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INTRODUCTION

The existence of colours in plants in bright contrast to the overwhelming greenness of the vegetative parts has challenged scientific research in various disciplines. The flower is the most important organ in which the widest and full range of colour variations are most obviously realized. Although the colour range in most garden plants is already very extensive, plant breeders are still searching for new and unusual colour forms, and a conscious selection for subtle shades, tints and colour patterns has been a continuing endeavour.

Among the pigments responsible for the varied and attractive colours of flowers are the water-soluble flavonoids. The compounds are characterized by the presence of a $C_6-C_3-C_6$ structure in which two benzene rings are joined together by a 3-C link, and the state of oxidation of the 3-C link distinguishes the various classes of flavonoids from one another.

There are several classes of flavonoids distributed in nature, but only four are directly involved in plant colouration. They are: flavones/flavonols, chalcones, aurones and anthocyanins, all occurring commonly as glycosides.

The flavones and flavonols, with the exception of a few with certain structural modifications, are responsible for a large portion of ivory, white or cream flowers which would otherwise appear translucent. The yellow colour in flowers is

due mainly to fat-soluble carotenoid pigments, but the chalcones, and the aurones which may or may not accompany the carotenoids are also responsible. On the other hand, the flavonols with certain modifications of hydroxylation, methylation and glycosylation patterns also contribute to the yellow colouration. Finally, the anthocyanins are the most widespread group, contributing to the red, scarlet, crimson, orange, pink, mauve, blue and purple colours of flowers.

The contribution of flavonoids to flower colours has been reviewed by many authors (Scott-Moncrieff, 1936; Blank, 1947; Paech, 1955; Reznik, 1956; Harborne, 1965, 1967a).

From these reviews and innumerable investigations in this field, it has been found that the majority of bright cyanic flower colours are produced by a surprisingly small number of anthocyanidin pigments. These, either singly or in combination, are responsible for many colour variations. The structural modifications of anthocyanidins involving hydroxylation, methylation and glycosylation can influence flower colour from red to blue. Mixture of two or more anthocyanins, their proportion and concentration also effect flower colour considerably.

pH is known to effect the colour of anthocyanins which *in vitro* is red in acid and blue in an alkaline medium. Karrer (1928) observed a correlation of higher mineral salt content in blue flowers relative to red flowers. However, a

general survey of the cell sap pH of a wide variety of plants by Hayashi and Isaka (1946) has shown that it is almost always acidic, i.e., about 4.2 to 5.5. Recent investigations on the structural modifications of the pigments as a function of pH have revealed that in the above-mentioned range of pH, the pigments within the cell should be virtually colourless. Explanations therefore have been sought to understand the appearance of a blue colour in such situations.

Several phenomena such as the formation of metal-complexes, co-pigmentation, adsorption of pigments to polysaccharides have been implicated in the blue colour forms. It has been also equally challenging to understand why the blue colour forms are totally absent in some flowers, such as the roses, despite the presence of required factors.

The flower pigmentation of *begonia tuberhybrida*, a popular ornamental plant, is interesting, in that the flowers appear in all shades of colour, except blue, purple, mauve, violet, or related shades.

In his work on the application of thin-layer chromatography for the identification of anthocyanidins, Nybom (1964) identified cyanidin in flowers of *begonia tuberhybrida* which were used as a test material among a number of other plants.

Harborne and Hall (1964), while surveying the anthocyanins of higher plants, have studied the various glycosidic patterns in the flowers and leaves of some 15 species and cultivars within the genus *Begonia*, without specifying the flower colour of the varieties selected. They found cyanidin and pelargonidin 3-glycosides to be the main anthocyanins which occurred, in most cases along with some quercetin and kaempferol glycosides. The anthocyanin glycosides include cyanidin 3-glucoside from *B. manicata*, pelargonidin 3-sophoroside in *B. sutherlandii*, cyanidin 3-sophoroside in *B. [illegible]* v. rosea, cyanidin 3-sambubioside in *B. [illegible]* as well as *B. manicata*. Certain other species contain either of these two cyanidin biosides or both, along with two newly identified cyanidin 3-triosides, namely cyanidin 3-xylosylrutinoside and cyanidin 3-glucosylrutinoside. The authors have not reported the occurrence of any other glycosidic patterns in *Begonia* species.

With the exception of the above reports, there is no other work on the flower pigments of *Begonia* plants. The range and type of colour variations in *Begonia tuberhybrida* therefore offers a challenge for determining the pigment composition and the effect of modifying factors upon flower colour.

The present research undertakes to investigate the variations in flower colour in *Begonia tuberhybrida*. Seven varieties

ranging from white, yellow, salmon, orange, orange-red and deep red were selected to study the following:

1. The contribution of anthocyanins to flower colour;
2. The factors affecting the colour variations, with particular attention given to explain the absence of the blue colour forms, and the predominance of the red and related shades.

Several proposals can be put forth to explain these colour variations, as follows:

1. Variations in the anthocyanidins present in each colour form.
2. Variations in the relative proportions of the anthocyanidins present.
3. Quantitative differences in the total anthocyanins among the cultivars.
4. Variations in the glycosidic forms of single or mixtures of anthocyanidins.
5. Effect of the pH of the cell sap.
6. Absence of co-pigmentation and metal chelation.
7. Effect of metabolic products in the cell sap that are most likely to affect its pH.

The aim of this study was to test the above proposals and determine the actual factors responsible for colour variations among distinct colour forms of *Begonia tuberhybrida*

To achieve this aim, the following research plan was formulated.

1. Study of the nature of anthocyanidins present and their relative concentration among the various colour forms.
2. Preliminary studies on the crude pigments and chromatographic survey of the anthocyanin glycosides.
3. Isolation and identification of the major anthocyanin glycosides based on chromatographic and spectral studies, followed by:
   (a) complete hydrolysis and identification of the aglycones and sugar moieties;
   (b) selective hydrolysis to determine the position of the sugar residues.
4. Determination of the cell sap pH.
5. Study of the evidence for the presence (or absence) of factors influencing co-pigmentation and metal chelation.
6. Examination of various metabolic components existing in the free state within the cell sap, namely organic acids, free sugars, phenolic acids and amino acids.

The above investigation was for the most part accomplished by chromatographic and spectrophotometric methods.

REVIEW OF LITERATURE

I. CHEMISTRY OF ANTHOCYANINS

The common anthocyanins existing in nature are glycosides of anthocyanidins, whose basic structure is the flavylium cation (Figure 1).

I.1. The Anthocyanidins

The naturally-occurring anthocyanidins and 3-deoxyanthocyanidins identified up to the present time are given in Table 1.

The visible colour of anthocyanidins varies with the hydroxylation or methylation of the benzene rings. Six are widespread in nature: pelargonidin, cyanidin, delphinidin, malvidin, peonidin, and petunidin. Of these, cyanidin is the most common. Examples of their contribution to flower colour in ornamental plants are given in Table 2.

In addition to the anthocyanidins listed in Table 1 are those which are formed by acid treatment of leucoanthocyanidins (Mathew, 1969), proanthocyanidins or 3-hydroxyflavones (Jurd, 1969), carexidin, the new 3-deoxyanthocyanidin (Clifford and Harborne, 1969) and the two new violet pigments in *Sphagnum nemoreum* (Nilsson, 1967).

Due to their instability, anthocyanidins are not normally found in nature in free state, except a few rare cases

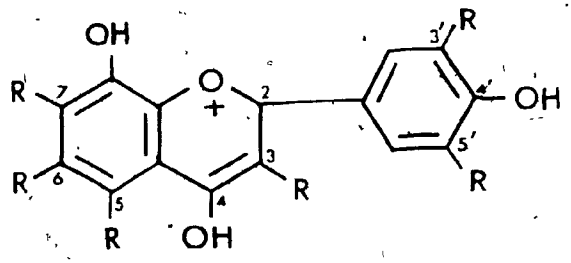


Figure 1.- The flavylium cation.

TABLE 1.- Naturally occurring anthocyanidins and 3-deoxyanthocyanidins*.

Pigment	Substitution pattern (R)						Visible colour
	3	5	6	7	3'	5'	
Apigeninidin	H	OH	H	OH	H	H	Yellow
Luteolonidin	H	OH	H	OH	OH	H	Orange
Tricetinidin	H	OH	H	OH	OH	OH	Orange-red
Pelargonidin	OH	OH	H	OH	H	H	Red
Aurantinidin	OH	OH	OH	OH	H	H	Orange-red
Margicassidin	OH	OH	alkenyl	OH	H	H	Magenta
Cyanidin	OH	OH	H	OH	OH	H	Magenta
Peonidin	OH	OH	H	OH	OMe	H	Magenta
Rosinidin	OH	OH	H	OMe	OMe	H	Magenta
Delphinidin	OH	OH	H	OH	OH	OH	Purple
Petunidin	OH	OH	H	OH	OMe	OH	Purple
Pulchellidin	OH	OMe	H	OH(?)	OH(?)	OH	Purple
Europinidin	OH	OMe	H	OH(?)	OMe	OH	Purple
Malvidin	OH	OH	H	OH	OMe	OMe	Purple
Hirsutidin	OH	OH	H	OMe	OMe	OMe	Purple
Capensinidin	OH	OMe	H	OH(?)	OMe	OMe	Purple

* Adapted from Timberlake and Bridle, 1975, p. 217.

TABLE 2.- Contribution of anthocyanidins in flower colours of ornamental plants.*

Plant, colour forms and typical variety	Pigment(s) present
SNAPDRAGON (*Antirrhinum*)	
Magenta	Pure cyanidin (Cy)
Orange-red	Cy and yellow aurone
Pink	Pure pelargonidin (Pg)
Orange-yellow	Pg and yellow aurone
SWEET PEA (*Lathyrus odoratus*)	
Deep purple,"Jupiter"	Malvidin (Mv)
Pale mauve,"Elizabeth Taylor"	Mv
Deep crimson,"harrow"	Peonidin (Pe) + some Cy
Carmine,"Carlotta"	Pe
Pink,"Mrs. R. Bolton"	Pg
Scarlet,"Air Warden"	Pg
HYACINTH (*Hyacinthus orientalis*)	
Deep red,"Scarlet O'Hara"	Pg + 10% Cy
Pink,"Pink Perfection"	Pg and Cy (6 : 4)
Mauve,"mauve Queen"	Pure Cy
Blue,"Delft Blue"	Delphinidin (Dp) + 10% Cy
Pale blue,"Spring Time"	Pure Dp
ROSE (*Rosa*)	
Mauve,"Reine de Violette"	Cy (co-pigmented)
Crimson,"Rose Bourbon"	Cy
Pink,"Roserai de l'Haye"	Pe and Cy (1 : 1)
Scarlet,"Will Scarlet"	Pe and Cy (2 : 1)

* Adapted from Harborne (1967a)

recently reported: malvidin in *Lathyrus sativus L.* (Pecket, 1960), cyanidin in *Hibiscus mutabilis* (Lowry, 1971).

I.2. The Anthocyanin Glycosides

There are 29 classes of anthocyanin glycosides known at the present, excluding the acylated anthocyanins. These comprise the 21 classes listed by Harborne (1967a) and some new ones recently compiled by Timberlake and Bridle (1975).

In spite of the considerable variations, some common characteristics are noted:

(i) There is always a sugar substitution at the 3-position which appears to be preferred, except in the case of the 3-deoxyanthocyanins where the sugar is usually in the 5-position. Some authors have recently reported a 7-monoside pattern (Sun and Francis, 1967; Tanchev and Timberlake, 1969) and a 4'-monoside pattern (Hedin *et al*, 1968).

(ii) If a second position in the molecule is glycosylated, it is the 5 or 7 position.

(iii) Substitution on a B-ring hydroxyl is not recorded, except the new 4'-monoside pattern mentioned above.

(iv) The sugar moieties include simple hexoses and pentoses (monosides) and di- and trisaccharides (biosides and

triosides), the latter being linear or branched. They are always attached to the aglycone through the C_1 position. D-glucose, occurring either alone or as part of a disaccharide or trisaccaride, is the most common sugar in glycosides. D-galactose and L-rhamnose are less frequent, and L-arabinose and D-xylose are rather rare. Of the 29 classes cited, the most frequently encountered is the 3-glucoside and to a lesser extent, the 3-rutinoside.

The occurrence in plants of acylated anthocyanins has been known for a long time. Generally speaking, a molecule of the organic acid is esterified to the sugar hydroxyl and the phenolic hydroxyl group is not involved. Most of the acylated anthocyanins described up to date (Harborne, 1967a; Timberlake and Bridle, 1975) are based on 3,5-diglucosides (except certain acylated 3-glucosides occurring in grapes) where the acid molecule -- p-coumaric acid in most cases, caffeic or ferulic in a few others -- is always attached to the sugar molecule in the 3-position.

II. STRUCTURE AND PROPERTIES

The monomeric anthocyanins are based on the flavylium salt structure. They are usually presented as having their positive charge associated with the heterocyclic ring. However, it is currently recognized that the charge is delocalized over the whole structure which is depicted

according to the formula of Bendz *et al* (1967) (Figure 2) but for the sake of convenience in discussing mechanisms, the more conventional formula as shown in Figure 1 will be used.

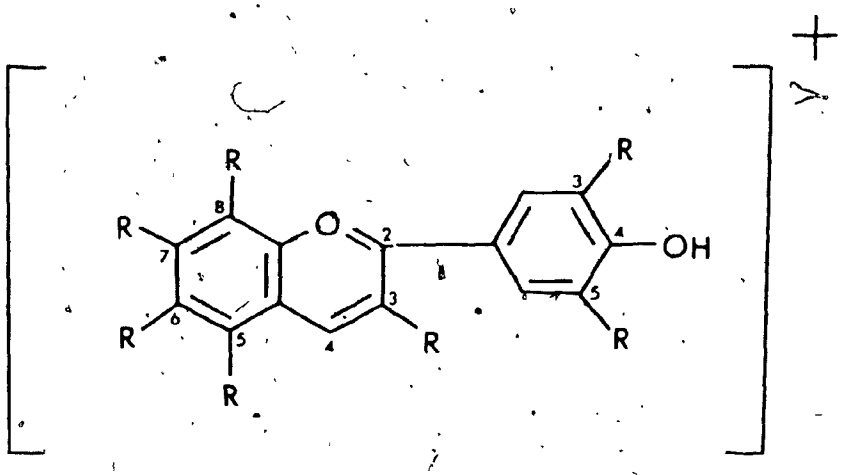


Figure 2

The flavylium cation

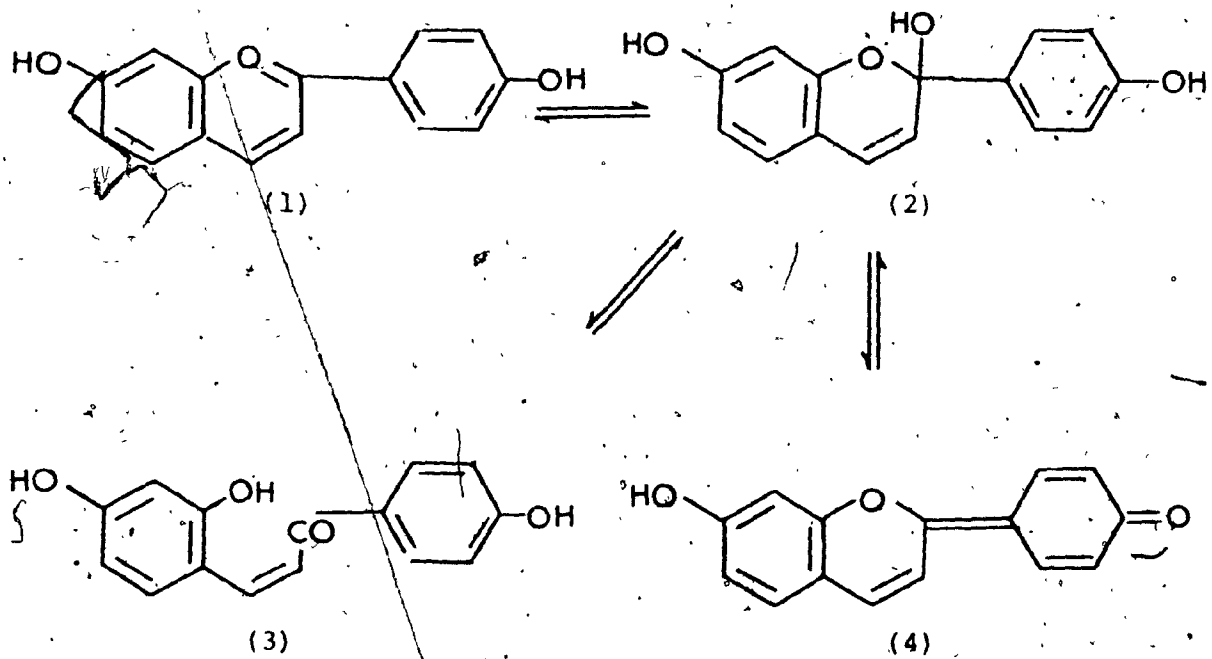
Among several chemical and physical properties related to the behaviour of anthocyanins *in vivo*, the structural transformations induced by pH variations and the formation of complexes with metals and co-pigments are the reactions most commonly encountered in nature. They result in the production of the full range of colour variations seen in flowers.

II.1. Structural Modifications as a Function of pH

The behaviour of anthocyanins as a function of pH varies according to their structure and depends especially on the

nature of the substitution at the carbon atom 3. The phenomenon has been well investigated and certain significant facts have been established during the last decade (Jurd, 1963; Jurd and Geissman, 1963; Harper, 1967; Harper and Chandler, 1967a, 1967b).

Basically, the flavylium salts are cationic in acid solution (1), these forms being responsible for the red colours of anthocyanins. With regard to anthocyanidin, when the pH is raised to near neutrality, more and more of the pigment is transformed to the colourless pseudobase form (2) which undergoes ring opening to form the corresponding chalcone (3).

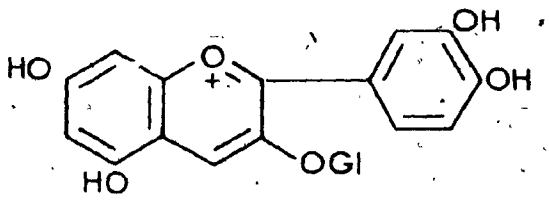


At pH above 7, the anthocyanidin can form blue anhydrobase (4) which is unstable unless the 3-hydroxyl group is glycosylated. Ionization of the phenolic hydroxyl groups occurs with further increase of pH, making the anthocyanidin more and more unstable, which then undergoes ring cleavage and aerial oxidation. Reacidification does not yield the original anthocyanidin.

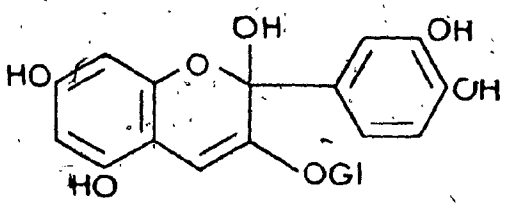
Jurd and Asen (1966) have studied the behaviour of cyanidin 3-glucoside in varying pH conditions and have demonstrated the influence of pH on the colour and stability of the pigment.

Within the pH range of 1 - 8, the intensity of absorption of the pigment markedly decreases as the pH is raised. Below pH 4, the red oxonium salt of cyanidin 3-glucoside has a λmax of 510 nm. In the pH range of 4 - 5, the solution is virtually colourless, the pigment being almost immediately and quantitatively converted through the transient anhydrobase form into a colourless carbino- or pseudobase. Above pH 5, the purple anhydrobase becomes more stable and has a λmax of 538 nm. It is most stable at pH 6.10. Between pH 6.1 to 7, partial ionization of anhydrobase takes place, the solution becomes stable and deep blue. Above pH 12, the molecule undergoes ring opening to convert to chalcone.

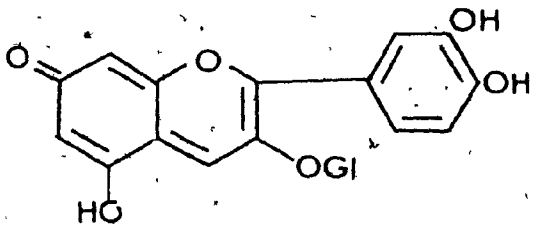
In nature, the pH range of 4 - 5 is the one most commonly found in the cell sap of coloured plant organs. It is understood that at this pH range, the anthocyanin is

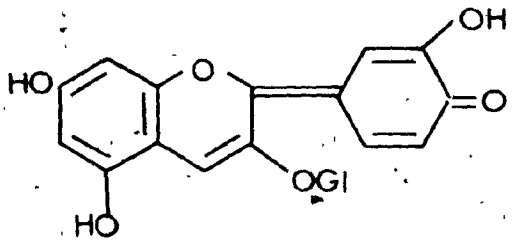


pH 4. Red oxonium salt of cyanidin 3-glucoside (λmax 510 nm)



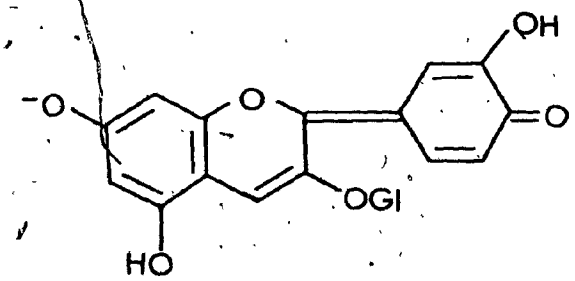
pH 4 - 5. Carbinobase or pseudobase (colourless)

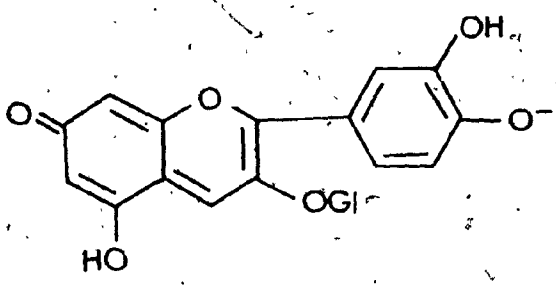




pH 5. Anhydrobase (purple)

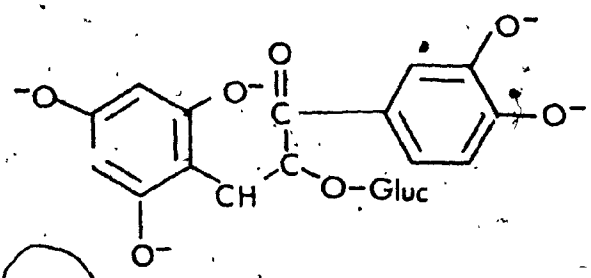
λmax 538 nm





pH 6.10 - 7. Partial ionization of anhydrobase

Blue - λmax 538 - 550



pH 12. Chalcone (yellow)

immediately converted into its colourless carbinobase or pseudobase, and by itself could not contribute significantly to the coloration of flowers or fruits. It is in this context that the phenomena of metal-complexing and co-pigmentation arise, to offer an explanation for the presence of certain forms of blueness which are not due to the existence of natural single pigments, such as delphinidin.

II.2. Effect of Co-pigmentation and Metal Chelation.

The structural changes that anthocyanins undergo to form stable blue pigments have been considered to be *in vivo* modifications produced by co-pigmentation, metal-complexing, or by a combination of both factors.

II.2.1. Co-pigmentation.

The explanation of the bluing of anthocyanins as a result of a colloidal bonding or "co-pigmentation" with flavones and related compounds was first offered by G.M. and R. Robinson in 1931. This has led to a number of recent investigations in which various compounds were added to pigment solutions at different pH, to study their effect (Jurd and Asen, 1966; Asen *et al*, 1972) as well as other investigations where pigments suspected to be formed by co-pigmentation were isolated and analyzed for their composition (Pecket, 1960, 1962; Harborne, 1963; Asen *et al*, 1970, 1973, 1975; Yazaki, 1976).

In the light of the above studies, the factors known to affect co-pigmentation of anthocyanins can be summarized as follows:

1. The phenomenon is basically influenced by the internal environment of the vacuole in which the pigments are present.

2. Glycosides of the six common anthocyanins all form co-pigment complexes with other flavonoids (flavones, flavonols, etc.), as well as other phenolics (tannins, phenolic acids, etc.). The phenomenon can occur with both the flavylium salt and the anhydrobase and is therefore independent of pH..

3. The formation of co-pigment--pigment complexes produces a bathochromic shift in the visible maxima of the anthocyanins, and also increases their intensity.

4. The degree of co-pigmentation is a function of the concentration of the anthocyanins and the molar ratio of the co-pigments to anthocyanins.

Based on the above characteristics, it is apparent that, under normal conditions found in the cell sap in nature, co-pigmentation could easily occur, making it a widespread phenomenon responsible for a large part of the blueness in many flowers.

II.2.2. Metal-complexing.

Compared to the co-pigmentation, metal-complexing is somewhat restrained: while co-pigmentation occurs with glycosides of all common anthocyanidins, metal chelation takes place only with anthocyanins that have at least two adjacent free hydroxyl groups on the second benzene ring "B" (Bayer *et al*, 1966), i.e., the glycosides of cyanidin, delphinidin and petunidin only.

Starting with the isolation of the blue cornflower pigment termed "protocyanin", Bayer (1958) analyzed and found it to be a chelate complex of cyanidin 3,5-diglucoside (cyanin) with iron and aluminum, and that the complex had spectral properties very similar to that of a synthetic anthocyanin-metal mixture. Since then, all kinds of metal complexes have been reported to occur in the cornflowers: a pigment complex of cyanin, magnesium and iron identified by Hayashi *et al* (1961); a complex of cyanin, iron, potassium and magnesium isolated by Saito *et al* (1961); a complex of iron, anthocyanin and three molecules of a "bisflavone" isolated from cornflower by Asen and Jurd (1967). Complexes in other plants have also been reported: a complex of aluminum, iron and delphinidin in the blue forms of *Lupinus* (Bayer, 1959) and a complex of iron, a tannin-like substance and cyanin in the blue spherules of the bluing petals of red rose reported by Yasuda (1970).

Metal complexes can occur along with co-pigmentation, but

when co-pigments are in high concentration, it is likely that the metal ions are preferentially bound by the flavonol glycosides and an anthocyanin pseudobase is formed (Bayer *et al*, 1966). On the other hand, a pH range of 2 - 6 and high concentration of anthocyanins are necessary conditions for metal complexing to occur (Asen *et al*, 1969). More specifically, Jurd and Asen (1966) have shown that the phenomenon depends not only on the pH, but also on the type of the organic acids which contribute to the buffering system of the cell sap. In their *in vitro* studies, the acetate buffer presented a favourable medium for complex formation to occur, whereas the citrate buffer did not allow such complexes to form. Apparently, the citrate ions themselves preferentially complex with metals and make them unavailable for reaction with pigments.

In resume, the requirements for bluing of anthocyanins by metal chelation are: (a) an anthocyanin which possesses an ortho-dihydroxyl system on its B ring; (b) a chelating metal; (c) high pH; and (d) absence or low concentration of other metal chelators, such as flavonols or citrate ions.

III. CONTRIBUTION TO FLOWER COLOUR

While the number of anthocyanidins in nature is very limited and the pH of the cell is such that many anthocyanin-containing flowers would be virtually colourless, flowers have been observed in a very wide range of colours, ranging

from red to violet. On one hand, many flowers with sharp differences in colour have been found to contain the same anthocyanins (Pecket, 1960; Ashtakala and Forward, 1971). On the other hand, the reverse phenomenon is known where flowers of similar colours contain different anthocyanins (Saito, 1967; Stewart *et al*, 1969).

Several causes have been found to explain the variations in flower colour in cyanic flowers:

1. The basic explanation is that the flower colour is the direct result of the nature of the aglycone present. In general, when metal complexing and co-pigmentation are not involved, the flowers take the colour of the existing aglycone. In the case of a mixture of anthocyanins, the flower colour is determined by the colour of the anthocyanidin that occurs in highest concentration. When several anthocyanidins exist in approximately the same concentration, they can combine to yield intermediate shades. Examples of all of the above cases can be found in Table 2.

The correlation between the nature of the aglycones and the colours they produce is as follows:

(i) Hydroxylation of the B-ring is the predominant factor. Generally speaking, flowers having a red-orange or orange coloration can be expected to contain pelargonidin (1 OH), those which are red have cyanidin (2 OH), and those which

are purple or blue contain delphinidin (3 OH). Furthermore, the total absence of an OH in position 3 causes a shift towards yellow colour.

(ii) Methylation of the hydroxyl groups leads to a hypsochromic shift, resulting in a change in colour towards the red. As a matter of fact, all the methyl substituted pigments are found to have a magenta or purple colour. The shift is small in the case of substitution of hydroxyl groups on the B-ring, proved by malvidin (λmax 542 nm) which is only slightly redder than delphinidin (λmax 546 nm), but it is important in the case of a hydroxyl in position 7 giving hirsutidin (λmax 536 nm).

2. Variations are related to the nature and position of the sugar residue or residues of the aglycones (the glycosidic patterns):

The spectral difference between an aglycone and its glycoside is a hypsochromic shift of about 12 nm. However, aglycones do not normally occur in the free state in petal tissue, except a few rare cases as mentioned earlier. In general, the colour differences between various glycosides of a given anthocyanidin are not remarkable, although the 3,5-diglycosides are reported to have a bluer colour than the 3-glycosides.

3. Variations are related to the concentration of anthocyanins: In this case, flowers displaying distinct colours

are found to contain the same anthocyanins, and the colour variations are determined by the concentration of the pigments. Ample evidence has been produced to substantiate this fact (Pecket, 1960; Ahuja *et al*, 1963; Ashtakala and Forward, 1971; Ashtakala and Schwartz, 1971).

Indirectly contributing to this effect is the level of free sugars in the cell sap, since the anthocyanin accumulation in plants is generally stimulated by sugars (Thimann and Edmonson, 1949; Thimann *et al*, 1951; Faust, 1965).

4. Adsorption of anthocyanins on polysaccarides, pectin or protein compounds in the cell sap is known to cause a blue coloration in flowers (Bayer *et al*, 1966; Lowry and Chew, 1974).

5. Effect of co-pigmentation and metal-complexing:

The effect of these two factors in the blue coloration of anthocyanins has been previously discussed. The blueness of flowers of *Lathyrus* hybrids (Pecket, 1962), "Prof. Blaauw" iris (Asen *et al*, 1970), *Limonium* cultivars (Asen *et al*, 1973), *Fuchsia hybrida* (Yazaki, 1976) have been explained on the basis of co-pigmentation. On the other hand, many blue metal complexes have been isolated and identified, some typical examples of them have been previously cited (Hayashi *et al*, 1961; Saito *et al*, 1961; Asen and Jurd, 1967; Yasuda, 1970, 1973, 1974).

6. Variations related to the pH of the cell sap:

This could be the most important factor which intervenes in the manifestation of anthocyanin pigments in flower colour.

In vitro, it has been clearly known that anthocyanins easily change their colour from red to blue with pH changing from acidic to alkaline. *In vivo*, the process is not as simple, and anthocyanins do not assume a blue coloration just because their environment is alkaline. In fact, such a condition does not exist in nature; as it has been shown that the cell sap of all plants is almost always acidic (Hayashi and Isaka, 1946).

The role of pH in colour determination and colour changing process in flower petals has been demonstrated by many workers. Asen *et al* (1973), by measuring the pH of epidermal peels of *Statice* flowers *in vivo*, have found that the colours were primarily influenced by the pH of the tissue. Nozollillo (1969) and Yazaki (1976) have shown clear evidence of the changing of colour in relation to pH in aging *Fuchsia* flowers.

In general, flowers take the colour of the existing aglycones when the cell sap is highly acidic, and under such conditions, the phenomena of co-pigmentation and metal-complexing in particular do not occur. In the pH range of 4 to 5, anthocyanins are virtually colourless, and both the phenomena of

co-pigmentation and metal-complexing are usually favoured, resulting in a display of blue colours, provided that other factors are available.

Important as such, the pH itself is under the influence of various metabolic products in the cell sap, notably the free organic acids, free amino acids and amides (Thomas *et al*, 1973; Yazaki, 1976). These metabolic products therefore are also factors which play a significant role in the contribution of anthocyanins to flower coloration.

IV. METHODS FOR ISOLATION, PURIFICATION AND IDENTIFICATION OF ANTHOCYANINS

IV.1. Isolation and Purification.

The anthocyanins are generally isolated from plant material by organic solvents such as acetone, alcohol or methanol. Since they are unstable in neutral or in weakly alkaline solution, the solvent must be lightly acidified to about pH 0.1 - 1.0 with HCl. Strong acidification would cause hydrolysis to occur, especially if the extraction is carried out at the boiling point of the solvent.

For purification, several methods based on adsorption have been introduced. The most successful method first carried out by Bate-Smith in 1948 and still occupying a dominant position in the field of flavonoid analysis is the method of separation by paper chromatography. Many extensive reviews have been written on the subject (Harborne, 1958,

1967b; Egger, 1961; Seikel, 1964; Zweig and Whitaker, 1971). The reasons for the continuing appeal of this technique are numerous: it is suitable for the separation of complex mixtures of all types of flavonoids and their glycosides; the ease of obtaining acceptable chromatographic separation and spectroscopically pure compounds; its convenience for isolating both small and relatively large amounts of flavonoids; and, finally, the low cost of equipment and materials.

Paper chromatographic analyses are commonly carried out on Whatman No. 1, No. 3 or 3MM papers. Many solvent systems have been adopted, based on the mixtures of strong mineral acid (HCl), weak acids (acetic acid, formic acid), water, and organic solvents (butanol, benzene, ethyl acetate, etc.) in different proportions.

For separation of flavonoids on a large scale and for preliminary purification, column chromatography is the best alternative. Columns of magnesol, polyamide, silicic acid, silica gel, cellulose and cation exchange resins have been used. The technique has been discussed in detail by Seikel (1962) and Harborne (1959a, 1967b).

On the other hand, thin-layer chromatography (TLC) is regarded as a method particularly useful for analyzing flavonoids on a microscale. The technique has been used in recent years for the identification of anthocyanins (Nybom, 1964; Asen, 1965; Mullick, 1969). A complete review of the application of TLC for anthocyanin study has been pro-

vided by Kirchner (1967) and Egger (1969). The shorter developing time, the better resolution, the more compact spots and the greater sensitivity are the advantages of this technique over the two previously cited. The materials used for TLC are generally similar to those used in column chromatography (polyamide, silica gel, cellulose, etc.).

Finally, among the less popular techniques for the separation and purification of anthocyanins are the methods of gas liquid chromatography (GLC), paper electrophoresis, the infra-red (IR), the nuclear magnetic resonance (NMR), the sublimination and the high pressure liquid chromatography (HPLC), a relatively new technique.

IV.2. Identification.

The anthocyanins, once purified, are characterized by their chromatographic and spectroscopic behaviour, as well as by their hydrolyzed products. From the review by Harborne (1967a), valuable criteria for identification purposes can be summarized as follows:

(a). Chromatographic Properties

The mobility of anthocyanins and anthocyanidins in different solvent systems, expressed as R_f value, is an important characteristic. It is related to the nature and number of the hydroxyl and methoxyl groups, the sugar residues or other substituents present in the molecule. The R_f value

of anthocyanidins in aqueous solvent such as Forestal or Formic acid decreases regularly with increasing hydroxylation, but increases with methylation. With glycosylation, the lowering of R_f value in organic solvents is reflected by the successive increase in the number of sugar residues, but in aqueous solvents, the R_f value increases. The above situation is reversed in the case of acylated anthocyanins.

The nature of the aglycone moiety in the anthocyanins modifies their R_f values slightly but in a very regular fashion. For example: R_f values of the 3-glucoside of the six common anthocyanidins in BAW are: 44 (pelargonidin), 41 (peonidin), 38 (cyanidin), 38 (malvidin), 35 (petunidin), and 26 (delphinidin). Those of the aglycones alone are: 80 (pelargonidin), 71 (peonidin), 69 (cyanidin), 58 (malvidin), 52 (petunidin), and 42 (delphinidin) respectively.

The colours of anthocyanidins and anthocyanins on paper chromatograms in visible and UV light can also be employed for identification purposes. Under visible light, the aglycones are distinguished by their natural colour. Under UV light, anthocyanidins fluoresce if the B-ring has a single p-hydroxyl group and the 5-hydroxyl is methylated or glycosylated. Thus, 3-glycoside anthocyanins show dull colour under UV light whereas 3,5-diglycosides fluoresce brightly.

(b) Spectral Properties

Anthocyanins and anthocyanidins exhibit two absorption maxi-

ma, one between 465 and 550 mn in the visible and another at about 270 - 280 nm in the UV regions in methanol or ethanol solvents. Acylated anthocyanins show a diagnostic peak between 310 - 335 nm in the long UV regions.

Anthocyanins which have a catechol nucleus in their structure (e.g., cyanidin, delphinidin and petunidin) all exhibit a bathochromic shift of 25 - 35 nm in the presence of aluminum ions at low pH.

An introduction of a sugar residue into the third position of anthocyanidins has a regular hypsochromic effect on spectra while the nature of the sugar substitution does not exert any effect.

Differences in the relative intensities of the peaks in the UV (400 - 460 nm) and the visible regions are specific for each anthocyanin or anthocyanidin, serving as an important criterion for identification purposes.

(c) Hydrolysis

Upon total hydrolysis by treatment with HCl, the anthocyanidin-sugar linkages and the sugar-sugar linkages are broken, releasing aglycones and single sugars. Those products can be identified by the usual procedures of paper chromatography.

During controlled or partial hydrolysis under appropriate conditions (Abe and Hayashi, 1956; Harborne, 1959b) the sugar molecules are liberated one after another in a particular sequence before the appearance of the aglycone, with

the formation of intermediate glycosides, the number and nature of which allows one to identify the original glycoside. This approach is particularly useful in the elucidation of complex glycosides.

The location of the sugar attachments can be determined with hydrogen peroxide oxidation (Chandler and Harper, 1961) which selectively attacks the aglycone-sugar linkage at the 3-position, yielding the intact sugar residue attached only at that position.

Alkaline hydrolysis is employed to obtain the acyl residues from acylated anthocyanins, and the products are identified by chromatography.

MATERIALS AND METHODS

I. FLOWERS

Begonia seeds (*Begonia tuberhybrida*) were purchased from W.H. Perron Co. in Montreal. The seeds were sown in flats with black soil and the young plants were later transferred into individual pots when they were about 10 cm high. The plants were kept in the greenhouse and exposed to continuous light from tungsten lamps.

Fully-open flowers with diameter ranging from 8 to 10 cm were harvested and immediately processed. Seven varieties were selected for this study, the flowers of each of which were uniformly coloured but together they represented a wide range of colour, from white to red. The varieties chosen are as follows (Plate 1):

Flower Colour	Equivalent Colour in Ridgway's Colour Chart(1912)
Red	Carmine
Orange-red	Scarlet red
Pink	Spinel Pink
Orange	Bitter Sweet Orange
Salmon	Orange Pink
Yellow	Lemon Yellow
White	White

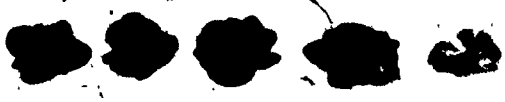


Plate 1.- Colour varieties of *Begonia tuberhybrida* used in this study.

From left to right : Red, Orange-red, Pink, Orange, Salmon, Yellow and White.

II. SOLVENT SYSTEMS FOR PAPER CHROMATOGRAPHY

For the sake of convenience, the various solvent systems used in this study are listed in Table 3, along with their compositions, and will be referred to throughout by their abbreviations, indicated in the table. The source of chemicals used in these solvent systems are as follows:

(a) Hydrochloric acid, acetic acid, formic acid, ammonium hydroxide: J.T. Baker Chemical Co., Phillipsburg, New Jersey, U.S.A.

(b) Benzene, n-butanol, pyridine, n-propanol, phenol: Fisher Scientific Co., Fairlawn, New Jersey, U.S.A.

III. STUDY ON THE ANTHOCYANIN PIGMENTS

III.1. Study of Anthocyanidins and their Relative Concentration

In order to study the effect of the nature and concentration of anthocyanidins on the colour variations, the anthocyanidins were isolated, characterized and their relative concentrations were compared, based on the optical density of the peak of maximum absorption in the visible region.

(a) Extraction and Preparation of Anthocyanidins

5 g of fresh flower petals of each colour variety was extracted in 1.5 ml of MeOH (Fisher Scientific Co.) - 0.1% HCl, filtered, and the volume was made up to 2.0 ml with the same

TABLE 3.- Solvent systems for paper chromatography.

Abbreviations	Composition	Proportion*	Layer used	Solvent used for
BAW	n-butanol-acetic acid-water	4 : 1 : 5	Upper	Anthocyanidins Sugars Amino acids
AWH	acetic acid-water-conc.HCl	15 : 82 : 3	Miscible	Anthocyanins
1% HCl	conc. HCl-water	3 : 97	Miscible	Anthocyanins
Forestal	conc. HCl-acetic acid-water	3 : 30 : 10	Miscible	Anthocyanidins
FWH	formic acid-water-conc. HCl	5 : 3 : 2	Miscible	Anthocyanidins
BBPW	n-butanol-benzene-pyridine-water	5 : 1 : 3 : 3	Upper	Sugars
BEW	n-butanol-ethanol-water	4 : 1 : 2.2	Upper	Sugar
BFW	n-butanol-formic acid-water	4 : 1 : 5	Upper	Organic acids
PrOH - NH_4OH	n-propanol-1M NH_4OH	7 : 3	Miscible	Organic acids
PhOH - H_2O	phenol-water	3 : 1	Miscible	Amino acids

*. Ratios for all solvents were volume to volume, except PhOH, which was weight to volume.

solvent. To this crude anthocyanin extract was added an equal volume of 2N HCl and complete hydrolysis was accomplished by heating the mixture in a boiling water bath at 100° C in subdued light for about 30 minutes. The hydrolyzate was cooled, filtered and the aglycones were extracted with 5.0 ml of iso-amyl alcohol.

(b) Purification and Characterization by Paper Chromatography and by Spectral Properties

An aliquot of 0.05 ml of each of the iso-amyl alcohol extract (equivalent to 0.05 g fresh weight of petals) was spotted on Whatman No. 1 chromatographic papers (22.5 x 27.5 cm) along with cyanidin isolated from red rose as a standard marker. The papers were developed by descending technique in three solvent systems: BAW, FWH and Forestal. The R_f values and colour reactions were recorded. Each anthocyanidin spot was then eluted in MeOH - 0.1% HCl, evaporated under vacuum to dryness and re-dissolved in 5 ml of MeOH (spectral grade -- Fisher Scientific Co.) - 0.01% HCl. The spectral absorption of the pigments were studied with a Unicam SP-800 automatic recording spectrophotometer, using the same solvent as the blank. The spectral shifts were produced by adding five drops of 5% $AlCl_3$ (Fisher Scientific Co.) (w/v) to the cuvette.

(c) Estimation of the Relative Concentration of Total Anthocyanidins

The optical density of the peak of maximum absorption in

the visible region was noted in order to compare the relative concentration of the anthocyanidins present in the various colour forms.

III.2. Spectral Studies of the Crude Pigments and Chromatographic Survey of Anthocyanin Glycosides

(a) Spectral Study of the Crude Pigments

The crude pigments from various colour forms could be considered to approximate the state of the pigments *in vivo*. In order to determine the variations among the crude pigments, 1.0 g of petal tissue of each colour variety was extracted in 0.7 ml of MeOH - 0.1% HCl and filtered. The volume was made up to 1.0 ml with the same solvent and five drops of the extract (equivalent to 0.05 g wet weight of flower) from the Red, Orange-Red and Pink was mixed in 10 ml MeOH (spectral grade) - 0.01% HCl while 10 drops of the extract (equivalent to 0.1 g wet weight of flower) from the other colour varieties were added to the same volume of the same solvent.

The samples were scanned in a Unicam SP-800 automatic recording spectrophotometer, using the same solvent as the blank. The characteristic bathochromic shifts were determined by adding 5 drops of 5% $AlCl_3$ (w/v) to the cuvette.

(b) Preliminary Survey of Anthocyanins by Two-Dimensional Chromatography

In order to examine the range of glycosides present, 0.5 ml of the above extracts (equivalent to 0.5 g, wet weight of petal tissue) was spotted on each Whatman No. 1 chromatographic paper and run in BAW by descending method in the short direction of the paper. When the papers were completely dry, they were irrigated by the descending method with AWH as the second solvent. The R_f values and colour reactions of the pigment spots were examined in each of the solvent systems and recorded. The chromatograms thus prepared, represented the pigment profile for each colour variety.

III.3. Isolation and Identification of the Major Anthocyanin Glycosides

(a) Extraction and Purification of Major Anthocyanins For Detailed Analysis

The pigment profile of the Red variety represented all the pigment spots which were found to occur in one or another of the other colour forms. The corresponding spots from the chromatograms of various *B. tuberhybrida* were identified to be the same pigments on the basis of their R_f values in the two solvent systems as well as their colour reactions. Thus, an analysis of the major anthocyanins of the Red variety also served for the identification of the major pigments present in other colour forms. For

this purpose, two-dimensional chromatograms were run, using a batch of 20 Whatman No. 3 chromatographic papers in the same manner as described above. 10 g of fresh petal tissue was used. In addition, the same procedure was repeated with 10 g of the Orange-Red flowers in order to provide sufficient material to identify the spot #3 which was present as a weak spot in the Red form.

The spots were eluted several times with methanol containing 0.1% HCl, filtered, and concentrated under vacuum to 2.0 ml. The pigments were purified further by streaking on Whatman No. 3 chromatographic papers and developed by the descending method in 1% HCl. Each major band was cut, eluted in MeOH - 0.01% HCl, filtered and evaporated to dryness under vacuum. The residue which was re-dissolved in 2.0 ml MeOH - 0.1% HCl (spectral grade) represented the purified anthocyanins used for the following analyses.

(b) Characterization of Purified Anthocyanins by Paper Chromatography and by Spectral Properties

1. Paper Chromatography

The purified anthocyanins as described above were run on one-dimensional chromatograms along with a mixture of authentic markers of cyanidin 3-sophoroside and pelargonidin 3-sophoroside (kindly provided by Dr. J.B. Harborne) using three solvent systems: BAW, AWH and 1% HCl in

descending direction at 20° C. The R_f values were measured shortly after the papers were dry. Colour characteristics were also recorded.

2. Spectral Properties

A few drops of each purified anthocyanin was mixed with 10 ml of MeOH (spectral grade) - 0.01% HCl and its absorption in the visible and UV regions was recorded with a Unicam SP-800 automatic recording spectrophotometer. Spectral shifts in the presence of $AlCl_3$ were measured after adding five drops of a solution of the salt (5% w/v) to the cuvette.

(c) Characterization by Complete Hydrolysis

1. - Identification of the Aglycones

An aliquot of 0.5 ml of each purified anthocyanin was hydrolyzed with an equal volume of 2N HCl for 30 minutes in the same manner as described previously. The aglycones were extracted in iso-amyl alcohol and chromatographed for R_f values in FWH, BAW and Forestal solvent systems and subsequently examined in the spectrophotometer for absorption properties as described previously.

2. Identification of the Sugar Moieties

The sugar moieties left in the colourless aqueous fraction were identified by paper chromatography. Prior to application on paper, the samples were treated repeatedly with a 10% solution of di-n-octymethylamine in chloroform to

remove HCl, in order to prevent the formation of arabinose by the action of mineral acid on the chromatographic paper. The samples were spotted on Whatman No. 1 chromatographic papers along with authentic solutions of glucose, galactose, arabinose, xylose, and rhamnose (Fisher Scientific Co.). The chromatography was carried out in a descending direction in BAW and BBPW solvent systems. The spots were developed by spraying with aniline hydrogen phthalate reagent made up by dissolving 9.2 ml aniline (J.T. Baker Chemical Co.), 6 g phthalic acid (J.T. Baker Chemical Co.), 490 ml n-BuOH, 490 ml Et_2O (Fisher Scientific Co.) and 20 ml H_2O, followed by heating for 5 minutes at 105° C. The R_f values and colour reactions of the various spots were recorded.

(d) Characterization by Selective Hydrolysis with Hydrogen Peroxide

The degradation of the pigment extracts with hydrogen peroxide was done according to the method of Chandler and Harper (1961).

0.2 ml of each purified anthocyanin extract was mixed with 40 ml of hydrogen peroxide (30% -- Fisher Scientific Co.) and allowed to stand at room temperature for 4 hours. The solution was treated with a few grains of palladium catalyst (Fisher Scientific Co.) to effect the decomposition of excess peroxide. After 20 hours, 50 ml of NH_4OH (0.88 N) was added and the solution warmed for 5 minutes in boiling water. The resulting solution was used to identify the sugar moieties released, by paper chromatography.

The solution was spotted on Whatman No. 1 chromatographic papers and run for 24 hours along with standard glucose and standard rutinose in the BAW and BBPW solvent systems. The rutinose was isolated from rutin by hydrolysis with 10% acetic acid (Chandler and Harper, 1961). The sugars were detected by spraying with aniline-hydrogen phthalate reagent, as described previously. The R_f values and colour reactions were recorded.

IV. STUDY OF SOME FACTORS MODIFYING THE COLOUR APPEARANCE OF ANTHOCYANIN PIGMENTS IN FLOWERS

IV.1. Measurements of the pH of the Flower Cell Sap.

The flower cell sap was obtained directly by pressing the flower petals in a mortar and pestle. The juice was filtered and transferred into a conical centrifuge test tube. The pH was determined directly on the freshly-pressed juice, using a pH meter (Beckman, model 3500). The measurements were made in triplicate from three different sets of flowers harvested and processed at three different times.

IV.2. Examination for the Phenomena of Co-pigmentation and Metal Chelation

In order to better understand the reasons for the absence of the blue colour forms, the factors considered to be the most influential for the occurrence of co-pigmentation and metal chelation were examined. These included the pH of the cell sap, its buffering system and the materials to

which the anthocyanins could be bound, namely the flavones and/or flavonols (for co-pigmentation) and the metal ions (for metal chelation).

The pH of the cell sap was measured as described in IV(a).

The buffering system was studied during the examination of the free organic acids in the cell sap. The flavonols were isolated and identified at the same time as the isolation and characterization of total anthocyanidins as described earlier. The metal ions were detected by the method of atomic absorption spectroscopy. Only one colour form (the Red) was used for this study.

The method involved drying a known weight of fresh petals (20 g) in a drying oven overnight at 110° C, followed by re-weighing and ashing in a muffle furnace for variable time periods at temperatures ranging from 100° C to 500° C. After cooling to room temperature, the sample was re-weighed. A volume of 2 ml of HNO_3 (16N -- T.J. Baker Chemical Co.) was added and the mixture warmed on a hot plate at about 80° C. The extract was then evaporated slowly to dryness and 2 ml of 12N HCl added, and again warmed for complete dissolution. The solution was then made up to 100 ml with de-ionized water.

The analysis for the metals was done on the flame atomic absorption spectrophotometer (Pye Unicam SP-1900) along with appropriate standards for each of the metals under examination.

[Appreciation is expressed to Prof. J.G. Dick, (Chemistry Dept.) for the AAS determinations]

IV.3. Examination of the Metabolic Products Affecting Flower Colour

(a) Examination of free organic acids in the cell sap.

Fresh petal tissue (10 g) of each colour variety was extracted repeatedly in boiling alcohol (95%), the extracts combined and concentrated down to 2.0 ml in a flash evaporator. An equivalent of 0.3 g of petal tissue was spotted on Whatman chromatographic No. 1 papers along with authentic solutions of succinic, citric, oxalic, malic, lactic and tartaric acids (Fisher Scientific Co.). The papers were developed two-dimensionally in n-PrOH - NH_4OH and BFW, then dried and sprayed with 0.04% bromophenol blue (Fisher Scientific Co.) in 0.01 M NaOH. The R_f values and relative spot intensities of the organic acids detected were recorded.

In view of reports that *Begonia* is particularly rich in free oxalic acid (Thomas *et al*,1973), a further test was done to detect oxalic acid.

To a solution of 2.0 ml of fresh petal juice of each colour variety was added a few drops of 7% $CaCl_2$ solution (Fisher Scientific CO.) and centrifuged. The supernatant was decanted and 2.0 ml of dstilled H_2O was added to the residue content, shaken and recentrifuged. The supernatant was discarded and the residue acidified with a few drops of 7% H_sSO_4 (T.J. Baker Chemical Com.). The solution was gently warmed and to this was added a 3% solution of $KMnO_4$ (Fisher Scientific Co.) drop by drop until the violet colour of the per-

manganate solution disappeared. A quick discharge of the violet colour implied the presence of oxalic acid in the juice.

(b) Determination of free amino acids in the cell sap.

The alcohol extracts obtained from the above procedure were also used for the free amino acid determination. An equivalent of 0.5 g of petal tissue was spotted on Whatman chromatographic No. 1 papers along with standard markers of some 20 frequently occurring amino acids (Eastman Kodak Co., Rochester, New York, U.S.A.). The chromatography was carried out two-dimensionally in BAW and PhOH - H_2O. The papers were dried and sprayed with 0.25% ninhydrin (Fisher Scientific Co.) in acetone (Anachemia , Montreal, Canada), dried again and heated for 10 minutes at 105 C. The R_f values and colour reactions of the spots were immediately recorded.

(c) Determination of free sugars in the cell sap.

Fresh petal tissue (10 g) of each colour variety was extracted in 10 ml MeOH - 0.1% HCl. The extacts were concentrated down to 2.0 ml in a flash evaporator. An equivalent of 0.5 g of fresh petal tissue was spotted on Whatman chromatographic No. 1 papers along with authentic solutions of sugar markers. The papers were chromatographed in BAW, BEW and BBPW and treated in the same manner as described earlier.

(d) Determination of the free phenolic acids in the cell sap.

Fresh petal tissue (10 g) of each colour variety was extracted

several times in 80% ethanol , the extracts combined and filtered. Free phenolic acids were extracted in ether, concentrated down to 2.0 ml and chromatographed two-dimensionally on silica gel TLC plates. The solvents employed were :

HOAc - chloroform* 1 : 9 v/v (first direction)

Ethyl acetate* - benzene 9 : 11 v/v (second direction).

The plates were sprayed with Folin reagent* and examined in the presence of NH_3 vapor.

* Fisher Scientific Co.

RESULTS.

I. STUDIES ON THE ANTHOCYANIN PIGMENTS.

I.1. Identification and relative concentrations of anthocyanidins

(a) Identification by paper chromatography and spectral properties

1. Paper chromatography :

The iso-amyl alcohol extract of all the cyanic cultivars yielded two anthocyanidin pigments (#2 and #4) - Table 4. In addition, two yellow pigments were seen from the extract of all the seven varieties (#1 and #3). Comparison with the standard markers as well as with R_f values and colour characteristics of known aglycones suggested the anthocyanidin pigments to be cyanidin and pelargonidin, and the yellow pigments to be quercetin and kaempferol. The distribution of aglycones and their relative concentrations among the various cultivars are presented in Table 5.

The obtained R_f values of the pigments in FWH relate very well with the literature values. In BAW, all the obtained values are consistently higher by 5 - 8 units, and hence can be adjusted to correspond to literature values. In Forestal, similar variations are also noted. However in both BAW and Forestal , the obtained values for cyanidin correspond exactly with authentic cyanidin marker isolated from red rose, therefore the identification can be assumed to be correct, subject to further confirmation by spectral

TABLE 4.- Chromatographic properties of pigment aglycones in the hydrolyzed extract of *Begonia tuberhybrida*.

Pigment	R_f values (x100) in*			Colour		Identification
	1	2	3	Visible	U.V.	
1	70	11	45	Yellow	Bright yellow	Quercetin
2	76	22	55	Magenta	Dull magenta	Cyanidin
3	89	27	61	Yellow	Bright yellow	Kaempferol
4	85	32	73	Orange-red	Dull orange-red	Pelargonidin
Authentic cyanidin	76	22	56	Magenta	Dull magenta	-
Literature values**						
Cyanidin	68	22	49	Magenta	Dull magenta	
Peonidin	71	30	63	Magenta	Dull magenta	
Pelargonidin	80	33	68	Orange-red	Dull orange-red	
Quercetin	64	--	41	Yellow	Bright yellow	
Kaempferol	83	--	55	Yellow	Bright yellow	
Apigenin	89	--	83	Yellow	Bright yellow	

* Solvent key : 1. BAW (n-BuOH - HOAc - H_2O 4 : 1 : 5 upper layer).
2. FWH (Formic acid - H_2O - conc. HCl 5 : 3 : 2).
3. Forestal (conc. HCl - HOAc - H_2O 3 : 30 : 10).

** taken from Harborne (1967a).

TABLE 5.- Distribution of pigment aglycones among the various *Begonia* cultivars.

Pigment	Distribution and relative intensity						
	Red	Orange-red	Pink	Orange	Salmon	Yellow	White
Cyanidin	+ + + +	+ + +	+	(+)	(+)	–	–
Pelargonidin	(+)	+ +	(+)	+ +	+	–	–
Quercetin	(+)	(+)	+	(+)	(+)	+	+
Kaempferol	(+)	(+)	+	(+)	(+)	(+)	(+)

* Abbreviations : + + + + very strong
+ + + strong
+ + moderate
+ weak
(+) trace

characterization. The reasons for the differences in experimental and literature R_f values could be temperature, saturation or other factors.

It can be seen up to this point that on basis of aglycone composition, two categories can be recognized :the Red, Orange-red,Pink,Orange and Salmon varieties characterized by both cyanidin and pelargonidin in addition to quercetin and kaempferol, and the Yellow and White showing total absence of anthocyanidins but the presence of both the yellow pigments. Thus the colour differentiation among the cyanic cultivars is not on the basis of the pigment aglycones, but due to other factors.

2. Spectral properties :

Because of the low concentration of the yellow pigments present in the various *Begonia tuberhybrida*, only the anthocyanidin pigments were subjected to the spectral studies.

Figures 3 and 4 show the spectra of pigments #2 and #4, with the main characteristics resumed in Table 6.

All the data, in comparison to standard cyanidin and the literature values, indicated that the pigment #3 was cyanidin and the pigment #4 was pelargonidin. These findings confirmed the previous identifications based on chromatographic properties.

(b) Relative concentration of anthocyanidins.

The optical densities of the two anthocyanidins represent their relative concentrations in 1.0 g wet weight of petal

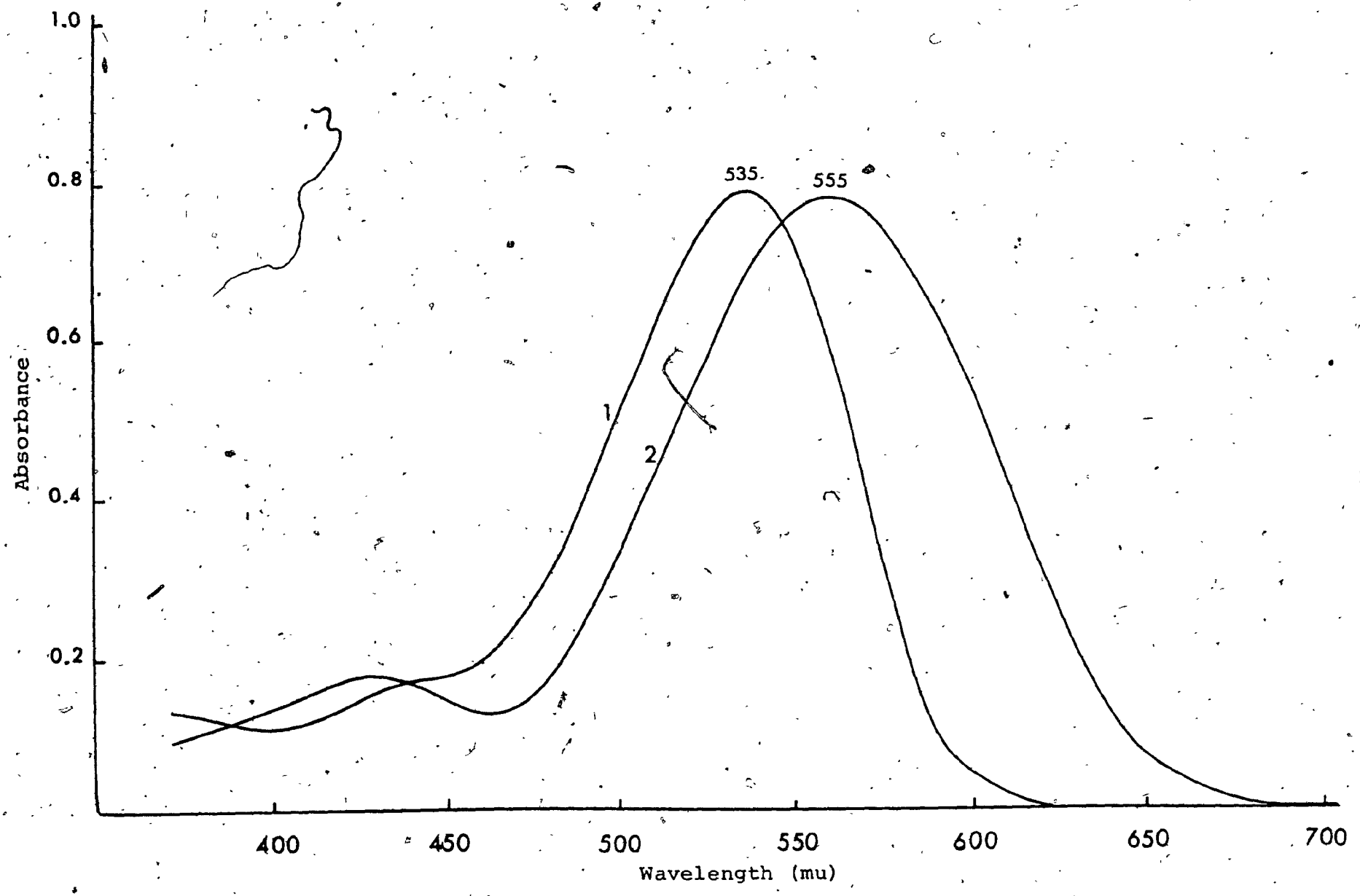


Figure 3.- Spectral properties of pigment spot #2 (identified as cyanidin)
Curve 1 :MeOH - 0.01% HCl . Curve 2 :MeOH - 0.01% + $AlCl_3$

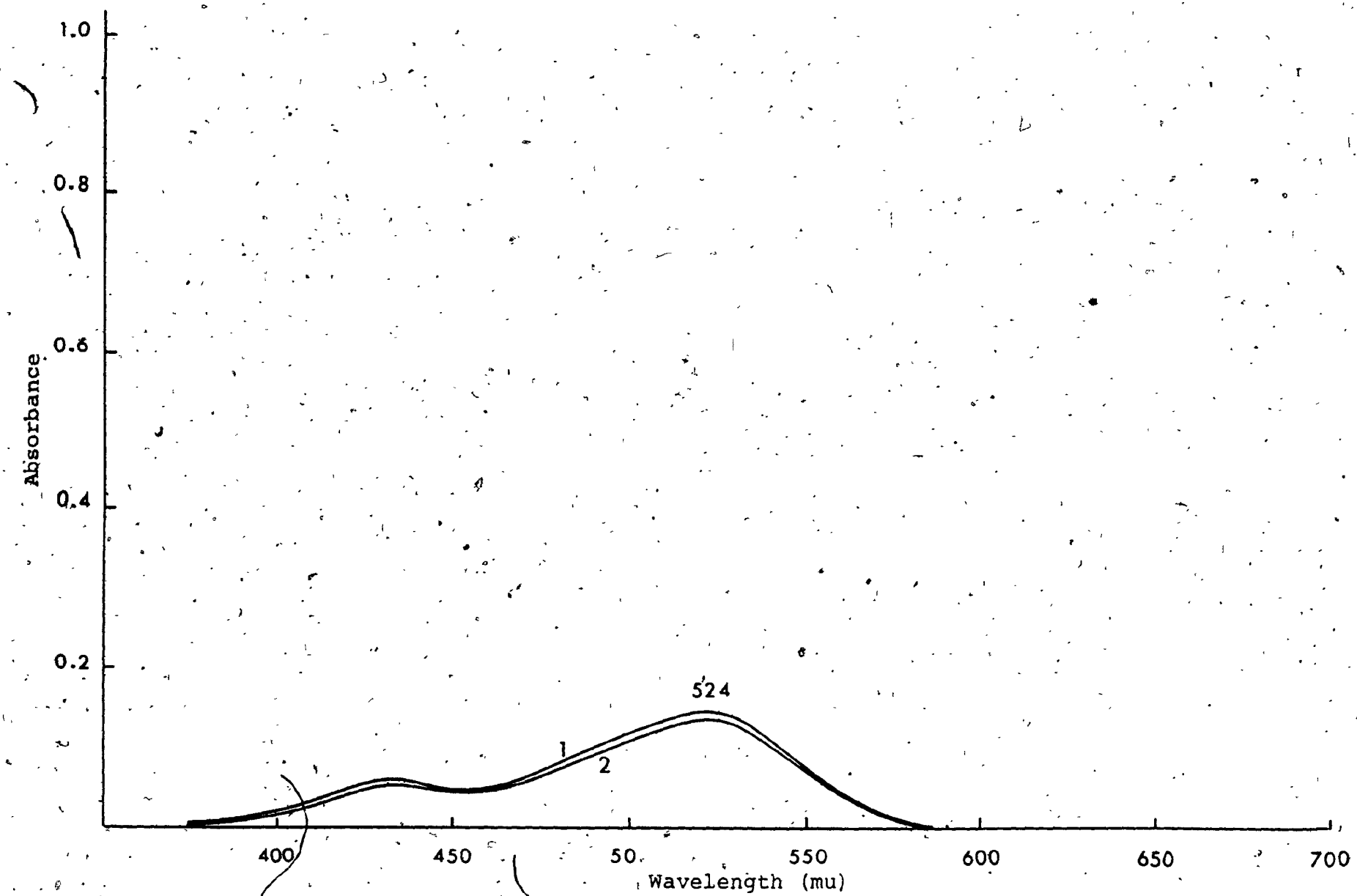


Figure 4.- Spectral properties of pigment spot #4 (Identified as pelargonidin)
Curve 1 : MeOh - 0.01% HCl. Curve 2 : MeOH - 0.01% HCl + $AlCl_3$

TABLE 6. Spectral characteristics of anthocyanidins of *Begonia tuberhybrida* petal tissue.

Pigment	MeOH-HCl λmax (nm)	$AlCl_3$ shift (nm)	$\frac{E_{440nm}}{E_{vis.max}}$	Identification
#2	535	+20	20	Cyanidin
#4	524	-	39	Pelargonidin
Authentic cyanidin	535	+20	20	
Literature values*				
Cyanidin	535	+18	19	
Peonidin	532	-	25	
Pelargonidin	524	-	39	

* taken from Harborne (1967a)

tissue of each colour form are presented in Table 7. The ratios of the two pigments are also included.

Based on these data, it was seen that the colour variations were correlated with the relative concentration of each pigment present in the flower tissue. The cyanidin content increased from Salmon through Orange, Pink, Orange-red to Red, the typical colour due to cyanidin, while the pelargonidin content increased as the colour varied from Pink through Red, Salmon, Orange-red to Orange, the typical colour due to pelargonidin. On the other hand, the effect of colour due to the interaction between the two pigments occurring together in each colour form was reflected by the ratios between them, and showed that in the Red, Orange-red and Pink varieties, the cyanidin was about 20 times, 7 times and 16 times more concentrated than pelargonidin respectively, while in the Orange and Salmon varieties, the pelargonidin was about 14 times and 8 times stronger than cyanidin respectively.

The above observations helped to establish the fact that distinct colour variations can exist with no qualitative differences in the major anthocyanidins present, but with significant differences in the pigment concentrations. Generally speaking, the flowers of red shade contained a stronger concentration of cyanidin than pelargonidin, the decrease in visible redness appeared to be related to the decreasing concentration of cyanidin. The same was true with orangeness manifested by pelargonidin in flowers of orange colour.

TABLE 7.- Relative concentrations of cyanidin and pelargonidin and their ratios in the various cyanic *Begonia tuberhybrida*.

Pigment	O.D. of anthocyanidin pigments and their ratio *				
	Red	Orange-red	Pink	Orange	Salmon
Cyanidin	15.6	12.2	4.8	0.2	0.2
Pelargonidin	0.8	1.8	0.3	2.8	1.6
Ratio of cyanidin content **	78	60	24	1	1
Ratio of pelargonidin content **	2.6	6	1	9.3	5.3
Ratio $\frac{\text{cyanidin}}{\text{pelargonidin}}$	20	7	16	-	-
Ratio $\frac{\text{pelargonidin}}{\text{cyanidin}}$	-	-	-	14	8

* in 1.0 g wet weight of petal tissue.

** pigment content expressed as ratio to the minimum present.

I.2. Spectral studies on the crude pigments and chromatographic survey of the anthocyanin glycosides

(a) Spectral indications of the nature and state of the crude pigments

The *in vitro* spectral absorption of the crude extract of the seven *B. tuberhybrida* varieties revealed a good correlation between spectral characteristics and the nature and concentrations of the major anthocyanins present (Figures #5 to #11 and Table 8).

Generally speaking, the spectra of the cyanic varieties showed the presence of those anthocyanins whose characteristics correlated with the visible colours of the flowers and the intensities of the pigments seemed to correlate with the colour intensity of the flowers. The peak of maximum absorption of the Red, Orange-red and the Pink varieties at 522 nm and 275 nm with a bathochromic shift upon addition of $AlCl_3$ suggested that the major anthocyanins involved were largely cyanidin glycosides. In the case of the Orange and Salmon varieties, the maximum absorption at 504 nm and 270 nm and an absence of a reaction with $AlCl_3$ indicated the predominant presence of pelargonidin glycosides. All the spectra of the cyanic varieties revealed a shoulder at 440 nm, indicating a predominance of the 3-glycosidic anthocyanins. In the acyanic varieties (Yellow and White), there was no evidence of naturally occurring anthocyanins, judged by the

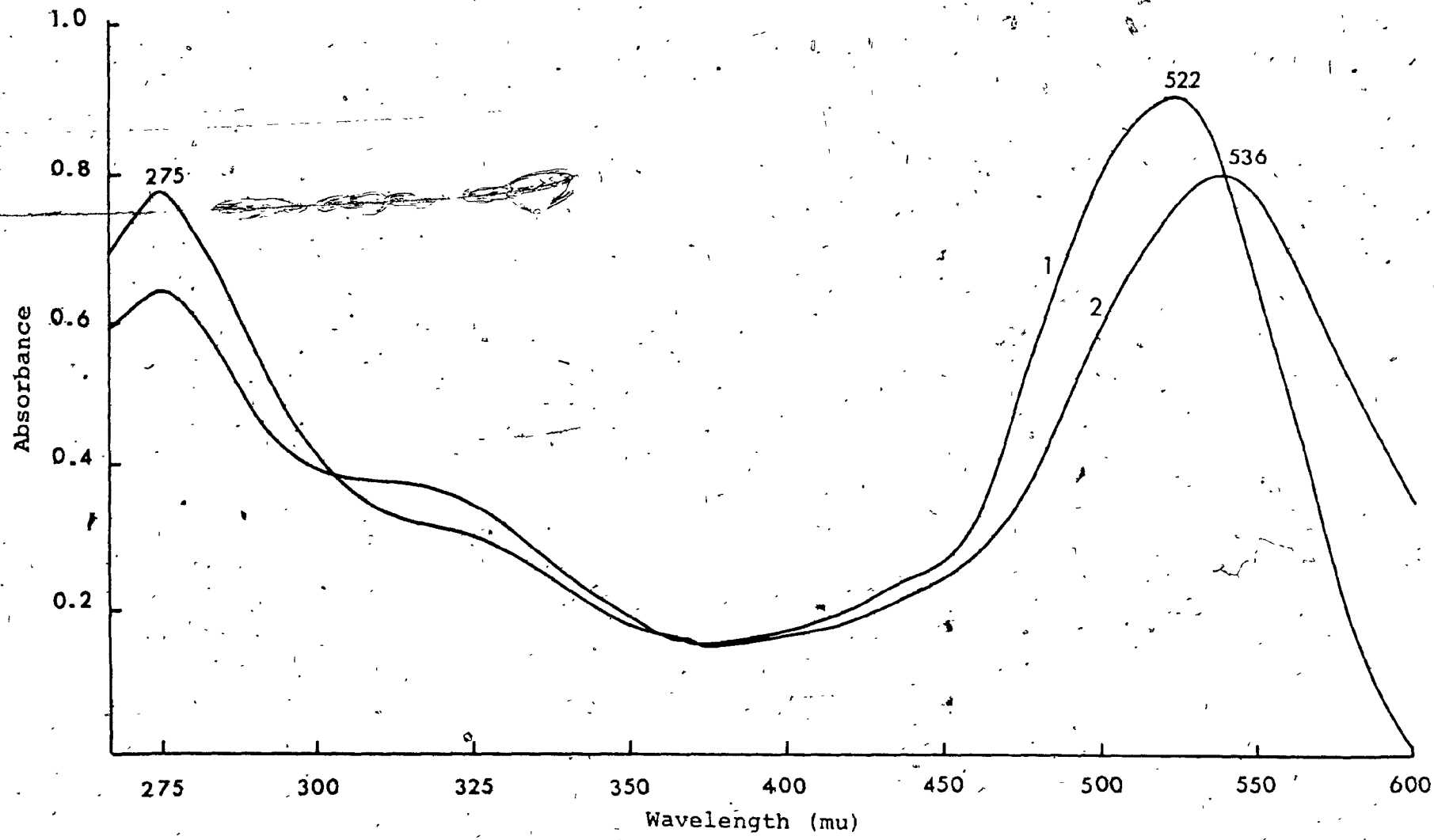


Figure 5.- Spectral characteristics of the crude pigments from the Red variety. Curve 1 : MeOH - 0.01% HCl. Curve 2 : MeOH - 0.01% HCl + $AlCl_3$

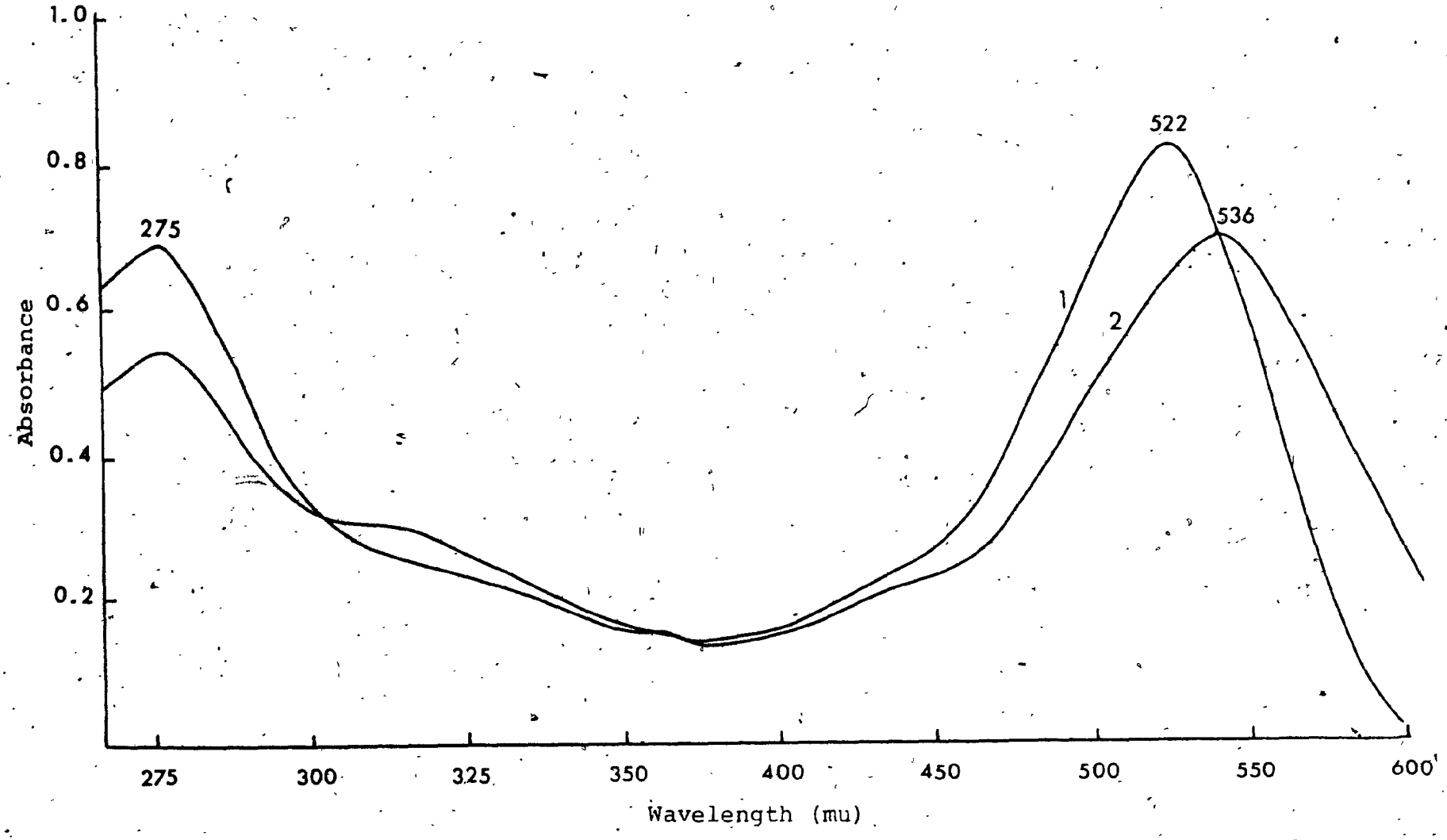


Figure 6.- Spectral characteristics of the crude pigments from the Orange-red variety
Curve 1 : MeOH - 0.01% HCl. Curve 2 : MeOH - 0.01% HCl + $AlCl_3$

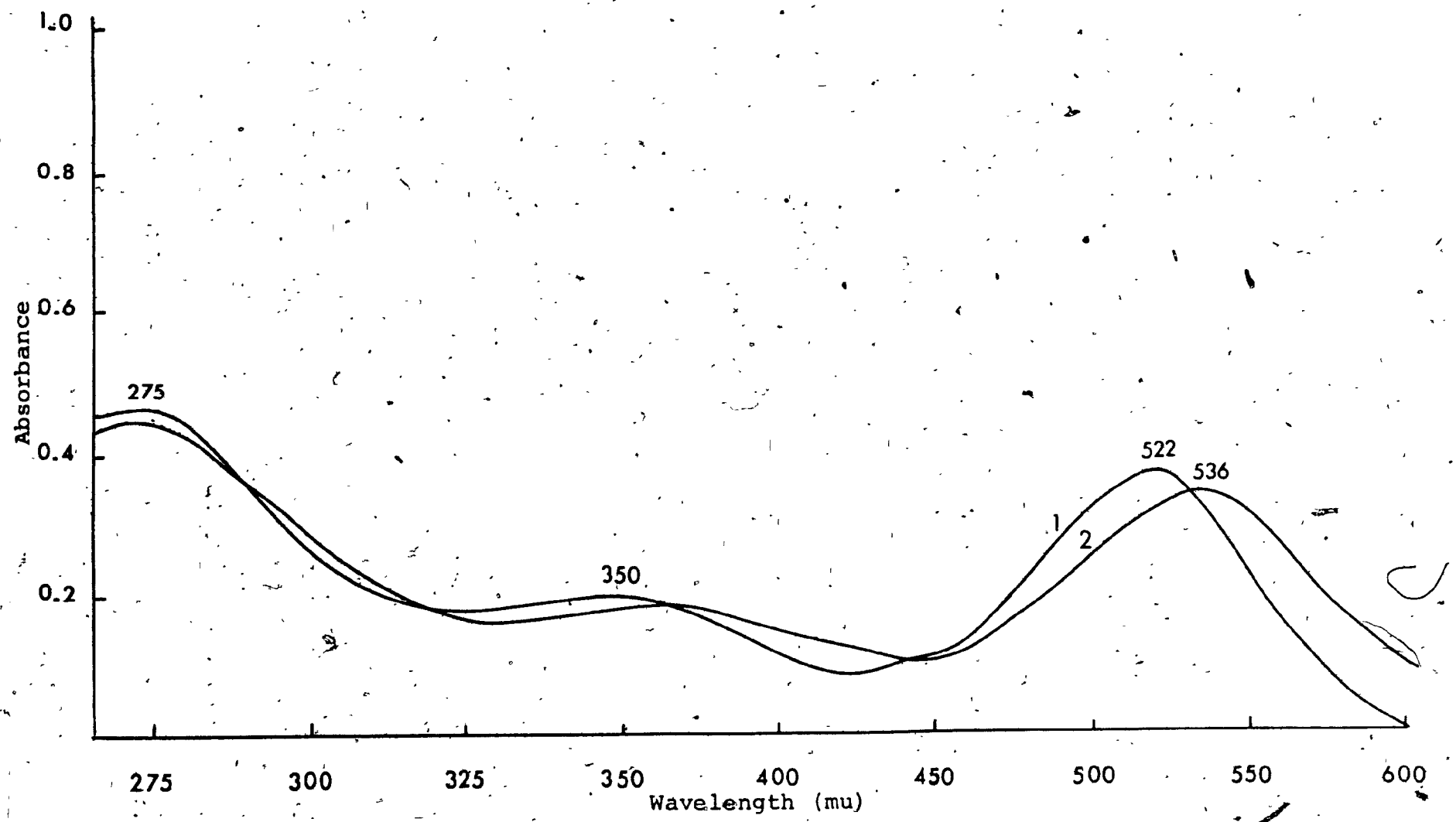


Figure 7.- Spectral characteristics of the crude pigments from the Pink variety. Curve 1 : MeOH - 0.01% HCl. Curve 2 : MeOH - 0.01% HCl + $AlCl_3$

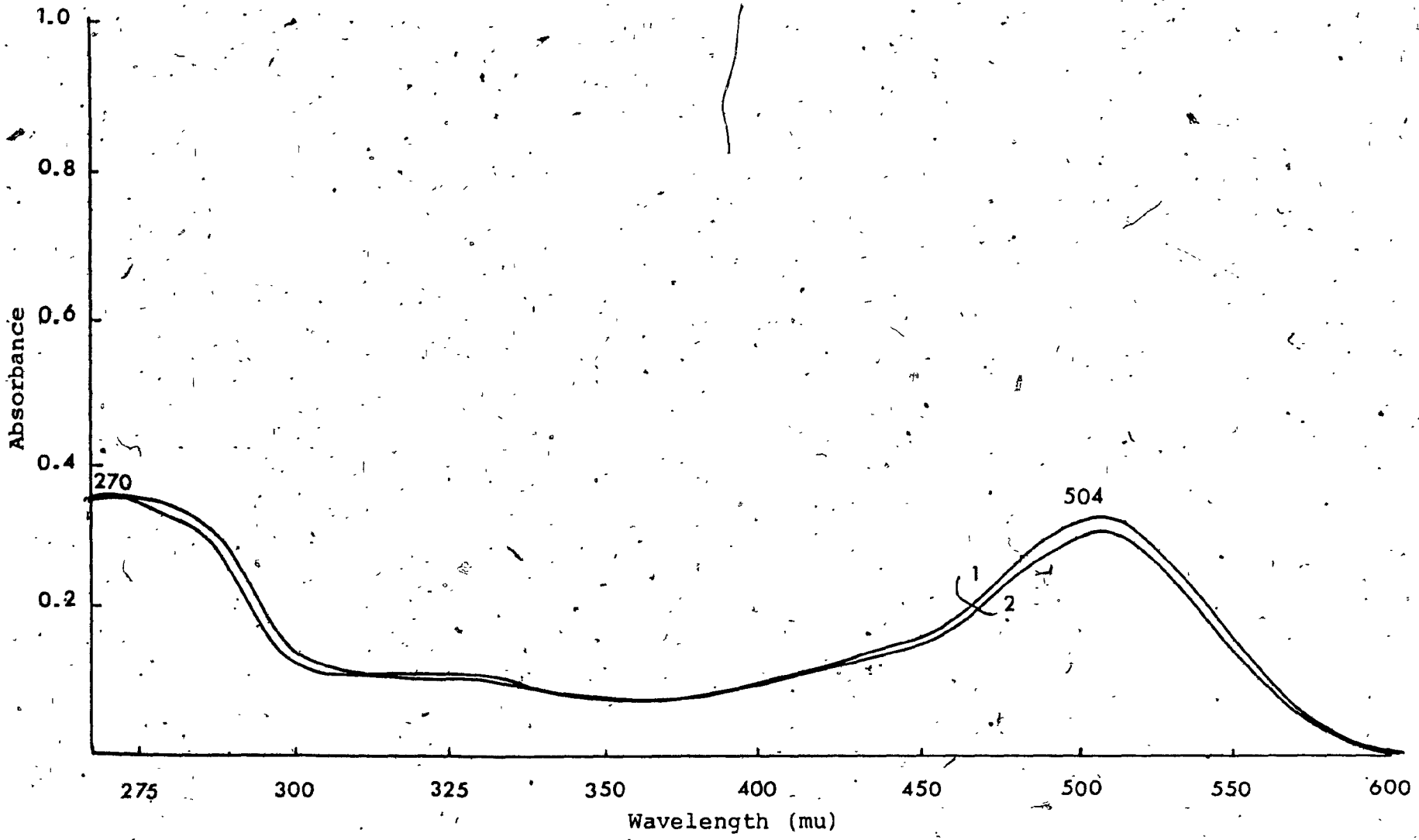


Figure 8.- Spectral charateristics of the crude pigments from the Orange variety. Curve 1 : MeOH - 0.01% HCl. Curve 2 : Me OH - 0.01% HCl + $AlCl_3$

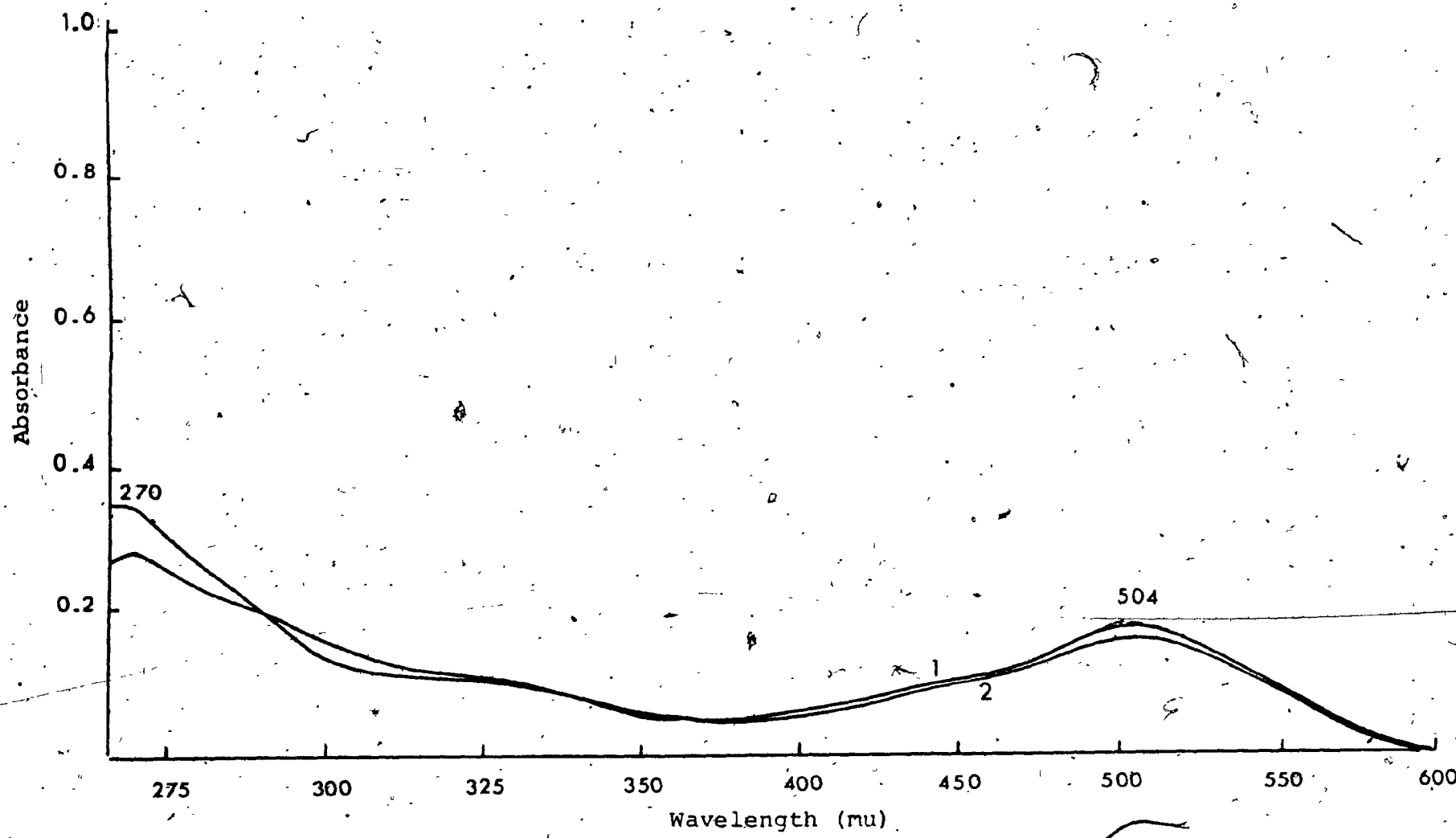


Figure 9.- Spectral characteristics of the crude pigments from the Salmon variety Curve 1 ; MeOH - 0.01% HCl. Curve 2 ; MeOH - 0.01% HCl + $AlCl_3$

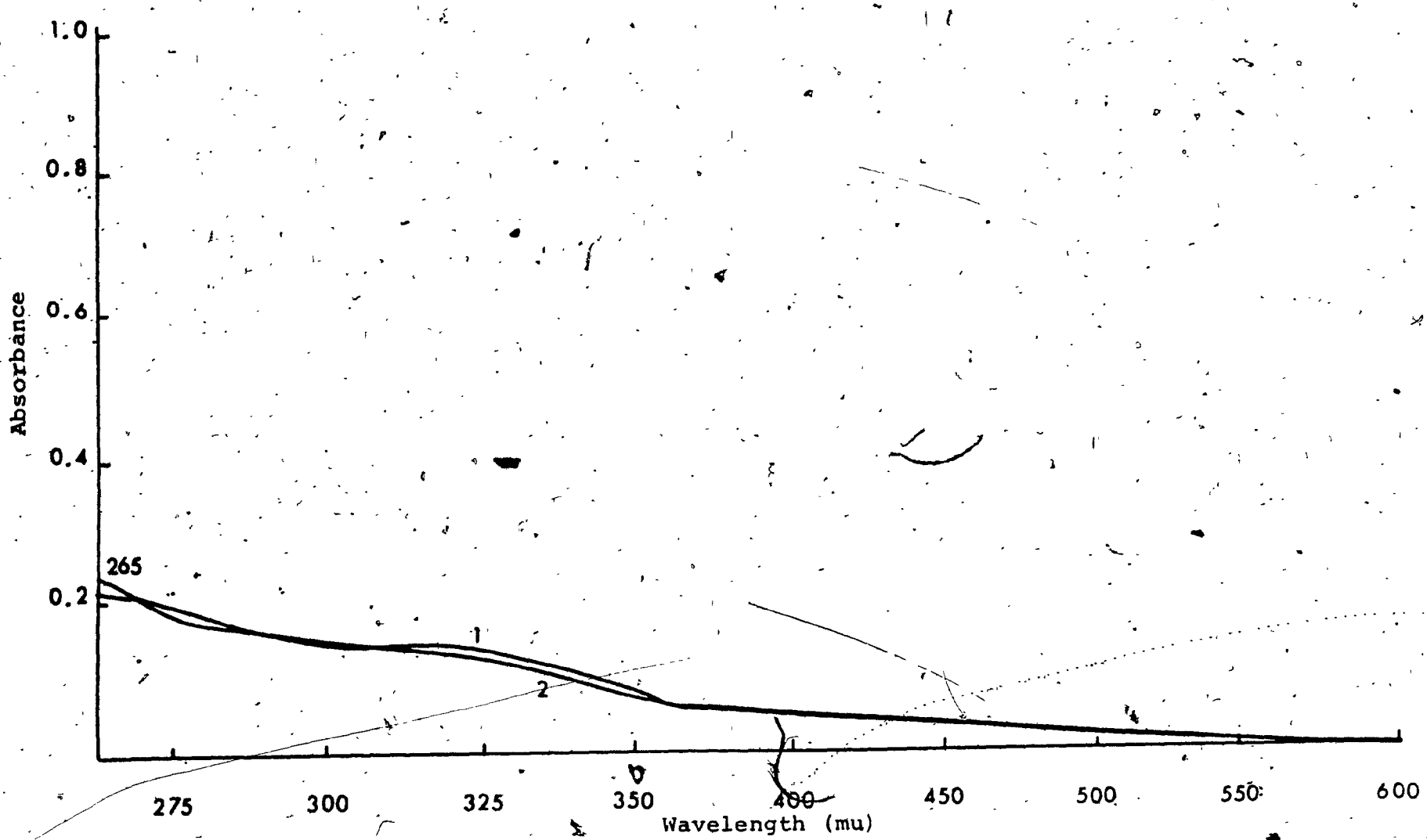


Figure 10.- Spectral characteristics of the crude pigments from the Yellow variety. Curve 1 : MeOH - 0.01% HCl. Curve 2 : MeOH - 0.01% HCl + $AlCl_3$

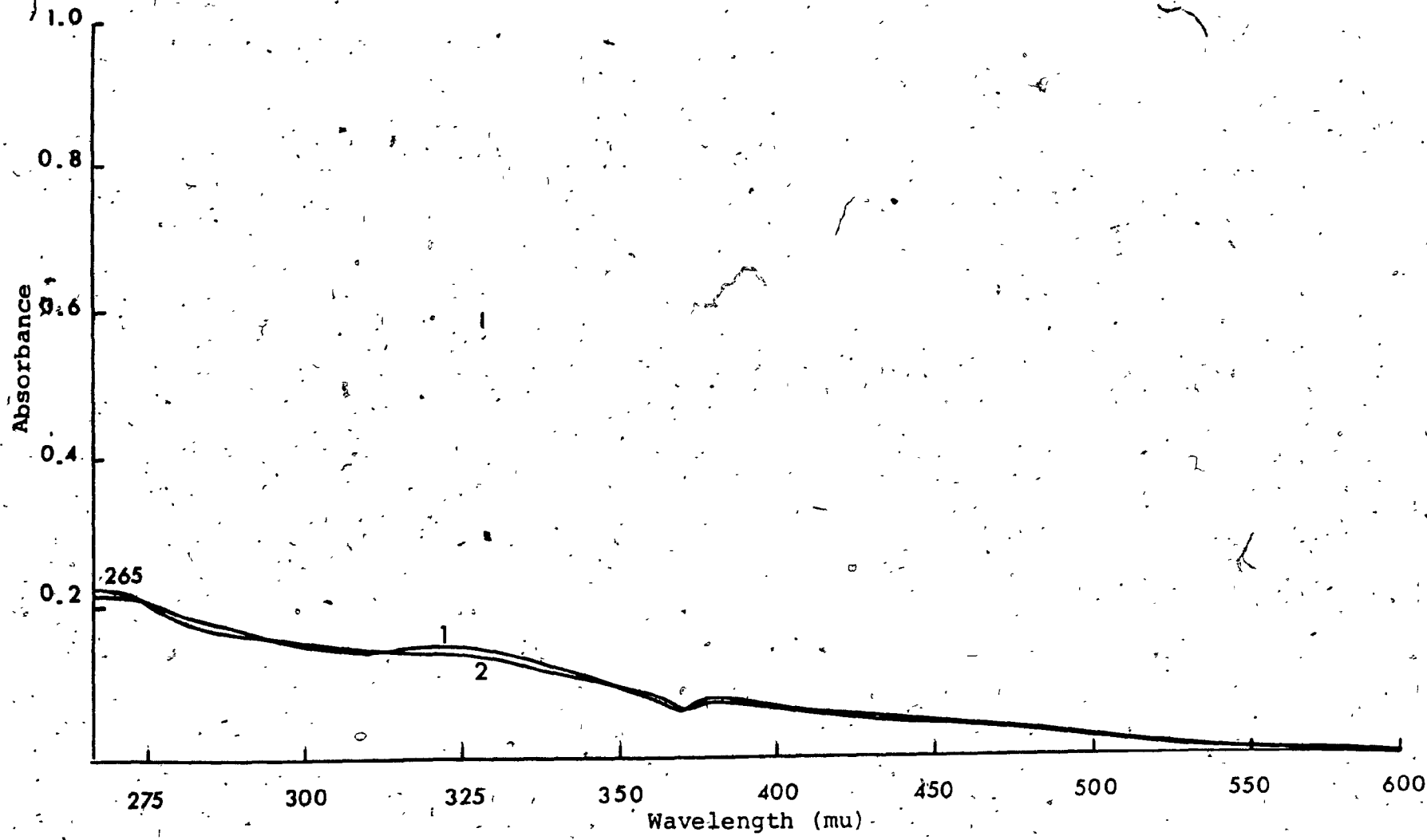


Figure 11.- Spectral characteristics of the crude pigments from the White variety. Curve 1 : MeOH - 0.01% HCl. Curve 2 : MeOH - 0.01% HCl + $AlCl_3$

TABLE 8.- Spectral properties of the crude pigments.

Variety	U.V. max. (nm)	Vis. max (nm)	O.D. at vis. max.*	Shoulder at 320-330 nm	Shoulder at 440 nm	$AlCl_3$ shifts (nm)
Red	275	522	18.0	+	+	+14
Orange-red	275	522	16.0	+	+	+14
Pink	275	522	6.0	+	+	+14
Orange	270	504	3.3	+	+	-
Salmon	270	504	1.8	+	+	-
Yellow	265	-	-	+	-	-
White	265	-	-	+	-	-

* in 1.0 g wet weight of petal tissue.

absence of any peak in the visible regions.

The presence of flavonols was weakly represented by a shoulder in all, with the exception of the Pink, where a clear absorption peak at 350 nm was observed.

Finally, a shoulder between 300 - 325 nm was noted in all the spectra, except the Pink where absorption in this region was obscured by the increased absorption towards 350 nm due to flavonols. This shoulder indicated the presence of either free or esterified phenolic acids in the cell sap, or acylated pigments, or all of them.

(b) Chromatographic profiles of total anthocyanins

Two-dimensional chromatography of the crude extracts of the White and Yellow flowers did not reveal any naturally occurring anthocyanins. Those of the Red, Orange-red, Pink, Orange and Salmon varieties are shown as diagrams in Figures #12 - #16. Altogether, there were 13 pigment spots seen, those marked with solid line are the major pigments and those marked with broken line are the minor ones. Table 9 presents a resume of the pigments, their R_f values, their distribution and relative concentrations in different varieties.

When examined under U.V. light, spots #3 and #8 which showed a visible orange-red colour, did not fluoresce, suggesting the presence of pelargonidin 3-glycoside pigments. Of the other magenta coloured spots, #4, #5, #7, #12 fluoresced, while the rest appeared dull, indicating the possible occurrence

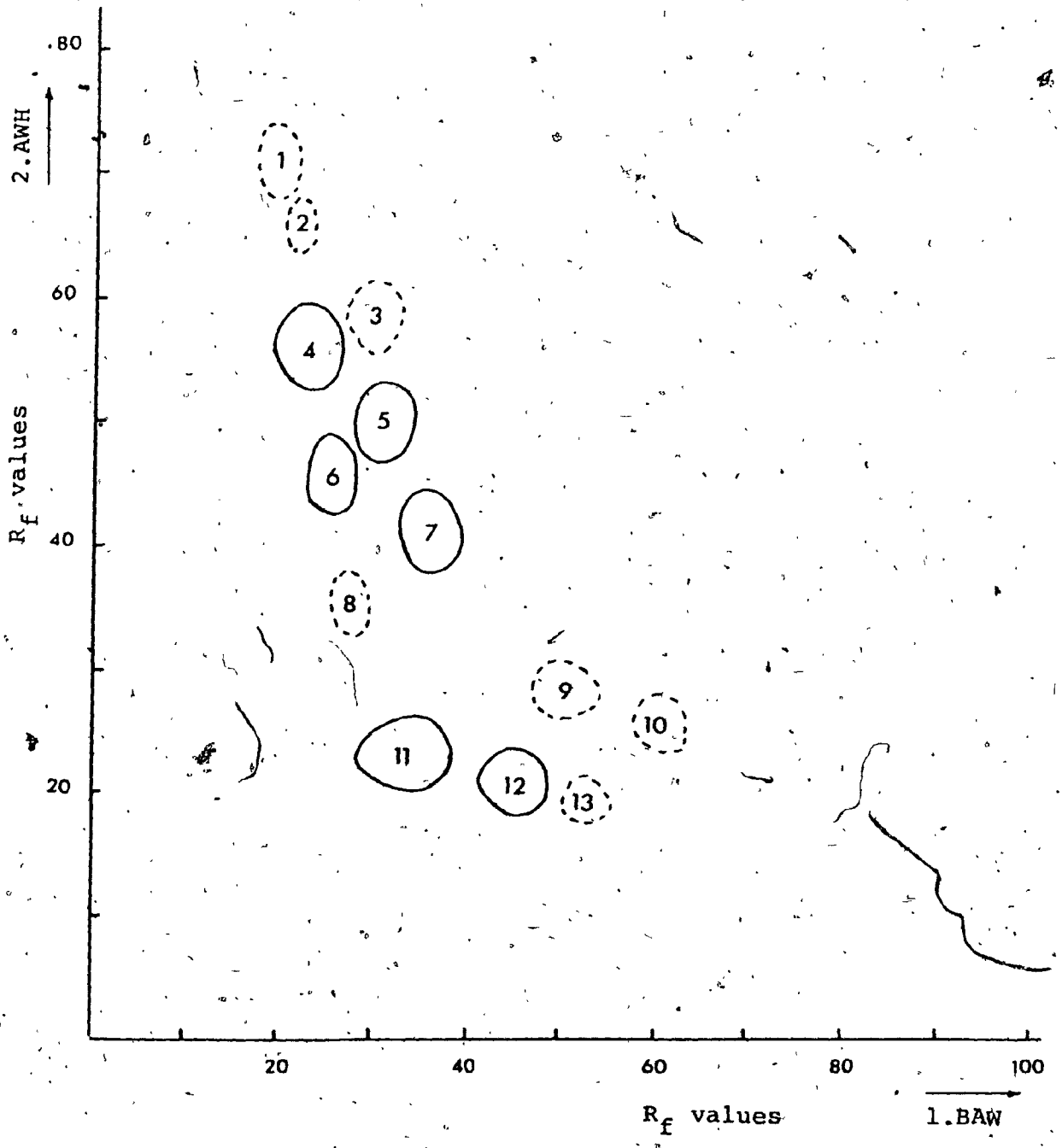


Figure 12.- Chromatographic profile of anthocyanins of the Red variety

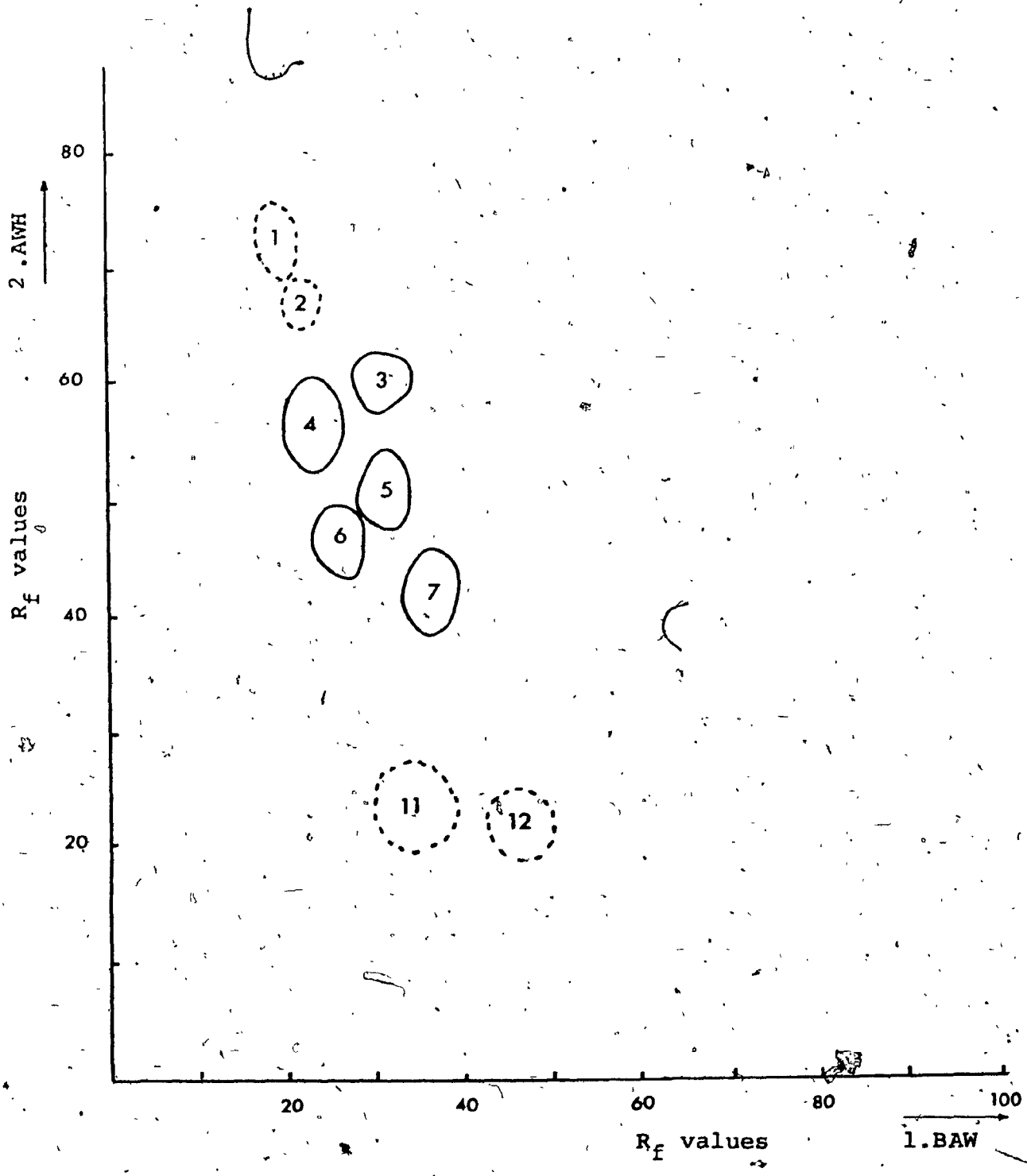


Figure 13.- Chromatographic profile of anthocyanins of the Orange-red variety.

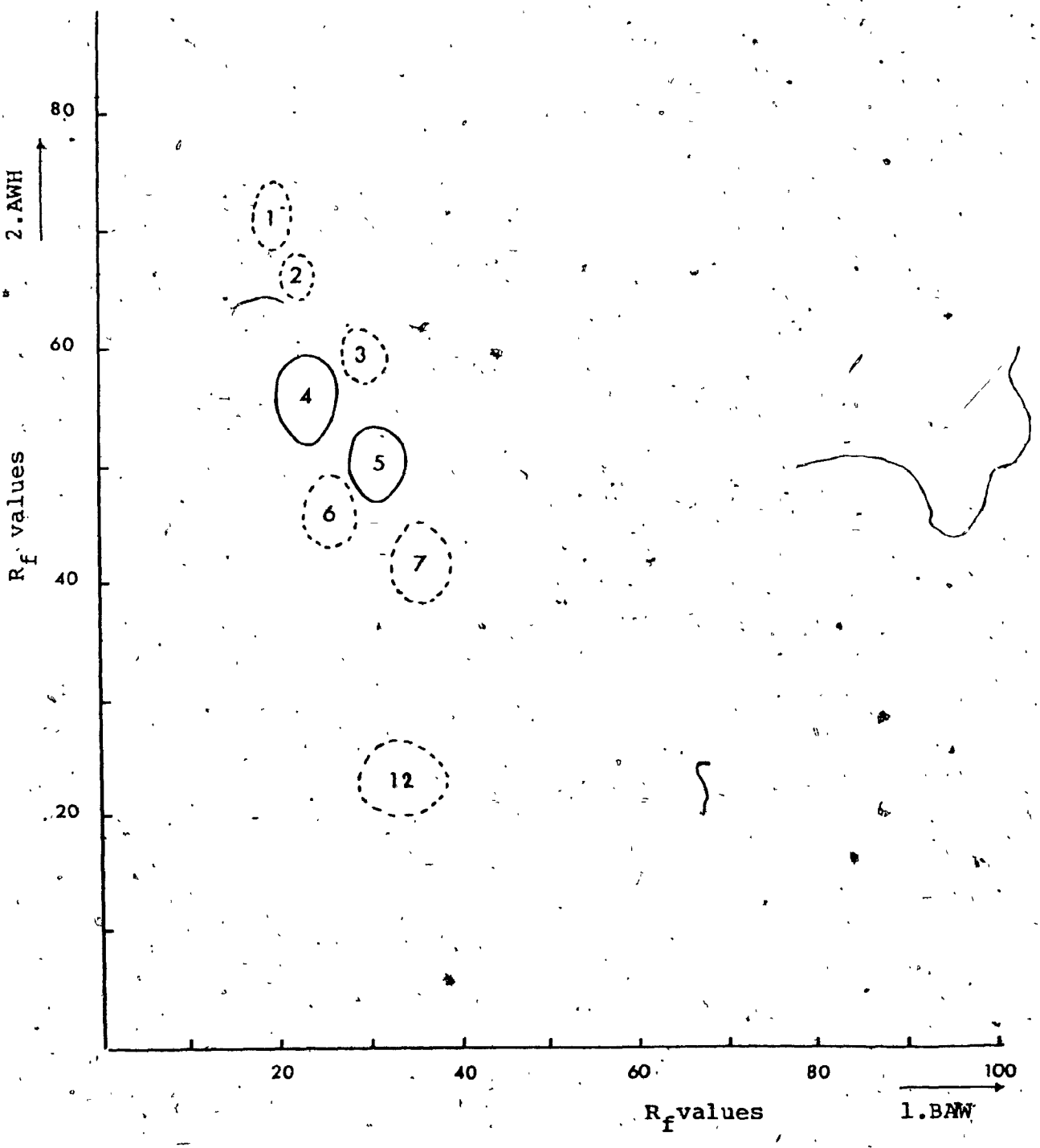


Figure 14. Chromatographic profile of anthocyanins of the Pink variety.

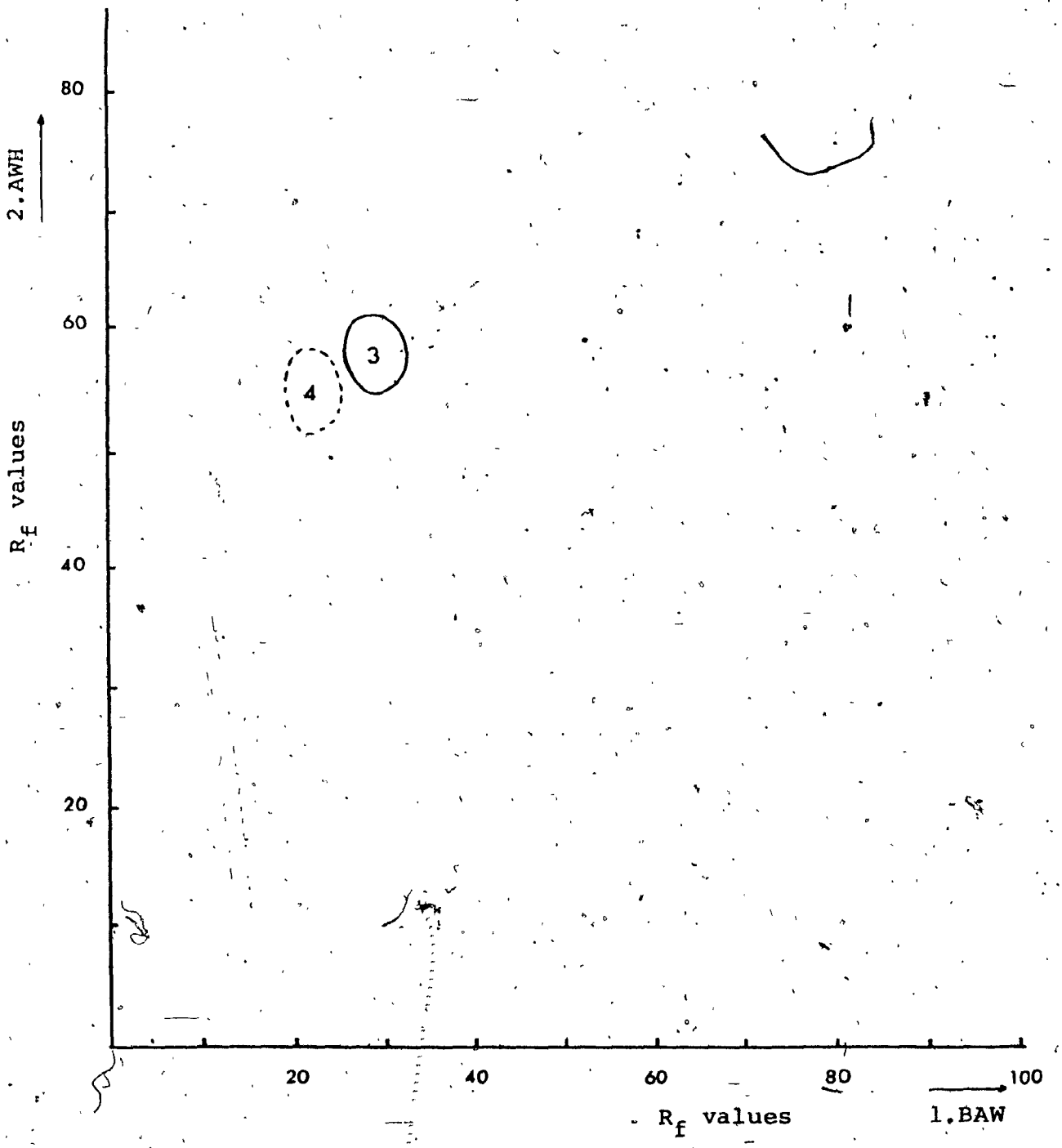


Figure 15.- Chromatographic profile of anthocyanins of the Orange variety.

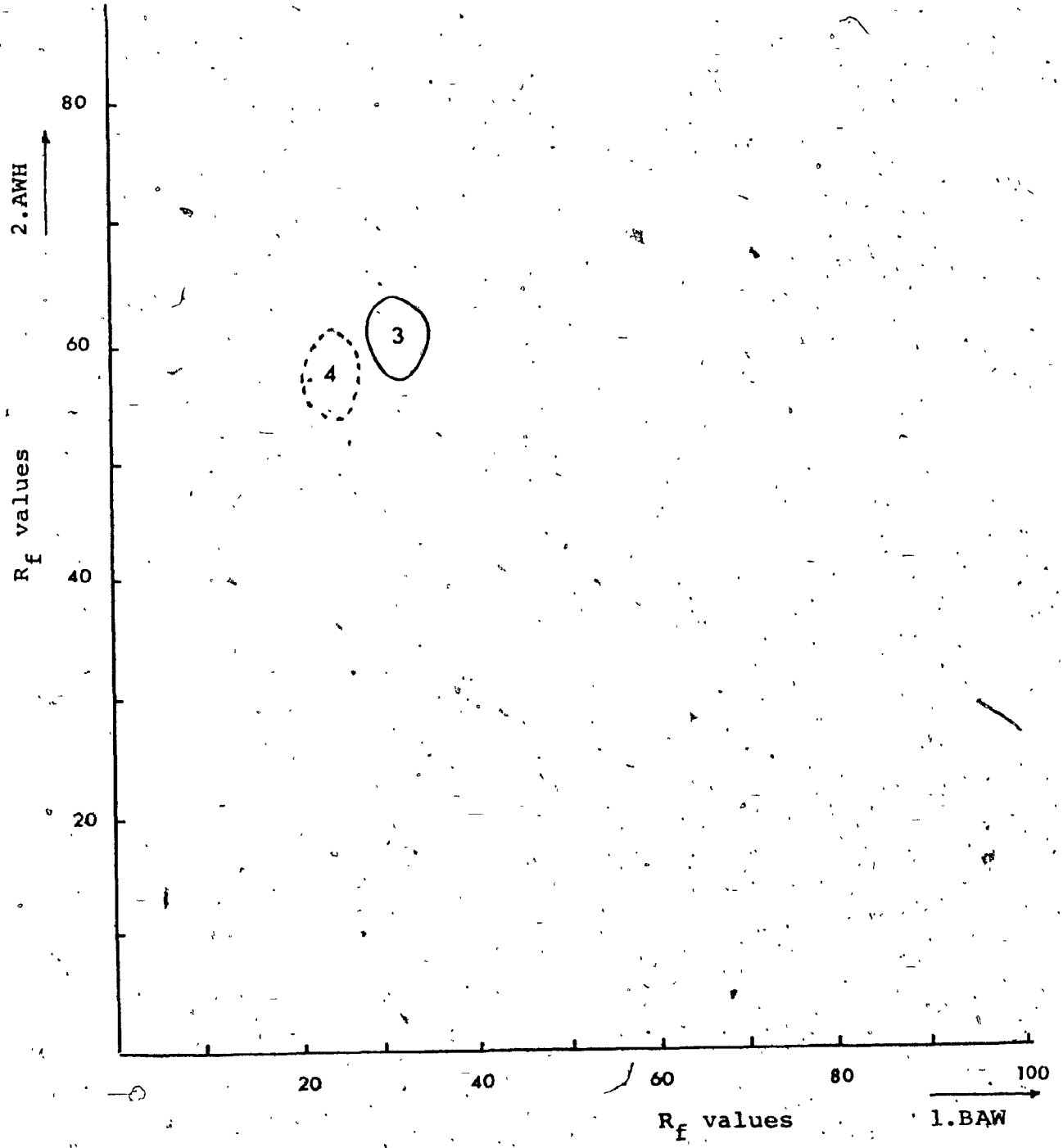


Figure 16.- Chromatographic profile of anthocyanins of the Salmon variety.

TABLE 9.- Anthocyanin pigments in various cyanic *B. tuberhybrida* separated by two-dimensional chromatography.

Pigment spot	R_f values (x100)*		Occurrence and relative intensity					Colour	
	1	2	R	O-r	P	O	S	Visible	U.V.
1	20	71	(+)	(+)	(+)	-	-	Magenta	Dull magenta
2	22.5	66	(+)	(+)	(+)	-	-	Magenta	Dull magenta
3	33	60	(+)	+	(+)	+ +	+	Orange-red	Dull orange-red
4	23	56	+	+ +	+ +	(+)	(+)	Magenta	Fluorescent magenta
5	34	50	+	+ +	+	-	-	Magenta	Fluorescent magenta
6	25	46	+	+	(+)	-	-	Magenta	Dull magenta
7	36.5	41	+ +	+ +	(+)	-	-	Magenta	Fluorescent magenta
8	26.7	35	(+)	-	-	-	-	Magenta	Dull magenta
9	50	28	(+)	-	-	-	-	Orange-red	Dull orange-red
10	61	26	(+)	-	-	-	-	Magenta	Dull magenta
11	35	23	+ + +	(+)	(+)	-	-	Magenta	Dull magenta
12	45	21	+ +	(+)	-	-	-	Magenta	Fluorescent magenta
13	52.5	19	(+)	-	-	-	-	Magenta	Dull magenta

(continued)

TABLE 9.-- Continued.

* Solvent key : 1. BAW (n-BuOH - HOAc - H_2O 4 : 1 : 5 upper layer)

2. AWH (HOAc - H_2O - conc. HCl 15 : 82 : 3)

** in 0.5 g wet weight of petal tissue.

Abbreviations : R : Red ; O-r : Orange-red ; P : Pink ; O : Orange ; S : Salmon.

+ + + very strong ; + + strong ; + moderate ; (+) weak.

of both cyanidin 3-glycosides and cyanidin 3,5-glycosides. The pigments as described above were however, probably not all in pure state, since the separation was done only in two solvent systems. Further purification in a third solvent system was therefore performed on the major pigments for the ultimate identification, in the manner described in the "Methods" Section.

I.3. Purification and Identification of the major anthocyanin glycosides.

(a) Isolation and Purification of major anthocyanin glycosides.

Paper chromatography of the major pigment spots in 1% HCl revealed that spots #3 and #11 were pure, but the spots #4, #5, #6, #7, #12 were not; each was the mixture of two pigments. Table 10 shows all the pigments observed, with their colour characteristics. The fluorescent pigments represented probably the cyanidin 3,5-glycosides (pigments #4b, #5b, #7b, #12b), none of which together with the non-fluorescent pigment #6b was subjected to further studies, because of their low concentrations. Complete identification of the purified major anthocyanins were based on :

- spectral properties
- R_f values in three solvent systems
- total hydrolysis with 2N HCl, and
- selective hydrolysis with hydrogen peroxide.

TABLE 10.- Purification of major anthocyanin pigments by re-chromatography in 1% HCl.

Pigment	Separation in 1% HCl			Colour		Pigments used for further studies (major pigments)
				Visible	U.V.	
#3	1 band	---	#3	Orange-red	Dull orange-red	#3
#4	Band 1	---	#4a	Magenta	Dull magenta	#4a
	Band 2	---	#4b	"	Fluorescent magenta	-
#5	Band 1	---	#5a	"	Dull magenta	#5a
	Band 2	---	#5b	"	Fluorescent magenta	-
#6	Band 1	---	#6a	"	Dull magenta	#6a
	Band 2	---	#6b	"	Dull magenta	-
#7	Band 1	---	#7a	"	Dull magenta	#7a
	Band 2	---	#7b	"	Fluorescent magenta	-
#11	1 band	---	#11	"	Dull magenta	#11
#12	Band 1	---	#12a	"	Dull magenta	#12a
	Band 2	---	#12b	"	Fluorescent magenta	-

(b) Spectral and chromatographic characterization of purified anthocyanins.

1. Spectral properties :

When studied under spectrophotometer, the seven pigments fall basically into two categories, one represented by the absorption spectrum shown in Figure #17 and the other in Figure #18. The first category includes pigment #3 and the second, pigment #4a, #5a, #6a, #7a, #11 and #12a.

Table 11 lists the absorption maxima of the pigments in visible and ultraviolet regions and the other characteristics which usually serve as important criteria for the identification of anthocyanin glycosides by spectral characterization.

The absorption maxima at 270 nm and 506 nm of the pigment and the absence of a spectral shift in the presence of $AlCl_3$ indicated that the pigment was a pelargonidin glycoside. The $\frac{E440}{E\ vis.\ max}$ and $\frac{E\ U.V.\ max}{E.\ vis.max}$ ratios being 40 and 62 respectively indicated that the 5-hydroxyl group on the A-ring was free. The lack of spectral absorption at 325 nm suggested an absence of acylation. All these characteristics suggested that the anthocyanin in question was a non-acylated pelargonidin 3- glycoside. It is not possible to determine the nature of the sugar residues by spectral characteristics.

With respect of the other pigments, i.e. #4a, #5a, #6a, #7a,

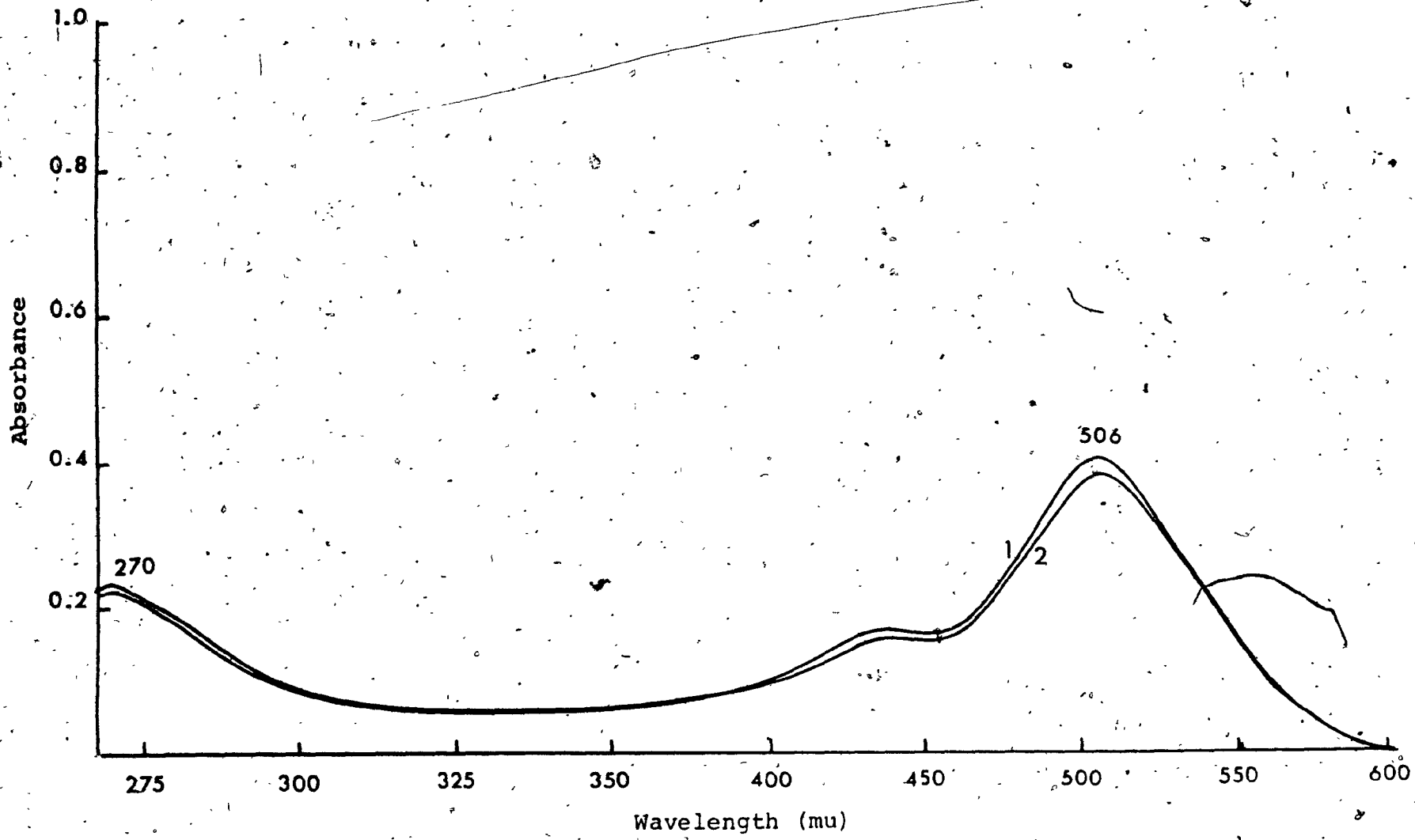


Figure 17.- Spectral absorption of pigment #3 (Identified as pelargonidin 3-glycoside)
Curve 1 : MeOH - 0.01% HCl. Curve 2 : MeOH - 0.01% HCl + $AlCl_3$

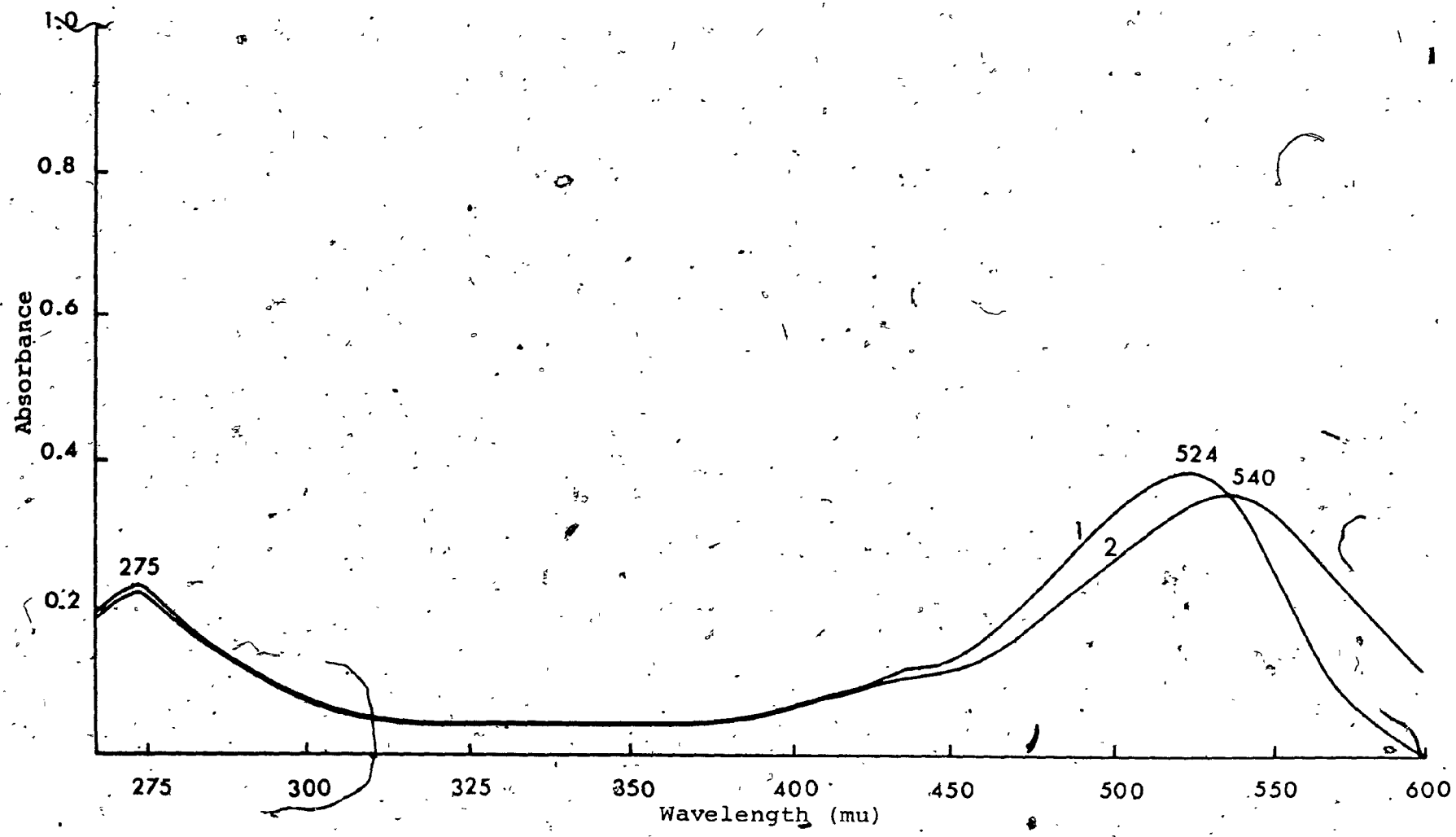


Figure 18.- Spectral absorption pattern of pigments #4a, #5a, #6a, #7a, #11 and #12a (identified as cyanidin 3-glycoside)
Curve 1: MeOH - 0.01% HCl. Curve 2 : MeOH - 0.01% HCl + $AlCl_3$

TABLE 11.- Spectral properties of purified anthocyanins.

Pigment	MeOH - HCl λmax(nm)	E U.V.max / E vis.max (as %)	E_{440nm} / E vis.max (as %)	E_{325nm}	$AlCl_3$ shift (nm)	Identification
#3	270,506	62	40	-	-	Pg 3-glycoside
#4a, 5a, 6a, 7a, 11, 12a	275,524	58	25	-	+18	Cy 3-glycoside
Literature values*						
Pg 3-glycoside	270,506	64	38	-	-	
Cy 3-glycoside	274,523	60	24	-	+	

* taken from Harborne (1967a).

#11 and #12a, the average absorption maximum was at 524 nm in the visible region and at 275 nm in the U.V. region. A shift of 20 nm was produced in the presence of $AlCl_3$. The average $\frac{E440}{E\ vis.\ max}$ and $\frac{E\ U.V.\ max}{E\ vis.\ max}$ ratios were 25 and 58 respectively, very close to the literature values of 24 and 60, characteristic of cyanidin 3-glycoside. No peak was seen in the 325 nm region, again indicating the absence of acylation. All the above evidence pointed strongly to all those pigments being cyanidin 3-glycosides.

2. Chromatographic properties :

Table 12 shows the R_f values of the individual anthocyanins in three solvent systems : BAW, 1% HCl and AWH. It also lists their colour appearance under visible and U.V. light. Similar data on the authentic markers of pelargonidin 3-sophoroside and cyanidin 3-sophoroside are also included. In comparison with the R_f values of authentic samples as well as literature values, pigment #3 was identified as pelargonidin 3-sophoroside. Other pigments were tentatively identified as follows : #4a as cyanidin 3-sophoroside, #5a as cyanidin 3-sambubioside, #6a as cyanidin 3-rutinoside, #11 as cyanidin 3-glucoside and #12a as cyanidin 3-galactoside. Futher characterization by total hydrolysis and hydrogen peroxide oxidation was necessary for confirmation of all pigments and in particular, the pigment #7a whose R_f values did not seem to match with any of the values reported by Harborne (Harborne, 1967a).

#11 and #12a, the average absorption maximum was at 524 nm in the visible region and at 275 nm in the U.V. region. A shift of 20 nm was produced in the presence of $AlCl_3$. The average $\frac{E440}{E\ vis.\ max}$ and $\frac{E\ U.V.\ max}{E\ vis.\ max}$ ratios were 25 and 58 respectively, very close to the literature values of 24 and 60, characteristic of cyanidin 3-glycoside. No peak was seen in the 325 nm region, again indicating the absence of acylation. All the above evidence pointed strongly to all those pigments being cyanidin 3-glycosides.

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TABLE 12.- R_f values and tentative identification of purified anthocyanins.

Pigment	R_f values (x100) in*			Colour		Tentative identification.
	1	2	3	Visible	U.V.	
#3	34	46	65	Orange-red	Dull orange-red	Pg 3-sophoroside
#4a	31	40	62	Magenta	Dull magenta	Cy 3-sophoroside
#5a	34	30	53	Magenta	Dull magenta	Cy 3-sambubioside
#6a	36	25	45	Magenta	Dull magenta	Cy 3-rutinoside
#7a	36	22	42	Magenta	Dull magenta	?
#11	37	10	26	Magenta	Dull magenta	Cy 3-glucoside
#12a	36	10	26	Magenta	Dull magenta	Cy 3-galactoside
Authentic Pg 3-sophoroside	34	46	65	Orange-red	Dull orange-red	
Authentic Cy 3-sophoroside	31	40	63	Magenta	Dull magenta	
Literature values**						
Pg 3-sophoroside	36	38	65	Orange-red	Dull orange-red	
Pg 3-glucoside	44	14	35	Orange-red	Dull orange-red	
Cy 3-sophoroside	33	44	61	Magenta	Dull magenta	
Cy 3-sambubioside	36	24	51	Magenta	Dull magenta	
Cy 3-rutinoside	37	19	43	Magenta	Dull magenta	
Cy 3-glucoside	38	7	26	Magenta	Dull magenta	
Cy 3-galactoside	37	7	26	Magenta	Dull magenta	

(continued)

TABLE 12 -- Continued.

Pigment	R_f values (x100) in*			Colour	
	1	2	3	Visible	U.V.
Cy 3-(2^G-xylosyl-rutinoside)	28	47	68	Magenta	Dull magenta
Cy 3-(2^G-glucosyl-rutinoside)	26	61	73	Magenta	Dull magenta

* Solvent key : 1. BAW (n-BuOH - HOAc - H_2O 4 : 1 : 5 upper layer)

2. 1% HCl (conc. HCl - H_2O 3 : 97)

3. AWH (HOAc - H_2O - conc. HCl 15 : 82 : 3)

** taken from Harborne (1967a)

Abbreviations : Pg : pelargonidin ; Cy : cyanidin.

(c) Characterization by Complete Hydrolysis

1. Identification of the Aglycones

The R_f values of the anthocyanidins of the purified pigments in BAW, FWH and Forestal along with other chromatographic data are shown in Table 13.

The experimental R_f values of the samples in FWH and Forestal solvent systems were lower than the literature values, but so were the values of the standard cyanidin marker run alongside. In BAW, all the experimental values obtained were slighly higher. The reasons for the differences in experimental and literature values could be temperature, humidity and other factors. Based on these chromatographic data and comparison with standard marker, the anthocyanidin of pigment #3 was identified as pelargonidin and those of pigments #4a, #5a, #6a, #7a, #11 and #12a were identified as cyanidin. The identification was further confirmed by the spectral absorption of the aglycones, with the main characteristics listed in Table 13.

2. Identification of the Sugar Moieties

The identification of the sugar moieties were achieved by paper chromatography. Table 14 records the R_f values of the sugar residues and their colour reactions to aniline hydrogen phthalate. The R_f values of the samples matched perfectly with those of the authentic sugars run alongside.

TABLE 13.- R_f values and spectral properties of the aglycones of purified anthocyanins.

Pigment	R_f values (x100) in*			Colour		MeOH - HCl vis.max (nm)	$AlCl_3$ shift (nm)	Identification.
	1	2	3	Visible	U.V.			
#3	58	28	82	O-r	Dull O-r	522	-	Pg
#4a, 5a,6a,7a,11, 12a	40	17	70	Mg	Dull Mg	535	+18	Cy
Authentic Cy	40	17	70	Mg	Dull Mg	535	+18	
Literature values**								
Pg	68	33	80	O-r	Dull O-r	520	-	
Cy	49	22	68	Mg	Dull Mg	535	+18	
Pe	63	20	71	Mg	Dull Mg	532	-	

* Solvent key : 1. Forestal (conc. HCl - HOAc - H_2O 3 : 30 : 10)

2. FWH (formic acid - H_2O - conc. HCl 5 : 3 : 2)

3. BAW (n-BuOH - HOAc - H_2O 4 : 1 : 5 upper layer)

** taken from Harborne (1967a)

Abbreviations : Pg : Pelargonidin ; Cy : Cyanidin ; Pe : Peonidine ; O-r : orange-red ; Mg : Magenta

TABLE 14.- Chromatographic properties of sugar moieties released by complete hydrolysis of purified anthocyanins.

Sample	R_f values (x100) in* 1	2	Colour reactions with aniline hydrogen phthalate	Identification
Authentic sugar :				
Glucose	12	30	Brown	
Galactose	11	27	Brown	
Arabinose	15	34	Pink	
Xylose	17	38	Pink	
Rhamnose	20	44	Yellow	
Sample spots				
Pigment #3	12	30	Brown	Glucose
" #4a	12	30	Brown	Glucose
" #5a	12 + 17	30 + 38	Brown + Pink	Glucose + Xylose
" #6a	12 + 22	30 + 44	Brown + Yellow	Glucose + Rhamnose
" #7a	11 + 20	27 + 44	Brown + Yellow	Galactose + Rhamnose
" #11	12	30	Brown	Glucose
" #12a	11	27	Brown	Galactose.

* Solvent key : 1. BAW (n-BuOH - HOAc - H_2O 4 : 1 : 5 upper layer)
2. BBPW (n-BuOH - benzene - pyridine - H_2O 5 : 1 : 3 : 3 upper layer)

Pigments #3, #4a and #11 released only glucose ; pigment #12 gave only galactose ; pigments #5a, #6a, #7a released two sugars each, which were identified as glucose and xylose, glucose and rhamnose, and galactose and rhamnose respectively.

(d) Position and Nature of the Intact Sugar Residues

Table 15 lists the R_g values (mobility relative glucose) in BAW and BBPW solvent systems of the intact sugar residues released from the 3-position of the anthocyanidin molecule. The sugars from various spots were identified on basis of their R_g values in both solvent systems and their colour reactions to aniline hydrogen phthalate.

All the sugars had values consistently either slightly higher (as in BBPW) or lower (as in BAW) than the reported values. However, in each solvent system, rutinose, wherever it occurred, correspond very closely with the standard rutinose run alongside. On basis of the above comparison, spot #3 was identified as sophorose, #4a also as sophorose, #5a as sambubiose, #6a as rutinose, #7a as robinobiose, #11 as glucose and #12a as galactose. Thus, the identification of major anthocyanin pigments based on various criteria previously described is as follows : pelargonidin 3-sophoroside (pigment #3) ; cyanidin 3-sophoroside (pigment #4a) ; cyanidin 3-sambubioside (#5a) ; cyanidin 3-rutinoside (#6a) ; cyanidin 3-robinobio-

TABLE 15.- Chromatographic properties of the intact sugar residues following selective hydrolysis with H_2O_2.

Sample	R_g values (x100) in * 1	2	Colour reactions with aniline hydrogen phthalate	Tentative identification.
#3	56	65	Brown	Sophorose
#4a	56	65	Brown	Sophorose
#5a	64	64	Pink-brown	Sambubiose
#6a	72	80	Brown	Rutinose
#7a	68	67	Brown	Robinobiose
#11	100	100	Brown	Glucose
#12a	97	95	Brown	Galactose
Standard glucose	100	100	Brown	
" rutinose	72	80	Brown	
Literature values **				
Glucose	100	100		
Rutinose	74	77		
Robinobiose	70	64		
Sambubiose	65	60		
Sophorose	58	61		
2^G-Glucosylrutinose	46	42		
2^G-Xylosylrutinose	55	57		

(continued)

TABLE 15.-- Continued

* Solvent key : 1. BAW (n-BuOH - HOAc - H_2O 4 : 1 : 5 upper layer

2. BBPW (n-BuOH - benzene - pyridine - H_2O 5 : 1 : 3 : 3 upper layer)

** taken from Harborne (1973)

side* (#7a) ; cyanidin 3-glucoside (#11) and cyanidin 3-galactoside (#12a).

Based on the intensities of the pigment spots as appeared on the two-dimensional chromatographic profiles of the cultivars, a resumé of occurrence and relative concentrations of the major anthocyanin pigments as identified above are presented in Table 16.

II. STUDIES OF FACTORS MODIFYING THE COLOUR APPEARANCE OF ANTHOCYANIN PIGMENTS IN *Begonia tuberhybrida*.

II.1 pH of the Flower Cell Sap

The pH measurements of the flower cell sap of the seven varieties are presented in Table 17. It was seen that the pH in all forms was highly acidic, with the variations ranging from 1.45 to 1.49 in the first two sets of measurements and from 1.50 to 1.53 in the other which was made with flowers harvested in a later season. One can thus state that the pH was marked with an extreme acidity with no remarkable differences among the various cultivars.

II.2 Occurrence of Flavonols and Chelating Metals

(a) Presence of Flavonols.

The presence of quercetin and kaempferol as established in the earlier study on the occurrence of aglycones in the va-

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* reported in Epacridaceae by Jarman and Crowden (1973) and in *Cornus canadensis* by Wang and Francis (1974).

TABLE 16.- Major anthocyanin pigments of *Begonia tuberhybrida* and their occurrence in various cyanic colour forms.

Pigment	Occurrence and relative concentration				
	Red	Orange-red	Pink	Orange	Salmon
Pg 3-sophoroside	+	+ +	+	+ + +	+ +
Cy 3-sophoroside	+ +	+ + +	+ + +	+	+
Cy 3-sambubioside	+ +	+ + +	+ +	-	-
Cy 3-rutinoside	+ +	+ +	+	-	-
Cy 3-robinobioside	+ + +	+ + +	+	-	-
Cy 3-glucoside	+ + + +	+	+	-	-
Cy 3-galactoside	+ + +	+	-	-	-

Abbreviations : Pg : pelargonidin ; Cy : Cyanidin

+ + + + very strong ; + + + strong ; + + moderate ; + weak.

TABLE 21.- Free phenolic acid in the cell sap.

Sample	R_f values (x100) in*		Colour reactions with**	Identification
	1	2		
Authentic phenolic acid:				
p-coumaric	00	-	Blue	
Caffeic	15	54	Blue	
Ferulic	78	20	Blue	
Vanillic	85	75	Blue after NH_3 fuming	
Gallic	07	45	Blue	
Sample spot	17	55	Blue	Caffeic acid

* Solvent key : 1. Acetic acid - chloroform (1 : 9)

2. Ethyl acetate - benzene (9 : 11)

** Folin reagent.

DISCUSSION

The colour variations in many garden cultivars are related to anthocyanins which are responsible for most pink, red, mauve and blue colours. However, the number of colour variations that are known cannot be attributed to the relatively few anthocyanidins that occur in nature. A great deal of effort was devoted in the past to the identification of anthocyanins but, until recently, little was known regarding their existence in the natural state within cells. The internal environment of the cell greatly influences their physical and chemical properties. The spectral studies of the pigments *in situ* carried out by S. Asen and his associates during the past decade have resolved some of the questions regarding not only colour variations in flowers but also the mechanism involved in the drastic colour changes observed in some flowers during maturation and senescence.

The anthocyanins and other accompanying flavonoids are distributed mostly in the epidermal tissue of the flowers and, in intensely coloured flowers, the pigments are also found in the sub-epidermal parenchyma tissue of the petal. The cells in both these tissues have more than 90% of their volume occupied by a centrally-located vacuole, containing a variety of substances in addition to flavonoid pigments. The chemical composition of the vacuolar sap varies from one flower to another, from one cell to another in the same

tissue of the flower and also at different stages of development. The dissolved substances include several mineral ions (potassium, sodium, magnesium and calcium) and anions (chlorides, sulphates and phosphates). Carbohydrates such as sugars and mucilages are present. Various nitrogenous substances (amino acids, amides, proteins, peptides and alkaloids) are quite common constituents of the vacuole. Specific substances such as betalaines are restricted to the Order *Centrospermales* and a few other unrelated families. Various organic acids such as citric, malic, oxalic, succinic and tartaric are also found. In addition to the flavonoid pigments (anthocyanins, flavones, flavonols, xanthones), other phenolics occur; namely phenolic acids in the free or bound state as esters and glycosides. Tannins also occur similarly. Crystalline constituents including salts of organic acids (calcium oxalate), pigments and certain amides such as asparagine are also common components of the vacuole (Voeller, 1964).

The biological significance of the vacuole is due to the presence of flower pigments in the vacuole, which constitute the main attractants for their insect pollinators.

Storage of many substances following accumulation seems to be the major function of the vacuole. In some cells, the amount of organic acids in the vacuole indicates the transport of positively charged cations and the negatively

charged anions of organic acids which are synthesized in the cytoplasm accompanying the cations into the vacuole in order to establish electrical equilibrium.

Thus, the internal environment of the vacuole in which the flower pigments are present can greatly influence their colours. The extracted pigments appear red in acid and blue in an alkaline medium. The cell sap pH is, however, almost always acidic, in the range of pH 2.8 - 6.2. A fairly acidic cell sap stabilises the red colour of oxonium salts of various anthocyanins and petal tissue appears red. The requirement of an alkaline cell sap, as speculated by Willstatter (1913) to be the cause for blue colours, involved the presence of alkaline metal salts causing the conversion of red oxonium salts into blue ionized anhydrobases.

Willstatter's assumption was, however, proven to be mistaken, since, due to the presence of dicarboxylic acids of citric acid cycle, flower petals are almost always acidic and never alkaline. In an alkaline sap, the phenoxides cannot be stable. The pH of the cell sap is equally significant whether it is involved in the production of red colours in an acid medium or in its implication in the formation of violet or purple anhydrobases at a pH value between 4.0 - 6.2, which, however, convert to colourless carbino- or pseudobases. Since both the red oxonium form and the purple anhydrobases are unstable at higher pH, anthocyanins

alone cannot contribute significantly to flower colour at pH above 4.0.

The appearance of blue colour as well as its several shades such as mauve, violet, purple, etc., is due to one of the several possibilities described below. A knowledge of the factors responsible for the blue shades is significant in any attempt to explain the total absence of these colours in many garden ornamentals such as *Canna*, *Rosa*, *Begonia* and *Antirrhinum*. At the same time, the conditions underlying the existence of predominantly red shades and related colour variations can be properly examined. The specific examples that have been selected here are therefore pertinent to the discussion on flower colour in *B. tuberhybrida* as established in this research.

Presence of delphinidin alone can result in a blue colour at the pH range encountered in flowers. Apparently, the violet-coloured quinonoid anhydrobases of this pigment are quite stable at a pH of 4 - 6 and contribute to the blue colour. The mauve or violet varieties of *Verbena* and *Brunsfelsia calycina* are due to pure delphinidin glycosides. At a higher concentration, the pgiment confers a purple-black colour to the tulip cultivar "Queen of the Night" as well as the purplish-black *Viola* (Harborne, 1965).

The pigment of the blue larkspur is a delphinidin glycoside

in the form of its neutral anhydrobase, free of metal or co-pigment, but adsorbed to a pectin-like polysaccharide (Bayer *et al*, 1966). Simulation of the natural pigment by addition of purified apple pectin to pure delphinidin from larkspur flowers increased the extinction and yielded a complex with the same spectral maxima as the natural pigment. It had a high molecular weight and was non-dialysable. Similar addition of cyanin to pectin did not yield such a complex.

Asen *et al* (1975) reported the occurrence of delphinidin 3,5-diglycoside acylated with p-hydroxybenzoic acid and contained kaempferol glycosides as co-pigments in a reddish-purple variety of larkspur, [illegible] Auct. (cv. "Dark Blue Supreme"). The colour change from the moderately reddish purple in the young flowers to a light bluish purple in the older flowers was accompanied by a pH change from 5.5 - 6.6 and deep blue crystals were found in the vacuoles of older flowers. In the younger flowers, no such crystals were seen, but the pigment appeared to be in an associated form in its natural state. Apparently the pigment was released from this association with age and became converted to crystals in the older flowers.

Delphinium ajacis has been the object of several investigations and the many conflicting reports on the state of anthocyanins in the "violet" flowers appear to be related to the significant changes occurring within the flowers during

maturation and senescence, and their effect on flower colour. The variations are also probably due to different plant material, the larkspur having a complex hybrid origin.

The bright blue colour of the "Prof.Blaauw" Iris, a hybrid of *Iris* [illegible] Bois and Rent., is a stable, non-metallic anhydrobase of delphinidin 3-(p-coumaryl rutinoside)-5-glucoside associated with pectin and complexed with several C-glycosyl flavones as co-pigments, at a pH of 5 (Asen *et al*, 1970). In their attempts to simulate the intact spectra, Asen *et al* found that the co-pigments complexed readily with the pigment at a pH as low as 1.2, causing a bathochromic shift of 40 nm. Increasing the pH to 5 resulted in an absorption spectrum that matched the pigment in the intact cell. Among the several compounds tested for co-pigmentation, tannins, aurones, flavones, flavonols, and flavanones were found to be most effective. Cinnamic acids also gave significant shifts (5 - 9 nm) but amino acids and alkaloids were totally ineffective.

The range of colours from reddish purple to blue in *Limonium* (Statice) cultivars was due to delphinidin glycosides co-pigmented with luteolin and iso-orientin, but a clear correlation with pH was observed, with the bluest colour form having the highest pH value of 4.70 and the red-violet variety with a pH of 3.88. Intermediate shades had intermediate pH values of 4.15 and 4.26 (Asen *et al*, 1973). Since the co-pigment--pigment ratios were relatively constant, pH was apparently the underlying factor.

Yazaki (1976) reported a similar effect in the colour change associated with aging in *Fuchsia* cultivars. The colour change from blue-violet in young petals to purple-red in older flowers is correlated with a pH change from 4.8 to 4.2, involving co-pigmentation of pure malvin as an anhydrobase co-pigmented with flavonol glycosides. Furthermore, the decrease in pH in older flowers was correlated with an increase in free organic acids, especially malic and tartaric acids. The amounts of free amino acids and amides, especially asparagine, were greater in younger flowers than in older ones. Apparently the conversion of asparagine to aspartic acid in older flowers contributed to acidic pH, in addition to other organic acids.

In both the above cases, co-pigmentation was involved, without metals, in the pH range of 4 - 5.

Co-pigmentation of cyanidin glycosides with six flavonol glycosides at a pH of 2.8 - 2.0 is the main factor responsible for the red colour in "Red Wing" Azalea (Asen *et al*, 1971). The orange sport containing the same anthocyanin at the same pH was, however, deficient in co-pigments. The *in vivo* spectra of the pigments from the two cultivars varied in their visible λmax, the red variety showing a bathochromic shift of 11 nm, evidently due to co-pigmentation effect.

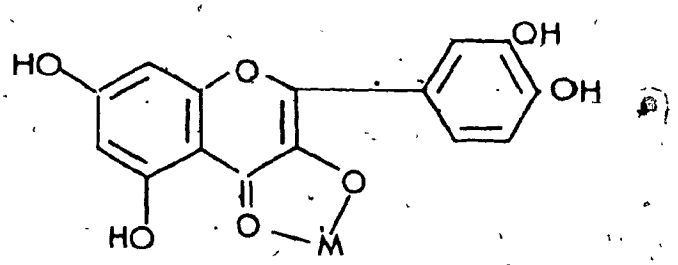
The conditions for metal chelation are more rigid, as described earlier in the literature review. Bayer's experiments

(Bayer *et al*, 1966) with synthetic complexes showed that neither the alkaline earth metals nor cobalt or nickel complexes of cyanin are stable and blue at pH 4 - 6. Bivalent ions of other transition elements also failed to form stable complexes. Deep blue chelates were obtained with iron, aluminum, zinc, titanium and chromium, but all except iron and aluminum occur in plants as traces only.

It is a well-known fact that red *Hydrangea* turn blue when sprinkled with iron-aluminum solution. These ions actually accumulate in the vacuoles but the phenomenon is not observed in rose when cut flowers are placed in iron-aluminum solution. Even the small amounts of iron and aluminum ions present in rose cells after 24 hours of uptake should be sufficient for complex formation but it does not occur.

The buffering system of the vacuole containing a variety of organic acid anions, metal ions and pigments controls the process of complex formation in a number of ways, including interactions between these components. When the metal ions are present in limited amounts, competition for these could occur among anthocyanins (with ortho di-hydroxyl groups), flavonols, flavones and citrate ions, provided these are in sufficient amounts. A preferential complex between citrate and aluminum was noted in simulation experiments (Bayer *et al*, 1966). The complexes so formed were stable and accounted for the failure of the blue colour to develop when cyanin was

added to the metal ion in citrate buffer at pH 4 - 6. Jurd and Asen (1966) observed the blue complexes and the subsequent bathochromic shift in the vis. λmax of the pigment only in acetate buffer at pH 5.45 and not citrate. Addition of citrate to acetate buffer reversed the shift and colour by abstracting the ions from the acetate buffer. The absence of blue ions is possibly due to preferential complexes of metals with more effective chelators such as citrate ions, flavonols and flavones. The stronger tendency of flavonols towards complex formation, relative to anthocyanins, is due to the presence of hydroxy keto grouping (Horhammer and Hansel, 1955).



The interaction between metal and co-pigment can have other interesting effects. Jurd and Asen (1966) have found that chlorogenic acid, a ubiquitous, natural polyphenol, affects

flower colour through complex formation with anthocyanins. In *Hydrangea*, aparently, both the red and blue varieties contain a moderate amount of this substance in their cells. However, in the blue variety, it appears to be present as a water-insoluble complex involving aluminium ions and delphinidin glycosides, and so cannot be detected, while substantial amounts are found in the red variety.

Bluing of roses during senescence was attributed to increasing pH, due to a large increase of free ammonia in the cells (Weinstein, 1957), and not to a change in total tannin content as suggested by Currey (1954). Yasuda (1970) has, however, shown the formation of tannin bodies in the epidermal cells of aging roses. These "spherules" seen in bluing roses were found to contain cyanin, a tannin-like sustance and iron.

The elucidation of factors causing colour variations has been possible mainly due to the development of sophisticated instrumentation techniques. The *in vivo* measurements of pigment spectra of single vacuoles especially by S. Asen and his colleagues at Beltsville Maryland, involved a specially devised microspectrophotometer. The pH measurements of single cell vacuoles were made with a special microelectrode. The approach used in their studies involved recording the absorption spectrum of the contents of the in-

tact cell, followed by simulation studies. The purified anthocyanin was mixed with one or more components (co-pigments or metals) known to be present, at the measured pH of the cell vacuole. The simulated pigment mixture yielded the same spectrum as the intact cell but was not necessarily identical with the mixture inside the vacuole. It is a reasonable approximation and perhaps the closest one can achieve. The *in vivo* spectrum is a more accurate representation of those cell contents that can effect the pigment curves and, consequently, the apparent colour due to the pigment. This is particularly so since the intact spectrum is subjected to the least disturbance or change. However, it has its own limitations, since not all substances occurring in the vacuole affect the pigment spectra directly but can have indirect effects. A corresponding chemical or biochemical analysis of the vacuolar contents would provide the actual identity and the amounts of various substances present as well as offer some clue as to the nature of their effects, if any. However, only the whole tissue can be extracted rather than the vacuole. There is no technique known at present of isolating the contents of a single vacuole and analyzing them. The isolation of the whole tissue would yield contents of all the cells, their vacuoles and cytoplasmic components and hence not be truly representative of the immediate environment in which the pigments are present.

In the light of the above evidence, we can now discuss the colour variations in *B.tuberhybrida* with respect to the following aspects, namely the predominance of the red colour and its shades, causes of colour variations in the narrow range of red/pink/orange and the total absence of blue colour among the cultivars. The epidermal cells in *Begonia* which contain the major portion of the pigments contain a large vacuole making up 90-95% of the cell volume, and a thin parietal layer of cytoplasm. While the vacuole is the only source of the flavonoid pigments, most of the other components in the free state are also presumably from the vacuole, although they may also exist in the cytoplasm.

In the absence of sophisticated instrumentation facilities, the closest approximation of the *in vivo* conditions in this study is represented by the spectra of the crude pigments measured in 0.01% MeOH - HCl. The pH of the cells of all cultivars at an equivalent stage of growth was about 1.50. The method used here was conventional and the value, although representing the pH of essentially the vacuolar sap, could reflect some contamination from cytoplasmic components. This pH is more acidic than is usually encountered in many plants. The factors that could possibly contribute to this pH will be discussed later, but it is obvious that this acidic pH value is the most important contributing factor to the occurrence of red colour forms in *B.tuberhybrida*, and the

colour variations are due to other factors exerting their effects through a cell sap that is pronouncedly acidic.

The spectra of the crude pigments fall into three categories. The Red, Orange-Red and Pink showed the same visible maxima (522 nm) suggesting the same major anthocyanin pigments. The spectral peaks represent the cumulative maxima of all the pigments present, namely cyanidin and pelargonidin in the visible regions and quercetin and kaempferol in the UV region. The visible maxima of 522 nm, due to both cyanidin and pelargonidin glycosides, is actually closer to the λmax of cyanidin than pelargonidin glycosides, indicating the predominance of cyanidin pigments. The bathochromic shift with $AlCl_3$ as well as the estimation of relative pigment concentration confirm this.

In the Red and Orange varieties, the presence of flavonols, quercetin and kaempferol, is indicated by the inflection at 350 nm. An analysis of the total aglycones confirmed their presence, although in minor amounts. On the other hand, the Pink variety exhibits a well-defined peak in the crude extract, substantiated by a greater amount of these flavonol aglycones in the hydrolyzed extract.

The spectra of all the three cultivars indicate a predominance of 3-glycosides (peak at 440 nm). Characterization of the major anthocyanins from all three indeed showed the presence of six cyanidin 3-glycosides, *viz.* cyanidin 3-sophoroside,

cyanidin 3-sambubioside, cyanidin 3-rutinoside, cyanidin 3-robinobioside, cyanidin 3-glucoside and cyanidin 3-galactoside, and one pelargonidin 3-glycoside, viz. pelargonidin 3-sophoroside. The relative concentration of these glycosides varies among the three cultivars. Since pelargonidin is the main factor for orangeness, a relatively higher concentration of this pigment in the Orange-Red variety is not surprising while the Pink and the Red have lesser amounts of this pigment but occurring in approximately equal concentrations in the two cultivars. On the other hand, the presence of six cyanidin glycosides in the Red variety with three of them occurring in higher concentration than in the Pink variety (cyanidin 3-rutinoside, cyanidin 3-robinobioside and cyanidin 3-glucoside) appears to differentiate the Red from the Pink variety. In addition, cyanidin 3-galactoside is totally absent in the Pink variety. The sugar moieties apparently have no contribution to colour variations and differences in the relative proportion of the individual cyanidin glycosides have probably no significance.

The spectra of the Orange and Salmon varieties are again due to the presence of both cyanidin and pelargonidin glycosides. The λmax (visible) at 504 nm indicates the predominance of pelargonidin glycosides, failing to yield a bathochromic shift with $AlCl_3$. Characterization of aglycones shows relatively higher concentration of pelargonidin which apparently supersedes and obscures the spectral

properties of cyanidin. Purified cyanidin 3-glycosides have a λmax (visible) at 522 nm, yielding a bathochromic shift of 20 nm with $AlCl_3$. Purified pelargonidin 3-glycoside has its visible λmax at 506 nm, lacking a shift with $AlCl_3$. The cumulative spectrum of all pigments in the crude state of Orange and Salmon varieties still yields a visible λmax at 506 nm with no shift. The proportion of pelargonidin to cyanidin *in vivo* seems to determine these effects on the crude spectra and causes the colour differences, not only between the Orange and Salmon but also between these two cultivars and the other cyanic forms, namely Red, Orange-Red and Pink. Kaempferol and Quercetin are present only in low amounts and the inflection at 440 nm is less pronounced than that in Red, Orange-Red and Pink varieties. The relatively higher proportion of pelargonidin glycoside apparently distinguishes the Orange from the Salmon.

The Yellow and White have only minor amounts of the two flavonols, namely quercetin and kaempferol.

It is apparent that, in all the six cyanic varieties, the flavonols are present without involving co-pigmentation. The major anthocyanin pigments, purified by re-chromatography, do not exhibit any inflection or peak at 350 nm. The visible λmax of cyanidin glycosides is at 524 nm and that of pelargonidin glycosides is at 506 nm and these are essentially the same maxima (522 and 504 nm respectively)

of the crude pigments, with the predominance of cyanidin and pelargonidin respectively. Any co-pigmentation effect could have caused a bathochromic shift in the visible λmax, in addition to a peak or shoulder at 350 nm due to the flavonols. No such shift was observed even in the Pink variety, with a well-defined peak at 350 nm. It is quite unlikely that some of the minor pigments which were not characterized could affect the visible colour significantly or be involved in co-pigmentation. The pH of the cell sap at about 1.50, although acidic, could permit co-pigmentation to occur, but the colour due to such pigment--co-pigment complex would be red rather than blue. The effect of these flavonols on the crude pigments could therefore be from co-existence with anthocyanin in the same vacuole rather than co-pigmentation which actually involves a loose chemical complex between the two pigment molecules. Purification of the pigments by re-chromatography cannot possibly dissociate the complex, the latter process requiring more severe procedures (Yazaki and Hayashi, 1967; Nozzolillo, 1970).

The spectra of the crude pigments from all the seven cultivars are characterized by another peak at 325 nm, indicating acylation. Again, the purified major pigment glycosides do not exhibit this particular absorption maximum. Although purification by re-chromatography is known to cause de-acylation in certain unstable acylated glycosides (Ashtakala and Forward, 1971), it is unlikely that the major pigments

analyzed here are acylated, since their R_f values in BAW on the two-dimensional chromatogram are not indicative of acylation. Evidently, the phenolic acids occur in the cell sap without involving acylation or co-pigmentation with anthocyanins. Subsequent analysis of vacuolar contents has indeed shown the presence of the phenolic acid, caffeic acid in the free state. The peak is quite noticeable in all the cultivars, except the Pink, where absorption in this region is obscured by the increased absorption towards 350 nm due to flavonols. The ratio of acid to pigment; $(\frac{\text{O.D. at 325 nm}}{\text{O.D. at vis } \lambda\text{max}})$, of the various cultivars is quite variable and the appearance of this peak suggests contamination with anthocyanins *in vivo*.

It should be mentioned that, while only the major pigment glycosides for each cultivar were re-purified and fully characterized and found to be free of acylation, one of the minor components (spot No. 13 in the two-dimensional chromatograms) with a highest R_f value in BAW could well be acylated since acylation increases R_f value in BAW. But it is unlikely that the presence of a minor acylated component could have a strong effect on flower colour. Similarly, two minor components, spots No. 1 and No. 2 appearing in the chromatographic profiles of the Red, Orange-Red and Pink varieties, are characterized by very low R_f values in BAW and appear pink or magenta. These are possibly the triglycosides of cyanin but their low concentration precludes

any significant contribution to flower colour. Harborne and Hall (1964) have reported the occurrence of two triglycosides in a few species of *Begonia*. In *B. tuberhybrida*, they appear to be insignificant.

In this study, a close correlation has been established between the pigments and flower colour based on spectral characteristics, chromatographic properties of the glycosidic pigments, analyses of the glycosides by complete hydrolysis and characterization of the aglycones and sugars, as well as selective hydrolysis by H_2O_2 oxidation and subsequent characterization of the sugar moieties. Although in the above discussion, the relative concentrations of the pigments were estimated by a visual comparison of the intensity of the aglycones and the glycoside spots on the chromatograms, the data on the quantitative estimation of the relative anthocyanidin concentrations in all the cultivars support the conclusions stated above in addition to providing further evidence for causes of colour variations in *Begonia tuberhybrida*

Increasing "redness" is seemingly due to increase in cyanidin content. Starting from lowest amounts in Orange and Salmon, following in sequence are Pink, Orange-red and Red in the respective ratio of approximatively 1:1:24:60:78 . On the other hand, increasing "orangeness" is apparently due to increasing pelargonidin content, the Pink variety containing

the lowest amount, followed in sequence by Red, Salmon, Orange-Red and Orange in the ratio of 1 : 2.6 : 5.3 : 6 : 9.3, respectively. The Orange and Salmon, with the same cyanidin content, are distinguished by their pelargonidin contents in the ratio of approximately 2:1. The Salmon and Orange-Red, with approximately the same pelargonidin content, differ in their cyanidin content by a ratio of 1:60 respectively. However, since both cyanidin and pelargonidin are present together in all cyanic cultivars, the relative proportions of the two pigments appear to be the determining factor in establishing colour differences among the cultivars. The redness is due to 20 times as much cyanidin as pelargonidin in the Red variety, followed by Pink with 16 times as much cyanidin as pelargonidin, and Orange-Red with 7 times as much cyanidin as pelargonidin.

A decreasing cyanidin:pelargonidin ratio establishes the colour sequence starting from Red - Pink - Orange-Red - Salmon to Orange (decreasing redness and increasing orangeness). Taking the Orange variety with the lowest cyanidin: pelargonidin ratio as 1, we arrive at a sequence of Red - Pink - Orange-Red - Salmon - Orange, while the two extreme colours Red and Orange contain the largest amount of the typical pigments, namely cyanidin and pelargonidin, contributing to these colours respectively, the intermediate colours are a result of specific distribution of the two pigments. The Pink is close to the Red in its cyanidin:

pelargonidin ratio (80%), but the Orange-Red has about 35% of this ratio for the Red, while the Salmon has about 0.8%. Some of the colour forms with subtle shades would probably fall in between these ratios.

The Yellow and White are pigmented mainly with the flavonols quercetin and kaempferol. However, the extractable amount of flavonols from the same quantity of flower tissue as used in the anthocyanidin study was not sufficient for analysis and no attempts were made to determine their quantities other than visual comparison on chromatograms.

Although the contribution from carotenoid pigments was not investigated in this study, no chromoplasts were observed in the cytoplasm of the epidermal or sub-epidermal cells under the microscope. Apparently, the contribution of carotenoids to colour variations in *Begonia tuberhybrida* is insignificant.

The acidic cell sap at a pH of about 1.50 is thus responsible for the red and shades of red in the flowers of *B. tuberhybrida*, the red oxonium salts of the anthocyanins being stable at this pH. The colour variations within this range among the cyanic cultivars are due to varying proportions of cyanidin and pelargonidin glycosides, an increasing cyanidin content causing increasing redness. While several cyanidin glycosides occur along with a single pelargonidin glycoside as the major pigments of Red, Orange-Red and Pink cultivars, the

Orange and Salmon are characterized by one cyanidin and one pelargonidin glycoside as the only major pigments. It therefore appears that in *B. tuberhybrida*, it is not merely an increasing pelargonidin content, but an actual decrease in cyanidin content that causes a tendency towards orangeness.

The factors contributing to the acidic cell sap appear to be mainly the organic acids contained in it. Plant cell sap is rich in organic acids, with pH values less than 5.5. Thomas *et al* (1973), reporting on the acid metabolism of certain plants, has cited pH values of 4 - 5 for leaves of many succulents; 3.26 - 3.67 for rhubarb petioles; 2.88 for *Valencia* oranges; 2.82 for leaves of *Pelargonium*, 1.53 for *Begonia* leaves and 1.50 for *Oxalis* leaves. These values are genotypic characteristics. (For comparison, the pH of 0.1N HCl is 1.07). The concentration of some of the above acids may reach 1N. However, the plant acids are weak acids and among these, oxalic acid is stronger than the others, contributing to the low pH of *Begonia* and *Oxalis* leaves.

Other substances contributing to the total acidity of the plant sap include fatty and amino acids, certain proteins, aromatic and other acids. However, the concentration of hydrogen ion in the cell sap is more significant than the total acidity in the consideration of colour variations of anthocyanins. In addition to the total acidity and percentage dissociation of the various acidic substances, the presence of buffer substances are also significant in controlling the hydrogen ion concentration in the cell sap.

Experimental reports showed that salts of malic and citric acids also play a major role in the buffering system of sap (Thomas *et al*, 1973). The hydrogen ion concentration of sap which contains these salts is likely to be higher than one lacking them. Yazaki (1976) found a higher citrate and malate content in young *Fuchsia* flowers with lower pH, relative to aged, bluing petals.

Analysis of the cell sap from all the *B. tuberhybrida* revealed the presence of four organic acids : citric, malic, oxalic and succinic in relatively large amounts, of these, citric acid seemed to be in highest concentration. The presence of free titrable acids in the *Begonia* is due to the operation of crassulacean acid metabolism (Ranson, 1965), involving dark fixation of CO_2, the gas entering the epidermal cells through the cuticle The metabolism in accumulation of acids by dark fixation of CO_2 was observed during maturation and senescence (Weinstein and Laurentcot, 1958). Malate, succinate, fumarate and citrate were seen to accumulate in petals during the early stages.

The metal ions detected in the *B. tuberhybrida* tissue included aluminium at less than 5 ppm, iron at 2 ppm and magnesium at 70 ppm. There are several reasons for assuming that pigment - metal chelations do not occur in *B. tuberhybrida*. The first and most important factor is the pH, which

at 1.50 maintains the cyanidin in its oxonium form, thereby preventing a chelation with the metals. Secondly, none of the above metals other than iron and aluminium are likely to form a stable blue complexes with cyanidin (Bayer *et al*,1966). Although Hayashi *et al* (1958) isolated a blue compound containing a magnesium-delphinidin glycoside complex from *Comelina communis*, simulation experiments by Bayer failed to yield complexes of delphinidin with alkaline earth metals at pH 2 - 9 (Bayer, 1958, 1960). Thirdly, the high citrate concentration would preferentially abstract the ions from the medium for the formation of citrate - metal complexes. Finally, kaempferol and quercetin could preferentially form chelates with metals. Both these substances are more powerful chelators than anthocyanins due to the presence of hydroxy-keto grouping in the molecule.

Although the absolute concentration of cyanidin was not determined, the existence of factors that prevent the pigment - metal complex formation seemed to be far too many, so that even if the pigment - metal ratio was within the required range, the occurrence of blue colour in these cultivars would still not be possible.

Other factors influencing the pH of the cell sap are the accumulation of certain basic amino acids and amides. The accumulation of amides and free amino acids precedes the ammonia formation during early stages of senescence of roses (Weinstein, 1957). Increased amounts of free amino acids and amides, particularly asparagine were observed in the

younger petals of *Fuchsia*, which in the older stages was converted to aspartic acid, increasing the pH value (Yazaki, 1976).

Although in the present study, no attempt was made to study the metabolic changes accompanying senescence, an analysis of the free amino acids in the cell sap of *B. tuberhybrida* flowers at the youger stage of development was considered useful in examining their contribution, if any, to flower colour. The major free amino acids found in all the cultivars were : lysine, serine, alanine, phenylalanine and glutamic acid. The only amino acid that could have contributed to the acidic pH in addition to the oraganic acids, would be the glutamic acid.

In his study on biogenesis of anthocyanins, Thimann and Edmondsen (1949) observed that sucrose stimulates anthocyanin production in *Spirodela oligorrhiza*. Leaf discs floated on dilute sugar solutions are known to induce greater anthocyanin formation. Increase in sucrose concentration up to 3% w/v in the medium enhanced anthocyanin content up to 35 times in detached flowers of *Impatiens* (Klein and Hagen, 1961). Increased anthocyanin accumulation in peel of apple was also seen to be correlated with various treatments known to increase the pentose phosphate pathway (Faust, 1965).

Sugars could thus exert their effect quite early in the biosynthesis of flavonoids. An analysis of the vacuolar contents *B. tuberhybrida* revealed the presence of two free sugars, namely glucose and fructose,with glucose occurring in much

larger amounts than fructose. Whether the free sugars themselves exert any effect on flower colour is not clear. A soluble pool of free amino acids, sugars and other substances represents a storage of these substances which the plant can utilize if needed, by retrieving them from the vacuole. Whether the sugars are retrieved back into the cytoplasm for flavonoid synthesis is not certain. Chromatography of the crude anthocyanins yielded a number of free sugars in *Iris* (Ashtakala and Forward, 1971) which apparently, occurred as contaminants.

The absence of blue colour in *B. tuberhybrida* appears to be mainly due to a highly acidic cell sap caused by an active Crassulacean acid metabolism and production of substantial amounts of citric, malic, oxalic and other acids. The presence of metal ions, even if sufficient for chelate formation, are apparently ineffective in complexing with cyanidin glycoside due to the highly acidic cell sap, at which the pigment would exist, not as anhydrobase, but as flavylium salt. Chelation involves the anhydrobase form only at a higher pH (4 - 6).

Co-pigmentation with flavonols which are present is not impossible although the phenomenon is not apparent. Co-pigmentation could occur with both the cyanidin and pelargonidin at the existing pH of 1.50 but the complexes would be redder, not blue.

It appears therefore that in *B.tuberhybrida*, the pH is the most

important factor determining the actual range in which the colour variations are manifested.

Bluing of petals with senescence, on injury or drying, was observed in *B. tuberhybrida*. A microscopic examination of petals showing the blue tinge, exhibited several filamentous structures appearing deep blue. These structures assumed a star shaped appearance with further progressing of aging. Although no further attempts were made to investigate the phenomenon, the structural changes observed here are similar to those seen in the red rose by Yasuda (1970). A massive tannin body was seen to form in the later stages, assuming an ellipsoidal shape, and containing cyanidin, iron and tannin. The clear, blue tinge seen in *B. tuberhybrida* flowers was found to start from the edge of the petals and spread inwards to the center and was insignificant in the Red, Orange-red and Pink varieties, but not in the Orange or Salmon which retained their original colours, although fading. The Yellow and White similarly showed no change towards blue with age. It is thus probable that only cyanidin was involved in some complex formation which caused the blue tinge in those flowers in which it was present as the predominent pigment, so as to be evident. It may be speculated that a pH change towards alkaline occurs in *B. tuberhybrida* during senescence, similar to that occurring in rose (Weinstein, 1957). A fall in citrate concentration accompanying the pH change during senescence is also another possibility that could permit abstraction of metal ions from citrate complexes and allow a pigment - metal complex to occur

during senescence (Weinstein and Laurencot, 1958). It is perhaps equally likely that the flavonoid content decreases with age, allowing preferential complexes of metals with anthocyanins. In the absence of experimental evidence in *B. tuberhybrida*, these changes can only be speculated, to account for bluing effects in these flowers predominantly pigmented with cyanidin.

In *Begonia*, as in many other garden ornamentals where blue colour forms are totally unknown, it is noteworthy that delphinidin is totally absent. When wild plants are brought under cultivation, mutations seem to have occurred in the direction delphinidin -- cyanidin -- pelargonidin and those cultivars which do not have wild species producing delphinidin are limited in the colour range they produce. Thus, in *Dahlia variabilis*, *Rosa* spp, *Canna indica*, no wild species are known with blue colours. In *Antirrhinum majus*, where again no true blue colour is known, the pigments include cyanidin and pelargonidin but no delphinidin. However two wild species have been known to contain delphinidin : *A. cornutum* and *A. nuttallianum* (Harborne, 1963b), suggesting a remote chance of producing a blue cultivar of Snapdragon There is no evidence of a blue "wild" species of *Begonia*. If it is possible to breed a *B. tuberhybrida* that has less citrate, higher pH and one that does not produce flavonols, the blue colour forms may be realized.

Harborne and Hall (1964) analyzed a variety of glycosides in

several *Begonia* species (excluding *B. tuberhybrida* used in this study) without relating them to flower colour. *Begonia* has a complex hybrid origin and there are pronounced morphological variations among the various species and their cultivars. The glycosides reported included : cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-sophoroside, cyanidin 3-xylosylrutinoside, cyanidin 3-glucosylrutinoside and pelargonidin 3-sophoroside. In this present study, the presence of some of these glycosides has been confirmed in *B. tuberhybrida*. In addition, three more glycosides have been isolated, namely cyanidin 3-galactoside, cyanidin 3-rutinoside, cyanidin 3-robinobioside

Thus the genus *Begonia* has a potential for synthesizing a variety of anthocyanin glycosides including two triglycosides containing branched trisaccharides and a variety of 3-monoglycosides and 3-biosodes. It is apparent from this research that the range of glycosidic forms exploited by *Begonia* is even greater. It is interesting to note that there are only two major anthocyanidins found among all the *Begonia* species studied so far : pelargonidin and cyanidin, the latter appearing much more commonly. The addition of glycosidic varieties does not seem to have contributed to colour variations significantly, the chief determinant factors being the relative proportion of the two aglycones.

The present study has clearly established the contribution of anthocyanidin to colour variations, the range of which is limited by a highly acidic pH brought about by organic acids

and a relatively high concentration of citrate in the medium. This study represents a careful examination of factors for establishing a basis of colour variations in terms of flavonoid pigments and several vacuolar components that are most likely to modify the pigments *in vivo* and therefore, the visible colour.

SUMMARY.

This research represents the first study of colour variations in flowers of *Begonia tuberhybrida* in relation to several vacuolar components in addition to flavonoid pigments.

The colour variations in *Begonia tuberhybrida* are limited to a narrow spectrum, ranging from shades of red through pink to orange. Blue, purple or related colours are totally unknown. The varieties chosen for this study included white, yellow , red, pink, orange-red, salmon and orange colour forms, and the factors underlying these variations were studied from two points of view :

a. to establish the cause of colour variations among the selected cultivars.

b. to define the conditions responsible for total absence of of blue or related colours.

The results from this study can be smmarized as follows :

1. The colour forms fall into two categories : cyanic which includes the Red, Pink, Orange-red, Orange and Salmon varieties with abundant anthocyanin, and the acyanic which includes the White and Yellow, containing no naturally occurring anthocyanin.

2. The pH of all varieties was highly acidic at 1.50 and is the major factor in determining the absolute colour range encountered in the cyanic forms.

3. The pigment aglycones of all the cyanic cultivars included two anthocyanidins : cyanidin and pelargonidin, and two

flavonols in minor amounts : quercetin and kaempferol. The acyanic forms are characterized by the presence of the two flavonols in minor amounts.

4. The relative proportion of cyanidin to pelargonidin was the main determinant factor of colour variations among the cyanic forms, which could be grouped into two categories : a) the Red, Pink and Orange-red characterized by a higher cyanidin to pelargonidin ratio while b) the Orange and Salmon had a higher pelargonidin to cyanidin ratio. The two extreme colour forms, Red and Orange, were due to the highest concentrations of cyanidin and pelargonidin respectively , the typical pigments contributing to these colours. The intermediate shades were due to specific proportions of the two pigments.

5. Several cyanidin glycosides and a pelargonidin glycoside characterized the Red, Pink and Orange-red varieties, whereas the Orange and Salmon were characterized by a single cyanidin glycoside and a single pelargonidin glycoside.

6. The major anthocyanin glycosides of the Red, Pink and Orange-red varieties included a pelargonidin 3-sophoroside and five cyanidin glycosides tentatively identified as follows : cyanidin 3-sophoroside, cyanidin 3-sambubioside, cyanidin 3-rutinoside, cyanidin 3-robinobioside and cyanidin 3-glucoside. In addition, cyanidin 3-galactoside was also present in the Red and Orange-red varieties. The Salmon and Orange varieties were characterized by a single pelargonidin 3-sophoroside and a cyanidin 3-sophoroside.

7. Chromatographic studies on the crude pigments of the cyanic forms suggested the presence *in vivo* of a mixture of pelargonidin and cyanidin 3-, 3,5-glycosides and probably triglycosides, the latter two categories occurring in minor or trace amounts. All the above pigments occurred in variable amounts among the various selected cultivars.

8. Spectral studies on the crude pigments of the cyanic forms indicated the existence *in vivo* of a mixture of predominantly 3-glycosides of anthocyanins, along with flavonol(s) and phenolic acids(s). The anthocyanins appeared to coexist with the above compounds without involving either copigmentation or acylation.

9. The presence of free organic acids : citric, malic, oxalic and succinic appeared to be the major factor contributing to the highly acidic pH of the cell sap. Glutamic acid, occurring in the free state, also possibly contributed to the low pH value .

10. From the evidence obtained in this study, several causes for the absence of blue colour among the cultivars are suggested : absence of delphinidin ; a highly acidic cell sap which stabilizes the red oxonium salt, preventing the formation of metal complexes ; the presence of quercetin and kaempferol which are more effective metal chelators than anthocyanins ; and the relatively high content of citrate which can preferentially chelate with the available aluminium, iron and magnesium ions detected in the tissue, rendering them

unavailable for anthocyanin.

The bluing of petals observed during senescence of *B. tuberhybrida* flowers suggested changes in the metabolism and vacuolar components, possibly involving some of the above aspects.

11. The presence of cyanidin 3-galactoside. cyanidin 3-rutinoside and cyanidin 3-robinobioside in *Begonia tuberhybrida* extends the range of glycosidic variations known in the genus *Begonia*.

12. Colour variations in *Begonia tuberhybrida* are not correlated to differences in pH, flavonol pigments, free organic acids, free amino acids, free phenolic acids and sugars, suggesting similar metabolism conditions in all the cultivars. The relative proportions of the two anthocyanidin pigments is the primary underlying factor of all colour differences among the cyanic cultivars.

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