SUPPLEMENTARY MATERIAL

Effect of variety on content of bioactive phenolic compounds in common elder (*Sambucus nigra* L.)

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Abstract

The inflorescence of common elder (*Sambucus nigra* L., Adoxaceae) is known to be rich in phenolic compounds. The content of five selected phenolic compounds (rutin, chlorogenic acid, isoquercitrin, isorhamnetin-3-*O*-rutoside and dicaffeoylquinic acid) was determined in methanolic extracts from flowers and floral stems by HPLC in samples obtained from twenty varieties of *Sambucus nigra* cultivated in Czech Republic. In all samples there were determined rutin (11 - 54 mg/g), chlorogenic acid (23 - 46 mg/g), isoquercitrin (0.6 - 18 mg/g), isorhamnetin-3-*O*-rutoside (3-10 mg/g), calculated on air-dried material. The content of dicaffeoylquinic acid was 0-13 mg/g of air-dried material. The amount of the analysed

compounds in floral stems was lower than in the flowers. The results are a unique set of information on the content of main phenolics in the inflorescence of the cultured elderberry varieties.

Keywords: *Sambucus nigra*, flavonoids, phenolic acids, HPLC-MS

1. Experimental

1.1. Reagents and materials

Methanol LiChrosolv gradient grade and acetonitrile LiChrosolv gradient grade were purchased from Merck KGaA (Germany), acetonitrile Optima LC/MS from Fisher Scientific (Belgium), orthophosphoric acid from Sigma-Aldrich (Germany), chlorogenic acid, rutin, isoquercitrin (syn. quercetin-3-*O*-glucoside) and 3,5-dicaffeoylquinic acid from Sigma-Aldrich (Germany).

All varieties of *Sambucus nigra* L. were grown in the Research and Breeding Institute of Pomology Holovousy Ltd., Czech Republic (50°22'29" lat., 15°34'38" long., 321 m alt.). Twenty elderberry varieties were evaluated. Three varieties were ornamental ('Aurea', 'Black Beauty', 'Madona'), the rest were fruit varieties ('Albida', 'Allesö', 'Bohatka', 'Dana', 'Heidegg 13', 'Haschberg', 'Körsör', 'Mammut', 'Pregarten', 'Riese aus Voßloch', 'Sambo', 'Sambu', 'Samdal', 'Sampo', 'Saml', 'Tulbing', 'Weihenstephan'). Three bushes of each variety were planted. The collection was situated on heavy loamy clay soil with a minimum thickness of 60-80 cm. The bedrock consists of claystone (Matějček et al. 2015). Average annual temperature of the locality is 8.14°C. Average annual rainfall is 655 mm and average rainfall during vegetation period is 379 mm. The orchard was irrigated, mulched between rows, and hand-weeded. No pesticides were used. Spacing of the plants was 3 × 2 m. The orchard was established in spring 2009 (Matějček et al. 2013).

The investigated trees are part of the fruit trees gene pool in Research and Breeding Institute of Pomology Holovousy Ltd. The voucher specimen numbers: Aurea G 18/1-3, Black Beauty G 0/2, Madona G 16/1-3, Albida G 1/1-3, Allesö G 4/1-3, Bohatka G 2/1-3, Dana G 9/1-3, Heidegg 13 G 17/1-3, Haschberg G 5/1-3, Körsör G 15/1-3, Mammut G 6/1-3, Pregarten G 3/1-3, Riese aus Voßloch G 12/1-3, Sambo G 11/1-3, Sambu G 14/1-3, Samdal G 10/1-3, Sampo G 13/1-3, Saml G 7/1-3, Tulbing G 0/1 and Weihenstephan G 8/1-3.

1.2. Sample preparation

Plant materials were collected in 2014. The samples contained from four to five fresh inflorescences which were dried at laboratory temperature in the shade for one week. Dried inflorescences were divided into flowers and floral stems. All material was homogenized and stored in plastic container at 4 °C until extraction. Dry plant material was extracted with 70% methanol at 50 °C for 30 min. After centrifugation the sediment was washed twice with 70% methanol and centrifuged. Supernatants were pooled and stored at -18°C until measurements by HPLC and LC / MS. Two samples were prepared from every dry plant material.

1.3. HPLC and LC/MS measurement

HPLC was used for quantification of phenolic compounds and LC/MS was used for their identification.

HPLC: HP 1050 (Hewlett-Packard, USA), DAD detector (HP 1040, Hewlett-Packard, U.S.A.). Samples were separated on Phenomenex Luna C18 (2), 3 µm, 2×150 mm column. The data was scanned in the range from 190 to 600 nm.

The mobile phase A: 5% acetonitrile + 0.1% orthophosphoric acid. The mobile phase B: 80% acetonitrile + 0.1% orthophosphoric acid. The gradient of phase B increased from 2% to 42% during 40 min. Flow rate was 0.25 ml/min and temperature on column was 25 °C. Injection volume was 5 µL.

The quantity of analytes was determined by external standard method using appropriate standard. Amount of isorhamnetin-3-*O*-rutinoside was calculated according to the calibration curve of isoquercetin. Each sample was injected ones and it was evaluated independently. The content of all analytes was expressed in mg of compound per g of dry plant material.

LC/MS: LCQ Accela Fleet (Thermo Fisher Scientific, San Jose, CA, USA) with atmospheric pressure chemical ionization (APCI) and a photodiode array detector. APCI capillary temperature was 275 °C, APCI vaporizer temperature 400 °C, sheath gas flow 58 L min⁻¹, auxiliary gas flow 10 L min⁻¹, source voltage 6 kV, source current 5 µA, and capillary voltage -4 V.

Samples were separated on the same column as in the case of HPLC and in the same gradient conditions, but in mobile phase was used formic acid instead of orthophosphoric acid.

1.4. Data analysis

Data processing was performed using ChemStation (Agilent Technologies, USA). The basic calculations and statistical tools (MS Office Excel) were used for confirmation of linearity, calibration and other calculations. All results were calculated on air-dried material (ADM). The analytical data was assessed statistically using program Statistica CZ 6.1 (Statsoft CR) and for PCA analysis program the Canoco (Ter Braak & Šmilauer 2002) was used.

The basic parameters of the analytical method were calculated for all analytes according to Dadakova et al. 2011. The limit of detection, limit of quantification, inter- and intra- day precision and recovery was determined (Table 3S). The LOD and LOQ were calculated as triple and tenfold of RSD of eight independent analyses of spiked matrix material. Intra-day precision was evaluated by repeated five analyses during one day. The data for inter-day precision were obtained by determination of the content of the analytes over a 4-day period. The recovery of analytes was determined for each sample by standard addition (100 and 200 % of analysed amount).

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Figure S1: Chromatogram (HPLC) of extract of flowers *Sambucus nigra* variety (Bohatka).

Chromatographic condition: see Experimental. (1) chlorogenic acid, (2) rutin, (3) isoquercitrin (4), isorhamnetin-3-O-rutinoside, (5) 3,5-dicaffeoylquinic acid.

Figure S2: Comparison of analysed compounds in flowers and floral stems

Figure S3: PCA plot for contents of phenolic compounds in flowers.

Figure S4: PCA plot for contents of phenolic compounds in floral stems.

Table S1: Flowers of *S. nigra*: content (mg/g ADM) of chlorogenic acid, rutin, isoquercitrin, isorhamnetin-3-*O*-rutinoside and 3,5-dicaffeoylquinic acid (n=2).

Varieties	Used abbreviation	Chlorogenic acid	Rutin	Isoquercitrin	Isorhamnetin- 3- <i>O</i> -rutinoside	3,5-dicaffeoylquinic acid
Albida	Ada	29.5±1.9	35.3±2.0	1.5±0.1	9.8±0.0	7.4±0.2
Allesö	Aso	32.9±1.3	17.5±0.2	2.1±0.1	3.2±0.7	5.7±0.2
Aurea	Aea	27.6±0.9	17.7±0.7	4.0±0.2	5.8±0.2	ND
Black Beauty	Bbty	33.2±0.6	11.2±0.0	2.2±0.0	3.0±0.0	4.4±0.2
Bohatka	Bka	26.0±0.2	19.3±0.4	7.9±0.2	4.1±0.3	4.6±0.1
Dana	Dna	23.5±0.5	21.3±1.4	7.8±0.3	4.6±0.2	6.5±0.2
Heidegg 13	Heg	35.1±1.2	20.5±0.9	16.6±0.9	3.2±0.2	7.7±0.6
Haschberg	Herg	33.8±0.5	49.6±0.2	0.7±0.1	7.1±0.1	5.5±0.1
Körsör	Kor	36.5±0.2	25.7±1.4	6.8±0.0	3.3±0.2	12.8±0.9
Madona	Mna	31.1±0.8	23.7±0.3	4.0±0.1	10.1±0.4	10.2±0.2
Mammut	Mut	33.9±1.3	35.9±0.2	2.9±0.0	10.8±0.6	7.9±0.8
Pregarten	Pen	31.2±2.9	30.3±1.1	2.3±0.3	4.9±0.6	6.6±0.1
Riese aus Voßloch	Rse	46.3±4.8	24.9±3.1	18.6±1.6	3.9±0.3	8.7±0.8
Sambo	Sbo	29.6±0.7	21.9±0.3	1.2±0.0	3.2±0.1	4.3±0.3
Sambu	Sbu	38.0±1.1	19.3±0.6	11.5±0.4	5.5±0.2	13.2±0.3
Samdal	Sal	29.6±0.1	21.6±0.3	5.5±0.3	4.5±0.1	9.0±0.5
Sampo	Spo	39.4±0.7	17.9±0.9	11.9±0.1	5.5±0.2	13.3±0.1
Samyl	Syl	31.7±0.5	14.9±0.2	5.9±0.0	3.2±0.1	10.1±0.2
Tulbing	Ting	29.5±1.5	44.8±1.6	1.5±0.1	7.8±0.2	12.5±0.3
Weihenstephan	Wan	34.2±0.6	54.1±2.3	0.7±0.0	7.2±0.1	7.7±0.4

Table S2: Floral stems of *S. nigra*: content (mg/g ADM) of chlorogenic acid, rutin, isoquercitrin, isorhamnetin-3-O-rutinoside and 3,5-dicaffeoylquinic acid (n=2).

Varieties	Used	Chlorogenic	Rutin	Isoquercitrin	Isorhamnetin-	3,5-dicaffeoylquinic
	abbreviation	acid			3-O-rutinoside	acid
Albida	Ada	13.0±1.7	27.8±3.8	0.6±0.1	1.3±0.2	2.5±0.3
Alleső	Aso	11.6±1.6	14.3±0.8	0.4±0.1	0.7±0.1	1.7±0.4
Aurea	Aea	6.3±0.6	9.0±0.3	0.2±0.0	0.3±0.1	0.5±0.0
Black Beauty	Bbty	16.1±0.7	10.3±0.2	1.1±0.1	0.7±0.0	2.1±0.1
Bohatka	Bka	16.8±0.6	18.1±1.3	1.8±0.2	1.3±0.2	2.4±0.6
Dana	Dna	14.6±1.1	17.6±4.0	1.6±0.2	1.1±0.2	1.8±0.4
Heidegg 13	Heg	26.6±8.4	22.8±5.8	5.2±1.7	1.5±0.1	1.7±0.3
Haschberg	Herg	6.6±0.5	13.0±0.8	0.2±0.0	0.4±0.1	1.1±0.0
Körsör	Kor	30.6±2.2	19.1±0.7	0.9±0.1	0.3±0.0	4.1±0.3
Madona	Mna	21.1±5.4	33.7±4.1	1.7±0.3	1.7±0.1	7.0±0.9
Mammut	Mut	9.2±4.2	11.8±4.6	0.4±0.0	0.5±0.0	0.6±0.3
Pregarten	Pen	13.3±1.9	28.6±1.5	0.4±0.1	0.5±0.1	1.2±0.1
Riese aus Voßloch	Rse	23.2±1.0	27.3±1.2	6.0±0.3	2.6±0.2	3.9±0.2
Sambo	Sbo	10.0±2.0	11.4±1.9	1.6±0.4	0.4±0.0	1.4±0.2
Sambu	Sbu	10.5±2.5	10.0±0.6	0.8±0.1	0.3±0.0	2.1±0.0
Samdal	Sal	8.1±2.7	7.9±1.4	0.2±0.0	0.3±0.1	1.1±0.1
Sampo	Spo	21.2±1.0	18.9±0.0	1.9±0.1	0.6±0.0	3.2±0.8
Samyl	Syl	17.7±2.9	19.9±2.8	2.1±0.5	0.6±0.0	2.2±0.4
Tulbing	Ting	23.5±1.9	31.9±3.3	0.6±0.1	0.5±0.1	3.5±0.5
Weihenstephan	Wan	8.1±0.5	16.0±0.6	0.1±0.0	0.4±0.0	1.1±0.1

Table S3: Parameters of analytical method

Analyte/Parameter	LOD	LOQ	Precision (R.S.D.%)		Recovery
	mg/kg		intra-day	inter-day	(%)
Chlorogenic acid	0.42	2.0	1.85	3.35	95
Rutin	2.40	10.0	2.36	4.05	93
Isoquercitrin	0.61	2.6	2.01	3.30	95
Isorhamnetin-3-O-rutinoside	0.85	2.9	1.66	3.44	91
3,5-dicaffeoylquinic acid	0.55	2.1	1.95	3.21	94