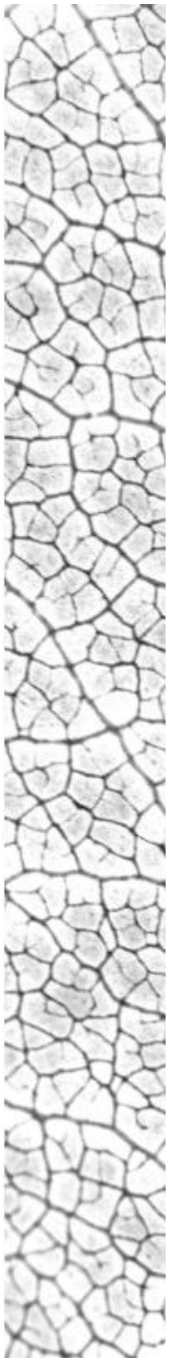

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Non-traditional sources of anthocyanins: flowers of *centaurea cyanus* (cornflower)

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Abstract
By reverse-phase HPLC with diode-matrix and mass spectrometric detection the species composition of anthocyanins of cornflower petals (*Centaureacyanus*) of the three colors: blue, red and maroon was determined. For the first time, the anthocyanin composition of cornflower flower extract of dark maroon color, was found to include including the same anthocyanins as cornflower flowers of traditional blue color, but with another ratio. The main component is cyanidin-3-O-(6"-malonylglucoside)-5-glucoside (37-58 mol. %) at outstanding overall anthocyanin accumulation reaching 3.5 ±0.4 and even 9.5 ± 1.0 g per 100 g of fresh and dried plant material, correspondingly. Meanwhile the anthocyanin set of blue cornflowers includescyanidin-3,5-diglucoside (23-25 mol.%), cyanidin-3-O-(6"-malonylglucoside)-5-glucoside (15 – 17 mol.%), cyanidin-3-O-(6"-succinylglucoside)-5-glucoside (49 – 56 mol. %) and cyanidin-3-glucoside (0.3-4.1 mol.%), which corresponds to the literature data, but additionally also isomeric malonated cyanidin-3,5-diglucoside (0.5 – 1.1 mol. %) and cyanidin-3-O-(6"-malonylglucoside) (3.3 – 6.7 mol.%) were found. The anthocyanin composition of red flowers is formed by similar substituents of anthocyanins, built on a pelargonidinebachbone with a different ratio between the components: pelargonidin-3,5-diglucoside (25 – 36 mol. %), pelargonidin-3-O-(6"-malonylglucoside)-5-glucoside (37 – 47 mol. %), pelargonidin-3-O-(6"-succinylglucoside)-5-glucoside (7 – 31 mol. %) aa well as two products with unknown malonation position of pelargonidin-3,5-diglucoside (in total up to 5 mol.%). It was found that the stationary phase of Kromasil 100-5C4 is more effective for separation of all components compared to the stationary phase of Symmetry C18 and hydrophilic chromatography withKromasil 60-5DIOL phase.

Keywords: anthocyanins, *Centaureacyanus*, flowers, three types of color, malonated and succinated 3,5-diglucosides, HPLC, stationary C4 and C18 phases, separation selectivity

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INTRODUCTION

Blue cornflower (Cenraureacyanus L.) is an annual herbaceous meadow and a popular ornamental plant among gardeners. In the State Pharmacopoeia, dried marginal funnel-shaped flowers of blue cornflower (Flores Centaureaecyani) are offered as a diuretic (State Pharmacopeia of the USSR 1990, Bayandina and Zagurskaya 2015, Shahabi et al. 2016). The blue color of the flowers is provided by the biosynthesis of anthocyanins. Moreover, the name "anthocyanins" is due to this flower - from the Greek " anthos "(flower) and" kyanose " (blue, blue), revealing an extensive range of water-soluble dyes of plant origin (Ghosh and Konishi 2007). And there were problems with determining the structure of the anthocyanins of this flower. So, initially, Wilstetter and Everest (1913) determined that the main component of the anthocyanin complex of blue cornflower flowers is cyanidin-3,5-diglucoside (cyanine) (Takeda and Tominaga 1983). Note that it is namely cyanine that should be determined in the flowers according to the State Pharmacopoeia of the USSR (1990). In the late 1950s, a unique complex was isolated from the flowers, called protocyanin, responsible for the blue color of the flowers, but the structure of the anthocyanins included in the complex was not paid attention to. Later, in Takeda and Tominaga (1983), the authors concluded that the main anthocyanin of proticyanin is cyanidin-3-succinylglucoside-5-glucoside (centaurocyanin). Long-term studies of protocyanin

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Table 1. Retention and parameters of electron absorption specta and mass-spectra of flowers extract anthocyanins of *Centaureacyanus*

No[1]	Anthocyanin type	t_R, min	λ_{max}, nm	M/z
1	Cy3,5diG	5.55	514	611.1; 449.1; 287.1
2	Cy3(*x*MalG)5G	7.22	515	697.2; 577.1; 287.1
3	Cy3(6MalG)5G	8.59	515	697.2; 577.1; 287.1
4	Cy3G	9.25	515	449.1; 287.1
5	Cy3(6SuccG)5G	12.39	515	711.2; 591.1; 287.1
6	Cy3(6MalG)	23.97	515	591.1; 287.1
7	Pg3,5diG	8.01	499	595.1; 433.1; 271.1
8	Pg3(*x*Mal-G)5G	11.20	499	681.2; 561.1; 271.1
9	Pg3(6MalG)5G	13.73	500	681.2; 561.1; 271.1
10	Pg3G	14.43	501	433.1; 271.1
11	Pg3(*y*MalG)5G	18.13	500	681.2; 561.1; 271.1
12	Pg3(6SuccG)5G	20.99	500	6952; 575.1; 271.1

[1] – Numbering and conditions are presented in **Fig. 1**

eventually led to the identification of a complex supramolecular structure of this unique complex formed by centaurocyanin, the flavonoid apigenin-7-O-glucuronide-4-O-(6”-malonylglucoside), polysaccharide, and metal ions-iron (III), magnesium, and calcium (Takeda et al., 2005; Escher et al., 2018), and in Takeda et al., (2005) the flavonoid was determined by HPLC, and method for anthocyanin identification was limited only to spectrophotometric studies. In Escher et al., (2018), it was stated that the structure of anthocyanin was confirmed by HPLC with mass spectrometric detection, but the mass spectra given in this paper are hardly satisfactory, and the chromatogram pattern in the paper was not found at all. Only in Lockowandt et al., (2019) a chromatogram of anthocyanins of cornflower blue flowers is presented, where the authors found not one anthocyanin, but four simultaneously: cyanidin-3,5-diglucoside (5.2), cyanidin-3-O-(6”-malonylglucoside)-5-glucoside (3.25), cyanidin-3-O-glucoside (2.89) and cyanidin-3-O-(6”-succinylglucoside)-5-glucoside (15.7 mg per 1 g of dry extract) (Alkhamaisah et al. 2019, Singh et al. 2017).

To date Centaureacyanus flowers are known with not only blue colors, but also red and even maroon. However, information about the anthocyanins of these flowers is very limited. Thus, in the work (Takeda et al., 1988), pelargonidin-3-O-(6”-malonylglucoside)-5-glucoside was found in red flowers, and a very high level of total anthocyanin accumulation is known about maroon flowers-up to 5.72% in cyanidin-3,5-diglucosideequivalent for dried flowers (Bayandina & Zagurskaya, 2015).

The aim of the research to use the method of reversed-phase HPLC with diode array and mass spectrometric detection to determine the composition of Cenraureacyanus anthocyanins of three colors-traditional blue, red and maroon, grown in the conditions of Belgorod.

MATERIAL AND METHODS

Flower petals were dried in a laboratory dry-air thermostat TS 1/20 SPU at 30ºC. Extracts were prepared by infusing fresh or dried flower petals in 0.1 M HCl aqueous solution. Before chromatography, extracts were separated from the residue by filtering through a paper filter than purified by solid-phase extraction using DiapakC18 cartridges (BioChemMack ST, Moscow). To do this, the cartridges were activated by passing 5 ml of acetone, conditioned by passing 15 ml of 0.1 M HCl aqueous solution, the extract was sorbed until the appearance of colored fractions eluted, and the anthocyanins were desorbed with a solution containing 30 vol. % formic acid and 30 vol. % acetonitrile in water. The resulting concentrate was diluted with water in a ratio of 1: 3.

Separation was performed on Agilent 1200 Infinity equipment with diode array and mass spectrometric detectors. The reverse-phase HPLC separation was performed with chromatographic columns: a) 150×4.6 mm Symmetry C18 (3.5μm), b) 100×4.6 mm Kromasil 100-5C4 – for serial studies, and c) 150×2.1 mm Kromasil 100-5C18 – for mass spectrometric detection. Dead time was determined by retention of oxalic acid. Eluents contained 10 vol. %of formic acid and specified vol. fraction of acetonitrilein water. The mobile phase rates for columns were a) 0.8 ml/min; b) 0.4 ml/min; and c) 250 μl/min. Hydrophilic chromatography used a column g) 250×4.6 mm Kromasil 60-5DIOL and mobile phases containing 0.5 vol. % phosphoric acid, 12.5-25 vol. % of water in acetonitrile. The mobile phase rate was 1 ml / min; the column temperature was40ºC. Chromatograms were recorded and processed by the Agilent ChemStation software. The results of identification of anthocyanins are presented in **Table 1**.

Quantitative determination of the overall anthocyanin accumulation in plant material was determined by differential spectrophotometric method (Monica Giusti and Wrolstad 2001).

During acid hydrolysis, the extract was mixed with a 20% solution of sulfuric acid in water and boiled in a water bath for 30 minutes.

RESULTS AND DISCUSSION

Fig. 1 presents chromatograms of extracts of dry *Centaureacyanus* flowers in three colors-traditional blue (A) and pink (B) and dark maroon (C).On chromatogram

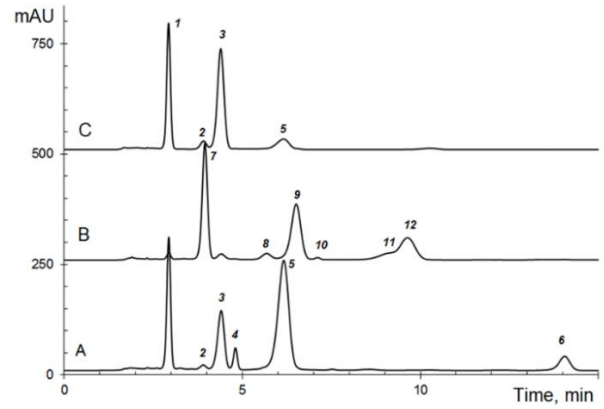

Fig. 1. Separation of anthocyanins of three differently colored *Centaureacyanus* flower petals

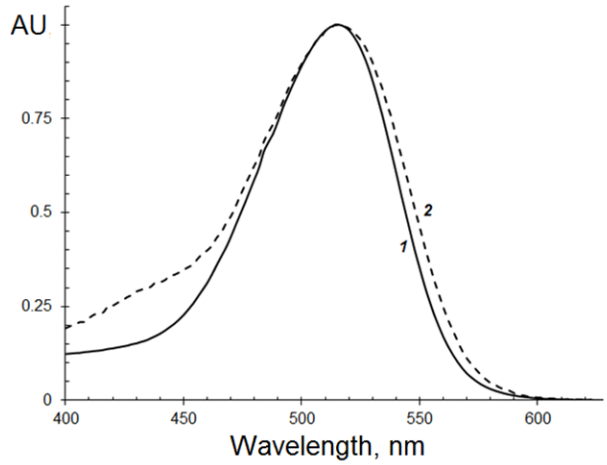

Fig. 2. Difference of electronic absorption spectra of 3,5-disubstituted (*1*) and 3-substituted (*2*) cyanidin derivatives

A, cyanidin-3,5-diglucoside,Cy3, 5diG (1), cyanidin-3-O-(6”-malonylglucoside)-5-glucoside, Cy3(6MalG)5G (3), cyanidin-3-O-(6”-succinylglucoside)-5-glucoside, Cy3(6 SuccG)5G (5), and cyanidin-3-glucoside, Cy3G (4) were found being known from literature data, but in addition to them, one more isomer of compound 3 (malonated to an unknown position), Cy3(XMalG)5G (2), and cyanidin-3-O-(6”-malonylglucoside), Cy3(6MalG) (6) are visible in lower concentrations. All the listed compounds were identified (except for the acylation position, which was accepted according to the corresponding literature data) by mass spectra and electron absorption spectra, **Table 1**. The Cy3,5diG compound was identified by matching the retention time with the main rose anthocyanin (Deineka et al. 2015). Note that the electron absorption spectra are sensitive to the glycosylation of the aglycone position 5 and differ from 3-glycosides by the absence of a weak absorption band with a maximum of about 430 nm (**Fig. 2**) (Harborne 1958).

An attempt was made to hydrolyze cornflower anthocyanins to remove the glucoside substituent from position 5 of the aglycone. However, we obtained products from which it follows that malonic and succinic acids are cleaved off most quickly when boiling with sulfuric acid, so Cy3,5diG, Cy3G and cyanidin-5-glucoside, Cy5G, and cyanidin itself were found among the products of hydrolysis of cornflower blue anthocyanins. Therefore, we were not able to clarify the position of acylation of 3-glucosides by retention times in comparison with malonated anthocyanins from the anthocyanin Bank in our laboratory.

The results obtained for the blue cornflower are generally consistent with the literature data on both qualitative and quantitative composition, **Table 2**, but the set of anthocyanins should be expanded with the components found in this work. Note also that in the case of malonic acid acylation, the presence of isomers is not surprising, since the similarisomer set for position of malonation was found for other anthocyanins (Deineka et al. 2016). It is also important that all the anthocyanins listed above were found in extracts not only of dry flowers, but also of fresh ones (this work has been performed for several years).

The red-colored anthocyanins of *Centaureacyanus* flowers were fundamentally different from anthocyanins discussed above – they were mainly built on pelargonidine backbone, **Fig. 1**. among the components of the extract, pelargonidin-3,5-diglucoside (7), Pg3,5diG, pelargonidin-3-O-(6”-malonylglucoside)-5-glucoside (9), Pg3(6MalG)5G, and its isomer with an unknown acylation position, Pg3(xMalG)5G (8), pelargonidin-3-O-(6”-succinylglucoside)-5-glucoside (12), Cy3(6SuccG)5G, and pelargondin-3-glucoside (10), Pg3G. The chromatogram also shows two

Table 2. Anthocyanin types content of differently colored *Centaureacyanus* flower petals (by peak areas)

No[1]	Anthocyanin type	Annthocyanin relative content, mol.% ± 0.2					
		Blue		Red		Dark maroon	
		Fresh.	Sush.	Fresh.	Sush.	Fresh.	Sush.
1	Cy3,5diG	24.7	20.0	0.6	0.9	21.3	24.0
2	Cy3(*x*MalG)5G	0.5	0.9	2.2	3.2	2.6	5.8
3	Cy3(6MalG)5G	14.9	14.9	-	-	50.8	37.0
4	Cy3G	0.3	0.3	-	-	-	-
5	Cy3(6SuccG)5G	55.8	55.8	-	-	21.3	27.2
6	Cy3(6SuccG)	3.3	3.3	-	-	-	-
7	Pg3,5diG	-	-	25.3	27.0	-	-
8	Pg3(*x*MalG)5G	-	-	1.4	2.7	-	-
9	Pg3(6MalG)5G	-	-	36.9	44.2	-	-
10	Pg3G	-	-			-	-
11	Pg3(*x*MalG)5G	-	-	31.0	3.2	-	-
12	Pg3(6SuccG)5G	-	-		14.0	-	-

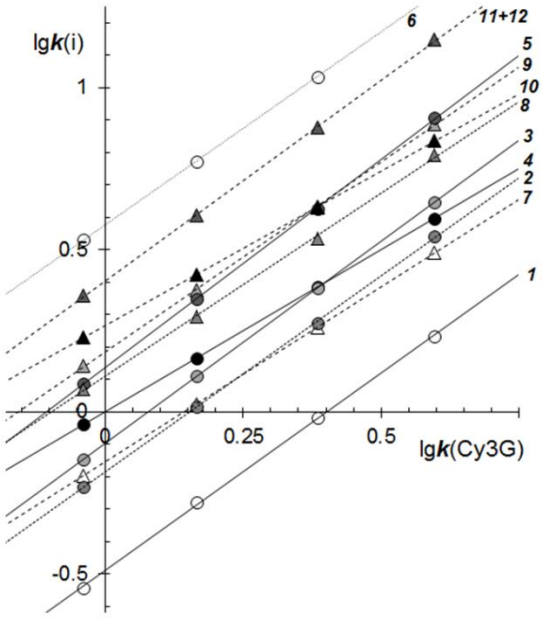

Fig. 3. Separation map of cyanidin and pelargonidin derivatives
Solute numbering as in **Fig. 1**

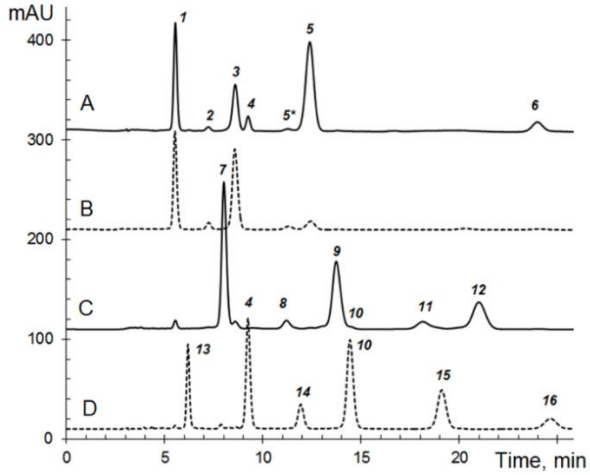

Fig. 4. Separation of anthocyanins of *Centaureacyanus* flower petals
Petals coloration: A – blue; B – red; C – darkmaroon; D – mixture of the six 3-glucosides. Column: 100×4.6 mm Kromasil 100-5C4; mobile phase: 6 vol.% CH_3CN and 10 vol. % HCOOH in water, 0.4 ml/min. Detection 515 nm. Anthocyanins ***1*** **–*12*** asin Fig.1; 3-glucosides of: ***13*** – delphinidin; ***14*** – petunidin; ***15*** – peonidin; ***16*** – malvidin

cyanidin-based anthocyanins, Cy3,5diG and Cy3(6MalG)5G. At the same time, an unexpected feature draws attention to itself - the appearance of a poorly separated from Ps3(6SuccG)5Gmalonated in another unknown position pelargonidin-3,5-diglucoside (11), Pg3(yMalG)5G.

Usually, the flowers of one plant species of one genus, which differ in color, are characterized by a change in the ratio between the derivatives of various aglicons (Deineka et al. 2016, Kulchenko et al. 2018). For this reason, the conditions under which all possible components of complex mixtures are separated are of interest. To determine such conditions, it is possible to perform separation in a large number of different compositions of mobile phases of the selected eluent system, but the most economical and effective method is the analysis of separation maps (Deineka 2006). The maps are constructed using the method of relative retention analysis - based on data from several isocratic separations for the selected eluent system. For such maps, it is possible to extrapolate retention parameters to mobile phase compositions that are outside the range of measurements used. A map of the separation of anthocyanins of blue and red cornflower flowers with cyanidin-3-glucoside as a reference substance is shown in **Fig. 3**.

The map reveals and predicts inversions of retentions of many pairs of compounds at different compositions of the mobile phase during separation under isocratic conditions in the eluent system " acetonitrile – 10vol.% formic acid-water" at a temperature of 40°C. At the same time, it is almost impossible to choose a gradient mode that allows to separate all the components. Temperature changes in this case also had little effect on the selectivity of anthocyanin separation. Based on the above results, to better separate the anthocyanins of *Centaureacyanus* flowers of different colors, it was necessary to radically change the separation conditions. However, the attempt to separate the above components under conditions of hydrophilic chromatography in the diol phase, which is effective in separating the same type of cyanidin and pelargonidin derivatives due to the difference in the number of OH groups in them (Deineka et al. 2016), was generally ineffective. Thus, malonated and succinated derivatives of the same agliicon were not separated from each other, and when Cy3,5diG was acylated, the peak was superimposed on the non-acylated Pg3,5diG.

The best results of the separation efficiency were obtained under reverse-phase HPLC, but when replacing the C18 phase of the Symmetry brand with the C4 phase of the Kromasil brand (**Fig. 4**).

In this case, the most interesting was the complete separation of the Pg3(6SuccG)5G and Pg3(yMalG)5G. Moreover, similar to the last of these compounds appeared for cyanidine derivatives in the case of an extract of cornflower flowers of maroon color – the existence of such a pair in the previously described separation conditions was even difficult to guess. Interestingly, among the anthocyanins of blue flowers, the content of this isomer is very small, i.e. it is not included in the supramolecular protocyanin.

According to the presented results (taking into account the UV spectra recorded in the cuvette of the spectrophotometric detector), it follows that the maroon cornflower anthocyanins are formed qualitatively by the same components as the anthocyanins of blue flowers.

However, a significant difference is in their quantitative ratio – the main one is not Cy3(6SuccG)5G, but Cy3(6MalG)5G, **Table 2**. If we assume that a malonated anthocyanin cannot form a protocyanin that provides blue color to the flowers, then the maroon color of the flowers may be the result of mixing of the blue color of the protocyanin with a red color of malonated anthocyanin being typical for objects containing mainly cyanidin derivatives without the formation of supramolecular structures.

Maroon cornflowers turned out to be uniquely rich sources of anthocyanins: their content in terms of cyanidin-3-glucoside chloride was 3.5 ±0.4 and 9.5 ± 1.0 g per 100 g of fresh (determined in 2017) and dry (determined in 2020) petals, respectively. This is the highest content of anthocyanins in plant objects for the entire long-term experience of our laboratory for the determination of anthocyanins.

CONCLUSION

Thus, the color of the petals of cornflower blue and red flowers is mainly due to rarely found in nature succinated 3,5-diglucosides of cyanidine and pelargonidine, respectively. The color of the flowers is blue and maroon due to the same cyanidine derivatives, but in a different ratio. Enhanced biosynthesis of anthocyanins in maroon flowers, the amount of which exceeds 3% for fresh petals, is not compensated by enhanced synthesis of other components of blue-colored supramolecular protocyanin.

The stationary phase of Kromasil 60-5 C 4 is most effective in separating the found anthocyanins in their combined presence under reverse-phase HPLC conditions.

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