ISSN 0036-0244, Russian Journal of Physical Chemistry A, 2021, Vol. 95, No. 8, pp. 1729–1734. © Pleiades Publishing, Ltd., 2021.
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PHYSICAL CHEMISTRY OF SEPARATION PROCESSES: CHROMATOGRAPHY

Chromatographic Separation and Determination of Anthocyanins under Conditions of Reversed Phase Chromatography, When Used As Mobile Phases of Acetonitrile–Formic Acid/Phosphoric Acid–Water Systems

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Received November 8, 2020; revised November 8, 2020; accepted December 15, 2020

Abstract—A study is performed of the effect of composition of the mobile phases of acetonitrile–formic acid/phosphoric acid–water systems and the concentrations of acetonitrile and acid have on the chromatographic behavior of anthocyanins under the conditions of reversed phase HPLC.

Keywords: reversed phase HPLC, anthocyanins, acetonitrile, formic acid, phosphoric acid, parameters of retention, efficiency

DOI: 10.1134/S0036024421080094

INTRODUCTION

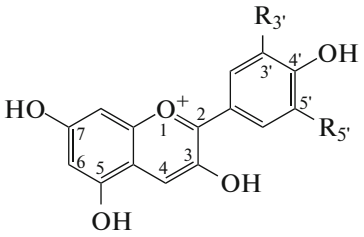
Acetonitrile is one of the best organic modifiers that determine the retention of sorbates in reversed-phase chromatography with aqueous organic mobile phases. This is due to its unique physicochemical properties, which include transparency in a wide range of wavelengths, low viscosity and miscibility with water, and low volatility. A disadvantage of this solvent is its high toxicity to humans and the environment [1]. Due also to its high cost, there is a clear trend of moving away from acetonitrile in favor of more environmentally friendly solvents, known under the term "green" chromatography [2].

Acetonitrile is most often used in the separation of anthocyanins, but the solution must in this case be additionally acidified with a number of acids, of which formic acid is used most often [3]. Acidification is needed to convert all forms of anthocyanins into flavylium form, an analytical form colored in tones of red [4], which allows us to determine these compounds in real multicomponent plant extracts containing a large number of other uncolored extracts. Although it is known that all types of anthocyanins transform almost completely into flavylium form only when pH ≤1, additions of formic acid range from 10 vol % (which yields a pH of less than 1.5) to 1 vol %, according to the literature [3]. The high content of volatile, caustic, and malodorous formic acid also cannot be considered an environmentally friendly factor. Replacing formic acid with one more environmentally acceptable is therefore also a problem of green chromatography. Results from changes in the chromatographic behavior of anthocyanins when formic acid was replaced with phosphoric acid were observed in [5]. However, the broadening of peaks in the chromatograms was largely ignored. At the same time, analysis of the observed dependences of the retention of anthocyanins on their structure [6, 7] allowed a "float" mechanism of retention to be proposed for anthocyanins [8], which explains the differences in the sensitivity of the structure to that of the flavylium and carbohydrate parts of anthocyanins.

When determining the effectiveness of new solvents as substitutes for acetonitrile, we must know the most important patterns in the behavior of sorbates in aqueous acetonitrile mobile phases. The aim of this work was to characterize as completely as possible acetonitrile–formic acid/phosphoric acid–water mobile phases for the separation of anthocyanins under conditions of reversed-phase HPLC, so they could be used as reference material for determining the effectiveness of other, more environmentally friendly organic modifiers.

EXPERIMENTAL

Five 3-glucosides of the main anthocyanidins (delphinidin, cyanidin, petunidin, peonidin and malvidin) were extracted from eastern redbud leaves (*Cercis canadensis* L., [9]). 3-Glucoside of pelargonidin was extracted from the fruits of the common barberry [10]. The extracts were purified of accompanying polymeric



Scheme 1. Structure of the aglycones of anthocyanins (anthocyanidins).

and oligomeric extractives on DIAPAK C18 concentrating cartridges (BioKhimMak ST, Moscow).

After dynamic solid-phase purification, anthocyanins in solutions were separated on an Agilent 1200 Infinity chromatograph equipped with a diode array detector and a thermostatted 150 × 4.6 mm Symmetry C18 (3.5 μm) column. The detection wavelength was 515 nm. Dead time was determined by retention of oxalic acid. Separation was performed in the isocratic mode using mobile phases with fixed contents of acetonitrile, formic, or phosphoric acids in distilled water. Chromatograms were recorded and processed with Agilent ChemStation software.

The anthocyanidins used in this work were delphinidin (3,5,7,3',4',5'-hexahydroxyflavylium), denoted as Dp; cyanidin (3,5,7,3',4'-pentahydroxyflavylium), Cy; petunidin (3,5,7,3',4'-pentahydroxy-5'-methoxyflavylium), Pt; peonidin (3,5,7,4'-tetrahydroxy-3'-methoxyflavylium), Pn; and malvidin (3,5,7,4'-tetrahydroxy-3', 5'-dimethoxyflavylium), Mv (see Scheme 1). The 3-glucosides of anthocyanidins were in this case denoted as, e.g., Cy3G.

Lipophilicity parameter miLogP was calculated interactively at the Molinspiration website.

RESULTS AND DISCUSSION

Separating 3-Glucosides of the Six Major Anthocyanidins

The separation of six 3-glucosides of the main anthocyanidins using two types of mobile phase compositions on the same stationary phase is shown in Fig. 1.

The order of the elution of all six components in any of the acceptable mobile phase compositions of the systems selected for the symmetry C18 stationary phase was the same as for the stationary phases studied in [6, 7]. It remained virtually the same upon changing the concentration of acetonitrile. An exception was for very slow eluents, where the degree of separation of the derivatives of pelargonidin and petunidin fell gradually until the retention times were inverted. At the same time, the retention times (as well as the retention factors) of some sorbates were proportional to the lipophilicities calculated with the miLogP program and shown in parentheses after the anthocyanin denotation in the series

$$t_R\text{Dp3G}(-3.08) < t_R\text{Cy3G}(-2.79) < t_R\text{Pt3G}(-2.78) < t_R\text{Pg3G}(-2.08) < t_R\text{Pn3G}(-2.49) < t_R\text{Mv3G}(-2.47).$$

Correlated changes in retention times and miLogP are observed only for the sequential addition of OH-groups to ring B, indicating an obvious increase in the hydrophilicity of substances with such structural changes. However, miLogP incorrectly estimates the

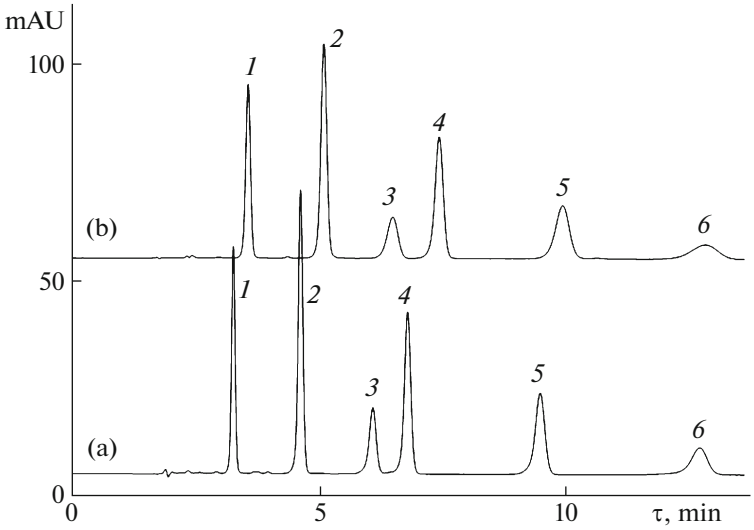


Fig. 1. Separation of the 3-glucosides of six major anthocyanidins under two conditions: (a) mobile phase, 10 vol % formic acid and 9.5 vol % CH_3CN in water; (b) mobile phase, 0.5 vol % formic acid and 12 vol % CH_3CN in water. Flow rate, 0.8 mL/min; temperature, 40°C. Detection at 515 nm. Substances: (*1*) Dp3G, (*2*) Cy3G, (*3*) Pt3G, (*4*) Pg3G, (*5*) Pn3G, (*6*) Mv3G.

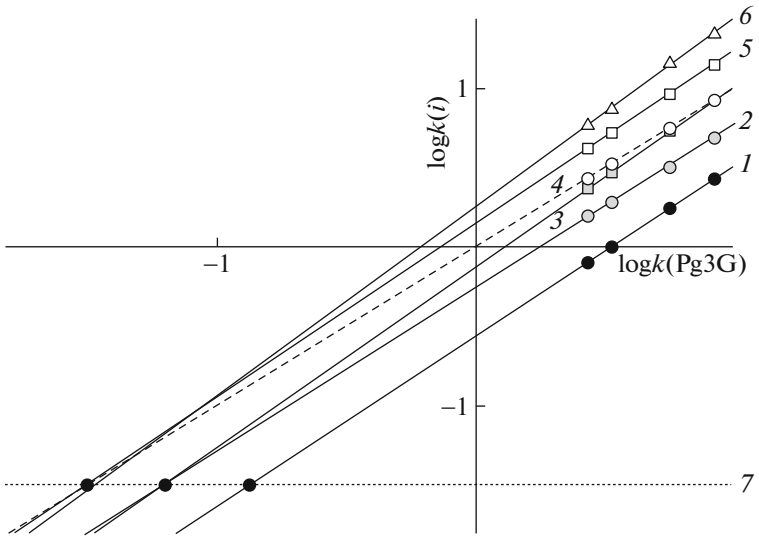


Fig. 2. Map of the separation of the 3-glucosides of six main anthocyanidins in the CH_3CN–(10 vol %) HCOOH–H_2O. The anthocyanins are denoted as in Fig. 1.

change in retention upon adding CH_3O-groups, as the program clearly underestimates the change in lipophilicity of substances in such cases. However, this is not surprising for a one-parameter scoring system that does not consider the balance of hydrophilic–hydrophobic properties. It is therefore useful to consider the map of anthocyanin separation (built by means of relative retention) in Fig. 2, where the retention of anthocyanins (as the decimal logarithm of the retention factor) was determined using a regression equation for relative retention with Pg3G selected as the reference compound:

$$\log k(i) = a \log k(\text{Pg3G}) + b. \quad (1)$$

The separation map shows that adding OH-groups results in downward movement of the trend lines, which corresponds to an increase in hydrophilicity. At the same time, however, the general drop in retention is accompanied by a notable increase in the slope of the trend lines (Table 1), due to a rise dispersion interactions upon an increase in the number of atoms in the structures. This can be interpreted as an increase in the hydrophobic component of the hydrophilic–hydrophobic balance. Adding CH_3O-groups in this case always shifts the trend lines upward and slightly raises the slope of the trend lines, which corresponds to an increase in the hydrophobic component. Strictly speaking, the hydrophilic component should also grow to some extent, since OCH_3-groups can form hydrogen bonds, but only as acceptors. Estimating the degree of this growth is problematic, but the possibility of ignoring the contribution from the methoxy group to the hydrophilic component in the first approximation allows us to classify the trend lines according to the number of OH-groups, using the position of the points of convergence into conditions extrapolated to very fast mobile phases. The behavior of trendline is close to that of the homologs (but with the addition of CH_3O-groups instead of CH_2 groups). Upon an increase in the hydrophilic component due to the number of OH-groups, the points of convergence shift to the right in line *7* of Fig. 2.

Effect of the Concentration of Formic Acid

As indicated above, acid was introduced into the mobile phase to convert all forms of anthocyanins into favylium form. According to literature data, however, only at pH ≤1 are all anthocyanins in solutions in this form. Such pH values lie outside the range of conventional reversed phase stability (pH 2–8) [11, 12], since 10 vol % of formic acid provides a pH of only ~1.3. This also lies outside the recommended range of pH for conventional stationary phases, but it is the concentration of acid used to separate anthocyanins in many works [3]. The frequent use of formic acid at

Table 1. Parameters of Eqs. (1) of the relative retention of anthocyanins for mobile phases based on acetonitrile acidified with two types of acids

No.	Anthocyanin	HCOOH		H_3PO_4	
		a	*b*	*a*	*b*
1	Dp3G	1.072	–0.566	1.139	–0.585
2	Cy3G	1.028	–0.260	1.053	–0.262
3	Pt3G	1.134	–0.126	1.129	–0.141
4	Pn3G	1	0	1	0
5	Pn3G	1.089	0.148	1.072	0.128
6	Mv3G	1.188	0.258	1.152	0.225

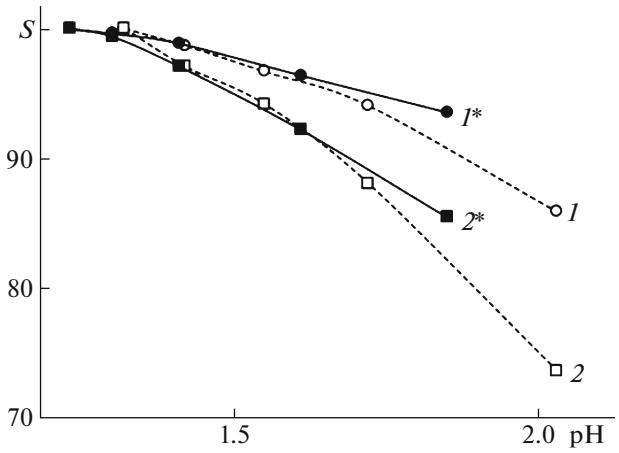


Fig. 3. Changes in the proportion of flavylium form upon a change in the pH (proportion of acids over peak areas) of the mobile phase for (*1*) Cy3G in eluents upon adding formic acid, (*1**) Cy3G in eluents upon adding phosphoric acid, (*2*) Mv3G in eluents upon adding formic acid, and (*2**) Mv3G in eluents upon adding phosphoric acid.

considerably lower concentrations in the composition of mobile phases was quite unexpected because, according to data obtained in our laboratory [7], this should broaden anthocyanin peaks and thus create problems in separating substances with similar chromatographic mobility. In this work, special attention was given to the effect the concentration of formic acid had on chromatographic processes.

Figure 3 presents data on the change in the proportion of the flavylium form of anthocyanins in the mobile phase, calculated from the change in the peak areas of identical portions of the sample upon a change in pH due to reducing the content of formic acid from 10 to 1 vol % (pH was determined for solutions with a fixed concentration of formic acid and no additions of acetonitrile). The proportion of flavylium forms fell for five of the 3-glucosides of the main anthocyanidins (no Pg3G was added in this series of experiments, due to problems in separating it from Pt3G), and not equally: the graphs for cyanidin-3-glucoside and malvidin-3-glucoside limit the set of the obtained data from above and below. At the same time, the fraction of the flavylium form of malvidin-3-glucoside fell most rapidly (up to 74%), which can be interpreted as a consequence of the higher apparent constant of hydration of the malvidin derivative. When comparing these results to literature data, we should remember that the relationship between the forms can be far from equilibrium for HPLC, since changes in the spectra of anthocyanins in solutions are usually observed within several hours. It should also be considered that the state of equilibria can differ in the stationary and the mobile phases, since it depends on the lipophilicity of the medium (the concentration of organic components) [13].

Table 2 shows the change in efficiency (in terms of the number of theoretical plates) of the chromatographic system upon a similar reduction in the concentration of formic acid in the mobile phase. Figure 2 shows data calculated using a formula not normally recommended:

$$N = 5.54\left(\frac{t_R(i)}{\Delta_{1/2}(i)}\right), \quad (2)$$

where 5.54 (8 ln 2) is a coefficient; $t_R(i)$ is the retention time of sorbate i; and $\Delta_{1/2}(i)$ is the peak width at half maximum. According to formula (2), which considers changes in sorbate retention factors $k(i)$,

$$N = 5.54\frac{k(i)}{k(i)+1}\left(\frac{t_R(i)}{\Delta_{1/2}(i)}\right). \quad (3)$$

It was shown in [14] that formula (3) is a true discrete solution to a discrete problem in the theoretical plates technique. Its use in HPLC is mandatory, since formula (2) is an asymptotically limiting formula for high values of the retention factor, and is usually applicable in gas chromatography.

The results from calculations using formula (3) are much closer to one another than those calculated with formula (2). The efficiency of the chromatographic system for all 3-glucosides in this case fell considerably upon reducing the concentration of formic acid in the mobile phase. The reduction was several times greater than the one in the peak areas, but the reasons for this require a special series of studies and are not considered in this work. Reducing the concentration of formic acid below 10–8 vol % is in any case difficult to recognize as a justified step in preparing the mobile phase.

Effect of the Speed of the Mobile Phase

The equivalent height of theoretical plate fell by approximately 1.5 times for all 3-glucosides upon reducing the mobile phase feed rate to 0.1–0.2 mL/min. However, this considerably increased the time required for one chromatogram. A rate of 0.8 mL/min was used as a compromise solution. The feed rate of the mobile phase could in some cases be made faster (if the degree of separation of neighboring anthocyanins was sufficient) or slower (if adjacent peaks partially overlapped).

Effect of Temperature on the of Efficiency Separation

It was established that the efficiency of the chromatographic system (in terms of the number of theoretical plates) fell by 9 (for Pn3G) to 20 (for Mv3G) percent upon lowering the temperature from 40 to 30°C. Raising the temperature contributed to an increase in this characteristic, but 40°C is a good compromise solution, considering the thermal instability of anthocyanins [15].

Table 2. Dependence of the efficiency of chromatographic systems (according to the number of theoretical plates) on the concentration of acids in the mobile phase

No.	Anthocyanin	Efficiency at the specified concentration of formic acid, vol %				
		10	7.75	5.5	3.25	1
1	Dp3G	4134	3648	2825	1817	737
2	Cy3G	6493	5802	4779	3361	1505
3	Pt3G	5685	4473	3070	1752	641
4	Pn3G	8048	6686	5054	3249	1300
5	Mv3G	5834	4337	2816	1652	572
No.	Anthocyanin	Efficiency at the specified concentration of phosphoric acid, vol %				
		2	1.5	1	0.5	0.25
1	Dp3G	4450	3926	3465	2494	1719
2	Cy3G	6920	6243	5712	4577	3248
3	Pt3G	6462	5490	4476	2814	1691
4	Pn3G	9010	7979	6807	4639	2967
5	Mv3G	6696	5188	3964	2141	1360

Result from Replacing Formic Acid with Phosphoric Acid

As was found in [5], the concentration of acetonitrile must be increased to achieve the same retention times upon substituting phosphoric acid for formic, since the eluting ability of phosphoric acid is approximately zero. As can be judged from the parameters of the equations of relative retention presented in Table 1, the six 3-glucosides elute in a slightly shorter range of retention times. Adding OH-groups to ring B increases retention slightly (compared to adding formic acid), while adding CH_3O-groups reduces it somewhat. The separating power of the mobile phase based on acetonitrile thus fell when phosphoric acid was substituted for formic, but to a relatively small extent.

The efficiency of the chromatographic system also fell upon an increase in pH (i.e., at a reduced concentration of phosphoric acid), as is seen Fig. 4 and

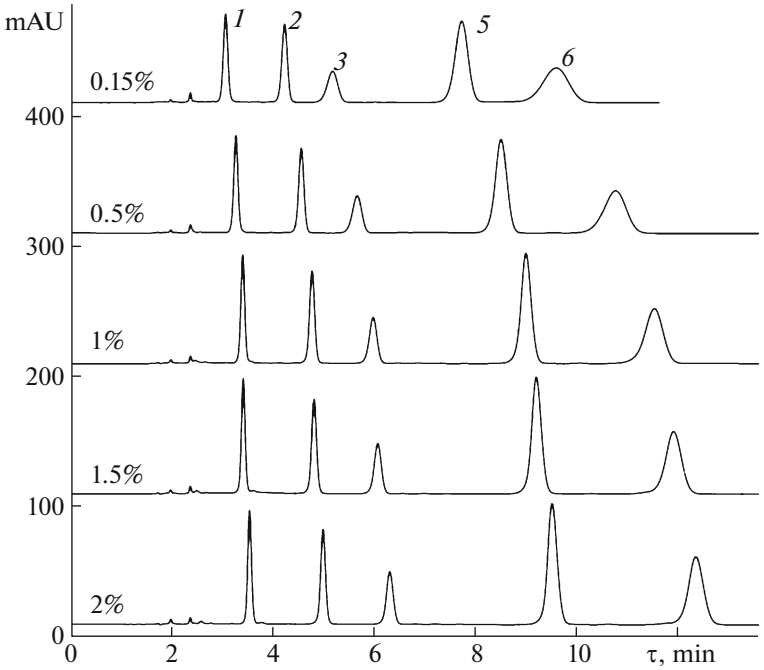


Fig. 4. Change in the width of the peaks of 3-glucosides of the five main anthocyanidins upon a change in the concentration of phosphoric acid (vol %) with an acetonitrile content of 12 vol %, 0.8 mL/min. Peaks are numbered as in Fig. 1.

Table 2. This is natural, due to the smaller proportion of the flavylium form upon such a substitution. However, while there are some differences in the behavior of anthocyanins in Fig. 3 and Table 2, no major changes were observed upon substituting phosphoric acid for formic. This substitution can therefore be considered a positive way of changing the composition of the mobile phase in the direction of "green" chromatography.

CONCLUSIONS

It was established that the correlations of the log-retention factor versus miLogP were satisfactory when OH-groups were added to ring B, but incorrect when CH_3O-groups were added to this ring. It was shown that the two-parameter system of relative retention describes the experimental results better when the hydrophilic–hydrophobic balance is considered. A drop in efficiency (in terms of the number of theoretical plates) upon reducing the concentration of acid was associated with a reduced fraction of the flavylium form of anthocyanins in the mobile phase, as was confirmed by a reduction in the areas of anthocyanin peaks. Lowering the concentration of formic acid below 10 vol % and that of phosphorus below 1 vol % thus led to a substantial loss of efficiency in the chromatographic system. It was established that the efficiency of the chromatographic system fell upon an increase in the number of substituents in the B ring of the flavylium moiety. It was shown that replacing the pungent and volatile formic acid with the more environmentally friendly phosphoric acid is acceptable in terms of chromatographic parameters.

FUNDING

This work was supported by the Russian Foundation for Basic Research, project no. 20-33-90031 "Postgraduates."

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