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Anthocyanidin 3-glucosides and *in Vitro* Unstable Anthocyanins from *Hibiscus syriacus*

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Chromatographic analyses of floral anthocyanins in *Hibiscus syriacus* L. showed the occurrence of six common anthocyanidin 3-glucosides and unknown acylated anthocyanins of the 3-glucosides. The characteristics of the unknown anthocyanins in extraction solvents and chromatographic solvents were further investigated. The acylated anthocyanins produced the esterified intermediates during deacylation in any concentration of hydrochloric and trifluoroacetic alcohols, but they were relatively stable in the acetic and formic solvents. The TLC solvents containing HCl also produced the esterified intermediates.

INTRODUCTION

Until recently, the acyl groups of anthocyanins were thought to be confined to aromatic acids, such as caffeic, *p*-coumaric or the simple aliphatic acetic acid (Harborne, 1967 ; Fong et ***al., 1974*** ; Hrazdina, 1982). However, it is now clear from both a limited survey of floral pigments (Harborne, 1986) and detailed investigations of individual anthocyanins (Cornuz et al., 1981 ; Bridle et al., 1984) that anthocyanins are also acylated in nature with aliphatic dicarboxylic acids, such as malonic (Bridle et ***al.,*** 1984), malic (Terahara and Yamaguchi, 1986), oxalic (Strack et ***al.,*** 1986) and succinic acids (Sulyok and Laszlo-Bencsik, 1985).

The important feature of these types of acylation is the instability of the acyl linkage ***in vitro,*** compared with other types of acylation. If such acylated anthocyanins are extracted by standard procedures using methanolic HCl, intermediate esters are formed, and the main reaction is the loss of the acyl group within a short time (Takeda et al., 1986). However, only a few fragmentary facts are known about the instability in the long procedure of methodology.

A previous work on the floral anthocyanins of ***Hibiscus*** *syriacus* has shown the occurrence of 3-glucosides of delphinidin, cyanidin, petunidin and malvidin (Egolf and Santamour, 1975). But the authors found the occurrence of six common anthocyanidin 3-glucosides and unknown MeOH-HCl labile anthocyanins in this species.

This paper describes the identification of six common anthocyanidin 3-glucosides and demonstrates the characteristics of labile anthocyanins in several extraction and chromatographic solvents as a basis of further structural determination.

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MATERIALS AND METHODS

In a preliminary work on the flower anthocyanins of *H. syriacus*, many cultivars were surveyed using HPLC. On the basis of the results we selected a few cultivars which contain enough amount of all anthocyanins for this experiment.

Standard procedures (Harborne, 1967) were followed in the extraction, purification and chromatographic identification of 3-glucosides. Anthocyanins were also analyzed on a Shimpak CLC-ODS reversed phase column (0.45 *x* 15 cm) by the slightly modified method of Wulf and Nagel (1978). The solvent system was 10% MeOH-HCOOH (9 : 1) in $HCOOH-H_2O$ (1 *: 9)* (10 min), and then the concentration was immediately increased to 22%. After 10 min, it was further increased to 45% gradually. The flow rate was 1 ml/min, and the detector wavelength was 530 nm.

For the studies of the labile anthocyanins, fresh petals were extracted with 5% methanolic formic acid over night at 5°C. The extract was concentrated to a small volume and passed through a Sephadex LH-20 column (1.0 *X* 20 cm) in the 5% formic acid to remove some mucilage. Anthocyanin fraction was adsorbed on Sep Pak C-18 cartridge and washed with 5% formic acid. Then, it was eluted with a small volume of 5% methanolic formic acid. The eluate was separated into several vials of equal volume and dried under N_2 gas. A few of the dried samples were used to characterize the instability on TLC. The others were dissolved in several solvents and sustained for 24 hours to investigate the instability in the solvents using HPLC.

To characterize the decomposition speed of acylated anthocyanin in methanolic HCl, acylated malvidin 3-glucoside was isolated from the blue cultivars of *H. syriacus* using MeOH-HCOOH (95 : 5). It was dissolved in 1% methanolic HCl and injected to HPLC at definite intervals. During the experiment the anthocyanin solution was kept at room temperature (15 $-$20°C).

RESULTS AND DISCUSSION

Identification of glycosides

Preliminary HPLC performance of the complete hydrolysates of fresh petals showed distinct 6 peaks of which four peaks were main and other two were minor. The six peaks were identical to those of the authentic markers of six common anthocyanidins. This result gave us the information about the presence of at least six anthocyanin glycosides.

Thin layer chromatography of anthocyanin extract from fresh petals gave six spots as shown in Fig. 1. Spots 1, 2, 3, and 4 were major pigments and 5 and 6 were minors. The spray of $AlCl_3$ in MeOH resulted in bathochromic shift of spots 1, 2 and 3. The six anthocyanins were isolated by paperchromatography (47 X60 cm) and successively by Sephadex LH-20 column chromatography. Partial hydrolyses of the six isolated anthocyanins produced no intermediate pigments, indicating that they are all monoglycosides. Figure 2 is a partial hydrolysis chromatogram of pigments 5 and 6 which were newly isolated from *H. syriacus*. Complete hydrolyses of the six anthocyanins produced only glucose as sugar component (Table 1) and six common anthocyanidins as aglycones, thus indicating the basic structure of the spots 1 to 6 are all mono-glucosides.

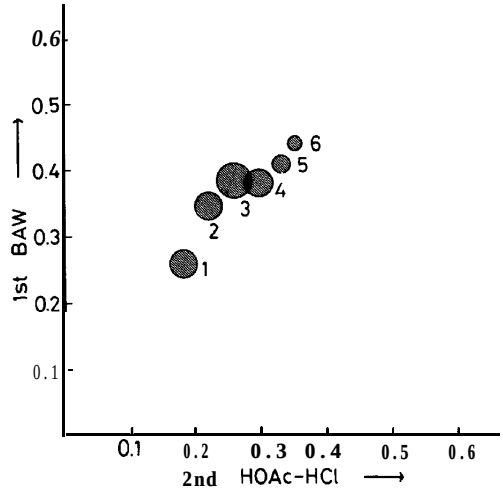

Fig. 1. Two dimensional thin layer chromatogram of the MeOH-HCl extract from petals of ***Hibiscus*** *syriacus.*

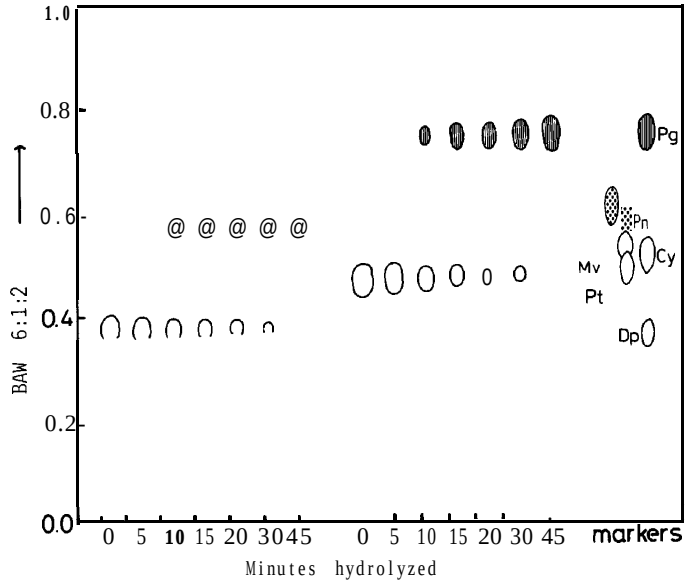

Fig. 2. Chromatogram of partial hydrolyses of peonidin and pelargonidin 3-glucosides which were newly isolated from ***Hibiscus*** *syriacus.*

The six isolated anthocyanins were further co-chromatographed on TLC and HPLC with authentic markers obtained from *Vitis* and ***Rosa.*** Rf values, retention times and the spectral data of them are summarized in Table 2. Rf values and retention times were identical to authentic markers of 3-glucosides of delphinidin (spot 1), petunidin (spot 2), cyanidin (spot 3), malvidin (spot 4), peonidin (spot 5) and pelar-

Table 1. Chromatographic properties of hydrolyzed sugars of each anthocyanin.

Sugar from spot No.	Rf value (x 100) in*				Color with AHP**
	ETN	BAW2	EAA	BAW	
1	53	21	24	26	brown
2	53	21	24	26	brown
3	53	21	24	26	brown
4	53	21	24	26	brown
5	53	21	24	26	brown
6	53	21	24	26	brown
Authentic glucose	53	21	24	26	brown

*On microcrystalline cellulose plates following solvents were used : ETN ; ethanol : water : NH_4OH (16 : 3 :1), BAW2 ; n-butanol : acetic acid : water (4 : 1 : 1), EAA ; ethanol : acetic acid : water (3 : 1 : 1), BAW ; n-butanol : acetic acid : water (4 : 1 : 5).
**AHP ; aniline hydrogenphthalate.

Table 2. Rf values, retention times and spectral properties of anthocyanins isolated from *Hibiscus syriacus*.

spot No.	Rf values (×100) in*				Retention time	Color shift in $AlCl_3$	in 0.01 % HCl-MeOH		Identical marker
	BAW	BuHCl	1% HCl	HOAc -HCl			Vis. Max. (nm)	E440/Vis. Max. (%)	
1	26	11	3	17	8.62	+	540	19	Dp 3-G
2	34	14	4	21	13.48	+	538	22	Pt 3-G
3	38	24	7	26	11.29	+	532	24	cy 3-G
4	40	15	6	30	17.15	−	537	23	Mv 3-G
5	46	31	8	32	16.21	−	526	26	Pn 3-G
6	48	36	12	34	14.47		507	42	Pg 3-G

* On microcrystalline cellulose plates following solvents were used : BAW ; n-butanol : acetic acid : water (4 : 1 :5), BuHCl ; n- butanol : 1N HCl (1 :1), 1 % HCl ; conc HCl : water (3 : 97), HOAc-HCl ; water : acetic acid : conc HCl (82: 15: 3).

gonidin (spot 6). The spectral data also indicated the characteristics of 3-glucosides. Thus, the six anthocyanins isolated from *H. syriacus* were identified as 3-monoglucosides of six common anthocyanidins.

Acylated anthocyanins and their instability in *vitro*

During the cultivar survey of *H. syriacus* with HPLC, we found that there are many unknown anthocyanins (peaks 7 to **12** in Fig. 3) which are relatively stable in methanolic HCOOH but very unstable in MeOH-HCl (Fig. 3). Furthermore, MeOH-HCl extraction produced many unexpected peaks (arrows in Fig. 3) which were not seen in the extract with MeOH-HCOOH. The peak areas of these unknown pigments (7-12) and unexpected pigments (arrows) continuously decreased with times proceeding at room temperature, whereas the peak areas (l-6) of 3-glucosides (Table 2) increased. Observations of the quantitative changes confirmed us that the unknown anthocyanins **(7-12)** are the aliphatic acylated anthocyanins and the unexpected pigments (arrows) are the esterified intermediates of them during the procedure of deacylation.

The instability of acylated anthocyanins in several solvents were further inves-

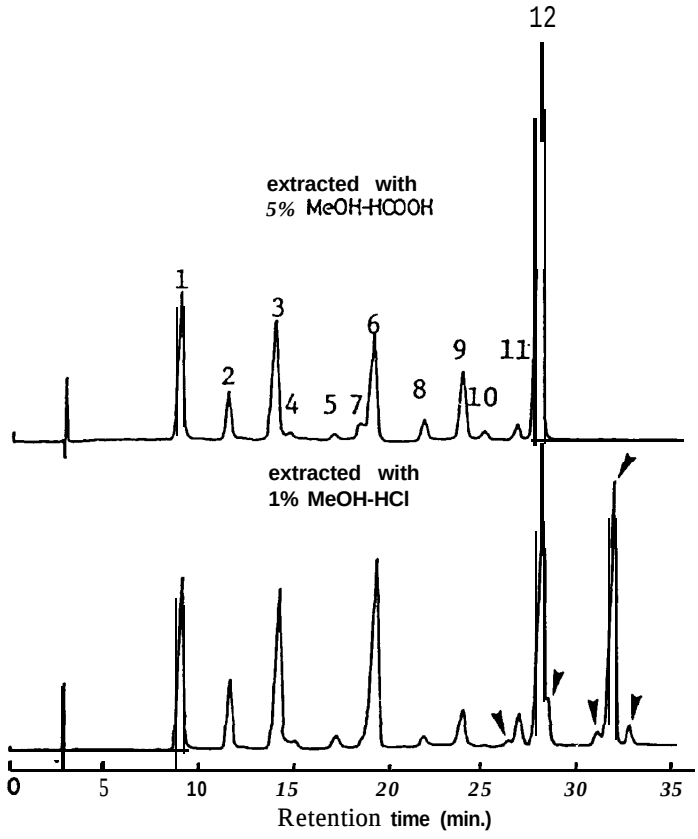

Fig. 3. Difference of the HPLC resolutions in the same petal extracts of different solvents. Arrows indicate the esterified intermediates.

tigated and the results were summarized in Table 3. Esterification of anthocyanins were occurred in any concentration of MeOH-HCl, EtOH-HCl and MeOH-TFA (trifluoroacetic acid), while a small amount of esterified intermediates appeared in MeOH-HCOOH and MeOH-CH_3COOH of which only 10% solvents produced about 2% intermediates of total acylated anthocyanins. Goto et ***al.*** (1984) replaced HCl by TFA for the extraction of aliphatic acylated anthocyanins, but our results indicate that TFA is not suitable as a solvent for the purpose.

Instability on TLC

Figure 4 shows the stable separation of the 12 anthocyanins on TLC. The first directional solvent used was the organic layer of n-butanol/glacial acetic acid/water = 4 : 1 : 5 (v/v/v), and the second was the aqueous mixture of water/glacial acetic acid/ HCl = 82 : 15 : 3 (v/v/v). Spots l-6 were 3-glucosides of delphinidin, petunidin, cyanidin, malvidin, peonidin and pelargonidin as in order of the number, and the spots A-F were the acylated anthocyanins of the 3-glucosides as in order of the alphabet. This correspondence was confirmed by HPLC of extracts of the spots. Acylated

Table 3. Esterification of acylated anthocyanins in various extractants.

Extractant	Total acylated pigments (μg)	Esterified pigments (μg)	Rate of esterification (%)
1 % MeOH-HCl	10.7	4.6	43
0.5 % MeOH-HCl	10.7	3.3	30
0.1 % MeOH-HCl	10.7	0.7	7
0.01% MeOH-HCl	10.7	0.3	3
1 % EtOH-HCl	13.8	2.6	19
0.5 % EtOH-HCl	13.8	1.9	14
0.1 % EtOH-HCl	13.8	0.9	6
0.01% EtOH-HCl	13.8	0.4	3
10 % MeOH-FA	8.9	0.2	2
5 % MeOH-FA	8.9	0.0	0
2.5 % MeOH-FA	8.9	0.0	0
1 % MeOH-FA	8.9	0.0	0
10 % MeOH-AA	7.8	0.3	2
5 % MeOH-AA	7.8	0.0	0
2.5 % MeOH-AA	7.8	0.0	0
1 % MeOH-AA	7.8	0.0	0
10 % MeOH-TFA	10.2	1.7	17
5 % MeOH-TFA	10.2	1.2	12
2.5 % MeOH-TFA	10.2	1.0	10
1 % MeOH-TFA	10.2	0.7	7

*FA ; formic acid, AA ; acetic acid, TFA ; trifluoroacetic acid

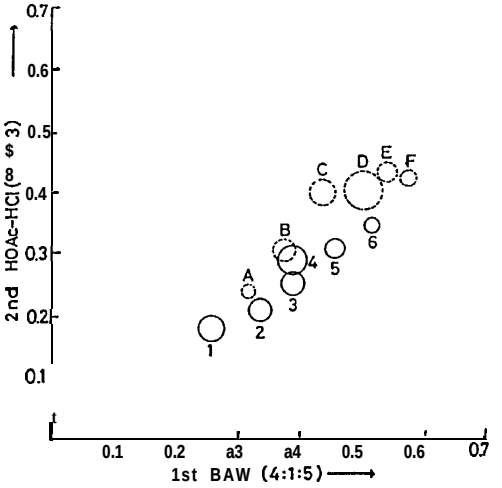

Fig. 4. Two dimensional thin layer chromatogram of the MeOH-HCOOH extract in the solvent system of BAW and HOAc-HCl.

anthocyanins did not show any unstableness in this solvent system.

The chromatogram of the same extract developed with another solvent system is

shown in Fig. 5. The first directional solvent was the organic layer of n-butanol/2N HCl=1 : 1 (v/v), and the second was the same as that in Fig. 4. Besides the spots 1 -6 and A-F which were the same as in Fig. 4, twelve unexpected spots appeared. Each two spot appeared on the vertical lines of each acylated anthocyanin A-F. These spots were scraped off and extracted with a small volume of cold methanol, and injected into HPLC. Spots 1′-6′ were identified as 3-glucosides which coincide with the anthocyanins of spots l-6 respectively. Spots A′-F′ showed distinctly delayed peaks than those of spots A-F in HPLC, but large amount of each spot was deacylated during extraction. Thus, spots A'-F' revealed distinct intermediate characters in the procedure of deacylation of the acylated spots A-F.

Speed of deacylation

Speed of the deacylation of malvidin 3-acylglucoside (spot D), which was isolated from so-called blue flowers of ***H. syriacus,*** was examined in 1% MeOH-HCl. As shown in Fig. 6, the reaction of deacylation was very fast in the first day ; i. e. about 70% within a day at room temperature, and it continued slowly thereafter. Almost all of the pigment was deacylated after three days.

Because of the reason that anthocyanins are generally unstable in neutral or weakly acidic aqueous solvent, quinonoidal base of anthocyanin hydrate easily form the corresponding colorless pseudobase via flavilium ion (Brouillard and Delaporte, 1977), classical methodology has taken MeOH-HCl as the extraction solvent. It is now, however, clear that anthocyanins of ***H. syriacus*** or aliphatic acylated anthocyanins of other plants must be extracted with weak acids such as formic or acetic acids instead of HCl. In addition, analyses of the labile anthocyanins should be conducted as fast as possible in any acidic alcohol. Besides the well known phenolic acids (hydroxy benzoates and hydroxy cinnamates), aliphatic acids have been extensively detected very recently as acyl moieties of anthocyanins. To date, acetic,

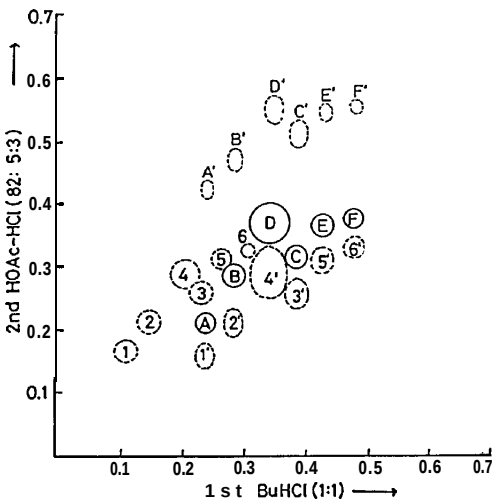

Fig. 5. Two dimensional thin layer chromatogram of the MeOH-HCOOH extract in the solvent combination of BuHCl and HOAc-HCl.

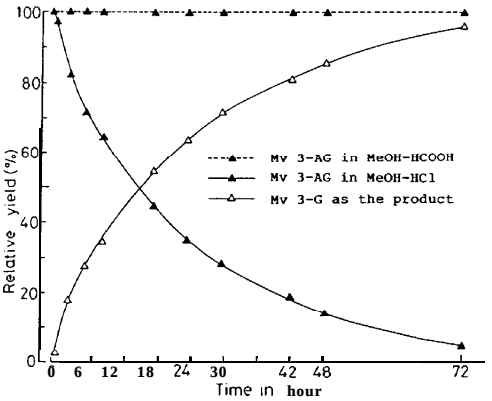

Fig. 6. Transformation of the malvidin 3-acylglucoside (Mv 3-AG) to malvidin 3-glucoside (Mv 3-G) by the treatment with 1% MeOH-HCl.

succinic, oxalic, malic and malonic acids have been identified, among which malonic acid appears to be the most commonly occurring ones (Harborne, 1986, etc.).

These acylated anthocyanins have been proved to make intermediate methyl ester, and the main reaction is the loss of acyl group within quite a short time. The appearance of acylated anthocyanins and their intermediate esters on TLC and HPLC of extracts of *H. syriacus* distinctly indicates the presence of dicarboxylic acid in anthocyanins of *H. syriacus*. Detailed studies on the structural nature of these anthocyanins are in progress.

On the other hand, our findings of two new glycosides, pelargonidin and peonidin 3-glucosides, in this species require reconsideration of breeding potentials concerning the flower color. Since pelargonidin derivatives have been recognized as important pigments in horticultural plants (Arisumi, 1963 ; 1964) for their spectral properties of orange-red or scarlet colors and this color is not present in *H. syriacus*, the presence of this pigment gives us encouraging meaning for breeding new colors in this species.

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