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Structure of Cornel Cornel Fruits Anthocyanins (*Cornus Mas*)

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Abstract—The composition of anthocyanins of dogwood fruits was determined using reverse-phase HPLC with spectrophotometric and mass spectrometric detection. The procedure for determining the structure of individual components of extracts including 3-galactosides of delphinidine, cyanidine and pelargonidine and 3-robinobiosides (3-rhamnosylgalactosides) of cyanidine and pelargonidine is described. It was found that the content of delphinidin-3-galactoside, as well as both 3-robinobiosides, may be insignificant when cyanidin and pelargonidin prevail in the composition of 3-galactosides. For the studied fruits, the level of anthocyanin accumulation can reach (depending on the intensity of coloring) 46 mg per 100 g of fresh fruits, while the concentration of anthocyanins in the peel of fruits is significantly higher – more than 390 mg per 100 g. When developing an exhaustive extraction of anthocyanins, a peculiarity of dogwood fruits was established, consisting in the fact that the extraction of anthocyanins with 0.1 M aqueous hydrochloric acid solution, unlike a large number of other natural sources studied, is practically impossible. The problem can be solved only by extraction with acidified water-alcohol mixtures with an ethanol content in the extractant of more than 30 vol.%, which indicates a high affinity of the components of the pulp of fruits to anthocyanins.

Keywords: *Cornus mas*, fruits, anthocyanins, determination, reverse-phase HPLC, extraction features

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INTRODUCTION

The genus *Cornus* (dogwood) has about 40 species, the fruits of plants can have different colors, but the most characteristic of them is red. The red color of the fruit is due to the accumulation of the most important water-soluble natural antioxidants–anthocyanins. There are a large number of works devoted to the study of anthocyanins of dogwood fruits, but the results of these studies are extremely contradictory.

In the first studies of anthocyanins of *Cornus mas* fruits, the biosynthesis of 3-galactosides of delphinidin, cyanidin and pelargonidin, as well as two 3-rhamnosyl galactosides, cyanidin and pelargonidin, was established in them [1, 2]. These results were confirmed in relatively recent publications [3, 4], and in [4] it was found that anthocyanins of the fruits of medicinal dogwood (*C. officinalis*) and its hybrid with common dogwood are formed mainly by derivatives of pelargonidine. However, different results were obtained in [5]. The authors of the cited work investigated anthocyanins of fruits of four species of the dogwood genus: *C. mas*, *C. officinalis*, *C. controversa*, and *C. kousa*. Using the HPLC method with mass spectrometric detection and analysis of ^{1}H NMR spectra of preparatively isolated anthocyanins, the authors found that only 3-galactopyranosides of delphinidin (I), cyanidin (II) and pelargonidin (III) are synthesized in *C. mas* fruits. The fruits of *C. officinalis* contain the same anthocyanins, but in different proportions. In the fruits of *C. controversa*, in addition to anthocyanins (I–III), several unidentified anthocyanins are found on the chromatogram shown in the work, including anthocyanins with peak areas exceeding the peak areas of anthocyanins (I–III). And only unidentified compounds were found in *C. kousa* fruits, despite the use of a mass spectrometric detector. In [6], in the fruits of another dogwood species, *C.suecica*, cyanidin 3–*O*-β-(2"-glucopyranosyl-*O*-β-galactopyranoside) containing a very rare disaccharide substituent was found as

the main anthocyanin (49%). Other components were cyanidin-3-*O*-β-(2"-glucopyranosyl-*O*-β-glucopyranoside (3-sophoroside) (31%), cyanidin-3-galactoside (16%) and cyanidin-3-glucoside (4%). In [7], in the study of dogwood fruits (*Cornus mas* L.), a rather strange set of anthocyanins was found: firstly, delphinidin was identified among them. But it is known that aglycones are unstable [8] and therefore practically do not occur in the plant world. Secondly, the other two components are quite common for the plant world—cyanidin-3-rutinoside and peonidin-3-glucoside, although their combination is also unusual. The fact is that the formation of cyanidin-3-rutinoside indicates the activity of rhamnosyl-6″-transferase (RT) in fruits, which for some reason did not turn a peonidine derivative into rutinoside, although such selectivity for aglycones is uncharacteristic for this enzyme. For the five studied plant genotypes from Turkey, the level of accumulation of the amount of anthocyanins ranged from 239 to 342 mg per 100 g of fruits. The discrepancy in the results of the determination of anthocyanins of common dogwood fruits is also confirmed in the work [9], in which cyanidin-3-galactoside, pelargonidin-3-glucoside and pelargonidin-3-rutinoside were found from anthocyanins. Peonidin-3-glucoside, cyanidin-3-galactoside and cyanidin-3-rutinoside found in the fruits of autochthonous varieties of common dogwood in Bosnia and Herzegovina [10] also look like a strange set, as, indeed, the composition of the fruits of this plant from Serbia [11], including 3-galactosides of cyanidin and delphinidin, as well as pelargonidine 3-glucoside. The anthocyanin composition of common dogwood fruits found in [12] is unusual, including delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, pelargonidin-3-galactoside, pelargonidin-3-glucoside. Cyanidin-3-glucoside and cyanidin-3-rutinoside were also found in the fruits of common dogwood by Romanian researchers [13].

Dogwood does not belong to the traditional plants grown in the Central Chernozem region, but recently there have been many offers on the local market for seedlings of this plant. Moreover, dogwood fruits began to appear regularly on sale, noticeably different from wild fruits in the Caucasus in size and intensity of color.

The purpose of this work is to determine the anthocyanins species composition of cultivated dogwood varieties available on the Belgorod market.

EXPERIMENTAL

Dogwood fruits were purchased at the Belgorod market and at a specialized exhibition.

For the extraction of anthocyanins, 0.1 M aqueous hydrochloric acid solution and ethanol were used in the specified ratios.

The total content of anthocyanins was determined by differential spectroscopy [14].

To determine anthocyanins by HPLC, extracts were purified using solid-phase extraction on DIAPAK C18 concentrating cartridges (BiohimMak ST, Moscow) [15].

The separation of anthocyanins by reverse-phase HPLC was carried out on an Agilent 1260 Infinity chromatograph with a diode-matrix detector and a mass spectrometric detector. The separation was carried out on a 150 × 4.6 mm Symmetry C18 column, 3.5 microns.

Acetonitrile (HPLC gradient grade, Fisher Chemical, Belgium), formic acid (85%, REACHIM) and distilled water were used to prepare the eluents.

Anthocyanin extracts from plant objects from the laboratory collection were used in the work.

RESULTS AND DISCUSSION

The first studies to determine the anthocyanins of dogwood fruits purchased at the Belgorod market and at the traditional agricultural exhibition were carried out in 2017 and for reliability, the results were rechecked in the 2021 season.

Chromatograms of some extracts of dogwood fruits are shown in Fig. 1.

The studies carried out in this work showed that the species composition of anthocyanins of all the samples studied included five main components, for which electronic absorption spectra were recorded in the cuvette of a diode-matrix detector and mass spectra when scanning positively charged ions in electrospray mode with partial fragmentation, allowing to obtain

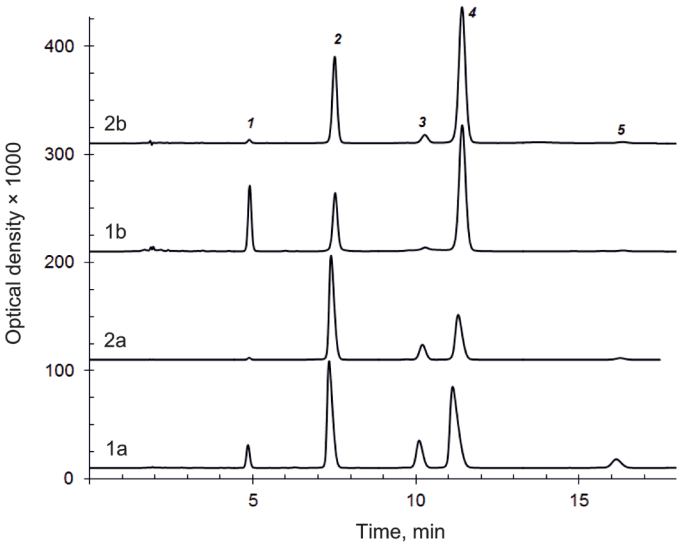

Fig. 1. Chromatograms of the peel extracts (1a, 2a) and fruits pulp (1b, 2b) of two dogwood samples. Conditions: 150 × 4.6 mm symmetry C18 column, 3.5 microns; mobile phase 6 vol. % acetonitrile, 10 vol. % formic acid in water, 0.8 mL/min. The wavelength is 515 nm. Substances: 1, Dp3Gala; 2, Cy3Gala; 3, Cy3Robi; 4, Pg3Gala; 5, Pg-3-Robi.

the *m/z* of the corresponding aglycones [16] (Table. 1). These methods of eluate control allowed us to establish that the first peak (most often having a small area) is a delphinidin 3-glucoside. The second peak, related to the main components, belongs to 3-hexoside, but already cyanidine. The third peak is identified as cyanidine 3-rhamnosylhexoside. Peaks 4 and 5 similarly relate to 3-hexoside and 3-rhamnosylhexoside of pelargonidine. To determine hexosides, we take into account that only two of them—glucoside and galactosidic (not counting rhamnoside, easily differentiated by mass spectra), which are common for natural anthocyanins.

To confirm the presence of 3-glucosides of delphinidin (Dp3Glu), cyanidin (Cy3Glu), and pelargonidin (Pg3Glu), it is convenient to use an extract of barberry fruits containing these substances [17], along with others according to literature data and the results of numerous studies performed in our laboratory. However, as follows

Table 1. Parameters of anthocyanin peaks of common dogwood extract and the proportion of peak areas for some samples

Peak no. (Fig. 1)	1	2	3	4	5
tR (min)	4.94	7.5	10.38	11.43	16.52
λ (max), nm	520	514	515	500	501
m/z	465.1, 303.0	449.1, 287.0	595.1, 287.0	433.1, 271.0	579.1, 271.0
samples	anthocyanin proportion by peak areas, mol %				
1	19.7	22.1	2	52.4	1.4
2	15.5	22.3	1.9	57.8	1.4
3	12.6	22.2	1.8	61	1.4
4	8.3	20.8	1.6	66.4	1.5
5	8.3	29.8	3.4	57.3	0.7
6	0.6	30.7	3.8	64.3	0.6
7	0.5	29.1	3.2	66.8	0.2
8	0.1	27.6	3	69.2	0.1

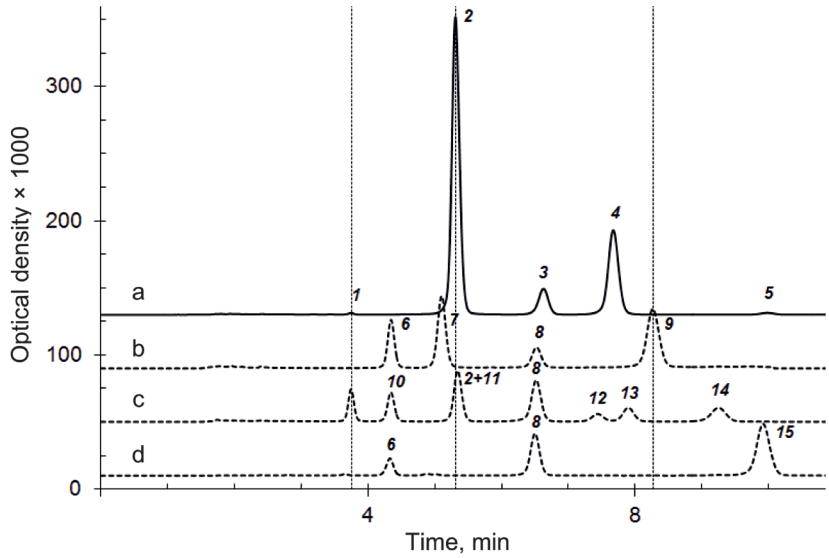

Fig. 2. Chromatograms of extracts of common dogwood (a), black currant (b), blueberry (c), scarlet tulip (d). Conditions: 150 × 4.6 mm symmetry C18 column, 3.5 microns; mobile phase 7.2 vol. % acetonitrile, 10 vol. % formic acid in water, 0.8 mL/min. The wavelength is 515 nm. Substances: Substances: 1, Dp3Gala; 2, Cy3Gala; 3, Cy3Robi; 4, Pg3Gala; 5, Pg-3-Robi; 6, Dp3Glu; 7, Dp3Rut; 8, Cy3Glu; 9, Cy3Rut; 10, Dp3Glu; 11, Dp3Ara; 12, Pt3Gala; 13, Cy3Ara; 14, Pt3Glu; 15, Pg3Glu.

from Fig. 2, the retention of these substances (with the control of electronic spectra) does not coincide with the retention of 3-hexosides of dogwood fruit extracts.

In some mobile phase compositions, the retention times of peak 3 and Cy3Glu may coincide (Fig. 2). Perhaps this was the reason for the incorrect attribution of peak 3 to Cy3Glu, the presence of which was reported in the literature cited above. But in other compositions of mobile phases, the coincidence of retention times disappears, and finally, there are significant differences in the mass spectra of these compounds. It remains to be recognized that substances 1, 2, and 4 are delphinidine 3-galactosides, cyanidine, and pelargonidine, respectively, Dp3Gala, Cy3Gala, Pg3Gala. Note that the elution order of the same type anthocyanidins glycosides is exactly the same as indicated above, and does not depend on the separation conditions used. This is the primary control that allows you to reject publications by definition of anthocyanins (and there are quite a lot of them!).

To confirm the attribution of peaks 1 and 2 to 3-galactosides (Dp-3-Gala and Cy3Gala), we can use a comparison of the retention times of these peaks with the peaks on the chromatograms of blueberry extracts (Fig. 2). The qualitative composition of blueberry anthocyanins (containing 15 types of anthocyanins) does not depend on the growing conditions and the variety [18], and the attribution of peak 2 to cyanidin-3-galactoside is easily confirmed by the coincidence of the retention times of this peak with the main anthocyanin of aronia prunus [19] (or Michurin [20]), and even the main anthocyanin of apples [21]. Unfortunately, to confirm the attribution of peak 4 to the 3-galactoside of pelargonidine, we cannot offer an available plant object. In addition, in order to exclude accidental coincidences of retention times, it is necessary to check the coincidence of retention times in two different compositions of the mobile phases of the selected eluent system.

It is also possible to make sure that peaks 3 and 5 do not belong to the cyanidin 3-rutinosides, Cy3Rut, and pelargonidin, Pg3Rut (by mismatch of retention times). Thus, Cy3Rut manifests itself as the fourth of the four main peaks of extracts of black currant fruits (regardless of the variety and place of cultivation [22]). At the same time, Pg3Rut can be isolated from extracts not only of scarlet-colored tulip flowers [23], but also from felt cherry fruits [24]. A direct comparison of the retention times

Table 2. Results of anthocyanin extraction from dogwood fruits of three color intensity variants

Fruit coloring	Amount, mg/100 g of fresh material	Additionally extracted with respect to the 1st extraction, %			
		1	2	3	4
Pink	4.45 ± 0.32	100	44.5	28.8	21.0
Red	20.7 ± 4.1	100	54.8	20.8	15.9
Dark red	46.1 ± 5.1	100	43.8	17.7	12.7

Table 3. Extraction of anthocyanins of dogwood fruits with the addition of ethanol to the extractant

Volume fraction of ethanol in the extractant, %	Fruit coloring	Additionally extracted with respect to the 1st extraction, %			
		1	2	3	4
10	Pink	–	21.2	14.1	6.90
	Red	100	30.9	13.6	3.90
	Dark red	100	45.5	26.3	5.96
20	Pink	100	0.5	0	0
	Red	100	30.9	7.9	0
	Dark red	100	36.1	12.0	0
30	Pink	100	0.1	0	0
	Red	100	25.2	0	0
	Dark red	100	17.8	0	0

of peaks 3 and 5 with the retention of components of other plant sources with a constant species composition is impossible due to the unavailability of such objects.

Thus, in this work, the anthocyanin composition of *C. mas* fruits turns out to be largely constant and contains cyanidin and pelargonidin 3-galactosides and 3-rhamnosylgalactosides (robinobiosides), Cy3Robi, Pg3Robi; the content of delphinidin-3-galactoside can vary widely (up to trace amounts) (Table 1).

When determining the total level of anthocyanin accumulation in the fruits of the common dogwood, the features of this object were found. So, usually, for the complete extraction of anthocyanins from plant sources, a maximum of three-fold extraction using 0.1 M of HCl aqueous solution is sufficient (depending on the ratio of the extractant object: the mass of plant material). In the case of *C. mas* fruits, it was not possible to achieve exhaustive extraction (when there was a colorless mass in the residue) even with four consecutive extractions of the starting material (Table 2)—the residue had a color due to the presence of anthocyanins.

In Table 2, the results are presented as follows: the amount of anthocyanins extracted in the first extraction is taken as 100%, and in subsequent stages it is determined as a fraction of the first value. If, at the same time, with an increase in the extraction number, the readings remain sufficiently high, then the extractability of anthocyanins is problematic. So, in Table 2, even at the fourth extraction with 0.1 M aqueous hydrochloric acid solution, the extraction exceeds 10–20% of the results of the first extraction for fruits of weak (pink) color, medium (red) and dark red color. Subsequent extraction is difficult to carry out, since the plant material passes into a finely dispersed state.

The addition of an organic solvent (ethanol) leads to a noticeable acceleration of extraction, especially with an alcohol content of 30% (Table 3). In this case, all anthocyanins are quantitatively extracted in two extractions.

Table 4 shows the results of the extraction of anthocyanins from the peel of fruits, the color (and the level of accumulation of anthocyanins) of which is significantly higher than in the pulp. In this case, the addition of

Table 4. Extraction of anthocyanins from the peel of common dogwood fruits with an average anthocyanin content of 395 ± 40 mg/100 g

Volume fraction of ethanol in the extractant, %	Additionally extracted with respect to the 1st extraction, %				
	1	2	3	4	5
0	100	18.0	4.9	2.2	0.9
30	100	6.5	0.5	0	0
50	100	6.6	0	0	0
80	100	5.5	0.7	0.2	0

alcohol up to 50% also allows the most efficient extraction of anthocyanins – already in two consecutive extractions.

The need to add ethanol indicates that the flesh of dogwood fruits has a high affinity for anthocyanins.

CONCLUSIONS

Thus, delphinidin 3-galactosides (a minor component in a number of studied samples), cyanidin and pelargonidin (the main components) and 3-robinobiosides (3-rhamnosylgalactosides) of cyanidin and pelargonidin (also in small quantities) are synthesized in the fruits of common dogwood. The level of anthocyanin accumulation can reach (depending on the intensity of coloring) 46 mg per 100 g of fresh fruits, while the concentration of anthocyanins in the peel of fruits is significantly higher—more than 390 mg per 100 g.

For the exhaustive extraction of anthocyanins from the fruits of common dogwood, it is necessary to use acidified water-alcohol extractants due to the high affinity of the pulp components to anthocyanins.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATES

This article does not contain any studies involving patients or animals as test objects.

Informed consent was not required for this article.

CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

AUTHOR CONTRIBUTION

All authors contributed to manuscript preparation and participated in the discussions.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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