FLAVONOIDS OF BLACK GRAPE VARIETIES

GROWN IN SOUTH AFRICA

By

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Thesis accepted for the Master's Degree in Agriculture

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December 1963.

ACKNOWLEDGEMENTS

The author expresses his gratitude to Dr. C.F. Garbers and Mr. C.T. de Waal for their interest and many helpful suggestions in in the course of this investigation.

Furthermore, the author thanks the University of Stellenbosch for granting him permission to complete this study while being in their employ.

The gift samples of anthocyanins obtained from Dr. J.B. Harborne and the sample of Ultramide, made available by Badische Anilin and Soda Fabrik, Germany, through the courtesy of Messrs. Taeuber & Corssen, is highly appreciated.

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CHAPTER I

INTRODUCTION

The constitution of anthocyans first became known as a result of investigations conducted by Wilstätter and Everest (I) on the pigment cyanin from the cornflower. The success of this fundamental work stimulated further interest in the colouring matter of plants.

In later researches, the methods developed by Robinson and Robinson (2) for the rapid analysis of anthocyanins in plant extracts, depended primarily on the purification of the colourant through the picrate or chloride and observations of colour changes coupled with the distribution of pigments between organic and aqueous solvent phases. Chemical analysis of the product, in particular the methoxyl index, gave an indication of the nature of the anthocyan. In a plant species containing several and or complex glycosides, the difficulty in separating the different constituents arises and here the methods of Robinson and Robinson (2) have proved to be of limited applicability in their scope.

The limitations of the above methods were largely overcome by the use of paper partition chromatography which also presented a different approach to the investigation of plant pigments. In 1948, Bate-Smith (3), for the first time successfully separated anthocyans, thus proving that they are ideally suited to this technique by reason of their wide range of polarity characteristics. He also published

2./ the....

the R_f values for most of the natural anthocyans known at that time as well as for synthesised samples. More recently other workers, notably Geissman (4) and Harborne (5) published R_f values in several more solvent systems.

Many chemical analysis of substances occurring in plants have been motivated to a considerable extent by an interest in these compounds as dyes or colouring matters or for the potential use as therapeutic agents. It is noteworthy that in recent years there is appearing a renewed interest in the field of biochemical genetics, since paper chromatography has largely overcome the limiting factors such as shortage of plant material and the need of identifying all the pigments including those present in trace amounts. Anthocyanins were the first group of plant pigments in which the relationship between single genes and simple biochemical differences was demonstrated (6).

The flavonoid pigments, a group of C_{15} polyphenols commonly known as the anthocyans, form the principal phenolic substances of red grapes and wines. The oenological interest in these substances thus becomes evident. They are responsible for the colour and partly for the hardness and astringency of wines. From an organoleptic point of view they are at least as important as the other substances which affect the bouquet and palatability of wines.

Although much work has been conducted in this field, investigation of the grape pigments is much less advanced. The many complex

3./ and....

and hitherto largely unknown changes occurring during the ageing of wine, directly involves the colouring matter derived from the grape and necessitates a clear picture of the various individual constituents comprising the colour.

The purpose of this investigation is the application of paper-chromatographic, spectrophotometric and other techniques, which have been successfully applied to the analysis of the colouring matter of other plants, relevant to the analysis of the grape pigments. Attention will be devoted, in particular, to the separation, purification and identification of all the pigments occurring in black grape varieties used in the production of red wines. An account will also be given on the properties of the anthocyans and other stereochemically related compounds occurring in grapes and wine.

4./ CHAPTER II....

CHAPTER II

COLOURING MATTER OF THE VITIS SPECIES

The pigment of the dark blue European grape Vitis vinifera, has been shown by Wilstätter and Zollinger (7) to consist largely of the crystalline chloride $C_{23}H_{25}O_{12}Cl$. This was recognised as oenin, the flavylium chloride of a dimethyl ether of delphinidin monoglucoside. The anthocyanidin was termed oenidin, however, it was not stated at the time that oenidin is identical with malvidin.

In 1923 Anderson (8) reported that the Norton grape contained, in addition to oenin, a small amount of pigment which appeared to be a diglucoside. In later studies of the pigment of several varieties of American grapes, Anderson and Nabenhauer (9) concluded that the anthocyans isolated from dark blue grapes such as Concord, Norton and Clinton, representing Vitis labrusca, Vitis aestivalis and Vitis riparia respectively, consist primarily of monoglucosides of monomethyl delphinidin (ampelopsin). The pigment occurring in Seibel grapes, a hybrid of Vitis aestivalis - Vitis rupestris with Vitis vinifera, was identical to oenin found by Wilstätter and Zollinger in the dark blue European grape.

Anderson and Nabenhauer (10) also reported methoxyl values for American and crosses of American - European grapes which were lower than those found by Wilstätter and Zollinger (7) for oenin. The values were too high for one methoxyl group and too low for two methoxyl

5./ groups....

groups. The conclusion was that the anthocyans of the American grapes were mixtures of oenin and monomethoxy delphinidin monoglucoside. The values could, however, just as readily be accounted for by a mixture of oenin and delphinidin monoglucoside or a mixture of oenin, delphinidin monoglucoside and monomethoxy delphinidin monoglucoside.

The presence of delphinidin and its 3'-methyl ether in addition to oenin was established by Levy et al. (II) in the Fogarina grape. About the same time Robinson and Robinson (I2) found that the skins of a red South African variety contained a cyanidin monoglucoside.

Apart from the unambiguous identification of some of the principal constituents, the limitations of the earlier investigations is evident from conflicting reports. With the advent of chromatography the identification of the substances thus separated could be effected by several new techniques e.g., by comparison with R_f values of known substances, utilisation of certain laws relevant to the chromatographic behaviour of anthocyans, spectrophotometry and selective oxidation and removal of saccharide groups.

In a survey (I3 - I5) on a large number of species of European, American and hybrid grapes, it was established that derivatives of the cyanidin and delphinidin series constitute the colouring matter. The number of these substances varies with the species from six for Vitis monticola to seventeen for Vitis lincenumii. With regard to the glycosidic character of these substances it has been established

6./ that....

that diglucosides do not occur in Vitis vinifera and certain American species e.g., Vitis berlandieri. The presence of diglucosides is characteristic of Vitis riparia and Vitis rupestris. In the genetic sense of the word, the diglucoside character is dominant and the monoglucoside character recessive.

Vitis riparia and Vitis rupestris are the species used most frequently in hybridisation and this explains why most of the hybrids have the diglucoside character. A cross between Vitis riparia and Vitis vinifera will thus yield a hybrid population FI in which the diglucoside character is dominant, however, if the FI hybrid is crossed again with Vitis vinifera, a new F2 hybrid population results of which 50% will have the diglucoside character and 50% the monoglucoside character.

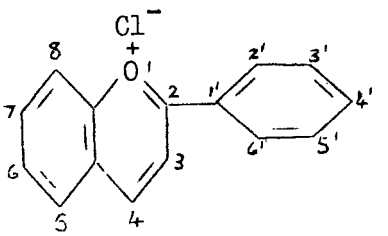
7./ CHAPTER III....

CHAPTER III

ANTHOCYANS AND RELATED FLAVANS

III,A. General Nature and Classification

Pyran and pyrylium derivatives are widely distributed in nature e.g., in the carbohydrates, chromones, flavones, anthocyanins and alkaloids. The anthocyanins are hydroxy derivatives of 2-phenyl--benzopyrylium or flavylium salts.



2-phenyl-benzopyrylium salt

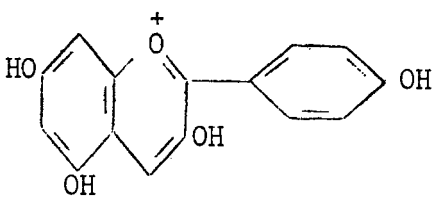
The term flavonoid designates all those compounds based on the $C_6 - C_3 - C_6$ carbon skeleton, consisting of two aromatic rings linked by an aliphatic 3-carbon chain. The various groups of flavonoid compounds differ mainly in the oxidation state of the C_3-portion of the molecule and on this basis they may be divided into two main groups viz. the anthocyanins and flavans which lack a 4-carbonyl grouping and the anthoxanthins which possess a 4-carbonyl grouping.

The anthocyans are the most important group of the flavonoid compounds imparting red, purple or blue colours to flowers and fruits whereas the anthoxanthins are yellow pigments and the flavans colourless compounds.

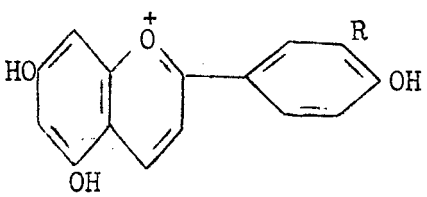
8./ III,B. The....

III,B. The Naturally Occurring Anthocyanins

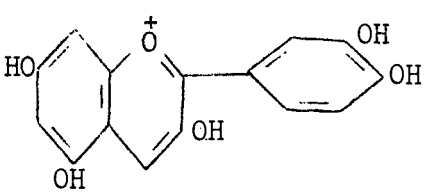
The individual anthocyanins occurring in nature differ within the type by the number and position of the glycosidic and methoxyl groups. They are flavylium salts derived from the three main types represented by pelargonidin, cyanidin and delphinidin (I6).



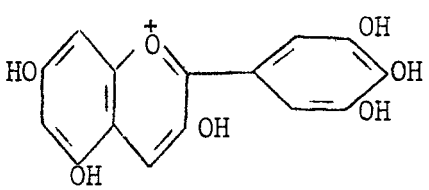
pelargonidin



apigeninidin R = H
luteolinidin R = OH



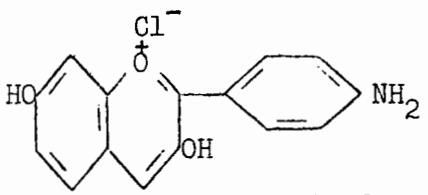
cyanidin
peonidin (3'-methyl ether)
rosinidin (7,3'-dimethyl ether)



delphinidin
petunidin (3'-methyl ether)
malvidin (3',5'-dimethyl ether)
hirsutidin (7,3',5' trimethyl ether)

9./ The....

The glycosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin occur commonly in nature whereas those of rosinidin, hirsutidin, apigeninidin and luteolinidin are rare and occur only in a few plants (16 - 19). The latter two pigments, representing the fourth type of anthocyanidin was discovered in gesnerin (Gesneridin is identical with apigeninidin.) and the aglycone gesneridin, in which the 3 - hydroxyl group of the other anthocyanins is absent (20 - 21). Other anthocyanins which are not derived from the three main types are the nitrogenous anthocyanins such as the betanin group (22) and the colouring matter of the Iceland poppy (23).



aminoflavylium salt

III,C. General Properties of Anthocyans

The anthocyans occur in nature in combination with sugars and are known as anthocyanins. On hydrolysis they yield an aglycone or anthocyanidin and a pentose or hexose, notably glucose, rhamnose, galactose, xylose and arabinose (5). In addition, anthocyanins frequently occur as acylated or esterified derivatives, the common acids being hydroxy cinnamic and benzoic acid. The acid esterifies either a phenolic hydroxyl group in the flavylium nucleus or a

10./ hydroxyl....

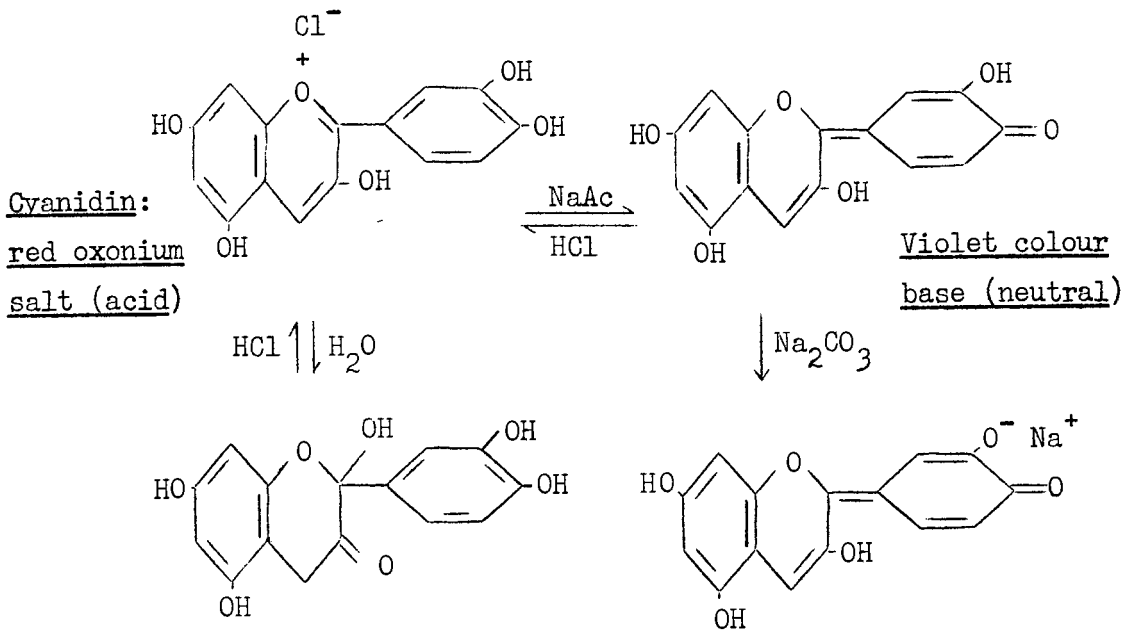
hydroxyl group in the sugar residue (5, 20,24)

Anthocyanins are crystalline substances, soluble in water and hydroxylic solvents but insoluble in non hydroxylic solvents such as ether and benzene. They are amphoteric and their salts with strong acids such as hydrochloric acid are fairly stable. The anthocyanidins on the contrary are less stable and even at low pH solutions of anthocyanidins fade rapidly when exposed to light.

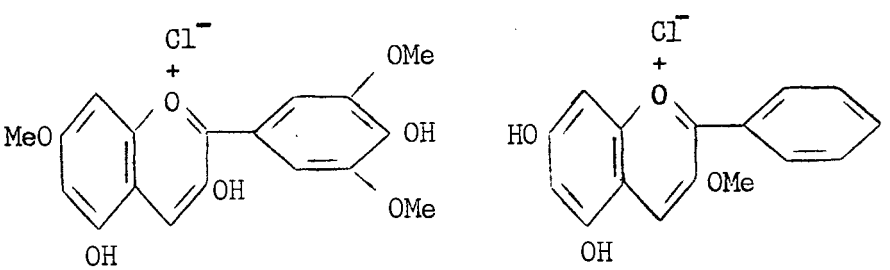
The importance of their amphoteric character is demonstrated by the variation in colour of an anthocyanin or anthocyanidin with changes in pH of the solution e.g., cyanidin is present in the cornflower as the blue potassium salt, but the free base is violet and the salts such as the chloride magenta red. At pH value 3 or less, cyanidin is red, at pH value 8.5 violet and at pH value II.0 blue. Generally the acid salts of the anthocyanidins are red, the metallic salts blue and the neutral substances purple or violet. These colour changes (25) depicting cyanidin as the red oxonium salt, violet colour base and blue sodium salt of the colour base are shown on page II. The carbinol base or pseudo - base is colourless.

Support for these formulae (p. II) comes from the colours of certain other anthocyanidins and anthocyanins. Galanginidin chloride has no hydroxyl group in the 4' - position and therefore cannot change into the quinoid form and in consequence cannot form a blue or violet salt with alkali.

II./ Hirsutidin....

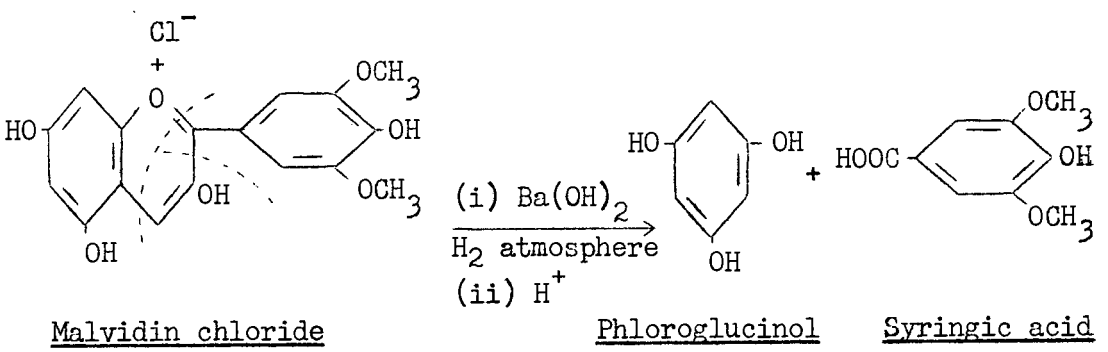


Hirsutidin chloride has no free hydroxyl group in the 3' or 5'-positions and can form a violet colour base but no blue alkali salt. For the formation of a pure blue colour, a quinoid structure and a salt forming auxochrome are evidently required. Confirmation is provided by the blue colour of the sodium salt of malvidin which has a hydroxyl group in the 7-position and the violet colour of the sodium salt of hirsutidin with a 7-methoxyl group (26).



III,D. Constitution of Anthocyans

The structure of an anthocyanidin is determined by the nature of the products obtained from potassium hydroxide fusion (7). Phloroglucinol or a methylated phloroglucinol and a phenolic acid are always obtained. The method, however, suffers from the disadvantage that the anthocyanidin is not only degraded, but is often also demethylated. This difficulty was overcome by Karrer (27) who degraded the anthocyanidin with aqueous barium or sodium hydroxide (IO%) in an atmosphere of hydrogen.



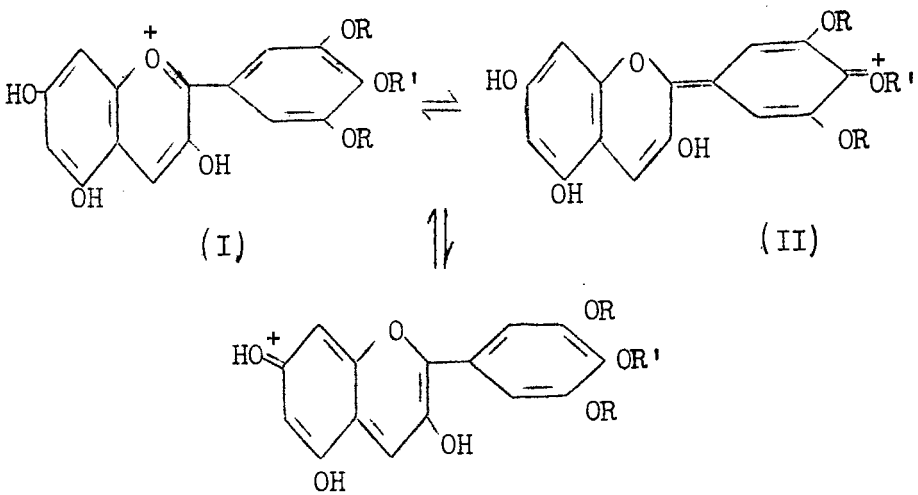
Malvidin chloride Phloroglucinol Syringic acid

(i) Hydroxylation

The three main types of anthocyans have a 3,5,7-trihydroxy flavylium system in common and differ by the number of hydroxyl groups in the 2-phenyl ring. In the anthocyanins the 4'-hydroxyl group is always free if present. It is not known whether the invariable freedom of this group from both methylation and glycosidation is due to its "protection" during the early

I3./ stages....

stages of pigment synthesis or to some other factor. It is also apparent that the 4'-position is uniquely situated (II) with respect to the positive charge in the oxonium ion (I) and could undergo ready displacement of the 4'-group (R') if it were already present, or conversely, be reluctant to undergo glycosidation or alkylation (4).



(ii) Methylation

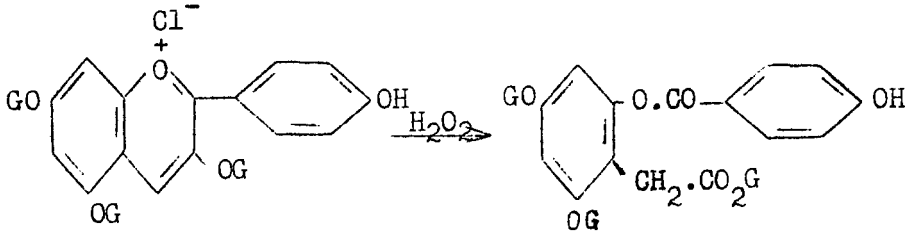
Information on the position of methoxyl groups has been obtained by the methods developed by Karrer (27). In the anthocyanins methylation is restricted to the 3' and 5'-hydroxyl groups in all but one known case, hirsutidin. The methylated anthocyanins are 3' and

14./ 3',5' mono....

3', 5' mono and dimethyl ethers of cyanidin and delphinidin. The existence of a 7 - methoxyl group in the case of rosinidin has not yet been fully confirmed (I6).

(iii) Glycosidation

The position of the sugar residues can be ascertained by methylating the anthocyanin and removing the sugar group or groups by hydrolysis with hydrochloric acid . The anthocyanidin is then degraded with barium hydroxide and the positions of the free hydroxyl groups in the products thus obtained, indicate the positions of attachment of the saccharide groups , however, in some cases the interpretation of the results was uncertain. Karrer et al. (27, 28) showed that hydrogen peroxide (I5%) attacks anthocyanins as follows:



If the anthocyanin has e.g., a glucose residue in the 3 - position , then this residue is readily

I5./ hydrolysed....

hydrolysed by dilute ammonia after peroxide treatment. If the glucose residue is in the 5 or 7-position, it is only removed by heating with dilute hydrochloric acid. Thus position 3 can be distinguished from position 5 or 7, but the latter two cannot be distinguished from each other. Conclusive evidence for the positions of the sugar residues was afforded by the synthesis of the anthocyanins (III,E).

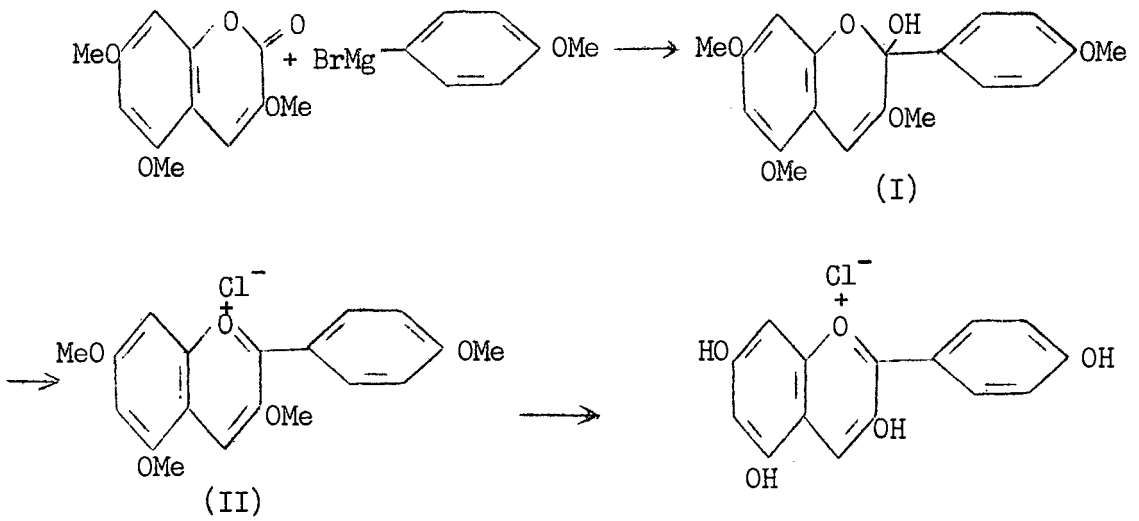
Robinson et al. (29) showed that anthocyanins with a free hydroxyl group in the 3-position are rapidly decolourised by ferric chloride whereas those with a sugar residue in this position are stable. As a result of these and other methods (VI,J), it is now known that the attachment of sugar residues may occur at one or more of positions 3,5 and 7.

III,E. The Synthesis of Anthocyans

The first general synthesis of an anthocyanidin was accomplished by Wilstätter and Zechmeister(30). Thus pelargonidin chloride was synthesised by treating 3,5,7-trimethoxycoumarin with anisylmagnesium bromide which on subsequent hydrolysis yielded the pseudobase (I) which reacted with hydrochloric acid giving the flavylium chloride (II). The latter was demethylated with hydriodic acid and subsequent treatment with silver chloride gave pelargonidin chloride.

I6./ The....

The method was difficult and had a very limited application.

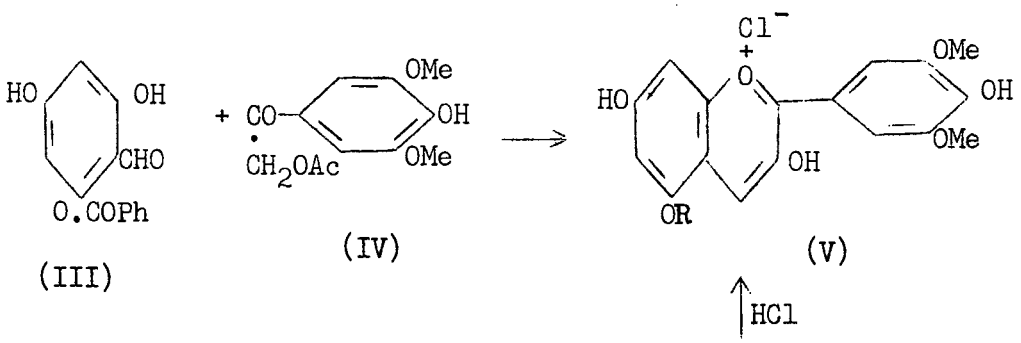


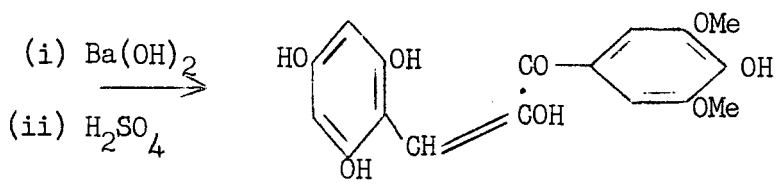
pelargonidin chloride

A new technique for the synthesis of anthocyanidins was later developed by Robinson et al. (3I) and although their original methods led to products in which the substituent groups were either all hydroxyl or methoxyl groups, modifications were introduced whereby all anthocyanidins could be prepared (2I, 32-34).The basic reaction involves the condensation between O-hydroxybenzaldehyde and acetophenone e.g., a monobenzoylphloroglucinaldehyde (III), from phloroglucinaldehyde and benzoyl chloride, when condensed with an ω-acetoxyacetophenone (IV) yields a benzoylated anthocyanidin (V,R = COPh). This condensation is accompanied by deacetylation. Treatment with barium hydroxide removes the benzoyl residue but opens the pyrylium ring to give a chalcone (VI) as the barium salt. Ring closure to

I7./ malvidin....

malvidin chloride (V,R = H) was effected by addition of sulphuric acid equivalent to the barium salt, followed by hydrogen chloride.

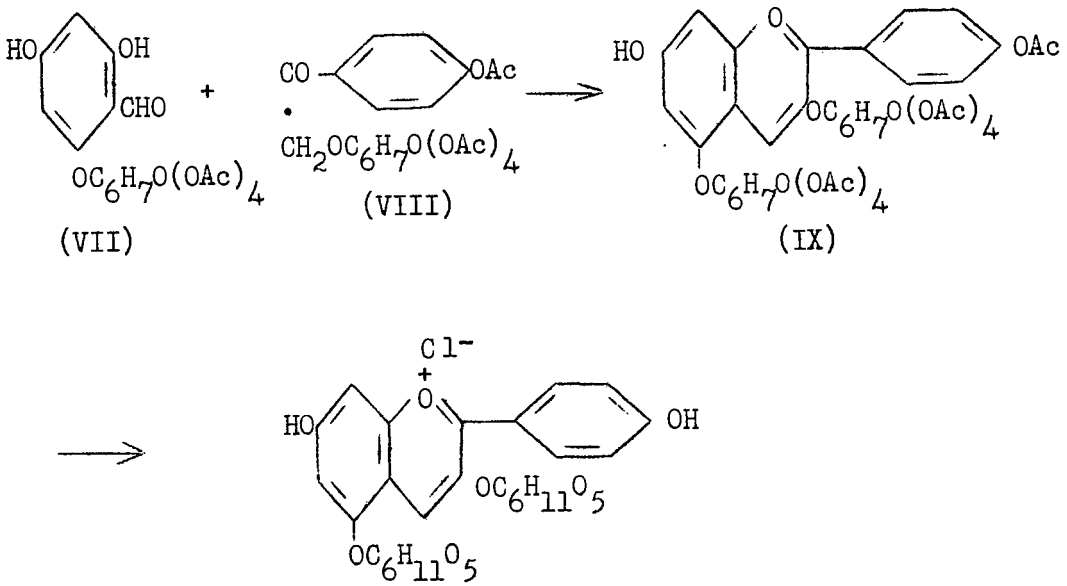




(VI)

By the use of suitable glucosated intermediates it was possible to prepare the isomeric β-glucosides of any particular anthocyanidin. Phloroglucinaldehyde with acetobromoglucose yields 2-O-tetra--acetyl-β-glucosidophloroglucinaldehyde (VII), which in the presence of ethyl acetate and hydrogen chloride, condenses with ω-O-tetra--acetyl-β-glucosidoxy-4-acetoxyacetophenone (VIII) to give the acetylated pelargonidin (IX), from which pelargonin chloride (X) can be obtained (see: p. I8).

I8./ III,F. Chromatographic....



(VII) (VIII) (IX)

(X) Pelargonin chloride

III,F. Chromatographic Properties of Anthocyans

Bate-Smith and Westall (35) showed that the R_f values of the C_{15} polyphenols can be correlated with the number of substituent groups. This relationship of which the amino acids (36) provide a notable example, follows from the relationship predicted by Martin (37). The R_f values of anthocyans were thus found to be influenced by the various substituent groups as follows:

(i) Hydroxylation

An increase in the number of hydroxyl groups in the anthocyanidin molecule, decreases the R_f value in both alcoholic and aqueous solvents. The R_f value therefore

I9./ decreases....

decreases with an increase in the polarity of the compound in polar solvents.

(ii) Methylation

Methylation reverses the effect of hydroxylation and the R_f value rises with successive methylation of hydroxyl groups, irrespective of their position of attachment. As a rule, the rise in R_f per unit hydroxyl methylated is about one third of the rise which would result from the complete removal of the hydroxyl group.

(iii) Glycosidation

There is a direct relationship between the R_f value and the number of sugar residues, which is quite independent of the nature of the anthocyanidin. In aqueous solvents the effect of glycosidation is to increase R_f values, in BAW or Bu-HCl (see: list of solvents and abbreviations) the exact reverse occurs. Glycosidation of a hydroxyl group e.g., with glucose in whatever position, usually causes a large decrease in R_f value with respect to the corresponding anthocyanidin, in butanolic solvents. Glycosidation of a second hydroxyl group causes a further decrease in R_f which is almost as large as that caused by the first sugar group.The attachment of a second sugar group to one already present e.g.,

20./ a 3-diglucoside....

a 3-diglucoside, causes a lesser fall in R_f than e.g., a 3,5-diglucoside. Glycosidation with rhamnose has an irregular effect on R_f value and the expected decrease is counterbalanced by the effect of the terminal methyl group in increasing the R_f value.

(iv) Acylation

This causes an increase in R_f value in butanolic solvents, but lowers the R_f in aqueous solvents. This effect is the reverse of that shown by glycosidation. The chromatographic behaviour of acylated anthocyanins will be further discussed in the section dealing with the acylated grape pigments (VIII,A).

III,G. The Flavan-3-ols

The natural occurrence of this group of polyphenols was illustrated by the work of Roberts and Wood (38). They examined green tea leaf polyphenols by paper chromatography and concluded that (+)-catechin and (+)-gallocatechin occur naturally in the tea leaf. The presence of the corresponding ± compounds was attributed to partial epimerisation. (-)-Gallocatechin gallate originating from (-)-epigallocatechin gallate by epimerisation was also identified. Catechins therefore do not appear in nature as glycosides, but either in the free state or esterified with gallic acid.

It is interesting to note that the catechins present in green

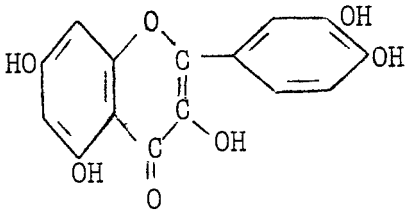
21./ tea....

tea leaves are also those constituting the grape tannins (39,40). In young wines they are responsible for the astringency, however, in older wines a crust developes due to oxidation and other changes in the tannins which make them insoluble. Their basic structure is so similar to that of the anthocyanidin pigments that a common biogenesis seems very probable.

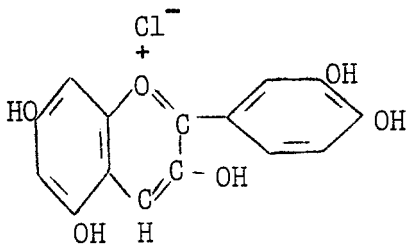
Catechins are the most easily oxidised of all the flavonoid compounds. They lack the conjugation between the A and B rings owing to the complete reduction of the pyrane ring.

The earlier investigations led to conflicting formulae for the catechins but the formulation of catechin as a reduction product of quercetin (4I) was substantiated by later workers , particularly Freudenberg and his collaborators. The pentahydroxy flavan structure for catechins has been confirmed in a number of ways. If examples of these compounds are considered in which the phloroglucinol and catechol nuclei are linked by a 3-carbon chain , the most highly oxidised is the flavonol quercetin and the most highly reduced is the catechin with cyanidin chloride and luteolin as intermediate stages. Thus cyanidin chloride with catalytic reduction, requires two molecules hydrogen for conversion to (±)-epicatechin (42). The reverse process was effected by Appel and Robinson (43). As a result of these investigations the catechins were designated as phenolic flavan-3-ols.

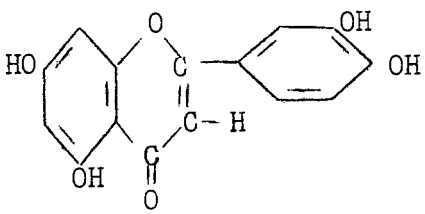
22./ The....



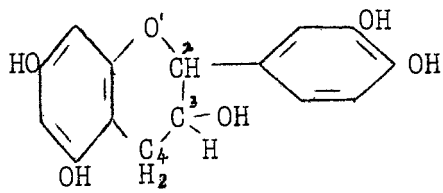
quercetin



Cyanidin chloride



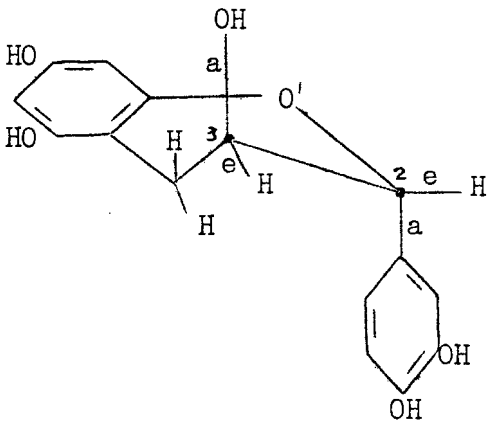
luteolin



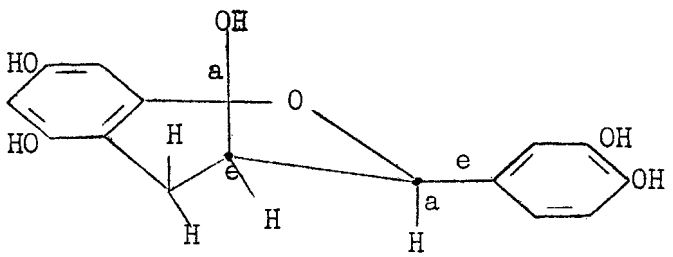
catechin

The stereochemical relationship of the diastereo-isomeric catechins and epicatechins has also been established (44,45). This assignment of configuration requires that the epimerisation of catechin to epicatechin, involves inversion of the 2-aryl and not the 3--hydroxyl group (see: conformations p. 23). Birch et al. (45) also reported that hydroxyl stretching frequencies of (+)-catechin and (-)-epicatechin tetramethyl ethers, indicate strong intramolecular hydrogen bonding of 3(a)-hydroxyl groups. Catechins thus have the preferred conformation 2(a)-aryl, 3(a)-hydroxyl and the epicatechins 2(e)-aryl, 3(a)-hydroxyl which also accounts for their chemical properties.

23./ The....



Catechin (Trans compounds)



Epicatechin (Cis compounds)

The oxidation products of monomeric catechins have also been investigated (46,47). Roberts and Meyers (48) studied the enzymic oxidation of substrates isolated from the tea leaf and the results of individual oxidation studies indicated that the phenolic oxidation products found in black tea, could originate either from (-)- epigallocatechin or (-)-epigallocatechingallate or from a mixture of these two substances. In their search for such products (48) they isolated theaflavin together with thearubigin from dry leaves, the former being considered a well defined chemical substance.

24./ It....

It should be noted that the monomeric catechins are themselves non tannins although they are strongly astringent. It is only in the polymeric form that they become true tannins.

III,H. Flavan-3:4-diols

Rosenheim (49) isolated a water soluble substance from young vine leaves which on treatment with hot hydrochloric acid (20%) yielded a coloured material regarded as anthocyanidin. He called these generators of anthocyanidins leucoanthocyanidins.

Robinson and Robinson (50) found that leuco pigments are widely distributed in plants e.g., the green leaves of the Corinth vine and the colourless (green) flesh of Alicante and Almeria grapes gave cyanidin on acid treatment. The coating of the seeds were also found to be rich in leucocyanidin. Bate-Smith (51) established the almost ubiquitous occurrence among higher plants and a remarkable feature of the results was that with a few exceptions, the leucoanthocyanidins (-nins) yielded only cyanidin and delphinidin when digested with hydrochloric acid.

A positive phlobaphene test is characterised by the formation of an insoluble reddish brown precipitate on treatment of the material under investigation with hot dilute hydrochloric acid. The precipitate dissolves in butanol or pentanol to give a red solution, which will reveal a small amount of anthocyanidin on chromatography. In aqueous medium polymer formation is the main reaction and very little

25./ anthocyanidin....

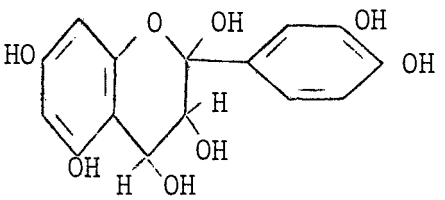
anthocyanidin is formed. Bate-Smith (51) found that if the anthocyanidin pigmentation is not excessive, leucoanthocyanins might still be detected by a comparison with a control prepared from an aqueous extract of tissues, hydrolysed with 2N hydrochloric acid. Roux (52) remarked that these conditions of generating the anthocyanidins is satisfactory to demonstrate the widespread distribution of leuco compounds, but in the presence of excess of other flavonoids, simultaneous formation of phlobaphene occurs. The latter not only obscures the colour of the anthocyanidin, but may cause interference on paper chromatograms due to a brown phlobaphene streak.

Roux (52) supplemented the above methods for the easy recognition of leucoanthocyanins by the use of toluene-p-sulphonic acid as chromogenic spray after two-dimensional paper chromatography. Improved conditions for generating anthocyanidins from leucoanthocyanidins were also developed by Pigman et al. (53), using hydrochloric acid in wet isopropanol solution under reduced pressure, which restricts phlobaphene formation due to the limited accesability of oxygen.

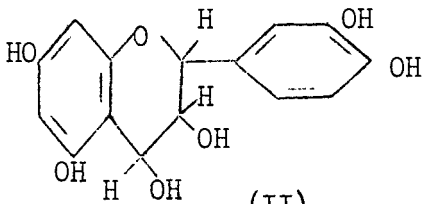
The formation of anthocyanidins from their leuco compounds, demands more vigorous conditions than are normally required for the regeneration of the flavylium salts from the related carbinols, consequently, despite the close relationship of the two series, leucoanthocyanidins cannot be identical with the ordinary pseudo bases. Robinson and Robinson (50) accordingly proposed the following structure (I) for

26./ leucocyanidin....

leucocyanidin which takes the form of a hydrated carbinol at the same oxidation state as that of the corresponding flavylium salt.



(I)



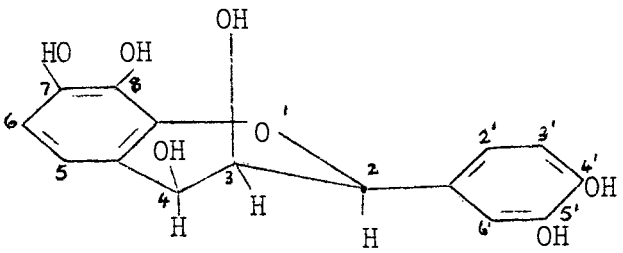
(II)

Although the flavan-3:4-diols were for many years considered to be the prototypes of all condensed tannins, Bate-Smith and Swain (54) pointed out that the phlobaphene test is not given by the catechins and the colour of the precipitate which formed on treatment with hot dilute mineral acid was cream or greyish brown. Bate-Smith (55) tentatively suggested the following formula (II) but no further evidence was then available to choose between the two.

Definite information as to the nature of leuco compounds was first obtained by King and Bottomley (56) through the isolation of a new phenolic compound ($C_{15}H_{14}O_7$) melacacidin (III) from Australian Blackwood. On boiling with hydrochloric acid it afforded a cherry red solution due to an anthocyanidin which was shown to be a 3,7,8,3',4'--pentahydroxy flavylium chloride (57).

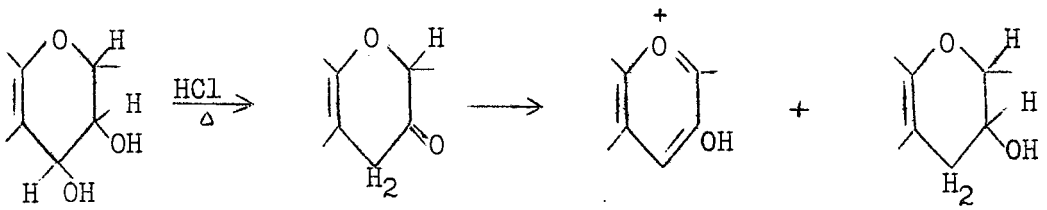
About the same time Forsyth (58) examined cocoa bean polyphenols. He studied the action of mineral acids on cocoa leucocyanidin

27./ and....



(III)

and detected catechin and epicatechin among the reaction products. The tannin reaction of the so called leucoanthocyanins (-idins) might therefore be due to catechins formed during the conversion of the leucoanthocyanidins to anthocyanidins by a compensating reduction. Thus the conversion of flavan-3:4-diols to anthocyanidins is regarded as dehydration of the 3:4-diol group on the heterocyclic ring i.e., loss of hydroxyl at the 4-position and hydrogen at the 3-position, followed by disproportionation to give the oxidised flavylium salt and the reduced catechin (58,59).

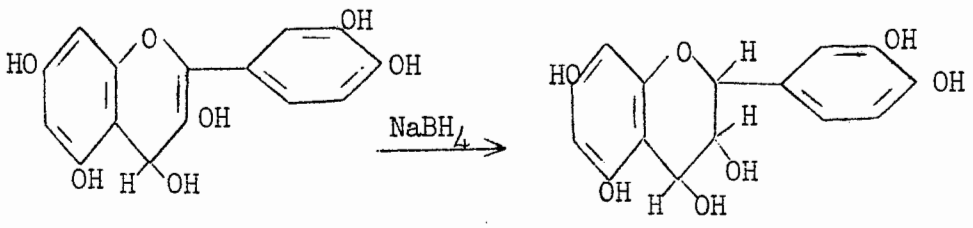


Flavan-3:4-diol ketoflavan flavylium salt catechin

Structural proof for the constitution of leucocyanidin was substantiated by the work of Swain (60) who synthesised 5,7,3',4' --tetrahydroxy flavan-3:4-diol from dihydroquercetin by

28./ reduction....

reduction with sodiumborohydride and showed that on treatment with hot dilute hydrochloric acid, cyanidin and a brown phlobaphene precipitate is produced.



dihydro quercetin leucocyanidin

From the oenological viewpoint the leuco compounds, in par - ticular leucocyanidin, have important properties. They are strongly astringent and therefore contribute to the organoleptic character of wines. Another important attribute of these substances is their vitamin P activity (61).

Red wines contain a significant quantity of leucocyanidin which is present in the same general order as that of the anthocyanins proper. It has been reported that the leucocyanidin present in wine and originating from the skins is negligible. These workers demonstra - ted that the seeds of grapes are rich in leucocyanidins which seemed a logical explanation (62).

III,I. Flavones

These yellow pigments occur naturally in the free state viz.

29./ anthoxanthidins....

anthoxanthidins, as glycosides or associated with tannins. Two pigments belonging to this group have been characterised in white wines (63). Both are glycosides of the aglycone quercetin viz. quercetrin, a monoglucoside (with rhamnose as the sugar) and rutin, a diglucoside (with one rhamnose and one glucose adduct).

III,J. Co-Pigments

Wilstätter and Zollinger (64) observed that the addition of tannin to a solution of oenin chloride in dilute hydrochloric acid, intensified the colour and produced a tone having a much more bluish red. Gallic acid had a similar but weaker effect. Robinson and Robinson (2, 20) reported that co-pigments exist for all types of anthocyanins and remarked that great changes in the colour of varieties or species are not brought about by changes in the pH of the cell sap, but rather by changes in the nature of the anthocyanin.

Although co-pigments have not been identified for all types of anthocyanins, it is generally assumed that tannins, flavone or flavonol glycosides and the formation of complexes with organic substances (and possibly with metals such as iron), play an important part.

III,K. Relationship Between Chromatographic Behaviour and Oxidation State of Flavonoid Compounds

The latest theory on the relationship between the oxidation state and the mobility during chromatography of the C_{15} polyphenols, stresses the importance of the stereochemical factors of these

30./ substances....

substances. The explanations put forward are in agreement with certain experimental evidence (65).

As a result of extensive paper chromatographic studies on the behaviour of phenolic substances in water or aqueous solvents containing up to 2% acetic acid, it has been established that flavonoids with an approximately planar structure have a zero R_f in water. This theory was first put forward by Roux (66) who stated that during "adsorptive" separations in water, the mobility of the C_{15} compound is apparently dependent on the non planar nature of the compound.

In water the R_f values of flavonol glycosides are significantly greater than zero and it was originally assumed that the increased R_f was due to combination with water soluble sugar residues. It was, however, found that both kaempferol 4'-arabinoside and quercetin 4'-glucoside have zero R_f values. Zero R_f was also recorded in water for quercetin 7-glucoside (4).

The introduction of a sugar residue in the 4' or 7'-position of a flavonol does not destroy the planarity of the molecule, whereas a sugar in the 3-position causes steric hindrance and prevents it from lying in the same plane. Glycosidation thus only increases the R_f value in aqueous solvents if in the 3-position, so that it appears that the increase is a consequence of loss of planarity and not to combination with a large water soluble group.

The view was also put forward that a planar structure may

31./ be....

be a factor which determines the ability of a flavonoid to function as a substrate for plant polyphenol oxidases. It was shown by Baruah and Swain (67) that potato polyphenol oxidase catalysed the oxidation of quercetin, myricetin and luteolin but that the 3-glucosides of quercetin and myricetin were not oxidised. Barauh and Swain furthermore demonstrated that the 7-diglucoside of luteolin also undergoes oxidation in the presence of potato oxidase. This confirmed and extended the earlier work by Roberts and Wood (68). The results suggest that plant oxidases act preferentially on flat molecules, a planar structure facilitating adsorption by the active enzyme surface.

The catechins, flavan-3:4-diols and flavanones possess a non planar structure and have positive R_f values in water but are readily oxidised enzymatically. The flavans are particularly susceptible to oxidation but it is significant that the catechins and gallocatechins are not as readily oxidised as the corresponding epicatechins and epigallocatechins (48). It has been shown that the epicatechins have the preferred conformation 2 (e)-aryl, which renders them more nearly planar than the catechins and this conformation could therefore explain the lower mobility and greater ease with which they are oxidised.

Flavonoid compounds which are nearly planar e.g., anthocyanidins, flavonols, aurones and chalcones, do not migrate in water. This apparent affinity for the cellulose has been ascribed to planarity

32./ and....

and to special adsorption effects associated with a planar structure (65,66,69). After further investigations concerning the chromatographic behaviour of some stereochemically interrelated flavonoids in aqueous medium , Roux (70), concluded that it appears more likely that planarity and low solubility are also associated properties in the C_{I5} - group.

33./ CHAPTER IV....

CHAPTER IV

PREPARATION AND STORAGE OF SAMPLES

IV,A. General

Plant tissues usually contain either general or spesific glycosidases, as well as enzymes capable of modifying cellular constituents e.g., polyphenol oxidases of fruits. Autolytic processes may also ensue subsequent to collection of the fresh material, resulting in either the hydrolysis of glucosides, or the destructive oxidation of sensitive compounds. Autolysis may thus result in the production within the cells of aglycones corresponding to glycosides originally present and subsequent isolation of these hydrolytic artefacts may lead to erroneous results.

Immediate and rapid drying of plant material usually preserves it in a form substantially equivalent to the fresh material and thoroughly dried and stored material (4) may be kept without danger for extended periods.

IV,B. Preparation and Storage of Grape Skins

A representative cluster, selected from each variety to be studied, was washed under running tap water to remove dust particles. The skins were removed from the berries with forceps and washed several times with distilled water until free from loose flesh particles. The skins were patted dry between sheets of filter paper and stored as follows:

34./ (i) Storage....

(i) Storage of fresh skins

Pyrex test tubes (25 x 200 mm.) were filled about one third with fresh skins. The tubes were constricted near the open end and the upper portion removed. A short piece of $\frac{1}{4}$ inch tubing was joined to each capsule and the latter evacuated and then filled with nitrogen. After repeating this procedure six times, the capsules were finally sealed under vacuum and stored in a deep freeze (-8° C.).

(ii) Storage of dried skins

The fresh skins, after removal from the berries and washing, were immediately frozen for a few hours and then desiccated for twelve hours over anhydrous calcium chloride at a pressure of 25 - 30 mm. The dried product was ground to a fine powder in a mortar and stored in stoppered specimen tubes in the dark. The samples which were stored frozen under vacuum were treated in a similar manner when required. Both methods gave excellent results.

IV,C. Ripeness of Samples

A portion of each sample was crushed and the juice used for the determination of sugar by measuring the refractive index with a Hilger Abbe Refractometer. The total acidity of the juice was

35./ determined....

determined by titration with 0.3333 N sodium hydroxide to pH value 7.0.

TABLE I

Percentage Sugar and Total Acidity of Samples

Variety	% Sugar	Total acidity as gm. tartaric acid /litre
Hermitage	20.4	4.5
Cabernet sauvignon	22.4	6.2
Schiraz	2I.3	7.4
Pinotage	23.0	6.7
Pontak	I9.4	5.7
Alicante bouschet	I9.7	6.0

36./ CHAPTER V....

CHAPTER V

THE AGLYCONE COMPONENTS

V,A. Introduction

Many glycosides when present in plant extracts, will yield the same aglycone when subjected to acid hydrolysis. It is thus advisable to investigate the parent aglycones first, since they are then usually relatively few in number.

The anthocyanins were extracted with dilute hydrochloric acid (I9,7I,72), since this permits hydrolysis to be performed directly on the extract. After hydrolysis the aglycones were extracted from the hydrolysate, purified, separated and identified in two different solvent systems.

V,B. Preparation of Anthocyanin Extract

A portion (0.20 gm.) of the powdered skins was transferred to a thick walled test tube. Purified fine white sand (ca 0.5 gm.) was added and the mixture moistened with a few drops hydrochloric acid (I%). The mixture was ground to a fine paste and a further portion of the acid (I0 ml.) was added (2,3,5). Air inside the tube was displaced with nitrogen and the tube stoppered. Extraction was allowed to continue for twelve hours in the dark at room temperature. The aqueous extract was subsequently decanted and stored under nitrogen in a deep freeze (-8° C.). The residue was extracted with a further portion of the acid (5 ml.) for another twelve hours.

37./ V,C. Quantitative....

V,C. Quantitative Acid Hydrolysis of Anthocyanins

The combined anthocyanin extract was filtered through a Wil-statter filter, using a paper disc cut from Schleicher and Schull No. 595 filter paper and transferred to a glass stoppered test tube. The extract was made I N with respect to hydrochloric acid, by addition of 4 N hydrochloric acid. Air inside the tube was displaced with nitrogen and the tube securely stoppered. The mixture was heated on a water bath in semi-darkness for one hour at IOO° C. (73). Under these conditions hydrolysis rapidly occurs at each glycosidic link in the flavonoid (74).

(i) Extraction of aglycones from hydrolysate

The hydrolysate was cooled and filtered through a Wil-statter filter to remove insoluble polymeric material (also known as phlobaphene) produced during the acid hydrolysis (52,54,58,75,76). The aglycones were extracted with portions n-hexanol (I.0 ml.) and separated from the aqueous layer (73). The combined alcoholic extract was diluted with twelve volumes benzene and the precipitated anthocyanidins re-extracted with portions aqueous hydrochloric acid (I.0 ml., I%) and separated from the benzene layer (2).

(ii) Evaporation of anthocyanidin extract under reduced pressure

The combined hydrochloric acid extract was evaporated to

38./ dryness....

dryness in the dark at room temperature over anhydrous calcium chloride and solid sodium hydroxide, at a pressure of 25 - 30 mm. All anthocyanidin and anthocyanin solutions which were either concentrated or evaporated to dryness, were treated in a similar manner.

V,D. Solvents for the Separation of Anthocyanidins

Anthocyanidins which are applied as coloured spots fade during irrigation with the upper phase of BAW. The probable explanation is that the anthocyanidin which is applied to the paper as the oxonium chloride, ionises during the conditions of the run and that the free base travelling faster than the chloride ion, gradually forms a colourless compound (35).

The choice of solvents for anthocyanidins is thus limited by the fact that they are less stable than the anthocyanins and in order to prevent them from fading during chromatography, it is necessary to maintain low pH. This was originally achieved by using the upper phase of Bu-HCl (35). Separation of anthocyanidins in this solvent is, however, not satisfactory due to tailing of the pigment (7I).

A solvent developed by the Forestal Laboratories and now well known as forestal solvent was found to give better defined spots and more consistent results (5I). Forestal solvent, being an aqueous system, is not sensitive to temperature and it is relatively insensitive to composition of solvent. Consequently, equilibration with the

39./ solvent....

solvent vapour is not necessary to obtain consistent results (77).The use of a variant of the Forestal solvent, 80% formic acid - hydrochloric acid - water (5:I:4 v/v) giving promising results, has also been reported (78).

V,E. Separation of Anthocyanidins in Forestal Solvent

The anthocyanidins were dissolved in the minimum volume BEH. The concentrated solution was spotted at 0.7 cm. intervals on a line drawn 3 cm. from one end of Whatman No. 3 MM chromatography paper and dried in a current of air at room temperature. The papers were suspended in a chromatography tank and immediately irrigated with Forestal solvent by the ascending technique. Delphinidin chloride and cyanidin chloride were run on either side as marker substances. All papers were irrigated in the same machine direction and development was allowed to continue for 24 hours in the dark at room temperature. After removal from the tank the solvent front was marked and the papers dried in a current of air at 30° C. The anthocyanidins separated into three discrete pigment bands termed AI, A2 and A3, with AI having the lowest and A3 the greatest mobility (Plate I).

V,F. R_f Values

The R_f values were in all instances calculated as the ratio of the distance travelled by the solute (from origin to centre of spot or band) to that of the solvent front. Average values from a large number of determinations on the components separated in Forestal

40./ solvent....

solvent are presented in Table II, together with their respective colours in visible and U.V. light. The chromatograms were developed at temperatures varying from 2I to 23° C. with fluctuations of not more than 0.5° C. for individual runs.

TABLE II

R_f Value and Colour of Anthocyanidins in Forestal Solvent

Component	R_f	Visible	U.V. Light
Delphinidin	.339	Purple	Mauve
AI	.333	Purple	Mauve
A2	.5I0	Purple	Mauve
Cyanidin	.507	Magenta	Pink
A3	.667	Purple	Mauve

V,G. Elution of Anthocyanidins from Chromatograms

Flavonoids are usually eluted from paper chromatograms by cutting the areas to be eluted into strips having a pointed end. The other end which should have a clear margin, is immersed in a trough containing the solvent, as for descending elution and the eluate is collected in appropriate containers. The procedure is conducted in a

4I./ closed....

closed vessel and may take several hours depending on the length of the paper strips and the solvent used (5,79,80).

Preliminary experiments revealed that the anthocyanidins are best eluted with methanol containing hydrochloric acid (1%). Elution according to the above procedure could be accomplished in approximately three hours from paper strips five inches long, however, during this period the colour of the eluate faded almost completely in spite of elution being conducted in the dark.

(i) Modified procedure for elution

Immediately after drying the forestal chromatograms, the individual pigment bands were cut into $\frac{1}{4}$ inch squares and transferred to small glass stoppered erlenmeyer flasks. Sufficient methanol containing hydrochloric acid (1%) was added to cover the cuttings. Air inside the flasks was displaced with nitrogen and the flasks securely stoppered and shaken in the dark with an automatic flask shaker. Elution was repeated with successive small portions of the solvent and was practically completed within 30 minutes. The eluates from each band were combined and filtered to remove paper particles.

(ii) Evaporation in vacuo at low temperature

Fading of the colour and the formation of brown to black products resulted during evaporation of the

42./ anthocyanidin....

3

2

I

4

PLATE I

Separation of Anthocyanidins in Forestal Solvent

1. Pigment band A I
2. Pigment band A 2
3. Pigment band A 3
4. Cyanidin chloride

I 2 3 4 5 6 7 8

PLATE II

Identification of Anthocyanidins in Butanol : Acetic Acid : Water

1. Delphinidin chloride
2. Band A I (ex Forestal)
3. Petunidin chloride
4. Band A 2 (ex Forestal)
5. Cyanidin chloride
6. Malvidin chloride
7. Band A 3 (ex Forestal)
8. Peonidin chloride

anthocyanidin solutions in vacuo at room temperature. The eluate from band A3 was particularly prone to this phenomena. When redissolving the dry pigment some of these impurities are unavoidably transferred to the chromatograms and produce streaking on development.

These difficulties were, however, overcome by taking the anthocyanidin solutions to dryness in vacuo in a refrigerator (4° C.).

V,H. Identification of Anthocyanidins

Anthocyanidins may be successfully chromatographed in BAW if the paper is previously washed with dilute hydrochloric acid (5). Preliminary experiments revealed that papers irrigated with aqueous hydrochloric acid (4%) and dried at room temperature immediately prior to use, gave satisfactory results.

The dry pigments were dissolved in the minimum volume BEH and spotted on dry acid washed Whatman No. I chromatography paper. Authentic specimens were spotted with each component as shown on Plate II. The chromatograms were irrigated by the descending technique, using the thoroughly separated upper phase of BAW which had been equilibrated for three days prior to use (35). Development was continued for I4 to I6 hours in the dark at room temperature.

Pigment band AI contained only one component which was identical with delphinidin whereas band A2 separated into two components

43./ with....

with R_f values corresponding to petunidin and cyanidin. Band A3 likewise separated into two components, the one of lower R_f being identical with malvidin. The component of higher R_f from fraction A3 was tentatively identified as peonidin since the only other anthocyanidins (5) having comparable R_f values in this solvent, have distinctly different colours.

It is recommended that chromatograms developed with BAW be equilibrated with the vapour of the aqueous phase, for 24 hours, before irrigation commences (5,35). During equilibration, however, considerable fading of the anthocyanidins resulted and the R_f values of both authentic and unknown samples were inconsistent. Chromatograms developed immediately after application of the pigments gave slightly higher R_f values but fading was negligible and the R_f values very consistent. The R_f values presented in Table III were obtained from chromatograms developed at temperatures varying from 21.5 to 23.5° C. Variations for individual chromatograms did not exceed 0.5° C.

BAW is not regarded as a satisfactory solvent for the separation of a mixture containing the following anthocyanidins: delphinidin, petunidin , cyanidin, malvidin and peonidin. In BAW the difference in mobility between any two of these anthocyanidins is, however, of such magnitude, that they will separate as two discrete spots; a mixture of cyanidin and malvidin being the only exception. Separation between the latter two pigments is best accomplished in Forestal

44./ solvent....

solvent.

The absence of cyanidin as a glucoside will be confirmed in the next section dealing with the glucosides and its presence in the hydrolysate is due to the conversion of the corresponding 5,7,3',4'--tetrahydroxyflavan-3:4-diol (50,52,75).

TABLE III

R_f Value of Anthocyanidins in Butanol - Acetic Acid - Water

Component	Equilibrated for 24 hours	Without equilibration	Visible	U.V.Light
Delphinidin	.486	.489	Purple	Mauve
AI	.470	.500	Purple	Mauve
Petunidin	.583	.597	Purple	Mauve
A2 (i)	.56I	.598	Purple	Mauve
Cyanidin	.694	.720	Magenta	Pink
A2 (ii)	.695	.728	Magenta	Pink
Malvidin	.672	.679	Purple	Mauve
A3 (i)	.666	.676	Purple	Mauve
A3 (ii)	.752	.772	Pink	Pink

45./ V,I. Determination....

V,I. Determination of the Relative Concentration of Anthocyanidins

Examination of the aglycones of the six Vitis vinifera varieties showed that they contain the same five anthocyanidins, however, the proportions of each varying with the different varieties. In order to express the relative importance of each constituent , photometric recording (62) of the bands from one-dimensional Forestal chromatograms (Fig.I), followed by integration of the area under the curve was used.

This technique has certain limitations. One-dimensional chromatography does not separate all the constituents and the values obtained do not permit calculation of molecular concentration but instead the proportions of the total optical density of the colour which is contributed by each constituent.

It has been shown that bands A2 and A3 on the Forestal chromatograms each contain two aglycones which were subsequently separated in BAW. As a result of their unstable nature it is virtually impossible to prevent a certain amount of fading during manipulations after the initial separation. Moreover, it became apparent that the highly methylated derivatives e.g., malvidin, fade more rapidly than e.g., delphinidin and photometric recording of the BAW chromatograms would therefore give values which are not in accordance with the relative concentration of aglycones originally present in the hydrolysate and Forestal chromatograms.

46./ (i) Procedure....

(i) Procedure for the determination of the relative concentration

A portion of the dried skins (0.I gm.) was extracted and hydrolysed as described. The resulting anthocyanidins were dissolved in BEH (0.I0 ml.). An aliquot (20 µl.) of this solution was spotted at 0.5 cm. intervals on a line 4.5 cm. long, drawn 8 cm. from one end of Whatman No. 3 MM chromatography paper. The diameter of the spots did not exceed 0.5 cm. This method of placement was preferred to that of banding the pigment solution on the starting line, since the latter method invariably resulted in the bands moving slightly skew on development of the chromatograms.

The chromatograms were irrigated with Forestal solvent as described. Immediately after drying they were cut into strips (3.0 cm. wide) for measurement of the pigment band densities. A Beckmann photoelectric densitometer with a filter system transmittant to light of 500 mµ peak intensity was used.

The relative concentration of the various pigment bands are presented in Table IV. It is significant that the incidence of delphinidin is much lower in Alicante bouschet and Pontak than in the other varieties.

47./ Table IV....

FIG. 1

PHOTOMETRIC RECORDING OF FORESTAL CHROMATOGRAM

A1. Delphinidin
A2. Petunidin + Cyanidin
A3. Malvidin + Peonidin

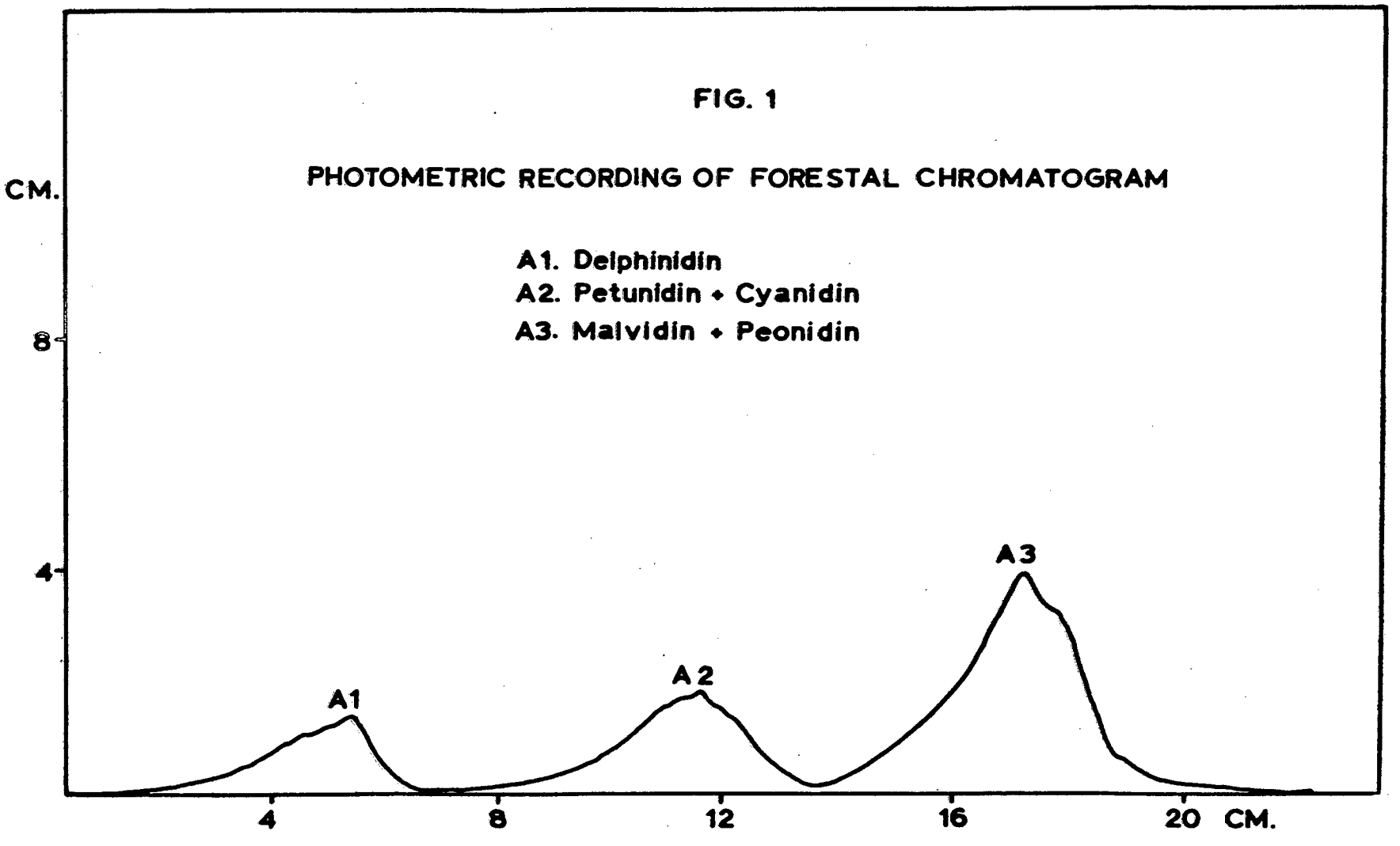


TABLE IV

Relative Concentration of Aglycones

Variety	% Delphinidin	% Petunidin + Cyanidin	% Malvidin + Peonidin
Pontak	4	30	66
Alicante bouschet	4	23	73
Schiraz	10	26	66
Hermitage	10	37	53
Pinotage	12	25	63
Cabernet sauvignon	17	24	59
Average reported values for Vitis vinifera (62)	12	22	66

48./ CHAPTER VI....

CHAPTER VI

THE NORMAL ANTHOCYANINS

VI,A. General

Extracts of plant organs containing anthocyanins can be examined directly by paper chromatography but in such instances the R_f values of the anthocyanins thus separated are not completely reliable. The number of constituents and their position on the chromatogram are affected by the presence of impurities such as flavone glycosides and other alcohol or water soluble substances e.g., free sugars.

The first step in the detailed examination of an anthocyanin is that of isolating it in a pure state. The usual chemical methods of isolation and purification are often tedious and wasteful (81) and do not always yield a pure product (82). As an alternative method paper chromatography provides a rapid procedure of preparing pure anthocyanins on a small scale without much loss.

The general procedure involves the preparation of an extract from the plant material which is then applied directly to several sheets of thick filter paper for chromatography. The anthocyanin bands are then cut out, the pigment eluted severally and the process repeated with the same or other solvent mixtures.

VI,B. Removal of Chlorophyll and Wax from Dry Skins

All powdered skin samples used were exhaustively extracted in the cold with successive small portions petroleum ether (B.P. 30-50° C.).

49./ The....

The mixture was shaken in a test tube and after allowing the material to settle on its own, the supernatant layer was decanted. After the final extraction the contents was transferred to a funnel and the ether allowed to drain. The material was then spread on a sheet of filter paper to dry.

VI,C. Preparation of Anthocyanin Extract

A portion of the plant material (0.2 gm.) was extracted as described using methanol containing hydrochloric acid (10 ml., 1%) as solvent (16,78,83). Extraction was continued for two hours in the dark at room temperature, with frequent shaking. The pigment solution was filtered, concentrated to 4.0 ml. under reduced pressure and again filtered.

VI,D. Effect of Hydrochloric Acid on R_f Value of Anthocyanins

The presence of hydrochloric acid in solutions, influence the R_f value of anthocyanins, which move behind the acid front. In Bu-HCl the hydrochloric acid front can be seen (in U.V. light) occupying a position represented by an R_f value between 0.8 - 0.9 whereas in BAW it is approximately 0.4 (24,35).

During chromatography in BAW an anthocyanin applied as the oxonium chloride, is equilibrated with the acetic acid at higher pH value and is partly converted to the colour base. The salt then travels at a slower rate than the colour base, so that the R_f of anthocyanins applied in hydrochloric acid solution, is slightly less than

50./ that....

that applied in neutral solution. The hydrochloric acid has, however, an advantage, in giving better defined spots (3). In this investigation a uniform practise was adopted by dissolving all anthocyanins in solutions containing hydrochloric acid (I%).

VI,E. Solvent Systems for the Separation of Anthocyanins

Chromatography of anthocyanins is usually conducted in solvent systems containing acid since they are cations and are only stable at acid pH. For solvents which do not contain mineral acid e.g., BAW, it is important that sufficient hydrochloric acid be present in the original extract to keep the anthocyanin in chloride form.

The solvent systems used are mainly of two types, either an aliphatic alcohol such as n-butanol or water containing hydrochloric or acetic acid. In order to determine the number of individual pigments present, the anthocyanin extract was chromatographed in the following solvents:

(i) HOAc - HCl (5)

The anthocyanins separated as a broad zone in this solvent (R_f about 0.27). Three components, all purple in colour, could be discerned but due to the elliptical nature of the spots and tailing, separation between the different constituents was most unsatisfactory.

(ii) Bu - HCl (5)

The anthocyanins separated into six components with

5I./ increasing....

increasing R_f values respectively as follows: 0.I49, 0.I90, 0.250, 0.320, 0.376, 0.720. Components I and 2 (both purple) were rather incompletely separated whereas 3 and 4 (dark purple and pink respectively) were well resolved. Components 5 and 6 (both light purple) formed indistinct diffuse spots.

During the initial stages of chromatography, the two components of highest mobility were present as well defined spots but as irrigation proceeded they gradually spread forming diffuse zones. The spots formed by components I - 4 were slightly elliptical with a certain amount of tailing.

(iii) BAW

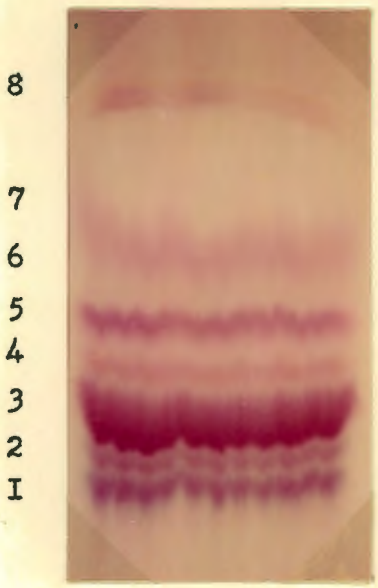
Separation into eight discrete pigment bands termed FI to F8 (in sequence of ascending R_f value) was accomplished in this solvent (Plate III). Although some of the pigment bands were rather close to one another, they were well defined and without any visible tailing. BAW has the further advantage over Bu-HCl, in that much more of the pigment extract may be spotted on the starting line without overloading the chromatograms.

Examination of the BAW chromatograms in U.V. light

52./ showed....

PLATE III

Initial Separation of Anthocyanins in Butanol : Acetic Acid : Water



I. Pigment band F I
2. " F 2
3. " F 3
4. " F 4
5. " F 5
6. " F 6
7. " F 7
8. " F 8

showed that the area occupied by the anthocyanins on the chromatogram was free from U.V. fluorescent impurities.

VI,F. Preparative Separation of Anthocyanins in BAW

The concentrated extract was spotted on several sheets of Whatman No. 3 MM paper, in a manner similar to that described for the Forestal solvent. Several applications were made until the spots appeared almost black when viewed against visible light. The chromatograms were developed immediately by the ascending technique for 24 hours in the dark at room temperature. Ascending chromatography was preferred since the slower movement of the solvent effected a slightly better resolution of the large number of pigment bands. The aqueous phase of the solvent was present in the tank to maintain a saturated atmosphere.

Average R_f values for the initial separation at 15° C. are presented in Table V. No attempt was made to develop these chromatograms under standard conditions, moreover, the R_f values were substantially affected by the amount of extract spotted on the starting line. The values should therefore be regarded as indicative only of the degree of resolution.

VI,G. Location of Pigment Bands

Normally all eight pigment bands were visible in ordinary light, however, U.V. light may be used with advantage to locate the

53./ margins....

margins of individual bands. The pigment present in band F6, which occurs in very low concentration, was not always clearly demarcated and was located by cutting a narrow strip from either side of the chromatogram. After exposure of these strips to ammonia vapour band F6 appeared yellow in visible light (probably due to impurities) whereas the remaining anthocyanins showed varying intensities of blue. After several weeks on the paper chromatogram F6 turned blue after exposure to ammonia vapour.

TABLE V

R_f Value of Anthocyanins Present in Crude Plant Extract

Pigment	R_f	Visible light	Ammonia vapour Visible light	U.V.Light
FI	.237	Blue	Light blue	Dull blue
F2	.26I	Blue-purple	Light blue	Dull blue
F3	.299	Dark purple	Dark blue	Dark purple
F4	.343	Pink	Light blue	Dull blue
F5	.390	Dark purple	Dark blue	Dull purple
F6	.420	Light pink	Yellow	Dull blue
F7	.456	Light purple	Light blue	Dull blue
F8	.609	Light purple	Light blue	Dull blue

54./ VI,H. Detection....

VI,H. Detection of the Normal Anthocyanins

The anthocyanin bands F I-8, obtained from the initial separation in BAW, were cut out, the cuttings combined and eluted with WMA (5, I6). The eluate was evaporated to dryness in vacuo and the dry pigment hydrolysed with 2 N sodium hydroxide soln. (VIII, C). The resulting pigment was chromatographed in BAW and Bu-HCl. Only pigment bands F I-4 separated in these two solvents and were henceforth regarded as the normal glycosides (anthocyanins).

VI,I. Purification of the Normal Anthocyanins

(i) Bands F I and F 2

The individual pigment bands were cut out and eluted with WMA. This solvent was preferred to aqueous methanol since the time required for quantitative elution was considerably shorter. The eluate containing the pigment was evaporated to dryness, redissolved in MHB and chromatographed repeatedly in BAW until free from the other pigment bands. This was followed by chromatography in HOAc--HCl, Bu-HCl and again BAW. This procedure will also remove other polyphenols when present (IX, D).

(ii) Bands F 3 and F 4

Repeated chromatography in BAW effectively removed all the other pigment bands but F 3 and F 4 remained contaminated with F 5 and F 6 respectively, the amount of F 5

55./ and....

and F 6 showing a slight decrease with each successive rechromatography. After removal of the other pigments (excluding F 5 and F 6), bands F 3 and F 4 were individually hydrolysed with 2 N soduim hydroxide solution. The resulting pigment was obtained in a pure condition after chromatography in HOAc-HCl, Bu-HCl and BAW.

VI,J. Procedures for the Identification of Anthocyanins

The complete identification of an anthocyanin depends on the determination of the type, position and number of the saccharide groups in the molecule. In anthocyans so far encountered in nature, the sugars vary from simple monosaccharides to unidentified trisaccharides and are attached to one or two of the three oxygen atoms at the 3,5,7-positions.

The identification of anthocyanin monoglycosides usually present no difficulties, since glycosidation always occurs at the 3-position. When more than one sugar is present at one or more of these positions, it is often more difficult to locate the individual positions of attachment without ambiquity.

(i) Paper chromatography

An unknown anthocyanin may be characterised by careful comparison of its R_f value in several solvent systems, with that of well characterised glycosides of the same anthocyanidin. In the event of it being identical

56./ with....

with one of the authentic samples, co-chromatography will confirm this. If the unknown compound is not identical with any of the known pigments, the data should still provide an indication of the number of sugar residues present.

(ii) Spectral investigation

Spectral investigation of anthocyanins provide information to distinguish between the three main groups, represented by pelargonidin, cyanidin and delphinidin and also between the two main glycosidic classes. The spectral characteristics of a particular anthocyanin is related to the anthocyanidin from which it is derived. The three main groups are spectrally well differentiated e.g., pelargonidin: λ max. 520 mμ, cyanidin: λ max. 535 mμ, and delphinidin: λ max. 544 mμ (I6).

Glycosidation of these anthocyanidins cause a shift of the absorption maxima in the visible region towards shorter wavelengths. The amount of this hypsochromic shift is largely unaffected by the nature of the sugar residue, but depends on which and how many of the hydroxyl groups are glycosidated. Glycosidation in the 3-position causes the largest shift e.g., pelargonidin: $\Delta\lambda$I5 mμ, cyanidin: $\Delta\lambda$I2 mμ and delphinidin: $\Delta\lambda$I0 mμ. Glycosidation in

57./ the....

the 5-position only causes a hypsochromic shift of about 7 mu. Thus only very small differences in the spectral maxima can be detected between related groups of anthocyanins which contain sugars in the 3-position, and those which have sugars in both the 3 and 5-positions (I6).

It has been observed that the absorption spectra of all anthocyans with sugar or benzoyl residues on the 5-hydroxyl group, show characteristic differences from those in which the 5-hydroxyl group is free. The latter group all show a distinct shoulder to the main absorption peak in the 4I0 - 450 mμ region (Fig. II). Another important characteristic is that the ratio of the optical density at 440 mμ, to that at the wavelength of maximum absorption in the visible region, as a percentage, is relatively constant for all 3-glycosides related to one particular group, but differ significantly between the three main groups e.g., pelargonidin 3--glycosides 39%, cyanidin (including peonidin) 3-glycosides 23%, and delphinidin (including petunidin and malvidin) 3-glycosides I8%.

In addition the above percentages, for a 5 or 3,5--glycoside is about half that of the corresponding

58./ anthocyanidin....

anthocyanidin in which the 5-hydroxyl group is free (16).

U.V. light fluorescence will provide further evidence regarding the two main glycosidic classes, since most anthocyanins with sugars in both the 3 and 5-positions show strong U.V. light fluorescence (5).

The presence of O-dihydroxyl groups in anthocyans, which is also useful for identification purposes, may be detected by the use of aluminium chloride and measurement of the resulting spectral shift of the main absorption peak (16,84).

(iii) Partial acid hydrolysis of anthocyanins

Anthocyanins can be further characterised by studying on paper chromatograms the simpler glycosides produced during partial acid or enzyme hydrolysis (73,85). The number and identity of the simpler glycosides provide information regarding the number and position of the sugar residues in the original glycoside . Thus a 3-diglucoside will, in addition, yield an intermediate 3-glucoside, whereas a 3,5-diglucoside gives a 3 and 5--monoglucoside. Anthocyans containing three sugar residues give two or four simpler glycosides (86).

Partial acid hydrolysis of a purified anthocyanin is usually performed in dilute hydrochloric acid at 100° C.

59./ for....

for 10 to 15 minutes (24,73,79) and the resulting solution chromatographed directly in BAW, Bu-HCl, HOAc--HCl or other suitable solvents for the separation of anthocyanins. The intermediate glycosides are then examined individually by the techniques described and by quantitative acid hydrolysis and identification of the sugars liberated.

More recently, attention has been drawn to the fact that variations occur in the stability of sugar-sugar links and the sugar-aglycone links (74). This limits the general applicability of partial hydrolysis techniques, using hydrochloric, formic or acetic acid, since the favoured points of attack may vary from flavonoid to flavonoid.

New techniques which have been developed and adapted for work on a microscale, include the following (74):

(a) Spesific identification of saccharides attached to the 3-position by oxidation with hydrogen peroxide.

(b) Identification of all saccharides attached to cyclic systems which are readily ruptured on oxidation with potassium permanganate.

(c) All saccharides present in phenolic and enolic glycosides, which would arise from rupture of the molecule

60./ at....

at double bonds by ozonolysis.

These techniques have the advantage that the reaction products containing the liberated sugars, may be used directly for paper chromatography.

(iv) Enzymatic hydrolysis

The release of an intact polysaccharide group, using the enzyme rhamnodiastase, has been reported (74). Another method of locating a monosaccharide, when both a polysaccharide and a monosaccharide are present, depends on the preferential hydrolysis of phenyl-monosaccharides by anthocyanase, an enzyme prepared from certain aspergillus species (85).

(v) Determination of aglycone:sugar ratios

This procedure entails quantitative hydrolysis of the anthocyanin and determination of the aglycone concentration spectrophotometrically by comparison with standard solutions of the same anthocyanidin (73,79), or through reference to published values (87).

The sugars present in the hydrolysate, after separation by paper chromatography, may be determined by one of the following techniques: Somogyis copper micro method (88), the anthrone reagent (89), the presulphonated reaction (90) or by a modification of the method of

61./ Wilson....

Wilson (9I) and spectrophotometric determination of the molar concentration of the coloured products of the individual sugars, after treatment with aniline phosphate (92) on the paper chromatograms.

VI,K. Identification of Pigments FI - 4

Prior to spectral and chromatographic investigation, the purified anthocyanins were finally tested for purity by chromatography in Bu-HCl, BAW and HOAc-HCl on Whatman No. I paper. All pigments migrated as one discrete spot in these solvents. Quantitative acid hydrolysis of the individual pigments gave one aglycone each, corresponding to delphinidin, petunidin, malvidin and peonidin.

(i) Measurement of the absorption spectra

For the purpose of measuring the absorption spectra of the anthocyanins, the purified dry pigments were dissolved in methanol containing hydrochloric acid (3 ml., 0.0I%) and the solutions diluted if necessary to give an optical density reading in the range 0.6 - I.0 (I6). Measurements of the absorption spectra were made with a Zeiss PMQ II Spectrophotometer.

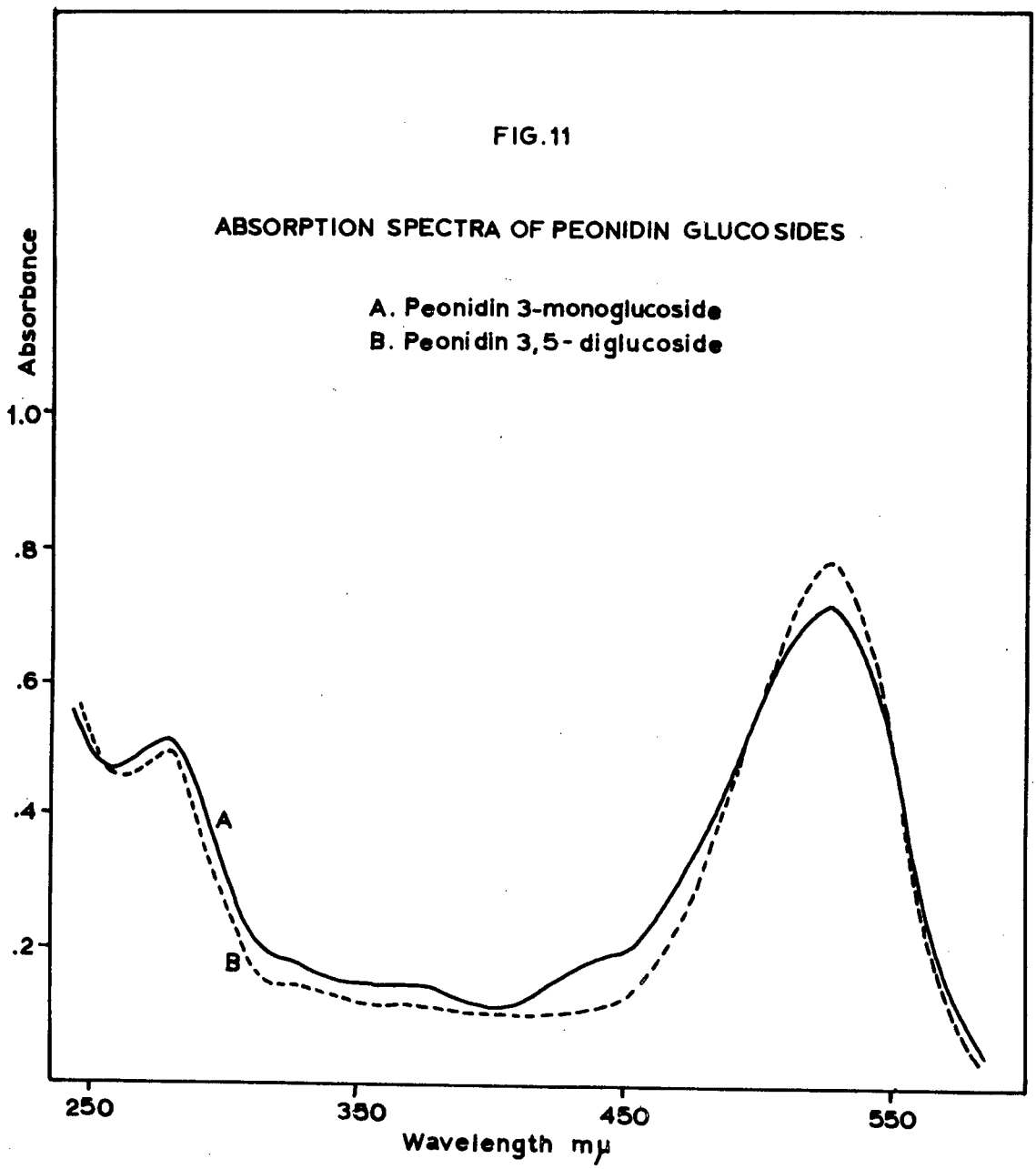
Pigments FI-3 exhibited similar absorption in the visible region, with λ max. at about 535 mμ. Pigment F4 showed maximum absorption at about 525 mμ (peonidin 3--glucoside: λ max. 523 mμ). This however, does not

62./ distinguish....

FIG. 11

ABSORPTION SPECTRA OF PEONIDIN GLUCOSIDES

A. Peonidin 3-monoglucoside
B. Peonidin 3,5-diglucoside



distinguish peonidin 3 -monoglucoside from other cyanidin derivatives having a sugar attached in the 3-position. Fortunately these two anthocyanins have distinctly different chromatographic properties and colours and as will be shown, the latter does not occur as a glycoside in grape skins.

In addition, FI-4 all showed a distinct shoulder to the main peak in the 410 - 450 mμ range(Fig. II) thus indicating that sugars are attached to the 3-position only. The percentage ratio of the optical density at 440 mμ, to that at the wavelength of maximum absorption in the visible region viz. 17.5% for FI-3 and 25% for F4, is in agreement with published values and is a tentative indication that the former group is related to the delphinidin and the latter to the cyanidin series (VI,J. (ii)).

(ii) Paper chromatography

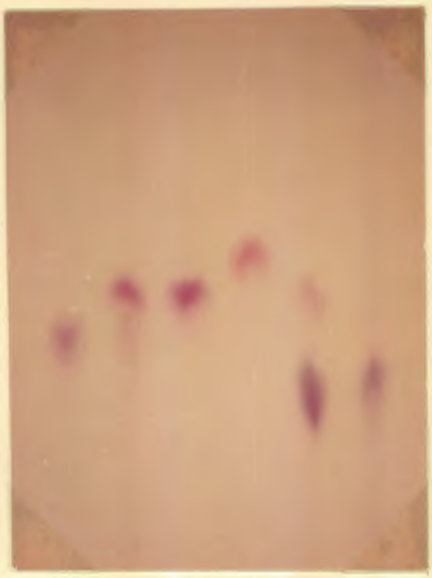
The purified dry pigments were dissolved in the minimum volume MBH and sufficient of the concentrated solution was spotted on Whatman No. I paper to produce a well coloured spot. The anthocyanin specimens (delphinidin 3-monoglucoside containing malvidin 3-monoglucoside and malvidin 3-monoglucoside containing petunidin

63./ 3-monoglucoside....

PLATE IV

Identification of Pigments F I-4 in Butanol : Acetic Acid : Water

I. Pigment F 2
2. (lower)Petunidin 3-monoglucoside
2. (upper)Malvidin 3-monoglucoside
3. Pigment F 3
4. Pigment F 4
5. (lower)Delphinidin 3-monoglucoside
5. (upper)Malvidin 3-monoglucoside
6. Pigment F I

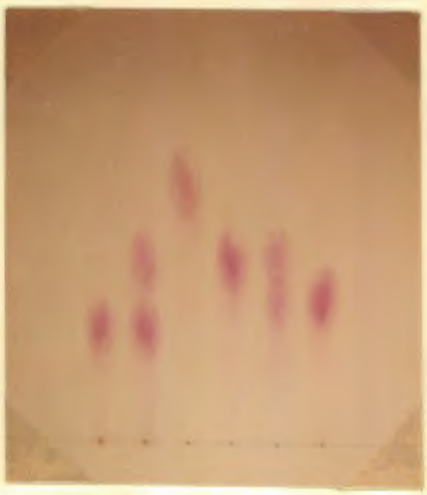


I 2 3 4 5 6

PLATE V

Identification of Pigments F I-4 in Butanol : 2N HCl

I. Pigment F I
2. (lower)Delphinidin 3-monoglucoside
2. (upper)Malvidin 3-monoglucoside
3. Pigment F 4
4. Pigment F 3
5. (lower)Petunidin 3-monoglucoside
5. (upper)Malvidin 3-monoglucoside
6. Pigment F 2



I 2 3 4 5 6

3-monoglucoside) were likewise dissolved in MBH and spotted as illustrated on Plates IV and V. The chromatograms were equilibrated for eight hours with the vapour of the aqueous phase, before irrigation with the organic phase was commenced. Chromatography was conducted in BAW and Bu-HCl by the ascending technique.

FI, F2 and F3 were identical with delphinidin, petunidin and malvidin 3-monoglucoside respectively, with regard to R_f value and colour in the two solvent systems. Identification of F4 as peonidin 3-monoglucoside was based on colour, R_f value and spectral characteristics mentioned above. The identification of the sugars are discussed in Chapter VII.

On account of their chemical constitution and polarity characteristics, delphinidin, petunidin, malvidin and peonidin as well as their corresponding 3-monoglucosides, will always separate in order of ascending R_f value in both aqueous and butanolic solvents.

Chromatograms which were developed at the same temperature, indicated that the presence of one glucose group in the 3-position, produced an average decrease of 42,5% in the R_f value with respect to the R_f value

64./ of....

of the corresponding anthocyanidin. The R_f values of the 3,5-diglucosides (Table XIII) showed an average decrease of 36.5% with respect to the corresponding 3-monoglucosides (see: III,F. (iii)).

TABLE VI

R_f Value and Colour of Pigments FI-4 in BAW and Bu-HCl

Pigment	R_f in BAW (15° C.)	Colour	R_f in Bu-HCl (24.5° C.)	Colour
FI	.288	Blue	.146	Purple
Delphinidin 3--monoglucoside	.279	Blue	.145	Purple
F2	.334	Blue-purple	.188	Purple
Petunidin 3--monoglucoside	.335	Blue-purple	.182	Purple
F3	.387	Mauve	.239	Mauve-purple
Malvidin 3--monoglucoside	.388	Mauve	.236	Mauve-purple
F4	.424	Pink	.328	Pink

VI,L. Confirmation of the Absence of Cyanidin Glycosides

Monoglycosides of cyanidin, when present together with the

65./ anthocyanins....

anthocyanins already identified, would occupy a position on the chromatogram intermediate between F2 and F3 . Bands FI-4, obtained during the initial separation, were cut from the chromatograms without discarding the intermediate zones. The cuttings were combined, eluted, the eluate evaporated to dryness and rechromatographed in HOAc-HCl and BAW. After the final purification the combined eluate was again evaporated to dryness. The dry pigment was redissolved in I N hydrochloric acid (2.0 ml.) and hydrolysed as described. The resulting anthocyanidins were chromatographed in Forestal solvent and BAW. Cyanidin was not detected. Other workers (I5) stated that Vinifera grapes (i.e., European countries) contain up to 2% cyanidin 3-monoglucoside. At this concentration, however, cyanidin may be readily detected if present.

VI,M. The Relative Concentration of Anthocyanins

A pigment extract prepared from methanolic hydrochloric acid was chromatographed by the ascending technique in BAW for 30 hours. In order to restrict deacylation of the esterified pigments, preparation of the extract and chromatography was conducted at a low temperature (IO^{o} C.). After drying the developed chromatograms, they were cut into strips for measurement of the pigment band densities with a photoelectric densitometer (V,I).

For the purpose of expressing the relative concentration of the individual pigment bands, the area under the curve (Fig, III) was determined planometrically and each peak calculated as a percentage

66./ of....

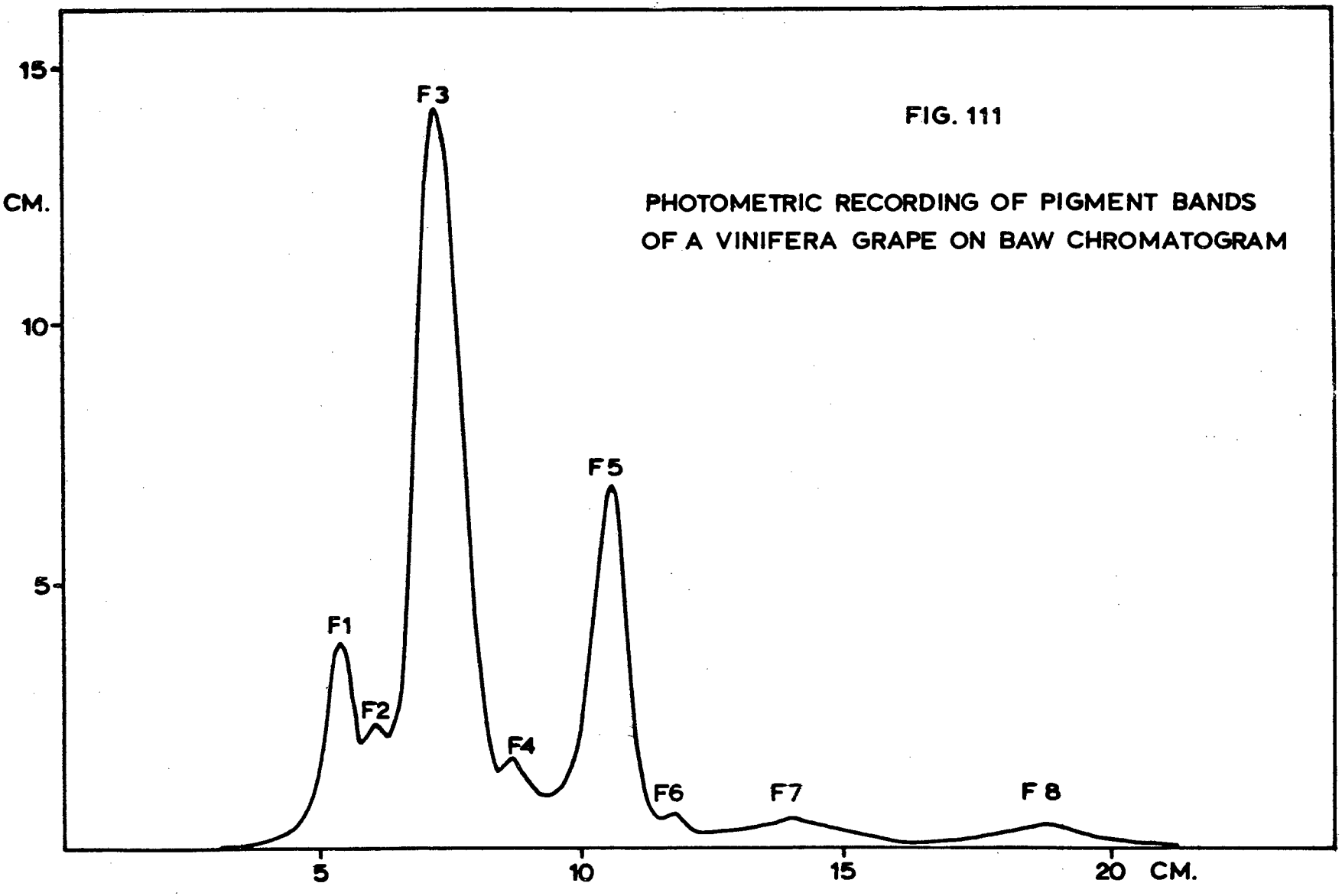


FIG. 111

PHOTOMETRIC RECORDING OF PIGMENT BANDS OF A VINIFERA GRAPE ON BAW CHROMATOGRAM

of the total.

The limitations pertaining to this method for expressing the relative concentration have been discussed (V,I). In addition, inter-contamination, chiefly between adjacent pigment bands and unavoidable deacylation of the esterified pigments (which consequently affect the concentration of the respective normal glucosides), occur to some extent. For the present purpose, the method nevertheless appears to be the best way of recording varietal differences.

TABLE VII

Average Relative Concentration of Individual Pigment Bands

Variety	Percentage							
	F1	F2	F3	F4	F5	F6	F7	F8
Alicante bouschet	3.2	4.3	62.4	15.4	3.1	1.1	7.1	3.4
Pontak	4.8	6.3	61.9	3.7	9.8	1.6	5.1	6.8
Schiraz	8.5	6.8	32.8	5.1	21.1	4.0	14.1	7.6
Hermitage	8.7	4.4	36.7	4.8	22.3	3.0	9.4	10.7
Pinotage	10.4	8.4	48.8	4.7	16.0	1.2	6.0	4.5
Cabernet sauvignon	11.4	8.9	43.2	6.9	24.3	1.2	3.1	1.0

67./ CHAPTER VII....

CHAPTER VII

THE SUGAR COMPONENTS

VII,A. General

The sugars produced from anthocyanins on acid hydrolysis may be identified by the usual chromatographic procedures (93-96) or by measurement of the absorption spectra of the coloured products of individual sugars, after treatment with sulphuric acid and resorcinol (90). Electrolytes cause serious interference during paper chromatography of sugars and consequently necessitates removal of salts, acids and bases. This is most readily accomplished by the use of ion exchange resins (74,94,97), or in the case of acids by an aliphatic amine (98).

VII,B. Removal of Free Sugars

Anthocyanin samples used for sugar analysis were chromatographed in acetic acid (15%) during the purification process, to remove free sugars such as dextrose and levulose which could have been present in the skins (5,99). The purified samples were eluted from the chromatograms and filtered through a sintered glass filter of fine porosity to remove all filter paper particles.

VII,C. Quantitative Acid Hydrolysis of Anthocyanins

The filtered eluate was taken to dryness, redissolved in I N hydrochloric acid (2 ml.) and heated under nitrogen at 100° C. for two hours. The hydrolysate was filtered through a sintered filter

68./ using....

using a glass fibre paper disc to remove traces of polymeric material which was occasionally found to be present. After cooling, the aglycones were extracted with successive small portions n-pentanol (I ml.) until the aqueous layer remained a faint pink colour due to traces of unhydrolysed anthocyanin (I00).

VII,D. Removal of Electrolyte from Hydrolysate

The hydrochloric acid present in the hydrolysate was removed by washing with four successive portions (2.5 ml.) di-n-octylmethylamine (I0% v/v) in chloroform (73,I00). The amount of amine was calculated as a slight excess, however, traces of unhydrolysed anthocyanin conveniently served as an indicator. Traces of the amine and its hydrochloride, both of which are slightly soluble in water (0.0I and 0.5% respectively) were removed by washing with three successive portions (5 ml.) chloroform (98). The aqueous solution (pH 6.2-6.5) was filtered through a sintered filter and concentrated to small bulk under reduced pressure in an acid free atmosphere. An infra-red lamp was used as a source of heat.

VII,E. Chromatography of the Sugars of Individual Anthocyanins

Aliquots (25-I00 µl.) of the aqueous sugar concentrate were spotted on Whatman No. I paper. Authentic galactose, glucose, arabinose and rhamnose were used as reference sugars. The standard sugar solutions were made up in I% concentration in aqueous ethanol (I0% v/v) and were stable for several weeks if kept in the refrigerator. Before

69./ chromatography....

chromatography aliquots of the standard sugar solutions were diluted to give a concentration of 2.5 % per µl. and 10 µl. of each sugar solution was spotted with the unknown samples.

The chromatograms were developed by the descending technique using Bu-BPW, CW and EPW as solvents (72,73,94). Irrigation was continued until the solvent front had advanced 45 - 50 cm. from the starting line.

VII,F. Location of Sugars on the Chromatograms

After removal from the tank, the chromatograms were dried at room temperature in a current of air for one hour, followed by drying in an oven at 50° C. for twenty minutes. The sugar spots were located by spraying the dried chromatograms with aniline hydrogen phthalate (0.1 M in moist butanol) and heating at 105° C. for 3-5 minutes (101). Both glucose and arabinose were detected, the incidence of the latter varying from traces to as much as 50% in some samples (Fig.IV).

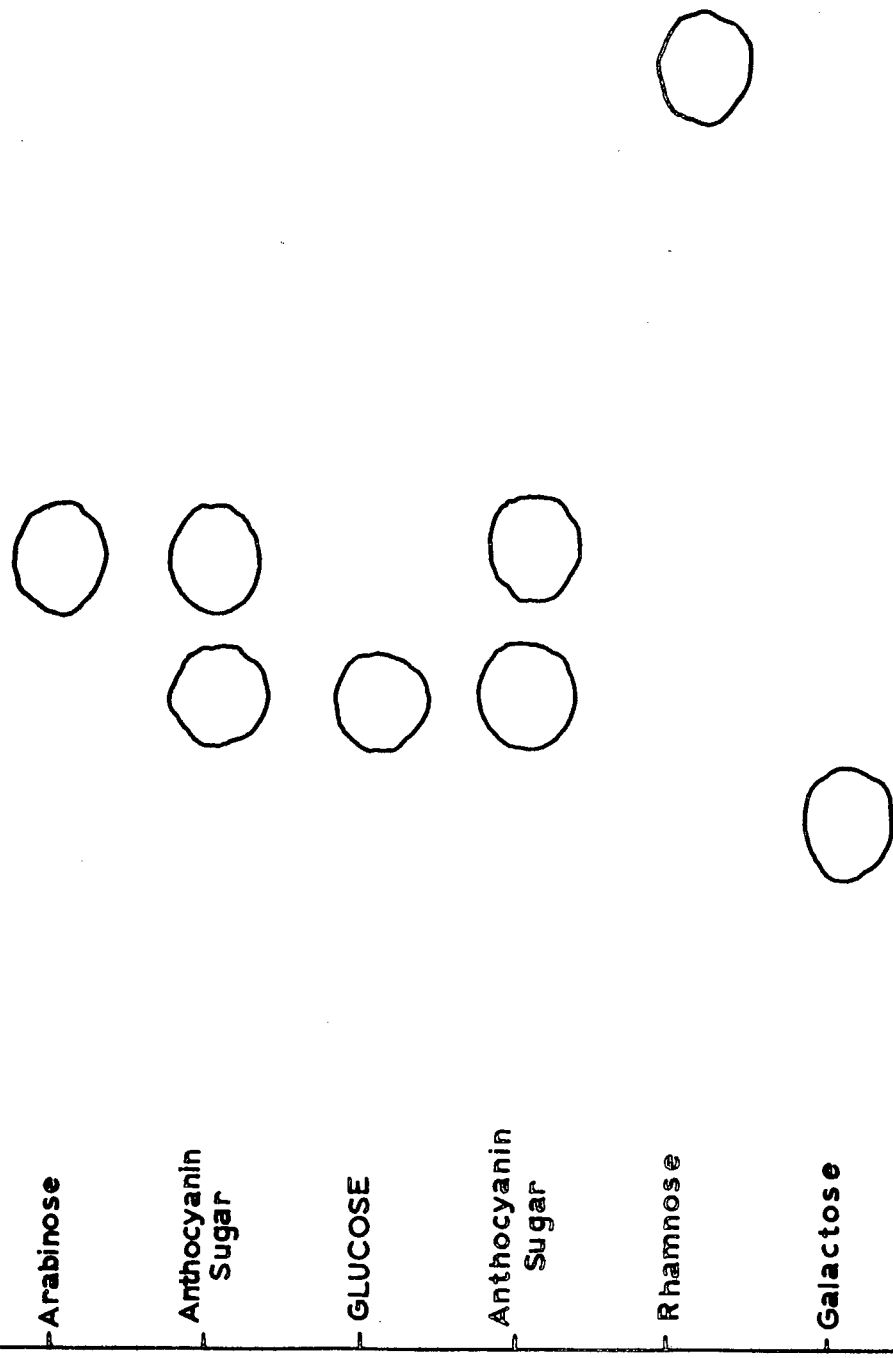
Two possibilities could account for the presence of both these sugars. Both could have been present as glycosidic sugars, however, experimental evidence (chromatographic and spectral examination) indicated that the anthocyanins investigated were all 3-monoglucosides, in which case the arabinose originated from another source.

The production of arabinose (102) is peculiar to anthocyanins which have been purified by paper chromatography and results from the action of mineral acid (present in solvent systems) on filter

70./ paper....

FIG. 1V

SEPARATION OF SUGARS IN Bu-BPW (21°C.)



paper. The arabinose detected above, could thus have originated during the purification of the individual anthocyanins.

VII,G. Identification of all the Sugars in the Combined Anthocyanin Hydrolysate

Production of arabinose is avoided during chromatography in aqueous acetic acid and the faster running free sugars are also separated from the anthocyanin pigments (5). If, however,the presence of plant glycosides (other than anthocyanins) are suspected, further purification is necessary.

An extract prepared from the skins (0.2 gm.) in methanol containing hydrochloric acid (I%) was chromatographed in aqueous acetic acid (I5%). The anthocyanins appeared as a broad zone without being resolved into individual components. The entire anthocyanin band was cut out, eluted with WMA and the process repeated a second time in the same solvent. All papers used for chromatography were previously washed with acetic acid (I5%). The final eluate containing the anthocyanins was evaporated to dryness and the pigment hydrolysed as described. The resulting aqueous sugar solution was chromatographed in Bu-BPW and CW. Glucose was the only sugar detected in the combined anthocyanin hydrolysate. The arabinose previously detected in the anthocyanin samples which were purified in the conventional manner (see: VI,I. and VIII,B) thus resulted from the action of the mineral acid (present in the solvent systems) on the filter paper.

7I./ VII,H. Removal....

VII,H. Removal of Arabinose from Sugar Solutions

The sugar solution , after hydrolysis of the individual anthocyanins, was spotted at 0.7 cm. intervals on the starting line on Whatman No. I paper and chromatographed in Bu-BPW. This solvent gives well defined round spots and a high degree of resolution between glucose and arabinose, provided the solvent front is allowed to migrate at least 45 - 50 cm. from the starting line, the sugar concentration per spot is kept in the 25 γ range and the diameter of individual spots does not exceed 0.7 cm.

After drying, three strips, each 3/I6 th. inches wide were cut through the entire length of the chromatogram and the sugars located by treatment with aniline hydrogen phthalate. The strips were replaced in their respective positions on the chromatogram and the zone occupied by the glucose was marked, cut out, eluted with distilled water and concentrated to ca 0.5 ml. Aliquots of this solution were finally tested for purity by chromatography in Bu-BPW and BAW.

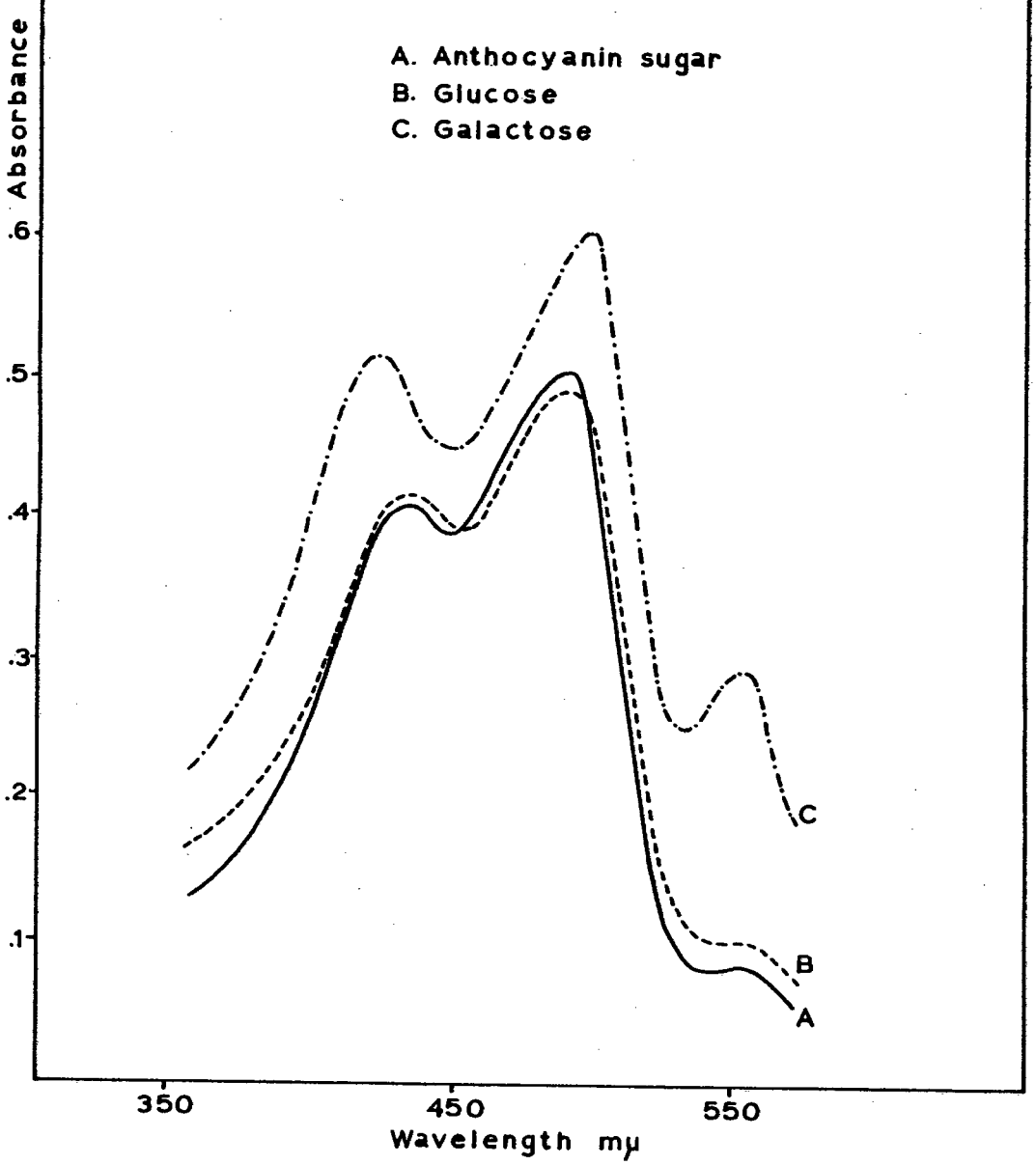
VII,I. Spectrophotometric Identification of Sugars

When simple sugars are dehydrated with sulphuric acid, a furan type aldehyde is produced which condenses with I-naphthol to produce coloured complexes (Molish reaction). The different colour formations with the naphthol reaction with various monosaccharides are probably due to aldehydes other than the furan type and are not the same for all the hexoses and ketoses (I03).

72./ The....

FIG. V

ABSORPTION SPECTRA OF THE COLOURED PRODUCTS OF SUGARS



The unsulphonated resorcinol reaction was also found satisfactory to detect these aldehydes and measurement of the absorption spectra of the coloured products permits identification of monosaccharides such as arabinose, rhamnose, xylose, galactose, mannose and glucose (90). It is fortunate that these sugars are also those present in naturally occurring anthocyanins.

(i) Procedure for the spectral identification of sugars

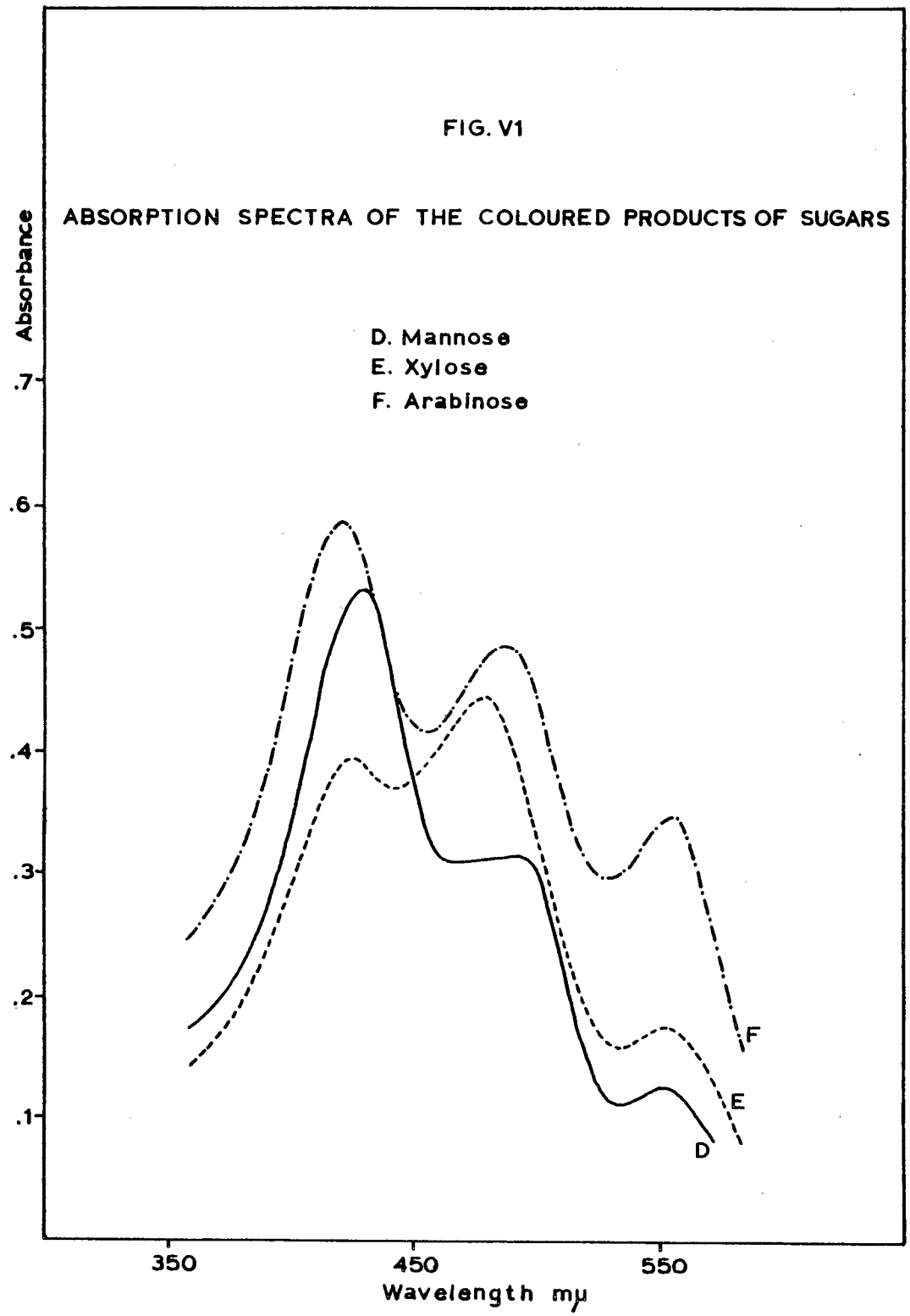
Measured portions (50, 100, 150 and 200 µl.) of the aqueous sugar solution, obtained after removal of arabinose, were each made up to 2.0 ml. with distilled water. Each portion was transferred to a test tube containing resorcinol (5.0 mg.) and mixed. Sulphuric acid (5.50 ml.) was rapidly added, with mixing and the tube shaken for 30 seconds. (The sulphuric acid reagent was prepared by adding 100 ml. chem. pure acid to 10 ml. distilled water and cooling to room temperature.).

Authentic sugar samples were treated in a similar manner and the reference solution was devoid only of sugar. The standard sugars were made up to contain 25 or 50 γ per ml. of solution. After colour development, the solutions were kept in the dark at room temperature for 45 minutes and their absorption spectra measured in the range 360 - 590 mµ. For the unknown samples, a dilution

73./ was....

FIG. V1

ABSORPTION SPECTRA OF THE COLOURED PRODUCTS OF SUGARS



was selected having an optical density in the range 0.3 to 0.8 at the wavelength of maximum absorption.

All anthocyanin sugars (Fig.V) examined in this manner, gave spectral curves identical with glucose (λ max. 489 mμ, inflexions at 430 and 555 mμ). The two other sugars producing somewhat similar spectra viz. galactose and xylose are spectrally well differentiated from glucose. With galactose (λ max. 495 mμ, inflexions at 422 and 550 mμ) the two smaller peaks give a much greater absorption compared with glucose at the same wavelength, whereas with xylose (λ max. 480 mμ, inflexions at 430 and 550 mμ) the intensity at 430 mμ compared to that at the wavelength of maximum absorption, is approximately of the same order as glucose, but at 550 mμ it shows a well defined peak compared to a mere inflexion in the case of glucose (Fig. VI).

TABLE VIII

R_f Value of Neutral Sugars in Various Solvents (21° C.)

Sugar	BAW	CW	Bu-BPW	Colour on treatment with aniline hydrogen phthalate
D(+)-Galactose	.160	.424	.186	Green-brown
D(+)-Glucose	.168	.481	.229	Green-brown
Anthocyanin sugar	.163	.481	.228	Green-brown
D(-)-Arabinose	.223	.497	.279	Purple-brown
Rhamnose	.347	-	.447	Green-brown

74./ CHAPTER VIII....

CHAPTER VIII

THE ESTERIFIED OR ACYLATED ANTHOCYANINS

VIII,A. Chromatographic Behaviour of Acylated Anthocyanins

A further important aspect in determining the glycosidic nature of an anthocyanin, is to ascertain whether it is acylated or not and to determine the nature of the acid involved.

Benzoic and cinnamic acid or their derivatives, occur naturally in combination with anthocyanins. In such instances the introduction of a benzoyl residue in the parent molecule, considerably decreases the polarity of the compound, thus causing an increase in the mobility in butanolic solvents.

During the preparation of an extract (which usually contains acid) it is unavoidable to prevent partial hydrolysis of the ester and during subsequent chromatography the original compound yields two spots, the major constituent (higher R_f) being represented by the ester and the minor constituent (lower R_f) that of the de-esterified anthocyanin. Even if the major spot is separated chromatographically from the minor spot, two spots will appear on rechromatography (5,104). Acylated anthocyanins are thus less stable compounds than the corresponding anthocyanins.

VIII,B. Purification and Chromatographic Behaviour of Pigments F5 - 8

It has been shown that alkali hydrolysis of pigment bands FI-8 resulted in the disappearance of bands F5-8. These pigment bands

75./ were....

were consequently suspected as being esterified derivatives of the normal glucosides already identified. On rechromatography of the individual pigment bands F5-8, the following were noted:

(i) Band F5

The principal contaminants of this pigment band were found to be F6 and F7. The latter two were removed by chromatography in BAW, HOAc-HCl and finally again BAW. On rechromatography in BAW the purified pigment yielded, in addition, a minor constituent which corresponded in R_f to malvidin 3-monoglucoside. F5 was also obtained in a pure condition during the purification of pigment F3 (VI,I. (ii)).

(ii) Band F6

This band was severely contaminated by both F5 and F7 and could not be resolved in any of the usual anthocyanin solvents. On rechromatography in BAW, pigments F5, F6 and F7 reappeared, together with deacylated products corresponding to malvidin and peonidin 3-monoglucoside, the latter originating from F6. This pigment was obtained in a pure state during the purification of pigment band F4 in BAW prior to alkali hydrolysis, in which case it is entirely free free from F5 and F7 (VI,I (ii)).

76./ (iii) Band....

(iii) Band F7

This pigment was obtained in a relatively pure condition during the initial separation of the anthocyanin extract in BAW. It was purified in the same solvents as F5. On rechromatography in BAW, the purified pigment yielded, in addition, a minor constituent which corresponded in R_f to malvidin 3- monoglucoside.

(iv) Band F8

This constituent, being the compound of highest R_f was well separated from the other pigment bands and was consequently obtained in a relatively pure condition during the initial separation of the anthocyanins. On rechromatography in BAW, the bulk of this pigment was converted to F7, together with a deacylated product corresponding in R_f to malvidin 3-monoglucoside. As a result of the very large proportion of this pigment being converted to F7, it was necessary to use the material from F8 as such for all further investigations.

From the above it would appear that pigments F5, F7 and F8 originate from the same glucoside viz. malvidin 3-monoglucoside and that F7 and F8 are related but distinctly different from F5, since on partial de-esterification F8 yields both F7 and F3, whereas F7

77./ and....

and F5 yield only F3.

Acylated anthocyanins in general and those based on the delphinidin series in particular, do not separate as satisfactorily as the corresponding glycosides, when present together in plant extracts. Instances similar to F6 have been reported where acylated derivatives of pelargonidin and peonidin, giving clearly separated bands on paper chromatograms, remain contaminated by one another (5).

It should be noted that on successive rechromatography of acylated pigments, a substantial portion is lost by deacylation, the amount of which appears to be largely affected by temperature and may vary from traces at 10 - 15° C. to as much as 50% or more at temperatures exceeding 25° C. Sufficient material should therefore be prepared to compensate for such losses.

The acylated pigments of grapes based on peonidin (F6) and malvidin (F5, F7 and F8), are not individually separated in the solvent HOAc-HCl. Their R_f values in this solvent are also virtually similar to that of the corresponding glucosides. This solvent (HOAc-HCl) was, however, included during the purification wherever possible, to effect removal of other impurities which could have been present.

VIII,C.Alkali Hydrolysis of Acylated Anthocyanins

When acylated anthocyanins are dissolved in a solution of sodium hydroxide in the cold, hydrolysis occurs at the ester linkage (III,C), thereby leaving the glycoside intact (5,20,73). The purified

78./ acylated....

acylated pigment was hence eluted from the chromatogram with WMA, filtered and evaporated to dryness. The dry pigment was dissolved in 2 N sodium hydroxide (2.0 ml.) and transferred to a glass stoppered test tube. After displacing the air with nitrogen, the tube was stoppered and left in the dark at room temperature for two hours. The hydrolysate was acidified with hydrochloric acid (0.55 ml.), cooled and left in the dark for ten minutes to allow for complete regeneration of the anthocyanin.

(i) Extraction of Acid Component from Hydrolysate

The acidified hydrolysate was saturated with chemically pure sodium chloride (to decrease solubility of acid component in aq. layer) and extracted with three portions (I.0 ml.) diethyl ether (distilled from sodium).The ethereal extracts were combined and used for examination by paper chromatography and spectrophotometry.

(ii) Extraction of Glucoside from Hydrolysate

The anthocyanin was extracted from the remaining saline solution with successive small portions (I ml.) n-pentanol. The combined pentanol extract was diluted with five volumes benzene and the anthocyanin re-extracted with successive small portions aq. hydrochloric acid (I ml., I%). The hydrochloric acid extract was evaporated to dryness, redissolved in the minimum volume Bu-MH and filtered

79./through....

through a sintered filter to remove salt crystals.

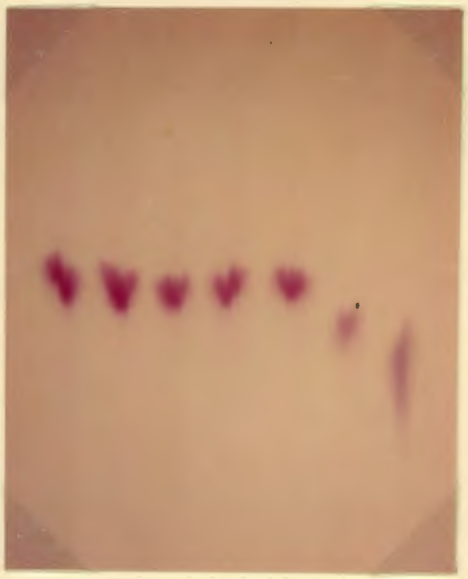
VIII,D. Purification and Identification of the Glycosides Resulting from Alkali Hydrolysis of Pigments F5 - 8

The Bu-MH extract was spotted on Whatman No.3MM or No.I paper, depending on the quantity of pigment and chromatographed in HOAc--HCl and BAW, to free the pigment from traces of sodium chloride which resulted in slightly low R_f values. Identification was conducted by chromatography and co-chromatography on Whatman No. I paper, using the 3-monoglucosides of delphinidin, petunidin, malvidin and peonidin as reference compounds. The identity of the deacylated pigments resulting from F5, F7 and F8 was confirmed as being malvidin 3-monoglucoside and F6 as peonidin 3-monoglucoside, in both BAW and Bu-HCl (Plates VI, VII).

During purification after alkali hydrolysis, the glucosides isolated from F7 and F8 were accompanied by traces of peonidin 3-monoglucoside on the BAW chromatogram, the latter pigment being discarded during purification. This contaminant was not observed during purification of the individual acylated pigments. Further experiments revealed that it could not be removed by chromatography in the solvents mentioned.

The presence of traces of acylated peonidin derivatives, other than F6, occurring in both F7 and F8, can hardly be regarded as contamination. If such pigments, related to F7 and F8 with respect to the acid component were present, they should occupy positions on the

80./ chromatogram....

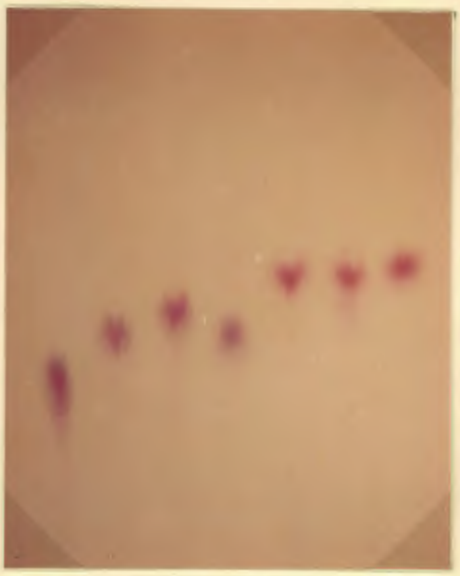


I 2 3 4 5 6 7

PLATE VI

Identification of Glucosides
Resulting from Pigments F5, F7 and F8,
in Butanol : Acetic Acid : Water

1. Pigment F 7
2. Malvidin 3-monoglucoside
3. Pigment F 5
4. Malvidin 3-monoglucoside
5. Pigment F 8
6. Petunidin 3-monoglucoside
7. Delphinidin 3-monoglucoside



I 2 3 4 5 6 7

PLATE VII

Identification of Glucoside
Resulting from Pigment F 6,
in Butanol : Acetic Acid : Water

1. Delphinidin 3-monoglucoside
2. Petunidin 3-monoglucoside
3. Malvidin 3-monoglucoside
4. Petunidin 3-monoglucoside
5. Peonidin 3-monoglucoside
6. Pigment F 6
7. Peonidin 3-monoglucoside

chromatogram immediately to the front of bands F7and F8. These areas on the chromatogram were subsequently termed F7X and F8X respectively.

The cuttings from a large number of chromatograms were collected (since they exhibit only a trace of colour), eluted and the pigment subjected to alkali hydrolysis. Chromatography of the deacylated pigments resulting from F7X and F8X again revealed same as for above, but with peonidin 3-monoglucoside occurring in significantly higher concentration with respect to malvidin 3-monoglucoside. It therefore seems probable that such derivatives do exist, though at very low concentration , which does not permit investigation of the individual pigments.

VIII,E. Identification of the Acid Components of Acylated Pigments

The acid components of acylated anthocyanins may be examined by the usual chromatographic procedures (24,40,I05) or by spectral means (I6). The hydroxy and methyl ether derivatives of benzoic and cinnamic acid are colourless compounds and U.V. light and various chromogenic sprays are employed in conjunction with paper chromatography to detect and identify these compounds (40,I05).

VIII,F. Paper Chromatographic Investigation of the Acid Components of Pigments F5 - 8

The ethereal extract containing the acid component was carefully evaporated to dryness under reduced pressure and the residue dissolved in absolute ethanol (ca I00 µl.). The ethanol solution was

8I./ spotted....

spotted on Whatman No. I chromatography paper. p-Hydroxycinnamic acid was used as reference compound and phloroglucinol as standard marker. The standard substances were dissolved in ethanol and applied at a concentration of 20 γ per spot. Duplicate papers were developed by the descending technique in BAW.

After drying, the developed chromatograms were suspended for a few minutes in a tank containing ammonia vapour, followed by examination in U.V. light immediately afterwards. The papers were freed from ammonia in a current of air at room temperature for I5 minutes. One chromatogram was sprayed with diazotised p-nitroaniline (40) and the other with diazotised benzidine (40). After drying in a current of hot air, the papers were oversprayed with aq. sodium carbonate (I0%). p-Hydroxycinnamic acid was detected as the acid component, only in pigments F7 and F8 (Table IX).

It should be noted that pigments F5 and F6 were obtained in a pure condition from pigments F3 and F4 respectively, during purification (VI,I. (ii) and VIII,B. (ii)).

VIII,G. Spectral Investigation of the Acid Components of Pigments F5 - 8

Preliminary evidence as to whether an anthocyanin is acylated or not, may be obtained from the U.V. absorption spectra. All normal anthocyanins show a weak absorption peak in the U.V. region at about 270 mμ and it is only in the visible region that differences in

82./ the....

TABLE IX

U.V. Light Fluorescence and Colours of Polyphenols

Produced by Various Spray Reagents

Treatment	Phloro-glucinol	F5	F6	F7	F8	p-Hydroxy-cinnamic acid
R_f in BAW	.738	ND	ND	.910	.922	.919
U.V.Light + NH_3	bB	ND	ND	lB	lB	lB
Diazotised p-nitroaniline + NaAc	YO-OR	ND	ND	OBr--YBr	OBr--YBr	OBr--YBr
Ditto + Na_2CO_3	O-YBr	ND	ND	GB--GrB	GB--GrB	GB--GrB
Diazotised benzidine	BP-dP	ND	ND	YBr--OBr	YBr--OBr	YBr--OBr
Ditto + Na_2CO_3	dP	ND	ND	V--PBr	V--PBr	V--PBr

b = Bright, d = Dark, l = Light, B = Blue, Br = Brown

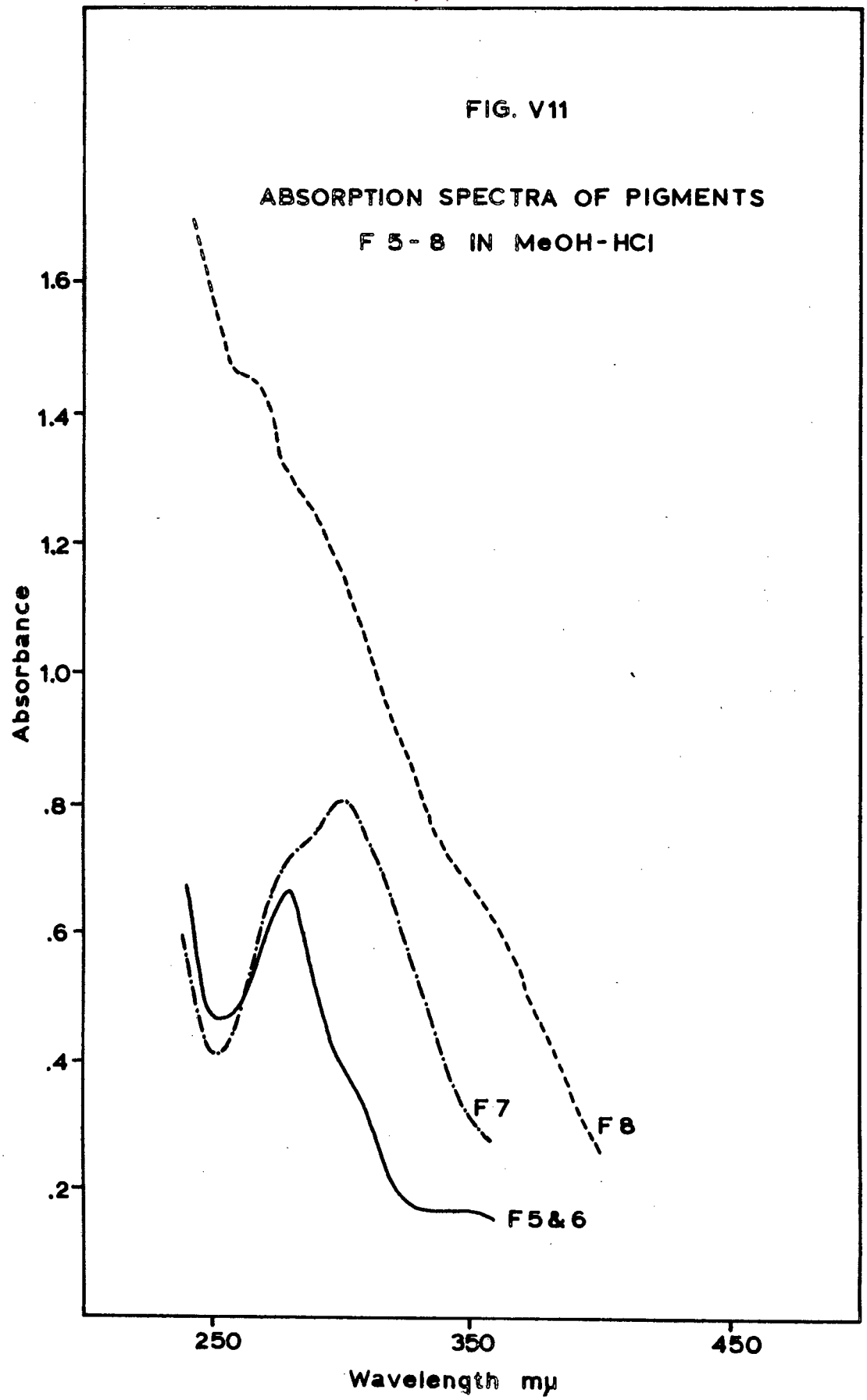
G = Green, Gr = Grey, O = Orange, P = Purple

R = Red, V = Violet, Y = Yellow, ND = Tested, not found

the absorption maxima are apparent. It has been reported that the spectra of anthocyanins acylated with p-hydroxycinnamic acid, show two peaks in the ultraviolet at 289 and 310 mμ respectively, due to the

83./ superimposition....

FIG. V11



superimposition of the absorption of the acid upon that of the pigment absorption (I6).

The acylated anthocyanins F5-8 were purified as described (VIII,B). The dry pigment was dissolved in methanol containing hydrochloric acid (0.0I%) and filtered. The solutions were diluted to give an optical density in the range 0.6 - I.0 at the wavelength of maximum absorption in the visible region and their absorption spectra measured between 240 and 350 mμ. For all measurements in the U.V. region, the reference solution was prepared from appropriate blank chromatograms.

It is evident from the spectra given in Fig. VII, that F7 and F8 show abnormal patterns, whereas F5 resembles that of a normal anthocyanin. The U.V. absorption spectrum for F6 is not given , since it was found identical to F5. These data are of extremely limited diagnostic value, relevant to the acylated anthocyanins of the vinifera grapes, since they do not provide any conclusive evidence as to the nature of the acid involved.

VIII,H. Spectral Investigation of the Ethereal Extract Resulting from Alkali Hydrolysis of Pigments F5 - 8

The U.V. absorption spectra of the ethereal extracts resulting from alkali hydrolysis (Fig. VIII) revealed beyond doubt the existence of p-hydroxycinnamic acid (Fig. XI) in F7 and F8. It should be noted that with p-hydroxycinnamic acid in methanolic hydrochloric acid,

84./ maximum....

FIG. V111

ABSORPTION SPECTRA OF ACID COMPONENTS OF PIGMENTS F5, F7 AND F8 IN ETHER

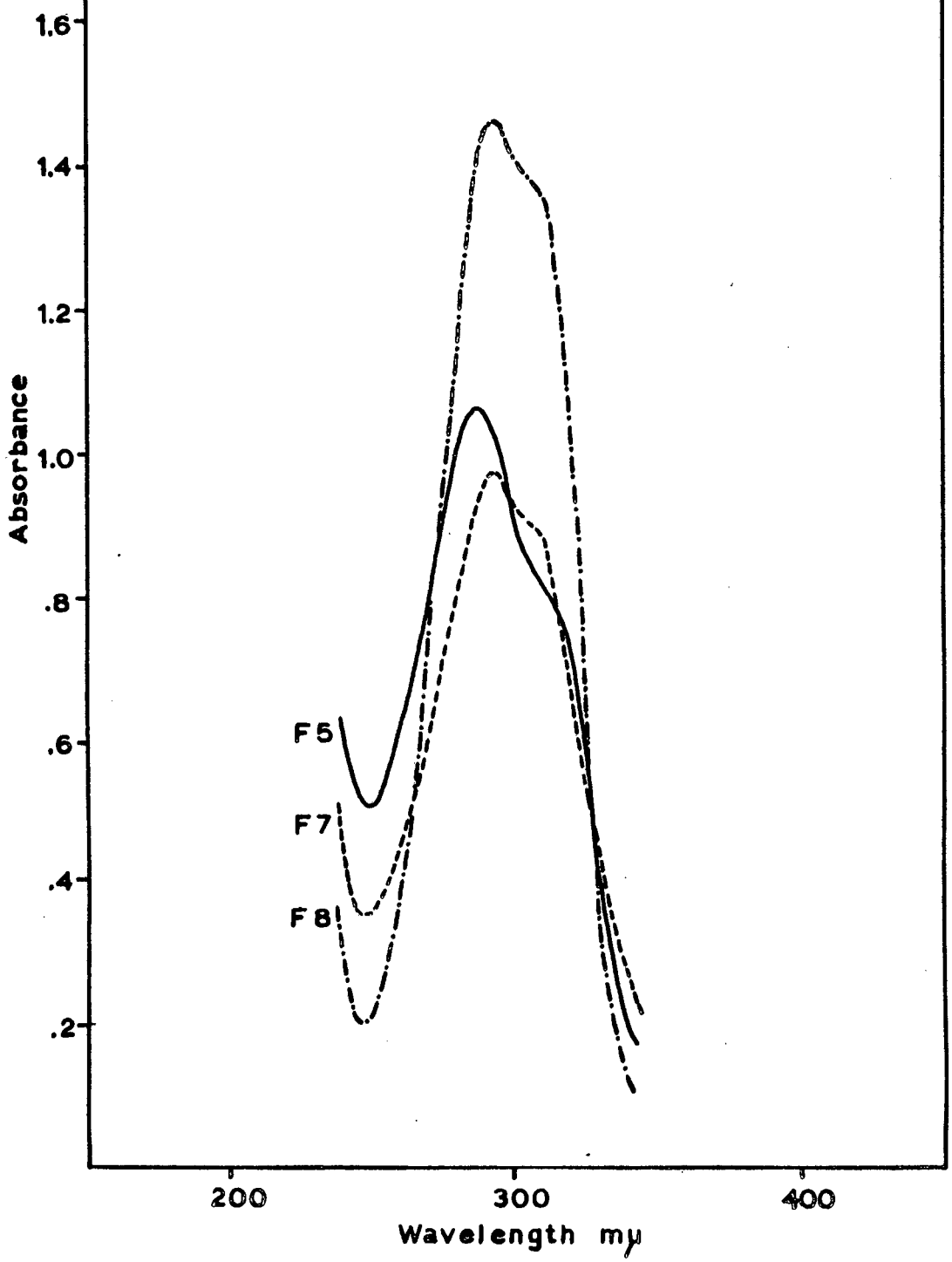
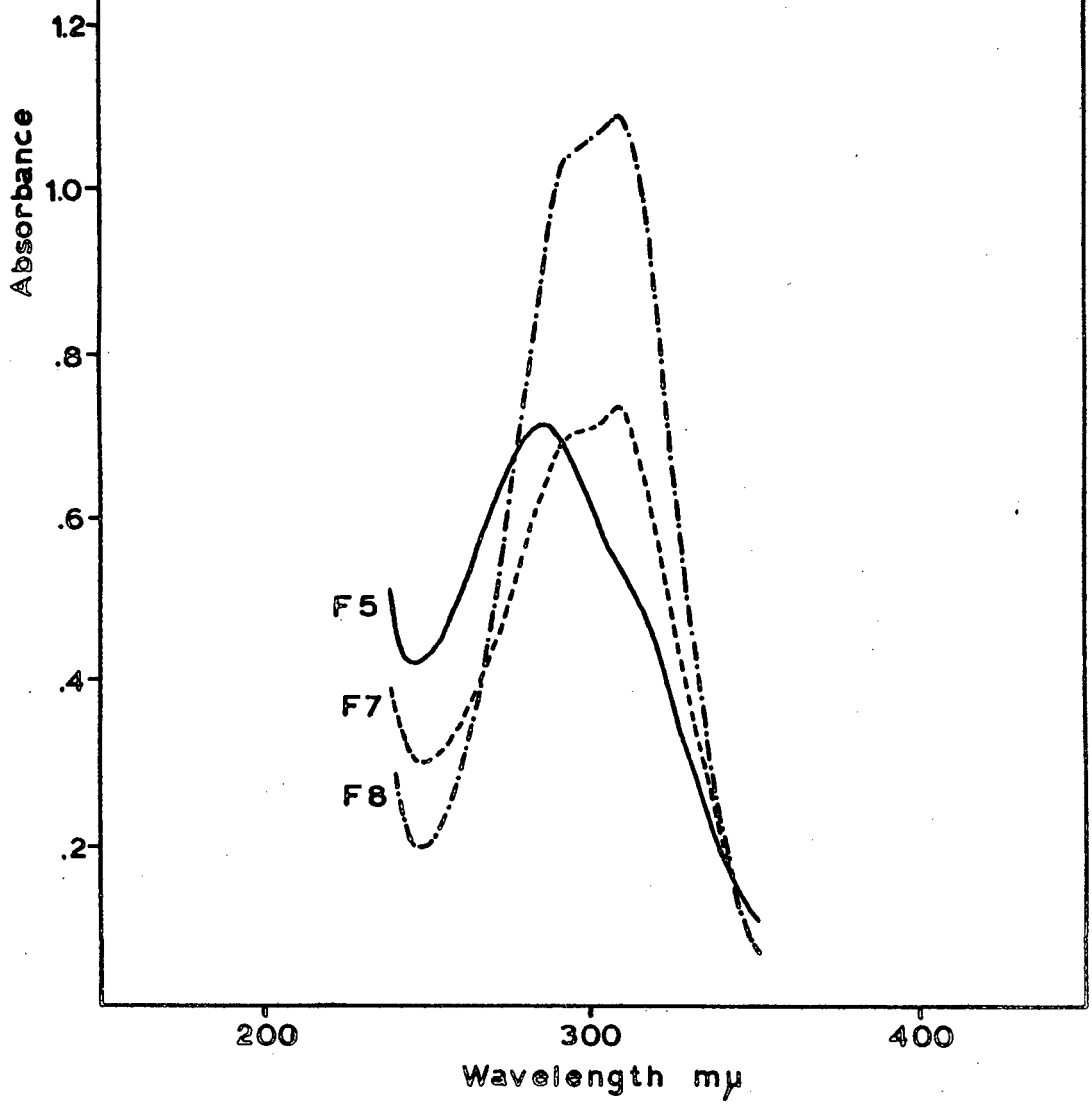


FIG. 1X

ABSORPTION SPECTRA OF ACID COMPONENTS OF PIGMENTS F5, F7 AND F8 IN MeOH-HCl



maximum absorption occurs at 310 mμ, with a slight shoulder adjacent to the main peak at about 295 mμ. In ether, however, it shows λ max. at 292.5 mμ with an inflection at 310 mμ. This behaviour also distinguishes the compound extracted from F5 and having λ max. at about 285 mμ in ether.

The ethereal extracts were subsequently taken to dryness. The residues were redissolved in methanolic hydrochloric acid and the spectra measured again (Fig. IX). In the case of F7 and F8 , the main peak shifted to 310 mμ whereas with F5 it remained at about 285 mμ.

VIII,I. Measurement of the Spectral Shift of the Acid Components of Pigments F5 - 8

Measurement of the spectral shift in MeOH-NaOH was conducted in the above solution (methanol containing 0.01% hydrochloric acid)by addition of two drops chem. pure 2 N sodium hydroxide to the cell solution (3 ml.) and mixing (106).

The p-hydroxycinnamic acid present in F7 and F8, showed maximum absorption at 332.5 mμ (Fig. X) which represents a bathochromic shift of 22.5 mμ. A pure sample of the acid gave λ max. 312 mμ in MeOH-HCl and λ max. 335 mμ in MeOH-NaOH ($\Delta\lambda$ 23 mμ) which is in agreement with reported values (106). Measurements performed on several grape extracts, revealed that the maximum absorption for p-hydroxycinnamic acid in MeOH-HCl, varied from 310 to 312 mμ, however, the spectral shift remained constant ($\Delta\lambda$ 22.5 mμ).

85./ The....

FIG. X

ABSORPTION SPECTRUM OF p-HYDROXYCINNAMIC ACID RESULTING FROM PIGMENTS F7 AND F8

A. Measured in MeOH-HCl
B. Measured in MeOH-NaOH

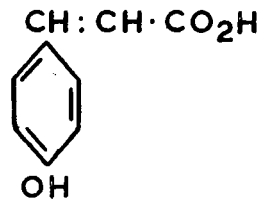
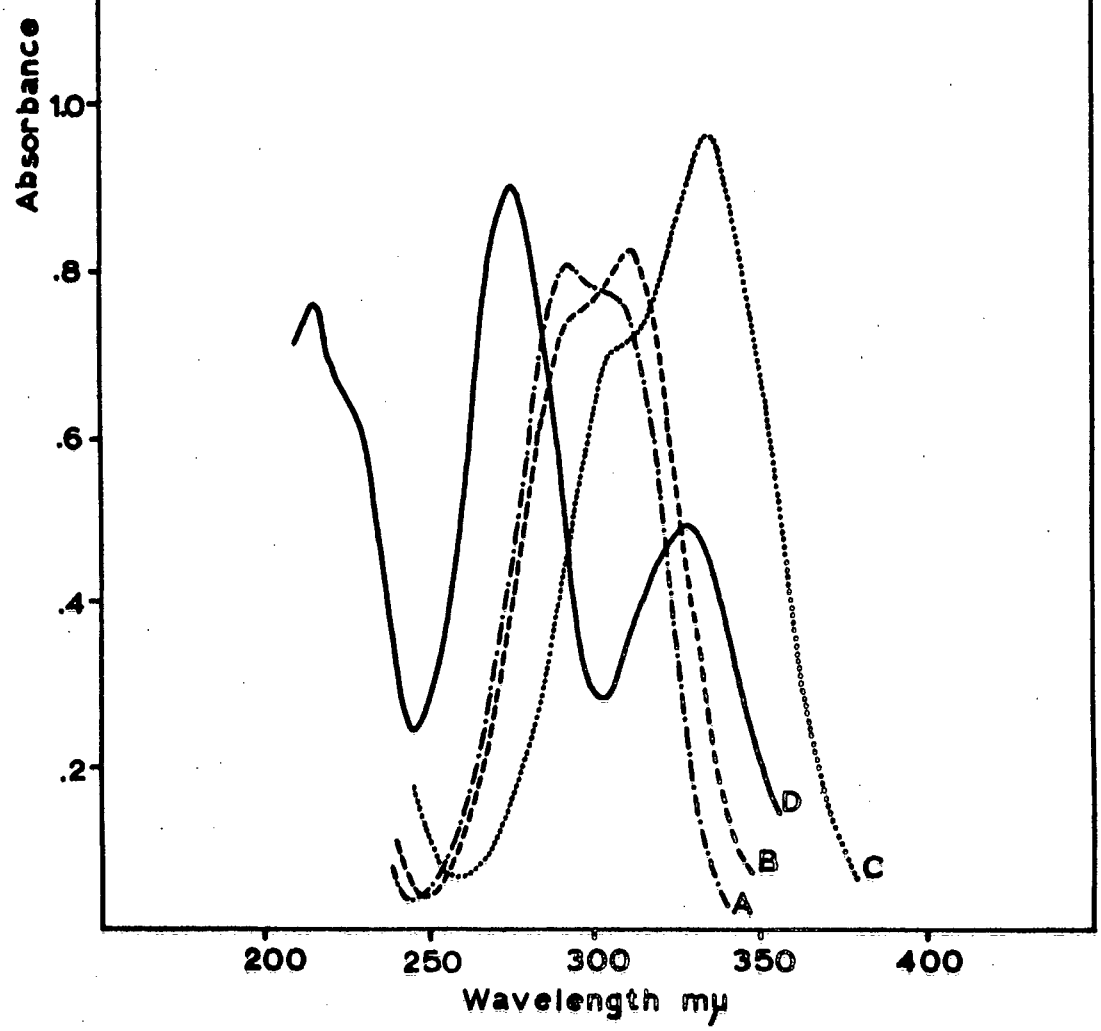


FIG. X1

ABSORPTION SPECTRA OF PURE CINNAMIC ACIDS

A. p-Hydroxycinnamic acid in Ether
B. " " " MeOH-HCl
C. " " " MeOH-NaOH
D. O-Hydroxycinnamic acid in MeOH-HCl



The substance extracted from F5 showed greater variation, having λ max. at about 285 mμ (± 5 mμ) in MeOH-HCl. A corresponding variation resulted in the maxima at about 302.5 mμ in MeOH-NaOH but the bathochromic shift ($\Delta\lambda$ 17.5 mu) remained constant for each individual sample (Fig. XII).

Measurement of the absorption spectra in ether, MeOH-HCl and MeOH-NaOH, revealed that the substance present as "acid" component in F5 is not related to cinnamic acid or its derivatives such as chlorogenic, caffeic, hydrocaffeic, ferulic, sinapic and O-hydroxycinnamic acid, nor is it related to benzoic acid and derivatives such as protocatechuic, p-hydroxybenzoic, gallic and syringic acid.

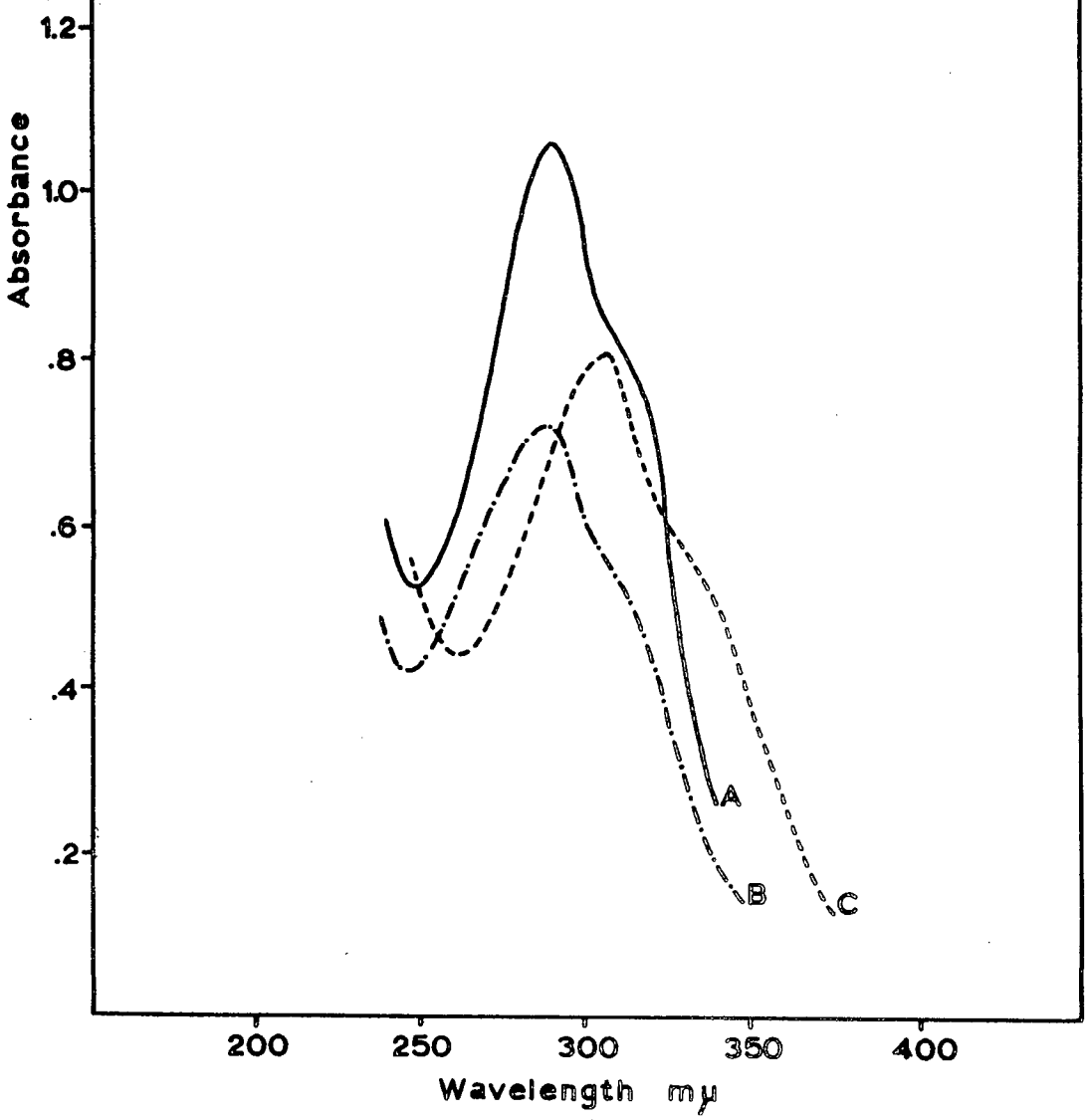
Catechins and leucoanthocyanins have absorption spectra which show the maxima typical of polyhydric phenols in which no carbonyl conjugation is present and the wavelength of maximum absorption is found in the 280 mμ region. It is shifted in alkaline solution to 290--300 mμ. This behaviour is also to be expected of a substance such as catechin in which the substituted phloroglucinol and catechol residues are insulated from each other and thus absorb independently and substantially as do the respective parent phenols (4). Reported values indicate that whereas maximum absorption for leucoanthocyanins and D-catechin occur at the same wavelength viz. 280 mμ in EtOH, the former shows a bathochromic spectral shift varying from 8 - II mμ in EtOH-NaOH and the latter I8 mμ in the same direction.

86./ In....

FIG. X11

ABSORPTION SPECTRUM OF COMPONENT RESULTING FROM ALKALI HYDROLYSIS OF PIGMENT F 5

A. Measured in ETHER
B. " MeOH-HCl
C. " MeOH-NaOH



In further experiments the ethereal extract,obtained after alkali hydrolysis of F5 was chromatographed in BAW using (+)-catechin and (-)-epicatechin as reference compounds. The developed chromatograms were dried and sprayed with diazotised benzidine and diazotised p-nitroaniline. By means of these two chromogenic sprays, the two principal constituents detected , gave colour reactions identical with the reference compounds. Since epicatechins do not occur naturally, its presence can be accounted for by epimerisation of the corresponding (+)-compound.

The concentration of the catechins detected chromatographically, appeared abnormally low compared to the intensity of absorption of the catechin, as determined spectrophotometrically in the ethereal extract. Several factors should, however, be considered.Catechins are susceptible to oxidation in alkaline medium, considering also, that only a few gammas of material was investigated. (+)-Catechin and (-)-epicatechin show similar absorption in the U.V. region and the absorption spectra obtained from the ethereal extract (Figs. VIII,IX,XII) account for both substances whereas by paper chromatography they are individually separated.

The spectra given in Figs. VIII, IX and XII also deviate somewhat (excluding λ max.) from that of the pure substance (Fig. XIII) which is almost certainly due to the presence of other unidentified substances detected on the chromatograms. It is also possible that the

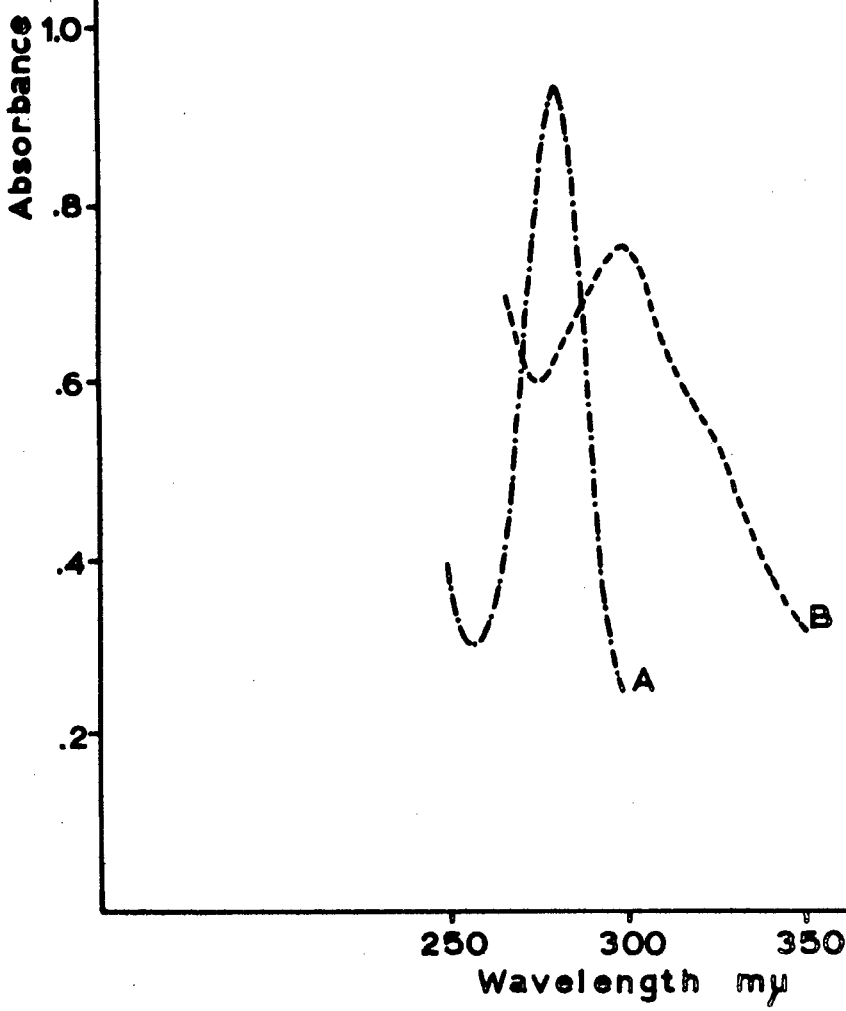
87./ intensity....

FIG. X111

ABSORPTION SPECTRUM OF D(+)-CATECHIN

A. Measured in MeOH-HCl

B. " MeOH-NaOH



intensity of absorption of the main peak is influenced by these impurities. It will be noted that the R_f values of the catechins which were obtained after alkali hydrolysis of pigment F5, are somewhat lower than the R_f values of the pure substances, which is attributed to the lower concentration of the former in relation to the latter (Table X).

The possibility of the catechins originating from the grape skins, was also considered. Comparison of the R_f values in Tables V and X, show that the catechins have substantially higher R_f values in BAW than F5 and F6 and would therefore be effectively removed in this solvent. In HOAc-HCl (+)-catechin also has a higher R_f (.443) than F5 (.382) whereas for (-)-epicatechin it is approximately of the same order (.369).

TABLE X

R_f Value of Catechins and Colours Produced by Various Sprays

Compound	BAW (I5°C.)	HOAc-HCl (I5°C.)	Diazotised benzidine	Diazotised p-nitroaniline
(+)-Catechin	.682	.443	Orange	Yellow
F5 (a) Ether extract	.678	-	Orange	Yellow
(-)-Epi-catechin	.572	-	Orange	Yellow
F5 (b) Ether extract	.560	.369	Orange	Yellow

88./ VIII,J. The....

VIII,J. The Anthocyanin : p-Hydroxycinnamic Acid Ratio in Pigments F7 and F8 : Effect of Substituent Acid Groups on R_f Value

It has been demonstrated that pigments F7 and F8 have the same anthocyanin (malvidin 3-monoglucoside) and acid component (p-hydroxycinnamic acid) in common. During the separation of the crude plant extract in BAW, pigment band F7 represented an increase in R_f of 0.157 over pigment band F3, whereas the increase in R_f of band F8 over band F3 (0.310) is approximately doubled (Table V).

If it is assumed that the number of substituent acid groups per anthocyanin molecule in pigment F7 is I, and in F8 is 2, these values, when plotted against the respective increases in R_f value over the corresponding acid free anthocyanin, i.e., F3, give a straight line, and lends support to this assumption. These molar ratios were furthermore substantiated by the following investigations:

VIII,K. Preliminary Investigation to Determine the Increased Acid Content of Pigment F8

The acylated pigments (F7 and F8) used for the determination of the anthocyanin:acid ratios were purified immediately after separation of the crude plant extract, using the solvents mentioned, however, elution was conducted under nitrogen. After the final purification the dry pigment was dissolved in the minimum volume MeOH-HCl. The solutions of both pigments (F7 and F8) were further diluted with MeOH-HCl to give similar optical density readings in the range 0.8 to

89./ I.20 at....

I.20 at the wavelength of maximum absorption in the visible region. A measured quantity (3.0 ml.) of each pigment solution was evaporated to dryness and subjected to alkali hydrolysis. After acidifying and saturation with sodium chloride, the acid component was thrice extracted with portions (I ml.) diethyl ether. Further extraction with ether (3 ml.) revealed no U.V. absorption, thus indicating that the acid component was quantitatively removed by the first three extractions. The ethereal extracts were combined, adjusted to 3.0 ml. (combined extract was 2.80 ml.) and transferred to I.0 cm. stoppered quartz cells. The absorbance was measured immediately at 292.5 mμ followed by measurement of the complete spectrum of p-hydroxycinnamic acid to ascertain whether the latter was free from interference.

The procedure was repeated with several pigment samples and in all instances the p-hydroxycinnamic acid present in F8 gave an optical density reading which was approximately 30% higher than for F7. This increase was considered satisfactory, bearing in mind that during manipulation of the small volume of ether containing about 2 - 5 γ of the acid per ml. solution, losses could easily result.

VIII,L. The Anthocyanin : Acid Ratio in Pigment F7

The ratio of the optical density at 3I0 mμ to that at the wavelength of maximum absorption in the visible region (expressed as a percentage) is a measure of the molar ratio of the acid to the anthocyanin (I6). Such values have been reported for various anthocyanins

90./ acylated....

acylated with one molecule p-hydroxycinnamic acid e.g., pelargonidin: 67%, petunidin: 66%, malvidin: 7I% (I6).

It has been shown that measurement of the U.V. spectra of pigments F7 and F8 in MeOH-HCl revealed no characteristic peaks at about 3I0 mμ, which could be used to calculate the molar ratio in relation to the intensity of the pigment absorption in the visible region. As a result of these obstacles, other methods had to be resorted to and the differential spectrum method, when applied to F7, gave promising results.

The spectrum of the purified acylated pigment was measured in methanol containing hydrochloric acid (0.0I%) between 240 and 590 mμ. The pigment solution was evaporated to dryness and hydrolysed with alkali. The resulting anthocyanin was purified as described and its spectrum measured in the same region in MeOH-HCl.

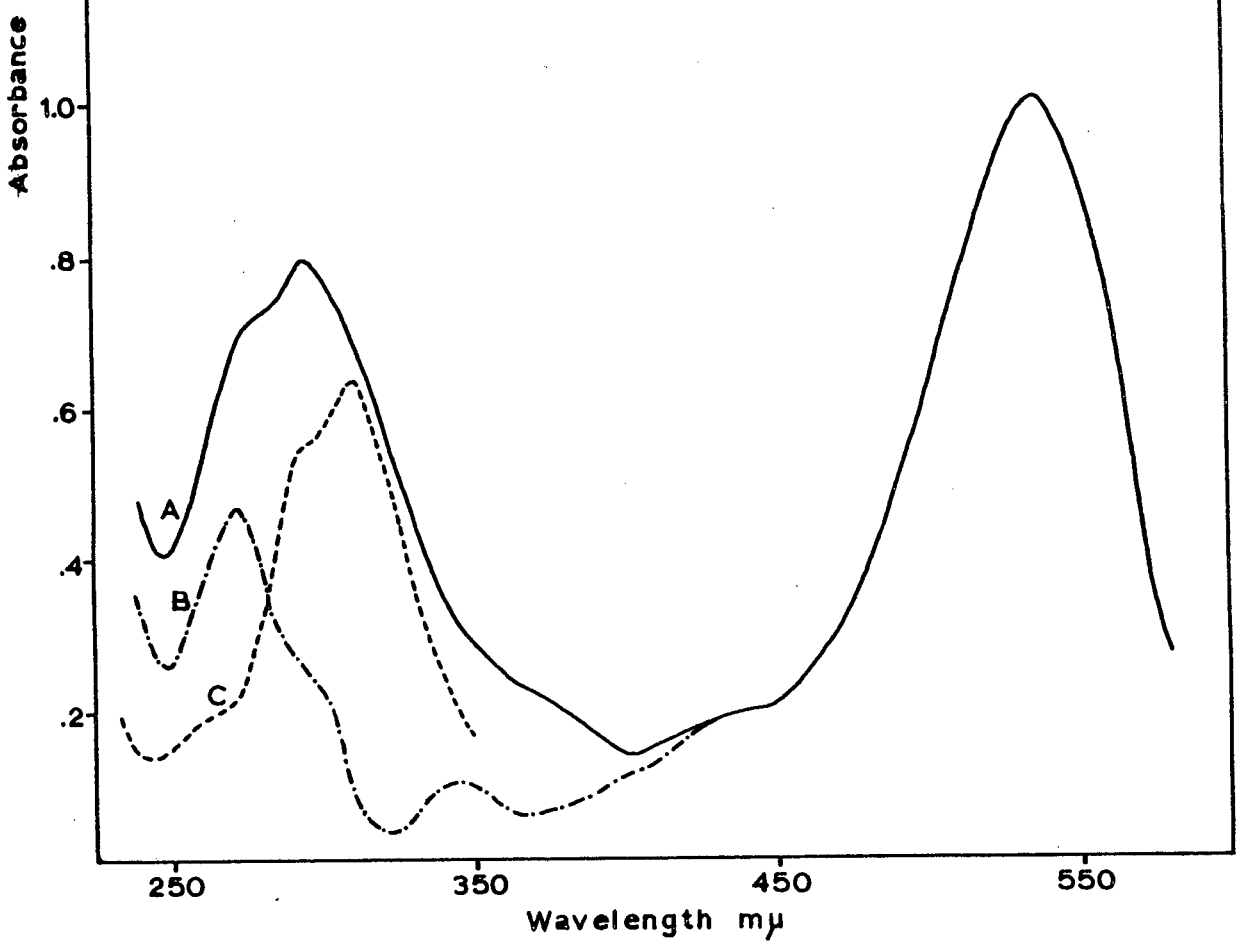
The optical density readings for both spectra were calculated to coincide at a convenient optical density at the wavelength of maximum absorption in the visible region. The differential spectrum for p-hydroxycinnamic acid (found: λmax.3I2.5 mu) was then calculated by subtracting the values thus obtained for the anthocyanin, from those of the ester (Fig. XIV).

The ratio of the optical density at 3I2.5 mμ, to that at the wavelength of maximum absorption in the visible region (535 mμ), averaged 64% for several determinations. This percentage, therefore,

FIG. X1V

ABSORPTION SPECTRUM OF PIGMENT F 7 IN MeOH-HCl SHOWING DIFFERENTIAL SPECTRUM CAUSED BY ACYL CHROMOPHORE

A. Spectrum of ester
B. .. malvidin 3-monoglucoside
C. .. p-hydroxycinnamic acid



being in agreement with published values (I6), denotes pigment F7 as having an anthocyanin:acid ratio of I:I.

VIII,M. The Anthocyanin : Acid Ratio in Pigments F7 and F8

For anthocyanins acylated with two molecules of p-hydroxy-cinnamic acid (or other related aromatic acids), the ratios (VIII,L) are approximately doubled (I6) since a second chromophore system, in equimolecular concentration is introduced.

For unknown reasons, the procedure used to determine the molar ratio in F7, could not be applied to F8. After much experimentation it was decided to disrupt the ester linkages in the acylated pigment and to measure the combined spectrum of the anthocyanin plus the acid component. The latter was subsequently removed and the spectrum of the remaining anthocyanin measured again. The differential spectrum of the acid was calculated as before.

VIII,N. Procedure for the Determination of the Anthocyanin : Acid Ratio in Pigments F7 and F8, in the Hydrolysate

Pigments F7 and F8 were eluted from the chromatograms with WMA under nitrogen. The eluate was filtered and diluted with WMA to give an optical density reading in the range 0.20 to 0.25 at the wavelength of maximum absorption in the visible region. A portion (8-I0 ml.) of each pigment solution was evaporated to dryness and redissolved in 2 N sodium hydroxide (2.0 ml.). After hydrolysis under nitrogen at room temperature for three hours, the hydrolysate was heated at I00°C.

92./ for....

for 30 seconds. After cooling to room temperature, the solution was acidified with hydrochloric acid (0.55 ml.). The acidified solution was filtered through a sintered filter and the filter washed with distilled water (0.45 ml.). The absorption spectrum of the filtered solution (3.0 ml.) was measured between 240 and 590 mμ in quartz cells.

The solution was subsequently saturated with pure soduim chloride and extracted with three portions diethyl ether (2 ml.) which quantitatively removed the p-hydroxycinnamic acid. The remaining saline anthocyanin solution was again filtered through a sintered filter and used for spectral measurement. The reference solution was prepared from an appropriate blank chromatogram and was devoid only of the pigment and its acid component.

The intensity of absorption in the U.V. region appears to be unaffected in aqueous hydrochloric acid and is rather similar to that in MeOH-HCl, however, in the visible region, the absorption is much lower compared to methanolic solutions and there is also a slight shift in the wavelength of maximum absorption in the visible region, towards shorter wavelengths.

The differential spectra obtained, using the above procedure were exceptionally good and free from interference, with p-hydroxycinnamic acid having λ max. at 310 mμ ($\pm$ I mμ).In the case of F8 (Fig. XVI) the intensity of absorption of the acid was double that recorded for F7 (Fig. XV), thus indicating an anthocyanin: acid ratio of I:2.

93./ VIII,0. Position....

FIG. XV

ABSORPTION SPECTRUM OF PIGMENT F7 IN HYDROLYSATE SHOWING DIFFERENTIAL SPECTRUM CAUSED BY ACYL CHROMOPHORE

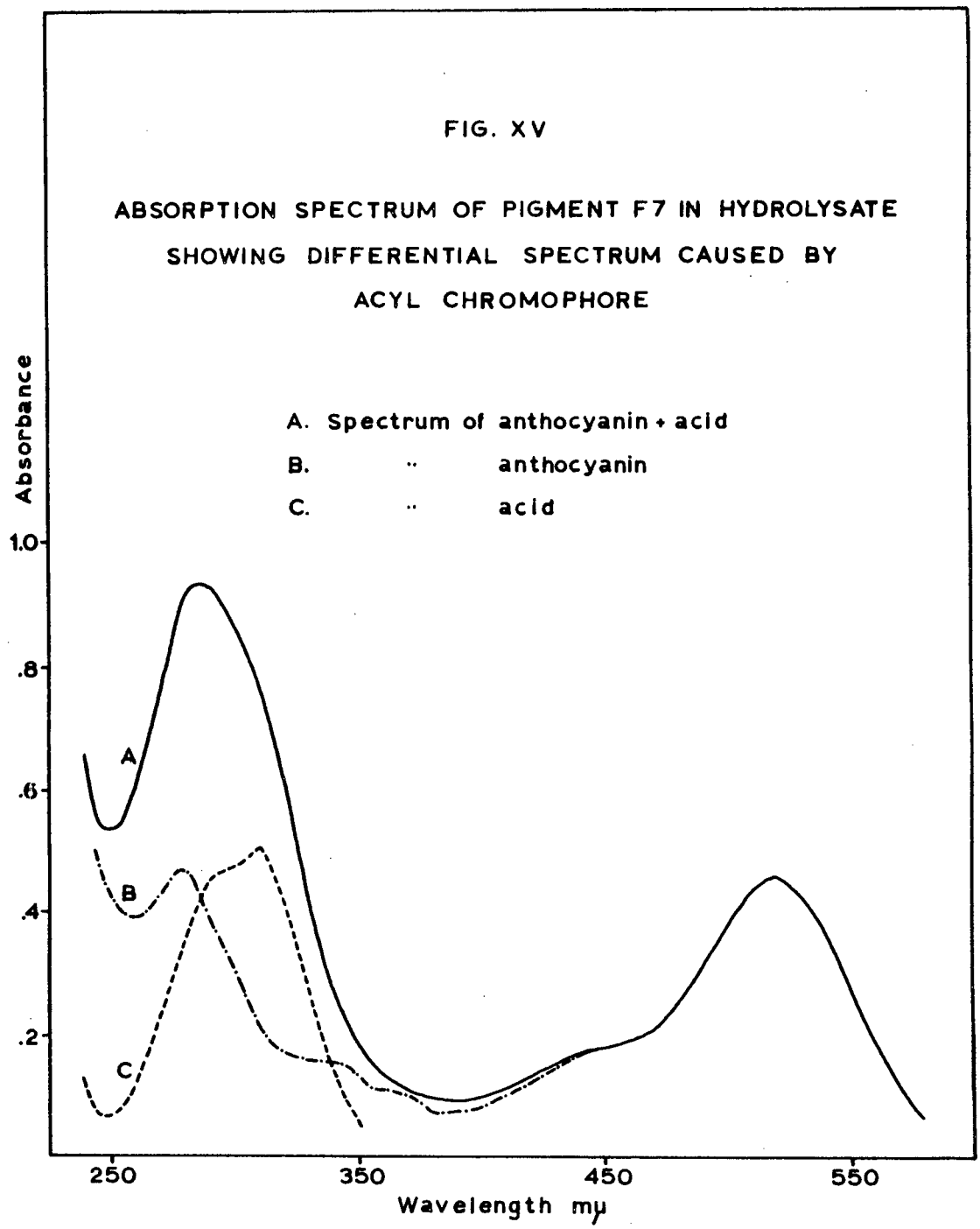
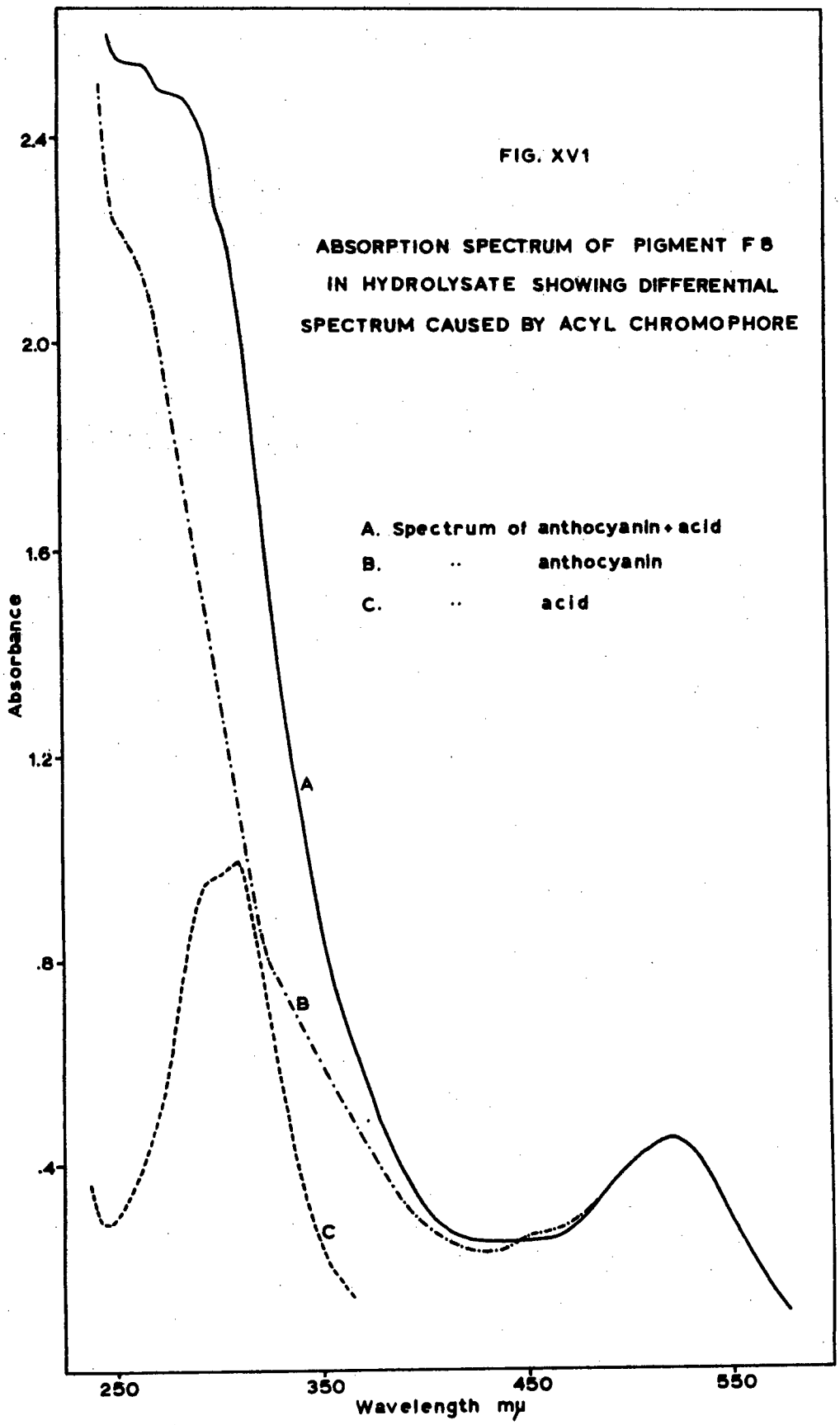


FIG. XV1

ABSORPTION SPECTRUM OF PIGMENT F 8 IN HYDROLYSATE SHOWING DIFFERENTIAL SPECTRUM CAUSED BY ACYL CHROMOPHORE



VIII,0. Position of Attachment of p-Hydroxycinnamic Acid

It will be noted from Figs. XIV and XV that F7 shows a distinct shoulder to the main peak in the 410 - 450 mμ region with regard to both the ester and the deacylated anthocyanin pigment. This shoulder is absent in the esterified pigment F8 and reappears after removal of the acid component (Fig. XVI).

The absence of this shoulder in the 410 - 450 mμ region, is characteristic of anthocyanins which carry sugar or benzoyl residues in the 5-position (16) and it is tentatively suggested that in F8 the second molecule of the acid is attached to the 5-position since this anthocyanin has only one glucose adduct in the 3-position.

94./ CHAPTER IX....

CHAPTER IX

ANALYSIS OF A DRY RED WINE

The procedures outlined in the previous sections, with certain modifications, were successfully applied to a ten month old dry red wine prepared from Cabernet sauvignon grapes. Before investigation of the individual wine pigments, a pigment extract prepared from the wine was chromatographed in 0.6% citric acid (I5), using a pigment extract containing only the monoglucosides of grapes as standard. The developed chromatogram revealed only one non fluorescent pigment band, thereby confirming the total absence of diglycosides in the wine.

IX,A. Investigation of the Aglycones

Excess (40 ml.) of a saturated solution of lead acetate was added to the wine (40 ml.) and the lead-anthocyanin complex removed by centrifugation (4,I07). The supernatant liquid was decanted, the precipitate suspended in methanol and again centrifuged. The procedure was subsequently repeated twice with distilled water . The final precipitate was suspended in distilled water (4 ml.) and decomposed by addition of a slight excess hydrochloric acid. The lead chloride precipitate was centrifuged down and the supernatant anthocyanin solution filtered through a Wilstätter filter. An equal volume 2 N hydrochloric acid was added to the filtrate and the solution heated for two hours under nitrogen(in semi-darkness) at I00°C.

The anthocyanidins were extracted, purified as described

95./ (V,C. (i)) and....

(V,C. (i)) and were separated and identified in Forestal solvent and BAW. The chromatograms were in all respects identical to those obtained for the vinifera grapes, however, the incidence of cyanidin in relation to the other aglycones appeared to be much higher.

IX,B. Investigation of the Glucosides; Preparation of Anthocyanin Extract

Preliminary experiments were conducted using anthocyanin extracts prepared from neutral and basic lead acetate solutions. Paper chromatography in BAW showed that both extracts contained the same anthocyanin pigment bands, however, the incidence of brown streaking, resulting from polymeric material present, was markedly less in extracts obtained from the neutral salt precipitate.

The anthocyanins were consequently precipitated by addition of excess saturated neutral lead acetate solution to wine (40 ml.). The precipitate was consecutively washed with water and methanol. The final precipitate was suspended in methanol (8 ml.) and decomposed with a slight excess hydrochloric acid. The lead chloride was centrifuged down, the methanolic extract filtered, concentrated in vacuo (4 ml.) and filtered again.

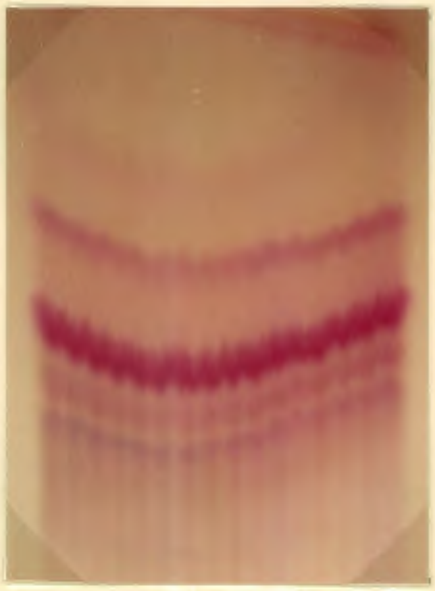
IX,C. Purification of Extract on Polyamide Column

Polymeric material remaining in the extract was removed by column chromatography, using polyamide as adsorbant (76,107,108). The nylon resin used was Ultramide BM, a linear polycondensation product

96./ of....

PLATE VIII

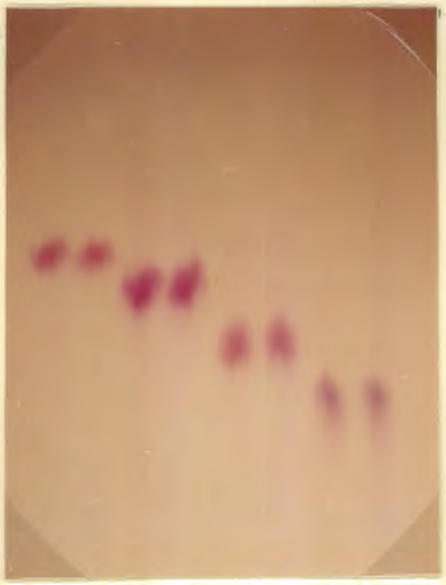
Separation of Wine Pigments in Butanol : Acetic Acid : Water after Purification on Polyamide Column



I. Pigment band W I
2. " W 2
3. " W 3
4. " W 4
5. " W 5
7. " W 7
8. " W 8

PLATE IX

Identification of Pigments W I-4 in Butanol : Acetic Acid : Water



I. Peonidin 3-monoglucoside
2. Pigment W 4
3. Malvidin 3-monoglucoside
4. Pigment W 3
5. Petunidin 3-monoglucoside
6. Pigment W 2
7. Delphinidin 3-monoglucoside
8. Pigment W I

of ε-caprolactam.

The resin (I0 gm.) was dissolved in formic acid (I00 ml.) at 90°C. with stirring. After cooling the solution to 60°C., methanol (200 ml.) and celite (25 gm.) was rapidly added. More methanol (200 ml.) was added and stirring continued for I5 minutes. The slurry was filtered and the adsorbant suspended repeatedly in distilled water (ca 500 ml.) with vigorous stirring until free from formic acid. A portion of the adsorbant was finally suspended in water and poured into a glass column (I0 x I cm.) containing a glass wool plug. The adsorbant was allowed to settle on its own, giving a column with an effective length of 5.0 cm.

The anthocyanin extract was diluted with an equal volume hydrochloric acid (I%) and a portion of the diluted solution (2-3 ml.) applied to the column. The column was washed with hydrochloric acid (5 ml., I%) and the anthocyanins eluted with methanol containing hydrochloric acid (I%). On elution the anthocyanins moved down the column as one band, the polymeric material being firmly adsorbed at the top. The portion of the eluate containing the anthocyanin band was collected and evaporated to dryness.

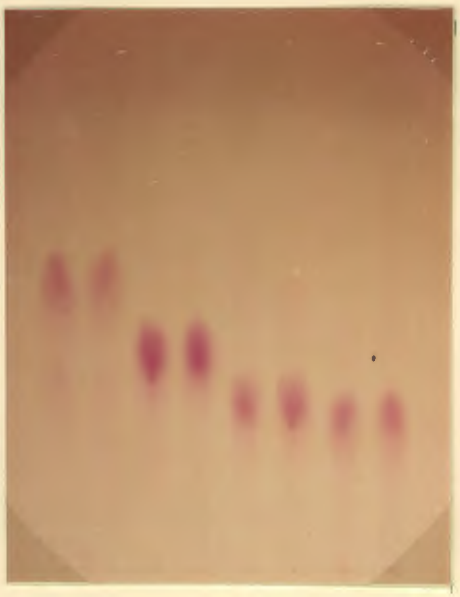
IX,D. Separation and Purification of the Individual Anthocyanins

The dry pigment was dissolved in MHB and chromatographed in BAW on Whatman No. 3MM paper. A very successful separation into six visible pigment bands termed WI, W2, W3, W5, W7 and W8 was achieved.

97./ (Numbers....

PLATE X

Identification of Pigments W I-4 in Butanol : 2N HCl

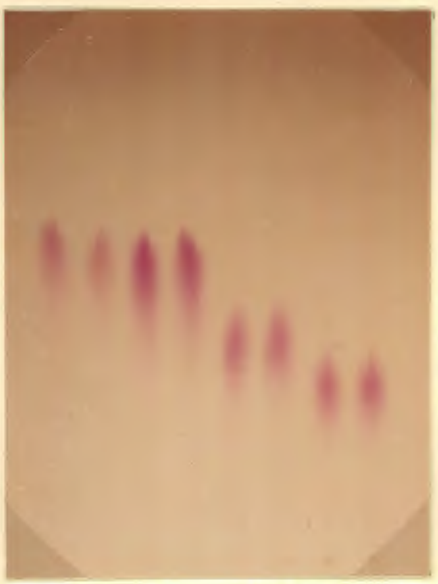


I 2 3 4 5 6 7 8

1. Peonidin 3-monoglucoside
2. Pigment W 4
3. Malvidin 3-monoglucoside
4. Pigment W 3
5. Petunidin 3-monoglucoside
6. Pigment W 2
7. Delphinidin 3-monoglucoside
8. Pigment W I

PLATE XI

Identification of Pigments W I-4 in HOAc-HCl



I 2 3 4 5 6 7 8

1. Peonidin 3-monoglucoside
2. Pigment W 4
3. Malvidin 3-monoglucoside
4. Pigment W 3
5. Petunidin 3-monoglucoside
6. Pigment W 2
7. Delphinidin 3-monoglucoside
8. Pigment W I

(Numbers refer to corresponding pigment bands of vinifera grapes.) No coloured material remained at the origin, however, U.V. fluorescent impurities were detected in the vicinity of band WI, between W3 and W5 and towards the solvent front. Pigment bands WI-3 were purified and freed from these impurities by paper chromatography in HOAc-HCl, Bu--HCl and BAW. Alkali treatment was necessary to free pigment band W3 from W5. Pigment band W4 was not visible on individual chromatograms and was obtained by eluting and purifying the cuttings from several chromatograms. The purified pigments WI-4 were identified by chromatography in BAW, Bu-HCl and HOAc-HCl, Plates IX, X and XI respectively, using the monoglucosides of delphinidin, petunidin, malvidin and peonidin as reference compounds.

IX,E. The Acylated Anthocyanins

The esterified pigments W5 and W7 were purified as described for the corresponding vinifera grape pigments (VIII,B). Band W6 could not be detected and bearing in mind the very low incidence of W4, its absence was to be expected. The pigments were subjected to alkali hydrolysis and the ethereal extract used for spectral examination. The extract was subsequently evaporated to dryness and re-examined in MeOH-HCl and MeOH-NaOH. W5 and W7 were identical to the corresponding grape pigments.

Pigment band W8, obtained during the initial separation of the purified wine extract, was accompanied by brown material on the

98./ chromatogram....

chromatogram. As a result of the unstable nature of this acylated pigment, further purification was not possible. The presence of p-hydroxycinnamic acid was confirmed by chromatography after alkali hydrolysis.

It would appear that the acylated pigments are partially deesterified during fermentation and vinification, however, a very significant amount was still present in this ten month old wine.

TABLE XI

R_f value of Anthocyanins in Various Solvents (27°C.)

Component	BAW	Bu-HCl	HOAc-HCl
Delphinidin 3-monoglucoside	.352	.I87	.I99
WI	.354	.I86	.I98
Petunidin 3-monoglucoside	.4I8	.22I	.248
W2	.4I2	.222	.25I
Malvidin 3-monoglucoside	.482	.288	.347
W3	.480	.286	.346
Peonidin 3-monoglucoside	.5I3	.369	.352
W4	.5I6	.368	.353

99./ CHAPTER X....

CHAPTER X

INVESTIGATION OF A HYBRID GRAPE (JACQUEZ)

It is generally assumed that this grape is a natural hybrid, probably originating from the species Vitis aestivalis, Vitis cineria and Vitis vinifera. Since it is an American - European hybrid, the anthocyanin pigments consist of diglycosides and monoglycosides, the former being a dominant character of such hybrids (I5,62).

X,A. Separation of the Different Glycosidic Groups

A methanolic extract containing hydrochloric acid (I%) was prepared from the dry skins and chromatographed by the ascending technique in 0.6% citric acid (I5) on Whatman No. 3MM paper. Irrigation was continued until the solvent front had advanced 35 to 40 cm. from the starting line. The anthocyanins separated into five bands, all purplish red in colour and were numbered JA to JE in sequence of increasing R_f value. Bands JA and JB were poorly resolved, but JC, JD and JE exhibited a satisfactory separation (Plate XII).

When using a butanolic solvent as irrigant, the diglycosides have lower mobilities than the corresponding monoglycosides, however, in aqueous solvents such as citric acid, HOAc-HCl or the aq. phase of BAW, the order of R_f is reversed (I09).

Comparison of the Jacquez extract with authentic malvidin 3--monoglucoside and 3,5-diglucoside in 0.6% citric acid and HOAc-HCl, revealed that bands JB and JD corresponded in R_f value and colour in

U.V. light to the standard substances respectively. All pigment bands were subsequently individually eluted from the citric acid chromatograms with WMA and the eluates evaporated to dryness. (Due to the incomplete separation between bands JA and JB, these two fractions were combined and the pooled fraction was termed JAB.). The residues were dissolved in MHB and rechromatographed in HOAc-HCl.

X,B. Investigation of Pigment Bands JA and JB

The pooled fraction JAB was rechromatographed in BAW. The anthocyanins separated into six well resolved pigment bands termed JAB I, 2, 3, 4, 5 and 7 in sequence of increasing R_f value. (Numbers refer to corresponding pigment bands of Vinifera grapes.). Pigments JAB I-4 were individually purified by repeated chromatography in BAW and Bu--HCl. Alkali treatment was necessary to free JAB 3 from JAB 5. Bands JAB 5 and JAB 7 were purified by repeated chromatography in BAW and HOAc-HCl.

The purified pigments JAB I-4 were dissolved MeOH-HCl and their spectra measured between 400 and 590 mμ. The ratios of the optical density at 440 mμ, to that at the wavelength of maximum absorption in the visible region (as a percentage) were as follows: JAB I: 18.8%, JAB 2: 17.5%, JAB 3: 19.5% and JAB 4: 22.0%. These percentages tentatively classified pigments JAB I-3 as 3-glycosides of the delphinidin group and JAB 4 as a 3-glycoside of the cyanidin group (VI,J.(ii)). Chromatography in BAW, Bu-HCl and HOAc-HCl as well as their

101./ characteristic....

TABLE XII

R_f Value and Colour of Anthocyanins in 0.6% Citric Acid

Pigment	R_f	Colour visible light	Colour U.V. light
JA	.099	Purple-red	Dull blue-purple
JB	.I68	Purple-red	Dull blue-purple
JC	.24I	Purple-red	Bright purple-red
JD	.436	Purple-red	Fluorescent tile-red
JE	.594	Purple-red	Fluorescent tile-red

characteristic colours, identified these pigments as the 3-monoglucosides of delphinidin, petunidin, malvidin and peonidin respectively, using the monoglucosides of the Vinifera grapes as reference compounds.

The acylated pigments JAB 5 and JAB 7 were hydrolysed with a 2 N sodium hydroxide soln. (VIII,C). The substance present in the ethereal extract resulting from JAB 5 was spectrally identical with that found in pigment F 5 of the Vinifera grapes. The acid component from JAB 7 was identified as p-hydroxycinnamic acid and occurred in an anthocyanin:acid ratio of I:I (VIII,L). After alkali hydrolysis, the glycosides resulting from JAB 5 and JAB 7 were both identified as malvidin 3-monoglucoside. Pigment JAB 6 was not detected.

I02./ Investigation....

PLATE XII

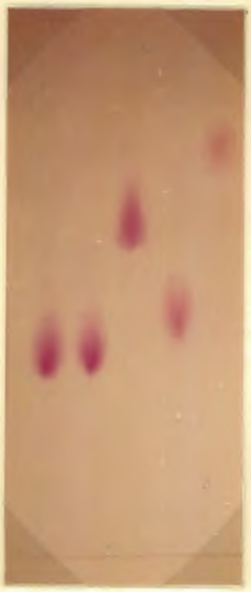
Separation of Jacquez Pigments in 0.6% Citric Acid

1. Pigment band JA
2. " JB
3. " JC
4. " JD
5. " JE

PLATE XIII

Identification of Pigments JD I-2 in Butanol : Acetic Acid : Water

1. Malvidin 3,5-diglucoside
2. Pigment JD I
3. Malvidin 3-monoglucoside
4. Pigment JD 2
5. Peonidin 3-monoglucoside



X,C. Investigation of Pigment Band JC

Monoglucosides resulting from bands JA and JB were present as contaminants in this pigment band and were removed by repeated chromatography in HOAc-HCl and BAW. In BAW the main pigment band exhibited a mauve to purple colour and R_f about .335 on Whatman No. 3MM paper. Immediately to the front of the main pigment band, a reddish-pink zone was noticeable. In U.V. light the main band appeared dull purple whereas the pink zone exhibited a bright pink fluorescence.

The entire band (including the pink zone) was eluted and the eluate evaporated to dryness. The dry pigment was redissolved in MHB and chromatographed in Bu-HCl. Two definite pigment bands separated in this solvent and were termed JC I and JC 2 in sequence of increasing R_f value. The individual pigment bands were further purified by chromatography in the same solvent. The R_f values on No. 3MM paper were .239 and .303 respectively. On the Bu-HCl chromatograms JC I appeared bright purple in U.V. light, whereas JC 2 showed a pink fluorescence.

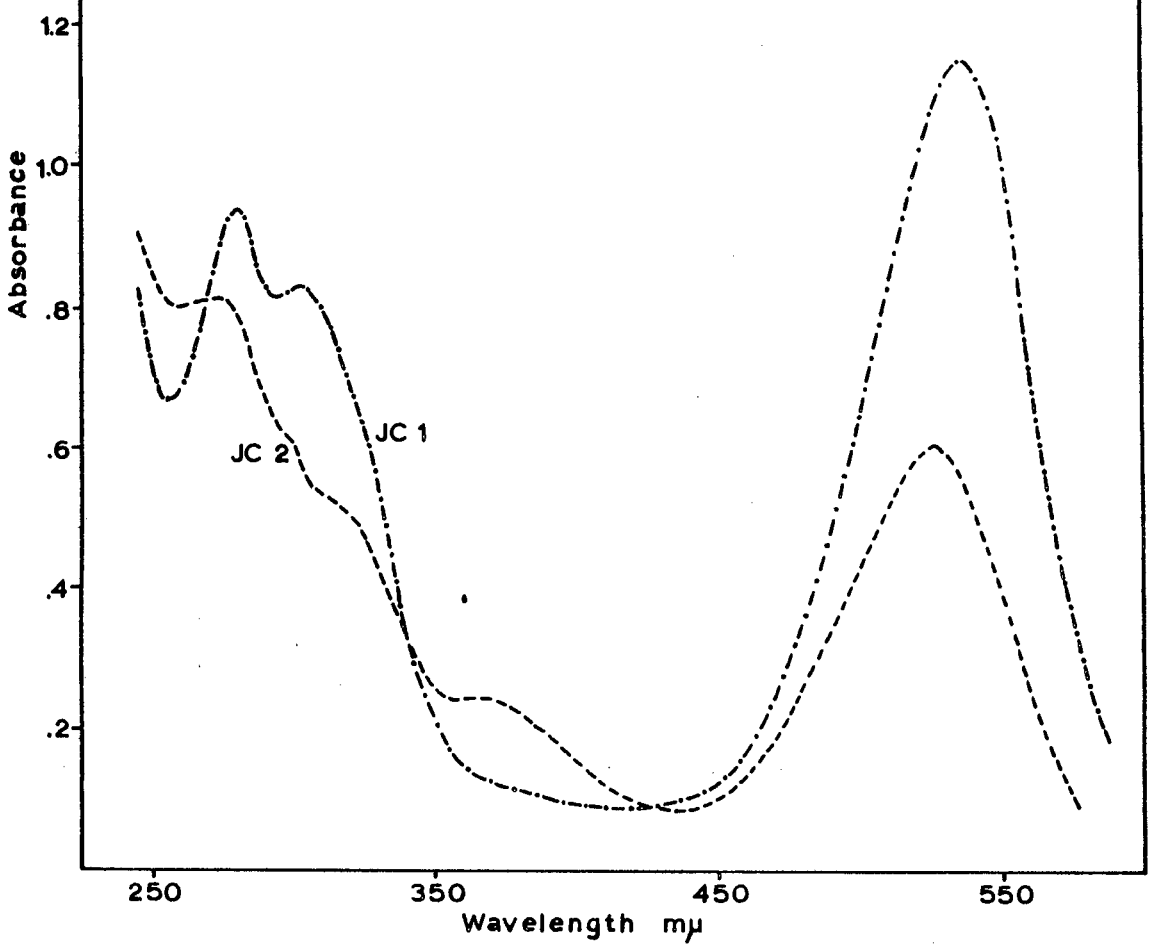
During purification of the individual pigments in Bu-HCl, the appearance of a minor component of very low R_f was noticed. The minor component resulting from JC I was purple to mauve and that from JC 2 pink. In U.V. light these two components were fluorescent tile-red and pink respectively.

Spectral investigation of JC I and JC 2 in MeOH-HCl, revealed abnormal patterns in the U.V. region, indicative of acylated pigments.

I03./ Pigment....

FIG. XV11

ABSORPTION SPECTRA OF PIGMENTS
JC 1 AND JC 2 IN MeOH-HCl



Pigment JC I showed two small peaks at 280 and 300 mμ respectively, whereas JC 2 exhibited a very weak peak at about 272.5 mμ and a slight inflexion at about 315 mμ (Fig. XVII). Both pigments were subsequently hydrolysed with alkali and in both instances the acid component was identified as p-hydroxycinnamic acid, by spectral examination of the ethereal extract.

(i) Purification and identification of the glycosides resulting from JC I and JC 2 after alkali hydrolysis

Diglucosides are much more polar compounds than the corresponding monoglucosides and are thus not extractable from the saline hydrolysate by higher alcohols. The saline solution after removal of the acid component, was evaporated to dryness in vacuo. The pigment was redissolved in Bu-MH and the solution filtered to remove salt crystals. After repeating the procedure a second time, the pigment was purified by chromatography in HOAc--HCl and BAW or Bu-HCl.

The purified pigments were redissolved in MeOH-HCl and their spectra measured between 400 and 590 mμ. The ratio of the optical density at 440 mu to that at the wavelength of maximum absorption in the visible region (as a percentage), was 10.3% for JC I and 13.1% for JC 2. Reported values for glycosides of the delphinidin

104./ and....

and cyanidin groups, which have sugars in either the 5 or the 3 and 5-positions are I0% and I2% respectively (I6), which is about half that given by the corresponding glycosides in which the 5-position is free (VI,J. (ii)). Pigments JC I and JC 2 were thus tentatively classified as glycosides belonging to the delphinidin and cyanidin groups respectively, having sugars at least in the 5-position.

Both pigments were subsequently chromatographed in BAW and Bu-HCl, using malvidin 3,5-diglucoside as reference compound. JC I was identical with the reference compound with respect to R_f value, colour and U.V.fluorescence in both solvent systems. JC 2, on account of its characteristic pink colour and pink fluorescence in U.V. light was suspected as being the 3,5-diglucoside of peonidin. This was substantiated by plotting the R_m values (35) of peonidin, its 3-monoglucoside, and JC 2 , against the number of substituent sugar groups. R_f values are related to the nature and number of substituent groups in the C_{15} skeleton respectively, in such a manner that in many instances the R_m value i.e., log $(\frac{I}{R_f} - I)$, changes by equal increments with each successive addition of a particular substituent

I05./ group....

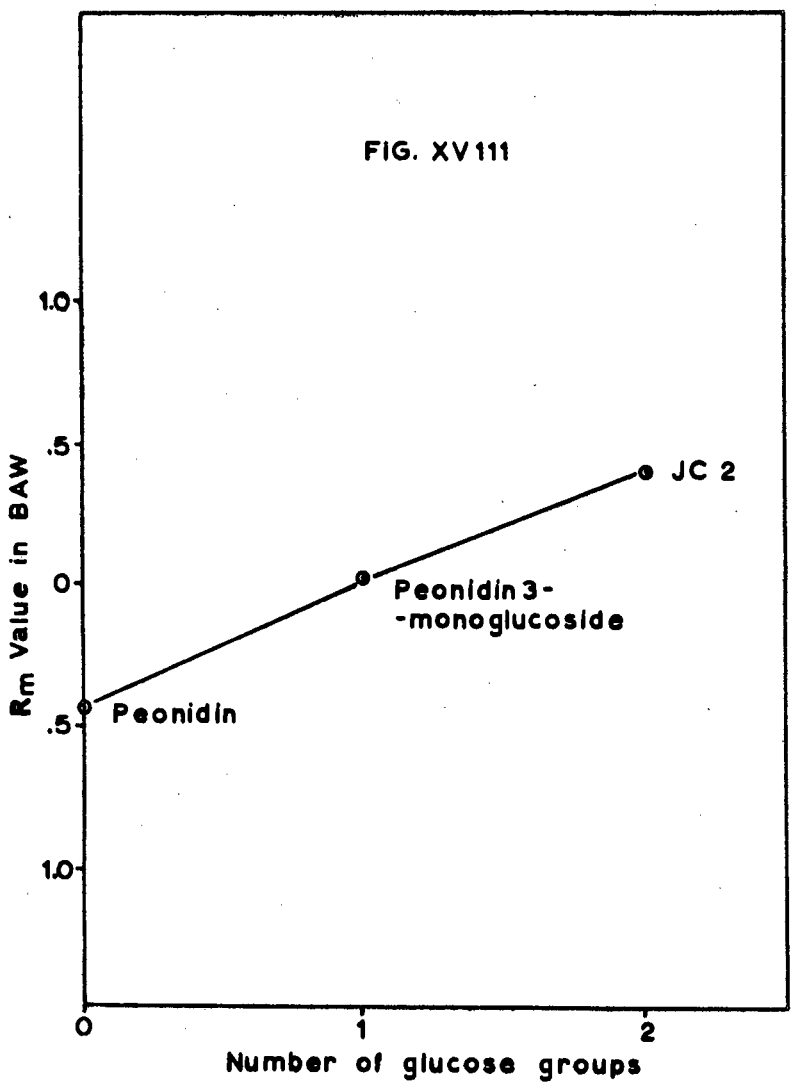


FIG. XV 111
1.0
.5
0
.5
1.0
Rm Value in BAW
JC 2
Peonidin 3-
-monoglucoside
Peonidin
0
1
2
Number of glucose groups

group of any one kind. The R_f values used for this purpose were obtained from chromatograms which were all developed by the descending technique in BAW, at the same temperature. The almost linear relationship obtained (Fig. XVIII), as well as chromatographic and spectral evidence, thus contributed to the identification of pigment JC 2 as peonidin 3,5-diglucoside.

(ii) The anthocyanin:acid ratio in pigments JC I and JC 2

Application of the differential spectrum method in MeOH-HCl to pigments JC I and JC 2 was most unsatisfactory and only in the case of the former pigment (Fig. XIX), could the spectrum of the acid component be obtained. Though this differential spectrum was by no means satisfactory (λ max. 315 mμ), the ratio of the optical density at 315 mμ, to that at the wavelength of maximum absorption in the visible region (as a percentage), averaged 55% for several determinations. This is about 10% lower than the reported values (VIII, L), but still indicates an anthocyanin:acid ratio of I:I.

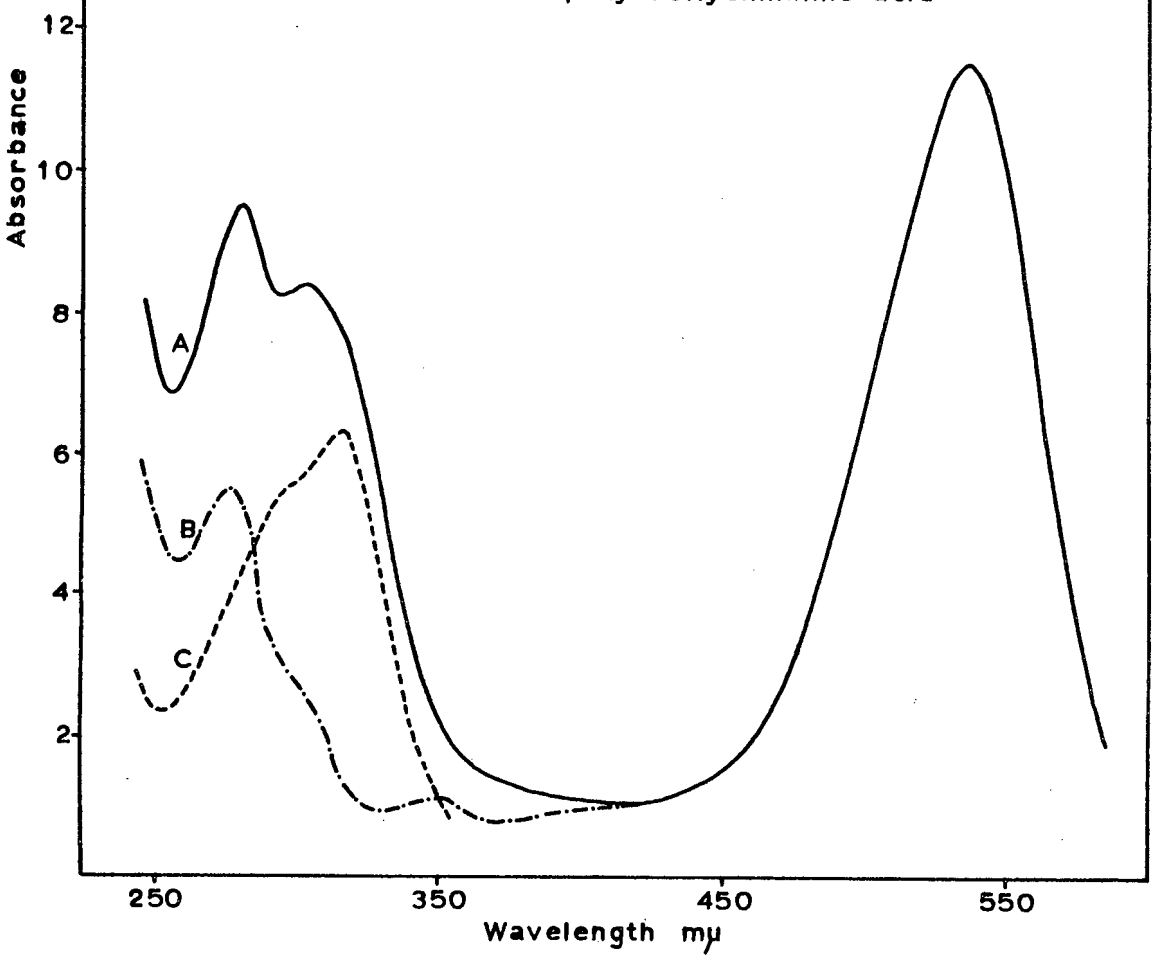
The same procedure, when applied directly to the hydrolysate, gave promising and more consistent results for both pigments (Figs. XX and XXI). The ratio of the

106./ optical....

FIG. X1X

ABSORPTION SPECTRUM OF PIGMENT JC1 IN MeOH-HCl SHOWING DIFFERENTIAL SPECTRUM CAUSED BY ACYL CHROMOPHORE

A. Spectrum of ester
B. " malvidin 3,5-diglucoside
C. " p-hydroxycinnamic acid



optical density at 3I0 mμ, to that at the wavelength of maximum absorption in the visible region (as a percentage), was I3% higher in JC 2 than in JC I. This variation might be due to differences in the intensity of the pigment absorption in the visible region, as JC I and JC 2 are different pigments (X,C. (i)). The anthocyanin:acid ratio was thus regarded as I:I in both instances.

From the experiments described in this thesis, it would appear that the spectroscopical investigation of the esterified glucosides, provide a means for distinguishing between the esterified 3-monoglucosides and 3,5-diglucosides of the same anthocyanidins. If the spectra are determined in aqueous hydrochloric acid (i.e., in the hydrolysate) and if a molar ratio of acid to the anthocyanin component is assumed as being I:I, the intensity of absorption of the 3-monoglucoside is approximately 50% of the intensity of absorption of the anthocyanin in the 3,5-diglucoside at the wavelength of maximum absorption in the visible region (Figs. XV and XX). This may be due to solvent effect and merits further investigation.

I07./ TABLE....

FIG. XX

ABSORPTION SPECTRUM OF PIGMENT JC 1 IN HYDROLYSATE SHOWING DIFFERENTIAL SPECTRUM CAUSED BY ACYL CHROMOPHORE

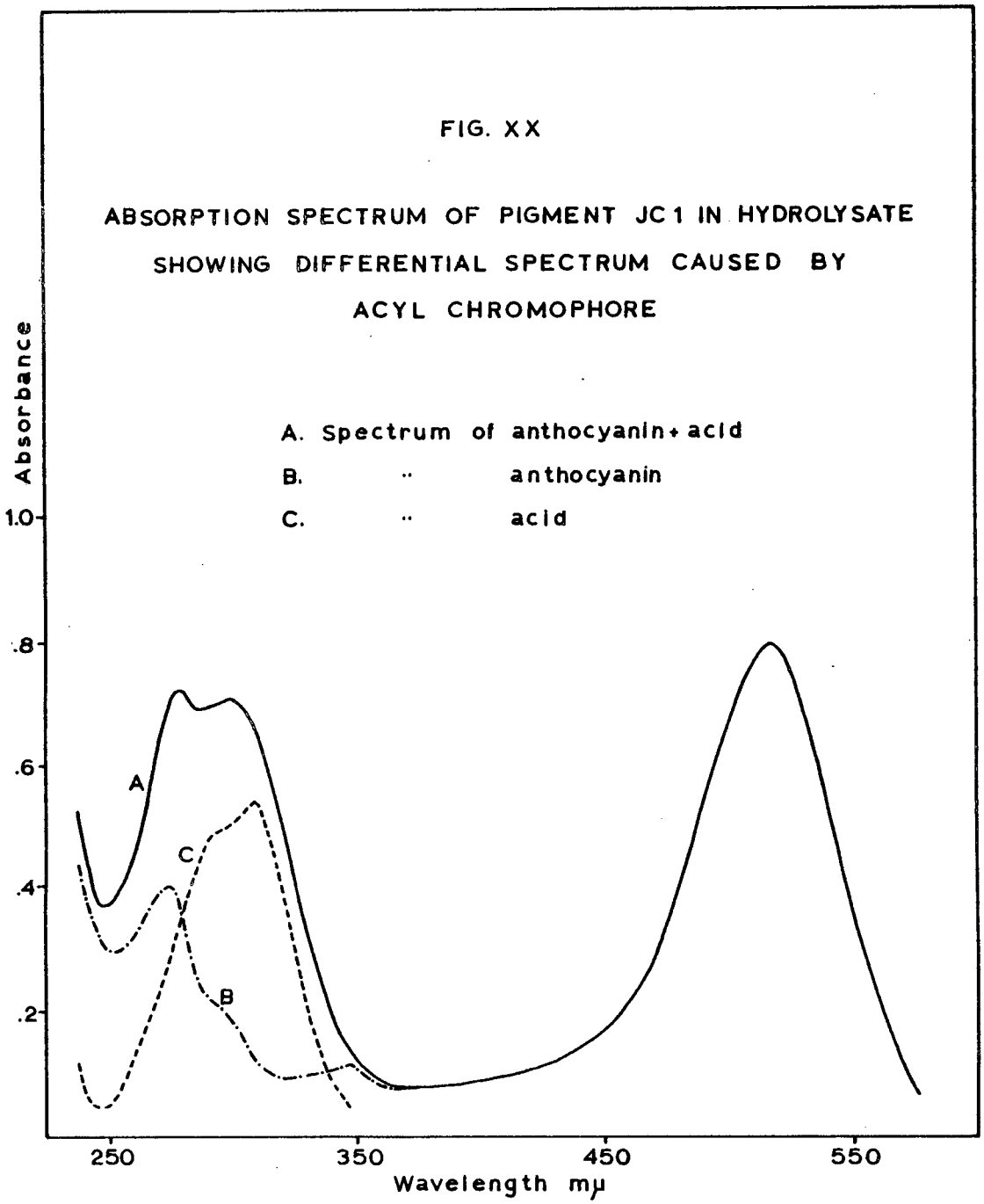


FIG. XX1

ABSORPTION SPECTRUM OF PIGMENT JC 2 IN HYDROLYSATE SHOWING DIFFERENTIAL SPECTRUM CAUSED BY ACYL CHROMOPHORE

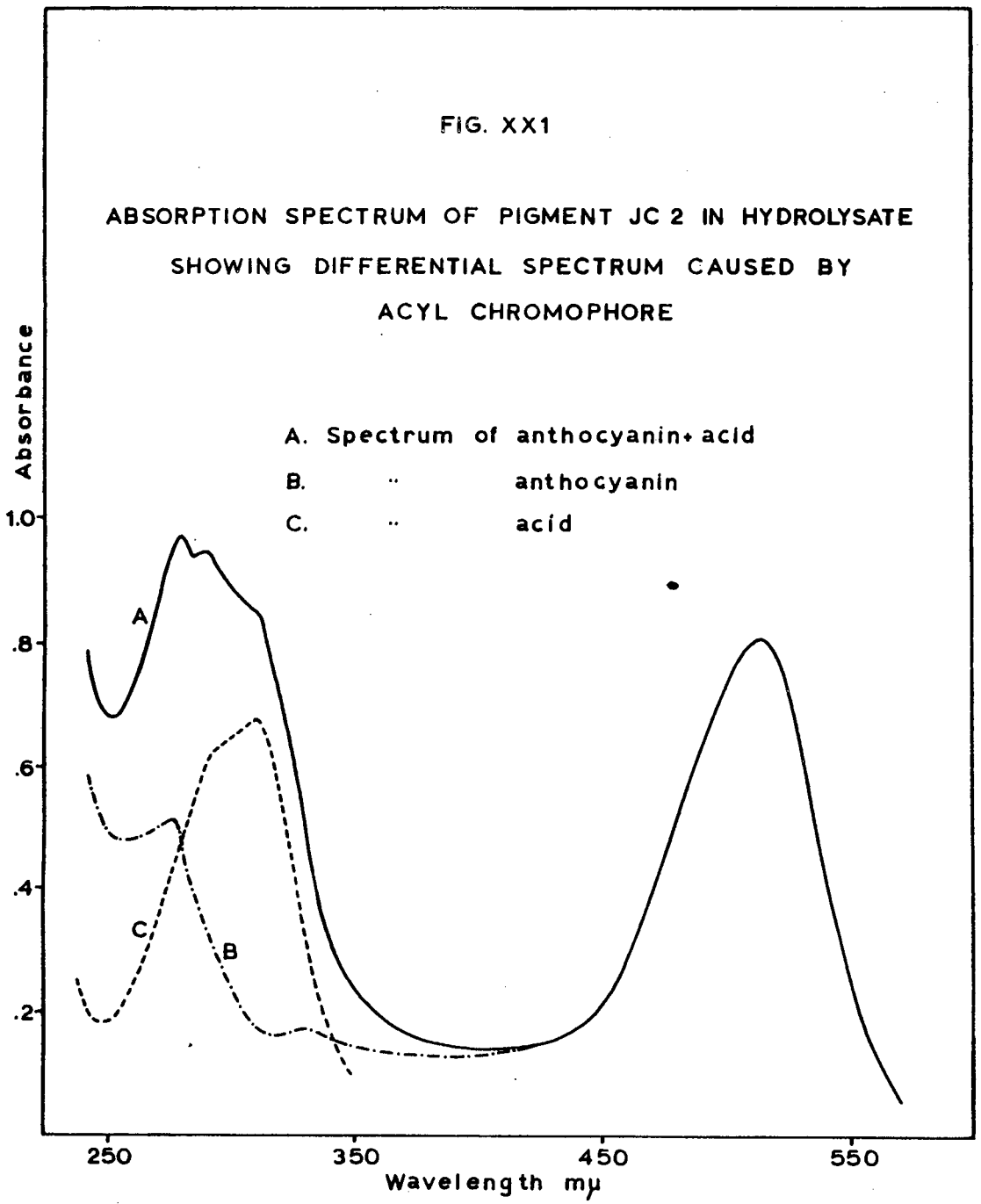


TABLE XIII

R_f Value and Colour of Diglucosides after Alkali Hydrolysis

Pigment	R_f BAW (25°C.)	Visible	U.V.	R_f Bu-HCl (25°C.)	Visible	U.V.
Malvidin 3,5-diglucoside	.262	P-M	d P	.093	P-M	ftR
JC I	.265	P-M	d P	.097	P-M	ftR
JD I	.265	P-M	d P	.098	P-M	ftR
JD 3	.265	P-M	d P	.098	P-M	ftR
JD 5	.265	P-M	d P	.098	P-M	ftR
JD 2 (Peonidin 3,5-diglucoside)	.295	PK	fPK	.I32	PK	fPK
JD 4	.294	PK	fPK	.I28	PK	fPK
JD 6	.294	PK	fPK	.I33	PK	fPK
JC 2	.290	PK	fPK	.I33	PK	fPK

P = Purple, M = Mauve, PK = Pink, d = Dull, f = Fluorescent, tR = Tile Red

X,D. Investigation of Pigment Band JD

This pigment constitutes the principal U.V. light fluorescent band on the citric acid chromatogram. Repeated chromatography in 0.6% citric acid and HOAc-HCl could not free this pigment from traces of

I08./ band....

band JE. Chromatography in BAW gave six components, termed JD I-6 in sequence of increasing R_f value (Table XIV). Bands I and 2; 3 and 4; as well as 5 and 6 were only partially resolved, whereas the three individual pairs were well separated from one another. Bands JD I and JD 2 were the two principal components and were present in about equal concentration.

Pigments JD I and JD 2 were combined and rechromatographed in BAW, however, bands JD 3-6 also reappeared on the chromatogram.Further rechromatography in BAW showed that bands JD 3-6 could not be removed entirely. JD I and JD 2 were again combined and hydrolysed with alkali (VIII,C). After removal of salt they were separated in Bu-HCl and then individually purified in the same solvent.

Chromatography of JD I and JD 2 in BAW, before and after alkali treatment, showed that the R_f values remained constant, thus indicating that no acid components were present in these two anthocyanins. (Pigments JC I and JC 2 both showed a decrease in R_f value after alkali hydrolysis.). JD I and JD 2 were identified as malvidin and peonidin 3,5-diglucoside respectively, by spectral and chromatographic means as described for pigments JC I and JC 2.

Bands JD 3 and JD 4 could not be separated in Bu-HCl or the other anthocyanin solvents and was thus combined. On rechromatography in BAW, each yielded a minor component of low R_f value. The minor component having the lowest R_f value was purple to mauve and obviously

109./ resulted....

resulted from partial deacylation of JD 3 which was also purple. The minor component of higher R_f value and having a pink colour, likewise originated from JD 4 (Table XIV).

The combined fraction JD 3 and JD 4 was subsequently hydrolysed with 2 N sodium hydroxide soln. (VIII,C) and the resulting pigments separated and purified as described for JD I and JD 2. They were identified as malvidin and peonidin 3,5-diglucoside respectively.Spectral investigation of the ethereal extract, obtained after alkali hydrolysis of the combined fractions JD 3 and JD 4, revealed a component identical with that present in F5 of the Vinifera grapes (Fig. VIII).

The amount of material present in bands JD 5 and JD 6 did not permit rechromatography experiments in BAW. After alkali hydrolysis of the combined pigment bands, JD 5 and JD 6 were identified as malvidin and peonidin 3,5-diglucoside respectively. The component present in the ethereal extract was spectrally identical with that detected in the combined fraction JD 3 and JD 4.

X,E. Investigation of Pigment Band JE

On rechromatography of this individual pigment band in HOAc--HCl, a relatively large portion (25 - 50%) was converted to pigment band JD. Subsequent chromatography of band JE in BAW gave six components, termed JE I-6 in sequence of increasing R_f value. These pigments were in all respects identical with those obtained from band JD in BAW. Band JD, which originated from band JE on rechromatography in

IIO./ HOAc-HCl....

HOAc-HCl, likewise contained the same pigments.

It has been shown that pigment band JD contained malvidin and peonidin 3,5-diglucoside as the two principal components (JD I and JD 2 respectively). The presence of "esterified" derivatives of these two pigments in band JD was therefore regarded as contamination resulting from band JE. The malvidin and peonidin 3,5-diglucosides detected in band JE, after rechromatography in BAW, consequently resulted from partial de-esterification of the "esters" present in this band.

In conclusion, therefore, it would appear that band JD contains only the two principal normal diglucosides (JD I and JD 2), whereas their corresponding "esters" occur in band JE. The two malvidin 3,5-diglucoside "esters" are thus JE 3 and JE 5, whereas the two peonidin 3,5-diglucoside "esters" are JE 4 and JE 6.

TABLE XIV

Separation of Band JD in BAW on Whatman No. 3MM Paper

Pigment	R_f	Colour visible light	Colour U.V. light
JD I	.260	Mauve	Dull blue-purple
JD 2	.285	Pink	Fluorescent pink
JD 3	.321	Mauve	Dull purple
JD 4	.355	Pink	Fluorescent pink
JD 5	.382	Mauve	Dull purple
JD 6	.410	Pink	Fluorescent pink

III./ CHAPTER XI....

CHAPTER XI

CONCLUSION

Investigation of the glucosides of six Vitis vinifera varieties, after quantitative acid hydrolysis and chromatography in Forestal solvent and in BAW on acid treated paper, revealed that the same five anthocyanidins viz. delphinidin, petunidin, cyanidin, malvidin and peonidin were present. The proportion of each aglycone varied with the different varieties. Cyanidin was shown not to be present as a glycoside and hence originated from the corresponding flavan-3:4-diol.

Contrary to reports (56) that leucocyanidin present in wine and originating from the skins, is negligible, its presence in the skins in the same general order as the anthocyanidins proper, has been established.

The conventional methods for eluting flavoniod pigments from paper chromatograms were found unsatisfactory due to fading of the anthocyanidin solutions. The procedure was modified to effect elution under nitrogen in the dark in a much shorter time. Methanolic solutions of anthocyanidins containing hydrochloric acid (I%) were evaporated to dryness under reduced pressure at $4^{\circ}C$. in order to restrict fading and the formation of degradation products.

With regard to the glycosidic pigment pattern of the Vinifera varieties and in particular the variety Cabernet sauvignon, the identity of eight pigments were established. Four of these pigments

II2./ constitute....

constitute the normal glycosides and were identified as the 3-monoglucosides of delphinidin, petunidin, malvidin and peonidin (in sequence of increasing R_f value), by spectral and chromatographic means.

BAW was the only solvent suitable for the initial separation of both the normal and esterified glucosides, whereas Bu-HCl and HOAc--HCl were useful for further purification and identification of individual pigments.

Comparison of the relative concentration of the individual pigments of the six Vinifera varieties, showed that Alicante bouschet and Pontak differ significantly from the others. In both varieties, the incidence of delphinidin 3-monoglucoside is about half or even less than that of the other varieties. The malvidin 3-monoglucoside content, conversely, is approximately double those of the other varieties. In addition, Alicante exhibited the highest percentage recorded for peonidin 3-monoglucoside, being about three times higher than the average for the remaining five varieties. Differences in the relative concentration of the esterified pigments were also apparent.

It is known that during the course of ageing, a gradual demethylation of the methylated anthocyanins occur. Alicante and Pontak yield wines which loose their colour more readily than the other varieties investigated and in this regard their pigment pattern (particularly those pigments mentioned above) is certainly of interest.

Paper chromatography of the sugars of the purified glycosides

II3./ revealed....

revealed the presence of both glucose and arabinose. The latter was shown by examination of all the sugars present in the combined anthocyanin hydrolysate, to have originated during the purification process, from the action of mineral acid (present in solvent systems) on the filter paper. The arabinose was effectively removed by chromatography in Bu-BPW and the glucose identified by paper chromatography and spectral investigation of the coloured products produced by the unsulphonated reaction.

Particular attention was devoted to the esterified glucosides. An "acylated" malvidin and peonidin 3-monoglucoside, in which the presence of catechin was substantiated by spectral and chromatographic evidence, have tentatively been identified. Although the chromatographic behaviour of these two pigments are identical to that of the other acylated pigments containing an aromatic acid as the acid component, they cannot be classified as being acylated in the true sense of the word. It is tentatively suggested that they occur in association with catechins or alternatively that the latter acts as a co-pigment.

In addition, two further malvidin 3-monoglucosides acylated with p-hydroxycinnamic acid in different molar ratios (I:I and I:2), were characterised. Existing techniques (without resorting to quantitative determinations) were found unsatisfactory for the investigation of these two acylated pigments. A new spectrophotometric technique,

II4./ not....

not previously reported for acylated anthocyanins was developed and permits identification and determination of the anthocyanin : acid ratio in one operation. This technique was successfully applied to acylated diglucosides occurring in Jacquez grapes.

Experimental evidence also suggests the existence of two further peonidin 3-monoglucosides, esterified in the same manner with p--hydroxycinnamic acid i.e., with anthocyanin : acid ratios of I:I and I:2. They were, however , present in trace amounts which did not permit detailed investigation.

The procedures used for the investigation of the Vinifera grape pigments were successfully applied to the identification of the anthocyanins present in a dry red wine. This, however, necessitated the preparation of an anthocyanin extract through the lead-anthocyanin complex. Polymeric material present in this extract was removed by chromatography on an Ultramide-celite column.

It is significant that esterified pigments were still present after fermentation and vinification in this ten month old wine. It has been reported that p-hydroxycinnamic acid adds sulphite to the double bond in the side chain (43), and if this can be substantiated, the acylated anthocyans play an important part, particularly in young wines, regarding the bound sulphurous acid.

In recent years certain countries have raised objections to the presence of diglucosides in export wines and for this reason a

II5./ detailed....

detailed investigation on the pigments occurring in Jacquez grapes (which represents the most commonly used self bearing rootstock) was included. Being a hybrid, this grape contains both mono and diglucosides. Fourteen individual pigments were characterised after separation of the different glycosidic groups. Six of these pigments were identical with the monoglucosides of the Vinifera grapes, whereas the remainder constituted the normal and acylated 3,5-diglucosides of malvidin and peonidin.

It is assumed that one parent of Jacquez was Vitis aestivalis, in which cyanidin 3-monoglucoside accounts for about 3I% of the total pigmentation (I4). This pigment was, however, not detected or alternatively, may have been present in trace amounts.

II6./ CODE....

CODE LETTERS AND DESCRIPTION OF PIGMENTS

Vinifera grape	Wine	Jacquez	Aglycone	Glycosidic nature	Acid component	Anthocyanin: acid ratio	Occurrence
F I	W I	JAB I	Delphinidin	3-monoglucoside	-	-	G
F 2	W 2	JAB 2	Petunidin	"	-	-	G
F 3	W 3	JAB 3	Malvidin	"	-	-	G
F 4	W 4	JAB 4	Peonidin	"	-	-	G
F 5	W 5	JAB 5	Malvidin	"	(C)	-	(C)
F 6	-	-	Peonidin	"	(C)	-	(C)
F 7	W 7	JAB 7	Malvidin	"	PC	I:I	E
F 7X	-	-	Peonidin	"	PC	I:I	E
F 8	W 8	-	Malvidin	"	PC	I:2	E
F 8X	-	-	Peonidin	"	PC	I:2	E
		JC I	Malvidin	3,5-diglucoside	PC	I:I	E
		JC 2	Peonidin	"	PC	I:I	E
		(JD/JE I	Malvidin	"	-	-	G
		(JD/JE 2	Peonidin	"	-	-	G
		(JD/JE 3	Malvidin	"	(C)	-	(C)
		(JD/JE 4	Peonidin	"	(C)	-	(C)
		(JD/JE 5	Malvidin	"	(C)	-	(C)
		(JD/JE 6	Peonidin	"	(C)	-	(C)

(C) = Associated with catechin
E = As ester
G = As glucoside
PC = p-Hydroxycinnamic acid

II7./ SOLVENTS....

SOLVENTS AND ABBREVIATIONS USED

Abbreviation	Composition	Proportion v/v	Layer used
BAW	n-Butanol : acetic acid : water	4:I:5	Upper layer
Bu-HCl	n-Butanol : 2N HCl	I:I	"
Forestal	Water : acetic acid : HCl	I0:30:3	Miscible
BEH +	n-Butanol : ethanol : water	80:I9:I	"
HOAc-HCl	Water : acetic acid : HCl	82:I5:3	"
WMA	Water : methanol: acetic acid	25:70:5	"
MHB +	Methanol : 5% aq. HCl : n-butanol	70:20:I0	"
Bu-BPW	n-Butanol : benzene : pyridine : water	5:I:3:3	Upper layer
CW	s-Collidine : water	I:I	"
EPW	Ethyl acetate : pyridine : water	3:I:3	"
Bu-MH +	n-Butanol : methanol : HCl	I9:80:I	Miscible

+ These solvents were composed for this investigation.

II8./ ORIGIN....

ORIGIN OF SPECIMENS

Delphinidin	(Fluka A.G., Chemische Fabrik, Buchs, Schweiz.)
Malvidin 3,5-diglucoside	"
p-Hydroxycinnamic acid	"
O-Hydroxycinnamic acid	"
(-)-Epicatechin	"
(+)-Catechin	"
Chlorogenic acid	"
Caffeic acid	"
Sinapic acid	"
Protocatechuic acid	"
Hydrocaffeic acid	"
Syringic acid	"
Ferulic acid	"
Delphinidin 3-monoglucoside	(Dr. J.B. Harborne, John Innes Institute, Bayfordbury, Hertford, England.)
Petunidin	"
Petunidin 3-monoglucoside	"
Malvidin	"
Malvidin 3-monoglucoside	"

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