snacks and ground elderberry fruits/flowers were prepared by mixing dry components in weight ratios of 100:0, 95:5, 90:10 and 80:20. The blended samples were moistened up to 15% moisture by spraying with water and mixing continuously for 10 min. Blends were processed in a TS-45 single screw extrusion-cooker (ZMCh Metalchem, Gliwice, Poland) according to previously described method (Oniszczuk et al., 2015c). Samples were stored in polyethylene bags at room temperature.

2.4. Ultrasound assisted extraction (UAE)

Extraction was performed in an ultrasonic bath (Bandelin Electronic, Germany, 20 kHz, 100 W) for 30 min (three cycles for 10 min) at 60 °C and 40 °C. Each of 2 g portions of powdered sample was extracted with 40 mL of ethanol (Oniszczuk et al., 2015c). Extracts were filtered, combined and evaporated until dry. The residues were dissolved in 10 mL of methanol. The procedure was repeated three times.

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2.5. Accelerated solvent extraction (ASE)

ASE was carried out with a Dionex ASE 200 instrument (Sunnyvale, CA, USA) with solvent controller. Each of 2 g portions of powdered sample was extracted with ethanol at two temperatures – 100 and 120 °C, at a pressure of 60 bar, for 30 min (three cycles for 10 min). Extracts were combined and evaporated until dry. The residues were dissolved in 10 mL of methanol. The procedure was repeated three times (Oniszczuk et al., 2015b).

2.6. Solid phase extraction (SPE)

Crude extracts were purified using solid phase extraction. 5 mL of every sample (in methanol) was passed through a previously conditioned SPE C18 column (Bakerbond C18, 3 mL containing, 500 mg packing, J.T. Baker, the Netherlands).

Polyphenols were eluted with 5 mL of 30% aqueous methanol and next with 10 mL of 60% aqueous methanol. The samples were evaporated to dryness and dissolved in 10 mL of methanol (Oniszczuk et al., 2015c).

2.7. LC-ESI-MS/MS analysis of phenolic compounds

The samples were analyzed by high-performance liquid chromatography and electrospray ionization mass spectrometry (HPLC-ESI-MS/MS) according to previously described method (Oniszczuk et al., 2015c). For chromatographic separation, an Agilent 1200 Series HPLC system (Agilent Technologies, USA) equipped with a binary gradient solvent pump, a degasser, an autosampler and column oven was used. Phenolic compounds were separated at 25 °C, on Zorbax SB-C18 column (2.1 × 50 mm, 1.8-μm particle size; Agilent Technologies, USA), using 3 μL injections.

MS detection was performed in a 3200 QTRAP Mass spectrometer (AB Sciex, USA) equipped with an electrospray ionization source (ESI) and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 1.5 software (previously described method) (Oniszczuk et al., 2015c). ESI worked in the negative-ion mode at the following conditions: capillary temperature 500 °C, curtain gas at 20 psi, nebulizer gas at 50 psi, and negative ionization mode source voltage −4500 V. Nitrogen was used as curtain and collision gas.

For each compound, the optimum conditions of Multiple Reaction Mode (MRM) were determined in the infusion mode. All standard solutions and samples were injected three times. The analytes were identified by comparing retention time and m/z values obtained by MS and MS^2 with the mass spectra from corresponding standards tested under the same conditions. Detailed information about qualitative analysis parameters is described in our previous paper (Oniszczuk et al., 2015c). The calibration curves obtained in MRM mode were used for quantification of all analyzed compounds. The limits of detection (LOD) and quantification (LOQ) for analytes, data for calibration curves and linearity ranges are presented in Table S1 in the Supplementary material.

2.8. Radical-scavenging activity of the analyzed extracts

The radical-scavenging activity of the extracts was determined spectrophotometrically using the DPPH· free radical (Kedare and Singh, 2011; Marteau et al., 2013). The concentration of the methanolic solution used for the experiment was 0.1 mM (4 mg of the free radical and 100 mL of methanol). The reference sample of DPPH· was prepared by mixing 2.0 mL of the solution and 1.0 mL of methanol. Measurement of snacks extracts was done after mixing 2.0 mL of DPPH· solution and 1.0 mL of the extracts. Each measurement was repeated three times at the wavelength 517 nm at room temperature. The final result was the average of three replications. The antiradical activity was calculated with the following formula (Oniszczuk et al., 2015a):

$$\%\ \text{DPPH radical scavenging ability} = \frac{A_0 - A_1}{A_0} \times 100\ [\%]$$

where A_0 - absorbance of the reference sample, A_1 - absorbance of the sample with tested extracts.

2.9. TLC-DPPH· test

The TLC-DPPH· test was used for confirming the antiradical capacity of the extracts. Samples were applied to HPTLC plates with applicator Desaga S-30 and developed in vertical chambers according to previously described method (Oniszczuk et al., 2015a). After development the plates were dried for 20 min. The TLC plates were immersed for 5 s. in 0.1% (w/v) DPPH· - methanol solution, dried in the dark for 5 min and then scanned every 5 min for one hour. The test was repeated three times.

The results of TLC– DPPH· test were documented by flatbed scanning, saved in the form of JPG documents. For image analysis Sorbil TLC Videodensitometer software (Sorbpolymer, Russia) was used. Suitable width of each track line was set and the evaluation of the chosen track to measure peak area was performed. The software evaluated a band in each track on a TLC image on the assumption that the size and the intensity of a bright spot depended on the quantity of a substance in the band. After changing the video scan images into chromatograms, the total areas under the peaks in one track (one extract) were compared with the area obtained for rutin in concentration 0.5 mg mL^{-1} (reference standard with activity taken as 1) (Oniszczuk et al., 2015c).

3. Results and discussion

3.1. Polyphenolic content

In the present work the qualitative and quantitative analysis (LC-ESI-MS/MS) of phenolic extracts obtained from corn snacks enriched with elderberry fruits or flowers (5%, 10%, 20% of the additive) and antiradical properties of these extracts were evaluated.

In fruit extract nine phenolic acids were found. Flower extract contained ten acids (additionally gentisic acid). In corn snacks without additives nine phenolic compounds were identified. These were protocatechuic, 4-OH-benzoic, caffeic, *p*-coumaric, salicylic, ferulic acids, rutin, isoquercetin and apigenin-7-glucoside. In the extract from elderberry flowers and extract from snacks containing 20% of elderberry flowers fifteen polyphenols were found. They were additionally gallic, gentisic, vanillic, sinapic acids, kaempferol-3-rutinoside and astragalin (Table 1 and 2, Fig. 1). Detailed analysis of extracts

Table 1 Content of phenolic acids in extracts from elderberry flowers and fruits and corn snacks with addition (0%, 5%, 10%, 20%) of elderberry flowers or fruits (n = 3).

Extraction method	Sample	Additive	Yield of phenolic acids ± SD[a] (μg g^{-1} of dry weight)									
			Gallic	Protocat.	Gentisic	4-OH-benzoic	Vanilic	Caffeic	*p*-Coumaric	Salicylic	Ferulic	Sinapic
ASE 120 °C	Snack	Flowers	N	B[c]	N[b]	B	N	0.51 ±	0.69 ±	B	B	N
			–	–	–	–	–	0.020	0.004	–	–	–
		Fruits	N	B	N	B	N[b]	0.33 ±	0.77 ±	B[c]	B	N
			–	–	–	–	–	0.001	0.000	–	–	–
	5%	Flowers	B	2.24 ±	B	0.98 ±	B	1.02 ±	4.08 ±	B	0.55 ±	N
			–	0.067	–	0.021	–	0.004	0.201	–	0.008	–
		Fruits	N	1.02 ±	N	0.61 ±	B	0.48 ±	0.89 ±	B	0.12 ±	N
			–	0.041	–	0.011	–	0.012	0.022	–	0.001	–
	10%	Flowers	0.79 ±	7.15 ±	0.30 ±	1.96 ±	0.23 ±	1.24 ±	4.72 ±	0.06 ±	0.71 ±	B
			0.011	0.156	0.003	0.017	0.000	0.012	0.007	0.001	0.003	–
		Fruits	B	3.21 ±	N	1.28 ±	B	0.54 ±	1.42 ±	B	0.22 ±	N
			–	0.028	–	0.007	–	0.009	0.013	–	0.000	–
	20%	Flowers	2.27 ±	11.98 ±	0.70 ±	5.82 ±	0.52 ±	2.09 ±	5.43 ±	0.09 ±	1.05 ±	0.21 ±
			0.082	0.323	0.002	0.043	0.002	0.102	0.094	0.002	0.009	0.001
		Fruits	1.91 ±	9.29 ±	N	3.13 ±	0.22 ±	1.00 ±	2.69 ±	0.22 ±	0.39 ±	B
			0.004	0.032	–	0.005	0.002	0.003	0.009	0.002	0.011	–
	Extract	Flowers	9.05 ±	63.14 ±	2.95 ±	24.98 ±	2.48 ±	11.27 ±	20.31 ±	0.42 ±	6.13 ±	1.09 ±
			0.008	0.618	0.092	0.056	0.112	0.076	0.091	0.007	0.054	0.023
		Fruits	3.68 ±	59.91 ±	N	5.97 ±	0.91 ±	2.59 ±	5.82 ±	0.342 ±	1.12 ±	0.15 ±
			0.032	0.197	–	0.223	0.011	0.029	0.085	0.005	0.009	0.003
ASE 100 °C	Snack	Flowers	N	B	N	B	N	0.61 ±	0.72 ±	B	B	N
			–	–	–	–	–	0.002	0.006	–	–	–
		Fruits	N	B	N	B	N	0.41 ±	0.80 ±	B	B	N
			–	–	–	–	–	0.002	0.002	–	–	–
	5%	Flowers	B	2.87 ±	B	1.11 ±	B	1.12 ±	3.99 ±	B	0.59 ±	N
			–	0.106	–	0.003	–	0.011	0.028	–	0.013	–
		Fruits	N	1.08 ±	N	0.72 ±	B	0.51 ±	1.02 ±	B	0.17 ±	N
			–	0.001	–	0.009	–	0.015	0.021	–	0.002	–
	10%	Flowers	0.84 ±	7.23 ±	0.29 ±	1.96 ±	0.21 ±	1.34 ±	4.87 ±	0.06 ±	0.82 ±	B
			0.002	0.008	0.001	0.009	0.002	0.009	0.095	0.001	0.006	–
		Fruits	B	3.42 ±	N	1.48 ±	B	0.58 ±	1.76 ±	B	0.22 ±	N
			–	0.108	–	0.017	–	0.012	0.011	–	0.001	–
	20%	Flowers	2.04 ±	11.71 ±	0.74 ±	5.57 ±	0.57 ±	2.23 ±	5.65 ±	0.10 ±	1.13 ±	0.23 ±
			0.132	0.098	0.008	0.331	0.000	0.032	0.193	0.001	0.023	0.001
		Fruits	2.04 ±	10.27 ±	N	3.26 ±	0.26 ±	1.12 ±	3.58 ±	0.27 ±	0.42 ±	B
			0.031	0.036	–	0.027	0.004	0.043	0.071	0.008	0.014	–
	Extract	Flowers	9.29 ±	62.68 ±	2.87 ±	24.62 ±	2.23 ±	12.08 ±	20.22 ±	0.39 ±	6.02 ±	1.12 ±
			0.349	0.716	0.130	0.603	0.022	0.361	0.059	0.091	0.248	0.003
		Fruits	3.71 ±	61.89 ±	N	6.21 ±	0.93 ±	2.73 ±	6.16 ±	0.401 ±	1.14 ±	0.18 ±
			0.029	0.789	–	0.157	0.008	0.057	0.235	0.013	0.043	0.001
UAE 60 °C	Snack	Flowers	N	B	N	B	N	0.52 ±	0.86 ±	B	B	N
			–	–	–	–	–	0.001	0.003	–	–	–
		Fruits	N	B	N	B	N	0.32 ±	0.81 ±	B	B	N

Table 1 (*continued*)

Extraction method	Sample	Additive	Yield of phenolic acids ± SD[a] (μg g^{-1} of dry weight)									
			Gallic	Protocat.	Gentisic	4-OH-benzoic	Vanilic	Caffeic	*p*-Coumaric	Salicylic	Ferulic	Sinapic
			–	–	–	–	–	0.009	0.032	–	–	–
	5%	Flowers	B	3.09 ±	B	1.28 ±	B	1.05 ±	4.19 ±	B	0.61 ±	N
			–	0.049	–	0.007	–	0.004	0.132	–	0.025	–
		Fruits	N	1.20 ±	N	0.84 ±	B	0.38 ±	1.10 ±	B	0.19 ±	N
			–	0.021	–	0.012	–	0.002	0.042	–	0.003	–
	10%	Flowers	0.91 ±	7.51 ±	0.32 ±	2.19 ±	0.29 ±	1.21 ±	5.13 ±	0.08 ±	0.83 ±	B
			0.001	0.042	0.005	0.102	0.003	0.045	0.156	0.000	0.017	–
		Fruits	B	3.84 ±	N	1.60 ±	B	0.41 ±	1.91 ±	B	0.26 ±	N
			–	0.084	–	0.010	–	0.002	0.021	–	0.001	–
	20%	Flowers	2.79 ±	12.12 ±	0.87 ±	6.07 ±	0.64 ±	1.92 ±	5.81 ±	0.12 ±	1.27 ±	0.27 ±
			0.084	0.421	0.012	0.212	0.007	0.026	0.099	0.001	0.032	0.009
		Fruits	2.18 ±	10.42 ±	N	3.28 ±	0.31 ±	0.93 ±	3.70 ±	0.31 ±	0.53 ±	B
			0.085	0.237	–	0.122	0.008	0.005	0.101	0.006	0.011	–
	Extract	Flowers	10.32 ±	66.00 ±	3.43 ±	26.28 ±	2.80 ±	10.42 ±	21.06 ±	0.47 ±	6.80 ±	1.29 ±
			0.014	0.261	0.023	0.134	0.054	0.156	0.251	0.002	0.096	0.007
		Fruits	4.01 ±	65.25 ±	N	6.54 ±	1.01 ±	2.34 ±	6.40 ±	0.419 ±	1.28 ±	0.29 ±
			0.032	0.258	–	0.109	0.007	0.043	0.057	0.003	0.051	0.009
UAE 40 °C	Snack	Flowers	N	B	N	B	N	0.49 ±	0.74 ±	B	B	N
			–	–	–	–	–	0.016	0.012	–	–	–
		Fruits	N	B	N	B	N	0.28 ±	0.77 ±	B	B	N
			–	–	–	–	–	0.003	0.002	–	–	–
	5%	Flowers	B	2.95 ±	B	1.19 ±	B	0.97 ±	4.07 ±	B	0.49 ±	N
			–	0.046	–	0.001	–	0.008	0.033	–	0.009	–
		Fruits	N	0.98 ±	N	0.70 ±	B	0.38 ±	0.99 ±	B	0.10 ±	N
			–	0.002	–	0.002	–	0.009	0.029	–	0.001	–
	10%	Flowers	0.80 ±	7.28 ±	0.25 ±	1.99 ±	0.24 ±	1.14 ±	5.00 ±	0.05 ±	0.76 ±	B
			0.011	0.078	0.002	0.023	0.001	0.048	0.202	0.001	0.012	–
		Fruits	B	3.48 ±	N	1.33 ±	B	0.40 ±	1.63 ±	B	0.21 ±	N
			–	0.098	–	0.012	–	0.006	0.011	–	0.000	–
	20%	Flowers	2.23 ±	12.01 ±	0.81 ±	6.01 ±	0.61 ±	1.83 ±	5.76 ±	0.09 ±	1.19 ±	0.19 ±
			0.113	0.38	0.012	0.043	0.017	0.037	0.213	0.001	0.008	0.000
		Fruits	2.10 ±	9.97 ±	N	3.21 ±	0.19 ±	0.91 ±	3.62 ±	0.28 ±	0.39 ±	B
			0.030	0.032	–	0.112	0.001	0.041	0.008	0.003	0.011	–
	Extract	Flowers	10.17 ±	64.82 ±	3.28 ±	25.42 ±	2.39 ±	9.98 ±	20.92 ±	0.31 ±	6.45 ±	1.22 ±
			0.097	0.176	0.038	0.279	0.115	0.219	0.545	0.008	0.145	0.014
		Fruits	3.91 ±	63.82 ±	N	6.03 ±	0.92 ±	2.23 ±	6.22 ±	0.412 ±	1.19 ±	0.21 ±
			0.121	0.081	–	0.085	0.002	0.107	0.301	0.009	0.041	0.003

[a] SD – standard deviation (n = 3).
[b] N– peak not detected.
[c] B – peak detected, concentration lower than the LOQ but higher than the LOD.

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Table 2 Content of flavonoids in extracts from elderberry flowers and fruits and corn snacks with addition (0%, 5%, 10%, 20%) of elderberry flowers or fruits (n = 3).

Extraction method	Additive	Sample	Yield of flavonoids ± SD[a] (μg g^{-1} of dry weight)				
			Rutin	Isoquercetin	Kaempferol- 3-rutinoside	Astragalin	Apigenin -7-glucoside
ASE 120 °C	Snack	Flowers	1.54 ± 0.039	B[c] –	N[b] –	N –	0.29 ± 0.010
		Fruits	1.13 ± 0.0062	B –	N –	N –	0.31 ± 0.004
	5%	Flowers	1.84 ± 0.090	0.22 ± 0.001	0.17 ± 0.001	0.10 ± 0.001	0.21 ± 0.011
		Fruits	1.23 ± 0.077	0.07 ± 0.000	0.02 ± 0.001	B[c] –	0.26 ± 0.009
	10%	Flowers	3.11 ± 0.003	0.38 ± 0.014	0.33 ± 0.006	0.31 ± 0.002	0.19 ± 0.000
		Fruits	1.31 ± 0.094	0.14 ± 0.005	0.04 ± 0.001	B –	0.25 ± 0.007
	20%	Flowers	5.13 ± 0.132	0.56 ± 0.003	0.81 ± 0.004	0.55 ± 0.004	0.10 ± 0.003
		Fruits	2.73 ± 0.021	0.54 ± 0.000	0.18 ± 0.001	B –	0.25 ± 0.001
	Extract	Flowers	48.86 ± 0.159	7.74 ± 0.179	7.99 ± 0.071	5.09 ± 0.074	N –
		Fruits	16.29 ± 0.23	3.63 ± 0.009	1.41 ± 0.001	0.15 ± 0.002	N –
ASE 100 °C	Snack	Flowers	1.83 ± 0.032	B –	N –	N –	0.32 ± 0.012
		Fruits	1.38 ± 0.003	B –	N –	N –	0.39 ± 0.002
	5%	Flowers	2.04 ± 0.100	0.24 ± 0.002	0.29 ± 0.007	0.19 ± 0.001	0.29 ± 0.013
		Fruits	1.48 ± 0.004	0.09 ± 0.002	0.03 ± 0.001	B –	0.31 ± 0.001
	10%	Flowers	3.14 ± 0.009	0.41 ± 0.011	0.39 ± 0.011	0.41 ± 0.003	0.21 ± 0.003
		Fruits	1.60 ± 0.079	0.16 ± 0.002	0.05 ± 0.001	B –	0.27 ± 0.005
	20%	Flowers	5.27 ± 0.032	0.62 ± 0.008	0.84 ± 0.006	0.62 ± 0.0141	0.20 ± 0.003
		Fruits	2.93 ± 0.021	0.45 ± 0.009	0.28 ± 0.000	0.09 ± 0.000	0.25 ± 0.001
	Extract	Flowers	49.98 ± 0.698	8.03 ± 0.099	8.31 ± 0.278	5.55 ± 0.167	N –
		Fruits	17.87 ± 0.001	3.74 ± 0.073	1.59 ± 0.002	0.39 ± 0.005	N –
UAE 60 °C	Snack	Flowers	1.98 ± 0.001	B –	N –	N –	0.33 ± 0.014

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Table 2 (*continued*)

Extraction method	Additive	Sample	Yield of flavonoids ± SD[a] (μg g^{-1} of dry weight)				
			Rutin	Isoquercetin	Kaempferol- 3-rutinoside	Astragalin	Apigenin -7-glucoside
		Fruits	1.47 ± 0.001	B –	N –	N –	0.34 ± 0.012
	5%	Flowers	2.19 ± 0.045	0.29 ± 0.008	0.30 ± 0.011	0.11 ± 0.001	0.30 ± 0.004
		Fruits	1.52 ± 0.000	0.12 ± 0.002	0.03 ± 0.001	B –	0.28 ± 0.002
	10%	Flowers	3.48 ± 0.094	0.44 ± 0.013	0.43 ± 0.015	0.32 ± 0.005	0.28 ± 0.008
		Fruits	1.76 ± 0.027	0.17 ± 0.000	0.06 ± 0.000	B –	0.27 ± 0.005
	20%	Flowers	5.82 ± 0.071	0.72 ± 0.019	0.90 ± 0.003	0.51 ± 0.013	0.25 ± 0.013
		Fruits	3.69 ± 0.003	0.56 ± 0.003	0.47 ± 0.001	B –	0.27 ± 0.003
	Extract	Flowers	52.53 ± 0.451	8.20 ± 0.191	8.95 ± 0.054	4.86 ± 0.045	N –
		Fruits	18.90 ± 0.041	3.91 ± 0.091	1.65 ± 0.003	0.27 ± 0.002	N –
UAE 40 °C	Snack	Flowers	1.77 ± 0.081	B –	N –	N –	0.32 ± 0.011
		Fruits	1.32 ± 0.032	B –	N –	N –	0.28 ± 0.002
	5%	Flowers	1.87 ± 0.051	0.22 ± 0.003	0.21 ± 0.005	0.09 ± 0.000	0.28 ± 0.013
		Fruits	1.28 ± 0.011	0.10 ± 0.000	0.03 ± 0.001	B –	0.27 ± 0.004
	10%	Flowers	3.19 ± 0.002	0.38 ± 0.009	0.31 ± 0.005	0.31 ± 0.001	0.18 ± 0.001
		Fruits	1.68 ± 0.005	0.11 ± 0.008	0.05 ± 0.001	B –	0.23 ± 0.004
	20%	Flowers	5.71 ± 0.027	0.68 ± 0.004	0.79 ± 0.011	0.48 ± 0.011	0.19 ± 0.003
		Fruits	3.01 ± 0.005	0.48 ± 0.001	0.41 ± 0.001	B –	0.20 ± 0.0008
	Extract	Flowers	48.87 ± 0.938	7.73 ± 0.069	8.22 ± 0.097	4.53 ± 0.096	N –
		Fruits	16.13 ± 0.031	3.87 ± 0.191	1.62 ± 0.0000	0.13 ± 0.001	N –

[a] SD – standard deviation ($n = 3$).
[b] N – peak not detected.
[c] B – peak detected, concentration lower than the LOQ but higher than the LOD.

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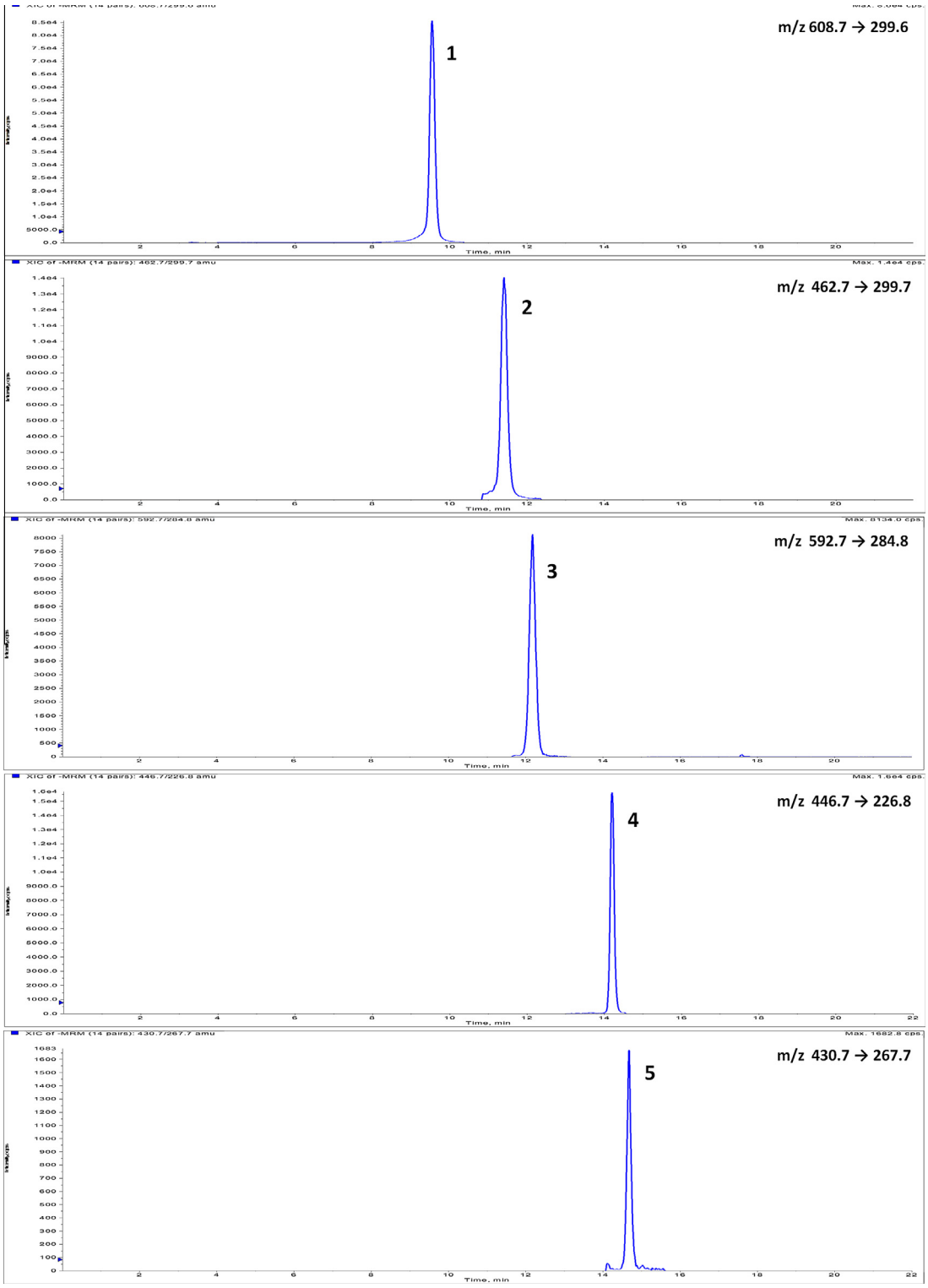

Figure 1 Exemplary LC–MS/MS extracted ion chromatograms acquired in a *MRM* mode for flavonoids from snacks enriched with elderberry flowers. 1. Rutin (*m*/*z* 608.7 → 299.6), 2. Isoquercetin (*m*/*z* 462.7 → 299.7), 3. Kaempferol-3-rutinoside (*m*/*z* 592.7 → 284.8), 4. Astragalin (m/z 446.7 → 226.8), 5. Apigenin-7-glucoside (*m*/*z* 430.7 → 267.7). See Experimental section for more details.

prepared with the use of ASE 120 °C method, revealed significant differences in quantitative and qualitative components of studied samples. Changes in percentile addition of fruits or flowers to the snacks allowed to indicate essential qualitative and quantitative changes between these two additives. In the case of 5% addition of flowers, contents of all identified substance are much higher than in case of extracts from snack with fruits. Additionally, gentisic and salicylic were identified

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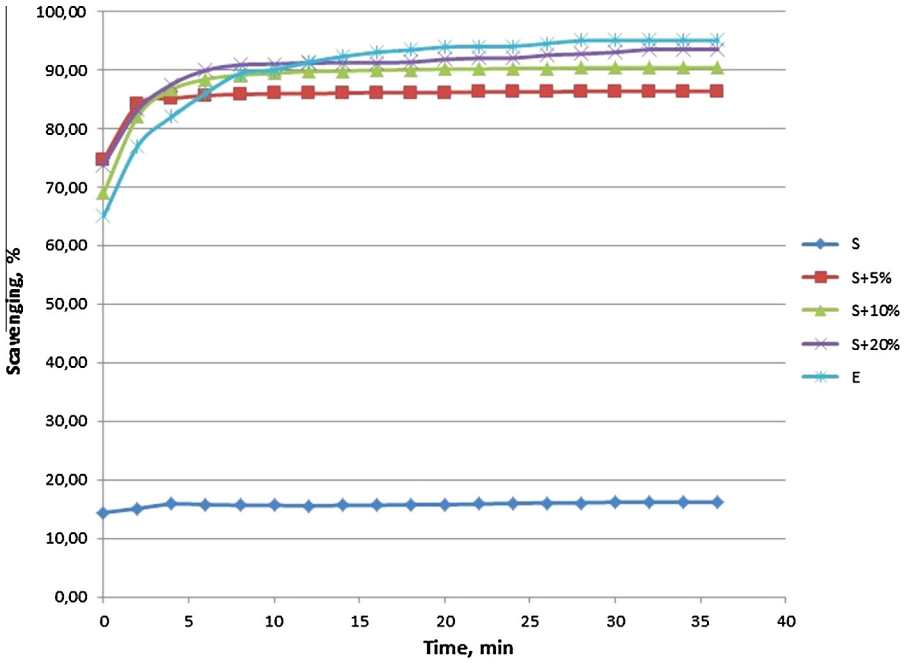

Figure 2 Free radical scavenging activity of extract of corn snacks (S), snacks enriched with 5%, 10%, 20% elderberry flowers (S + 5%, S + 10%, S + 20%, respectively) and extract of elderberry flowers (E), toward DPPH· in methanol. See Experimental section for details.

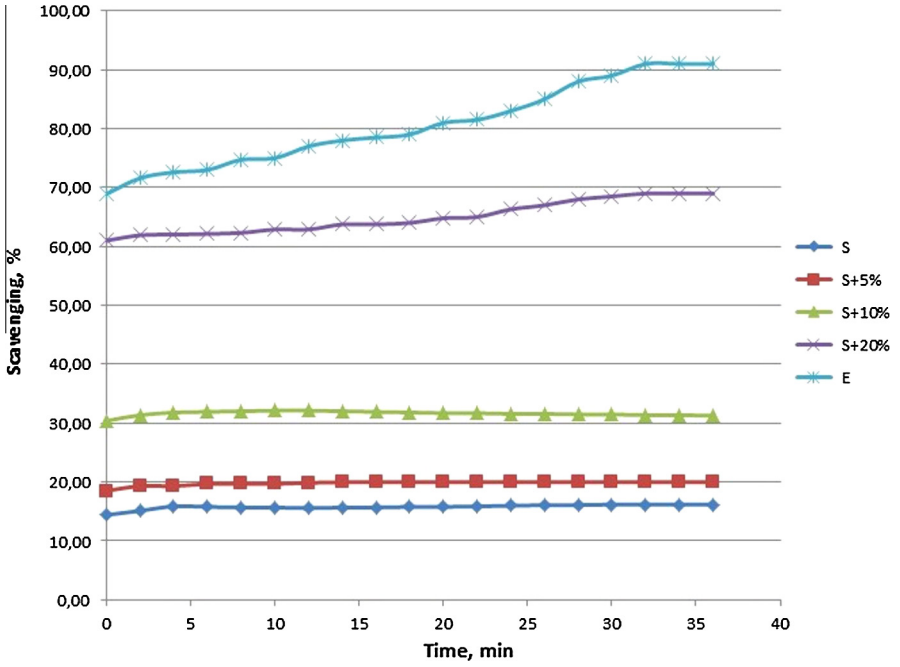

Figure 3 Free radical scavenging activity of extract of corn snacks (S), snacks enriched with 5%, 10%, 20% elderberry fruits (S + 5%, S + 10%, S + 20%, respectively) and extract of elderberry fruits (E), toward DPPH· in methanol. See Experimental section for details.

in 10% addition of flowers which were not recognized in the case of the same addition of elderberry fruits. Similarly changes have been noted for extracts obtained by UAE method. LC-ESI-MS/MS analysis explicitly indicated that snacks with elderberry flowers are significantly richer in flavonoids and phenolic acids than snacks' extracts with fruits addition. Elderberry flowers and products with addition of elderberry flowers contain several times more polyphenols than elderberry fruits and corresponding products enriched with fruits.

Phenolic acids in the elderberry fruit products were qualitatively less diverse than those present in snacks with flowers. Particularly, the content of phenolic acids in snacks with 5% and 10% addition of *Sambucus* fruit was found to be relatively

Table 3 Antiradical activity of samples in relation to rutin (rutin in concentration of 0.5 mg mL^{-1} possesses activity taken as 1). Extraction method – UAE 60 °C.

Time	Activity in relation to rutin (area under the common peak/area under rutin peak) ± SD[a]				
	Elderberry fruits				
	Corn snacks	Elderberry fruits extract	Snacks with 5% addition of elderberry fruits	Snacks with 10% addition of elderberry fruits	Snacks with 20% addition of elderberry fruits
15 min	0.01 (±0.000)	3.17 (±0.004)	0.08 (±0.001)	0.23 (±0.009)	0.85 (±0.014)
30 min	0.04 (±0.002)	3.68 (±0.363)	0.12 (±0.000)	0.31 (±0.012)	1.21 (±0.083)
60 min	0.07 (±0.001)	3.79 (±0.188)	0.14 (±0.001)	0.32 (±0.018)	1.28 (±0.003)
	Elderberry flowers				
	Corn snacks	Elderberry flowers extract	Snacks with 5% addition of elderberry flowers	Snacks with 10% addition of elderberry flowers	Snacks with 20% addition of elderberry flowers
15 min	0.01 (±0.000)	3.82 (±0.031)	0.96 (±0.012)	1.15 (±0.026)	1.55 (±0.032)
30 min	0.04 (±0.002)	4.53 (±0.235)	1.38 (±0.023)	1.54 (±0.032)	1.87 (±0.004)
60 min	0.07 (±0.001)	4.61 (±0.090)	1.41 (±0.141)	1.62 (±0.048)	2.12 (±0.049)

[a] SD – standard deviation ($n = 3$).

poor. This obviously results from lower levels of polyphenols in fruit extracts in comparison with flowers.

The content of phenolic acids and majority of flavonoids increased in snacks proportionally with addition of elderberry, while content of apigenin-7-glucoside is related to all samples of snacks and decreases insignificantly with addition of the inflorescence. This phenomenon results from the absence of this compound in *S. nigra* extracts.

3.2. Optimization of extraction method

Very important step of the quantitative and qualitative analysis of plant material or food is selection and optimization of the extraction method. Similar to our previous work, the most effective extraction method of phenolic compounds from functional food products was ultrasound assisted extraction at 60 °C. UAE can offer high yield of analyzed compounds in short times, simple manipulation, reduced volume of solvent, lower energy input, high reproducibility and meets the requirements of ''Green Chemistry (Chemat et al., 2008; Chen et al., 2008). Moreover, no evidence for polyphenols degradation during examined UAE conditions could be observed. The ultrasonic degradation of phenolic acids is slower in comparison with more volatile aromatic compounds that diffuse more readily into the cavitation bubble for pyrolysis. Moreover, their degradation occurs at higher frequencies (required for the generation of the hydroxyl radical) than the one used in this experiment (20 kHz) (Chowdhury and Viraraghavan, 2009). The comparison between the ultrasound-assisted extraction, and different extraction method developed by other researches showed the superiority of UAE for extracting phenolic compounds from functional food (Chemat et al., 2008), for example resveratrol from cookies and jams (Guamán-Balcázar et al., 2016; da Silva et al., 2016). This method, followed by SPE, was proved as the most precise and accurate for food products (Oniszczuk et al., 2015c). In order to determine the accuracy of the method, recovery study was performed. Crude extracts were spiked with standard solution (three concentration levels). Solid phase extraction and LC-ESI-MS/MS analysis of phenolic compounds were carried out as for real samples. Recoveries ranged from 89.1% (astragalin) to 101.8% (protocatechuic acid), demonstrating the accuracy of the method. For caffeic acid and astragalin only, the most efficient technique was accelerated solvent extraction at 100 °C.

3.3. Antiradical activity

The next step of experiment was determination of free radical scavenging activity of the analyzed functional food, using spectrophotometry, by the $DPPH^{\cdot}$ assay. The findings of the spectrophotometric studies revealed that radical-scavenging activity of the analyzed snacks was positively correlated with the content of elderberry. The high antiradical potential was observed for crude extracts from elderberry flowers or fruits and for all snacks enriched with elderberry flowers (Figs. 2 and 3). The product with a 20% addition of fruits revealed moderate free radical scavenging properties, while snacks containing 10% and 5% of fruits exhibited only marginal antiradical activity. Considering extracts from snacks with flowers as additive high free radical scavenging activity is observed for all samples (5%, 10% and 20%) which exhibited over 85% of free radical scavenging ability after 10 min. Similar to fruit samples, in this case the activity was dependent on content of flowers but the differences were not as significant as in the case of fruits. In order to compare the influence of additives

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on antiradical activity of snacks, extracts of snacks without fruits or flowers were determined. The obtained results revealed lack of antradical ability of the sample. The results of the experiment have shown, that functional food enriched with elderberry fruits exhibits much lower antiradical activity than snacks with addition of elderberry flowers. This is due to lower content of polyphenols in elderberry fruits than elderberry flowers.

Antiradical capacity was also conducted using TLC-DPPH$^{\cdot}$ method. TLC-DPPH$^{\cdot}$ enables separation and detection of active constituents simultaneously, what is not possible when using standard spectrophotometric protocol. Moreover, results can be saved in the form of JPG image files and analyzed by means of image processing software in unrestricted way.

The TLC–DPPH$^{\cdot}$ analysis exhibited comparable trend as the spectrophotometric studies. Antiradical action of analyzed samples increased with the addition of elderberry in snacks (Table 3, Fig. S1 in the Supplementary material). Samples enriched with elderberry flowers exhibited high antiradical properties, while extracts with addition of elderberry fruits characterized lower activity. This finding was based on a comparison of the activity of the separated tracks (all spots of the one extract) in relation to rutin's radical scavenging properties.

High antiradical properties of active samples were observed just after the first five minutes of the experiment, while maximum radical scavenging activity was observed after 60 min (Table 3). Similarly, as in our previous study (Oniszczuk et al., 2015a,c), the results confirmed that high-temperature short time (HTST) extrusion-cooking process did not deactivate polyphenolic antioxidants, which were presented in extracts from medical plant and in functional food enriched with addition of this plant. Özer et al. (2006) reported that total phenolic values in snack food had insignificant changes after extrusion. The results of other study demonstrated, the antioxidant activity and phenolic compounds content in most of the analyzed products increased as a result of extrusion processing due to an increase in phenolic bioavailability mediated by fiber bound phenolic release (Morales et al., 2015). This technique seems to be one of the most appropriate method for obtaining the maximum nutritive value of several plant materials. Versatility of technical and technological solutions allows applying various raw materials and additives (Wójtowicz et al., 2013).

Extrusion-cooking is an excellent method of food processing due to the fact that it may produce articles containing all nutritive compounds enhanced by the addition of natural, biologically active components. Snacks enriched with elderberry flowers, presented first time in this paper, may be a convenient, health-promoting product useful in the prevention of lifestyle diseases.

4. Conclusions

The results of the above study indicate, that extruded snacks enriched with elderberry flowers, have great potential to be a good source of natural antioxidants. HTST extrusion-cooking had no negative impact on antiradical activity of polyphenols, which are present both in the plant snacks and in extracts from corn snacks enriched with addition of elderberry flowers. The most effective extraction method of phenolic compounds from functional products, proved to be an ultrasound assisted extraction at 60 °C.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.arabjc.2016.09.003.

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