ISSN 0036-0244, Russian Journal of Physical Chemistry A, 2022, Vol. 96, No. 8, pp. 1768–1772. © Pleiades Publishing, Ltd., 2022.
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PHYSICAL CHEMISTRY OF SEPARATION PROCESSES: CHROMATOGRAPHY

Comparing Two Versions of a Separation Map in Reversed Phase Liquid Chromatography

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Received September 26, 2021; revised November 15, 2021; accepted February 15, 2022

Abstract—Two types of separation maps are compared for the first time, based on (1) the dependence of the logarithm of the retention factor on the volume fraction of the organic modifier and (2) the means of relative retention analysis. The two types of map agree with each other. Parameters of quadratic equations for the dependence of retention on the volume fraction of the organic modifier, extrapolated to the zero content of the organic modifier in the mobile phase, must be used in order to compare the energies of interaction between sorbates and sorbent. It is established that separation maps of the second type with linear trend lines are convenient for comparing the interaction energies of sorbates with organic modifiers of mobile phases. Additional possibilities of using this approach in special cases are shown. It is concluded that environmentally harmful acetonitrile can be replaced with more benign acetone to separate 3-glucosides of the main six anthocyanidins.

Keywords: reverse phase HPLC, two types of separation maps, consistency, physical meaning of parameters of approximation, green chromatography

DOI: 10.1134/S0036024422080076

INTRODUCTION

Reverse phase HPLC (RP HPLC) is today's most commonly used chromatographic procedure [1]. At the same time, its use is complicated by the existence of more than 600 brands of commercially available stationary phases, the properties of which depend on the technology of preparation with some differences even between columns of the same brand, but from different batches. There are thus problems in comparing these phases to determine the possibility of replacing or optimizing the selectivity of the separation of complex mixtures of sorbates.

Selectivity when separating a pair of compounds in chromatography refers to the ratio of adjusted times, volumes, or sorbate retention factors i and j [2]:

$$\alpha = \frac{V'_R(j)}{V'_R(i)} = \frac{t'_R(j)}{t'_R(i)} = \frac{k_R(j)}{k_R(i)}, \quad (1)$$

if

$$k_R(j) > k_R(i),$$

where α is selectivity (the separation factor); V'_R and t'_R are adjusted volumes or retention times; and $k_R(i)$ or $k_R(j)$ are the retention factors of components i and j, respectively.

Selectivity is difficult to consider as a reliable characteristic, since this parameter usually depends on the composition of the mobile phase, even for given sorbates and a given stationary phase. It is then not uncommon for a change in the ratio of the components of the mobile phase to invert the order of elution of some pairs of sorbates. It therefore usually makes no sense to compare the selectivity of the separation of sorbates on different stationary phases in one or a limited number of mobile phase compositions of even a chosen system. It is thus inaccurate to say that, e.g., anthocyanidin diglycosides are retained under conditions of RP HPLC more weakly than monoglycosides [3].

The current procedure based on linear ratios of the energy of solvation (LSER, [1]) in a wide range of mobile phase compositions is time consuming, so the differences between the experimental and calculated values are considerable for even trial series of sorbates [4].

On the other hand, the change in the retention of sorbates upon increasing the concentration of the organic modifier of the mobile phase in a narrow range of modifier concentrations is usually described by the linear Snyder equation [5]

$$\log k(i) = a_0(i) - a_1\varphi(\mathrm{OM}), \quad (2)$$

where is the logarithm of sorbate i retention factor depends linearly on φ (the volume fraction of the organic modifier (OM)), but $a_0(i)$ is an asymptotic

Table 1. Dependence of the retention of four anthocyanins on the composition of the mobile phase of the system CH_3CN–HCOOH (10 vol %)–water

No.	Sorbate	Parameters of Eq. (3)			R^2
		a_0	a_1	a_2	
1	Delphinidin-3-glucoside, Dp3G	1.3628	–0.1878	0.0028	0.99995
2	Delphinidin-3-rutinoside, Dp3R	1.6524	–0.2169	0.0035	0.99996
3	Cyanidin-3-glucoside, Cy3G	1.6365	–0.1942	0.0036	0.99995
4	Cyanidin-3-rutinoside, Cy3R	1.9659	–0.2258	0.0044	0.99996

characteristic—the logarithm of the sorbate retention factor in mobile phases with $\varphi = 0$. In a wide range of mobile phase compositions, however, the experimental data are better described by the quadratic equation [6]

$$\log k(i) = a_0 - a_1\varphi(\mathrm{OM}) + a_2\varphi^2(\mathrm{OM}). \quad (3)$$

The parameters of Eq. (3) for four anthocyanins are given in Table 1.

On the other hand, according to the displacement model in [7], n moles of organic modifier are released according to the equation

$$\log k(i) = a_i - n(i)\log c(\mathrm{OM}), \quad (4)$$

during the sorption of the sorbate on the stationary phase in a certain range of compositions of the mobile phase.

The same equation describes the retention of the substance used as a reference:

$$\log k(\mathrm{R}) = a_\mathrm{R} - n(\mathrm{R})\log c(\mathrm{OM}). \quad (5)$$

Excluding the concentration of OM from Eqs. (4) and (5), we obtain the equation of relative retention:

$$\log k(i) = \frac{n(i)}{n(\mathrm{R})}\log k(\mathrm{R}) + a_i - \frac{n(i)}{n(\mathrm{R})}a_\mathrm{R}, \quad (6)$$

$$\log k(i) = a_0 + a_1\log k(\mathrm{R}). \quad (7)$$

Although the numbers of released molecules of the organic modifier are not constant throughout the range of mobile phase compositions, experiments show their ratio a remains constant in a wide range of compositions.

In this work, we propose using separation maps that are a graphic representation of the dependence of the logarithms of the retention factors of a group of substances to be separated on the composition of the mobile phase (technique 1) or the retention of one of these substances (technique 2), determined for different compositions of the mobile phases of a given eluent system. The consistency of both types of mobile phases is determined for the first time, and a procedure is proposed for comparing the selectivity of a chosen stationary phase in different mobile phases.

EXPERIMENTAL

We used extracts of anthocyanins from a laboratory collection obtained by soaking plant materials in a 0.1 M aqueous solution of hydrochloric acid at room temperature away from direct sunlight. The extracts were stored in a plastic container inside a freezer. The anthocyanins were separated on an Agilent 1200 Infinity unit equipped with a diode array detector. Chromatograms were recorded at 520 nm. The chromatographic column was a 150 × 4.6 mm Symmetry C18 (3.5 μm) model, and the column oven temperature was 40°C. Dead time was determined using oxalic acid. Chromatograms were recorded and processed with the ChemStation program.

The anthocyanins were abbreviated as Dp (delphinidin (3,5,7,3',4',5'-hexahydroxoflavilium)), Cy (cyanidin (3,5,7,3',4'-pentahydroxoflavilium)), Pt (petunidin (3,5,7,3',4'-pentahydroxo-5'-methoxyflavylium)), Pn (peonidin (3,5,7,4'-tetrahydroxo-3'-methoxyflavilium)), and Mv (malvidin (3,5,7,4'-tetrahydroxo-3',5'-dimethoxyflavillium)). Glucosides were denoted by the letter G; rutinosides (rhamnosylglucosides), by the letter R.

RESULTS AND DISCUSSION

Processing our experimental data according to Eq. (3) allowed us to construct the first type of separation map for a given stationary phase and a chosen eluent system. Such a separation map is shown in Fig. 1 for four anthocyanins: 3-glucosides and 3-rutinosides (rhamnosylglucosides) delphinidin and cyanidin (Dp3G, Dp3R, Cy3G, and Cy3R) of black currant fruits.

An important feature of this technique of retention analysis is that sorbate retention can be extrapolated to $\varphi = 0$ in the mobile phase. However, the parameters obtained in this way are very conditional, since many C18 stationary phases are subject to phase collapse at low contents of the organic modifier [8]. Rigorous experimental verification of the results from extrapolation is thus by no means always possible. However, these values no longer depend on the concentration and type of the organic modifier—they depend only on the properties of the stationary phase itself. The

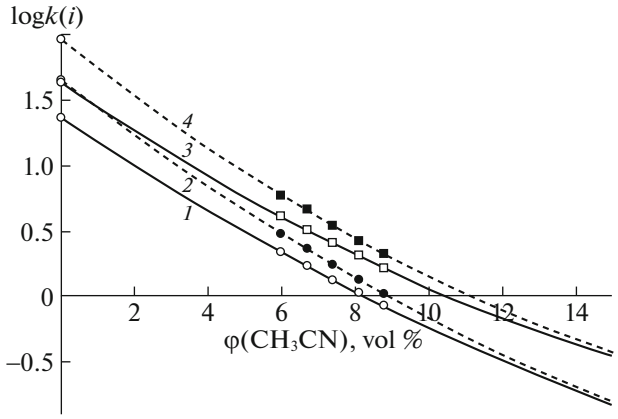

Fig. 1. First type of separation map based on the equation for the dependence of the logarithm of the retention factor on the volume fraction of the organic modifier. The substances are (*1*) Dp3g, (*2*) Dp3R, (*3*) Cy3G, and (*4*) Cy3R. The eluent system is CH_3CN–10 vol % HCOOH–water at 40°C.

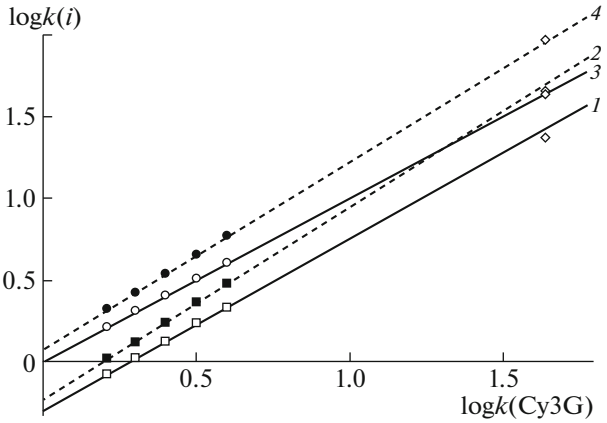

Fig. 2. Second type of separation map based on the equation for relative retention. The substances are (*1*) Dp3g, (*2*) Dp3R, (*3*) Cy3G, (*4*) Cy3R. The eluent system is CH_3CN–10 vol % HCOOH–water at 40°C.

important order of retention of a group of sorbates, which depends only on the interaction between the sorbate and the stationary phase (from an aqueous solution of an acidic modifier), must therefore be determined from the extrapolation logarithms of retention when there is no organic modifier in the mobile phase:

$$t_R(\text{Dp3G})_{aq} < t_R(\text{Cy3G})_{aq} < t_R(\text{Dp3R})_{aq} < t_R(\text{Cy3R})_{aq}.$$

It is this order that must be used to compare the properties of different stationary phases with respect to the selected sorbates. The type of the eluent system (i.e., the choice of the organic modifier when obtaining the initial data) no longer matters.

The change in this order in mobile phases containing an organic modifier (e.g., acetonitrile) depends on the energy of interaction between this sorbate and both the mobile and the stationary phase, which is also saturated with the organic modifier (acetonitrile). When acetonitrile is added to the mobile phase, the retention of both 3-rutinosides falls faster than for 3-glucosides, so the order of elution is quickly altered:

$$t_R(\text{Dp3G})_i < t_R(\text{Dp3R})_i < t_R(\text{Cy3G})_i < t_R(\text{Cy3R})_i.$$

It is this order of elution of these four sorbates that is usually observed in the compositions of mobile phases in almost all published works on separating anthocyanins from black currant fruits [9–14].

There can be several changes in the order of elution of two (or more) substances. To determine each one, we need only indicate the initial order and the dependence of the retention of each component on the composition of the mobile phase. The quadratic dependence according to Eq. (3) cannot be used to consider the second characteristic. It is better to map the separation of the second type according to Eq. (7), using sorbate Cy3G as a reference (Fig. 2).

Note that when constructing a selectivity map, we can ignore the accuracy of preparing the mobile phase (including the change of the batch of solvent). To build the map, we need only use two different compositions of the mobile phases, which greatly reduces the required time.

In addition to points constructed using the same experimental data as in the first type of separation map, the diamonds in the upper right part of Fig. 2 were plotted using extrapolation values a_0 found with Eq. (3). The proximity of these points to the trends according to Eq. (7) (which can also be estimated from the data given in Table 2) testifies to the agreement between the two types of separation maps.

At the same time, the second approach allows us to construct another separation map of the second type using two different samples: Cy3G for Cy3R and Dp3G for Dp3R (Fig. 3), in the same range of mobile phase compositions. This map shows that the change in the retention of 3-glucosides upon moving to 3-rutinosides (the rhamnosyl radical being attached to the existing glucosidic one) is described by close dependences for the two anthocyanidins (delphinidin and cyanidin). This is important when identifying peaks in chromatograms with a limited set of standard compounds. The dependence of the retention of 3-rutinosides on that of 3-glucosides of five anthocyanidins is also described by a linear dependence for the same composition of the mobile phase and the same stationary phase [15]. Finally, another way of using comparative diagrams for chromatograms recorded with the same composition of mobile phases is known as a

Table 2. Relative retention of three anthocyanins using cyanidin-3-glucoside as a reference substance in the mobile phase of the system CH_3CN–HCOOH (10 vol %)–water

No.	Sorbate	Parameters of Eq. (7)			a_0^*/a_0^{**}
		a_0	a_1	R^2	
1	Delphinidin-3-glucoside, Dp3G	–0.294	1.017	0.99996	1.363/1.419
2	Delphinidin-3-rutinoside, Dp3R	–0.229	1.175	0.99998	1.652/1.694
3	Cyanidin-3-glucoside, Cy3G	0	1	—	1.637/1.637
4	Cyanidin-3-rutinoside, Cy3R	0.081	1.144	1.00000	1.966/1.953

a_0^* from Eq. (3); a_0^{**} calculated according to Eq. (7).

direct transfer of the technique of similar series proposed by M.Kh. Karapetyanets on the retention of the same type of derivatives of different bases (e.g., anthocyanidins) [16].

Let us use the proposed separation maps to compare the retention of isomeric 3-glucosides of six major anthocyanidins (delphinidin, cyanidin, petinidin, pelargonidin, peonidin, and malvidin) under conditions of reverse phase HPLC. Figure 4 shows the separation of these anthocyanins on a Symmetry™ C18 column in two eluent systems—one based on traditional acetonitrile and another in which acetonitrile is replaced with green (i.e., more environmentally friendly) acetone by acidifying the mobile phases with formic acid (10 vol %).

With the linear approximation in this figure for the same anthocyanins in different eluent systems, these lines tend to intersect on the right side of the separation map. The lines for malvidin and peonidin intersect at points with the same abscissa, which can be interpreted as obtaining compositions with zero organic modifier content with equivalent properties of the mobile phases of both eluent systems. For these substances and Pg3G, the coordinates of the points of intersection coincide with those obtained using Eq. (3) in the eluents of the systems acetone–10 vol % formic acid–water and acetonitrile–10 vol % formic acid–water, as proposed above. The order of the energy of interaction between anthocyanins and the C18 phase thus becomes the same in both eluent systems, since to the concentrations of organic modifiers for the mobile phases are in this case zero:

$$t_R(\text{Dp3G})_{aq} < t_R(\text{Cy3G})_{aq} < t_R(\text{Pg3G})_{aq} < t_R(\text{Pt3G})_{aq} < t_R(\text{Pn3G})_{aq} < t_R(\text{Mv3G})_{aq}$$

and this order in real compositions of mobile phases contains only one inversion:

$$t_R(\text{Dp3G}) < t_R(\text{Cy3G}) < t_R(\text{Pt3G}) < t_R(\text{Pg3G}) < t_R(\text{Pn3G}) < t_R(\text{Mv3G}).$$

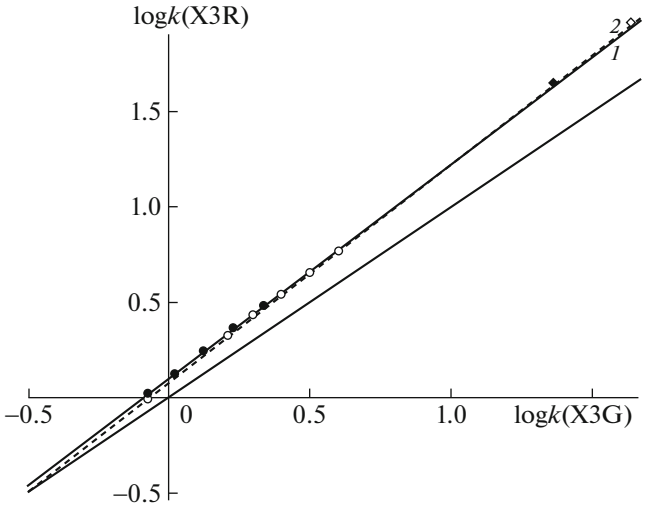

Fig. 3. Correlations between the retention values of the 3-rutinosides and 3-glucosides of delphinidin and cyanidin for the eluent system CH_3CN–10 vol % HCOOH–water. The lines are for (*1*) Dp3R vs. Dp3g and (*2*) Cy3R vs. Cy3G.

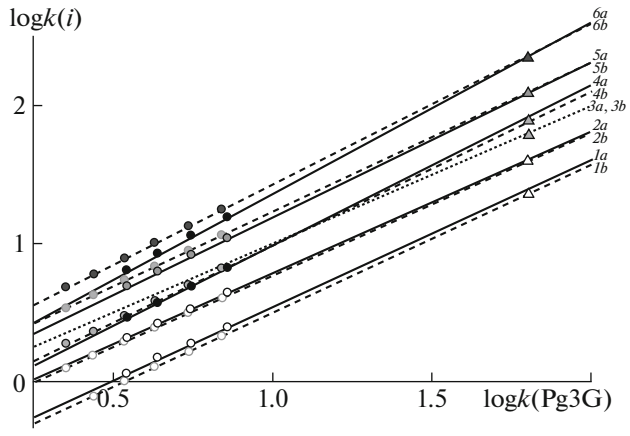

Fig. 4. Separation map of the second type for the 3-glucosides of the main natural anthocyanidins in two eluent systems: CH_3COCH_3–10 vol % HCOOH–water (with indices *a*) and CH_3CN–10 vol % HCOOH–water (with indices *b*). The substances are (*1*) Dp3G, (*2*) Cy3G, (*3*) Pg3G, (*4*) Pt3G, (*5*) Pn3G, and (*6*) Mv3G.

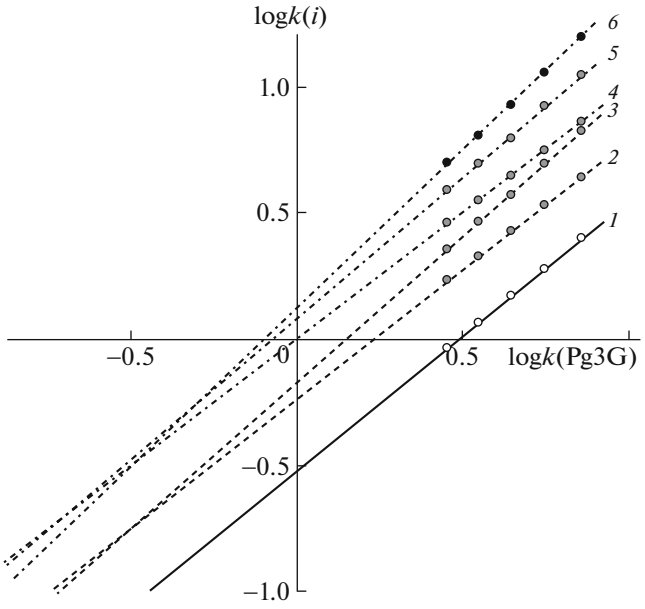

Fig. 5. Grouping of trend lines according to the number of hydroxyl groups in the structure of anthocyanins. The eluent system is CH_3COCH_3–10 vol % HCOOH–water. See Fig. 4 for the numbering of sorbates.

Finally, when extrapolating anthocyanin retention to mobile phases with strong elution (moving to the left on the separation map), the lines of anthocyanin approximation are grouped according to the number of OH groups in the aglycone in acetone-based eluents (Fig. 5), just as they are in acetonitrile-based eluents [17].

FUNDING

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

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