DOI: 10.6060/ivkkt.20236605.6784

УДК: 543.645

КОНТРОЛЬ СЕЛЕКТИВНОСТИ РАЗДЕЛЕНИЯ 3-ГЛЮКОЗИДОВ И 3,5-ДИГЛЮКОЗИДОВ АНТОЦИАНИДИНОВ ВИНОГРАДА: ОПРЕДЕЛЕНИЕ АНТОЦИАНОВ ПЛОДОВ ВИНОГРАДОВ, ВЫРАЩЕННЫХ В БЕЛГОРОДСКОЙ ОБЛАСТИ

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Впервые предложен новый вариант разделения 3-глюкозидов и 3,5-диглюкозидов пяти основных для плодов виноградов антоцианидинов – дельфинидина, цианидина, петунидина, пеонидина и мальвидина в условиях обращенно-фазовой ВЭЖХ. Показано, что замена традиционно используемого ацетонитрила на экологически более приемлемый этанол при подкислении не муравьиной, а ортофосфорной кислотой позволяет существенно изменить селективность разделения двух типов глюкозидов при их совместном присутствии. Предложенный вариант разделения позволяет дифференцировать виноградов вида Vitis vinifera, в кожуре плодов которых синтезируются только 3-глюкозиды перечисленных выше антоцианидинов, и виноградов иных видов или гибридных сортов винограда. Для полного обзора антоцианового состава необходимо использование градиентного режима, поскольку ацилирование антоцианов уксусной и пара-кумаровой кислотами существенно изменяет липофильность антоцианов. Тип антоцианов анализировали по ранее предложенной системе, учитывающей активность трех типов ферментов: 1) 5-О-гликозилтрансферазы, участвующей в образовании 3,5-диглюкозидов, 2) 3′,5′-гидроксилазы ответственной за гидроксилирование кольца В; 3) антоциан О-метилтрансферазы для превращения производных цианидина в производные пеонидина, как и производных дельфинидина в гликозиды петунидина и мальвидина. Метод был использован для определения антоцианов 43 сортов виноградов, выращенных в Белгороде в фермерских и частных хозяйствах. Среди исследованных виноградов обнаружены сорта с плодами винограда с накоплением только 3-глюкозидов, как и одновременно 3-глюкозидов и 3,5-диглюкозидов, с дельфинидиновым и цианидиновым типами антоцианов, и с различной степенью метилирования. Рассчитанные параметры всех сортов виноградов представлены в двух таблицах и приведено краткое обсуждение некоторых сортов.

Ключевые слова: плоды виноградов, антоцианы, ОФ ВЭЖХ, подвижные фазы на основе этанола, три критерия классификации виноградов

Для цитирования:
Саласина Я.Ю., Дейнека В.И., Блинова И.П., Олейниц Е.Ю., Дейнека Л.А., Макаревич С.Л. Контроль селективности разделения 3-глюкозидов и 3,5-диглюкозидов антоцианидинов винограда: Определение антоцианов плодов виноградов, выращенных в Белгородской области. *Изв. вузов. Химия и хим. технология*. 2023. Т. 66. Вып. 5. С. 72–79. DOI: 10.6060/ivkkt.20236605.6784.

For citation:
Salasina Ya.Yu., Deineka V.I., Blinova I.P., Oleinits E.Yu., Deineka L.A., Makarevich S.L. Control of the selectivity of the separation of grape anthocyanidin 3-glucosides and 3,5-diglucosides: determination of anthocyanins in grape fruit grown in the Belgorod region. *ChemChemTech* [*Izv. Vyssh. Uchebn. Zaved. Khim. Khim. Tekhnol.*]. 2023. V. 66. N 5. P. 72–79. DOI: 10.6060/ivkkt.20236605.6784.

CONTROL OF THE SELECTIVITY OF THE SEPARATION OF GRAPE ANTHOCYANIDIN 3-GLUCOSIDES AND 3,5-DIGLUCOSIDES: DETERMINATION OF ANTHOCYANINS IN GRAPE FRUIT GROWN IN THE BELGOROD REGION

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For the first time, a new version of the separation of 3-glucosides and 3,5-diglucosides of the five main anthocyanidins for grape fruits - delphinidin, cyanidin, petunidin, peonidin and malvidin under reverse-phase HPLC conditions was proposed. It has been shown that the replacement of the traditionally used acetonitrile with environmentally more acceptable ethanol upon acidification with ortho-phosphoric rather than formic acid makes it possible to significantly change the selectivity of the separation of two types of glucosides at their joint presence. The proposed separation option allows differentiating between Vitis vinifera grapes, in the fruit peel of which only 3-glucosides of the above anthocyanidins are synthesized, and grapes of other species or hybrid grape varieties. For a complete review of the anthocyanin composition, it is necessary to use a gradient mode, since the acylation of anthocyanins with acetic and p-coumaric acids significantly changes the lipophilicity of anthocyanins. The type of anthocyanins was analyzed according to the previously proposed system, which takes into account the activity of three types of enzymes: 1) 5-O-glucosyltransferase that leads to 3,5-diglucosides synthesis; 2) 3′,5′-hydroxylase responsible for ring B hydroxylation; 3) anthocyanin O-methyltransferase for converting cyanidin into peonidin derivatives as well as delphinidine into petunidin and malvidin glycosides. The method was used to determine anthocyanins in 43 grape varieties grown in Belgorod on farms and private farms. Among the studied grapes, varieties were found with grape fruits with the accumulation of only 3-glucosides, as well as 3-glucosides and 3,5-diglucosides simultaneously, with delphinidin and cyanidin types of anthocyanins, and with different degrees of methylation. The calculated parameters for all grape varieties are presented in two tables and a brief discussion of some varieties is given.

Key words: grape fruits, anthocyanins, RP HPLC, ethanol-based mobile phases, three criteria for grape classification

INTRODUCTION

Grape is one of the most popular fruit crops cultivated worldwide; according to literature data world grape production reached 25.62 million metric tons in 2021/2022 season [1]. *Vitaceae* (the grape family) consists of about 14 genera and 900 species primarily distributed in tropical regions [2]. Grapes are explored for mainly winemaking - about 80%, 13% is sold as table while the remaining grapes are used to produce raisins, juice and the other products [3]. Since ancient times in Europe (in Mediterranean region)

namely *Vitis vinifera* species are cultivated. But in the second half of the 19th century, the European vineyards were attacked with accidentally introduced from North America phylloxera and the problem to withstand to the attacks was resolved by employing American grapevines as rootstocks for *V. vinifera* [4].

The dark coloration with blue or red shades of grape fruits is obliged to the biosynthesis (ordinary in the skin) of anthocyanins that may be utilized to prepare natural food colorants (E163). Meanwhile there is a distinct difference between anthocyanin types as *V. vinifera* and non-*V. vinifera* species [5]. For the former the synthesis of 3-O-glucosides (3Glu) of five anthocyanidines: delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin (Mv) is characteristic, Fig. 1. For the latter addition to 3Glu-types of 3,5-O-diglucosides (3,5diGlu) of the same anthocyanidins was found [6-10].

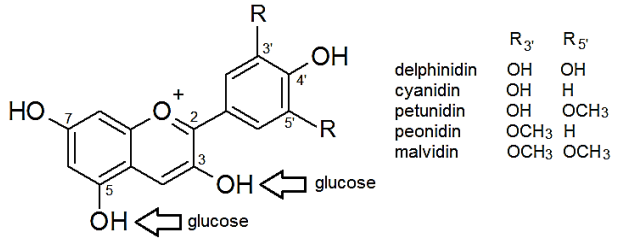

Fig. 1. Structures of five main grape fruit anthocyanidins and positions of glucosylation

Рис. 1. Структуры пяти основных антоцианидинов плодов винограда и положения глюкозилирования

Thus, the detection of 3,5-*O*-diglucosides can be used for differentiation of *V. vinifera* and *V. rotundifolia*, *V. labrusca*, *V. coignetiae*, *V. rupestris*, *V. amurensis* as well as interspecific hybrid grapes. The ten 3-glucosides and 3,5-diglucosides are supplemented with set of anthocyanins acylated with acetic and *para*-coumaric acids (sometimes also acylated with caffeic one) that have highly enhanced lipophilicity. Thus, a problem of anthocyanins separation for a subsequent quantification is not a simple task. Reversed-phase HPLC (RP HPLC) in gradient elution mode is a method ordinary used for grape anthocyanins analysis [11-14]. Though acylated anthocyanins are interesting for preparation of differently colored encapsulated anthocyanins [15] the problem of *Vitis* species differentiation may be established by the determination only of non-acylated compounds. The five 3-glucosides as well as the five 3,5-diglucosides are easily separated by acidified mobile-phases with acetonitrile or methanol as organic modifiers eluting in the order of retention times growth:

Dp3Glu – Cy3Glu – Pt3Glu – Pn3Glu – Mv3Glu,
Dp3,5diGlu – Cy3,5diGlu – Pt3,5diGlu – Pn3,5diGlu – Mv3,5diGlu.

In the case of joint presence of 3-glucosides and 3,5-diglucosides some separation problems arise in isocratic as well as in gradient elution with mobile phases containing acetonitrile and formic acids as mobile phase modifiers are used [13, 14, 16]. Meanwhile acetonitrile is a toxic solvent for men and environment, thus giving rise to find more ecologically suitable conditions for RP HPLC.

Thus, the objective of this study was to find more ecologically friendly mobile phase modifiers for separation of the ten non-acylated anthocyanins for differentiation of *V. vinifera* and hybrid varieties of the grapes grown in Belgorod.

EXPERIMENTAL TECHNIQUES

Samples of grape were obtained from peasant farms of Belgorod and the region. Grape fruits accumulate anthocyanins mainly in fruit skin like black currant [17] and some other fruits. Thus, anthocyanins were extracted by maceration of grape fruit skins in 0.1 M HCl water solution for 20 h. The samples were partially cleaned up by solid phase extraction on DIAPACK C18 cartridges as follows: cartridges were activated by passing 3 ml of acetone, conditioned by passing 9 ml of 0.1 M HCl water solution. After sorption of anthocyanins from the extracts, anthocyanins were desorbed from cartridges by passing 3 ml of solution 30 vol. % of CH_3CN, 30 vol.% of HCOOH and 40 vol. % of water. The obtained solution was diluted with water 1:2 by volume for subsequent injection into the chromatograph.

For gradient elution two mobile phases were used - phase A, 8 vol.% of ethanol and 1 vol.% of H_3PO_4 in water; phase B, 20 vol.% of ethanol and 1 vol.% of H_3PO_4 in water. Gradient mode: 0 min 0% B; 10 min 10% B, 30 min 100 % B, 31 min 0% B; 40 min 0% B.

Separations were performed on an Agilent 1200 Infinity chromatograph with a diode array detector (DAD). A chromatographic column 150×4.6 mm Symmetry^{TM}C18, 3.5 μm was used for separation of anthocyanins.

Utilization of DAD permits to differentiate 3-glucosides by characteristic electronic absorption spectra that are almost the same for Cy3Glu and Pn3Glu (solutes are mentioned according to the order of elution) with absorption maxima near 515 nm, Fig. 2. Spectra of Dp3Glu, Pt3Glu and Mv3Glu have absorption maxima shifted bathochromically to 524-526 nm region, while the methylation of OH-groups in B-ring leads to small sequential 1.0 nm shift of the maxima. 3,5-diglucosides are also easily differentiated by absence of local maxima at 420-450 nm in electronic ab-

sorption spectra fixtures. Moreover, peaks of 3,5-diglucosides have extra broadening compared to that of 3-glucosides [18].

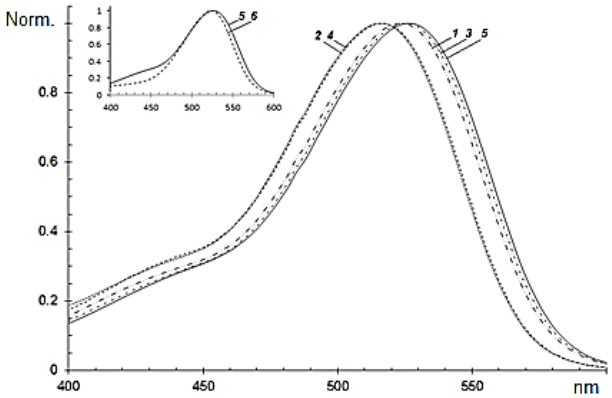

Fig. 2. Electronic absorption spectra of anthocyanins in the mobile phase. Compounds: 1 – Dp3Glu, 2 – Cy3Glu, 3 – Pt3Glu, 4 – Pn3Glu, 5 – Mv3Glu, 6 – Mv3,5diGlu.
Рис. 2. Электронные спектры поглощения в подвижной фазе. Антоцианы: 1 – Dp3Glu, 2 – Cy3Glu, 3 – Pt3Glu, 4 – Pn3Glu, 5 – Mv3Glu, 6 – Mv3,5diGlu

The mobile phases were prepared using acetonitrile (HPLC Gradient grade, Fisher Chemical, Germany), ethanol (95%, Hippocrates LLC, RF) *ortho*-phosphoric acid (85%, RUSHIM, RF), distilled water.

RESULTS AND DISCUSSION

The most popular mobile phases for anthocyanins separations are based on acetonitrile as an organic modifier and formic acid for pH correction to transfer anthocyanins into flavylium form. The separation map for 3-glucosides and 3,5-diglucosides of five common for grape fruits anthocyanidins for mobile-phase system "10 vol. % of formic acid–(6-10 vol. %) acetonitrile – water" and Symmetry™C18, 3.5 μm column is proposed in Fig. 3.

It is evident that for the system there is a problem for separation Pt3Glu and Mv3,5diGlu. The problem is complicated because of the latter compound' peak broadening being the intrinsic property of peaks of solutes with 3,5-diglycosylation. This problem has a great value since the presence of namely malvidin-3,5-diglucoside is commonly the main criteria for detection of hybrid or non-*vinifera* grape cultivars. The problem has some ways of decisions [16, 18, 19], but lowering the temperature of separation and exchange of formic acid with phosphoric acid buffer [18] permits not to separate all the ten compounds. Thus another approach must be found to solve the problem.

Ethanol seems to be the most «green» solvent for the substitution of ecologically unfavorable acetonitrile [20]. But the exchange of acetonitrile with ethanol demands also to exchange formic acid for *ortho*-phosphoric one to prevent esterification reaction. To maintain high mobile phase acidity 1 vol. % of the acid must be added. The separation map for mobile phase system "10-20 vol. % of ethanol – 1 vol.% of *ortho*-phosphoric acid in water" is presented in Fig. 4.

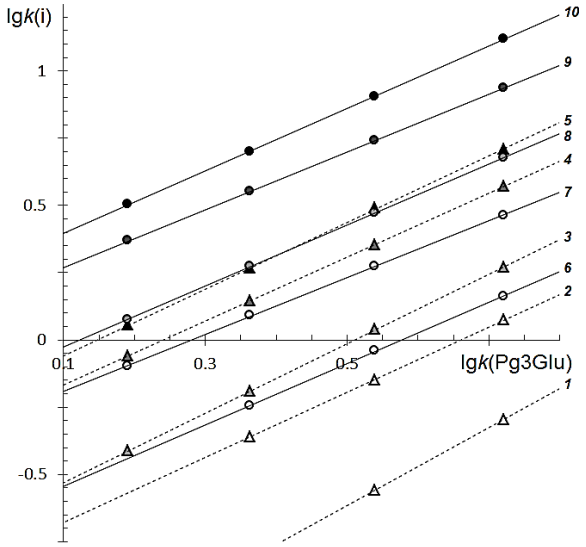

Fig. 3. Separation map of 3-glucosides and 3,5-diglucosides in mobile phase system "6 – 9.6 vol.% of CH_3CN, 10 vol.% of HCOOH in water". Compounds: 1 – Dp3,5diGlu; 2 – Cy3,5diGlu, 3 – Pt3,5diGlu, 4 – Pn3,5diGlu, 5 – Mv3,5diGlu, 6 – Dp3Glu; 7 – Cy3Glu, 8 – Pt3Glu, 9 – Pn3Glu, 10 – Mv3Glu
Рис. 3. Карта разделения 3-глюкозидов и 3,5-диглюкозидов в системе подвижных фаз "6 – 9,6 об.% CH_3CN, 10 об.% HCOOH в воде". Соединения: 1 – Dp3,5diGlu; 2 – Cy3,5diGlu, 3 – Pt3,5diGlu, 4 – Pn3,5diGlu, 5 – Mv3,5diGlu, 6 – Dp3Glu; 7 – Cy3Glu, 8 – Pt3Glu, 9 – Pn3Glu, 10 - Mv3Glu

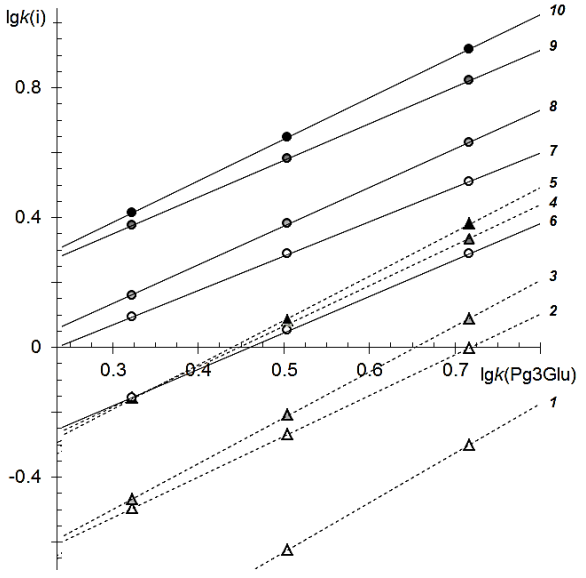

Fig. 4. Separation map of 3-glucosides and 3,5-diglucosides in mobile phase system "12 – 16 vol.% of CH_3CH_2OH, 1 vol.% of H_3PO_4 in water". For compounds numbering see Fig. 2
Рис. 4. Карта разделения 3-глюкозидов и 3,5-диглюкозидов в системе подвижных фаз "12 – 16 об.% CH_3CH_2OH, 1 об.% H_3PO_4 в воде". Нумерация соединений как на Рис. 2

In this case, selectivity of 3Glu and 3,5diGlu compounds separation changes markedly. At low ethanol concentration (10 vol. %) in a mobile phase Mv3,5diGlu elutes after Pt3Glu and Pn3,5diGlu. At high mobile phase elution strength solutes are grouped according to number of OH-groups in B-ring with tendency for reversal of the elution order in pairs Mv3Glu + +Pn3Glu and Pt3Glu + Cy3Glu. Nevertheless, the elution mode in mobile phases, based upon ethanol is highly favorable for grape type differentiation by HPLC method.

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Thus, for investigation of *V. vinifera* grape anthocyanins simple gradient modes with CH_3CN, CH_3OH or C_2H_5OH may be composed, but for non-*V. vinifera* and hybrid grape cultivars the mode of elution must be carefully selected, e.g. gradient mode proposed in the experimental part. The separation of grape fruits anthocyanins in this mode of elution is presented in Fig. 5.

The developed in this paper method was applied for analysis of anthocyanins composition in fruits of 43 grape cultivars grown and harvested in Belgorod region. For grape classification we used criteria developed in paper [21] for peak areas of non acylated anthocyanins.

The first criterion is devoted to estimate the inheritance (or absence) of 5-*O*-glucosyltransferase in anthocyanins biosynthesis from non-*V. vinifera* species by equation (1):

$$\alpha_1 = \frac{\sum_5 S_{3Glu}(i)}{\sum_5 S_{3,5diGlu}(i) + \sum_5 S_{3Glu}(i)} \cdot 100, \% \qquad (1)$$

where $\sum_5 S_{3,5diGlu}(i)$ - is sum of areas of peaks of all five anthocyanidins, glucosylated at positions 3 and 5; $\sum_5 S_{3Glu}(i)$ - is sum of areas of peaks of all five anthocyanidins, glucosylated at position 3. For *V. vinifera* grapes $\alpha_1 = 100$.

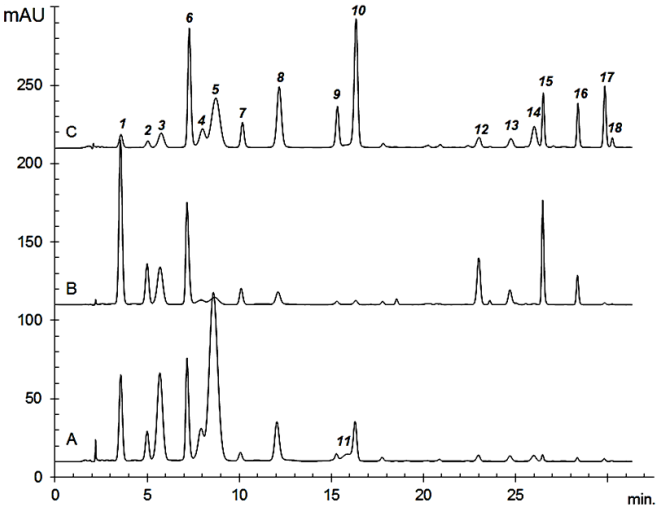

Fig. 5. Separation of hybrid grape anthocyanins in gradient mode with eluents, based upon ethanol and phosphoric acid in water. Extracts of fruits of grape cultivars: A – Mucuzani; B – Chernyj sultan; C – Regent. For compounds numbering see Fig. 2

Рис. 5. Разделение антоцианов гибридных сортов в градиентном режиме с элюентами на основе этанола и фосфорной кислоты. Экстракты плодов винограда сортов: А – Мукузани, В – Черный султан, С – Регент. Нумерация соединений как на Рис. 2

The second criterion differentiates grapes on delphinidin or cyanidin types pointing out relative activity of flavonoid 3',5'-hydroxylase (F3'5'H):

$$\alpha_2 = \frac{\sum_3 S_{3Glu}(i) + \sum_3 S_{3,5diGlu}(i)}{\sum_3 S_{3Glu}(i) + \sum_3 S_{3,5diGlu}(i) + \sum_2 S_{3Glu}(j) + \sum_2 S_{3,5diGlu}(j)} \cdot 100, \% \qquad (2)$$

where $\sum_3 S_{3Glu}(i) + \sum_3 S_{3,5diGlu}(i)$ - is sum of areas of peaks of delphinidin, petunidin and malvidin 3-glucosides and their 3,5-diglucosides, $\sum_2 S_{3Glu}(j) + \sum_2 S_{3,5diGlu}(j)$ - is sum of areas of peaks of cyanidin and peonidin 3-glucosides and their 3,5-diglucosides.

According to our experience the grape of species with α(F3'5'H) > 50% have dark blue coloration while in the case of α(F3'5'H) > 50% coloration is dark or light red.

The third criterion will determine the relative degree of activity of anthocyanin *O*-methyltransferase (AOMT):

$$\alpha_3 = \frac{\sum_2 S(Pn)_{ij} + \sum_2 S(Pt)_{ij} + 2\cdot\sum_2 S(Mv)_{ij}}{2\cdot\sum_2 S(Dp)_{ij} + \sum_2 S(Cy)_{ij} + 2\cdot\sum_2 S(Pt)_{ij} + \sum_2 S(Pn)_{ij} + 2\cdot\sum_2 S(Mv)_{ij}}\cdot 100\,,\ \% \qquad (3)$$

where $\sum_2 S(X)_{ij}$ - is sum of areas of peaks of 3-glucoside and 3,5-diglucoside of anthocyanidins indicated in brackets. Common European *V. vinifera* grapes belong to malvidin type due to high values of parameters α_2 and α_3.

The results of grape anthocyanins determination are summered in Table 1 and Table 2. In the Tables parameters α_1-α_3 are calculated as well as the mole fraction of the main anthocyanin. The first table includes grapes without inheritance of glycosylation of OH-group in position 5 of the aglycons. The Table 2 contents grape cultivars with typical for hybrid cultivars anthocyanin types.

Table 1

Characterization of anthocyanin biosynthesis in fruits of some grape cultivars without inheritance of 3,5-diglucosides biosynthesis

***Таблица 1.* Характеристики биосинтеза антоцианов в плодах некоторых сортов виноградов без наследования биосинтеза 3,5-диглюкозидов**

No.	Grape cultivar	The main anthocyanin mole fraction, %	α_1	α_2	α_3
1	Chernaya magiya	Mv3Glu (46.1)	100	93.2	61.4
2	Furor	Mv3Glu (86.6)	100	91.5	96.6
3	Bagira	Mv3Glu (80.0)	100	88.6	93.2
4	Charli (Antracit)	Mv3Glu (43.9)	100	87.5	62.4
5	Kodryanka	Mv3Glu (72.2)	100	85.7	88.8
6	Tornado	Mv3Glu (70.7)	100	85.1	88.3
7	Atos	Mv3Glu (59.1)	100	80.2	80.9
8	Lorano	Mv3Glu (64.3)	100	80.2	86.0
9	Yupiter	Mv3Glu (31.6)	100	75.6	53.0
10	Magiya	Dp3Glu (38.6)	100	66.8	32.6
11	Yasya	Mv3Glu (42.9)	100	58.7	80.1
12	Gurzufskij rozovyj	Pn3Glu (28.5)	100	44.1	48.8
13	Kishmish Luchistyj	Cy3Glu (36.9)	100	32.2	44.9
14	Polonez	Cy3Glu (50.4)	100	30.2	31.1
15	Koronnyj	Cy3Glu (57.1)	100	21.6	29.9
16	Chernyj zhemchug	Pn3Glu (63.5)	100	20.2	80.9
17	Pamyati uchitelya	Pn3Glu (72.4)	100	20.0	88.5
18	Preobrazhenskij	Pn3Glu (75.6)	100	12.2	84.9
19	Podarok Vinokuru	Pn3Glu (79.6)	100	7.4	85.3
20	Ametist	Pn3Glu (91.1)	100	0.7	91.7

In spite of 3,5-diglucosides absence in anthocyanin set of fruits presented in Table 1 most of the species are mostly of hybrid nature. Thus, grape cultivar No. 1 ("Chernaya magiya") entirely has not 3,5-diglucosides but is relatively resistant to mildew, gray mold and oidium, as a hybrid type cultivar. This property is highly valuable for grape cultivation in our region. But there is a difference from conventional "malvidin" V. vinifera grapes due to reduced AOMT activity. In fruits of also hybrid "Bagira" grape cultivar mole fraction of Mv3Glu is appreciably higher and the grape has high frost resistance but loose stability towards phylloxera. Anthocyanins biosynthesis is mainly located in the fruit skin. As a rule, delphinidin (or malvidin) grape types have dark blue coloration while intensity of red coloration is significantly reduced for cyanidin (peonidin) types.

Table 2

Characterization of anthocyanin biosynthesis in fruits of some grape cultivars with inheritance of 3,5-diglucosides biosynthesis

***Таблица 2.* Характеристики биосинтеза антоцианов в плодах некоторых сортов виноградов с наследованием биосинтеза 3,5-диглюкозидов**

No.	Grape cultivar name	The main anthocyanin mole fraction, %	α_1	α_2	α_3
1	Spokusa	Mv3Glu (49.7%)	99.0	69.9	81.0
2	Dubovskij rozovyj	Cy3Glu (52.9%)	97.8	29.5	24.9
3	Fioletovyj rannij	Mv3Glu (29.6%)	96.9	70.2	57.1
4	Severnyj plechistik	Mv3Glu (29.6%)	96.7	69.7	57.9
5	Preobrazhenie	Cy3Glu (76.3%)	96.4	14.4	21.2
6	Zarya Nesvetaya	Pn3Glu (81.6%)	96.2	8.2	90.9
7	Asya	Pn3Glu (81.6%)	95.2	38.5	88.3
8	Zejbel'	Mv3Glu (28.2%)	88.0	91.2	53.5
9	Andryusha	Pn3Glu (64.1%)	85.4	9.54	85.0
10	Nadezhda AZOS	Mv3Glu (33.8%)	78.8	92.3	65.6
11	Markett	Dp3Glu (28.9%)	76.9	92.8	53.1
12	Atos	Pn3Glu (20.9%)	71.1	43.3	69.6
13	Regent	Mv3Glu (22.7%)	67.3	86.1	63.5
14	Vas'kovskij 5	Mv3Glu (25.6%)	67.0	73.6	50.8
15	Livadijskij chernyj	Mv3,5diGlu (27.3%)	59.9	94.5	63.4
16	Monarh	Mv3,5diGlu (44.8%)	48.5	96.2	78.0
17	GIS 1-31	Mv3,5diGlu (35.1%)	48.0	78.6	78.5
18	Nadezhda rannyaya	Mv3,5diGlu (40.8%)	47.4	84.2	90.1
19	Kaberne kortis	Mv3,5diGlu (44.3%)	40.1	89.7	79.5
20	Taezhnyj (*V. amurensis*)	Mv3,5diGlu (51.9%)	25.3	89.2	71.6
21	Neretinskij	Mv3,5diGlu (70.2%)	25.1	97.6	90.9
22	Seedless Chernyj sultan	Dp3,5diGlu (38.5%)	35.2	85.2	16.4
23	Mukuzani ("Oberlin Noir")	Mv3,5diGlu (47.1%)	24.6	95	64.1

The first 7 grape species in the Table 2 are closed to *V. vinifera* types though they belong to different types according to criteria α_2 and α_3 having Mv3Glu, Cy3Glu or Pn3Glu as a major anthocyanin. The grape "Livadijskij chernyj" begins the list of cultivars with Mv3,5diGlu as a predominant anthocyanin. The set includes "Taezhnyj" cultivar which is an example of *V. amurensis*. The most interesting cultivar was "Mukuzani". According to non-official information available in Internet cites the cultivar was accidentally found in Georgia though the "Saperavi" cultivar is used to make wine under the "Mukuzani" brand. Meanwhile according to our investigation "Mukuzani" is evidently hybrid cultivar with the lowest value of criteria α_1 between all cultivars studied in current paper.

CONCLUSION

The new RP HPLC method is developed for separation of 3-glucosides and 3,5-diglucosides of five common grape fruit anthocyanidins – delphinidin, cyanidin, petunidin, peonidin and malvidin. The method as applied for investigation anthocyanin set in grape fruit skins of 43 grape cultivars grow in Belgorod region. The results are discussed according three previously proposed criteria.

The authors declare the absence a conflict of interest warranting disclosure in this article.

Авторы заявляют об отсутствии конфликта интересов, требующего раскрытия в данной статье.

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Поступила в редакцию 12.12.2022
Принята к опубликованию 08.02.2023

Received 12.12.2022
Accepted 08.02.2023