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INCREMENTAL APPROACH TO HPLC ANALYSIS OF ANTHOCYANS

V. I. Deineka,[1] A. M. Grigor′ev,[2] V. M. Staroverov,[2] and O. N. Borzenko[1]

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Relative analysis and relative incremental analysis, which can substantially simplify the determination of anthocyanidine glycosides without using standards, were used to determine anthocyans from plant materials by reversed-phase HPLC. The compositions of anthocyans from several plants grown in Belgorod district were investigated.

Key words: HPLC, anthocyans, retention, increments.

Traditional methods of determining anthocyan components from plant materials are labor-intensive and tedious. The use of mass-spectrometric detectors in combination with chromatographic methods greatly simplifies this procedure. However, many issues remain unresolved. On the other hand, HPLC is known to be highly effective in separating components of complex mixtures and to give retention times with good reproducibility. This makes it possible to determine a variety of substances in complex mixtures not only qualitatively but also quantitatively. Thus, this method is incorporated into the pharmacopoeia of many countries.

It is also known that HPLC is a powerful tool for determining even unknown derivatives using an incremental method [1].

We investigated the retention times of anthocyans of well-defined composition [2, 3] and applied this method to the analysis of specimens grown in Belgorod district.

Good agreement was found for the compositions (chromatograms) of the natural dyes in *Cerasus vulgaris* (Lyubskaya variety), *Sambucus nigra*, *Fragaria ananassa* (Zenga Zengana variety), *Rubus idaeus*, and *Ribes nigrum* (Belorussian sweet variety). The differences are greatest for the anthocyan composition of black current, *R. rubrum* (Fig. 1a). In materials grown in Belgorod district, Cy-3-(2^{X}Rut) and not Cy-3-(2^{G}Rut) dominated. The dominance of glycosides containing xylose was confirmed by the observation among the pigments of cyanidine 3-sambubioside.

We used the method of relative analysis in the comparison. The retention times of the cyanidine glycosides (*i*) [log k′(*i*)] as functions of the retention time of cyanidine-3-glucoside [log k′(Cy-3-Glu)] were strictly linear with relatively small least-squares deviations of the data as the eluent composition was changed (Table 1). The slope of the lines increased markedly on going from monoglycosides to glycosides with disaccharides and trisaccharides. This is important for identifying anthocyans and comparing their retention times with the reported values. Therefore, even in arguable instances (simultaneous elution of certain pairs of derivatives of a single aglycon), changing the eluent composition can reliably lead to their identification.

The composition of various aglycon derivatives can be identified by using experimental data obtained from a set of anthocyanidines in extracts of available materials (grapes and strawberries) and literature data:

1) the retention times (log k′) increase in the order of the monotypic glycosides Dp-3-Gly ≪ Cy-3-Gly ≪ Pt-3-Gly < Pg-3-Gly ≪ Pn-3-Gly ≪ Mv-3-Gly;

2) different UV absorptions: if chromatograms are recorded using two wavelengths (515 and 495 nm), the signal increases (by 15-25%) only for pelargonidine derivatives with a noticeable (5-10%) decrease of detector response for the remaining bases. The detector response for cyanidine and peonidine glycosides decreases slightly (by 10-20%); for delphinidine, petunidine, and malvidine, is almost constant (±5%) if the wavelength is increased to 525 nm.

1) Belgorod State University, 308015, Belgorod, ul. Pobedy, 85, e-mail: Deyneka@belgtts.ru; 2) FGUP BG NII TMP, 308002, Belgorod, ul. Michurina, 39a. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 137-139, March-April, 2003. Original article submitted March 26, 2003.

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TABLE 1. Retention Times of Cyanidine 3-Glycosides as Functions of Retention Time of Cyanidine 3-Glucoside, log k′(*i*) = *a* + *b*·log k′(Glu)

3-Glycoside	a	b	S, standard deviation for n = 7
Gala	-0.093	0.937	0.0034
Ara	0.145	0.948	0.0064
Ru	0.064	1.144	0.0043
Sam	-0.071	1.087	0.0062
Sopho	-0.218	1.053	0.0071
2GRu	-0.235	1.248	0.0053
2XRu	-0.057	1.226	0.0034

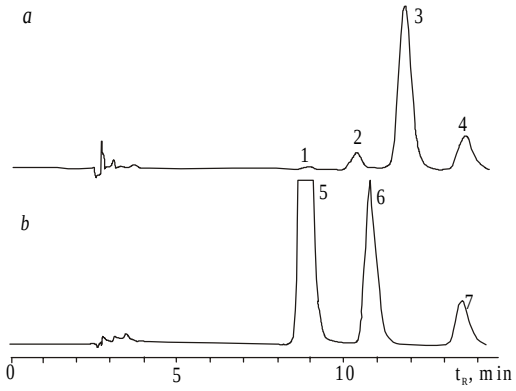

Fig. 1. Separation of cyanidine 3-glycosides of *Ribes rubrum* (a) and *Amelanchier ovalia* (b) extracts. 2^GRut (1), Sam (2), 2^X Rut (3), Rut (4), Gala (5), Glu (6), Ara (7). Eluent: CH_3CN (9 mL) and HCOOH (10 mL) in H_2O (100 mL), 1 mL/min, λ = 515 nm.

Relative incremental analysis, which was used successfully to calculate relative retention times of triglycerides from that of the fatty acids [4], was an even more stringent method of comparison. The increments for glycosides (*i*) [differences of log k′(*i*) and log k′(Glu)] as functions of a reference increment, e.g., log k′(Rut) - log k′(Glu) of the same aglycon, were investigated by this method.

According to the literature, *Amelanchier* anthocyans consist of cyanidine 3,5-diglucoside (main), 3-glucoside, and 3-arabinoside, etc. [5]. Three cyanidine glucosides, one of which is Cy-3-Glu (Fig. 1b), were observed in extracts of *A. ovalia* fruit grown in Belgorod district. The behavior of the retention times of the two other glycosides was studied for a relatively small slope (Table 1) and using relative incremental analysis in coordinates of log k′(*i*) - log k′(Glu) as a function of log k′(Rut) - log k′(Glu). This confirmed that the more weakly and strongly retained components are cyanidine 3-galactoside and 3-arabinoside, respectively. The agreement of the retention times obtained if a chromatographic column with substantially different retention characteristics [4] was used is evident (Fig. 2).

The retention times log k′(Dp-3-Rut) as a function of log k′(Dp-3-Glu) and log k′(Cy-3-Rut) as a function of log k′(Cy-3-Glu) were similar and can be written as the general formula $y = 0.060 + 1.123 \cdot x$ ($s = 0.0047$ for n = 8) with a small decrease of slope on going from delphindine to cyanidine derivatives. The signals corresponding to Pt-3-Rut and Pn-3-Rut in the extract of one of the black current varieties confirm the assumption that the results can be extrapolated to analogous pairs of other anthocyanidine derivatives. This agrees with the literature [6] on preparative separation of these components. Extrapolation of this function to pelargonidine components enabled the identification of Pg-3-Rut in the extract of red-tulip leaves, which are very common in amateur nurseries of our region. The assignment was confirmed by the spectral properties of the signals and the formation of the corresponding 3-glucosides upon hydrolysis.

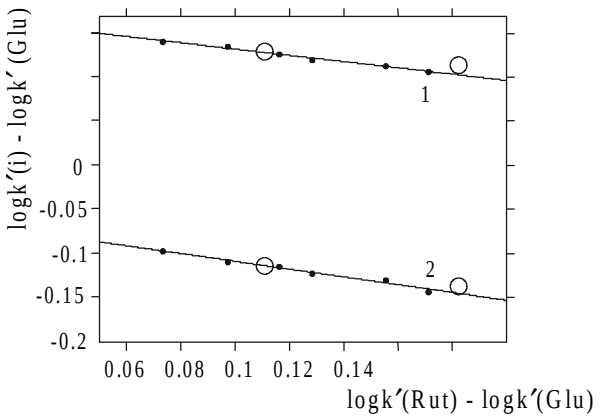

Fig. 2

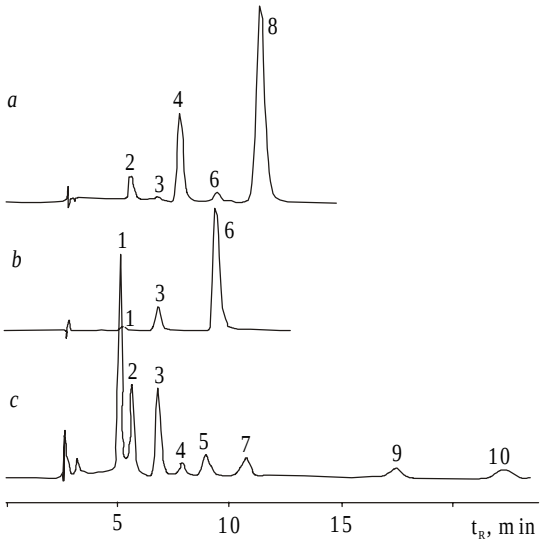

Fig. 3

Fig. 2. Relative incremental analysis of retention of 3-Ara (1) and 3-Gala (2) derivatives of cyanidine (o from [3]).
Fig. 3. Separation of anthocyans of *Tulipa* (3a), *Berberis vulgaris* (3b), and *Mahonia aquifolium* (3c) extracts: Dp-3-Glu (1), Dp-3-Rut (2), Cy-3-Glu (3), Cy-3-Rut (4), Pt-3-Glu (5), Pg-3-Glu (6), Pt-3-Rut (7), Pg-3-Rut (8), Mv-3-Glu (9), Mv-3-Rut (10). Eluent: CH_3CN (10 mL) and HCOOH (10 mL) in H_2O (100 mL), 1 mL/min, λ = 515 nm.

Anthocyan components of several plant materials grown in Belgorod region were investigated using the resulting rules. We present results from some of these.

The color of red tulip is due to the presence of delphinidine, cyanidine, and pelargonidine 3-rutinosides (Fig. 3a) with a substantial increase in this order of the relative fraction of anthocyans. The discrepancy of these investigations and the literature [7] is not surprising because the composition is fundamentally different for tulips of a different color (work on this continues).

The main components responsible for the color of *Berberis vulgaris* are delphidine, cyanidine, and pelargonidine 3-glucosides (Fig. 3b). This also does not fully agree with the literature [8].

The fraction of the pelargonidine component in the extract of *Berberis tunbergi* fruit is even higher. The anthocyan composition of another plant of this same family, *Mahonia aquifolium*, was much more complicated. The color of the fruit is due to delphinidine, cyanidine, petunidine, and malvidine glucosides and rutinosides with a complete lack of pelargonidine derivatives. Detectable quantities of peonidine derivatives also were not observed (Fig. 3c).

The anthocyans from *Amelanchier ovalia*, *Aronia melanocarpa*, *Crataegus sanguinea*, and *Malus domestica* were practically identical. The main component was Cy-3-Gala. The compounds Cy-3-Ara and Cy-3-Glu were present in much smaller amounts.

EXPERIMENTAL

Anthocyans were extracted from plant material by mixtures of CH_3CN, HCOOH, and H_2O. After filtration the samples were diluted to the composition of the eluent because injecting samples with a relatively high organic content (especially ethanol) significantly distorted the shape of the signal. It should be noted that we did not attempt to extract all the anthocyans. One fruit or flower could be used for the investigations.

Chromatographic studies were performed using Gilson equipment with a Holochrome spectrophotometric detector and a column (250×4.0 mm) packed with Ultrasphere ODS. Samples (20 μL) were injected using a Rheodyne 7125 valve-injector.

Chromatograms were recorded and processed using a YUNIKHROM 97 system (OOO "New Analytical Systems"). The flow rate for the chromatographic studies was 1 mL/min (H_2O—CH_3CN—HCOOH). The capacity factor (k′) was calculated as usual [1]. The column dead volume was determined using sodium nitrate.

The anthocyans were abbreviated using a system analogous to that in the literature [3]: Dp, delphinidine; Cy, cyanidine; Pt, petunidine; Pg, pelargonidine; Pn, peonidine; Mv, malvidine; Gly, glycoside; Glu, glucoside; Rut, rutinoside; Ara, arabinoside; Gala, galactoside; 2^X, xylosyl substituent in the 2″-position of the carbohydrate; 2^G, glucosyl substituent in the same position.

Several methods that substantially simplify the analysis were proposed to idenfity anthocyans of plant materials using reversed-phase HPLC. The principal anthocyan pigments of flowers and fruit of several plants growing in Belgorod district were determined using the developed method.

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