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Research Article

Co-pigmentation of black chokeberry (*Aronia melanocarpa*) anthocyanins with phenolic co-pigments and herbal extracts

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ABSTRACT

The co-pigmentation of black chokeberry (*Aronia melanocarpa*) anthocyanins with ten phenolic co-pigments was studied. Tested compounds provoked different co-pigmentation effect, manifested by hyperchromic and batochromic shifts. The co-pigmentation was accompanied by a magnification of color intensity and decrease of color hue, both related to a more pleasant color. The hyperchromic effect was the most significant for rosmarinic acid (51.02%), syringic acid (43.24%) and catechin (39.73%). However, it was observed at the highest pigment/co-pigment ratio (1:50), not achievable in plant matter. Targeting the potential practical application of co-pigmentation, we tested eight herbal extracts for their co-pigmentation ability with aronia anthocyanins. The use of herbal extracts led to a significant hyperchromic effect at much lower pigment/co-pigment ratios, compared to pure compounds. The use of selected herbal extracts as co-pigments opens realistic prospects for development of aronia functional foods with improved sensory properties and biological effects, due to enhanced color and anthocyanin stability.

1. Introduction

Anthocyanins are among the most important and widely spread plant pigments, belonging to the class of flavonoids. They present in many flowers, fruits and vegetables, as well as in foods and beverages (red wine, fruit juices and nectars, jams, etc.), giving them red, blue or purple color (Harborne & Grayer, 1988). Anthocyanins are of a great importance for food science and technology, since attractive color of foods is an important quality parameter that affects customers' preferences. Color conservation of anthocyanins strongly depends on their concentration and structure, pH, solvents, temperature, presence of oxygen, enzymes and other concomitant substances. They are more stable in acidic conditions, at low pH and their ionic nature allows them to change molecular structure according to pH, resulting in different colors and hues (Brouillard, 1983). Besides their role for the sensory acceptance of foods, anthocyanins have various beneficial effects for human health. They reduce the fragility and permeability of capillaries (Wagner, 1985) and reveal anti-inflammatory, hypotonic, collagen-stabilizing (Lila, 2004), and cardioprotective effects (Bell & Gochenaur, 2005). Anthocyanins were successfully applied for prevention and treatment of glaucoma (Ghosh & Konishi, 2007) and for improvement of vision (Canter & Ernst, 2004). The strong and intense color of foodstuffs and beverages, due to the presence of anthocyanins, and their biological activities, make the presence of these pigments in foods, functional foods and beverages particularly desired. However, due to their relatively unstable nature, the preservation of anthocyanins during processing and storage is a challenge for food scientists and technologists. Co-pigmentation is one of the main mechanisms for natural stabilization of anthocyanins. The co-pigmentation phenomenon occurs when pigment (anthocyanins) and co-pigment (colorless substance) form non-covalent complexes. It is observed as a bathochromic shift and hyperchromic effect, which stabilizes and modulates anthocyanin color, contributing to a more intense color (Asen, Stewart, & Norris, 1972; Trouillas et al., 2016). Phenolic acids and flavonoids are among the best co-pigments and special attention is paid to the practical applications of co-pigmentation in food chemistry and food industry, for both stabilizing anthocyanins in foodstuffs and for intensification of their color. The co-pigmentation has been studied mostly in regards to color changes in wine, during vinification and aging (González-Manzano, Dueñas, Rivas-Gonzalo, Escribano-Bailón, & Santos-Buelga, 2009; Zhang, Liu, He, Zhou, & Duan, 2015; Heras-Roger, Alonso-Alonso, Gallo-Montesdeoca, Díaz-Romero, & Darias-Martín,

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2016; Bimpilas, Panagopoulou, Tsimogiannis, & Oreopoulou, 2016; Zhang et al., 2018; Rivero, Jara-Palacios, Gordillo, Heredia, & González-Miret, 2019). Several studies indicate that anthocyanins from various fruits (acai, strawberries, plums, raspberries, lingonberries, cranberry, etc) could be stabilized by the addition of phenolic compounds or plant extracts, as well (Rein & Heinonen, 2004; Mollov, Mihalev, Shikov, Yoncheva, & Karagyozov, 2007; Shikov, Kammerer, Mihalev, Mollov, & Carle, 2008; Pacheco-Palencia & Talcott, 2010; Hernández-Herrero & Frutos, 2015; Müller-Maatsch, Bechtold, Schweiggert, & Carle, 2016; Gras, Nemetz, Carle, & Schweiggert, 2017; Weber, Boch, & Schieber, 2017; Chitgar, Aalami, Kadkhodaee, Maghsoudlou, & Milani, 2018).

Fruits of *Aronia melanocarpa*, known as black chokeberry are not so popular among consumers, although they have shown to be one of the richest sources of polyphenols, including anthocyanins among fruits (Kulling & Rawel, 2008; Denev, Kratchanov, Ciz, Lojek, & Kratchanova, 2012). Cyanidin-3-*O*-galactoside and cyanidin-3-*O*-arabinoside are the predominant anthocyanins, representing more than 90% of the anthocyanins in aronia berries, whereas cyanidin-3-*O*-glucoside and cyanidin-3-*O*-xyloside are presented as minor components (Oszmiański & Wojdylo, 2005). The biological activities of aronia anthocyanins include antioxidant, antimutagenic, antidiabetic, cardioprotective, hepatoprotective and anticarcinogenic effects (Denev et al., 2012). Because of that, nowadays, chokeberries are important industrial crop, used for production of juices, nectars, wines, jams and food-grade coloring agent. Even though chokeberries are thoroughly studied for their chemical composition and health benefits, we only found one study on the co-pigmentation of aronia anthocyanins, revealing that chlorogenic acid, at concentrations greater than that of anthocyanins, enhanced the color intensity of aronia juice (Wilska-Jeszka & Korzuchowska, 1996).

From the literature review, it is evident that many phenolic compounds act as co-pigments for anthocyanins in different food matrices. However, in most of the cases co-pigment/pigment ratio is quite high and irrelevant to their native ratios in the plant matter. Based on these studies, we hypothesized that chokeberry anthocyanins will form co-pigment complexes with different phenolic compounds (besides chlorogenic acid) and the problem with the high concentration of co-pigments could be overcome by the use of popular herbs, rich in particular phenolic co-pigments. Therefore, the aim of the present work was to investigate the impact of 10 different phenolic co-pigments on the color intensity of aronia anthocyanins in model systems. Further, herbal extracts, rich in particular polyphenols were tested for their co-pigmentation ability with aronia anthocyanins, as well. The study provides comprehensive information about the influence of various co-pigments found naturally in fruits and herbs on color intensity of chokeberry anthocyanins. The results could be used for the development of aronia-based functional foods and beverages with improved color intensity and stability.

2. Material and methods

2.1. Chemicals

Cyanidin-3-*O*-galactoside chloride, cyanidin-3-*O*-arabinoside chloride and cyanidin-3-*O*-glucoside chloride were purchased from Extrasynthese S.A. (Genay Cedex, France). Chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, protocatechuic acid, catechin, rutin, naringin, naringenin, epicatechin, myrecetin, quercetin-3-*O*-glucoside, quercetin, kaempherol, rosmarinic acid, vanillic acid, synapic acid, syringic acid, Amberlite XAD7 and Sephadex LH20 were obtained from Sigma-Aldrich (Steinheim, Germany). The Folin-Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). All other solvents used were of analytical grade and purchased from local distributors.

2.2. Plant materials

Aronia fruits were supplied from the licensed farmer Todor Petkov (Kazanlak, Stara Zagora district, Bulgaria), in the stage of full maturity, in August 2017. Fresh fruits were put in polyethylene bags, frozen immediately and stored at −18 °C until extracted.

Herbs: dry elder (*Sambucus nigra*) flowers, lavender (*Lavandula angustifolia*) aerial parts, lemon balm (*Melissa officinalis*) aerial parts, rosemary (*Rosmarinus officinalis*) aerial parts, green tea (*Camellia sinensis*) leaves, lady's mantle (*Alchemilla glabra*) aerial parts, meadowsweet (*Filipendula ulmaria*) aerial parts were purchased from local pharmacies in Plovdiv, Bulgaria. Lavender residual wastes after distillation of the essential oil were provided by Zelinikovo distillery, Bulgaria.

2.3. Extraction and purification of aronia anthocyanins and proanthocyanidins

Extraction and purification of aronia anthocyanins and proanthocyanidins were performed by the following procedure: Briefly, 100 g frozen fruits were defrosted at room temperature, mixed with 300 ml ultra pure water and homogenized in a laboratory blender. The homogenized sample was transferred into extraction tubes and subjected to extraction in thermostatic shaker water bath (NUVE, Turkey) for one hour at 60 °C. After that, the mixture was centrifuged (20 min, 6200 g) and the obtained supernatant was used for purification and isolation of aronia polyphenol preparations. Purification of anthocyanins and proanthocyanidins was performed in two steps – with Amberlite XAD7 and with Sephadex LH 20.

Column chromatography with Amberlite XAD7 was performed on SUPELCO VISIPER system, connected to a vacuum pump (KNF lab) with glass cartridges (volume 50 cm^3). Amberlite XAD7 was soaked with ultra pure water and then loaded into the column. After that, column was rinsed with ultra pure water and crude extract (50 ml portion) was applied. The impurities (sugars, organic acids, etc.) were eluted with ultra pure water and 10 ml fractions of the eluate were collected until no soluble dry solids were detected. After that, aronia polyphenols were eluted with 200 ml 96% ethanol. The column was washed with 100 ml water and another 50 ml portion of the extract was applied and processed by the same way. The procedure was repeated several times in order to purify the whole amount of the crude extract. All polyphenol-containing ethanol fractions were combined and the ethanol was rotary evaporated at 50 °C under vacuum.

We used the modified procedure of Howell et al. (2005) in order to further purify the anthocyanins and to isolate proanthocyanidins. Briefly, 20 ml of the semi-purified extract were applied to 50 cm^3 cartridges filled with 5 g of Sephadex LH − 20, equilibrated overnight in ethanol:distilled water (50:50) (v/v). After that, the column was washed with 80 ml ethanol:distilled water (50:50) (v/v) in order to remove anthocyanins. The procedure was repeated several times in order to process the whole amount of the extract. All ethanol:distilled water (50:50) fractions were collected and combined and ethanol was evaporated via rotary evaporation at 50 °C under vacuum. The obtained water extract, containing anthocyanins was freeze-dried (Christ Alpha 1–4 LDplus, Martin Christ GmbH, Germany) to obtain dry anthocyanin-rich extract (ARE). The HPLC analysis revealed that anthocyanin content of ARE is 40.02 g/100 g extract, with the following anthocyanin composition: 29.98 g/100 g cyanidin-3-*O*-galactoside, 7.16 g/100 g cyanidin-3-*O*-arabinoside and 2.16 g/100 g cyanidin-3-*O*-glucoside. The retained proanthocyanidin fraction in the column was eluted with 80 ml 80% acetone in distilled water (80:20) (v/v). The aqueous acetone fractions were combined and acetone was rotary evaporated at 40 °C under vacuum. The obtained water extract was freeze-dried in order to obtain dry aronia proanthocyanidins.

2.4. Preparation of herbal extracts

For the preparation of herbal extracts, the plant materials were powdered in a laboratory mill to pass through a 500 μm sieve. After that, 4 g of the milled herb were added to 200 ml distilled water and infused for 10 min at 90 °C, in accordance with the procedure of Kratchanova, Denev, Ciz, Lojek, and Mihailov (2010). The mixtures were filtered and centrifuged, supernatants were frozen and freeze-dried in Alpha 1–4 LDplus laboratory freeze dryer to powders.

2.5. Preparation of model solutions of pigment and co-pigments

A stock solution of the pigment (aronia anthocyanins) with a concentration of 2×10^{-4} M was prepared in McIlvaine buffer (0.1 M, pH 3.4) on the basis of the total amount of anthocyanins (40.02 g/100 g, determined by HPLC) in ARE, taking the molecular weight of cyanidin-3-*O*-galactoside as the predominant anthocyanin. The pH of the model systems was 3.4, which is very close to the native pH of aronia juice (pH 3.2–3.7). The scheme for preparation of model solutions of pigment and co-pigments is presented in Supplementary Table S1. Stock solutions of co-pigments (caffeic acid, catechin, chlorogenic acid, *p*-coumaric acid, epicatechin, gallic acid, protocatechuic acid, rosmarinic acid, syringic acid and vanillic acid) in concentrations 2×10^{-4} M, 4×10^{-4} M, 1×10^{-3} M, 1×10^{-2} M were prepared in McIlvaine buffer (0.1 M, pH 3.4). Model solutions of aronia anthocyanins and phenolic co-pigments were obtained by mixing equal volumes of the corresponding stock solutions. In order to prevent a self-association effect, the final concentration of aronia anthocyanins in the model systems was 1×10^{-4} M in all cases. Stock solutions of aronia proanthocyanidins and herbal extracts were prepared on the basis of mass, instead of on molar concentration. For that, final pigment concentration was set to be 48.5 mg/l (equal to 1×10^{-4} M solution, based on cyanidin-3-*O*-galactoside molecular weight). Model solutions of aronia anthocyanins with proanthocyanidins and herbal extracts were prepared according to this concentration, taking in mind the pigment/co-pigment ratio (Supplementary Table S1). In addition, a control model solution without co-pigment was added by mixing equal volumes of anthocyanins stock solution and McIlvaine buffer.

2.6. Spectrophotometric measurements

Spectral changes were followed by recording the absorption spectra of model solutions from 400 to 700 nm with Halo RB−10 spectrophotometer (Dynamica Scientific, United Kingdom), using 1 cm path length cuvettes. Before measurements, samples were incubated in a thermostatic shaker water bath (NUVE, Turkey) for 30 min at 25 °C. The spectrophotometric measurements were used for calculation of the co-pigmentation effect, color evaluation and calculation of thermodynamic parameters.

2.7. Calculation of co-pigmentation effect

The co-pigmentation effect, (expressed in %) was calculated by the following formulas according to Malaj, De Simone, Quartarolo, and Russo (2013):

As the hyperchromic effect at 520 nm $(\Delta A)\% = \frac{A - A_0}{A_0} \times 100$ where:

Ao – absorption of the sample without co-pigment at 520 nm
A – absorption of the sample with co-pigment at 520 nm

As the bathochromic shift of absorption maximum with addition of co-pigments:

$$\Delta\lambda_{max} = \lambda_{max} - \lambda_{max\,0}$$

where:

λ_{max0} – absorption maximum of the sample without co-pigment
λ_{max} – absorption of the sample of pigment with co-pigment

After spectrophotometric measurements, the equilibrium constant of the reaction and Gibbs free energy were calculated according to the equations:

$$1n[(A - Ao)/Ao] = 1n[K] + n \times 1n[C]$$

where: A and Ao are the absorption maximum values of the anthocyanin solution with and without co-pigment; C is the molar co-pigment concentration; K is the equilibrium constant and n is the stoichiometric ratio of the reaction. The dependence of $\ln[(A - Ao)/Ao]$ on the co-pigment concentration, $\ln[(A - Ao)/Ao] = f(\ln[C])$, is a straight line with a slope and intercept equal to n and ln[K].

Gibbs free energy ΔG (kJ mol^{-1}), was calculated using the equation:

$$\Delta G = -RT\,1n\,K$$

where: R is the universal gas constant ($R = 8.314\ J\,K^{-1}\ mol^{-1}$), T is the absolute temperature (K), K is the equilibrium constant (Dangles & Brouillard, 1992).

2.8. Color evaluation

Color intensity and color hue were calculated according to Bimpilas et al. (2016) via the following formulas:

Color intensity: $CI = A_{420} + A_{520} + A_{620}$

Color hue: $T = A_{420}/A_{520}$

where: A_{420}, A_{520} and A_{620} are the absorbance values at 420 nm, 520 nm and 620 nm, respectively.

2.9. HPLC determination of anthocyanins

For the characterization of anthocyanin content of ARE, anthocyanins were determined on HPLC system Agilent 1220 (Agilent Technology, Palo Alto, Ca), with a binary pump and UV–Vis detector (Agilent Technology, USA). A wavelength of 520 nm was used. Anthocyanins were separated using Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25 °C. The following mobile phases were used: 5% formic acid (A) and 100% methanol (B) at a flow rate of 1.0 ml/min. The gradient condition started with 15% B and linearly increased to 30% B at 20 min. The results were calculated using standard curves built with each individual anthocyanin (see Section 2.1) and expressed as g/100 g extract.

2.10. HPLC analysis of phenolic compounds

The main polyphenol compounds of herbal extracts were quantified according to Denev et al. (2014) on Agilent 1220 HPLC system (Agilent Technology, USA), equipped with a binary pump and UV–Vis detector. A wavelength of 280 nm was used. The separation of phenolics was performed on Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25 °C. The mobile phases constituted of 0.5% acetic acid (A) and 100% acetonitrile (B) at a flow rate of 0.8 ml/min. The gradient condition started with 14% B, between 6 min and 30 min linearly increased to 25% B, then to 50% B at 40 min. The results were calculated using standard curves built with each individual compound and expressed as mg/100 g extract.

2.11. Total polyphenol content analysis

Total polyphenols were determined according to the method of Singleton and Rossi (1965) with the Folin-Ciocalteu's reagent. Gallic acid was employed as a calibration standard and results were expressed in mg gallic acid equivalents (GAE) per 100 g of herb.

Table 1
Wavelength of the absorption maximum (λ_{max}), hyperchromic effect (ΔA), bathochromic shift ($\Delta \lambda_{max}$) and color hue (T) of chokeberry anthocyanins interacting with co-pigments at different molar ratios.

Co-pigment	Molar ratio pigment/co-pigment	λ_{max}, nm	Hyperchromic effect (ΔA)	Batochromic shift (Δ λ_{max})	T
Caffeic acid	1:1	512	1.0	0	0.47
	1:2	512	1.4	0	0.46
	1:5	514	3.1	2	0.45
	1:10	514	6.2	2	0.45
	1:50	518	19.9	6	0.44
Catechin	1:1	512	1.5	0	0.48
	1:2	512	3.1	0	0.47
	1:5	512	5.4	0	0.48
	1:10	514	11.6	2	0.47
	1:50	516	39.7	4	0.54
Chlorogenic acid	1:1	512	1.4	0	0.45
	1:2	512	2.7	0	0.45
	1:5	514	4.6	2	0.45
	1:10	514	10.6	2	0.45
	1:50	522	39.7	10	0.42
p-Coumaric acid	1:1	514	0.1	2	0.44
	1:2	512	0.9	0	0.44
	1:5	514	1.5	2	0.43
	1:10	514	3.2	2	0.42
	1:50	518	18.1	6	0.40
Epicatechin	1:1	512	1.0	0	0.46
	1:2	512	2.2	0	0.46
	1:5	514	4.9	2	0.45
	1:10	514	9.0	2	0.45
	1:50	518	32.4	6	0.44
Gallic acid	1:1	512	0.5	0	0.46
	1:2	512	0.9	0	0.46
	1:5	512	2.1	0	0.46
	1:10	514	5.0	2	0.45
	1:50	514	22.1	2	0.43
Protocatechuic acid	1:1	512	0.5	0	0.44
	1:2	512	1.7	0	0.43
	1:5	514	3.7	2	0.44
	1:10	514	5.0	2	0.44
	1:50	514	14.2	2	0.42
Rosmarinic acid	1:1	512	0.5	0	0.45
	1:2	514	2.1	2	0.45
	1:5	514	6.6	2	0.44
	1:10	516	17.2	4	0.43
	1:50	522	51.0	10	0.42
Syringic acid	1:1	512	1.5	0	0.45
	1:2	514	3.4	2	0.45
	1:5	514	8.0	2	0.45
	1:10	514	13.9	2	0.46
	1:50	516	43.2	4	0.48
Vanillic acid	1:1	514	0.8	2	0.43
	1:2	514	2.1	2	0.43
	1:5	514	4.2	2	0.43
	1:10	514	5.6	2	0.43
	1:50	516	20.1	4	0.42

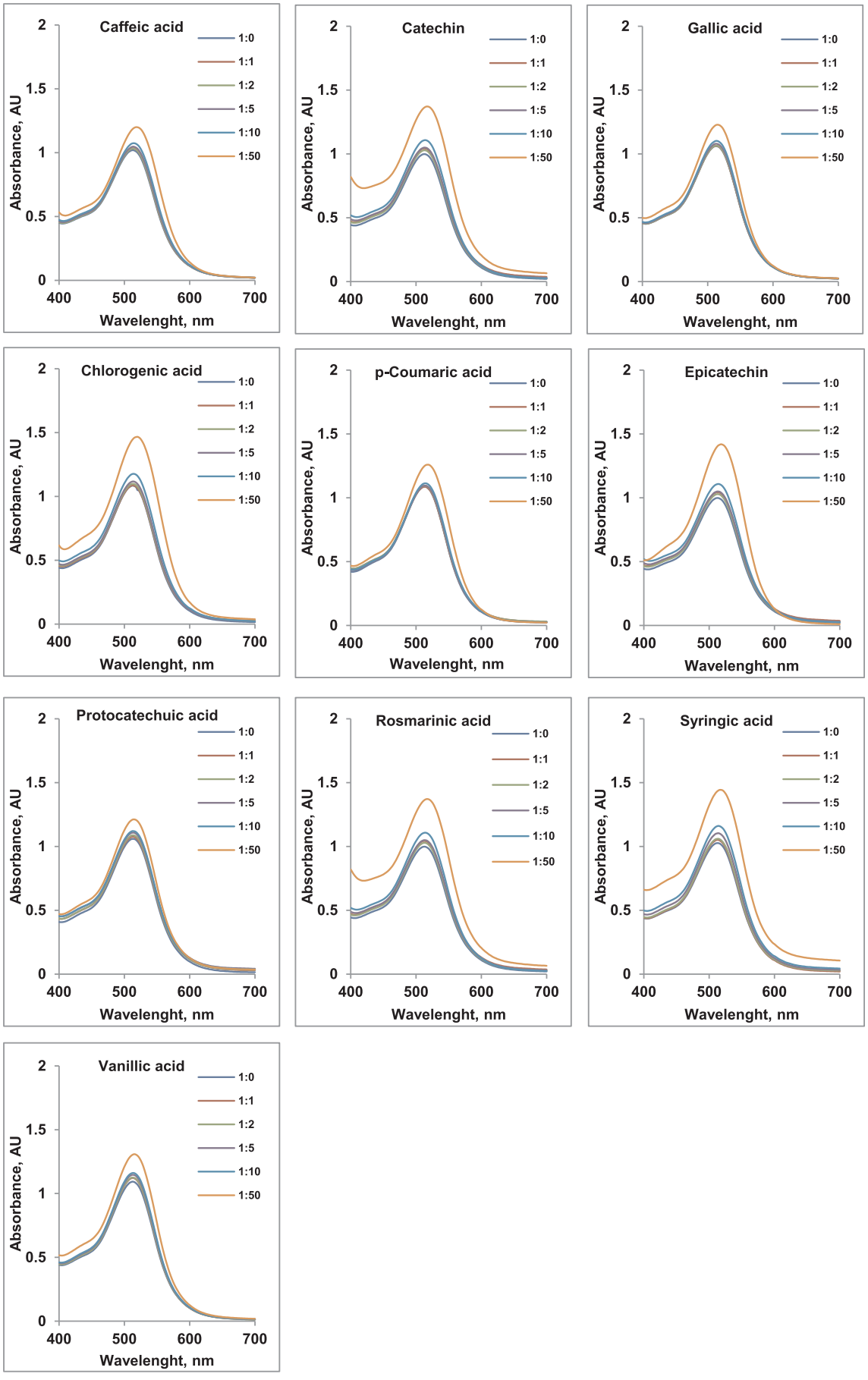
2.12. Statistical analysis

The results are expressed as the means from at least two independent experiments. Comparisons with the control were performed by analysis of variance (ANOVA), followed by the Newman-Keuls Post-hoc test. P values less than 0.05 were considered as significant.

3. Results and discussion

3.1. Spectral characteristics of aronia anthocyanins with phenolic co-pigments

As already mentioned, the most studied anthocyanin in regards to co-pigmentation is malvidin-3-*O*-glucoside (oenin) – the principle anthocyanin of wine. The available co-pigmentation studies with aglycones different from malvidin, and particularly cyanidin are considerably less (Gras, Bause, Leptihn, Carle, & Schweiggert, 2018; Sun, Cao, Bai, Liao, & Hu, 2010). In our study, we investigated the ability of aronia anthocyanins to form co-pigment complexes with ten phenolic substances. As shown in Table 1 and visualized on Fig. 1, all of the tested compounds provoked a hyperchromic effect when mixed with chokeberry anthocyanins. The increased concentrations of co-pigments resulted in increase of absorbance, indicating intermolecular interactions between the pigments and co-pigments, and in all cases, the strongest effect was observed for the highest ratio – 1:50. The effect differed significantly among the tested compounds, being the most significant for rosmarinic acid (51.02%) and syringic acid (43.24%). Catechin (39.73%), chlorogenic acid (39.71%) and epicatechin



(caption on next page)

Fig. 1. Change of absorbance spectra (400–700 nm) of black chokeberry anthocyanins interacting with phenolic co-pigments at different molar ratios. Curves with higher absorbance maximums correspond to higher co-pigment-pigment ratios.

(33.20%) were good co-pigments, as well. It is known, that the selected co-pigments do not have considerable absorbance in the visible spectrum and the increase of the absorbance of the model systems would be mainly due to the co-pigmentation effect.

Besides the hyperchromic effect, bathochromic shifts were recorded, as well. They varied between 0 and 10 nm, depending on the used co-pigment and molar ratio. The biggest shift of 10 nm was recorded for chlorogenic acid and rosmarinic acid, at a molar ratio of 1:50. Rosmarinic acid, syringic acid, chlorogenic acid and flavan-3-ols have already been identified as good co-pigments in different model systems or foods. Comparing the co-pigmentation between five anthocyanins and five phenolic acids, Eiro and Heinonen (2002) revealed that rosmarinic acid is a very strong co-pigment in malvidin-3-*O*-glucoside solutions, triggering significant bathochromic shift and hyperchromic effect. Additionally, chlorogenic acid and rosmarinic acid proved to be very good co-pigments for purple sweet potato anthocyanins, which are predominantly acylated (Gras et al., 2017). Interestingly, Malaj et al. (2013) reported that the co-pigmentation of oenin with phenolic acids depends on the degree of *O*-methoxylation and revealed that syringic acid was better co-pigment in comparison to vanillic and *p*-coumaric acids. Our study showed the same rank order for the co-pigmentation of chokeberry anthocyanins with these phenolic acids. Since π–π stacking interactions are one of the major driving forces of co-pigmentation, it is not surprising that hydroxycinnamates were better co-pigments to aronia anthocyanins than hydroxybenzoic acid with the same number of hydroxyl or methoxyl groups (Trouillas et al., 2016). From the obtained results, it is evident that the interaction of used polyphenols with aronia anthocyanins triggered both hyperchromic effect and bathochromic shifts. Both effects were the most significant at the highest pigment/co-pigment ratios, which however are not achievable in plant matter.

It was interesting to compare the hyperchromic effect and color intensity (CI) change, both accompanying the co-pigmentation phenomenon (Fig. 2). The co-pigmentation between chokeberry anthocyanins and tested polyphenols had a favorable effect on their color, as it is evident from the calculated color intensity (Fig. 2) and color hue (Table 1). The co-pigmentation led to a significant increase of CI, which was visible even with a naked, non-trained eye. In the same time, color hue decreased, which is again related to a perception of a more pleasant color. The increase of color intensity correlated well with the observed hyperchromic effect.

The hyperchromic effect (increased absorbance at λ_{max}) observed as a result from the co-pigmentation between black chokeberry anthocyanins and different co-pigments was related to a high equilibrium constant of the process (Table 2). High equilibrium constant values are indicative for the strength of the co-pigmentation complex. Our results are similar to those from previous studies on the interaction of oenin with different co-pigments. Malaj et al. (2013) reported K values for the interaction of oenin with vanillic and syringic acid of 39.90 and 48.12, respectively. In a more recent study, Zhang et al. (2015) reported values of 37.98 and 54.38 at pH 3.65 for the same substances. The change of Gibbs free energy could be referred to the stability of the investigated system. All ΔG values are negative, which is an indicator for the spontaneous nature of the co-pigmentation process and high stability of the co-pigment complex at room temperature. The highest equilibrium constant and the lowest ΔG value were recorded for rosmarinic acid, indicating again that this compound is the most efficient co-pigment towards aronia anthocyanins. In all cases the stoichiometric ratios were in the range 0.75–0.97, which is in a close agreement to previous studies with different anthocyanins (Sun et al., 2010; Malaj et al., 2013; Zhang et al., 2015).

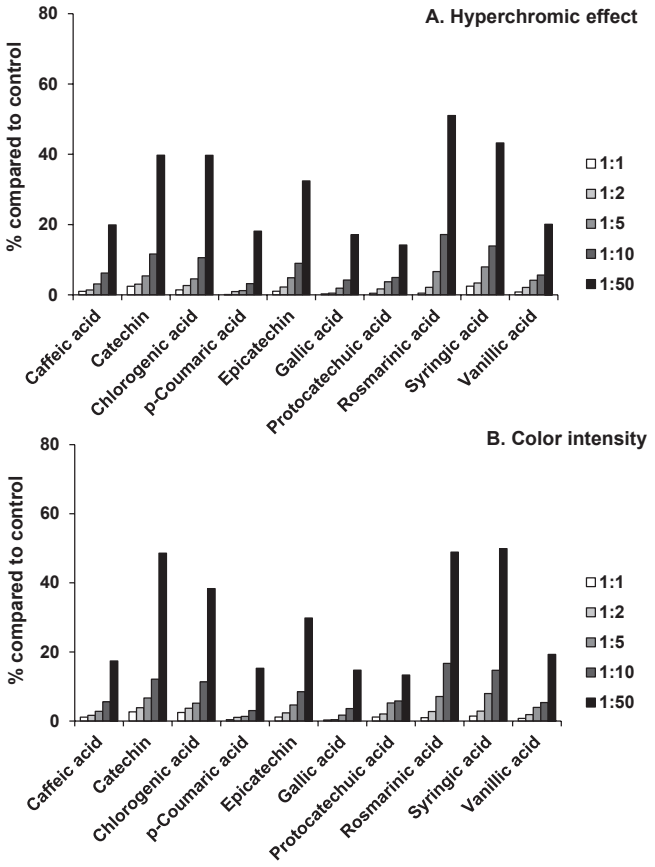


Fig. 2. Hyperchromic effect and color intensity change of aronia anthocyanins triggered by different phenolic co-pigments.

Table 2
Equilibrium constants (**K**), stoichiometric ratios (**n**) and change in Gibbs free energy of the co-pigmentation process between aronia anthocyanins and different phenolic co-pigments at 25 °C.

	n	K	ΔG, (kJ mol^{-1})
Caffeic acid	0.80	27.94	−8.19
Catechin	0.85	36.27	−8.90
Chlorogenic acid	0.97	38.09	−9.02
Epicatechin	0.81	29.57	−8.25
Gallic acid	0.94	37.9	−9.01
p-Coumaric acid	0.78	11.02	−5.94
Protocatechuic acid	0.75	7.51	−4.99
Rosmarinic acid	0.98	108.8	−11.61
Syringic acid	0.84	43.49	−9.35
Vanillic acid	0.81	20.26	−7.45

3.2. Co-pigmentation of aronia anthocyanins with proanthocyanidins and herbal extracts

The approach to use high co-pigment concentrations is commonly accepted and used mostly for studying the mechanisms of the co-pigmentation phenomenon and to determine which compounds are prone to form co-pigment complexes with given anthocyanin or anthocyanins. However, it should be noted that such high co-pigment/pigment ratios are not applicable from a practical point of view. Moreover, fortification of food products with high concentrations of phenolic compounds is unacceptable from a toxicological point of view and not allowed by the acting food legislation. That is why, targeting the potential practical

Table 3
Wavelength of the absorption maximum (λ_{max}), hyperchromic effect (**ΔA**) and batochromic shift ($\Delta\lambda_{max}$) of chokeberry anthocyanins interacting with aronia proanthocyanidins and herbal extracts.

Copigment	Mass ratio pigment/ co-pigment	λ_{max}., nm	Hyperchromic effect (ΔA), %	Batochromic shift ($\Delta\lambda_{max}$), nm
Elder flowers	1:1	512	0.1	0
	1:2	512	2.2	0
	1:5	514	7.2	2
	1:10	516	14.8	4
Green tea	1:1	514	0.9	2
	1:2	514	6.0	2
	1:5	515	11.6	3
	1:10	516	30.7	4
Lady's mantle	1:1	513	0.8	1
	1:2	515	3.3	3
	1:5	518	10.4	5
	1:10	520	16.8	8
Lavender	1:1	512	4.8	0
	1:2	514	8.6	2
	1:5	516	19.3	4
	1:10	518	34.7	6
Lavender wastes	1:1	512	1.5	0
	1:2	514	6.0	2
	1:5	514	16.1	2
	1:10	516	33.6	4
Lemon balm	1:1	512	2.5	0
	1:2	514	5.4	2
	1:5	516	12.3	4
	1:10	516	20.7	4
Meadow sweet	1:1	513	4.09	1
	1:2	515	9.32	3
	1:5	519	18.0	7
	1:10	522	28.4	10
Proanthocyanidins	1:1	512	0.9	0
	1:2	512	1.3	0
	1:5	514	5.2	2
	1:10	514	12.9	2
Rosemary	1:1	512	2.5	0
	1:2	512	3.1	0
	1:5	512	3.9	0
	1:10	514	8.2	2

application of the co-pigmentation in aronia-based functional foods, we used different herbal extracts to enhance the color of aronia anthocyanins. Another interesting natural source of co-pigments are wastes from essential oil-bearing plants, such as lavender wastes, which are rich source of phenolic compounds, after the distillation of the essential oil (Vasileva et al., 2018). Mixing of aronia anthocyanins with herbal extracts (maximal pigment/co-pigment ratio – 1:10, instead of 1:50) led to a significant co-pigmentation effect, accompanied by magnification of the color intensity, as well (Table 3, Fig. 3). The hyperchromic effect was the most significant at 1:10 ratio with lavender flowers and lavender flower waste (after distillation of the essential oil), reaching 34.7% and 33.6%, respectively. Even at 1:5 ratio, the magnitude of the absorbance at 520 nm with the lavender extracts was 19.3%. The increase of color intensity was even more significant when compared to the hyperchromic effect. For example, the hyperchromic effect for lavender and lavender wastes led to a color intensity magnification of more than 70%. Similarly to the pure compounds, herbal extracts induced bathochromic shift, beside the hyperchromic effect. However, the effect was less pronounced, being the most significant for meadowsweet extract – 10 nm.

Interestingly, the observed hyperchromic effect did not correlated with the polyphenol content of the studied extracts (presented in Supplementary Table S2, together with the major phenolic

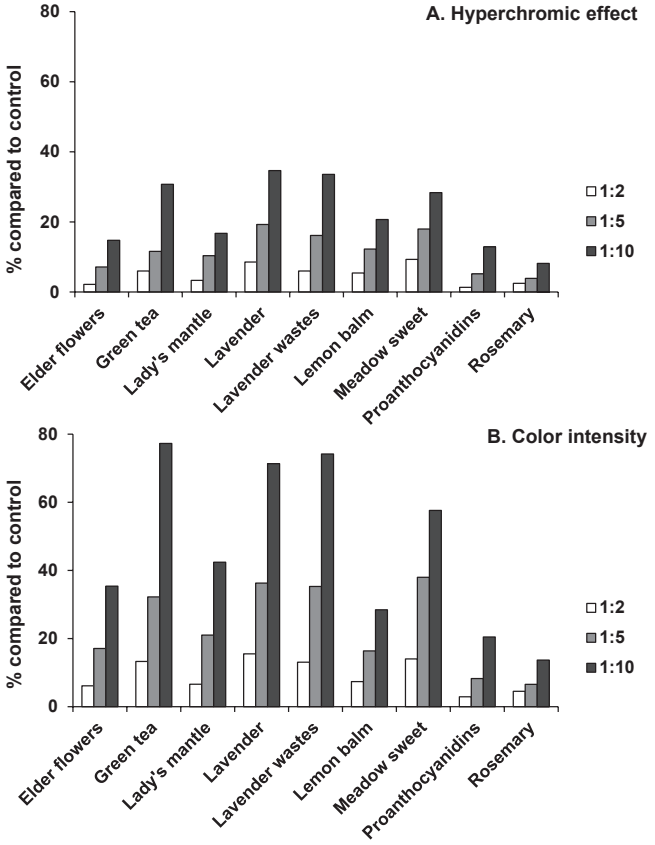


Fig. 3. Hyperchromic effect and color intensity change of aronia anthocyanins influenced by the addition of aronia proanthocyanidins and herbal extracts.

constituents). This indicates that the qualitative phenolic composition of the extracts is more important than the total polyphenol content. Herbal extracts triggered significant hyperchromic effects at much lower concentrations in comparison to pure compounds. This is likely due to the presence of many colorless co-pigments, which become colored under the co-pigmentation process. Moreover, at the used pH, not only the flavylium cation exists in the co-pigmentation complexes but also the quinonoid base, which may affect the hyperchromic effect especially in complex mixtures. The obtained results open realistic prospects for the practical application of herbal extracts for stabilization of aronia anthocyanins in foods and drinks. Anthocyanin content of freshly produced aronia juices (1030 mg/l–2674 mg/l) and nectars (365 mg/l–1182 mg/l) (Denev et al., 2018), and current results indicate that 1:5 pigment/co-pigment ratio, could be achieved by fortification of aronia juices and nectars with 0.51%–1.34% and 0.18%–0.59% extracts, respectively. This is completely achievable from practical point of view. However, further studies evaluating the co-pigmentation of aronia anthocyanins with herbal extracts, during the processing and long-term storage of aronia foods and drinks are required, since co-pigmentation is not an immediate phenomenon, but is progressively established during food storage (González-Manzano et al., 2009).

It was interesting to determine which phenolic constituents of aronia reveal the best co-pigmentation affinity towards its anthocyanins. The amount of anthocyanins in fresh aronia berries is in the range 284–686 mg/100 g fresh weight, which is slightly higher, but comparable to the content of hydroxycinnamic acids (chlorogenic acid and neochlorogenic acid) and several fold higher than that of epicatechin. The native content of proanthocyanidins in aronia berries is up to 3–4 fold higher than the content of anthocyanins (Denev et al., 2018). As it could be seen from Fig. 3, at 1:10 ratio, chokeberry proanthocyanidins provoked 12.9% hyperchromic effect, compared to 10.6% for chlorogenic acid. This means that aronia proanthocyanidins are the best

phenolic co-pigments, native for the berries. Moreover, their contribution for the color and stability of aronia anthocyanins could be higher, due to their substantial amount in the fruits. As it was demonstrated, the major phenolic components of aronia – proanthocyanidins, hydroxycinnamic acids and epicatechin are good co-pigments, which could explain the relatively high stability of aronia anthocyanins and their color, in comparison to other berries with non-acylated anthocyanins. Moreover, the combined action of these co-pigments could explain the interesting finding of Wilska-Jeszka and Korzuchowska (1996), who observed that purified anthocyanins from aronia had smaller co-pigmentation effects in comparison to aronia juice, indicating the participation of other natural co-pigments, different from chlorogenic acid, in the co-pigmentation process.

4. Conclusion

The results from the study reveal that various phenolic compounds (particularly rosmarinic acid, syringic acid, catechin and epicatechin) act as good co-pigments to aronia anthocyanins, besides chlorogenic acid. The investigated compounds provoked different co-pigmentation effect in the model systems, accompanied by a color intensity magnification. The effect was profound at high co-pigment/pigment ratios, not characteristic for plant matter and unachievable from a practical point of view. However, the approach to use high co-pigment concentrations revealed the phenolic compounds, prone to form co-pigment complexes with aronia anthocyanins and pointed us to several popular herbs, rich in these polyphenols. The use of herbal extracts and particularly from lavender, lavender waste (after distillation of essential oil), green tea and meadowsweet led to a significant hyperchromic effects at much lower pigment/co-pigment ratios, compared to pure compounds. The use of these extracts as co-pigments opens realistic prospects for the development of aronia functional foods with improved sensory properties and biological effects, due to enhanced color and anthocyanin stability.

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Conflict of interests

Authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.11.125.

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