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Lanka V. L. N. Sastry
Iowa State College

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NATURE OF PIGMENTS IN CONCORD GRAPES AND THEIR BEHAVIOUR DURING HEAT PROCESSING AND STORAGE

by

Lanka V. L. N. Sastry

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Head of Major Department

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I. INTRODUCTION

Pleasing flavor and agreeable appearance are the qualities which characterize fruit juices as important food commodities. It is estimated that approximately 90.8 million cases of canned fruit and vegetable products were produced in the United States during 1949-1950 (94). Grape juice is one of the most popular fruit beverages and its production in the U. S. during 1949-50 was about 2.8 million cases. Because of its characteristic flavor and aroma, most of the bottled grape juice on the market is prepared from Concord grapes. The color of the clear fruit juice offers psychological appeal and is ranked as an important factor for judging quality. The intense color of Concord grape juice has been attributed principally to the presence of the water soluble anthocyanin pigments, modified to some extent by the other components of the juice. These desirable colored substances are derived from the skins.

Numerous studies have been conducted in the past to find the nature of the pigments in many varieties of grapes and in certain cases conclusive evidence could not be adduced as to their exact identity. A considerable amount of work has been reported on the variation in the color content of grape juice with different processing methods. Numerous efforts have also been made to demonstrate both the stability and deterioration of Concord grape juice on storage under various conditions. While some success has been achieved, the interpretation of these changes has not been complete.

The present investigation was intended to demonstrate the nature of the pigments present in Concord grapes with emphasis on the water soluble

pigments. A study was made to determine the effects of various factors on the stability of the colored components in the juice during varying thermal processes in glass containers. The behaviour of the principal color components of the juice on storage under controlled experimental conditions has been investigated. A survey has been made to discover some of the factors affecting the stability of the color components pertaining to conditions promoting oxidation, both with pigment solutions and grape juice samples.

II. REVIEW OF LITERATURE

The coloring principles of many plants and plant products are determined by the nature of the pigments which form a part of their structure. There exist numerous functions of these pigments in the plant during the different stages of its growth. The plant pigments are roughly divisible into two major classes (96). "The fat-soluble plastid pigments represent one group. They are associated with the protoplasmic structure of the plants." (27, p. 1316). The second group includes those pigments which are water soluble and exist in solution in the cell sap.

A. Fat Soluble Pigments

The plastid pigments can be divided into two subgroups; the chlorophylls and carotenoids. Chlorophyll is the name "given by Pelletier and Caventou to the green coloring material present in the chloroplasts of green plants" (27, p. 1294). Chlorophyll and its derivatives are insoluble in water but are sparingly soluble in most of the common organic solvents. The carotenoids comprise a group of yellow or orange substances; the xanthophylls, carotenes and their derivatives (86, p. 128). They are insoluble in water but are soluble in organic solvents.

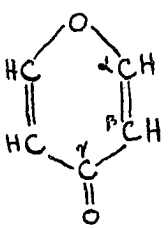
B. Water Soluble Pigments

The vast majority of the water soluble blue, red and yellow colors of the higher plants are compounds belonging to the group of anthocyanin and

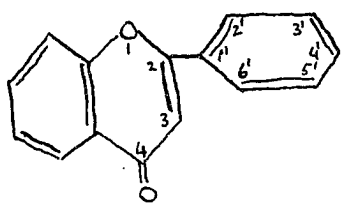
anthoxanthin pigments (96). The classical work of Willstätter and Everest (97) on the isolation of the pigments of blue corn flowers marked a beginning in the state of our knowledge of these pigments. Their molecular structures have been established and supported by synthesis.

1. Chemical structure

The anthocyanins form a class of vegetable bases, in which the basic properties are due to the oxonium oxygen in quinoid combination and hence form stable oxonium salts, a property which distinguishes them from the anthoxanthins. The anthocyanins occur in nature as glycosides or as the sugar free pigments, namely anthocyanidins. The anthoxanthins exist also mainly as glycosides. The basic nucleus of the flavones is γ-pyrone, the anhydride of an unsaturate 1,5-dihydroxy-3-ketone. Earliest investigations of Willstätter and co-workers (97,98), Karrer and co-workers (41, 45) and Robinson and co-workers (55, 76) contributed to the elucidation of the basic anthocyanidin nucleus. The anthocyanidins are derivatives of 2-phenylbenzopyrilium given below in the form of the chloride. In the plant, however, the flavilium nucleus is combined with other anions, probably in a large measure with those of plant acids, tannins etc. (29, 41, 44)

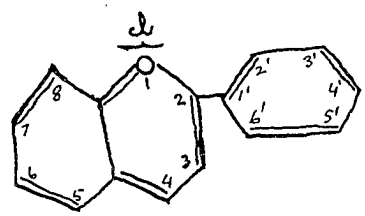


γ-pyrone



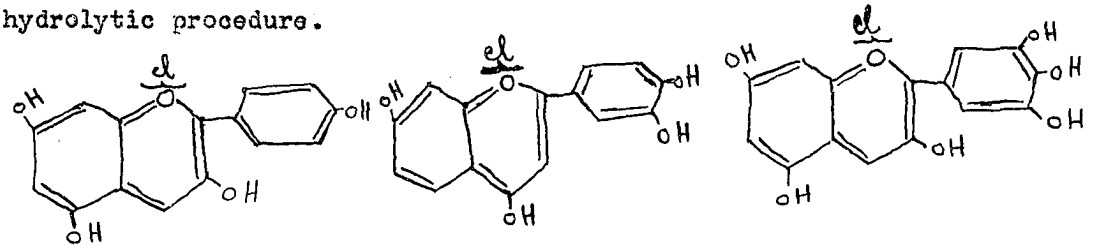
2-phenylbenzopyrone

(flavone)



2-phenylbenzopyrilium chloride
(flavilium chloride)

The anthocyanins differ among themselves in the 2-phenyl ring. Three primary anthocyanidins can be differentiated on this basis. The nature of the substituents in the 2-phenyl ring of these types is determined by hydrolytic procedure.



Pelargonidin chloride

Cyanidin chloride

Delphinidin chloride

Few cases are known where anthocyanidins occur as such in nature (99). They appear usually as mono or diglycosides. So far glucose, rhamnose, galactose and gentiobiose have been isolated as the sugar components present in glycosidic form. In the monoglycoside the sugar is attached in the 3-position and in a diglycoside, the sugar molecule is either coupled with the first sugar molecule or attached to the anthocyanidin in the 5-position. A number of plants contain anthocyanins combined with an organic acid, either in ester combination with one of the hydroxyl groups of the anthocyanidin or attached to a hydroxyl group of the sugar component (41, 44). The methyl esters of the anthocyanidins for example Peonidin (3' - methyl cyanidin), Malvidin (3' - 5' dimethyl delphinidin), Hirsutidin (7 methyl - 3' - 5' dimethyl delphinidin) and Petunidin - (3' - methyl delphinidin), are shown to occur in nature. Nitrogenous anthocyanins have also been isolated though their structural formulae are not established as yet (6, 53, 73, 76).

2. Biogenesis of anthocyanins

The nature and quantity of the pigments have been assumed to be genetically controlled and in a number of cases "complementary genes" are apparently involved (52, p. 55). The contention that the anthocyanins were formed from flavonols and similar substances has been supported by a number of authors (7, 46). Another school of thought has assumed a biogenesis of the anthocyanins from structural elements smaller than the flavones and other allied substances. The C_{15} system (C_6-C_3-C_6) is regarded as built up from the hexoses and trioses by means of aldol condensation (80).

3. Methods used for isolation of anthocyanins

a. As picrate. All anthocyanins are soluble in water as shown by their presence in the cell sap of vacuoles. They are quite soluble in the hydroxylic solvents, but they are insoluble in such nonhydroxylic solvents as ether, benzene, or chloroform. Willstätter and Everest (97) observed that the diglycosides were insoluble, the monoglycosides partly soluble and the aglycones very soluble in isoamyl alcohol. The formation of easily crystallisable oxonium salts with acids was utilized in the isolation and purification of these pigments. Willstätter and his colleagues extracted the anthocyanin salts by means of methanolic-hydrochloric acid, acetic acid or other similar solvents, and precipitated a crude, often syrupy product with ether. The process was repeated, with variation of solvent, until the anthocyanin could be precipitated as the chloride or picrate. Precipitation of picrate from dilute acid solutions afforded good separation of the anthocyanin from all acid soluble impurities. The

addition of anhydrous ether to a solution of the anthocyanin picrate in methanolic-hydrochloric acid precipitated the anthocyanin as the chloride. Final purification was accomplished by allowing the separation as the chloride from MeOH-HCl solution.

b. As lead salt. Reynolds et al (74) separated the anthocyanin from crude methanolic-HCl extracts as the insoluble lead salt; the lead salt was later decomposed with a mixture of propyl alcohol, methanol and hydrochloric acid. The pigment was precipitated from the acid alcohol solution by addition of anhydrous ether, recrystallization (from the methanolic-HCl solution) being affected as the chloride on standing. The anthoxanthins which were also precipitated as insoluble lead salts along with the anthocyanins were removed by shaking the pigment extracts with anhydrous ether.

c. Chromatographic techniques. Chromatography has been helpful for the isolation of mixtures of anthocyanins obtained by other methods. Karrer and Strong (42) separated peonin chloride into cyanin chloride and pure peonin chloride, by using a column of activated alumina. Nebesky and others (61) employed the same type of purification to secure purified extracts of anthocyanin pigments of strawberries and currants from their crude aqueous extracts.

4. Methods used for identification of anthocyanins

a. Color changes of anthocyanins. A decrease in the number of hydroxyl groups contained in the pigment molecule has been observed to cause an increased redness. Methylation of the pigment molecule increased the redness while the diglycosides tended to be bluer than the monoglycosides. Fear and Nierenstein (25) observed that color changes of anthocyanins and

anthocyanidine in the presence of acids and alkalis, must be standardized with respect to pH, temperature and time of contact with the reagents. Some investigators employed this property to use them as indicators (72). Robertson and Robinson (75) studied the behaviour of the anthocyanin pigments in buffered solutions over a varied pH range. Robinson and Robinson (77) pointed out that increased reliance could be placed on comparisons of the color of pigments provided they were observed at the boiling point of the solution. Under these conditions the order from orange red to blue red was that of the anthocyanins based on pelargonidin, peonidin, malvidin, and delphinidin. The necessity for these precautions arose from the existence in the solutions of substances (copigments) which intensify and modify the color. Willstätter and Zollinger (100) observed that the addition of tannin to a solution of oenin chloride in dilute HCl, intensified the color and produced a change in tone giving a much bluer red. They stated that the interference of tannin effect was specific for oenin and was not observed to a comparable extent in the case of cyanin solutions. The same copigmentation effect was reported as a result of the presence of anthoxanthins in anthocyanin solutions (51).

b. Characteristic color reactions and distribution between immiscible solvents. The color reactions of the anthocyanins with different reagents together with the knowledge of their distribution between immiscible solvents has proved to be very useful in the identification of the anthocyanins.

Bancroft and Rutzler (8) outlined a qualitative method for the identification of the anthocyanins and anthocyanidins in the presence of chlorophylls, carotenoids, and anthoxanthins. Robinson and Robinson (77, 78, 79) mentioned a few color reactions whereby the anthocyanins and antho-

cyanidins could be distinguished. These color reactions were compiled and presented in Tables 1 and 2, included in which is the work reported by some other authors (45, 55, 98, 100).

The ferric chloride reaction was carried out by adding sodium carbonate solution to the clear pigment extract till the color changed towards violet or blue; acid conditions were then restored by addition of just sufficient 0.5 per cent HCl. In the positive test addition of a drop of neutral ferric chloride would produce a deep violet coloration replacing and more intense than the anthocyanin color. Any residual red tinge represented a negative result.

Oxidation test: A portion of the dilute extract was shaken with half of its volume 10 per cent NaOH. To estimate what amount of anthocyanidin was destroyed, the solution was made acidic and extracted with amyl alcohol. The color of the amyl alcohol layer would provide an indication of the recovery of the anthocyanidin after treatment of the anthocyanidin with sodium hydroxide in the presence of air.

Extraction with delphinidin reagent: The delphinidin reagent consisted of anisole (five volumes) in a mixture of ethyl isoamyl ether (one volume) containing 5 g. of picric acid in 100 ml. This reagent was used to extract the anthocyanin dissolved in 1 per cent aqueous hydrocholoric acid.

Extraction with cyanidin reagent: The cyanidin reagent was prepared by mixing cyclohexanol (one volume) and toluene (5 volumes). This reagent was employed to extract the anthocyanin dissolved in an equal volume of 1 per cent aqueous hydrochloric acid.

Table 1

Color Reactions of Anthocyanidin Glycosides

	Pelargonidin 3:5-diglucoside	Pelargonidin 3-glucoside	Delphinidin 3-glucoside	Delphinidin 3:5-digluco-	Cyanidin 3-glucoside	Malvic 3:5-di glucos
Addition of Na acetate to original solution	Bright bluish red	Dull brownish violet red	Blue violet to blue	Blue violet to blue	Violet red	Bright violet
+ Na_2CO_3 and then NaOH	Violet with Na_2CO_3 and greenish blue on addition of NaOH stable to NaOH	Violet red stable to NaOH	Blue	Blue	Rich blue with Na_2CO_3 Unstable to NaOH	Bright greeni blue
+ $FeCl_3$			Violet	Bluish violet in aqueous and violet blue in alcoholic solution		

Table 1

ions of Anthocyanidin Glycosides

din ide	Delphinidin 3:5-digluco-	Cyanidin 3-gluco-side	Malvidin 3:5-di-glucoside	Oenidin 3:5-di-gluco-side	Peonin 3:5-di-gluco-side	Peonidin 3-gluco-side	Oenidin 3-glu-coside	Petunidin 3:5-di-glucoside
et	Blue violet to blue	Violet red	Bright violet	Red violet	Bright brownish violet red			Blue with violet tinge
	Blue	Rich blue with Na_2CO_3 Unstable to NaOH	Bright greenish blue	Violet blue unchanged by NaOH	Blue with Na_2CO_3	Rich violet unchanged by NaOH		Pure greenish blue. Addition of NaOH causes rapid decomposition to yellow
	Bluish violet in aqueous and violet blue in alcoholic solution			In alcoholic solution, darkening and dulling of color and a change towards violet			In alcohol a dulling of color and a change towards violet. In water no color.	

Table 2

Properties of Anthocyanidins (45, 55, 77, 78, 79, 9

	Pelargonidin	Cyanidin	Delphinidin	Petunidin	Ma
Color of eq. soln.	Red	Violet red	Bluish red		Vi
Soln. of chloride in water	Readily soluble	Only slightly sol. in dil. HCl	Very soluble		Slig solu
$FeCl_3$ reaction	Not definite	Intense blue	Intense blue		No r
Behaviour toward Fehlings	Reduces when warmed	Reduces in the cold	Reduces in the cold	Reduces in the cold	Redu boil
Behaviour in aq. soln.	Color fades on standing	Color disappears on heating (Isomerisation)	Slow fading in the cold; when heated rapid fading (Isomerisation)	Color disappears in very dilute soln. when heated	
Color change in soda soln.	Blue	Violet, then blue	Violet, then blue		Viol gree
Oxidation test	Relatively stable	Relatively stable	Destroyed at once	Destroyed at once	Rela stab
Extraction with isoamyl + sod. acetate, then $FeCl_3$	Violet red; no change after addition of $FeCl_3$	Reddish violet changing to blue after addition of $FeCl_3$	Blue	Violet blue with sod. acetate; changes to pure blue after addition of $FeCl_3$	Slig viol amyl chan FeCl
Delphinidin reagent	Completely extracted	Extraction to a considerable extent of soln. is not too dilute	Not extracted	Partially extracted	Comp extr
Cyanidin reagent	Largely extracted	Slightly extracted	Not extracted	Not extracted	Very extr fair

Table 2

nthocyanidins (45, 55, 77, 78, 79, 98, 100)

Delphinidin Bluish red	Petunidin	Malvidin Violet red	Peonidin Violet red	Hirsutisin Violet red	Oenidin
Very soluble		Slightly soluble	Readily soluble	Slightly soluble	
Intense blue		No reaction	Not definite; only faint	No reaction	No change
Reduces in the cold	Reduces in the cold	Reduces when boiled	Reduces when boiled	Reduces when boiled	
Slow fading in the cold; when heated rapid fading (Isomerisation)	Color disappears in very dilute soln. when heated		Color disappears on heating	Color disappears in very dil. solution when heated	
Violet, then blue		Violet, then greenish blue	Violet, then blue	Violet, then greenish blue	
Destroyed at once	Destroyed at once	Relatively stable	Relatively stable	Relatively stable	Relatively stable
Blue	Violet blue with sod. acetate; changes to pure blue after addition of $FeCl_3$	Slight blue violet in amyl, no change with $FeCl_3$			Bluish violet on addition of Na acetate. Faint change toward blue with $FeCl_3$ added
Not extracted	Partially extracted	Completely extracted	Completely extracted		Completely extracted
Not extracted	Not extracted	Veryslightly extracted; faint blue	Largely extracted		Faint mauve coloration

Nikiforowsky (66) suggested that the addition of a few drops of aluminum chloride solution to the ethanol extract (more than 50 per cent conc.) gave a blue or red coloration which was quite permanent and was not destroyed by boiling. The red color was obtained with pelargonin, and blue with cyanin and delphinin. Before adding aluminium chloride, chlorophyll and its accompanying pigments were removed by shaking with a few drops of benzene and the excess acidity neutralized with alcoholic potassium hydroxide. Flavonols would not interfere in the reaction while tannins seemed to give only a yellow color.

Tauber (89) described a color reaction for the identification of some natural pigments and phenols. It consisted in treatment of a pigment extract with 3 per cent hydrogen peroxide. After agitation for one minute 0.3 ml. of 0.2 N NaOH was added and the color noted. The color was again recorded after subsequent acidification with 0.5 ml. of 0.2 N H_2SO_4. Under these conditions carotenoids gave no color. With blue grape pigments it was observed that the pigment solution, after treatment with hydrogen peroxide, gave a yellow color which changed to red after acidification with sulfuric acid.

c. Absorption spectra. The anthocyanins and the anthocyanidins studied so far exhibited strong absorption powers over the range of 2,000 to 6,000 Å and an absorption maximum was present in the visible range.

Schou (82) determined the absorption spectra of the anthocyanins and the anthocyanidins in ethanolic solution. In addition to other bands, pelargonidin had a band at 5045 Å, cyanidin at 5105 Å, and delphinidin at 5225 Å, peonidin at 5110 Å, syrinigidin at 5200 Å, and malvin (syringidin

diglucoside in acid solution) at 5190 Å. Further investigations on the spectra of the flavilium salts were carried out by Hayashi (34) and these were mostly confined to the ultraviolet region. The relationship between light absorption and hydroxyl and sugar substitution were investigated in these studies. Tasaki (88) reported similar observations on the absorption spectra of the anthocyanins, flavones and catechins in ethanol solution in the ultraviolet range.

Johnson, Foreman and Mayer (35) determined the ultraviolet absorption of polyphenolic substances from various fruits using a Beckman Quartz spectrophotometer. Two absorption bands, one at 280 mμ and the other at 322-324 mμ were observed. The band at 280 mμ was attributed to two or more substances, D-catechin and its derivatives, while the other band at 322-324 mμ was due to a caffetannin.

d. Identity of pigments in grapes. The study of the grape anthocyanins over the past half a century has shown that these pigments are as a rule the glucosides of delphinidin and its methyl ethers. Willstätter and Zollinger (99) showed that the pigment in the dark blue European grape, Vitis vinifera, consisted largely of the monoglucoside oenin isolated as oenin chloride, and indicated that diglucoside and free oenidin were also associated with the monoglucoside in the grape. On the other hand, investigations on American grapes by Anderson (3) indicated that three varieties of these grapes, namely Vitis aestivalis Michx. (Norton), V. labrusca (Concord), and V. riparia Michx., consisted of an anthocyanin which on hydrolysis yielded a monomethyl ether of delphinidin, or possibly a mixture of delphinidin dimethyl ether and delphinidin in equimolecular proportions. It was also stated that there was evidence for the presence

of some diglucoside and some free anthocyanidin in these grape varieties. The anthocyanidin obtained by hydrolysis of the pigment was similar to oenidin, but it differed in that it contained a lower percentage of methoxyl. The pigment differed from oenin in its color reaction with ferric chloride. The anthocyanin from Siebel grapes (5) was shown to be a monoglucoside and appeared to be identical with oenin, the glucoside derived from Vitis vinifera. The pigment in Clinton grapes (4) was found to be a monoglucoside and the chloride was difficult to crystallize. The anthocyanidin obtained on hydrolysis consisted largely of a monomethyl ether of delphinidin but the methoxyl values were too high indicating that it contained some dimethyl ether of delphinidin. Levy, Posternack and Robinson (55) found in the Fogarina grape a small amount of delphinidin and its 3' - methyl ether in addition to oenidin. Cornforth (20) isolated from Vitis hypoglauca, an Australian wild grape, an anthocyanin which was shown to be largely oenin (3'-5' dimethyl 3-monoglucoside of delphinidin). Browne (16) isolated the anthocyanin from Muscadine grapes and showed that it consisted of 3'-5' diglycoside of petunidin and no evidence of a monoglucosidal anthocyanin was found.

Mackinney (57) stated that very low concentrations of chlorophyll were found by spectrophotometric methods in Sultanina grapes and raisins.

5. Methods used for separation of anthocyanins

Most of the classical methods of purifying substances present in the same phase have involved phase changes or separation by diffusion with semi-permeable membranes in which a high degree of enrichment was reached in a single operation.

a. Dialysis. One of the earliest experiments utilizing dialysis for the purification of anthocyanins was reported by Portheim and Scholl (70). It was stated that by dialyzing the aqueous extract of pulped plant tissues through an animal membrane, the coloring matter was obtained in a state of relative purity. Since then no attempts seem to have been undertaken to utilize dialysis either for the separation or the purification of the anthocyanins.

b. Distribution in immiscible solvents. Rosenheim (81) observed that butanol was a good organic solvent to extract the anthocyanins from acid solutions. Nonhydroxylic solvents were utilized to extract and also to identify the pigments by taking advantage of the differences of distribution coefficients between the organic solvents and water. Nebesky and others (61) extracted most of the anthocyanin pigments from strawberries and currants from crude aqueous solutions by use of isobutyl alcohol in equal quantities.

c. Chromatographic techniques. Isolation and identification of chemical substances occurring in minute quantities were facilitated with the advent of adsorption techniques. In 1906 the Russian botanist, Tswett (93) described the new technique for separating the components from complex mixtures. The basis of this technique lies in the partition of solutes between a stationary solid adsorbent and a moving liquid phase. It consisted of pouring a small quantity of the solution of pigments (petrol-ether extract of dried leaf material) on the top of a vertical column (precipitated chalk) followed later with the pure solvent, whereupon a series of colored bands were formed down the length of the column. The sequence of the various substances responsible for the different color

bands was explained as due to differences in adsorption coefficients. Tswett mainly dealt with colored substances and named this technique the chromatographic method.

The use of chromatography was revived in 1931, when Kuhn and Lederer (43) described the separation of carotenoids from several natural sources by adsorption analysis on fibrous alumina. From that time chromatography developed in many directions and was employed as a separation procedure in many branches of science and reference was made to a few of the recent reviews and publications (59, 86, 101).

(1) Solid columns. Considerable difficulty has been experienced in the preparation of the anthocyanins and the resolution of their mixtures by the use of chromatographic adsorption methods. Price and Robinson (73) noted that variation of the color of the pure anthocyanins with changes in the hydrogen ion concentration produced by the adsorbent might lead to the formation of more than one band on the adsorbent column. Karrer and Strong (42), using a column of activated alumina separated peonin chloride, considered to be pure, into cyanin chloride and pure peonin chloride. Cyanin chloride was washed down the column on repeated elution with dilute HCl, while peonin chloride was not eluted from the column under these conditions. Karrer and Weber (43) used a column of hydrated calcium sulfate, and separated althaein chloride, the pigment from black mallow, into delphinidin-dimethyl ether (unadsorbed on the column) and two bands of pigments which were adsorbed on the column. These two bands could be eluted with warm, dilute HCl but were not identified with certainty as the separation was incomplete.

Using a talc-siliceous mixture (3:1 ratio by volume) Aronoff and Aronoff (6) separated the pigments of the red beet, which were identified as nitrogenous anthocyanins. A yellow material, vaguely distributed, was also indicated.

(2) Partition chromatography-filter paper strips. During the last few years with the success of partition chromatography, notably the two dimensional procedure, advantage has been taken of paper to detect micro quantities of materials. Martin and Synge (58) observed that certain separations could be affected by using water saturated silica gel as one phase of a chromatogram, the other being some fluid immiscible with water, silica merely acting as a mechanical support. Separations in a chromatogram of this type depend upon the differences in partition between the two liquid phases of the substance to be separated, and not on differences in the adsorption between liquid and solid phases. The authors also dealt with the theoretical aspects and the practical limitations of their theory.

This method was used for the micro determination of mono amino acids in protein hydrolysates, by using silica as the supporting substance, and washing the column with chloroform saturated with water containing 1 per cent butanol.

Gordon, Martin and Synge (28) pointed out that by substitution of paper for silica more promising results were obtained. Following further work along these lines Consden, Gordon and Martin (19) demonstrated the presence of each of the amino acids contained in 200 μg. of wool (hydrolyzed extract), which were shown to be present by other methods. Solvents partially soluble in water gave satisfactory results. Though the absolute

partition coefficients might be greatly changed due to variation in temperature, the ratios of the partition coefficients of the respective amino acids were almost unaltered. They also defined the R_f value of the solute as the ratio of distance moved by the band of the solute to the distance traversed by the advancing front of liquid. The authors described the apparatus which, in essence, facilitated the flow of solvent, down a strip of filter paper at the top of which was placed a spot of solution of mixed amino acids. For greater clarity two dimensional analysis was conducted using a standard sheet of paper (18 x $22\frac{1}{2}$"). The identification of the individual components was achieved by spraying the paper with the specific color developing reagents. They noted that the reproducibility of R_f values depended upon the following factors: paper, temperature, quantity of solute, extraneous substances, degree of saturation with water, supply of solvent and distance between starting point and source of solvent. Mention was made that the quantity of solute had little effect on R_f value though the size of the spot varied considerably.

Instead of allowing the solvent to descend, Williams and Kirby (95) found that allowing the solvent to ascend by capillary action accomplished consistent results with a more simplified apparatus. They noted that the R_f values determined by both methods agreed closely though the values obtained by the ascending method were frequently a little lower. In addition the use of the ascending method would avoid the bends of the filter paper needed in the descending method.

Bate-Smith (11) in a study of the anthocyanins and related substances in petal extracts using paper chromatography evaluated the use of phenol, collidine, butanol-acetic acid-water (40:10:50) as solvents. He noted

that use of 1 per cent HCl extracts of petals allowed formation of less diffused spots than those from aqueous extracts. He also mentioned that if the acid were not added to the extract of anthocyanins and if instead aqueous medium was used, the main spot was accompanied by fainter spots probably due to a tautomeric color base. The position of a spot giving the color reaction of a particular anthocyanidin type when sprayed with ammoniacal silver nitrate together with an examination of the color in ultra violet light yielded useful information as to its identity.

In a later note Bate-Smith (9) reported that the relative distances travelled by the anthocyanins in butanol-acetic acid were markedly affected by HCl and that there were other advantages in the presence of this acid in the system. The aglycones, especially cyanidin, were unstable when run on filter paper unless considerable HCl was present. Greatest stability of aglycones was observed with butyl alcohol equilibrated with 2 N HCl.

In a recent review on the chromatography of anthocyanins, flavones and other phenolic compounds, Bate-Smith (10) stated that chloride ion affected the R_f value of the anthocyanins. The R_f values of a number of phenolic compounds, flavones, the anthocyanins and their aglycones were reported. It was observed that the C_{15} compounds (flavones, anthocyanins, etc.) having the same number of hydroxyl groups had approximately the same R_f value, and the R_f value was found to diminish with each additional OH^- group. The glycosidic combination with sugars other than rhamnose caused the same fall in R_f as addition of hydroxyl but the formation of a bioside caused a lesser fall in R_f value than that of a glycoside. Methylation of a hydroxyl caused a rise in R_f. It was also mentioned that using a mixture of m-cresol:acetic acid and water (50:2:48) there was a regularity of

behaviour with chemical constitution. With this solvent the spots were more widely spaced and the R_f values of substances containing the same number of hydroxyl groups fell in a different order from that in butanol, acetic acid and water. This solvent facilitated the identification of unknown substances by two-dimensional chromatography, but the C_{15} compounds tended to form elliptical spots, covering a large area when run two-dimensionally.

d. Ion exchange materials. Recent developments in the preparation and use of adsorbents stimulated intensive work in the application and use of ion exchange compounds. Though ion exchange compounds have been used in water refining in the past, the work of Adams and Holmes (1) on the insoluble resins formed as a result of condensation of phenols with formaldehyde, brought to light newer and more widespread developments in this field. Ion exchange might be defined as "the reversible interchange of ions between a liquid phase and a solid phase which does not involve any radical change in the solid structure" (60, p. 43). A number of resinous products containing reactive groups are at present available commercially (87).

6. Methods used for estimation of anthocyanins.

In most experiments the color changes in fruits and fruit products have been roughly evaluated by direct visual inspection of the product under examination. The colors have been graded by comparing the experimental samples with a series of standard color samples, numerical ratings being arbitrarily assigned to the latter (65).

a. Colorimeters. Lovibond red, yellow and blue or brown tintometers have often been used to estimate the browning of fruits and their products. Joslyn et al (40) utilized this technique to measure changes in orange juice. Tintometers have been adopted to follow color changes in strawberry, currant and raspberry juices (13, 92). Kramer and Smith (42) reviewed the methods of measuring color in canned foods and enumerated the relative advantages of utilizing disc colorimeters and spectrophotometers to measure the color of various canned products.

b. Spectrophotometers. In the last few years various workers have made use of the spectrophotometer for the measurement of color. In a study on the change in color of grape juice as a result of storage, Pederson (68) determined the per cent transmission values using the spectrophotometer after diluting the grape juice to suitable volume. Sondheimer and Kertesz (84) stated that attempts to separate anthocyanins quantitatively from other colored materials, present in the strawberry juice, by chromatographic adsorption and differential solubility in organic solvents were unsuccessful. They mentioned that the difference in optical densities at 500 mμ, at pH's 3.4 and 2.0, was proportional to the anthocyanin concentration in the solution.

Processing of Grapes and Juice Storage Studies

1. Influence of time and temperature of process on extraction of anthocyanin pigment from grapes.

The influence of the temperature and the time of heating on the quality of color in the resulting fruit juices prepared from fresh fruits was early realized though an extensive study of these factors was not made for

a long time. The qualitative effect of these factors was studied by Hartmann and Tolman (33). They pointed out that for V. labrusoa varieties a temperature of 150°F. should not be exceeded during the heating process as otherwise an excessive amount of tannin is extracted from the seeds at high temperatures. The increase in the color with the temperature was attributed to a better extraction of the pigment from the skin of the grape berry. In qualitative experiments with V. vinifera varieties in California, Cruess (21) observed that the color did not dissolve in the juice unless the grapes were heated sufficiently to cause the "color to flow" (p. 402). He found that the color would dissolve in the juice slowly at 105° to 130°F. and almost instantly at 160° to 170°F.

Using the grapes of Petite Sirah (a V. vinifera variety of red grapes grown in California), Joslyn, Farley and Reed (38) observed that the juice of heated samples contained a violet to dark purple pigment in addition to a red pigment. The concentration of the violet to dark purple pigment in the heated samples increased much more rapidly than that of the red pigment. A slight increase in color was noticed in raising the temperature from 104° to 122°F., while increase of temperature from 122° to 158°F. caused a more rapid increase. Raising the temperature from 158°F. to 194°F. resulted in a very large change and no appreciable changes were noted when attempts were made to raise the temperature from 194°F. to 212°F. The temperature of 158°F. was found to be critical and at this temperature the color was found "to flow" as stated by Cruess (21). The color obtained by heating for 5 minutes at 158°F. matched with that obtained for 18 minutes at 140°F., about 40 minutes at 122°F., 60 minutes at 104°F. and about 2 minutes at 176°F. The Hess-Ives tintophotometer was used for the determina-

tion of the color employing transmitted light with cells of 3 mm. thickness.

Using 120 varieties of ripe grapes Shoemaker (83) compared their colors based on the natural unheated juice. The results were, however, different from the grape juice which was heated. The colors of the natural juices were compared by filling test tubes half full and arranging them in a line with the darkest at one end and the lightest colors at the other. The comparison was made to consider the various shades of color as red, then the brown and lastly the clays. The juices were divided as hot (i.e. the grapes were crushed, and heated to 140°F. before pressing), and cold (the grapes were simply crushed and pressed). The juices from both the lots were heated to 176°F., filtered, bottled and sterilized at 167°F. before examination.

The 120 varieties were divided into 10 color groups as dark maroon purple, purplish maroon, light purplish red, brownish red, very dark brown, medium brown, pinkish brown, light brown, very light brown and clay brown. The author noted that the skin color did not necessarily indicate the color of the juice. Unheated Concord grapes contained little red color, while when heated in preparing the juice, a purplish color resulted. A number of varieties, particularly those listed in the "blackish purple" and the "dark maroon purple" groups showed more color in the juice than those which were not heat treated.

Amerine and Dematties (2) studied the effect of respiration, various gas treatments and heat on the rate of extraction of the color from uncrushed Carignane grapes. They observed that the anthocyanin pigments, responsible for the color of the red wines were present in the epidermal cells of the grapes in most varieties of Vitis vinifera (L.) although a few varieties such as Alicante Bouschet and Salvador also had a pigmented juice.

The Carignane grapes were packed in separate wide mouth glass jars surrounded by oxygen, nitrogen, ethylene, and CO_2 and were stored for varying periods of time ranging from 9 to 86 hours. They were then crushed in a beaker, and heated for 20 seconds while stirring on a water bath, and filtered as rapidly as possible. The color contents of the resulting juices were compared with the juices from fresh grapes, prepared similarly but without the gas treatment. No conclusive results on the color extraction were noted due to storage under the various gas treatments prior to processing.

Heat treatments of the whole fruit were tried prior to crushing and initial heat treatment. The best results for color extraction were obtained by dipping the whole grapes in hot water at 203° to 208°F. from 1 to 3 minutes before crushing and processing. Practically all of the pigments in the skins diffused into the pulp immediately after such initial heat treatments. At the time of normal pressing for red sweet wines the mass of the treated samples contained from 50 to 100 per cent greater pigmentation than the check samples.

Konlechner (47) indicated that if the mashed red grapes were heated the total extraction of the coloring matter in the juice samples rapidly increased with temperature. Heating to 80° to 90°F. was found to be preferable as too much tannin was extracted at higher temperatures.

Neubauer (63) worked out a process to extract greater quantities of the pigment into the juice. The process consisted in extracting the whole grapes or the freshly prepared mass with (1) superheated steam at 266° - 294°F. for 0.5 to 2 minutes or (2) with dry ice. The color content was estimated to be 2 to 4 times higher than when usual methods were used.

In experiments involving improvements in the manufacture and the preservation of grape juice Pederson and Tressler (69) used temperatures of 140° - 145°F. for the extraction of color, heat treatment being continued till the bright purple red color of the grapes was diffused throughout the mass.

Tischer (90) adopted a high temperature process for the extraction of Concord grape juice using a steam retort, in the absence of air. Juice extractions with varying processing times (5 to 100 minutes) and temperatures ranging from 190° - 250°F. were used and the juice removed from the heated atmosphere at it was formed. The juice remaining in the cooked grape mass was centrifuged. A basket centrifuge was employed for this purpose. The times of processing varied up to 100 minutes at 190°F. and to 15 minutes at 250°F. No serious losses of juice quality were indicated in the different juices as evidenced by chemical analyses, and triangular difference palatability tests. The maturity of grapes was shown to have a slight effect on the amount of the tannin and coloring matter. On visual examination it was noted that the juices resulting from high temperature processes were fairly red in color, whereas those from the low temperature processes were purple. Inclusion of the stems during processing or the higher processing temperatures employed did not result in higher tannin contents.

2. Color changes in grape juice on storage

Fruit juices have been shown to fall among the most perishable of foodstuffs. Berthelot (15) and Pasteur (67) observed changes in the color of wines during storage and attributed them to the action of light and

oxygen. Joslyn and Marsh (39) indicated that the browning of citrus juices involved oxidation as a primary step followed by secondary condensation reactions complicated by the presence of color pigments, terpenes, proteins and other substances.

Neubert and Veldhuis (64) observed that the period of stability of apple juice depended on the particular lot of juice and that the inert sediment from clarified apple juice was related to tannins. The formation of inert material appeared at the same time as the development of yellow color. Carpenter, Pederson and Walsh (18) noted that while bottled Seitz-filtered juice normally remained sterile indefinitely, it later became hazy and some sediment was deposited, indicating that the high temperature of pasteurization was not a factor in the deterioration of fruit juices.

Carpenter (17) showed that certain wavelengths of light tended to accelerate the deterioration of apple and Kraut juices, whereas others did not. The red end of the spectrum darkened the color of the juice, while the blue end had a tendency to fade the color. Juices exposed to green light retained more nearly their original color than those exposed to light of any other color.

In studying the visual color changes occurring in Concord grape juice during storage, Tressler and Pederson (91) observed that these changes could be reduced by eliminating air from the bottle and further noted that light had a detrimental effect. It was suggested that the juice color might change because of its reaction with the alkali of glass, a theory which seemed to gain support by the staining of the bottle occurring on storage. It was stated that the detrimental effect of the high pasteurization temperatures (180°F. as compared to 165°F.) might be due to a large

part to the speeding up of the rate of oxidation and if oxygen were entirely eliminated from contact with the juices, no differences would be noted. Slightly less sediment was obtained in the deaerated samples than those which were not deaerated. The deterioration was detected by a clouding in the juices, especially the clear samples. Shortly afterwards a sedimentation of a brown material occurred, and in advanced deterioration a subsequent clearing was noticed leaving an amber colored juice. Light of short wavelengths was shown to accelerate the changes as shown by the experiments in which colored cellophane was used to wrap the bottles. The samples were stored in a window with a southern exposure.

Pederson and Tressler (69) noted that the juice had to be bottled hot to exclude air after pasteurization. On storage, the color of the juice changed from reddish purple to a brick red and then to a dull brown, simultaneously with the clouding and sedimentation. Along with a decrease in the protein content and the acidity there was an increase in the total water insoluble pigment.

The agents responsible for deterioration of color in fruit products were summarized by Joslyn (37). He pointed out that the problem of color retention in fruit products was complicated by a number of reactions which might be involved in bleaching, discoloration and browning. The natural pigments might change in tint during the preparation, processing or storage of fruit products, and the discoloration or browning might occur as a result of the formation of the pigmented substances by the decomposition of certain chemical constituents of the fruit.

Hamburger and Joslyn (30) suggested that in filtered citrus juices the principal reducing agents were ascorbic acid and flavonols, and their

reaction might be influenced by the sugars, soluble nitrogenous constituents and traces of galacturonic acid. Powers and Esselen (71) stated that effects of light were only of minor importance in the deterioration of glass packed foods in comparison with the deteriorative changes caused by heat and oxygen. Beattie, Wheeler and Pederson (13) noted an increased destruction of the ascorbic acid content during storage in the presence of air and greater decreases in the color when ascorbic acid was added to fruit juice. It was further suggested that since ascorbic acid was oxidizable and the pigments reducible, they probably may react with each other. Esselen, Powers and Fellers (24) reported that no improvements to any great extent were noted in grape juice samples on storage after addition of ascorbic acid.

Pederson, Beattie and Stolz (68) examined the variation in the color content of stored grape juice samples with a Beckman spectrophotometer and recorded the adsorption values in the visible range after convenient dilution of the clear juice. The optical density near 500 mμ was lowered on storage while increases were noted around 400 - 420 mμ, due probably to the formation of soluble brown compounds formed simultaneously with the decrease in the principal color of the juice. The results did not suggest, however, that the brown color arose directly from the deteriorating principal color, except possibly in strawberry juice. A brown, alkali soluble precipitate was noted in the grape and apple juices and the precipitate increased progressively with storage. The oxidation type of reaction preceded the type of deterioration resulting from the reaction between reducing sugars and amino acids. Qualitative tests showed that the reducing sugar-amino acid type of precipitate was quite strongly fluorescent in contrast

to the quinone type, but no studies were made to identify the precipitate.

Lewis, Esselen and Fellers (56) stated that the browning of foods was not restricted to the Maillard reaction but was also shown to occur due to the interaction of glucose with the carboxylic acids in general. Stadtman (85) reviewed in great detail the work pertaining to the nonenzymatic browning in fruit products.

Nebesky and others (61), studying the effects of the various factors on the stability of the color in a variety of fruit juices during storage, observed that cherry, grape and tomato juices were much more stable and exhibited less color deterioration than strawberry, raspberry and currant juices. The colors of the juices were examined after convenient dilution with water by determining the light transmittance characteristics using a Coleman model 11 spectrophotometer. Addition of ascorbic acid accelerated color deterioration in the form of progressive bleaching in the other juice samples, except in blueberry and grape juices. Sugar and pH values played only minor roles in regard to the stability of the fruit juices.

D. Processing and Storage Studies on Purified Anthocyanin Pigment Solutions (from Strawberry and Currant)

Working with purified pigment solutions from strawberries and currants, Nebesky and others (61) observed that storage temperatures and the presence of oxygen accelerated color deterioration with both pigments, while a low storage temperature preserved the color. Light and ascorbic acid had a rapid bleaching effect on strawberry pigment, which was not noticed in the juice of this fruit. The strawberry pigment solutions unlike the currant pigment solutions developed a faint precipitate after storage for a week.

This precipitate increased on further storage. "This precipitate may have occurred as a result of acid hydrolysis which converted the anthocyanin (glucoside) to the water soluble anthocyanidin" (p. 273). Probably the pigments in currants were more stable than those from strawberries. Sugar had little effect on the stability of the color in the pigment solutions. Increasing the sugar or the citric acid concentration enhanced the stability of the color of strawberry fountain syrup during storage.

E. Nature of Tannins in Grapes and Antioxidant Properties of Tannins

Guilliermond (29) observed that in certain plant cells granules were formed which seemed to consist of anthocyanin-tannin compounds. Cruess (21) stated that the tannins in grapes belong to the so-called catechol tannin group as they yielded catechol or orthodihydroxy acids on alkaline hydrolysis. Johnston et al (36) stated that about 0.01 to 0.6 per cent hydroquinone, toluhydroquinone, catechol or resorcyl aldehyde might be added to inhibit oxidation and preserve the aromatic, flavoring and coloring principles of fruit juices. Lea (54) reported that among others gallic acid and pyrocatechol could act as powerful antioxidants in butter fat while tannic acid showed weaker activity. Cruess and Armstrong (23) pointed out that tannic acid, digallic acid and also grape seed tannin were good antioxidants for walnut meats.

III. EXPERIMENTAL PROCEDURES

A. Description of Equipment

1. Processing studies

Throughout this investigation glass containers were used for processing. Glass jars (capacity 1 pint), glass tumblers (12 oz.) and the metal and molded caps (63-A-H-N and 68-T) used for closing the glass containers were secured from Anchor Hocking Glass Corporation. The automatic vacuum closing machine supplied by Anchor Hocking Glass Corporation and provided with appropriate chucks was used for closing the containers under a vacuum of about 20". The retort used for processing was cylindrical in shape, with inside height of 51 inches and a diameter of 24 inches. The temperature of processing was regulated by use of a Foxboro temperature controller. The controller consisted of an on-off type, air operated thermal system. Compressed air and high pressure steam were supplied through the college lines. A Brown Electronik eight point, strip chart, potentiometer type temperature recorder and a self-balancing potentiometer were used to register the temperatures inside and outside the containers during the process. Molded plastic, copper-constantan thermocouples, of length 2", were employed as the sensing elements.

A polar planimeter (No. 132, Ser. No. 5973) was used for the estimation of the areas under the processing curves plotted on linear graph paper.

2. Estimation of color and pH of solutions

The pH of the samples throughout this investigation was determined by use of a Leeds & Northrup pH meter model 7663A-1 equipped with a glass electrode (std 1199.12). For the estimation of the pigments, a Coleman spectrophotometer model 11 was used while working in the visual range (4000-6000 mμ). When the absorption studies were made in the ultraviolet range a Beckman photoelectric Quartz spectrophotometer model D-U was employed.

3. Drying and centrifuging

To obtain clear solutions for volumes, up to one liter, an International centrifuge (size 2, No. L6930) was used. For the separation of the juice from the processed grapes, a basket centrifuge, silver plated on the inside was employed (94). The drying of the samples at low temperatures was done by use of the lyophilization apparatus designed by Ralph W. Kline and described in detail by Hanson (31).

A Weber vacuum oven provided with a Lectrodryer was used for the drying and concentration of solutions. The vacuum oven was connected to a vacuum pump through a trap of about $\frac{1}{2}$ liter capacity to condense the evaporating fluids. The trap consisted of an iron pipe of 2" diameter provided with inlet and outlet pipes of 3/4" diameter. The iron pipe contained a copper coil of 1/4" diameter to allow the refrigerant to flow through. The outside of the trap was covered with asbestos. The cooling was achieved by means of a refrigerator compressor.

4. Ultra violet light exposure studies and preparation of buffer solutions

A General Electric, 15 watt ultra violet lamp of length, 17.5", was fitted into a flourescent fixture and used in studies of the effects of exposure to ultraviolet light. The lamp emitted radiations mainly in the region of 2537 Å.

Buffer solutions were used throughout the study where it was desired to regulate the pH during the course of the experiment. McIlvaine's (50, p. 696) standard buffer solutions which included a pH range of 2.2 to 8.0 were employed, unless otherwise stated. The buffer systems were prepared by mixing definite quantities of stock solutions consisting of 0.1 molar citric acid and 0.2 molar disodium phosphate. In experiments involving dialysis, where buffer solutions with a wider pH range were needed, Clark and Lubs buffer mixtures (50, p. 699-700) were used.

B. Qualitative Identification of Pigments Present in Concord Grapes

For the qualitative identification of the water soluble and insoluble pigments present in grapes, the method suggested by Bancroft and Rutzler (8) was employed. The pigments present in 2.5 gm. of lyophilized skins were extracted into methanol solution by grinding the skins with 150 ml. of methanol and 3 gm. of sea sand. The methanol extract was centrifuged and a portion of the clear extract was added to an ether-water system made such that the two liquid layers were of equal volume. A green color in the ether layer fading gradually on adding dilute hydrochloric acid showed the presence of chlorophyll.

When aqueous hydrochloric acid (5 ml. 1 per cent) was added to the ether-water system, a pink color in the aqueous layer indicated the presence of anthocyanins or anthocyanidins. After separating the two layers, 10 ml. of aqueous ammonia (5 per cent) was added to the ether layer. The presence of a yellow color in the water layer was indicative of the presence of flavones and a yellow color exclusively or chiefly in the ether layer showed presence of the "water soluble yellow pigments" (8, p. 2946). After separating the layers, heptane was added to the ether layer, the ether was evaporated off and 10 ml. of 90-95 per cent methyl alcohol was added. A yellow color in the heptane layer denoted carotenes and a yellow color in the water layer indicated the presence of xanthophylls, subject always to possible errors due to the presence of water soluble yellow pigments. At each stage the absorption values of the different layers were recorded using the Coleman spectrophotometer.

C. Procedures Used for Separation of Anthocyanins

1. Dialysis

Visking tubing ($5\frac{1}{2}$" long x 2.5" diameter) was fastened with thread at the bottom end to form a container and the inside was washed with distilled water. A known volume of solution to be dialysed was pipetted into it and 1 ml. of toluene was added to prevent fermentation. The top end was fastened with a string attached to an angle iron metal framework. The framework and the attached bags were continuously raised and lowered by means of a connecting rod attached to a motor driven eccentric pulley.

To prevent the bags from floating when they were immersed in the solutions, a small Visking tubing containing glass beads was hooked on to the bottom end. The bag and its contents were completely immersed at all times in the outside solution, contained in a 1 litre measuring cylinder by varying the length of the supporting thread. After dialysis the solutions in the measuring cylinders were concentrated to convenient volumes in a vacuum oven at a temperature of 122°F. under reduced pressure. The absorption values were recorded with a spectrophotometer at a pH of 1.0.

Grape juice was dialyzed by immersion in solutions of different pH values (1.0 - 8.0). To determine if either the pectin or sugar present in grape juice exerted any influence during dialysis, the experiments were repeated using grape juice free from pectin and sugar. Pectin and sugar were removed by hydrolysis with pectinol and yeast fermentation respectively. The effects of various sugars added to the juice before dialysis were also investigated. Purified pigment solutions prepared from grape skins were also dialyzed under conditions identical with those used for dialyzing juices.

2. Solubility of anthocyanin pigments in different solvents

The pigments present in 20 gm. of skins, containing approximately 25 per cent dry matter, were extracted by crushing in a mortar with 100 ml. of the following solvents: 95 per cent ethanol, n-butanol, isobutyl alcohol, glacial acetic acid, and mixtures of ethanol and butanol, butanol and isobutyl alcohol, isobutyl alcohol and ethanol, formic acid and ethanol, glacial acetic acid and ethanol mixed in equal volumes. The extracts were then transferred to 250 ml. Erlenmeyer flasks fitted with tight corks and shaken thoroughly in a laboratory shaking machine for one hour. After allowing

the solutions to stand over night they were filtered through Buchner funnels under suction and each of the residues was re-extracted using 100 ml. of each of the solvents. The process was repeated nine times when practically no additional color could be extracted. The color content in each individual extract was determined, using the spectrophotometer, after evaporating 4 ml. of the extract at 104°F. under reduced pressure and dissolving the dried extract in 100 ml. of 2 per cent hydrochloric acid.

3. Chromatography

a. Solid columns. A glass tube with uniform bore (6" long x 2.5" diameter) was employed as the container for the adsorbent column. It was fitted with a rubber stopper provided with a hole at the bottom and an S-shaped small bore glass tube passed through this hole. This small bore glass tube was connected to an aspirator for drawing suction via a glass test tube provided with a two-holed rubber stopper.

The tube was filled to 4" in height with the adsorbent over cotton placed at the bottom of the main glass tube to prevent the adsorbent from passing through the outlet. A uniform distribution of adsorbent was obtained by pouring a slurry of the adsorbent mixed with the solvent down the sides of the tube, mild suction being drawn at the same time. Water was used as the solvent if the adsorbent was insoluble in water. Butanol was used when a water soluble adsorbent was employed. The solution containing the solute to be resolved was poured down the sides of the tube. The chromatogram was then developed with the pure solvent.

The adsorbed substances were eluted with 2 per cent dilute hydrochloric acid when acid insoluble adsorbents were used and with butanol

when employing acid soluble adsorbents. The per cent transmittance values of the eluted solutions were determined with a spectrophotometer.

b. Filter paper strips. Whatman No. 4 filter paper was cut into strips (22.5" length, 0.75" width) and initially the solvent used for separation was allowed to flow to the top of the paper strips by capillary action, by immersing the lower end of the strip in the solvent contained in a 1 liter measuring cylinder. This procedure facilitated the removal of some yellow colored soluble impurities present in the paper as these impurities moved along with the solvent front. The ends of the strips containing the colored impurities were then cut off.

A few drops of the pigment solution, the number depending on its concentration, were pipetted on to the paper strip about $1\frac{1}{2}$" from the bottom without allowing it to spread unduly on the paper. After allowing the paper to air dry, it was dipped in the flowing solvent mixture contained in the bottom of a 1 liter cylinder to a depth of about 1". The chief precaution to be taken was that the test spot should not dip into the solvent mixture but should be at least half an inch above the solvent level. The paper was held in position in the top of the cylinder by bending it over the edge of the cylinder. Further, to facilitate holding the paper strip in a vertical position without rolling, pieces of glass rods were inserted in slits at the bottom of the paper strip.

After allowing the solvent to flow upwards for a 24-hour period, the paper strips were taken out and allowed to air dry after marking the level of the solvent front. The R_f values of the individual bands, if more than one was present, were noted by determining the ratio of distances traversed by the bands and the solvent front. In cases where it was not possible to

achieve good resolution of the individual bands, the chromatogram was repeatedly developed after allowing the paper strip to air dry. In this case only apparent R_f values for a suitable number of passes could be obtained. In some cases where the main objective was to note the presence of different spots or bands obtained on resolution, the measuring cylinder was replaced by a glass bottle (10" height, 4.5" diameter) and paper strips (11" length, 0.75" width) were used. Three runs, each of 4 hours duration were usually conducted, the paper being air dried after each run. This procedure seemed to give a better resolution of the individual bands than when separation was attempted for one single pass for a duration of 24 hours.

4. Ion exchange materials

The apparatus employed for this purpose consisted of a glass tube (12" length, 1.7" inside diameter) carrying at the bottom end a straight small bore tube (2" length, .25" internal diameter) fitted through a rubber stopper. The small bore glass tube acted as an outlet for the pigment solutions which passed through the ion exchange material. The ion exchange material was contained in the larger glass tube to a height of about 10". The ion exchange material XE-75 was secured from Rohm and Haas Company. This material was recommended by the manufacturers to be used for the separation of colored substances.

D. Isolation of Anthocyanin Chloride from Concord Grapes

The method used for the isolation and crystallization of the pigment from Concord grapes was essentially the one followed by Anderson (3) in his investigations with Concord grapes.

E. Preparation of Purified Anthocyanin Pigment Solution

The method utilized to prepare purified pigment solution from the skins or from the juice was the one used by Reynolds, Robinson and Scott-Moncrieff (74). It consisted of precipitation of the pigment as the insoluble lead salt. No attempts were made to crystallize the pigment and precautions were taken to free it from all impurities by repeated precipitation with anhydrous ether from a 1 per cent methanolic HCl solution of the pigment. The purified pigment solution was adjusted to pH 3.4 with dilute ammonia (5 per cent) and evaporated to dryness in a vacuum oven at a temperature of 104°F. under reduced pressure. The aqueous extract of the dried substance was used as mentioned in the investigation.

F. Preparation of Tannin-free Grape Juice

The tannins were removed from the grape juice by the method employed by Johnson, Foreman and Mayer (35), with a few modifications. To a mixture of 100 ml. of grape juice and 25 ml. of 95 per cent alcohol, saturated neutral lead acetate was added with stirring until no further precipitation occurred. The mixture was centrifuged after allowing the contents to remain at 104°F. over night.

Dilute sulphuric acid was added to the clear supernatant liquid and the mixture was left at 104°F. over night to precipitate soluble lead salts as insoluble lead sulphate. The solution containing the insoluble lead sulphate was filtered and the clear filtrate was adjusted to pH 3.4 with dilute ammonia (10 per cent). The filtrate was concentrated in a vacuum oven at 104°F. under a reduced pressure of 1 mm. until the volume was reduced to less than 50 ml. The concentrated solution was then made up to 50 ml. with a McIlvaine's buffer solution of pH 3.4. This extract served as tannin free juice for ultra violet exposure studies.

The insoluble lead precipitate was decomposed by adding a solution of 1 N H_2SO_4 until no further precipitation of lead as sulphate occurred. The solution was cooled over night and then centrifuged using the International centrifuge for 10 minutes at 1500 R.P.M. The clear filtrate containing tannins and colored substances was adjusted to a pH of 3.4 with dilute ammonia (10 per cent) and then concentrated in a vacuum oven at 104°F. under a reduced pressure of 1 mm. till the volume was reduced to less than 50 ml. It was then made up to 50 ml. with a buffer solution of pH 3.4. This extract was added to the tannin free juice in ultra violet exposure studies.

G. Preparation of Extract of Tannins and Other Polyphenolic Substances from Grape Stems

Grape stems from grapes stored at 0 ± 1°F. were dried at 104°F. under a pressure of 1 mm. The dried stems were mixed with 10 gms. of sea sand and crushed in a mortar. The crushed mass was extracted by boiling with 500 ml. of water. The sea sand and the water insoluble stem material were

separated by filtration of the boiled mixture through a Buchner funnel under suction. Saturated neutral lead acetate was added to the clear filtrate to precipitate the tannins and polyphenolic substances as insoluble lead salts. The remainder of the procedure was identical with the one followed in the extraction of tannin-free juice. The extract was utilized for ultra violet exposure studies.

H. Procurement and Storage of Concord Grapes

Fresh ripe grapes were harvested in the morning from the Horticultural Farm at Iowa State College, and were processed within a period of 24 hours. To keep the grapes in as fresh a condition as possible for the investigations to be conducted during off season, the fresh grapes filled into tin cans (9.5" diameter, 13" height) were stored in the cold storage room at 0 ± 1°F.

I. Design of Processing Experiment

Preliminary studies indicated that increased color concentrations were obtained with higher process temperatures and longer process times. The main experimental design was based on these preliminary observations. The process times were chosen such that their logarithmic values were approximately in an arithmetic progression, and the process temperatures were likewise fixed, on the assumption that the effect of process times was logarithmically related to process temperatures. The process times and temperatures expressed logarithmically and the times and temperatures employed are presented in Tables 3 and 4 respectively.

Table 3

Process Times at Each of Three Process Temperatures
Expressed Logarithmically

Process trial	Logarithm of temperature		
	2.22	2.32	2.42
1	1.9	1.5	1.1
2	1.8	1.4	1.0
3	1.7	1.3	0.9
4	1.6	1.2	0.8
5	1.5	1.1	0.7

Table 4

Process Times at Each of Three Temperatures
Used to Process Grapes

Process trial	Process temperature		
	170°F.	210°F.	250°F.
1	79.0 min.	31.5 min.	12.5 min.
2	63.0 min.	25.0 min.	10.0 min.
3	50.0 min.	20.0 min.	7.9 min.
4	39.7 min.	15.8 min.	6.3 min.
5	31.5 min.	12.5 min.	5.0 min.

The statistical design also included a comparison of the effects on the pigment concentrations of the juice samples of varying process temperatures, for equal process times. This point was exemplified by use of 31.5 min., as the processing time for the processing temperatures of 170° and 210°F; and 12.5 min. as the processing time for the processing temperatures of 210° and 250°F. The order in which the processes were conducted was randomly chosen to minimize experimental errors arising from the temporary storage of the raw product at room temperature before processing. Further, three separate replications of each of the processes were done, using grapes filled into three separate glass jars each time. The juice samples were filled into eight test tubes leaving a headspace of about 1 inch. The treatments included addition of toluene into four test tubes while the other four test tubes were pasteurized at 180°F. for 1 minute. Nitrogen gas was filled into the headspace of two of the pasteurized samples. The samples were sorted out and half the samples were left exposed to light from electric bulbs and the other half of the samples were left in the dark at a constant temperature of 70° ± 0.5°F. Samples of the juice from each process of each of the three replicates were examined for their pigment concentrations at intervals of two months for a period of six months, using the Coleman spectrophotometer.

J. Procedure Used for Processing Concord Grapes

The stemmed fresh grapes were filled into glass containers. While both the types of glass containers (1 pint capacity and 12 oz. capacity) were used in the preliminary studies, in further work pertaining to storage

treatment, etc. only the jars (1 pint capacity) were utilized. The grapes inside the containers were crushed with a pestle and the jar was filled until a headspace of about half an inch remained. The jars were closed with metal and molded caps under vacuum with an automatic closing machine. One of the jars in each group was provided with a cap fitted with a thermocouple inserted to the center, to measure the temperature of the contents inside the container. The glass jars were placed in the bottom of the retort in an erect position and to facilitate this, a heavy mesh metal screen was placed on the tops of the jars, so that the jars would not be displaced during processing. The retort was filled with cold water (45°F. to 55°F.) from the main supply until the retort was about half full and the glass jars were completely immersed in the water to prevent breakage due to uneven heating of the glass jars. A thermocouple was also kept in contact with the outside of the container to record the processing temperature. The thermocouples inside and outside the container were connected to the temperature recording apparatus by suitable leads. The lid of the retort was tightly closed and steam was introduced. Steam supply was continued for the requisite process time measured from the time when the retort reached the required process temperature. The steam inlet was closed after processing and cold water was run into the retort gradually to avoid breakage of glass jars due to sudden cooling. Cold water was circulated until the temperature inside the jars was lowered to about 100°F. Throughout this period the temperatures inside the glass jar and the surrounding water temperatures were recorded on the Brown electronik instrument. The glass jars were left over night in a constant temperature chamber at 50°F. to prevent possible mold spoilage of the juice samples

inside the jars as a result of the time lag involved between processing and transfer of clear juice sample to containers.

K. Collection of Grape Juice

The contents of the three jars in each process were mixed and then centrifuged in a basket centrifuge for 10 minutes at the rate of 1500 R.P.M., with cheese cloth serving as the filter. The clear juice emerging was collected in beakers, and weighed. The juice sample was then filled into 8 sterilized pyrex test tubes (6" length, 0.6" internal diameter) leaving a headspace of about $3\frac{1}{2}$".

L. Transfer of Grape Juice to Containers and Storage Treatments

Into each of four of the test tubes containing the juice sample, 1 ml. of toluene was added to prevent fermentation. The contents were shaken thoroughly after closing the test tubes with cork stoppers. The cork stoppers were pre-treated by dipping into melted wax in an effort to prevent mold contamination from the stoppers. The other four test tubes containing the juice samples were immersed to about 1" from the top of the test tubes in water at 180°F. These juice samples were pasteurized at this temperature for 1 minute after the contents in the center of the test tube reached a temperature of 180°F. as noted by a thermocouple placed at the center. The test tubes were cooled rapidly in cold water and closed with sterilized No. 1 rubber stoppers. The headspaces of two of the pasteurized tubes of each process were filled with nitrogen, which was introduced for about 30 seconds at a pressure of about 5 pounds. The tubes

were closed with rubber stoppers as the passage of nitrogen was discontinued. The samples were then sorted and half the samples were exposed to light from electric bulbs (75 Champion 115) and the other half placed in the dark covered by aluminum foil. All the samples were stored in a constant temperature room (70° ± 0.5°F.). One ml. volumes of the fresh juice samples were pipetted out of each test tube by means of sterile pipettes, centrifuged and the color from the clear filtrates estimated by use of the Coleman spectrophotometer.

Three replications of each process were made from fresh samples of grapes as mentioned in the design of the experiment, within a period of 5 days.

M. Studies on Pigment Concentrations in Grape Juice Samples after Storage

The pigment concentrations of the stored samples were determined at intervals of 2 months during a period of 6 months. The absorption values of each sample over the visible range (4000 - 6000 Å) were recorded using the Coleman spectrophotometer after suitable dilution (50 - 100 times) with 2 per cent aqueous HCl.

N. Processing Studies on Grapes Held in Storage

Processing and storage studies were conducted with grapes which were stored in the refrigerator at 40°F. for 1 month. These studies included determination of the effects of storage periods and treatments on the juice samples, obtained as a result of processes conducted at 210°F. and 250°F.

The process times used were longer than those employed with fresh grape samples. The process times and temperatures used are given in Table 5.

Table 5

Process Times at Each of Two Process Temperatures in Extension of Processing Studies to Longer Process Times

Process trial	Process temperature	
	210°F.	250°F.
1		12.5 min.
2	31.5 min.	31.5 min.
3	63.0 min.	63.0 min.
4	100.0 min.	100.0 min.

O. Processing and Storage Studies on Anthocyanin Chloride Solutions

The anthocyanin chloride (prepared by using Anderson's method) was dissolved in McIlvaine's buffer solution of pH 3.4 and the solution centrifuged to separate the insoluble residue formed as a result of storage. Using 25 ml. of the clear solution contained in glass jars the processing was done at times and temperatures identical with those used with fresh grapes. After cooling, the pigment solutions were stored at 70°F. The solutions obtained as a result of each process were examined for pigment concentrations before and after subsequent storage periods.

P. Ultraviolet Exposure Studies on Grape Juice and Anthocyanin Chloride Solutions

Test solutions (10 - 25 ml.) adjusted to varied pH values were pipetted into petri dishes (3.55" inside diameter) with the tops removed. The solutions were then exposed to the ultraviolet light. The samples were held at a distance of approximately 14" from the ultraviolet lamp. The petri dishes containing the solutions were initially weighed using a Torsion balance (Style 269, Class A) with a precision to hundredths of a gram. From each petri dish 1 ml. of the sample was withdrawn at intervals and the color estimated at pH 1.0 after dilutions to known volumes (50 - 100 times). Before withdrawing solutions for pigment estimations, the weights of the petri dishes were adjusted by addition of the buffer solutions, such that the weights of the solutions withdrawn were accounted for. This precaution corrected the losses due to evaporation of water on exposure of the pigment solutions to ultraviolet light. An identical procedure was employed while working with pigment solutions or with grape juice samples.

Q. Methods Used for Estimation of Anthocyanins

It was observed that the optical density at a wavelength of 515 mu would bear a linear relationship to the pigment concentration over a limited range when clear pigment solutions were examined at a pH of 1.0 or less. The clear solutions, obtained after centrifugation, were diluted with 3 per cent HCl to known volumes depending on the concentration of the pigment. The absorption values over the entire visible range (400 - 600 mμ)

were recorded using the spectrophotometer and the reading at 515 mμ was utilized to estimate the pigment concentration as the absorption of the pigment was found to be maximum at this wavelength. The interferences of the products obtained on thermal degradation of the pigment and the discolorations of stored juices were determined. The effects of the components other than the anthocyanin pigments present in grape juice, e.g. sugar, pectin, tartaric, malic and tannic acids, on the color estimation procedure, were also determined.

R. Methods Used for Estimation of Tannins and Identification of Sugar Present in Glycosidic Form in the Anthocyanin Molecule

For the estimation of tannins, the method of Hartman (32) was adopted with the modifications suggested by Tischer (90). The method of French, Knapp and Pazur (26) was used to identify the sugar present in the anthocyanin in glycosidic form. The pigment solution was hydrolyzed by boiling with dil. HCl for 15 minutes and the anthocyanidin was removed from the boiled acid solution by repeated extraction with isoamyl alcohol. The acid solution containing the sugars was adjusted to pH 3.4 with dilute ammonia (10 per cent) and then evaporated to dryness in a vacuum oven at a temperature of 95°F. under a pressure of 1 mm. The dried extract was dissolved in distilled water, and the sugar present was identified by use of paper chromatography.

S. Methods Used for Separation of the Aglycone, Its Mono- and Di-glycosides

The method employed was essentially the one suggested by Willstätter and Everest (97). It consisted in extracting a dilute acid solution (2 per cent) of the mixed pigment solution with equal volumes of isoamyl alcohol. The two layers were separated after thorough shaking. The aqueous layer was extracted repeatedly with isoamyl alcohol until no color could be extracted into the isoamyl alcohol layer. The mixed isoamyl alcohol extracts contained practically all the anthocyanidin and its monoglycoside. The diglycoside was left in the water layer.

The water layer was washed with ether to remove traces of isoamyl alcohol and was evaporated under reduced pressure to a convenient concentration after reducing the acidity with dilute ammonia. The isoamyl layer was reduced in volume by evaporation under reduced pressure. The concentrated solution was extracted with equal volumes of 20 per cent HCl until no further color was obtained in the acid layer. The mixed acid extracts contained the monoglycoside in the form of the chloride and the isoamyl extract contained the aglycone. The aglycone was brought into acid solution by adding large volumes of benzene and dilute HCl (2 per cent). The isoamyl alcohol passed into the benzene layer leaving the aglycone in dilute acid. The solutions were concentrated by evaporation under reduced pressure after reducing the acidity with dilute ammonia (10 per cent).

IV. EXPERIMENTAL RESULTS AND DISCUSSION

A. Fractionation Methods Used to Identify the Pigment Components of Grapes

1. Qualitative identification

The qualitative tests for the various pigments present in 2.5 gms. of lyophilized skins were performed according to the method suggested by Bancroft and Rutzler (8). These tests indicated the presence of chlorophyll, water soluble yellow pigments and carotenes in addition to the free anthocyanidin and its glycosides. The absorption values of each pigment component were determined using the Coleman spectrophotometer and indicated in Figure 1. Using fresh grape juice no positive evidence could be obtained for the presence of any substantial amounts of the anthoxanthins. The shapes of the absorption curves of the fat soluble pigments are all similar. The fat soluble pigments exhibit their maximum absorption in the ultra violet range, and the optical densities of these pigment solutions diminish gradually as the wavelength is increased in the visible range. The water soluble pigments show their absorption maxima at 515 mμ when they are present in acid solution. The isoamyl layer obtained, after extraction of the acid solution of the water soluble pigments with isoamyl alcohol, shows an absorption maximum at 550 mμ. This change in absorption maximum is presumably due to the effect of the solvent. As indicated in Figures 4 and 5, both the anthocyanidin and its monoglycoside exhibit maximum absorption at 515 mμ in acid solutions.

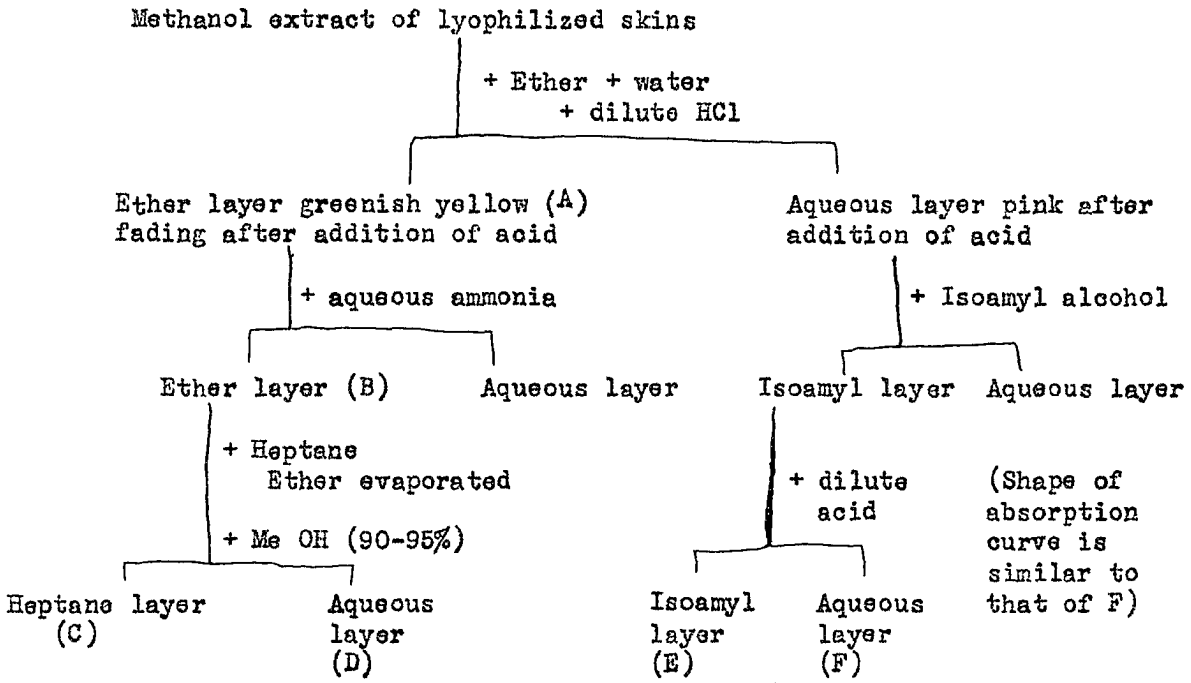
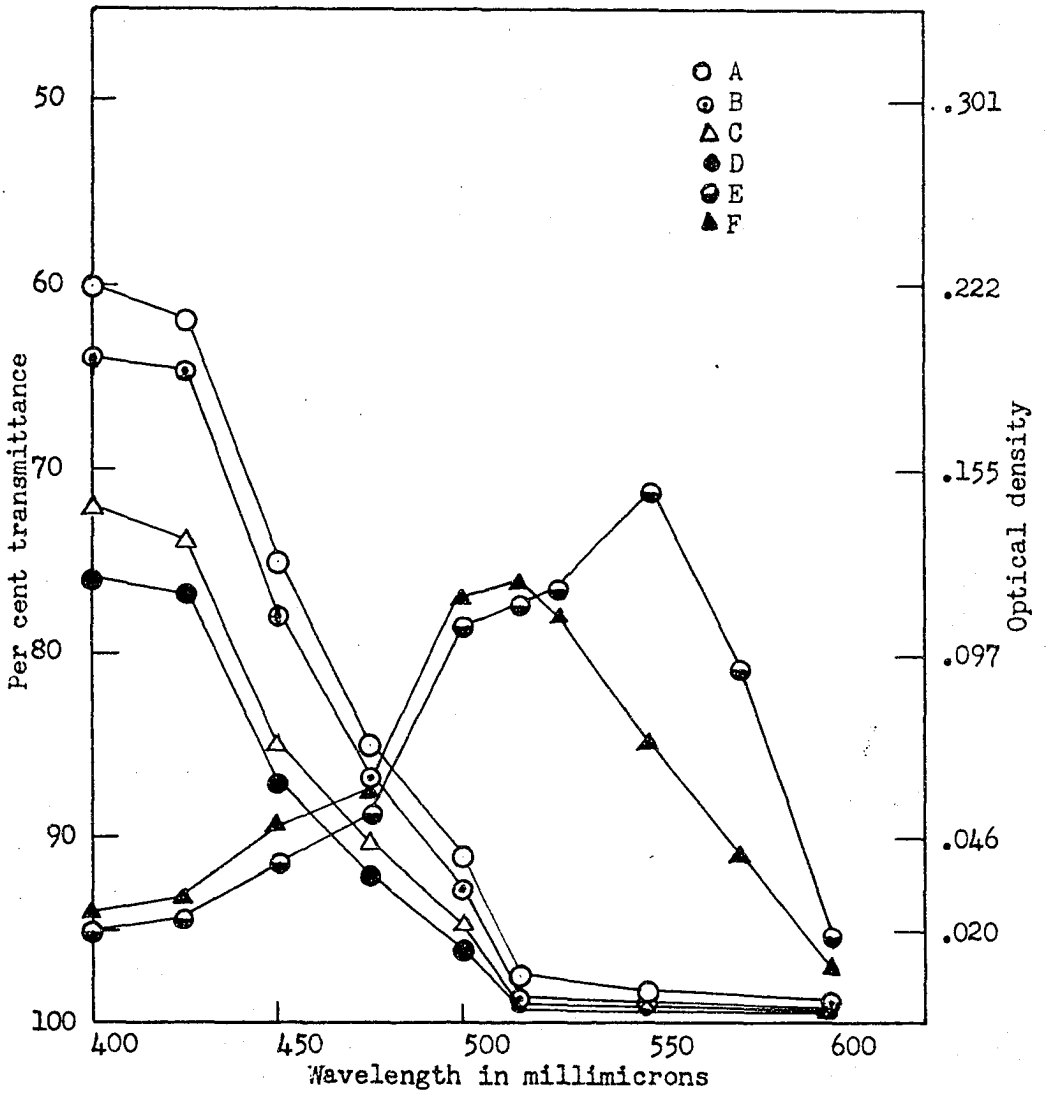


Figure 1. Absorption values of pigment solutions obtained by fractionation of pigments from lyophilized skins (Bancroft and Rutzler's method)


A
B
C
D
E
F
50
60
70
80
90
100
.301
.222
.155
.097
.046
.020
Per cent transmittance
Optical density
400
450
500
550
600
Wavelength in millimicrons

2. Dialysis

The Welch and Teagarden juices (commercial juices) were dialysed in addition to the pigment extract from skins. Volumes of 20 ml. of each brand of the juice sample and 20 ml. of the diluted pigment extract were pipetted into the Visking tubes and the samples were dialysed using distilled water outside the tubes. Separate experiments involving dialysis were also conducted using Clark and Lub's buffer solutions of pH 1, 2.5, 4, 5.5, 7 and 8.5 in the measuring cylinders. The solutions in the measuring cylinders were examined at the end of 24, 48, 72 and 96 hours. The results indicated that about 85 per cent of the pigment dialysed out of the bag in the first 24 hours, with considerably lesser quantities dialysing out at later periods. In each case the absorption maxima of the outside solutions appeared at 515 mμ when acid solutions were examined. The effect of addition of water soluble carbohydrates, e.g. dextrose, fructose, galactose and sucrose (3 gms. to 20 ml.) on the dialysis of the pigment was investigated to find if the sugars exerted any influence during the course of dialysis. No evidence for more than one pigment component was demonstrable from the results. The shapes of the absorption curves were identical in all cases. Similar results were obtained even when the juices were dialysed after removal of sugar by yeast fermentation, and removal of pectin by hydrolysis with pectinol. No positive evidence for the presence of more than one band was indicated when the solutions inside and outside the bags were chromatographed using a mixture of alumina and dicalite, as the adsorbent. The solute adsorbed on the column was eluted with dilute HCl and the adsorption values of the acid solutions were identical in every case.

3. Solubility differences in various solvents

Extracts of the grape skins with the solvents used were obtained by extracting 20 gms. of grape skins (25 per cent dry matter) repeatedly with 100 ml. of the solvent. In each case 4 ml. of the extract were evaporated to dryness under reduced pressure at a temperature of 104°F. The adsorption values of these dried extracts were determined after dissolving in 100 ml. of dilute acid (2 per cent) and the results were included in Table 6. The results indicate that extractions of the skins with mixtures of butyl or isobutyl alcohol with ethyl alcohol yield larger amounts of pigments into the solution than individual extraction with either butyl or isobutyl alcohol.

The interference of the fat soluble pigments was eliminated by the evaporation of the solvent extract and subsequent solution in dilute acid. In every case the absorption maximum was observed to be at 515 mμ. This procedure of fractionation using different solvents did not provide any positive evidence for the presence of more than one pigment component, though a general idea of the differential solubilities of the pigment in different solvents was furnished which might prove to be of aid to isolate pigments from natural sources.

4. Chromatography

a. Solid columns. Adsorption alumina, mixtures of alumina and Dicalite, mixed in a ratio of 1:2 by volume, magnesium sulphate, potassium dihydrogen tartarate, norite, silicic acid and calcium sulphate were among the various adsorbents used to separate the pigments present in the juice sample or in the 95 per cent methanolic extract of skins. Butanol and

Table 6

Pigment Concentrations[1] of Anthocyanin Extracts Obtained
Grape Skins Repeatedly with Various Solvents

Solvent used	Number of e				
	1	2	3	4	5	6
N-Butyl alcohol	17.5	3.30	0.388	0.248	0.170	0.090
95% Ethyl alcohol	35.0	5.85	0.699	0.248	0.084	0.070
Isobutyl alcohol	12.7	2.59	0.320	0.232	0.082	0.063
Ethyl alcohol + Butyl alcohol (1:1)	28.7	3.50	0.388	0.220	0.087	0.085
Butyl alcohol + Isobutyl alcohol	17.4	2.92	0.301	0.215	0.087	0.054
Butyl alcohol + Ethyl alcohol (1:1)	27.7	2.35	0.315	0.262	0.096	0.073
Formic acid + Ethyl alcohol (1:1)	35.0	5.95	0.363	0.237	0.090	0.065
Glacial acetic acid + Ethyl alcohol (1:1)	35.0	3.46	0.578	0.310	0.200	0.100
Glacial acetic acid	28.7	4.76	0.388	0.242	0.056	0.043

[1]Expressed as optical densities measured at 515 mμ and at pH 1.0.

Table 6

[1] of Anthocyanin Extracts Obtained by Extracting
.ns Repeatedly with Various Solvents

	Number of extraction						
	4	5	6	7	8	9	Total
18	0.248	0.170	0.090	0.07	0.04	0.04	21.85
19	0.248	0.084	0.070	0.05	0.02	0.02	42.04
10	0.232	0.082	0.063	0.05	0.02	0.01	16.06
18	0.220	0.087	0.085	0.04	0.02	0.02	33.07
11	0.215	0.087	0.054	0.02	0.01	0.01	21.02
15	0.262	0.096	0.073	0.04	0.03	0.03	30.90
13	0.237	0.090	0.065	0.04	0.02	0.02	41.79
18	0.310	0.200	0.100	0.07	0.04	0.03	39.80
18	0.242	0.056	0.043	0.02	0.01	0.01	34.23

5 mμ and at pH 1.0.

isobutyl alcohol extracts of the pigments were also used for chromatography especially in cases where the absorbents were water soluble. Elution from the adsorption columns was accomplished either by use of dilute HCl (3 per cent) or the organic solvents (butanol and isobutyl alcohol). In each case the column containing the colored component was separated before elution or by allowing the eluant to pass through the column under reduced pressure. When alumina was used as the adsorbent, the colored substance was adsorbed at the top and no separate bands could be noticed. Even when the adsorption capacities of alumina were reduced by mixing with Dicalite the experiments yielded identical results. The blue band formed on the alumina column was eluted by dilute HCl and this acid extract showed its absorption maximum at 515 mμ. Norite adsorbed the pigment completely but elution of the pigment was unsuccessful. The other substances did not prove to be of any apparent value as adsorbents.

When the petroleum ether and benzene extract (9:1 by volume) of the lyophilized skins was chromatographed using sucrose, a faint green band appeared in the middle of the column, while a yellow band was observed using the same extract with magnesia as adsorbent. These observations indicated the possible presence of chlorophyll, carotene and the water soluble yellow pigments in grape skins. Further work to identify the fat soluble pigments was not pursued as these pigments were apparently not extracted into the processed juices.

b. Filter Paper strips. By using a few drops of the methanolic-HCl (1 per cent) extract of lyophilized skins, separation was attempted employing paper strips and several flowing solvent mixtures. The non-aqueous layer of the ternary mixtures of isoamyl alcohol, glacial acetic acid and water, and butanol, acetic acid and water (4:1:5 by volume in

each case) were used as the flowing solvents at a temperature of 70° ± 0.5°F. A greenish yellow band moved along with the solvent front while separation of the red pigments was rather hazy with ternary mixtures containing isoamyl and isobutyl alcohol (in the above mixtures). With butanol, acetic acid and water, a separation into two red bands and a hazy one at the top portion of the paper was noticed. The R_f values of the different bands were determined after allowing the paper to air dry. The lower band which turned to purple indicated an R_f value of 0.11 while the upper band which remained red gave an R_f value of 0.19. It was observed that the red band at the top with an R_f value of 0.56 was destroyed in the course of about 24 hours while the other two bands were quite stable. Purified pigment solutions free from fat soluble pigments did not indicate the presence of the greenish yellow band, observed when the methanolic-HCl extract of skins was separated on the paper. On exposure to HCl fumes both the bands obtained on separation of purified pigment solution turned red. Exposure of the bands to ammonia fumes changed the colors to bluish green, with the red band regaining its original color in the course of a few hours. Evidently in the latter the ammonia escaped during this period. On spraying dilute ferric chloride solution onto the paper after separation, the color of the lower band appeared purple and the upper one reddish violet.

When the dilute acid (2 per cent) extract of the purified pigment solution was hydrolysed by boiling for 15 minutes and the solution after hydrolysis chromatographed using butanol, acetic acid and water, the lower bands were not noted, and a hazy trail of red pigment with an R_f value near 0.56 appeared on the chromatogram showing that the purple and red

bands (with R_f values 0.11 and 0.19) were degraded on hydrolysis to the red band with an R_f value (0.56), which, however, disappeared in the course of 24 hours.

The bands of the red and purple color from many paper chromatograms were cut and the separate strips were dipped in dilute HCl and the absorption values of the resultant acid solutions were determined with the spectrophotometer, and indicated in Figure 2. The shapes of both of the absorption curves are similar with an absorption maximum at 515 mμ. The curves also indicate that in the pigment extracts, the pigment component responsible for the red band predominates over the pigment component causing the purple band.

Further attempts to identify the two bands were made by separating the pigment extract into the diglycoside and monoglycoside by repeated extractions with isoamyl alcohol. The R_f values of the di- and mono-glycosides were determined individually under identical conditions. It was noted that the R_f value of the lower purple band equalled that of the diglycoside while that of the monoglycoside corresponded with the upper red band. These findings suggested that the upper band was the monoglycoside and the lower band the diglycoside of the anthocyanidin while the band at the top represented the sugar free anthocyanidin. Further support for these assumptions was gained from the purple color of the lower band and the red color of the upper band, in agreement with the observations of Robinson and Robinson (77) that, in general, the diglycosides tend to be bluer than the corresponding monoglycosides.

Using fresh grape juice acidified with dilute HCl the same number of bands in the middle and a hazy band at the top were noticed. The R_f values

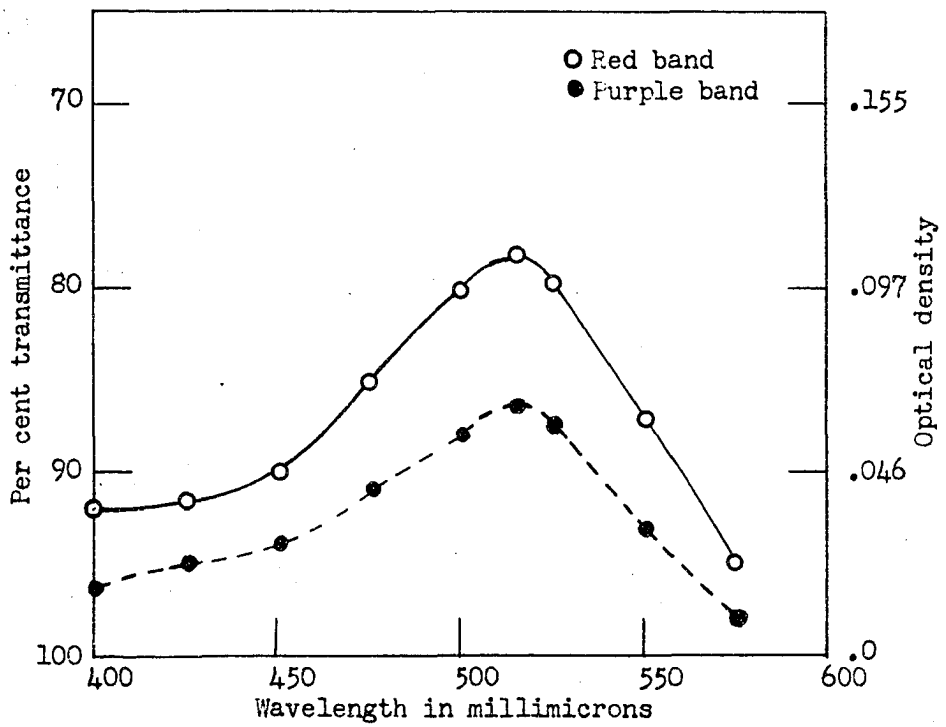


Fig. 2. Transmittance values of acid solutions of red and purple bands after separation of mixed pigment extracts by paper chromatography.

of the purple and red bands coincided with those obtained with purified pigment solutions. The bands obtained with the juice were, however, slightly broader than the ones obtained with purified pigment solutions, probably due to the effects of the other juice components. The resolution in the case of grape juice was improved by allowing the flowing solvent to run a second time through the column after the column was dried. In this case only apparent R_f values could be determined. Substituting Whatman No. 1 filter papers for Whatman No. 4 filter papers appeared to increase the R_f values slightly (0.12 and 0.21). If water extracts of the pigments were used instead of the acid extracts some hazy bands appeared at the top of the paper due probably to the neutral form of the color base. A comparison of the R_f values with those reported by Bate-Smith (10, p. 68) indicated that the monoglycoside was probably oenin as the R_f values seemed to agree closely. The purified di- and mono-glycosides separated from each other were concentrated and convenient volumes of the concentrated solutions were added to 15 ml. of buffer solutions of pH's 1.0, 1.5, 2.0, 2.5, 3.0, 3.4, 4.0, 7.0, and 8.0. The absorption values throughout the entire visible range were determined at the various pH values with the spectrophotometer and presented in Figs. 3 and 4. The absorption values of anthocyanidin obtained by hydrolysis of the monoglycosides were presented in Fig. 5. A close observation of the Figures 3, 4, 5 and 6, indicates that at the same concentration, as the pH is increased, the optical densities are found to decrease up to a pH of 5.0. The absorption maximum is noted at 515 mμ while with increasing pH the absorption maximum is shifted towards 400 mμ. These variations are found to be less striking for the monoglycoside solutions than with the changes observed with solutions of the diglycoside. The color of the anthocyanidin is found to be

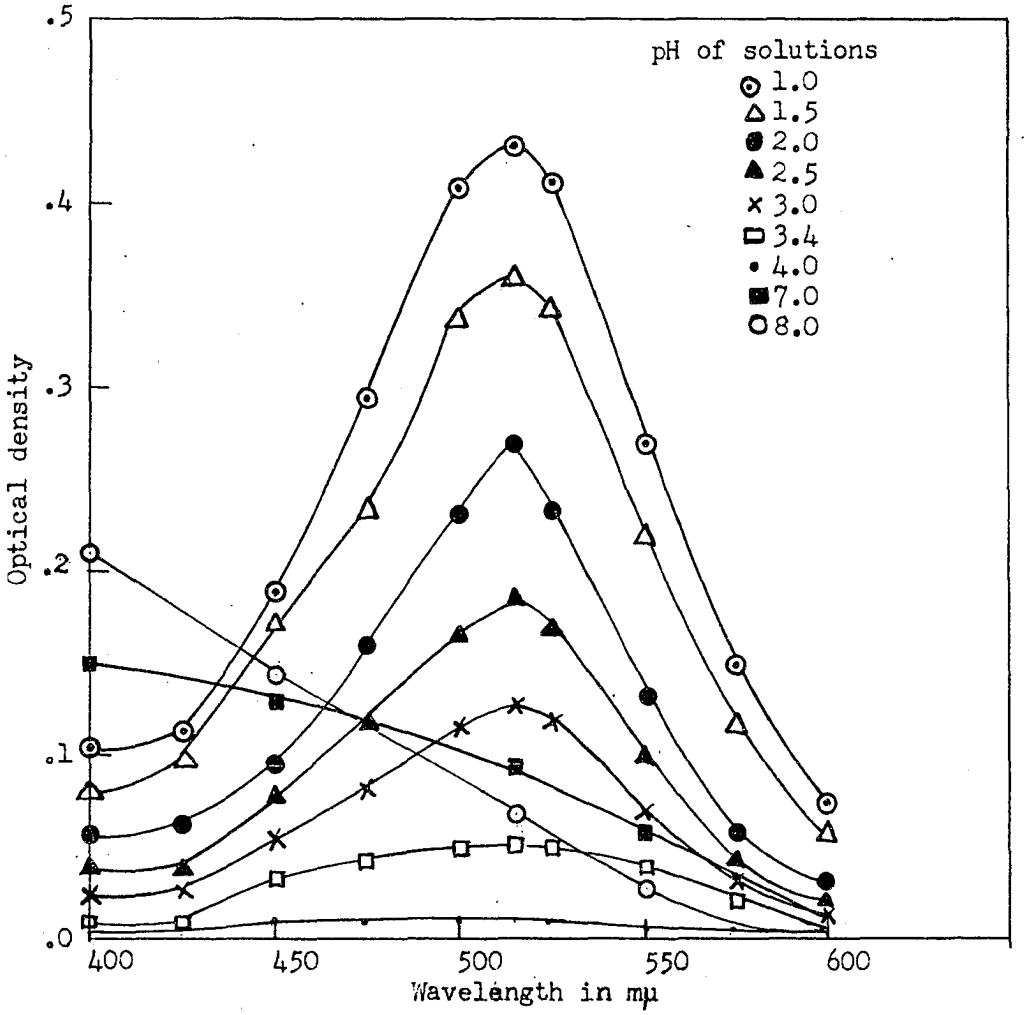


Fig. 3. Effect of pH on absorption values of solutions of anthocyanin chloride (diglycoside).

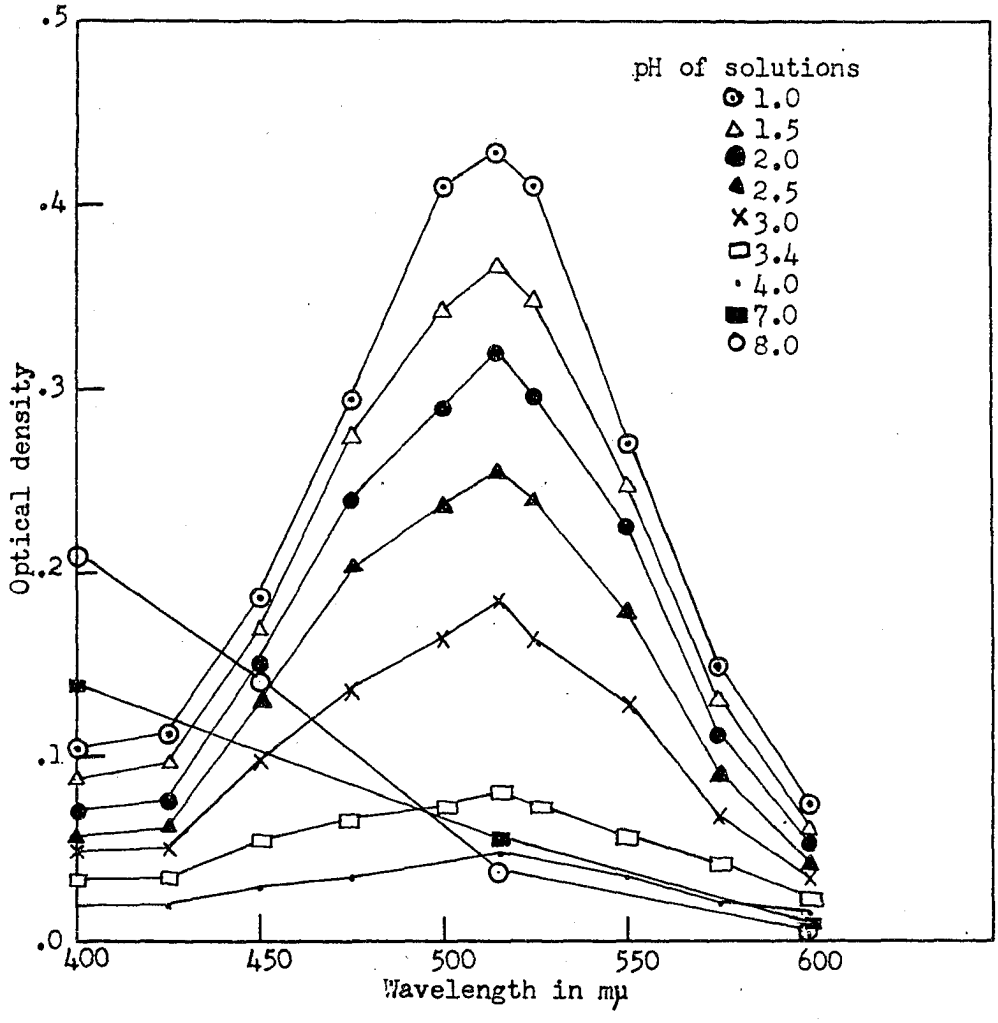


Fig. 4. Effect of pH on absorption values of solutions of anthocyanin chloride (monoglycoside).

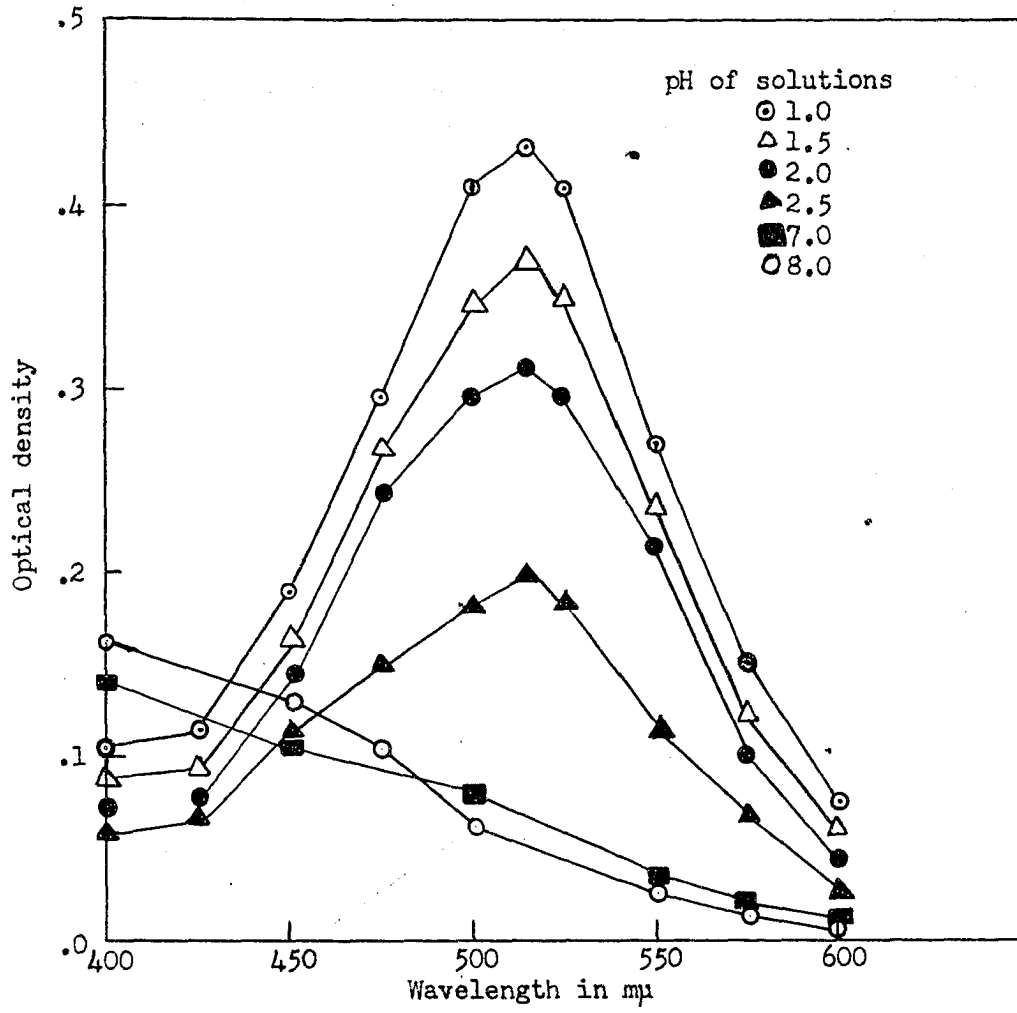


Fig. 5. Effect of pH on absorption values of anthocyanidin chloride.

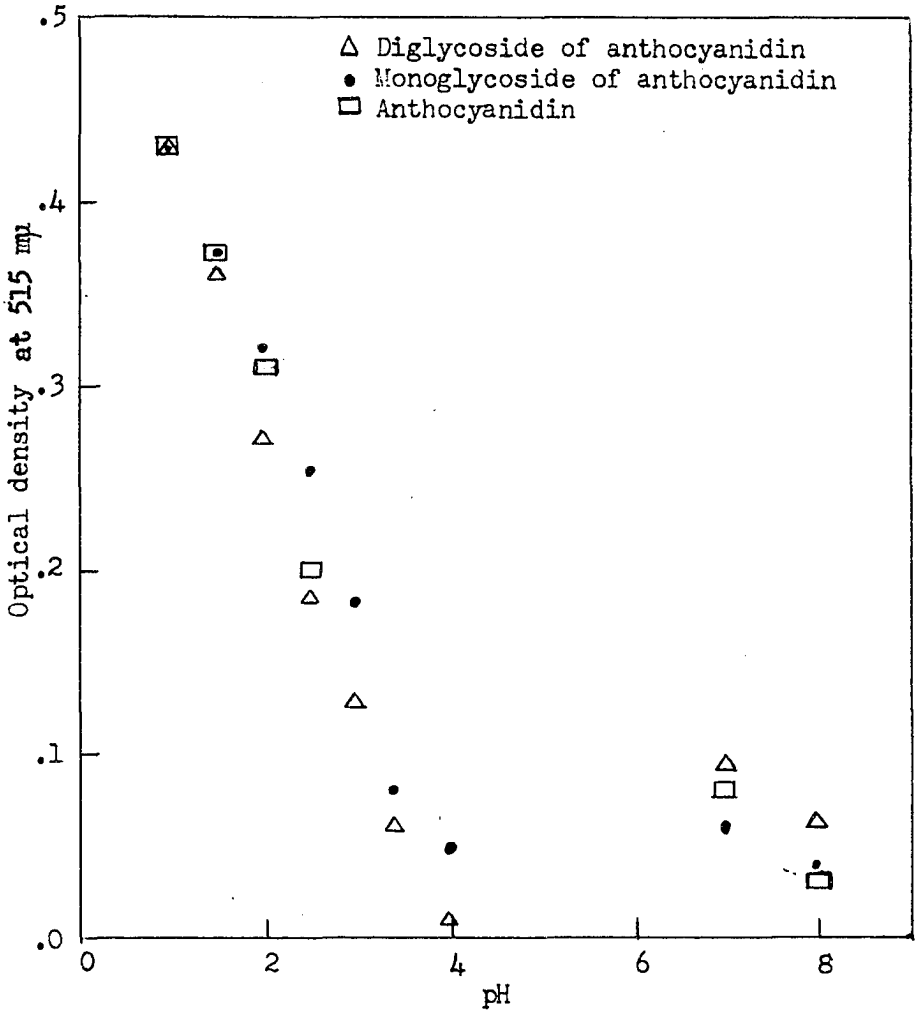


Fig. 6. Effect of pH on absorption values of solutions of anthocyanidin, its mono- and di-glycosides. (Values obtained from Figs. 3, 4 and 5)

faded as the pH increased to 3.0 and at pH's 7.0 and 8.0 the absorption maxima have shifted to 400 mμ in the visible range.

5. Ion exchange materials

Ion exchange material, XE-75 supplied by Rohm & Haas Company, meant for use with colored substances, was used in this investigation. When a 96 per cent alcoholic extract of the lyophilized skins was passed through a column of this resinous substance, the red pigments were adsorbed on the column while a yellow solution passed through. The absorption values of the yellow solution are shown in Fig. 7. The maximum absorption is noted to be at 400 mμ, and these absorption values diminished as the wavelength increased to 600 mμ.

It was further observed that the red pigment adsorbed on the column could not be eluted completely by acid. The acid extract showed an adsorption maximum at 515 mμ. The use of ion exchange resin did not offer any evidence of the presence of more than one anthocyanin pigment. The resinous substance could not, however, adsorb the red pigments from 3 per cent dilute acid solutions.

B. Isolation, Properties and Color Reactions of Anthocyanin Chloride Pigment from Concord Grapes

1. Isolation

The method used by Anderson (3) wad adopted to isolate the pigment from Concord grapes. The yields of anthocyanin chloride were very low. About 10 gms. of crude anthocyanin chloride were obtained from 4000 gms. of fresh skins and the yields were reduced while crystallization of the

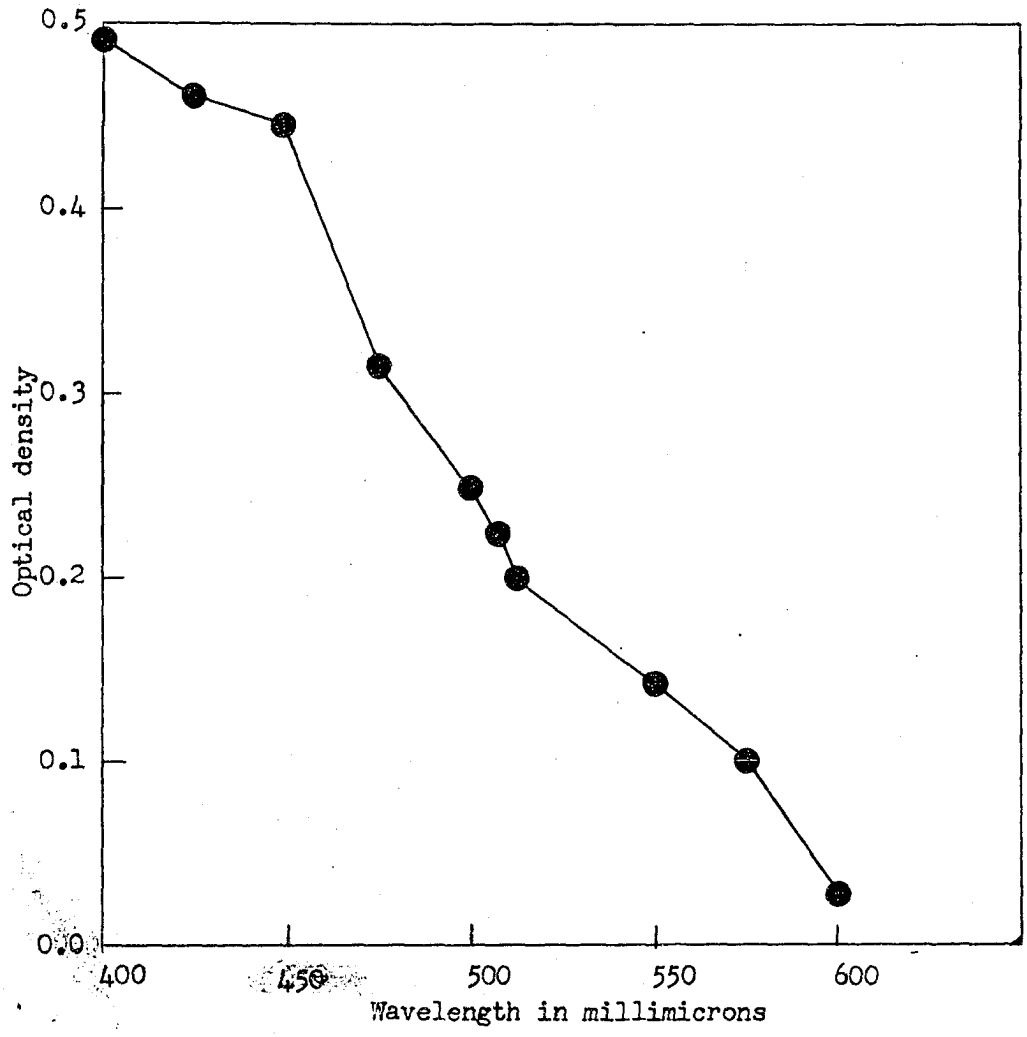


Fig. 7. Absorption values of alcoholic solutions of yellow pigments remaining unadsorbed by ion exchange material, XE-75.

anthocyanin chloride was affected. The anthocyanin chloride could not be obtained in the crystalline state in spite of varying techniques employed.

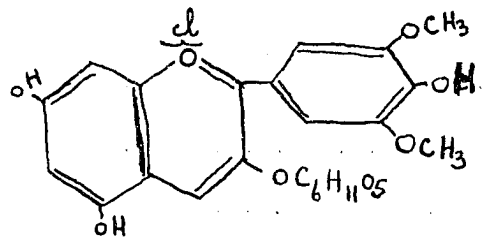
To 3 g. of the crude glycoside dissolved in 20 ml. of methanol were added, 25 ml. of ethanol containing 7 ml. of 21 per cent hydrochloric acid in absolute alcohol. The clear filtrate was allowed to stand for 3 days at room temperature in a loosely covered dish. Amorphous masses of the pigment precipitated out and the substance could not be obtained in a crystalline state even though the conditions for crystallization were varied. The solution of the crude glycoside was allowed to stand at 40°F. after addition of ethanolic hydrochloric acid. The crude glycoside was allowed to stand both at room temperature and at 40°F. in solutions of dilute hydrochloric acid, ethyl alcohol and water separately. All attempts to crystallize the substance failed, yielding in every case brownish red, tiny, amorphous granules in clusters. To free them from all impurities these granules were washed thoroughly with anhydrous ether. The substance did not show any trace of metals or nitrogen. The percentage composition of the individual elements was determined and reported below.

Found - C - 52%, H - 4.66% , Cl - 6.5%

Found - water - 7.4%

From these results the empirical formula was assigned to be $C_{23}H_{25}O_{12}Cl$ and this agreed closely with the calculated values. Calculated - C - 52.2%, H - 4.73%, Cl - 6.71% and H_2O - 7.84%.

The percentage composition agreed fairly closely with that reported by Anderson (3). From these results the pigment appears to be similar to the one obtained by Anderson and reported as oenidin 3-monoglycoside except for its low methoxyl content.



Chloride of Oenidin 3-monoglycoside (20)

However, in this investigation no attempts were made to elucidate the structures of the anthocyanin or the sugar free anthocyanidin.

The sugar present in the pigment molecule in the glycosidic form was identified by paper chromatography (26) after hydrolysing the pigment to the aglycone and sugar. The apparent R_f value of the sugar present in the pigment after three passes exactly equalled that of glucose (0.63) under identical conditions, confirming the presence of glucose in glycosidic form.

2. Properties and color reactions

To aid in the identification of the isolated pigment the properties and color reactions characteristic of anthocyanins were observed. The anthocyanin chloride was soluble in water giving a dull brownish red solution, turning to bright red on addition of dilute acid. It was very soluble in methanol and ethanol yielding carmine and intense purple solutions respectively. The macroscopic appearance was brownish red. Addition of sodium acetate to an aqueous solution caused the color to change to violet with a purple tinge. Addition of dilute sodium carbonate solution to an aqueous extract of pigment changed the color to bluish green turning to yellow on further addition of sodium hydroxide. With ferric chloride in water solution a light purple color was noted, changing to brown on standing, while in alcoholic solution a violet color was observed fading very

slowly and turning to red. Addition of a few drops of aluminium chloride to a neutral solution of the pigment in 95 per cent ethanol turned the color to blue. The R_f value of the pigment in dilute HCl solution was determined to be 0.21 using the nonaqueous layer of the butanol, acetic acid and water mixture (4:1:5) and Whatman No. 4 filter paper at 70° ± 0.5°F.

The absorption values of the pure anthocyanin chloride both in the ultra violet and the visible region were recorded by means of the Beckman quartz spectrophotometer and plotted in Fig. 8. It may be noted that the pigment shows an absorption maximum at 280 mμ in the ultra violet range in addition to that at 515 mμ in the visible range. It was also observed that the hydrolysis of the anthocyanin chloride to the aglycone did not change the absorption values when the solutions were examined at pH 1.0 or less.

The dry anthocyanidin, obtained by hydrolysis of the glycoside was black. The anthocyanidin was soluble in water and the violet red color of the aqueous solution faded slowly on standing. On addition of dilute sodium carbonate solution, the color was changed to violet and then to greenish blue. The color was destroyed on shaking a solution of the pigment with 10 per cent sodium hydroxide. There was no apparent change in color observed on addition of ferric chloride and the color faded slowly. Addition of sodium acetate solution to an isoamyl alcohol extract changed the color to bluish violet and this color changed to blue on addition of ferric chloride. It was partially extracted by the delphinidin reagent and not at all extracted by the cyanidin reagent. The above reactions indicated that it answered most of the reactions of oenidin though few of the characteristic reactions of petunidin were shown (from Tables 1 and 2).

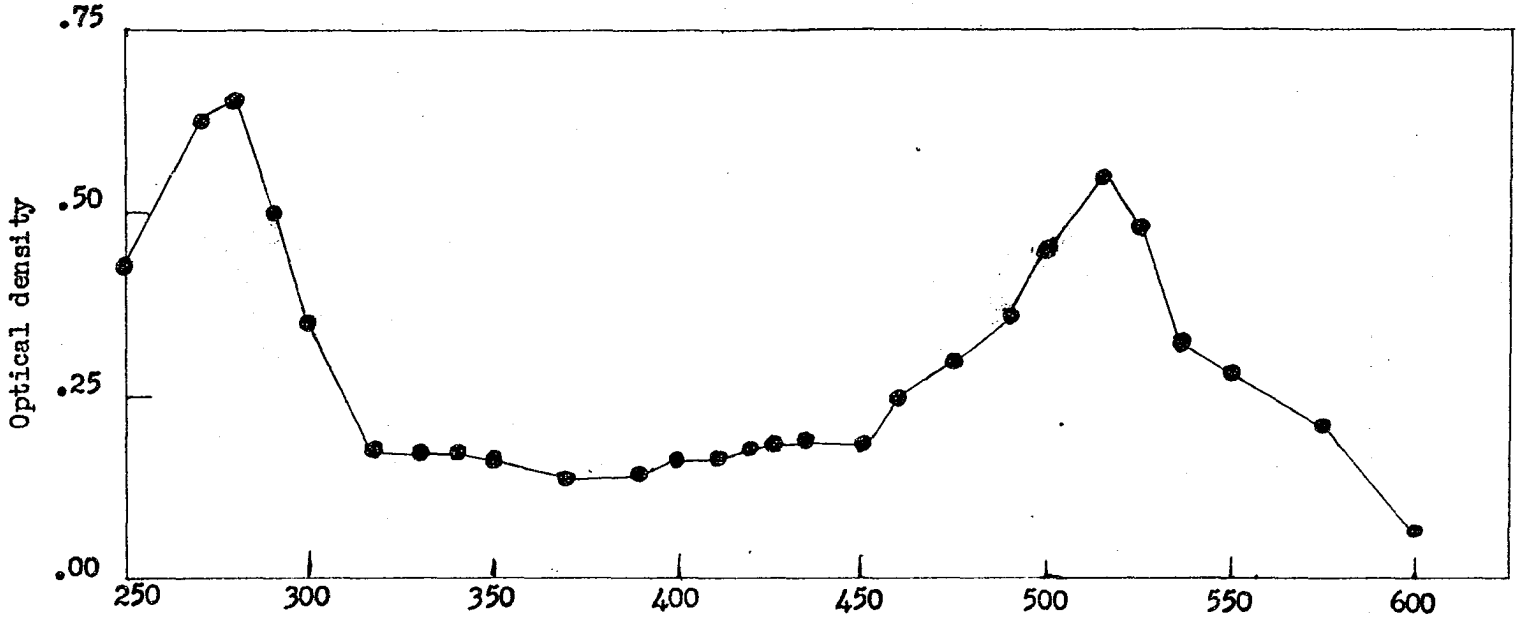


Fig. 8. Absorption values of acid solution of anthocyanin chloride over wavelength range of 250-600 mμ.

C. Method Used for Estimation of Anthocyanins

The property that the anthocyanin solutions possessed the maximum color intensity in solutions of pH 1.0 or less was referred to in an earlier section (from Fig. 6). Throughout this investigation the estimation of the pigment concentration was made by determining the optical densities of the clear pigment solutions at pH 1.0 or less at a wavelength of 515 mμ using a Coleman spectrophotometer.

These studies were carried out by dissolving 40.8 mgs. of pure pigment in 300 ml. of dilute HCl (3 per cent). Serial dilutions of this solution were made by diluting 10, 20, 30, 40, 50 and 75 ml. of the solution to 100 ml. with dilute HCl (3 per cent). The absorption values of these solutions were recorded with the spectrophotometer and presented in Fig. 9. The constant 'K' of the spectrophotometer was calculated from these results by using the formula $C = KD$ where 'C' is the concentration of the pigment in the solution, and D the optical density depending on the transmission values and K the constant dependent on the spectrophotometer used, employing glass cuvettes (13 x 13 x 100 mm.) The value of K was found to be 0.0112 for a concentration of 13.6 mg. of pure pigment in 100 ml. of solution of pH 1.0.

An experiment was conducted to find out if the optical densities of grape juice observed at 515 mμ and pH 1.0 increased linearly with serial dilution. One ml. of freshly prepared juice was diluted to 10, 20, 30, 50, 80, 100 and 125 ml. with dilute HCl (3 per cent). The optical densities were recorded and presented in Fig. 10.

To investigate if the soluble brown pigments formed during storage would interfere with the absorption values at 515 mμ 1 ml. of preserved

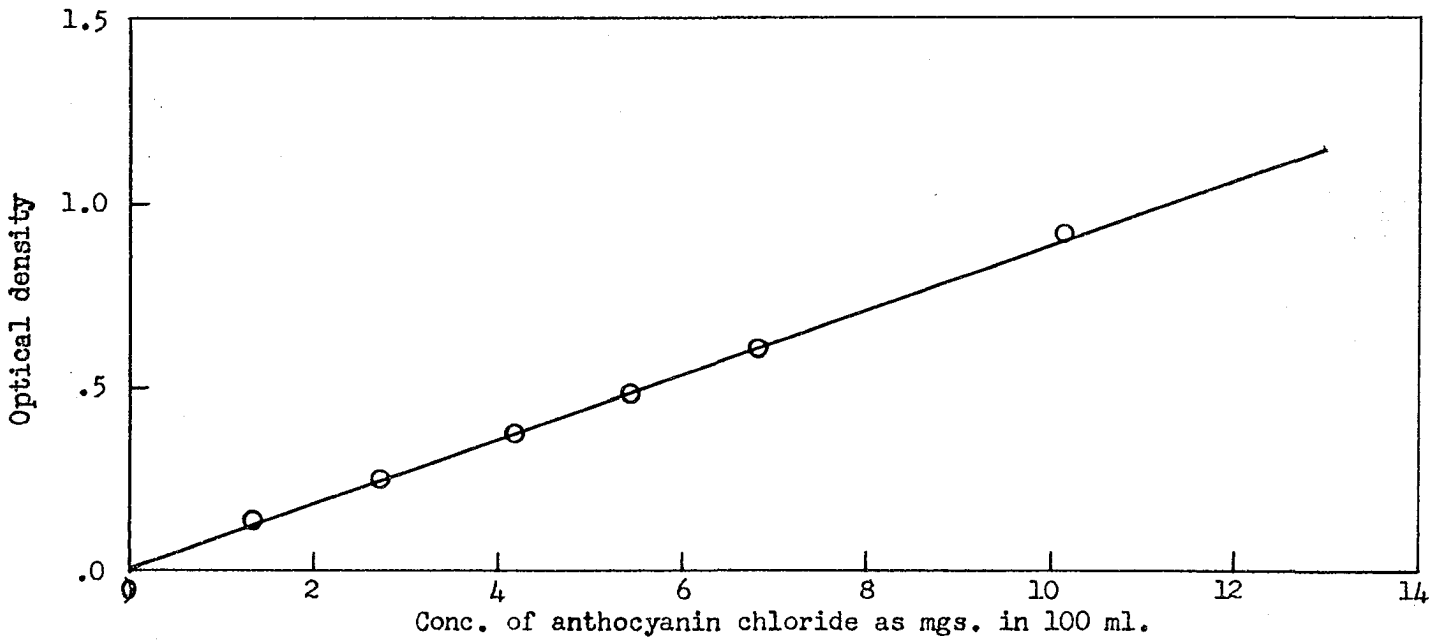


Fig. 9. Effect of concentration on optical density of solutions of anthocyanin chloride measured at 515 mμ and at pH 1.0.

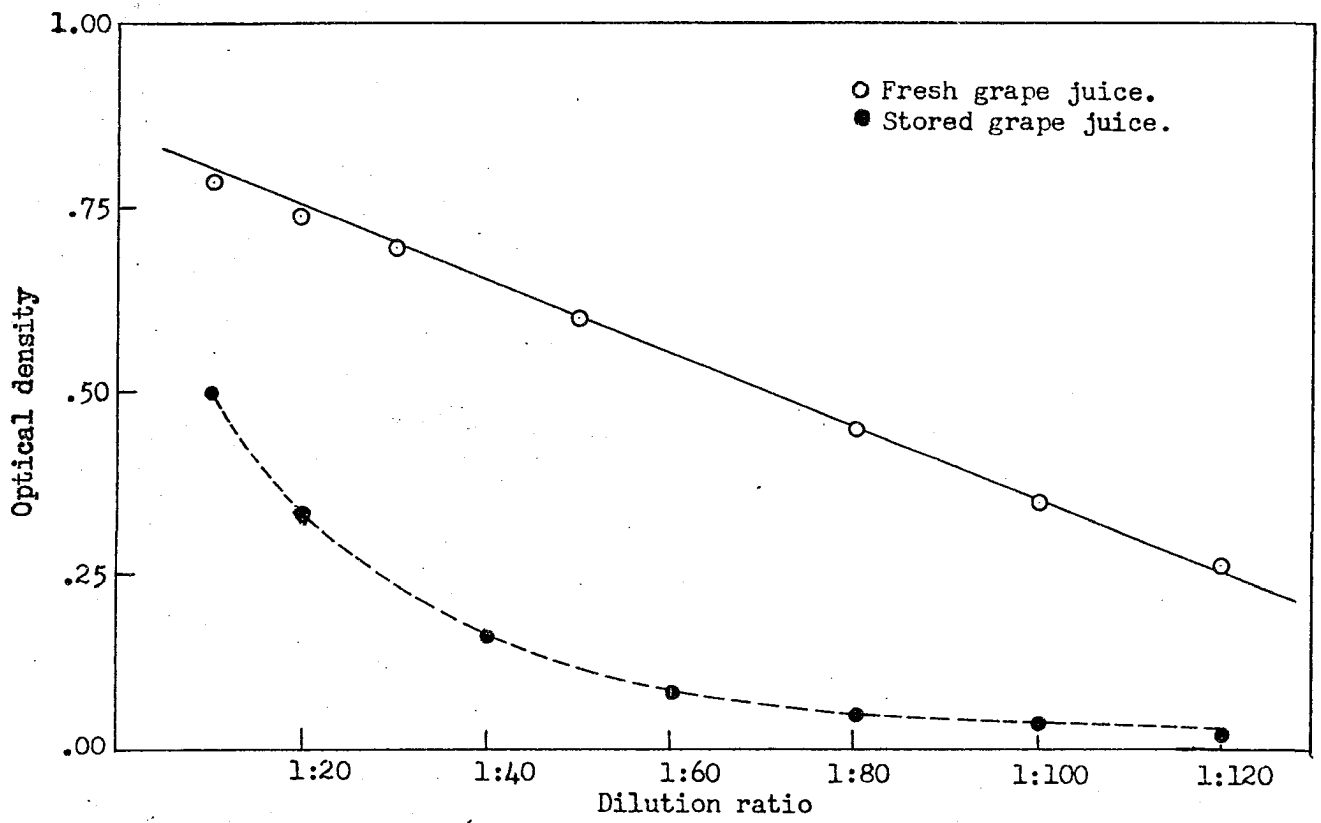


Fig. 10. Effect of serial dilution of fresh and stored grape juice samples on optical density measured at 515 mμ and at pH 1.0.

juice, the anthocyanin pigment of which was almost completely destroyed, was diluted to 10, 20, 40, 60, 80, 100 and 120 ml. with dilute HCl (3 per cent). The absorption values of these solutions at 515 mμ and at a pH of 1.0 were presented in Fig. 10. The absorption values of the stored juice were recorded throughout the entire visible range. It is found that the interference due to brown pigments is least observed at a wavelength of 515 mμ. Slight turbidity was noted on addition of dilute acid to adjust the pH to 1.0 and the interference of the turbidity was, however, obviated largely by dilutions to convenient volumes (50 - 100 times).

The influence of the thermal breakdown products on the estimation of the pigment solutions was next investigated. The optical densities obtained by diluting 1 ml. of the clear pigment solutions to 20, 40, 60, 80, 100 and 120 ml. with dilute HCl (3 per cent) after thermal processing at 250°F. for 100 minutes were recorded and reported in Fig. 11. It may be seen from the data that the influence of the soluble products formed on thermal breakdown of the pigment solutions is negligible when measured at the wavelength of 515 mμ.

The interference of the grape juice components, e.g. sugar, pectin, tartaric, malic and tannic acids, on the estimation of color was noted by determining the optical densities after adding a mixture of the components to the pure pigment solutions at levels (95, 62) present in the grape juice. The values recorded at a pH of 1.0 were reported in Fig. 12.

It may be observed from the data that the optical densities of the clear solutions observed at a wavelength of 515 mμ and a pH of 1.0 give a fair indication of the amount of anthocyanin pigment present in grape juice or in solutions of pure pigment isolated from grapes, when the samples are diluted to limited volumes.

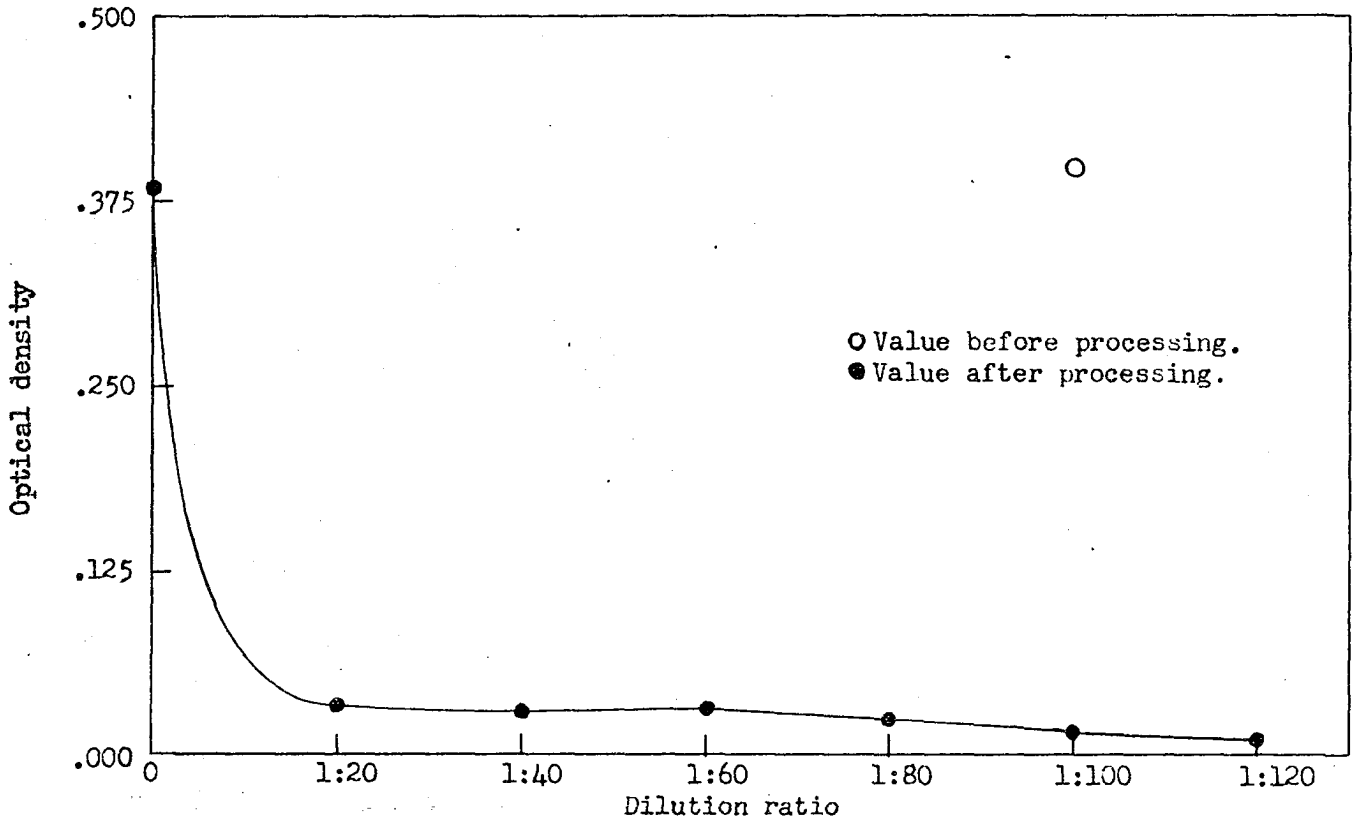


Fig. 11. Effect of serial dilution on optical density measured at 515 mμ and at pH 1.0 of anthocyanin chloride solutions before and after processing at 250° F for 100 minutes.

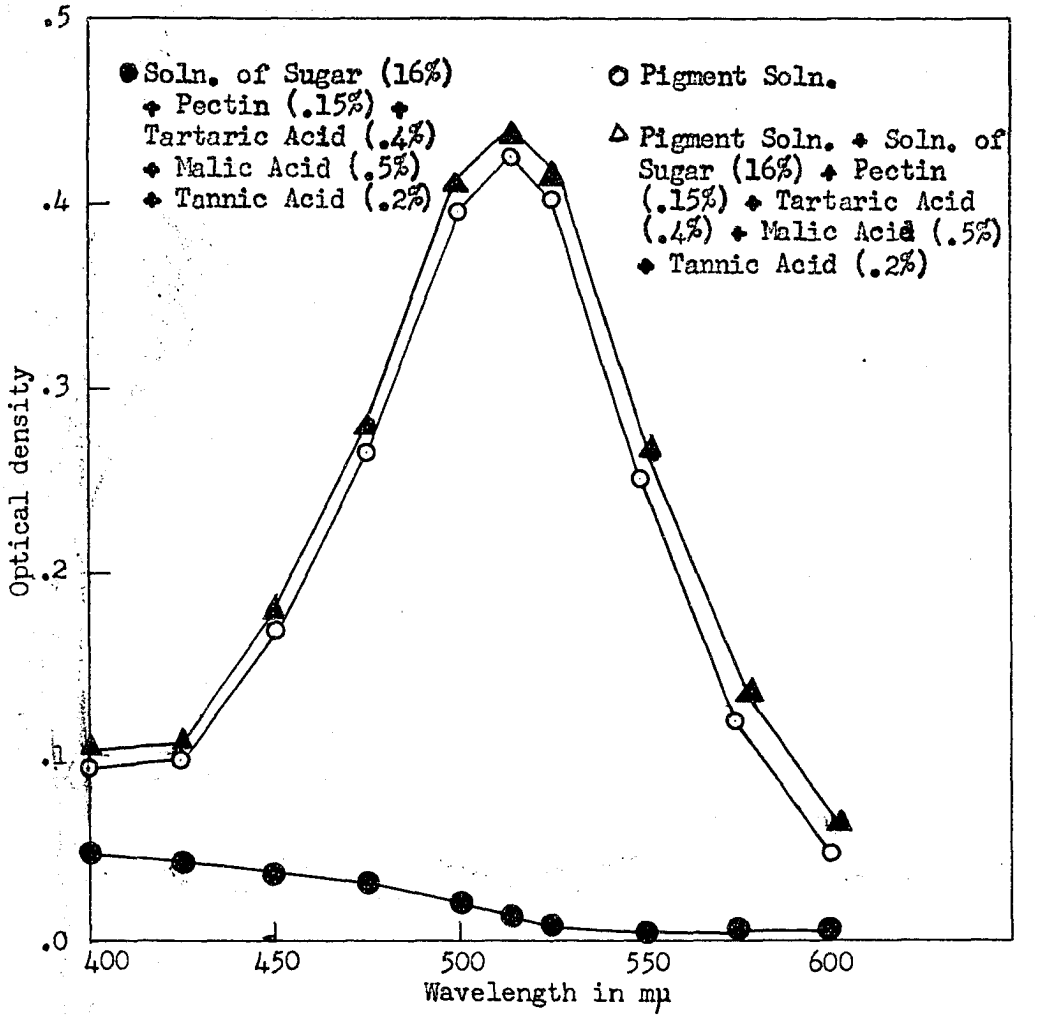


Fig. 12. Absorption Values of Grape Juice Components Measured at pH 1.0 of solutions (1:100).

D. Processing and Storage Studies

1. Processing studies

Preliminary process studies were conducted with frozen grapes stored at 0° ± 1°F., employing both glass jars and glass tumblers. The grapes were thawed but not crushed before processing. The volumes of the juices yielded were determined by draining the juice through a funnel (5" inside top diameter) for 5 minutes. The color content was estimated using the centrifuged juice. The results are presented in Tables 7 and 8. Preliminary process studies were also done in glass jars using uncrushed fresh grapes. The results of the juice yields and the color content were included in Table 9.

The results indicated that when uncrushed grapes (frozen and well as fresh) are processed the juice yields increase with higher processing temperatures. The pigment concentration of the juices shows a similar increase with the processing temperature. With frozen grapes turbidity was observed in the juices prepared at 250°F.

The temperatures and times of processing used for processing the fresh grapes and the subsequent storage treatments to which the juice samples were subjected were included in Tables 4 and 10.

The percentage yields of the juice and the areas under the processing curves were included in Tables 11 and 12. An examination of the data shows that while there is a progressive increase in the yields with increasing process times at 170° and 250°F. process temperatures, no such trends are noticeable with processes at 210°F. The concentration of color in the juice samples immediately after processing does not indicate any regulari-

Table 7

Effect of Thermal Processes on Pigment Concentrations[1] and Yields of Juice Samples from Frozen, Uncrushed and Thawed Grapes Processed in Glass Tumblers

Process temperature (°F.)	160			220			250		
Process time (minutes)	5	10	15	5	10	15	5	10	15
Optical density	.134	.193	.233	.362	.398	.388	.368	.301*	.264*
Percentage yield	32.0	32.1	32.2	34.4	32.1	-	42.0	45.1	46.1

[1]Expressed as optical densities measured at 515 mu at pH 1.0 of solutions (1:100).

*Turbid.

Table 8

Effect of Thermal Processes on Pigment Concentrations[1] and Yields of Juice Samples from Frozen, Uncrushed and Thawed Grapes Processed in Glass Jars

Process temperature (°F.)	160			220			250		
Process time (minutes)	5	10	15	5	10	15	5	10	15
Optical density	.149	.161	.204	.31	.319	.323	.369	.319*	.252*
Percentage yield	26.7	26.4	28.3	34.7	37.5	37.2	39.3	42.3	44.6

[1]Expressed as optical densities measured at 515 mu at pH 1.0 of solutions (1:100).

*Turbid.

Table 9

Effect of Thermal Processes on Pigment Concentrations[1] and yields of Juice Samples from Fresh and Uncrushed Grapes Processed in Glass Jars

Process temperature (°F.)	160				220				250			
Process time (minutes)	5	10	15	30	5	10	15	30	5	10	15	30
Optical density	.054	.116	.103	.146	.276	.301	.31	.222*	.328	.276*	.252*	.204*
Percentage yield	1.360	3.970	5.600	9.30	12.400	21.000	25.20	37.600	29.400	32.000	41.300	42.400

[1]Expressed as optical densities measured at 515 mμ at pH 1.0 of solutions (1:100).

*Turbid.

Table 10

Effect of Thermal Processes, Storage Periods and Treatments on Pigment Concentrations[1] of Pasteurized Grape Juice Samples

Storage treatment		Storage period	Temperature (°F.) 170 Time of processing (minutes)					210 Time of processing (minutes)					250 Time of processing (minutes)				
Exposure	Headspace	(months)	31.5	39.7	50.0	63.0	79.0	12.5	15.8	20.0	25.0	31.5	5.0	6.3	7.9	10.0	12.5
Light	Air		.271	.243	.287	.267	.317	.243	.307	.335	.276	.338	.286	.271	.299	.331	.289
Dark	Air		.271	.243	.287	.267	.317	.243	.307	.335	.276	.338	.286	.271	.299	.331	.289
Light	Nitrogen	0	.271	.243	.287	.267	.317	.243	.307	.335	.276	.338	.286	.271	.299	.331	.289
Dark	Nigrogen		.271	.243	.287	.267	.317	.243	.307	.335	.276	.338	.286	.271	.299	.331	.289
Light	Air		.136	.133	.135	.118	.138	.109	.142	.159	.122	.151	.134	.139	.138	.151	.128
Dark	Air		.151	.143	.146	.131	.145	.117	.161	.173	.139	.166	.149	.149	.152	.165	.135
Light	Nitrogen	2	.151	.148	.150	.139	.158	.128	.159	.174	.138	.165	.152	.157	.158	.170	.143
Dark	Nitrogen		.160	.154	.149	.141	.166	.132	.168	.184	.149	.171	.159	.164	.162	.174	.144
Light	Air		.076	.068	.072	.063	.070	.057	.067	.082	.065	.075	.081	.071	.071	.085	.071
Dark	Air		.077	.080	.084	.082	.088	.070	.088	.099	.081	.095	.095	.091	.091	.103	.092
Light	Nitrogen	4	.096	.092	.097	.085	.097	.075	.081	.108	.086	.097	.098	.098	.099	.110	.089
Dark	Nitrogen		.110	.110	.102	.099	.112	.084	.109	.119	.096	.113	.112	.106	.106	.121	.098
Light	Air		.048	.037	.05	.032	.041	.043	.048	.051	.041	.056	.032	.061	.061	.075	.056
Dark	Air		.061	.052	.063	.047	.052	.051	.063	.066	.053	.073	.046	.068	.072	.088	.061
Light	Nitrogen	6	.063	.054	.068	.049	.056	.056	.069	.072	.058	.081	.054	.073	.078	.094	.067
Dark	Nitrogen		.081	.075	.086	.063	.067	.065	.09	.086	.070	.103	.073	.086	.083	.114	.072

[1]Expressed as optical densities measured at 515 mμ at pH 1.0 of solutions (1:100).

Table 11

Yields of Grape Juice After Thermal Processes

Temperature (°F.)	Time (min.)	Yields in percent Lot 1	Lot 2	Lot 3	Average yield
170	31.5	50.5	54.7	55.6	53.6
170	39.7	52.3	55.0	57.5	54.9
170	50.0	55.5	59.0	66.5	60.0
170	63.0	55.5	58.7	67.2	60.5
170	79.0	56.0	64.5	65.0	61.8
210	12.55	58.0	62.7	67.0	62.6
210	15.8	67.0	65.5	63.7	65.4
210	20.0	58.7	68.0	60.2	62.3
210	25.0	64.6	66.0	68.0	66.2
210	31.5	58.7	65.5	61.5	61.9
250	5.0	57.0	62.6	68.0	62.5
250	6.3	63.2	64.0	70.1	65.8
250	7.9	56.0	66.0	64.5	62.3
250	10.0	68.2	72.0	63.0	67.7
250	12.5	71.0	74.8	75.5	73.8

Table 12

Areas Under Processing Curves, Measured from Base Temperature of 100° F.

Temperature (°F.)	Time (min.)	Area in square inches Lot 1	Lot 2	Lot 3	Average area
170	31.5	8.60	8.66	8.79	8.68
170	39.7	9.93	10.33	9.90	10.05
170	50.0	14.99	16.91	16.30	16.07
170	63.0	20.26	18.56	20.40	19.74
170	79.0	25.76	23.80	25.75	25.10
210	12.5	6.95	7.77	7.58	7.43
210	15.8	11.36	10.95	10.85	11.06
210	20.0	12.16	12.79	12.06	12.34
210	25.0	15.30	16.87	19.60	17.26
210	31.5	19.20	19.21	19.40	19.27
250	5.0	6.91	5.58	9.90	7.46
250	6.3	10.19	9.44	10.12	9.92
250	7.9	10.63	10.99	10.17	10.60
250	10.0	11.00	11.20	10.74	10.96
250	12.5	15.70	16.60	16.25	16.18
180	1.0	2.04	2.20	1.94	2.06

ties with change in process time or temperature. The shapes of absorption curves of all the samples are similar, the maximum being noted at 515 mμ and at a pH of 1.0.

The data seem to indicate that when grapes are processed above 170° F., under the conditions imposed in this investigation, no greater concentrations in color content can be obtained. However, on visual examination it was perceived that the samples processed at 250° F. were red in color while those processed at lower temperatures remained purple. It was also found that the pasteurization of the clear juice for 1 minute at 180° F. did not bring about any changes in the pigment concentration of the juice samples. All the three replicates showed fair agreement among one another in regard to juice yields and pigment concentrations.

Separate processing experiments were conducted to determine the effect of longer process times at process temperatures of 210° F. and 250° F. using grapes stored at 40° F. for one month as no fresh grapes were available. Examination of the data in Tables 13 and 14 indicates that the yields are higher when the processing is done at 250° F. compared to the yields obtained after processing at 210° F. No differences in yields are realized when the time of processing is increased beyond 63 minutes at a process temperature of 250° F. However, in these processes the concentration of the color in the juices shows wide variations with change in process, as indicated in Table 15. The juices prepared by processing for times greater than 63 minutes at 250° F. were brownish red and were slightly turbid. The color in these juices was destroyed

Table 13

Yields of Grape Juice[1] after Thermal Processes Extended to Longer Process Times

Temperature (°F.)	Time (min.)	Yields in percent Lot 1	Lot 2	Lot 3	Average yield
210	31.5	61.0	64.5	66.0	63.8
210	63.0	62.0	63.8	66.0	63.9
210	100.0	66.7	67.8	69.0	67.8
250	12.5	66.0	70.0	69.5	68.5
250	31.5	71.0	73.0	74.0	72.7
250	63.0	80.0	78.7	79.0	79.6
250	100.0	87.7	78.5	80.0	79.1

[1]From grapes stored at 40° F. for one month.

Table 14

Areas Under Processing Curves,[1] Measured from Base Temperature of 100° F. in Process Studies Extended to Longer Process Times

Temperature (°F.)	Time (min.)	Area in square inches			Average area
		Lot 1	Lot 2	Lot 3	
210	31.5	18.92	19.61	19.42	19.32
210	63.0	35.97	35.20	36.50	36.23
210	100.0	61.44	61.66	60.30	61.10
250	12.5	15.19	14.75	15.01	14.98
250	31.5	31.60	31.36	32.40	31.79
250	63.0	56.10	55.04	55.40	55.51
250	100.0	88.90	88.10	89.40	88.80

[1]Processes on grapes stored at 40° F. for one month.

Table 15

Effect of Thermal Processes, Storage Periods and Treatments on Pigment Concentrations[1] of Pasteurized Grape Juice Samples[2]

Storage treatment		Storage period	Temperature (°F.) 210 Time of processing (minutes)			250 Time of processing (minutes)		
Exposure	Headspace	(months)	31.5	63	100	12.5	31.5	63	100
Light	Air		.348	.329	.329	.338	.348	.215	.087
Light	Nitrogen		.348	.329	.329	.338	.348	.215	.087
Dark	Air	0	.348	.329	.329	.338	.348	.215	.087
Dark	Nitrogen		.348	.329	.329	.338	.348	.215	.087
Light	Air		.210	.175	.155	.155	.118	turbid	turbid
Light	Nitrogen		.210	.182	.164	.182	.129	turbid	turbid
Dark	Air	2	.210	.200	.162	.164	.150	turbid	turbid
Dark	Nitrogen		.210	.212	.164	.168	.146	turbid	turbid
Light	Air		.107	.100	.100	.084	.146	turbid	turbid
Light	Nitrogen		.125	.104	.107	.107	.146	turbid	turbid
Dark	Air	4	.120	.100	.104	.084	.146	turbid	turbid
Dark	Nitrogen		.125	.107	.107	.100	.146	turbid	turbid
Light	Air		.061	.058	.064	.045	.146	turbid	turbid
Light	Nitrogen		.070	.064	.070	.053	.146	turbid	turbid
Dark	Air	6	.073	.070	.072	.049	.146	turbid	turbid
Dark	Nitrogen		.079	.076	.078	.06	.146	turbid	turbid

[1]Expressed as optical densities measured at 515 mμ at pH of 1.0 of solutions (1:100).

[2]From grapes stored at 40° F. for one month.

due to longer process times. Comparable times of processing at 210°F. are, however, not found to be detrimental to the color. The shape of the absorption curves of the juices prepared with process times more than 63 minutes at 250°F. are found to be different from others. The optical density of these juices at 400 mμ is almost equal to that at 515 mμ, as shown in Figure 13. Apparently with the destruction of the anthocyanin pigment the values at 515 mμ are lowered and the degradation products of the anthocyanin seem to possess higher absorption values at 400 mμ.

2. Studies on pigment concentrations of grape juice samples as a result of storage

The concentration of the pigments in each clear juice sample was estimated after storage periods of 2, 4 and 6 months and presented in Table 11. Since no apparent differences were noticeable among replicates, the average values of the pigment concentration of the three replicates for each process were reported in terms of their optical densities at the wavelength of 515 mμ. The pigment concentrations in the juice samples after storage for six months, could not be determined exactly as larger dilutions of the juice samples with dilute acid were employed to reduce the interference of the turbidities developed. It is noted from Figure 13 that the shapes of the absorption curves of the juice samples are changed with increasing storage period. However, no differences could be found among juice samples stored for the same period. The differences in optical densities between 400 mμ and 515 mμ were found to decrease

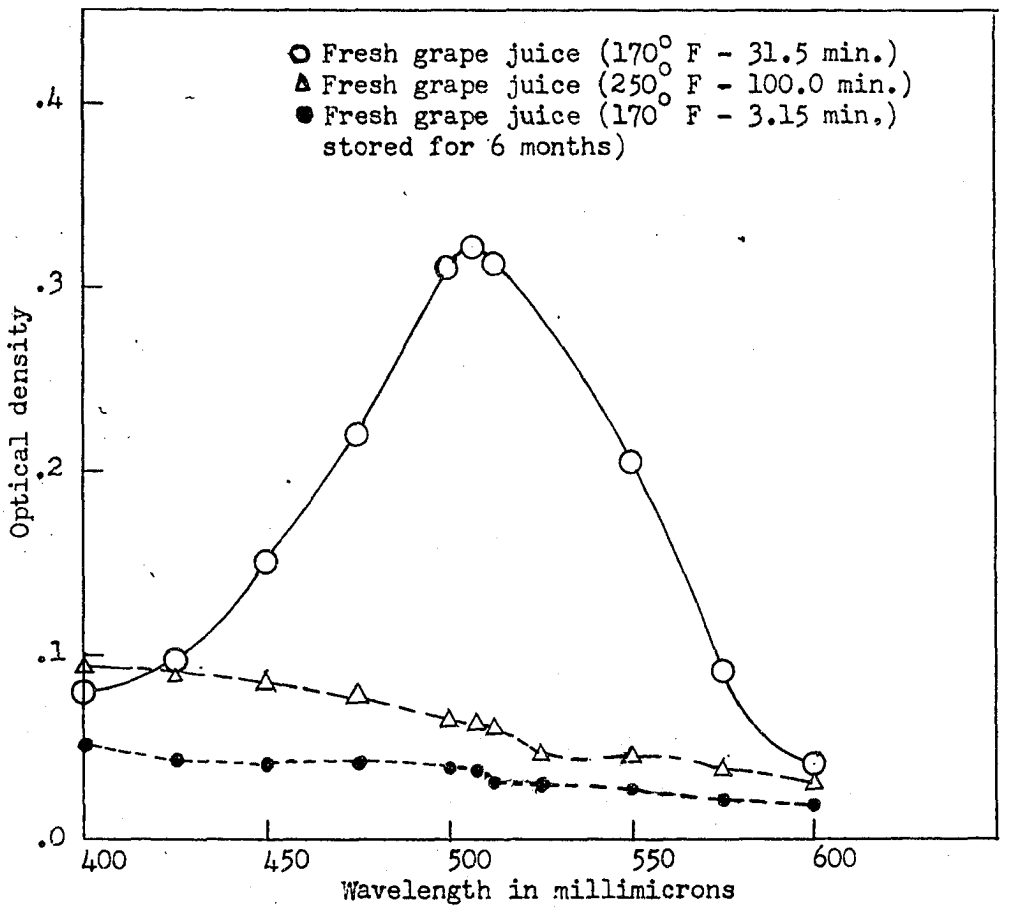


Fig. 13. Effect of thermal processing and storage on pigment concentrations of grape juice samples. Pigment concentrations are expressed as optical densities measured at 515 mμ and at pH 1.0 of solutions (1:100).

gradually with increasing storage period, i.e. with increasing loss of the anthocyanin pigment. The results also indicate that the pigment concentrations were lower in every case when nitrogen formed the headspace and the samples were stored in the dark. However, the data definitely lead to the conclusion that the period of storage exerted the maximum influence on the deterioration of color compared to the storage treatments as indicated in Figures 14, 15 and 16, presented by using the data in Table 10. The temperatures or the times of processing appeared not to influence the pigment concentrations of the stored juice samples.

A brown sediment settled to the bottom of the test tubes containing the juice samples, and the sediment increased with the period of storage. No effort was made to secure a quantitative estimate of the brown sediment. The losses of the pigment concentrations in the clear juice samples were predominant in the first two months while further storage periods did not involve comparable deterioration. During the storage period the juices had undergone changes from purple to light brownish red. The data indicate that the nature of the gas in the headspace does not bring about any considerable changes in the juice as the amount of air dissolved in the juices is apparently sufficient to deteriorate the pigments possibly by oxidative changes. Further support for this assumption is drawn from the persual of results obtained with juice samples stored under toluene to prevent spoilage. These results were presented in Table 16.

The pigment concentrations of the stored samples of the resulting juices, obtained by processing grapes stored at 40° F., are presented

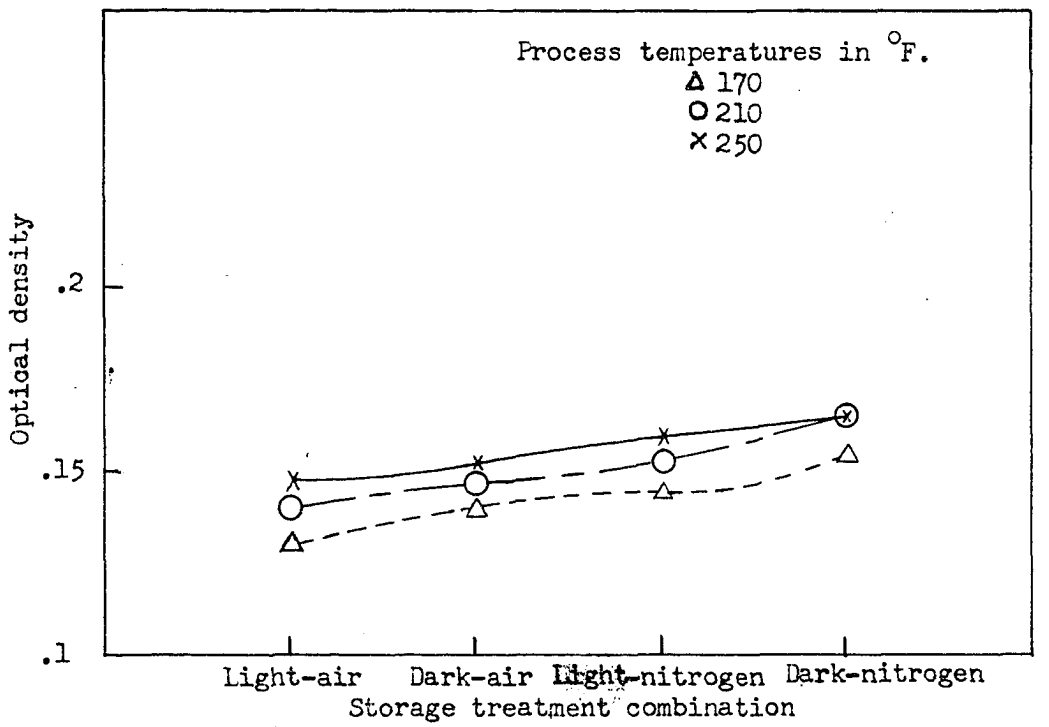


Fig. 14. Variation of pigment concentrations of grape juice samples with storage treatments averaging over other variables except process temperature. Pigment concentrations are expressed as average values of optical densities (from Table 10).

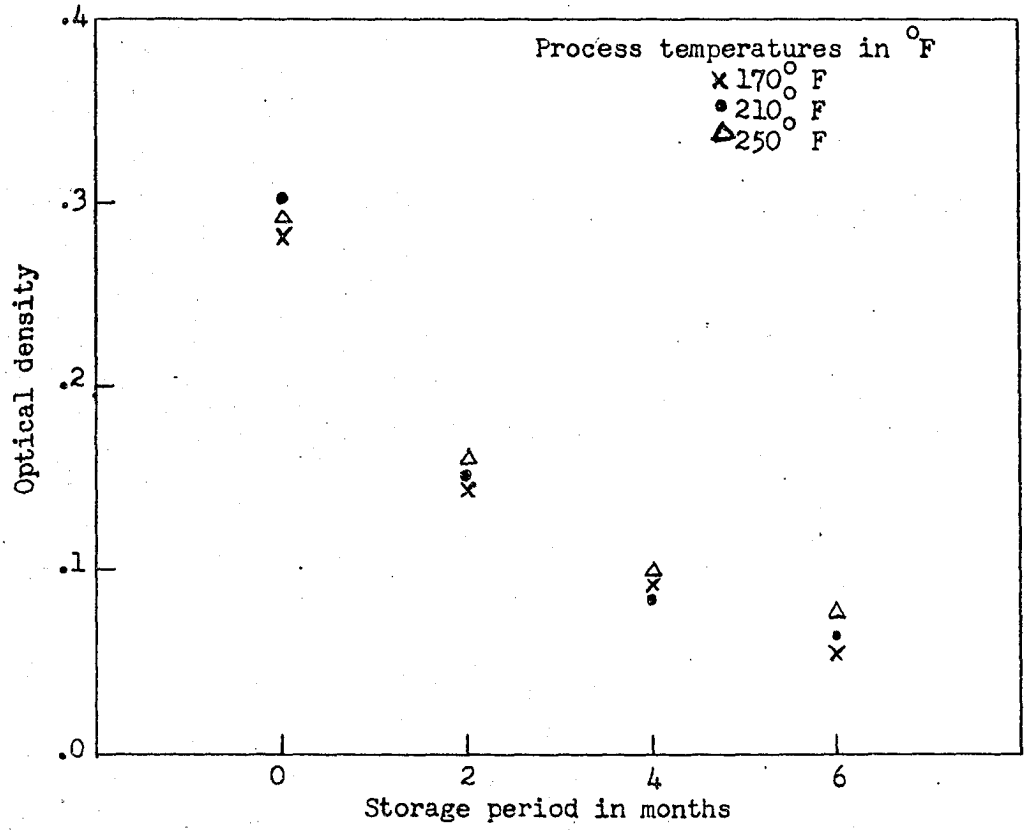


Fig. 15. Variation of pigment concentrations of grape juice samples with storage period averaging over other variables except process temperature. Pigment concentrations are expressed as average values of optical densities (from Table 10).

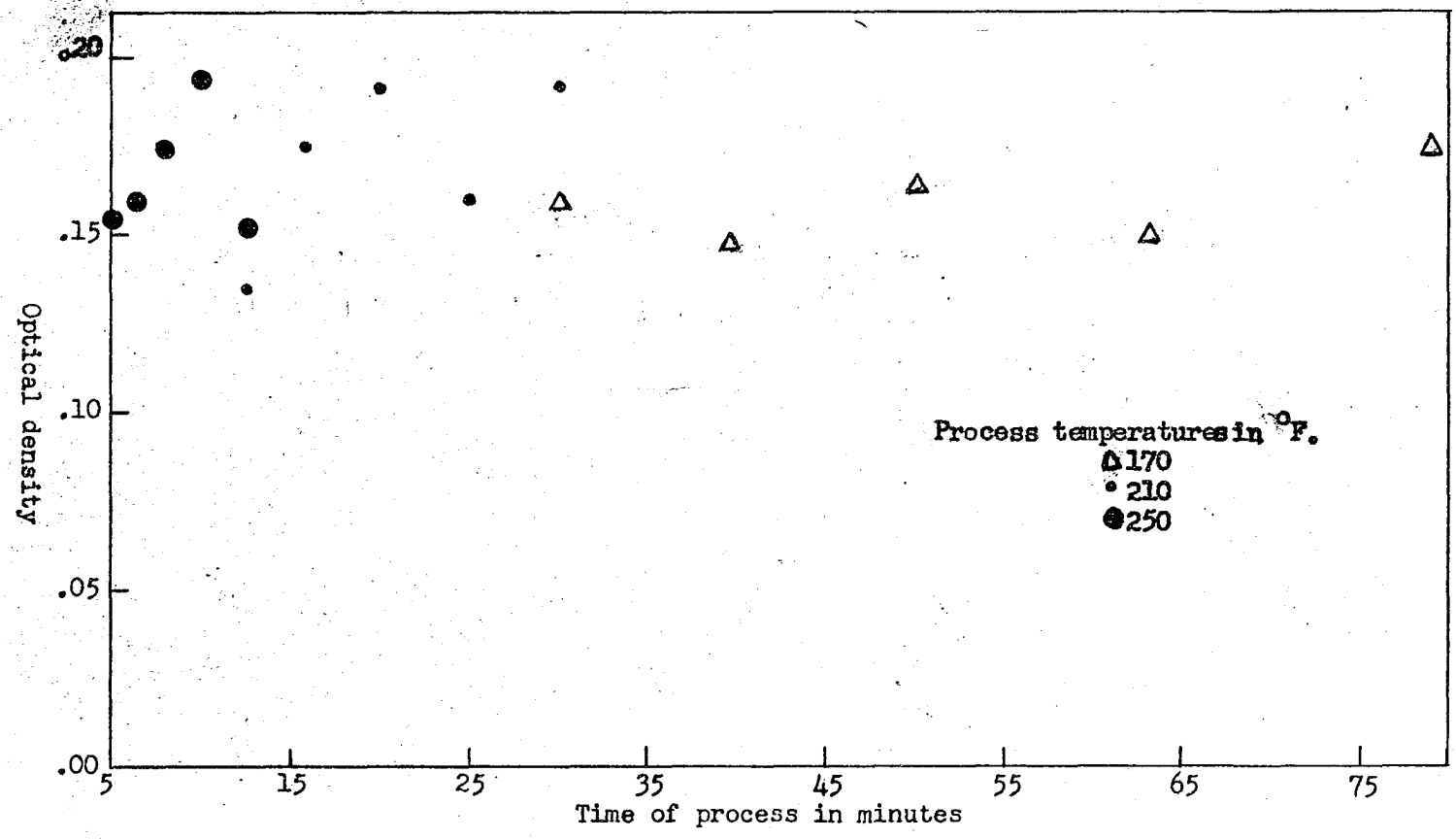


Fig. 16. Variation of pigment concentrations of grape juice samples with process times averaging over other variables except process temperature. Pigment concentrations are expressed as average values of optical densities (from Table 10).

Table 16

Effect of Thermal Processes, Storage Periods and Treatments on Pigment Concentrations of Grape Juice Samples,[1] Preserved by Addition of Toluene

Storage treatment		Storage period	Temperature of processing, °F.													
			170					210					250			
			Time of processing (minutes)					Time of processing (minutes)					Time of processing (minutes)			
Exposure	Headspace	(months)	31.5	39.7	50.0	63.0	79.0	12.5	15.8	20.0	25.0	31.5	5.0	6.3	7.9	10.0	12.5
Light	Air	0	.271	.243	.287	.267	.317	.243	.307	.335	.276	.338	.286	.271	.299	.331	.289
Dark	Air	0	.271	.243	.287	.267	.317	.243	.307	.335	.276	.338	.286	.271	.299	.331	.289
Light	Air	2	.114	.126	.126	.117	.131	.119	.119	.137	.114	.149	.131	.126	.131	.161	.103
Dark	Air	2	.126	.143	.143	.127	.149	.126	.137	.155	.126	.167	.143	.155	.149	.172	.131
Light	Air	4	.073	.075	.083	.084	.076	.083	.091	.096	.088	.101	.080	.087	.086	.088	.069
Dark	Air	4	.081	.087	.091	.090	.089	.092	.109	.115	.093	.115	.086	.101	.096	.109	.086
Light	Air	6	.059	.062	.062	.064	.073	.051	.069	.075	.053	.068	.054	.059	.071	.079	.068
Dark	Air	6	.068	.071	.084	.081	.079	.081	.091	.084	.072	.085	.065	.091	.085	.089	.074

[1]Expressed as optical densities, measured at 515 mμ at a pH of 1.0 of solutions (1:100).

in Table 15. The data indicate that storage periods play the chief role in the deterioration of the pigments in the juice samples. The pigment concentration in the juice samples prepared by processing for 63 minutes or more at 250°F. could not be estimated as the samples became turbid with storage, the cause of which might possibly be the initial extraction of larger quantities of pectin or tartarates and their subsequent precipitation on storage.

3. Changes occurring in pigment components of grape juice samples on storage as shown by paper chromatography

A study of the changes occurring in the pigment components during storage was made by use of the chromatographic technique using paper strips. With freshly prepared juices it was noted that two major bands were prominent on the paper column. The lower band with an R_f value of 0.12 was purple in color and the upper band with an R_f value of 0.21 was red in color. It was suggested previously in this discussion that the lower one was the diglycoside and the upper one the monoglycoside of the anthocyanidin. With each juice sample the R_f values were determined under identical conditions and the average values were found to be 0.12 for the purple band and 0.21 for the red band. Spraying ammoniacal silver nitrate on the papers indicated faint brown bands, moving along with the solvent front and reducing silver nitrate in the cold, and these brown bands were observed only with juices processed at temperatures of 250°F. for process times greater than 10 minutes. However, at the sites where the spots were initially placed, brown discolorations reducing ammoniacal silver nitrate in the cold were noted showing that brown discolorations

could not be moved by the solvent front.

At the end of each storage period each juice sample was separated by use of paper chromatograms run under identical conditions. The intensities of the two bands were reduced progressively with storage though the lower band became much less intense than the upper one. A brown band also appeared at the top of the column moving along with the solvent front and this band was particularly noticeable with the juice samples obtained after processing at higher temperatures.

On exposure of the papers to ammonia two yellow bands at the top, one below the other, became prominent. The presence of another colorless band turning yellow when exposed to ammonia fumes was revealed and this band possessed an R_f value of 0.52. All these bands reduced ammoniacal silver nitrate in the cold in the course of a few minutes. The brown discolorations at the site where the samples were placed on the paper columns, increased with storage and no special flourescent properties were observed when these were examined under the ultra violet light, though the colors of the bands looked brighter than under daylight. Spraying dilute ferric chloride solution on the developed and air dried chromatogram indicated a change of the color of the top band, moving along with the solvent, into an intense yellow which faded rapidly. A light greenish band of R_f value 0.52, changing to grey in the course of one hour, was also noticed on spraying ferric chloride solution on the chromatogram. From these observations it was surmised that the formation of the new bands during storage was due to polyphenolic substances formed probably as a result of the deterioration or destruction of the anthocyanin pigments or the con-

densation products formed therefrom. The possibility, however, existed that the bands formed after storage might be the result of the formation of browning pigments as a consequence of the Maillard type of reaction between reducing sugars and amino acids.

4. Processing and storage studies on Anthocyanin pigment solutions

An investigation was attempted to compare the effects of process times and temperatures on anthocyanin chloride solutions. The process times and temperatures employed were identical with those used to process grapes. The pigment concentrations in the pigment solutions were determined. The pigment solution was prepared by dissolving 4.25 grams of the pigment isolated from grapes by the method suggested by Anderson, in two litres of Mcilvaine's buffer solution of pH 3.4. Equal volumes of 25 ml. of the pigment solution were processed in glass jars for times and temperatures, included in Table 17. The concentrations of the clear resulting pigment solutions before and after the storage periods of two, four and six months were determined by diluting 1 ml. of the clear solution with 2% dilute HCl to volumes varying from 50 to 100 ml. The results were recorded in Table 17. It was observed that as the temperatures of processing increased to 250° F., dark brown precipitates were formed and at processing times longer than 60 minutes the solutions became brownish yellow with practically all the anthocyanin pigment destroyed. Even at lower temperatures of processing a gradual loss of pigment was noted with increasing process time. On storage the pigment concentrations were lowered with the simultaneous formation of gelatinous sediments in the

Table 17

Effect of Thermal Processes and Storage Periods on
Concentrations[1] of Pigment Solutions of pH 1

Storage period (months)	Temperature of processi: 170 — Time of processing (minutes)						210 — Time of processing (minutes)					
	0	31.5	39.0	50.0	63.0	79.0	12.5	15.8	20.0	25.0	31.5	63.0
0	.378	.329	.325	.319	.316	.312	.293	.287	.273	.265	.252	.186
2	.162	.123	.116	.101	.121	.130	.086	.086	.077	.046	.024	.030
4	.062	.052	.046	.043	.043	.050	.042	.043	.031	.032	.021	-

[1]Expressed as optical densities measured at 515 mμ at pH 1.0 of solutions (1:50).

Table 17

nal Processes and Storage Periods on Pigment
ations[1] of Pigment Solutions of pH 3.4

Temperature of processing (°F.)														
210							250							
Time of processing (minutes)							Time of processing (minutes)							
12.5	15.8	20.0	25.0	31.5	63.0	100.0	5.0	6.3	7.9	10.0	12.5	31.5	63.0	100.0
.293	.287	.273	.265	.252	.186	.175	.217	.201	.192	.180	.170	.090	.060	.040
.086	.086	.077	.046	.024	.030	.020	.041	.036	.025	.019	.005	.004	.004	.003
.042	.043	.031	.032	.021	-	-	.031	.025	.028	.008	.005	-	-	-

15 mμ at pH 1.0 of solutions (1:50).

solutions. These sediments were insoluble in dilute HCl (3%) but were soluble in alkalies forming dark colored solutions with absorptions maxima around 400 mμ in the visible range of 400 - 600 mμ. These sediments were probably the result of the polyphenolic condensation products of the oxidized pigments, the formation of which was apparently accelerated as a consequence of heat and storage. The losses with pure pigment solutions on heat and storage treatment were remarkably greater compared to the losses when they were present in the juices, along with the other juice components which include the sugars, pectin, tannins and organic acids. The pure pigment in the solid state formed water insoluble dark brown residues on storage at room temperature.

5. Ultraviolet exposure studies on grape juice and anthocyanin pigment solutions

Further investigations were meant to discover the nature of the substance present in grape juice which was responsible for the protective influence on the pure pigment solutions during storage. Experiments were conducted on exposure of the pigment solutions to ultraviolet rays which apparently accelerated destruction of the pigment, and made short period studies feasible.

Each of 5 ml. of concentrated purified pigment extract were diluted with 25 ml. each of McIlvaine's buffer solutions of pH 2.4, 2.9, 3.4, 3.9, and 4.4. Volumes of 25 ml. of each of these solutions were pipetted into petri dishes and the weights of the petri dishes were recorded along with the contents. The dishes were exposed to ultraviolet light at room

temperature and after intervals of 8, 16, 24, and 32 hours, 1 ml. was pipetted out from each petri dish and the optical densities of the clear solutions estimated at pH 1.0 after diluting them to suitable volumes varying from 50-100 with dilute HCl. Before withdrawing the solution from the petri dish for the color estimation correction for the loss of water by evaporation was made by adjusting the weights of the petri dishes by addition of the buffer solutions, account being taken of the volume withdrawn. In each case the absorption values in the entire visible range were noted, though only the values recorded at 515 mμ were included in Figure 17. It was observed that as more of the pigment was destroyed by increased exposure to ultraviolet rays, the optical density in the range of 400 mμ showed a higher value than the value at 515 mμ. The color in the resulting solution after complete destruction of the anthocyanin was yellowish brown.

An examination of the results shows that the rate of deterioration of the pigment solution is slower in the solution of pH 2.4 during the first eight hours, while further exposure seems to bring about rapid changes. After the end of 32 hours the pigment losses are practically the same in all the different solutions. The concentration of the pigments dissolved in buffer solutions and left exposed to daylight, were reported in Figure 18. It is noticed that with these samples no obvious differences in pigment concentration are detected as a result of change of pH of the pigment solution (2.4 - 4.4). However, inconsiderable pigment losses appeared on exposure of the pigment solutions to daylight.

A similar experiment was conducted using 25 ml. Concord grape juice

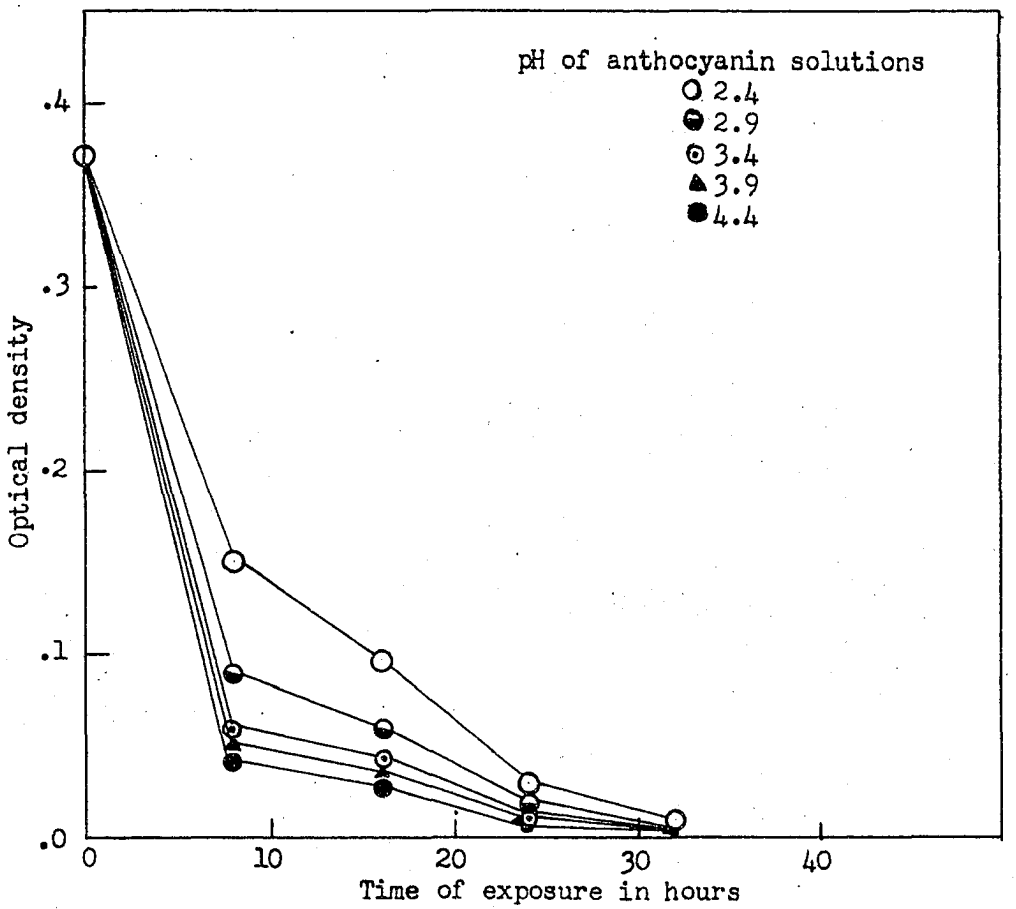


Fig. 17. Effect of exposure to ultra violet light on pigment concentrations of anthocyanin chloride solutions (pH 2.4-4.4). Pigment concentrations are expressed as optical densities at 515 mμ and at pH 1.0 of solutions (1:100).

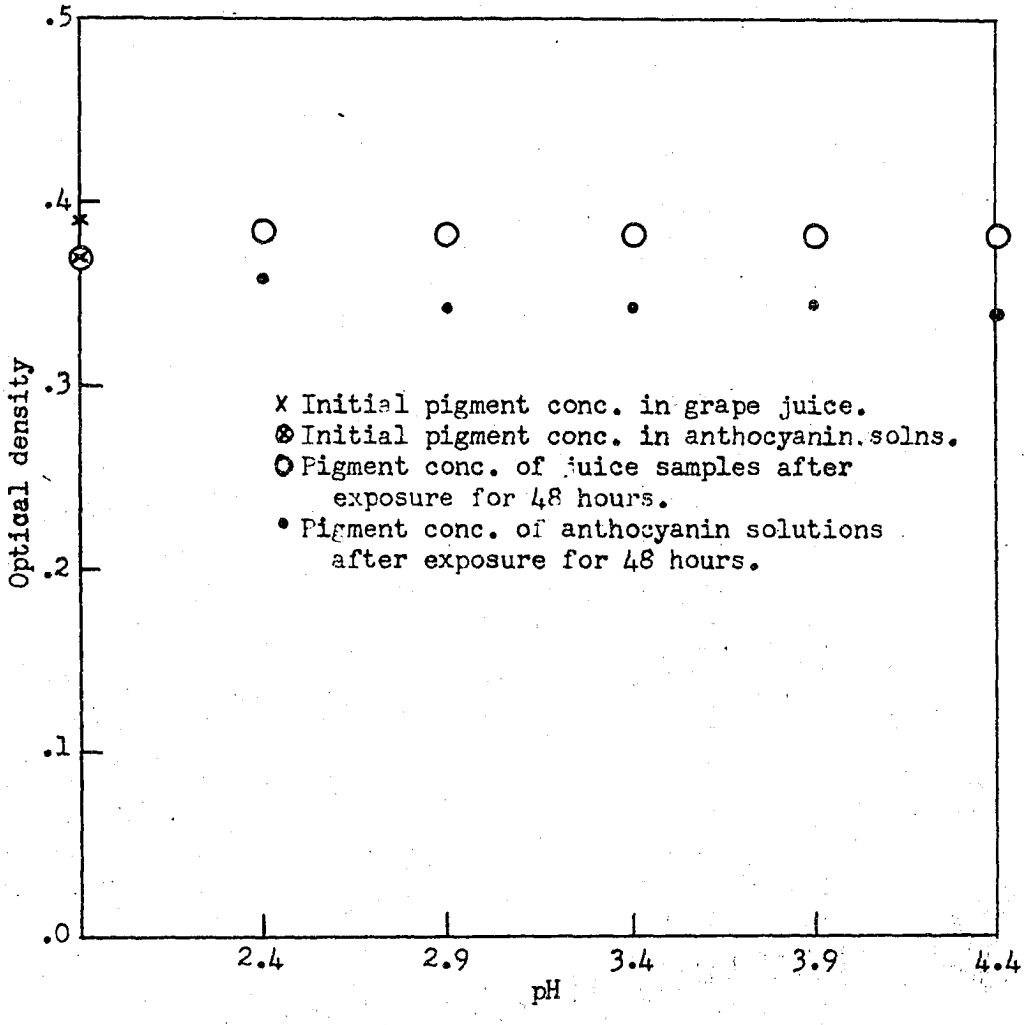


Fig. 18. Effect of exposure to daylight on pigment concentrations of anthocyanin solutions (pH 2.4-4.4) and grape juice samples (pH 2.4-4.4). Pigment concentrations are expressed as optical densities measured at 515 mμ and at pH 1.0 of solutions (1:100).

(pH 3.4) after adjusting the pH to 2.4, 2.9, 3.4, 3.9 and 4.4 with 0.5% dilute HCl and 25% ammonia. The results are given in Figure 19. It is observed that the losses of pigment concentration in the juice samples increase on exposure to ultraviolet light, with increasing pH of the juice samples. The pigment in the juice samples is not destroyed to the same extent as noted with pure pigment solutions. Possibly the pigment in the juice is protected by the other components of the juice or the pigment exists in combination with some of the components of the juice in a form resisting the effects of ultraviolet exposure.

The different components present in grape juice, e.g. sugar (16%), tannin (0.2%), pectin (0.15%), tartaric (0.4%) and malic acids (0.5%) (91, 62) were added to 20 ml. of purified pigment solutions (pH 3.4) at the levels present in the juice. Pure tannic acid (di-galloyl gallic acid) was substituted for tannin. The mixed solutions of pigment and the other juice components were exposed to ultraviolet light. The results are presented in Figure 20. The results indicated that tannic acid showed a protective effect on the pigment during the first few hours of exposure, though continued exposure brings about greater losses. In the samples in which tannic acid was added to the pigment solution, it was observed that on exposure to ultraviolet light, the optical densities are higher at 400 mμ than those samples treated otherwise. However, exposure of pure tannic acid solution to ultraviolet light indicates that the optical density at 515 mμ is negligible and should not cause any interference in the optical density values of the samples under examination at the dilutions employed.

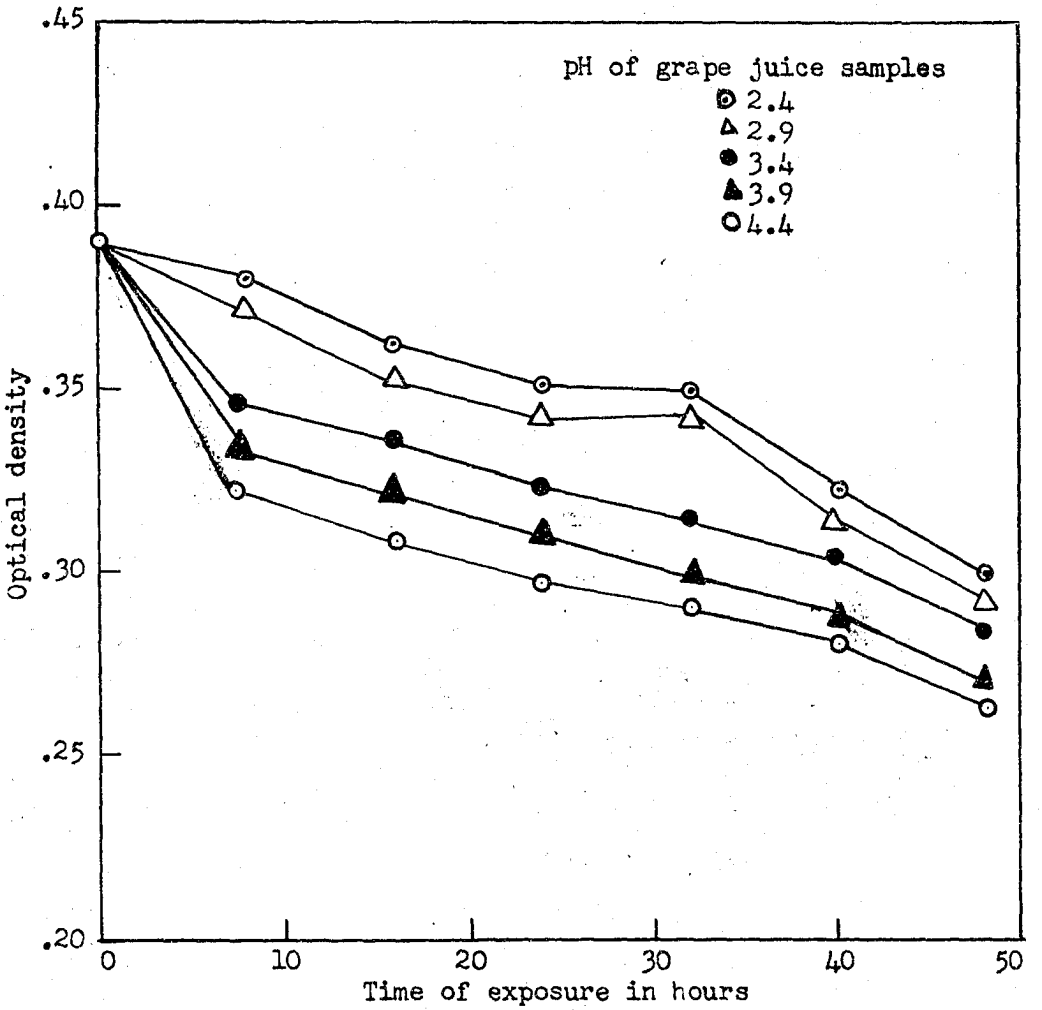


Fig. 19. Influence of exposure to ultraviolet light on pigment concentrations of grape juice samples (pH 2.4-4.4). Pigment concentrations are expressed as optical densities measured at 515 mμ and at pH 1.0 of solutions (1:80).

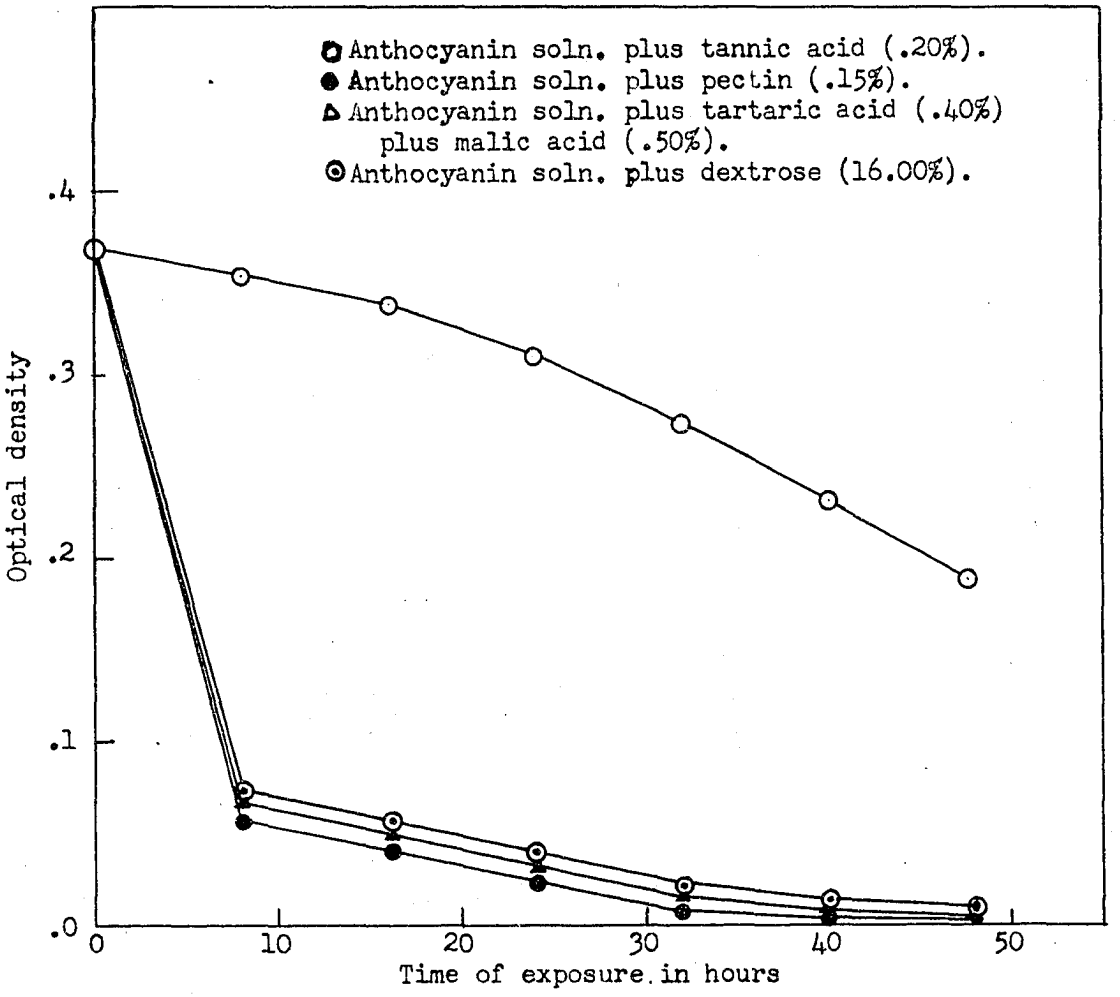


Fig. 20. Influence of grape juice components on pigment concentrations of anthocyanin solutions (pH 3.4) exposed to ultraviolet light. Pigment concentrations are expressed as optical densities measured at 515 mμ and at pH 1.0 of solutions (1:80).

To gain an insight into the effect of tannins on grape pigments exposed to ultraviolet light, studies were made with and without the addition of tannic acid to grape juice free of tannins. The results presented in Figure 21 indicate that tannic acid has a protective influence in the first eight hours of exposure though prolonged exposures reduce this effect considerably. The protective influence might possibly be accounted for on the presumption that the tannin was present in the juice in a different state and that added tannic acid could not truly represent that state.

Further experiments were carried out to find out the influence of tannin extract of stems, and the tannin extract (also containing the anthocyanin pigments of grape juice) from juice when added to juice free from tannins and other polyphenolic substances. A solution of the tannin extract was also exposed to ultraviolet light to estimate its interference on the absorption values. The results contained in Figure 21 indicate that the tannin extract from grape juice shows the best protective effect for the pigments when the juice samples are exposed to ultraviolet light. The results also show that the tannin extract from grape stems is superior to pure tannic acid in protecting the pigments from destruction as a result of exposure of the pigment solutions to ultraviolet light. Possibly the juice contains other polyphenolic substances, in addition to tannins, which have effects on the color similar to the tannins. It is equally possible that the anthocyanin pigment and the tannins are present in the grape juice samples in a form, difficult to be destroyed on exposure to ultraviolet light.

Description of the Symbols Used in Figure 21

○ grape juice free from tannins and anthocyanin pigments + extract of tannins and anthocyanin pigments obtained from grape juice.

△ grape juice free from tannins and anthocyanin pigments + pigment soln. + tannin extract from stems (equiv. 0.2% tannin).

● grape juice free from tannins and anthocyanin pigments + pigment soln. + tannic acid (0.2%).

X grape juice free from tannins and anthocyanin pigments + pigment soln.

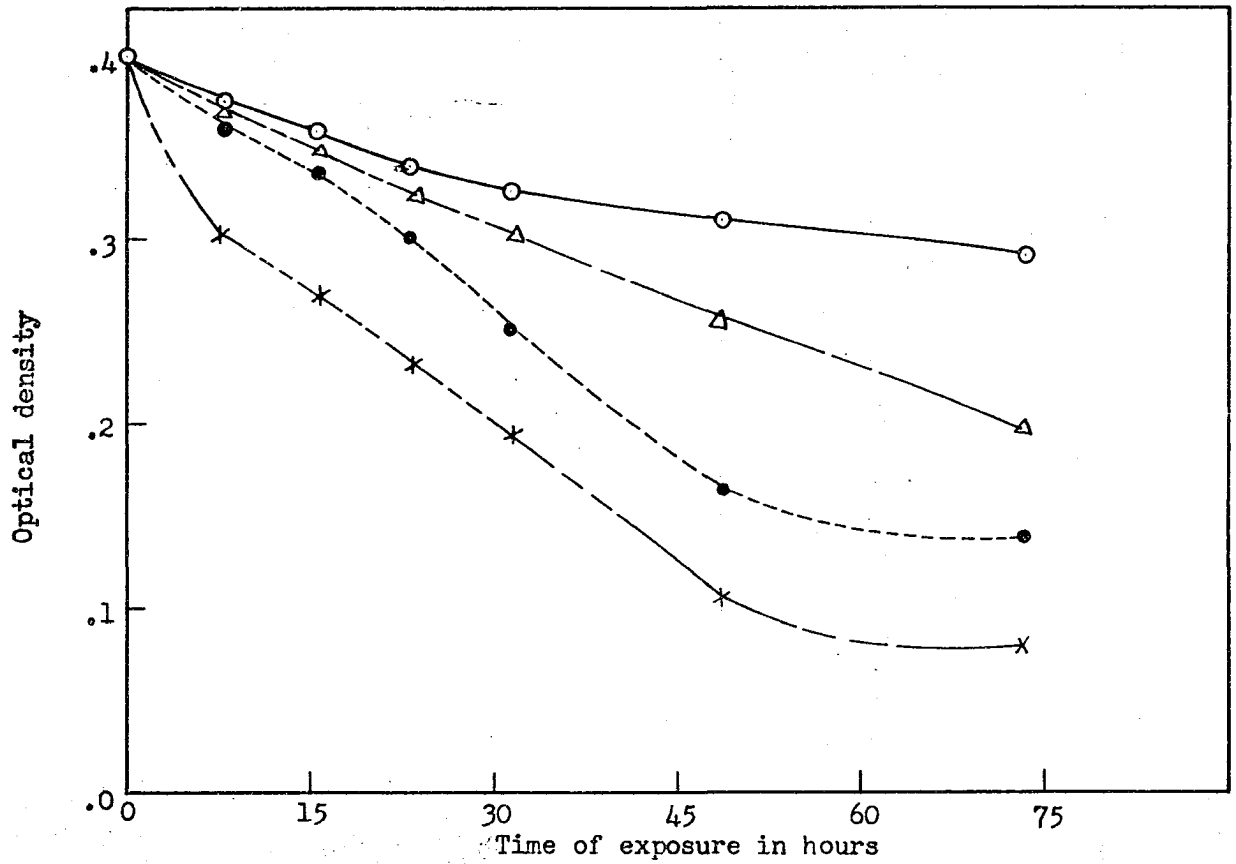


Fig. 21. Influence of natural tannin extracts and tannic acid on grape juice free from tannins. Pigment concentrations are expressed as optical densities measured at 515 mμ and at pH 1.0 of solutions (1:80). The solutions were exposed to ultraviolet light.

V. SUMMARY AND CONCLUSIONS

An investigation was made to find the nature of the pigments present in concord grapes with emphasis focused on the water soluble pigments. For this purpose fractionation techniques involving dialysis, solubility differences of the pigments in various solvents, chromatography and ion exchange were utilized. To determine the nature of the pigments, advantage was taken of the absorption values of the different pigment extracts using the Coleman Spectrophotometer. Dialysis experiments using samples of grape juice and extracts of lyophilised skins did not give any indication of the presence of more than one anthocyanin pigment in grapes. Experiments involving solubility differences of the water soluble pigments in the various solvents used (ethanol, butyl alcohol, isobutyl alcohol and their mixtures) also indicated a single anthocyanin pigment in grapes. Qualitative tests of the methanolic extract of the grape skins showed the presence of chlorophyll, water soluble yellow pigments and carotenes along with the anthocyanin pigments. These observations were supported by the chromatographic techniques wherein magnesia and alumina were employed as the adsorbents.

Chromatograms run by using filter paper (Whatman No. 4) strips as supporting material and the nonaqueous layer of the mixture of butanol, acetic acid and water (4:1:5 by volume) as the partitioning solvent showed a greenish yellow band moving along with the solvent front and this band was found to be due to fat soluble pigments. The paper chromatogram also

revealed the presence of two water soluble pigments which formed purple and red bands on the chromatogram possessing R_f values of .11 and .19 respectively. A hazy red band with an R_f value of about .56 was also noted. This red band faded in intensity in the course of 24 hours. These bands with R_f values of .11 and .19 were identified as di- and monoglycosides of the anthocyanidin while the band with R_f value of about .56 was identified as the free anthocyanidin on the basis of hydrolysis and separation procedures. The R_f values also added support to the identification of the pigments. The absorption values of solutions of the individual pigment components, when observed in buffer solutions of varying pH values added further proof to this assumption.

The major anthocyanin pigment present in Concord grapes was isolated from fresh ripe skins by employing the method suggested by Anderson. In essence, the method consisted of precipitating the pigment as picrate from dilute acid extract of pressed skins and conversion of the picrate later into the chloride. Attempts to crystallize the pigment were not successful. However, the substance was found to be free from metallic and organic impurities. The emperical formula of the substance ($C_{23}H_{25}O_{12}Cl$) and the color reactions indicated that the pigment may be Oenidin 3-monoglycoside. The R_f value of this substance in dilute HCl solution was determined to be .21 using the nonaqueous layer of mixture of butanol, acetic acid and water (4:1:5) and Whatman No. 4 filter paper at 70 $\pm$ .5° F. and this value added support to the identification of the pigment. The sugar present in glycosidic form was identified as glucose from paper chromatogram studies.

The pigment concentration in anthocyanin chloride solutions and grape juice samples was estimated throughout this study by determining the optical density of clear solutions at pH of 1.0 and at 515 mμ using a Coleman spectrophotometer. Under these conditions the optical density was found to be proportional to the concentration of anthocyanin chloride in its solutions.

The later part of this investigation was intended to find the changes in the anthocyanin pigments present in grape juice samples and anthocyanin chloride solutions resulting from varying thermal processes and storage periods. The pigment concentrations of grape juice samples, prepared by processing fresh grapes in glass jars for varying lengths of time at temperatures of 170° F., 210° F., and 250° F., were estimated by using the Coleman spectrophotometer. The process times were chosen such that their logarithmic values were approximately in an arithmetic progression. The process temperatures were likewise fixed on the assumption that the effect of process times was logarithmically related to process temperatures. Longer process times were employed for lower process temperatures. The concentrations of anthocyanin pigment in the grape juice samples did not indicate any significant variations as a consequence of processing at different times and temperatures.

To prevent fermentation of juice samples during storage half the number of the samples of each process were pasteurized at 180° F. for one minute and to the other half one ml. of toluene was added. Half of the grape juice samples of the two groups mentioned were left exposed to light from electric bulbs and the other half were kept in the dark covered with

aluminum foil. Into half of the pasteurized samples nitrogen was filled into the headspace and all the samples were stored at a constant temperature of 70 $\pm$ 0.5° F. The pigment concentrations in the juice samples were determined at intervals of two months over a six month period. The results indicated that the pigment concentrations in the juice samples were reduced with increasing storage period. Among the different treatments imposed in the investigation, storage period showed the maximum influence in the deterioration of the pigment. At the end of each storage period the pigments present in each of the juice samples were separated by paper chromatography. The two major bands of purple and red color, observed with fresh juice samples, became less intense with increasing storage. On storage a brown band also appeared at the top moving along with the solvent front. Exposure of the developed chromatogram to ammonia fumes revealed the presence of two yellow bands at the top, one below the other and a yellow band with an R_f value of .52. Ammoniacal silver nitrate was reduced in the course of a few minutes when it was sprayed on these bands. Discolorations were also shown on spraying neutral ferric chloride solutions on these bands indicating that the bands were due to phenolic substances. All available evidence pointed to the conclusion that these phenolic substances are probably formed by oxidative changes brought about in the anthocyanin pigments, and these changes are obviously augmented by increased storage periods.

The juice samples prepared by using grapes stored at 40° F. for one month and processed for longer process times at temperatures of 210° F. and 250° F. showed changes of deterioration on storage identical with

those observed with juice samples processed from fresh grapes. The anthocyanin pigment was destroyed if processes at 250° F. were conducted for process times longer than 63 minutes.

Anthocyanin chloride solutions were processed in glass jars using times and temperatures identical with those used for processing grapes. The results indicated increasing destruction of the pigment in the solutions when the processes were for longer times and at higher temperatures. On storage the pigment in the processed anthocyanin chloride solutions was destroyed with the simultaneous formation of insoluble brown residues. The destruction of pigment on processing and subsequent storage was found to be more striking in the case of anthocyanin chloride solutions than in the grape juice samples.

An effort was made to find the component normally present in grape juice which is responsible for protecting the pigment from oxidation as easily as the anthocyanin chloride solutions. In an attempt to accelerate the oxidative changes the samples of grape juice and pigment solutions were exposed to ultraviolet light. Grape juice samples of pH 2.4, 2.9, 3.4, 3.9 and 4.4 were exposed to ultraviolet light for a period of 48 hours. Similarly anthocyanin chloride solutions of pH 2.4, 2.9, 3.4, 3.9 and 4.4 were exposed to ultraviolet light. The results indicated that in the initial stages of exposure to ultraviolet light, the losses of pigment increased with increasing pH while increased periods of exposure practically destroyed all the anthocyanin pigment. However, these pigment losses were not as appreciable with grape juice samples as those with the anthocyanin chloride solutions.

Of the grape juice components, tannins exerted the maximum influence in preventing destruction of the anthocyanin pigment present in grape juice samples of pH 3.4 on exposure to ultraviolet light. Dextrose, citrus pectin, tartaric and malic acids did not seem to prevent the pigment from being destroyed. The extract of tannins obtained from grape juice seemed to possess better antioxidant properties than the extract of tannins from grape stems or pure tannic acid (galloyl gallic acid) solutions. The results also indicated that in addition to tannins, other polyphenolic substances present in grape juice exert similar anti-oxidative effects preventing destruction of the anthocyanin pigment from degradations.

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