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ENCAPSULATION OF THE ELDERBERRY FRUIT ANTHOCYANINS BY SPRAY DRYING*

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Spray-dried forms of anthocyanins were prepared in a maltodextrin matrix. The anthocyanins were extracted by maceration of elderberry fruits in 0.1 M HCl followed by extract filtration through a paper filter. The extract was partially purified by solid phase extraction in a column filled with styrene-divinylbenzene copolymer beads (Sepabeads SP859/L) or in syringe cartridges with C18 silica (BioChemMack ST, Moscow, RF) followed by re-extraction with aqueous HCl and alcohol mixtures. The procedure of reversed-phase HPLC determination of the extracted anthocyanin type is discussed in comparison to HILIC method in DIOL silica columns. The latter method was shown to be preferable to avoid systematic errors in the anthocyanin type determination. Solid-phase extraction in syringe cartridges gave samples with very high anthocyanins concentration (up to 70 g of cyanidin-3-glucoside chloride equivalent). When using the copolymer sorbent, the concentration was somewhat lower: up to 27 g/L. After spray drying red powder samples with anthocyanins content more than 2% were prepared, although freeze drying gave only dark resin. The red color of the samples indicates the space separation of flavylium ions in the solid state by radicals of the maltodextrin background.

Keywords*: elderberry, fruits, anthocyanins, spray drying, extraction, solid phase extraction, reversed-phase HPLC, HILIC.*

Introduction

Black elder, *Sambucus nigra* L., is not very popular in the Russian Federation, although the flowers of this plant are approved in official medicine and applied to prepare a diaphoretic drug in case of catarrhal diseases in the form of infusion [1]. In addition, the blue-black fruits of black elder are used in many countries of the world as rich sources of anthocyans, as food dyes [2] and as a source of pharmaceutical compositions [3]. The black color of the fruits is due to the very high level of anthocyan accumulation in them. Due to this property black elder can be ranked among the richest available sources of anthocyans in Russia. According to [4], black elder fruits of various kinds and stages of maturing can contain from 600 to 1,250 mg of anthocyans per 100 g of fresh fruits. Bosnian scientists [5] found that the content of anthocyans in the local flora is 670 mg per 100 g of fresh fruits, and in a number of Danish kinds of black elder of various stages of maturing a wide range of variability of this indicator was found: from 190 to 2020 mg per 100 g of fresh fruits [6].

The basis of the anthocyan complex of the fruits was found to be constant. It includes four cyanidine glycosides: 3-sambubioside (Cy3Sam), 3-glucoside (Cy3Glu), 3-sambubioside-5-glucoside (Cy3Sam5Glu) and 3,5-diglucoside (Cy3,5diGlu) [4–6]. Besides, small amounts of

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cyanidine-3-rutinoside [4] and even pelargonidin derivatives [7] can also be found. It was established in a study carried out in our laboratory a few years ago [8] that perspective sources of anthocyan are not only black elder, but also American elder, *S. canadensis* L., one of introduced species of the Botanical garden of the Belgorod National Research University. In addition, this plant synthesizes another basic cyanidine derivative, cyanidin-3-(6-*para*-coumaroyl-2-xylosylglucoside). Acylated anthocyans are compounds more stable with respect to various factors [9], which is important for practical use of anthocyans as dyes in the medical and food industry. Unfortunately, this type of elder did not withstand the adverse weather conditions of the last several years in Belgorod. For this reason only fruits of black elder were available for us.

The world literature shows that the juice or residue extract after separating the juice [6, 10], as well as processing the juice into solid forms by lyophilization [11] or spray drying [12] are used to obtain dyes based on black elder anthocyans.

However, we found that using plant juices as dyes for preparing drinks sometimes leads to the formation of undesirable precipitates. Therefore, the problem of this study is obtaining purified concentrates based on black elder fruits extracts.

Experimental

We used berries of *Sambucus nigra* L. elder grown in the Botanical garden of the Belgorod National Research University as a source of anthocyans. Anthocyans from the plant material were extracted with a 0.1 M solution of hydrochloric acid without grinding the berries. Instead, infusion for 24 h was used. The obtained extract was separated from the solid residue by filtering through a paper filter. The extraction was repeated several times, and the concentration of anthocyans in the obtained solutions was checked.

The combined extracts were purified from unnecessary substances by solid-phase extraction on styrene – divinylbenzene copolymer (Sepabeads SP859/L) and on oktadecylsilicagel (DIAPAK S18 cartridges). In order to obtain solid forms the anthocyans were desorbed by a mixture of ethanol with aqueous hydrochloric acid.

In order to obtain solid forms the anthocyan concentrate was mixed with an aqueous solution of maltodextrin (DE 18-20) in a preset ratio for obtaining dry forms by two methods.

1) *Spray drying*. The solutions were sprayed in a flow of air at 120°C with use of the EYELA SD-1000 spray drier.

2) *Lyophilization (high-vacuum freeze drying).* The solutions were frozen in a freezer at –20°C and freeze-dried with the use of a "LABCONCO FreeZone 2.5" lyophilic dryer at a condenser temperature of –40°C.

The combined concentration of anthocyans (calculated for cyanidin-3-glucoside chloride) in the studied samples was determined by differential spectrophotometry [13] with the use of a Shimadzu UV-2550 spectrophotometer.

In order to check the species composition of the anthocyan complex of the staring material and of the obtained dry forms we used two variants of HPLC: an Agilent 1260 chromatograph with diode-matrix (1260 DAD VL) and mass-spectrometric (Quadrupole LC/MS 66130) detection. In case of reverse phase chromatography anthocyans were separated in the isocratic mode in a Symmetry®C18 chromatographic column (4.6×250 of mm, 5 μm, eluent: CH_3CN–HCOOH–H_2O 10:10:80 vol. %, 1 ml/min) at a column thermostat temperature of 40°C. In order to create separation maps the content of acetonitrile in the mobile phases was changed in the range 6÷10 vol. %. The isocratic mode was used also in the conditions of hydrophilic chromatography with a Kromasil 60-5DIOL chromatographic column (4.6×250 mm, eluent: CH_3CN–H_3PO_4–H_2O 80:0.5:19.5 vol. %, 1 ml/min) at a column thermostat temperature of 40°C. In all the cases the chromatograms were registered at 515 nm.

Results and Discussion

Analytical studies

According to the results of determining individual the anthocyans by reverse-phase HPLC with mass-spectrometric and UV-detection (Table 1) the anthocyan complex of the fruits contained four components: Cy3Sam5Glu, Cy3,5diGlu, Cy3Sam and Cy3Glu (peaks 1, 2, 3 and 4 on the chromatogram in Figure 1). Note the good separation of the peaks of two main anthocyans, Cy3Sam and Cy3Glu in the chosen conditions, although these compounds can fail to separate according to a number of publications [4, 14, 15]. In addition, this fact was found with the use of mass-spectrometric detection [4, 14], but not spectrophotometric [15].

Table 1. Characteristics of anthocyans of *Sambucus nigra* L. fruits extracts according to reverse-phase HPLC with mass-spectrophotometric and UV detection

	Cy3Sam5Glu	Cy3,5diGlu	Cy3Sam	Cy3Glu
Characteristics of anthocyans				
t_R, min (RP HPLC*)	4.91	6.12	10.76	6.08
λ_{max}, nm	515	514	517	516
M/z	743.2 (287.0)	611.2 (287.0)	581.2 (287.0)	449.1 (287.0)
Composition of the anthocyan complex, mol. % (according to peak areas, n = 2)				
Initial concentrate	12.9±0.2	1.4±0.4	53.0±0.1	32.8±0.2
Solid form**	11.3±0.3	2.4±0.3	41.9±0.2	41.4±0.2

* HPLC conditions: column 4.6×250 mm Symmetry®C18, 5 μm; mobile phase: CH_3CN–HCOOH–H_2O (10:10:80 vol. %) (1 ml/min); column thermostat temperature: 40°C.
** See experiment 1 from Table 3.

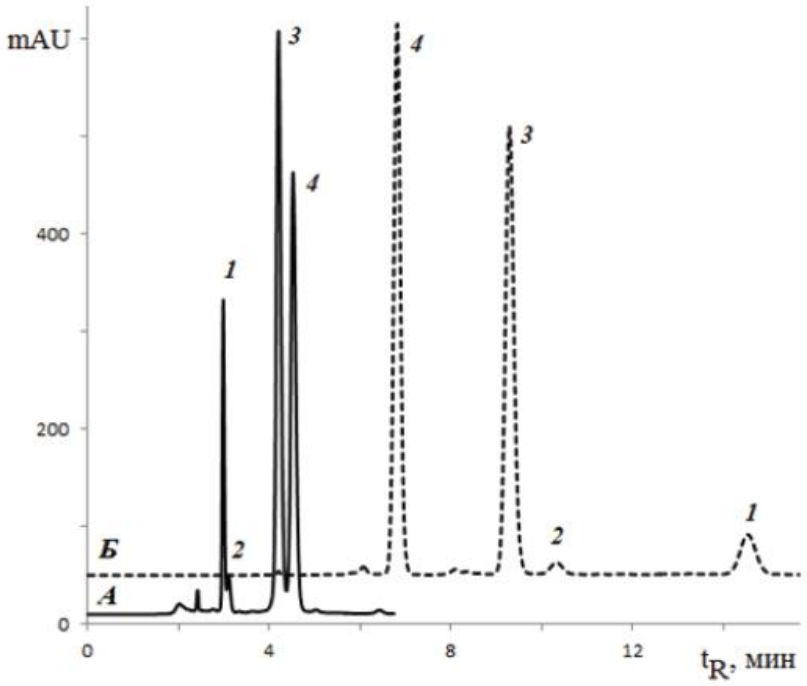

Figure 1. Separation of anthocyans of black elder fruits.

Conditions A. Column: 4.6×250 mm Symmetry®C18, 5 μm, eluent: CH_3CN–HCOOH–H_2O (10:10:80 vol. %).

Conditions B. Column: 4.6×250 mm Kromasil 60-5Diol, eluent: CH_3CN–H_3PO_4–H_2O (80:0.5:19.5 vol. %).

Mobile phase feed rate: 1 ml/min; column thermostat temperature: 40°C; detection at 515 nm.

Substances: 1 – Cy3Sam5Glu; 2 – Cy3,5diGlu; 3 – Cy3Sam; 4 – Cy3Glu.

In order to exclude possible errors when identifying the species composition of the anthocyans a separation map of anthocyans was created according to the method of relative retention analysis [16]. In order to create this map we used 3 various compositions of the mobile phase: 4, 6 and 10 vol. % of CH_3CN at the constant content of formic acid: 10 ob. % (Figure 2). As a result it was found that in case of mobile phase containing 6 vol. % of acetonitrile the chromatogram has only two peaks (instead of four), because the pairs of compounds Cy3Glu and Cy3Sam, on one hand, and Cy3,5diGlu and Cy3Sam5Glu, on the other hand, are not separated. The retention map in this range shows inversion of retention of the compounds of these pairs. Thus, when recording chromatograms of the anthocyan complex of black elder fruits in the conditions of reverse-phase HPLC, some components can be eluted simultaneously, which can lead to errors even in the determination of the number of components.

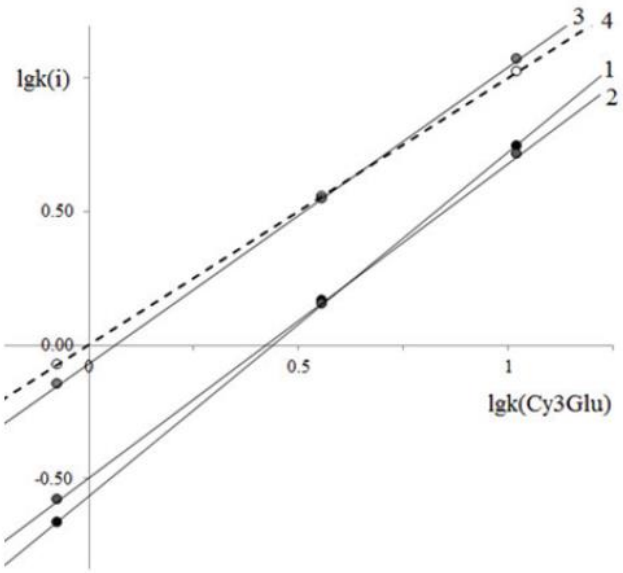

Figure 2. Separation map of anthocyans of black elder fruits.

Column: 4.6×250 mm Symmetry®C18, 5 μm, eluent: CH_3CN–HCOOH–H_2O (CH_3CN – 4, 6 and 10 vol. %; HCOOH – 10 vol. %), 1 ml/min, 40°C; detection at 515 nm. Substances: 1 – Cy3Sam5Glu; 2 – Cy3,5diGlu; 3 – Cy3Sam; 4 – Cy3Glu.

In order to exclude such errors we suggested using hydrophilic chromatography (Figure 1 B). The retention of substances in this case is of essentially different nature: anthocyans with a large number of polar functional groups (carbohydrate residues) in their structure are retained most strongly. Thus, the order of elution should be and actually it is as follows:

Cy3Glu (monoglycoside) <Cy3Sam ≤ Cy3,5diGlu (diglycosides) <Cy3Sam5Glu (triglycoside).

There are no inversions on the separation map for this variant of chromatography. Therefore, this method is a good addition to the variant of reverse-phase chromatography for ensuring reliability in the determination of the species composition of multicomponent anthocyan complexes.

Extraction of anthocyans from fresh fruits

In order to reduce the extent of extraction of unnecessary substances, anthocyans from the plant material were extracted by infusing unground fruits in 0.1 M hydrochloric acid. Next day the extract was decanted from the fruits. The residue was filled with a new portion of the extractant, and the maceration was repeated. As a result it was found that maceration done twice is sufficient for extracting the major part of anthocyans (more than 95%), and the content of anthocyans in the fruits taken from different bushes was very much different: from high (more than 500 mg per 100 g of fresh fruits) to very high (about 1.5 g per 100 g). (See Table 2.)

Extracts containing from 0.05 to 0.25 g of anthocyans in 1 l were obtained with the use of this technique.

Table 2. Extraction of anthocyans from unground fruits of black elder upon maceration

No	Samples of plants from the botanic garden	Sample mass, g	Extract volume (ml) and yield of anthocyans (g/100 g)				
			1-st extraction		2-nd extraction		Sum
			V_1	c_1	V_2	c_2	$c_1 + c_2$
1	Bush 1, old offshoots	2.06	100	0.361	50	0.153	0.514
2	Bush 2, old offshoots	2.06	100	0.456	50	0.183	0.639
3	Bush 3, young offshoots	2.10	100	0.650	50	0.089	0.739
4	Bush 3, young offshoots	2.08	102	0.646	50	0.119	0.765
5	Bush 4	2.03	100	1.121	50	0.344	1.465
6	Bush 5	2.04	100	1.353	50	0.176	1.529

Partial purification by solid-phase extraction

The extract was purified from unnecessary substances by solid-phase extraction. The obtained extract was passed through a layer of "Sepabeads SP859/L" sorbent (layer diameter 24 mm, height 15 cm) in an "Axioma® PSK-24-270" glass column (Biochemmack, Moscow). In order to desorb the anthocyans a mixture consisting of 99 parts of 95% ethanol and 1 part of 35% aqueous HCl was used. The obtained solution was mixed with 0.1 M HCl, and ethanol was distilled off in a vacuum rotary evaporator. The content of anthocyans in the purified extract calculated for cyanidin-3-glucoside chloride was 2–5 g/l. This solution was used to prepare solutions for subsequent drying.

It was found by HPLC that the obtained purified extract practically did not contain UV-absorbing unnecessary substances. This extract was used for obtaining solid forms by spray drying and lyophilization. The content of individual anthocyans in the obtained concentrate is presented in Table 1.

Note that if chemically modified silica gels (C18, Merck) are used for sorption, it is possible to collect fractions containing up to 70 g of anthocyans in 1 l of the reextract from such sorbent by a mixture of ethanol and 0.1 M aqueous HCl in the ratio 1:1. In case of "Sepabeads SP859/L" the maximum concentration of anthocyans in the most concentrated portions of the eluate was several times less: up to 27 g/l. However, the polymeric sorbent is more stable with respect to the highly acidic medium of the extract.

Spray drying

The compositions of the mixtures and some technological parameters of drying the ready forms are presented in Table 3. The method allowed obtaining fine free-flowing powders. The sized of their particles estimated by optical microscopy were from 2 to 15 μm. The obtained powders were easily and quickly dissolved in water forming solutions of deep dark-red color. The content of anthocyans in them was determined by spectrophotometry after dissolution of samples in 0.1 M HCl.

Table 3. Results of analysis of samples obtained by spray drying

No	Composition of mixture for spray drying			Temperature, °C		Content of anthocyans in solid forms, c, g/100 g		–Δc, %
	Mass, g		Mixture volume, ml					
	matrix	anthocyans		inlet	outlet	preset	obtained	
1	10	0.211	200	120	80	2.11	2.04	3.6
2	10	0.148		120	80	1.49	1.43	4.1
3	10	0.106		120	75	1.07	1.06	0.8
4	10	0.053		120	80	0.54	0.53	0.6
5	15	0.053		120	80	0.72	0.66	7.2
6	10	0.211		135	95	2.11	1.82	14.2
7	10	0.106		135	95	1.07	1.01	5.3

It follows from the presented data that losses of anthocyans upon drying can be rather small: less than 5%. |However, increasing dispersion temperature from 120 to 135°C leads to a noticeable growth of losses. It is possible to obtain samples with a very high content of anthocyans: more than 2%. The product obtained in such conditions has a pleasant bright-red color. Note that solutions of anthocyans of black elder fruits even with a concentration several times smaller have almost black color. This can be due to stacking of flavylium ions into large supramolecular structures in concentrated solutions. In this case the red color of obtained solid forms can indicate spatial separation of flavylium ions by the glucose residues of the oligomeric matrix.

In order to test the effect of high temperature on the dispersion on the anthocyan composition, a sample of the powder was dissolved in 0.1 M hydrochloric acid. Anthocyans from the solution were concentrated (to remove the polymeric matrix) on a cartridge filled with C18 sorbent and then desorbed with a solution containing 30 vol. % of CH_3CN, 30 vol. % of HCOOH and 40 vol. % of water. The extract was diluted with water by a factor of 3 before introducing into the chromatograph. Analysis of the obtained chromatograms showed changes in the extract. They

consist mostly in partial hydrolysis of complex glycosides, which led to a certain growth of cyanidin-3-glucoside content in the ready product.

Finally, an attempt of obtaining similar samples by lyophilization was unsuccessful: instead of red powder a black tarlike product was formed.

Conclusion

Thus, the suggested technique enables obtaining anthocyans of black elder fruits encapsulated in a maltodextrin matrix with the content of anthocyans 2% in the form of a red free-flowing powder with 2–15 μm particles and minimal changes of the anthocyan composition.

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