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A biosystematic study of Allium amplectens Torr

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A BIOSYSTEMATIC STUDY

OF *Allium amplectens* Torr.

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by

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

ILLUSTRATION PAGE

LIST OF TABLES

TABLE PAGE

LIST OF APPENDICES

I. INTRODUCTION

Biochemical systematics, and more specifically, chemical plant toxonomy, is a field that has only begun to develop. In the last two decades it has been recognized that chemistry can play a very important role in determining the phylogeny of plants. It is necessary to find chemical characters that can be compared in various taxa of plants. Such chemical characters, or markers, have been found in the flavonoids. Chemical constituents of the morphologically more "primitive" taxa may be compared with the more "advanced"; and in this way, valuable phylogenetic information may be obtained.

In general the evolutionary trend in the flavonoids is toward a more highly substituted chemical structure in the more "advanced" plant taxa. The more specialized families - like Leguminosae, Gesneriaceae and Compositae - exhibit the most complex flavonoid structures. Less highly developed families contain the flavonoids with the simpler hydroxylation and glycosylation patterns as seen in the gymnosperms, ferns, horsetails, and mosses. Flavonoids are apparently completely absent in more primitive organisms - the bacteria, fungi, and algae. Thus a phylogenetic tree can be arranged relative to the flavonoids in plants (Figure 1).

A biosynthetic pathway has been established for flavonoids; that is, the degree of chemical complexity of isolated flavonoids

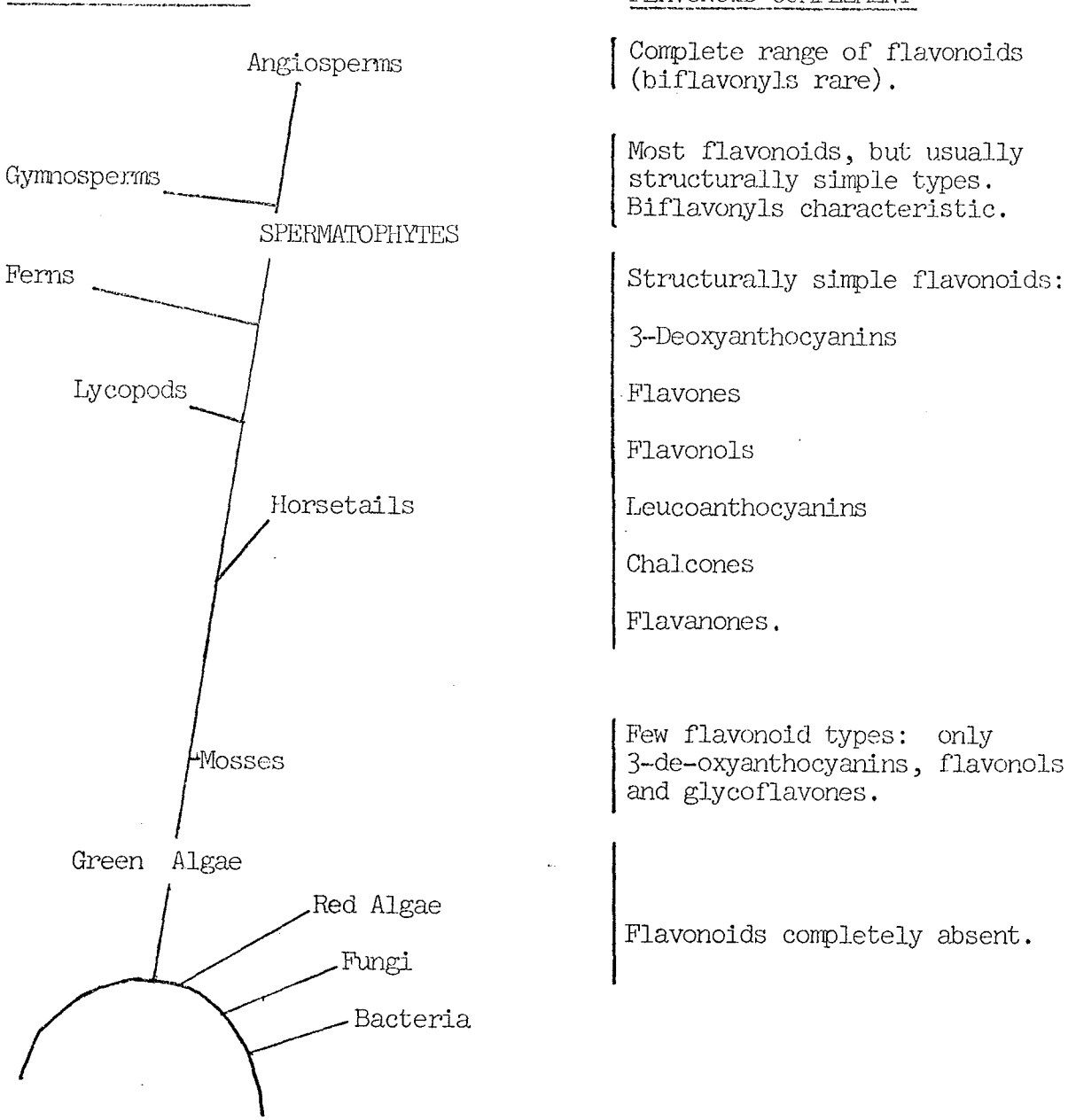
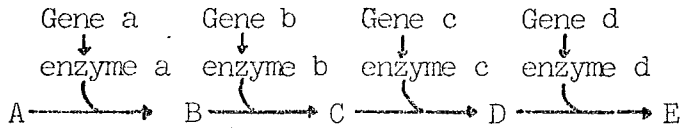


Figure 1: Evolution of flavonoids in plants.
(From Harborne, 1967, page 312).*

* One text that has been used extensively as a reference is J.B. Harborne, Comparative Biochemistry of the Flavonoids, 1967, Academic Press. Whenever pertinent, the page numbers for this reference will be noted.

can be ordered in their established biosynthetic pathway and thus the evolutionary advancement of plants may be determined. For example, in a biosynthetic pathway:



if E is the isolated flavonoid it may be accepted that the plant synthesizing E has more enzymes at its disposal for synthesis than a plant with only the A flavonoid. It is generally agreed that the plant with the E flavonoid is the more "advanced" organism. Chemical characteristics are more useful than morphological characteristics in some instances, in the determining of phylogenetic relationships. Comparative biochemistry has great potential for the plant taxonomist and phytogeneticist, but as stated previously it is a very new field with a great deal of groundwork still to be completed before an overall picture of phylogenetic relationships can be developed.

Recently, attempts have been made to carry out analysis of various chemical characters in groups of plants where there is disagreement as to their classification. One such study has been going on for several years involving the genus Allium. (Liliaceae). Allium is a circumboreal genus composed of approximately 500 species. According to Ownbey (personal communication) 80 species occur in the New World. New World species form a distinct group and, except for two, A. schoenoprasum and A. tricoccum, do not appear closely related to the

Old World species. The North American group has been divided into nine well-defined alliances on the basis of morphology and cytotaxonomy (Ownbey and Aase, 1955; Saghir et al, 1966).

Initial chemotaxonomic studies have been completed on the two larger alliances involving forty-eight of the taxa. These investigations utilized thin-layer chromatography (TLC), paper chromatography, and to a lesser degree, ultraviolet-visible spectrophotometry. The chemotaxonomic studies have considered anthocyanin pigments as the chemical character. Anthocyanins are just one of 12 classes of flavonoid compounds. The Allium falcifolium alliance is comprised of 31 taxa. Fourteen different anthocyanins have been reported in this alliance (Mingrone, 1968). Nine of the anthocyanins occur in more than half of the alliance taxa and three anthocyanins (Spots 1,2, and 3, on 2 dimensional paper chromatography) occur in all but one taxon (See Mingrone, 1968, for the numbering system of the spots from the Allium falcifolium alliance).

The Allium acuminatum alliance contains seventeen taxa. McNeal (1970) reported nineteen different anthocyanin spots for this alliance. Two anthocyanin spots (spots 1 and 3) occur in all of the A. acuminatum alliance taxa. All 14 anthocyanins reported by Mingrone (1968) for the A. falcifolium alliance have been found in one or more of the A. acuminatum alliance taxa by comparative paper chromatography - using R_f values. Two spots, 1 and 3, occur in all taxa of the A. acuminatum alliance. Of the twelve remaining anthocyanins reported by Mingrone, for the A. falcifolium alliance, ten of these occur in more than half

of A. acuminatum taxa. The theory that a close evolutionary relationship exists between the New World Allium species is, therefore, reinforced by the constancy of anthocyanin spots across alliance boundaries. The anthocyanidin, the anthocyanin aglycone, of the ten most common spots has been identified, using paper chromatography and thin layer chromatography, as cyanidin (Chu, 1972).

This investigation attempted to verify the identification (Chu, 1972) of the anthocyanin in A. amplectens by spectral means. An attempt was also made to identify the sugars, and possibly their locations on the heterocyclic ring system, of the anthocyanins.

II. LITERATURE REVIEW

A. Flavonoid Compounds

The flavonoid group of compounds has a carbon skeleton of two substituted benzoid rings (or one benzenoid and one quinonoid ring) connected by a three carbon olefinic chain. The C_6-C_3-C_6 structure is shown below in 2 different forms:

Figure 2 (Grisebach, 1965; Fieser & Fieser, 1956).

Different groups of flavonoid compounds are distinguished by additional oxygen-heterocyclic rings and by the addition of hydroxyl groups in various patterns. The largest group of flavonoids is characterized by those containing a pyran ring  linking the three-carbon chain with the benzene rings. The numbering system for these flavonoid derivatives is given in Figure 3.

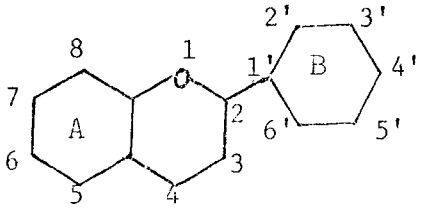


Figure 3 Numbering system for flavonoid derivative.

The flavonoid compounds that have the above carbon skeleton are

divided into classes by the oxidation state of the three carbon chain (C-2, C-3, C-4) (Figure 4).

In Figure 4, the structures of the more prominent flavonoid classes are shown in order from most reduced to most oxidized state.

Five other variations in the three carbon link (C-2, C-3, C-4) pattern exist but because of rarity will not be discussed. Hydroxyl groups are normally found on the aromatic rings and may sometimes be substituted to give methoxyl groups -O-CH_3 or glycosides -O-Gly. (A glycoside is the acetal product of the reaction of a monosaccharide with an alcohol, in this case called the aglycone).

B. Anthocyanins

Anthocyanins are one of twelve classes of flavonoids. They are the common pigments of red, violet, and blue in "higher" plants. It has been shown that the apparent "color" of a given pigment depends on the pH of the solution in which it is dissolved, and hence represents a certain degree of protonation of that pigment (Figure 5). Thus the cyanin cation (pH $<$ 3) is observed to be red, its color base - an electrically neutral molecule - (pH $\simeq$ 7) to be violet, and its anion (pH $>$ 11) to be blue (Fieser & Fieser, 1956). They are found throughout the plant parts - flower petals, leaves, stems, and bulbs. Anthocyanins occur throughout the plant kingdom, except in the fungi. Normally anthocyanins exist in plants as the cation portion of the salts of naturally occuring organic acids. This is because the normal pH of vacuoles, where the pigment is in solution, is acidic.

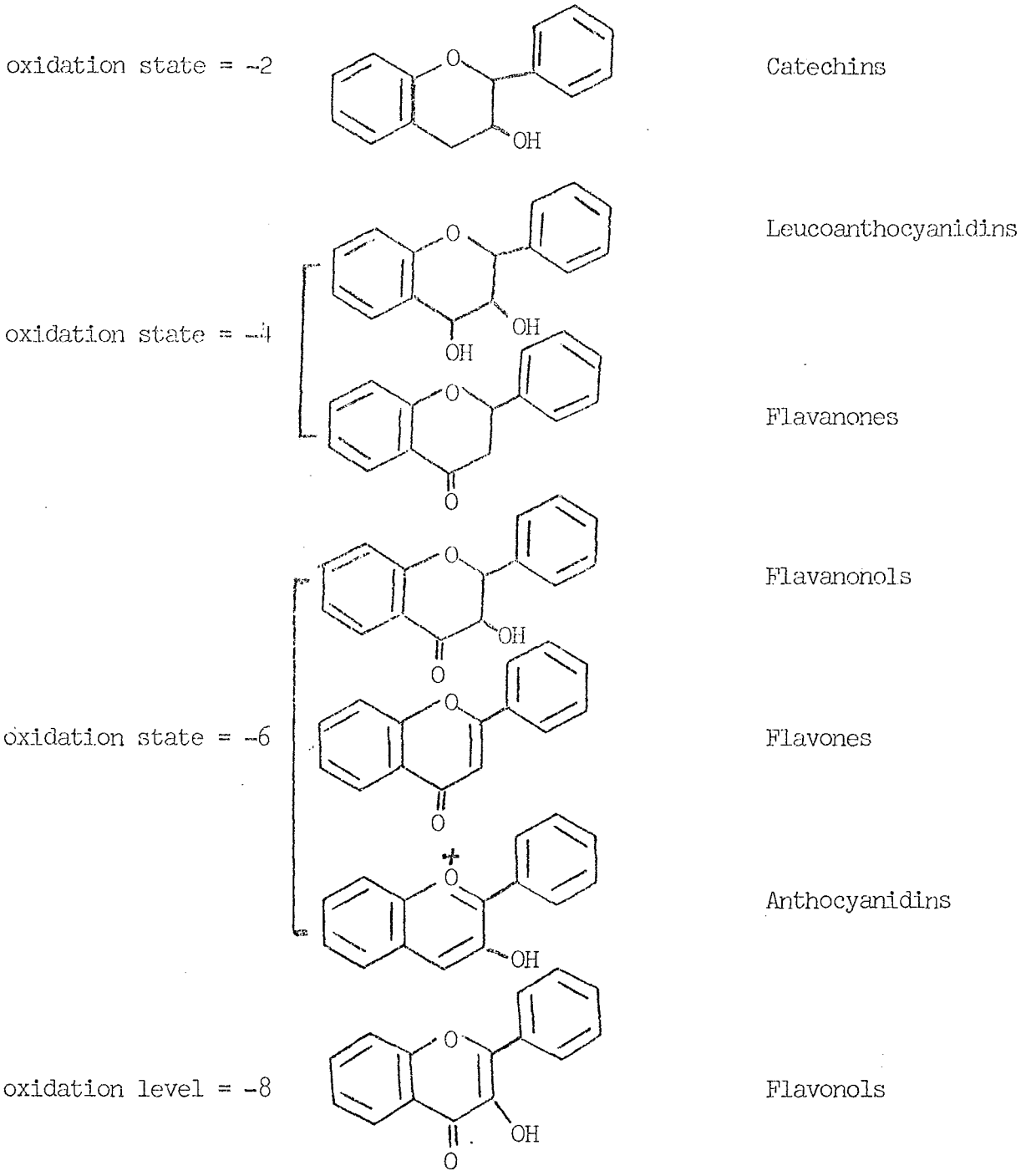


Figure 4

Structures of the more prominent flavonoid classes shown in order from most reduced to most oxidized state.

The positive charge of the ion is delocalized over the entire molecule and does not reside at just the heterocyclic oxygen (Griseback, 1965), and thus the molecule is best represented by a number of resonance hybrids of varying stability. Anthocyanins occur normally as glycosides, unlike other classes of flavonoids. Traces of anthocyanidins (the aglycone of an anthocyanin) are sometimes identified free in plants but these are believed to be artifacts. Anthocyanins are easily oxidized and destroyed by air if they are in a quinone form as would be found at a basic pH (Figure 5). It is for this reason that care must be taken when working with anthocyanins to maintain a slightly acidic solution. However, too strong an acidic solution could cause hydrolysis of the sugar moiety, especially at warmer temperatures (warmer is in this case above about 4°C).

Anthocyanins are also destroyed by light, other oxidative conditions, and in vivo by enzymes (glycosidases or anthocyanases and catecholases). The glycosidases, or anthocyanases, hydrolize the anthocyanin to its residual aglycone and sugar (Huang, 1956). The aglycone spontaneously decomposes to a colorless product, probably a decomposition mixture of variously hydroxylated benzoic acids and phenols and their oxidation products (Figure 6). The catecholases oxidize anthocyanins to a colorless product. Thus, anthocyanins are sensitive to light, temperature, pH, enzymes, and several other environmental conditions. Their color may be preserved for long periods of time, however, with storage in a cool, completely dark, slightly acidic, relatively oxygen free atmosphere. (As witnessed by the preservation of the color in red wines for many years).

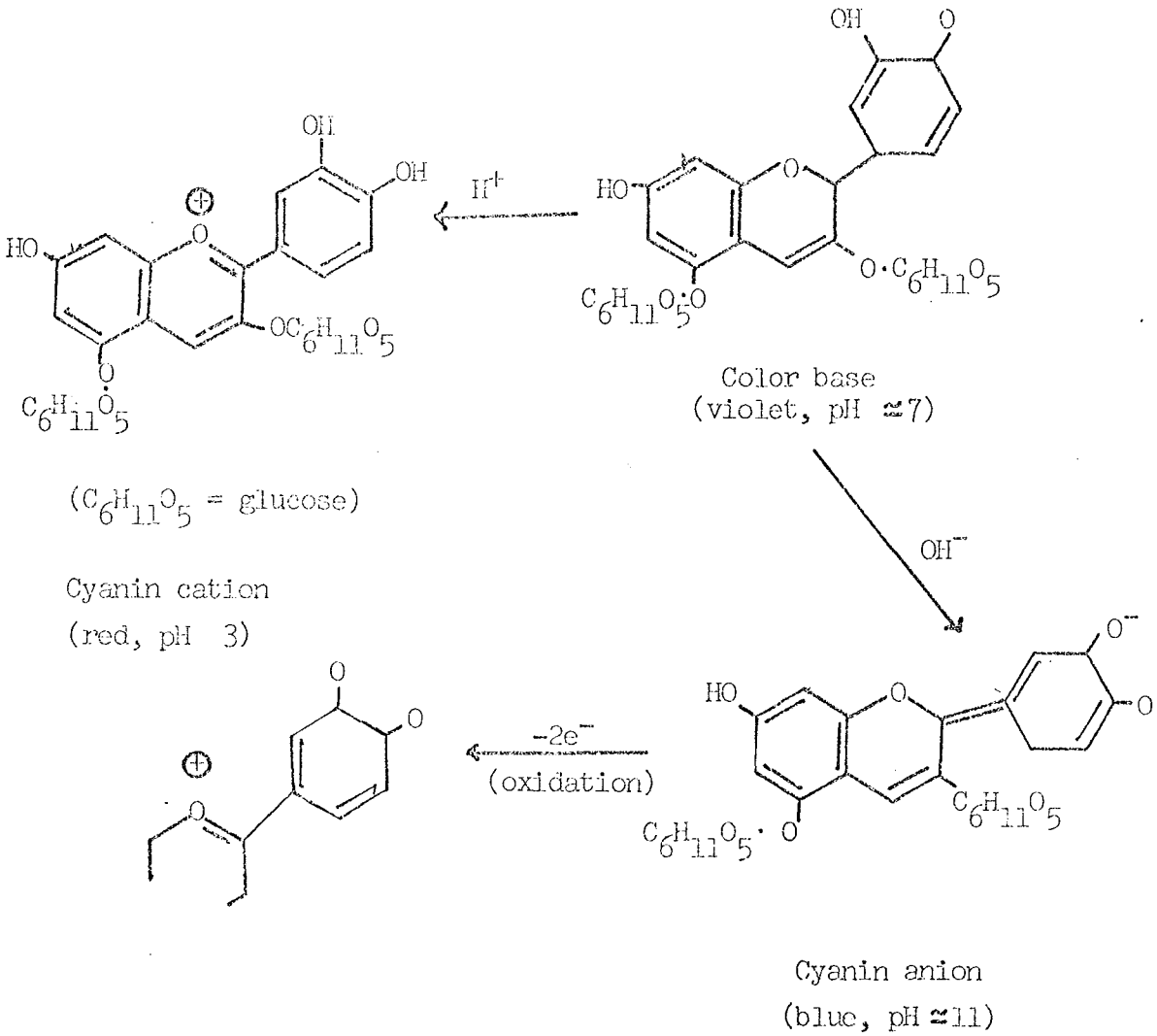
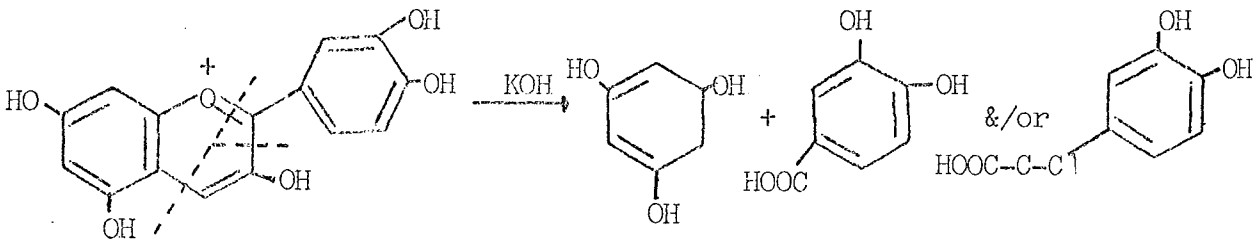


Figure 5 Cyanidin at different pH's.
(Feiser & Feiser, 1956)



Phenolic* Compound

[phloroglucinol (1,3,5-trihydroxybenzene)]

Benzoic &/or Phenylcarboxylic derivatives

Figure 6 Alkali fusion of anthocyanidin and resultant breakdown products. (Adapted from Fieser & Fieser, 1956).

* NOTE: Would obtain resorcinol (1,3-dihydroxybenzene) if either the 5 or 7 position did not have a hydroxyl, or would obtain some other substituted benzene if the 5 &/or 7 position was substituted with an acyl acid or methoxy group.

The glycosylation and methylation patterns of anthocyanins are restricted relative to the other flavonoids. Methylation is normally found only at the 3' and/or 5' hydroxyl group positions. Hirsutidin is an exception with a methoxy group at the 7 position in addition to 3' and 5' methoxy groups. Glycosylation usually follows a regular pattern. If only one sugar is present it will be at the 3 position (except for apigenidin glycosides, where a 3-hydroxyl group is missing and glycosylation occurs first at position 5). If two sugars are present they will usually be found at the 3 and 5 positions or as a disaccharide at the 3 position. A 3,5-diglycoside is the most common anthocyanin form (Grisebach, 1965). (Very rarely three monosaccharides will be found on anthocyanins, and in these cases they will be at the 3,5 and 7 positions). Of the plants surveyed by Harborne (1967) the 5 and 7 position sugars have always been identified as glucose. Except the cyanidin 3-rhamnoglucosido-7-xyloside derivative discovered by Fahselt (1970) in Dicentra spectabilis (Fumariaciae).

The anthocyanins are of basically three types, depending on the degree of hydroxylation of the B-ring; perlargonidin (the 4'-hydroxy derivative), cyanidin (the 3',4'-dihydroxy derivative) and delphinidin (the 3',4',5'-trihydroxy derivative). Derivatives of these 3 basic types are produced by methylation of the B-ring hydroxyls and by removal of oxygen (Figure 7).

The anthocyanins are the most widespread group of coloring pigments in plants. They are water-soluble pigments that are responsible for nearly all the pink, red, mauve, violet, and blue colors in the flowers

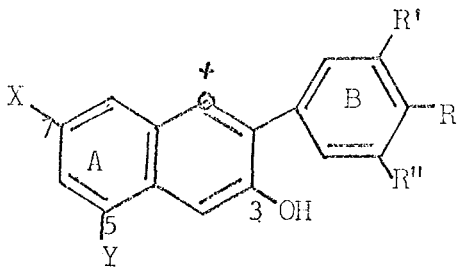


Figure 7 Structures of the naturally occurring anthocyanidins (Adapted from Harborne, 1967, p. 5).

1. **Pelargonidin R=OH; R'=R''=H; X=Y=OH (Pg)
   Aurantinidin R=OH; R'=R''-H; OH also at C-6 or C-8; X=Y=OH (Au)

2. **Cyanidin R=R'=OH; R''=H; X=Y=OH (Cy) (3,3',4',5,7-Pentahydroxyflavylium)
   **Peonidin X=Y=R=OH; R'=OCH_3; R''=H; (Pn)
   Rosinidin X=R'=OCH_3; R=Y=OH; R''=H (Rs)

3. **Delphinidin R=R'=R''=X=Y=OH (Dp)
   **Petunidin R'=OCH_3, R=R''=X=Y=OH (Pt)
   Pulchellidin R=R'=R''=X=OH; Y=OCH_3 (Pl)
   Europinidin R=R''=X=OH; R'=Y=OCH_3 (Eu)

4. **Malvidin R=X=Y=OH; R'=R''=OCH_3 (Mu)
   Hirsutidin R=Y=OH; R'=R''=X=OCH_3 (Hs)
   Capensinidin R=X=OH; R'=R''=Y=OCH_3 (Cp)

5. Apigeninidin R=X=Y=OH; R'=R''=H (Ap)
   Luteolinidin R=R'=X=Y=OH; R''=H (Lt)
   Tricetinidin R=R'=R''=X=Y=OH (Tr)
   Columnidin R=R'=X=Y=OH, R''=H, OH at 6 or 8 position (Co)

6. 3,7,4'-Trihydroxyflavylium R=X=OH; R'=R''=Y=H
   Fisetinidin R=R'=X=OH; R''=Y=H
   Robinetinidin R=R'=R''=X=OH; Y=H

7. 3,7,8,3',4'-Pentahydroxyflavylium R=R'=X=OH; R''=Y=H;
   OH at 8 position

** The six most commonly isolated anthocyanidins.

and leaves of higher plants. The color of the anthocyanin pigment is altered by the addition or removal of hydroxyl, methyl and/or glycosyl groups and by the ionic or electrical state of the molecule - hence color depends on the electron distribution within the molecule and on the pH of the solution. With respect to substituent effects, hydroxyl groups attached to the B-ring apparently have the most effect on color. Methoxyl substitution for the hydroxyl causes a dull bluish shade. With respect to pH effects on flower color, Shibata et al (1949) have shown that the pH of the cell sap of flowers varies by only a small amount (±0.5-1.0 pH unit). In other words, the pH of red and blue flowers is almost the same and one would conclude that pH effects are minor (Grisebach, 1965). Other factors such as glycosylation, exposure to light, etc., can also affect anthocyanin color (Blank, 1958; Hayashi, 1962).

Because the anthocyanins are responsible for a majority of the color variations found in flowers, a great deal of research has been done to identify the pigments present in ornamental flowers and in common fruits and vegetables. Numerous anthocyanins isolated from many species are sufficiently well characterized by chemical means to serve as standards for identification purposes (Harborne 1958, 1967; Hayashi, 1962). A summary listing the plants, variety, color and pigments present can be found in Harborne (1967, pg. 286, 297).

The economic importance of the color reactions of anthocyanins cannot be ignored. Marketable products made more attractive by pigment research are becoming more common -- to list a few examples:

1. Brighter colors in rasberries, radishes, potatoes, cabbage, and strawberries.

2. Methods to retain the anthocyanin color while storing and processing fruits and berries. (Example: In jam-making, the anthocyanin pigment is destroyed by autolytic enzyme action and by oxidation.)

The wine and tea industries have been involved in anthocyanin research for years (MacCarrone & Russo, 1969; G. Niketic-Aleksic and G. Hrazdina, 1972; L. Mattick, L. Weirs and W. Robinson, 1969). Flavonoids, specifically flavanones and chalcones, contribute to taste and flavor of citrus fruits. Anthocyanin contributions to flavor and palatability of foods have been reviewed by Swain, 1962, and Goldstein and Swain, 1963.

Leucoanthocyanidins can occur in several forms in plant tissues. It is the chemical conversion of these forms in ripening fruits (peach, pear, plum, persimmon, and banana) that is accountable for the loss of the astringent taste. Leucoanthocyanidin forms also have the ability to tan proteins resulting in the formation of precipitates in wines and beers. (Harborne, 1967; Mattick, Weirs and Robinson, 1969).

C. Sugars

The solubility and stability of anthocyanins is partially dependent on the attached sugar moieties. The order of attachment of these glycosides at the 3,5 and sometimes 7 positions has been mentioned previously. The rate of attachment is usually $3 > 5 > 7$. Most of the structural variation of anthocyanins that occurs in nature is due

to changes in the type, quantity, and position of attachment of sugar moieties, not to variations in the aglycone structure.

Pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin are the six most commonly isolated anthocyanidins. The glycosides attached to the anthocyanin molecules have been divided into four types (Harborne, 1967, pg. 13-29).

1. Monosides (monosaccharides)
2. Biosides (disaccharides)
3. Triosides (trisaccharides)
4. Acylated glycosides

1. Monosides

Four 3-substituted monosides are known to occur in anthocyanins: the 3-glucoside, the 3-galactoside, the 3-rhamnoside, and the 3-arabinoside.

One xyloside has been reported in Lavandula pedunculata (Labiatae) flowers; the sugar position, however, is not certain (Maroto, 1950). In all substantiated reports, the monosides are always found in nature attached to the 3-position. It is believed this 3-position is related to pigment stability.

The 3-glucosides are the most common anthocyanins found. Robinson and his co-workers (1934) have synthesized this monoside. 3-Glucosides are the anthocyanin of autumnal leaves and of many flowers, fruits, and vegetables. Some individual sources of 3-glucosides include the strawberry (pelargonidin), blackberry (cyanidin), grape (malvidin), and Verbena (Verbenaceae) flower (delphinidin).

The 3-galactosides are less common than the 3-glucosides. It

is difficult to distinguish the two monosides and several mistakes in identification have occurred (Sondheimer & Karash, 1956; Harborne & Sherratt, 1957, 1961; Harborne, 1967). The 3-galactosides are most common in the berries of Vaccinium species (Ericaceae: cranberries, bilberries, blueberries, etc.) with five of the six common anthocyanins involved; pelargonidin has not been identified in these taxa.

The 3-arabinosides occur in many of the 3-galactoside sources, however in much smaller concentrations. Pelargonidin-3-arabinoside is not known, but the other five common aglycones have been found as their 3-arabinosides. L-arabinose and D-galactose are very similar stereochemically. It is this relationship that could account for the co-occurance of arabinosides and galactosides in the Theobroma (Sterculiaceae), Vaccinium, Rhododendron (Ericaceae) and grasses (Gramineae) (Harborn, 1967, page 23). More work needs to be done on this group of compounds.

The 3-rhamnosides are rare. They can be found in combination with any of the six common aglycones and are commonly found in conjunction with other glycosides. The most common source of the 3-rhamnosides is the sweet pea flower, Lathyrus odoratus (Leguminosae).

2. Biosides

The biosides can occur in two ways: as the 3-diglycosides of which there are five types reported, or as the 3,5-dimonosides of which there are five known classes.

The two bioside types are distinguishable by their partial hydrolysis products and R_f values.

The former five types of 3-diglycosides are the 3-rhamnosylglucoside, the 3-xylosylglucoside, the 3-xylosylgalactoside, the 3-gentiobioside, and the 3-sophoroside. The structures for these disaccharides are shown in Figure 8.

The most common class is that of the 3-rhamnoglucosides which contains rutinose or 6-α-L-rhamnosido-D-glucose (rutinoside). Cyanidin 3-rutinoside, also called antirrhinin, is the most well known bioside. It was isolated from Antirrhinum majus (Snapdragon; Scrophulariaceae) flowers by Scott-Moncrieff in 1930. 3-Rutinoside has been identified with five of the six common aglycones. Malvidin-3-rutinoside has not been isolated as yet, but probably does exist in nature.

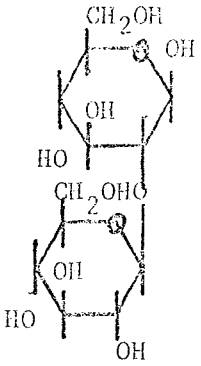
A cyanindin-3-xylosylglucoside has been identified in elderberries as the 2-β-D-xylosido-D-glucose derivative — also called sambubiose (Leichel & Reichwals, 1960).

The 3-diglucosides or 3-glucosylglucosides are found in two forms:

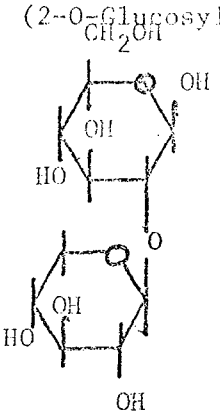
a) as gentiobiose which has a β-1,6 linkage and is rare
b) as sophorose which has a β-1,2 linkage and is fairly common.

Delphinidin is the only common anthocyanin in which neither a 3-gentiobioside nor a 3-sophoroside has not been described in the literature.

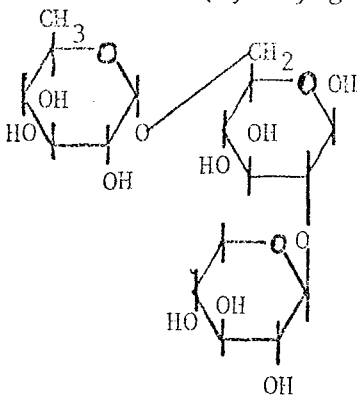
The latter five classes of 3,5-dimonosides are: the 3,5-diglucosides, the 3,7-diglucosides, the 3-rhamnoside-5-glucoside, the 3-galactoside-5-glucoside, and the 3-arabinoside-5-glucoside. No valid report has been made where the 5-position had a sugar other



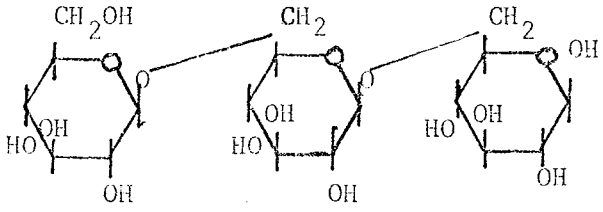
Sophorose
(2--O-Glucosylglucose)



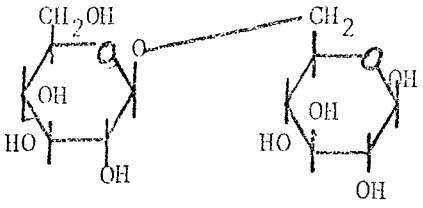
Sambubiose
(xylosylglucose)



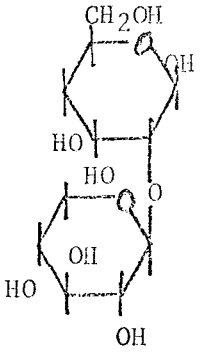
2^G-Xylosylrutinose



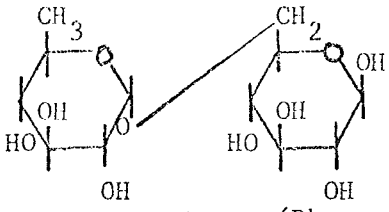
Gentiotriose



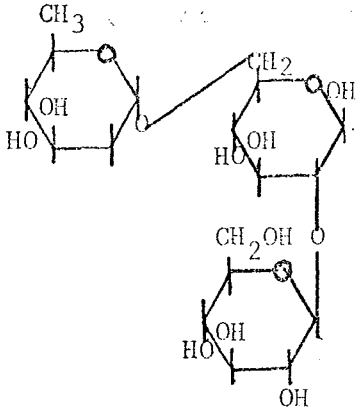
Gentiobiose
(6--O-Glucosylglucose)



Lathyrose
(xylosylgalactose)



Rutinose (Rhamnosylglucose)



2^G-Glucosylrutinose

Figure 8 Di- and trisaccharides present in anthocyanins. (Adapted from Harborne, 1967, p. 24).

than glucose (Harborne, 1967).

All of the common anthocyanidins have been reported with 3,5-diglucosides in nature.

The only 3,7-diglucoside in nature thus far reported is cyanidin (Birkofer, et al., 1963).

The 3-rhamnoside-5-glucosides occur with all six of the common anthocyanidins in the sweet pea flowers. Also in the sweet pea flowers are the only two reported 3-galactoside-5-glucosides which are pelargonidin and peonidin. These two are in trace amounts thus making their identification tentative (Harborne, 1967).

Recently a cyanidin 3-arabinoside-5-glucoside was isolated from petals of a Rhododendron (Ericaceae) cultivar (Asen and Budin, 1966). This is the only reported instance of this bioside.

3. Triosides

Triosides can occur in a variety of combinations since there are 3 possible positions of attachment, the 3,5 and/or 7 positions, and a fairly large number of sugar moieties that can be attached.

One trioside, 3-rutinoside-5-glucoside, has been found in nature quite widely with all six of the common anthocyanidins. 3-Sambubioside-5-glucoside and 3-sophoroside-5-glucoside occur with pelargonidin and cyanidin in a variety of plants in both an acylated and unacylated form. The remainder of the triosides are uncommon and occur in relatively few genera.

4. Acylated glycosides

Acylated anthocyanins have been reported in the literature for a long time. Their structures have been difficult to determine. Chromatography, however, has been an important aid in their characterization. Three acyl groups derived from p-coumaric (most commonly), caffeic and ferulic acids are now recognized in anthocyanins. These organic acids are attched by an ester linkage to one of the hydroxyl groups of the terminal sugar located at the 3-position. The known acylated anthocyanins usually have only one acyl group (very rarely two). Most commonly, the anthocyanins are 3,5-diglycosides with the 3-position glycoside being either glucoside, rutinoside, sambubioside, or sophoroside; and less commonly they are 3-glucosides.

D. Isolation and Purification

The term anthocyanin was first used for these pigments in 1935 by Marquart, and is taken from the Greek words, anthos, meaning flower, and kyanos, meaning blue.

The color changes of anthocyanins (red-violet-blue) have been mentioned previously. In flowers of higher plants the color changes are known to be controlled by single gene loci. Anthocyanins in color mutants of garden flowers provide excellent material for studying the relationship of biochemical synthesis to gene action. A great deal of knowledge on the biochemical genetics of higher plants has been derived from such studies.

It is generally agreed that the primary function of anthocyanins is to aid in attracting animals (such as birds and insects) to the plant to aid in pollination and seed dispersal. In relation to such animal vectors, it is known that flower and fruit colors have adaptive values (Harborne, 1957 pgs. 280-284; Harborne, 1965). Allium amplectens is quite illusive from this point. Why are so many anthocyanins necessary in a single species? In some species of Allium the major concentration of anthocyanins is in the bulb coat--which is underground! Possibly other functions can be attributed to the anthocyanins of Allium. Perhaps it will be shown in the future that there is an adaptive value to Allium in the possession of a large number of different anthocyanins, instead of a large concentration of just a few anthocyanins. The increased number of different anthocyanin pigments has not kept the genus from flourishing. As can be seen, it is a very widespread Western Northern American species (Figure 9).

A few of the other functions that have been attributed to anthocyanins are (Harborne, 1967, pg. 280-303; Grisebach, 1965):

1. growth regulation in roots and shoots
2. disease resistance
3. enzyme inhibition (Fairbairn, 1959)
4. antibiotic effect to protect the plant against attack by parasites

The anthocyanins have presented quite a challenge to the chemist. They are difficult to isolate in a pure state, particularly in anything more than micro-quantities. A melting point is difficult to determine. On analysis it is found that anthocyanins form hydrates, and when dried,

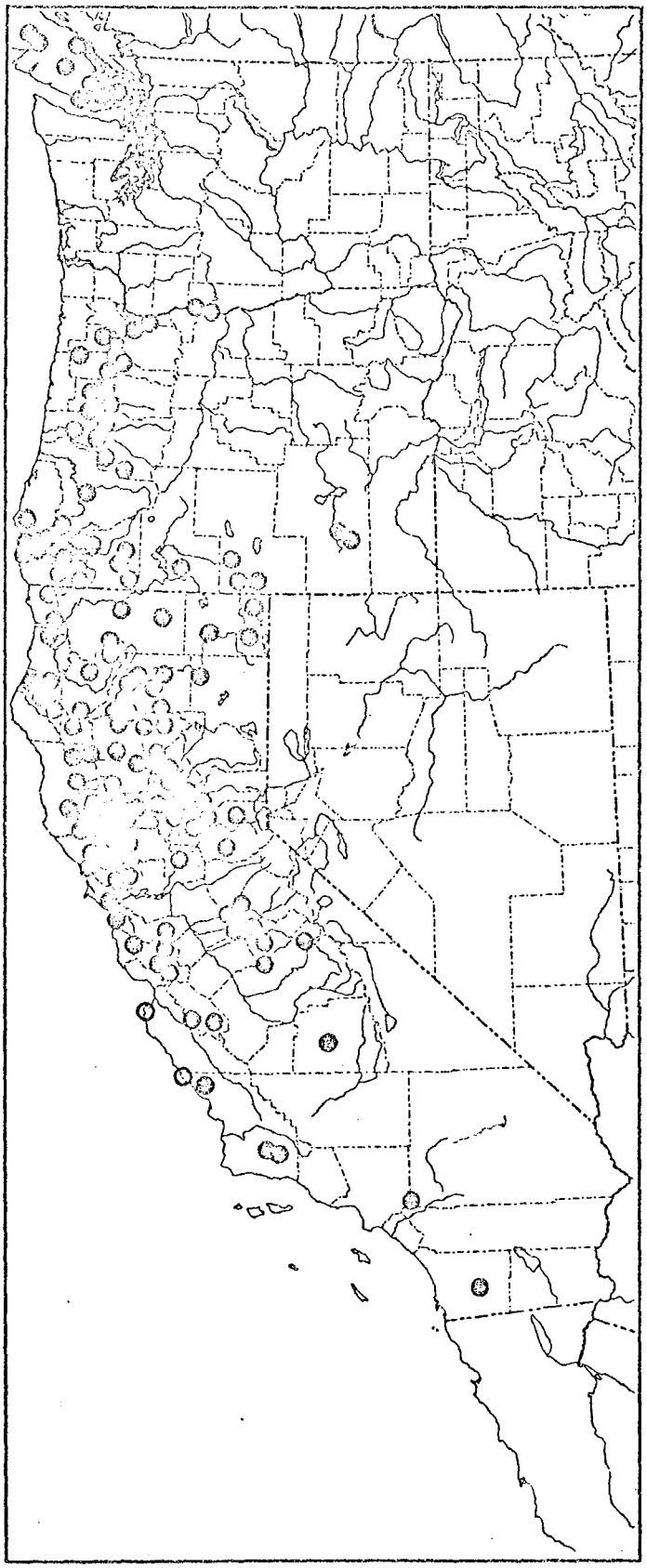


Figure 9 Distribution of Allium amplectens (McNeal, 1970).

eliminate hydrogen chloride.

Anthocyanins are relatively water soluble pigments that are light, pH and temperature sensitive *in vitro*. Upon acid hydrolysis the resultant aglycone, or anthocyanidin, is insoluble in water, a point which can be used to separate it from the hydrolized sugar moieties. The first chemical studies on anthocyanins by Willstater and Everest (1913), Karrer and Widmer (1927, 1928, and 1929) and by Robinson, *et al.* (1934, 1935) were concerned with establishing the chemical structures of the six commonly found anthocyanins. It was shown by Lawrence, *et al.* (1939) that changes in the sugar, and other substituent groups of the anthocyanin nucleus, and not the actual aglycone structure itself accounted for the different anthocyanins. Accurate methods for studying anthocyanin and sugar structure did not exist at this time. The introduction of paper chromatography for separating anthocyanins (Bate-Smith, 1948) and for identifying sugars (Partridge and Westall, 1948) was a major breakthrough in this field of chemistry. Absorption spectroscopy has also been applied to the characterization of these pigments (Harborne, 1957).

Resolution of pigment mixtures, which are present in some plant species, is now possible by several other methods besides paper chromatography. Method refinements, such as the utilization of different solvent mixtures, are now common in the literature, but the basic techniques for isolation, purification and identification of anthocyanins have already been developed.

Several other chromatographic techniques, other than paper, have

been used in the separation of anthocyanins. According to Harborne (1967) none seem to offer any advantage over paper chromatography and some apparently are not as good.

Column chromatography has been used by several different groups to separate anthocyanins from a crude extract (Chandler & Harper 1958). The results are not always reproducible, however, and the elute from the column is very dilute, which presents problems when working with low concentrations of anthocyanins. When working with a relatively large quantity of only a few (less than 4) anthocyanins, a column can produce satisfactory results. Using less time and a smaller quantity of solvent a column can resolve a relatively larger quantity of anthocyanins from a crude extract. The column is not reliable for resolving mixtures with more than about 4 anthocyanins (Harborne, 1967). The most frequently used column constituents are cellulose, silica gel, magnesol and polyamide. Columns have been used in some purification procedures and Fahselt (1970) used one for deacidification after hydrolysis.

Paper electrophoresis has been successfully used to separate two anthocyanins from an extract (Markakis, 1960). This method according to Harborne (1967) has no advantage over the simpler descending paper chromatography technique.

Thin layer chromatography (TLC) has been developed recently (Birkofer, 1962; Hess & Meyer, 1962) and is subject to some controversy. Harborne (1967, pg. 15) suggests that it "offers a satisfactory alternative to paper". Gupta (1968) successively used silica gel

and cellulose (both by Eastman Kodak) to separate both anthocyanin and anthocyanidin mixtures. He compared paper techniques to thin layer techniques and found the R_f values to be reproduceable and comparable on both. He tested sixteen common paper solvents on TLC and found that for mixtures of anthocyanidins all were suitable (i.e. gave good separation) for the plants studied in his investigation. For mixtures of anthocyanins, however, only three paper solvents were found suitable for resolution on TLC. [For silica gel EMFW was found to be suitable (ethyl acetate-methyl ethyl ketone-formic acid-water, 5:3:3:1 v/v). For cellulose two solvents were found to be suitable, n-BAW and water-acetic acid-HCl (82:15:3 v/v)].

Various new techniques are being applied to TLC of anthocyanins. Time is still needed to develop more standard TLC techniques. Advantages of TLC are that it provides good separation and sensitivity; it requires less time and less solvent to be developed; and smaller quantities of pigment can be analyzed than on paper. TLC sheets can be easily photocopied or stored for future reference (Pitteri, 1969).

Techniques used for the isolation and identification of sugar mixtures include; one- and two-dimensional paper chromatography, circular paper chromatography, paper electrophoresis, and thin layer chromatography. (Block, Durrham & Zweig, 1958; Randerath, 1966).

Paper chromatography was invented about thirty years ago. Unlike TLC, the techniques of paper chromatography are fairly well established. Published R_f tables are available for thousands of compounds, usually in several different solvent systems. Bate-Smith (1948) first applied

paper chromatographic techniques to anthocyanins. It became obvious that the technique was a very useful tool in the separation and identification of anthocyanins. R_f values are important in anthocyanin identification. The R_f value is equal to the distance the solute migrates divided by the distance the solvent front migrates.

No two glycosides have the same R_f values in different solvent systems. Anthocyanin behavior has been studied in a variety of solvent systems (Harborne, 1958; Abe & Hayashi, 1956). A direct relationship between the R_f value for a specific solvent and the anthocyanin structure was first noted by Bate-Smith & Westall (1950).

The degree of hydroxylation, methylation, glycosidation and acylation of the anthocyanin molecule affects the R_f value, in different solvents, in specific patterns:

Hydroxylation: An increased number of hydroxyl groups cause a decrease in R_f values in both aqueous and alcoholic type solvent systems.

Methylation: An increased number of methoxyl groups on the molecule cause increased R_f values in both aqueous and alcoholic type solvent systems. It should be noted that the increased R_f values caused by methylation are somewhat less than the decreased R_f values of hydroxylation.

Glycosidation: There is a direct relationship between the R_f value and the number of sugar moieties on the anthocyanin molecule. An increased number of sugar moieties acts to increase the R_f values in aqueous solvent systems, and to decrease values in alcoholic solvent systems.

Acylation: Has the reverse effect of glycosidation. The R_f values in aqueous solvents are decreased, and in alcoholic solvents are increased.

E. Identification

Identification of anthocyanins, anthocyanidins, and their sugar and acyl residues should include data from several different techniques. Chromatographic comparison of the compound in several different solvent systems with a known, or standard marker, is one method used (Harborne & Sherratt, 1957). Partial acid hydrolysis has been used as one method in which to determine the position and number of sugar residues that were attached to the anthocyanin molecule (Abe & Hayashi, 1956). The colors of chromatographed anthocyanins in visible and ultraviolet light have also been used for identification. Many of the anthocyanins fluoresce (Harborne, 1967, pg. 8). Harborne (1958) used spectral data, UV and visible, to characterize anthocyanins. Tables of standardized spectral data are available in the literature (Mabry et al., 1970). The effect of various chemicals (i.e. Aluminum chloride, sodium methoxide, sodium acetate and boric acid) on the spectra is also a useful technique to distinguish chemical characteristics of the molecules (Geissman, Jorgensen & Harborne, 1953; Geissman & Jurd, 1955). Enzyme analysis has been used in some situations as an aid in identification. Chemical synthesis is rarely used due to the microquantities of material available. Anthocyanins are now commonly identified using a combination of R_f values and spectral (UV & visible) data. Glycosides are determined

by direct comparison with standard markers on TLC.

Just as a relationship between the R_f value and the anthocyanin structure may be characterized; so it is possible to characterize the relationship between spectra and anthocyanin structure. A few of the main patterns relating spectra to structure as as follows: (adapted from Harborne, 1967, pg. 9)

1. Addition of hydroxyl groups to the B-ring causes bathochromic shifts (i.e. towards longer wavelengths), as seen when comparing pelargonidin (520 mμ), cyanidin (535 mμ) and dephinidin (546 mμ).
2. Methylation of the hydroxyl groups on the B-ring causes small hypsochromic shifts (i.e. towards shorter wavelengths), as seen when comparing delphinidin (546 mμ) with malvidin (542 mμ).

F. Biosynthesis

The inheritance of flower pigments has been a topic of study for many decades. Flower color is largely due to flavonoids, mostly anthocyanins. The variations in color are a result of changes in inheritance of the genes controlling color. Flower pigment genetics was first studied by Bateson at the turn of the century. During the 1930's biochemists became involved in these genetic studies. Many examples of single gene differences affecting flower color were found. Scott-Moncrieff (1936) related these differences to simple modifications in the synthesis of anthocyanins. The culmination of these studies

was in 1945 when Beadle stated the one gene-one enzyme hypothesis while working with Neurospora.

Continued studies of pigment inheritance in higher plants have elucidated biosynthetic information. This information has lead to the discovery of the probable discrete steps in the biosynthetic pathway of flavonoids. Recent methods used for studying biosynthetic pathways involve feeding radioactively labeled precursors into plants and tracing their paths. The amount of time for incorporation of the labeled precursor into flavonoids is measured at intervals. With great care in the interpretation, the position of various labeled molecules can be identified in the biosynthetic pathway (Figure 10).

Neish and others (Neish, 1964) have concluded that the C_{15} skeleton of flavonoids is derived from two separate pathways; one from acetate and the other from shikimic acid (Figure 11). Controversy still exists over the exact steps of the pathway. Below is a summary of the possible biosynthetic pathway.

1. The A-ring arises by the condensation of 2 malonyl CoA units and acetyl CoA. (This pattern of synthesis of aromatic rings is similar to the pattern found in fungi).
2. The B-ring and the C_3 unit is derived from a C_6-C_3 precursor, which is indirectly derived from sedoheptulose - via the shikimic acid pathway. This C_6-C_3 precursor is presumed to require activation - in some unknown way.
3. Thus a C_{15} - intermediate has been formed. Once formed it is modified in various ways to yield the different classes

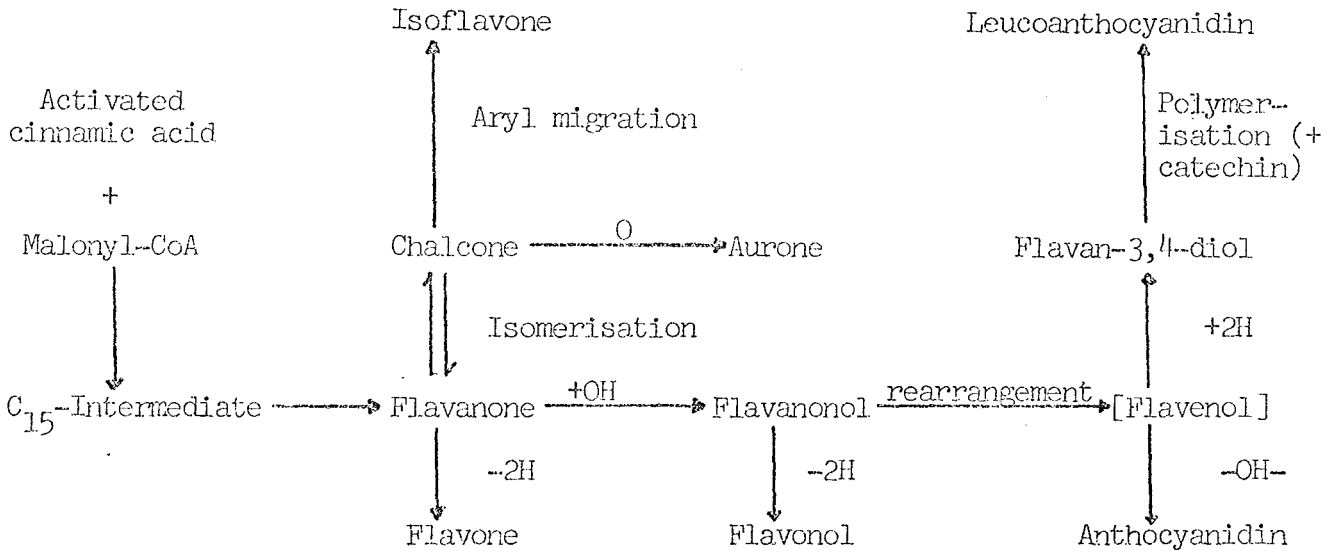


Figure 10 Biosynthetic pathways to the flavonoids.
(Harborne, 1967, pg. 267).

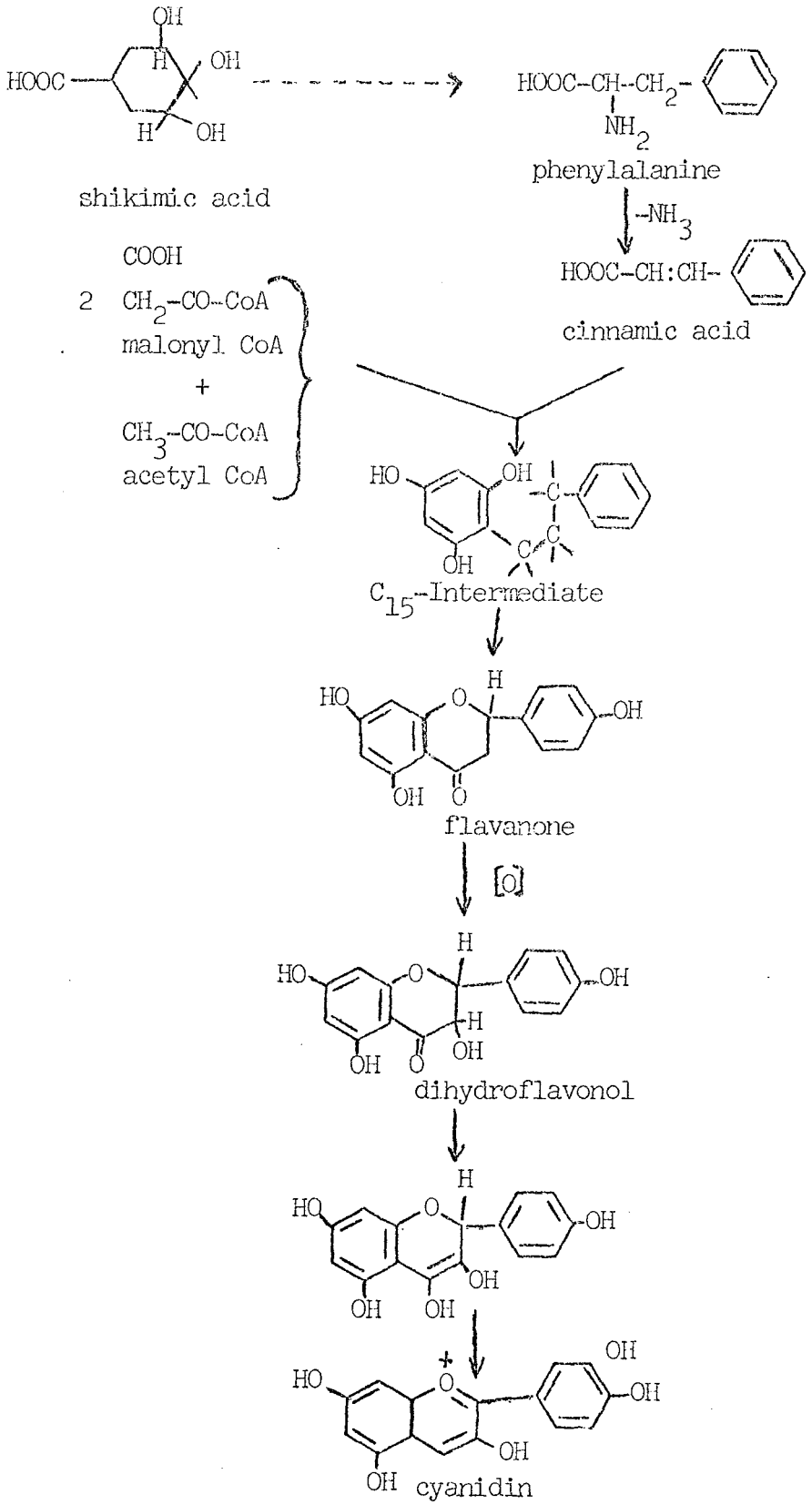


Figure 11 Biosynthetic pathways to anthocyanidin. (Chu, 1972).

of flavonoids. The C_{15} - intermediate has not yet been identified. Controversy exists as to whether all the flavonoid classes have one common intermediate, or if possibly interconversion between the classes can occur without the intermediate step (Grisebach & Patschke, 1961).

4. Methylation and glycosidation occur at the end of synthesis. The possibility still remains that some methylation or glycosidation can occur at the C_{15} - intermediate stage. Enzymes that catalyze O-methylation of caffecic acid have been found in petunias (Hess, 1964a) but these particular enzymes apparently are not involved in anthocyanidin methylation. The glycosylation of anthocyanidins is presumed to require its own group of enzymes - anthocyanases.

III. METHODS AND MATERIALS

The identification of anthocyanins may be divided into six basic steps: 1) extraction, 2) separation, 3) isolation, 4) analysis of aglycone, 5) sugar identification, and 6) spectral analysis.

1. Extraction

This step refers to the removal of the flavonoid pigments from the plant material. The *Allium amplectens* plants were obtained by 2 methods: 1) cultivated in an experimental garden in Stockton or 2) obtained on field trips to the Sierra foothills where *Allium amplectens* grows in abundance.

The entire plant was dried before extraction. To extract, the entire plant was broken into small pieces, placed in a beaker and just enough solvent (1% HCl in methanol) added to cover the pieces. The beaker was kept in the dark at approximately 4°C for 24 hours. The extract was then filtered and concentrated. To concentrate the extract the flask was placed in an ice bath under a current of forced air. The extract was spotted onto paper while it was being concentrated.

2. Separation

As mentioned previously various techniques have been used to separate anthocyanins. The method of choice is usually paper chromatography. Thin layer and column chromatography have also been used,

but paper chromatography seems to offer more advantages over these other two methods (Geissman, 1962, pgs. 34-69; Mabry, et al., 1970).

For separation of the anthocyanins, the extract was spotted on Whatman 3MM chromatographic paper (46 x 57 cm sheets of medium flow rate). The spot was approximately 2.5 cm in diameter, and was in the lower left corner about 10 cm from each edge. The chromatogram was developed by descending technique in 2 directions. The solvent for the first direction was n-butanol; glacial acetic acid : glass distilled water (4:1:5 v/v/v); the upper phase of this solution was used after equilibration for 3 hours. This mixture is commonly referred to as n-BAW. The solvent for the second direction is 15% glacial acetic acid in distilled water, commonly designated as HOAc. The first system is an alcoholic solution and the second is aqueous. The effect of alcoholic and aqueous solvent systems on the R_f value has been mentioned previously. Development in first direction takes approximately 20 hours at room temperature (i.e. 20-25°C). The papers are then dried, rotated 90° and refolded for descending development in the second direction. The second direction takes approximately 6 hours at room temperature. The temperature affects the developing time and, therefore, the quality of separation. If the solvent front moves too quickly the spots will 'streak' or 'tail' causing poor separation of the spots. If the front moves too slowly then the R_f's for the normally slower moving spots may be affected.

After the second direction the papers are again dried under the hood. The spots are viewed under visible light and circled with

a pencil. The R_f of the spots is determined, and one chromatogram from the lot is exposed to ammonia fumes to determine color reactions.

3. Isolation

The darker spots were cut from the paper and eluted. Some of the spots were too close together to be separated, therefore, spots 2-1-15 were eluted together, as were spots 3-4-14. The spots to be used for spectral analysis were eluted in spectral methanol for 20 minutes and then evaporated to dryness.

The spots to be used for aglycone and sugar analysis were eluted in .1% HCl in MeOH for 24 hours. The eluate was then poured into an evaporating dish and the spots eluted for a second 24 hour period. Elution and evaporation of the eluate to dryness was done in the dark at 4°C. The eluate was always taken to complete dryness and then covered for later use or resuspended for further steps.

Further purification of the 3 spot complexes, 2-1-15 and 3-4-14, could be done at this point. The 3 spot complex was eluted with .1% HCl in MeOH and applied as a 3 inch stripe at one end of a half piece of Whatmann 3MM paper. The paper was developed in n-BAW for 20 hours. Three distinct bands were usually obtained and eluted from the paper. The correspondence of spot number to band number can be obtained by comparing the spot R_f values from the first direction run (dev. in n-BAW), with the band R_f values (also dev. in n-BAW). It was concluded (Chu, 1972) that spot 15 from 2-1-15 was band 1 (the band closest to the origin); spot 1 was band 2; and spot 2 was band 3 (farthest from the origin). For spots 3-4-14 only 2 bands were usually visible.

Band 1 was probably spot 3; and band 2 was spot 14. This purification technique was later discontinued. Too much material was lost with this added step and the advantages of the step became minimal.

4. Analysis of Aglycone

After elution from paper for 24 hours, the eluate is filtered and placed in a screw cap test tube. To the eluate is added, in a volume of about 1/4 the sample solution, concentrated hydrochloric acid. The screw cap test tube is boiled in a water bath for 4-6 hours. This is to insure complete hydrolysis of all the O-bonded sugars. C-C bonded sugars are not affected by hydrolysis. The hydrolysate is then taken to complete dryness to remove all the acid. The aglycone is then extracted from the sugar. The extraction procedure that follows produced 5 samples (A, B, C, D & E) that are spotted on cellulose and silica gel thin layer plates (commercially prepared by Eastman Kodak).

To obtain sample A, MeOH:H_2O (1:20 v/v) is stirred over the dry hydrolysate. The aqueous phase is washed three times in a separatory funnel with petroleum ether (sample B). The sugars and any un-hydrolysed anthocyanin remain in the aqueous phase. Any residual anthocyanidins will move to the ether phase along with any breakdown products.

Samples C and D are obtained by adding approximately 5 ml aliquots of both the MeOH:H_2O (1:20 v/v) solution and petroleum ether directly to the evaporating dish containing the dried hydrolysate. The aliquots are poured into a separatory funnel - the lower, water phase, is sample

C and the upper, ether phase, is sample D. The procedure is repeated 3 times so that there is at least 15 ml of both sample C and D.

The last sample, E, is residue. The evaporating dish is left with a residue that is insoluble in either the MeOH:H_2O or ether solutions. Approximately 1-2 ml of .1% HCl in methanol was added to the dish. The residue immediately dissolved.

Each of the samples were taken to complete dryness and resuspended in 1-2 drops of appropriate solvent. The drops were spotted on a thin layer chromatograph. Both silica gel and cellulose pre-coated plates were used (See Appendix I). Also spotted on the same plate were known anthocyanidins and known sugars (See Appendices II & III).

The thin-layer plates were developed by the ascending technique in an Eastman Kodak TLC sandwich apparatus. Several different solvent systems were used in an attempt to get the best separation (See Appendix IV). After development the plates were air dried and viewed under visible, long-wave UV and short-wave UV lights. The plates were then sprayed with a visualizing reagent (See Appendix V), and developed in an oven at 100°C for 5-10 minutes. Following development they were again viewed under the visible and UV lights. The R_f values and color reactions were noted, and compared with the known compounds on the same plate, and with published data.

5. Sugar Identification

The sugars remain in the lower, water phase during extraction. The water extract was taken to dryness in a glass petri dish. The

visible sugar residue was resuspended in water and spotted on TLC plates, one drop at a time. Authentic known sugars were co-chromatographed on each TLC plate. The plates were developed in various solvent systems and visualized with several different sprays (See Appendices IV & V).

The TLC plates were viewed under visible and UV lights before and after spraying. After spraying the plates were heated at 100°C for 5-10 minutes or until the sugars were visible. Color reactions were noted and R_f values calculated.

6. Spectral Analysis

Spectra were taken on three different spectrophotometers, two of which were drum recording instruments. All data reported here is from either a Perkin-Elmer 202 or a Hitachi 3PS-3T. Wavelength calibration was carried out with a Didymium glass filter which has λ_{max}'s at 403.0, 529.5 and 586.0.

Spectra were run at medium-speed and on a scale at 0-1.0 absorbance. Spectral grade methanol without added reagent was used as reference. Three cuvetts of 1 cm path length were used during analysis: 1 for reference, 1 for the standard known solutions, and 1 for the unknown anthocyanin solutions. All spectra were run in UV (210-360 nm) and visible (340-700 nm) light ranges. Any necessary calibrations were made on the Hitachi when changing between the two wavelength ranges.

A stock solution of the standard known anthocyanidin was prepared by dissolving a small amount of the compound in approximately 15 ml of spectral grade methanol. The concentration was adjusted so that the optical density of the major absorption peak gave readings between

0.6 and 0.8.

The stock experimental anthocyanin solution was obtained by eluting spots cut from the second chromatographic run. The paper was cut into small pieces and eluted in spectral grade methanol for about 20 minutes. The eluate was filtered and taken to complete dryness. The residue was resuspended in approximately 10 ml of spectral methanol. The stock experimental solution was usually more dilute than the standard solution, however, all peaks were still visible.

Mabry, et al (1970) suggest that methanol may elute some UV-absorbing compounds from chromatographic paper. Thus, when preparing the reference solution, a piece of blank chromatographic paper was shaken with spectral methanol and the solution filtered. In this investigation however, when this reference was compared with a spectral methanol reference, no difference (i.e. in absorption) was noted in the UV range.

In determining the spectra of a particular unknown spot the spectrum of spectral methanol was taken first and compared with a reference of spectral methanol. This first spectrum served as a check for any absorptions due to the solvent or cuvette. Next the methanol spectrum neat (i.e. containing only MeOH and the sample) of both the standard and experimental stock solutions was run. A few drops of 4N hydrochloric acid in spectral methanol were added to each neat stock solution and the spectra run again. The Neat/HCl solution was then discarded.

Three drops of stock sodium methoxide solution (See Appendix VI) for preparation of all stock solutions used in spectral analysis)

were added to each of the stock solutions, the spectrum was run immediately after the addition. The spectrum was rerun after approximately 5 minutes as a check for flavonoid decomposition. The NaMOe solutions were then discarded.

Next the aluminum chloride ($AlCl_3$) spectrum was run immediately after 6 drops of stock $AlCl_3$ solution were added to each of the stock solutions. After the $AlCl_3$ spectrum was run, three drops of stock HCl solution were added to each cuvette containing the $AlCl_3$ solution, the spectrum was recorded immediately. The $AlCl_3$/HCl solution was then discarded.

The next spectrum run was with sodium acetate, NaOAc, in each of the stock flavonoid solutions. Anhydrous, powdered NaOAc was added in excess of solubility to the stock solutions, these were then shaken until approximately a 2mm layer of NaOAc remained at the bottom of the cuvette. The spectrum was run immediately after this addition, and again 10 minutes later to check for flavonoid decomposition. After the second spectrum was run, powdered anhydrous boric acid (H_3BO_3) was added until the solution was saturated. The spectrum was recorded and the NaOAc/H_3BO_3 solution was discarded.

The ratio $O.D._{440}/O.D._{max}$ (or E_{440}/E_{max}) was calculated so that relative peak intensity values could be compared with characteristic values published in the literature. The λ_{max} for the absorption peaks was also determined.

No effort was made to run the unknown anthocyanin eluate on other spectral equipment (such as NMR, IR, Mass Spec., or G.C.) due to the unavailability of published data on anthocyanins for any of these machines.

The chemical effect of the various reagents added to the stock solutions will be discussed later. Each of the reagent effects provides information about the presence and position of hydroxyl groups that may be applied towards the determination of the flavonoid type. The actual spectra of authentic cyanidin and pelargonidin, and the spectra of the 2--1-15 complex with each of the reagents are in Appendix VII.

IV. RESULTS AND DISCUSSION

A. Chromatography

McNeal reported in 1970 that Allium amplectens had 15 different spots when run on two-dimensional paper chromatography using t-BAW (or TBA) (t-butanol:acetic acid:water, 3:1:1, v/v) and 15% acetic acid (HOAc) as the two solvent systems. The spots reported were (using the Mingrone (1968) system of numbering); 1,2,3,4,5,6,7,8,9, 11,13,14,16 and 18 (Figure 12). Since then McNeal (personal communication) has also located spots 12,15 and 19.

Chu reported (1972) that only ten of these spots were detected when using n-BAW and 15% acetic acid for solvent systems. The ten spots detected by Chu were; 1,2,3,4,8,9,12,13,14 and 15.

In this investigation the following spots were detected using n-BAW and 15% acetic acid for the two solvent systems; 1,2,3,4,5,6,8,9, 12,14,15,16,19 (Figure 13, Chu). For this investigation the only spots worked with were; 1,2,3,4,14 and 15. Of these six, the only spots that could be obtained in a high enough concentration for spectral and sugar analysis were; 1,2, and 15. The R_f values of these three spots on paper are shown in Table 1.

It was found that partial hydrolysis occurred in spot complex 2-1-15 even when great care was taken to avoid it. Steps taken to mitigate hydrolysis were: storage in the dark at 4°C, a minimum of time in solvents, a minimum of time between initial extraction and

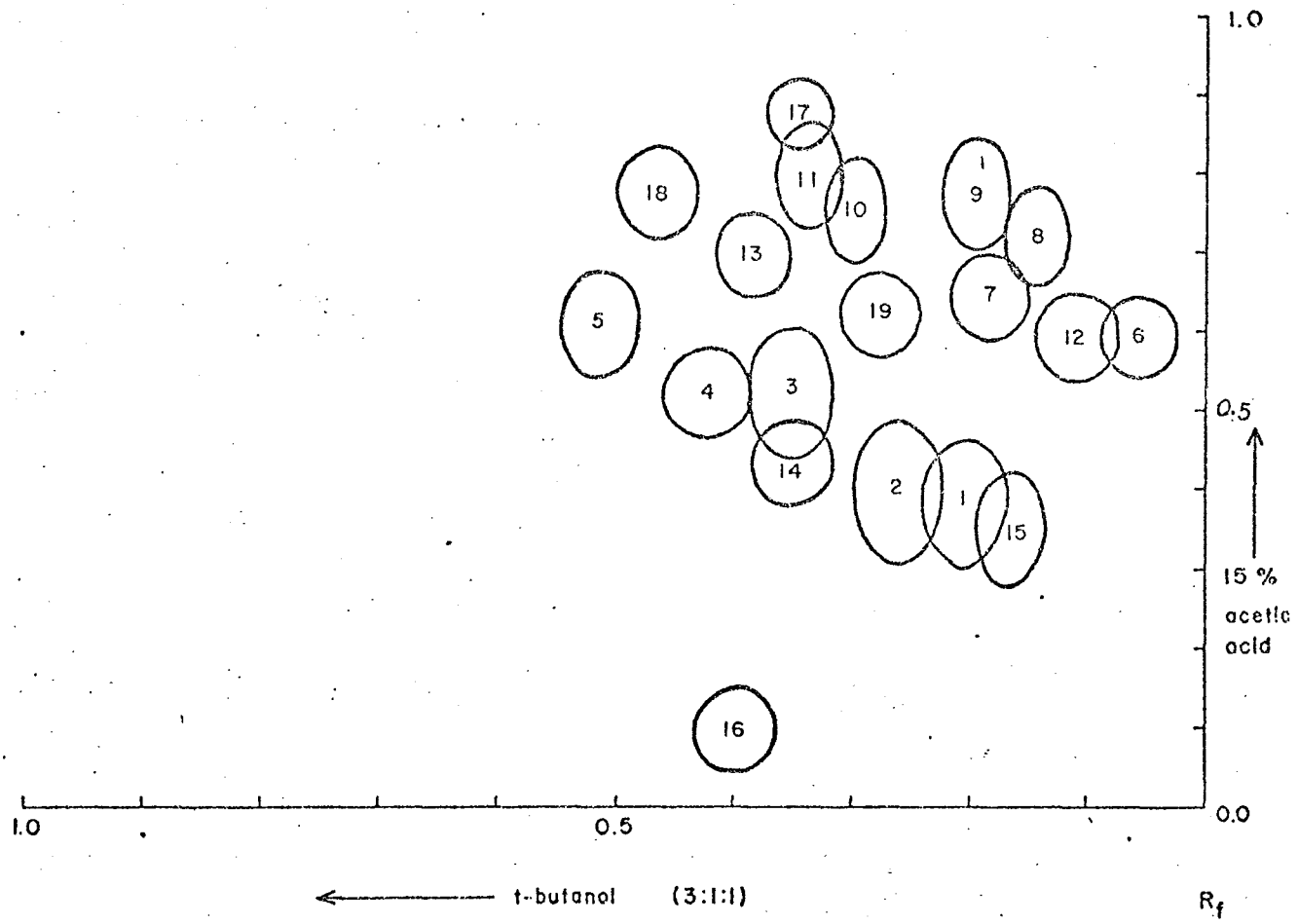


Figure 12 The distribution of spots of Allium amplectens when chromatographed in two dimensions on paper, with t-BAW as the first solvent system and 15% acetic acid as the second.

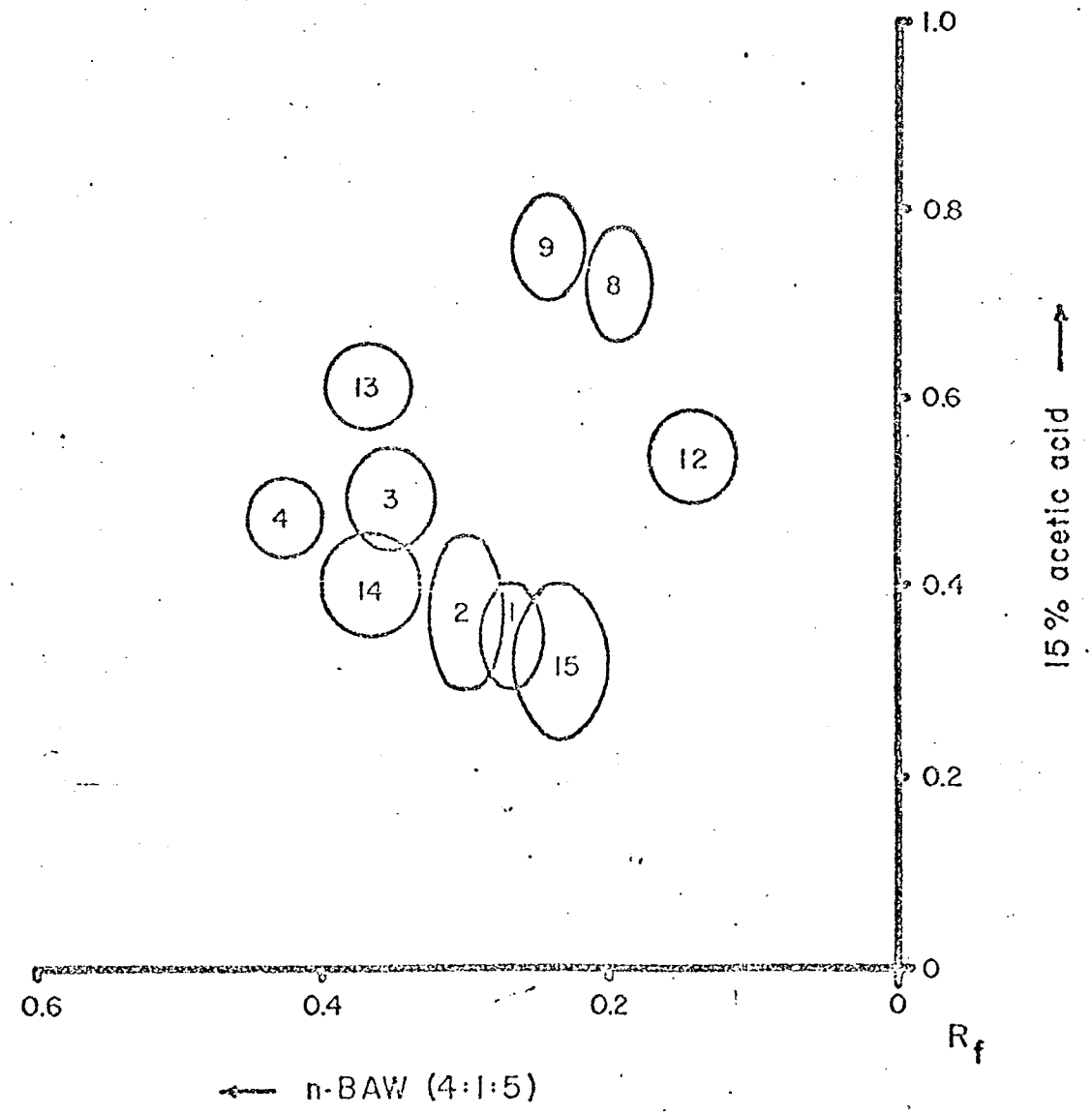


Figure 13 Composite chromatogram of the anthocyanin components of the Allium amplectens alliance (Chu, 1972).

TABLE 1

R_f Values of Spots 1,2 and 15 on Paper Chromatograms.

Spot Number	Solvent Systems	
	n-BAW	15% Acetic Acid
1	29	38
2	30	40
15	25	34

purification procedures and between purification and the final analyses for aglycone and sugars.

The procedure was altered continuously throughout the investigation in an attempt to obtain results with fewer partial hydrolysis and breakdown products. Consequently the results reported (in Tables 2 through 15) will reflect this procedural evolution. Data is reported initially for bands 1,2 and 3. Later this changed to data for the spot complex 2-1-15; thus, the third paper chromatography purification step was eliminated. Data for the 2-1-15 spot complex before and after hydrolysis are reported.

The data after hydrolysis frequently include what are believed to be breakdown products; phenolic, benzoic and phenyl carboxylic compounds (see Lit. Review pg 10). R_f values for some of the possible breakdown products are listed in Table 16.

It should be noted that the R_f values of anthocyanidins are less than anthocyanins for the same spot in aqueous solvent systems. The anthocyanidin R_f values are greater than the anthocyanin values in alcoholic solvent systems. These patterns are due to removal of sugar moieties from the hydrolyzed anthocyanins. Removal of acyl groups or methoxyl groups would exhibit a different set of patterns in aqueous and alcoholic solvent systems. (For a detailed explanation see the Lit. Review, pgs. 25 and 26).

It has been shown that different types of flavonoids usually occur in characteristic areas on a chromatogram. A schematic diagram from Mabry et al. (1970) of some of the flavonoids more commonly encountered

in his research is shown in Figure 14.* The characteristic patterns for these common flavonoids can be compared with the two-dimensional chromatogram of Allium amplectens in Figure 12. (Note: both diagrams represent chromatograms run in the same solvent systems on the same type of paper and are drawn to the same scale.)

Although anthocyanins are not specifically shown in the diagram a comparison can be made between the two Figures 12 and 14 with special reference being made at spot complexes 2-1-15 and 3-4-14. For the 2-1-15 complex a 7-O-diglycoside is suggested. Because the diagram is not specific for anthocyanins the position of attachment is not necessarily at the 7-position. The inference at an O-diglycoside is probably still valid since the diagram is for flavonoids.

The geometrical relationship of one spot relative to another may also be indicative of structural differences in compounds. Below is a list of some patterns mentioned by Mabry et al. (1970), that exist among the common flavonoids:

1. 5-Deoxy flavonoids run consistently slower in t-BAW and slightly faster in 15% acetic acid, relative to the 5-hydroxy flavonoid equivalents.

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*The more commonly encountered flavonoids mentioned are flavones, flavonols, isoflavones, flavanones, chalcones, dihydroflavonols, aurones, and their O-glycosides. The information presented is for the more commonly encountered flavonoids which possess a 5,7-dihydroxylated A-ring and either a 4'-mono-or a 3',4'-di-hydroxylated B-ring. The glycosides refer to the 3-and 7-mono-and 3,7-di-glycosides, unless stated otherwise on the diagram. Slightly different chromatographic properties may be found for flavonoids with other oxygenation and/or glycosylation patterns.

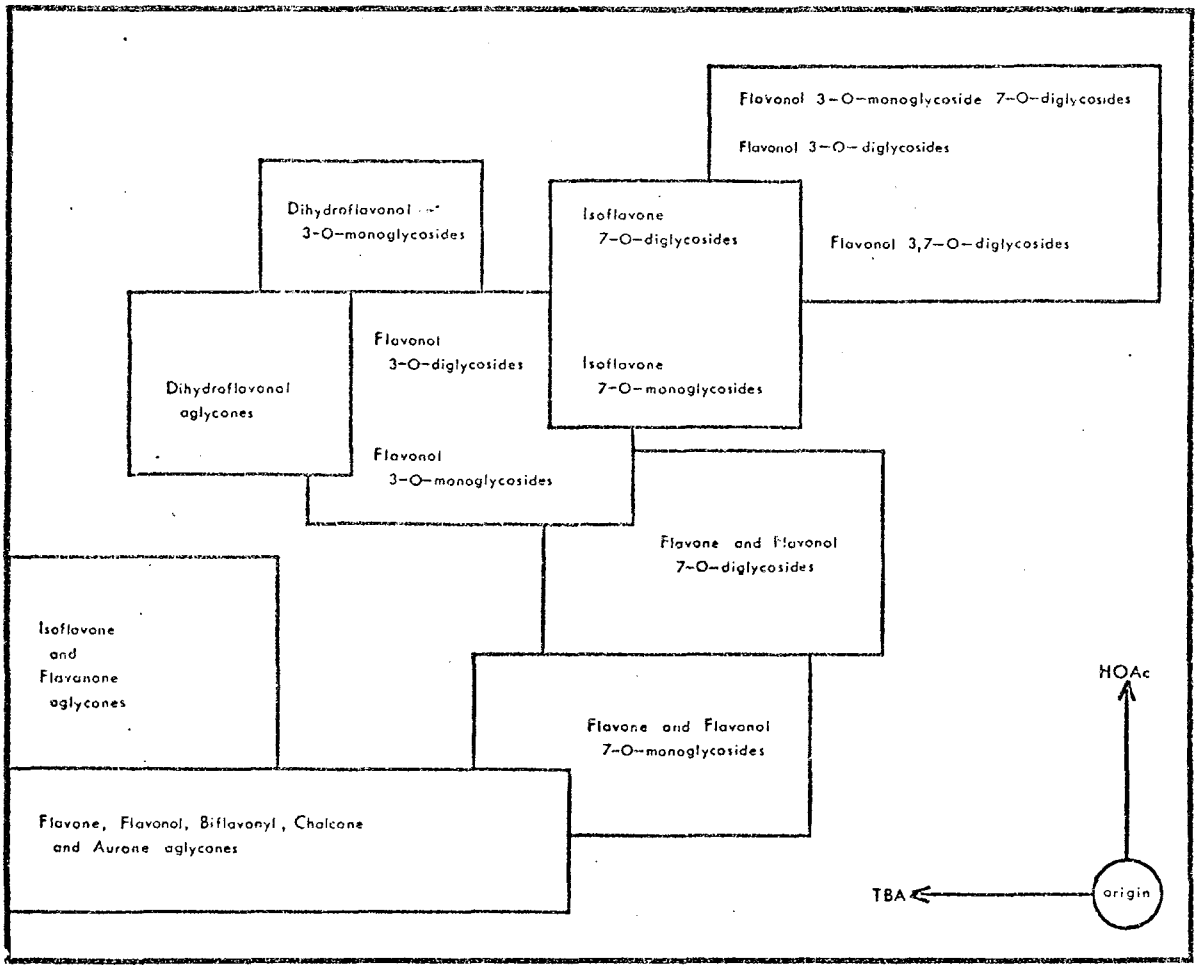


Figure 14 The distribution of flavonoids on a TBA (t-BAW)/HOAc (15% acetic acid), two-dimensional paper chromatogram.

2. 4'-Monohydroxy flavonoids run faster in both t-BAW and 15% acetic acid than do the 3',4'-dihydroxy equivalents.
3. For monoglycosides and diglycosides of the same aglycone, the diglycoside consistently runs slower in t-BAW and faster in 15% acetic acid than the monoglycoside. This relationship has been shown to be consistent for apigenin and luteolin (both anthocyanins) and for other types of flavonoids.

It should be noted that the average R_f's of spots in the Tables are not the same as the spots from paper chromatography. The numbering system of Mingrone (1968) was used for identifying spots on two dimensional paper chromatograms. On TLC, the initial spot when developed separates into several distinct spots. The R_f value was measured from the center of each distinct spot and then listed on the tables under a numbered column. The column numbers are not meant to correlate from Table to Table (i.e. the average spot R_f's in column 3, Table 3, are not necessarily for the same spot as the R_f's in column 3, Table 4). A typical spot as it would appear before and after development on TLC is shown in Figure 15.

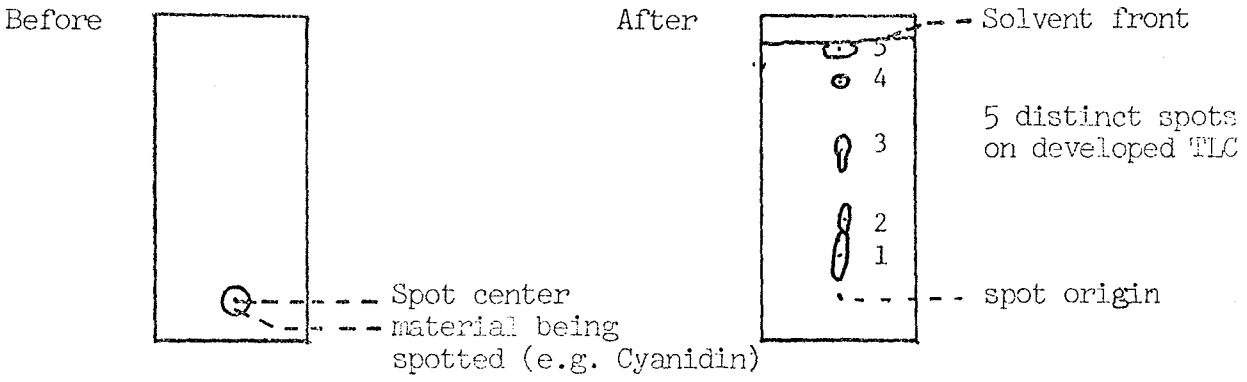


Figure 15 A typical spot before and after development on TLC.

Tables 2 through 5 represent development in the same solvent. The R_f values on them can, therefore, be compared to identify the anthocyanidin and possibly the sugars associated with it.

In Table 2 the R_f's for galactose and glucose in this solvent system are almost the same. These two sugars are difficult to separate in any solvent system. Glucuronic acid had two distinct spots. Uronic acids are easily converted to the lactone, thus the two spots are probably glucuronic acid and its lactone. Galacturonic acid may also have had a lactone spot, but due to a small concentration on the TLC, it was not visualized.

The overall averages in Tables 3,4 and 5 are very similar. Authentic cyanidin has one spot with an average R_f of 61.7 that apparently is either not in 2-1-15 or Band I, or has a different R_f. Both 2-1-15 and Band I have spots at 50.9-52.6 that could be this authentic cyanidin spot. The R_f could vary due to the presence of a sugar. The R_f's of glucose and galactose (and also possibly arabinose and rhamnose) are close enough to those of 2-1-15 that any one could be a sugar of the complex. The R_f of 79.2 from 2-1-15 is most likely a breakdown product due to hydrolysis.

The results from Tables 2 through 5 indicate that cyanidin is the anthocyanidin of the 2-1-15 complex, and either glucose and/or galactose (maybe arabinose and/or rhamnose) are the sugars present.

Tables 6 and 7 indicate that the sugar of 2-1-15 is probably glucose. The R_f of glucose if 44.2 and 2-1-15 has a spot with an average R_f of 43.1.

TABLE 2

Average R_f's of TLC Spots From Authentic Sugars on Silica Gel in the MEK:Ac:MeOH (6:2:2 v/v) Solvent System.

Sugar	Average R_f's of Spots
Arabinose	56.4
Galactose	47.2
Glucose	48.4
Mannose	—
Rhamnose	68.5
Glucuronic acid	48.14 & 74.5
Galacturonic acid	45.9

TABLE 3

Average R_f's of Spots of Authentic Cyanidin on Silica Gel TLC with Various Treatments in MEK:Ac:MeOH (6:2:2 v/v) Solvent System.

Treatment	Average R_f's of Spots					
	1	2	3	4	5	6
Cyanidin:						
without hydrolysis	12.7	22.9	---	41.2	---	---
with hydrolysis	11.6	18.6	33.1	44.0	61.7	98.6
Overall average of Cyanidin	12.2	20.8	33.1	43.1	61.7	98.6

TABLE 4

Average R_f's of Band 1 (spot 15) of 2-1-15 Complex on Silica Gel TLC with Various Treatments in the MEK:Ac:MeOH (6:2:2 v/v) Solvent System.

Treatment	Average R_f's of Spots					
	1	2	3	4	5	6
Band I without hydrolysis:	12.6	---	31.9	43.5	53.1	---
Band I with hydrolysis:						
Ether extract	---	17.1	---	---	---	98.5
MeOH:H_2O extract	---	16.0	38.6	49.0	---	---
No extraction	---	---	29.6	41.5	52.2	---
Average of hydrolysis	---	16.6	34.1	47.6	52.2	98.4
Overall average of Band I (with and without hydrolysis)	12.6	15.2	32.8	44.7	52.6	98.5

TABLE 5

Average R_f's of Spots from 2-1-15 Complex on Silica Gel TLC with Various Treatments in the MEK:Ac:MeOH (6:2:2 v/v) Solvent System

Treatment	Average R_f's of Spots					
	1	2	3	4	5	6
2-1-15:						
without hydrolysis	---	31.9	---	49.2	---	---
with hydrolysis (no extraction)	---	---	42.1	55.1	80.3	93.3
Extracts of hydrolyzed 2-1-15:						
A (MeOH:H_2O)	---	25.2	---	47.2	---	97.8
B (ether)	17.8	---	---	---	---	98.3
C (MeOH:H_2O)	---	---	---	---	---	---
D (ether)	---	---	---	---	---	96.4
E (MeOH:HCl)	---	---	---	---	78.0	98.5
Average of MeOH extracts	---	25.2	---	47.2	---	97.8
Average of ether extracts	17.8	---	---	---	---	98.3
Overall average of hydrolyzed 2-1-15 (without and with extraction)	17.8	25.2	44.3	53.0	79.2	97.1
Overall average of 2-1-15 (without and with hydrolysis)	17.8	27.9	42.5	50.9	79.2	97.2

TABLE 6

Average R_f's of Authentic Sugars on Silica Gel TLC in Ethyl Acetate:Pyridine:Water (12:5:4 v/v) Solvent System.

Sugar	Average R_f's
Arabinose	61.2
Galactose	37.8
Glucose	44.2
Mannose	52.7
Rhamnose	80.2
Xylose	72.5

TABLE 7

Average R_f's of Spots From 2-1-15 Complex on Silica Gel TLC in Ethyl Acetate:Pyridine:Water (12:5:4 v/v) Solvent System.

	Average R_f's of Spots				
	1	2	3	4	5
2-1-15	9.9	14.5	18.0	25.5	43.1

Tables 8,9 and 10 with development in Forestal gave little conclusive evidence. They indicate that the cyanidin of 2-1-15 could be contaminated either with any of several breakdown products, or with another anthocyanidin. The possibility of breakdown products has been mentioned before and is considered the most likely. Forestal appears to be a poor solvent for resolving different sugars. No conclusions could be made on the identity of the sugars from this solvent.

Tables 11 through 13 were developed on cellulose in n-BAW. This was a good solvent for separating sugars and anthocyanidins. Results from this solvent indicate that cyanidin is the anthocyanidin in the 2-1-15 complex. The possibility of contamination of 2-1-15 with delphinidin, malvidin or pelargonidin does not appear probable. Breakdown products of the 2-1-15 complex are apparently the only major contaminants. Partial hydrolysis does occur with time in 2-1-15 even when stored dry in the cold and dark. The partial hydrolysis is indicated by a comparison of fresh 2-1-15 with old 2-1-15 (Table 13). The old 2-1-15 had 5 more spots than the fresh 2-1-15 (i.e. spots in columns 1,2,3,9 and 11).

The spots in columns 1 and 2 could be glucose which was no longer attached to the molecule. Partial hydrolysis having removed the substituent at the most unstable position first. The average R_f's of glucose and cyanidin plus glucose compare with the average R_f's of the 2-1-15 complex. It is more difficult to indicate other sugars that may be present in 2-1-15 since R_f's of cyanidin are so similar to sugar R_f's in this solvent. The presence of an average R_f of

TABLE 8

Average R_f's of Spots from Authentic Sugars on Cellulose TLC in Forestal (Acetic Acid:HCl:H_2O, 30:3:10 v/v) Solvent System.

Sugar	Average R_f's of Spots	
	1	2
Arabinose	82.3	
Fucose	86.5	
Galactose	80.6	
Glucose	80.2	
Lyxose	87.3	
Mannose	81.2	
Rhamnose	87.6	
Ribose	72.6	90.7
Xylose	82.0	
Galacturonic acid	70.3	84.1
Glucuronic acid	77.4	86.4

TABLE 9

Average R_f's of Spots from Authentic Anthocyanidins on Cellulose TLC in Forestal (Ac:HCl:H_2O, 30:3:10 v/v) Solvent System.

Anthocyanidin	Average R_f's of Spots	
Cyanidin	48.0	72.4
Delphinidin	25.5	---
Malvidin	68.2	---
Pelargonidin	69.6	---

TABLE 10

Average R_f's of Spots from 2-1-15 Complex on Cellulose TLC with Various Treatments in Forestal (Ac:HCl:H_2O, 30:3:10 v/v) Solvent System.

Treatment	Average R_f's of Spots					
	1	2	3	4	5	6
2-1-15 without hydrolysis:	45.8	62.6	65.9	---	---	---
2-1-15 with hydrolysis and extraction:						
A (MeOH:H_2O)	42.1	58.0	65.7	78.6	---	96.9
B (ether)	---	---	---	73.0	88.5	---
C (MeOH:H_2O)	---	---	---	73.5	86.0	---
D (ether)	---	---	---	---	84.0	---
E (HCl:MeOH)	---	55.0	68.4	78.6	---	96.1
Overall average of extracts	42.1	56.5	67.1	75.9	84.7	95.9
Overall average of 2-1-15	44.0	58.5	66.7	75.9	84.7	95.9

TABLE 11

Average R_f's of Spots from Authentic Sugars on Cellulose and Silica Gel TLC's in n-BAW (n-Butanol:Acetic:H_2O, 4:1:5 top layer) Solvent System.

Sugar	Average R_f's of Spots				
	Cellulose			Silica	
Fucose	49.5	---		44.7	---
Galactose	28.3	---		8.7	27.3
Glucose	11.7	19.0	36.3	9.0	31.7
Rhamnose	38.2	56.9		47.9	
Glucuronic acid	13.0	45.7		---	---

TABLE 12

Average R_f's of Spots from Authentic Anthocyanidins with Various Treatments (addition of sugars) on Cellulose TLC in n-BAW (n-butanol:Acetic Acid:H_2O, 4:1:5 top layer) Solvent System.

Anthocyanidin & Treatment	Average R_f's of Spots										
	1	2	3	4	5	6	7	8	9	10	11
Delphinidin			40.7								
Malvidin							72.4				
Pelargonidin							74.3			94.5	
Pelargonidin & Glucose	15.6	22.2	38.8	---	---	---	---	---	92.3	---	98.6
Cyanidin	11.5	---	39.5	46.5	59.6	66.4	---	79.1	92.7	---	98.5
Cyanidin & Glucose	12.0	19.7	37.3	---	60.4	65.8	---	81.3	92.3	---	97.5
Cyanidin & Rhamnose	---	---	---	---	61.8	---	---	---	91.3	---	98.5
Cyanidin & Glucuronic acid	10.9	29.0	40.5	---	63.5	---	---	---	87.0	---	---

TABLE 13

Average R_f's of Spots of 2-1-15 Complex on Cellulose TLC with Various Treatments in n-BAW (n-Butanol:Acetic Acid:Water, 4:1:5 top layer) Solvent System.

Treatment	Average R_f's of Spots										
	1	2	3	4	5	6	7	8	9	10	11
2-1-15: Without hydrolysis fresh*	---	---	---	39.5	42.1	47.3	56.4	60.8	---	92.6	---
old*(partial hydrolysis)	10.8	16.4	30.0	38.1	---	48.5	58.7	65.1	85.2	92.2	98.6
fresh & glucose	---	19.9	30.9	---	43.3	51.0	60.5	68.8	---	---	---
old & glucose	---	19.0	27.7	35.4	42.4	47.7	---	63.1	87.6	94.5	---
average 2-1-15 & glucose	---	19.5	29.3	35.4	42.9	49.4	60.5	66.0	87.6	94.5	---
extracted without hydrolysis:											
MeOH:H_2O extract	---	---	---	---	43.9	47.5	59.0	65.5	---	---	---
ether extract	---	---	---	---	44.9	---	---	63.7	86.2	94.3	---
average of extracts	---	---	---	---	44.4	47.5	59.0	64.6	86.2	94.3	---
Overall average of 2-1-15:	10.8	17.6	29.8	37.8	43.5	49.0	58.3	63.7	86.0	92.2	98.6

* Fresh indicates little or no storage time; old indicates stored for a period of time before being spotted -- partial hydrolysis has occurred.

TABLE 14

Average R_f's of Spots from Authentic Anthocyanidins on Silica Gel TLC in n-BAW (n-Butanol:Acetic Acid:H_2O, 4:1:5 top layer) Solvent System.

Anthocyanidin & Treatment	Average R_f's of Spots								
	1	2	3	4	5	6	7	8	9
Delphinidin	----	----	----	52.7	----	----	----	----	----
Pelargonidin	----	----	----	----	57.2	----	82.2	----	92.1
Cyanidin	19.0	----	47.0	----	----	71.8	----	88.0	----
Cyanidin & glucose	----	25	41.3	----	----	----	----	----	----

TABLE 15

Average R_f's of Spots from 2-1-15 Complex on Silica Gel TLC with Various Treatments in n-BAW (n-Butanol:Acetic Acid: Water, 4:1:5 top layer) Solvent System.

Treatment	Average R_f's of Spots						
	1	2	3	4	5	6	7
2-1-15 fresh	---	---	---	35.1	47.4	59.6	---
2-1-15 old (partial hydrolysis)	11.6	16.8	23.6	37.9	46.4	64.5	89.7
2-1-15 & glucose	9.1	18.9	27.9	35.1	44.2	73.0	---
Overall average of 2-1-15	10.4	17.9	24.7	36.8	46.0	65.2	89.7

TABLE 16

R_f Values of Possible Breakdown Products Chromatographed in n-BAW Solvent System. (Adapted from Block, Durrum and Zweig, 1958).

Possible breakdown products	R_f
Aromatic acids	
O-Hydroxy benzoic acid	95
Protocatechuic acid	85
Gallic acid	68
Phenols	
Pyrogallol	77
Phloroglucinol	76
2-(2,4-Dihydroxyphenyl azophenol)	90
Catechol	91
Resorcinol	91
Hydroxyquinone	88
Orcinol	91

43.5 in 2-1-15 could possibly be due to a sugar (perhaps rhamnose with average R_f's of 38.2 and 56.9, see Table 2) since a similar R_f is not present in either cyanidin or glucose. Another possible sugar is galactose (average R_f of 28.3). The 2-1-15 spots in column 3, Table 13, have an average R_f of 29.8, neither cyanidin nor glucose have similar averages. Columns 9,10 and 11 (Table 13) appear to be spots due to breakdown products, compare these R_f's with Table 16.

Cellulose TLC is better for separation with n-BAW than silica gel. The solvent front moves very slowly on the silica gel thus affecting R_f values. Visualizing spots on silica gel also requires a higher concentration of material. The results for silica gel in n-BAW are, therefore, not as complete as with cellulose.

All the average R_f's of 2-1-15 compare with cyanidin and glucose R_f's except for two spots - 36.8 and 65.2. The 36.8 is most likely due to a sugar since the R_f is too low to be a breakdown product. The spot at 65.2 could be the spot that authentic cyanidin has - with an average R_f of 71.8 (refer to Table 14). Galactose may be present, but its average R_f's (8.9 and 27.3) are very similar to those of cyanidin and glucose and it is difficult, therefore, to determine with certainty.

B. Spectral

As mentioned previously there are two major absorption peaks in the methanol spectra of most flavonoids. Anthocyanins have their major absorption peaks in the regions of 250-300 nm and 450-550 nm. The absorptions in the visible range, 450-550 nm, are referred to as

Band I. Band II is the absorption in the UV range, 250-300 nm. Band I is considered to be associated with absorptions due to the B-ring (cinnomoyl system), and Band II with absorptions due to the A-ring (benzoyl system) (Figure 16).

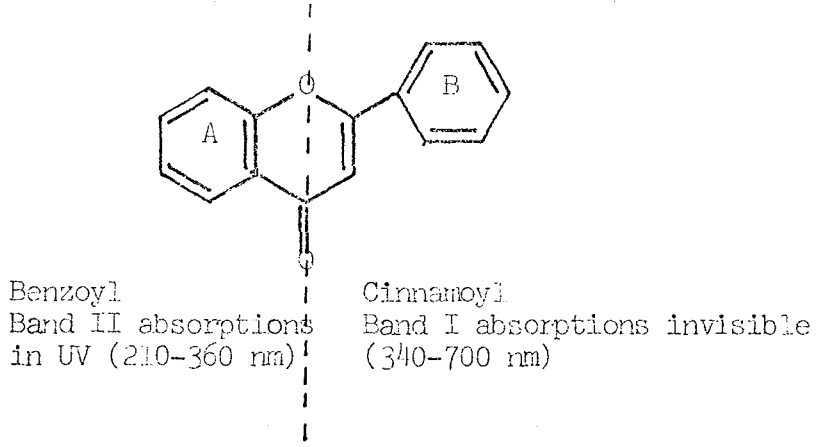


Figure 16 Diagram showing spectral absorption ranges of the flavonoid molecule (Adapted from Mabry et al, 1970).

Various reagents added to the neat solutions of the anthocyanins form complexes that absorb at different wavelengths. Spectral shifts towards shorter wavelengths are termed hypsochromic, and towards longer wavelengths are bathochromic.

Results for the spectral portion of this investigation were determined by comparison of spectra of the 2-1-15 spot complex, with spectra of authentic cyanidin (3,3',4',5,7-Pentahydroxy flavylium) and with spectral data in the literature. Interpretations were made with the aide of Mabry et al (1970), and Harborne (1957).

Mabry et al. (1970) do not deal specifically with anthocyanins. They discuss in depth general trends found to hold for a majority of flavonoids. Harborne (1957) details specific information for

anthocyanins, but does not go into depth on reagent effects. A combination of this general and specific information was used to interpret spectra. Results are given for; the theoretical reagent effects, what the Band shifts indicate, and the actual shifts for authentic anthocyanidin samples and the 2-1-15 complex.

The methanol spectra provide information on flavonoid types and degree of oxidation of the heterocyclic ring systems. The position of the Band I peak between 475-550 nm is characteristic of anthocyanins. For other flavonoids Band I occurs characteristically at other positions, for example, flavones are between 304-350 nm and flavonols between 352-385 nm.

Band I for authentic cyanidin has an absorption maximum at 534 nm according to Harborne (1957; 1967, pg. 7). A maximum of 537-538 nm was found in this investigation. The 2-1-15 complex had an average maximum of 528.5 nm, thus, a hypsochromic shift of approximately 6.5 nm between cyanidin and 2-1-15.

The hypsochromic shift in this case is indicative of hydroxyl groups being glycosidated. The magnitude of the shift depends on how many and which positions are glycosidated. The 3-glycosides of cyanidin have a λ_{max} at 523 nm, a hypsochromic shift of approximately 12 nm. The hypsochromic shift for a glycoside in the 5-position is approximately 7 nm. There is little difference, however, between anthocyanidins with sugars at the 3- and 5-positions and just the 3-position. To distinguish between these two glycosides it is necessary to observe Band I and the E_{440}/E_{max} ratio values. When the 5-hydroxyl

group is free (no sugar substituent), a characteristic shoulder to the main peak (between 410--450 nm) will frequently be observed. The E_{440}/E_{max} value for the 5-substituted anthocyanidin will be approximately one-half the value of the free 5-hydroxyl anthocyanidin.

The lack of a shoulder on Band I at the 2-1-15 complex, and the hypsochromic value of 6.5 nm indicates that the 3-hydroxyl is free and the 5-hydroxyl is probably substituted with a glycoside. The E_{440}/E_{max} value of the authentic cyanidin sample was roughly double the 2-1-15 value, also an indication that the 5-hydroxyl is substituted.

The first reagent added to the neat stock solutions was sodium methoxide, NaOMe. Sodium methoxide is a strong base and ionizes hydroxyl groups. It has been used by Mabry et al (1970) on flavones and flavonols to detect free 3- and/or 4'-hydroxyl groups. A large bathochromic shift of Band I, approximately 40-65 nm, is considered evidence for the presence of a free 4'-hydroxyl group. A decrease in peak intensity usually indicates a free 3-hydroxyl and a substituted 4'-hydroxyl.

Band I in authentic cyanidin showed bathochromic shifts of 79 nm for the main peak and 34 nm for the shoulder in the presence of NaOMe. Authentic pelargonidin (3,4',5,7-tetrahydroxyflavylium) had bathochromic shifts of Band I and its shoulder of 93 nm and 38 nm respectively. An overall increase in magnitude of the shifts (Δ14 nm) with a decrease of one free hydroxyl position (i.e. from the free 3,3',4'-hydroxyls in cyanidin to the free 3,4'-hydroxyls in pelargonidin).

Band I of the 2-1-15 complex experienced a bathochromic shift of 66 nm. This evidence from addition of the NaOMe reagent indicates

that the 2-1-15 complex has present free hydroxyls at the 3,3' and 4' positions.

Sodium acetate, NaOAc, was the next reagent added to the neat stock solutions. NaOAc is a weaker base than sodium methoxide. Consequently, it should only ionize the more acidic hydroxyl groups. For flavones and flavonols these groups would be the hydroxyls at the 3,4'- and 7-positions. For anthocyanins the 5-position may also be considered as more acidic.

Sodium acetate can be specific for the detection of 7-hydroxyl groups. This is because ionization of the A-ring mainly affects Band II, whereas, ionization of 3,3'- and 4'-positions mainly affects Band I. Decomposition of the flavonoid is possible if an alkali-sensitive grouping is present. For flavones and flavonols, alkali-sensitive groupings consist of hydroxyl groups in any of the following patterns; 3,3',4'- ; 3',4',5'- ; 5,6,7- ; or 5,7,8-. If one of these patterns is present decomposition may occur quite rapidly. Consequently, determination of the presence or absence of free 7-hydroxyl groups can be quite difficult.

The characteristic spectra for flavones and flavonols with NaOAc, will experience a bathochromic shift in Band II of 5-20 nm in the presence of a free 7-hydroxyl group. Band I will also shift if there is a free 7-hydroxyl group, whether or not a 4'-hydroxyl is present (Mabry et al, 1970). Those flavones and flavonols that possess a 4'-hydroxyl and no free 3- or 7-hydroxyls, usually exhibit a shoulder on Band I (on the longer wavelength side) in NaOAc.

Band II was very difficult to obtain data from due to its apparent decomposition in the presence of NaOAc. Both authentic cyanidin and 2-1-15 exhibited decomposition in Band II. What remained of the absorption peaks showed little or no shift. Authentic pelargonidin also had decomposition, however, there appeared to be a bathochromic shift of approximately 7 nm. No conclusions can be drawn about the 7-position from this NaOAc effect on Band II.

Band I experienced a shift in NaOAc. There was a bathochromic shift of approximately 62 nm for authentic cyanidin (the shoulder also shifted bathochromatically 34 nm). Authentic pelargonidin also shifted bathochromatically, 70 nm for the main peak and 34 nm for the shoulder. A bathochromic shift was also observed for the 2-1--15 complex of about 49 nm (it had no apparent shoulder). It is difficult to determine from the NaOAc reagent effects whether the 7-position has a free hydroxyl or not. The data tends to reinforce previous data, however, that the 3,3' and 4'-positions do have free hydroxyls.

Boric acid was added to the solution that already contained NaOAc. Boric acid will chelate with ortho-dihydroxyl groups present on the flavonoid rings. An example of a chelated complex is shown in Figure 17.

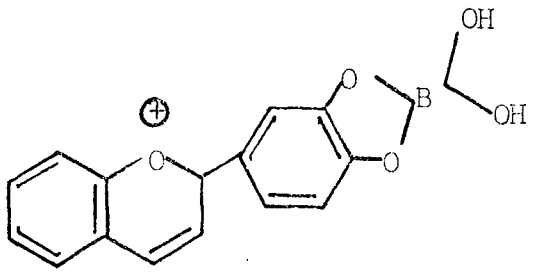


Figure 17 Boric acid chelated with orthodihydroxyls of an anthocyanidin. (Adapted from Mabry et al, 1970).

A bathochromic shift in Band I of approximately 12-30 nm is characteristic of B-ring ortho-dihydroxyl groups, for flavones and flavonols (Mabry et al, 1970). It is suspected that a similar type of shift would also occur for other types of flavonoids.

Authentic cyanidin exhibits a bathochromic shift in Band I of 36 nm, while pelargonidin has a bathochromic shift of 60 nm. For 2-1-15 no obvious absorption peaks were discernable. This was probably because either the stock 2-1-15 solution was too dilute, or the NaOAc decomposed the flavonoid so rapidly that no absorption of the chelated complex could occur.

Aluminum chloride was the next reagent added to the neat stock solutions. Aluminum chloride forms acid stable complexes with hydroxyl groups in the C-3 and C-5 positions. $AlCl_3$ has been used as a diagnostic reagent for the detection of anthocyanins with ortho-dihydroxyl systems in the B-ring. The acid stable complexes that are formed in flavones and flavonols with the C-3 and C-5 positions probably cannot be formed in anthocyanins. The lack of a carbonyl at the 4-position being the reason. The possible types of complexes formed with $AlCl_3$ and HCl are shown in Figure 18.

The complexes formed between the ortho-dihydroxyl system and $AlCl_3$ decompose in acid. It is expected that the ortho-dihydroxyl system of anthocyanins should form complexes of the same type as are found in other types of flavonoids.

To detect the ortho-dihydroxyl system in flavonoids the $AlCl_3$ spectra should be compared with the $AlCl_3$/HCl spectra. Band I will

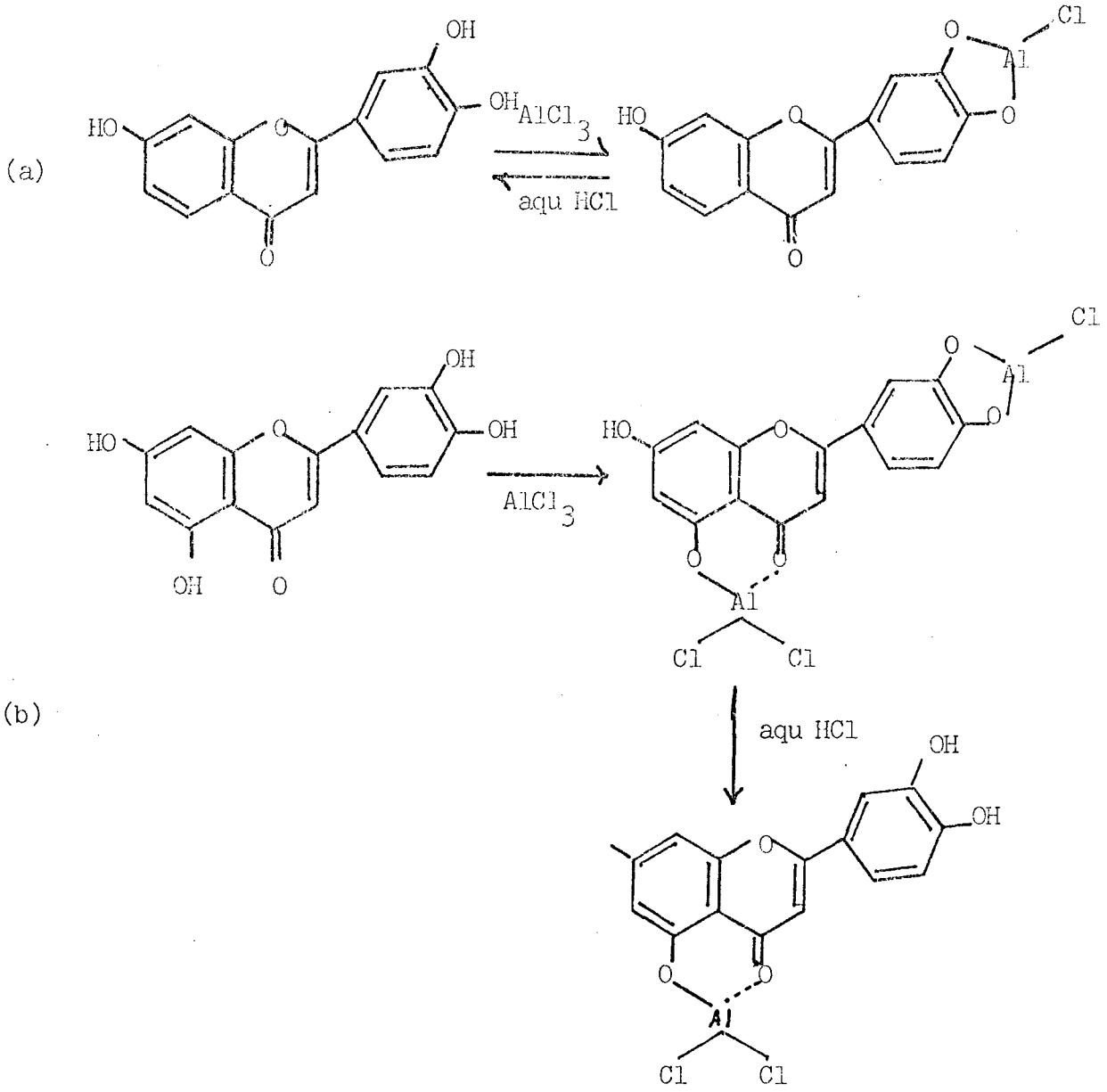
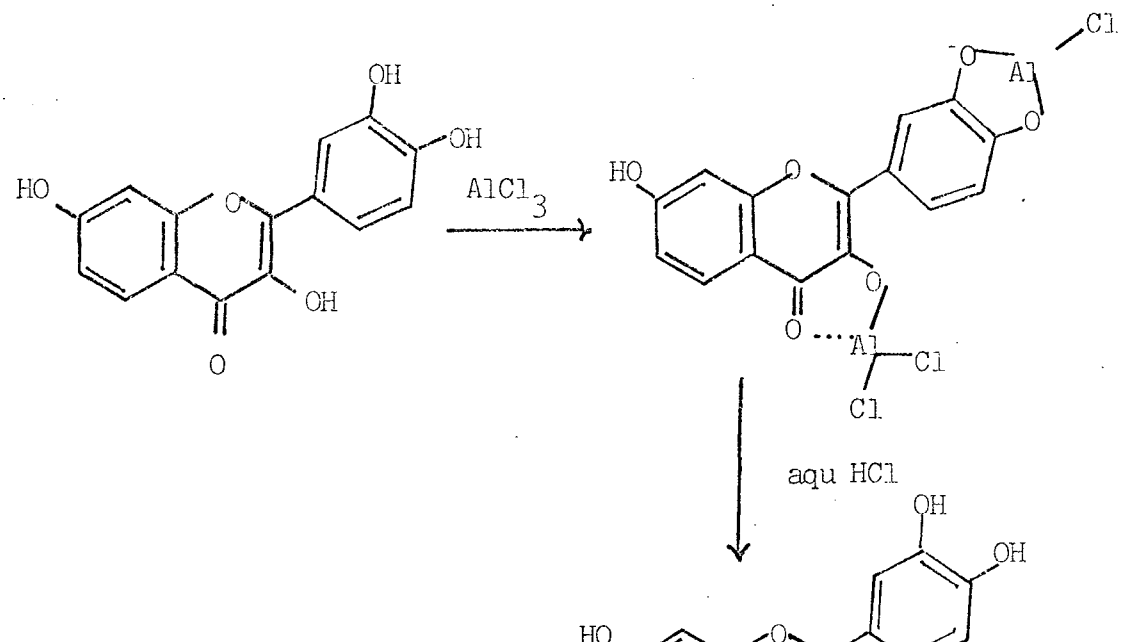


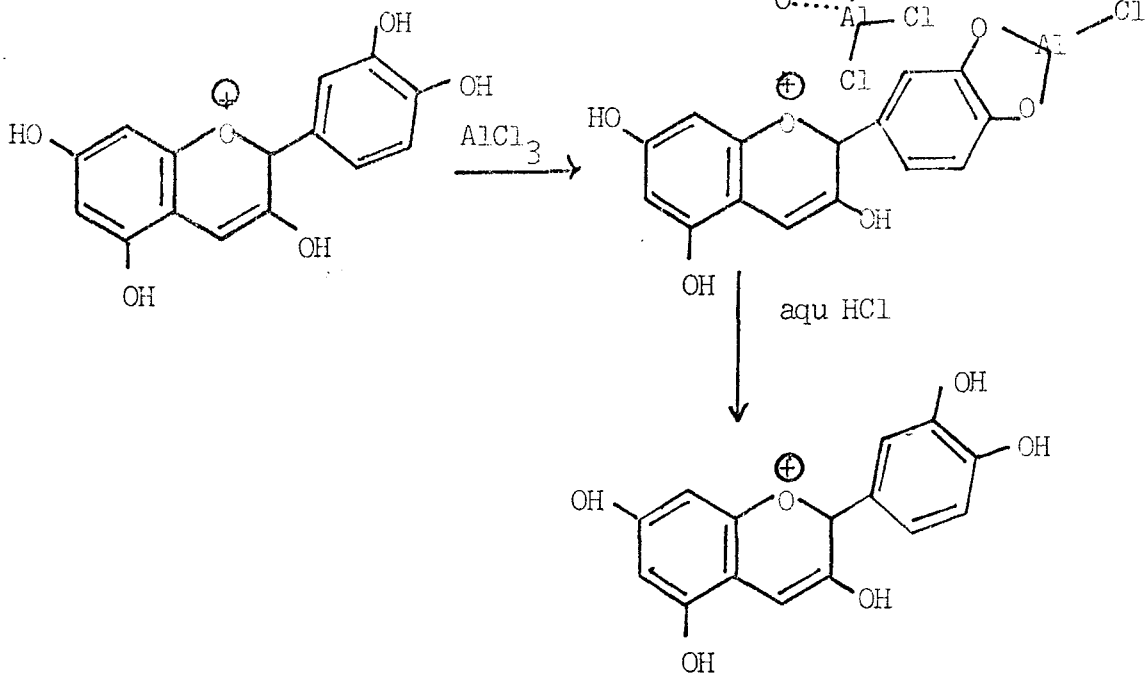
Figure 18 Schemes illustrating the types of complexes that $AlCl_3$ could form with certain flavones, flavonols (a,b,c) and anthocyanidins (d) in the presence or absence of acid.*

(Adapted from Mabry et al, 1970, pg. 72).

(c)



(d)



*Note the difference in structure between the flavones and flavonols and anthocyanidins is the reason that no C-3 or C-5 hydroxyl information can be obtained with $AlCl_3$, but ortho-dihydroxyl information can be obtained.

shift bathochromatically in $AlCl_3$ approximately 30-40 nm in the presence of an ortho-dihydroxyl system. If three hydroxyls are adjacent to each other the shift will only be about 20 nm. With the addition of acid the $AlCl_3$ complex should decompose and Band I will shift hypsochromatically back to its original (i.e. neat) position. If, for flavones and flavonols, the hypsochromatic shift is greater than this, then the excess nm shift is due to the presence of free 3- and/or 5-hydroxyl groups. Since the $AlCl_3$ cannot form acid stable complexes with any free 3- or 5-hydroxyls present in anthocyanins it is then expected that when acid is added Band I should return to its original position. The neat methanolic spectrum should be regenerated in the $AlCl_3$/HCl if only anthocyanins are present. If other flavonoid types are present with the carbonyl at the 4-position then the neat methanolic spectrum should not be regenerated unless both the 3- and 5-hydroxyl groups are either absent or substituted.

The addition of $AlCl_3$ to the authentic cyanidin stock solution caused a 35 nm bathochromic shift of Band I. The shoulder that was in the neat methanolic spectra was not apparent with the $AlCl_3$. The spectra of authentic pelargonidin supports the fact that the bathochromic shift is an indicator of ortho-dihydroxyl systems in anthocyanins. Bands I and II of pelargonidin did not shift when either $AlCl_3$ or HCl were added. A bathochromic shift of 47 nm was experienced by the 2-1-15 complex when $AlCl_3$ was added.

The addition of HCl to the $AlCl_3$ complex caused a regeneration of the neat methanolic spectra in both authentic cyanidin and 2-1-15.

Thus it is suspected that an ortho-dihydroxyl system exists on the B-ring. Apparently the 2-1-15 sample was not contaminated with other flavonoid types. A summary of the spectral reagent results will be found on Table 17.

TABLE 17

Summary of Spectral Reagent Results

	Band*		Change in nm	Shift Direction
NaOMe	I	Cyanidin	79	bathochromic shift (shoulder ▲34 bathochromic
	I	2-1-15	66	bathochromic
	I	Pelargonidin	93	bathochromic (shoulder ▲38 bathochromic
	II	Cyanidin	52	bathochromic
	II	2-1-15	5	hypsochromic
	II	Pelargonidin	8.5	bathochromic
NaOAc	I	Cyanidin	62	bathochromic (shoulder ▲34 bathochromic
	I	2-1-15	49	bathochromic
	I	Pelargonidin	70	bathochromic apparent decomposition (shoulder ▲31 bathochromic)
	II	Cyanidin	No Data	
	IIb	approx. no shift	IIa 21	bathochromic
	II	Pelargonidin	7	bathochromic - obvious decomposition
NaOAc/H_3BO_3	I	Cyanidin	36	bathochromic (shoulder ▲74 bathochromic no obvious decomposition
	I	2-1-15	No Data	
	I	Pelargonidin	60	bathochromic (shoulder ▲18 bathochromic obvious decomposition
	II	Cyanidin	7	bathochromic
	II	2-1-15	2.5	bathochromic
	II	Pelargonidin	7	bathochromic

TABLE 17 (continued)

$AlCl_3$	I	Cyanidin	35	bathochromic (no shoulder)
	I	2-1-15	47	bathochromic
	I	Pelargonidin	2	bathochromic (shoulder - no change)
	II	Cyanidin	6	hypsochromic (develop a shoulder)
	II	2-1-15	3.5	bathochromic (shoulder ◁ 9 bathochromic)
	II	Pelargonidin	No Shift	
$AlCl_3/HCl$ (compared with Neat)				
	I	Cyanidin	Same as Neat	
	I	2-1-15	Same as Neat	
	I	Pelargonidin	Same as Neat	
	II	Cyanidin	Same as Neat	
	II	2-1-15	Same as Neat	
	II	Pelargonidin	Same as Neat	

V. SUMMARY AND CONCLUSIONS

The conclusions of this investigation include data from chromatography and spectral analysis. The chromatography data indicates that possibly several sugars are present on the 2-1-15 spot complex.

Glucose is present, and galactose is also quite probable. This is based on TLC data using R_f values. Glucose and galactose were difficult to separate chromatographically. Furthermore, when separation was achieved the R_f's of galactose were similar to those of Cyanidin as seen with n-BAW. Of the other sugars worked with the chromatography data indicated that arabinose and/or rhamnose might also be found on the 2-1-15 spot complex.

The spectral data indicated that Cyanidin is the anthocyanidin of the 2-1-15 complex. There did not appear to be any other anthocyanidins present in 2-1-15, as demonstrated by the reagents $AlCl_3$ and HCl in spectral analysis.

The spectral results also indicated that the 3-position was free. If this is combined with the partial hydrolysis data from chromatography, it indicates that glucose could be at the 3-position. Apparently for 2-1-15 the 3-position substituent is sensitive to acid hydrolysis and, therefore, the spectral data always indicated the 3-position was a free hydroxyl. The chromatography indicated that partial hydrolysis was occurring while the 2-1-15 was in the acid solution drying. The other possible sugars could be located at the 5 and/or 7 positions,

No conclusive data was obtained to indicate the number of sugars present or if the sugars present were monosides, biosides or triosides. Neither the spectral or chromatography data ever indicated that an acyl substituent might be present on the 2-1-15 complex. Infra-red data could give conclusive evidence for this.

To obtain more definite information on the anthocyanidin molecule of Allium amplectens more sophisticated equipment and materials would be necessary (e.g. NMR, Mass Spec., IR, nitrogen storage, temperature and light controlled chromatography room, more authentic sugar samples, etc...)

The 2-1-15 complex appears to be a very sensitive molecule and is, therefore, very difficult to work with, unless many variables in the environment can be controlled (temperature, light, time, O_2). The sensitivity of 2-1-15 was demonstrated with chromatography by the partial hydrolysis of the complex when stored even for minimal periods of time. Spectral data also indicated the 2-1-15 complex to be very sensitive. Decomposition of an older sample was easy to demonstrate when a fresh sample was available for comparison. The extreme sensitivity of this complex made results difficult to obtain.

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LITERATURE CITED

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APPENDICES

APPENDIX I

Types of Thin-Layer Chromatography Plates

The two types of Thin-Layer Chromatography plates used in this investigation were both commercially precoated* and were as follows:

Silica gel Eastman Chromagram sheet type 6061 (without fluorescent indicator)

Cellulose Eastman Chromagram sheet type 6064 (without fluroescent indicator)

Dimensions of both types were 20 x 20 cm.

* Eastman Kodak Company, Distillation Products Industries, Rochester, NY.

APPENDIX II

Authentic Anthocyanidins

Cyanidin Chloride 3,3',4',5,7-Pentahydrosyflavylium Hydroxide Lot 99731.

Delphinidine Chloride (HCl) Lot 74282

Malvin Chloride

Pelargonidin Chloride Lot 84970

Obtained from:

K&K Laboratories, Inc. Plainview, NY/Hollywood, California.

APPENDIX III

Authentic Sugars*

D(-) Arabinose
L - Fucose
D(+) Galactose
Glucose

D - Lyxose
D(+) Mannose

L(-) Rhamnose
2-Deoxy-D-Ribose
D-Ribose
D(+) Xylose

D-Galacturonic acid
D-Glucuronic acid

*Pfanstiehl Laboratories, Inc. is a laboratory that specialized in supplying rare sugars for research: Pfanstiehl Laboratories
1219 Glen Rock Ave.
Waukegan, Illinois

APPENDIX IV

Solvents Used in Paper Chromatography and TLC

Paper Chromatography:

n-BAW (BAW) - n-butanol:acetic acid:water* 4:1:5 top layer after equilibrating 3 hours.

15% acetic acid (HOAc) - acetic acid:water 15:85 v/v

t-BAW (TBA) - t-butanol:acetic acid:water 3:1:1

TLC:

MEK:Ac:MeOH (MEK) - methyl ethyl ketone:acetic acid:methanol 6:2:2 v/v/v

- ethyl acetate:pyridine:water 12:5:4 v/v/v

Forestal - acetic acid:hydrochloric acid:water, 30:3:10 v/v/v

n-BAW - same as for paper

*only glass distilled water was used

APPENDIX V

Visualizing Reagents Used for TLC

1 g p-anisidine hydrochloride
.1 g sodium hydrosulfite
10 ml methanol
100 ml n-butanol

aniline phthalate
930 mg aniline
1.6 g phthalic acid
100 ml water-saturated n-butanol

ammoniacal silver nitrate ($AgNO_3$)
mix equal volumes together of:

.1 N $AgNO_3$
5 N NH_4OH

APPENDIX VI

Stock Reagent Solutions used in Spectral Analysis

Sodium methoxide (NaOMe). Freshly cut metallic sodium (2.5 g) was added cautiously in small portions to dry spectroscopic methanol (100 ml). The solution was stored in a glass container.

Aluminum chloride ($AlCl_3$). Five grams of fresh anhydrous reagent grade $AlCl_3$ were added cautiously to spectroscopic methanol (100ml).

Hydrochloric acid (HCl). Was mixed with concentrated reagent grade HCl to 4N with distilled water.

Sodium Acetate (NaOAc). Anhydrous powdered reagent grade NaOAc was used.

Boric acid (H_3BO_3). Anhydrous powdered reagent grade H_3BO_3 was used.

APPENDIX VII

Spectra of Authentic Cyanidin and Pelargonidin and
Spectra of 2-1-15 Complex with each of the Stock Reagents.

1 authentic cyanidin and pelargonidin in visible range

2 authentic cyanidin in UV range

3 2-1-15 complex in visible range
blue = 20 min. extract without acid
red = 24 hr. extract with acid

4 2-1-15 complex in UV range (Red) Neat

5 2-1-15 complex in visible range with NaOMe

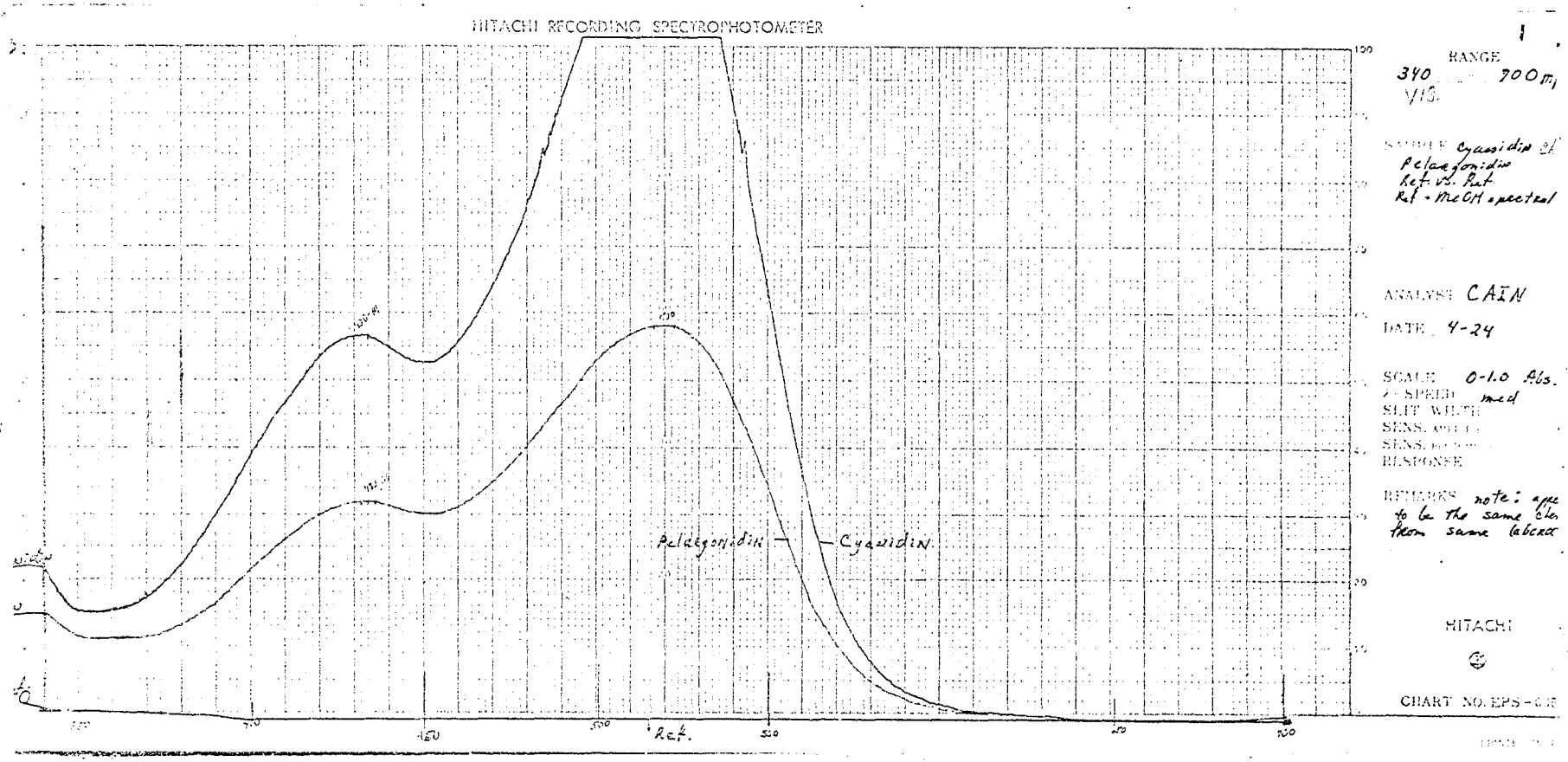
6 2-1-15 complex in UV range with NaOMe

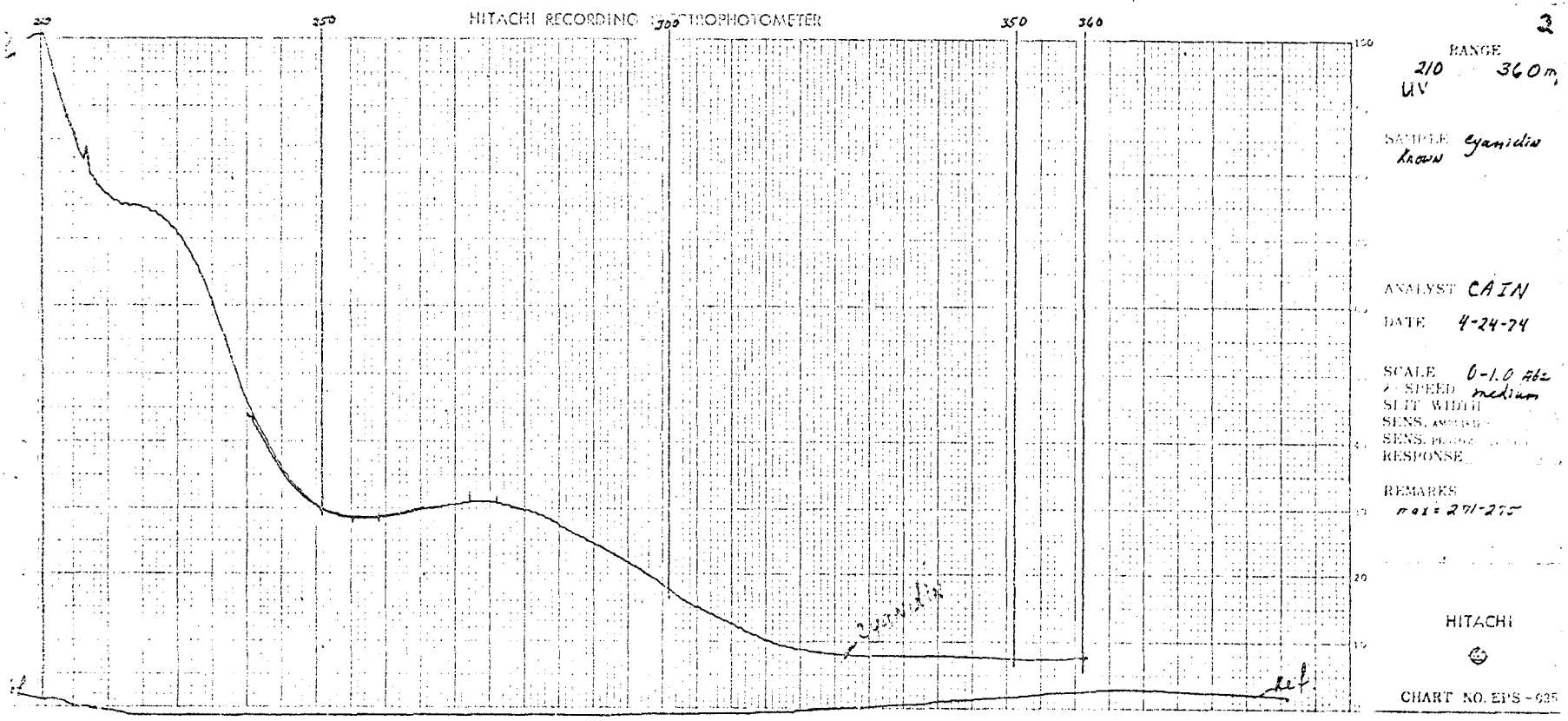
7 2-1-15 complex in visible range with $AlCl_3$ and $AlCl_3$/HCl

8 2-1-15 complex in UV range with $AlCl_3$ and $AlCl_3$/HCl

9 2-1-15 complex in visible range with NaOAc and NaOAc/H_3BO_3 (blue)

10 2-1-15 complex in UV range with NaOAc and NaOAc/H_3BO_3 (blue)


HITACHI RECORDING SPECTROPHOTOMETER
Pelargonidin
Cyanidin
Ref.
RANGE
340 — 700 mμ
VIS.
SAMPLE Cyanidin of Pelargonidin
Ref vs. Ref
Ref - MeOH spectral
ANALYST CAIN
DATE 4-24
SCALE 0-1.0 Abs.
SPEED med
SLIT WIDTH
SENS.
RESPONSE
REMARKS note: appe to be the same cle from same labora
HITACHI
CHART NO. EPS-


HITACHI RECORDING SPECTROPHOTOMETER
200
250
300
350
360
150
100
50
0
RANGE
210 360 mμ
UV
SAMPLE known cyanidin
ANALYST CAIN
DATE 4-24-74
SCALE 0-1.0 Abs
SPEED medium
SLIT WIDTH
SENS. A
SENS. R
RESPONSE
REMARKS
max = 271-275
HITACHI
CHART NO. EPS-035

HITACHI RECORDING SPECTROPHOTOMETER

3

RANGE 340 700 mμ
VIS

2-1-15

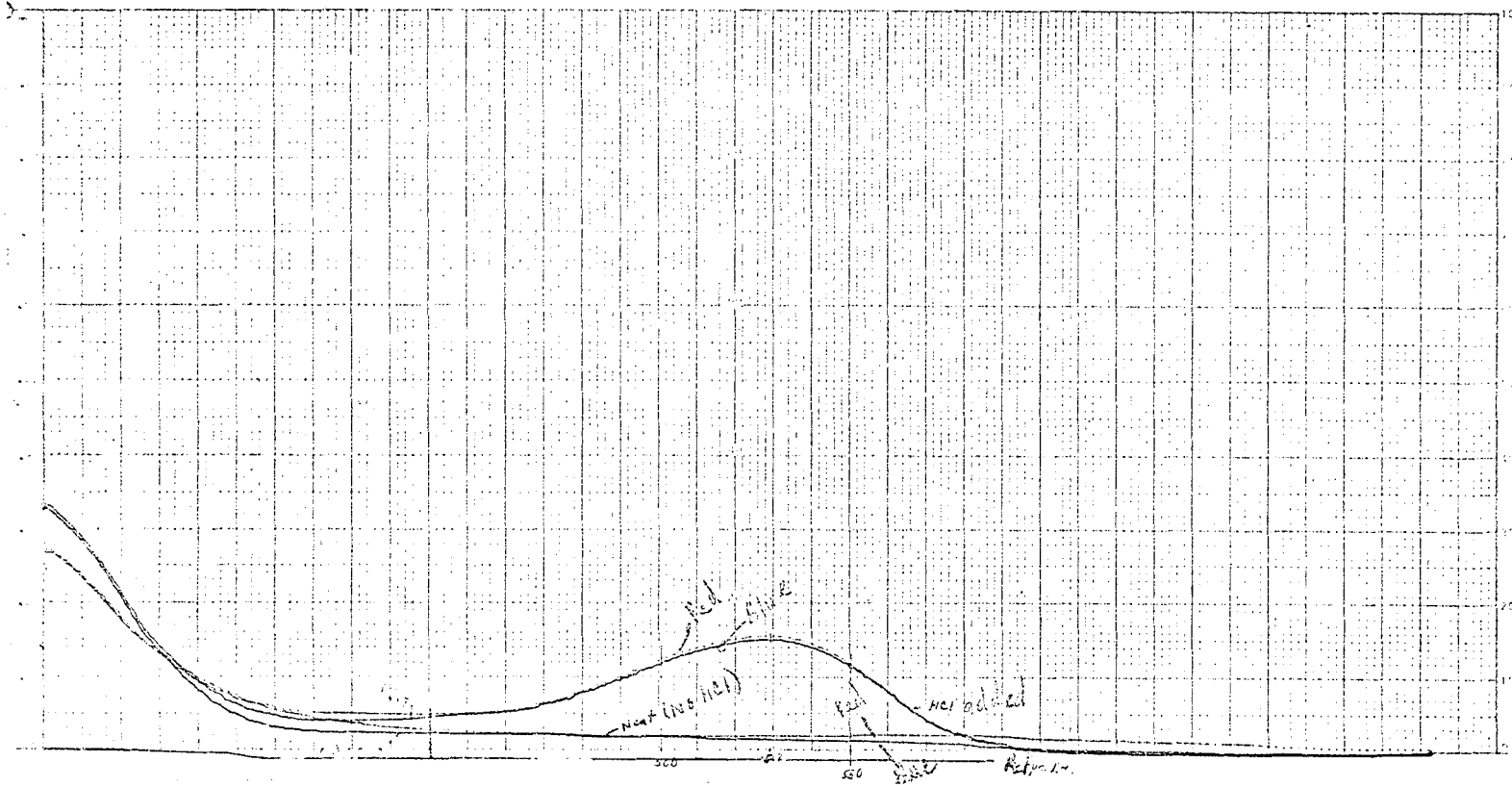
6hc = 20 min extract 3
Rd = 24 hr. extract 3 ac

Neat
Neat & 2 drops .01% [illegible]

4-24-74

SCALE 0-1.0 Abs.
SPEED Medium

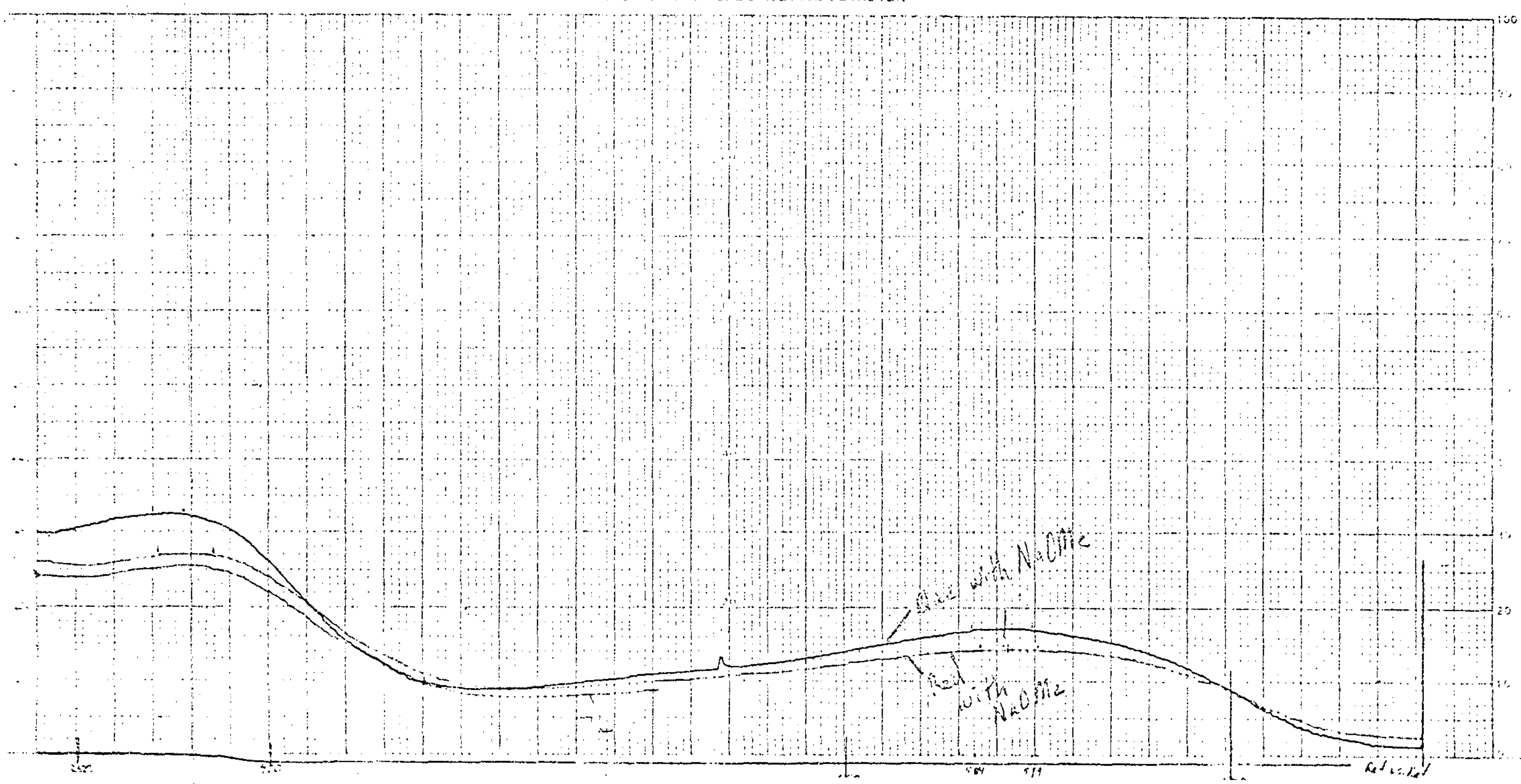
REMARKS
$E_{340}/E_{max} = .325$
max = 529



HITACHI

CHART NO. EPS-035

HITACHI RECORDING SPECTROPHOTOMETER



5

340 RANGE 700 n
VIS

SAMPLE (blue = 20 min extract)
Red = 24 hr. extract

2-1-15 with NaOMe
Immediate

ANALYST

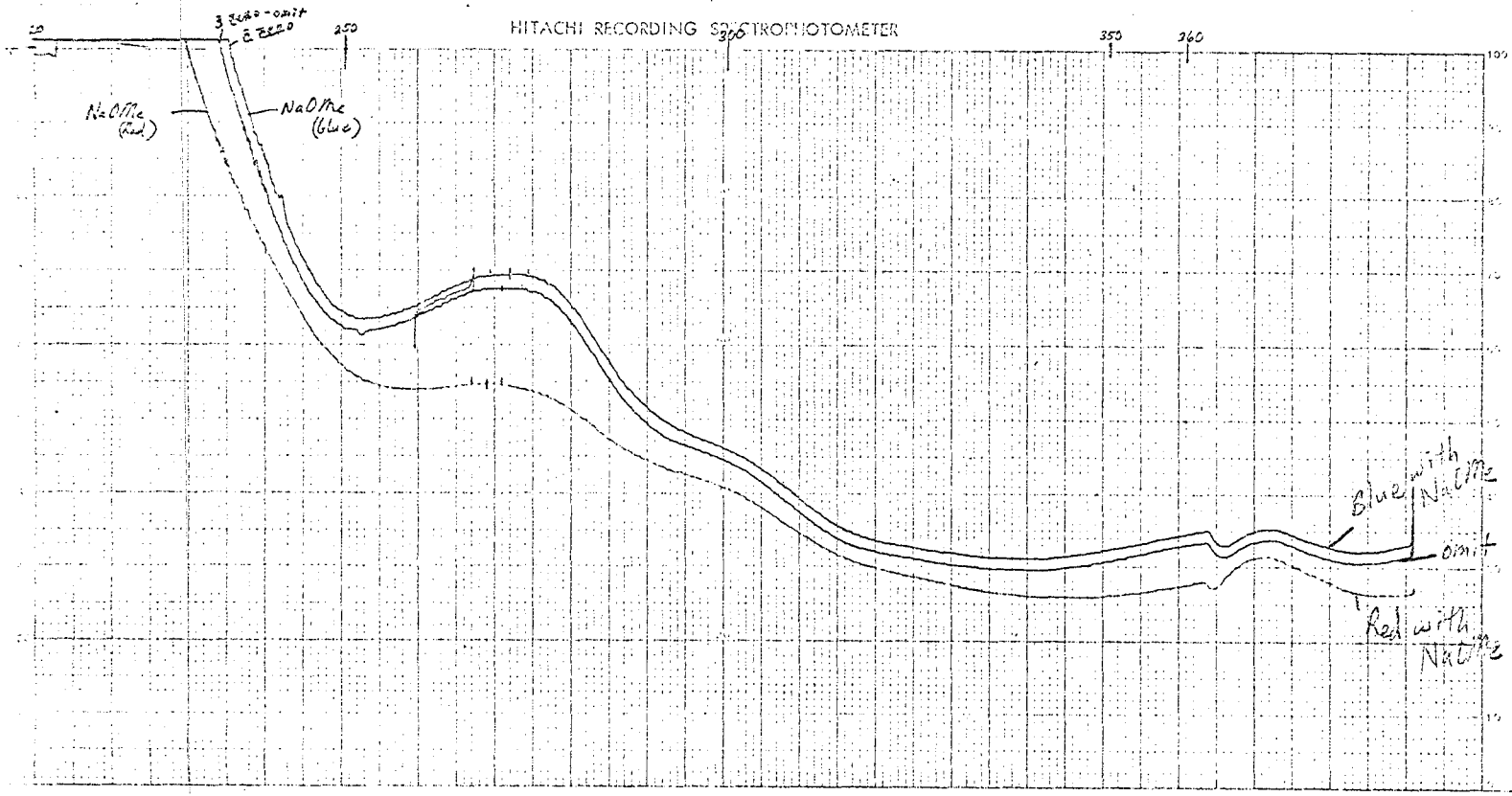
DATE

SCALE 0 - 1.0 Abs.
λ-SPEED medium
SLIT WIDTH
SENS.
SENS.
RESPONSE

REMARKS

HITACHI

CHART NO. EPS-035



6

RANGE 210 — 360 UV

SCALE 2-1-15 (E-C with 3 drops NaOMe immediately

ANALYST CAIN

DATE 4-24-74

SCALE 0-1.0 Abs.
SPEED medium
SLIT WIDTH
SENS. (UV)
SENS. (VISIBLE)
RESPONSE

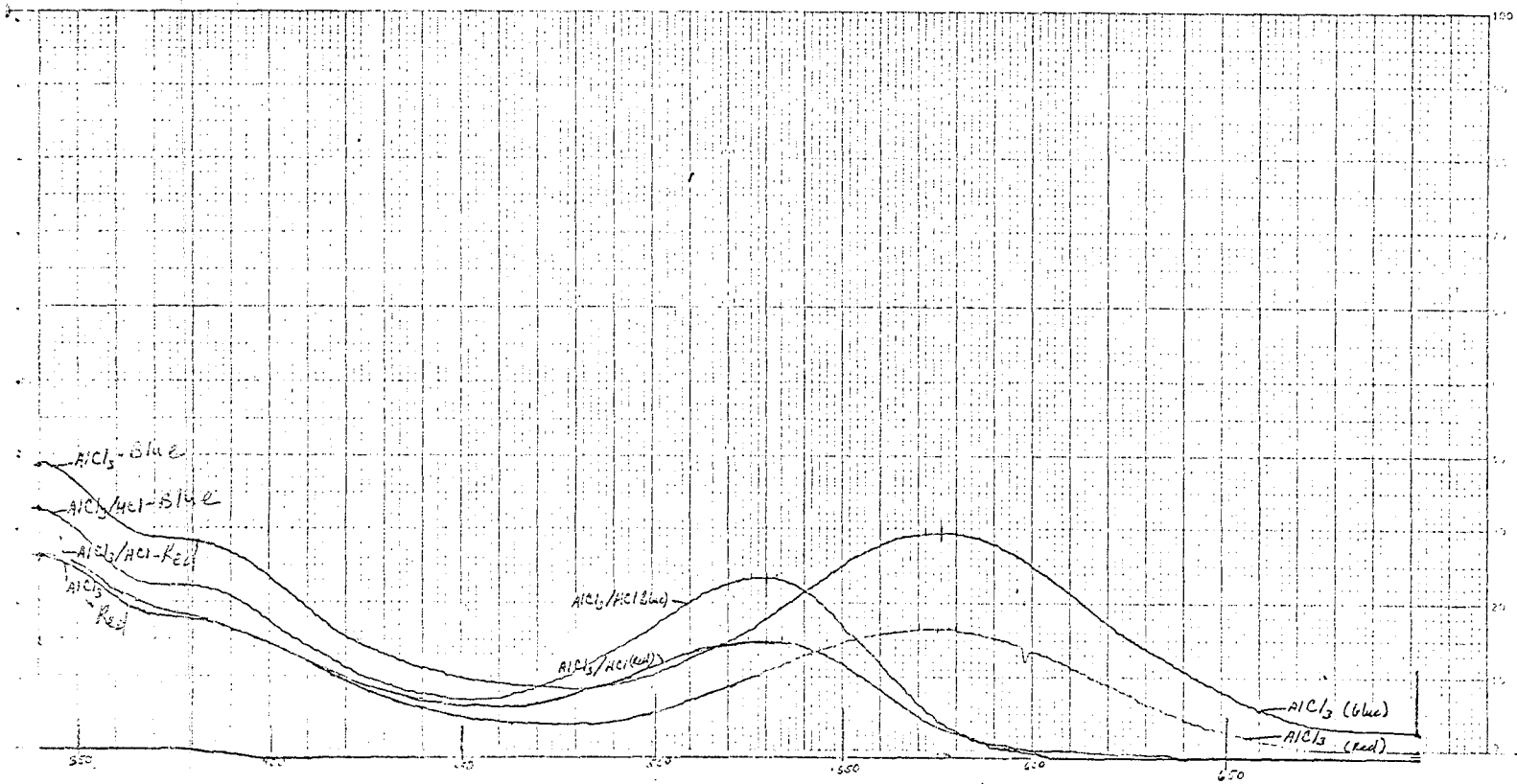
REMARKS
NaOMe max Blue = 271. (269-29
Red = 268. (266.5-29

HITACHI

CHART NO. EPS 665

HITACHI RECORDING SPECTROPHOTOMETER

7



RANGE 340 — 700 nm

VIS

2-1-15

with 3 of 1 cp =

$AlCl_3$

$AlCl_3/HCl$

DATE 4-24

SCALE 0-1.0 Abs

SPEED medium

SLIT WIDTH

SENS.

SENS.

RESPONSE

REMARKS

$AlCl_3$ max blue = 576

red = 575

$AlCl_3/HCl$ max blue = 536

red = 530

E_{max}/E_{max}

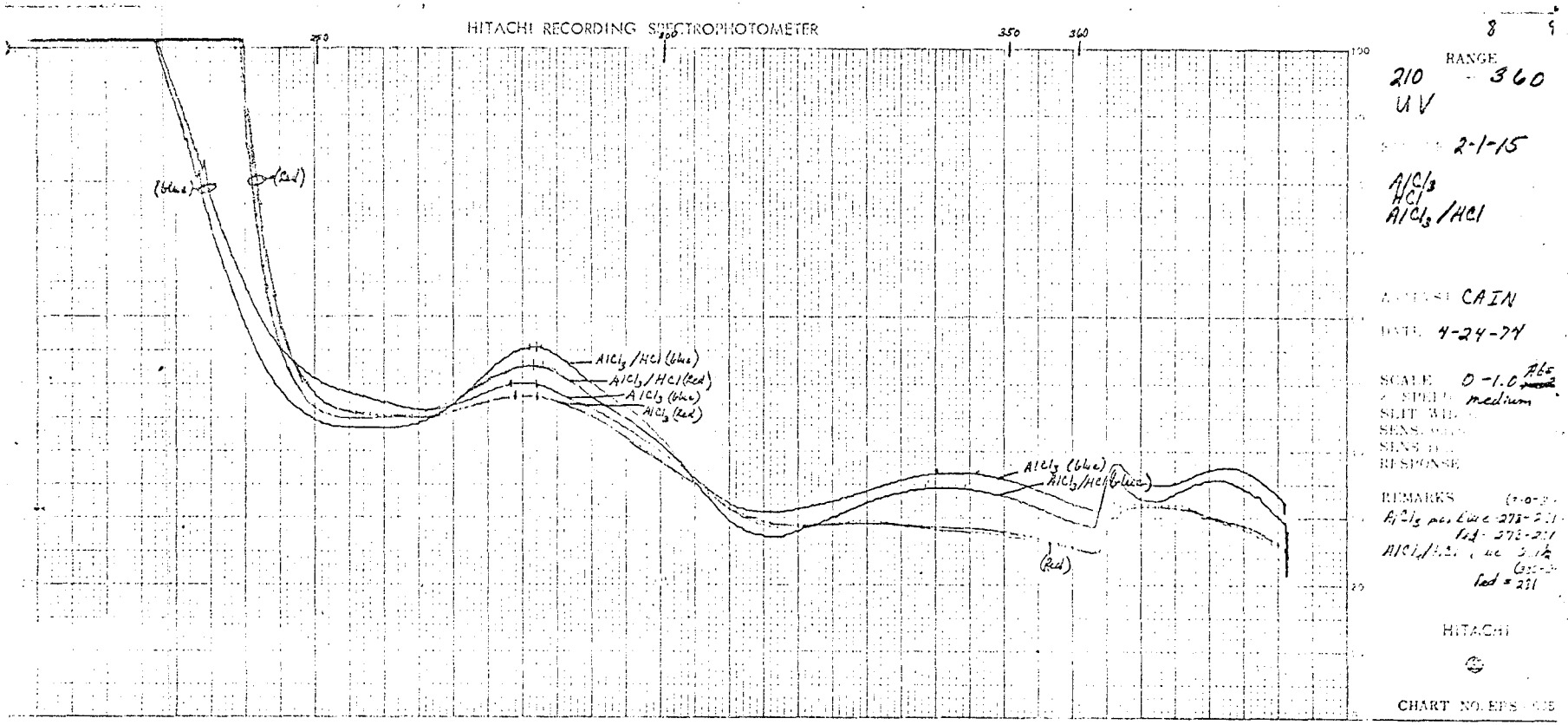
$AlCl_3$ blue = .87

red = .94

$AlCl_3/HCl$ blue = .86

red = .91

CHART NO. EPS-[illegible]


HITACHI RECORDING SPECTROPHOTOMETER
350
360
100
(blue)
(Red)
AlCl3/HCl (blue)
AlCl3/HCl (Red)
AlCl3 (blue)
AlCl3 (Red)
AlCl3 (blue)
AlCl3/HCl (blue)
(Red)
RANGE
210 - 360
UV
2-1-15
AlCl3
HCl
AlCl3/HCl
CAIN
DATE 4-24-74
SCALE 0-1.0 Abs
medium
REMARKS
HITACHI
CHART NO.

HITACHI RECORDING SPECTROPHOTOMETER

9

340 RANGE 700
VIS

2-1-15 Glu
20 min. extract

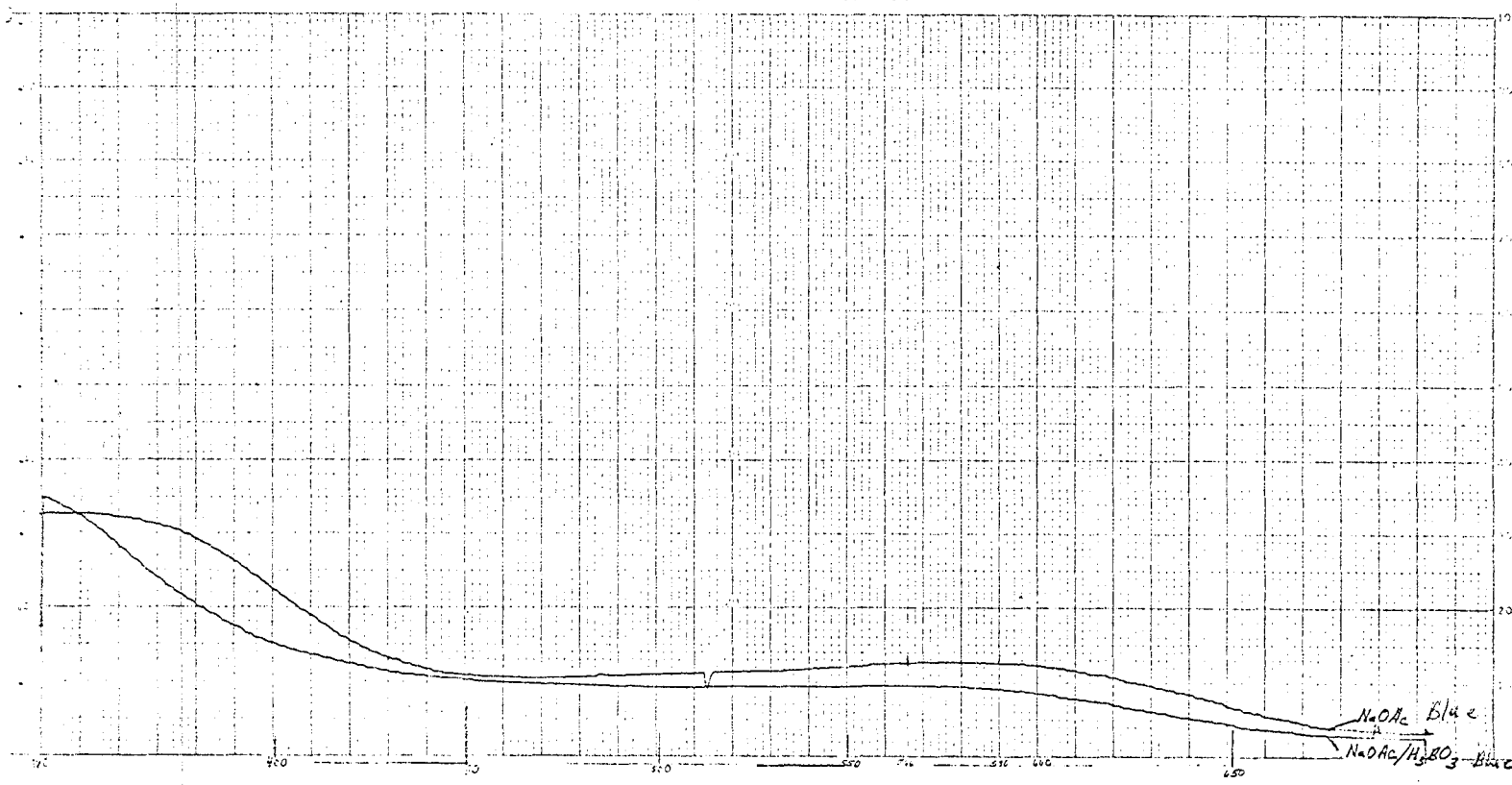
NaOAc
$NaOAc/H_3BO_3$

ANALYST CAIN

DATE 4-24-74

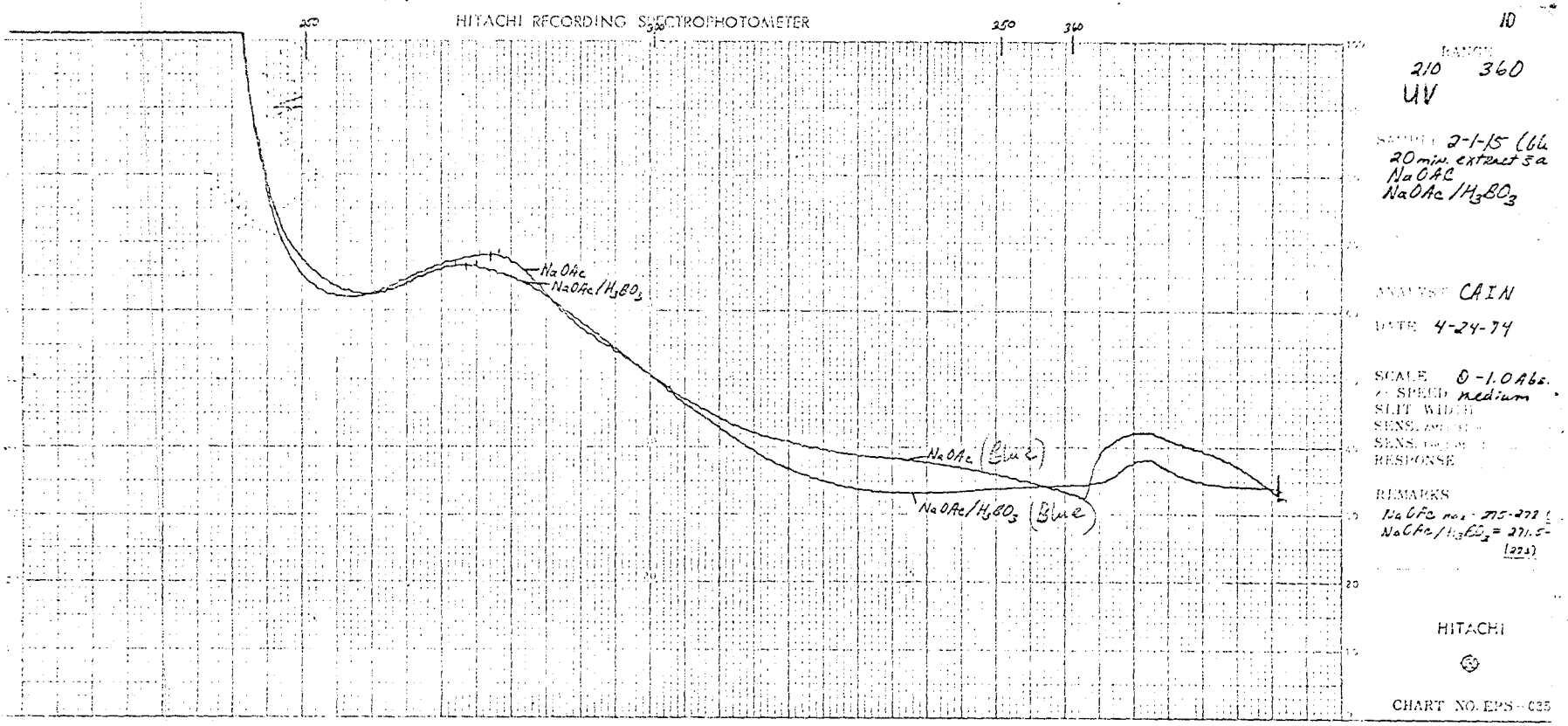
SCALE 0-1.0 Abs.
Y-SPEED medium
SLIT WIDTH
SENS. ADJUST
SENS. RESPONSE
RESPONSE

REMARKS
Helix max = 566-570



HITACHI

CHART NO. EPS-035


HITACHI RECORDING SPECTROPHOTOMETER
250
300
350
360
NaOAc
NaOAc/H3BO3
NaOAc (Blue)
NaOAc/H3BO3 (Blue)
10
RANGE
210 360
UV
SAMPLE 2-1-15 (66
20 min. extract 5a
NaOAc
NaOAc/H3BO3
ANALYST CAIN
DATE 4-24-74
SCALE 0-1.0 Abs.
SPEED medium
SLIT WIDTH
SENS.
SENS.
RESPONSE
REMARKS
NaOAc max: 275-277
NaOAc/H3BO3 = 271.5-
(273)
HITACHI
CHART NO. EPS-035