Research Article

Regularities of Anthocyanins Retention in RP HPLC for “Water–Acetonitrile–Phosphoric Acid” Mobile Phases

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The influence of exchange of HCOOH (System 2) by phosphoric acid (System 1) for acidification of the "acetonitrile–water" mobile phases for reversed-phase HPLC of anthocyanins was investigated in the framework of relative retention analysis. The differences and similarities of anthocyanins separation were revealed. It has been shown that some common features of the quantitative relationships may be used for preliminary anthocyanins structure differentiation, according to the number of OH-groups in anthocyanidin backbone as well as to a number of saccharide molecules in glycoside radicals in position 3 of the anthocyanin without MS detection.

1. Introduction

Anthocyanins are powerful water-soluble antioxidants of flavonoids class with health promoting effect [1, 2]. The coloured flavylium form of anthocyanins is a reason to regard them as natural food colorants [2]. The latter explains a high scientific and technological interest to the substances. Anthocyanins are synthesized in plant tissue, mainly in fruits, flowers and for some species in leaves as a rule as a complex mixture of compounds with different structures [3]. Anthocyanins are glycosides of anthocyanidins (Figure 1), with great varieties of more than 600 anthocyanin structures found in plant sources [4] though only six structures of the latter cover the majority of the structures due to glycosylation type variability [5].

Reversed-phase HPLC is a common method for analysis of complex mixtures of plant anthocyanins [6, 7]. The specificity of the method is usage of rather strong acidic mixtures of water and organic water-miscible solvent. Acidification is necessary to transfer the substances into charged and coloured flavylium form, due to the fact that anthocyanins may be easily detected at the presence of large amounts of other colourless substances.

The retention of substances in HPLC depends upon type and even trademark of stationary reversed phase, composition of mobile phase, and temperature as well as solute structure. Mobile phases of water mixtures with acetonitrile or methanol are acidified with HCOOH [8], acetic [9], phosphoric [10], and trifluoroacetic [11] acids as well as the mixtures (without any explanation) of some of them [12–14] for anthocyanins separation. However, as far as we know only "HCOOH–acetonitrile–water" mobile phases were investigated extensively to elucidate the regularities of anthocyanins retention [8, 15–17]. According to Snyder’s selectivity triangular water, acetonitrile and HCOOH are solvents of different groups, VIII, VIb, and IV correspondingly [18], so the withdrawal or exchange of some solvents may lead to alteration of solutes separation selectivity.

The aim of the present paper is the investigation of regularities of anthocyanins retention in RP HPLC with mobile phases composed of water, acetonitrile and phosphoric acid and comparing that with retention in the more commonly used mobile phases being water-acetonitrile mixtures acidified with HCOOH (10 vol. %).

2. Experimental

2.1. Chemicals and Reagents. All anthocyanins under investigation were extracted from the plant sources and isolated...
The isolation of individual anthocyanins was performed by Shimadzu equipment LC-20 with spectrophotometric detection on chromatographic column 10 × 250 mm SUPELCOSIL C18 (5 mcm) in eluents of water–acetonitrile, 10 vol. % HCOOH system.

2.4. Analytical HPLC. The fractions of semipreparative separations were controlled and chromatographic behaviour of anthocyanins was investigated with utilisation of Agilent Infinity 1200 equipment with diode array (DAD) and MS (6130 Quadrupole LC/MS) detectors. Chromatographic column: 4.6 × 250 mm Symmetry C18 in two mobile phase systems.
3.2. Solutes with Different Anthocyanidin Structures and the Same Glycosylation Type. The separation map in the framework of relative retention analysis [17] with pelargonidin-3-glucoside as a reference solute is presented in Figure 3. Each point on the plot has coordinates \( x \), logarithm of capacity factor of Pg3Glu and \( y \), that for corresponding solute in the same mobile phase. Points for the same solute and different mobile phase compositions settle down on straight lines according to equation of relative retention (3) (Table 1):

\[
\log k(i) = a \cdot \log k(Pg3Glu) + b.
\]  

Pg3Glu was taken as a reference solute for the simplest ring B structure. Parameters of (3) are the valuable characteristics of corresponding solutes. For example, addition of OH-group into position 3' of ring B (for a transfer from Pg3Glu to Cy3Glu) leads not only to decrease of retention (and parameter \( b \)) because of solute hydrophilicity increase but also to increase of parameter \( a \) as a consequence of additive van der Waals interactions of these O and H atoms with stationary phase atoms. It is easy to see that addition of OH- and CH\(_3\)O-groups to positions 3' and 5' of ring B leads to close to additive increase of capacity factor logarithm. This property is true for the same solute retention in System 2 [15]; thus the sequence of anthocyanins’ elution on the chromatograms of mixtures of 3-glucosides delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin for both systems remains unchanged (Figure 4). Meanwhile the exchange of phosphoric acid by HCOOH one results in slight selectivity alterations: some decrease of relative retention for OH-substitutions and an increase of that for OCH\(_3\)-substitutions are evident (Figure 5).

Thus, points for Mv3Glu and Pn3Glu for System 2 settle down above the lines for the same anthocyanins for System 1, while the opposite case is found for relative retention of Dp3Glu and Cy3Glu. Accordingly, System 2 has somewhat higher selectivity for separation of substances with different flavylum ions hydrophilicity.

The lines of relative retention approximated to the region of zero points [21] (left down corner of the plot on Figure 3) are differentiated according to the number of OH-groups.
Table 1: Chromatographic characteristics of 3 glucosides of six common anthocyanidins.

| N  | Solute        | Parameters of (3) | R² | λ<sub>max</sub><sup>1</sup>, nm | m/z    |
|----|---------------|-------------------|----|-------------------------------|--------|
| 1  | Dp3Glu        | 1.123 ± 0.010     | −0.618 | 0.99995                      | 523.0  | 465.1; 287.0                  |
| 2  | Cy3Glu        | 1.057 ± 0.008     | −0.273 | 0.99998                      | 515.0  | 449.1; 287.0                  |
| 3  | Pt3Glu        | 1.139 ± 0.011     | −0.146 | 0.99997                      | 524.0  | 479.2; 317.0                  |
| 4  | Pg3Glu        | 1                | 0     | —                            | 500.5  | 433.2; 271.0                  |
| 5  | Pn3Glu        | 1.070 ± 0.010     | 0.136  | 0.99997                      | 515.0  | 463.1; 301.0                  |
| 6  | Mv3Glu        | 1.150 ± 0.010     | 0.236  | 0.9999                       | 525.0  | 493.1; 331.0                  |

<sup>1</sup>Mobile phase: 10 vol.% CH<sub>3</sub>CN and 0.5 vol.% H<sub>3</sub>PO<sub>4</sub>.

3.3. Solutes with the Same Anthocyanidin Structure and Different Glycosylation Type. The separation map of relative retention of some cyanidin-3-glycosides with Cy3Glu as a reference solute in System 1 is presented in Figure 6. It becomes evident that not only the absolute retention but also relative retention of different cyanidin-3-glycosides depends not only upon solute structure, but also upon mobile phase composition; coelution of some solute pairs including the reversal of the elution order may occur after alteration of component concentrations in the mobile phase of the same eluent system and stationary phase.

By the way the drawing of the resolution map may escape mistakes connected with estimation of number of solutes and choice of appropriate mobile phase composition for complex mixtures analysis by HPLC methods.

In the case of System 1 parameter <sup>a</sup> of (3) may be utilised for preliminary estimation of complexity of glycoside structure in position 3 of anthocyanidin backbone: <sup>a</sup> has a value in the region 1.000 ± 0.020 for monoglycosides (Cy3Gala and Cy3Ara), 1.122 ± 0.011 for diglycosides (Cy3Sopho, Cy3Sam, Cy3AGlu, and Cy3Rut), and 1.300 ± 0.030 for trisaccharides (Cy3GRut, Cy3XRut) (Table 2). The values are close to that reported for retention of cyanidin-3-glycosides in RP HPLC in solvent System 2 [17, 23] proving the property to be a common regularity of the solute chromatographic behaviour at least for the systems under investigation.

Finely, the exchange of HCOOH by phosphoric acid also leads to slight decrease of relative retention of di- and trisaccharides, Figure 7, but no significant alterations of solutes separation selectivity were found.
Table 2: Parameters of relative retention (3) of some cyanidin-3-glycosides.

| Solutes                  | System 1 |   | System 2 [23] |   | m/z     |
|--------------------------|----------|---|--------------|---|---------|
|                          | a        | b | $R^2$        | a | b       |
| Cyanidin-3-monoglycosides|          |   |              |   |         |
| Galactoside, Cy3Gala     | 0.983 ± 0.012 | -0.122 | 0.9998 | 0.976 | -0.135 | 449.1; 287.0 |
| Glucoside, Cy3Glu        | 1        | 0 | —            | 1 | 0       | 449.1; 287.0 |
| Arabinoside, Cy3Ara      | 0.941 ± 0.011 | 0.140 | 0.9998 | 0.932 | 0.141 | 419.1; 287.0 |
| Cyanidin-3-diglycosides  |          |   |              |   |         |
| Glucosylglucoside, Cy3Sopho | 1.130 ± 0.012 | -0.251 | 0.9997 | —   | —       | 611.1; 287.0 |
| Xylosylglucoside, Cy3Sam | 1.112 ± 0.008 | -0.063 | 0.99997 | 1.148 | -0.066 | 581.1; 287.0 |
| Rhamnosylglucoside, Cy3Rut | 1.133 ± 0.007 | 0.084 | 0.99999 | 1.131 | 0.099  | 595.2; 287.0 |
| Arabinosylglucoside, Cy3AGlu | 1.126 ± 0.010 | -0.137 | 0.99995 | 1.138 | -0.110 | 581.1; 287.0 |
| Cyanidin-3-triglycosides |          |   |              |   |         |
| Glucosylrutinoside, Cy3GRut | 1.313 ± 0.013 | -0.221 | 0.9997 | 1.308 | -0.235 | 757.2; 287.0 |
| Xylosylrutinoside, Cy3XRut | 1.273 ± 0.008 | -0.036 | 0.99996 | 1.285 | -0.057 | 727.2; 287.0 |

4. Conclusions

The exchange of HCOOH (System 1) for phosphoric acid (System 2) leads to substantial increase of anthocyanins retention in mobile phases, acidified mixtures of water and acetonitrile.

Selectivity of resolution of the same glycosides of six common anthocyanidins is only slightly greater in System 2, by the way, though the sequence of elution remains the same for reasonable solutes retention times.

Analysis of anthocyanins relative retentions on the separation map (in the region of zero points) may be explored for estimation of the number of OH-groups in anthocyanidin backbone.

Selectivity of resolution of the different glycosides of the same anthocyanidin (an example of the most common natural aglycone, cyanidin) is also close to that for System 1.

But the advantage of relative retention analysis is a sensitivity to structure of carbohydrate radicals; parameter $b$ for mono-, di-, and trisaccharides is differing enough permitting determination of complexity of glycosyl radical in the 3 positions of anthocyanidin without utilization of MS detection. Moreover, parameter $a$ is highly sensitive to sugar isomers structures.

Thus, eluent systems under investigation have close properties, though System 2 seems to be somewhat more efficient.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] G. Mazza, “Anthocyanins and heart health,” *Annali dell’Istituto Superiore di Sanità*, vol. 43, no. 4, pp. 369–374, 2007.
[2] J. He and M. Monica Giusti, “Anthocyanins: natural colorants with health-promoting properties,” *Annual Review of Food Science and Technology*, vol. 1, no. 1, pp. 163–187, 2010.
[3] S. Deroles, “Anthocyanin biosynthesis in plant cell cultures: a potential source of natural colourants,” in *Anthocyanins. Biosynthesis, Functions, and Applications*, K. Gould, K. M. Davies, and C. Winefield, Eds., New York, NY, USA, pp. 107–168, Springer Science+Business Media, LLC, 2009.
[4] I. Konczak and W. Zhang, “Anthocyanins—more than nature’s colours,” *Journal of Biomedicine and Biotechnology*, vol. 2004, no. 5, pp. 239–240, 2004.
[5] F. Delgado-Vargas, A. R. Jiménez, and O. Paredes-López, “Natural pigments: carotenoids, anthocyanins, and betalains—characteristics, biosynthesis, processing, and stability,” *Critical Reviews in Food Science and Nutrition*, vol. 40, no. 3, pp. 173–289, 2000.
[6] M. M. Giusti and P. Jing, “Analysis of anthocyanins,” in *Food Colorants: Chemical and Functional Properties*, C. Socaciu, Ed.,
Section 6, Analysis of Pigments and Colorants, pp. 479–506, CRC Press, Taylor & Francis, New York, NY, USA, 2008.

[7] C. T. da Costa, D. Horton, and S. A. Margolis, “Analysis of anthocyanins in foods by liquid chromatography, liquid chromatography—mass spectrometry and capillary electrophoresis,” *Journal of Chromatography A*, vol. 881, no. 1-2, pp. 403–410, 2000.

[8] J.-P. Goiffon, M. Brun, and M.-J. Bourrier, “High-performance liquid chromatography of red fruit anthocyanins,” *Journal of Chromatography*, vol. 537, no. 1-2, pp. 101–121, 1991.

[9] H. Oroumi and N. Hassibi, “Study the correlation between some climate parameters and the content of phenolic compounds in roots of *Glycyrrhiza glabra*,” *Journal of Medicinal Plant Research*, vol. 5, no. 25, pp. 6011–6016, 2011.

[10] E. É. Nicoü, S. Savard, and K. Belkacemi, “Anthocyanins in wild blueberries of Quebec: extraction and identification,” *Journal of Agricultural and Food Chemistry*, vol. 55, no. 14, pp. 5626–5635, 2007.

[11] C.-G. Qin, Y. Li, W. Niu, Y. Ding, X. Shang, and C. Xu, “Composition analysis and structural identification of anthocyanins in fruit of waxberry,” *Czech Journal of Food Sciences*, vol. 29, no. 2, pp. 171–180, 2011.

[12] J. Lee, C. Rennaker, and R. E. Wrolstad, “Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods,” *Food Chemistry*, vol. 110, no. 3, pp. 782–786, 2008.

[13] K. R. Markham, K. S. Gould, C. S. Winefield, K. A. Mitchell, S. J. Bloor, and M. R. Boase, “Anthocyanic vacuolar inclusions—their nature and significance in flower colouration,” *Phytochemistry*, vol. 55, no. 4, pp. 327–336, 2000.

[14] R.-Z. Yang, X.-L. Wei, F.-F. Gao et al., “Simultaneous analysis of anthocyanins and flavonols in petals of lotus (*Nelumbo*) cultivars by high-performance liquid chromatography-photodiode array detection/electrospray ionization mass spectrometry,” *Journal of Chromatography A*, vol. 1216, no. 1, pp. 106–112, 2009.

[15] J.-P. Goiffon, P. P. Moully, and E. M. Gaydou, “Anthocyanic pigment determination in red fruit juices, concentrated juices and syrups using liquid chromatography,” *Analytica Chimica Acta*, vol. 382, no. 1-2, pp. 39–50, 1999.

[16] V. I. Deineka and A. M. Grigor’ev, “Determination of anthocyanins by high-performance liquid chromatography: regularities of retention,” *Journal of Analytical Chemistry*, vol. 59, no. 3, pp. 270–274, 2004.

[17] V. I. Deineka and A. M. Grigor’ev, “Relative analysis of the chromatographic retention of cyanidin glycosides,” *Russian Journal of Physical Chemistry A*, vol. 78, no. 5, pp. 796–799, 2004.

[18] B. Spangenberg, C. F. Poole, and C. Weins, *Quantitative Thin-Layer Chromatography. A Practical Survey*, Springer, New York, NY, USA, 2011.

[19] A. de Villiers, D. Cabooter, F. Lynen, G. Desmet, and P. Sandra, “High performance liquid chromatography analysis of wine anthocyanins revisited: Effect of particle size and temperature,” *Journal of Chromatography A*, vol. 1216, no. 15, pp. 3270–3279, 2009.

[20] K. Valko, L. R. Snyder, and J. L. Glach, “Retention in reversed-phase liquid chromatography as a function of mobile-phase composition,” *Journal of Chromatography A*, vol. 656, no. 1-2, pp. 501–520, 1993.

[21] V. I. Deineka, “Relative retention analysis in HPLC: the correlation between incremental relationships,” *Russian Journal of Physical Chemistry A*, vol. 80, no. 4, pp. 605–608, 2006.