

THE UNIVERSITY OF MANITOBA

STABILIZATION OF ANTHOCYANINS

by

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TABLE OF CONTENTS

	Page
List of Tables	v
List of Figures	viii
Abstract	x
I. INTRODUCTION	1
II. CERTAIN PROPERTIES OF ANTHOCYANINS THAT HAVE A BEARING ON THEIR STABILITY	3
1. Chemical factors affecting stability	3
2. Other factors affecting anthocyanin stability in plants	15
III. IDENTIFICATION AND PURIFICATION OF ANTHOCYANINS	17
1. Identification of anthocyanins	17
2. Isolation of anthocyanins in large quantities	18
3. Sour cherry anthocyanins	28
3.1. Anthocyanins found by other researchers	28
3.2. Anthocyanins found in present study	28
IV. THE ISOLATION AND INVESTIGATION OF A BLUE COLORED COMPLEX FROM CANNED BLUEBERRY JUICE	32
1. Introduction	32
2. Literature Review	33
2.1. Differences in pH	33
2.2. Co-pigmentation	34
2.3. Cation complexes	34
2.4. Complex, macromolecular forms of anthocyanins isolated and identified in plants	36
3. Experimental	40
3.1. Isolation of the colored complex	40
3.2. Alternative method of isolation of the colored complex	40
3.3. Characterization tests	41
3.4. Attempts to isolate anthocyanin complexes from fresh frozen blueberries and raspberries	44
3.5. Comparison between the AIS of canned blueberries, strawberries and raspberries	44

	Page
4. Results	45
4.1. Characterization tests	47
4.2. Attempts to isolate anthocyanin complexes from fresh and processed fruit	58
5. Discussion	60
V. COMPLEX FORMATION REACTIONS OF ANTHOCYANINS	66
1. Introduction	66
2. Literature Review	66
2.1. Experimental evidence for complex formation	66
2.2. Comparison of anthocyanin stability under various conditions	67
2.3. Chelation of aluminium and ferric ions by anthocyanins	68
2.4. Naturally occurring components which may take part in complexing reactions	69
3. Experimental Reagents	70
4. Dialysis Experiments	72
4.1. Experimental	73
4.2. Results and discussion	74
5. Spectrophotometric experiments	79
5.1. Experimental	79
5.2. Results and discussion	81
6. Electrophoresis experiments	99
6.1. Experimental	99
6.2. Results and discussion	100
7. Paper chromatography experiments	101
7.1. Experimental	101
7.2. Results and discussion	102
8. Photodensitometric methods applied to certain artificial systems	103
8.1. Experimental	104
8.2. Results and discussion	107
8.3. Effect on anthocyanin-pectin systems on addition of ferric ions	110

	Page
VI. ANTHOCYANIN STABILITY IN PROCESSED MONTMORENCY CHERRIES	112
1. Introduction	112
2. Literature Review	114
3. Experimental	115
3.1. Design of experiment	116
3.2. Chemical analysis	116
3.3. Statistical analysis	119
4. Results and discussion	119
4.1. Pigment content of the juice	119
4.2. Ratios of cyanidin 3-rutinoside to cyanidin 3-glycosylrutinoside	123
4.3. Degradation Index	125
4.4. Pectin present in the syrup and in the berry	126
4.5. Correlations between a number of parameters in processed cherries	132
VII. FINAL DISCUSSION	135
VIII. SUMMARY AND CONCLUSIONS	138
LITERATURE CITED	142
APPENDIX 1	151
Identification of anthocyanins	151
APPENDIX 2	164
Standard chromatographic method for anthocyanin separation on columns of insoluble polyvinylpyrrolidone	164
APPENDIX 3 TO 15	165-181

LIST OF TABLES

	Page
TABLE 1. Anthocyanin composition of selected botanical material investigated in this study	19
TABLE 2. Published anthocyanin composition of selected botanical material.	22
TABLE 3. Separation of large quantities of individual anthocyanins by P.V.P. column chromatography	26
TABLE 4. Order of anthocyanins migration in 1% HCl as found by von Elbe and by Harborne (starting with the least mobile)	31
TABLE 5. A comparison of the electrophoretic mobilities of some cyanidin glycosides.	31
TABLE 6. Chemical composition of the syrup and of the AIS of canned blueberries, strawberries and raspberries . ..	46
TABLE 7. Tests made on paper chromatograms of samples taken from complex treated with 20% HCl.	48
TABLE 8. Concentration of certain cations present in the blue complex isolated from blueberries and in a commercial sample of pectin as determined by x-ray fluorescent spectroscopy.. .. .	50
TABLE 9. Effect of the addition of a number of chemicals to processed blueberry syrup on the color of the syrup and on the color of the precipitate obtained on the addition of ethanol... .. .	75
TABLE 10. Effect on dialysis rate of a mixture of pectin and cranberry anthocyanins as measured by the absorbancies of the dialysate	78
TABLE 11. Absorption maxima and pH values of a three component system of galacturonic acid-aluminium-cyanidin 3-glucoside	81

	Page
TABLE 12. Absorption maxima and pH value of the three component system of galacturonic acid-aluminium-delphinidin 3-glucoside	84
TABLE 13. Spectral Characteristics and color of some anthocyanins at different pH levels on the addition of hydroxyproline and pectin	93
TABLE 14. Color reactions of three anthocyanins at two pH levels with and without hydroxyproline and pectin after one week storage	95
TABLE 15. Spectral characteristics and color at certain pH values of some anthocyanins with various combinations of hydroxyproline, pectin, Fe^{+++} and Al^{+++}	97
TABLE 16. Color present after one week of storage of anthocyanins with various combinations of Al^{+++} , Fe^{+++} , hydroxyproline and pectin	99
TABLE 17. Mean values of the total delphinidin glycoside present after reaction with pectin in the presence and absence of Al^{+++}	107
TABLE 18. The effect on color and absorption maxima of mixtures of Al^{+++} , high methoxyl pectin and cyanidin and delphinidin glycosides.. .. .	108
TABLE 19. Mean percentage of total free delphinidin glycosides after reaction at pH 2.7 with pectin with or without metal cations	109
TABLE 20. Mean percentage of total free delphinidin glycoside after reaction at pH 4.3 with pectin with or without metal cations	109
TABLE 21. Average concentration of anthocyanins after reaction with pectin, Fe^{+++} and/or Al^{+++} cations at pH 2.7 and 4.3	111

	Page
TABLE 22. Mean pigment content expressed in absorbance in 0.25 N HCl at three storage lengths in processed Montmorency juice	122
TABLE 23. Mean treatment values of pigment content as determined by absorbance in 0.25 N HCl of processed Montmorency cherry syrup	122
TABLE 24. Comparison of the pigment ratio means of fresh Montmorency cherries and those of the processed fruit at three different storage levels . ..	124
TABLE 25. Mean values of the Degradation Index calculated for processed Montmorency cherry juice after three storage periods	125
TABLE 26. Mean values of the Degradation Index for five treatments applied to processed Montmorency cherry juice	126
TABLE 27. Mean pectin content of processed Montmorency cherries after three storage periods	129
TABLE 28. Means of five treatments of the pectin present in processed Montmorency cherry juice	129
TABLE 29. Mean pectin content in processed Montmorency cherry juice at three lengths of storage	131
TABLE 30. Identities and sources of supply for the plant material used	153
TABLE 31. Paper chromatographic solvents systems	154
TABLE 32. R_f values and absorption spectrum characteristics for anthocyanins examined in this study	159
TABLE 33. Reference data used to identify anthocyanins	161
TABLE 34. Comparison of R_g values of sugar moieties identified in anthocyanins in this study with other published values	163

LIST OF FIGURES

Figure		Page
1	Spectral curve of processed blueberry juice (syrup) after dilution 10 to 100 with pH 3.1 adjusted water	54
2.	Spectral curve of the blue complex isolated from the processed blueberry juice (syrup) dissolved in pH 3.1 adjusted water	54
3	Spectrum of the isolated blueberry complex and the anthocyanins liberated from the complex by 20% HCl.	56
4	Absorbancies calculated by least square fit for the effect of some added chemicals on the rate of dialysis of processed blueberry juice	77
5	Delphinidin 3-glucoside 1) in pH 2.3 buffer; 2) in buffer with added galacturonic acid; 3) in buffer with added galacturonic acid and AlCl ₃ ; 4) in buffer with aluminium chloride	83
6	Cyanidin 3-glucoside 1) in pH 2.3 buffer; 2) in buffer with added galacturonic acid; 3) in buffer with added galacturonic acid and AlCl ₃ ; 4) in buffer with AlCl ₃	83
7	Delphinidin 3-glucoside 1) in pH 4.5 buffer; 2) in buffer with added galacturonic acid; 3) in buffer with added galacturonic acid and AlCl ₃ ; 4) in buffer with AlCl ₃	86
8	Spectra of cyanidin 3-glucoside 1) in pH 2.1 buffer with added FeCl ₃ ; 2) in buffer with added AlCl ₃ ; 3) same as 1 but recorded after one day; 4) same as 2 but recorded after one day.	89

Figure		Page
9	Spectra of delphinidin 3-glucoside 1) in pH 2.1 buffer with added FeCl_3 ; 2) in buffer with added AlCl_3 ; 3) same as 1 but recorded after one day; 4) same as 2 but recorded after one day.. ..	89
10	Spectra of cyanidin 3-glucoside recorded after 3 days 1) in pH 3.4 buffer; 2) in buffer with added hydroxyproline	91
11	Spectra of delphinidin 3-glucoside recorded after 3 days 1) in pH 4.3 buffer; 2) in buffer with added hydroxyproline	91
12	Mean pigment content as determined by absorbance measured in 0.25 N HCl after three storage periods ..	121
13	Treatment means for the measurement of pigment content in processed cherry juice	121
14	Mean total pectic substances (mg per 100 g drained berries) after three storage periods	128
15	Treatment means for the measurement of pectin in processed cherry syrup	128
16	Mean pectic substances (mg per 100 ml of syrup) after three storage periods	130

ABSTRACT

Van Teeling, Cornelis G., Ph.D. The University of Manitoba

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Stabilization of Anthocyanins

Research on the stability of anthocyanin (I) pigments was carried out. The reaction of purified 3-glucosides of pelargonidin (II), cyanidin (III), and delphinidin (IV) with hydroxyproline (V), pectin (VI) and galacturonic acid (VII) in the absence or presence of aluminium (VIII) and ferric (IX) ions were studied at various pH values. The results showed that under certain conditions the stability of the pigments to storage was increased. Ions of (IX) had a destructive effect on all the pigments tested but the glycosides of (IV) were most seriously affected. In a system containing glycosides of (III) and (IV) together with (VI) and (VIII), those of (IV) appeared to form complexes or associations of molecules more readily than those of (III). Evidence for complex formation or molecular association between (I) and various combinations of (V), (VI) (VII) and (VIII) was obtained by spectrophotometry, dialysis, paper chromatography, paper electrophoresis, and photodensitometry.

A complex with a molecular weight between 77,000,000 and 490,000,000 was isolated by ethanol precipitation from the syrup of canned blueberries. The complex contained glycosides of (IV) and petunidin (X) as well as cations, proteinaceous material and large quantities of a polysaccharide resembling (VI).

In an experiment to reduce pigment loss, (V) and (VI) were introduced into the tissues of Montmorency cherries by injection and by

vacuum infiltration. In the treated processed cherries, there was some evidence that the diffusion of pigment into the syrup from the fruit differed with different treatments.

I Introduction

The color of food is one of a number of attributes which provides pleasure and variety to the human diet. The consumer associates color of food with quality and freshness. Nature has supplied a wide selection of colors, such as the yellows produced by the carotenoid pigments and the vivid reds and blues found in fruit that are due to the anthocyanin pigments. Anthocyanin pigments belong to the flavonoid class of polyphenolic compounds which have a basic C₆-C₃-C₆ carbon skeleton.

The anthocyanins are water soluble pigments which are most stable under acidic conditions. In general, anthocyanins are unstable and decompose through ill-defined chemical pathways. Decomposition of anthocyanins may be accompanied by the formation of insoluble brown colored products, which adversely affect the appearance of the food product.

The problem may be illustrated by using canned strawberries as an example. Canning results in disruption of the organization of the cells of the strawberries, and anthocyanins are released from the tissue. Some pigment may remain in the berry, but a great proportion diffuses out of the berries and into the covering syrup. The pigments in the berries and in the syrup may retain a reddish tinge, but analysis shows that a large proportion of the color is due to the decomposition products. There are, therefore, two problems:

1. Prevention of loss of pigment from the berry tissue.
2. Prevention of loss of pigment due to chemical reactions.

These could be summarized in the phrase "maintenance of the positional and of chemical stability".

Food processors have found qualitative differences between

different fruits in the retention of color by the processed product. Strawberries yield the worst product, while blueberries probably provide the best. Anthocyanin stability is also important in colored beverages such as juices made from anthocyanin-containing fruit. Cranberry and grape juice are examples of this type of product. Jams and jellies can lose so much pigment that artificial food color may be required to give a product that is acceptable to the consumer. Acceptable maraschino cherries can only be produced by the substitution of artificial colors.

The use of artificial food colors bears an element of risk since some have been implicated in certain human disorders. The trend is towards prohibition or severe limitation in the use of artificial food colors. This trend will probably increase as more evidence is gathered about artificial food colors, and it is therefore imperative that research be carried out into methods for the protection and conservation of anthocyanin pigments.

Information obtained on the stability of anthocyanins usually pertains to selected systems of one or two anthocyanin structures, either in fruit or in artificially constituted model solutions. The effect of ascorbic acid, certain sugars, head space gases and metal ions on pigment stability during and subsequent to processing have been investigated. These experiments have yielded much valuable information but have the limitation that the investigations can usually not be extended to cover all the six common aglycone structures or to all the possible combinations of the numerous factors involved.

The approach taken in this research was to stabilize the anthocyanins through interaction with components found in the plant cell.

Through the complexation with the plant cell constituents, it was hoped that the anthocyanin pigments would be anchored or fixed in the tissue and thus yield a highly colored product. This form of reaction seemed quite possible but would probably be expected to vary with the structure of the individual anthocyanins.

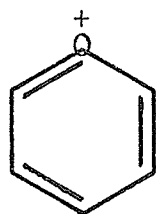
The author has made conscious efforts to include as much data and experimental techniques as possible. This was done to facilitate the re-interpretation of data by other anthocyanin researchers when new ideas are presented for scrutiny. Furthermore, the approach taken in this thesis is novel and different from the conventional study of anthocyanins mentioned above.

II Certain Properties of Anthocyanins that Have a Bearing on their Stability

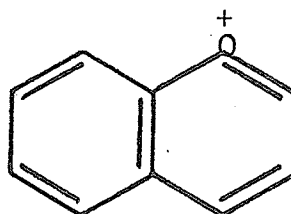
1. Chemical factors affecting anthocyanin stability

Specialized review articles are available on anthocyanin chemistry. (Hayashi (66), Jurd (72), Harborne (58), Swain (127), Pridham (102), and Harborne (61).) The anthocyanins are also described in a number of standard organic textbooks. (Fieser and Fieser (43), Acheson (1), Cram and Hammond (31).) These sources have been liberally used in this short discussion of the anthocyanins.

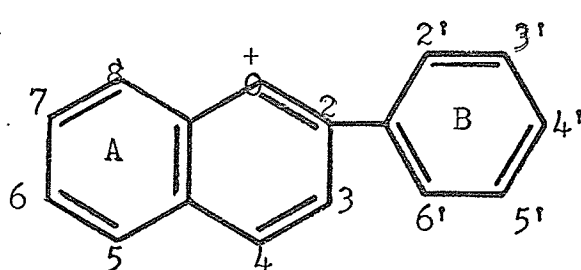
The aromatic pyrylium cation I is the basis of the benzopyrylium salt II. The addition of a phenyl group at the 2 position produces the flavylium skeleton III (2-phenylbenzopyrylium salt). The flavylium skeleton is shown with the proper numbering of the positions with the letters A and B denoting the two aromatic rings.



1

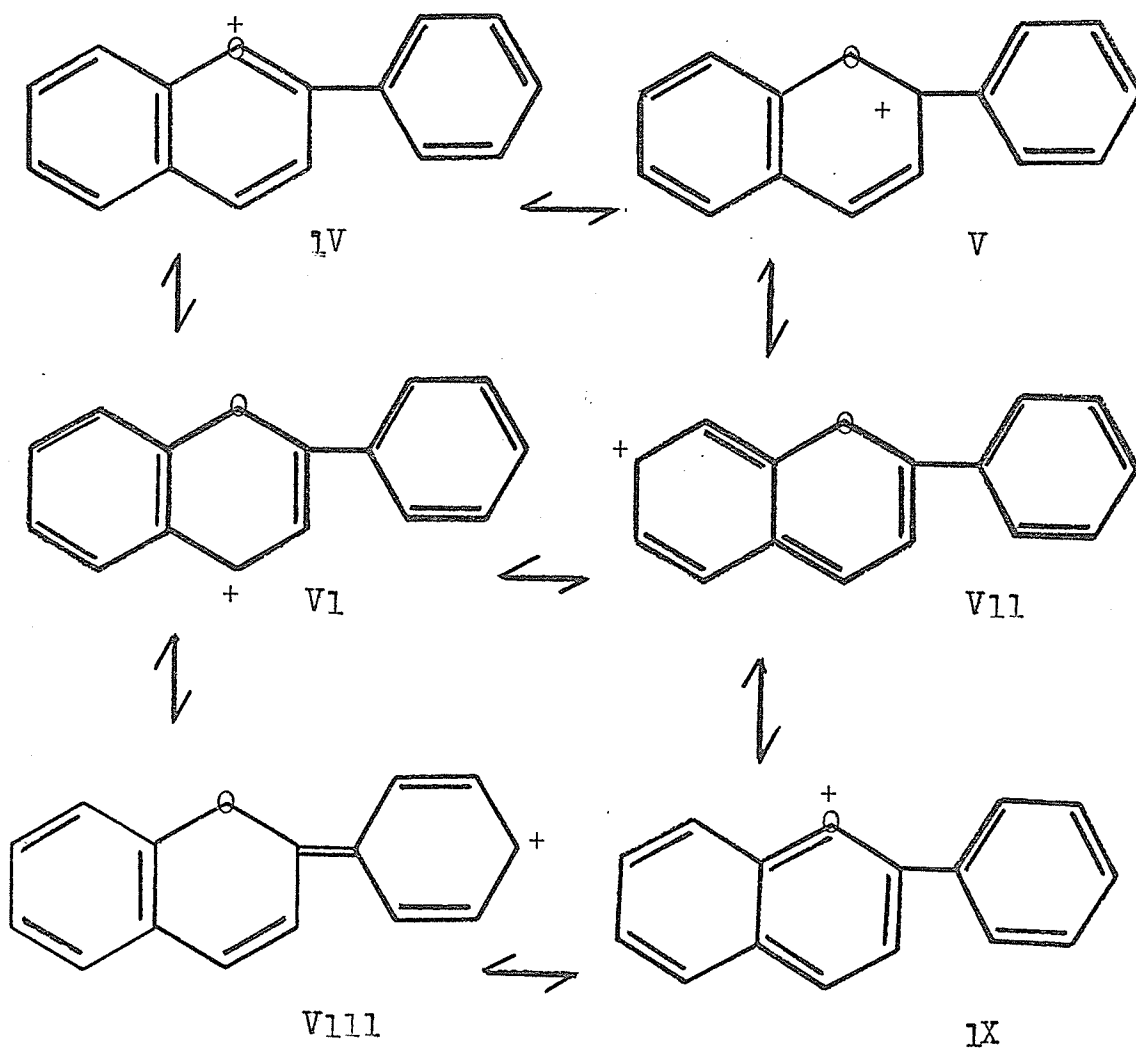


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A number of resonating structures may be proposed to represent the flavylium ion IV to IX (31) (43).

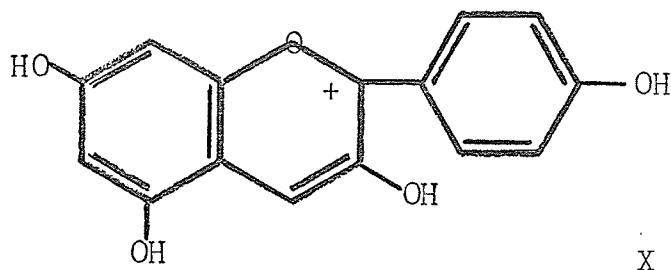


The striking color of the flavylium salts may be related to the distribution of the positive charge throughout the different canonical forms of the resonance ring system. Both IV and IX are oxonium ion forms, while in the others, the positive charge is associated with the carbon atoms. Since the oxygen has a greater ability to carry the positive charge than the carbon, the IV and IX forms are thought to predominate. The IV form, which has a naphthalene type structure, would be expected to be more stable than the IX form which has a quinonoidal system. Evidence for the carbonium ion V has been found in the nitration at 3', or meta, to the 2 carbon atom (43) and by reaction with H_2O_2 (76) (103) (121) or by SO_2 (24) (73) (133). The older books on this subject stress the oxonium form of the anthocyanins. Modern workers, for example Harborne (59), show the formula as a carbonium ion and assume the presence of an anion. This formula will be used in this thesis.

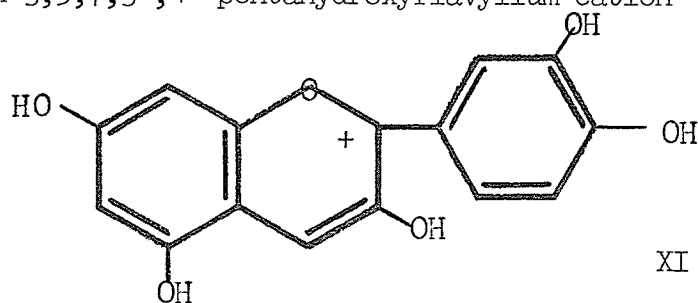
The anthocyanidins or the aglycones of anthocyanins are highly substituted flavylium salts differing in the position and number of free or methylated hydroxyl groups. Certain restricted but recurring patterns are possible reflecting the biosynthetic origins. Thus, the A ring contains hydroxyl substituents at the 3, 5, or 7 position usually non-methylated. The B ring has either free or methylated hydroxyl groups at position 3', 4', or 5'.

Harborne (59) has listed 20 structures which are classed as anthocyanidins. However, in the common food plants only six anthocyanidins may be present, X to XV.

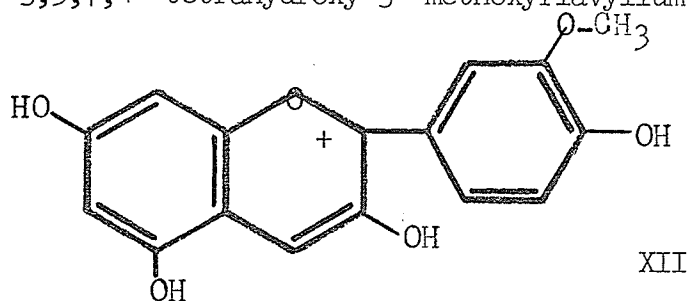
Pelargonidin 3,5,7,4' tetrahydroxyflavylium cation



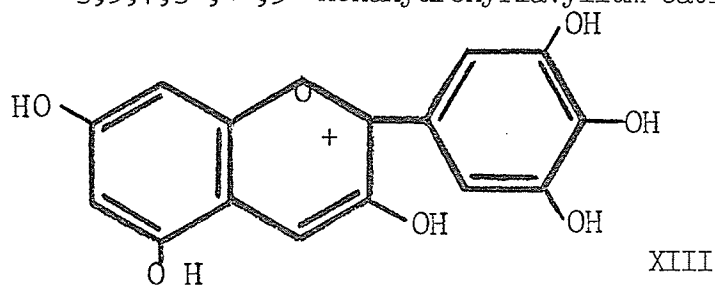
Cyanidin 3,5,7,3',4' pentahydroxyflavylium cation



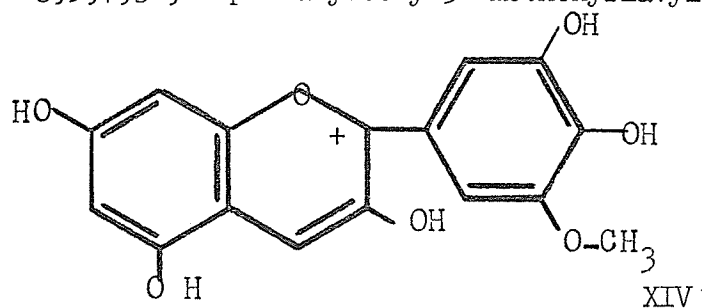
Peonidin 3,5,7,4' tetrahydroxy-3'-methoxyflavylium cation



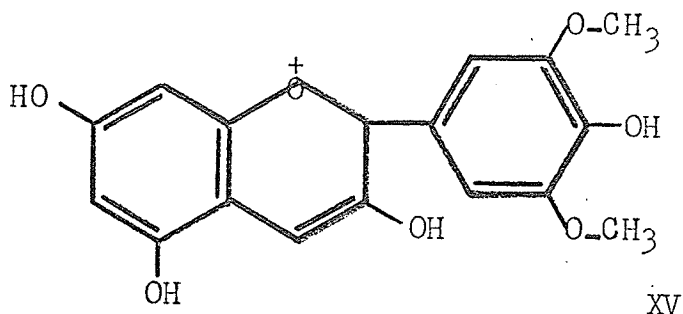
Delphinidin 3,5,7,3',4',5' hexahydroxyflavylium cation



Petunidin 3,5,7,3',4' pentahydroxy-5'-methoxyflavylium cation



Malvidin 3,5,7,4' tetrahydroxy-3',5'-dimethoxyflavylium cation



The structures of the aglycones have a strong influence on the visible colors and on the spectra. With increased hydroxylation on the B ring, the color changes from orange red of pelargonidin, maximum absorption at 520 nm to delphinidin, blue in color, with a maximum at 546 nm. Methylation causes a slight shift of the absorption maxima towards lower wavelengths. The solvent affects absorption maxima and hence color with a shift toward the blue when the series water, methanol and ethanol is considered.

The anthocyanidins are insoluble in aqueous solvents and are unstable except at low pH. In plant cells, the anthocyanidins exist as glycosides. The glycosides are water soluble and they are more stable than the aglycones.

In the glycosides, the sugar moieties are joined by semi-acetal type links to the hydroxyl groups at certain positions on the flavylium ions. Glycosidation is usually restricted to the 3 position (the 3-monoglycoside, the 3-biosides or 3-triosides); 3,5 position (the diglycosides) although 5 and 7 monoglycosides are known. The glycosidic sugars most commonly present are: the simple hexoses, glucose, galactose, rhamnose and the pentose sugars, xylose and arabinose. The anthocyanidin-sugar link is usually β but rhamnosides and arabinosides have α links.

Dissacharides are well known and are restricted to 1-2 and 1-6 linkages. Glucose is usually at the reducing end, attached to the aglycone. Trisaccharides with straight chains or branched chains have also been found. The presence of the α and β linkages in the sugar-aglycone or sugar-sugar bonds have been proven by enzymatic studies. The different types of linkages produce a large effect on the R_f values of the anthocyanins. For example, cyanidin 3-gentiobioside contains two glucose molecules linked together by β , 1 $\rightarrow$ 6 link and has R_f values of .20 in BAW and .46 in AWH. Cyanidin 3-sophoroside has a β , 1 $\rightarrow$ 2 link between glucose molecules with consequent R_f values of .33 in BAW and .61 in AWH.

Glycosidation of the aglycones has a hypsochromic effect on the spectra. Thus pelargonidin has an absorption maximum at 520 nm while the pelargonidin 3-glucoside has an absorption maximum at 506 nm both in 0.01% HCl in methanol. A more complete discussion of the effect of structure on spectral properties may be found in the review articles cited at the beginning.

Acylated anthocyanin glycosides have been found in a number of fruits. The acyl component of the pigment is easily lost and requires lower levels of acidity at the extraction stage. Acyl groups may migrate to alternate positions and thus it has been difficult to establish the acylated anthocyanin structure (41). At present, substitution is thought to be at the 4 or 6 position of the sugar attached to the 3 position of the aglycone (3) (16). Migration has been shown to occur between these two positions on the sugar and thus some uncertainty still exists.

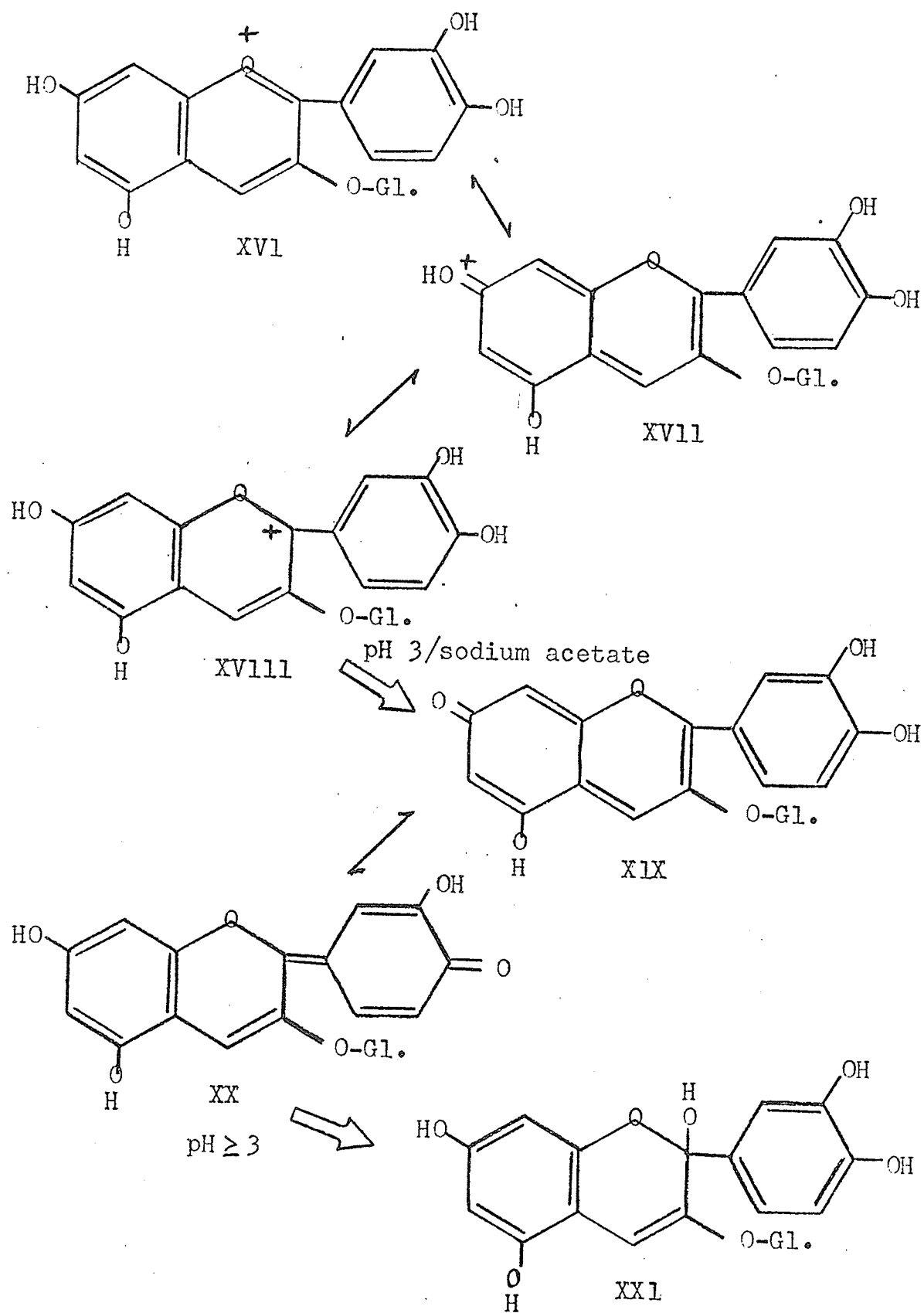
The most common acylating group appears to be p-coumaric acid although caffeic and ferulic acid may also occur frequently (84).

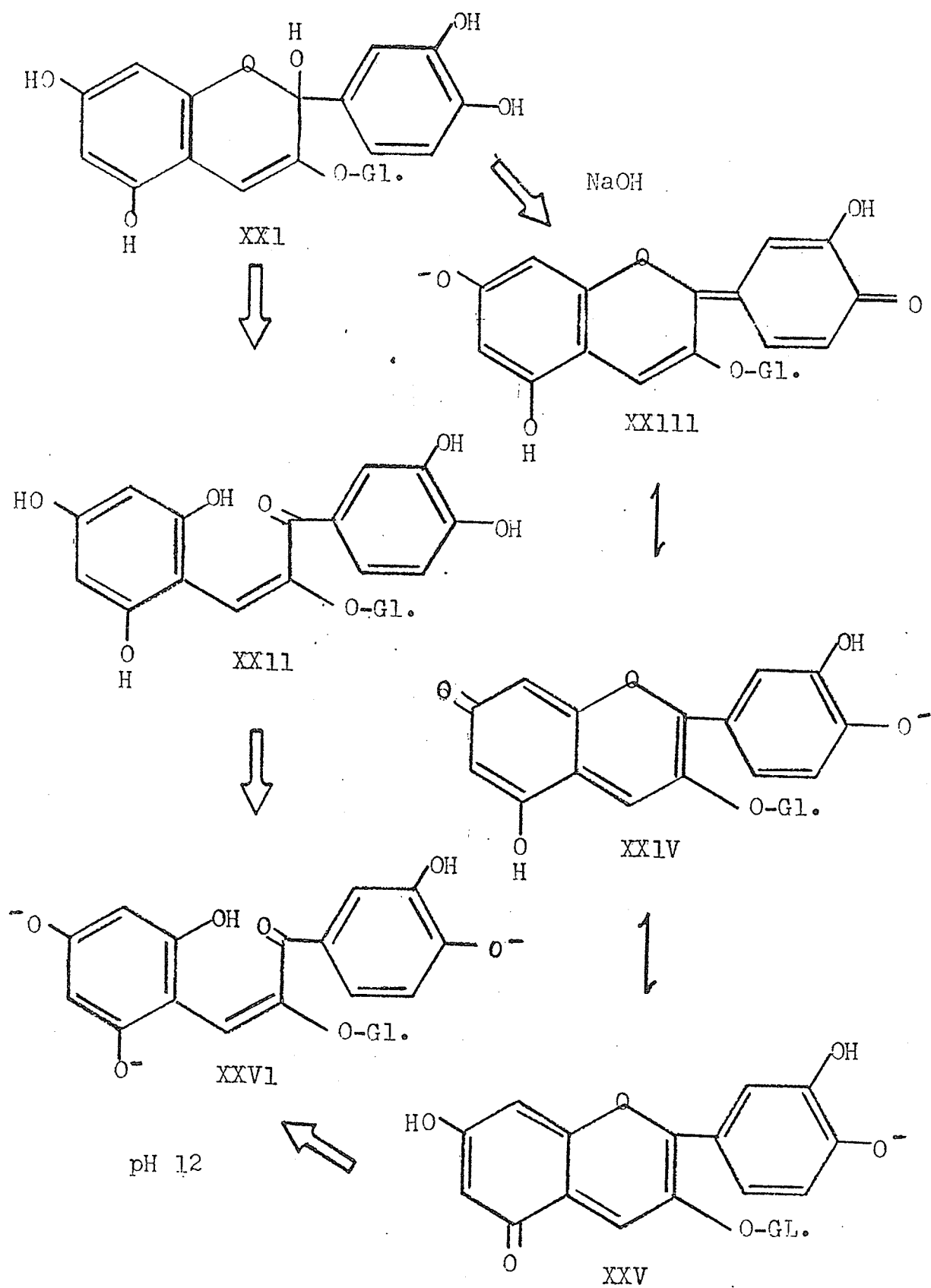
Various other compounds considered to have acylating functions were malic, tartaric, chlorogenic, gallic, sinapic and p-hydroxybenzoic acids (77). Albach et al. (2) have provided a review and experimental methods for the identification of acylated pigments. The outstanding characteristic of the acylated anthocyanins is an absorption peak or shoulder in the 305-320 nm region.

The structural formulae for the anthocyanidins X to XV are shown as the charged, deeply colored oxonium ions. This form occurs at low pH 1-2. The cation form, of which cyanidin 3-glucoside XVI will be used as example, is best considered as a resonance hybrid of various charged structures XVI, XVII, and XVIII. As the pH is raised, the highly colored oxonium form will establish an equilibrium with its quinoidal color base XIX and XX (75) (105). This occurs only if the oxonium salt has at least one free hydroxyl group at 5,7,3', or 4' positions to produce the quinoidal form. On standing this form is hydroxylated to produce the carbinol pseudo base XXI (75) which, being less conjugated, is colorless. The carbinol pseudo base may form an open chain chalcone structure XXII (105). As the pH is raised, these equilibria would shift progressively toward the chalcone open chain.

If the anthocyanins are placed in slightly alkaline solution (pH 8-10), highly colored blue, ionized anhydro bases are formed which are relatively stable. The resonating structures XXIII to XXV indicate these compounds. The fully ionized anhydro bases may change rapidly at pH 12 to produce the fully ionized chalcone XXVI (106).

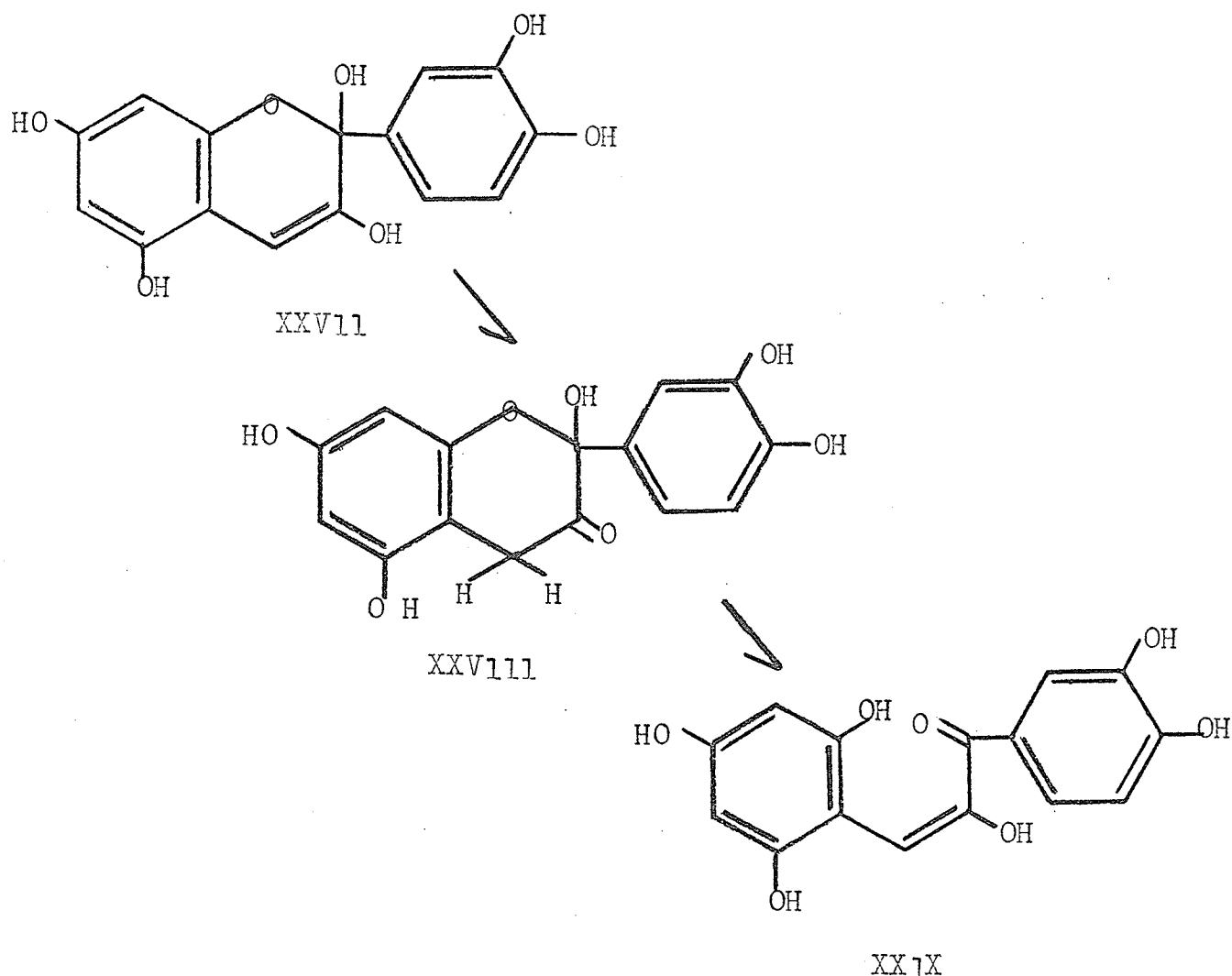
The outline presented consists of a series of equilibria, and





with rapid changes of conditions, not all the intermediates shown in the scheme need actually be present. Evidence for intermediary products is usually obtained from the presence of isosbestic points in the spectrum. Timberlake and Bridle (131) found a number of isosbestic points between pH 1.7 and pH 3.9 in the spectrum of cyanidin (2 isosbestic points) and pelargonidin (1 isosbestic point). This suggested that the structure may affect the sequence of reactions. Thus in the natural 3-substituted anthocyanin, the equilibrium is established between the carbonium ion and the carbinol base and not with the chalcone. In other flavylum salts, the chalcones are in equilibrium at pH 5 (75).

The nature of the decomposition of anthocyanins is still not satisfactorily resolved due, in part, to the character of the decomposition products. Polymerization, of some forms of anthocyanin-derived polyphenol, is the most likely mechanism. Huang (58) postulated that degradation occurred in two stages. The first stage was the enzymatic removal of the sugar attached to the aglycone. The aglycone, being much more labile than the anthocyanin, is decomposed into the colorless form. Two modes of decomposition of the anthocyanidin have been suggested. It is only possible for the pseudo base form of the aglycone XXVII to exist in the keto form XXVIII and in the chalcone form XXIX. The keto form is more susceptible to oxidative breakdown (69). A second mode is suggested by the shift in equilibrium from the oxonium to the anhydro form when the glycoside sugar is removed. The increase in the anhydro base form leads to increased fading and decomposition by oxidation and subsequent splitting of the molecule (120).



Equilibria may be characterized by the calculation of parameters such as pK value. Various authors have published pK values of a number of anthocyanins (78) (132)(141). The pK value is indicative of the acid strength. It measures the extent of the reaction $R^+ + 2H_2O \rightarrow ROH + H_3O^+$. Using the pK values obtained, Timberlake and Bridle (132) showed that the 3,5-diglycosides were stronger acids than the 3-glycosides. Thus at a specific pH more color would be shown by the 3-glycoside than the 3,5-diglycosides. Enzymatic decomposition through the phenol oxidase system is possible only for the anthocyanidin. It has been shown that the anthocyanins are resistant to this enzyme system (8).

Tinsley and Bockian (134) found that the degradation of the anthocyanin is dependent on the amount of the pseudo base present. Lukton et al. (85) reached this same conclusion. They suggested that the pseudo base is directly oxidized, after which a brownish precipitate is formed. Markakis et al. (88) postulated that, during processing at elevated temperatures, the pyrylium ring may be hydrolytically opened with the formation of substituted, brown, insoluble polyphenolic matter.

The degradation of the flavylium salts in the presence of furfural type compounds has been investigated by Blackburn et al. (17). They found that oxidative coupling with the pseudo base form was possible in the presence of oxygen.

Some factors affecting the breakdown of anthocyanins in processed foods are pH, temperature, (both of storage and of processing), presence or absence of oxygen, ascorbic acid and sugars. Several research papers provide summations of this area (34) (78) (85) (100) (122) (134). Rate constants of decomposition of a number of anthocyanins under various conditions have been studied (18) (78) (134) (141) (158) (164). Generally first order kinetics were found for the degradation.

There are few indications of the relative stability of the aglycone-sugar or sugar-sugar linkages in the anthocyanin. Chandler and Harper (26) have investigated the ease of hydrolysis of a number of flavonoids with different acids. They found that certain aglycone-sugar linkages would be hydrolysed in preference to the disaccharide bonds, depending on the flavonoid. Starr and Francis (125) found that the galactoside pigments (a hexose) were more stable in cranberry cocktail than the arabinoside (a pentose). No differences in stability were

indicated between cyanidin and peonidin types of aglycone structures. This aspect is of importance in studies where manipulations are carried out on the anthocyanin pigment. One report of different stabilities of anthocyanidin structures was given by Robinson et al. (110). In their experiments, certain anthocyanins were introduced into white wines and the stability of each added pigment was measured. They found diglycosides to be more stable to decolorization than the corresponding monoglycoside. Malvidin and peonidin were more stable with delphinidin being least stable. Cyanidin 3-glucoside has been shown to be more rapidly degraded than pelargonidin 3-glucoside (141). Keith and Powers (78) found that pelargonidin 3-glucoside was more stable than malvidin and petunidin 3-glucoside. Timberlake and Bridle (132) have shown that the stability of the anthocyanidin cations depends upon the substitution pattern. Substitution of hydroxyl groups at the 7 and 5 position results in increased stability while stability was decreased by hydroxylation at the 3 position. This agrees with the stabilities of the 3-deoxyanthocyanins, such as apigeninidin and luteolinidin. These anthocyanins do not have any substituent at the 3 position, and even show stable color at alkaline pH. Jurd (73) deals with the structural changes under acidic condition of a number of flavylum substitution patterns.

2. Other factors affecting anthocyanin stability in plants

Besides a study of the chemistry of the anthocyanins, the investigation of anthocyanin stabilization requires a familiarity with plant physiology. In what form do the anthocyanins exist in the cell? Harborne (59) states that anthocyanins "are cations and are presumed to

occur in living cells in association with anions of organic acids".

It has been generally accepted that the anthocyanins are found in the vacuolar sap of the plant cell (22) (42); that they are dissolved in the cell sap with the cytoplasmic membrane being impermeable to them (92); or that they are dissolved in the cytoplasm (28). Anthocyanin content may vary a great deal but typical values for a number of fruits have been established (19) (34) (48) (120) (141).

Since the structural form of the anthocyanin is determined by pH, this property becomes of utmost importance. Approximate pH values have been obtained from large vacuolated plant cells. The determination of pH by microelectrode methods, which is the most accurate, is difficult in the smaller vacuolated fruit cells (30). The pH of the expressed cell sap is only an average over the whole fruit and may be expected to be a highly buffered solution. pH values of expressed cell sap have been published. Some representative values are; for strawberries 3.29 - 3.62 (147) and cornflowers 3.8 to 5.0 (13). Hayashi and Isaka (121) reported that the cell sap of fruit, flowers, or leaves would average around 5.5, having a range of 2.8 - 6.2.

It has been pointed out that the anthocyanins in some species are located in narrow and specific regions such as the epidermis (Montmorency cherry, apple, cranberry). The pH of these cells may not be that of the expressed cell sap, and microelectrode determination of these pigmented cells would be required. If the assumption is made that some anthocyanin containing flowers have cell sap pH 3.0 to 5.0, there must be a large proportion of pigments in the pseudo base/carbinol base forms. Both forms are prone to decomposition and are not greatly colored. Since

these flowers do retain bright colors, it may be suggested that there are differences in the behaviour of anthocyanins in vivo and in vitro. However, Swain (127) points out that small pH changes may cause greater changes in color at the higher pH levels found in plants.

The anthocyanins, so well characterized in the laboratory are extracted under acidic conditions which may modify them from the form in the cell. Hayashi and his co-workers (64) (94) (113) have obtained anthocyanins from a number of flowers in the crystalline state without using acidic extraction. These pigments were blue in color and they were described as "(anthocyanin)-OH". It is not clear whether these complexes occur in vivo or if they are formed during neutral extraction. The suspicion must be kept in mind that the anthocyanins obtained in the laboratory may only be artifacts of the forms occurring in Nature. This was suggested by the evidence obtained by Saito (114) who measured the transmission spectra of flower tissue between layers of opal glass. Four distinctly different patterns based on the number and location of maxima of absorption could be discerned. Only one was similar to the conventional anthocyanin spectra.

III Identification and Purification of Anthocyanins

1. Identification of anthocyanins

One object of this study was to investigate the properties of as many of the aglycone structures as possible. Large quantities of each individual aglycone glycoside would provide for the best experimental conditions and allow for confirmatory tests. As only a few anthocyanins are commercially available, they had to be obtained from plant tissues and

certain procedures were used for their isolation, purification, and identification.

The techniques used in this study are given in Appendix 1. Descriptions of the experimental methods and their interpretations may be found in a number of review articles (58) (59) (66) (72) or in research papers (27) (117) (143).

On the basis of the various test results, a number of anthocyanins were identified. Table 1 lists the anthocyanins identified in this study and it may be compared with Table 2 which lists anthocyanins found by other researchers in equivalent material.

2. Isolation of anthocyanins in large quantities

The best approach to obtaining large quantities of anthocyanins seemed to lie in a column chromatographic method. The adsorbent chosen was polyvinylpyrrolidone (PVP) on the basis of the initial study of Gallop (51). This method was modified and extended so that a large number of anthocyanins became available. Table 3 shows that certain individual anthocyanins could be eluted as homogeneous bands from the column. It was found that the anthocyanins had a tendency to decompose on storage, even in the dry state under nitrogen. For this reason, the anthocyanins were separated on PVP columns when required.

The experimental procedure for column chromatography using PVP is given in Appendix 2.

TABLE 1. Anthocyanin composition of selected botanical material investigated in this study

Fruit	Variety	Pigment	Preferable method of separation	Ratio of pigment concentration % ⁵	CV ³ %
Strawberry	Redcoat	Cyanidin 3-glucoside	P.V.P. or AWH paper chrom.	17.7	15
		Pelargonidin 3-glucoside		82.3	3
	Ogallala	Cyanidin 3-glucoside	AWH paper chrom.	15.6	14
		Pelargonidin 3-glucoside		76.4	4
		Pelargonidin 3-rutinoside		8.0	10
	Sparkle	Cyanidin 3-glucoside	AWH paper chrom.	17.2	15
		Pelargonidin 3-glucoside		74.5	3
		Pelargonidin 3-rutinoside		8.3	8
Red Raspberry	Boyne ¹	Cyanidin 3-glucoside	AWH paper chrom.	8.8	11
		" 3-rutinoside		25.2	9
		" 3-sophoroside		20.7	10
		" 3-glucosylrutinoside		45.3	3

Black Raspberry	Unknown	Cyanidin 3-glucoside	P.V.P. or AWH paper chrom.	25.5	4
		" 3-rutinoside		60.6	3
		" 3-xylosylrutinoside		13.9	15
Cranberry	Early Black	Cyanidin 3-galactoside	BFW paper chrom.	26.0	8
		" 3-arabinoside		18.5	18
		Peonidin 3-galactoside		45.1	4
		" 3-arabinoside		10.4	10
Grape	Flame Tokay	Cyanidin 3-glucoside	P.V.P. or AWH paper chrom.	not measured ⁴	
		Peonidin 3-glucoside			
	Emperor	Cyanidin 3-glucoside	P.V.P. or AWH paper chrom.	not measured ⁴	
		Malvidin 3-glucoside			
		Peonidin 3-glucoside			
Poinsettia	Mikkelson	Pelargonidin 3-glucoside	P.V.P. or AWH paper chrom.	17.9	4
		Pelargonidin 3-rutinoside		32.1	7
		Cyanidin 3-glucoside		25.5	11
		" 3-rutinoside		24.5	7
	Eche	Pelargonidin 3-glucoside	P.V.P. or AWH paper chrom.	20.8	9
		" 3-rutinoside		31.5	12

Poinsettia	Eche	Cyanidin 3-glucoside		25.3	8
		" 3-rutinoside		22.4	9
Sour Cherry	Montmorency	Cyanidin 3-rutinoside	P.V.P.	27.3	14
		" 3-glucosylrutinoside		72.7	5
Blackcurrant	Buddenborg ²	Delphinidin 3-glucoside	P.V.P. or AWH paper chrom.	14.2	10
		" 3-rutinoside		8.6	9
		Cyanidin 3-glucoside		39.9	5
		" 3-rutinoside		37.3	6

1. The varieties Madawaska and Trent contained the same anthocyanins as the Boyne variety.
2. The varieties Kerry and Climax contained the same anthocyanins as the Buddenborg variety.
3. Coefficient of variation = $\frac{s}{\bar{x}}$
4. Could not be measured as some of the anthocyanins were present in trace amounts only.
5. Ratio of pigment concentration in % determined as the average of six chromatograms which were measured photodensitometrically.

TABLE 2. Published anthocyanin composition of selected botanical material.

Fruit	Botanical name	Variety	Pigment	Reference
Strawberry	Fragaria x ananassa (Duch)	Sparkle or Redcoat	Pelargonidin 3-glucoside	Fuleki (50)
			Cyanidin 3-glucoside	
			two pelargonidin monosides	
			one " bioside	
			one " trioside	
	Fragaria vesca		Pelargonidin 3-glucoside Cyanidin 3-glucoside	Sondheimer and Karash (123)
Black Raspberry	Rubus occidentalis	Bristol	Cyanidin 3-glucoside	Nybom (96)
			3-rutinoside	
			3-xylosylrutinoside	
	Rubus occidentalis		Cyanidin 3-glucoside 3-rutinoside 3-xylosylrutinoside 3-sambioside	Harborne and Hall (57)
	Rubus occidentalis	Monger	Cyanidin 3-glucoside 3,5-diglucoside	Daravingas and Cain (34) (35)

Black Raspberry	<i>Rubus occidentalis</i>	Monger	Cyanidin 3-diglucoside " 3-rhamnoglucoside- -glucoside	
Poinsettia	<i>Euphorbia pulcherrima</i>	Eche	Cyanidin 3-glucoside " 3-rutinoside Pelargonidin 3-glucoside 3-rutinoside	Asen (7)
Blackcurrant	<i>Ribes nigrum</i>	Whitebud	Delphinidin 3-glucoside " 3-rutinoside Cyanidin 3-glucoside " 3-rutinoside	Chandler and Harper (27) Blundstone (19)
Red Raspberry	<i>Rubus idaeus</i>	October	Cyanidin 3-sophoroside " 3-glucoside	Harborne and Hall (57)
	<i>Rubus idaeus</i>	September	Cyanidin 3-glucoside " 3-rutinoside " 3-sophoroside " 3-glucosylsophoroside	Harborne and Hall (57)
Cranberry	<i>Vaccinium macrocarpon</i>	Early Black	Cyanidin 3-galactoside " 3-arabinoside Peonidin 3-galactoside " 3-arabinoside	Zapsalis and Francis (143)

Cranberry	Vaccinium macrocarpon	Howes	Cyanidin 3-galactoside	Fuleki (50)
			" 3-arabinoside	and
			" 3-glucoside	Fuleki and
			Peonidin 3-galactoside	Francis (49)
			" 3-arabinoside	
			" 3-glucoside	
Grapes	Vitis vinifera	Flame Tokay	Cyanidin 3-glucoside	Akiyoshi <u>et al.</u> (5)
			Peonidin 3-glucoside	
	Vitis vinifera	Emperor	Cyanidin 3-glucoside	Akiyoshi <u>et al.</u> (5)
			Peonidin 3-glucoside	
			Malvidin 3-glucoside	
Blueberry	Vaccinium angustifolium (Ait) (low bush)		two delphinidins	
			two petunidins	
			two cyanidins	Wood and Barker (140)
			two malvidins	
"	"		The 3 glucoside and 3 galactosides of delphinidin, malvidin, petunidin, peonidin and cyanidin plus some 3-arabinoside and some diglycosides	Francis <u>et al.</u> (45)
Cherry	Prunus cerasus	Montmorency	Cyanidin 3-glucosylrutinoside	Harborne
			" 3-rutinoside	and Hall (57)

Cherry	Prunus cerasus	Montmorency	Cyanidin 3-rhamnoglucoside a cyanidin 3-diglucoside	Li & Wagenknecht (83)
	Prunus cerasus	Montmorency	Cyanidin 3-gentiobioside	
			" 3-rhamnoglucoside " 3-glucoside	von Elbe <u>et al.</u> (136)

TABLE 3. Separation of large quantities of individual anthocyanins
by P.V.P. column chromatography

Pigment	Fruit	Position on column
Pelargonidin 3-glucoside	Strawberry (any variety)	1st band
Cyanidin 3-glucoside	Black raspberry	3rd band
	Red raspberry (var Boyne)	3rd band
	Strawberry (var Sparkle)	2nd band
	Grape (var Flame Tokay)	1st band
Cyanidin 3-sophoroside	Red raspberry (var Boyne)	2nd band
Cyanidin 3-xylosylrutinoside	Black raspberry	1st band
Cyanidin 3-rutinoside	Black raspberry	2nd band
	Cherries (var Montmorency)	2nd band
Cyanidin 3-glycosylrutinoside	Cherries (var Montmorency)	1st band
Pelargonidin 3-rutinoside	Poinsettia	1st band
Cyanidin 3-galactoside	Cranberry (Early Black)	3rd band
Peonidin 3-galactoside	Cranberry (Early Black)	1st band
Delphinidin 3-glucoside	Blackcurrant (var Buddenborg)	4th band
Malvidin 3-glucoside	Grape (var Ribier)	1st band

Petunidin 3-glucoside	Blueberry (commercially fresh frozen)	2nd band
Delphinidin 3-rutinoside	Blackcurrant (var Buddenborg)	2nd band

3. Sour cherry anthocyanins

In the course of the identification of the anthocyanins in sour cherries, variety Montmorency, (Prunus cerasus), it became apparent that discrepancies were present both in the number and the type of anthocyanin. This was further investigated since cherries formed the basis of a later processing experiment.

3.1. Anthocyanins found by other researchers

Li and Wagenknecht (83) found antirrhinin (cyanidin 3-rhamnoglucoside) and mecocyanin (a cyanidin diglucoside) in nearly equal proportions in sour cherries. Harborne and Hall (57) identified the 3-glucosylrutinoside, 3-sophoroside, 3-rutinoside and 3-glucoside of cyanidin in sour cherry varieties other than Montmorency. von Elbe et al. (136) found that Montmorency cherries contained cyanidin 3-gentiobioside (62%), cyanidin 3-rhamnoglucoside (35%) and cyanidin 3-glucoside (3%). These authors noted that decomposition products due to HCl hydrolysis could occur during isolation procedures with ion exchange resin (135).

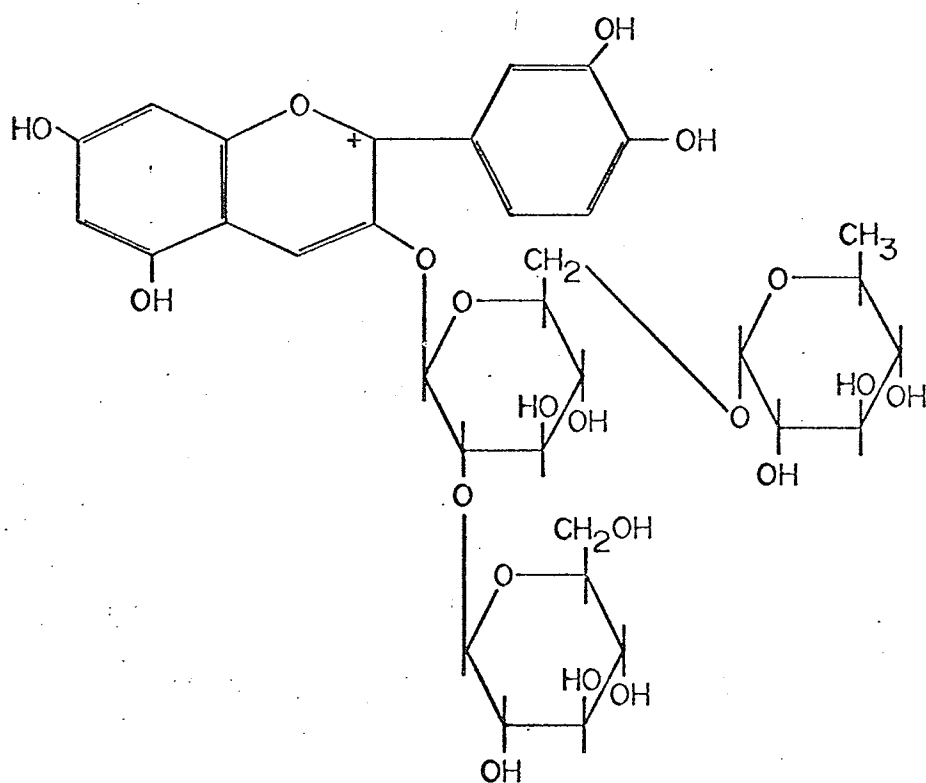
3.2. Anthocyanins found in present study

Variety Montmorency was found to contain only two glycosides, the 3-glucosylrutinoside and the 3-rutinoside of cyanidin. These two pigments were present in freshly prepared extracts separated: i) on paper chromatograms, ii) by PVP column chromatography, or iii) by electrophoresis. They were also present in the covering syrup of canned cherries after isolation by basic lead acetate precipitation. However, multiple bands occurred if the 3-glucosylrutinoside was isolated by ion exchange and the eluate concentrated. Five anthocyanins were then present. They were identified as cyanidin together with the 3-glucoside,

the 3-rutinoside, the 3-sophoroside and the 3-glucosylrutinoside of cyanidin. These products would be expected in view of the sugar-sugar or sugar-aglycone linkages that occur in cyanidin 3-glucosylrutinoside (XXX). Partial hydrolysis of this pigment provides the same five anthocyanins. This anthocyanin was readily decomposed even by storage in dry form under nitrogen. On partial hydrolysis the pigment disappeared after the first five minutes. Thus, cyanidin 3-glucosylrutinoside may be considered fairly labile.

von Elbe and his co-workers (116)(135) (136) reported the presence of three anthocyanins which did not include a triglycoside. One of the three was identified as a gentiobioside. The other two pigments, the 3-glucoside and 3-rutinoside, are commonly accepted as being present. It is suggested that the gentiobioside was incorrectly identified and may have been a trisaccharide. The trisaccharide pigments are readily distinguished on AWH paper chromatograms because of their very high R_f values between .65 and .80. The disaccharides have R_f values of lower magnitude. Furthermore, it may be noted that the densigram tracing presented by these authors (135) shows a different order of R_f values for paper chromatograms developed with 1% HCl than that obtained by Harborne (Table 4) and by the present writer.

von Elbe et al. (136) used an elegant method of electrophoresis



XXX

whereby cherry skins were applied directly to chromatography papers.

The skins after electrophoresis were devoid of color showing complete anthocyanin extraction. Three bands were obtained. This experiment was duplicated in the present study and only the two previously identified pigments were found. The mobilities are given in Table 5 where they may be compared with those obtained by von Elbe et al. (136). This approach has considerable merit as there should be no possibility of decomposition of the anthocyanin pigment. See method in Appendix 1, Section 1.4.

Table 4

Order of anthocyanins migration in 1% HCl as found by von Elbe and by Harborne (starting with the least mobile).

von Elbe <u>et al.</u> (135)		Harborne (59) (R_f in brackets)	
cyanidin		cyanidin	
"	3-glucoside	"	3-gentiobioside (.14)
"	3-rhamnoglucoside	"	3-glucoside (.17)
"	3-gentiobioside	"	3-rutinoside (.19)

Table 5

A comparison of the electrophoretic mobilities of some cyanidin glycosides.

Anthocyanin	Mobility $\times 10^5$	
	von Elbe <u>et al.</u> (136) N=6	obtained (in this study).
Cyanidin 3-glucoside	.92 $\pm$.06	.41 $\pm$.11 (n = 5)
" 3-sophoroside		1.57 $\pm$.33 (n = 14)

Table 5 (continued)

Anthocyanin	Mobility x 10 ⁵	
	von Elbe <u>et al.</u> (136) N=6	obtained (in this study)
Cyanidin 3-rutinoside	1.69±.06	.74 ± .17 (n = 36)
" 3-gentiobioside	3.11±.06	
" 3-glucosylrutinoside		2.08 ± .28 (n = 26)
" 3-diglycoside	2.58±.06	
Cyanidin		.12 ± .05 (n = 5)

The fact that von Elbe et al. (136) found three pigments in Montmorency cherries whereas only two were found by Li and Wagenknecht (83) and myself shows that there must be different strains within the Montmorency cherry variety. Harborne and Hall (57) found cyanidin 3-glucosylrutinoside, 3-rutinoside, 3-sophoroside and the 3-glucoside in the Wye Morello variety of sour cherries. The present research has shown that cyanidin 3-glucosylrutinoside is easily hydrolysed under acid conditions. It seems possible that some of the glycosides obtained by Harborne and Hall (57) were artifacts resulting from the hydrolysis during the isolation and purification procedure.

IV The Isolation and Investigation of a Blue Colored Complex from Canned Blueberry Juice

1. Introduction

The glycosides of the six common aglycone structures have absorption maxima in the visible region differing by about 36 nm (calculated from data presented by Harborne (59)). It seems unlikely that

this small wavelength range could explain the great diversity of fruit and flower color ranging from orange red to deep blue.

An even greater problem arises when it is realized that at physiological pH values of the plant, the anthocyanin pigments should be predominantly in the unstable and faintly colored pseudobase form. It may be reasoned that the vacuolar pH is much lower than the cell sap. However, the concept that the vacuole is a waste storage organ is now being questioned and this would lead to questioning the assumption of very low pH values in the vacuole. The problem is still present in that the pH values of canned fruit products vary around pH 3.5, which is not best for the stability of anthocyanins, nor does it maximise the highly colored oxonium form. These interrelated problems are investigated in this section.

2. Literature Review

Three theoretical explanations have been offered to explain the anthocyanin color and stability in the plant cell.

2.1. Differences in pH

Willstatter (180) isolated cyanidin 3, 5-diglucoside from the flowers of roses and cornflowers. He attributed the red color of the rose to the red oxonium salt which would be present in acidic media. The blue cornflower was postulated to contain the alkali metal salt, or the stable ionized anhydro base of the anthocyanins which are present at higher pH values. This would require a large species to species variation in the physiological pH of the plant cell sap which seems unlikely (63).

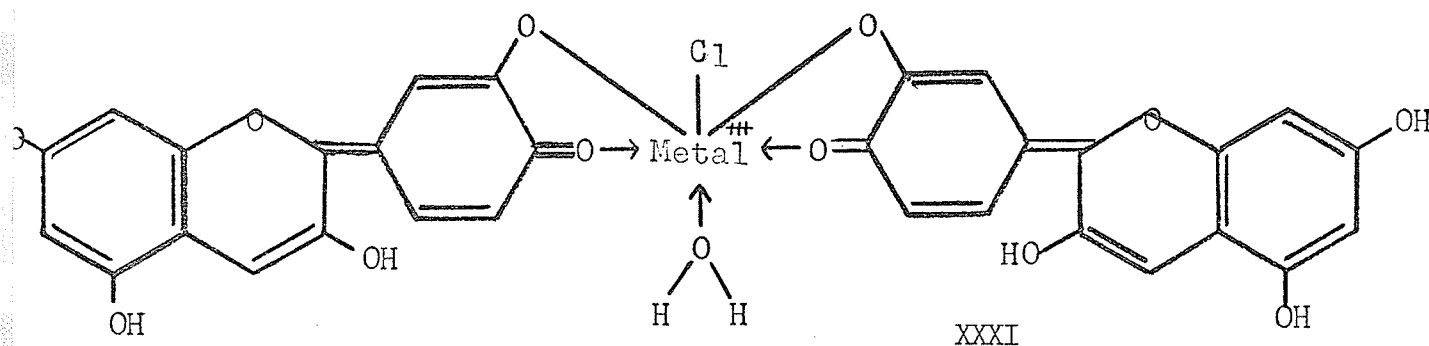
2.2. Co-pigmentation

Robinson and Robinson (107) (108) (109) explained the deep blue colors at acidic pH levels as a co-pigmentation effect with other substances. Co-pigmentation has the effect of producing a deeper blue than would be expected from consideration of the spectra of pure anthocyanins. Since these early reports, other workers have substantiated this effect (77) (82) (99). For example, pelargonidin chloride produced a bathochromic shift with caffeine of about 23 nm which decreased on heating but returned to the original value on cooling (77).

Harborne and co-workers (55) (56) have shown that small bathochromic shifts of 1-5 nm in the visible region of the spectrum occurred when cyanidin or malvidin glycosides were mixed with either quercetin or kaempferol (i.e., co-pigmentation). How this phenomenon occurs or what type of bonding is involved is not known. (For further reviews see (58) (61) (66)).

2.3. Cation Complexes

The third theory involves metal ion complexes of anthocyanins with or without other components. The complexing reaction of anthocyanins containing ortho hydroxyl groups is well known. Bayer et al. (12) have provided evidence for the structure of the anthocyanin- Al^{+++} complex, as shown in XXXI.



The complex is pH dependent, as has been quantitatively shown by Jurd and Asen (74). Maximum complexation was found to occur at pH 5.5. Absorbance of a solution of the complex decreases to about 70% of the initial value during the first hour, after which the rate of decomposition levels off to about 60% of the initial value. Ferrous and ferric salts (74) and molybdate, Sn^{+4} , Ti^{+3} , Cr^{+3} , and UO_2^{+2} also form blue complexes between pH 4 to 7, while none of the alkaline earth metals, cobalt or nickel form blue complexes (10) (13).

Cupric ions were found by Somaatmadja et al. (119) to produce a shift in the ultraviolet region of the spectrum, even with malvidin 3-glucoside. At pH 3.5 and 4.5, the complex existed in 1:2 cupric ion to malvidin 3-glucoside ratios and intermolecular bonding was thought to occur between the two 4' hydroxyl groups. At pH 3.9, the ratio was 2:1 which could be explained only by intramolecular bonds. At pH 5 to 6, the cupric ion and the anthocyanin were present in 1:1 proportions. Their evidence, obtained by polarographic methods, suggests that either methoxyl groups do not effect hydrogen bonding or that other groups than those on the B ring might be involved.

Harper (62) has found that the cupric ion formed a complex with the anthocyanins present in blackcurrant juice at pH 4.5. The bonding site was not the B ring. Quercetin, a flavonol, could complex with the cupric ion even at pH 2.3. Clarke and Geissman (29) also found that quercetin complexed certain metallic ions. The active sites were the 3' and 4' dihydroxyl grouping and the 3-hydroxyl 4-ketone groups.

Complexes containing metals, anthocyanins and a number of other components are also known. Jurd and Asen (74) indicated that in the

presence of aluminium salts, quercitrin, chlorogenic acid or methyl gallate will form blue colored, insoluble complexes with cyanidin 3-glucoside in acetate buffer at pH 5.45. The anthocyanins at that pH would be expected to be in the anhydro base form. Citric acid, if present, does not allow the formation of the complex, as it will chelate preferentially with the aluminium. These authors found that a mixture of quercitrin and cyanidin 3-glucoside had no effect on the spectrum at pH 5.5. The anthocyanin and aluminium formed a "cerise" colored complex with absorption maximum at 555 nm. The solution containing all three components showed an increase of 45% in absorbance and a bathochromic shift to 580 nm.

Harborne (58) has stated three requirements for blueing of anthocyanin by chelation.

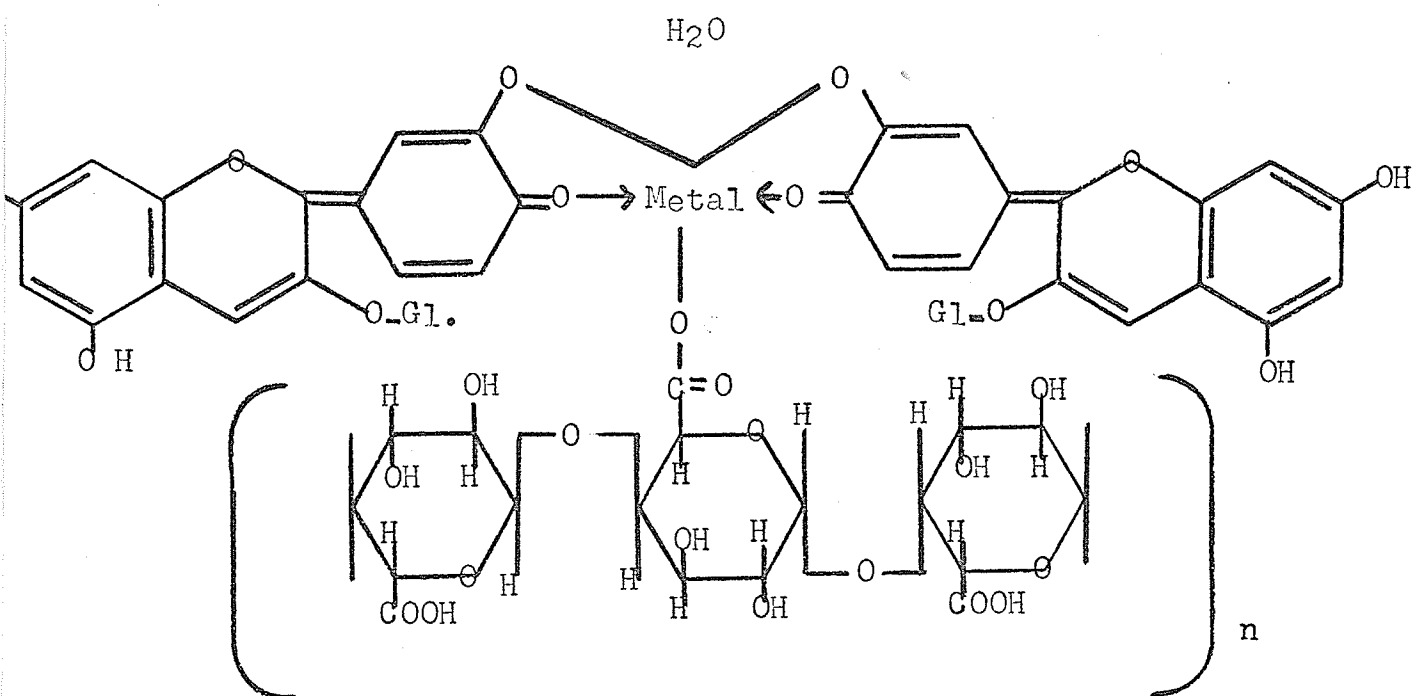
1. Anthocyanin must have catechol nucleus
2. A chelating metal of any valence
3. Availability of the chelating metals.

He also pointed out that in plants containing anthocyanins that have no ortho hydroxyl groups in the B ring, other mechanisms than chelation of metal ions must be used to explain the deep blue colors found in certain plant tissues.

- 2.4. Complexed, macromolecular forms of anthocyanins isolated and identified in plants.

A number of naturally occurring metal complexes of this type are known. Bayer et al. (13) have shown that a cyanidin 3, 5-diglucoside - Al^{+++} -pectin complex occurs in the blue cornflower. They named this pigment protocyanin. Extraction with mineral acid destroyed the

protocyanin. The complex was isolated by precipitation with ethanol, was non-dialyzable and was negatively charged, as shown by electrophoresis at pH 4.6. The molecular weight was found to be 6200 and the complex contained 19.2% of cyanidin and one equivalent of Fe^{+++} and Al^{+++} per mole of cyanidin, with traces of other material present. 80% of the protocyanin was shown to be a polysaccharide which was probably a pectic substance. The possible structure is shown below. XXXII.



XXXII

These results have been contradicted by Saito, Mitsui and Hayashi (111) and by Hayashi, Saito and Mitsui (65). These authors found

that protocyanin had a molecular weight of 20,000, contained 2 atoms of potassium and 8 molecules of cyanin. The aluminium reported to be present by Bayer et al. (13) was found only in trace quantities. Potassium ion content could be reduced on prolonged dialysis.

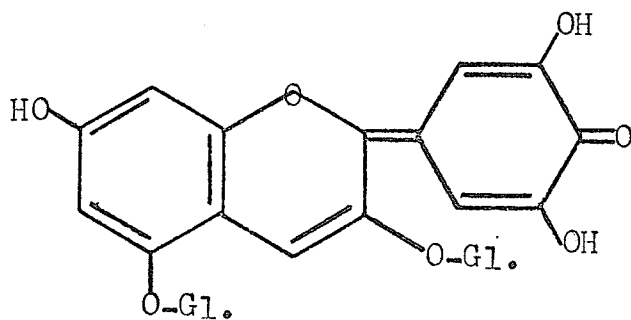
Asen and Jurd (6) identified a crystalline blue cornflower (Centaurea cyanus L.) pigment which consisted of 4 molecules of cyanidin 3, 5-diglucoside and 3 molecules of a "bisflavone" glycoside. The hydrolysis of the bisflavone produced 7-O-methyl apigenin and an unidentified flavone similar to vitexin. The authors named this complex "cyanocentaurin". The component formed stable, deep blue solutions in neutral aqueous media with broad absorption bands at 570-600 nm. Absorption at 510 nm was present, which is characteristic of anthocyanins, as well as a secondary maximum at 319 nm, which represents the bisflavone glycoside. Cyanocentaurin contained 1-2% iron.

Hayashi and co-workers (64) (94) described a blue complex of similar type from Commelia communis, which they called commelinin. In aqueous solvents, the compound had absorption maxima at 643, 591, 316 and 273 nm while in 2% HCl only the three absorption maxima were found; 528, 317 and 274 nm. Hydrolysis with 20% HCl produced 27.33% of delphinidin (as the chloride), 30.7% glucose, and 11.83% of p-coumaric acid. The potassium in this complex could be removed with 1% HCl without color loss. However the removal of the magnesium resulted in the destruction of the blue color. The following metal ions were found: .42% magnesium, 1.77% potassium and 0.29% sodium. In AWH (5:1:5), these authors found two fluorescent spots which could not be identified. Asen and Jurd (6) have suggested that commelinin may be the same compound as

cyanocentaurin.

Saito and his co-workers (111) (112) characterized a blue pigment also obtained from cornflowers. This pigment, after hydrolysis, was found to contain some peptides, carbohydrates, cyanin (8 parts), iron (1 part), potassium (24 parts), magnesium (2 parts) and 16% flavone. The molecular weight of the complex was 20,000. The potassium was shown to be dialysable. Potassium has been reported to be the only metal ion present in the violet pigment isolated from the Viola species. (67). Bopp (21) has postulated that the colloidal state of the complex may be a factor in the color change from blue to red found in Streptocarpus species.

Bayer et al. (13) have studied a blue pigment from the Larkspur which was sparingly soluble in water and non-dialysable. It was shown to be a pectin-delphinidin glycoside complex without structural metal ions. An apparently identical compound was synthesized in his laboratory. Complexes of this type would only form with delphinidin glycosides, not with the aglycone. Cyanidin glycosides could not replace delphinidin glycosides. The pH value was also critical; a pH of 4.7 was necessary at which the pigment would be expected to exist in the anhydro base form, XXXIII.



XXXIII

3. Experimental

3.1. Isolation of the colored complex.

Standard method.

Blueberries, Canada Fancy, 14 oz tins, processed by Berryland Canning Co. Ltd., of Haney, B.C., were bought from a local store. The cans were made from electrolytic tin plate having an enamelled inside. No additives, other than 30% sugar were used and the processing followed normal plant practice.

The juice was allowed to drain through cheesecloth and then centrifuged for 10 minutes at 2000 rpm. The pH and S.S content was determined, then five volumes of ethanol were added. After stirring the mixture for five minutes, the solution was filtered. The gelatinous precipitate thus collected was resuspended in distilled water and placed in dialysis tubing. Dialysis for 2 days against distilled water was carried out, after which the solute inside the tubing was reprecipitated with another five volumes of ethanol. The precipitate was separated by filtration and it was dried and used for a number of characterization tests.

3.2. Alternative method of isolation of the colored complex.

Two alternative methods for the isolation of the blueberry complex were developed.

Method A.

Some hydrated P.V.P. was added to the blueberry juice and stirred for one day. The mixture was allowed to settle and a blue supernatant liquid was noted. After filtration to remove the P.V.P. with the adsorbed anthocyanins, the filtrate was treated with five volumes of ethanol.

The gelatinous precipitate was filtered and dialysed.

Method B.

The blueberry juice was placed on a column of Zerolit 226, weak cationic ion exchange resin in the hydrogen form. A blue colored solution migrated rapidly through the column while the free anthocyanins were adsorbed in a thick band at the top of the column. The eluted blue colored solution was treated with five volumes of ethanol and the gelatinous material removed by filtration.

3.3. Characterization tests

These tests were carried out after the precipitation of the complex with ethanol, after dialysis for two days and a final reprecipitation.

3.3.1. Identification of anthocyanins and anthocyanidins.

The blue colored solid was ground to a powder and dissolved in 20% HCl and allowed to stand for 3 days. After this storage period, 5 volumes of ethanol were added and the nearly colorless gelatinous precipitate was removed by filtration. The ethanolic solution was concentrated to dryness in a rotary evaporator and after dissolving the residue in methanol, suitable volumes were spotted onto paper chromatograms. The papers were developed in BAW, AWH and 1% HCl solvents. The remaining solution was hydrolysed in formic acid and the aglycone(s) were determined by paper chromatography in Forestal solvent.

3.3.2. Identification of hot acid hydrolysis products.

Some of the solid complex was dissolved in 2N HCl and sealed in a glass ampule. The tube was heated in an oven for 24 hours at 100°C. The hydrolyzate which showed considerable brown insoluble material was

spotted on cellulose thin layer chromatography plates and chromatographed in two dimensions with BAW and pyridine-water (4:1) to separate amino acids. The spots were identified with ninhydrin solution and compared with a template made from chromatographs of known amino acids.

3.3.3. Effects of enzymatic attack on the complex.

The complex was dissolved in water adjusted to pH 4.5 with HCl and divided into six aliquots. To each of four aliquots, one of four enzymes was added. The enzymes were pectinase, Pectinol, pectin methyl-esterase and papain which was activated by Na_2S . The papain was compared to a blank which also contained the Na_2S while the sixth aliquot was compared with the pectic enzymes. The enzyme digested samples were centrifuged and the supernatant spotted with a number of reference compounds on paper chromatograms, which were developed in BAW and EtAc. After development, the papers were sprayed with Partridge reagent (98) to identify the sugars present.

3.3.4. Cation analysis of the dried complex.

The solid form of the blue complex and a sample of pectin obtained from J.T. Baker Chemical Comp. (Phillipsburg, New Jersey) were analysed for various ions by x-ray fluorescence spectroscopy. The ethanol precipitate from strawberries, raspberries and blueberries were also analysed by this method.

3.3.5. Quantitative analysis of the complex.

The total nitrogen was determined on the dried complex by a Kjeldahl digestion followed by microdiffusion. The total pectin content of the dried complex was measured by the carbazole colorimetric method (89) (90). The polyphenol composition of the complex was determined by a

modified Folin-Ciocalteu (44) method.

3.3.6. Approximate molecular weight determination.

Attempts were made to determine the molecular weight of the complex using the Beckman ultracentrifuge. Three runs were made with a standard cell assembly in a AN-4-181 rotor at 60,000 rpm for 32 minutes; 6,000 rpm at 20 minutes and 10,000 rpm for 10 minutes. The last two runs used the ultra-violet optical determinations while the first run used the Schlieren optical system.

3.3.7. Effects of a sequestering agent on the complex.

The complex was dissolved in water adjusted to pH 4.5 and 2 g of EDTA (disodium salt) was added. The solution was placed in dialysis tubing and dialysed against pH 4.5 water (adjusted to pH 4.5 with HCl) for 4 days. In a further experiment, a solution containing the complex was dialysed against a 0.5% EDTA solution adjusted to pH 4.5.

3.3.8. Spectroscopic properties of the complex.

The blue complex dissolved in distilled water, was filtered through a 0.45×10^{-6} m Millipore membrane. The complete spectrum between 850 nm and 200 nm was scanned using a SP 800 Unicam spectrophotometer. For comparisons, the spectra of juice diluted with distilled water, adjusted by HCl to the pH value of the juice, was also obtained.

The complex was dissolved in 0.1 N HCl, 2N HCl and distilled water and the spectras were determined. The pH of the complex solution in distilled water was gradually raised to pH 12 by the addition of 1N NaOH and color and spectrum noted at each pH level.

3.3.9. Chromatographic and electrophoretic properties of the complex.

The dissolved blue complex was spotted on a number of paper

chromatograms and chromatographed in BAW and AWH. A strip of each chromatogram was sprayed with Partridge and ninhydrin solution.

The dissolved blue complex was applied to sheets of Whatman No. 3MM papers and run on the Savant electrophoresis apparatus using citric acid pH 2.0 and acetate buffer pH 4.45 at various voltages and for various lengths of time.

3.3.10. Stability of complex to heat and light.

The dissolved complex was heated at about 94°C . for one hour in a boiling water bath. The absorbance and absorbance maximum before and after heating were determined to obtain the percent loss and to detect possible shifts. A sample was stored on a window ledge for 2 months and the absorbance and absorption maxima were measured. These two parameters were also determined after heating at 30, 45, 50 and 70°C in a circulating water bath for 4-6 hours.

3.4. Attempts to isolate anthocyanin complexes from fresh frozen blueberries and raspberries.

Fresh frozen blueberries purchased from a local store and raspberries, variety Boyne, were pressed through cheese cloth when nearly thawed, to obtain the juice. After the juice was centrifuged, each fruit sample was divided into two parts and stored for 2 days at refrigerator temperatures to equilibrate. After storage, one sample of each fruit was heated for 2 hours at 80° . All four samples were then precipitated with five volumes of ethanol. The precipitates were collected in the manner previously described.

3.5. Comparison between the AIS of canned blueberries, strawberries and raspberries.

The drained blueberries previously described were homogenized

with distilled water and allowed to stand for two days. The slurry was centrifuged and the supernatant was treated with 5 times its volume of ethanol. The color of the precipitate that formed was noted. Similar preparations were made from drained canned strawberries and raspberries.

4. Results

The canned blueberry juice pH 3.35, contained 20.5% soluble solids. Further analytical data may be found in Table 6.

After dialysis, only trace quantities of color were found in the water surrounding the dialysis tubing. Thus, the precipitation step seemed to provide a complex without much extraneous, freely diffusible material.

The percentage of the color of the juice due to the complex present in the canned product was determined by measuring the absorbance of suitable dilutions of the juice and the precipitated and redissolved complex. The percentage was calculated by dividing the absorbance of the complex by the total absorbance of the juice and expressed as a percentage. Four cans of differing code dates were tested and the percentage of color due to the complex varied between 24% and 50%. On a weight basis, 100 ml of juice produced 0.31 g to 0.38 g of the oven dried complex.

The blue complex was not adsorbed on P.V.P. or on a cation exchange resin. This led to the conclusion that the complex did not have a net positive charge.

The procedure adopted for isolating the blue colored complex in this laboratory, was very similar to that used by Asen and Jurd (6). They extracted blue cornflowers with water and precipitated the pigment

TABLE 6. Chemical composition of the syrup and of the AIS of canned blueberries, strawberries and raspberries

Syrup				AIS						
Fruit	pH	S.S%	Total pectin/100 ml as mg galacturonic acid	Total pectin /100g as galacturonic acid	Al ⁺⁺⁺	Na ⁺	Ca ⁺⁺	Mg ⁺⁺	K ⁺	Fe ⁺⁺⁺
Strawberry	3.30	23.1	382	437 mg	+	+	+	+	+	+
Raspberry	3.05	24.5	280	377 mg	trace	+	+	+	+	+
Blueberry	3.35	20.5	184	788 mg	trace	+	+	+	+	+

with ethanol. After a number of precipitations the pigment crystallized from ethanol. Both Bayer et al. (13) and Hayashi et al. (64) isolated blue complexes by ethanol precipitation.

4.1. Characterization tests

4.1.1. Identification of anthocyanins and anthocyanidins

To identify the components in the blue complex, a suitable method had to be found to decompose the complex. As complexes of this type are decomposed when extracted under cold acidic conditions, treatment with acid seemed the best approach. The precipitate, after HCl treatment, retained some red color even when washed twice with 100 ml of ethanol.

The spectrum of the ethanolic solution showed absorption maxima at 545 nm and 278 nm both characteristic of anthocyanins. In 0.01% HCl in methanol solution, an absorption maximum was found at 540 nm. The ratio

$A_{440\text{nm}}/A_{\text{max}} \times 100$ was 28 with a shift of 20 nm when 2 drops of AlCl_3 solution was added. This indicated that an ortho dihydroxy grouping was present on the anthocyanin component. After hydrolysis, the paper chromatogram showed two bands when developed with Forestal solvent. By comparison of R_f values, and relative R_f values to marker aglycones, delphinidin and petunidin were found to be present. These two aglycones are similar in that both have free ortho hydroxyl groups on the B ring and are substituted at the 5' position by a free hydroxyl group in the former case and a methoxyl group in the latter case.

Chromatographic separation with AWH, BAW and 1% HCl solvents showed that the hydrolysate contained four bands listed in Table 7.

TABLE 7. Tests made on paper chromatograms of samples taken from complex treated with 20% HCl.

Band	$\text{Fe}^{+++}/\text{K}_3 [\text{Fe}(\text{CN})_6] \equiv$	AlCl_3 Spray	R_f values		
			BAW	AWH	1% HCl
1.	+	-	.00	-	.05
2.	+	+	.05	0.17	.05
3.	+	+	.09	0.21	.05
4.	+	-	.12	0.24	.05

These results suggested that the pigments present in the complex were delphinidin and petunidin glycosides. Possibly leucoanthocyanins (anthocyanogens) were also present. Evidence for the latter was the colored band $R_f = .00$ in the BAW chromatogram which is typical of leucoanthocyanins. In view of the fact that acidic decomposition of the complex had been accomplished in the cold, there was no chemical reason why leucoanthocyanin should not have been present. The $\text{Fe}^{+++}/\text{K}_3 [\text{Fe}(\text{CN})_6] \equiv$ spray showed blue bands only in the anthocyanin positions, hence polyphenolic compounds did not occur at other places on the chromatograms. There was no evidence of blue, yellow or dark bands on the chromatograms when the latter were viewed under UV light, and this again suggests the absence of polyphenols other than the anthocyanins already mentioned.

4.1.2. Identification of hot acid hydrolysis products.

The 24 hour acid hydrolysate showed no evidence of the presence of carbohydrate material. If pectic substances were present, some evidence of these should have been found in the hydrolysate. Copious amounts of brown polymerized material suggested that the carbohydrate

material had decomposed, especially since uronic acids may be easily lost even under mild conditions. Thin layer chromatograms showed nine definite ninhydrin reacting spots. Six of these, appeared to have the following identities: alanine, serine, histidine, glycine, methionine and leucine.

4.1.3. Effects of enzymatic attack on complex.

When the complex was hydrolysed by the various enzymes, a blue colored precipitate was found after 24 hours incubation with all but the papain and pectin methylesterase. The supernatant was completely colorless, while the precipitate retained the blue color. This suggests that the pectic enzymes had hydrolysed very high molecular weight pectic substances into short chain polygalacturonic acid types that are normally sparingly soluble in water but which retained the anthocyanin pigment. Pectin methylesterase is specific for the ester linkage and did not show any precipitate.

The supernatant of the other pectic enzymes showed large amounts of galacturonic acid, glucose and arabinose when chromatographed in BAW and EtAc. Although some glucose was found to be present in the enzyme preparation, the evidence still strongly suggested the presence of polysaccharide material including pectic substances. Hydrolysis with papain did not produce any effects other than a change in color. A slight color difference of hue rather than intensity between the blank treatment (with Na_2S) and the enzyme activated with Na_2S , was present but not enough to suggest that proteins were involved in essential bonding functions.

These experiments were deficient in that it was not possible to submit samples of the enzyme hydrolysate to spectrophotometric examination. The insolubility of the pectin enzyme hydrolysed precipitate

prevented this. In the case of papain, the effect of sodium sulphide upon anthocyanins is not well known and hence conclusions made about the experiment must be interpreted with discretion.

4.1.4. Cation analysis of the dried complex.

Results obtained for various cations of the complex and the pectin used in this study are shown in Table 8. Pectin as supplied by J.T. Baker Chemical Co., contained a significant level of cations, especially trivalent cations, which could form complexes with anthocyanins.

It is difficult to draw any firm conclusions from the cation analysis. Perhaps some potassium may be adsorbed by an ion exchange mechanism on the pectic chains. Alternatively, since it is present in the greatest amount, it may be a required and necessary constituent of the complex. The complex found by Hayashi and Takada (67) also had a high content of potassium ions.

TABLE 8. Concentration of certain cations present in the blue complex isolated from blueberries and in a commercial sample of pectin as determined by x-ray fluorescent spectroscopy.

	Blue complex	Pectin
Al	.133%	.090%
Fe	.118%	.048%
Ca	.129%	.021%
K	<u>.673%</u>	<u>.028%</u>
Total	1.053%	.187%

4.1.5. Quantitative analysis of the complex.

The following quantities of each component was found in the blue complex. (See Table 8A).

TABLE 8 (Continued) Proximate analysis of the blue complex.

TABLE 8A.

Crude protein on the basis of 6.25 conversion factor	2.81%
Total pectin	60.0%
Polyphenolics	4.0%
Total cations	1.05%
S value	1,000
Ash	2.1%

4.1.6. Approximate molecular weight determination.

The S value bears a relationship to the molecular weight.

As the extremely high S value could not be used to find the molecular weight by the standard graphic method, a rough estimate of the molecular weight was obtained by comparison with known S values. T bacteriophage has an S value of 487 corresponding to a molecular weight of 37,500,000. Polyhedral silkworm virus has a S value of 1871 with a molecular weight of 916,200,000 (118). This provides an estimate of molecular weight between 77,000,000 and 490,000,000. No estimate of the homogeneity could be obtained other than that sedimentation occurred evenly.

4.1.7. Effects of sequestering agent on the complex.

To test the nature of the bonds linking possible ligand to cations, EDTA was added to the dissolved complex and the mixture placed in dialysis tubes and dialysed against water at pH 4.5. No color penetrated the dialysis tubing and the color of the complex/EDTA solution remained unchanged. At this pH, the EDTA should complex preferentially with Al^{+++} and Fe^{+++} . It seems from this small experiment that the cations present in the complex were unaffected by the sequestering action of EDTA. Alternatively, the cations may have been sequestered but, in

the latter case, they would be obviously unessential to the maintenance of the color and stability of the complex.

4.1.8. Spectroscopic properties of the complex.

The spectra of the blueberry syrup and complex were compared. A shoulder at 310 nm was present in the syrup which was absent from the complex. The syrup at pH 3.1 had an absorption maximum at 527 nm while the complex dissolved in pH 3.1 water had an absorption maximum at 561 nm. (Figure 1 and 2).

The spectrum of the complex was very similar to the spectra of the constituent anthocyanins after the latter had been complexed with Al^{+++} ions. This is shown in Figure 3 and suggests either aluminium is involved in the complex or that bonding between ligands occurs in a similar manner to the ligand-aluminium system.

The complex, dissolved in 2N HCl, was red in color with an absorption maximum at 520 nm. The complex when dissolved in 0.1 N HCl showed an absorption maximum at 550 nm. A slight fading with time was noted in this solvent.

The color of the complex changed as the pH was increased. Usually the intensity of the blue color decreased towards pH 6.0 to 7.0 where a greyish blue tinge was noted. At pH 8.5 a yellow color was noted which increased in intensity with increasing pH. The spectrum showed loss of the 560 and 275 nm peaks. The yellow pigment at the high pH values showed no diagnostic or distinctive maximas.

4.1.9. Chromatographic and electrophoretic properties.

It was found that the complex was stationary on paper chromatograms run with BAW solvent. However, in AWH two colored bands

FIGURE 1. Spectral curve of processed blueberry juice (syrup) after dilution 10 to 100 with pH 3.1 adjusted water.

FIGURE 2. Spectral curve of the blue complex isolated from the processed blueberry juice (syrup) dissolved in pH 3.1 adjusted water.

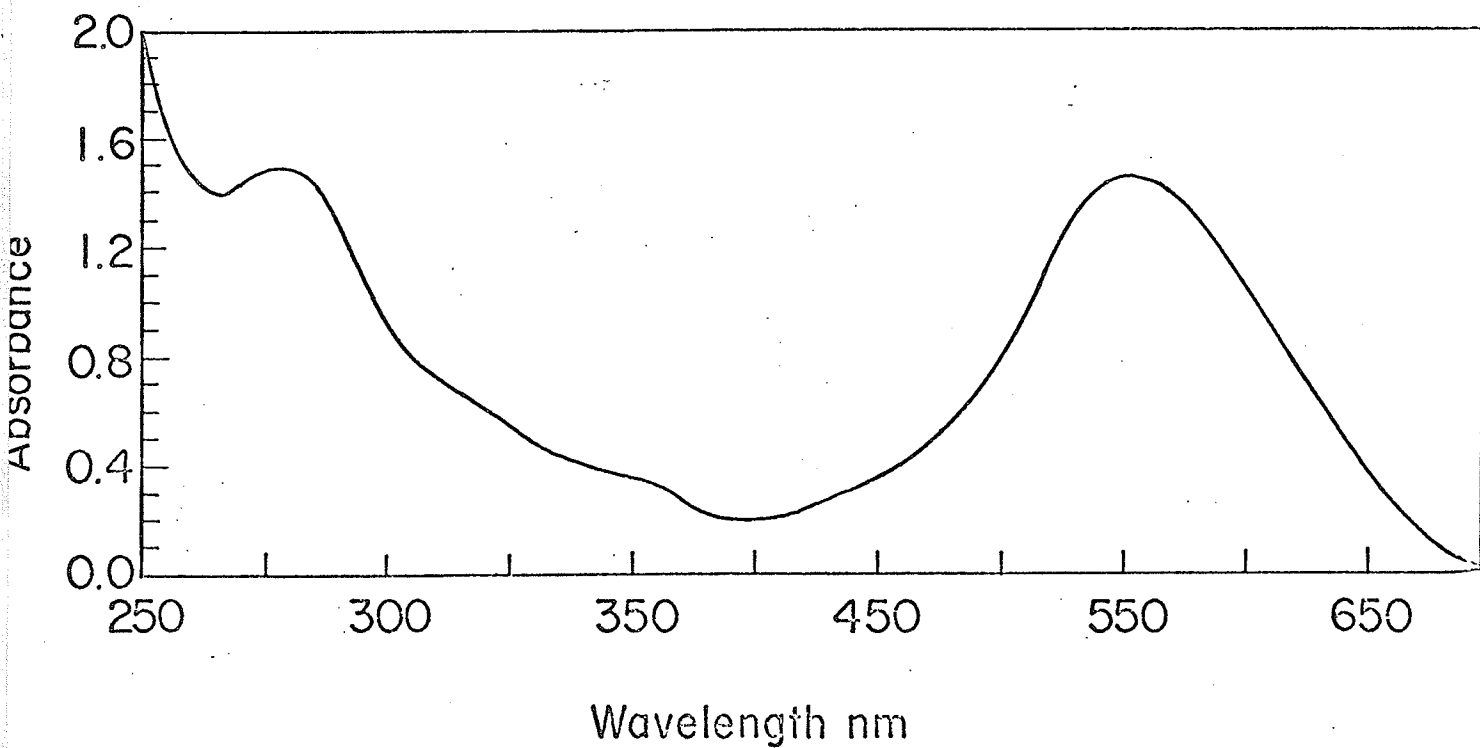
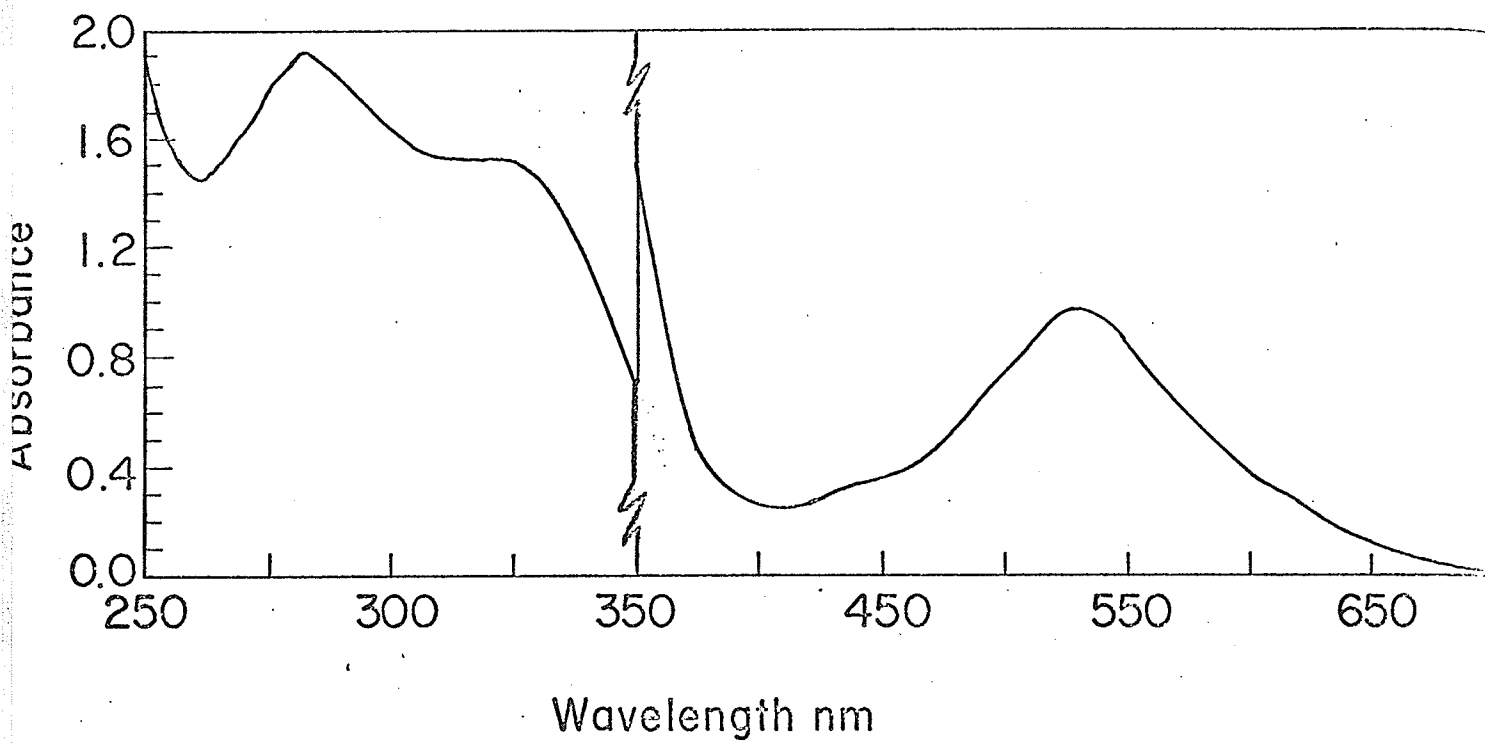
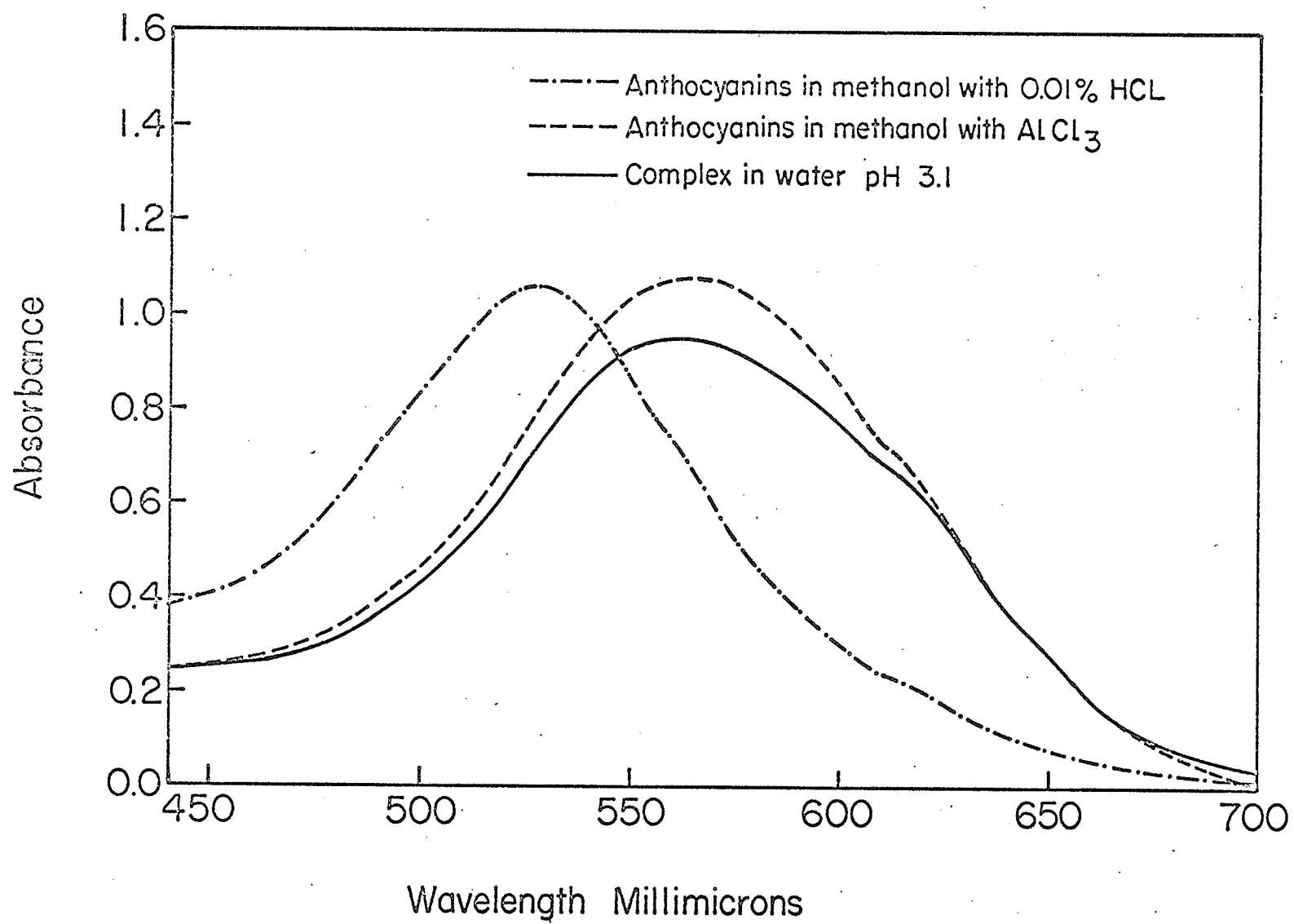


FIGURE 3. Spectrum of the isolated blueberry complex and the anthocyanins liberated from the complex by 20% HCl. (—) anthocyanin after the addition of 3 drops of AlCl_3 , (---) blueberry complex, (-.-.-) blueberry anthocyanins after the destruction of the complex.



migrated downward at R_f .17 and R_f .20. It was supposed that the acidity of this solvent had decomposed the complex yielding the free anthocyanins.

In order to identify the other breakdown products (polysaccharides or proteins) the papers were sprayed with Partridge and ninhydrin reagents. No evidence was found for the presence of ninhydrin reacting material or substances that react with Partridge reagent. Pectin, if present in the complex, would probably remain at the start line. In any case, this compound does not react with Partridge reagent and therefore would not be detected in this test.

The colored complex when applied to paper electropherograms did not migrate in either pH 4.45 or pH 2.0 buffers. Voltages of 300 to 2,000 volts for up to 6 hours were tried. Marker anthocyanin pigments did migrate under these conditions. One conclusion that could be drawn was that the complex was unchanged. But the lack of mobility might also be attributed to the large size of the molecules of the complex which could not move through the pores of the paper support.

4.1.10. Stability of complex to heat and light.

The stability of dilute solutions of the complex to heating was tested at a number of temperatures. A shift of 23 nm was produced at pH 2.5 when the complex was heated in a boiling water bath. After cooling, the absorbance maximum shifted back 5 nm. This seems to suggest some reversible mechanism operating in the blue complex. A fine precipitate was noted after storage for two weeks of the heated complex. The percent color loss of the complex in solution at pH 4.5 after one hour in a boiling water bath, was 58% measured by absorbance differences. No shift in absorption maximum was noted.

When the complex was heated for 6 hours at pH 3.5 in a 50°

water bath, 10% of the color was lost and a hypsochromic shift of 5 to 8 nm was present. At 70° a greater hypsochromic shift of 30 nm occurred after 3 hours of heating. It seems that the stability to heating depended on both the pH and temperature.

The complex stability to light at pH 3.1 was tested and a slow but steady decrease in absorbance occurred over a period of two months. The total decrease over this period of time was 20% of the original value.

4.2. Attempts to isolate anthocyanin complexes from fresh and processed fruit.

A logical question was whether the complex existed only in the syrup of canned blueberries. A water extraction (at pH 5.0) of drained canned blueberries was only faintly colored. After precipitation with ethanol, the thin gelatinous mass did not retain blue pigmentation. It seems that the water soluble blueberry color complex was not present in the berries. This is reasonable since little soluble pectin could be expected to be left in the fruit. Any complex existing in the processed fruit would be the result of bonding of anthocyanins to insoluble constituents of the tissue and it would not be extracted under the conditions used.

The heated and unheated samples of the expressed syrup of blueberries when added to 5 times the volume of ethanol did not produce any gelatinous precipitate. The raspberry samples when precipitated were light brown in color, with the heated sample having a slightly darker shade. From these results, it seems that no complex of the type found in processed blueberry syrup was present in the raspberries.

Absence of detectable quantities of complex from the syrup of fresh frozen blueberries could be explained in several ways;

1. The complex is produced only by certain strains or varieties of blueberries differing in anthocyanins content.
2. The complex is an artifact produced under the particular processing conditions.
3. The complex is produced only at a certain stage in the berry ripening.
4. The complex is destroyed by enzymatic or other means during freezing or thawing of the berries.

Canned strawberries and raspberries, randomly chosen from the supermarket shelves were investigated to see if they contained a stabilized anthocyanin complex. It was hoped that if complexation occurred only in the blueberries and was due to a specific component, this would be shown in the proximate analysis of the three processed fruits. Table 6 page 46 contains analytical results of this type and it will be seen that the only differences of any note are that blueberries had a lower content of total pectin and a very much higher content of alcohol insoluble solids than the other fruits. It was impossible to say whether these differences were significant.

To test if the lack of complexing could be attributed to differences in cation content, x-ray fluorescence analysis of the AIS was carried out on a qualitative scale. Results listed in Table 6 are rather inconclusive. The alcohol insoluble material that was unpigmented had a higher content of aluminium ions than the blueberry which contained a blue complex and this might be an argument against the essentiality of

aluminium for the formation of colored complexes.

In comparison with blueberries, the gelatinous precipitates of strawberries and raspberries obtained by treating the syrups with 5 volumes of ethanol retained very little color. The strawberry precipitate was brownish in color perhaps due to pigment polymerisation. After dialysis, little color remained in the dialysis tubing. The blueberry complex under these conditions, shows a strong blue color. This leads to the conclusion that no complex of the blueberry type was present in strawberry or processed raspberry products.

5. Discussion

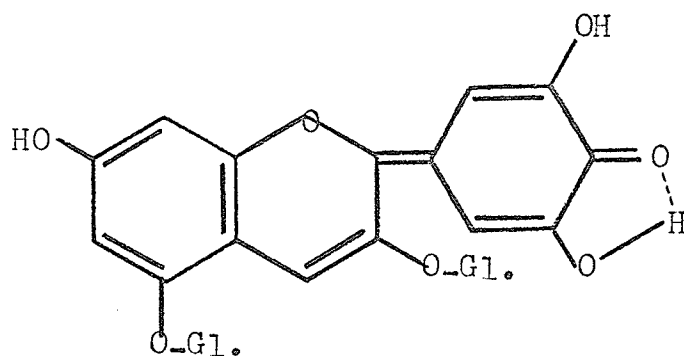
It is to be noted that no anthocyanins other than cyanidin and delphinidin glycosides have been reported to occur in complexes. It is thus significant that petunidin glycoside was present in the blue complex described in this thesis. This would seem to show that for complexing of anthocyanins in processed blueberry syrup, ortho dihydroxyl groups are required and also substitutions at the 5' position by either a hydroxyl or methoxyl group. (XIII and XIV). Malvidin (XV) or cyanidin (XI) glycosides, although present in the blueberry do not form complexes in the processed berry syrup even though one of the two criteria noted above was satisfied. (Malvidin has a 3', 5'-dimethoxy-4'-hydroxy B ring structure while cyanidin has a 3', 4'-dihydroxy structure.)

Various types of complexing are known (4) (52) (86). This area of research, including co-ordinate chemistry, is outside the scope of this thesis. Research along this line would seem beneficial as some of the more doubtful structural hypothesis could probably be eliminated. As pointed out by Bayer et al. (13) it is rather difficult to visualize

the structure of a blue compound isolated by Hayashi et al. (64) containing 1 atom of magnesium per 4 molecules of delphinidin glucoside. This molecule, assuming bonding through the ortho dihydroxyl group, would require a co-ordination number of 8 for magnesium. Magnesium should have a co-ordination number of four according to the rule that the co-ordination number equals twice the charge on the ion. This is confirmed in the chlorophyll molecule where magnesium has a co-ordination number of four. Complex formation between anthocyanins and alkaline earth cations at pH values of between 2 and 9 has not been detected (10) (12).

Copper has been found to complex with malvidin 3-glucoside and petunidin 3-glucoside (119). (Petunidin has a 3', 4'-dihydroxy-5'-methoxy substitution on the B ring). Polarographic methods were used and various complex ratios were found at different pH levels. However, the malvidins were found to complex just as well as petunidins which do have ortho-dihydroxyl groups. A number of possible structural formulae were suggested which show only complexing on the B ring. The only alternative is that complexing may possibly occur at the 5 and 7 positions. This, of course would give a greater size of chelate ring but complex formation through the 4-keto and 5-hydroxyl groups is known in certain other flavonoids.

In order for the complexing reaction to occur (assuming that the B ring is the primary site of reaction), it seems likely that the quinoid anhydro base XXXIV must be stable in weakly acidic to neutral media. There is some evidence for this (80) (137) and it may be that the other pigments are not stable or do not provide this form in sufficient quantities to form complexes. Comparison of pK values may be useful although few pK values are known for the petunidin, malvidin and delphinidin glycosides.



XXXIV

It seems imperative that in the discussion of complexing of anthocyanins, the chemical structure(s) present at a particular pH be used. The charge oxonium form cannot enter into complexing reactions. The complexing reaction would most likely occur in the nebulous pH region where a number of anthocyanin forms exist. The pH range of 4 to 7 would require study in order to establish the equilibrium between the different anthocyanin forms.

There are a number of reports which suggest that delphinidin glycosides may be the only types of anthocyanin present in the complexed form (9) (97). However these studies usually investigated differences between cyanidin and delphinidin types of structure and do not therefore, provide complete comparative information about the other anthocyanidin structures.

Other polyphenol complexes have been found which show some comparable properties to the anthocyanin complex. Thus apple juice sediment was found to contain complexes which had large amounts of amino acids, chlorogenic acid and polyphenolic compounds with a molecular weight

in the 100,000 to 200,000 range (71). Brown and Wright (23) showed that casein was mainly responsible for the formation of a soluble complex with tea polyphenols. Millin et al. (93) after dialysing black tea infusions found 10-12% non-dialysable soluble solids. The complex which had an absorption maximum at 450 nm, contained protein and polyphenols. Polygalacturonic acid, molecular weight 50,000 was also a component.

The blue complex found in the present study was probably formed under artificial conditions of processing and/or storage. Different quantities of the blue complex were found when a number of cans with different code dates were examined. Thus it would seem that processing conditions may affect the blue complex which may only form at temperatures in the region of 100°C. All the anthocyanin complexes that have been previously described were isolated from fresh unprocessed plant material.

Tin has been suggested as a possible complexing agent, especially since it may be present in large quantities in certain canned foods. However, X-ray fluorescence analysis did not show the presence of tin nor did the lacquered blueberry cans show any evidence of corrosion.

There seems to be some confusion in the naming of a number of the complexes that have been found. Thus Asen and Jurd (6) used cyanocentaurin as the trivial name for an iron complex containing cyanidin and a bisflavone. It may also be possible, according to these authors, that their pigment was identical to that found by Hayashi and co-workers (64) (94) which was called commelinin. This neglects the fact that the latter pigment was thought to contain magnesium rather than iron.

Classification and nomenclature of complexes in which

anthocyanins are associated with other molecular species is a difficult matter. It would appear that certain ligands are firmly bound into the complex while others are much more weakly bound. The latter may even be components adsorbed during the process of isolation. Thus complexes of different compositions might be isolated at different times from the same bulk sample of material. It would therefore seem inappropriate to apply a name to each complex as it is isolated. No attempt has been made to provide a name for the complex isolated from blueberry syrup.

The complexes described in the literature summary have been shown to contain any of the following constituents: delphinidin or cyanidin 3,5-diglucoside, p-coumaric acid, amino acids, pectic material, flavones/flavonols, iron, aluminium, potassium, and magnesium ions. Since all the complexed pigments are precipitated with ethanol, it is suggested that analysis for pectic substances should always be made. The molecular structure of pectic substances is thought by some to resemble a "brush heap" (79). A molecule of this type having large numbers of exposed carboxyl and hydroxyl groups would have a tendency to adsorb and also form co-ordination complexes with ions of the types reported above. Pectins are notoriously difficult to purify and it seems likely that various non-structural components will be found. Proteins and metal ions may be given as two examples of substances which are difficult to remove from pectin completely.

The present state of knowledge does not allow any firm conclusion as to which of the three mechanisms (1) pH differences, (2) co-pigmentation, (3) metal chelate formation, operates to produce stable

blue colors in flowers and fruit. If, as has been reported here, and reported by Bayer et al. (13) pectic substances were present, it will be difficult to state unequivocally if the metal ions present were structural components of the pectin, or required for bonding within the complex. It also seems desirable to investigate the known complexes for pectic or proteinaceous material. The blue complex found in this study cannot be listed under the three mechanisms described. Thus a fourth category should be established consisting of complexes characterized by the presence of macro-molecular components with or without structural cations.

Further quantitative determinations of the complex components would seem essential. This task will be rather difficult as at least two of the components are virtually impossible to measure in absolute terms. Concentrations of anthocyanins are best determined by extinction coefficients. However, the reported values show marked differences and they are not complete even for the common anthocyanins. In fact, it is extremely difficult to obtain highly purified samples of anthocyanins free from water, decomposition products, or adsorbed solvent. The structure and conformation of pectins have been incompletely investigated hence it is difficult to predict how they are associated within the complex. More sophisticated techniques and instrumentation may be of help in shedding new light on these questions.

The isolated blue complex was highly colored and it was felt by the author that it might have a potential use as a food colorant. For this reason, the heat and light stability tests were carried out. The complex seemed to be stable in the pH range 2 to 7. It showed some light sensitivity in pure solutions and it was labile at higher temperatures.

All efforts to produce equivalent complexes in the laboratory were unsuccessful. However, the fact that the blueberry complex was formed, probably during processing, out of naturally occurring components, suggested that a further search for the proper conditions of synthesis should be made.

V. Complex Formation Reactions of Anthocyanins

1. Introduction

The aim of the experiments reported in this section was to demonstrate possible interactions between anthocyanins and other naturally occurring plant constituents. It was thought that the results of these experiments would suggest methods for the stabilization of anthocyanin pigments in processed fruit. Since this approach was novel, no widely accepted, standard procedures were available. Thus various techniques such as dialysis, spectrophotometry, electrophoresis, chromatography and photodensitometry were applied to the problem in hand. As far as possible, several techniques were applied to the same problem, this approach was dictated by the pH dependent, rather labile nature of the anthocyanin substrates.

2. Literature Review

2.1. Experimental evidence for complex formation

The spectral shifts of the anthocyanin absorbance maxima in the visible or in the ultra violet region, suggest complexation (or interaction between molecules). The bathochromic shift of certain anthocyanins with AlCl_3 is a well known example. Jurd and Asen (74) have studied the interaction between aluminium ion, cyanidin 3-glucoside and each one of

the following: quercitrin, chlorogenic acid and methyl gallate. They showed that none of the three polyphenols could produce a blue color with cyanidin 3-glucoside (i.e. copigmentation) but all required the addition of aluminium to the mixture of polyphenol and cyanidin 3-glucoside to produce a spectral shift toward the blue end of the spectrum (i.e. chelation).

A small bathochromic shift of 1-5 nm was found when cyanidin and malvidin glycosides were allowed to interact in acidic solution with quercitin and kaempferol glycosides (55) (56). This contradicts the above results obtained by Jurd and Asen (74). Woof and Pierce (139) found a 5 nm shift when rutin and amylase were mixed.

A slight hypsochromic shift in absorbance maximum was observed by Daravingas and Cain (34) after the processing of red and black raspberries. The authors explained this shift as being caused by differences in the quantity of the individual anthocyanins.

Sondheimer et al. (124) have demonstrated that a shift of 23 nm (estimated from their published spectrum) occurred when 0.0185 millimoles/liter of pelargonidin chloride and 33 millimoles/liter of caffeine were mixed at pH 2.05.

Harborne (60) mentioned that complexes of anthocyanins with flavones may produce multiple spots on paper chromatograms. He did not document this, and Fuleki (50) has found no confirmation of this effect in his literature search. Fuleki noted that co-pigmentation effects do not occur at low pH values where these complexes are in the dissociated form (See (6) (13) (142)).

2.2. Comparison of anthocyanin stability under various conditions.

Grape juice samples were found to lose less color than the anthocyanin chloride solution when exposed to ultraviolet light (115). Black raspberry anthocyanins were more stable in a system containing citric acid and sugars than in the actual berry juice (34). Blundstone and Crean (18) proved that increased rate of decomposition of cyanidin 3-glucoside could occur if common constituents of the fruit syrup was added. Nebesky et al. (95) found that light decreased anthocyanin concentration in solutions of isolated pigment but not in the juice.

Various authors have indicated that the structure of the anthocyanins have definite relationships to their stabilities. Lamort (81) demonstrated that strawberry pigments, presumably pelargonidin glycosides, are much more stable to heat than the blueberry, currant, raspberry, or cherry pigments. It has also been shown that delphinidin glycosides are less prone to decolorization than cyanidin glycosides in weakly acidic or neutral conditions (137). Peonidin and malvidin glycosides were the most stable with delphinidin glycoside the least stable (110). These results have been confirmed by other authors (39) (104) but may only apply to the pigments in wines. Some of the early work of Robinson and his co-workers (15) (87) makes reference to differences in stabilities of some of the anthocyanins they synthesized.

2.3. Chelation of aluminium and ferric ions by anthocyanins.

As mentioned in 2.1., Jurd and Asen (74) found a shift only if cyanidin 3-glucoside, Al^{+++} and quercitrin were present. The complex formed was relatively insoluble in aqueous solvents. However, no information was obtained for Fe^{+++} or for other anthocyanins. Bayer and his co-workers (10) (11) (12) (13), have investigated the metal chelates of

cyanidin glycosides with Al^{+++} . Maximum complexation occurred at pH 5.5 and the color so produced was stable. Ferrous and ferric salts formed similar blue complexes at pH 5.5 with absorption maxima at 600 and 560 nm respectively. Complexes of 1:1, 1:2 and 1:3 ratios have been obtained for Al^{+++} and Fe^{+++} . The cyanidin- Fe^{+++} complexes formed stable blue colors.

Robinson and Robinson (107) used the color reaction of ferric chloride with the six common anthocyanidin structures as a means of identification. Delphinidin was found to produce a blue colored complex in the presence of sodium acetate but they did not mention the stability of this blue color. The possible harmful effect of iron on anthocyanins has been mentioned by Szechenyi (128). Other indirect evidence was obtained by Stein and Weckel (126) who reported loss of anthocyanins in frozen cherries. The latter authors attributed this to an iron tannate reaction.

2.4. Naturally occurring components which may take part in complexing reactions.

The analysis of the blueberry complex reported previously suggested that anthocyanins could form complexes with pectic substances and proteinacious substances. Cansfield (25) found that hydroxyproline was the only amino acid which interacted with the flavonoids. Woof and Pierce (139) have used α -amylase, ribonuclease and gelatin at 0.5 g/l as model proteins for complex formation with flavonoids. It was also noted that the hydroxy amino acid, serine, gave a more intense interaction than others.

Pectic substances have not been used in the study of pigment stabilization except by Sastry and Tischer (115). There is a wide range

of pectic substances. The basic unit, or mer, is galacturonic acid and it was thought to be a logical starting point. However, Tinsley and Bockian (134) have found that glucuronic acid had a marked effect on the stability of pelargonidin 3-glucoside. The uronic acids decompose under acidic conditions to furfural and substituted furfurals, which are known to react with anthocyanins. These authors feel that galacturonic acid would show a similar reaction to glucuronic acid.

Galacturonic acid reduced the amount of extractable copper due to the formation of a complex (130). Hydrogen bonding can readily occur between hydroxyl groups at C_2 and C_3 in the galacturonic acid units of protopectin. The glucose units in cellulose may also form hydrogen bonds. Cellulose is of interest as a possible immobilization site for anthocyanins as it is present as a structural network in plants and it is thus ideal for the fixation of pigments.

3. Experimental Reagents

Pectin obtained from J.T. Baker Co., methoxyl content 8.5%, total pectin as galacturonic acid 90.0%, potassium 0.028%, aluminium 0.090%, calcium 0.021%, iron 0.048%, equivalent weight 1690. Described in the text as high methoxyl pectin.

Pectin of citrus origin. Obtained from Matheson Coleman & Bell (low methoxyl). Methoxyl content 3.1%, 62% total pectin as galacturonic acid, equivalent weight 2550. Described in the text as low methoxyl pectin.

Buffer. Walpole buffer system of HCl and sodium acetate between pH 1.8 to 5.0 (53). This buffer was selected because it covered the 1-5 pH range used in these experiments. Buffers containing citrates

were unsuitable because citric acid chelates preferentially with the ferric ion as well as with other ions (74) (130).

Pectin solution. Various concentrations were used. To prepare solutions, the weighed, dry pectin was moistened with alcohol prior to the addition of the required volume of water. Pectin was solubilized by mixing in the cold, and also by heating under pressure in an autoclave. The autoclaved pectin solution had a faint brown color but was quite clear and free of opalescence; the pectin solution mixed in the cold was colorless and opalescent. A 1% pectin solution in buffer had an absorption peak at about 285 nm with a shoulder at 350 nm.

Aluminium Chloride. BDH, reagent grade, $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ was used. The appropriate quantity of the salt was weighed out and dissolved in buffer at the required pH. Usual concentration was 2.41 g per 100 ml of buffer or 0.5% solution ($\approx 0.02\text{M}$).

Ferric Chloride. BDH, reagent grade, anhydrous, ferric chloride was used. This was prepared in similar manner to the AlCl_3 . Usual concentration 108 mg/100 ml in buffer or 0.5% solution ($\approx 0.05\text{M}$).

Anthocyanins. The anthocyanins of blackcurrants were extracted with 0.25 N HCl in methanol. The extract was purified by PVP column chromatography using 0.25 N HCl in methanol as eluant. The purified anthocyanin extract contained the 3-rutinosides and 3-glucosides of delphinidin and cyanidin. This extract was evaporated to dryness on a rotary evaporator as required, and redissolved in neutral methanol to obtain a stock solution of anthocyanins.

Three pure anthocyanins were obtained in large quantities from PVP column chromatography (see Table 3) and were made up in 0.01% HCl in

methanol. Pelargonidin 3-glucoside, 37 mg/ml; cyanidin 3-glucoside 10 mg/ml; and delphinidin 3-glucoside 4 mg/ml, were the stock solution concentrations. The R_f values in AWH were 0.36, 0.25 and 0.15 respectively. The weight per ml was determined by drying suitable samples after purification.

Gelatin, Bacteriological grade. Usual concentration of 2.5 mg/ml dissolved in buffer. This produced absorption maxima at 264 and 300 nm. (Assumed molecular weight 70,000).

Galacturonic acid. Galacturonic acid monohydrate. Usual concentration 440 mg/100 ml in buffer.

Calcium Chloride. Anhydrous 110 mg/100 ml of buffer.

4. Dialysis Experiments.

A number of experiments were carried out, in which fresh and thermally processed fruit juice, pureed, canned fruit and mixed solutions of pure anthocyanins and pectins, were dialysed against water that had been adjusted to acidic pH values. The objects of these experiments were as follows:

1. To determine whether anthocyanins occurred in nondialysable forms. If nondialysable forms did occur then it was presumed that the pigments were complexed with high molecular weight material.
2. To determine whether nondialysable anthocyanin-pectin complexes could be formed under laboratory conditions. Pectins might behave as weakly acidic ion exchangers that might bind positively charged anthocyanin molecules to the polysaccharide matrix.
3. To find whether the rate of dialysis of anthocyanins through cellulose membranes could be influenced by the addition of certain reagents to the

material being dialysed. Thus calcium ions might displace adsorbed anthocyanin molecules from pectins because calcium ions themselves form very strong bonds with pectins. EDTA might release anthocyanins from anthocyanin-trivalent cation-pectin complexes by virtue of its power of sequestering cations.

All dialyses were carried out using Visking No. 36 DC dialysis tubing. The dialysis cells were made of four blocks of Perspex acrylic resin 76 mm x 76 mm x 12 mm thick. The middle two blocks had openings of 38 mm x 44 mm cut out at the top edges, which formed the troughs and these were separated by the dialysis membrane. The cell was assembled by using 5 bolts with wing nuts to tighten the four Perspex blocks. Volume of each compartment was about 20 ml.

4.1. Experimental

4.1.1. Dialysis of fruit material.

Some Early Black cranberries were pressed and the expressed sap (pH 2.8) was dialysed in 400 ml of water, adjusted to pH 2.8. Commercially available, canned raspberries, strawberries and blueberries were pureed and dialysed in the same manner.

4.1.2. Dialysis of model components

3% pectin solution at pH 4.0 was added to the cranberry anthocyanins dissolved in pH 4.0 water. The cranberry and Poinsettia anthocyanins were used after FVP column chromatography and they were shown to be free from other compounds by paper chromatography in a number of solvents. A number of dialysis trials were carried out on pectin/anthocyanin mixtures and on anthocyanin solutions diluted to the same extent with water. The experiment was repeated at pH 2.0.

4.1.3. Dialysis of syrup from canned blueberries.

A replicated experiment was designed to test the effect of a number of compounds on the rate of dialysis of processed blueberry juice.

Two days before the dialysis a bulk supply of the syrup from canned blueberries was divided into 100 ml samples. The six treatments are given below.

1. 0.500 g of EDTA
2. 5 g of urea
3. 0.500 g of CaCl_2
4. 0.1500 g of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$
5. Untreated
6. 0.200 g of FeCl_3

The solutions were dialysed in the dialysis cells, three at a time, against pH 3.0 water for 8 hours. The color of the blueberry juice samples were noted after storage and the color of the ethanol precipitate was noted after the addition of five volumes of ethanol as an indication of the breakdown of the complex.

4.2. Results and discussion

Anthocyanins dialysed rapidly from the fresh cranberry juice. The pigments from pureed, thermally processed fruit including: blueberries, strawberries, and raspberries, also passed through the membrane rapidly. The plant tissue could be completely freed of anthocyanin pigment by dialysis until only a slight coloration remained and the latter appeared to be due to browning polymers. However, prolonged dialysis of thermally processed blueberry juice failed to remove all of the color from it, although a certain amount of color actually passed through the membrane.

The addition of calcium chloride to the blueberry syrup prior to dialysis increased the rate at which pigment diffused through the membrane and reduced the quantity of color retained by the syrup. Aluminium ions had a more pronounced effect than calcium ions, while urea, EDTA and ferric ions had little or no effect. Figure 4 shows a least square line calculated from the data in Appendix 3. Table 9 contains the pH values of the syrup and the color changes of the ethanol precipitate.

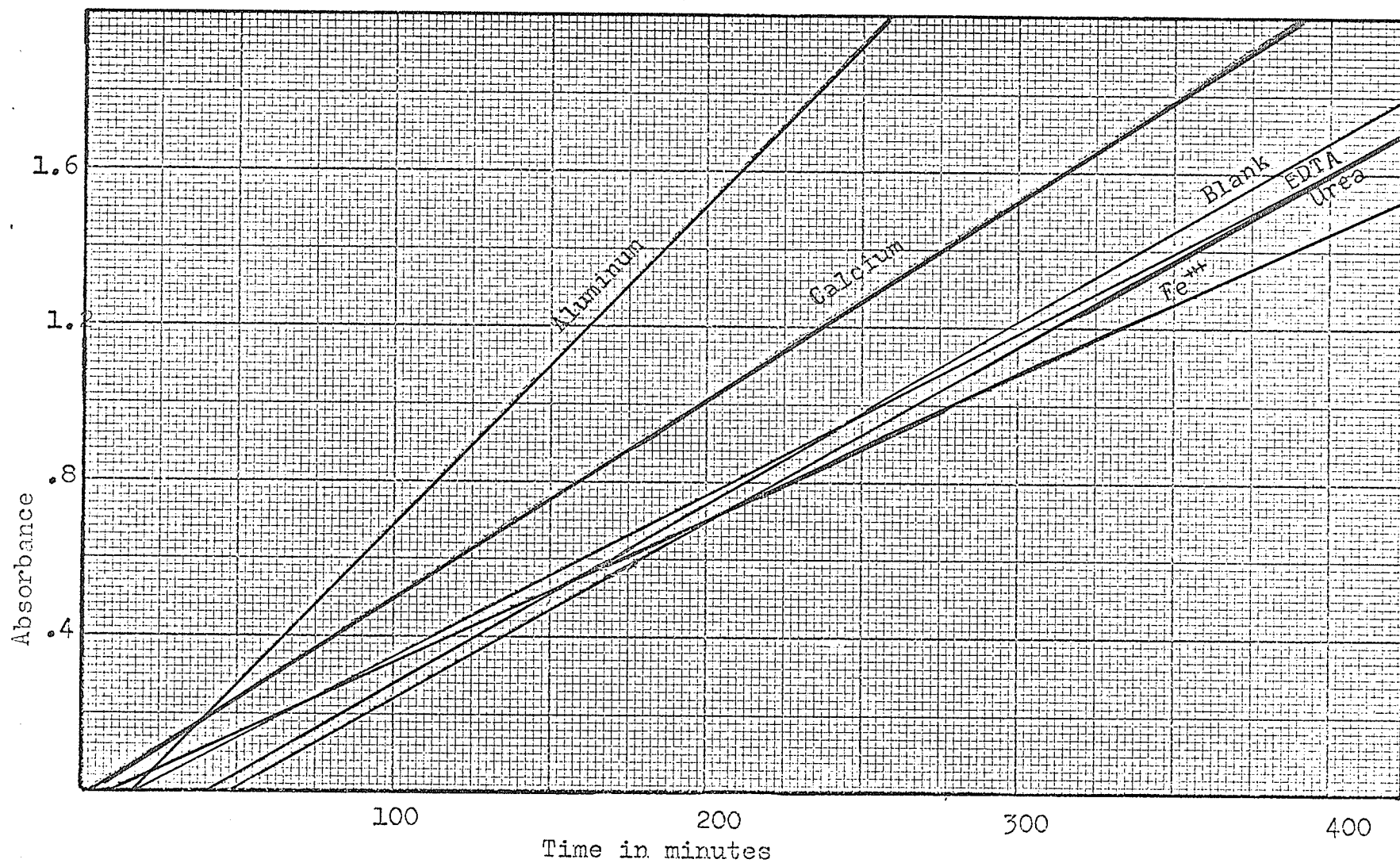
The increased rate of dialysis with Al^{+++} was unexpected in that the Al^{+++} pectin-pigment complex should decrease the dialysis of the anthocyanins. It is suggested that the aluminium-anthocyanin complex being uncharged or being of such a configuration, that dialysis through the membrane is facilitated. This explanation would not apply to the Ca^{++} cation as it is not known to chelate with the anthocyanin.

TABLE 9. Effect of the addition of a number of chemicals to processed blueberry syrup on the color of the syrup and on the color of the precipitate obtained on the addition of ethanol.

Solution	pH	Color of syrup	color of ppt.
Blank	2.85	blue (light)	normal blue
$CaCl_2$	3.65	reddish blue (light)	dull red; little color
Urea	3.50	dark blue	normal blue
$FeCl_3$	1.95	dark reddish	very light red
EDTA	3.10	dark reddish	dull red; little color
$AlCl_3$	2.19	dark blue	normal blue

A number of conclusions can be drawn from Table 9. For example addition of Ca^{++} to the blueberry syrup (pH 3.65) produced a light

FIGURE 4. Absorbancies calculated by least square fit for the effect of some added chemicals on the rate of dialysis of processed blueberry juice.



reddish blue color and a slight dull red pectin precipitate. This result would be expected if Ca^{++} had displaced the anthocyanins from the pectin. Figure 4 does show greater rates of dialysis after the addition of Ca^{++} . The results with FeCl_3 were rather similar. Interpretations for the other treatments can be made along these lines but further conclusions should be based on more quantitative data.

The addition of pectin to canned blueberry syrup reduced the rate of dialysis of pigment. Similar results were obtained with model systems where blueberry syrup was replaced by solutions of purified anthocyanin pigments. The pigments used for these experiments were the natural mixtures obtained from cranberries and Poinsettia bracts. The addition of pectin greatly reduced the dialysis rate of the anthocyanins. The viscosity may have influenced the rate of diffusion. However, after dialysis for four days, a distinct difference was still present and showed that pectin had caused the retention of some of the anthocyanins under equilibrium conditions. Typical absorbancy data are given in Table 10.

TABLE 10. Effect on dialysis rate of a mixture of pectin and cranberry anthocyanins as measured by the absorbancies of the dialysate

Elapsed time	Pectin	Blank
5 hours	.090	.133
25 hours	.117	.168
4 days	.350	.917

The exact theory for this dialysis was unclear and may be extremely complex. Dialysis is governed in part by the size of the

molecule and the anthocyanins (MW 400-600) pass through the membrane. Other factors may affect the rate of dialysis such as osmotic pressure, Donnan equilibrium and viscosity.

A number of experiments were attempted in efforts to determine whether selective association of the anthocyanins occurred with pectin. A suitable series of anthocyanins were mixed with pectin and the mixture was allowed to dialyse. The dialysate was chromatographed and the separated pigments were measured on the photodensitometer. Results indicated that the experimental variations were too great and it was not possible to draw any conclusions. Dialysis experiments and the photodensitometric experiment described in 8.1. were two techniques used in efforts to show selective association of the anthocyanins with pectin. The reproducibility of the dialysis technique requires refinement and will require proof for the assumption that the dialysis process is not dependent on hydroxylation and glycosidation of the anthocyanins.

5. Spectrophotometric Experiments

5.1. Experimental

A Unicam SP 800 double beam spectrophotometer was used in the following experiments. Unless otherwise stated, spectra were determined from 550 nm to 230 nm. Silica cells, which had a capacity of 3.5 ml, were used. They were matched against each other to produce equal pairs.

The following general arrangement was used to study the effects of a number of compounds.

- a. Solution of pigment in the appropriate buffer
- b. (a) plus an organic ligand
- c. (b) plus a cation

d. (a) plus a cation

Variables studied were (1) Anthocyanin; the 3-glucosides of pelargonidin cyanidin, delphinidin, and malvidin, the 3-rutinoside of cyanidin.

(2) Organic ligands; galacturonic acid, pectin, and polygalacturonic acid.

(3) Cations; aluminium (as AlCl_3) and ferric ion (as FeCl_3).

(4) pH values; 4.5, 2.3, 1.8.

A second type of experiment studied the effect of the addition of solid hydroxyproline and pectin to the three pure anthocyanins. A dilute solution of appropriate absorbance was prepared for each of the three anthocyanins at one of the following pH levels: 2.2, 3.4, and 4.2. The solution was placed in two cuvettes and the spectra of each cuvette measured against a cell containing the appropriate buffer. About 10 mg of solid hydroxyproline was added to one of the sample cells and the spectrum was redetermined. The same was done for the pectin except that due to its slow solubility in water, the pectin was added as 3 drops of a 1% solution in the appropriate buffer. After three days of storage, the spectra were redetermined for all samples.

The pH 2.2 series, which showed no change after three days, was then tested as follows: the spectra of the pectin and the hydroxyproline sample and their respective blanks were determined. 2 drops of 0.5% AlCl_3 in pH 2.2 buffer was added to the hydroxyproline containing sample. To the blank, 10 mg of solid hydroxyproline and 2 drops of 0.5% FeCl_3 were added. The two spectra were determined. This procedure of adding Al^{+++} and Fe^{+++} was also carried out with the pectin sample and its blank. This experiment showed the effect of cation, either Al^{+++} or Fe^{+++} on the anthocyanins in the presence of pectin or hydroxyproline at

three pH levels.

The last series of samples consisted of only the anthocyanin with one of the two metals at the three pH levels.

5.2. Results and discussion.

The evidence suggested that at pH 4.5, pelargonidin 3-glucoside, galacturonic acid and aluminium ions associated in such a manner that the absorption at the 508 nm maximum increased by 15%. After 28 days storage, the wavelength for peak absorption shifted from 508 to 504 nm and there was an increase in absorption at 275 and 300 nm.

Cyanidin 3-glucoside at pH 4.5, formed a complex with aluminium ions that was apparently unaffected by the addition of galacturonic acid to the system since there was no change in the wavelength of maximum absorption (558 nm). There was no evidence that complex formation occurred between this anthocyanin and galacturonic acid.

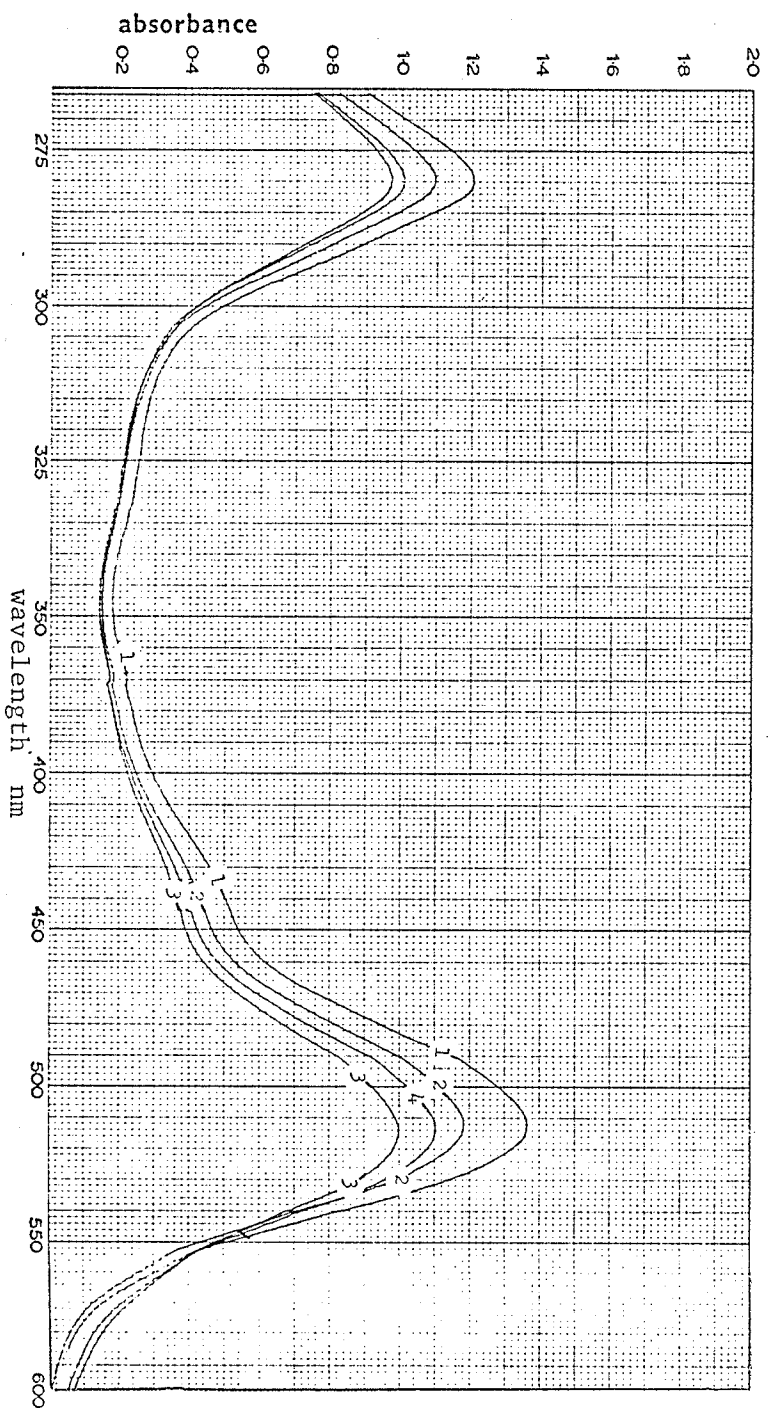
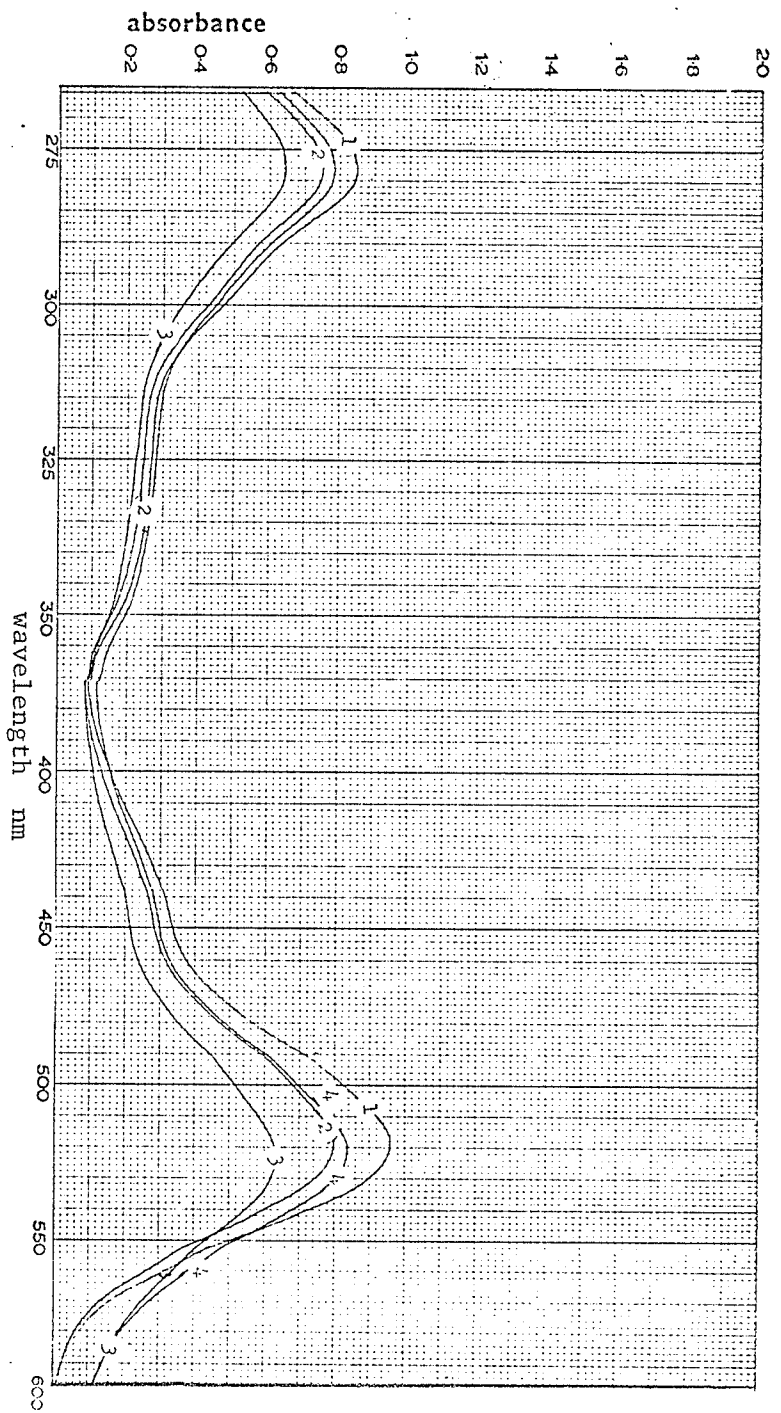
At pH 2.3 - 2.5 cyanidin 3-glucoside showed no spectrophotometric evidence of interaction with aluminium ions or galacturonic acid or a mixture of these. See Table 11 and Figure 6. However in the same pH

TABLE 11. Absorption maxima and pH values of a three component system of galacturonic acid-aluminium-cyanidin 3-glucoside.

	pH	Color	Abs. max. nm	
			Initial	after 5 days
Cyanidin 3-glucoside	2.3	Red	512	511
" + galacturonic acid	2.4	Red	512	511
" + " + Al ⁺⁺⁺	2.5	Blue-red	513	513
" + Al ⁺⁺⁺	2.5	Blue-red	514	513

FIGURE 5. Delphinidin 3-glucoside 1) in pH 2.3 buffer;
2) in buffer with added galacturonic acid;
3) in buffer with added galacturonic acid and AlCl_3 ;
4) in buffer with aluminium chloride.

FIGURE 6. Cyanidin 3-glucoside 1) in pH 2.3 buffer;
2) in buffer with added galacturonic acid;
3) in buffer with added galacturonic acid and AlCl_3 ;
4) in buffer with AlCl_3 .



region, delphinidin 3-glucoside formed a complex with aluminium alone and apparently a different complex with aluminium ion together with galacturonic acid. The shift of absorption maximum can be seen in Table 12 Figure 5. At pH 4.5, a complex was still formed between delphinidin 3-glucoside and aluminium ions but the ternary complex involving galacturonic acid was not formed (Figure 7).

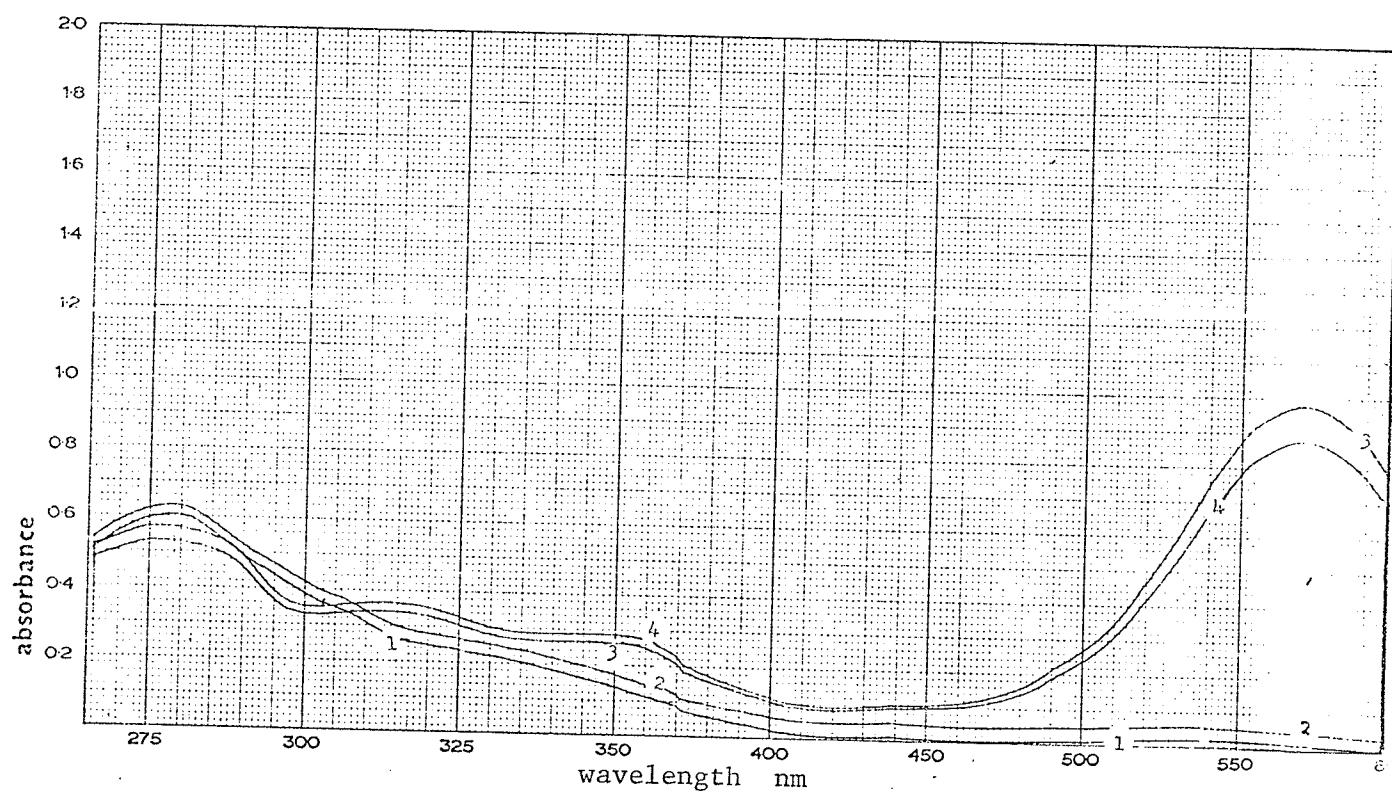
The color of malvidin 3-glucoside faded rapidly in pH 4.5 buffer. This anthocyanin formed a complex with aluminium ions but apparently no ternary complex with aluminium and galacturonic acid was formed.

TABLE 12. Absorption maxima and pH value of the three component system of galacturonic acid-aluminium-delphinidin 3-glucoside.

No.	pH	Color	Abs. max. nm.	
			Initial	After 1 day
1. Delphinidin 3-glucoside	2.3	Red	516	515
2. " + galacturonic acid	2.5	Red	517	517
3. " + " + Al ⁺⁺⁺	2.4	Red Blue	522	523
4. " + Al ⁺⁺⁺	2.3	Blue	519	519

In the above experiments attempts to investigate complex formation between anthocyanins, pectin or polygalacturonic acid and aluminium, were defeated by the insolubility of pectin and polygalacturonic acid in the presence of aluminium ions. A very careful study of these systems would be required to ensure that the components were present in exactly the right concentrations. There was insufficient time

FIGURE 7. Delphinidin 3-glucoside 1) in pH 4.5 buffer;
2) in buffer with added galacturonic acid;
3) in buffer with added galacturonic acid and
 AlCl_3 ; 4) in buffer with AlCl_3 .



to carry out such experiments.

With Fe^{+++} and delphinidin 3-glucoside, an initial blue color was produced which faded rapidly. This was a firm indication that Fe^{+++} causes rapid decomposition of delphinidin 3-glucoside. It was also noted that there were major differences in the ultraviolet spectra of the aluminium anthocyanin complex and the ferric anthocyanin complex. The increase in absorbance after 350 nm was especially pronounced. Cyanidin 3-glucoside did not produce a blue shift at this pH. Thus the ferric complexation with delphinidin occurred at a very acidic pH (2.1), while that of the cyanidin glycosides occurred at higher pH levels. See Figures 8 and 9.

Pelargonidin at the same pH retained little color when iron was present. The shoulder at 440 nm disappeared and increased absorption in the 300 nm area was observed. Thus even the most stable anthocyanin was decomposed in the presence of ferric iron. Neither Al^{+++} or Ca^{++} in the presence or absence of galacturonic acid had a modifying effect on the spectra obtained.

5.2.1. Stabilizing effect of hydroxyproline.

The stabilization effect of added hydroxyproline was determined with solutions of anthocyanins maintained at pH 2.2, pH 3.4, and pH 4.2 respectively. A complete, tabulated summary of the quantitative color and spectral changes after three days storage is given in Table 13. Similar data after a storage period of seven days is given in Table 14. Figures 10 and 11 demonstrate the anthocyanin protective effect by showing retention of absorbance at 500 - 550 nm region.

There was no spectrophotometric evidence for complex formation

FIGURE 8. Spectra of cyanidin 3-glucoside 1) in pH 2.1 buffer with added FeCl_3 ; 2) in buffer with added AlCl_3 ; 3) same as 1 but recorded after one day; 4) same as 2 but recorded after one day.

FIGURE 9. Spectra of delphinidin 3-glucoside 1) in pH 2.1 buffer with added FeCl_3 ; 2) in buffer with added AlCl_3 ; 3) same as 1 but recorded after one day; 4) same as 2 but recorded after one day.

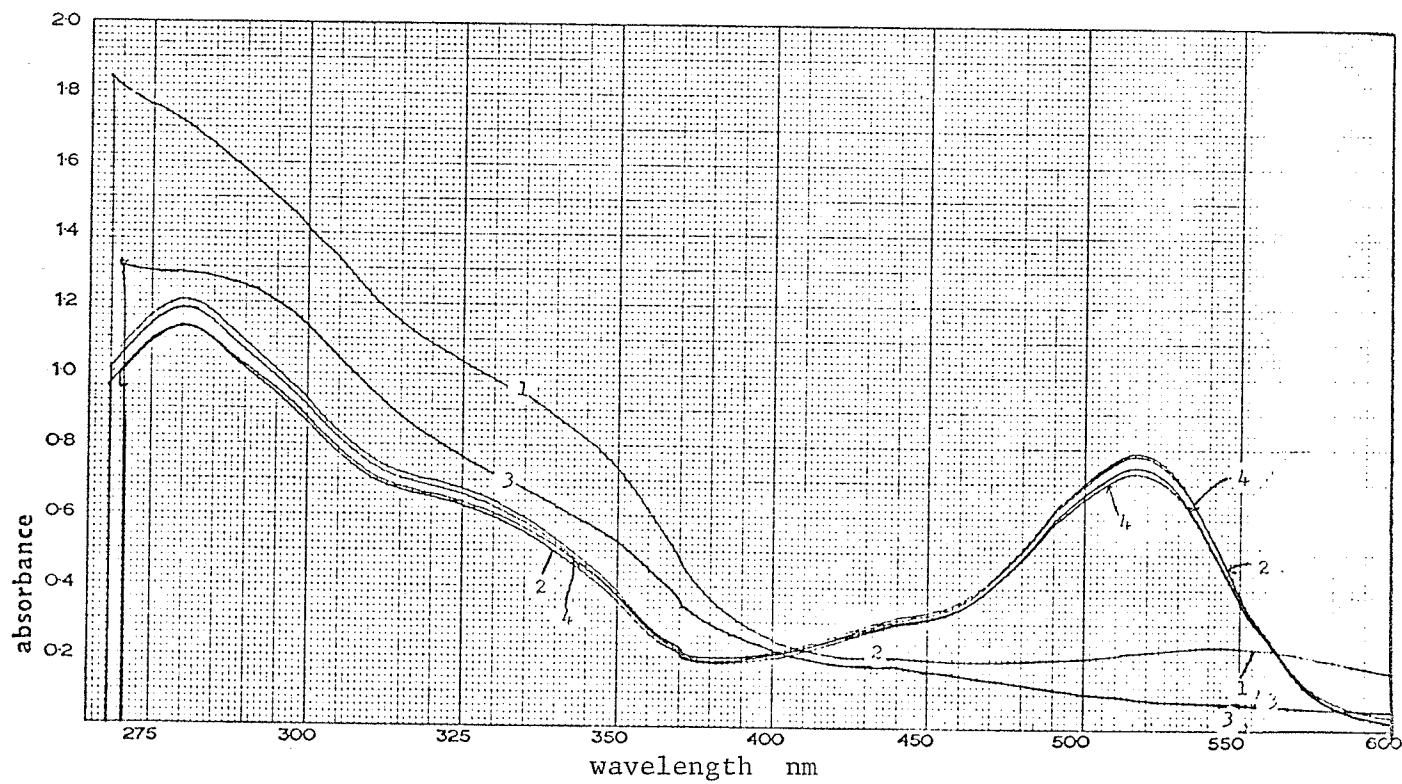
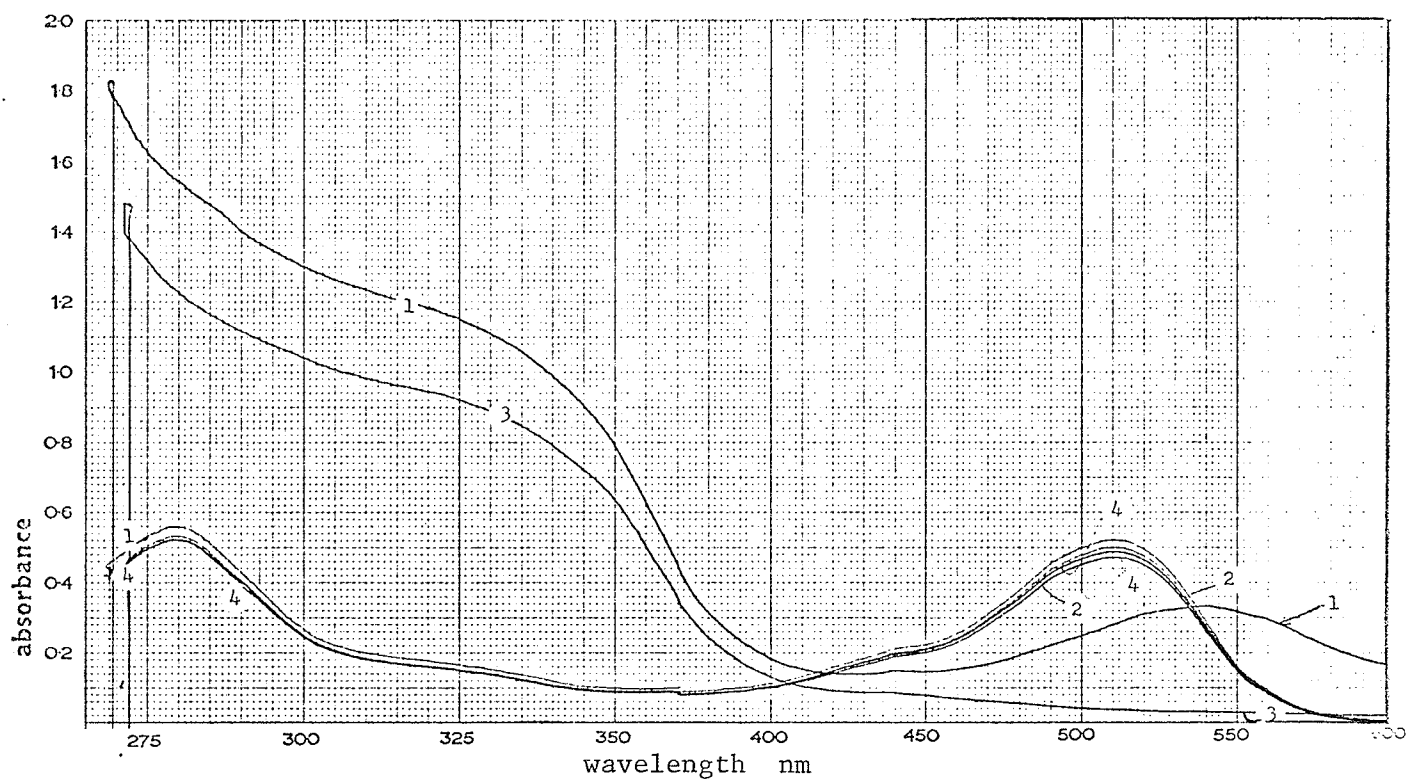
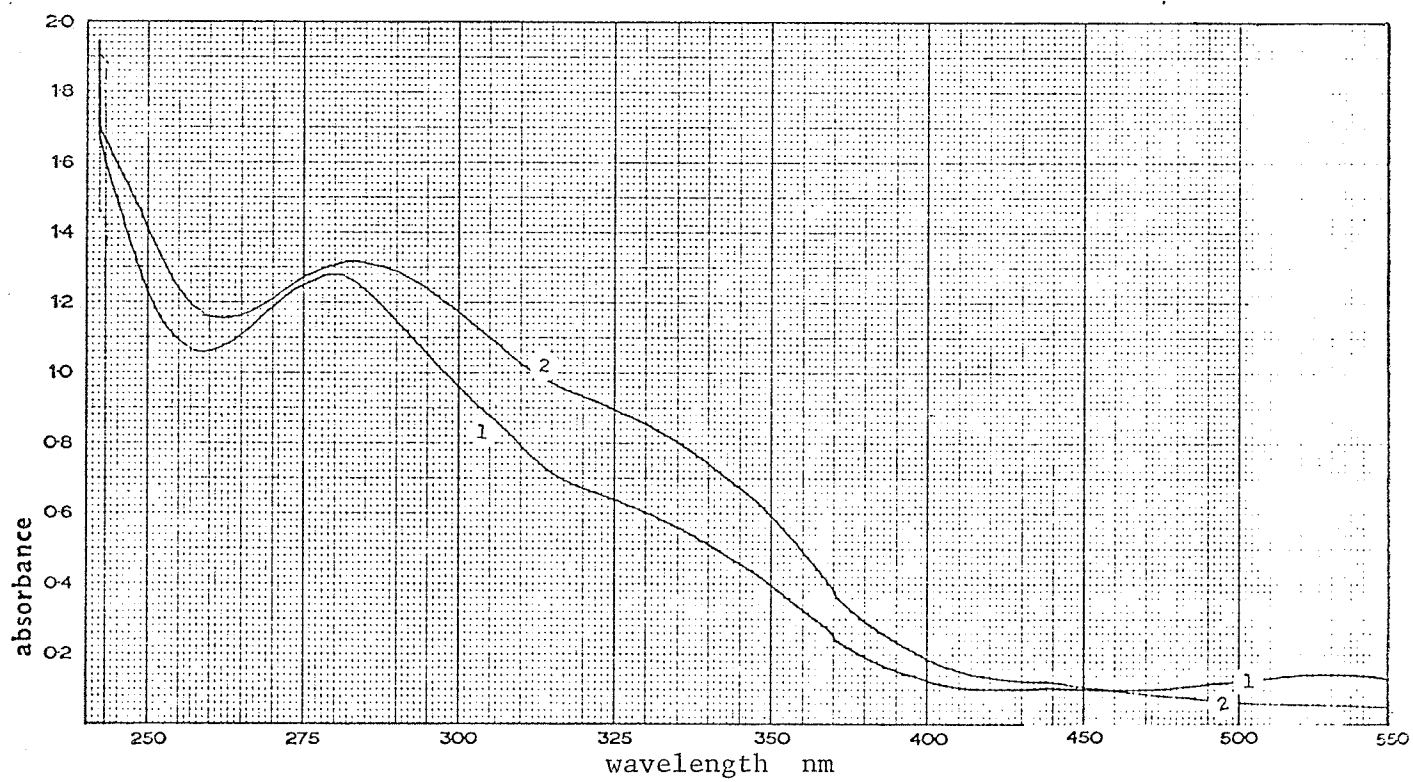
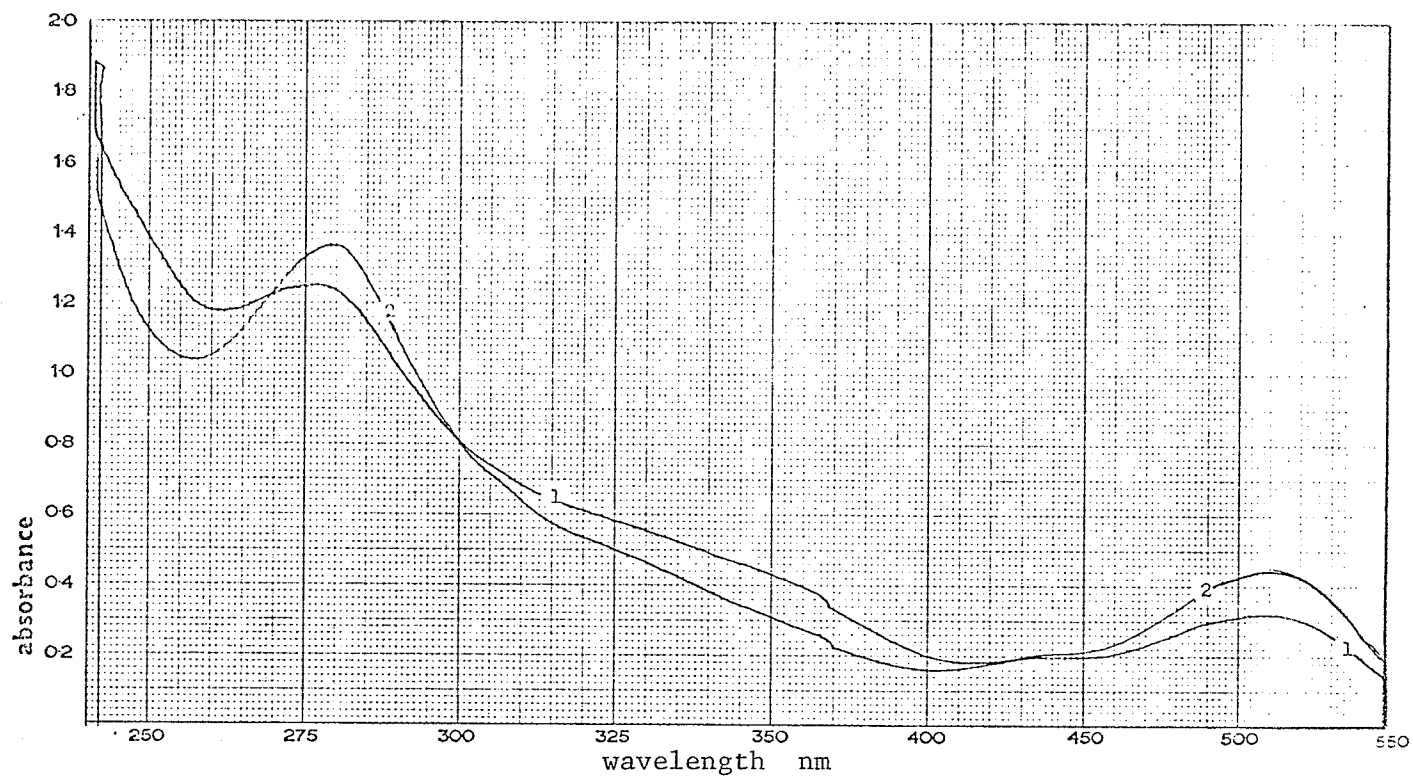


FIGURE 10. Spectra of cyanidin 3-glucoside recorded after 3 days 1) in pH 3.4 buffer; 2) in buffer with added hydroxyproline.

FIGURE 11. Spectra of delphinidin 3-glucoside recorded after 3 days 2) in pH 4.3 buffer; 1) in buffer with added hydroxyproline.



between pectin or hydroxyproline and the 3-glucosides of pelargonidin, cyanidin and delphinidin at pH values 2.2, 3.4 and 4.2 in the absence of cation. Addition of the two complexing reagents introduced a slight reduction in absorbance, most detectable in the case of hydroxyproline and at low pH values.

5.2.2. Effect of pectin on anthocyanin stability.

The experiments showed that there was no evidence of ternary complex formation or molecular association (detectable by spectrophotometry) between anthocyanins hydroxyproline or pectin and aluminium or iron. Again the destructive effect of iron on anthocyanin was noted. It was shown that the least affected anthocyanin was pelargonidin 3-glucoside (Table 13 and 14).

5.2.3. General observation.

From Table 13, no empirical color differences could be detected for any anthocyanin at pH 2.2. When the pH was raised, the anthocyanin containing the greatest possible number of hydroxyl groups on the B ring, e.g., delphinidin, showed the greatest difference in color with the two test compounds. At pH 4.2, decomposition of the anthocyanin color may be noted. The preservation of color when delphinidin 3-glucoside and hydroxyproline were mixed, demonstrated a stabilising function of the added compound.

An interesting observation was made when attempts were made to regenerate the anthocyanins, at pH 4.2, by the addition of acid. Delphinidin 3-glucoside did not recover the anthocyanin color, nor did it form a blue color on the addition of AlCl_3 . Cyanidin 3-glucoside was regenerated to some extent and did give a blue color with AlCl_3 .

TABLE 13. Spectral characteristics and color of some anthocyanins at different pH levels on the addition of hydroxyproline and pectin

Anthocyanin	Trt.	pH		
		4.2	3.4	2.2
Pelargonidin 3-glucoside	hydroxy-proline	same	same	same
	Blank	color	color	color
	Blank			
	Pectin	same color	same color	same color
Cyanidin 3-glucoside	Hydroxy-proline	red	dark red	
	Blank	faint orange	red	same color
	Blank			
	Pectin	same color	same color	same color
Delphinidin 3-glucoside	Hydroxy-proline	faint purple	reddish	
	Blank	faint rose	faint orange	same color
	Blank	faint rose	colorless	
	Pectin	faint grey	faint bluish	same color

TABLE 13. (Continued)

Anthocyanin	Treatment	pH		
		4.2.	3.4	2.2
Pelargonidin 3-glucoside	Hydroxy proline		495 and 277 nm	
	Blank	500,420,280 nm		496,278,270 nm
	Blank		494,277 nm	
	Pectin		496,277 nm	
Cyanidin 3- glucoside	Hydroxy proline			
	Blank			
	Blank	517,281 nm	510,278 nm	511,280 nm
	Pectin			
Delphinidin 3-glucoside	Hydroxy proline	530,280 nm		516,280 nm
	Blank	530,280 nm		517,280 nm
	Blank		518,282 nm	
	Pectin	527,280 nm		516,283 nm

TABLE 14. Color reactions of three anthocyanins
at two pH levels with and without hydroxy-
proline and pectin after one week storage

		4.2.	3.4
Pelargonidin 3-glucoside	Hydroxyproline		
	Blank	— orange	— orange
	Pectin		
Cyanidin 3-glucoside	Hydroxyproline	— blue	red
	Blank	— colorless	— faint orange slightly red
	Pectin		
Delphinidin 3-glucoside	Hydroxyproline	blue	reddish
	Blank	— colorless	— colorless
	Pectin		

Pelargonidin 3-glucoside was completely regenerated. Thus it seems that delphinidins are more readily decomposed, compared with the other anthocyanins, at the higher pH levels. The only other alternative explanation was that they were removed by some other phenomenon.

Table 15 shows the results obtained when either Fe^{+++} or Al^{+++} cations were added to the three anthocyanins at a number of pH levels. It will be noted that neither pectin nor hydroxyproline modified the spectra when Al^{+++} or Fe^{+++} was present. Also with delphinidin 3-glucoside, a shift from 280 to 285 was noted when pectin was present.

Cyanidin 3-glucoside produced a large shift with the ferric cation. Delphinidin 3-glucoside formed a blue color which decomposed rapidly. This was accompanied by a decrease in the 500-550 nm region typical of the anthocyanins, and increases in absorption below 400 nm. Pelargonidin 3-glucoside retained its peak at 500 nm but showed increased absorbance below 400 nm. Table 16 shows the qualitative changes after the samples had been stored for one week. In all samples where ferric ions were added, decomposition had occurred. The latter effect was least with pelargonidin 3-glucoside. Hydroxyproline with Al^{+++} showed more blue color than did pectin and Al^{+++} when mixed with one of the three anthocyanins.

The anthocyanins could not be regenerated with acid if ferric ions had been added. The addition of citric acid did not produce a color change. This seems to indicate that delphinidin 3-glucoside and cyanidin 3-glucoside are irreversibly decomposed at pH 2.2 in the presence of ferric ions and either hydroxyproline or pectin. Pelargonidin 3-glucoside retained a measure of color showing that it had a certain resistance to

TABLE 15. Spectral characteristics and color at certain pH values of some anthocyanins with various combinations of hydroxyproline, pectin, Fe^{+++} and Al^{+++} .

Treatment	pH	Pelargonidin 3-glucoside	Cyanidin 3-glucoside	Delphinidin 3-glucoside
Pigment +	2.2			
Hydroxyproline		493 nm	508,278 nm	514,280 nm
Hydroxyproline				
+ Al^{+++}	2.2			
Hydroxyproline		absorption after 400 nm		blue color but
+ Fe^{+++}	2.2	is different. 493 nm	534 nm	unstable 540 nm
Pectin + Fe^{+++}	2.2	absorption after 400 nm		blue color but
		is different 493 nm	533 nm	unstable 550 nm
Pectin + Al^{+++}	2.2			
Pectin		493 and 275 nm	508,278 nm	515,285 nm
Fe^{+++}	4.0	brownish orange 497 nm	grey 544 nm fades completely	grey dark blue-precipitate 470 nm
	3.1	497 nm faded brownish orange	colorless 544 nm	550 nm fades rapidly to colorless
	2.1	497 nm faded brownish orange	540 then colorless after one day	550 nm fades to colorless

TABLE 15 (Continued).

Treatment	pH	Pelargonidin 3-glucoside	Cyanidin 3-glucoside	Delphinidin 3-glucoside
Al ⁺⁺⁺	4.0	fade orange 497 nm	grey/red 540 nm stable	faint blue-shows a precipitate-565 nm
	3.1	orange 497 nm	red 514 nm	blue red 520 nm
	2.1	orange 497 nm	red 510 nm	red 515 nm
Pigment	4.0	faded orange 497 nm	light red 514 nm	colorless 520 nm
	3.1	orange 497 nm	red 510 nm	red 518 nm
	2.1	orange 497 nm	red 510 nm	red 515

decomposition although complete color recovery was not possible.

TABLE 16. Color present after one week of storage of anthocyanins with various combinations of Al^{+++} , Fe^{+++} , hydroxyproline and pectin.

Treatment	Pelargonidin	Cyanidin	Delphinidin
Hydroxyproline + Al^{+++}	Orange	Red	Dark purple
Hydroxyproline + Fe^{+++}	Faint orange	colorless	colorless
Pectin + Al^{+++}	Orange	Red	Dark purple
Pectin + Fe^{+++}	very faint orange	colorless	colorless

6. Electrophoresis experiments

6.1. Experimental

All electrophoresis was carried out on a Savant 3 KV apparatus in conjunction with a flat bed electrophoresis cell. No. 3MM Whatman chromatographic paper was used as support. The start line was 23 cm from one edge which was dipped into the anode buffer cell. The separations were run usually at 1500 volts for 3 hours in formic acid buffer (16 ml 88% formic acid per liter).

The anthocyanin under test was applied to the start line. A 50 μ l aliquot of hydroxyproline (50 mg/10 ml solution) was streaked 0.5 cm, or at a multiple of 0.5 cm, behind the band of anthocyanin (i.e., towards the anode). This caused the faster migrating hydroxyproline to pass through the anthocyanins. Pectin was applied in front of the anthocyanin. Changes in the rate of migration or in the colors of the anthocyanins were

noted.

6.2. Results and discussion.

When small 4 cm bands of the anthocyanins were spotted with and without hydroxyproline behind it, no modification of migration was noted. The migration was rather uneven. It seemed that end effects of the 4 cm bands predominated to produce poor results. When a long band of pelargonidin 3-glucoside was spotted across the paper with 4 cm bands of hydroxyproline behind it, some modification in the migration rate was noted. The rate of migration of pelargonidin 3-glucoside was reduced when hydroxyproline was allowed to migrate through the pigment. The resulting pigment was dark brown-orange with a cerise fluorescence under ultraviolet light. Under these conditions the pure pelargonidin 3-glucoside showed no fluorescence. This effect was maximized when the two compounds were 2 cm apart and electrophoresis carried out at 800 volts for $3\frac{1}{2}$ hours.

The hydroxyproline-pelargonidin 3-glucoside present on the paper support was made visible by spraying with ninhydrin solution. The pelargonidin 3-glucoside area on the paper was orange colored while the top area (toward the cathode) was a dark grey color. Farther up the paper, the main band of hydroxyproline was present. The dark grey area above the pelargonidin 3-glucoside was cut out and eluted with 1% HCl in methanol. The spectrum of this solution showed peaks at 510 and 475 nm, shoulders at 405, 322 and at 290 nm, while hydroxyproline and ninhydrin alone had an absorption maximum at 227 nm. The 510 nm peak, characteristic of pelargonidin 3-glucoside showed conclusively that the dark grey colored area, positive with ninhydrin, was indeed a complex containing anthocyanins.

Pectin and proline did not have any effect. Gelatin (50 mg/10 ml) 50 μ l per 4 cm band did not produce any effect on the migration of PVP purified cranberry anthocyanins, when electrophoresis was carried out at 400 volts for 10 hours. No color changes were noted under these conditions. Proline did produce compaction (i.e., less streaking) of the anthocyanin band as it migrated.

Cyanidin 3-glucoside showed a marked difference in the rate of migration of nearly one half with hydroxyproline. An interaction between delphinidin 3-glucoside and hydroxyproline was also detected, but it was less pronounced than the former.

The above evidence suggests some interaction between the anthocyanins and hydroxyproline irrespective of the number of hydroxyl groups on the B ring. Complexes might be more readily formed by varying the quantities of the two ligands applied to the paper. Masking by unreacted compounds would then be less of a problem, and a more distinct intermediate band might be found.

7. Paper chromatography experiments

7.1. Experimental

Two methods were tried in efforts to demonstrate complex formation by the presence of a third chromatographically distinct compound. The anthocyanins, the compound being tested, and the mixture of the two in the same ratio, were streaked in 4 cm long bands at the startline of a No. 3MM Whatman paper chromatogram. All papers were developed in BAW for 8 hours. The following compounds were tested:

gelatin 0.3 g/10 ml

hydroxyproline 180 mg/10 ml

The following anthocyanins were tested: pelargonidin 3-glucoside, cyanidin 3-glucoside and delphinidin 3-glucoside.

Hydroxyproline could be made visible by spraying with ninhydrin. Gelatin did not produce any color with ninhydrin and it was dipped in Azocarmine B or in Amido Black.

The other method used a complete sheet of Whatman No 3MM paper. The paper chromatogram was folded and the complete startline was spotted with the anthocyanin being tested. A line was drawn at an angle of 45° which was streaked with the compound being tested.

7.2. Results and discussions

When chromatographed in BAW solvent, no differences in migration rate were noted between mixtures and the two separate components. The color development of the proteinacious compounds did not show any other band. The anthocyanin bands were rather diffuse and thick even when low concentrations were applied to the paper and could thus possibly contain a small complexed band. Differences in color were noted. The pelargonidin 3-glucoside turned a darker orange-brown and had a cerise fluorescent border near the lower edge (nearer the start line) when co-chromatographed with hydroxyproline. Cyanidin and delphinidin 3-glucoside did not have the cerise fluorescence, although the color of the mixture was darker than the color of the pure anthocyanin.

On some chromatograms, there were indications that pure hydroxyproline (R_f on BAW.15) moved more slowly than the hydroxyproline that was present in a mixture with the anthocyanin. This was not a consistent result and may have been within the experimental error of the procedure.

Gelatin did not migrate on BAW chromatography and no retarding effect was found on the various anthocyanins. Pectin could not be made visible on paper chromatograms, but in comparison with the pure anthocyanin, there was no alteration of the rate of migration of anthocyanin applied as a mixture with pectin.

Experiments using diagonally spotted compounds such as pectin, Al^{+++} or hydroxyproline, would be likely to produce a distinct bend in the straight start line as the pigment migrated down, if, indeed a complex was formed. The paper chromatograms spotted with the three anthocyanins and cyanidin 3-rutinoside did not produce any bending effect of the migrating anthocyanins. It was noted that the color of the anthocyanins at the point at which the migrating anthocyanin band intersected the diagonal band of substrate, such as Al^{+++} , hydroxyproline and gelatin, changed to a slightly blue color. The change of color at the point of intersection of the two bands suggest some interaction between pigment and substrate. However, the complex, if it was formed, would appear to have had limited stability since there was no appreciable alteration in the direction of migration of the anthocyanin bands.

8. Photodensitometric methods applied to certain artificial systems.

Part of the research reported in this thesis was aimed at correlating the molecular structure of the anthocyanins with the positional stability in the processed fruit. During thermal processing and in the storage period subsequent to the latter, redistribution of pigments between plant tissue and surrounding liquor or syrup occurs. A study of the distribution of the anthocyanins in processed foods would therefore show whether selective retention of pigments by processed plant tissue

does occur.

A method was required to separate and measure individual anthocyanin pigments. A number of methods have been summarized by Fuleki and Francis (48). After preliminary experimental considerations, the photodensitometric procedure described in the latter reference was chosen. The experimental technique was investigated to test its reproducibility under the conditions of this laboratory.

8.1. Experimental

The following is an example of the experimental procedure used. Concentrations of reagents and pH values of buffer were changed as noted in subsequent experiments.

8.1.1. Experiment 1.

A 1%, 2% and 3% autoclaved solution of pectin was made in pH 2.7 buffer and a solution of 2.41 g of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ in 100 ml of the same buffer. Pigments used were the purified blackcurrant extract.

The following series of test tubes were prepared.

1. 10 ml buffer + 1 ml pigment
2. 10 ml buffer 1 ml pigment + 10 ml of 1% pectin + 1 ml AlCl_3
3. 10 ml buffer 1 ml pigment + 10 ml of 2% pectin + 1 ml AlCl_3
4. 10 ml buffer 1 ml pigment + 10 ml of 3% pectin + 1 ml AlCl_3
5. 10 ml buffer 1 ml pigment + 10 ml 1% pectin
6. 10 ml buffer 1 ml pigment + 10 ml 2% pectin
7. 10 ml buffer 1 ml pigment + 10 ml 3% pectin
8. 10 ml buffer + 1 ml pigment + 1 ml AlCl_3

After thorough mixing of the contents, the test tubes were stored in darkness for two days. Then 40 ml of ethanol was added to each test tube

and the content was shaken. The gelatinous precipitate was separated by centrifugation and the supernatant concentrated in a Buchler rotary vacuum evaporator.

The extract of each treatment was streaked 4 or more times on the startline of Whatman No. 3MM paper chromatograms. Chromatograms were developed with AWH for 8 hours. After drying the paper and fuming with HCl for ten minutes the four bands on each chromatogram were measured with a Densicord, Model 542 (Photovolt Corp., New York) under the following standard conditions:

Chart speed 2"/min.

Filter 525 nm

Aperture 0.1 x 6 mm.

Instrument response setting 5

Instrument warm up period 4 hours

Areas under the curves were found by planimeter and the percentage of each pigment determined. The results of band 1 and 3 (as measured from the origin of the paper chromatogram) were combined and the analysis of variance calculated for eight treatments and three replicates (e.g., three paper chromatograms from each aliquot taken from the treatment test tube). The results showed the percentage of delphinidin glycoside present in the ethanol extract.

8.1.2. Experiment 2

The following four solutions were made up:

1. High methoxyl pectin and cyanidin and delphinidin glycoside
(Blackcurrant anthocyanin extract)
2. Same as 1. but with 1 ml 0.1% AlCl_3

3. Cyanidin and delphinidin glycosides in buffer.
4. Same as 3. but with 1 ml 0.1% AlCl_3

After thorough mixing the pH of each test tube was adjusted to pH 1.8. The pH of each solution was gradually brought up to pH 4.0 by small incremental additions of NaOH. The absorption spectrum between 250 nm and 800 nm of each solution was recorded at pH 1.8, 2.6 and 4.0.

8.1.3. Experiment 3

A further experiment in complex formation between anthocyanins, pectin and cations was made. The different treatments are listed below. Each series was made up in pH 2.7 buffer and in pH 4.4 buffer.

1. Blackcurrant anthocyanin in buffer
2. Anthocyanins + 1 ml of AlCl_3 (0.24%)
3. Anthocyanins + 1 ml of FeCl_3 (0.5% solution)
4. Anthocyanins + 1% low methoxyl citrus pectin solution
5. Anthocyanins + 1% high methoxyl pectin solution + 1 ml of AlCl_3 solution
6. Anthocyanins + 1% high methoxyl pectin solution + 1 ml of FeCl_3 solution
7. Anthocyanins + 1% high methoxyl pectin solution + 1 ml each of FeCl_3 and AlCl_3 solution.

The pH values of the 14 test tubes solutions were checked before the ethanol precipitation. The procedure given in Exp. 1 was followed to obtain the concentration of free unabsorbed delphinidin glycosides.

8.2. Results and Discussion

8.2.1. Experiment 1.

Appendix 4 shows the concentrations obtained for band I delphinidin 3-glucoside; band II cyanidin 3-glucoside; band III delphinidin 3-rutinoside; and band IV cyanidin 3-rutinoside. The total amount of delphinidin was found by adding Band I and III and the results analysed statistically. At pH 2.7, treatments were found to be highly significant while the replications were not significantly different (Appendix 5).

TABLE 17. Mean values of the total delphinidin glycoside present after reaction with pectin in the presence and absence of Al^{+++} .

Treatment	Total % delphinidin glycoside
1. Pigment and buffer	41.07%
8. Pigment and buffer + Al^{+++}	39.00%
5. Pigment + 1% pectin	39.53%
6. Pigment + 2% pectin	34.70%
7. Pigment + 3% pectin	31.37%
4. Pigment + 3% pectin + Al^{+++}	29.07%
3. Pigment + 2% pectin + Al^{+++}	25.13%
2. Pigment + 1% pectin + Al^{+++}	18.23%
L.S.D. at 5% level 3.19	

Table 17 shows the treatment means placed in order of magnitude. The results seem to suggest that the greatest quantity of delphinidin glycoside was retained by the 1% pectin system in the presence of aluminium ion, i.e., system 2. Systems 1, 8 and 5 yielded very similar results. These,

in effect were blanks which showed that no other retention had occurred.

The reason why the lower concentration of pectin should show most retention of the delphinidin is not clear. It is suggested that the Al^{+++} was the limiting factor.

The pectin solutions were autoclaved for 10 minutes to solubilize the pectin. The autoclaving did produce a slightly brownish tinge which signified decomposition or hydrolysis of the pectin during heating. This effect of autoclaving was studied in a later experiment and no differences were found between the autoclaved and non-autoclaved pectin solutions in the effect on pigment interaction.

8.2.2. Experiment 2.

When the pH was changed incrementally to pH 2.6, various color changes were noted. Table 18 shows the pertinent data. It seems that the three components together do have a different effect from either of the two separate components in regard to color of solutions and absorbance maxima. However, changes in the absorbance maxima of the order of 2 nm are rather tenuous grounds for the assumption of complex formation. Certain molecular associations may produce only slight changes in the absorption spectrum especially in the visible region.

TABLE 18. The effect on color and absorption maxima of mixtures of Al^{+++} , high methoxyl pectin and cyanidin and delphinidin glycosides

Reagents	pH	Abs. max. nm	Color of solution
High methoxy pectin + pigments	2.6	515	red
" " " + Al^{+++}	2.5	517	bluish red
Pigments	2.5	515	red
Pigments + Al^{+++}	2.6	518	deep blue

8.2.3. Experiment 3.

The complete data obtained in this experiment is presented in Appendix 6. The analysis of variance for the two pH levels are shown in Appendix 7 and Appendix 8. There were differences between the means of the treatments at $p = 0.01$ level. The means are shown in Tables 19 and 20 for the two pH levels.

TABLE 19. Mean percentage of total free delphinidin glycosides after reaction at pH 2.7 with pectin with or without metal cations

Treatment	mean %
Pigment	48.41%
Pigment + Al^{+++}	50.21%
Pigment + citrus pectin + Al^{+++}	48.32%
Pigment + high methoxyl pectin + Al^{+++}	41.45%
L.S.D. at 5% level 2.01	

TABLE 20. Mean percentage of total free delphinidin glycoside after reaction at pH 4.3 with pectin with or without metal cations

Treatment	mean %
Pigment	35.31
Pigment + Al^{+++}	47.39
Pigment + citrus pectin	43.40
Pigment + high methoxyl pectin + Al^{+++}	41.96
L.S.D. at 5% level 3.11	

The blank pigment compositions at pH 2.7 and pH 4.3 should be comparable. However they are not as at pH 4.3 less delphinidin glycosides were present. This suggested that the differences found may not be

completely due to complexation, but partly to differences in stability or to measurement of the different components at this pH.

From Table 20, it can be seen that the percent of free delphinidin glycoside in pigment Al^{+++} at pH 4.3 is nearly the same as the blank pigment at pH 2.7. Thus it seems that the delphinidin- Al^{+++} complex stabilizes the color. Also pigment-high methoxyl pectin Al^{+++} at both pH levels are the same. If complexing had indeed taken place, then the pH had no effect on it. From the data presented in Tables 17 and 19 it would seem that high methoxyl pectin forms complexes with delphinidin glycosides and aluminium ions more readily than the low methoxyl pectin.

8.3. Effect on anthocyanin-pectin system on the addition of ferric ions.

A number of interesting results were noted when iron was present in the reaction mixtures. (See Table 21). At pH 2.7 in all the iron-containing test tubes, only cyanidin glycosides were present. In the case of the pH 4.3 series of reactions, only the two cyanidin glycosides were found when both the ferric ions and pectin were present. However, the blank treatment, that is, with only the ferric ions present, showed both cyanidin and delphinidin glycosides to be present. This experiment confirms the results obtained from the spectrophotometric experiment which showed that pectin and ferric ions led to the complete destruction of delphinidin glycosides. This destruction effect on the basis of these experiments would seem to be pH dependent as the delphinidin glycosides were stable at pH 4.3 when only Fe^{+++} was present.

TABLE 21. Average concentration of anthocyanins after reaction with pectin,
 Fe^{+++} and/or Al^{+++} cations at pH 2.7 and 4.3

pH	Treatment	Pigment			
		Delphinidin 3-glucoside	Cyanidin 3-glucoside	Delphinidin 3-rutinoside	Cyanidin 3-rutinoside
2.7	Pigment + Fe^{+++}	0	19.0	0	81.0
	Pigment + pectin + Fe^{+++}	0	26.6	0	73.4
	Pigment + pectin + Fe^{+++} + Al^{+++}	0	26.9	0	73.1
4.3	Pigment + Fe^{+++}	12.7	16.7	22.0	48.6
	Pigment + Fe^{+++} + pectin	0	27.5	0	72.5
	Pigment + Fe^{+++} + Al^{+++} + pectin	0	24.0	0	76.0

VI. Anthocyanin Stability in Processed Montmorency Cherries

1. Introduction

Studies reported in this thesis have demonstrated that anthocyanins may form complexes with pectin and with the amino acid hydroxyproline. It seemed possible that if anthocyanin containing fruits were impregnated with either pectin or hydroxyproline prior to thermal processing, loss of pigment from the tissues during processing might be retarded.

It must be clearly established where the anthocyanins are desired to be stabilized. There are three regions where this is possible. The anthocyanin could be immobilized in the thin epidermal cell layer. This region contributes greatly to final product color. The second possibility would be to allow the anthocyanins to diffuse into the fruit where it would then be immobilized. The third possibility would be to stabilize the anthocyanins after the latter had diffused out of the berry and were present in the juice. To the author's knowledge, no quantitative studies have been carried out to determine in which area the anthocyanins are least stable.

The preferred region for stabilization would obviously be the skin region. However, due to the nature of this fruit and the conditions under which it is processed, pigments may diffuse deep into the cortical tissue or out into the syrup. It seemed a feasible proposition to minimize diffusion into the syrup and to increase the color retained in the berry. This was based in the hope that the pigments retained in the cortical tissues would eventually contribute to the color of the product. It also seemed to the author, that the syrup would be a much better environment

for the pigment decomposition than the berry.

The treatments in this experiment were designed to increase the retention of pigment by the tissue of the cherry. If the treatments had been successful, the added compounds would have held the pigments within the tissues beneath the skin rather than in the narrow epidermal layer itself.

In these types of experiments, small changes in the various constituents may have great significance. For this reason the experimental design must be such that these small differences may be found. As noted before, cherries as well as blueberries and cranberries have the anthocyanins confined to a thin external epidermal layer (or skin). The remainder of the berry is whitish or yellowish as in the Montmorency cherry. Thus the amount of the anthocyanins added or processed is not proportional to the weight of the fruit but to the surface area of berry skin. Ideally, therefore, each processing container should have contained an equal area of berry surface, a condition that was actually impossible to achieve. The nearest approximation was to take an equal weight of berries because the latter were approximately equal in size and shape. The processing of the fruit introduced other variations. In this experiment the weights of the berries were equalized to within 2 g within each treatment. If exactly equal weights of berries were taken, it would have been necessary to cut portions of cherries for balancing purposes. The presence of cut cherries might well affect the results. Correction to constant weight of berries and volume of syrup was carried out when the samples were analysed.

The containers used for processing were filled as completely as

possible with syrup to reduce head space gases which are known to affect anthocyanin stability (132). In view of sample to sample variations in total cherry volume and slight differences in container volume, the volume of syrup added varied by about 6% from sample to sample.

2. Literature Review

Montmorency sour cherries can be used for canning or in the production of pie fillings. Both products have been criticized for the loss of color after processing (32) (46).

Sour cherries used for canning, may or may not have pits removed from them. Syrups range from 40° Brix for the highest grades to 10° for the lower grades. Cans are usually exhausted for at least ten minutes at 165 to 185°F. After closure, sterilization may be carried out at 212°F for 12 to 25 minutes depending on the can size (38).

Pectic substances undergo changes as the fruit ripens. Evidence suggests that the pectic components of fruit may be hydrolysed enzymatically or non-enzymatically to yield more soluble forms. This results from partial de-esterification and depolymerization with consequent reduced molecular weight (20). Processing decreases attack by enzymes upon pectic substances due to heat denaturation and deactivation of the enzymes. However, non-enzymatic reactions which promote solubilization of the pectins also do occur.

The changes in pectic substances in processed sour cherries have been investigated (33). The usual pattern seems to be that the total pectin decreases during storage, the loss being greater at elevated storage temperatures. Thus the insoluble protopectin in the berry is hydrolysed to the soluble form in the covering syrup.

Loss of anthocyanin pigments in processed fruit has been shown to be affected by certain cations (91) (122) (126) high temperatures (32) (34) (37) (91) (95) (101), and handling procedure (14). Measurement of color or anthocyanin pigments can be done by a number of instruments such as the Hunter color difference meter, Munsell colorimetry or spectrophotometrically. A simplified method for measuring anthocyanin pigment content uses a low pH buffer, pH 1.0, to dilute the juice in order to obtain measurable readings on the spectrophotometer (70) (100). At this pH, the highly colored oxonium form of the anthocyanins predominates and the resultant absorbance at the wavelength of maximum absorption indicates the relative amount of pigment present.

Fuleki and Francis (47) have proposed a Degradation Index (D.I.) which is based on differences in absorbance at pH 1.0 and pH 4.5. Anthocyanins show little absorption at the latter wavelength and most absorbance may be assumed to be due to decomposition products. Freshly prepared cranberry extracts showed a D.I. of 1.10. Starr and Francis (125) applied the D.I. to the stability of cranberry anthocyanins and obtained values of 1.24 at the end of about 30 weeks corresponding to 40% loss of total anthocyanins.

3. Experimental

Montmorency cherries dipped in 2000 ppm Baycovin (diethyl esters of pyrocarbonic acid) were supplied by Dr. Tibor Fuleki, Vineland Research Station. The berries, after storage for four days, subsequent to delivery, in the laboratory cool room, were destemmed and washed in distilled water.

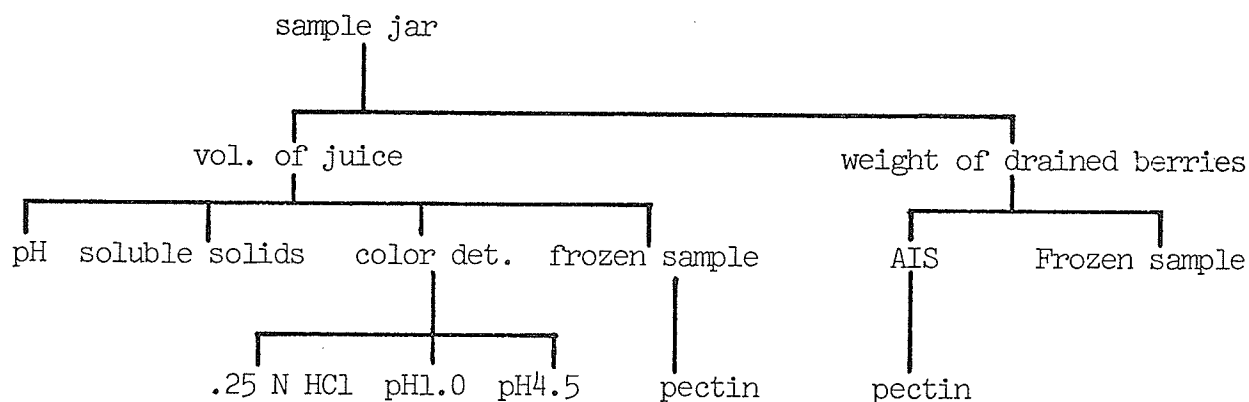
3.1. Design of experiment

An experiment was designed for five treatments with analyses carried out after three post-processing storage periods. There were three replicates of each treatment so that the total number of samples was 45. The five treatments are listed below:

- A. Cherries with no treatment.
- B. Cherries injected at 3 places with a total of 0.5 ml. of 3% pectin solution at pH 2.5.
- C. Cherries injected at 3 places with a total of 0.5 ml. of 3% solution of hydroxyproline at pH 2.5.
- D. Cherries immersed for 15 minutes in 3% pectin solution at pH 2.5 under vacuum.
- E. Cherries immersed for 15 minutes in 0.1% hydroxyproline solution at pH 2.5 under vacuum.

3.2. Chemical analysis

One sample of the fresh frozen cherries was set aside for the determination of pigment ratios. A schematic diagram of the analytical procedure used for the processed cherries is given below.



The cherries after each treatment, were placed in 12 oz. glass pickle jars so that the total weight in each jar did not vary by more than 2 g in each treatment. The jars were topped with hot 50% sugar syrup and exhausted to an internal temperature of 185°F (85°C) in a steam kettle until no more bubbles were noted. The jars were topped and the temperature was raised to 212°F. (100°C) and processed for 12 minutes. After removal from the kettle, the jar lids were tightened and the jars slowly cooled in running warm water, and then in cool water.

Analyses were made on the 13th, the 34th and the 62nd days after processing. The jars were placed for extra light protection in closed paper carton boxes and stored in a dark cupboard at room temperature.

The contents of each jar was poured onto a cheesecloth filter and allowed to drain for one hour. The drained weight of the cherries and the volume of juice were then recorded. The pH and the soluble solids were determined with a Radiometer Model M26 pH meter and a Carl Zeiss hand refractometer, respectively. Suitable dilutions of the juice were made with one of the following three diluents: 25 ml to 100 ml with 0.25 N HCl, 25 ml to 100 ml with pH 1.0 buffer, and 35 ml to 100 ml with pH 4.5 buffers. A sodium acetate/hydrochloric acid buffer system was used (47). The absorbancies of the dilutions were determined using a Unicam SP 800 spectrophotometer after the samples had been stored for two hours in darkness to allow the anthocyanins to fully reach equilibrium. The remainder of the juice was frozen and stored for future analysis.

A 20 g sample of the depitted berries was boiled in 200 ml of 95% ethanol for one hour. The berries were ground in a blender after

ten minutes to facilitate the extraction. The volume was kept constant during boiling by the addition of further ethanol. The alcohol-insoluble solids (AIS) were then vacuum filtered on a Buchner funnel and washed twice with 200 ml of fresh 95% ethanol. The AIS collected were air dried and finally dried in a vacuum oven at 50°C for four hours. The weight of AIS was recorded after the final drying and the samples were analyzed for total pectin content.

Another sample of the depitted, processed berries was blended with 50 ml of an 85:15 v/v mixture of 95% ethanol/1.5 N HCl and stored overnight to extract the anthocyanins. The filtered extract was spotted on paper chromatograms and developed with 2% HAc for 2 hours. This produced two, well separated anthocyanin bands previously identified as the 3-glucosylrutinoside and the 3-rutinoside of cyanidin. Strips, cut from the chromatograms, were scanned on a Densicord Model 542 recording photoelectric densitometer using conditions previously established. (8.1.1.) The weight of the paper beneath the tracing of the curve or peak was taken to indicate the relative concentration of the pigment in each band. The ratio of the two bands was then calculated for each sample. A sample of the fresh frozen cherries was also treated and analysed in this manner to determine the pigment ratios. The remaining depitted berries were frozen and stored for future analysis.

The total pectin in the berry AIS was determined by the standard method of McCready and McComb (90). The pectin content of the syrup was measured using the procedure applied by these authors to the analysis of frozen orange concentrate serum.

3.3. Statistical Analysis

The experiments were analysed statistically as a factorial experiment with five treatments, three periods of storage and three replications. A standard computer library package of factorial analysis, run on an IBM 360 computer, provided the analysis of variance tables to three decimal places.

4. Results and Discussion

The data obtained from the various analyses carried out are listed in Appendix 9.

4.1. Pigment content of the juice.

A distinction should be made between the terms color and pigment content. Color is a three dimensionally defined property. Measurement of the absorbancy at one wavelength defines only one of the three dimensions. Thus it is incorrect to use the term color except where it is measured on a Hunter Color Difference Meter or equivalent instrument. The measurement of absorbance at a particular wavelength corresponding to the maximum absorption of the anthocyanins is an indication of relative pigment content. This is the correct term to use.

Anthocyanin content was measured by the absorbance of solutions in 0.25 N HCl. The analysis of variance is given in Appendix 10. It indicated that length of storage was highly significant while the treatments were significantly different. This data has been corrected for variations in the weight of the berries and the volume corrected to equal the volume of the juice present in the untreated sample. As shown in Table 22 and Figure 12 pigment content decreased after increasing lengths of storage. This result is in agreement with other workers (33)(95)(125).

FIGURE 12. Mean pigment content as determined by absorbance measured in 0.25 N HCl after three storage periods.

FIGURE 13. Treatment means for the measurement of pigment content in processed cherry juice.

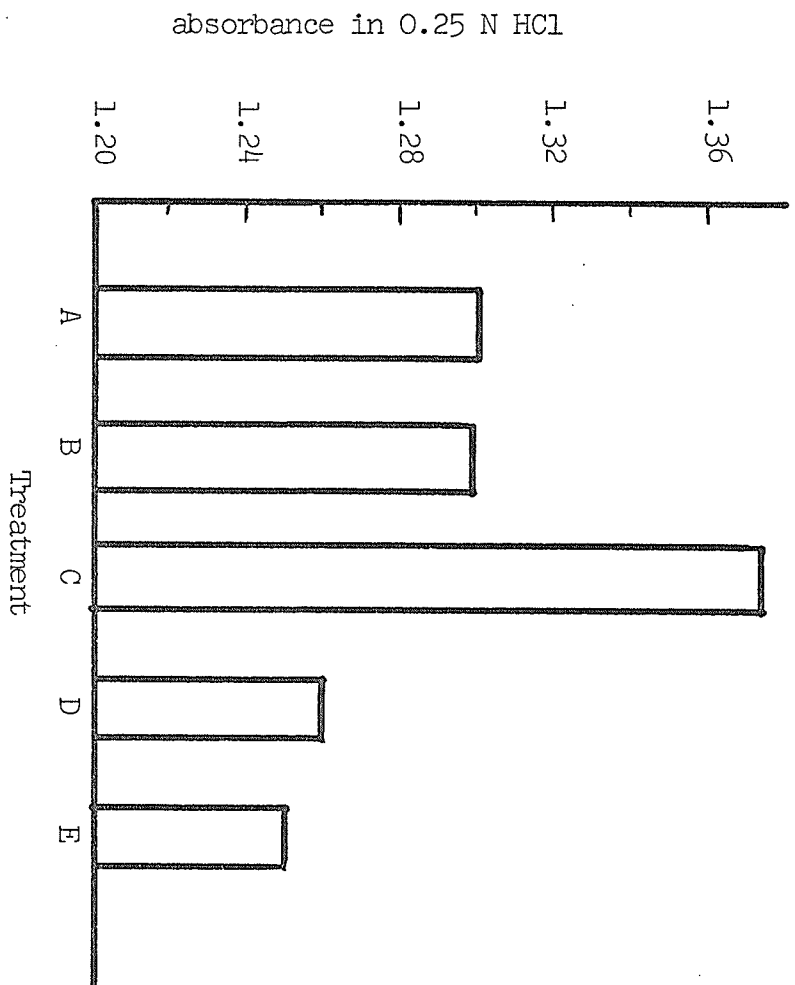
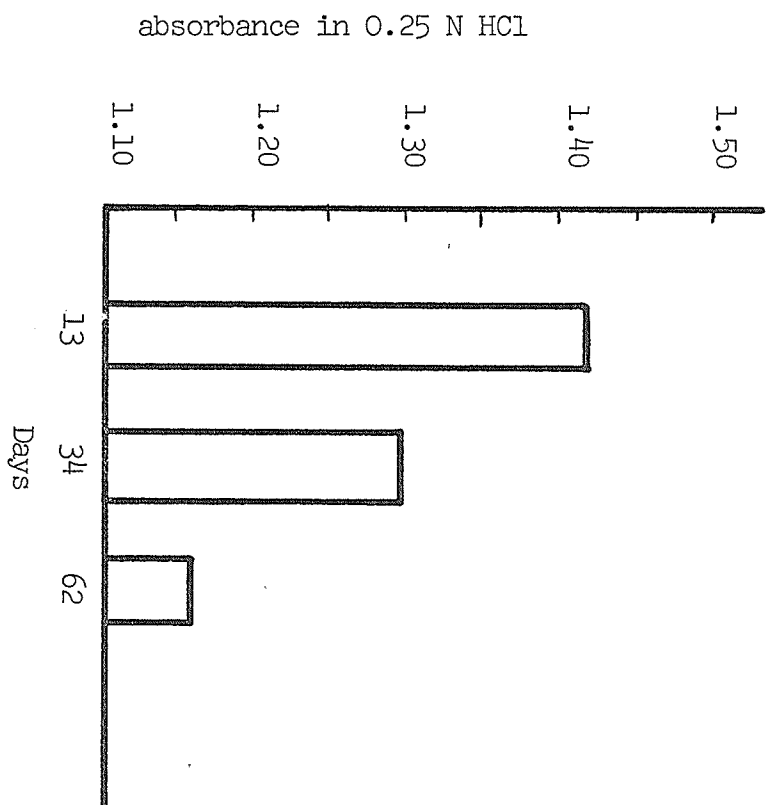


TABLE 22. Mean pigment content expressed in absorbance in 0.25 N HCl at three storage lengths in processed Montmorency juice

Storage period	Absorbance in 0.25 N HCl
1st. (13 days)	1.429
2nd. (34 days)	1.297
3rd. (62 days)	1.163
L.S.D. value; $P = 0.05 = .035$	

TABLE 23. Mean treatment values of pigment content as determined by absorbance in 0.25 N HCl of processed Montmorency cherry syrup

Treatment	Absorbance in 0.25 N HCl
A. (untreated)	1.296
B. (injected pectin)	1.303
C. (injected hydroxyproline)	1.371
D. (vacuum pectin)	1.259
E. (vacuum hydroxyproline)	1.253
L.S.D. value; $P = 0.05 = .069$	
$P = 0.10 = .043$	
$P = 0.25 = .028$	

The comparison of the treatment means in Table 23 (or Figure 13) shows that the injection of hydroxy proline produced a significant difference in the mean pigment values when compared with the untreated sample by the least significant difference technique. The means showed that two highest values were obtained with the injection treatment while the vacuum treatment produced the two lowest values. The greatly lower

values for the vacuum treatment with pectin or hydroxy proline seems to suggest that this process has the effect of decreasing pigment diffusion from the berry. This effect fails to be significantly different from the untreated L.S.D.; 0.042 value compared to the 0.069 at $P = 0.01$. However it is significant when the L.S.D. value is calculated at the $P = 0.10$ level.

Before conclusions are drawn from this experiment, it must be pointed out that it was impossible to separate the effect of increased retention in the berry and the possible increased or decreased destruction of the anthocyanins in the juice. The addition of the pectin and the hydroxyproline may well have accelerated the decomposition of the anthocyanins in the juice, although heating cyanidin glycosides in a 1% solution of pectin at pH 3.0 did not lead to appreciable decomposition. The low level of significance found, and the latter mentioned problem, make it difficult to ascribe protective effects to the two tested compounds with certainty.

4.2. Ratios of cyanidin 3-rutinoside to cyanidin 3-glycosylrutinoside

Statistical analysis of the pigment ratios (Appendix 11) showed that the differences between the means of the treatments were not significant at the $P = 0.05$ level nor were the results for the different storage periods significantly different. The pigments would therefore appear to decompose at equal rates and yield the same pigment ratio. Comparison with other experiments of this type shows that the coefficient of variation of 24% obtained in this section is somewhat higher but to the author within acceptable experimental limits.

The ratios of the pigments present in the fresh frozen cherries

were obtained using the same experimental procedure. On statistical comparison between the fresh berry ratios and those obtained after the three storage periods, it was shown that significant differences did occur between these four ratios (Appendix 12).

TABLE 24. Comparison of the pigment ratio means of fresh Montmorency cherries and those of the processed fruit at three different storage lengths

Storage	Pigment ratios
Fresh	2.731
1st. (13 days)	2.378
2nd. (34 days)	1.830
3rd. (62 days)	2.617
L.S.D. value at P = 0.05 level .476	

It is readily apparent that the mean for the second storage period (after 34 days) was significantly different from the remainder.

The ratio was calculated by dividing the cyanidin 3-glucosyl-rutinoside by cyanidin 3-rutinoside. Each peak was measured by taking the weight of graph paper underneath the peaks of the photodensitometric tracing. From the data of the four means in Table 24, the ratio value is decreasing to the end of the second storage period, after which is a rapid increase in the ratio. Until more is known about the chemistry of processed fruits it is impossible to give a chemical interpretation of these results.

A rather interesting deduction may be drawn from this data. At no time did any of the chromatograms show intermediate anthocyanin decomposition pigments, such as the cyanidin 3-glucoside or the 3-sophoro-

side. Thus the anthocyanin glycosides may not decompose through an anthocyanidin intermediary but may instead polymerize directly to produce the brown colored decomposition products. Alternatively, if hydrolysis does occur, then the products must react completely so that no detectable intermediates remain but this seems unlikely.

No comparable data was found in the literature for the Montmorency cherry anthocyanins. It has been shown in the case of red raspberry, that one band (possibly cyanidin 3, 5-diglycoside) was more stable than the other anthocyanins. The individual anthocyanins were measured by colorimetric methods. The authors did note that the apparent increase in the stability may have been due to the large quantities of this pigment that were present. (36).

4.3. Degradation Index (D.I.)

Highly significant differences were found between the different lengths of storage (Appendix 13). Table 25 indicates that the mean D.I. at the three storage periods increased as the length of storage increased. This was an expected result as the absorbance in 0.25 N HC l was demon-

TABLE 25. Mean values of the Degradation Index
calculated for processed Montmorency cherry
juice after three storage periods

Storage period	Mean D.I.
1st. (13 days)	1.216
2nd. (34 days)	1.274
3rd. (62 days)	1.351
L.S.D. value $P = 0.05$.0063	

strated to decrease over this time interval. Table 26 shows that the

treatments had no effect upon pigment degradation since the differences between the means were not significant at $P = 0.05$ level. It may be

TABLE 26. Mean values of the Degradation Index for five treatments applied to processed Montmorency cherry juice

Treatment	D.I.
A. (untreated)	1.284
B. (injected pectin)	1.275
C. (injected hydroxyproline)	1.276
D. (vacuum pectin)	1.283
E. (vacuum hydroxyproline)	1.282
L.S.D. value $P = 0.05$.009	

tentatively concluded that the various treatments did not exert any protective effect in the cherry syrup as measured by the degradation index. The two injection treatments did yield lower values than the other three treatments. The significance of this is not known although it is recommended that in future experiments the appropriate blank treatments (i.e., injection with water) are included.

4.4. Pectin present in the syrup and in the berry.

Analysis of variance for the total pectin content in the processed berry (Appendix 14) showed highly significant differences between storage periods. Mean pectin content over the whole experiment was 508.9 mg galacturonic acid/100 g of drained berry. The means decreased rapidly between the second and third storage period (Table 27 or Figure 14).

FIGURE 14. Mean total pectic substances (mg per 100 g
drained berries) after three storage periods.

FIGURE 15. Treatment means for the measurement of pectin
in processed cherry syrup.

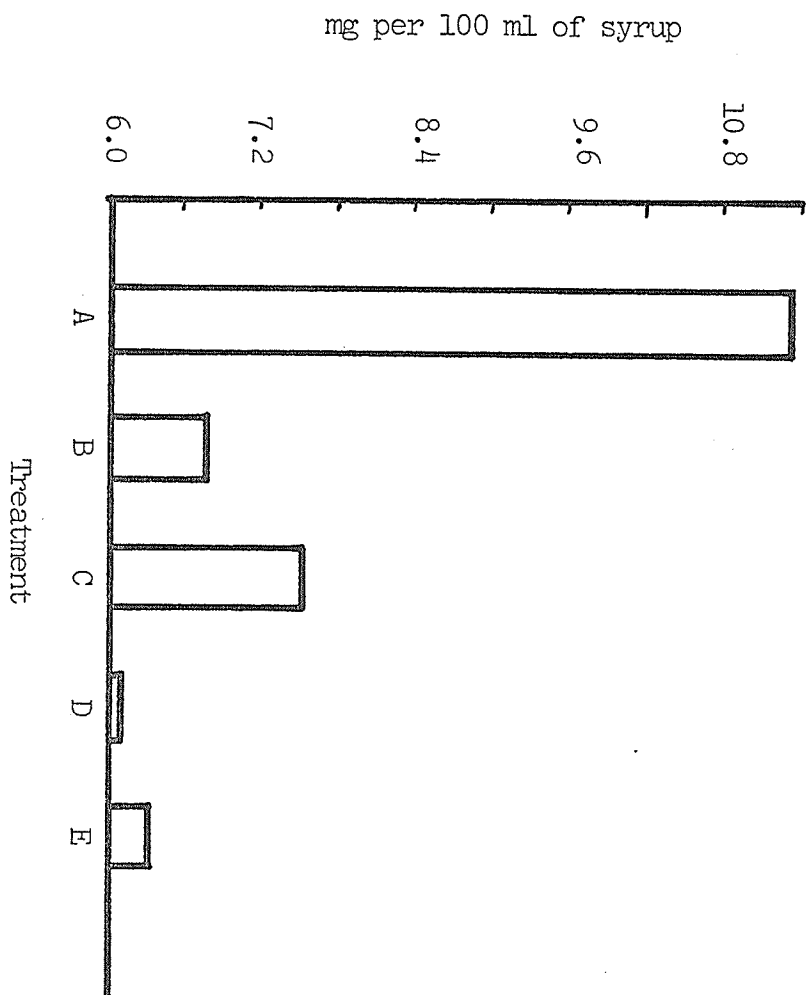
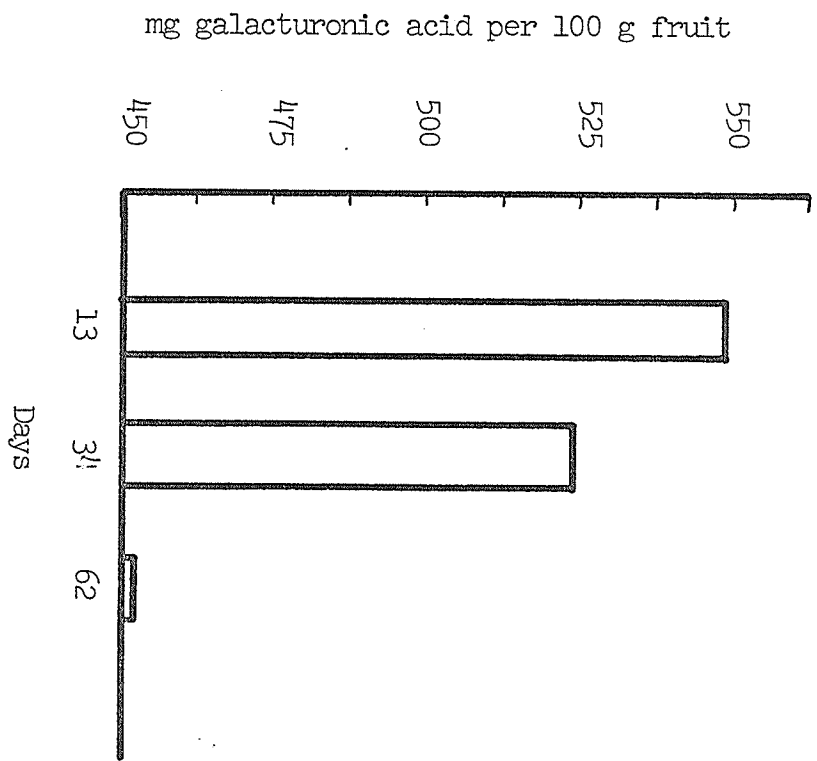


TABLE 27. Mean pectin content of processed Montmorency cherries after three storage periods

Storage period	Pectin content as mg galacturonic acid/100 g
1st. (13 days)	549.4
2nd. (34 days)	524.5
3rd. (62 days)	452.8
L.S.D. value $P = 0.05$ 22.30	

The lack of significant differences between the treatment means indicate that the addition of pectin did not lead to the recovery of this compound on analysis. It was shown that the AIS should have about .09 g of extra pectin in each sample taken for analysis. The pectin content as determined averaged around 550 mg. An extra 90 mg should definitely have shown up in this analysis. However, as with the AIS, no increase in pectin was noted after the pectin treatments (Appendix 14).

The means of the various treatments and storage lengths are given in Table 28 and Table 29 and graphically in Figures 15 and 16.

TABLE 28. Means of five treatments of the pectin present in processed Montmorency cherry juice

Treatment	pectin as mg.gal.acid/100 ml
A. (untreated)	11.3
B. (injected pectin)	6.8
C. (injected hydroxyproline)	7.4
D. (vacuum pectin)	6.1
E. (vacuum hydroxyproline)	6.3
L.S.D. value $P = 0.01$ 2.61	

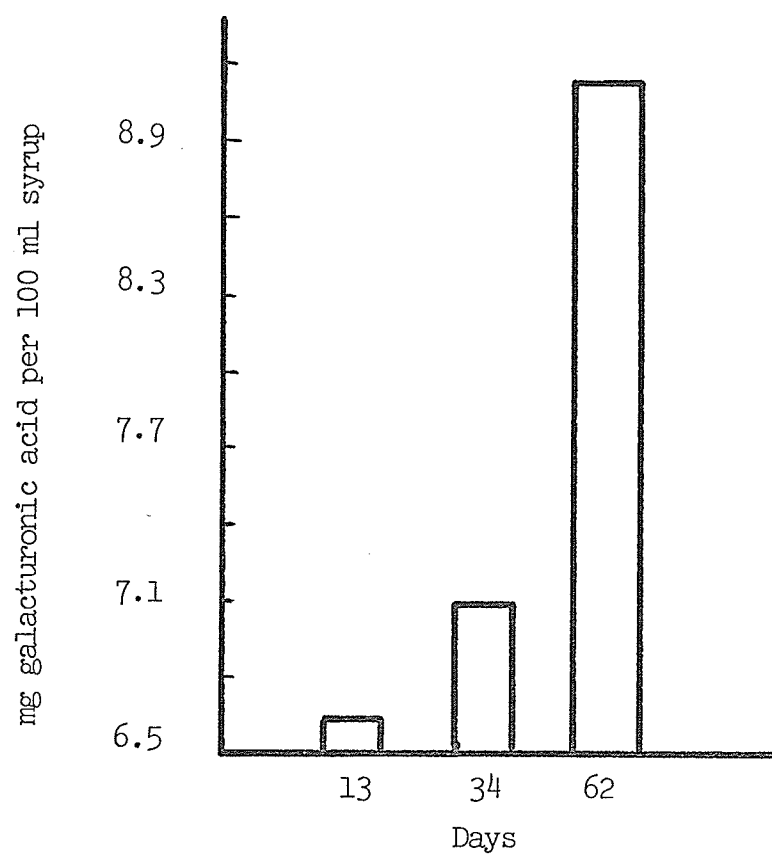


FIGURE 16. Mean pectic substances (mg per 100 ml of syrup) after three storage periods.

TABLE 29. Mean pectin content in processed
Montmorency cherry juice at three lengths
of storage

Storage period	mg galacturonic acid/100 ml of juice
1. (13 days)	6.6
2. (34 days)	7.0
3. (62 days)	9.2
L.S.D. value $P = 0.01$ 2.0	

The comparison of treatment means in Table 28 shows that again the untreated sample contained the highest amount of pectin. It seems unlikely that the added pectin was recovered. The evidence suggests that pectin had been converted to other compounds that were not detected by pectin analysis. The pectin determinations were corrected to a standard weight of berry and syrup.

Commercial pectins of the type used in this experiment do not resemble naturally occurring pectins very closely. They receive extensive purification treatment. The added pectin may therefore behave differently from naturally occurring pectin, both in possible retention of anthocyanin or in stability under processing conditions.

The four treatments resulted in lower values for the pectin in the treated berries and in the juice. The evidence contained in Table 28 seems to suggest that treatment of cherries with commercial pectin or hydroxyproline resulted in the lowering of pectin content. The reason for this is not apparent unless the treatment manipulations, such as injection or vacuum infiltration had major effects.

4.5. Correlations between a number of parameters in processed cherries.

From the data reported in Appendix 9, correlation coefficients could be calculated which might indicate associations between the variables. A number of correlation coefficients are listed below with the calculated "t" value. (** Significance at $P = 0.01$; *denotes significance at $P = 0.05$. t value for $N = 43$ at $P = 0.01$ 2.7045 and at $P = 0.05$ 2.0211.)

- A. Pigment content measured by absorbance in .25 N HCl
vs. total pectin in canned berries. $r = .39$, $t = 2.779^{**}$
- B. Pigment content measured by absorbance in .25 N HCl
vs. AIS of the canned berries. $r = .15$, $t = 1.116$
- C. Pigment content measured by absorbance in .25 N HCl
vs. pH of the syrup. $r = -.23$, $t = 2.197^{*}$
- D. Pigment content measured by absorbance in .25 N HCl
vs. D.I. $r = -.79$, $t = 8.448^{**}$
- E. Pigment content measured by absorbance in .25 N HCl
vs. soluble pectin as mg/100 ml syrup. $r = -.18$, $t = 1.435$
- F. Soluble pectin as mg/100 ml syrup vs. D.I.
 $r = .37$, $t = 2.947^{**}$
- G. Total pectin in canned berries mg galacturonic acid
vs. D.I. $r = -.61$, $t = 5.048^{**}$

The correlation coefficient in (E) could be compared with the r value calculated from the data presented by Dickinson and Gawler (40). For nine different storage times, a correlation coefficient of $r = .41$, $t = 1.189$ was calculated between pigment content of the syrup (mg/100 ml of

chrysanthemum) and pectin in the syrup (mg/100 ml of syrup determined as calcium pectate). The correlation coefficient obtained in this study, $r = -0.18$, is thus at complete variance with the previous result calculated from the data of Dickinson and Gawler (40). Unfortunately the color of the drained, canned berry was not obtained in this study. This value may be correlated with the pectin content of the berries to show if stabilization in the berries was possible. From the data presented here, no protective effect of pectin could be shown on the cyanidin glycosides present in the Montmorency cherry.

Both the pectin in the juice and in the berry affected the D.I. as highly significant r values were shown. The pectic substances decreased as the degradation index increased. This need not necessarily be a causal relationship.

In correlation between the pH of the juice and the pectin in the berry and in the juice, correlation coefficient of .04 and .02 were found respectively. Thus the addition of pectin and its subsequent "loss" cannot be explained by the pH levels which control the enzymatic or hydrolytic changes in the pectin. The negative correlation coefficient between color of the juice and pH, shows that as the pH is lowered more color is present. Two explanations may be offered. The decrease in pH may produce more colored oxonium forms of the anthocyanins. However, it may also be that a lower pH value, degradation reactions are considerably slowed. Meschter (91) has noted that at pH 1.85 the color of pelargonidin 3-glucoside is maximized.

The high sugar content of the covering syrup interferes with the chromatographic separation of the anthocyanins. It was impossible to

separate the two anthocyanins present in the juice in any chromatographic solvent directly. The pigment ratios could only be obtained from the ethanol extracted berries. Even this produced a higher coefficient of variation than was usual. It is suggested that a lower sugar content be used which is still compatible with commercial practice. Separation of sugars from pigment by ion exchange, PVP chromatography or possibly by lead acetate precipitation would require proof of quantitative recovery of individual anthocyanins.

The sugared syrup may aid the rupture of the anthocyanin cell layers due to the high osmotic pressure. Since this layer of cells is essential for an attractive product, it seems that the osmotic effect could be important. The higher the sugar content of the syrup, the greater is the likelihood of the formation of compounds like the furfurals that will condense with anthocyanins present to form brown colored compounds. The osmotic effect mentioned above should be properly investigated as a possible solution to the diffusion of the anthocyanins from the fruit. Daravingas and Cain (34) did note that increasing the sugar concentration from 0 to 50° Brix resulted in lower, recoverable pigments. The effect on individual anthocyanins is not known.

It should be remembered that these results obtained refer only to cyanidin 3-rutinoside and 3-glucosylrutinoside. The ideal fruit for this type of experiment would contain in nearly similar proportions two or three anthocyanidin structures which must be readily separable. Black-currants may be suggested as one such fruit, if large enough quantities could be secured.

VII. Final Discussion

Early in this investigation of anthocyanin stability, it became obvious that there were two types of stability that were of interest to the food scientist. These were described as positional stability and chemical stability.

Chemical stability has been investigated by a number of workers in both model systems and in specific fruit products. Some of this work is difficult to interpret because of particular difficulties associated with anthocyanin research. Investigation of structural and pH effects is extremely laborious due to the fact that six different anthocyanidins commonly occur as glycosides in foods and there are, of course, many different glycosides of each anthocyanidin. It can be stated that even with the extensive research published, no coherent explanation has been provided to account for the various reactions that have been observed.

Positional stability may be defined as: a reduced tendency to diffuse from the plant tissues into the surrounding syrup. To increase positional stability one would have to devise means of securing the anthocyanin molecules at the sites they occupy in the plant cell. This problem to the author's knowledge, has not previously been investigated. As the study progressed, it was felt that perhaps there was not as great a distinction between the two types of stability as was originally postulated. Whenever attempts were made to increase positional stability of anthocyanins, there would also be a chemical stabilizing effect.

The present research showed that certain anthocyanins could form complexes with pectin. The general requirements for complex form-

ation were the presence of aluminium ions and a suitable pH which was usually in the pH 2.7 region. The anthocyanins that formed complexes of this type, were delphinidin glycosides. There are literature reports of complexes that contain cyanidin glycosides while the complex isolated during the present research contained petunidin glycosides. All glycosides tested except those of pelargonidin, formed complexes with aluminium.

Evidence was obtained for the complex formation or molecular association between anthocyanins and the amino acid, hydroxyproline. Gelatin, a rich source of hydroxyproline did not appear to form complexes with anthocyanins under the conditions tested.

Experiments were carried out with cherries in which attempts were made to confine the pigment to the cherry tissue. The cherries were impregnated with hydroxyproline and pectin by either vacuum infiltration or by injection in order that the test compound could interact with the cherry anthocyanin. The results of this experiment were largely inconclusive. There was slight evidence that vacuum infiltrated hydroxyproline reduced the diffusion of pigment from the cherries but the results could also be explained by an increased rate of destruction of the pigment when in contact with hydroxyproline. The methodology of this type of experiment is such that only marked differences can be detected. This is due to the variability of cherries (in size, pigment content, density), difficulty of isolating pure pigments quantitatively and the difficulty in distinguishing between the effects of decreased mobility or increased decomposition.

A blue, complexed form of anthocyanin was found in processed blueberry syrup. This complex contained 60% of galacturonic acid, 2.8%

crude protein, 4% polyphenols, 2.1% ash, and both delphinidin and petunidin glycoside. It was not possible to determine whether the metal ions detected in the complex were integral components of the complex.

The results of the research done on the processed blueberries in this present study, and the reports of other complexes found in flowers of various species, are still too confusing and contradictory to draw a firm picture of the structural form of these complexes. There were a number of similarities between the blueberry complex described here and the complexes previously described in the literature. Both types of complex were precipitated with ethanol and decomposed under strongly acid conditions. There is only one report of the occurrence of pectin and anthocyanins without any metal. It is rather interesting to note that these authors found that only delphinidin glycosides gave this reaction (13).

The theory is proposed that one of the forms of the anthocyanin at pH 3 to 6 reacts with the other ligand(s) to produce a stable macro-molecule. The complexation is thought to occur through the B ring since the shift is to the blue end of the spectrum, e.g., bathochromic, indicative of a B ring phenomenon. In order to have the complex at the B ring, the hydroxyl groups must have a stable quinonoid group which can only be present above certain pH values. If the complex were to be formed at the physiological pH, the equilibrium of the anthocyanins must be such that appreciable amounts of the quinone form must be available for the reaction.

It is felt that theoretical analysis is required in the investigation of possible complexation reaction mechanisms. Co-ordination chemistry may be of help in the establishment of a foundation for further

studies. Secondly, the pH of the anthocyanin containing cell sap would be of great interest since it would shed light on possible stabilization mechanisms native to the plant cell.

If it is desired that anthocyanin complexes be formed in the fruit cells, the form, amount and location of the complexing compounds should be known. It would be interesting to know, for example, whether metal ions exist in plant cells in the free state or whether they occur as complexes with hydroxy acids such as citric acid.

It was found that ferric cations could destroy anthocyanins. Even pelargonidin 3-glucoside, perhaps the most stable anthocyanin was partially decomposed. The mechanism of this process is unknown. Decomposition of the metal chelated anthocyanin complex could be postulated but this does not explain the decomposition of pelargonidin 3-glucoside which does not chelate with any cations.

The harmful effect of ferric cations has not generally been recognised. This study indicates that iron may be a primary cause of breakdown of all the common anthocyanin structures. This topic merits further investigation. Also tin, both the stannic and stannous forms, showed some interesting reactions. Investigations into these two cations should not neglect the question as to whether the above mentioned cations are actually present or available in the fresh or processed fruit.

VIII. Summary and Conclusions

A blue colored complex was found in processed blueberry syrup. The complex contained the following metal ions: aluminium .13% iron .11% calcium .12% and potassium .16%. Also present was 2.8% crude protein,

60% pectin and 4% polyphenol. An s value of about 1000 was obtained by ultra centrifuge determination which corresponds to a molecular weight of between 37,000,000 and 490,000,000. The complex contained only delphinidin and petunidin glycosides and no other polyphenolic material. Acidity completely destroyed the blue colored complex. The action of pectinase showed that the color was precipitated with the products of the enzyme activity. Complexes of this type were absent from processed strawberries and raspberries and the covering syrups of these fruits. No complexes could be detected on fresh blueberries, raspberries, strawberries and cranberries. It seems that complex formation occurred only during the processing of the blueberries. This is the first report of the isolation of a complex of this type from a source other than flowers.

Anthocyanins dialysed rapidly through a cellulose membrane. Under the same conditions the complex isolated from blueberry syrup was non-dialysable. The addition of calcium to blueberry syrup containing free and complexed forms of anthocyanins caused an increase in the rate of dialysis of anthocyanins, while the addition of pectin decreased the dialysis rate. An even greater rate of dialysis was achieved on the addition of aluminium ions. No significant differences were found when urea, ferric chloride or EDTA were added.

Spectroscopic evidence was obtained that, within the range pH 2 to pH 3, delphinidin 3-glucoside could produce bathochromic color shifts in the presence of aluminium and galacturonic acid. There was no change in the spectrum of cyanidin 3-glucoside in this pH range. The addition of hydroxyproline and pectin to the 3-glucosides of pelargonidin, cyanidin,

and delphinidin at various pH levels caused only minor changes to the spectra. Major changes were noted after storage for three or more days when distinct anthocyanin protective mechanisms were noted. This effect was especially prominent at higher pH values where the anthocyanins were unstable. The changes were confined to the cyanidin and the delphinidin types of structures. Hydroxyproline had a greater effect than pectin, but this was dependent on the pH level.

The addition of ferric ions caused all three anthocyanins to lose color rapidly and irreversibly. Of the three, delphinidin 3-glucoside was found to be the most readily affected, with pelargonidin 3-glucoside being least affected.

An electrophoresis technique was developed which indicated that hydroxyproline may interact with pelargonidin, cyanidin and delphinidin 3-glucosides. Interaction was proven by modification of the anthocyanin color and changes in the mobilities. Pectin, being immobile under the electrophoretic conditions, did not exhibit any notable changes. An alternate attempt at showing interaction was by the paper chromatography of anthocyanins in conjunction with various test compounds. The three anthocyanins mentioned above showed only changes in color with no observable changes in the migration rates or R_f values.

Photodensitometry was used in efforts to test for selective retention of pigments by pectins. The largest amounts of delphinidin glycosides were retained at pH 4.5 by pectin when aluminium ions were present. At that pH, the treatments were found to have a stabilizing effect on the anthocyanins. When iron was introduced with pectin, delphinidin glycosides were completely lost. These results confirmed the

anthocyanin decomposing effect of the ferric cation previously investigated by spectrophotometric methods.

The analysis of processed sour cherries, variety Montmorency, provided proof that processed cherry juice contained only two pigments throughout the storage period. The color during the storage period decreased greatly. Thus the decomposition or polymerization of the anthocyanins does not proceed through the anthocyanidin or involve hydrolysis of the glycosides. The pigment ratios proved to be fairly consistent except for one storage period which showed a significant fall. The decrease in the ratios of the two pigments indicated that differential decomposition rates may occur. Vacuum infiltration of hydroxyproline into the berries decreased the amount of pigment found in the syrup after processing using $P = 0.10$ level of significance. No significant correlation was found between the color of the juice and the AIS of the berries or pectin in the juice. A value of $R = .39$ was found between the color of the juice and the total pectin in the berry which was highly significant ($P = 0.01$). The correlation between the total pectin in the berry and the calculated degradation index was $R = -0.61$ significant at $P = 0.01$.

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APPENDIX 1

IDENTIFICATION OF ANTHOCYANINS

1. Experimental

The sources and identities of the plant material are summarized in Table 30. In those cases where the material was purchased from local stores, no positive identification of varieties could be established. All fresh tissue, bearing anthocyanins, was frozen and stored at -10°C .

1.1. Preparation of extracts.

In order to provide reproducibility, 150 g of tissue were extracted. An exception was made for Poinsettia bracts where only 25 g sample was used. All material except the grapes were used in the fresh frozen state. The grapes were subjected to pressure in a hydraulic press to remove a proportion of the sugars and other extraneous material. After pressing, the grape skins were frozen and stored.

For extraction the required weight of tissue was partially thawed and blended with 400 ml of 1% HCl in methanol for two minutes. The slurry was stored overnight at 3°C and then filtered under vacuum using Whatman No. 4 filter paper. If the marc showed excessive color retention, another extraction was made. The filtrate obtained was allowed to settle for one day at 3°C and vacuum filtered through a layer of acid-washed Celite supported on a No. 4 filter paper. The solvent was partially removed in an all-glass circulatory cyclone evaporator (Quickfit Assembly 20EF) under reduced pressure. Final drying of the extract was done with a Buchler rotatory flask evaporator at 30°C until approximately 35-40 ml remained. The final extract was made up to 50 ml and stored at

-10°C. These extracts were used as a source of supply of authentic anthocyanins.

1.2. Approaches to the initial separation of anthocyanins.

The preliminary investigations into the number and types of anthocyanins in a fruit was carried out by paper chromatography of the fresh extract. Paper chromatography was done in Shandon Panglass (all glass) Chromatanks 22½ in. x 9 in. x 22½ in. using the solvent systems listed in Table 31. The papers were developed in a descending manner in the tanks, which were previously equilibrated with the solvent. The tanks were placed in a well ventilated, enclosed area. The Whatman No. 3MM or No. 1 chromatographic paper was cut into 22½ in. x 9 in. sheets. The No. 3MM paper was used when comparatively large volumes of extract were to be chromatographed.

Two dimensional chromatography was used for the identification of fruit anthocyanins. The developing solvents BAW and AWH were used most frequently. In one dimensional separation, the extract was streaked as bars at the top of the chromatogram and developed with BAW, BFW, AWH, and 1% HCl. After certain pigments had been conclusively identified, they were used as markers. The developed chromatograms were checked for spots in both visible and ultra-violet light, and the color and/or fluorescence noted in each case. Added information as to the type of hydroxylation on the B ring was obtained by spraying some of the chromatograms with a 5% alcoholic solution of aluminium chloride. Strips were cut from the paper chromatograms developed in each solvent and the color changes noted when fumed with ammonia vapor.

These preliminary results gave an indication of the numbers and

TABLE 30. Identities and sources of supply for the plant material used

Fruit	Variety	Source
Strawberry	Sparkle Redcoat Ogallala	Plant Science Department, University of Manitoba.
	Unknown varieties	Local store
Raspberries Red	Boyne Madawaska Trent	Canada Dept. of Agriculture Morden Research Station Manitoba
	Unknown varieties	Local store
Black	Unknown varieties	Plant Science Department University of Manitoba.
Cranberries	Early Black	Ocean Spray Company Hanson, Wisc. U.S.A.
	Unknown varieties	Local store
Grape	Flame Tokay Emperor Ribier	Purchased from fruit jobber
Poinsettia	Mikkelson Eche	Metropolitan Corp. of Greater Winnipeg
Blueberry	Unknown variety	Local store
Blackcurrants	Buddenborg Kerry Climax	Plant Science Department, University of Manitoba.
Sour Cherry	Montmorency	Vineland Research Station, Vineland, Ont.

TABLE 31. Paper chromatographic solvents systems

Abbreviation	Composition
BAW	n-butanol:acetic acid:water (4:1:5) upper phase
AWH	acetic acid:water:hydrochloric acid (15:82:3)
1% HCl	hydrochloric acid:water (3:97)
2% HAc	acetic acid:water (2:98)
Forestal	acetic acid:hydrochloric acid:water (30:3:10)
EtAc	ethyl acetate:acetic acid:water (3:3:1)
BFW	n-butanol:formic acid:water (4:1:5) upper phase
PYR	pyridine:water (4:1)

types of pigments that were present. The method used for isolating individual pigments was based on this preliminary information. For some pigments, polyvinylpyrrolidone (PVP) column chromatography produced the same number of bands that were present on the preliminary paper chromatograms. (i.e., black raspberry). For others, isolation of the pigments required paper chromatography in the most suitable solvent (i.e., cranberry with BFW; Poinsettia with AWH).

Paper chromatographic separation consisted of streaking a known volume of the standard extract as a thick bar at the tops of a number of papers. After development with the chosen solvent the separated bands were cut out and eluted with 0.1% HCl in methanol. The pigments were concentrated in a Buchler rotary flash evaporator at 30°C, and applied to a small column of PVP. After elution from the PVP, the pigments were dried under identical conditions to those used before. They were stored at room temperature under nitrogen in stoppered test tubes.

The techniques used for the separation of anthocyanins by PVP column chromatography and its use in the final purification of paper chromatographically separated pigments are described in Appendix 2.

1.3. Tests for the identification of anthocyanins.

The following tests were employed to identify each pure anthocyanin.

1.3.1. Spectroscopic data

The absorbance maximum was determined in 0.01% HCl in methanol with a Unicam SP 800 spectrophotometer. The spectrum was recorded again three minutes after the addition of 3 drops of a 5% AlCl_3 in ethanol solution. Any bathochromic shift of absorption maximum was noted. The

ratio of absorbance at 440 nm to absorbance at the wavelength of maximum absorption was also determined.

1.3.2. Chromatographic data

R_f values were determined in the three standard solvents BAW, AWH, and 1% HCl. (See Table 31). Color reactions in visible and ultra-violet light were noted after application of ammonia fumes and of the $AlCl_3$ solution. A "Chromato-Vue" ultra-violet light source manufactured by Ultra-violet Products Inc., was used.

1.3.3. Identification of the aglycone

The aglycones were obtained by the hydrolysis of the anthocyanins in 6N HCl and methanol (1:1.v/v ratio). The pigments, under nitrogen in test tubes fitted with a cold finger condenser, were heated for 30 minutes in a boiling water bath. The hydrolysates were spotted directly onto paper chromatograms and developed with Forestal solvent. (See Table 31). Results were based on R_f values directly and also by using cyanidin ($R_f = 0.50$) as marker pigment. The aglycones could be separated by extraction into amyl alcohol which could subsequently be removed by vacuum evaporation.

1.3.4. Identification of the sugar moiety

The sugars present in the anthocyanins were identified after hydrolysis with 3 ml of 10% acetic acid for 1 hour in a boiling water bath. A test tube fitted with a cold finger condenser was used which was flushed with nitrogen prior to hydrolysis. Zerolit 226, SRC 24 ion exchange resin in the hydrogen form was added to absorb the free aglycone. The supernatant was dried over sulphuric acid in a vacuum dessicator. The residue was dissolved in 10% isopropyl alcohol and spotted on paper

chromatograms with known marker sugars. The sugars were made visible by spraying with Partridge reagent (0.93 g aniline, 1.66 g phthalic acid in 100 ml of water saturated 1-butanol) and heating at 100°C for five minutes (98).

1.3.5. Partial hydrolysis

Partial hydrolysis utilized the same procedure as for the hydrolysis for the sugar component, except that a two neck 125 ml tapered reaction vessel was used. One neck was fitted with a reflux condenser while the other neck had a removable ground-glass stopper to facilitate the removal of samples at appropriate time intervals. This test was done on the glycosides that contained disaccharides and trisaccharides. For these pigments, under the conditions used, samples were withdrawn at one to two minute intervals for the first 20 minutes. After this interval, samples were taken every 5 minutes. The samples were spotted directly on paper chromatograms and developed in AWH or 1% HCl solvents (See Table 31).

1.3.6. Estimation of relative pigment concentration

Estimates of relative pigment concentrations in mixtures were obtained by separation of the component anthocyanins on paper chromatograms after development with suitable solvents. The paper strips were fumed with HCl for 5 minutes in an enclosed tank and scanned on a Photovolt Densitometer Model 542. The following standard instrument settings were employed: linear setting 5, filter 525 nm, carriage speed 2 in/min., slit 0.1 mm x 5 mm. The areas under the peaks on the resultant tracings were measured by planimeter and the percentage of the total area under each peak calculated.

1.4. Electrophoretic separation of cherry anthocyanins

Cherry anthocyanins were separated by using a Savant Instrument Inc. high voltage electrophoresis apparatus consisting of a 3.K.V. power pack and a F.P. 30A Flat Plate (45 cm) cell. Whatman No. 3MM chromatographic paper was spotted with the sour cherry extract. In place of the extract, thin (1.0 mm wide) slices of frozen cherry skin varying in length from 1 to 2 cm were applied directly to the paper. The extract or skins were applied 17 cm from the edge of the plate nearest to the anode of the cell. The method which worked best was to place the dry paper sheet (57 cm x 23 cm) on the water cooled support bed. The paper was then sprayed with buffer until it was just wet. The most successful buffers were: 8 ml of 90% formic acid/1000 ml of water to give a pH of 2.3 and 0.1M citric acid at pH 2.0. Applied voltages varied from 500 to 2000 volts and the times of application varied from 1 to 6 hours. Satisfactory results were obtained with 1000 volts (or 23v/cm) for 4 hours in either buffer. The mobilities expressed in cm per sec. per volt/cm $\times 10^5$, were calculated for a number of cyanidin glycosides and for the anthocyanins present in the sour cherry.

2. Results

The identification of the anthocyanins based on the tests described, are tabulated in Table 32, with the accompanying data. This table may be compared to Table 33 which lists the reference values collected by Harborne (54) (59). Table 34 is a compilation of data for the identification of sugars found in this study and by others.

TABLE 32. R_f values and absorption spectrum characteristics for anthocyanins examined in this study

Anthocyanin	$R_f \times 100$			Fore-stal	absorption maximum nm MeOH/HCl	Shift with $AlCl_3$ nm ³	Ratio $A_{440nm}/A_{max.}$
	BAW	1% HCl	AWH				
Pelargonidin 3-glucoside	30	17	36		511	0	39
Pelargonidin 3-rutinoside	27	30	50		512	0	37
Cyanidin 3-glucoside	23	10	25		531	14	19
Cyanidin 3-galactoside	20	07	22		530	14	23
Cyanidin 3-arabinoside	23	05	22		530	16	22
Cyanidin 3-rutinoside	26	24	42		531	12	20
Cyanidin 3-sophoroside	14	42	64		528	9	28
Cyanidin 3-sambioside	18	29	56				
Cyanidin 3-glucosylrutinoside	16	64	77		528	8	20
Cyanidin 3-xylosylrutinoside	19	52	71		530	7	21
Peonidin 3-glucoside	27	11	26				
Peonidin 3-galactoside	24	10	27		527	0	24
Peonidin 3-arabinoside	29	12	25				
Delphinidin 3-glucoside	19	04	15		537	5	35
Delphinidin 3-rutinoside	19	15	32				

TABLE 32 (Continued)

Anthocyanin	BAW	$R_f \times 100$ 1% HCl	AWH	Fore- stal	absorption maximum nm MeOH/HCl	Shift with $AlCl_3$ nm ³	Ratio $A_{440nm}/$ $A_{max.}$
Malvidin 3- glucoside	21	08	30		537	0	20
Petunidin 3- glucoside	17	06	20		538	9	22
Peonidin				66			
Petunidin				48			
Cyanidin			07	50			
Malvidin				62			
Pelargonidin				69			
Delphinidin				32			

TABLE 33. Reference data used to identify anthocyanins*

Anthocyanin	R _F x 100			absorption maxima nm MeOH/HCl	Shift	Ratio
	BAW	1% HCl	AWH Fore- stal			
Cyanidin 3-glucoside	38	17	26	525		22
Cyanidin 3-galactoside	37	07	26			
Cyanidin 3-arabinoside	42	05	27			
Cyanidin 3-sophoroside	33	34	61			
Cyanidin 3-sambioside	36	24	51			
Cyanidin 3-rutinoside	37	19	43	523		23
Cyanidin 3-glucosylrutinoside	26	61	73			
Cyanidin 3-xylosylrutinoside	28	47	68			
Pelargonidin 3-glucoside	44	14	35	506		38
Pelargonidin 3-rutinoside	37	22	44	508		40
Peonidin 3-glucoside	41	09	33	523		26
Peonidin 3-galactoside	39	10	32			
Peonidin 3-arabinoside	48	09	36			
Delphinidin 3-glucoside	26	03	18	535		18
Delphinidin 3-rutinoside	30	11	37	537		17
Malvidin 3-glucoside	38	06	29	535		18

TABLE 33 (Continued)

Anthocyanin	$R_f \times 100$				absorption maxima nm MeOH/HCl	Shift	Ratio
	BAW	1% HCl	AWH	Fore- stal			
Petunidin 3- glucoside	35	04	22		535		18
Pelargonidin	80			68	520	0	39
Cyanidin	68			49	535	18	19
Delphinidin	42			32	546	23	16
Petunidin	52			46	543	14	17
Peonidin	71			63	532	0	25
Malvidin	58			60	542	0	19

* All data from Harborne (54) or Harborne (59).

TABLE 34. Comparison of R_g values of sugar moieties identified in anthocyanins in this study with other published values

Sugar	R _g value					
	Found		Smith et al.(117)		Daravinas et al.(36)	
	BAW	EAW	BAW	EAW	BAW	EAW
Glucose	1.00	1.00	1.00	1.00	1.00	1.00
Galactose	.92	.91	.88	.87	.90	.94
Arabinose	1.28	1.36	1.18	1.23	1.17	1.43
Xylose	1.39	1.59	1.30	1.49	1.27	1.77
Rhamnose	2.00	2.06	1.72	2.16	1.61	1.96
Maltose	.50	.41	.47	.37		
Rutinose	.63	.60	.61	.61	.60	.55

APPENDIX 2

STANDARD CHROMATOGRAPHIC METHOD FOR ANTHOCYANIN SEPARATION
ON COLUMNS OF INSOLUBLE POLYVINYLPYRROLIDONE

After preliminary experiments, a standard method of column chromatography was established. Columns 15 cm x 18 mm OD were used. To reduce mixing volume, the columns were drawn to a narrow delivery neck which supported a filter paper and the adsorbent. The following procedure was carried out in the separation of individual anthocyanins in a fruit extract: three grams of PVP powder were hydrated for two hours and then poured into the prepared column. After allowing the adsorbent to settle under gravity, the column was washed with 100-150 ml of 0.25 N HCl. The required column of standard extract was layered carefully on top of the adsorbent and eluted with 0.25 N HCl until the pigments had migrated approximately 2 cm. The developing solvent was changed to 50% of methanol in 0.25 N HCl and elution of the colored bands completed. Fractions of appropriate size (1-5 ml) were collected with a Buchler Fractomat fraction collector.

For the purification of anthocyanidin extracts the same procedure was followed except that smaller amounts (1.5 to 2.0 g) of dry PVP could be used.

APPENDIX 3

ABSORBANCIES OF DIALYSATES OF BLUEBERRY JUICE THAT HAD
BEEN TREATED WITH CERTAIN CHEMICALS

Solution	Time after commencing the dialysis min.			Solution	Time after commencing the dialysis min.		
	145	275	355		100	280	410
FeCl ₃	.35	.64	.85	EDTA	.26	1.14	1.53
CaCl ₂	.53	1.05	1.38	Urea	.40	1.55	-
Urea	.26	.61	.86	FeCl ₃	.54	1.50	1.93
	95	215			110	230	320
Urea	.15	.75		AlCl ₃	.74	1.88	-
FeCl ₃	.28	.83		CaCl ₂	.62	1.45	1.92
FeCl ₃	.26	.80		Blank	.33	.98	1.43
	100	195	340		100	230	390
CaCl ₂	.58	1.12	1.93	AlCl ₃	.61	1.70	-
EDTA	.17	.45	.97	Urea	.23	.78	1.29
Blank	.25	.61	1.31	Blank	.26	.89	1.44

APPENDIX 4

PERCENTAGE OF FOUR ANTHOCYANINS AFTER POSSIBLE ABSORPTION
ON VARIOUS CONCENTRATIONS OF PECTIN OR PECTIN AND
ALUMINIUM (EXP. 1)

Treatment	Band	Replications				
		I	II	III	$\bar{x}$	s
Pigment in buffer	1.	10.5	10.5	9.6	10.2	.52
	2.	17.3	22.2	22.4	20.6	2.89
	3.	30.4	33.0	29.2	30.9	1.94
	4.	41.8	34.4	38.8	38.3	3.72
Pigment, Al ⁺⁺⁺ and 1% pectin	1.	4.1	5.2	4.5	4.6	.56
	2.	22.1	21.2	21.6	21.6	.45
	3.	14.8	12.1	14.0	13.6	1.39
	4.	59.0	61.4	59.8	60.1	1.22
Pigment, Al ⁺⁺⁺ and 2% pectin	1.	5.1	7.1	5.6	5.9	1.04
	2.	22.2	20.6	20.6	21.1	.92
	3.	19.2	21.2	17.2	19.2	2.00
	4.	53.4	51.0	56.6	53.7	2.81
Pigment, Al ⁺⁺⁺ and 3% pectin	1.	6.6	7.3	5.9	6.6	0.76
	2.	11.7	21.7	20.1	17.8	5.37
	3.	25.2	21.3	20.9	22.5	2.38
	4.	56.5	49.8	53.1	53.1	3.35
Pigment and 1% pectin	1.	10.4	8.8	9.8	9.7	.81
	2.	19.5	18.0	22.6	20.1	2.35
	3.	30.2	29.3	30.1	29.9	.49
	4.	40.0	44.0	37.5	40.5	3.28

APPENDIX 4 (Continued)

Treatment	Band	Replications				
		I	II	III	$\bar{x}$	s
Pigment and 2% pectin	1.	8.3	8.8	7.5	8.2	.66
	2.	21.4	17.8	19.4	19.5	1.80
	3.	26.3	28.3	24.9	26.5	1.71
	4.	44.1	45.2	48.2	45.8	2.12
Pigment and 3% pectin	1.	9.4	9.3	9.1	9.3	.15
	2.	16.2	15.6	17.7	16.5	1.08
	3.	20.1	24.3	21.9	22.1	2.11
	4.	54.2	50.8	51.4	52.1	1.81
Pigment and Al ⁺⁺⁺	1.	9.0	8.8	10.5	9.4	.93
	2.	15.5	17.9	19.2	17.5	1.88
	3.	28.2	31.1	29.4	29.6	1.46
	4.	47.3	42.2	40.0	43.5	3.38

APPENDIX 5

ANALYSIS OF VARIANCE FOR THE EFFECT OF EIGHT TREATMENTS OF
VARIOUS COMBINATIONS OF PECTIN AND ALUMINIUM CATIONS
ON TOTAL FREE DELPHINIDIN GLYCOSIDES

Source	df	S.S.	M.S.	F.
Replications	2	16.6953	8.3477	2.22
Treatments	7	1321.1953	188.7422	50.23**
Error	14	52.6016	3.7573	
Total	23	1390.4922		

** Significant at $P = 0.01$

APPENDIX 6

THE PERCENT CONCENTRATION OF FOUR ANTHOCYANINS AFTER
POSSIBLE ABSORPTION ON PECTIN AT TWO pH LEVELS

Buffer pH 2.7				
Treatment	Band*	Replications		
		I	II	III
Pigment	1.	15.9	19.9	18.3
	2.	17.8	17.8	19.7
	3.	31.1	30.3	30.6
	4.	35.2	32.8	31.4
Pigment + AlCl_3	1.	20.6	20.0	19.1
	2.	19.7	21.8	19.4
	3.	30.3	30.1	30.6
	4.	29.4	28.1	30.9
Pigment + citrus pectin + AlCl_3	1.	17.8	17.6	20.8
	2.	19.8	19.2	18.3
	3.	30.1	31.9	27.9
	4.	32.3	31.3	33.0
Pigment + pectin + AlCl_3	1.	13.0	14.5	11.9
	2.	23.0	22.6	23.7
	3.	29.0	27.8	28.1
	4.	35.0	35.1	36.3
Buffer pH 4.2				
Pigment	1.	12.2	11.0	10.0
	2.	20.3	19.5	17.3
	3.	23.4	22.8	26.7
	4.	44.1	46.7	46.0
Pigment + AlCl_3	1.	15.4	15.8	15.4
	2.	16.3	18.2	17.6
	3.	33.3	31.1	31.3
	4.	35.0	34.9	35.7

APPENDIX 6 (Continued)

Buffer pH 4.2				
Treatment	Band*	Replications		
		I	II	III
Citrus pectin + AlCl ₃ + pigment	1.	14.0	13.7	14.3
	2.	15.5	16.7	18.8
	3.	31.9	28.6	27.2
	4.	38.6	41.3	39.2
Pectin + AlCl ₃ + pigment	1.	14.3	14.3	16.9
	2.	18.6	16.0	18.0
	3.	25.9	26.8	27.8
	4.	41.2	42.9	37.3

- Band* 1. delphinidin 3-glucoside
 2. cyanidin 3-glucoside
 3. delphinidin 3-rutinoside
 4. cyanidin 3-rutinoside

APPENDIX 7

ANALYSIS OF VARIANCE OF THE FREE DELPHINIDIN GLYCOSIDE
CONCENTRATION AFTER REACTION WITH PECTIN WITH OR WITHOUT
ALUMINIUM CATION AT pH 2.7

Source	df	S.S.	M.S.	F.
Replications	2	.1563	.0781	.05
Treatments	3	134.4844	44.8281	30.12**
Error	6	8.9297	1.4883	
Total	11	143.5703		

** Significant at $P = 0.01$

APPENDIX 8

ANALYSIS OF VARIANCE OF THE FREE DELPHINIDIN GLYCOSIDE
CONCENTRATION AFTER REACTION WITH PECTIN WITH OR WITHOUT
ALUMINIUM CATIONS AT pH 4.3

Source	df	S.S.	M.S.	F.
Replications	2	6.250	3.125	.88
Treatment	3	227.496	75.832	21.27**
Error	6	21.3867	3.564	
Total	11	255.1328		

** Significant at $P = 0.01$

APPENDIX 9

DATA OBTAINED FOR A NUMBER OF PROPERTIES OF CANNED MONTMORENCY CHERRIES. FIVE DIFFERENT TREATMENTS OF THE CHERRIES WERE REPLICATED THREE TIMES WITH ANALYSIS CARRIED OUT AT THREE STORAGE DURATIONS

Storage period	Treatment	Replication	Volume of juice ml	Weight of berries g.	pH	Soluble solids % as sucrose	Absorbance in nm in .25N HCl	Degradation index	ratio $\frac{\text{Cy.3-G Rg}}{\text{Cy.3-Rg}}$	Alcohol insoluble solids g/20 g berries	pectin mg/100 ml of juice	pectin mg/100 g of drained berries
13 days	1	1	176	144.6	3.41	29.0	1.517	1.23	2.600	.7609	14.50	610.8
		2	177	146.6	3.47	30.0	1.381	1.22	2.347	.5700	10.16	510.2
		3	179	149.5	3.42	29.5	1.470	1.22	2.187	.7088	8.92	587.1
	2	1	201	137.0	3.43	32.0	1.162	1.21	2.432	.5751	4.33	547.8
		2	178	147.9	3.45	29.8	1.168	1.21	2.152	.6508	4.28	581.6
		3	207	131.4	3.46	32.0	1.132	1.21	2.147	.5841	5.15	494.4
	3	1	188	138.1	3.45	31.0	1.338	1.22	1.833	.5348	7.97	468.4
		2	202	135.9	3.45	31.8	1.338	1.21	2.002	.5444	5.06	507.2
		3	196	133.6	3.42	30.5	1.278	1.22	2.534	.5140	5.31	467.4

34 days

4	1	188	144.8	3.44	30.8	1.218	1.22	2.517	.5426	7.25	490.3
	2	196	139.7	3.49	31.3	1.142	1.21	2.542	.5617	5.37	537.6
	3	195	142.4	3.45	31.2	1.251	1.22	2.245	.5631	5.14	505.1
5	1	194	141.0	3.44	30.0	1.270	1.21	1.879	.5759	5.21	523.3
	2	192	143.3	3.49	30.0	1.258	1.21	2.761	.5328	4.61	503.5
	3	184	145.6	3.49	29.5	1.355	1.22	2.164	.5441	5.91	534.0
1	1	180	151.1	3.49	30.3	1.342	1.28	1.696	.6216	16.16	606.1
	2	183	147.1	3.54	30.5	1.280	1.27	1.934	.5872	9.56	567.9
	3	183	146.8	3.42	29.7	1.248	1.28	2.271	.5567	9.98	491.7
2	1	190	140.4	3.50	31.0	1.228	1.27	3.151	.5288	6.58	505.5
	2	195	136.7	3.52	31.0	1.172	1.27	2.251	.5872	8.92	563.7
	3	192	141.6	3.45	30.6	1.066	1.27	4.408	.5680	5.37	551.8
3	1	192	140.3	3.51	30.5	1.215	1.27	2.885	.5502	6.50	515.4
	2	193	143.0	3.54	30.3	1.305	1.26	3.007	.5804	4.16	542.9
	3	192	141.6	3.45	30.6	1.066	1.27	4.408	.5680	5.37	551.8
4	1	185	149.0	3.49	30.3	1.277	1.28	2.834	.6102	4.98	573.0
	2	179	150.3	3.54	30.0	1.282	1.29	1.974	.5297	7.51	559.8
	3	190	143.3	3.43	30.5	1.141	1.28	2.944	.5814	4.34	566.0
5	1	186	151.0	3.50	30.0	1.261	1.27	1.999	.5941	5.94	574.1
	2	182	148.7	3.56	30.0	1.240	1.27	4.016	.5286	4.92	523.3
	3	196	143.6	3.45	30.5	1.152	1.26	3.012	.5663	4.39	564.8

62 days	1	1	172	156.4	3.45	29.5	1.170	1.36	2.325	.5595	7.83	455.5
		2	180	150.2	3.48	30.2	1.110	1.36	1.846	.5469	12.35	429.2
		3	182	155.0	3.40	29.5	1.148	1.34	2.831	.5499	12.45	447.7
	2	1	201	138.0	3.47	31.5	.958	1.34	2.796	.5928	9.40	484.9
		2	197	140.9	3.51	31.5	1.000	1.34	2.568	.5320	5.94	450.0
		3	193	139.7	3.47	31.0	1.001	1.34	1.843	.5339	9.73	445.4
	3	1	192	142.9	3.47	31.0	1.036	1.37	2.024	.5437	7.52	438.3
		2	188	134.6	3.49	30.2	1.005	1.33	3.732	.5766	7.56	447.9
		3	190	138.9	3.50	30.5	1.052	1.34	2.326	.5771	17.96	447.5
	4	1	188	149.1	3.47	31.0	1.100	1.34	2.854	.5971	4.24	468.6
		2	180	145.4	3.46	30.0	1.100	1.36	2.070	.5374	11.61	432.4
		3	186	148.8	3.51	30.5	1.030	1.35	2.657	.5955	5.14	467.2
	5	1	186	146.0	3.48	30.2	--	1.35	1.390	.5841	5.73	464.4
		2	183	145.9	3.47	30.0	1.050	1.38	2.197	.5537	9.80	442.2
		3	160	154.3	3.48	29.0	1.198	1.37	3.012	.5664	10.25	471.6

APPENDIX 10

ANALYSIS OF VARIANCE FOR THE EFFECT ON COLOR OF JUICE AS MEASURED
BY ABSORBANCE IN 0.25 N HCl OF THREE STORAGE PERIODS AND FIVE
TREATMENTS IN CANNED MONTMORENCY CHERRIES

Source	d.f.	s.s.	M.S.	F.
Replications	2	.027	.014	
Storage	2	.534	.267	50.421**
Treatments	4	.080	.020	3.758*
Stor. x Trt.	8	.075	.009	1.781
Error	28	.148	.005	
Total	44	.864		
C.V. = 5.6%				

** Significant at $P = 0.01$

* Significant at $P = 0.05$

APPENDIX 11

ANALYSIS OF VARIANCE FOR THE EFFECT ON PIGMENT RATIOS OF THREE
PERIODS OF STORAGE AND FIVE TREATMENTS IN CANNED
MONTMORENCY CHERRIES

Source	df	S.S.	M.S.	F
Replications	2	358,400	179,200	
Storage	2	1,183,488	591,744	1.675
Treatments	4	1,384,704	346,176	.980
Stor. x Trt.	8	3,009,280	376,160	1.065
Error	28	9,891,072	353,252	
Total	44	15,826,944		

C.V. = 24.087%

APPENDIX 12

ANALYSIS OF VARIANCE FOR THE ANTHOCYANIN RATIO CALCULATED
FOR THE TWO MONTMORENCY CHERRY ANTHOCYANINS, FRESH
AND AFTER THREE STORAGE PERIODS

Source	df	S.S.	M.S.	F.
Replications	8	5575936.0	696992.0	2.18
Storage	3	4336400.0	1445466.0	4.52*
Error	24	7668576.0	319524.0	
Total	35	17580912.0		

* Significant at $P = 0.05$

APPENDIX 13

ANALYSIS OF VARIANCE FOR THE EFFECT ON THE DEGRADATION INDEX
OF THREE STORAGE PERIODS AND FIVE TREATMENTS IN CANNED
MONIMORENCY CHERRIES

Source	df	S.S.	M.S.	F.
Replications	2	.000	.000	
Storage	2	.138	.069	819.406**
Treatments	4	.001	.000	2.484
Stor. x Trt.	8	.001	.000	1.897
Error	28	.002	.000	
Total	44	.143		

C.V. = 0.7%

** Significant at $P = 0.01$

APPENDIX 14

ANALYSIS OF VARIANCE FOR THE EFFECT ON THE TOTAL PECTIN IN
CANNED MONTMORENCY CHERRIES OF THREE STORAGE PERIODS AND
FIVE TREATMENTS

Source	df	S.S.	M.S.	F.
Replications	2	794.0	397.0	
Storage	2	75,477.0	37738.5	42.457**
Treatments	4	6,110.0	1527.5	1.718
Stor. x Trt.	8	9,938.0	1242.25	1.398
Error	28	24,888.0	888.857	
Total	44	117,207.0		

C.V. 5.9

** Significant at $P = 0.01$

APPENDIX 15

ANALYSIS OF VARIANCE FOR THE EFFECT ON PECTIC SUBSTANCES IN
CANNED MONTMORENCY JUICE OF THREE STORAGE PERIODS
AND FIVE TREATMENTS

Source	df	S.S.	M.S.	F.
Replications	2	.951	0.476	
Storage	2	55.624	27.812	3.803*
Treatments	4	164.267	41.067	5.616**
Stor. x Trt.	8	53.019	6.627	0.906
Error	28	204.753	7.313	
Total	44	478.615		

C.V. 35.5%

** Significant at $P = 0.01$

* Significant at $P = 0.05$