STUDY OF IDENTIFICATION AND CHANGES IN QUANTITIES OF ANTHOCYANIDIN CONTENT IN APPLE LEAVES AND THE COMPONENT PART OF THE FRUIT

by

RAJESHWAR SINGH

B. Sc. (Agri), Bihar University, India, 1959

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Horticulture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1962

TABLE OF CONTENTS

TABLE OF CONTENTS (concluded)

INTRODUCTION

Most of the red, blue, and purple pigments of plants belong to the group known as anthocyanins. These compounds are glycosides which have been formed by a reaction between a sugar and a glycone (anthocyanidin)(11). This fact has been known for many years. Anthocyanins are water soluble, and are found in the cell sap of the plant tissues (11). Anthocyanidins, however, are insoluble in water, but soluble in other solvents like iso-amyl alcohol, n-butanol, ethanol, etc. (11).

The most recent techniques for isolation and identification of anthocyanidins are the application of paper chromatographic and spectrophotometric methods (7). At the present stage, these methods have been found most scientific and reliable for isolation and identification of anthocyanidins. When separating and identifying anthocyanidins, it is necessary to first remove the glycosides (sugars) from the anthocyanins by acid hydrolysis.

This is true because glycosides (glucose, galactose, antibiose, and rhamanose) not only affect the color, but they also make isolation of the anthocyanidins difficult.

The object of the present investigation was to separate, identify, and calculate the amount of anthocyanidin present in the leaf and component parts of the fruit, at constant intervals in the growth period of the apple. Although some work has been done on the formation of anthocyanin in apple fruit, research to discover the relative amounts of anthocyanidin in the leaf and component parts of the fruit has been neglected. The distinct

radiation-dependent phases are very important for the formation of anthocyanins in the apple. The first phase is an induction period of twenty hours without anthocyanin production. But anthocyanin formation is a linear function of the time of irradiation at constant irradiance in the second phase. It has already been pointed out that the action spectrum for anthocyanin formation in apple skin has a principal maximum near 6500 A° (50).

REVIEW OF LITERATURE

The various shades of blue, violet, purple, and red colors of stems, leaves, flowers, and fruits are due to the presence of the pigment anthocyanin. Anthocyanin is also present in some species of ferns, mosses, etc. Anthocyanins are water soluble, and are usually dissolved in the cell sap. The reds and purplish reds of autumn foliage are also the result of the presence of anthocyanins (11).

The word, anthocyanin, was first postulated by Marquart (38) who used blue, violet, and red pigments. He believed that anthocyanins are formed by the dehydration of chlorophyll of plant organs. Boyle's (1664) (12) publication of results of experiments indicated changes in color, on addition of acids and alkalies to extracts from stems, flowers, autumn foliage, and other plant parts.

Malpighi (37) accounted for histological studies of colored pigments. Grew (19) presented lectures before the Royal Society of London, England, discussing the color of plants. Macaire-Princep (36) pointed out that autumn leaf coloration indicated the presence of colored pigment. Candolle's (14) classification of

pigments indicated two series, Xanthic and Cyanic. Onslow (40) reviewing the work of Marquart, said that the word "anthocyanin" was retained in the same sense as the postulator had used. Other rival terms, now obsolete, such as erythrophyll, cyanophyll, and cyanin, have been used from time to time. Weigert (52) differentiated anthocyanin into two groups by doing qualitative tests. Kruths (32) give a detailed account of the biological significance of anthocyanins in plants. Buscalioni and Pollacci (13) pointed out the morphological and histological distribution of plant pigments.

Willstatter and his collaborators pointed out as reported by (11) that anthocyanins are formed in the plants with glycosides. He further stated that glycosides are hooked with the aglycones which are anthocyanidins. He did pioneer work on the various aspects of anthocyanins.

Karrer (23) and Robinson (45) investigated and revealed that anthocyanins are found in a chloride form called oxonium salt; hence there are eight different types of anthocyanidins known. Willstatter and Mallison reported as pointed out by (11) that by treating anthocyanidins with potassium hydroxide, two products are obtained; viz., phenol and substituted phenolcarbolic acid. They further stated that pelargonidin chloride is decomposed into phloroglucenol and p-hydrobenzoic acid. Willstatter et al. have pointed out as reported by Blank that the various anthocyanidins are derivatives of 2-phenyl benzopyrilium found in the form of chloride called flavilium chloride.

Willstatter further pointed out as reported by (11) that anthocyanin will be hydrolyzed into sugar (glycosides) and anthocyanidins (a glycone) by boiling with 20% hydrochloric acid.

Jonesco (22), Rosenheim (46), and Schriner et al. (47) state that anthocyanidins have been observed in plants only in rare cases. Blank (11) is of the opinion that the anthocyanin present in nature are partly mono- and di-glycosides. These glycosides are rhamnose, glucose, galactose, and gentiobiose. Robinson (44-45) reported anthocyanins present in nature into the following groups: (1) 3-monoglucoside and 3-mongalactoside, (2) 3-rhamnoglucoside, (3) 3-bioside, (4) 3, 5-diglucosides, (5) acylated anthocyanins.

Willstatter found as reported by (11) that 3, 5-diglucosides are very widely distributed in nature.

Blank (11), Robinson and Robinson (42-43), and Scott - Moncrieff (48) reported that the color of anthocyanin is affected by the variation in sugar. Blank (11) further reviewed his work, and stated that anthocyanins are water soluble, which is shown by the fact that they are found in the sap of vacuoles. Anthocyanidins, on the other hand, are insoluble in water. Molisch (39) discovered the most important property of anthocyanin is that it produces crystallizing products in the state of solution, when heated with acids.

Blank (11) states that anthocyanins are usually found in plants in a mixture of forms, and they may be separated either by fractional crystallization of picrates, or by the use of a chromatographic absorption technique. Karrer et al. (24-26) pointed out

the fact that in aqueous and alcoholic solutions, the anthocyanins and anthocyanidins possess neighboring phenolic hydroxyl groups, and show a color change toward violet and blue with ferric chloride. Karstens (28) also reported the same statement.

Blank (11) remarks that anthocyanins show increased blueness with an increase in the hydroxyl groups, and change from 3 to 3-5 sugar types. He further adds that methylation of one or more hydroxyl groups increases the redness of these pigments.

The approach of Robinson and Robinson (42-43) was quite different from Willstatter's, as far as synthesis was concerned. He used condensation of orthohydroxy-benzaldehydes with appropriate ketones followed by ring closure.

Willstatter and Mallison pointed out that the color of the plant origin is not due to anthocyanins only, but also due to many other factors (11).

Blank also believed that color variation may be due to the change in pH of the cell sap, variation in the ash content, copigmentation and mineral accumulations.

Karrer et al. (24-26) discussed the ash content of various red and blue flowers, and concluded that blue flowers contained more ash than red ones. Willstatter and Everest understood the significance of reactions of cell sap for differentiation of color (11). Robinson et al. (42-43) concluded that the blue varieties possess higher pH than red varieties.

Jonesco (22) believed that the changes in anthocyanin are related to the tannins and their resultant additive complex. Currey (16) actually found that the cause of bluing in rose petals is due

to the lack of tannin in the cell sap of the petals. Robinson and Robinson (42-43) did their work on this, and they designated it copigmentation.

Lawrence et al. (34-35) reported that the bluing phenomenon is not due to the salt formation, but is evidently a result of formation of weak additive complexes. Blank (11) reported that deviation in pH is usually insufficient to explain alteration in color. On the basis of chemical behavior of anthocyanins and anthocyanidins, Robinson and Robinson (41-43) postulated a number of quantitative tests.

Herzfelder (21) reported that as far as histological studies of anthocyanins are concerned, in the majority of cases, the anthocyanins are found in the cell sap.

Kosaka (30-31) observed the various factors which are responsible for the formation of anthocyanin in the plant. He stated that light intensity plays a great role in the formation of anthocyanins. Chi-Yen-Chia (15) observed a significant decrease of anthocyanin content in Amaranthus ororatus by decreasing illumination or intensity of light. Continuous illumination, however, caused a discontinuation of pigment formation in his experiments. The same type of results were obtained by Karstens (28) and Kuilman (33). These investigators support the theory that for synthesis of anthocyanin in plants, both the light reaction and the dark reaction are equally important.

Kuilman (33), Kosaka (30-31), and Flint (17) have reported the influence of temperature on the synthesis of anthocyanin. Weisse (53) believed that low temperature had an unfavorable in-

favorable influence on the synthesis of anthocyanins. Other workers, however, including Kuilman et al. (33) concluded that low temperature had a favorable influence on the formation of anthocyanins.

Gassner and Straib (18) pointed out that synthesis of anthocyanin in plants is related to the deficiency of phosphorus, potassium, and nitrogen. They concluded that red coloration due to increased production of anthocyanin is increased by addition of potassium in the nutrient solution, but that addition of nitrogen phosphorus decrease the anthocyanins.

A series of papers have been reviewed by Bate-Smith (5-8). First, he pointed out that the anthocyanins and their mono and diglucosides from their spots can be well differentiated by their RF values, and give a characteristic color reaction with ammonia vapor. He further pointed out, in great detail, the method for the detection of leuco-anthocyanins by chromatographic method. His papers revealed the factors which might affect the RF values, and listed the precautions to take in getting correct RF values. A series of workers, viz, Blank (11) and Karrer (27) have also separated and identified the anthocyanidins by this paper chromatographic absorption technique.

Bate-Smith and Lerner (9) applied a more scientific method in his technique to extract anthocyanidins from leaves and other organs of the plants. He worked on many species of plants and reported his results. He pointed out that except in Rosaceae and a few Leguminoseae (which appear to contain leuco-anthocyanidin) the leuco-anthocyanins appear to be restricted to leuco-cyanidins,

and leuco-delphinidin. He further published a detailed list of many families of Gymnosperms and Pteridophyta, indicating the plant and the anthocyanin content.

Many solvents have been used by the various workers for chromatographic separation and identification of anthocyanins. Bate-Smith (7) investigated a good solvent, known as Forestal solvent. He got this solvent from Dr. White of the Forestal Land, Timber, and Railway Co. Harpenden. Bate-Smith (7) reported on the RF values of various anthocyanidins extended by three different solvents, including the Forestal solvent. Bate-Smith and Westall (10) pointed out the use of a mixture of N-butanol and 2N H Cl at the ratio of 1:1, to maintain low pH of the solvent during chromatography, to prevent the anthocyanidins from fading out.

Halevy and Asen (20) successfully used the chromatographic method for identification of anthocyanidin in tulips, and results were consistent. They also reported their results of the identification of anthocyanidins in Euphorbia pulcherrima plants by paper chromatographic and spectrometric methods.

The absorption sepectrum has been used as a more reliable guide for the identification not only of anthocyanidin, but also as the basis for the calculation of amount of anthocyanidin in the plant organ. A series of workers, viz. Bate-Smith (7), Asen (3), Asen et al. (4), Halevy and Asen (20), Klein and Hagen (29) have used the absorption spectrum as a reliable guide for identification, and to secure other information about anthocyanidins. The absorption maximum does not differ very much for a given anthocyanin,

and its anthocyanidin.

The absorption maximum reported by the above workers is as follows: Cyanidin and Peonidin 545 millimicrons; Pelargonidin 530 millimicrons; Delphinidin and Malvidin 555 millimicrons.

Siegelman and Hendricks (49) used a direct method for the study of the amount of anthocyanin in turnip and red cabbage seedlings.

Asen (3) isolated and identified the anthocyanins of poinsettia plants by paper chromatographic and spectrophotometric methods. He used three different solvents, and got the different RF values as follows:

	Max. in Ethanol Containing .01 N Hydrochloric acid millimicrons	Acetic acid: Hydrochloric acid:Water (30:3:10 V/V)	M-Cresol:5N Hydrochloric acid:Acetic acid (1:1:1 V/V)	1-Butanol: 2N Hydrochloric acid (1:1 V/V)
Delphinidin	555	.38	.57	.35
Cyanidin	545	.60	.74	.69
Pelargonidin	530	.74	.82	.80

Siegelman and Hendricks (50) used the same direct method for examining the photocontrol of anthocyanin formation in apple skin. They found two distinct radiation-dependent phases. The first phase is an induction period of about 20 hours without anthocyanin production. In the second phase, anthocyanin formation is a linear function of the time of irradiation at constant irradiance. It was found that the action spectrum for anthocyanin formation in apple skin has a principal maximum near 6500A°; a subsidiary maximum near 600A°; and a weak action throughout the visible region.

Siegelman and Hendricks (51) further reported the time courses of aldehyde, alcohol, and anthocyanin synthesis in light and darkness, in apple skins of Jonathan and Arkansas varieties. They found that radiation suppresses aldehyde and alcohol synthesis. The time courses for anthocyanin synthesis indicates that two radiation-limited steps are involved in anthocyanin synthesis in apple tissue.

Ahmedullah (1) identified anthocyanidin in three varieties of Pelargonium hortorum by chromatographic and spectrophotometric methods. He calculated the RF values in Forestal solvent and identified the anthocyanidin.

Ahuja (2) identified anthocyanidin in two varieties of rose by chromatographic and spectrophotometric methods. He calculated the amount of cyanidin present in the above cultivars by direct method.

MATERIALS AND METHODS

The leaves and the fruit of the Red Delicious and Golden Delicious apple were used for this study because of their marked difference in skin and leaf color. Leaf and fruit samples selected for this study were free from diseases and from insect damage. Extreme caution was taken to minimize error, by testing these materials while they were fresh from the orchard. This work was conducted during the summer of 1961, and was completed within a three month period.

Extraction and Isolation of the Pigments

Hydrolysis. One gram each of leaf, one-half gram of skin and seed, and five grams of flesh samples of the two apple varieties were weighed on a chainomatic balance, and placed separately in 75 milliliter test tubes. Ten milliliters of 2N Hydrochloric acid were added to each test tube. These test tubes were placed in the steam bath for fifteen minutes. The contents of the test tubes were crushed with the electric homogenizer rod for two to five minutes, depending upon the type of sample. Following homgenization the rod and test tube was washed in a solution of 10 milliliters of 2N hydrochloric acid to avoid any loss of the sample or pigment. All samples were prepared in this manner.

The test tube containing homogenized leaf, skin or seed samples was placed in the steam bath for two hours for complete hydrolysis of the pigment. The test tubes with the flesh samples were processed one additional hour. The temperature at the time of hydrolysis of the leaf and component parts of both varieties of apple was about 90 degrees C. Exacting care and supervision was essential at this time, to prevent accidental damage to the specimen. In case damage was noted the specimen was rejected, and a fresh sample taken. After these above mentioned specific time intervals the test tubes were taken out of the steam bath, and cooled in 600 milliliter beakers, under running water.

Separation

Each cooled sample was transferred into a 125 milliliter separatory funnel. Each tube was washed twice with about 8 milliliters of distilled water, which was added to the funnel

contents, thus preventing any loss of pigment. Four milliliters of N-butyl alcohol was added to the funnel which was stoppered, then shaken carefully. The funnel was allowed to stand for five minutes until the two phases had formed. The hypo phase was drained into another separating funnel, and four milliliters of N-butyl alcohol added to the hypo liquid. This process was repeated several times until the hypolayer was perfectly clear.

The separated anthocyanidin was collected in the 25 milliliter measuring cylinder. The volume was 15 to 25 milliliters, depending upon the concentration of the extracted pigment. The extracted pigment was put into 15-milliliter centrifugal tubes, and centrifuged for 20 minutes under two different speeds: first, ten minutes at the speed of 1500 R.P.M.; the second, ten minutes at the speed of 3000 R.P.M. Each funnel and measuring cylinder used was washed with N-butyl alcohol, to insure that no anthocyanidin was left. All samples of each plant part of both varieties were extracted in this manner.

Identification

Chromatographic Method. The above extracted pigment from the samples of Red Delicious and Golden Delicious apples was spotted on Whatman No. 1 filter paper, with markings made about one centimeter in diameter. Using an insulin syringe, spots were made 4 centimeters apart on the starting line. This starting line, marked by black lead pencil, was drawn ten centimeters from the base of the paper.

The chromatographic sheets were prepared by streaking the

anthocyanidin across the width of the paper, in bands 1½ centimeters wide, extending from the starting line.

The spotted and streaked chromatographic sheets were rolled, clipped, and placed in the presaturated chromatographic glass chamber, 24 inches high and 12 inches in diameter. The chamber was saturated by fumes of a solvent, which was placed in the container at the bottom of the chambers eight hours prior to chromatographing. To insure an airtight condition, the lid of the chamber was sealed with modeling clay.

The chamber was kept in a cardboard box, to reduce photochemical action in the chromatographed anthocyanidin. About 175 milliliters of Forestal solvent (acetic acid: conc. hydrochloric acid: water: 30:3:10 V/V) was poured in the pan at the bottom of the chamber.

Ascending chromatography was carried on with this solvent at room temperature. The chromatogram was taken out of the chromatographic glass chamber after twenty hours and dried at room temperature. The RF values were calculated from the spotted chromatograms by dividing the distance traveled by the anthocyanidin from the starting line by the distance traveled by the solvent front. In addition to the chromatographic values spectrophotometric examination of the pigment was also made.

Spectrophotometric Method. Moist streaked chromatograms were used for this identification. The anthocyanidin bands were cut from the chromatograms into small pieces (one to two centimeter square area).

These antocyanidin-soaked chromatographic pieces were placed

in the test tubes, and six to eight milliliters of acidic methyl alcohol were added to each. Each test tube was corked tightly to insure an air-tight condition, and kept in a dark cabinet for about one and one-half hours. During this period, the tubes were shaken once or twice, to facilitate the elution of the pigment.

After the anthocyanidin was eluted from the chromatogram, the eluent was placed in the cells of the Beckman DU Spectrophotometer. The reading was taken against acidic methyl alcohol as a blank in the visible range of light, under different wave lengths of 510 to 580 millimicrons. The readings were plotted on graph paper, and maximum absorption spectra were obtained for both the Red Delicious and Golden Delicious varieties.

The extracted anthocyanidin was diluted with a n-butyl alcohol, and the readings were taken in the same manner with the Beckman DU Spectrophotometer, under wave lengths of 510 to 580 millimicrons. The same peak point was obtained in all samples of both varieties of apples for both methods. The anthocyanidin of the leaf and fruit of the apple in both varieties was identified by these methods.

Comparison with the Authentic Compound. Cyanidin chloride, which was obtained from the Mann Research Laboratory, New York, N. Y., was used as an authentic compound for comparison with the extracted cyanidin of the leaf and the fruit of the apples. A sample of this authentic compound was dissolved in acidic N-butyl alcohol and chromatographed in the same manner as above. The RF values and the maximum absorption obtained from this compound were compared with the extracted pigment from the leaves and fruit.

Estimation of Plant Pigment (Cyanidin)

Two methods, direct and chromatographic, were employed to estimate the quantity of cyanidin present in the leaf and component parts of the fruit. In the direct method, a measured amount of plant extracted cyanidin was diluted to a 50 milliliter concentration, and read at 545 millimicrons with a Beckman DU Spectrophotometer. When the chromatographic method was used, the chromatogram eluent was read at 545 millimicrons in a Beckman DU Spectrophotometer.

Extraction and Isolation. The plant samples were collected from trees at the Kansas State University horticultural farm at weekly intervals beginning for extraction and isolation of anthocyanidin. Complete hydrolysis for the maximum extraction of cyanidin was essential for the quantitative work. The extraction and isolation of the pigment was done as described for all samples of the two varieties.

Direct Spectrophotometric Measurement. The measured amount of extracted cyanidin from the leaf and component parts of the apple fruit (skin, flesh, and seeds) were put into test tubes. Each of the samples was diluted with N-butyl alcohol up to the concentration of 50 milliliters. The test tubes were shaken vigorously to insure homogeneous dilution.

The reading of the diluted homogeneous samples of extracted cyanidin was taken at 545 millimicrons with a Beckman DU Spectrophotometer. It was necessary to take the reading within the range of 0.1 to 0.8, to insure a consistant and reliable computation.

When some samples of the extracted cyanidin did not require further diluting, they were read in the spectrophotometer at the same concentration, and the spectrophotometric readings were adjusted to 50 milliliters concentration. In case of highly concentrated samples, they were diluted to 100 milliliters, and the spectrophotometric readings were transformed into 50 milliliter concentrations. This dilution was often necessary for samples of the skin of Red Delicious apples.

Comparison with the Standard Curve Made by Direct Spectrophotometric Method. The authentic cyanidin chloride purchased from Mann Research Laboratories was used to make the standard curve for comparison and calculations of the amount of cyanidin present. 9.5 milligrams of cyanidin chloride were weighed with a chainomatic balance, and dissolved in acidic n-butyl alcohol. The volume was increased to the mark in the 25-milliliter volumatic flask. Five samples of 1, 2, 3, 4, and 5 milliliters were drawn from the aliquot by pipettes, and each sample was transferred to the 50-milliliter volumetric flask. The content of each flask was diluted up to the mark, by adding acidic n-butyl alcohol. The diluted sample was insured to be homogeneous by vigorous shaking.

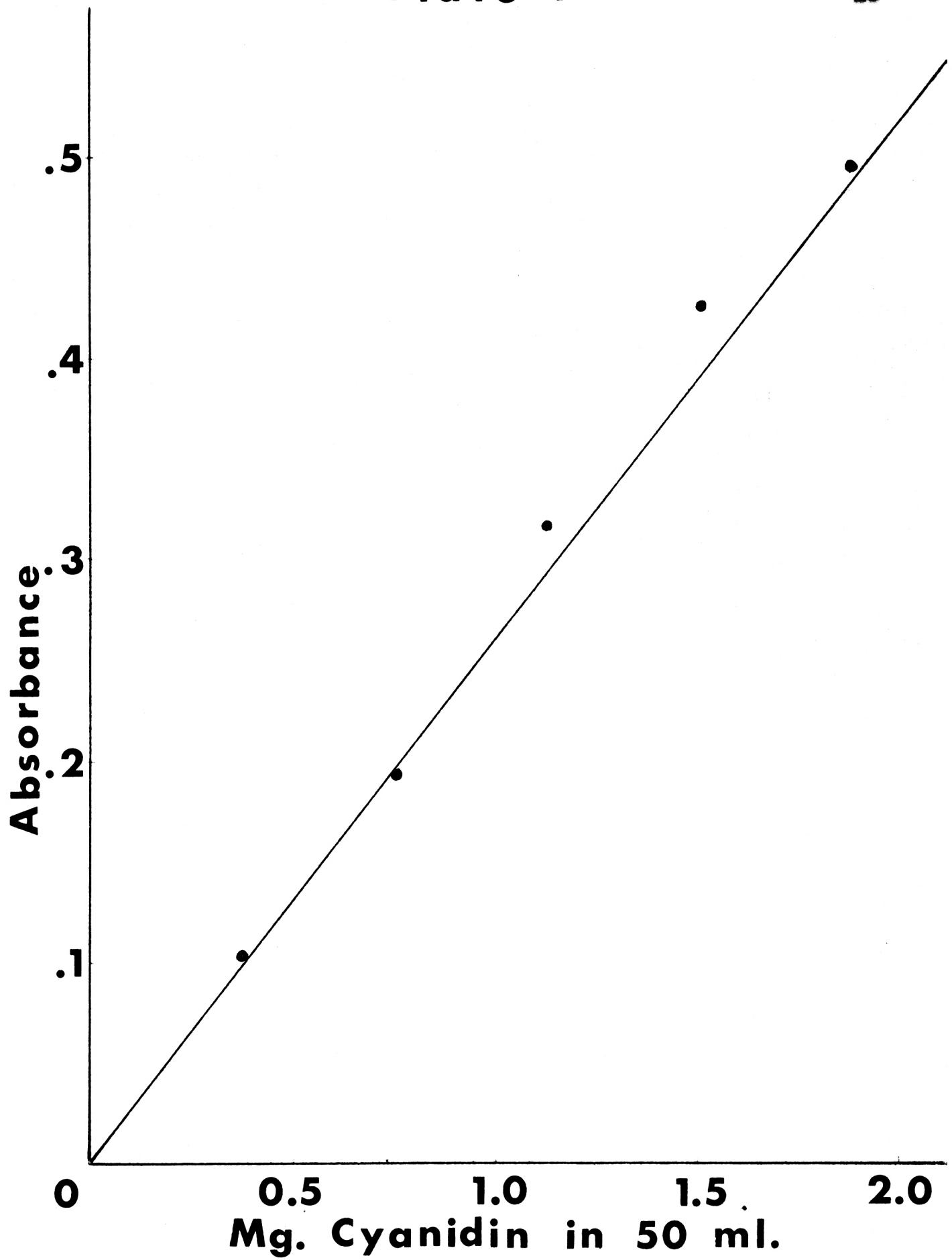
Each of the flask samples was read at 545 millimicrons in the Beckman DU Spectrophotometer against acidic n-butyl as a blank.

The spectrophotometric readings of the extracted pigment (cyanidin) of the leaves, skins, flesh and seed of the Red Delicious and Golden Delicious apple varieties were compared with this standard curve (Plate I). Using this curve, the quantity of cyanidin at different stages in the different plant organs was

EXPLANATION OF PLATE I

A standard curve of solutions of authentic cyanidin chloride established by the direct spectrophotometric method as determined with a Beckman DU Spectrophotometer.

Plate 1



calculated. The calculations for the amount of cyanidin present in the leaves, skins, flesh and seed were based on per gram fresh weight basis of the above samples.

Chromatographic Spectrophotometric Measurement. A measured aount of extracted cyanidin was streaked on Whatman No. 1 filter paper with an insulin syringe. The chromatograph was made in the same manner as described above. The concentration of cyanidin which was chromatographed on the filter paper, was equivalent to the above sample concentration used in the direct method. The equivalent amount of extracted cyanidin was chromatographed on the paper to facilitate the comparison between the direct and chromatographic methods. This comparison was expected to give some idea of the relative superiority and reliability of these two techniques.

The chromatograph was rolled, clipped, and placed in the presaturated chromatographic glass chamber containing Forestal solvent (acidic acid; conc. hydrochloric acid: water: 30:3:10 V/V) for 20 hours. After 20 hours, the chromatogram was taken out of the chamber, and moist colored bands were cut by scissors, into small pieces and placed in test tubes.

Into each test tube, four to ten milliliters of acidic methyl alcohol was added to elute the cyanidin. Each test tube was corked tightly and kept for two hours in the dark cabinet. During this period, each tube was agitated once or twice to facilitate the elution of the cyanidin.

An amount of acidic methyl alcohol was added for elution of cyanidin in each sample to make the spectrophotometric readings equivalent to the corresponding readings of the sample extracted

by the direct method.

The readings, of the eluent, were taken at 545 millimicrons with the Beckman DU Spectrophotometer compared to the blank cell containing acidic methyl alcohol. Each sample of the leaves, skins, flesh, and seed of Red Delicious and Golden Delicious varieties of apple was chromatographed and readings were taken. These spectrophotometric readings were transformed into 50 milliliter concentration. The derived readings were compared with the standard curve established by chromatographic spectrophotometric method of the authentic compound. The amount of cyanidin was calculated in each sample of the leaves, skins, flesh, and seed of the two apple varieties.

Comparison with the Standard Curve Made by the Chromatographic Spectrophotometric Method. The authentic compound (cyanidin chloride) solution referred to above was also utilized for making the standard curve. Five samples, of five different streaks were applied with an insulin syringe on the chromatographic paper. Each streak was chromatographed with one milliliter of the cyanidin chloride solution.

The chromatographic paper was rolled, clipped, and placed in the Forestal solvent of chromatographic glass chamber for 20 hours; then taken out, and dried. The moist color band of different samples was cut into small pieces, and kept in the five test tubes. In each tube, five different concentrations of acidic methyl alcohol were added. Tubes were corked, and kept in a dark cabinet for two hours. During this period, the tubes were shaken once or twice to facilitate the elution of the cyanidin.

Each of the tube contents were read in the Beckman DU Spectrophotometer at a wave length of 545 millimicrons. These solutions were transformed into 50 milliliters of five different concentrations, as shown in Plate II. These five readings were plotted on the graph paper. This plotted straight line showed a linear relationship with concentration.

The spectrophotometric readings of the extracted cyanidin of the leaves, skins, flesh, and seed of Red Delicious and Golden Delicious apple varieties were compared with this standard curve. Using this curve, the quantity of cyanidin was calculated, in the different stages of the various plant organs used in this research. The amount of cyanidin was calculated by this method on a per gram fresh weight basis for all samples.

RESULTS

Identification of Pigments

Chromatographic Identification. The extracted anthocyanidin of the leaves, skins, flesh, and seed of the two apple varieties, Red Delicious and Golden Delicious, were chromatographed in Forestal solvent, and RF values were calculated. These RF values are listed in Table 1.

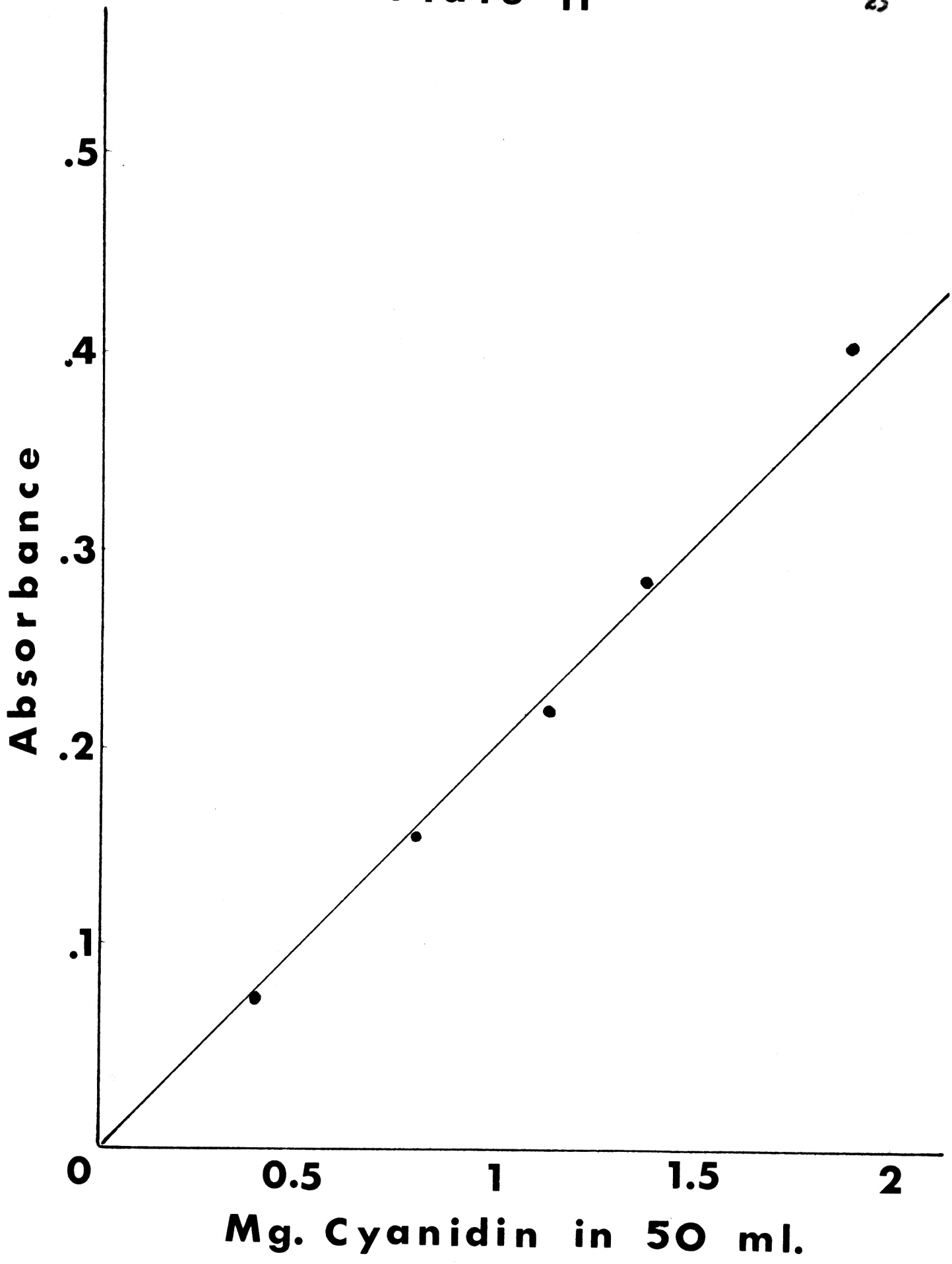
In a like manner, the RF value of authentic cyanidin chloride was calculated. The results are reported in Table 2, along with the authentic values of the same compound, as ascertained from the literature.

The comparison of RF values, of extracted anthocyanidin, and

EXPLANATION OF PLATE II

A standard curve of solutions of authentic cyanidin chloride established by the chromatographic-spectrophotometric method as determined with a Beckman DU Spectrophotometer.

Plate II



RF values of the authentic compound strongly suggested that the extracted pigment of the leaves, skins, flesh, and seed of the two varieties of apple, was cyanidin.

Spectrophotometric Identification. The color bands of the paper chromatograms of extracted anthocyanidin of the leaves, skins, flesh, and seed of the two apple varieties, was eluted with acidic methyl alcohol and readings were obtained with a Beckman DU Spectrophotometer at different wave lengths.

Similarly, the extracted anthocyanidins were diluted, and the readings taken with the Beckman DU Spectrophotometer at different wave lengths. These readings from 510 to 580 millimicrons were plotted, and curves obtained giving the maximum absorption or peak values at 545 millimicrons, are shown in Plates III and IV.

A curve of the wave length of a solution of the authentic compound, cyanidin chloride, was plotted, and the value recorded at 545 millimicrons, as shown in Plate V. Similar absorption values for this authentic compound were obtained by the various workers, Bate-Smith (7), Halevy (20), and Asen (3). The maximum absorption readings of the extracted anthocyanidin were comparable with the values obtained with the authentic compound and that reported in the literature. This would suggest that the anthocyanidin extracted from all the samples was cyanidin.

Table 1. Average RF values of paper chromatograms developed in Forestal solvent of extracted anthocyanidins of the leaves, skins, flesh, and seed of Red Delicious and Golden Delicious apple

Plant Organs	Red Delicious RF values	Golden Delicious RF values
Leaf	.57	.57
Skin	.56	.56
Flesh	.55	.56
Seed	.56	.57

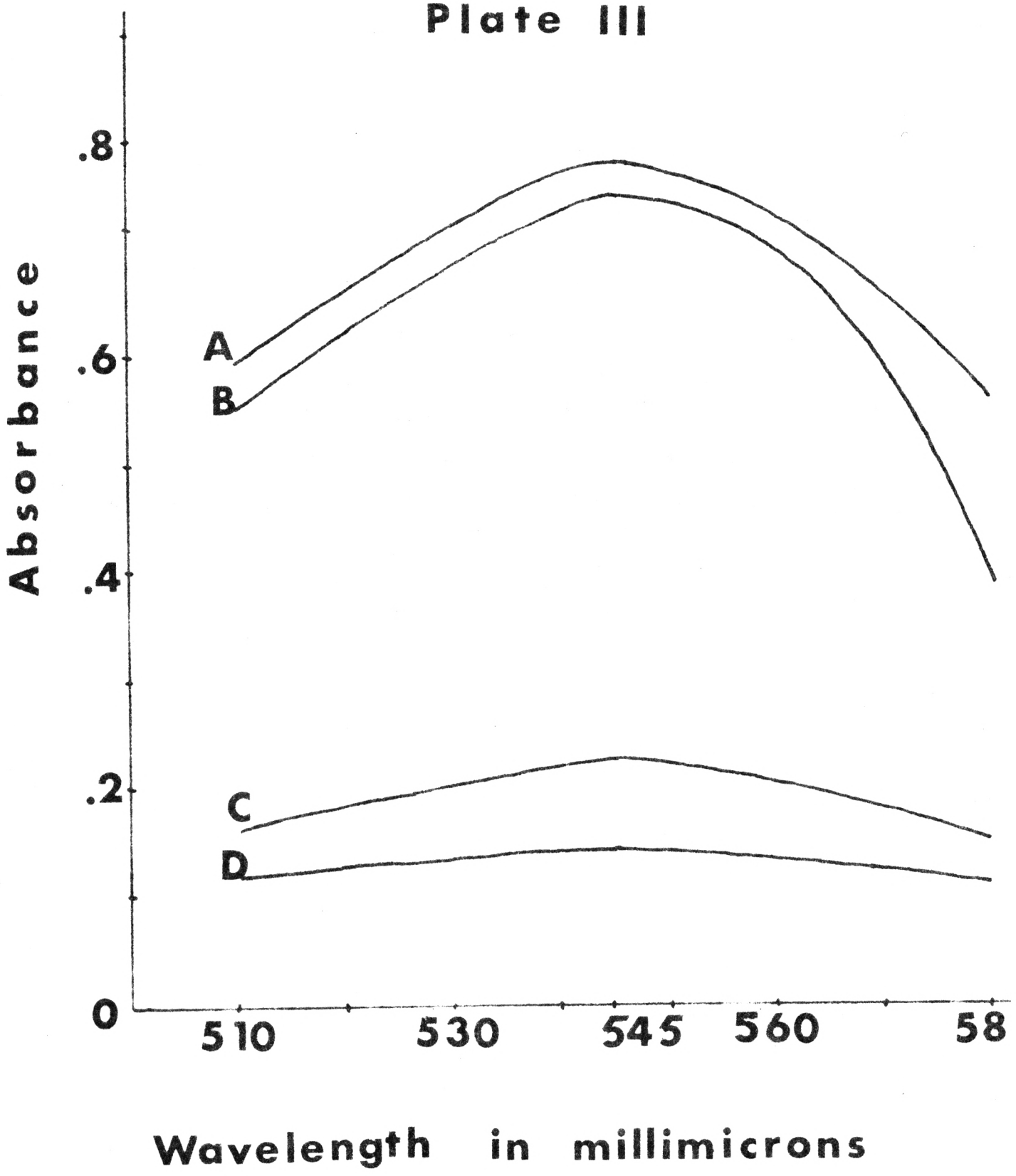
Table 2. Average RF values of chromatograms of the authentic compound cyanidin chloride in Forestal solvent as reported from the literature and determined in this study.

Material	RF values	
Authentic cyanidin	.56	Author
Chloride	.50	Bate-Smith
	.56	Asen
	.60	Halevy et al.
	.56	Ahuja

EXPLANATION OF PLATE III

A, B, C, D are maximum absorption curves of cyanidin from skins, seed, leaves, and flesh, respectively of Red Delicious apple. In each case the peak reading is at 545 millimicrons.

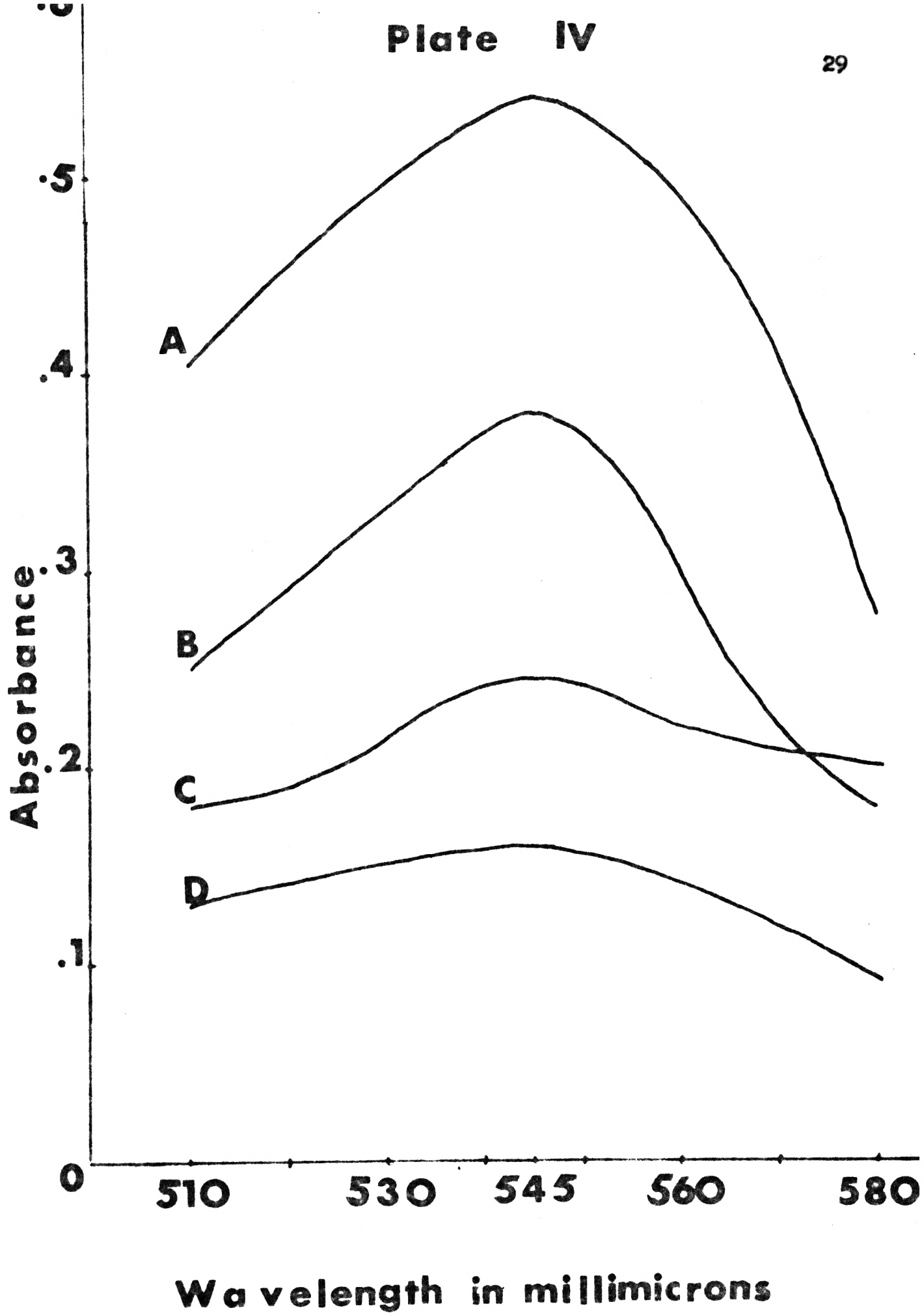
Plate III


Absorbance
.8
.6
.4
.2
0
A
B
C
D
510
530
545
560
58
Wavelength in millimicrons

EXPLANATION OF PLATE IV

A, B, C, D are maximum absorption curves of cyanidin from skins, seed, leaves, and flesh of Golden Delicious apple, respectively. In each case the peak reading is at 545 millimicrons.

Plate IV


.6
.5
.4
.3
.2
.1
0
Absorbance
A
B
C
D
510
530
545
560
580
Wavelength in millimicrons

EXPLANATION OF PLATE V

Maximum absorption curve of solutions of authentic cyanidin chloride in acidic n-butyl alcohol.

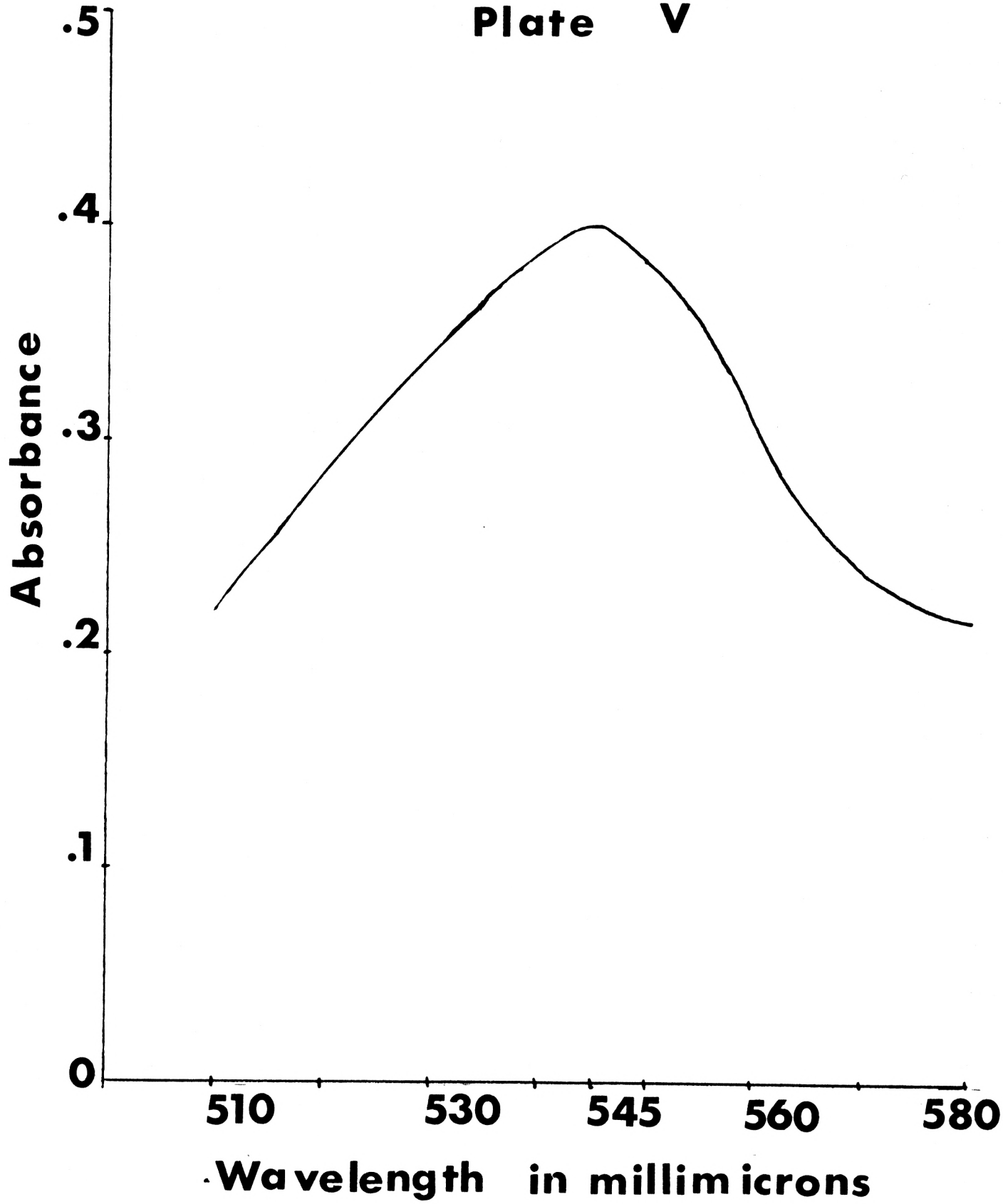


Plate V
.5
.4
.3
.2
.1
0
Absorbance
510
530
545
560
580
Wavelength in millimicrons

Table 3. Milligrams of cyanidin in one gram samples each of fresh leaves and skins of the Red Delicious and Golden Delicious apple.

Calendar dates	Week interval	Red Delicious				Golden Delicious			
		Leaf		Skin		Leaf		Skin	
		Chromato-graphic	Direct	Chromato-graphic	Direct	Chromato-graphic	Direct	Chromato-graphic	Direct
July 1	1	.761	1.770	4.020	4.676	.376	1.177	1.174	2.440
July 8	2	.996	1.946	4.770	5.292	.438	1.260	1.615	3.004
July 15	3	1.070	1.967	5.413	6.475	.527	1.569	3.675	4.725
July 22	4	1.100	2.062	6.843	7.836	.557	1.625	4.688	5.813
July 29	5	1.247	2.165	6.821	7.895	.687	1.715	4.972	6.016
Aug. 5	6	1.436	2.293	7.572	8.596	.856	1.882	5.203	6.122
Aug. 12	7	1.777	2.612	7.841	8.813	1.187	2.120	5.300	6.230
Aug. 19	8	1.892	2.850	8.281	9.270	1.462	2.420	5.375	6.341
Aug. 26	9	2.182	2.995	8.575	9.606	1.500	2.500	5.712	6.562
Sept. 2	10	2.432	3.105	9.429	10.020	1.947	2.956	5.944	6.925
Sept. 9	11	2.455	3.180	9.899	10.974	1.472	2.540	6.349	7.280
Sept. 16	12	1.367	2.222	11.275	13.400	.955	1.852	6.538	7.457
Sept. 23	13	1.312	2.003	15.912	16.840	.826	1.812	6.713	7.692
Sept. 30	14	1.282	2.063	15.937	17.050	.785	1.767	6.820	7.940

Table 4. Milligrams of cyanidin in one gram samples each of fresh seed and flesh of the Red Delicious and Golden Delicious apple.

Calendar dates	Week interval	Red Delicious				Golden Delicious			
		Flesh		Seed		Flesh		Seed	
		Chromato-graphic	Direct	Chromato-graphic	Direct	Chromato-graphic	Direct	Chromato-graphic	Direct
July 1	1	.010	.086	7.666	8.733	.009	.087	4.214	5.108
July 8	2	.012	.153	7.419	8.384	.011	.146	4.116	5.025
July 15	3	.012	.150	7.333	8.234	.011	.143	3.696	4.716
July 22	4	.012	.170	5.187	6.265	.011	.152	2.212	3.210
July 29	5	.011	.168	4.762	5.820	.011	.158	1.910	2.525
Aug. 5	6	.012	.177	4.246	5.281	.011	.168	1.317	2.178
Aug. 12	7	.011	.180	3.245	4.176	.012	.178	.940	1.875
Aug. 19	8	.011	.160	3.146	4.072	.006	.175	.743	1.658
Aug. 26	9	.007	.168	2.450	3.360	.010	.141	.687	1.20
Sept. 2	10	.011	.162	1.997	2.913	.010	.165	.466	.896
Sept. 9	11	.011	.175	1.570	2.675	.012	.178	.422	.770
Sept. 16	12	.005	.110	1.213	2.138	.005	.110	.325	.727
Sept. 23	13	.007	.111	.890	1.827	.007	.106	.306	.712
Sept. 30	14	.008	.150	.735	1.621	.006	.143	.279	.697

Table 5. Values of correlation coefficients (r) of amount of cyanidin in the leaves, skins, seed and flesh of the two apple varieties as determined by the direct and chromatographic methods at 14 weekly intervals.

		Red Delicious								Golden Delicious							
	Date of collection of samples	Leaf chromato-graphic	Leaf direct	Skin chromato-graphic	Skin direct	Seed chromato-graphic	Seed direct	Flesh chromato-graphic	Flesh direct	Leaf chromato-graphic	Leaf direct	Skin chromato-graphic	Skin direct	Seed chromato-graphic	Seed direct	Flesh chromato-graphic	Flesh direct
Red Delicious	Date of collection of samples	.369**	.264 N.S.	.882**	.884**	-.873**	-.871**	-.341*	-.069 N.S.	.417**	.416**	.812**	.832**	-.803**	-.818**	-.363**	-.019 N.S.
	Leaf chromatographic		.978**	.245 N.S.	.225 N.S.	-.609**	-.608**	-.053 N.S.	.500**	.976**	.966**	.579**	.559**	-.680**	-.677**	.159 N.S.	.585**
	Leaf direct			.118 N.S.	.103 N.S.	-.514**	-.511**	.014 N.S.	.532**	.951**	.938**	.488**	.464**	-.602**	-.589**	.176 N.S.	.613**
	Skin chromatographic				.994**	-.871**	-.876**	-.420**	-.124 N.S.	.270 N.S.	.295*	.807**	.828**	-.780**	-.804**	-.416**	-.121 N.S.
	Skin direct					-.876**	-.880**	-.447**	-.142 N.S.	.249 N.S.	.278*	.822**	.842**	-.788**	-.809**	-.447**	-.138 N.S.
	Seed chromatographic						.998**	.431**	-.088 N.S.	-.627**	-.636**	-.929**	-.935**	.967**	.974**	.290**	-.147 N.S.
	Seed direct							.431**	-.088 N.S.	-.626**	-.635**	-.931**	-.936**	.968**	.976**	.306*	-.143 N.S.
	Flesh chromatographic								.481**	-.080 N.S.	-.099 N.S.	-.340*	-.338*	.336*	.366**	.335*	.251 N.S.
	Flesh direct									.411**	.445**	.247 N.S.	.238 N.S.	-.242 N.S.	-.187 N.S.	.315*	.790**
Golden Delicious	Leaf chromatographic										.972**	.572**	.553**	-.688**	-.691**	.094 N.S.	.518**
	Leaf direct											.625**	.606**	-.714**	-.708**	.107 N.S.	.551**
	Skin chromatographic												.997**	-.955**	-.941**	-.232 N.S.	.270 N.S.
	Skin direct													-.950**	-.938**	-.236 N.S.	.255 N.S.
	Seed chromatographic														.987**	.252 N.S.	-.280 N.S.
	Seed direct															.258 N.S.	-.240 N.S.
	Flesh chromatographic																.519**
	Flesh direct																

Significant at .05 is ± .273
Significant at .01 is ± .354

N.S. = Non-significant
* = Significant 5% level
** = Significant at 1% level
\- = Negative correlation

Quantitative Estimation of Cyanidin

The quantities of cyanidin in milligrams per gram fresh weight of the leaves, skins, flesh, and the seed of the two apple varieties, Red Delicious and Golden Delicious were estimated at weekly intervals by direct and chromatographic methods. The results are presented in Tables 3 and 4.

The correlation coefficients of the amounts of cyanidin found were calculated as shown in Table 5.

DISCUSSION

Identification of Pigments

Chromatographic Identification. Extracted anthocyanidin of the leaves, skins, flesh, and seed of Red Delicious and Golden Delicious varieties of the apple were tentatively identified by comparing the RF values of the extracted samples, with RF values of anthocyanidin available in the literature (Table 3). The RF value of the authentic cyanidin chloride chromatographed in Foresal solvent, and found to be 0.56. The RF values of extracted anthocyanidin were thus compared with RF values of authentic cyanidin chloride.

Average RF values (Table 2) of an unknown anthocyanidin of different plant parts of the two apple varieties were found to be 0.55, 0.56 and 0.57. These values are in accord with the values (Table 2) of cyanidin found in the literature (Table 2).

Spectrophotometric Identification. The presence of the tentatively identified cyanidin in all the samples under study was confirmed by the spectroscopic method. The maximum absorption values (Plates II and IV) at 545 millimicrons of extracted cyanidin are in accord with the maximum absorpiton values given by Bate-Smith (7), Halevy (20), Asen (4), et al. The extracted cyanidin peak values (Plates III and IV) are also in accord with the authentic cyanidin chloride peak value (Plate V) used in this study.

Quantitative Estimation of Cyanidin

The quantity of cyanidin per gram fresh weight of the leaves of Red Delicious and Golden Delicious was found to increase steadily to the tenth and eleventh weeks and then to decrease regularly at each successive sampling date (Table 3). The average amount of cyanidin in the leaves of Red Delicious was consistently greater than for the Golden Delicious apple.

The quantity of cyanidin in the skin of both varieties increased gradually throughout the growth period except for the sharp increase noted in samples of Red Delicious in the three weeks before harvest. The change in the amount of cyanidin found in the seeds at the various sampling dates was an interesting feature of the study. The cyanidin content of seed was higher at the first sampling date than for any of the component parts sampled. This relationship continued for the first few weeks although the quantity of cyanidin in the seed decreased at each successive sampling date. From about July 22 to the end of the study, average amounts

were less than found in the skins of both varieties. Also beginning September 2, the leaves of Red Delicious had more cyanidin than did the seed of the same variety. From August 12 to the end of the experiment, leaf samples of Golden Delicious had more cyanidin than was found in seed of this variety.

No single observation can be made concerning cyanidin in the flesh of both varieties except that the quantity of cyanidin found in the flesh was very small in both varieties (Table 4). Further there was no discernible trend or pattern in the occurrence of cyanidin.

It can be suggested that cyanidin was first synthesized or manufactured in the leaves and then transported through the vascular system to the seed, flesh, and finally the skin. The decrease and increase of cyanidin in leaves and seed, and skin respectively, justify the above suggestion.

The data presented in Tables 3 and 4 were statistically analyzed and the correlation coefficients were calculated as given in Table 5.

The quantity of cyanidin in the Red Delicious leaves was positively highly significantly correlated with amounts found in the Golden Delicious leaves with both the chromatographic and the direct methods. These values are statistically significant at the one percent level.

The amount of cyanidin in leaves of the Golden Delicious was positively significantly correlated with the amounts found in skin of the same variety using chromatographic and direct methods of determination (Table 5). These values are significant at the one percent level.

The quantity of cyanidin in leaves of the Red Delicious and Golden Delicious were negatively highly significantly correlated with the amounts in seeds of each variety.

The occurrence of cyanidin in leaves was significantly correlated with the cyanidin of the flesh in both varieties only when direct method of determination was used (Table 5). These values are significantly different at the one percent level.

The quantity of cyanidin in the skin of both varieties were positively highly significantly correlated with each other regardless of the method used (Table 5).

It was found that the amount of cyanidin in the seed was negatively significantly correlated with the skin in both the varieties for each method of pigment determination (Table 5). These values are significantly different at the one percent level.

It may be suggested that the synthesis of cyanidin in the skin of the apple took place at the same time that the degradation of cyanidin occurred in the seeds of both the varieties.

The amount of cyanidin found in the skin each of the Red and Golden Delicious was negatively correlated with quantity found in flesh each of the same variety, as well as when compared with the other variety (Table 5).

The quantity of cyanidin in seeds of both the varieties were positively significantly correlated with each other. This also suggests that the changes in cyanidin occurs similarly in both apple varieties throughout the growth period.

The amount of cyanidin in the seed of the Red Delicious was positively significantly correlated with the quantity found in the

flesh when the chromatographic methods of determination were employed.

The quantity of cyanidin in the flesh of both apple varieties was found to be positively correlated (Table 5).

The negative correlation of amount of cyanidin of the seed with other component parts also indicates that all during the growth period of the apple fruit, the changes of cyanidin occur. These changes may be the result of degradation in the seed or synthesis of cyanidin in the skin. The same suggestion can be made with reference to the synthesis of cyanidin in the leaf.

The time of collection of the samples was found to be positively related to the occurrence of cyanidin in skins of both varieties regardless of method of determination used. Also a positive relationship was noted between date and cyanidin content in leaves of Golden Delicious for both methods and for Red Delicious and date when chromatographic method was used. Negative correlations were found for sample date and quantity of cyanidin in seed and flesh of both varieties (Table 5).

A close correlation was observed between the two methods of cyanidin determination used; the chromatographic and the direct. Four tables, 6, 7, 8, 9 of the leaf, skin, flesh and seed are taken from the main correlation coefficient (Table 5) to more clearly show the relationship of the two methods.

It is quite clear, according to Table 6, on the basis of cyanidin content of the leaves of the two varieties that both methods are significantly positively correlated with each other. All the values of "r" which are shown in Table 6 are above 90 percent and reveal a high correlation between the two methods.

Table 6. Correlation coefficients (r) of cyanidin content of the leaf of two apple varieties as determined by the direct and chromatographic methods.

	:Leaf Red :Delicious:Chromato.	:Leaf Red :Delicious:Direct	:Leaf Golden:Delicious :Chromato.	:Leaf Golden :Delicious :Direct
Leaf Red Delicious Chromatographic		.978 **	.976 **	.966 **
Leaf Red Delicious Direct			.951 **	.938 **
Leaf Golden Delicious Chromatographic				.972 **
Leaf Golden Delicious Direct				

Table 7. Correlation coefficients (r) of cyanidin content of the skin of two apple varieties as determined by the direct and chromatographic methods.

	:Skin Red :Delicious:Chromato.	:Skin Red :Delicious:Direct	:Skin Golden:Delicious :Chromato.	:Skin Golden :Delicious :Direct
Skin Red Delicious Chromatographic		.994 **	.807 **	.828 **
Skin Red Delicious Direct			.822 **	.842 **
Skin Golden Delicious Chromatographic				.997 **
Skin Golden Delicious Direct				

Significant at .05 is ± .273
Significant at .01 is ± .354

n.s. non-significant
* significant 5% level
** significant 1% level
\- negative correlation

Table 8. Correlation coefficients (r) of cyanidin content of the flesh of two apple varieties as determined by the chromatographic and direct methods.

	:Flesh Red :Delicious :Chromato.	:Flesh Red :Delicious :Direct	:Flesh Golden :Delicious :Chromato.	:Flesh Golden :Delicious :Direct
Flesh Red Delicious Chromatographic		.481 **	.335 *	.251 n.s.
Flesh Red Delicious Direct			.315 *	.790 **
Flesh Golden Delicious Chromatographic				.519 **
Flesh Golden Delicious Direct				

Table 9. Correlation coefficients (r) of cyanidin content of the seed of two apple varieties as determined by chromatographic and direct methods.

	:Seed Red :Delicious :Chromato.	:Seed Red :Delicious :Direct	:Seed Golden :Delicious :Chromato.	:Seed Golden :Delicious :Direct
Seed Red Delicious Chromatographic		.998 **	.967 **	.974 **
Seed Red Delicious Direct			.968 **	.976 **
Seed Golden Delicious Chromatograph				.987 **
Seed Golden Delicious Direct				

Significant at .05 is ± .273
Significant at .01 is ± .354

n.s. non-significant
* significant 5% level
** significant 1% level
\- negative correlation

Table 7 presents the relationship of cyanidin content of the skin of both varieties. The values also reveal that the two methods are highly significantly correlated at both the five and the one percent level.

The cyanidin content of flesh of both varieties was positively related for both methods of determination. The only non-significant relationship observed was between direct reading for Golden Delicious and chromatographic reading for Red Delicious.

The correlation coefficients of cyanidin content of the seed of both varieties were high for the two methods (Table 9).

As a result of the above values of the correlation coefficients, it suggests that both methods are nearly reliable for estimating the amount of anthocyanidin in plant parts (Tables 6, 7, 8, 9). But in few exceptions the correlation of the amount of cyanidin in the flesh with the leaves and seed does not qualify the above statement (Table 5).

SUMMARY

The method described by Bate-Smith (7) for the chromatographic identification of anthocyanidins was employed to identify the anthocyanidin of the leaves, skins, flesh, and seed of Red Delicious and Golden Delicious apples. All parts of the two varieties of apples were found to contain cyanidin in varying amounts.

Two methods, the chromatospectrophotometric and the direct spectrophotmetric, were used to estimate the quantity of cyanidin in the leaves, and various parts of the apple. A close relationship for amounts of cyanidin determined was found between methods

used with few exceptions. The quantity of cyanidin found in the leaves, skins, and seed of Red Delicious was much greater than observed in the Golden Delicious apple. The quantity of cyanidin in leaves of both varieties showed a steady increase for the first 10-11 weeks of the study followed by a gradual decline for the remainder of the experimental period.

The quantity of cyanidin in the skin of both varieties, increased greatly with the development of the fruit, whereas the quantity of cyanidin in the seed decreased continuously during the entire experimental period. The quantity of cyanidin in the flesh was low and fluctuated considerably.

It was also found that the quantity of cyanidin in the seed was highly significantly negatively correlated with amounts of this pigment in the skins and leaves of the two apple varieties. It may be suggested that the pigment was manufactured in the leaves and transported to the seed, flesh and skin throughout the experimental period and possibly also some degradation of cyanidin occurred in the seed at the same time the synthesis of the pigment took place in the skin of both varieties.

ACKNOWLEDGMENT

The author acknowledges his indebtedness to Dr. Ronald Wayne Campbell, Professor of Horticulture, for his numerous valuable suggestions and guidance in the prosecution of this study. The writer is further most grateful to his major adviser for suggesting a beautiful problem which has never lost its fascination.

The author wishes to express his most sincere appreciation to Dr. Howard L. Mitchell, Head, Department of Biochemistry, for his generous help and guidance throughout the course of this study and allowing the use of all the facilities and equipment in the Biochemistry Laboratory of Kansas State University.

Appreciation is extended to Dr. Stanley Wearden, Department of Statistics, for his guidance in Statistical analysis and presentation of the data in this thesis.

Gratitude is due Dr. Robert P. Ealy, Head, Department of Horticulture for reviewing this manuscript.

LITERATURE CITED

1. Ahmedullah, M.
Identification of anthocyanidin pigments in three varieties of pelargonium hortorum by chromatographic and spectrophotometric methods. Thesis of Master of Science, Dept. of Hort., Kansas State Uni., Manhattan. 1961.

2. Ahuja, K. G.
Identificationaand estimation of anthocyanidins in petals of rose cultivars 'Pink Cornet' and 'Happiness'. A dissertation of Doctor of Philosophy, Dept. of Hort., Kansas State Uni., Manhattan. 1962.

3. Asen, S.
Anthocyanins in bracts of Euphorbia pulcherrima as revealed by paper chromatographic and spectrophotometric methods. Plant Phys. Vol. 33. No. 1, pp. 14-17. Jan. 1958.

4. Asen, S., H. W. Siegelman and N. W. Stuart.
Anthocyanin and other phenolic compounds in red and blue sepals of Hydrangea macrophylla var. Merveille. Proc. Amer. Soc. Hort. Sci. 69:561-569. 1957.

5. Bate-Smith, E. C.
Anthocyanins, flavones and other phenolic compounds. Synp. Biochem., Soc. 3:62. 1949.

6. Bate-Smith, E. C.
Color reactions of flowers attributed to (a) flavonols and (b) carotenoid oxides. J. Exptl. Bot. 4:1-9. 1953.

7. Bate-Smith, E. C.
Leuco-anthocyanins. I. Detection and identification of anthocyanidins formed from leuco-anthocyanins in plant tissue. Biochem. J. 58:1, 122-125. 1954.

8. Bate-Smith, E. C.
Nature, London. 161:835. 1948.

9. Bate-Smith, E. C., and N. H. Lerner.
Leuco-anthocyanins. II. Systematic distribution of leuco-anthocyanins in leaves. Biochem. J. 58:126-132. 1954.

10. Bate-Smith, E. C. and R. W. Westall.
Biochem. Biophys. Acta, 4:417. 1950.

11. Blank, F.
The anthocyanin pigments of plants. Bot. Rev. 13; 241-317. 1947.

12. Boyle, R.
Experiments and considerations touching colours, London, from Onslow, M. W. The Anthoc. Jug. of Plts. pp. 3-58. 1864.

13. Buscalinoni, L. and G. Pollacci.
Le antocianine e il bro significo biologico nelle pianta, Milano, p. 379. 1903. (Onslow, M. W. The anthocyanin pigments of plants. Cambridge. 1925.)

14. Candolle, A. P. de.
Pflansenphysiologie oder Darstellung der Lebenskrafte und Lebensverrichtungen der Gewachse, Ubersetzt von I Roper, Stuttgart and Tubingen. 1833-1835. (Onslow, M. W. The antho cyanin pigments of plants, Cambridge, 1925.)

15. Chi-Yen-Chia.
The influence of environmental factors on the development of anthocyanin and the physiological significance of this pigment in Amaranthus odoratus. Bull. Chinese Bot. Soc. 3:119-120. 1937. Chemical abstracts, 1:467. 1940.

16. Currey, G. S.
The cause of blueing in red roses. Jour. Royal Soc. New South Wales, 61:307-314. 1927.

17. Flint, L. H.
Note on the quality of light and temperature in relation to the development of anthocyanin. Amer. Jour. Bot. 24:739. 1937.

18. Gassner, G. and W. Straib.
Untersuchunger uberdden Einfluss der Mineralsalzernahrung auf die Anthocyanbuldung an jungen Gesttenpflanzen. Ang. Bot. 19:225-245. 1937. (Blank, F. Bot. Rev., 13:241-317. 1947)

19. Grew, N.
The anatomy of plants with an idea of the anthocyanins in History of plants. Lectures read before the Royal Society, London. 1882.

20. Halevy, A. H. and S. Asen.
Identification of the anthocyanins in petals of tulip varieties Smiling Queen and Pride of Haarlem. Plant Phys. 34:5. 494-499. 1959.

21. Herzfelder, H.
Beitrage zur frage der Moosfarbungen. Beih. Bot. Zbl. 38:355-400. 1921. (Blank, F., Bot. Rev. 13:241-317. 1947.)

22. Jonesco, S.
Separation des tannins et des anthocyanidines renfermes dans les memes organes des vegetanx. Isolement d,ime nonvle anthocyanidine des feuilles rouges d, Acer platanoides. Extraction des lannins comp. Rend. Soc. Biol. Paris. 96:1022-1023. 1927. (Blank, F. Bot. Rev. 13:241-317. 1947.)

23. Karrer, P.
Anthocyane. In. Klein, Handbuch der pflanzenanalyse III/2:941-984. 1932. (Blank, F. Bot. Rev. 13:241-317. 1947.)

24. Karrer, P. et al.
Uber Pflanzenfarbstoffe. IV. Zur Kenntuns der Anthocyane and Anthocyanidine. Helve Chim. Acta. 10:729-757, 1927. (Blank, F. Bot. Rev. 13:241-317. 1947)

25. Karrer, P. and R. Widmer.
Untersuchungen uber Pflanzebfarbstoffe. I. Uber die Konstitution einiger Anthocyanidine. Helve Chim. Acta. 10:5-33. 1927. (Blank, F. Bot. Rev. 13:241-317. 1947.)

26. Karrer, P. and F. M. Strong.
Reindarstellung von Anthocyanen durchchromatographische. Helv. Chim. Acta. 19:25-28. 1936. (Blank, F. Bot. Rev. 13:241-317. 1947.)

27. Karrer, P. et al.
Umwandlungsprodukte einfacher Benzopyryliumverbindungen. Helv. Chim. Acta. 26:2116-2120. 1943. (Blank, F. Bot. Rev. 13:241-317. 1947.)

28. Karstens, W. K. H.
Anthocyanin and anthocyanidin formation in the seedlings of Fagopryum esculentum Moench. Rec. Trav. Bot. Neerl. 36:85-179. 1939.

29. Klein, A. L. and C. W. Hagen.
Anthocyanin production in the detached petals of Impatiens Balsamina L. Plant Phys. Vol 36:1. Jan. 1961.

30. Kosaka, H.
DieBezieHungen Zwischen der verschiedenen physiologischen Erscheinun gen der Pflanzen unde der an verschiedenen Vegetatioon-sorganen in Erscheinung tretenden Farbstoffen. I. Mitteilung. Uber die Beziehungen zwischen der Anthocyanbildung und dem Wachstum von Abutilon avicennae. Jour. Dept. Kyushu Univ. 2:208-240. 1929. (Blank, F. Bot. Rev. 13:241-317. 1947.)

31. Kosaka, H.
Uber den Einfluss desLichtes, der Temperatur und des Wassermangles auf die Farbung von Chrysanthemum-Bluten. Bot. Mag., Tokyo 66:551-560. 1932. (Blank, F., Bot. Rev. 13:241-317. 1947.)

32. Kruth, P.
Handbuch der Bluthenbiologie. Leipzig 1898. 'Handbook of Flower Pollination'. Translated by J. R. Ainsworth Davis, 3 vol. Oxford. Vol. I, 212-380. 1906.

33. Kuilman, L. R.
Physiologische Untersuchungen uber die Anthocyane. Rec. Trav. Bot. N'eerl 27:286-416. 1930. (Blank, F. Bot. Rev. 13:241-317. 1947.)

34. Lawrence, W. J. C. et al.
A survey of anthocyanins. Biochem. Jour. 32:1661-1667. 1938.

35. Lawrence, W. J. C.
Interaction of flavones and anthocyanins. Nature 129:834. 1932.

36. Macaire-Princep.
'Memoire sur la coloration automnale des deulles,' Mem. Soc. Phys., Geneva iv. pp 43-53. 1828. (Onslow, M. W. The Anthocyanin pigmentsoof plants. 19 .)

37. Malpighi, M.
Anatome Plantarum. London. 1675.

38. Marquart, L.
Cl., Die Farben der Bluthen, Eine chemisch physiologische abhandlung, Bonn. 1835. (Onslow, M. W. The anthocyanin pigments of plants. 1925.)

39. Molisch, H.
Uber amorphes und krystallisiertes anthokyan. Bot. Ziet. 63:145-162. 1905. (Blank, F., Bot. Rev. 13:241-317. 1947.)

40. Onslow, M. W.
The anthocyanin pigment of plants. Second ed., Cambridge at the University Press. 1925.

41. Robinson, G. M. and R. Robinson.
A survey of anthocyanins I. Biochem. Jour. 25:1687-1705. 1931.

42. Robinson, G. M. and R. Robinson.
A survey of anthocyanins. Biochem Jour. 26:1647-1664. 1932.

43. Robinson, G. M. and R. Robinson.
A survey of anthocyanins IV. Biochem. Jour. 28:1712-1720. 1933.

44. Robinson, R.
Natural colouring matters and their analogues. Rep. Bit. Assoc. 103rd Meeting pp. 45-56. 1933.

45. Robinson, R.
Chemistry of anthocyanins. Nature. 135:732-736. 1935.

46. Rosenheim, O.
Observation on anthocyanins. I. The anthocyanins of young leaves of grapevine. Biochem. Jour. 14:178-188. 1920.

47. Schriner, R. L. and R. J. Anderson.
A contribution to the chemistry of grape pigments. I. The anthocyanins in Ives grapes. Jour. Biol. Chem. 80:743-752. 1928.

48. Scott-Moncrieff, R.
A biochemical survey of some Mendelion factors of flower colour. Jour. Genetics 32:117-168. 1932.

49. Siegelman, H. W. and S. B. Hendricks.
Photocontrol of anthocyanin Formation in turnip and red cabbage seedlings. Plant Phys. Vol. 32, No. 5. 393-398. Sept. 1957.

50. Siegelman, H. W. and S. B. Hendricks.
Photocontrol of anthocyanin synthesis in apple skin. Plant Phys. 33:3:185-190. May 1958.

51. Siegelman, H. W. and S. B. Hendricks.
Photocontrol of alcohol, aldehyde and anthocyanin production in apple skin. Plant Phys. 33:6:409-413. Nov. 1958.

52. Weigert, L.
'Beitrage zur Chemie der rothen Pflanzenfarbstoffe, 'Jahresbericht und programm der k.k.onologischen und pomologischen Lehranstalt in Klosterneuburg. Wien. pp. i-xxxi. 1895. (Onslow, M. W. The anthocyanin pigments of plants. 1925.)

53. Weisse, A.
Uber Anderung der Blutenfarbe durch aussere Faktoren Verh. Bot. Ver. Brandeburg 65:27-36. 1923. (Blank, F. Bot. Rev. 13:241-317. 1947.)

STUDY OF IDENTIFICATION AND CHANGES IN QUANTITIES OF ANTHOCYANIDIN CONTENT IN APPLE LEAVES AND THE COMPONENT PART OF THE FRUIT

by

RAJESHWAR SINGH

B. Sc. (Agri) Bihar University, India, 1959

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Horticulture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1962

The method described by Bate-Smith (7) was employed to identify the anthocyanidins from the leaves, skins, flesh and seed of Red Delicious and Golden Delicious apples. Weighed samples of above plant parts were hydrolyzed with 2n-hydrochloric acid for a specific time over a steam bath. The anthocyanidins were extracted with n-butyl alcohol and chromatographed on Whatman No. 1 filter paper in Forestal solvent. After RF values were determined, the maximum absorption spectra of the anthocyanidins were then determined with a Beckman DU Spectrophotometer.

RF and maximum absorption values were compared with the values given in the literature as well as with those obtained from an authentic sample of cyanidin. The anthocyanidins of the leaves, skins, flesh, and seed of both the varieties were identified as cyanidin on this basis.

Two methods, direct spectrophotometric and chromatospectrophotometric, were employed to estimate the amount of cyanidin present in the leaves, skins, flesh and seed of both varieties. The quantity of cyanidin was estimated throughout the growth period of apples from July 1 to September 30 and the results were calculated by the direct spectrophotometric and chromatospectrophotometric methods.

It was found that the amount of cyanidin in the leaves, skins, and seed of Red Delicious was much greater than found in the same parts of Golden Delicious apple throughout the experimental period. The quantity of cyanidin in leaves of both varieties showed a steady increase during the first 10-11 weeks of the study followed by a gradual decline for the remainder of the experimental period.

The quantity of cyanidin in the flesh was low and fluctuated considerably. The pigment content in the skin increased greatly with the development of the fruit whereas the amount in the seed decreased continuously during the experimental period.

This may suggest that during the growth period the leaves manufacture the pigment which is transported to the skin, seed, and flesh.

Correlation coefficients were calculated to relate the amount of cyanidin found in different plant parts of the two varieties as measured by the two methods of pigment determination.

The amount of cyanidin in the leaves and skins of both varieties were found to be positively correlated with each other, whereas the amount of cyanidin in seed as related to these component parts were significantly negatively correlated. The significant negative correlations of cyanidin content between seed and skins of both varieties suggest the synthesis of cyanidin in the skin may take place at the same time as degradation of the pigment occurs in seed.

The cyanidin content of the flesh of both varieties was positively related for both methods of determination.

A close relationship for amounts of cyanidin determined was found between the methods used with few exceptions.