REVIEW

THE CHROMATOGRAPHIC IDENTIFICATION OF ANTHOCYANIN PIGMENTS

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INTRODUCTION

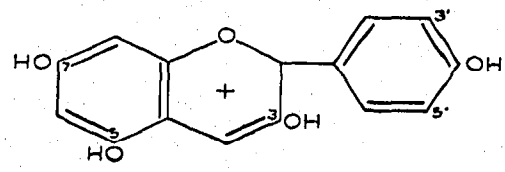
Chromatography is well recognised to-day as an indispensable technique in the study of all kinds of plant pigments. Only in the last ten years, however, has it been applied to the separation of the water-soluble pigments, such as the anthocyanins. Attempts to separate them by absorption chromatography on columns, a technique which had so successfully been applied to the carotenoid pigments[1], did not meet with much success[2,3]. It was not until the introduction of paper partition chromatography into this field by BATE-SMITH in 1948[4,5] that satisfactory chromatographic methods for separating the anthocyanins and related flavonoid pigments became available. Since then, paper chromatography has been used in the identification of individual pigments, especially the minor components, in complex mixtures of flavonoid compounds, such as are found in the flowers of *Antirrhinum majus*[6], *Dahlia variabilis*[7] and in many other plant extracts. As a result of these and other investigations (*e.g.*[8]) the R_F values of all the main classes of flavonoid compounds, including the anthocyanins, became available and some general reviews of these data have already appeared[9–11].

This review is concerned only with the chromatography of the anthocyanins, the compounds responsible for most of the red, purple and blue pigmentation in plants[12]. They are more difficult to characterise by conventional methods than the related flavones and the well known colour and distribution tests[13] are limited in their scope. For these reasons, chromatographic methods are of especial importance for characterising anthocyanins. Their value may be illustrated by the fact that the recent discoveries in plants of two new anthocyanin aglycones, *i.e.* anthocyanidins[14,15], and several new classes of anthocyanin[16,17] were only made possible by means of paper chromatography. It has also been shown in this laboratory[17,18] and elsewhere[19,20] that unknown pigments can be quickly and economically identified on a micro-scale by the aid of chromatographic techniques.

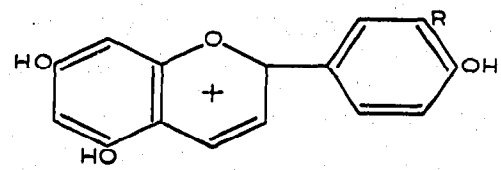
The paper chromatography of the anthocyanins will first be reviewed and some previously unpublished R_F values will be given and discussed. A description of the value and limitations of the chromatographic approach to anthocyanin identification will form the main part of this article. Finally, some examples will be given of the use that has already been made of chromatographic methods in studying the occurrence and distribution of anthocyanins in plants.

Before closing this introduction, it is necessary to consider what is involved in

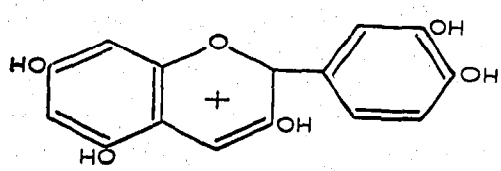
characterising anthocyanins. The main facts of their chemistry are now well established (for review, see, *e.g.* WAWZONEK[21]). They occur in nature in combination with sugars (as glycosides) and occasionally also in association with organic acids (as acylated or "complex" glycosides). There are only a few different anthocyanidins formed on acid hydrolysis of anthocyanins and all have the same basic chemical structure (see Fig. 1). In addition, the positions at which sugars may be attached to



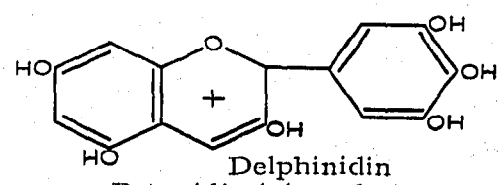
Pelargonidin



Apigeninidin (R = H)
Luteolinidin (R = OH)



Cyanidin
Peonidin (3′-methyl ether)
Rosinidin (7,3′-dimethyl ether?)



Delphinidin
Petunidin (3′-methyl ether)
Malvidin (3′, 5′-dimethyl ether)
Hirsutidin (7, 3′,5′-trimethyl ether)

Fig. 1. The naturally occurring anthocyanidins.

hydroxyl groups in the anthocyanin molecule are restricted and residues are only normally found in the 3-position or in the 3- and 5-positions. The characterisation of an unknown pigment thus depends on identifying the anthocyanidin produced on hydrolysis and on determining the nature, position of attachment and number of sugars present. In addition, examination for the presence of an acyl component must be made and if present, the organic acid concerned must be identified.

It will be shown here that chromatography can be used at each step in the identification of anthocyanins. It must however be emphasised that chromatographic methods should be combined with other techniques for the complete structural determination of anthocyanins. For example, spectral methods are important[3,22] and can be used most profitably in conjunction with chromatographic studies. Finally, it must be stressed that considerable experience is necessary for interpreting chromatographic data, which must be related to similar data obtained from reference compounds.

I. PAPER CHROMATOGRAPHY OF ANTHOCYANINS

The use of paper chromatography in the study of anthocyanins is a logical extension of the tests devised by ROBINSON AND ROBINSON[13] for distinguishing different glycosidic classes according to their distribution between water and amyl alcohol. BATE-SMITH[4,5] first showed that anthocyanins could be readily separated on filter paper developed with butyl alcohol–acetic acid–water and he published R_F values for most of the anthocyanins that were known at the time and that had been synthesised in the laboratory. Other workers[9,10,23] have since published R_F values for these and

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other anthocyanins and several more solvent systems have been employed. The general conditions for the paper chromatography of the anthocyanins are now well appreciated and are as follows.

Extraction

Extracts of plant organs containing pigment can be examined directly by paper chromatography. The solvents used for extraction are methanol or water containing 1% hydrochloric acid. Methanol is generally to be preferred since the extracts are more easily concentrated prior to their application to paper. This is important when extracts are used of plant organs which contain only small amounts of anthocyanin. Leaf extracts are normally washed with petroleum ether to remove chlorophyll, before being concentrated. The R_F values of anthocyanins, present in crude plant extracts, are not completely reliable since the position and number of the spots may be affected by the presence of other components, especially flavonol glycosides. Therefore, in order to obtain accurate R_F values it is essential to purify anthocyanins in plant extracts either by the more usual methods (*cf.*[21]) or by chromatography on thick Whatman paper (see section 2). Anthocyanins so obtained are best dissolved in 1% methanolic hydrochloric acid before being applied to paper.

Filter paper

Whatman No. 1 paper is commonly employed for the chromatography of anthocyanins. All R_F values quoted in this review were measured on this paper. The corresponding grades used by German and Japanese workers are Schleicher and Schüll 2043b MGL and Toyo No. 50 respectively.

Solvent systems

The solvent systems used are listed in Table I. Because anthocyanins are cations and are only stable at acid pH, chromatography is normally carried out in solvent systems containing acid. If solvents do not contain mineral acid, *e.g.* BAW, it is important

TABLE I

SOLVENT SYSTEMS FOR THE CHROMATOGRAPHY OF ANTHOCYANINS

Abbreviation	*Composition*	*Proportions (v/v)*	*Layer used*
BAW	*n*-butanol–acetic acid–water	4:1:5*	top**
BuHCl	*n*-butanol–2 *N* hydrochloric acid	1:1	top**, ***
—	*m*-cresol–acetic acid–water	50:2:48	top
isoPr.	isopropanol–2 *N* hydrochloric acid	1:1	miscible
PhOH	phenol–water	saturated	bottom
—	acetone–0.1 *N* hydrochloric acid	1:3	miscible
—	ethyl acetate–formic acid–water	8:2:3	top
1% HCl	water–12 *N* hydrochloric acid	97:3	miscible
HAc–HCl	water–acetic acid–12 *N* hydrochloric acid	82:15:3*	miscible
30% HAc	water–acetic acid	70:30*	miscible

* Other proportions of these mixtures can also be used.
** The time of use after mixing is important and affects the R_F value.
*** BuHCl should be left for 24 h before use and the paper must also be equilibrated in a tank containing the lower aqueous phase of BuHCl for 24 h.

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that sufficient hydrochloric acid is present in the original extract to keep it in chloride form as it travels down the paper[4]. As will be seen in Table I, the solvent systems are mainly of two types, based either on an alcohol (*e.g.* *n*-butanol) or on water. The relative merits of some of these systems are discussed by ABE AND HAYASHI[23]. The author has found BAW, BuHCl, 1% HCl and HAc–HCl to be a useful selection of solvents, each of which gives consistent results. The only solvent system in which it is essential to equilibrate the paper before development in the aqueous phase, is BuHCl. Special care must also be taken with BAW, since the length of time this solvent mixture is allowed to stand before use can affect the R_F values considerably. For measuring standard R_F values, an *n*-butanol–acetic acid–water mixture which has been aged for three days before use, is recommended[5]. For general use, a fresh mixture of these solvents is quite satisfactory and such mixtures have been used routinely in this laboratory.

Chromatograms are normally developed with these solvents by descent, but it has been claimed[24] that more reliable results are obtained with BAW if it is allowed to ascend the paper. Suitable solvent pairs for two-dimensional paper chromatography are BAW and 15% HAc or BuHCl and 1% HCl. Anthocyanins tend to fade and form large diffuse spots when chromatographed in two directions, so that this method has not been greatly used. Circular paper chromatography has also been used with anthocyanins[25].

Visualisation

Different anthocyanins appear as different coloured spots on chromatograms (see Table II) when seen in daylight and characteristically change colour on fuming the paper with ammonia[4]. Examination under ultra-violet light is also of value, since

TABLE II

R_F VALUES AND COLOURS OF PELARGONIDIN GLYCOSIDES

*Pelargonidin glycoside**	*R_F values in*				*Visible*	*Colours in ultra-violet*
	BAW	*BuHCl*	*1% HCl*	*HAc–HCl*		
3-monoglucoside	0.44	0.38	0.14	0.35	orange-red	dull red
3-monogalactoside	0.39	0.37	0.13	0.33		
3-rhamnoglucoside	0.37	0.30	0.22	0.44		
3-diglucoside I**	0.36	0.26	0.50	0.62	orange-red	dull red
3-diglucoside II***	0.30	0.17	0.21	0.47		
3-triglucoside	0.25	0.10	0.35	0.52	orange-red	dull red
3,5-diglucoside	0.31	0.14	0.23	0.45		
3-rhamnoglucosido-5-glucoside	0.29	0.13	0.40	0.58	orange	fluorescent yellow
3-diglucosido (1)-5-glucoside	0.25	0.10	0.60	0.68		
3-diglucosido (1)-7(or 4')-glucoside	0.18	0.04	0.73	0.73	orange-yellow	dull orange red

* Sources are given in refs. [17,22].
** Isolated from *Papaver rhoeas*.
*** Isolated from *Primula sinensis*, possibly the 3-gentiobioside.

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differences in ability to fluoresce provide a means to their identification[17,23]. Pelargonidin and peonidin 3,5- and 5-glycosides appear as intensely fluorescent yellow and pink spots, while the corresponding 3-glycosides are non-fluorescent. Similar, but less striking, differences have been noted with glycosides of the other anthocyanidins. The colours produced by anthocyanins after chromatograms developed in BAW or 15% HAc have been sprayed with a 5% ethanolic solution of aluminium chloride[26], or 2% aqueous ferric chloride[27] have also been noted. These sprays distinguish derivatives of cyanidin, delphinidin and petunidin (positive colour change) from those of the other anthocyanidins (no colour change).

R_F values

The R_F values of anthocyanins as determined in this laboratory are given in Tables II, III and IV. They have been chosen to illustrate the relationship that exists between chemical structure and R_F value in this series, and at the same time, to provide a useful collection of data for identification purposes. The results obtained

TABLE III

R_F VALUES AND COLOURS OF CYANIDIN AND PEONIDIN GLYCOSIDES

Glycoside	*R_F values in*				*Visible*	*Colours in ultra-violet*
	BAW	*BuHCl*	*1% HCl*	*HAc–HCl*		
Cyanidin:						
3-monoglucoside	0.38	0.25	0.07	0.26	magenta	dull magenta
3-monogalactoside	0.37	0.24	0.07	0.26		
3-rhamnoglucoside	0.37	0.25	0.19	0.43	magenta	dull magenta
3-xyloglucoside	0.36	0.24	0.24	0.51		
3-diglucoside	0.33	0.22	0.34	0.61		
3,5-diglucoside	0.28	0.06	0.16	0.40	magenta	bright red
3-rhamnoglucosido-5-glucoside	0.25	0.08	0.36	0.59		
Peonidin:						
3-monoglucoside	0.41	0.30	0.09	0.33	pink	dull pink
3,5-diglucoside	0.31	0.10	0.17	0.44	pink	fluorescent rose
3-rhamnoglucosido-5-glucoside	0.29	0.12	0.37	0.60		

here are in general agreement with values obtained by other workers[5,23] and include figures for a number of anthocyanins not previously available. No attempt has been made to run all chromatograms under standard conditions, but the results given are strictly comparable and are typical values from a large number of determinations.

Tables II, III and IV give the R_F values and colours of the most frequent anthocyanins, including the 3-monoglucosides and 3,5-diglucosides of all the common anthocyanidins. Table II, giving the R_F values of ten glycosides of pelargonidin, shows the range of R_F values that can be obtained for a series of different glycosides of the same anthocyanidin. It is also to be noted that no two glycosides have exactly the same R_F values. R_F values in BAW and 1% HCl of three different glycosides of the six main anthocyanidins are plotted graphically in Fig. 2, after the manner of

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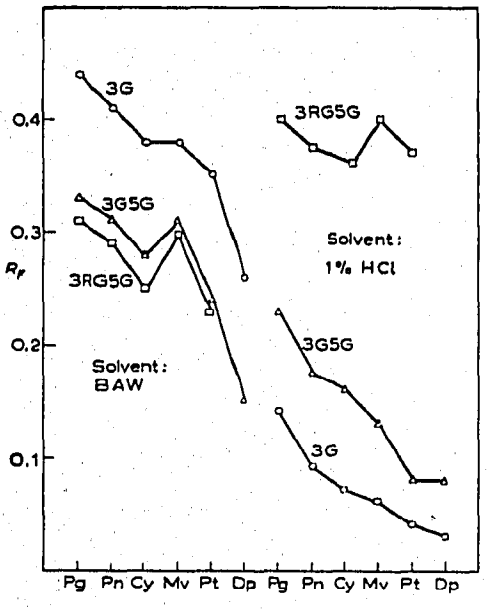
TABLE IV

R_F VALUES AND COLOURS OF DELPHINIDIN, PETUNIDIN AND MALVIDIN GLYCOSIDES

Glycoside	R_F values in				Visible	Colours in ultra-violet
	BAW	BuHCl	1% HCl	HAc–HCl		
Delphinidin:						
3-monoglucoside	0.26	0.11	0.03	0.18	purple	dull purple
3-rhamnoglucoside	0.30	0.15	0.11	0.37	purple	dull purple
3,5-diglucoside	0.15	0.03	0.08	0.32	purple	purple
Petunidin:						
3-monoglucoside	0.35	0.14	0.04	0.22	purple	dull purple
3-rhamnoglucoside	0.35	0.16	0.13	0.42	purple	dull purple
3,5-diglucoside	0.24	0.04	0.08	0.32	purple	bright
3-rhamnoglucosido-5-glucoside	0.23	0.06	0.37	0.61		purple
Malvidin:						
3-monoglucoside	0.38	0.15	0.06	0.29	mauve	dull mauve
3,5-diglucoside	0.31	0.03	0.13	0.42	mauve	fluorescent
3-rhamnoglucosido-5-glucoside	0.30	0.05	0.40	0.63		cerise



ABE AND HAYASHI[23], to illustrate the relationship between structure and R_F value. The glycosides are the 3-monoglucosides (3G), the 3,5-diglucosides (3G5G) and the 3-rhamnoglucosido-5-glucosides (3RG5G). This latter group of glycosides has only recently been discovered[16,17] and no R_F values have previously been given for its members.

Fig. 2. Graph of the R_F values of the 3-glucosides (3G), 3,5-diglucosides (3G5G) and 3-rhamnoglucosido-5-glucosides (3RG5G) of pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp).

R_F value and structure

Regular relationships between the R_F value and structure of an anthocyanin have been noted by BATE-SMITH AND WESTALL[28], by ABE AND HAYASHI[23] and by REZNIK[10]. Their results have been amplified by studies in this laboratory and the general conclusions have been shown to hold good for flavones and other polyphenols (see, *e.g.*[28,29]). The results are best considered in relationship to particular structural modifications.

(1) *Hydroxylation.* The greater the number of hydroxyl groups present in the anthocyanidin molecule, the lower the R_F value is, in both alcoholic (BuHCl and

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BAW) and aqueous (1% HCl and HAc–HCl) solvents. Thus corresponding glycosides of pelargonidin, cyanidin and delphinidin can always be placed in decreasing order of R_F value.

(2) *Methylation* reverses the effect of hydroxylation, so that the greater the number of methoxyl groups, the higher is the R_F value, again in both types of solvent (see Table IV especially). The increase in R_F value brought about by methylation is rather less than the decrease caused by hydroxylation. Thus the same glycosides of cyanidin and malvidin have rather similar R_F values (Tables III and IV). This effect is observed not only of methylation in the 3′- and 5′-positions in the anthocyanin molecule, but also of methylation in the 7-position, as shown by the figures for hirsutin[28] and rosinin[14].

(3) *Glycosidation.* There is a direct relationship between R_F value and the number of sugar residues which is quite independent of the nature of the anthocyanidin. In aqueous solvents, the effect of glycosidation is to increase R_F values; in BAW or BuHCl, the exact reverse is true (see Fig. 2). This is well illustrated by the figures for the pelargonidin 3-mono-, di- and triglucosides which occur in *Primula sinensis*[17] (Table II). It is also to be noted that this simple relationship is complicated if sugar residues are attached to more than one position in the molecule and also depends on the nature of the sugar–sugar linkage in the case of the 3-biosides[17].

(4) *Acylation* causes an increase in R_F value in solvents based on *n*-butanol, but lowers the R_F value in aqueous solvents. The effect of acylation on R_F value is the reverse of that shown by glycosidation. The R_F values of acylated anthocyanins will be further discussed under section 5.

2. SEPARATION AND PURIFICATION

(*a*) *By paper chromatography*

The first step in the detailed examination of an anthocyanin pigment is that of isolating it in a pure state. It must be separated from other compounds which are extracted from the plant at the same time, namely other anthocyanins, flavone glycosides (co-pigments) and water-soluble substances such as free sugars. The usual chemical methods of isolation are often tedious and wasteful (*cf.*[21]) and do not always yield a pure compound (see, *e.g.* SCOTT-MONCRIEFF[30]). Paper chromatography provides a convenient and rapid alternative means of preparing pure anthocyanins on a small scale without incurring much loss.

The general procedure is similar to that used for flavone glycosides[7,31] and has been described in outline already[17]. An extract of the plant material with methanolic hydrochloric acid is concentrated and applied directly to several sheets of thick filter paper (Whatman No. 3 or 3MM) and chromatography is carried out in BAW, BuHCl or, less frequently, 1% HCl. The anthocyanins appear as clear discrete coloured bands, which are then cut out and the paper eluted with methanol containing hydrochloric acid or acetic acid and the process is repeated twice with the same or a different solvent mixture. It is important to carry out these operations quickly, since solutions

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of anthocyanins tend to fade on exposure to light. Anthocyanin bands, when cut into strips five inches long, can be eluted with methanol in a very short time (1–2 hours), especially if they are cut from chromatograns which are only just dry. Pigments which have been left on filter paper strips for several weeks cannot always be completely recovered by elution, due to some irreversible adsorption having taken place. Anthocyanins which occur in very complex mixtures of flavonoids may require further purification on paper and some acylated pigments of closely related structure do not separate well under these conditions (see section 5).

Using these methods, a large number of anthocyanins have been successfully purified in this laboratory. In some instances, sufficient material has been obtained from paper chromatograms to yield the pigment in crystalline form. As many as eight different anthocyanins have been separated from a single plant extract, that of the stems of certain *Primula sinensis* genotypes[32]. Indeed the method is particularly recommended for separating the anthocyanins in plants which contain several such pigments. It has already been used successfully for this purpose with the pigments of grapes[20], potato tubers[15] and of many garden flowers.

(b) By column chromatography

Column chromatography provides a method of separating anthocyanins on a preparative scale, but the high degree of resolution that can be achieved on filter paper has not yet been duplicated on a column. Mixtures of two or three anthocyanins have been separated by this means[2,3,33,34], but the method is only partially successful with plant extracts containing more than three pigments[35].

Difficulty has been experienced in finding a suitable adsorbent for anthocyanins. They are so strongly adsorbed on to columns of basic salts, such as alumina, that it is not easy to elute them from the column. The choice of eluting solvent is also restricted to those containing *n*-butanol or a similar alcohol and aqueous or methanolic hydrochloric acid. The use of alumina and a specially chosen grade of calcium sulphate by KARRER AND STRONG[2] and KARRER AND WEBER[33] for separating mixtures of two anthocyanins has not found favour in the more recent work of LI AND WAGENKNECHT[3]. These latter authors successfully separated the 3-rhamnoglucoside and 3-diglucoside of cyanidin, present in sour cherries, on silicic acid columns by elution with BAW.

However, the most popular adsorbent for separating anthocyanins is cellulose, but it is clear that the success of the operation is dependent on the grade of cellulose powder[36] and the method of packing[34] employed. ENDO[36] separated the six anthocyanins present in the flowers of *Viola tricolor maxima* on cellulose, but only one particular batch of the powder was really successful. After separation, the column was extruded and the bands cut out and eluted with methanol. CHANDLER AND HARPER[35] in separating six pigments present in blackcurrants on cellulose, eluted the pigments directly from the column. The faster moving anthocyanins had however to be further purified by lead precipitation.

Column chromatography is also used for separating anthocyanins from other components of plant extracts in a preliminary purification in which no separation of

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individual pigments is attempted. The anthocyanins are strongly adsorbed on to the column, which can then be washed thoroughly with water to remove impurities. The pigments are then eluted from the column with methanolic hydrochloric acid. Columns of paper[37], lead hydroxide[38] and magnesol–celite (5:1) mixtures[39] have been used for this purpose.

3. CHROMATOGRAPHIC IDENTIFICATION OF ANTHOCYANIDINS AND SUGARS

On acid hydrolysis, anthocyanins give rise to anthocyanidins (aglycones) and sugars. The identification of the aglycone and sugar moieties of unknown pigments is most satisfactorily accomplished by chromatographic comparison on paper with standard markers. The type of procedure to be used has been described by HARBORNE AND SHERRATT[18] and NORDSTRÖM[19] and works well with the amounts of anthocyanin that can be obtained after separation and purification on paper.

The choice of solvent for the chromatography of anthocyanidins is limited by the fact that they are even less stable than the anthocyanins to pH and light. For example, anthocyanidins tend to fade when chromatographed in BAW. For this reason satisfactory solvents for anthocyanidins usually contain mineral acid and the "Forestal" solvent (acetic acid–conc. HCl–water, 30:3:10 v/v) has been widely used[40]. A similar type of solvent system (formic acid–conc. HCl–water, 5:2:3 v/v) is also useful, as with this mixture, anthocyanidins are particularly well separated from unhydrolysed anthocyanin, the presence of which can cause confusion on "Forestal" chromatograms. BAW may be used for anthocyanidins, if the filter paper used is previously washed with dilute hydrochloric acid. A number of other solvent systems have been suggested for anthocyanidins (*e.g.* BuHCl and *m*-cresol–acetic acid–conc. HCl, 1:1:1 v/v) but none of these can be considered to be completely satisfactory due to tailing of the pigment spots and the presence of "chloride" fronts on chromatograms developed with these mixtures. Anthocyanidins have also been separated on a larger scale by column chromatography on cellulose[41] or silicic acid[42].

The regular relationship that exists between R_F value and chemical structure

TABLE V

R_F VALUES AND COLOURS OF ANTHOCYANIDINS

	R_F values in			Visible	Colours in ultra-violet
	Forestal	Formic	BAW*		
Hirsutidin	0.78	0.36	0.66	purple	mauve
Malvidin	0.60	0.27	0.58	purple	mauve
Petunidin	0.46	0.20	0.52	purple	mauve
Delphinidin	0.32	0.13	0.42	purple	mauve
Rosinidin	0.76	0.39	—	magenta	pink
Peonidin	0.63	0.30	0.71	magenta	pink
Cyanidin	0.49	0.22	0.68	magenta	pink
Pelargonidin	0.68	0.33	0.80	red	orange-red
Luteolinidin	0.61	0.35	0.56	orange	red-brown
Apigeninidin	0.75	0.44	0.74	yellow	yellow-brown

* Run on acid-washed paper.

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with the anthocyanins is also found in the anthocyanidin series. The distance travelled in "Forestal" solvent is directly related to the number of free phenolic hydroxyl groups and the most highly hydroxylated anthocyanidin, delphinidin, has the lowest R_F value (Table V). On the other hand, methylation of the hydroxyl groups reverses this effect in a regular way, as can be seen in the series delphinidin, petunidin, malvidin and hirsutidin.

The sugars produced from anthocyanins on acid hydrolysis are identified by the usual chromatographic procedures (for review, see LEDERER AND LEDERER[43]).

The use of either di-*n*-octylmethylamine in chloroform[18] or of ion exchange resins[7] is recommended for the removal of mineral acid from the sugar hydrolysates before chromatography. It is important to distinguish between anthocyanins containing glucose and those containing galactose, so that at least one chromatogram should be developed with a suitable solvent system for 2–3 days, in order to separate these two sugars. At the same time, disaccharides, which are occasionally present in anthocyanin hydrolysates, are more easily detected. When two isomeric 3-diglycosides of the same anthocyanidin are known, for example of pelargonidin[17], the identification of the disaccharides found in this way becomes important.

One difficulty has arisen in this method peculiar to anthocyanins which have been purified by paper chromatography. It has been found[17] that arabinose is produced as an artifact by the action of mineral acid in the solvent system on filter paper. This arabinose then interferes with the normal detection of the sugars and gives misleading results. Other workers (NORDSTRÖM[19], ASEN, SIEGELMAN AND STUART[44]) have also been misled by the presence of arabinose in their sugar hydrolysates arising in this manner[17,37]. The production of arabinose can be avoided by carefully replacing the mineral acid used during the purification process by acetic acid. It has also been found advisable to wash the filter paper before use with dilute acetic acid to remove any soluble impurities.

Anthocyanins purified for sugar analysis should not be contaminated with free sugars or other plant glycosides. Free sugars are readily removed by routinely carrying out chromatography in an aqueous solvent (*e.g.* 15% HAc) during purification. Other plant glycosides are normally removed by using two or more solvent systems during purification, but it is not always possible to be certain that the anthocyanin is finally free from such contamination. Indeed, in two or three instances in this laboratory, incorrect sugar analyses have been attributed to this cause. The remedy is to repeat the sugar analysis on pigment which has been purified in a different set of solvent systems or in the same solvent systems in a different order. It has now become standard practice to do this in all cases where more than one sugar is detected. It should also be noted that anthocyanins which have been purified by column chromatography may be contaminated by free sugars[34,45].

4. DETERMINATION OF THE POSITION AND NUMBER OF SUGAR RESIDUES

The next step in the characterisation of an unknown anthocyanin, once its anthocyanidin and sugars have been identified, is to determine the position and number of

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these sugar residues or to find out to which glycosidic "class" the pigment belongs. Paper chromatography provides an excellent method of classifying anthocyanins in this way. Indeed, as a result of using this method, a number of new glycosidic classes have been found[16,17] and the strict classification of anthocyanins into the four "classes" described by the ROBINSONS[13] can no longer be made, since there are now at least ten such "classes" known. It is preferable to describe anthocyanins according to the actual sugars that are present, rather than to use the vaguer terms of the ROBINSONS' terminology, *i.e.* 3-monoside, 3-bioside, etc.

An unknown anthocyanin can be characterised by a careful comparison of its R_F values in several solvent systems with values of well characterised glycosides of the same anthocyanidin. If the unknown compound has the same R_F values as a well characterised glycoside, co-chromatography will confirm the identification. Even if it is not chromatographically identical with any of the known pigments, these data should provide a clear indication of the number of sugar residues present.

Anthocyanins can be further characterised by studying on paper chromatograms the simpler glycosides produced during acid[17,23] or enzymic hydrolysis[46]. The number

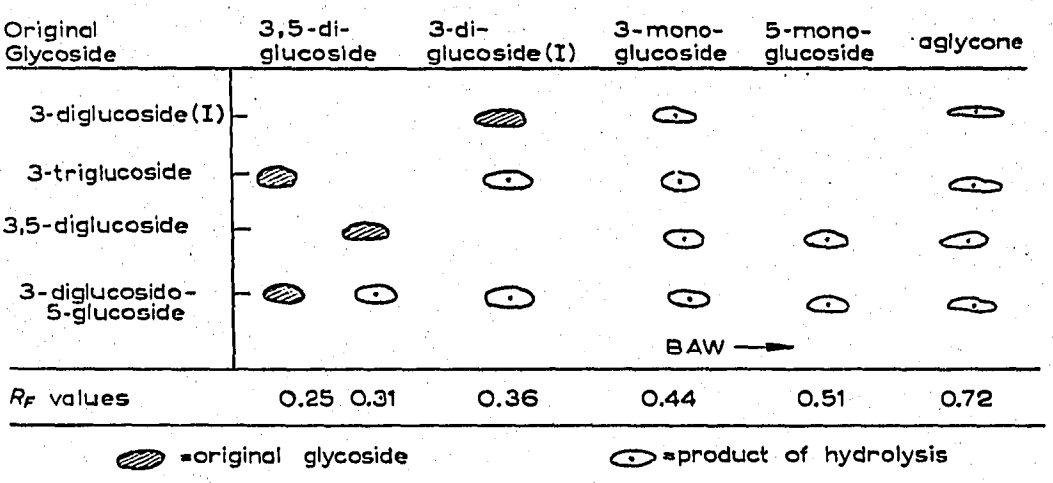

Fig. 3. A chromatogram of the products of controlled acid hydrolysis of some pelargonidin glucosides.

and identity of these simpler glycosides provide a valuable clue to the number and position of the sugar residues in the original glycoside. Thus 3-diglucosides and 3,5-diglucosides are distinguished by the fact that they give one and two simpler glycosides respectively during acid hydrolysis. Anthocyanins containing three sugar residues give two or four simpler glycosides in this way [16,17]. The individual components in the mixture of pigments present when acid hydrolysis is stopped before completion, can be separated and identified on paper chromatograms in the usual manner. The method works particularly well with derivatives of pelargonidin, as can be seen in Fig. 3, which illustrates the general principles of this approach. Alternative solvent systems for separating the glycoside mixtures are BuHCl and 1% HCl.

Identification by the above means depends on having available a number of pigments of known structure. Many of these can easily be obtained directly from plant

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sources, as described in the literature[13,21]. The sources of the new pigments isolated in this laboratory are described elsewhere[22]. The author was also fortunate in having a number of anthocyanins both natural and synthetic, which were left over from earlier work at this Institution. If no such specimens are available, it is advisable to check carefully the identity of plant material used for preparing authentic "markers", especially as different "varieties" of the same plant may differ in their pigment content. In addition, some of the earlier pigment identifications have had to be modified by later work[47].

The accuracy of chromatographic identifications made in the above manner depends on the following factors. (1) No exceptions to the very striking relationship between R_F value and number of sugar residues of the anthocyanin in several different solvent systems have yet been found. (2) Isomeric 3-diglucosides of pelargonidin have different chromatographic properties, so that it is possible to distinguish anthocyanins which only differ in the nature of the linkage between the two sugars that are attached. (3) Chromatographic methods will distinguish glycosides in which the sugars are attached in an unusual position. Thus the first 3,7- or 3,4'-glycoside to be found in nature[17], pelargonidin 3-diglucosido-7(or 4')-glucoside, is chromatographically distinct (see Table II). (4) The high degree of resolution that can be achieved on paper makes it possible to distinguish between 3-monoglycosides, where structural differences between the sugars attached are very small. Thus the pairs of compounds cyanidin 3-monogalactoside and 3-monoglucoside, and cyanidin 3-monoxyloside and 3-monoarabinoside separate in BAW sufficiently, although not into two discrete spots, to show that in such cases two compounds are present[45]. Pelargonidin 3-monoglucoside and 3-monogalactoside are similarly separable and the best results are obtained if the paper is developed with BAW for 36–48 hours, while allowing the solvent to drip off the end of the paper[17,48].

The present limitations of the chromatographic separation of anthocyanins are as follows. The choice of solvent system is limited to two or three main types. The excellent resolutions of different pigments that have been obtained with derivatives of pelargonidin and cyanidin, have not yet been obtained in the delphinidin series. Paper chromatography will not completely resolve acylated anthocyanins if they are of very similar structure.

5. ACYLATED ANTHOCYANINS

Before determining the glycosidic nature of an anthocyanin, it is important to know whether it is acylated or not, since acylation has a profound effect on R_F values. Information on this point can be obtained during the preliminary chromatographic studies. The most characteristic chromatographic feature of acylated pigments is the fact that when they are chromatographed in BuHCl, two spots are shown to be present, a main spot of high R_F value and a minor spot of low R_F. Even if the major component is separated chromatographically from the minor one, on rechromatography two spots will again appear. This point was first noted by DODDS AND LONG[49]

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and is due to the fact that the acyl linkage to the anthocyanin is more labile than the sugar linkages. It is therefore impossible to avoid some deacylation taking place during the preparation of an extract of any acylated pigment. On carrying out the deacylation with alkali and then reacidifying, the pigment appears as a single component of low R_F, the spot of high R_F having disappeared.

A simple test for acylation consists of comparing R_F values in different solvent systems of the pigment before and after alkaline treatment. If there is no change in R_F value, the pigment can be assumed to be free of labile acyl groups. All acylated pigments which have been examined so far do change their R_F value under these

TABLE VI

R_F VALUES OF ACYLATED ANTHOCYANINS

Acylated glycosides		*Acyl group*	*BAW*	*BuHCl*	*1% HCl*	*HAc–HCl*
Aglycone	*Sugars**					
Pelargonidin	3RG5G	*p*-coumaric	0.37	0.43	0.27	0.67
Cyanidin	3RG5G	*p*-coumaric	0.32	0.26	0.22	0.62
Peonidin	3RG5G	*p*-coumaric	0.34	0.31	0.22	0.62
Delphinidin	3RG5G	*p*-coumaric	0.31	0.24	0.19	0.59
Petunidin	3RG5G	*p*-coumaric	0.32	0.26	0.19	0.59
Malvidin	3RG5G	*p*-coumaric	0.36	0.28	0.20	0.64
Pelargonidin	3G5G	*p*-coumaric	0.40	0.46	0.19	0.53
Cyanidin	3G5G	*p*-coumaric	0.35	0.34	0.11	0.43
Delphinidin	3G5G	*p*-coumaric	0.30	0.22	0.05	0.32
Pelargonidin	3G5G	caffeic	0.37	0.37	0.17	0.48
Pelargonidin	3GG5G	*p*-coumaric	0.34	0.34	0.49	0.73
Pelargonidin	3GG5G	ferulic	0.34	0.26	0.49	0.73

* Abbreviations: 3RG5G = 3-rhamnoglucosido-5-glucoside; 3G5G = 3,5-diglucoside; 3GG5G = 3-diglucosido-5-glucoside.

conditions. The identification of the acyl component, which is most frequently *p*-hydroxycinnamic acid or a similar organic acid, can also most conveniently be carried out by chromatographic means. The procedures for doing this have been already described[50–53]. The detection of such a compound, after alkaline hydrolysis of the pigment, confirms the identification of that pigment as an acylated anthocyanin.

The R_F values and structures of some typical acylated anthocyanins are given in Table VI. The fact that they are fairly clearly distinguishable in chromatographic behaviour from simple glycosides is illustrated by a comparison of R_F values of simple and acylated derivatives of pelargonidin (see Tables II and VI). As has already been mentioned, acylated derivatives have high R_F values in butanolic solvents, but relatively low R_F values in aqueous solvents. The R_F values of the acylated anthocyanins, shown in Table VI are consistent with differences in structure and the expected regular relationships hold good. It is also to be noted that changes in the acyl group are sufficient to alter R_F values in one or more solvent systems.

One final point about the chromatography of acylated anthocyanins must be made. This is that, in general, they do not separate as satisfactorily as simple glycosides when present together in plant extracts. In some cases when two pigments such

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as the acylated 3-rhamnoglucosido-5-glucosides of pelargonidin and peonidin give clearly separated bands on chromatograms, it has been noticed that one of these bands is contaminated by the presence of the other pigment and one or more steps in purification are necessary to free the one pigment from the presence of the second pigment. Mixtures of acylated anthocyanins based on delphinidin, petunidin and malvidin are even more reluctant to separate on paper chromatograms.

6. EXAMPLES OF THE USE OF CHROMATOGRAPHIC METHODS

Chromatographic methods have largely replaced the ROBINSON colour and distribution tests for studying the widespread occurrence of anthocyanins in plants. Thus recent surveys of the anthocyanins in some tropical plants by FORSYTH AND SIMMONDS[27], in mountain plants by HAYASHI AND ABE[54] and in garden plants by REZNIK[10] have all been made by means of paper chromatography. The results are in general agreement with the earlier survey of LAWRENCE, PRICE, ROBINSON AND ROBINSON[12], in which the older methods were used. Chromatographic methods more clearly distinguish mixtures of pigments and the recent work has shown that the complexity of pigmentation in some plants is greater than was earlier supposed. The accuracy of the new methods is however no greater than that of the distribution tests, if identification is based solely on the measurement of R_F values and colour of spots in crude plant extracts. As has been mentioned, the R_F values of anthocyanins in such extracts can be affected by the presence of other components. In addition, a single anthocyanin may give rise to several spots on a chromatogram, if acid or enzymic hydrolysis of acyl or sugar groups takes place during the preparation of the extract. For these reasons, the wide range of values obtained by FORSYTH AND SIMMONDS[27] for cyanidin glycosides present in the plants of Trinidad, does not necessarily indicate a similar range of variation in glycosidic or acylated forms.

There is no doubt, however, that anthocyanins do occur in a greater number of different glycosidic combinations than the earlier work of ROBINSON AND ROBINSON[13] disclosed. The detailed chromatographic examination of more anthocyanins, on the lines of our recent investigation of pelargonidin derivatives[17], will surely reveal some further glycosidic variants.

Chromatographic procedures have also been used for studying in some detail the distribution of anthocyanins within a particular genus or order in connection with taxonomic investigations. A chromatographic examination of the anthocyanins of the petal, base and stamen of five species of *Papaver* has shown that the different species can be distinguished in this way[24]. This is because the distribution of the six anthocyanins in the flowering organs of *Papaver* plants is relatively complex. Chromatographic surveys of the anthocyanidins of the *Primulaceae*[14] and *Solanaceae*[55] have also yielded some results of taxonomic interest. At the same time, a new anthocyanidin, rosinidin was discovered[14] and on the basis of the chromatographic and spectral properties, a structure has been advanced for it. The identification of anthocyanidins

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formed from leuco-anthocyanins present in plant tissue has been made with the aid of chromatographic methods[40,56].

The most valuable application of chromatographic methods in the identification of anthocyanins has undoubtedly been and will continue to be in the field of biochemical genetics. The anthocyanins were the first group of plant substances in which the relationship between single genes and simple biochemical differences was demonstrated[57]. Although much work was done in this field by LAWRENCE[57], SCOTT-MONCRIEFF[58] and others, much remains to be done in more detailed and thorough investigations of the pigments present in the colour mutants of garden plants. The limiting factors in this type of investigation are shortage of plant material, the need for identifying all the pigments, including those present in trace amounts, and the difficulty of separating the complex mixtures encountered in the petals of hybrid plants. With the aid of paper chromatography, these difficulties can largely be overcome and considerable progress has been made recently in this field. Some of the results that have been obtained have been briefly summarised by the author[59]. A number of garden plants, in which flower colour variation occurs, have now been examined by chromatographic procedures. They include *Antirrhinum majus*[6], *Cyclamen europeaum*[60,61], *Dahlia variabilis*[7], *Impatiens balsamina*[62], *Primula sinensis*[63], *Streptocarpus*[64], *Solanum phureja*[15,49], and *Viola tricolor*[65].

Finally, chromatographic methods are useful for examining any anthocyanin of which the structure has not been fully determined by earlier investigators. There are many pigments which fall into this category. The presence in nature of new types of anthocyanidins is also revealed by chromatographic investigations[14]. Even if such pigments cannot be fully characterised by means of paper chromatography, it is at least possible to show that they are different from any of the known compounds. For example, the pigment of *Spirodela oligorrhiza*, thought to be a cyanidin glycoside by THIMANN AND EDMONDSON[66], has recently been re-investigated by GEISSMAN AND JURD[67] using chromatographic means. It is clear from these later results that the pigment is not directly related to any of the known anthocyanins.

From the above examples, it will be seen that chromatography has already established itself as a most valuable technique in the study of the anthocyanin plant pigments.

ACKNOWLEDGEMENTS

The author is grateful to Drs. E. C. BATE-SMITH, K. S. DODDS and H. S. A. SHERRATT for their helpful criticism of this manuscript.

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Received June 19th, 1958