ISSN 1061-9348, Journal of Analytical Chemistry, 2023, Vol. 78, No. 1, pp. 63–67. © Pleiades Publishing, Ltd., 2023.
Russian Text © The Author(s), 2022, published in Zhurnal Analiticheskoi Khimii, 2022, Vol. 77, No. 1, pp. 43–47.

ARTICLES

Replacement of Acetonitrile with Ethanol in the Determination of Anthocyanins by Reversed-Phase High-Performance Liquid Chromatography

V. I. Deineka[a], *, E. Yu. Oleinits[a], I. P. Blinova[a], and L. A. Deineka[a]

[a] *Belgorod State National Research University, Institute of Pharmacy, Chemistry and Biology, Belgorod, 308015 Russia*

**e-mail: deineka@bsu.edu.ru*

Received January 17, 2022; revised January 27, 2022; accepted January 29, 2022

Abstract—It was found experimentally that the replacement of acetonitrile with ethanol upon the acidification of the mobile phase with phosphoric acid is an effective version for replacing acetonitrile. At that, the order of elution of glycosides of the same type of the five main anthocyanidins (delphinidin, cyanidin, petunidin, peonidin, and malvidin) does not change in eluents convenient for analysis, and the range of retention times for a complete set of such anthocyanins becomes noticeably narrower in going from acetonitrile to ethanol. To determine the effect of aglycone structure on the retention of derivatives of the same anthocyanidin (cyanidin), a mixture of six glycosides was used: one monoglycoside (3-glucoside), two 3-diglycosides (sophoroside and sambubioside), and two 3-triglycosides (2"-glucosylrutinoside and 2"-xylosylrutinoside). This mixture is a real set of anthocyanins, requiring the careful selection of the mobile phase for separating all components. In this work, based on an analysis of separation maps, the composition of a mobile phase based on ethanol is determined that ensures the achievement of the effective separation of these anthocyanins.

Keywords: green chromatography, ethanol, anthocyanins, reversed-phase HPLC

DOI: 10.1134/S1061934822110053

Reversed-phase HPLC is one of the most commonly used methods in analytical laboratories. In this method, acetonitrile, as a solvent with unique and convenient properties for implementing the method, is often used as a mobile phase [1]. In addition to properties favorable for HPLC analysis, acetonitrile also has disadvantages, such as high cost and toxicity, which forces us to look for other solvents for replacing acetonitrile. In [1], a possibility of replacing acetonitrile for methanol was studied and positive results were obtained; however, the study was fragmentary: the results of the separation of anthocyanins from a limited set of samples at randomly selected compositions of mobile phases in a gradient mode with insufficiently acidified one of the components in each version (in using acetonitrile or methanol) were compared. The fact that the insufficient and uneven acidification of the mobile phase can lead to problems in the quantitative determination of anthocyanins was indicated in [2]. A systematic analysis of the change in the selectivity of anthocyanin separation in replacing acetonitrile with methanol revealed the features of anthocyanin retention depending on the structure of aglycone and glycosidic substituents and showed that methanol can effectively replace acetonitrile [3].

Among alternative solvents for reversed-phase HPLC, ethanol is usually singled out as less toxic than acetonitrile and methanol, a widely available and a cheaper component of mobile phases [4]. Examples (21 versions) of using ethanol as a component of mobile phases in pharmaceutical analysis were given in [4]. There are only a few works on the determination of anthocyanins with ethanol as an organic component of the mobile phase. In [5], there were no problems in the separation of anthocyanins, because in the fruits of blue honeysuckle, mulberry, and blackberry, anthocyanins are represented by almost solely cyanidin-3-glucoside. Similarly, in [6], one main anthocyanin was also identified (*para*-coumaroyl, a petunidin-3-rutinosyl-5-glucoside derivative). In [7], the components of a more complex mixture of three cyanidin derivatives from the red leaves of *Prunus cerasifera* var. *Atropurpurea* were separated, but the quality of the chromatograms was low.

The aim of this work was to study possibilities of mobile phases based on ethanol in the separation of complex mixtures of anthocyanins.

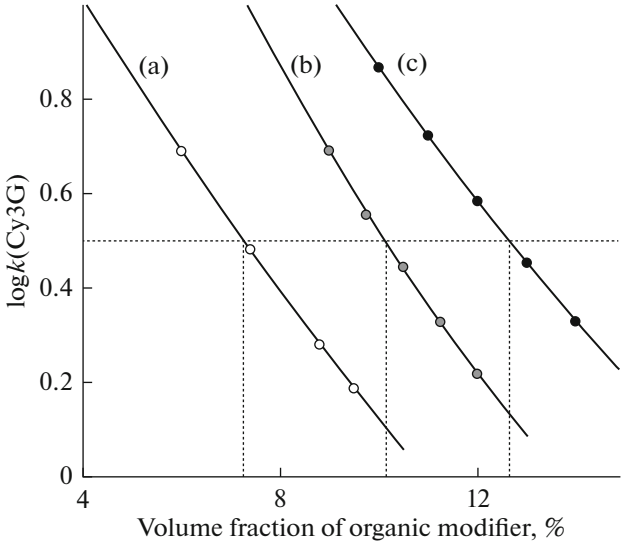

Fig. 1. Effect of organic modifier concentration on the retention of cyanidin-3-glucoside in three eluent systems: (a) 10 vol % formic acid–(6–10) vol % acetonitrile–water; (b) 1 vol % orthophosphoric acid–(10–20) vol % acetonitrile–water; and (c) 1 vol % orthophosphoric acid–(10–15) vol % ethanol–water.

EXPERIMENTAL

Anthocyanin extracts were prepared by soaking a plant material (fruits of grapes and red currants) in a 0.1 M aqueous solution of hydrochloric acid for 24 h and stored in a household refrigerator. The extracts were purified by solid-phase extraction using Diapak C18 cartridges (BioChemMac ST, Moscow, Russia).

To control the species composition of anthocyanins in the samples, an Agilent 1260 Infinity chromatographic system with a diode array detector was used. A 150 × 4.6 mm Symmetry C18, 3.5 μm chromatographic column was used at a column oven temperature of 40°C. The following systems were used in the work:

(a) 10 vol % formic acid–(6–10) vol % acetonitrile–water;

(b) 1 vol % orthophosphoric acid–(10–20) vol % acetonitrile–water;

(c) 1 vol % phosphoric acid–10–15 vol % ethanol–water.

All chromatograms were recorded in the isocratic mode in several different compositions of mobile phases for each eluent system.

Chromatograms were recorded, stored, and processed using the Agilent ChemStation software.

RESULTS AND DISCUSSION

To compare the retention of anthocyanins using mobile phases with various organic modifiers, separation maps were compared, which made us possible to draw a conclusion about the features of chromatographic behavior in the entire eluent system rather than using one randomly selected composition of the mobile phase.

The data on the retention of cyanidin-3-glucoside (**Cy3G**) on the separation map on the coordinates log$k(i)$–ϕ (volume fraction of the organic modifier) are shown in Fig. 1. One can see that, for all three eluent systems, the dependence of the logarithm of the Cy3G retention factor on the composition of mobile phases is described by the quadratic equation [8]:

$$\log k\,(Cy3G) = a_0 + a_1\phi + a_2\phi^2.$$

At that, a decrease in the elution power of the mobile phase upon transition from system (a) to system (b) is explained by the fact that formic acid is not only an acidic modifier for converting anthocyanin forms mainly into the flavylium form, but also a modifier that determines the retention of anthocyanins together with acetonitrile. A transition to eluent system (b) is associated with the need in creating a correct comparison system for system (c) because of the instability of the properties of the system 10 vol % formic acid–ethanol–water in view of the acylation of alcohol with formic acid. A comparison shows that ethanol turns out to be a component with a noticeably lower elution power compared to acetonitrile: to achieve log$k$(Cy3G) = 0.5, mobile phases containing 7.25, 10.12, and 12.62 vol % organic modifier for systems (a), (b) and (c), respectively, are required.

Dependence of anthocyanin retention on the structure of aglycone (anthocyanidin that formed anthocyanin). According to the float mechanism proposed in our laboratory [9], the dependence of retention on the structure is fundamentally different for aglycone penetrating into the bonded phase and for glycosidic substituents remaining on the surface of the reversed phase.

The dependence of retention on the aglycone structure was studied using relative retention maps [10] for five 3-glucosides (**3G**) as examples (delphinidin, **Dp**; cyanidin, **Cy**; petunidin, **Pt**, peonidin, **Pn**; and malvidin, **Mv**), occurring, e.g., in extracts of fruits of grapes *Vitis vinifera* [11], as well as in extracts of many other plant samples in the form of derivatives of various aglycones of a similar type. Figure 2 compares the separation of anthocyanins in two eluent systems (a) and (b). One can see that the order of elution of five 3-glucosides turns out to be identical for any composition of the mobile phase of the compared eluent systems that are convenient for determining these anthocyanins (in terms of time consumption):

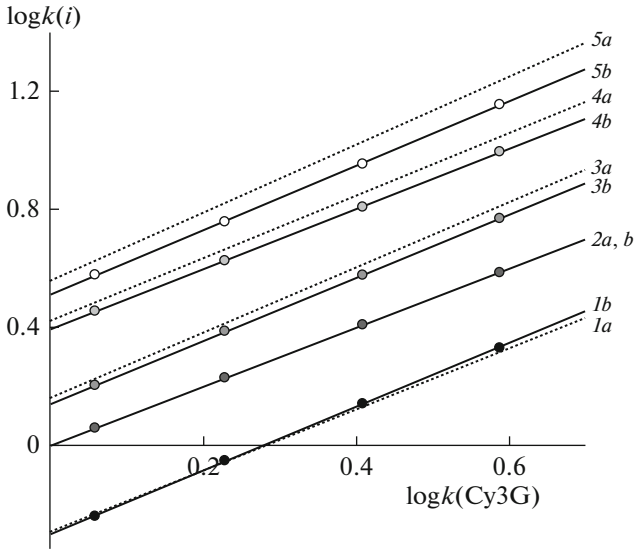

Fig. 2. Comparison of the retention of five 3-glucosides in two eluent systems (a) and (b). Anthocyanins: (*1*) Dp3G, (*2*) Cy3G, (*3*) Pg3G, (*4*) Pn3G, and (*5*) Mv3G.

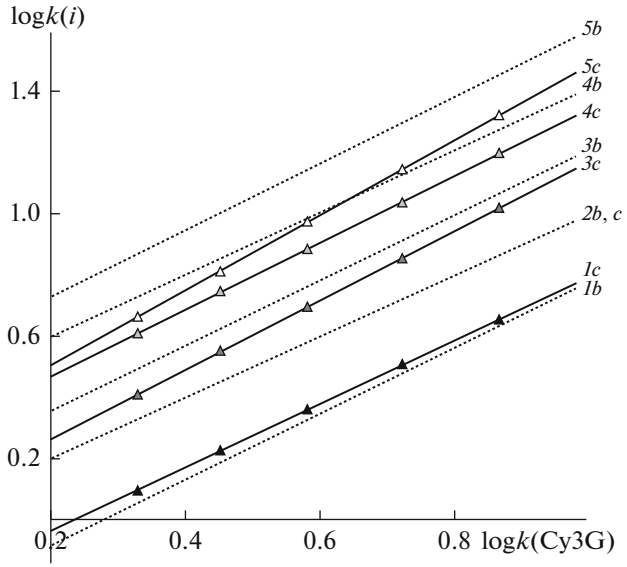

Fig. 3. Comparison of the retention of five 3-glucosides in two eluent systems (b) and (c). Anthocyanins: (*1*) Dp3G, (*2*) Cy3G, (*3*) Pg3G, (*4*) Pn3G, and (*5*) Mv3G.

$$t_R\,(\mathrm{Dp3G}) < t_R\,(\mathrm{Cy3G}) < t_R\,(\mathrm{Pt3G}) < t_R\,(\mathrm{Pn3G}) < t_R\,(\mathrm{Mv3G}).$$

At that, the replacement of formic acid with orthophosphoric acid slightly reduces the range of retention times between Dp3G and Mv3G, which is convenient for determining the ratio between the concentrations of five non-acylated anthocyanins sensitive to the grape variety [11].

The result of replacing acetonitrile with ethanol (while retaining orthophosphoric acid as the mobile phase acidifier) is illustrated in Fig. 3.

It is obvious that the relative decrease in the retention of 3-glycosides containing methoxy groups in the B ring of the aglycone (derivatives of malvidin, peonidin, and petunidine) significantly increases with increasing ethanol concentration in the mobile phase when acetonitrile is replaced by ethanol. The addition of an OH group to ring B (transition from Cy3G to Dp3G) leads to the opposite effect. In this case, the elution order of five 3-glucosides remains identical as indicated above, but with increasing ethanol concentration, the difference in the retention times of Mv3G and Pn3G decreases, creating problems in the separation of these components.

Dependence of anthocyanin retention on the structure of the glycoside radical. To analyze the effect of the structure of the glycoside radical on the retention of anthocyanins, six cyanidin derivatives were selected: one 3-monoglycoside (Cy3G), three 3-diglycosides: 3-sophoroside (Cy3Sopho); 3-sambubioside (Cy3Sam) and 3-rutinoside (Cy3Rut) and two 3-triglycosides: 3-(2"-glucosylrutinoside) (Cy3GRut) and 3-(2"-xylosylrutinoside) (Cy3XRut). These anthocyanins are found in the fruits of some cultivars and species of cherries popular in Russia [12], some cultivars of red currant [13], in a reduced number of derivatives in the fruits of black raspberry [14], black elderberry [15], etc.

Figure 4 compares separation maps of these compounds in eluent systems (b) and (c). It is obvious, that the analysis of a mixture of all six components requires the careful selection of the composition of the mobile

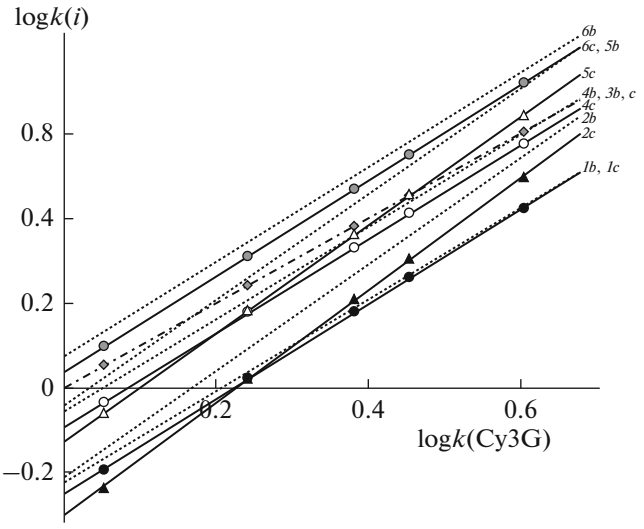

Fig. 4. Comparison of the retention of six different cyanidin 3-glucosides in two eluent systems (b) and (c). Anthocyanins: (*1*) Cy3Sopho, (*2*) Cy3GRut, (*3*) Cy3G, (*4*) Cy3Sam, (*5*) Cy3XRut, and (*6*) Cy3Rut.

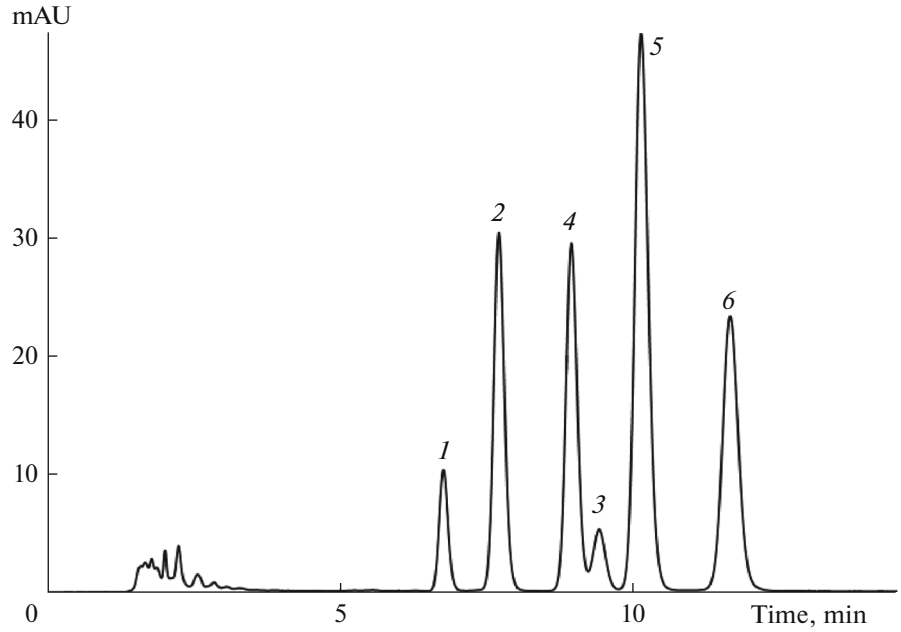

Fig. 5. Separation of six different cyanidin 3-glucosides in a mobile phase of composition 11.2 vol % ethanol–1 vol % orthophosphoric acid–87.8 vol % water. Mobile phase flow rate 0.8 mL/min, temperature 40°C, column 150 × 4.6 mm Symmetry C18, 3.5 μm. Anthocyanins: (*1*) Cy3Sopho, (*2*) Cy3GRut, (*3*) Cy3G, (*4*) Cy3Sam, (*5*) Cy3XRut, and (*6*) Cy3Rut.

phase for each system because of the large number of line intersections in the map (i.e., inversions of the elution order). In both cases, the slope of the straight lines increases as the composition of the glycosidic radical becomes more complex from a monoglycoside to a triglycoside. It is also obvious that the statement that when a glycosidic radical is added to the structure of an already existing substituent cannot be correct, because elution order depends on the composition of the mobile phase. However, the replacement of acetonitrile with ethanol leads to a noticeable decrease in the retention of 3-triglycosides and 3-diglycosides compared to monoglycoside, which can be explained by the better solvation of glycosidic substituents with ethanol compared to acetonitrile.

In addition, it was found that the high viscosity of eluents containing more than 80% water, noted as an "uncomfortable" property of ethanol, has little effect on the inlet pressure to the column compared to acetonitrile – the pressure increased only from 150 to 170 bar. The efficiency (number of theoretical plates) was also comparable for the eluent systems (b) and (c).

Thus, ethanol is an effective alternative to environmentally unfavorable acetonitrile in the determination of anthocyanins, both of similar glycosides of various aglycones, and of various glycosides of one and the same aglycone.

FUNDING

This work was supported by a grant from the Russian Foundation for Basic Research "Postgraduate students", project no. 20-33-90031/20.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Bordonaba, J.G., Crespo, P., and Terry, L.A., *Food Chem.*, 2011, vol. 129, p. 1265.
2. Deineka, V.I., Oleinits, E.Yu., Kulchenko, Y.Yu., Blinova, I.P., and Deineka, L.A., *Russ. J. Phys. Chem. A*, 2021, vol. 95, p. 1729.
3. Deineka, V.I., Oleinits, E.Yu., Blinova, I.P., and Deineka, L.A., *J. Anal. Chem.*, 2021, vol. 76, p. 939.
4. Yabré, M., Ferey, L., Somé, I.T., and Gaudin, K., *Molecules*, 2018, vol. 23, 1065.
5. Sang, J., Ma, Q., and Li, C.-Q., *Anal. Methods*, 2017, vol. 9, p. 25.
6. Zhang, Y., Chen, F.F., and Sang, J., *Food Anal. Methods*, 2018, vol. 11, p. 2141.
7. Chen, F.F., Sang, J., Zhang, Y., and Sang, J., *Int. J. Food Sci. Technol.*, 2018, vol. 53, p. 1494.
8. Schoenmakers, P.J., Billiet, H.A.H., Tijssen, R., and de Galan, L., *J. Chromatogr. A*, 1978, vol. 149, p. 519.

9. Deineka, V.I., Deineka, L.A., Saenko, I.I., and Chulkov, A.N., *Russ. J. Phys. Chem. A*, 2015, vol. 89, p. 1300.

10. Deineka, V.I., *Russ. J. Phys. Chem. A*, 2006, vol. 80, p. 426055.

11. Revilla, E., García-Beneytez, E., Cabello, F., Martín-Ortega, G., and Ryan, J.-M., *J. Chromatogr. A*, 2001, vol. 915, p. 53.

12. Deineka, L.A., Chulkov, A.N., Deineka, V.I., Sorokopudov, V.N., and Shevchenko, C.M., *Nauchn. Ved. Belgorod. Gos. Univ. Ser. Estestv. Nauki*, 2011, vol. 9 (104), no. 15/1, p. 364.

13. Goiffon, J.P., Mouly, P.P., and Gaydou, E.M., *Anal. Chim. Acta*, 1999, vol. 382, p. 39.

14. Dossett, M., Lee, J., and Finn, C.E., *J. Funct. Foods*, 2010, vol. 2, p. 292.

15. Silva, R.F.R., Barreira, J.C.M., Heleno, S.A., Barros, L., Calhelha, R.C., and Ferreira, I.C.F.R., *Molecules*, 2019, vol. 24, 2359.

Translated by V. Kudrinskaya