Arch. Biol. Sci., Belgrade, 67(3), 829-840, 2015 DOI:10.2298/ABS141204042S

IDENTIFICATION OF SEED COAT PHENOLIC COMPOUNDS FROM DIFFERENTLY COLORED PEA VARIETIES AND CHARACTERIZATION OF THEIR ANTIOXIDANT ACTIVITY

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Abstract: The phenolic composition of seed coats in four differently colored pea varieties (*Pisum sativum* L.) was investigated using UHPLC-LTQ OrbiTrap MS. The obtained findings revealed that the seed coats of the examined pea genotypes possess a unique phenolic composition compared to previously studied European cultivars. In total, 41 phenolic compounds have been identified. The seed coats of the studied cultivars contained certain amounts of rosmarinic acid, rutin, galangin, morin, naringin, hesperetin and pinocembrin as well as ten flavonol glycosides that had not been reported previously. Additionally, the total phenolic content, antioxidant activity and metal chelating capacity of extracts was determined using Folin-Ciocalteu's method, 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay, ferric ion-reducing capacity and ferrous ion-chelating capacity assay, respectively. Dark colored genotypes MBK 168 and MBK 173 possessed the highest total phenolic contents as well the strongest antioxidant activities. On the other hand, bright colored genotypes MBK 88 and MBK 90 exhibited the strongest metal-chelating capacities. The examined pea seed coats may be considered as important potential contributors to human health due to the presence of bioactive phenolic constituents. In addition, our results could be used as a guideline for breeding new pea cultivars with high antioxidant activities applicable in the formulation of functional food products.

Key words: *Pisum sativum* L.; seed coat; UHPLC-LTQ OrbiTrap MS; phenolic content; antioxidant

Received December 4, 2014; **Revised** December 15, 2014; **Accepted** December 17, 2014

INTRODUCTION

There is growing interest in the dietary intake of natural antioxidants as a way of preventing different health problems related to oxidative stress, such as inflammation, neurodegenerative diseases, cancer, cardiovascular diseases, etc. (Halliwell and Gutteridge, 1990). Plants are considered a valuable source of natural antioxidants, primarily the compounds belonging to the group of secondary metabolites such as various classes of phenolic compounds. Pea seeds are a rich source of many nutrient compounds represented by proteins, starch, fibers, vitamins and minerals. Non-nutrient phenolic compounds, including simple phenolics, flavonoids and condensed tannins, are also present in significant amounts, especially in varieties with dark colored seed coats (Troszyńska and Ciska, 2002; Dueñas et al., 2006; Agboola et al., 2010). In recent years the phenolic contents and antioxidant activities of raw and processed pea seeds have been extensively studied (Troszyńska and Ciska 2002; Xu

and Chang, 2009; Han and Baik, 2008; Stanisavljević et al., 2013). Pea seed coats, which constitute approximately 8-10% of the seed mass, are considered to be important by-products of the pulse crop processing (Dueñas et al., 2006; Oomah et al., 2011). Considering recent advances in the technology of pneumatic separation of seed coats from cracked legume seeds (Innocentini et al., 2009), the exploitation of these seed parts as a source of dietary fibers, phenolics and other bioactive compounds becomes very promising. Data on the phenolic composition of pea seed coats have been limited and restricted to a few varieties. In the previous study of Dueñas et al. (2004), the presence of 25 phenolic compounds was reported in the seed coats of two dark colored varieties of pea. Glycosides of flavones, flavonols, tetrahydroxydihydrohalcone and hydroxybenzoic acids were identified as the predominant compounds of the seed coat in the examined cultivars. The mentioned study also indicated that the greatest differences in phenolic profiles between varieties were observed in the composition of the seed coats. In the present study, we have focused primarily on differently colored varieties originating from Croatia, which are extensively used for breeding but rarely in human diet. Our aim was to examine the possibility of their exploitation as a source of compounds, primarily polyphenols, with potentially health-benefitting properties. These preparations could be used in the design of novel functional food products. The main objective of our study was to examine the phenolic composition of seed coats of four differently colored genotypes. In our research, we applied methanol extraction of seed coats, followed by the identification and quantification of individual phenolic compounds using ultra high performance liquid chromatography (UHPLC), coupled with a hybrid mass spectrometer which combines the linear trap quadrupole (LTQ) and orbitrap mass analyzer. The obtained extracts were also used for determination of the total phenolic content (TPC) as well as antioxidant (AO) activity by conventional *in vitro* approaches: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric ion-reducing capacity (FRC) and ferrous ion-chelating capacity (FCC) assays.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile and formic acid (both of them MS grade) were purchased from Merck (Darmstadt, Germany). Ultrapure water (ThermoFisher TKA MicroPure water purification system, 0.055 μS cm^{-1}) was used to prepare standard solutions and blanks. The syringe filters (13 mm, PTFE membrane 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA). All phenolic standards were purchased from Fluka AG (Buch, Switzerland). Folin-Ciocalteu's reagent, DPPH radical, ferricyanide, ferrichloride, and Ferrozine were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Seed material

The pea varieties used in this study (Fig. 1) were marked with the following catalog numbers: MBK 88 (breeding line in process of recognition), MBK 90 (breeding population), MBK 168 (breeding population) and MBK 173 (breeding population) from the collection of the Agricultural Institute, Osijek (Croatia). The pea varieties used for experiments were sown in three replications in March 2012, on plots (5 m × 2 m) with 12.5 cm inter-row distance and 4 cm sowing depth. Plants were grown under the same agricultural conditions in open experimental fields of the Agricultural Institute in Osijek, situated in the eastern Croatian region of Slavonia on the soil type belonging to *Eutric* Cambisols. The plants reached maturity at the end of July when they were harvested manually and seeds were collected. Samples from each replication (300 g) were chosen individually and randomly. Collected seeds were air dried at 25°C prior to analysis.

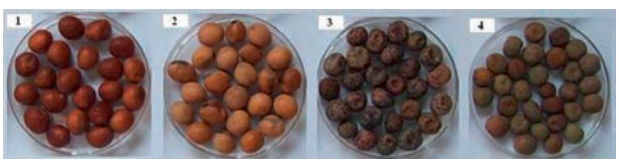

Fig. 1. Examined pea varieties: 1 – MBK 88, 2 – MBK 90, 3 – MBK 168, 4 – MBK 173.

Extraction of seed material

The dry seeds were cracked and dehulled manually. The separated seed coats were powdered using a basic mill (60 mesh sieves). Powdered samples (1 g), in triplicates, were extracted in 50-mL conical flasks with a tenfold volume of methanol/water/acetic acid mixture (80:19:1) for 6 h in first extraction. Extraction was carried out at room temperature with constant shaking. Two additional extractions were conducted with the same solvent at the solid/solvent ratio 1:5 and lasted for 12 h and 6 h, respectively. Extracts were combined and aliquots of 2 mL were kept at -80°C (no longer than three days) prior to analysis. Three accessions of each variety were extracted and analyzed separately for total phenolic content and antioxidant assays; for the UHPLC-LTQ OrbiTrap MS the triplicates were pooled prior to analysis.

UHPLC-LTQ OrbiTrap MS – determination of individual phenolic compounds

A 1 000 mg L^{-1} stock solution of a mixture of all standards was prepared in methanol. Dilution of the stock solution with methanol yielded the working solution at concentrations of 0.025, 0.050, 0.100, 0.250, 0.500, 0.750, and 1.000 mg L^{-1}. All stock and working solutions were stored in the dark at 4°C and were stable for at least 3 months. Calibration curves were obtained by plotting the peak areas of the compounds against the concentration of the standard solution. Calibration curves revealed good linearity, with R^2 values exceeding 0.99 (peak areas vs. concentration).

Chromatographic separations were performed using the UHPLC system consisting of a quaternary Accela 600 pump and Accela Autosampler (ThermoFisher Scientific, Bremen, Germany). The analytical column used for the separation of abscisic acid and phenolics was a Hypersil gold C18 (100 × 2.1 mm, 1.9 μm) from Thermo Fisher Scientific. The mobile phase consisted of (A) water solution of 0.1% formic acid and (B) acetonitrile and 0.1% formic acid. A linear gradient program at flow rate of 0.300 mL min^{-1} was used: 0-1 min 5% B, 1-12 min from 5% to 95 % (B), 12-12.1 min from 95% to 5 % (B), then 5% (B) for 3 min. The injection volume was 5 μL.

The UHPLC system was coupled to a linear ion trap-orbitrap hybrid mass spectrometer (LTQ OrbiTrap MS) equipped with a heated-electrospray ionization

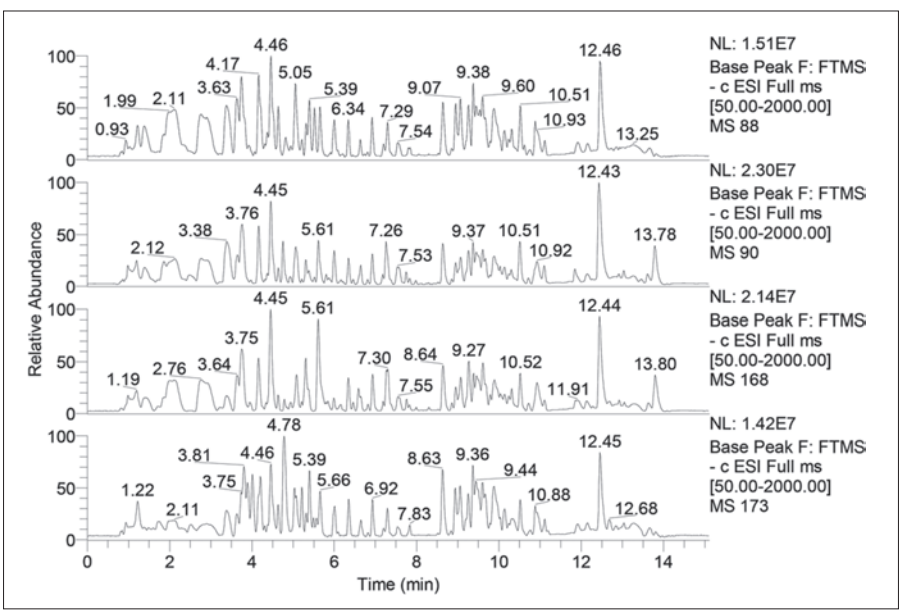

Fig. 2. Total ion chromatograms (TICs) of pea seed coats in negative ionization mode.

probe (HESI-II, ThermoFisher Scientific, Bremen, Germany). The mass spectrometer was ope rated in negative mode. Parameters of the ion source were as follows: source voltage was 4.5 kV, capillary voltage was 35 V, tube lens voltage was 35 V, capillary temperature was 300°C, sheath and auxiliary gas flow (N_2) was 32 and 7 (arbitrary units). MS spectra were acquired by full range acquisition covering 50-2000 *m/z*. For fragmentation study, a data-dependant scan was performed by deploying the collision-induced dissociation (CID). The normalized collision energy of the CID cell was set at 35 eV.

Phenolics were identified according to the corresponding spectral characteristics: mass spectra, accurate mass, characteristic fragmentation and characteristic retention time. Xcalibur software (ver. 2.1) was used for instrument control, data acquisition and data analysis. The generated MS/MS spectra were processed by Tox-ID software (ver. 2.1.1). The molecule editor program,

Table 1. Phenolic content of pea seed coats (mg kg-1DW), confirmed using the available standards.

	Compound	MBK 88	MBK 90	MBK 168	MBK 173
Phenolic acids	Gallic acid	1.96	1.89	14.23	9.37
	Protocatechuic acid	127.22	26.31	66.95	61.87
	Gentisic acid	5.01	3.59	3.07	2.97
	p-Hydroxybenzoic acid	Nd	Nd	5.88	6.42
	Chlorogenic acid	1.59	1.71	1.49	1.46
	Caffeic acid	2.49	1.59	2.44	2.83
	p-Hydroxyphenylacetic acid	3.49	2.51	2.36	2.17
	p-Coumaric acid	2.14	3.23	2.70	2.74
	Ferulic acid	5.33	6.20	3.79	3.26
	Sinapic acid	4.56	4.39	Nd	3.65
	Syringic acid	1.87	Nd	Nd	Nd
	Rosmarinic acid	1.81	1.80	Nd.	Nd.
Flavanols	Catechin	3.08	Nd	1.45	1.46
	Epicatechin	14.59	2.16	2.89	2.15
	Catechin gallate	2.69	Nd	Nd	Nd
	Gallocatechin	Nd	Nd	99.10	62.28
	Epigallocatechin	5.96	Nd	325.27	239.25
Flavonols	Quercetin	13.66	38.88	6.19	5.25
	Rutin	11.51	4.56	1.03	0.97
	Kaempferol	6.49	4.71	4.49	3.77
	Galangin	4.19	4.08	4.06	4.02
	Morin	4.93	4.52	Nd	Nd
Flavones	Luteolin	9.40	5.19	7.83	6.46
	Apigenin	3.62	3.046	3.14	3.38
Flavanones	Naringin	1.68	1.55	1.93	1.44
	Hesperetin	3.15	2.87	2.81	Nd
	Pinocembrin	3.44	3.43	Nd	Nd

Nd- not detected.

ChemDraw (ver. 12.0), was used as a reference library to calculate the accurate mass of the compounds of interest.

Determination of total phenolic content (TPC)

Total phenolic content was measured according to the method of Singleton and Rosi (1965). The absorbance of the samples was detected at 765 nm and results were expressed as mg of gallic acid equivalents per g of seed flour (mg GAE g^{-1}), using gallic acid calibration curve 10-1000 μg mL^{-1} with a linearity range $r = 0.991$.

DPPH scavenging assay

Scavenging activity against the DPPH radical was assayed according to the method of Pownall et al. (2010), with certain modifications. Different sample dilutions (500 µl) in 50 mM phosphate buffer, pH 7.0, were mixed with 500 µL of 100 µM DPPH reagent dissolved in methanol and incubated for 20 min in the dark. Immediately after, absorbance at 517 nm was recorded against th phosphate buffer/methanol mixture as blank. The control consisted of an equal volume of DPPH and phosphate buffer. For each sample dilution, the % of scavenged DPPH radical was calculated as follows:

$$(\%)\ \text{scav.} = (Ac_{517}\text{-}As_{517})/Ac_{517} \times 100,$$

where Ac_{517} and As_{517} represent the absorbance of the control and sample, respectively. Results were calculated using a Trolox standard curve (0.1-1mg mL^{-1}, r = 0.991) and expressed in mg of Trolox equivalents per g of dry sample (mg TE g^{-1}).

Ferric ion-reducing capacity assay (FRC)

The reducing power of the extracts was determined according to the method of Pownall et al. (2010), with slight modifications. Different sample dilutions (500 µL)in 50 mM phosphate buffer, pH 7.0 were mixed with 250 µL of 1% potassium ferricyanide solution and incubated for 20 min at 50°C. After the incubation, 500 µL of 10% trichloroacetic acid was mixed with 500 µL of the incubated sample, 100µL of 0.1% ferric chloride and 500 µL of distilled water. Following additional incubation for 10 min, absorbance was immediately measured at 700 nm against the blank consisting of phosphate buffer and the appropriate volume of solvent, treated in the same manner. The results were expressed as absorbance units at 700 nm, which was considered as a measure of reducing power.

Ferrous ion-chelating capacity assay (FCC)

The capacity of the seed coat extracts to chelate Fe^{2+} ions was determined according to the method of El and Karakaya (2001), with certain modifications. The extracts (200 µL) were mixed with 740 µL of distilled water and 20 µL of 2 mM $FeCl_2$ solution prior to 30 min incubation at room temperature. After incubation, 40 µL of 5 mM ferrozine solution was added to initiate the reaction. The mixture was then left to stand at room temperature for an additional 10 min. The absorbance was recorded at 562 nm. The distilled water was used as control instead of the sample. The chelating capacity of extracts was calculated using following equation: Fe^{2+} chelating capacity (%) = $[(A_{562c}\text{-}A_{562s})/A_{562c}] \times 100$, where c and s are the control and sample, respectively.

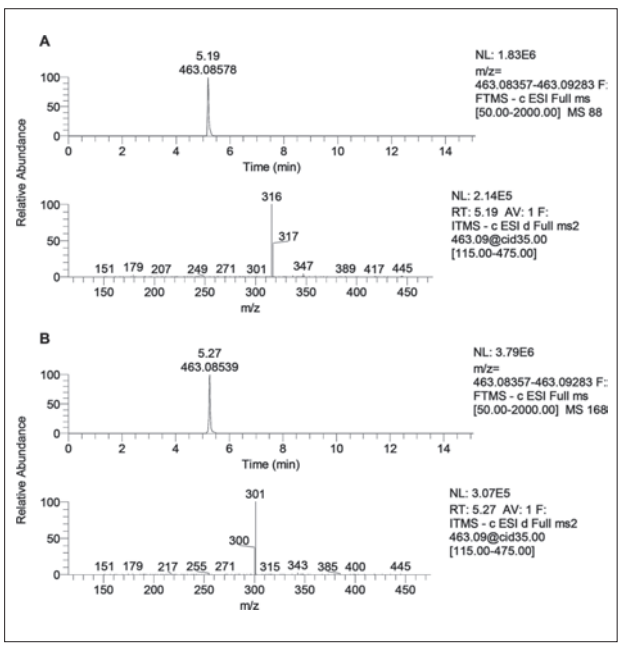

Fig. 3. Extracted ion chromatograms (EICs) of two identified compounds with same masses and very similar retention times: (A) myricetin deoxyhexoside and (B) quercetin hexoside.

Table 2. Phenolic compounds detected in the pea seed coat extracts in negative ionization mode.

Peak No	Compound	Molecular formula, $[M-H]^-$	t_R, min	Calculated mass, $[M-H]^-$	Exact mass, $[M-H]^-$	Δ mDa	MS/MS Fragmentation, (% MS/MS Base Peak)	MBK			
								88	90	168	173
1	Gallic acid[a]	$C_7H_5O_5^-$	1.48	169.01425	169.01331	0.94	125(100)	+	+	+	+
2	Gallocatechin-gallocatechin	$C_{30}H_{25}O_{14}^-$	1.73	609.12498	609.12210	2.88	591(10), 483(15), 441(100), 423(70), 305(30)	+	-	-	+
3	Gallocatechin[a]	$C_{15}H_{13}O_7^-$	2.31	305.06668	305.06500	1.68	287(10), 261(50), 221(80), 219(70), 179(100)	-	-	+	+
4	Gallocatechin-gallocatechin-gallocatechin	$C_{45}H_{37}O_{21}^-$	2.34	913.18328	913.18030	2.98	727(100), 609(20), 559(30), 305(15)	+	-	-	+
5	Protocatechuic acid[a]	$C_7H_5O_4^-$	2.50	153.01933	153.01862	0.71	109(100)	+	+	+	+
6	Gallocatechin-gallocatechin	$C_{30}H_{25}O_{14}^-$	2.73	609.12498	609.12183	3.15	591(10), 483(15), 441(100), 423(80), 305(40)	+	-	-	+
7	p-Hydroxybenzoic acid[a]	$C_7H_5O_3^-$	3.54	137.02442	137.02390	0.52	93(100)	-	-	+	+
8	Gentisic acid[a]	$C_7H_5O_4^-$	3.67	153.01933	153.01857	0.76	109(100)	+	+	+	+
9	Epigallocatechin[a]	$C_{15}H_{13}O_7^-$	3.81	305.06668	305.06506	1.62	287(10), 261(50), 221(80), 219(70), 179(100)	+	-	+	+
10	Dihydroquercetin hexoside	$C_{21}H_{21}O_{12}^-$	3.95	465.10385	465.10156	2.29	303(100), 285(15)	+	+	+	+
11	Catechin[a]	$C_{15}H_{13}O_6^-$	4.00	289.07176	289.07050	1.26	245(100), 231(10), 205(40), 179(20)	+	-	+	+
12	p-Hydroxyphenylacetic acid[a]	$C_8H_7O_3^-$	4.12	151.04007	151.03908	0.99	107(100)	+	+	+	+
13	Chlorogenic acid[a]	$C_{16}H_{17}O_9^-$	4.15	353.08781	353.08597	1.84	191(100), 179(5)	+	+	+	+
14	Catechin-catechin	$C_{30}H_{25}O_{12}^-$	4.22	577.13460	577.13214	2.46	559(10), 451(30), 425(100), 407(50), 289(20)	-	+	+	-
15	Caffeic acid[a]	$C_9H_7O_4^-$	4.37	179.03498	179.03415	0.83	135(100)	+	+	+	+
16	Dihydroquercetin dihexoside	$C_{27}H_{31}O_{17}^-$	4.39	627.15667	627.15442	2.25	465(100), 303(10)	+	+	+	+
17	Dihydrokaempferol hexoside	$C_{21}H_{21}O_{11}^-$	4.49	449.10894	449.10687	2.07	431(10), 287(100), 269(40), 259(30)	+	+	+	+
18	Epicatechin[a]	$C_{15}H_{13}O_6^-$	4.50	289.07176	289.07050	1.26	245(100), 231(10), 205(40), 179(20)	+	+	+	+
19	Kaempferol dihexoside	$C_{27}H_{29}O_{16}^-$	4.77	609.14611	609.14374	2.37	447(10), 429(70), 285(100), 284(50)	-	+	+	-
20	Quercetin dihexoside	$C_{27}H_{29}O_{17}^-$	4.79	625.14102	625.13916	1.86	505(20), 463(20), 445(50), 301(70), 300(100)	+	+	+	+
21	Kaempferol trihexoside	$C_{33}H_{39}O_{21}^-$	4.94	771.19893	771.19562	3.31	651(30), 609(100), 429(90), 285(60)	+	+	+	+
22	Rutin[a]	$C_{27}H_{29}O_{16}^-$	5.01	609.14611	609.14362	2.49	343(10), 301(100), 300(35)	+	+	+	+
23	p-Coumaric acid[a]	$C_9H_7O_3^-$	5.04	163.04007	163.03923	0.84	119(100)	+	+	+	+
24	Catechin gallate[a]	$C_{22}H_{17}O_{10}^-$	5.17	441.08272	441.08109	1.63	331(15), 289(100), 271(10), 193(5), 169(25)	+	-	-	-
25	Myricetin deoxyhexoside	$C_{21}H_{19}O_{12}^-$	5.19	463.08820	463.08578	2.42	347(10), 317(50), 316(100), 179(5)	+	-	-	+
26	Quercetin hexoside	$C_{21}H_{19}O_{12}^-$	5.27	463.08820	463.08539	2.81	343(5), 301(100), 300(30)	-	+	+	-
27	Sinapic acid[a]	$C_{11}H_{11}O_5^-$	5.37	223.06120	223.06001	1.19	208(100), 179(50), 177(30), 164(40)	+	+	-	+
28	Ferulic acid[a]	$C_{10}H_9O_4^-$	5.45	193.05063	193.04947	1.16	178(30), 149(40), 134(100)	+	+	+	+
29	Naringin[a]	$C_{27}H_{31}O_{14}^-$	5.48	579.17193	579.16895	2.98	459(100), 313(10), 271(40), 235(10)	+	+	+	+
30	Quercetin deoxyhexoside	$C_{21}H_{19}O_{11}^-$	5.61	447.09329	447.09073	2.56	301(100), 300(25)	+	+	+	+
31	Rosmarinic acid[a]	$C_{18}H_{15}O_6^-$	5.71	359.07724	359.07550	1.74	223(10), 179(25), 197(25), 161(100)	+	+	-	-

Table 2 continued:

32	Syringic acid[a]	$C_9H_9O_5^-$	5.73	197.04555	197.04457	0.98	182(40), 153(30), 135(100)	+	-	-	-
33	Kaempferol deoxyhexoside	$C_{21}H_{19}O_{10}^-$	6.00	431.09837	431.09607	2.30	315(10), 285(100), 284(40), 255(5)	+	+	+	+
34	Morin[a]	$C_{15}H_9O_7^-$	6.01	301.03538	301.03339	1.99	273(40), 257(50), 227(80), 151(100)	+	+	-	-
35	Quercetin[a]	$C_{15}H_9O_7^-$	6.59	301.03538	301.03333	2.05	273(20), 257(20), 179(100), 151(70)	+	+	+	+
36	Luteolin[a]	$C_{15}H_9O_6^-$	6.61	285.04046	285.03885	1.61	241(100), 199(70), 175(85), 151(30)	+	+	+	+
37	Apigenin[a]	$C_{15}H_9O_5^-$	7.22	269.04555	269.04416	1.39	225(100), 201(25), 183(20), 151(30)	+	+	+	+
38	Hesperetin[a]	$C_{16}H_{13}O_6^-$	7.22	301.07176	301.07010	1.66	286(100), 257(40), 242(60), 199(10)	+	+	+	-
39	Kaempferol[a]	$C_{15}H_9O_6^-$	7.30	285.04046	285.03882	1.64	285(100), 229(50), 185(50), 151(65)	+	+	+	+
40	Pinocembrin[a]	$C_{15}H_{11}O_4^-$	8.50	255.06628	255.06506	1.22	213(100), 187(15), 151(35)	+	+	-	-
41	Galangin[a]	$C_{15}H_9O_5^-$	8.68	269.04555	269.04422	1.33	241(50), 227(80), 213(90), 197(100)	+	+	+	+

[a]Confirmed using available standards, all the other compounds were identified based on MS/MS data. Peak number, target compound, molecular formula, mean expected retention times (min), calculated mass, exact mass, mean mass accuracy error (mDa), and MS/MS fragments (% MS/MS base peak); + stands for detected and – stands for not detected compound

Statistical analysis

The experiments were carried out in triplicate and data were expressed as means ± standard deviations. The data were subjected to ANOVA and Duncan's multiple range tests using the software package SPSS (version 19.0; SPSS Inc., Chicago) to determine significant differences at $P < 0.05$. Principal component analysis (PCA) was conducted using Past3 software, available online.

RESULTS AND DISCUSSION

Phenolic composition

In total, we identified 41 phenolic compounds based on MS/MS data. Additionally, 27 compounds were confirmed and quantified using the available standards (Table 1). Considering the composition of phenolic acids, 12 acids were quantified and 8 of them were detected in the seed coats of all the tested varieties (gallic, protocatechuic, chlorogenic, *p*-hydroxyphenylacetic, gentisic, caffeic, ferulic and *p*-coumaric acids). Syringic acid and rosmarinic acid were present only in the bright colored genotypes (MBK88 and MBK 90) in contrast to p-hydroxybenzoic acid which was detected only in the dark colored varieties (MBK 168 and MBK 173). Phenolic acids were the most abundant class of phenolic compounds present in the seed coats of the bright colored varieties. In both varieties, protocatechuic acid was the most abundant (127.2 mg kg^{-1} in MBK 88, and 26.3 mg kg^{-1} in MBK 90). This is in agreement with the previously published data of Dueñas et al. (2004), who also determined a high content of phenolic acids in seed coats of two pea varieties, although in their case glycosides of flavones and flavonols were characterized as the predominant compounds. In the dark colored varieties, protocatechuic acid was the most abundant acid (66.9 mg kg^{-1} in MBK 168, and 61.9 mg kg^{-1} in MBK 173), followed by gallic acid, which was also present in significant amounts. Chlorogenic acid and *p*-hydroxyphenylacetic acid, which were equally distributed in all examined varieties, are for the first time reported to be present in the seed coats of pea, although they were previously detected in whole seed extracts of green and yellow pea (Xu and Chang, 2009). Compared to previously published data (Troszyńska and Ciska, 2002; Dueñas et al., 2004), we

identified the same acids, with an exception of vanillic acid, which has not been detected in our specimens.

Compounds from almost all subclasses of flavonoids were detected in the majority of varieties. Five flavanols were identified (catechin, epicatechin, catechin gallate, gallocatechin, and epigallocatechin). The highest percentage of phenolics in the seed coat of dark colored varieties was that of epigallocatechin (325.3 mg kg^{-1} in MBK 168, and 239.3 mg kg^{-1} in MBK 173), the second was gallocatechin, with 99.1 mg kg^{-1} and 62.3 mg kg^{-1}, respectively. A high amount of epigallocatechin was recorded in seed coats of some dark colored pea varieties originating from France, while epicatechin, catechin and gallocatechin were characterized as minor compounds (Dueñas et al., 2004). On the contrary, in the study of Xu and Chang (2009), who examined North American varieties of green and yellow peas, catechin was the dominant flavonol present in seeds, while epigallocatechin and epicatechin were absent. In the seed coats of the bright colored varieties examined in our study, epigallocatechin and gallocatechin were present either in very small amounts in (MBK 88), or were completely absent (MBK 90). Only MBK 88 contained a significant amount of epicatechin (14.6 mg kg^{-1}). The flavonols quercetin and rutin were present mainly in the seed coats of the bright colored varieties, while kaempferol and galangin were almost equally distributed in all examined varieties. Morin was detected only in the bright colored seed coats. Quercetin, with a content of 38.9 mg kg^{-1}, was the most abundant phenolic compound in MBK 90. Considerable amounts of flavones (luteolin and apigenin) were detected in all varieties with no major differences among the varieties. As regards flavanones, naringin was found, with equal distribution among the varieties. On the other hand, hesperetin was absent in MBK 173, while pinocembrin was present only in bright colored genotypes.

In the absence of standards, identification of another 14 peaks in the chromatograms was based on the search for the $[M-H]^-$ deprotonated molecule and its fragmentation. The exact mass search and the study of the fragmentation pathways described in the literature enabled us to obtain as much structural information as possible. In this way, it was possible to identify flavonol glycosides and proanthocyanins. The main

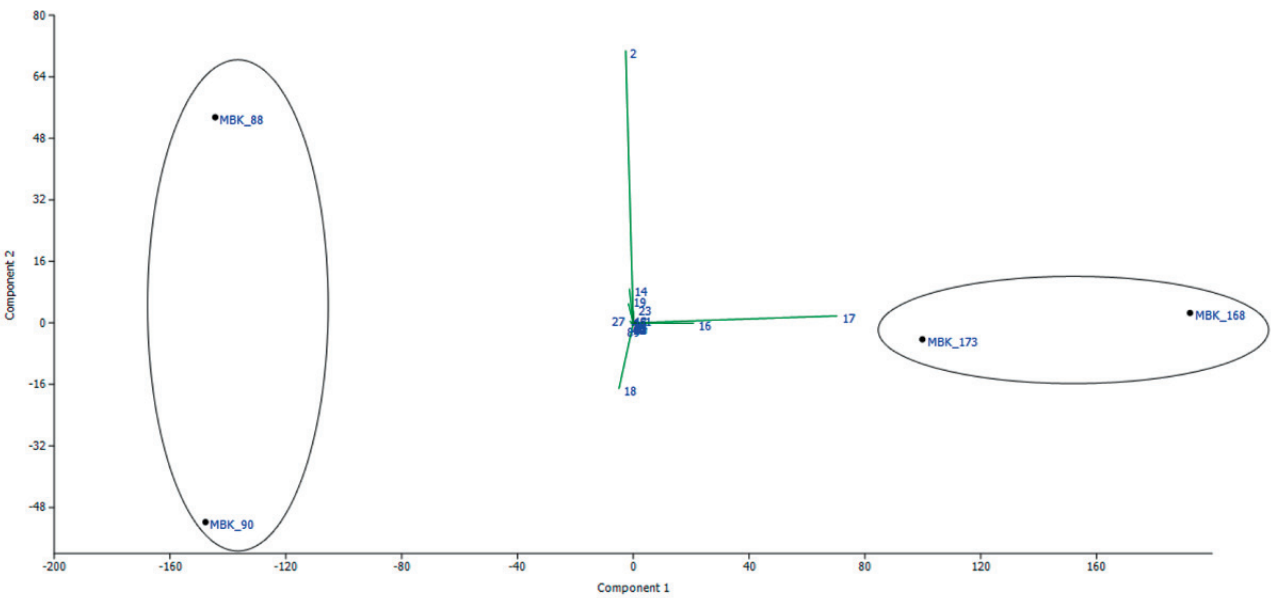

Fig. 4. Principal component analysis. Scores of the samples defined by the first two principal components. 1-27 Identified phenolic compounds, in the order presented in Table 4.

chromatographic and MS/MS data of the identified phenolics are summarized in Table 2. Chromatograms of the investigated samples showed similar profiles. The selected base peak chromatograms of all of four pea seed coat samples are shown in Fig. 2.

In the analyzed pea seed coat extracts, it was possible to identify flavonol monoglycosides, diglycosides, triglycosides, and deoxyglycosides. A total of 10 flavonol glycosides were identified. The presence of quercetin, kaempferol and myricetin glycosides was largely based on the MS/MS data (Table 2). In the case of the pseudomolecular ion at *m/z* 463, the occurrence of two peaks was observed for the same extracted exact mass. The typical UHPLC-MS extracted ion chromatograms (EICs) for *m/z* 463 are presented in Fig. 3. The chromatograms shown in Fig. 3A were the extracted peak of myricetin deoxyhexoside at 5.19 min. Their MS/MS spectra gave a MS/MS base peak at *m/z* 316 and secondary peak at *m/z* 317 ([M-H-deoxyhexosyl]$^-$), which corresponds to the aglycone part of molecule (myricetin). The compound at 5.27 min produced the MS/MS base peak at *m/z* 301 ([M-H-hexosyl]$^-$), the secondary peak at *m/z* 300 is characteristic of quercetin hexoside (Fig. 3B). It was interesting that in two samples (MBK 88 and MBK 173) only myricetin deoxyhexoside was found, whereas in the other two samples only quercetin hexoside was found (Table 2). Compared to available data (Dueñas et al., 2004), these flavonol glycosides were for the first time reported to be present in the seed coats of European cultivars, although some of them were detected in whole seed extracts of North American varieties (Xu and Chang, 2009). As for proanthocyanidins, flavan-3-ol oligomers, three dimers and one trimer were found (Table 2). Identification of the unknown proanthocyanidin was achieved by comparing the MS fragmentation patterns with literature data (Friedricrih et al., 2000).

The data collected in analysis of individual phenolic compounds have been subjected to principal component analysis (PCA) in order to determine the differences in phenolic profiles mong the examined varieties. The results from the principal component scores plot (Fig. 4) showed that the first component represented 94.02% of the total variance, while the second component explained 4.84% of total variance. A prominent differentiation of dark colored varieties (MBK168 and MBK 173) and bright colored varieties (MBK 88 and MBK 90) was observed along principal component 1. It was also observed that two bright colored varieties differed along the principal component 2. As can be seen from Table 4, which presents the loadings of the variables, it was evident that epigallocatechin and gallocatechin were the main contributors to principal component 1, while protocatechuic acid and epicatechin were the dominant features in principal component 2.

Total phenolic content of seed coat extracts

The total phenolic content (TPC) of the seed coats from examined varieties is shown in Table 3. Significant differences ($p < 0.05$) among the varieties was determined. TPC ranged between 2.57 mg GAE g^{-1} in

Table 3. Total phenolic content, antioxidant activity and metal-chelating capacity of pea seed coat extracts.

Varieties	TPC (mgGAE g^{-1})	DPPH (mM TE g^{-1})	FRC (A_{700nm})	FCC (% chel.)
MBK 88	14.35 ± 0.66a	1.73 ± 0.06a	0.063 ± 0.004a	48.8 ± 1.1a
MBK 90	2.57 ± 0.12b	0.72 ± 0.03b	0.019 ±0.002b	75.8 ± 0.7b
MBK 168	30.56 ± 1.3c	2.55 ± 0.19c	0.312 ±0.007c	29.6 ± 0.8c
MBK 173	21.56 ± 0.96d	2.37 ± 0.11c	0.240 ±0.01d	35.5 ± 0.7d

Results are presented as means ± SD (n=3). Mean values in a column followed by the same small case letter are not significantly different by Duncan's multiple range test ($P<0.05$). TPC – total phenolic content; DPPH – 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay; FRC-ferric-ion reducing capacity assay; FCC- ferrous-ion chelating capacity assay

MBK 90 and 30.56 mg GAE g^{-1} in MBK 168. As was expected, the varieties with darker seed coat (Fig. 1) showed a higher TPC value, which is in compliance with previously published data (Xu and Chang, 2007). As the data on TPC in pea seed coats were limited and only a few studies have been undertaken to date, the comparison of our results with literature data is quite difficult. In the study of Xu and Chang (2007), who determined the total phenolic content in the whole seed flour of over 30 legumes, values ranged from 0.57 mg GAE g^{-1} of whole dry seed in navy beans to 9.6 mg GAE g^{-1} in lentils, and the average TPC values for green and yellow peas were between 0.81 and 0.94 mg GAE g^{-1} of whole seed flour. Compared to our results, the TPC values for the peas in this study were far lower, which was expected since we used only seed coats that contained the majority of phenolic compounds. In one of the rare studies conducted on pea hulls (seed coats), Oomah et al. (2011) reported a total phenolic content of yellow pea hulls ranging from 2.6 to 9.1 mg of catechin equivalents per g of dry material, depending on the extraction solvent, which was comparable to our results. In a study on black soybean seed coats from 60 varieties (Zhang et al., 2011), TPC values ranged between 0.512 and 60.58 mg GAE g^{-1}. The TPC of lentil seed coats from different varieties was in the range of 24.63-87.16 mg of catechin equivalents per gram of dry weight (Oomah et al., 2011). Consequently, based upon these results it could be concluded that the seed coats of peas investigated in our study have TPC values comparable to other extensively cultivated pulse crops, such as lentils or soybeans.

Table 4. Loadings of the features in the first three principal components

		PC1	PC2	PC3
	% of variance	94.02	5.84	0.14
1	Gallic acid	0.0347	0.0006	0.1053
2	Protocatechuic acid	-0.0357	0.9605	0.1143
3	Gentisic acid	-0.0041	0.0138	0.0242
4	*p*-Hydroxybenzoic acid	0.0198	-0.0022	-0.1523
5	Chlorogenic acid	-0.0006	-0.0010	0.0056
6	Caffeic acid	0.0017	0.0081	-0.0393
7	*p*-Hydroxyphenylacetic acid	-0.0023	0.0096	0.0219
8	*p*-Coumaric acid	5.6902×10^{-5}	-0.0103	0.0015
9	Ferulic acid	-0.0070	-0.0072	0.0795
10	Sinapic acid	-0.0105	8.8745×10^{-5}	-0.1730
11	Syringic acid	-0.0031	0.0180	0.0101
12	Rosmarinic acid	-0.0059	0.0006	0.0350
13	Catechin	-0.0002	0.0292	-0.0121
14	Epicatechin	-0.0184	0.1195	0.1049
15	Catechin gallate	-0.0043	0.0259	0.0144
16	Gallocatechin	0.2821	-0.0018	0.6961
17	Epigallocatechin	0.9548	0.0246	-0.1554
18	Quercetin	-0.0670	-0.2316	0.5632
19	Rutin	-0.0228	0.0679	0.1100
20	Kaempferol	-0.0044	0.0178	0.0648
21	Galangin	-0.0003	0.0012	0.0035
22	Morin	-0.0154	0.0053	0.0900
23	Luteolin	0.0003	0.0041	0.0690
24	Apigenin	-0.0004	0.0053	-0.0155
25	Naringin	0.0005	0.0015	0.0281
26	Hesperetin	-0.0038	0.0050	0.2019
27	Pinocembrin	-0.0112	0.0011	0.0665

Antioxidant activities of seed coat extracts

Table 3 shows the antioxidant activity of each extract determined by DPPH and FRC assay, as well as the ferrous-ion chelating capacity measured by FCC assay. DPPH scavenging activity ranged from 0.72 mM TE g^{-1} in the MBK 90 genotype to 2.55 mM TE g^{-1} in MBK 168, showing a good correlation with TPC values ($r = 0.971$). The comparison of our results with other studies was rather difficult due to different assay procedures and units in which antioxidant activity was expressed. Nonetheless, our results are comparable to those obtained for whole pea seeds (Amarowicz et al., 2004), as they were expressed in the same units. Dark colored varieties showed significantly higher antioxidant activities than the bright colored, which does not differ from previous studies conducted on various legume species (Xu and Chang, 2007; Xu and Chang, 2008; Troszynska and Ciska, 2002). Similar to the radi-

cal scavenging activity determined by DPPH assay, the ferric-ion reducing capacity (FRC) of the extracts had the same trend. FRC decreased in the following order: MBK 168>MBK 173>MBK 88>MBK 90. This assay revealed a strong correlation between the total phenolic content of the examined extracts (r=0.95) and DPPH scavenging activity (r=0.92). The strong positive correlations between AO activity and TPC have been previously reported for numerous legume seed extracts (Amarowicz et al., 2003; Xu and Chang, 2007; Troszynska et al., 1997). On the other hand, the results of the FCC assay showed negative correlation with TPC values (-0.972), DPPH assay (-0.998) and FRC assay (-0.903). FCC was the only assay in which an inverse behavior of dark and bright colored varieties was determined. A possible explanation is that in the ferrous chelating assay some other non-phenolic compounds, such as phytic acid, had a major contribution to chelating capacity. This could be supported by the recent report of Abizari et al. (2012), who determined that the bioavailability of Fe^{2+} in whole cowpea meal depends on the phytic acid/iron molar ratio rather than polyphenol concentration, directly indicating that phytic acid may be the main iron-chelating agent in cowpea and an inhibitor of its absorption.

To summarize, there are significant differences in both phenolic content and antioxidant activity among the seed coat extracts from different genotypes. Our results clearly demonstrate that seed coats of the examined pea genotypes possess a unique phenolic composition compared to previously studied cultivars. Seed coats of the studied genotypes contained certain amounts of rosmarinic acid, rutin, galangin, morin, naringin, hesperetin and pinocembrin, as well as ten flavonol glycosides that, to the best of our knowledge, have not been reported previously. The dark colored genotypes MBK 168 and MBK 173 possessed the highest phenolic contents as well the strongest antioxidant activities. On the other hand, the bright colored genotypes exhibited the strongest metal-chelating capacities. Considering recent advances in studies of the inheritance of antioxidant activity and its association with seed coat color in other legume species (Nzaramba et al., 2005), our results could be used as a guideline for breeding new pea cultivars with high antioxidant activities which could be effectively used in the formulation of functional food products.

Acknowledgments: This work was financially supported by The Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant No. 173005 and Grant No. 172017. We express our appreciation to the professional contribution to Ms. Radmila Dodić, Sworn-to-Court Translator, for editing this document.

Authors' contributions: Nemanja Stanisavljević designed the experiments and wrote the paper. Živko Jovanović and Marija Ilić performed preparation of extracts, and antioxidant activity assays. Dragana Dabić, Maja Natić and Živoslav Tešić perfomed identification of individual phenolic compounds and statistical analysis. Tihomir Čupić provided seed material and grew the plants, Svetlana Radović edited the manuscript.

Conflict of interest disclosure: The authors declare that have no conflict of interest in this submission.

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