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RP HPLC determination of some uncommon anthocyanins

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Abstract

Anthocyanins of *Catharanthus roseus* petals were found to be composed of pairs (3-galactosides and 3-rhamnosylgalactosides) mainly of 7-methylated anthocyanidins of "dephinidin series" (7-methyldelphinidin, 7-methylpetunidin and hirsutidin); though the same derivatives of "cyanidin series" are present in low concentrations. Flowers of *Caesalpinia pulcherrima* contain two 3-glucosides: of cyanidin and 5-methylcyanidin. The migration of CH_3-radical from 3'-OH group to 7-OH position results in increase of retention, while for the migration to 5-OH group a decrease of retention was observed. Different position of ring A OH-groups methylation can be predicted also by controversial shift of λ_{max} of the solutes.

Keywords: Reversed-phase HPLC, uncommon anthocyanins, 7-OH methylation; 5-OH methylation; regularities of retention alteration, electronic spectra.

Установлено, что антоцианы лепестков цветков *Catharanthus roseus* образованы парами (3-галактозидами и 3-рамнозилгалактазидами) в-основном антоцианидинами дельфинидинового ряда с метилированием ОН-группы в положении 7: 7-метилдельфинидином, 7-метилпетунидином и 7-метилмальвидином (хирсутидином); впрочем, аналогичные производные антоцианов цианидинового ряда также присутствуют, но в небольших концентрациях. Антоцианы высушенных цветков *Caesalpiniapulcherrima* содержат два 3-глюкозида: цианидина и 5-метилцианидина. Перемещение CH_3-радикала из 3'-ОН группы к 7-ОН группе сказывается в заметном увеличении удерживания, но при аналогичном переносе на ОН-группу в положение 5 наблюдается уменьшение удерживания. Различный тип метилирования ОН-групп кольца А приводит к противоположным смещениям λ_{max} антоцианов.

Ключевые слова: ОФ ВЭЖХ, необычные антоцианы, 7-ОН метилирование; 5-ОН метилирование; закономерности в изменении удерживания, электронные спектры

Introduction

Anthocyanins are prominent natural colorants with a powerful antioxidant activity among all main water-soluble antioxidants. Anthocyanins are found in the cell vacuole, mostly in flowers and fruits but also in leaves, stems, and roots. In these parts, they are found predominantly in outer cell layers such as the epidermis and peripheral mesophyll cells. These compounds are synthesized almost exclusively in the form of glycosides, where sugar moiety of a different complexity is attached to a base structure – anthocyanidin. Anthocyanins may be utilised as chemosystematics markers, but the diversity of compounds exceeding 500 different structures is confined mainly to the abundance of sugar moiety (or moieties) composition. Only six anthocyanidin structures

are responsible for the majority of anthocyanin complexes of common plant sources. These are delphinidin (Dp), petunidin (Pt) and malvidin (Mv) - the “delphinidin series” of solutes with increasing degree of circle B OH-groups methylation; cyanidin (Cy) and peonidin (Pn) - the “cyanidin series” of solutes with also increasing degree of circle B OH-groups methylation; pelargonidin (Pg) – without OH in B circle to be methylated. It should be mentioned that methylation of 4’-OH group does not occur because of easy transformation of flavilium form to quinoidal one [1], Fig.1.

The scheme below explains also the reason of rare occurrence of anthocyanins with substitution of 7-OH group as well as instability of bonding of any moieties to 5-OH group. Meanwhile some other anthocyanidins are known to be the base for rare anthocyanins of some plants [2], Table 1.

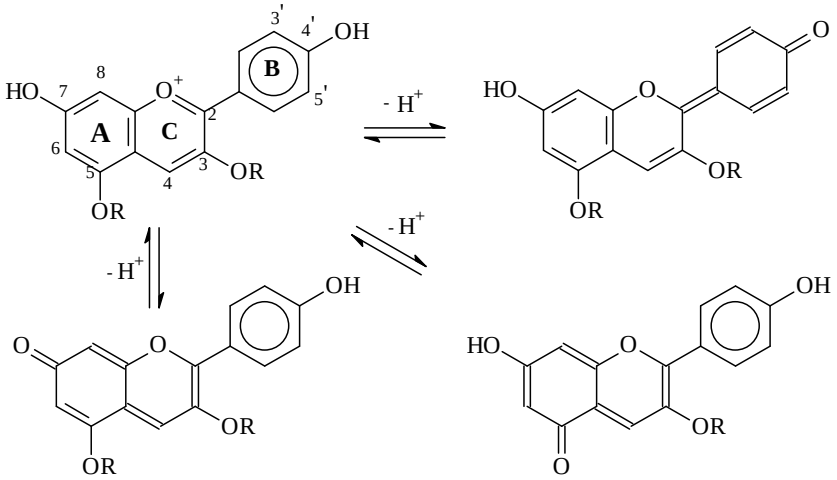


Fig. 1. Flavilium ion – quinoidal bases routs of transformation

Table 1. List of known antocyanidins

Anthocyanidin	Substitution pattern						
	3	5	6	7	3’	4’	5’
Common structures							
Pelargonidin, Pg	OH	OH	H	OH	H	OH	H
Cyanidin, Cy	OH	OH	H	OH	OH	OH	H
Peonidin, Pn	OH	OH	H	OH	OCH_3	OH	H
Delphinidin, Dp	OH	OH	H	OH	OH	OH	OH
Petunidin, Pt	OH	OH	H	OH	OCH_3	OH	OH
Malvidin, Mv	OH	OH	H	OH	OCH_3	OH	OCH_3
Rarer structures							
Aurantinidin	OH	OH	OH	OH	H	OH	H
6-Hydroxycyanidin	OH	OH	OH	OH	OH	OH	H
Rosinidin	OH	OH	H	OCH_3	OCH_3	OH	H
Pulchellidin	OH	OCH_3	H	OH	OH	OH	OH
Europinidin	OH	OCH_3	H	OH	OCH_3	OH	OH
Hirsutidin	OH	OH	H	OCH_3	OCH_3	OH	OCH_3
Capensinidin	OH	OCH_3	H	OH	OCH_3	OH	OCH_3
Apigenidin	H	OH	H	OH	H	OH	H
Luteolinidin	H	OH	H	OH	OH	OH	H
Tricetinidin	H	OH	H	OH	OH	OH	OH

A lot of published investigations of anthocyanin complexes of different origin included the separation of individual compounds by chromatographic methods. But the

particularities of solute retention in the most of papers are out of attention. It has led to mistakes in peaks assignment [3]. Meanwhile the anthocyanins retention is surprisingly sensitive to glycosylation type [4] depending upon the type of sugar moiety as well as upon the position of attachment. Thus, the consideration of anthocyanins retention regularities is a powerful alternative method for a solute structure definition.

The aim of a present investigation was to verify some anthocyanins complexes based upon uncommon anthocyanidins and to find regularities of relative retention [5].

Experimental

Catharanthus roseus was grown up in laboratory from seeds; fruits of mango were acquired in the nearest shop, while *Caesalpinia pulcherrima*dried flowers (of orange and pink colours) were taken from Vietman. For extraction of anthocyanins the plant material was macerated in 0.1M water HCl solution for 3-6 hours, after filtering extract was separated from co-extractable substances by solid-phase extraction on DIAPAC C18 (BioCheMac ST, Moscow) cartridges.

HPLC investigation was performed of Agilent 1200 Infinity series equipped by two detectors: DAD and MS. Two chromatographic columns were explored - 250×4.6 mm Symmetry®C18 (5 mcm) for retention investigation, and 150×2.1 mm Kromasil 100-5C18 – for MS spectra registration. Mobile phases were composed of distilled deionized water (UPVA 5, Belgorod, RF), formic acid (China), acetonitrile (HPLC gradient grade). Mobile phase flow velocity – 1 ml/min for the first column and 0.15 ml/min for MS spectra registration. All chromatographic runs were performed at column thermostat temperature 40^{o}C.

Results and discussion

According to the two published opposite results of *Catharanthus roseus* petals anthocyanins investigation the main compounds may be:

1) 3-*O*-glucosides and 3-*O*-(6-*O*-*p*-coumaroyl) glucosides of hirsutidin, malvidin and petunidin [6];

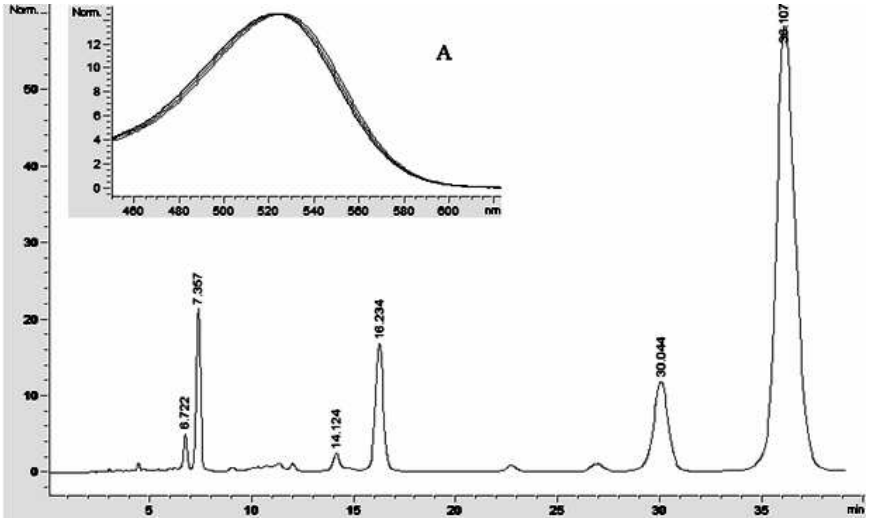
2) rosinidin-3-*O*-[6-*O*-(α-rhamnopyranosyl)-β-galactopyranoside], and also 7-O-methylcyanidin 3-*O*-[6-*O*-(α-rhamnopyranosyl)-β-galactopyranoside] [7].

Separation of *C. roseus* petals anthocyanins in present investigation is presented on Fig.2. According to combination of MS spectra, times of retention and electronic spectra the compounds are rhamnosylhexosides of uncommon anthocyanidins, Table 2.

Table 2. Parameters of *Catharanthus roseus* petals anthocyanins

N[a]	Retention time, t_R[b]	Peak assignment	λ_{max}, nm	MS spectra[c], m/Z
1	6.72	7MeDp3Gala	521.0	-
2	7.36	7MeDp3RhamnGala	522.5	317.1; 625.2
3	14.12	7MePt3Gala	522.5	-
4	16.23	7MePt3 RhamnGala	524.2	331.1; 639.2
5	30.04	7MeMv3Gala	524.0	345.1; 507.2
6	36.11	7MeMv3RhamnGala	526.0	345.1; 653.2
	9.087	5MeCy3Glu[d]	517.5	301.0; 463.1

[a] - number of peak on Fig.1; [b] - for mobile phase 8 vol.% of formic acid and 10 vol.% of acetonitrile in water, 1 ml/min;[c] - fragmentor voltage 200V, [d] - from *Caesalpiniapulcherrima petals*.



Column 250×4.6 mm Symmetry C18, 5 mcm; mobile phase: 10 vol.% of HCOOH and 8 vol.% of CH_3CN, 1 ml/min; detection at 515 nm

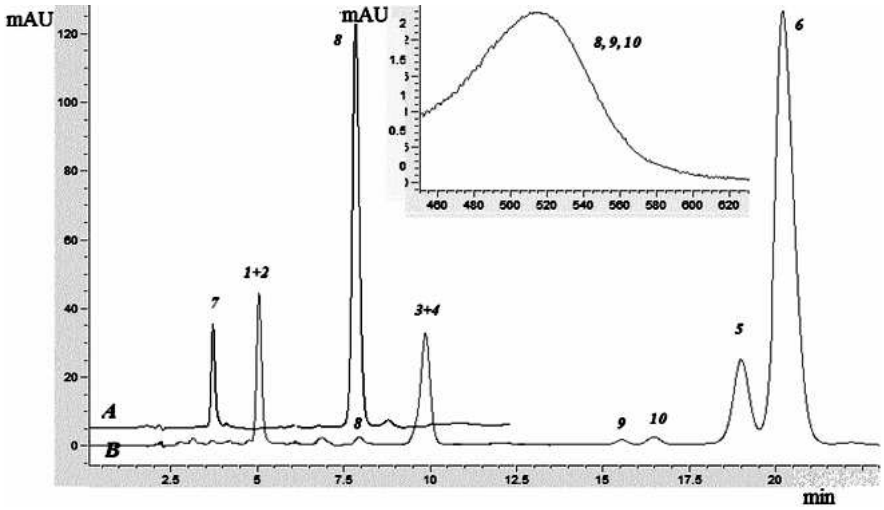
Fig. 2. Separation of *Catharantus roseus* petal anthocyanins

The consecutive bathochromic shift of λ_{max} for peak series N2, N4 and N6 (Fig.2A, Table 2) is characteristic for "delphinidin series" of anthocyanins. Accordingly, the most probable structures of the anthocyanin aglycones should be the same derivatives of delphinidin, petunidin and malvidin with unusual methylation type. The type of methylation could not be determined by available for us methods, but our results are in a full agreement with conclusions of paper [7], where 7-methylation of the anthocyanidins was confirmed by NMR. Thus, the anthocyanins under investigation may be derivatives of 7-methyldelphinidin (7MeDp), 7-methylpetunidin (7MePt) and 7-methylmalvidin (7MeMv). The series of anthocyanins for the same plant petals proposed in paper [6] seems to be scarily possible as well as a proposed 6"-coumaroyl acylation of 3-glucoside moiety. Indeed, MS spectra of 3-rhamnosylgalactisides (as well as 3-rhamnosylglucosides) and 3(6"-coumaroylglucosides) of the anthocyanidins are not distinguishable, but the characteristic for *p*-coumaric acid band (at 290-310 nm) was absent in electronic spectra of substances N2, N4 and N6.

Unfortunately, the methods explored in present paper also do not let tounambiguously differentiate between 3-rhamnosylgalactosides (proposed as a sugar moieties in [7]) and 3-rhamnosylglucosides. Meanwhile we may take into account that mango fruit red coloration is due to a biosynthesis of two anthocyanins – cyanidin-3-glucoside and 7-methylcyanidin-3-galactoside [3].

Comparison of the two chromatograms (Fig.3) makes it possible to find small peaks of 7-methylcyanidin-3-rhamnosilgalactoside, coeluting with 7-methylcyanidin-3-galactoside in the conditions explored; the peaks (N8 from mango fruit and N8 of catharanthus petals) identity is confirmed by analysis of electronic spectrum, shifted hypsochromically to 515 nm.

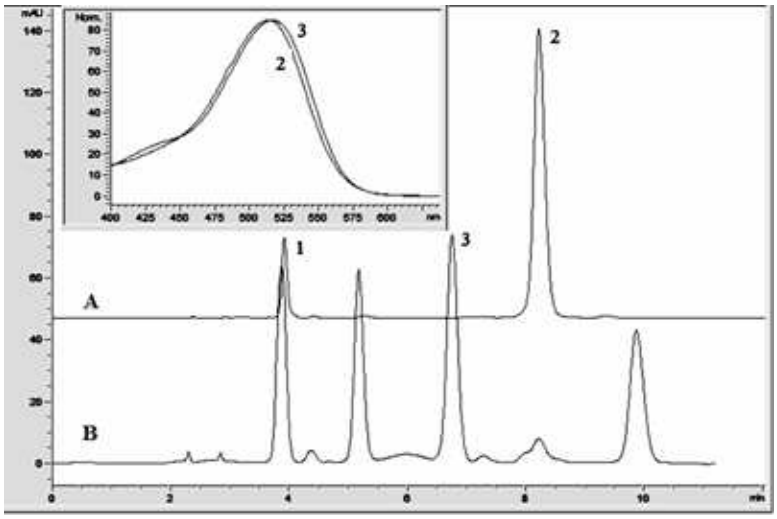
Thus, the anthocyanins complex has component not only of «delphinidine series» but also of «cyanidin series», though in reduced quantities. Hence, 7MeCy3Gala must be accomplished by 7-methylpeonidin derivatives. The latter is readily found on the chromatogram (peaks N9 – 7MePn3Gala and peak N10 – 7MePn3RhamnGala) with electronic spectrum of the same shift. The absence of any shift in series of the same derivatives Cy → Pn is according to our data a property of solutes of «cyanidin series».



Column 250×4.6 mm Symmetry C18, 5 mcm; mobile phase: 10 vol.% of HCOOH and 10 vol.% of CH_3CN, 1 ml/min; detection at 515 nm

Fig. 3. Separation of anthocyanins of mango fruit vs anthocyanins of *C. roseus*

Comparison of retention of 7MeCy3Gala with that of Pn3Gala (from cranberry fruits anthocyanins [8]) is presented on chromatograms of Fig. 4 while relative retention plot is given in Fig. 5.



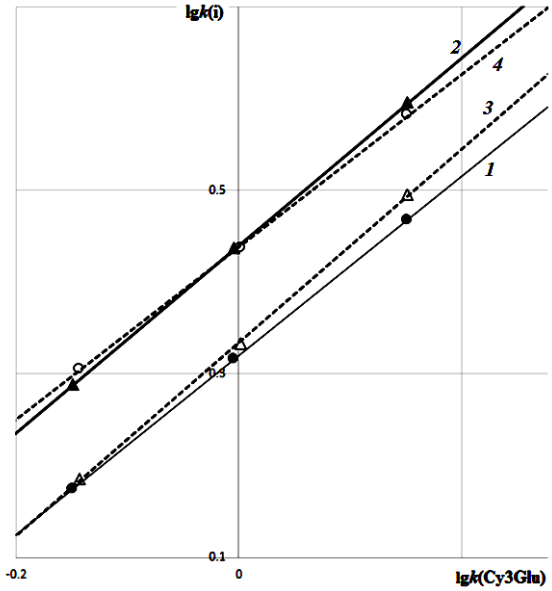
Column 250×4.6 mm Symmetry C18, 5 mcm; mobile phase: 10 vol.% of HCOOH and 9 vol.% of CH_3CN, 1 ml/min; detection at 515 nm; Solutes: 1 – Cy3Gala; 2 – 7MeCy3Gala; 3 – Pn3Gala.

Fig. 4. Separation of anthocyanins of mango fruit and of cranberry fruits

According to presented data migration of CH_3-group from 3'-OH-group towards 7-OH-group results in pronounced increase of solute retention, the finding can be fully translated upon all anthocyanidins. Meanwhile, such transformation leads to a small (~3.5 nm) hypsochromic shift of λ_{max} in electronic spectra. Another uncommon anthocyanin was found in theanthocyanin complex of petals of *Caesalpinia pulcherrima*, Fig.6.

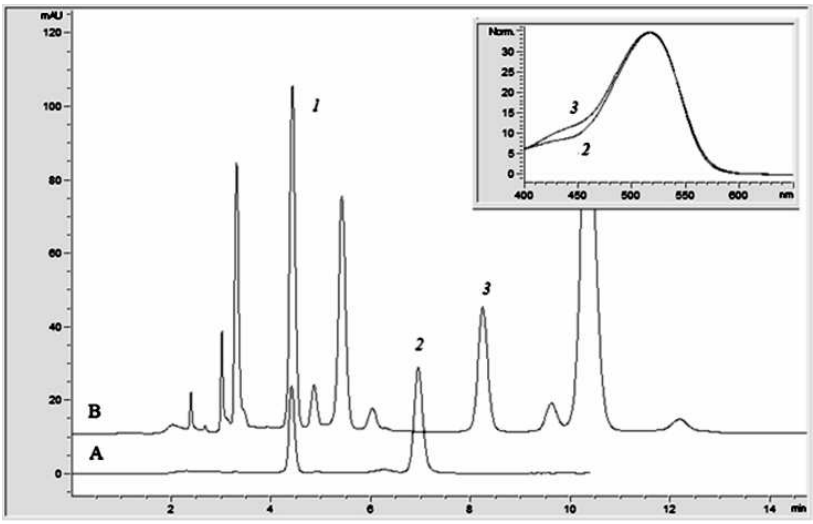
One peak of the two anthocyanins is easily determined as cyanidin-3-glucoside (by retention time, electronic and MS spectra), while the second one according to MS spectra should be formed by methylation of cyanidin-3-glucoside, but not a peonidin-3-glucoside. This compound has markedly lower retention in comparison to peonidin-3-glucoside

(Fig.5)and has absorption maxima in electronic spectra with small (1.5 nm) bathochromic shift.



Column 250×4.6 mm Symmetry C18, 5 mcm; mobile phase: 10 vol.% of HCOOH and 8 ÷ 10 vol.% of CH_3CN. Solutes: 1 – Pn3Gala; 2 – Pn3Glu; 3 – 5MeCy3Glu; 4 - 7MeCy3Gala.

Fig. 5. Relative retention plot for four anthocyanins vs Cy3Glu



Column 250×4.6 mm Symmetry C18, 5 mcm; mobile phase: 10 vol.% of HCOOH and 9 vol.% of CH_3CN. Solutes: 1 – Cy3Glu; 2 – 5MeCy3Glu; 3 – Pn3Glu.

Fig. 6. Separation of *Caesalpinia pulcherrima*petals and *Mahonia aquifolia* fruits anthocyanins

An evident decrease of absorption in region 400-450 nm (Fig.5A) is indicative for 5-substitution. Thus, a second substance can be defined as 5-methylcyanidin-3-glucoside.The flowers of two types of coloration (orange and pink) have the same types of anthocyanins, but in the latter case concentration of 5MeCy3Glu was greater than in a former case.

Conclusion

Anthocyanins of *Catharanthus roseus* petals are composed of pairs (3-galactosides and 3-rhamnosylgalactosides) of 7-methylated anthocyanidins of dephinidin series; that for anthocyanidins of a cyaniding series also can be detected in much lower concentrations.

Flowers of *Caesalpinia pulcherrima* contain mainly two anthocyanins: 3-glucosides: of cyanidin and 5-methylcyanidin.

Migration of CH_3-radical from 3'-OH group to 7-OH position results in increase of retention, while for the migration to 5-OH group leads to decrease of retention.

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