AN ABSTRACT OF THE THESIS OF

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Title PROBLEMS ENCOUNTERED IN MEASURING THE LEUCO-ANTHOCYANIN CONTENT OF PEARS

Abstract approved _____
(Major professor)

This study explored some of the difficulties involved in measuring the leucoanthocyanin content of Bartlett pears by a currently used method which involves extracting the leucoanthocyanin from the plant tissue, converting it to anthocyanidin and measuring the resulting pigment colorimetrically. Both the extraction and the conversion of leucoanthocyanin were investigated.

The effectiveness of five solvents, 70% acetone, 95% (ethanolic) ethanol, methanol, 40% aqueous acetone and 0.1 N HCl in 40% aqueous acetone, was studied. Acetone extracted approximately 60% of the leucoanthocyanin, ethanol extracted one-fourth and methanol extracted one-third.

The conversion was studied by varying the normality of the developer (HCl in n-butanol), the dispersing medium and the source of leucoanthocyanin (marc, slurry or synthetic leucocyanidin). The conversion appeared to be dependent on interrelations among all

three of these factors.

For developing the anthocyanidin from marc previously extracted with ethanol, a combination of a dispersing medium of 70% acetone and a normality of 0. 6 was better than ethanol and a normality of either 0. 025 or 0. 6. Seventy percent acetone and 0. 025 N gave the smallest conversion. For developing the anthocyanidin from the slurries, 0. 025 N HCl in n-butanol was used, as browning occurred due to phlobaphene formation with higher normalities. This normality plus a dispersing medium of 70% acetone gave greater yields of anthocyanidin than did ethanol, methanol or aqueous acetone and 0. 025 N HCl in n-butanol. The conversion of synthetic leucocyanidin was examined by using combinations of 70% acetone, 95% ethanol, methanol or aqueous acetone with both 0. 6 N and 0. 025 N HCl in n-butanol. Seventy percent acetone and 0. 025 N gave the greatest conversion whereas ethanol or methanol and 0. 6 N HCl in n-butanol gave the least.

There were similarities in the conversion to anthocyanidin of the leucoanthocyanin from the three sources but discrepancies also. Paper chromatograms indicated that another anthocyanidin in addition to cyanidin was formed during the conversion.

Results of this study suggested that the method currently used to measure leucoanthocyanins would be improved by using a slurry of pear dispersed in 70% acetone (ethanolic) and by using 0. 025 N HCl in n-butanol to effect the conversion.

PROBLEMS ENCOUNTERED IN MEASURING THE LEUCOANTHOCYANIN CONTENT OF PEARS

by

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PROBLEMS ENCOUNTERED IN MEASURING THE LEUCOANTHOCYANIN CONTENT OF PEARS

INTRODUCTION

Measurement of the leucoanthocyanin content of Bartlett pears was attempted by Phillips in 1963 (32, p. 19). Using the method of Swain and Hillis (51, p. 64), she tried to show a relationship between the quantity of this compound and the extent of the pink discoloration in the canned pears. Luh et al. (31, p. 55) had previously shown that the pink color in canned pears was due to the formation of anthocyanidin. Because colorless leucoanthocyanins form anthocyanidins when heated in the presence of acid, leucoanthocyanins are implicated. Phillips compared the extent of pink discoloration in over-processed pears with the concentration of leucoanthocyanin in the fresh tissue. She found that pears having the highest concentration of leucoanthocyanin together with the lowest pH values were the pinkest and those having the lowest concentration of leucoanthocyanin together with the highest pH values were least pink. For the other pears the relationship between the leucoanthocyanin content, pH and pinkness in the fruit was not clear cut.

Perhaps one factor which may have contributed to the inconclusiveness of that study was the method of measuring the leucoanthocyanin. In preliminary work for the present study, it was apparent

that the method used by Phillips (32, p. 15) failed to extract completely the leucoanthocyanin. Furthermore, Swain and Hillis had estimated that their method would measure only 25% of the leucoanthocyanin present in the extract (50, p. 65). Thus results from this currently used method are representative of only a small portion of the true leucoanthocyanin content of the fruit. As knowledge of the chemistry of leucoanthocyanins has been rapidly unfolding in recent years, it was anticipated that an application of new findings might be used to improve the method for measuring the leucoanthocyanin content of Bartlett pears.

REVIEW OF LITERATURE

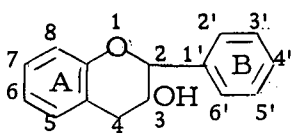
Nomenclature

Rosenheim (37, p. 185) first applied the term leucoanthocyanin to a colorless compound which when heated in the presence of acid yielded anthocyanidin. The term anthocyanidin denotes a sugar-free aglycone while that of anthocyanin signifies that the compound is a glycoside of the polyphenol. Thus to be consistent with the terminology used for anthocyanins, the term leucoanthocyanin should indicate a glycoside. At the present time, however, leucoanthocyanins are thought to be only rarely glycosylated (49, p. 537; 11, p. 218), so the term leucoanthocyanin is in most instances technically incorrect. A second drawback to the use of these terms is that both it and leucoanthocyanidin connote a simple phenolic compound, the basic structure of which consists of not more than fifteen carbon atoms. Many of these compounds are now believed to occur as polymers (41, p. 1454). Furthermore, these polymers are probably present in most mature plants along with the monomers (19, p. 372).

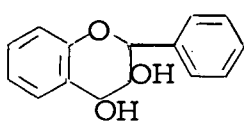
Swain, commenting on the confusion in terminology, suggested that the term leucoanthocyanidin should be applied to monomers whose structure had been determined and that of leucoanthocyanin reserved for those compounds whose structures were unknown, the latter term not necessarily implying the presence of a sugar molecule

(50, p. 110-111). Clark-Lewis (11, p. 212), however, maintained that the term leucoanthocyanidin was more appropriate as the compounds the structures of which had been studied did not contain sugar residues.

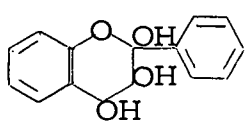
In recent years the more specific terms of flavan-3-ol, flavan-3:4-diols, and flavan-2:3:4-triols have been used with increasing frequency to refer to structures I, II, and III as shown below (49, p. 536-537):



I



II



III

The an of the word flavan indicates the saturated central ring of the flavonoid molecule and the 2-, 3-, and 4-ols indicate the number and positions of hydroxyls on this ring. Swain, in 1962, further suggested that monomers as a class should be called flavanols, polymers called flavolans and that those polymers which yield flavylium salts (anthocyanidins) should be called flavylans (49, p. 537).

Adding to the confusion, tannin, an ambiguous term associated with polymers of such compounds as gallic acid and ellagic acid (2, p. 125), is also used in connection with flavolans. Currently, it is recognized that many of the plant substances formerly called

tannins are, in the case of tea, in actuality catechins (flavan-3-ols) (49, p. 535) and, in the case of edible fruits and quebracho tannin, leucoanthocyanins (flavan-3:4-diols) (19, p. 372; 40, p. 162). Goldstein and Swain suggested that in the interest of precision only those polymeric compounds having tanning properties, that is the ability to form strong complexes with proteins and other polymers, should be classed as tannins. Thus, in accordance with their suggestion, flavolans may be called tannins.

In the work reported here, the term leucoanthocyanin is used because the actual structure of the flavylium-yielding compound in pears is unknown.

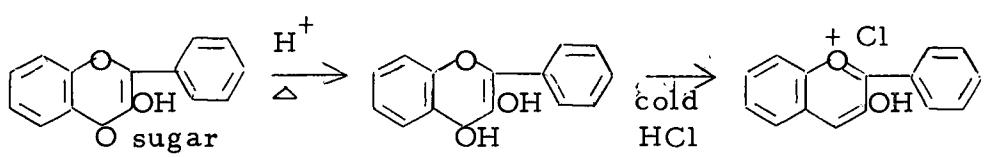
Chemical Nature of Leucoanthocyanins

Knowledge about the leucoanthocyanins has been obtained with difficulty. This has been especially true for the monomers which are very unstable (24, p. 474), showing a ready tendency to polymerize with themselves or other phenolic nuclei after atmospheric oxidation or exposure to acidic conditions. Roux has stated that "there are few instances where so little fact has given rise to so much speculative theory" (41, p. 1454).

The first worker to investigate the leucoanthocyanins was Rosenheim (50, p. 109). He was looking for the biochemical precursor of the red pigment in grape leaves. In addition to the red

pigment, which he identified as oenidin, he isolated a white amorphous substance which on heating in the presence of acid yielded an anthocyanidin.

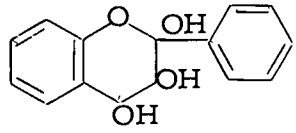
Because it appeared to make no difference whether the conversion took place in the presence of carbon dioxide or oxygen, Rosenheim proposed that the leucoanthocyanin was a glycoside of a pseudobase. Normally a pseudobase in the presence of cold dilute acid is converted to the corresponding red anthocyanidin. Rosenheim reasoned that the compound was colorless and remained so due to the presence of a sugar group attached to the hydroxyl group at carbon four. Only when the sugar residue was removed by the action of heat and acid was the pseudobase free to shift to the red anthocyanidin. According to Rosenheim's hypothesis the reaction proceeded as follows (37, p. 185):



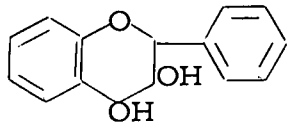
Glycoside of the Pseudobase | Pseudobase (Colorless) | Anthocyanidin (Red)

The Robinsons in 1933 (34, p. 206) questioned that leucoanthocyanins were glycosylated pseudobases. They observed that naturally occurring leucoanthocyanins were more stable to cold strong acids (10-15% aqueous HCl) than was a synthetic methylated pseudobase of cyanidin which was easily converted into cyanidin with cold 5% aqueous

HCl. They proposed the following structure for leucoanthocyanidin (34, p. 207):



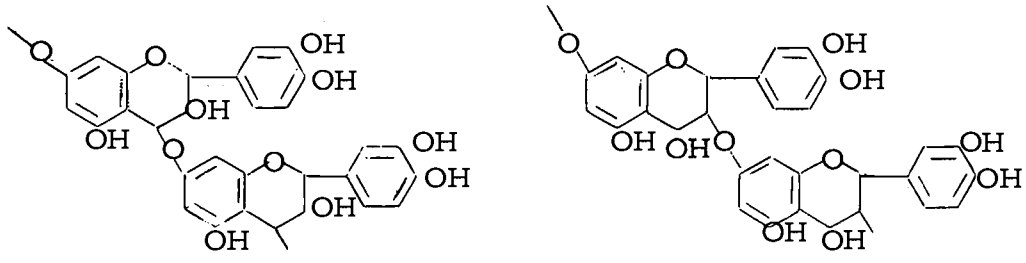
Rather than the above formula of a flavan-2:3:4-triol, a more recently accepted structure for leucoanthocyanidin is that of a flavan-3:4-diol as shown below (1, p. 5):



This proposed structure was recognized to be in a lower oxidative state than the anthocyanins. The first bit of evidence in support of this structure was that the use of picric acid, an oxidizing agent, increased the conversion of leucoanthocyanin to anthocyanidin (50, p. 111). A second point in favor of the structure with the lower oxidative state is that those isolated leucoanthocyanidins whose structures have been determined are 3:4-diols or derived from them (11, p. 220).

While the molecular weight of a monomeric flavan-3:4-diol is

around 300, the molecular weights of leucoanthocyanin polymers range from 600 to 2500 (44, p. 541). Little is known with certainty in regard to the type or types of linkage between monomers of a flavolan polymer. In the case of flavan-3:4-diols it is significant that the polymeric leucoanthocyanins are identified by the formation of anthocyanidins on heating with acid as are monomeric ones. The two types of ether linkages between carbons 3 and 7 or between carbons 4 and 7 proposed by Roux (21, p. 571), as shown below, are compatible with the formation of anthocyanidin when the leucoanthocyanin dimer is heated in the presence of acid.



However, other types of linkage may be involved, as Roux and Paulus (47, p. 322) have found that polymerization does seem to reduce the amount converted to anthocyanidin. For trimers, the conversion was reduced by one third as compared to monomers. Further polymerization, however, had little influence on the conversion.

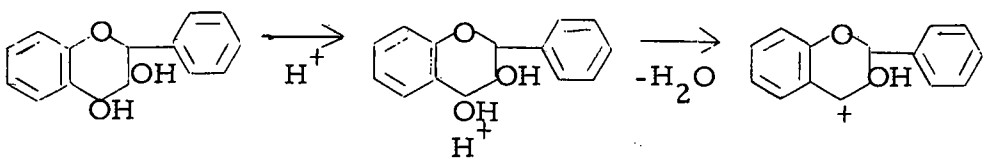
As mentioned previously, some of the polymeric compounds are tannins. For a polymer to have maximum tanning properties an intermediate molecular weight is most desirable. Evidently a

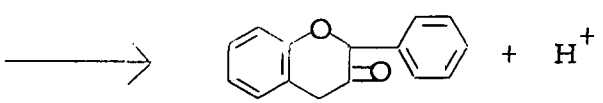
complex between a flavolan and a protein is formed when the flavolan has a sufficient number of hydroxyl groups and is able to form hydrogen bonds with receptor sites on the protein in such a way as to build up a reasonably stable cross-linked structure (19, p. 371). If the molecular weight of the flavolan is too small, the cross linking will not be effective enough to bring about precipitation of the protein. On the other hand, if the flavolan is too large, the "flavolan molecule may be either insoluble or too large to fit between suitably oriented protein molecules" (19, p. 371). A good example of the importance of the molecular size in tanning compounds can be demonstrated by astringency in unripe fruits, a sensation in the mouth thought to be brought about by a reduced lubricant action due to the cross linking of glycoproteins and tannins. Goldstein and Swain have demonstrated that concomitant with the loss of astringency in ripening fruits there is increased polymerization of flavolans (19, p. 382).

Conversion of Leucoanthocyanins to Anthocyanidins

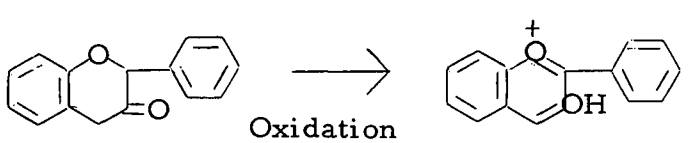
The method proposed for measuring leucoanthocyanin involves converting the colorless compound to the corresponding red anthocyanidin by heating in acid and then measuring this pigment colormetrically. According to the presently accepted theory, the conversion of a flavan-3:4-diol (leucoanthocyanidin) to anthocyanidin involves both dehydration and oxidation (43, p. 42). Birch has suggested that acid

effects the dehydration, with both cis and trans diols giving rise to monohydroxy compounds (7, p. 184). Presumably the conversion of a flavan-3:4-diol when heated in the presence of acid goes in the following manner:





Later, Bauer et al. (6, p. 434) suggested that the 3-keto-flavan is an important intermediate in the conversion of a flavan-3:4-diol to anthocyanidin. In the conversion of the 3-keto-flavan to the flavylium salt further oxidation must take place as shown:



When flavan-3:4-diols are converted to their corresponding anthocyanidins the yields reported have varied from 1 to 80%. One factor which affects the percent converted is the medium in which the conversion takes place. Swain and Hillis (51, p. 66) recommended that an alcoholic medium be used rather than an aqueous one. When the conversion was brought about in aqueous solvents, two products were formed as indicated by two peaks in the absorption curve, one at 450 mμ and the other at 540 mμ. The peak at 540 mμ corresponded to anthocyanidin whereas the peak at 450 mμ was attributed to the formation of condensation products. When an alcoholic medium was used in the conversion, only the peak at 540 mμ was observed. To prevent loss of volume during the conversion, Swain suggested that n-butanol with a boiling point of 117.7°C. be used instead of 2-propanol with a boiling point of 82.3°C.

The optimum conditions for converting Spruce leucoanthocyanin to anthocyanidin was studied by Pigman et al. (33, p. 6). These authors used a number of combinations of 2-propanol, water and HCl. When the developer contained 20% water by volume and the normality ranged from 0.1 to 1.8, the conversion of leucoanthocyanin was greatest at a normality of 0.6. When the developer contained 10% water by volume, the conversion of leucoanthocyanin was greater when the normality was 0.3 rather than 0.6. Under anhydrous conditions, with the normality varying from 0.3 to 0.015, the conversion was

greatest at a normality of 0. 03. Under anhydrous conditions and at a normality of 0. 03 the conversion was twice as great as was the case when the normality was 0. 3 or when the developer contained 20% water and the normality was 0. 6.

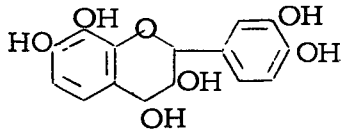
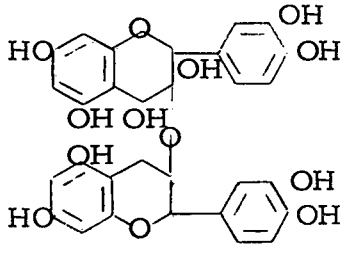
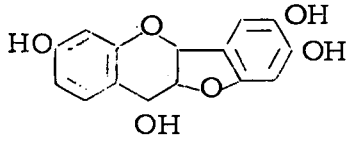
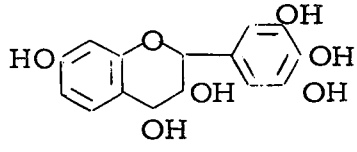
Data on the conversion of four natural leucoanthocyanidins, the formulas of which are known, are summarized in Table I.

Melacacidin, the first natural leucoanthocyanidin to be isolated, yielded, under the conditions used for the conversion, only 10% anthocyanidin (29, p. 3387).

Forsyth isolated a leucoanthocyanidin from cacao beans. In 1953 he noted that when it was held at 100°C. in 0. 1 N aqueous HCl for 5 minutes this leucoanthocyanidin yielded 31% of its weight as epicatechin and 6% as catechin (13, p. 726). In addition, a reddish brown precipitate formed. In 1960, Forsyth and Roberts (15, p. 374) elucidated the structure of this leucoanthocyanidin, showing it to be a flavan-3:4-diol and a flavan-3-ol (catechin) united by an ether linkage. Thus its structure accounts for the appearance of both catechin and epicatechin. When this compound was heated in acidified butanol, they reported a yield of 14% cyanidin from the flavan-3:4-diol, hence the name leucocyanidin. As only half of the dimer is capable of being converted to anthocyanidin, the conversion would be closer to 28% for a monomer (15, p. 376).

The conversion of peltogynol, a derived flavan-3:4-diol, was

TABLE I. DATA FOR THE CONVERSION OF LEUCOANTHOCYANIDINS TO ANTHOCYANIDINS

Leucoanthocyanidin	Plant Source	Converting Medium	% Conversion	Reference
Melacacidin 	heartwood of *Acacia melanoxylon*	3.3 N HCl in n-butanol	10	29
Leucocyanidin 	cacao bean	1.2 N HCl in n-butanol (26% aqueous)	14	15
Peltogynol 	heartwood of *Peltogyne porphyrocardia*	2 N aqueous HCl under nitrogen	1	10
		2 N aqueous HCl under oxygen		80
Leucorobinetinidin 	quebracho wood	3 N aqueous HCl	3	43
		0.5 N HCl in 85% 2-propanol	20	
		0.025 N HCl in 2-propanol	37-42	

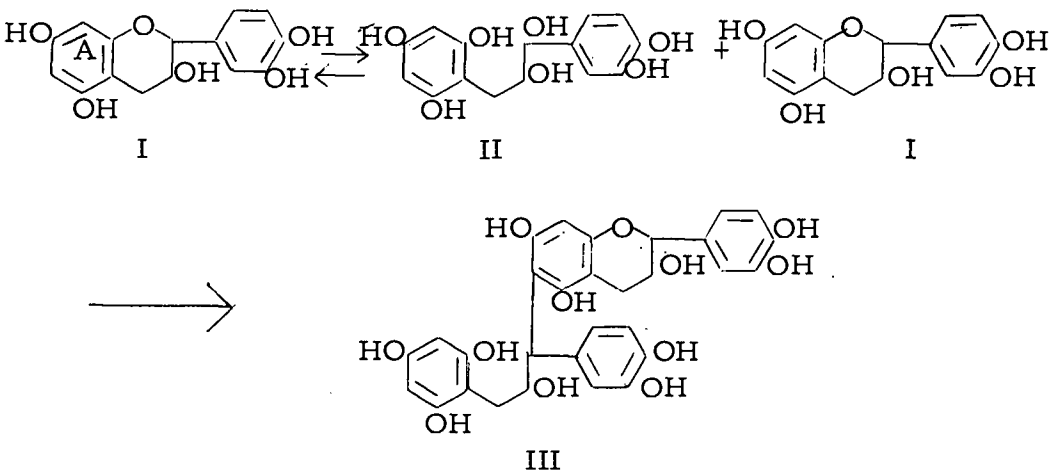
influenced markedly by the presence of oxygen (10, p. 3177). In 2 N aqueous HCl under nitrogen the conversion was 1% while in the presence of oxygen the conversion was 80%. Chan et al. also reported that when alcohol was used instead of water the conversion of peltogynol to its corresponding anthocyanidin was reduced.

In contrast to the above and in agreement with the work of Swain and Hillis previously mentioned (51, p. 66), Roux and Bill (43, p. 42) reported a 3% conversion of leucorobinetinidin to anthocyanidin in 3 N aqueous HCl which was increased to 20% in 0. 5 N HCl in 80% 2-propanol. Under anhydrous conditions and with a lower normality of HCl (0. 025), the yield was further increased. They found, as had Pigman et al. (33, p. 7), that this yield was higher than it was with 80% propanol and a higher normality of acid.

It has been proposed that a leucoanthocyanin does not completely convert to anthocyanidin in the presence of boiling mineral acids due to a secondary reaction, that of the formation of phlobaphene (5, p. 377). The term phlobaphene is frequently found in the literature on tannins and leucoanthocyanins. Phlobaphenes are described as reddish brown precipitates that are found in plant extracts which have been boiled in mineral acid (5, p. 377). As to whether leucoanthocyanins, catechins, or a combination of the two are responsible for their production is an unsettled question.

There seems to be little question that heating catechins in boiling

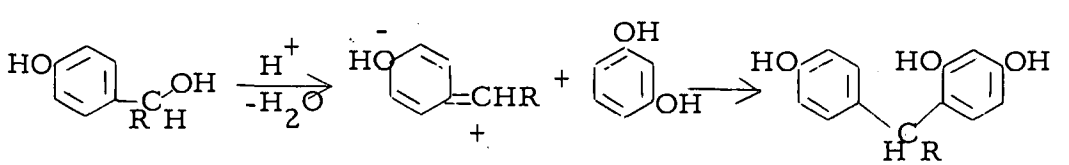
mineral acid yields condensed products. Bate-Smith reported in 1953 that catechins form phlobaphenes of a deep golden color (1, p. 4). Freudenberg has been foremost in establishing the steps involved in the condensation of catechin. He suggested that this condensation is brought about by acid as follows (16, p. 208):



I II I

III

This mechanism was verified by Brown et al. (9, p. 3757) with smaller molecules. They used resorcinol as the active group of the A ring of the catechin molecule (I) and methoxybenzyl alcohols to represent formula II. In this way the possible modes of condensation were limited and these authors established that methoxybenzyl alcohols are capable of reacting as carbonium ions and thus capable of condensing with phenols. The proposed mechanism for the condensation of these molecules is as follows:



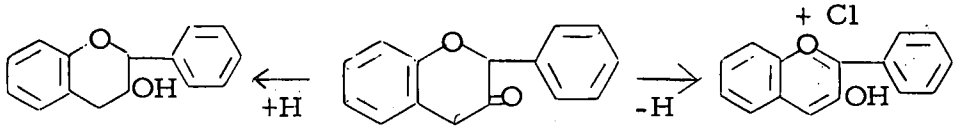
They state that "the activating influence of a p-hydroxyl group appears to be responsible for the attack of a benzyl alcohol on the nucleophilic centre in the presence of acid" (9, p. 3758).

In 1953 Bate-Smith and Swain proposed that leucoanthocyanins alone were responsible for the appearance of phlobaphenes (5, p. 377). Bate-Smith and Swain supported their theory with two main points. First, the phlobaphenes were, in many cases, quite red. This redness they attributed to leucoanthocyanins rather than to catechins. Roux's work (46, p. 480) supported this concept. He found that the presence of a hydroxyl group on carbon four of the leucoanthocyanidin was essential for the development of red color, thus ruling out catechin as its source. In addition, he also observed that flavan-4-ols when exposed to sunlight turned red. But when the hydroxyl at carbon four was missing the ability of the compound to turn red was lost. Roux stated that "this proves unequivocally that the 4-hydroxyl group is contributory to the redness induced in flavan-4-ols, flavan-3:4-diols, and polymeric flavan-3:4-diols" (42, p. 438).

The second part of Bate-Smith's and Swain's evidence was based on work with two newly isolated leucoanthocyanidins, one from Pinus martimus and the other a cacao leucocyanidin. They noted that these two leucoanthocyanidins had properties of tannins in that they formed complexes with hide powder. One property which supposedly is characteristic of tannins is that they also form phlobaphenes when

boiled with mineral acid. Furthermore, under aqueous conditions of conversion, the yield of anthocyanidin from the leucoanthocyanidin was only 10% of the theoretical, the largest amount of leucoanthocyanidin being converted to phlobaphene.

As to whether leucoanthocyanins alone give rise to phlobaphenes, it should be noted that at the time their work was done (1953) Bate-Smith and Swain were apparently unaware of the presence of catechin in cacao leucocyanidin. Perhaps this same oversight was responsible for the oxidation of 3-ketoflavans to flavylium salts being attributed to disproportionation in a number of papers (29, p. 3387; 27, p. 1368; 28, p. 1401; 6, p. 434), three of which give one of Forsyth's early articles (12, p. 26) on cacao leucocyanidin in support of this theory. Disproportionation involves the oxidation of one molecule of a compound while simultaneously another molecule of the same compound is reduced as shown below:



Catechin 3-Ketoflavan Anthocyanidin

Nevertheless, Roux and Paulus (46, p. 479) later demonstrated that when flavanols were dissolved in boiling water and immediately treated with cold 10 N HCl, a red brown condensation product was

developed instantaneously from flavan-4-ol, a light pink condensation product was developed in 30 seconds from flavan-3:4-diol (leucoanthocyanidin), and a buff condensation product was developed in 10 minutes from flavan-3-ol (catechin). It would appear then that under the proper conditions both catechins and leucoanthocyanidins can give rise to phlobaphenes.

The conversion of leucoanthocyanin to anthocyanidin thus appears to be affected by the medium in which the conversion takes place (alcoholic or aqueous), by the acidity of the medium and by the extent of polymerization of the leucoanthocyanin. In addition, the possibility of phlobaphene formation during the conversion must be considered. Despite the drawbacks, the conversion of leucoanthocyanins to the anthocyanidins is the only effective means now known for measuring leucoanthocyanins (19, p. 379).

Extracting the leucoanthocyanin prior to converting it to anthocyanidin also presents problems.

Extraction of Leucoanthocyanins

A number of solvents have been used to extract leucoanthocyanins. Much of the work has been done on wood and bark by chemists interested in tannins as well as leucoanthocyanins. What is applicable to tannins is in many instances true for polymeric leucoanthocyanins if, as Roux states, leucoanthocyanins are prototypes of condensed

tannins (40, p. 162).

Table II shows some of the solvents that have been used for the extraction of leucoanthocyanins (tannins) from various plant sources.

TABLE II. SOLVENTS USED TO EXTRACT LEUCOANTHOCYANINS

Investigator	Solvent	Plant Source	Reference
Roux, D. G.	ethyl acetate	black wattle bark	45
Swain, T. and W. E. Hillis	methanol and 50% aqueous methanol	Prunus domestica (plums)	51
Forsyth, W. G. C.	1% HCl	Cacao bean	12
	80% ethanol		15
Ito, S. and Y. Oshima	methanol	Japanese persimmon	25
Joslyn, M. A. and R. Peterson	1% HCl in methanol	Bartlett pear	26

In his work with black wattle tannin, Roux in 1949 found that it was fairly soluble in ethyl acetate containing 1% ethanol as impurity, soluble in ethanol and acetone, and very soluble in methanol (38, p. 401). Several years later he and Sykes observed that 50% aqueous acetone was the best solvent available for tannins (48, p. 19).

Roux (38, p. 403) gave the following explanation for differences in solubility of tannins in the above solvents. These compounds have

both electrophilic and nucleophilic sites on the molecule, especially if they are polymeric. The molecules, therefore, tend to hydrogen bond to each other or to other similar compounds. The best solvents will open these bonds. Although water is a dipolar molecule and is able to break these bonds, the water molecule can act as a bridge to establish new bonds between the tannin molecules. On the other hand, the lower alcohols, methanol and ethanol, are better extractants for such compounds because they contain not only a polar group but also an aliphatic group. This aliphatic group does not allow reassociation between the tannin molecules after the bonds have been opened.

Roux (45, p. 44) recommended ethyl acetate as a good solvent if a fairly pure sample of tannin is desired. From black wattle bark, ethyl acetate extracted 62% of the total tannin which was 92.5% pure. Methanol, on the other hand, extracted 96.5% of the total tannin but the extract contained 30% non-tannin substances. A disadvantage of the use of 50% aqueous acetone was that it extracted a high proportion of gums (48, p. 19).

Paper chromatograms of successive extracts of black wattle bark with ether, ethyl acetate, ethanol and methanol showed that these solvents removed the following types of compounds. Ether and ethyl acetate extracted 3% and 60% respectively of the moveable polyphenols, the monomers. Ethanol and methanol extracted the phenols that showed little or no movement on the chromatogram (39, p. 230).

Further examination of wattle tannins on chromatograms revealed that the R_f values were inversely related to the molecular weights. For example, those with zero R_f values in butanol, acetic acid and water (6:1:2 v /v) had a molecular weight equal to or above 2400 while those compounds that moved had correspondingly lower molecular weights.

In regard to the extraction of leucoanthocyanins from fruits, less work has been done than with woods. Forsyth (12, p. 25) and Joslyn (26, p. 318), working with cacao beans and Bartlett pears, respectively, used weakly acidic alcoholic solvents to extract the leucoanthocyanin. There are two main disadvantages to using acidic solvents. One is that the monomers have a tendency to undergo polymerization in the presence of acids and bases (11, p. 242). Undoubtedly this was one of the reasons that Forsyth later used 80% methanol to isolate cacao leucocyanidin dimer (15, p. 374). The second objection is that if the leucoanthocyanin in the extract is to be measured by its conversion to anthocyanidin, polymerization may influence the amount converted.

Ito and Oshima (25, p. 156) isolated tannin from Japanese persimmon and found leucodelphinidin, which they believed was glycosylated, to be a main constituent. The tannin was soluble in water, methanol, ethanol, and propanol but insoluble in ethyl acetate, acetone, light petroleum ether, chloroform and glacial acetic acid.

Swain and Hillis (52, p. 139) measured the leucoanthocyanin content of plums. Their data showed that approximately two-thirds of the leucoanthocyanin was extracted from plum tissues treated with six successive portions of boiling absolute methanol followed by grinding in 50% methanol. Forsyth (12, p. 25) also had estimated that two-thirds of the total tannin in cacao bean could be extracted with aqueous acetone, alcohol or dilute acids.

Why some leucoanthocyanin is extracted while the rest remains firmly bound to the plant is partially explained by the different types of leucoanthocyanin found in any one plant. Swain and Hillis (19, p. 373), after analyzing the fruit, leaves, bark and wood of Prunus domestica, concluded that there were three types of leucoanthocyanins: those that are soluble in organic solvents, those that are soluble in aqueous organic solvents and those that are not extractable. Paper chromatographic analysis of the extracts indicated that monomers and perhaps oligomers made up the first group. The second group consisted of polymers which showed no movement on the chromatograms. Presumably the third group which was insoluble was composed of even larger polymers.

It appears likely that it is the "tanning" properties of leucoanthocyanins which seriously interfere with their extraction. Roux achieved such a high extraction of leucoanthocyanin from wood by leaving thin slices of bark in the solvent for a period of four to five

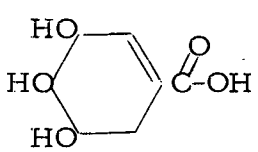
days. This procedure differs from that for extracting phenols from fruits in two ways. First, the fruit must be heated to deactivate enzymes which catalyze oxidation of the polyphenols. This step may cause a reaction of tannin materials with proteins (20, p. 390). Second, the fruit is usually ground, minced or blended which may also reduce the extractability of the leucoanthocyanins. For example, Roux (39, p. 232) reported that when the slices of wood were ground prior to extraction with the different solvents, he obtained no compounds which were mobile on the chromatogram. This suggests that the leucoanthocyanin may have reacted with other constituents in the bark. In this connection Swain (49, p. 537) has suggested that the reason why some leucoanthocyanins are insoluble is that they "form aggregates with polysaccharides or proteins of the plant residue." Perhaps this reaction is responsible for earlier workers reporting that leucoanthocyanins were glycosides. In this respect, Swain in 1962 goes so far as to say that of the leucoanthocyanin glycosides thus far reported, the sugars were probably adsorbed on the phenolic substances during extraction (49, p. 537).

Although estimations of the amount of leucoanthocyanin remaining in plant tissue after extraction have been reported, few details for this determination have been published. Forsyth et al. (14, p. 182), however, did give the method used to measure the leucoanthocyanin content of cacao beans. After exhaustively extracting the beans with

acetone, they developed the anthocyanidin from the residual leucoanthocyanin in the dried powder by refluxing in 2 N HCl in n-butanol.

Biological Significance of Leucoanthocyanins

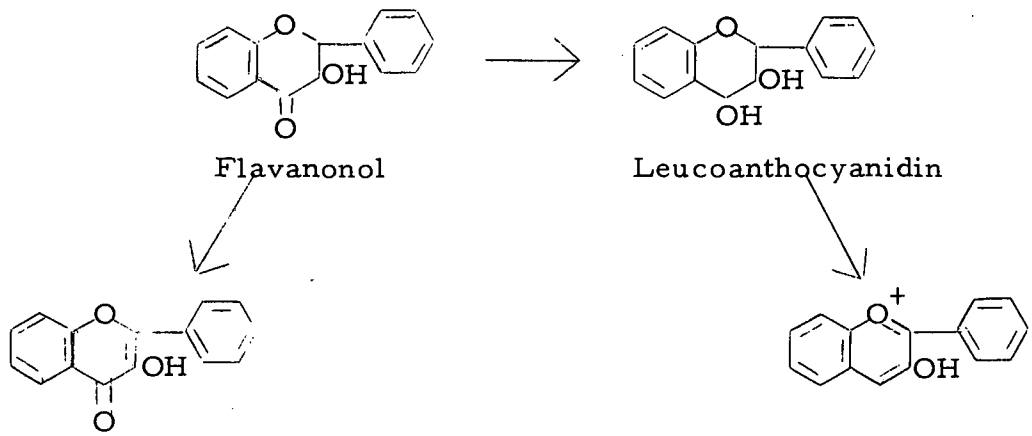
Since knowledge of the chemistry of leucoanthocyanins is incomplete, it is no wonder, as Roux has stated, "that theories of their biogenesis are even more hypothetical then their chemistry" (41, p. 1454). Although the biochemical role of leucoanthocyanins in plants is not well understood, the primary precursors for the C_6-C_3-C_6 compounds have been established as the result of tracer experiments (36, p. 200). It has been concluded that shikimic acid



originating from glucose contributes to the B ring, and the A ring is made up of three head-to-tail groupings of acetate molecules. Presumably the various types of flavonoid compounds present in a plant arise from these basic structures.

Whether leucoanthocyanins are end products of metabolism or are intermediates in the formation of such flavonoids as anthocyanidins or catechins has been a source of controversy. For example,

Rosenheim, in 1920, was motivated to study leucoanthocyanins because he anticipated finding some biochemical relationship between them and anthocyanidins. His work led him to suggest that perhaps leucoanthocyanins were the intermediate products formed by the plant during the reduction of flavonols to anthocyanidins. This theory still persists. Bogorad (8, p. 424) in 1958 included in his summary of the possibilities of biosynthetic schemes for flavonoids a very similar reaction as shown below:



A typical example of the type of experimental evidence that demonstrates a close relationship between leucoanthocyanins and anthocyanins was obtained by Hillis (22, p. 597) in a study of eucalypt tissues. He noted that the young leaves contained appreciable quantities of both anthocyanin and leucoanthocyanin. The anthocyanin was found throughout the leaf whereas the leucoanthocyanin was found primarily in the tips of the leaves which are considered to be areas of intense metabolism. As the leaves matured both compounds decreased

simultaneously.

On the other hand, a study of the general distribution of leucoanthocyanins by Bate-Smith and Lerner (3, p. 131) did not show any apparent connection between the metabolism of leucoanthocyanins and anthocyanins. Leucoanthocyanins were found only in certain kinds of plants. For example, in dicotyledons the presence of leucoanthocyanins was more frequent in woody plants than in the more herbaceous types. In contrast, anthocyanins seem to be more widely distributed throughout the plant world.

Another fact which indicates that leucoanthocyanins and anthocyanins arise from separate biochemical pathways is that anthocyanins are usually glycosylated whereas this is rarely if ever true for leucoanthocyanins. In addition, it is probably significant that the first leucoanthocyanins isolated were melacacidin, mollisacacidin and peltogynol for which no known corresponding anthocyanins have been found (11, p. 241).

As mentioned previously, a biochemical role for leucoanthocyanins that is more widely accepted is that the leucoanthocyanins are prototypes of polymeric compounds known as tannins (41, p. 1454). Hillis (22, p. 598) postulated that the monomeric leucoanthocyanins are synthesized in the tips of the leaves, translocated through the sapwood and deposited as polymeric leucoanthocyanins or tannins in the heartwood. In support of Hillis, Roux (41, p. 1456) found that monomeric

and lower molecular weight leucoanthocyanins predominated in the sapwood and higher molecular weight tannins in the heartwood.

These reddish brown polymeric compounds are often referred to as tannin reds (16, p. 212) or phlobaphenes (18, p. 3) or phlobatannins (36, p. 178) or condensed tannins (41, p. 1454). The lack of precise terms for these compounds is indicative of how little is known about them. However, the names do reflect some of the current thinking about their formation. Freudenberg (16, p. 213), who referred to them as phlobaphenes, suggested that these polymeric compounds are formed in the heartwood in a way similar to the formation of phlobaphenes when plant extracts are heated in the presence of acid. On the other hand, Roux (41, p. 1456) has stated that "little parallel exists between the oxidative conversions or the condensation induced by hydrochloric acid of 'flavanoid' or 2-phenylbenzopyran structures and the biogenesis of naturally occurring tannins which are likely to result from enzyme action only."

Whatever their mode of formation, it is thought that these polymers are of service to the plant. White (53, p. 383) has speculated that they tan the surfaces of seeds, spores and bark giving them chemical and physical resilience. Being involved in the browning of fruits "tannins provide the mature seed with a humus-like environment favorable to germination and growth" (53, p. 383). Another interesting supposition is that tannins may contribute to the longevity

of such trees as the redwoods by preventing spore germination of various fungi and bacteria.

Along with contributing to the well-being of plants, leucoanthocyanins and their polymers, tannins, play a significant role in the acceptability of certain foods. In addition to being related to the pink discoloration of canned pears, they are largely responsible for the astringency of wines and other such beverages prepared from fruit. Leucoanthocyanins also are involved in the occasional cloudiness of these drinks due to precipitation of protein (2, p. 125).

Because of the many uncertainties about the biochemical role of leucoanthocyanins and because of their possible relationship to the color and flavor of food products of plant origin, a more accurate method for measuring the leucoanthocyanins is needed.

EXPERIMENTAL WORK

General Procedure

One aspect of the experimental work in this project was to investigate some of the factors which affect the conversion to anthocyanidin of the leucoanthocyanin in Bartlett pears.[1] In the work on pears here reported all conversions were effected in n-butanol-HCl but both the normality of acid and the heating period were varied. A second aspect of the work was to compare the effectiveness of a few of the more promising solvents mentioned in the literature. An attempt was made also to measure the leucoanthocyanin remaining in the pulp after extraction. The pulp was resuspended in the same type of solvent used for extraction and one milliliter of this suspension was heated in n-butanol-HCl to convert any unextracted leucoanthocyanin to anthocyanidin. In addition, the effect of the solvent used for extraction on the conversion to anthocyanidin of the leucoanthocyanin from the pulp and of a synthetic leucocyanidin polymer was studied.

Preparation of slurry, extract and marc of pear. To obtain a sample, a radial section, approximately one-sixth of each pear, was cut and the skin, core, and any discoloration removed with a

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[1] Obtained from the Medford area, 1963.

stainless steel paring knife. Each sample, approximately 15 grams, was then sliced into a beaker containing 25 milliliters of alcohol and weighed to the second decimal place on a triple-beam balance. The sample was then boiled for five minutes to deactivate enzymes. The heated sample was ground with an additional 50 milliliters of extracting solvent in a micro cup of an electric blender[2] for five minutes at low speed and for an additional one minute at high speed. The contents of the blender cup were transferred to a 100-milliliter volumetric flask and made to volume with the extracting solvent. The sample of ground whole pear suspended in the extracting solvent is hereafter referred to as the slurry.

When the leucoanthocyanin content of the extract was to be determined, the extract was prepared as follows. The slurry of whole pear was poured into two 50-milliliter centrifuge cups and centrifuged at 3200 r. p. m. for ten minutes. The supernatant, hereafter referred to as the extract, was decanted into a 100-milliliter volumetric flask. Approximately 25 milliliters of the same type of solvent used to prepare the slurry, part of which was used to rinse the flask that had contained the slurry, were poured into the centrifuge cups containing the pear pulp. The contents of the centrifuge cups were stirred and then centrifuged for five minutes at 3200 r. p. m. The supernatant was

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[2]Waring Blender, Waring Products Corporation, New York 36, New York.

decanted into the flask containing the extract and the contents of the flask made to volume with the solvent. To prepare the marc for measuring its leucoanthocyanin content, the pulp remaining in the centrifuge cups was dispersed with the same solvent used for the extraction. This final suspension of marc was transferred to the flask that had originally contained the slurry and made to volume.

Conversion of leucoanthocyanin to anthocyanidin. To prepare the reagent used for converting leucoanthocyanin to anthocyanidin, concentrated HCl was diluted to the desired normality (0.6, 0.1, 0.5 and 0.025) with n-butanol. For the conversion itself, ten milliliters of the reagent were pipetted into a 7" x 1" test tube provided with a ground-glass, solid penny head stopper. One milliliter of the slurry, extract or marc being tested was pipetted into the tube containing the reagent. For the slurry or marc a graduated measuring pipette with a broken tip was used to measure the one milliliter. When slurry or marc was used, special care was taken to avoid having pulp on the sides of the test tube above the level of the reagent. For developing the anthocyanidin, the tube was placed unstoppered in a boiling water bath. After three minutes, the stopper was secured with rubber bands and the top of the bath covered with foil to exclude light. After a selected heating period, the stopper was removed from the test tube and the test tube was removed from the water bath and cooled in the

dark until the contents reached room temperature. The pear pulp was removed from the contents of the tubes containing marc or slurry by filtering through glass wool.

Measuring the concentration of anthocyanidin. The absorbancy of the solution of anthocyanidin developed from the leucoanthocyanin was determined with a colorimeter[3], using a green filter with a wavelength of 500 to 570 mμ. The blank was prepared in the same manner as the test solution except that the blank was not heated. The concentration of anthocyanidin was read from a calibration curve of cyanidin chloride in 0. 025 N HCl in n-butanol.

The slope of the calibration curve was determined in the following manner. Ten milligrams of cyanidin chloride were weighed on an analytical balance and made to 50 milliliters with absolute ethanol. Working standards made with 0. 025 N HCl in n-butanol were prepared from this stock solution. These contained 2, 4, 8, 10, 20, 40 and 60 micrograms per milliliter, respectively. These working standards were prepared in duplicate, the absorbancy of each was read, and these readings were plotted against the concentration.

To see whether the normality of HCl in n-butanol made a difference in the slope of the curve, working standards were made also

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[3]Klett-Summerson Photoelectric Colorimeter, Model 3935, Klett Mfg. Company, New York, New York.

in 0. 6N HCl in n-butanol. The normality of the acid made no difference. Finally, whether the cyanidin chloride standard was dissolved in 95% ethanol, methanol, 70% acetone or 40% acetone prior to preparing the working standards in 0. 025 N HCl in n-butanol made no difference in the readings.

The calibration curve is given in Figure 1.

Some of the Conditions Affecting the Conversion of Leucoanthocyanin to Anthocyanidin in Pear Marc

Effect of normality of HCl in n-butanol. In the work here reported normalities ranging from 0. 6 to 0. 025 were used for the conversion to anthocyanidin of the leucoanthocyanin in Bartlett pears. For the conversion of leucoanthocyanin in an alcoholic medium, Swain and Hillis (33, p. 64) in 1959 recommended the use of 0. 6 N HCl in n-butanol. Roux and Bill (43, p. 42), however, suggested that under anhydrous conditions lowering the normality to 0. 02 to 0. 033 increased the conversion from approximately 20% to 37-42%.

Aside from the possibility of a greater conversion of leucoanthocyanin, a second reason for testing the effects of lower normalities was that in the preliminary work the extracts from the pears heated with 0. 6 N HCl in n-butanol yielded brownish-orange solutions. This off-color apparently was due to the formation of phlobaphene. However, when the normality was 0. 1 or below, the resulting solutions

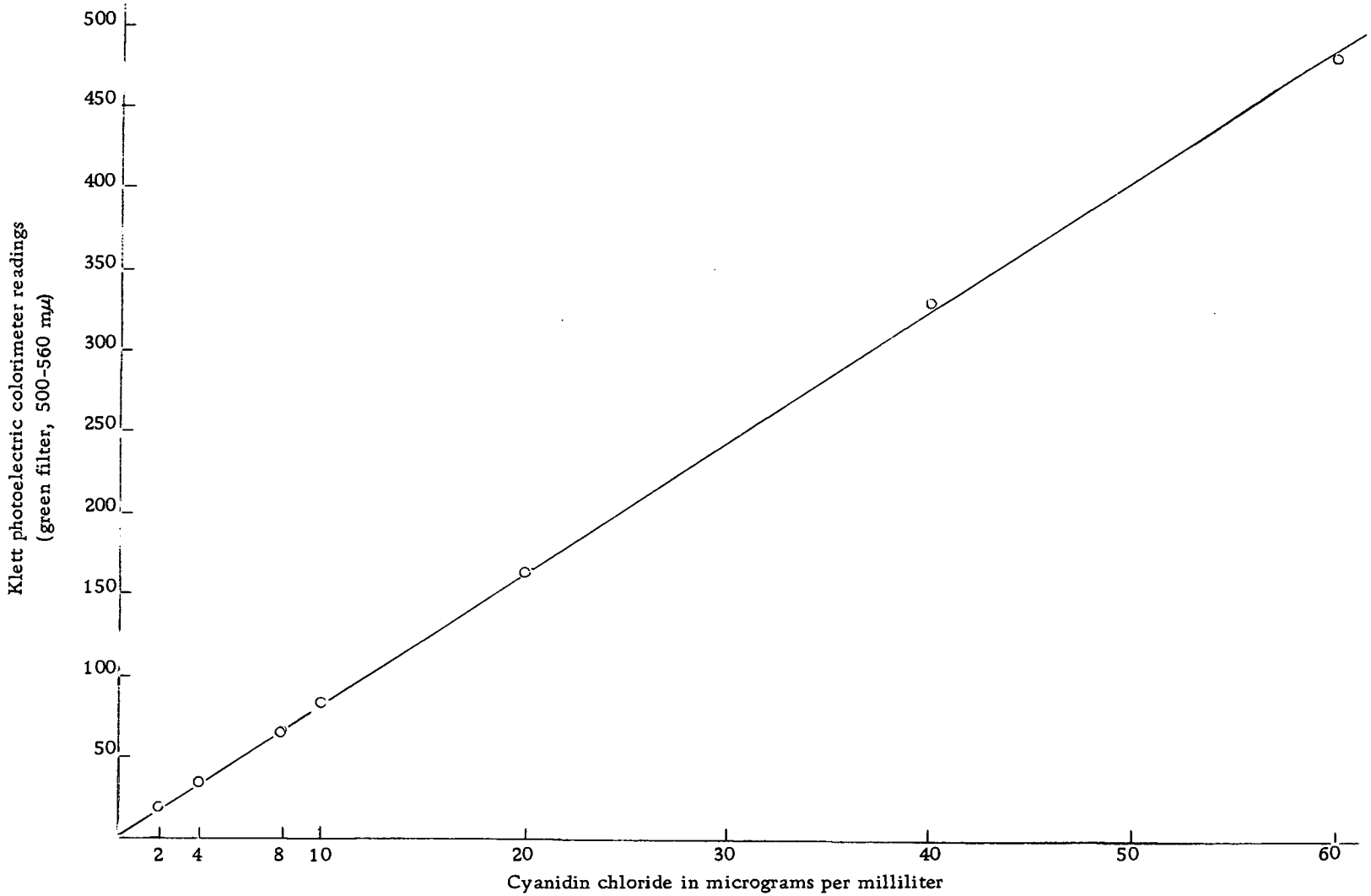

Figure 1. Standard curve for (leuco)anthocyanin

were a typical anthocyanidin pink. In contrast to the extract, the marc yielded a true anthocyanidin color even with 0. 6 N. On the basis of these observations and of a statement made by Swain and Hillis (52, p. 139) indicating that other phenolic compounds are extracted more readily than leucoanthocyanins, it was assumed that the marc contained a fairly pure source of leucoanthocyanin uncontaminated with other phenolic constituents which would influence the test. Consequently, the marc was used to study the effect of the normality of the acid on the conversion of leucoanthocyanin to anthocyanidin.

To prepare the marc, three samples from one pear were extracted with 70% aqueous ethanol. After centrifugation, the marc from one of the samples was dispersed in 70% ethanol, the solvent used by Phillips (32, p. 15) in her work on the leucoanthocyanin content of Bartlett pears. The marcs from each of the other two samples were first dispersed in a small amount of 95% ethanol. Then, 70 milliliters of acetone were added to each marc and the contents of the 100-milliliter volumetric flasks made to volume with 95% ethanol. This medium is hereafter referred to as 70% acetone. The acetone was used because in the preliminary work the development of anthocyanidin appeared to be increased in those marcs dispersed in acetone. Difficulties encountered in dispersing the marc in acetone alone were alleviated by adding some ethanol before the acetone was added.

Using the marc dispersed in ethanol, conversion was effected with 0.6, 0.1, 0.05 and 0.025 N HCl in n-butanol. In addition, the leucoanthocyanin in one of the marcs dispersed in acetone was converted to anthocyanidin using normalities of 0.6, 0.1 and 0.025 HCl in n-butanol.

For each normality all the conversions were carried out in triplicate. The heating period was 40 minutes and five replications were made, giving a total of 15 determinations at each normality.

TABLE III. EFFECT OF NORMALITY OF ACID AND OF DISPERSING MEDIUM ON CONVERSION OF LEUCOANTHOCYANIN TO ANTHOCYANIDIN (as mcg. cyanidin chloride per ml. of pear marc)

Normality of HCl in n-butanol	Dispersing Medium	
	70% Ethanol	70% Acetone
0.6	4.3	11.6
0.1	5.5	3.8
0.05	7.3	-
0.025	8.0	2.7

The average micrograms of anthocyanidin developed per milliliter of pear marc in 70% ethanol were 4.3, 5.5, 7.3 and 8.0 with normalities of HCl in n-butanol of 0.6, 0.1, 0.05 and 0.025, respectively (Table III). Their respective standard deviations were 0.66, 0.46, 0.39 and 0.67. The data indicate that for the marcs dispersed

in 70% ethanol, the lower the normality the greater the conversion of leucoanthocyanin to anthocyanidin.

For the marc in acetone, the average micrograms of anthocyanidin developed per milliliter were 11.6, 3.8 and 2.7, with normalities of HCl in n-butanol of 0.6, 0.1 and 0.025, respectively. Their respective standard deviations were 1.35, 0.71 and 0.68. Thus, when the marc was dispersed in acetone rather than in ethanol, the conversion to anthocyanidin was greatest at the highest normality.

Effect of heating period. It was noted that when the marc was dispersed in acetone instead of ethanol the anthocyanidin appeared to develop more rapidly. Therefore, the rate of development of anthocyanidin was determined on marc dispersed both in acetone and in ethanol. For this part of the work marc from the third sample of the pear originally extracted with 70% ethanol and then dispersed in acetone was used. To bring about the conversion, 0.6 N HCl in n-butanol was used and heating periods were 10, 20, 30 and 40 minutes. For conversion in ethanol, the remainder of the marc in acetone was centrifuged, the acetone decanted, and the marc dispersed and made to 100 milliliters with 70% ethanol. The leucoanthocyanin was then converted using 0.025 N HCl in n-butanol with heating periods of 20, 30, 40 and 50 minutes.

For each heating period, the conversions were carried out in

triplicate. Three replications were made of the time sequences for the marc both in acetone and in ethanol. Thus there were nine determinations for each heating period. The average concentration of anthocyanidin chloride developed per milliliter of pear marc for each heating period and dispersing medium is given in Table IV. The values for the marc in ethanol were adjusted to account for the marc that had been used when it was dispersed in acetone.

TABLE IV. EFFECT OF HEATING PERIOD ON CONVERSION OF LEUCOANTHOCYANIN TO ANTHOCYANIDIN (as mcg. cyanidin chloride per ml. of pear marc)

Heating Time	70% Acetone* 0.6 N HCl in n-Butanol	95% Ethanol* 0.025 N HCl in n-Butanol
Minutes		
10	8.8	-
20	9.0	3.1
30	9.2	4.3
40	9.4	5.2
50	-	6.0

* Dispersing Medium

When the marc was dispersed in acetone and heated in 0.6 N HCl in n-butanol, the concentration of anthocyanidin chloride in micrograms per milliliter of marc when heated for 10, 20, 30 and 40 minutes was 8.8, 9.0, 9.2 and 9.4, respectively. Their respective

standard deviations were 0.62, 1.03, 0.89 and 0.46. Thus the longer the heating time the greater the yield of anthocyanidin. Approximately 94% of the conversion had occurred in ten minutes and 96% had occurred at 20 minutes as compared with 40 minutes of heating.

When some of the same marc was dispersed in ethanol and heated for 20, 30, 40 and 50 minutes in 0.025 N HCl in n-butanol, the yield of anthocyanidin chloride was 3.1, 4.3, 5.2 and 6.0 micrograms per milliliter, respectively. Their respective standard deviations were 0.44, 0.27, 0.30 and 0.33. These smaller standard deviations for the marcs in ethanol compared to those in acetone reinforced the author's impression that it was easier to measure a milliliter of marc dispersed in the ethanol. The yield of anthocyanidin from the marc in ethanol continued to increase with heating up to 50 minutes. In contrast to the marc in acetone, however, the conversion to anthocyanidin was negligible after ten minutes, approximately 50% after 20 minutes and 92% after 40 minutes as compared to the amount developed after 50 minutes of heating.

In addition, it can be seen from Table IV that the yield of anthocyanidin from the marcs dispersed in acetone was substantially greater than from the marcs in ethanol. The leucoanthocyanin content of the supernatant from the marc in acetone was negligible; consequently, it was concluded that the increased development of anthocyanidin in this medium was not due to the fact that the acetone

was extracting the leucoanthocyanin from the marc. That the dispersing medium had an effect on the amount of leucoanthocyanin converted to anthocyanidin was further tested in the following experiment.

Effect of certain combinations of dispersing medium and normality of HCl in n-butanol on a single marc. A sample of pear was extracted with 70% ethanol and the marc was successively dispersed in 70% ethanol, in absolute ethanol, and in 70% acetone. While in each dispersing medium, the leucoanthocyanin content of twelve subsamples was determined. The pigment in the marc in 70% ethanol was developed in 0. 025 N HCl in n-butanol for 40 minutes, that in absolute ethanol was developed in 0. 025 N HCl in n-butanol for 40 minutes and that in 70% acetone was developed in both 0. 025 and 0. 6 N HCl in n-butanol for 20 minutes.

Table V contains the average concentration in micrograms of anthocyanidin developed per milliliter of pear marc for each combination of dispersing medium and normality of HCl in n-butanol. The values for the acetone and for the absolute ethanol were adjusted to account for the marc that already had been used.

The average concentration of anthocyanidin in micrograms per milliliter of marc dispersed in 70% ethanol and developed in 0. 025 N HCl in n-butanol was 7. 7. For marc dispersed in absolute ethanol and heated in 0. 025 N HCl in n-butanol the concentration of

TABLE V. EFFECT OF DISPERSING MEDIUM ON CONVERSION OF LEUCOANTHOCYANIN TO ANTHOCYANIDIN (as mcg. cyanidin chloride per ml. of pear marc)

Dispersing Medium	Normality of HCl in n-butanol	Amount of Anthocyanidin	Conversion %
70% Ethanol	0.025	7.7	64
Absolute Ethanol	0.025	8.0	67
70% Acetone	0.025	3.5	28
70% Acetone	0.6	12.1	100*

* Conversion arbitrarily set at 100%.

anthocyanidin was 8.0 micrograms per milliliter. For marcs dispersed in 70% acetone and heated in 0.025 N HCl in n-butanol and in 0.6 N HCl in n-butanol the concentrations were 3.5 and 12.1 micrograms per milliliter, respectively. Their respective standard deviations were 0.39, 0.36, 0.22 and 0.40. The conversions in absolute ethanol and in 95% ethanol were essentially the same and were twice as great as for the same marc in 70% acetone developed in the same normality, 0.025. But the conversion in ethanol was only two thirds of that in 70% acetone when 0.6 N HCl in n-butanol was used. It was concluded that not only does the normality of HCl in n-butanol affect the conversion but so does the dispersing medium, as differences in the yield of anthocyanidin were not due to differences in solubility.

Two interesting comparisons can be made using data from Tables III, IV, and V which suggest the precision achieved when the leucoanthocyanin content of pears is measured in this manner. In Table III the yield of anthocyanidin from the marc in acetone heated in 0.025 N HCl in n-butanol was 23% of that from the marc heated in 0.6 N HCl in n-butanol while in Table V the yield was 28%. A second comparison can be made from Tables IV and V. In Table IV the yield of anthocyanidin from the marc in 70% ethanol heated in 0.025 N HCl in n-butanol for 40 minutes was 55% of that from the same marc in acetone heated in 0.6 N HCl in n-butanol for 20 minutes whereas in Table V the yield was 64%.

Effectiveness of Certain Solvents for the Extraction of Leucoanthocyanin

A survey of the literature indicated that ethanol, methanol, acetone and various aqueous and acidic combinations of these solvents along with ethyl acetate were often used in the extraction of leucoanthocyanin or related compounds from various plant sources. Ethyl acetate was not used as a solvent for leucoanthocyanin in pears as in the preliminary work it appeared to give a poor extraction. Furthermore, this solvent had a drying effect on the pulp making the slurries extremely difficult to handle. Three solvents that were compared were 70% acetone, 95% ethanol and methanol. In a second

series, the most effective solvent of the three was used alone along with an aqueous combination and an aqueous acidic combination.

Acetone (70%), 95% ethanol and methanol. To make the comparison of the effectiveness of these three solvents valid so that ripeness, which might be related to the extent of polymerization of the leucoanthocyanin in the pear, would not affect the results, the three extractants were used on each pear. Aside from the problem of ripeness, another fact that needed to be considered in sampling was that the leucoanthocyanin is not distributed uniformly throughout the pear flesh. In preliminary work, pear slices heated in butanol-HCl became deeply red in small, irregular areas. Goldstein and Swain (19, p. 376) characterized these as tannin cells, also found in the banana and persimmon.

In an attempt to compensate for the uneven distribution of leucoanthocyanin in pear tissue, each of four pears were divided by radial sections into six sub-samples weighing approximately 15 grams. Prior to sampling, the skin and core had been removed. Joslyn (26, p. 318) found that tissues from or near the core of pears gave a strong positive test for leucoanthocyanin when heated in n-butanol-HCl. Slurries were made using two of the sub-samples from each pear with each of the three solvents. The leucoanthocyanin content of each slurry was determined by heating four aliquots in 0.025 N HCl

in n-butanol, as previously described. Consequently, the leucoanthocyanin content for each pear was based on eight determinations for each solvent. As four pears were used, the mean value for each extractant was based on 32 determinations. Those slurries in acetone were heated for 20 minutes and the ones in ethanol and in methanol were heated for 40 minutes. Although previously 0. 6 N HCl in n-butanol was found to be better for developing anthocyanidin from the marcs dispersed in acetone, the lower normality of 0. 025 was used for slurries in this medium because the higher normality of 0. 6 yielded orange-pink solutions after heating for ten minutes which became brown on further heating.

After the leucoanthocyanin content of each slurry was measured, the extract and the marc were prepared from each and their leucoanthocyanin contents were determined (See Table VI). The value for each extract was then compared with the value for the corresponding slurry and expressed as percent extraction. Presumably these percents are indicative of the solubility of the leucoanthocyanin in each of the solvents, provided, of course, that the amount of leucoanthocyanin converted to anthocyanidin was the same in both the extract and slurry in any one solvent and that the leucoanthocyanin contents of the sub-samples were identical.

By mistake, the slurries in acetone from pear 1 were developed with 0. 6 N HCl in n-butanol and due to the brownish discoloration the

TABLE VI. LEUCOANTHOCYANIN CONTENT OF PEAR SLURRIES, EXTRACTS AND MARCS IN THREE SOLVENTS (as mg. of cyanidin chloride per 100 g. of fresh pear) developed in 0.025 N HCl in n-butanol

	70% Acetone		95% Ethanol		Methanol	
Pear	mg/100g	Extraction %*	mg/100g	Extraction %*	mg/100g	Extraction %*
1 Slurry	___		71.0		82.2	
Extract	___	___	21.2	29.9	31.3	38.1
Marc	___		56.5		53.7	
2 Slurry	88.3		61.6		51.0	
Extract	53.9	61.0	19.4	31.5	22.8	44.7
Marc	31.8		45.3		33.5	
3 Slurry	87.1		78.1		71.8	
Extract	59.2	68.0	17.0	21.8	22.8	31.8
Marc	28.4		57.0		45.6	
4 Slurry	94.4		77.1		79.3	
Extract	70.2	74.4	20.7	26.9	23.8	30.0
Marc	27.8		58.5		48.5	
Mean of Slurry	89.9		71.9		71.1	
Mean of Extract	61.1		19.6		25.2	
Mean of Marc	29.3		54.3		45.3	
Sum of Extract and Marc (means)	90.4		73.9		70.5	
Mean %* in Extract		67.8		27.5		35.4

* Based on amount in slurry.

readings were invalid. The average leucoanthocyanin content of the slurries in acetone expressed as milligrams of anthocyanidin developed per 100 grams of pear was 88.3, 87.1 and 94.4 for pears 2, 3 and 4, respectively. The mean value for the three pears was 89.9 and the standard deviation was 4.31. In 95% ethanol the values were 71.0, 61.6, 78.1 and 77.1 for pears 1, 2, 3 and 4, respectively, with a mean of 71.9 and a standard deviation of 4.93. In methanol the corresponding values were 82.2, 51.0, 71.8 and 79.3 with a mean of 71.1 and a standard deviation of 28.3. Thus a greater amount of anthocyanidin was developed in the slurries in acetone than in the slurries in either ethanol or methanol, values for the latter two being approximately 80% of those in acetone. The higher value with 70% acetone was unexpected as the data on the marcs given in Table V showed that those marcs in 70% acetone yielded less than half of the anthocyanidin than did those in ethanol when both were heated in 0.025 N HCl in n-butanol. One difference which might be a factor was that the marcs dispersed in acetone had been extracted previously with 70% ethanol.

For the extracts in acetone, the average leucoanthocyanin content expressed as milligrams of anthocyanidin developed per 100 grams of pear was 53.9, 59.2 and 70.2 for pears 2, 3 and 4, respectively. The mean for the three pears was 61.1 and the standard deviation was 4.14. In 95% ethanol the values for the extracts were 21.2, 19.4,

17.0 and 20.7 for pears 1, 2, 3 and 4, respectively, with a mean of 19.6 and a standard deviation of 1.27. In methanol the values were 31.3, 22.8, 22.8 and 23.8, respectively, with a mean of 25.2 and a standard deviation of 3.1. Thus over twice as much anthocyanidin was developed in the extracts made with acetone as was developed in the extracts with methanol and three times as much as was developed in the extracts with ethanol.

The percent of pigment extracted by 70% acetone was 61.0, 68.0 and 74.4 for pears 2, 3 and 4, respectively, with a mean of 67.8. The percentages extracted by 95% ethanol were 29.9, 31.5, 21.8 and 26.9 for pears 1, 2, 3 and 4, respectively, with a mean of 27.5. The percentages extracted by methanol were 38.1, 44.7, 31.8 and 30.0, respectively, with a mean of 35.4. It would appear that acetone extracted two-thirds of the leucoanthocyanin whereas ethanol and methanol extracted approximately one-fourth and one-third, respectively.

The values for the percentages extracted were analyzed for variance by the randomized block method (31, p. 207) and found to be significantly different for each of the solvents. (The calculated F value was 48.6 with 2 and 6 degrees of freedom.) In addition, the new multiple range test (31, p. 238) showed that the extraction by acetone was significantly greater than that by methanol and the extraction by methanol was significantly greater than that by 95% ethanol.

The amount of leucoanthocyanin remaining in each marc was also

measured. The average values in milligrams of anthocyanidin per 100 grams of pear for marcs dispersed in 70% acetone were 31.8, 28.4 and 27.8 for pears 2, 3 and 4, respectively. The mean for the three pears was 29.3 and the standard deviation was 6.97. In 95% ethanol the values were 56.5, 45.3, 57.0 and 58.5 for pears 1, 2, 3 and 4, respectively with a mean of 29.3 and a standard deviation of 4.83. In methanol the corresponding values were 53.7, 33.5, 45.6 and 48.5 with a mean of 45.3 and a standard deviation of 6.32. As acetone extracted more leucoanthocyanin, the higher values for the marcs from ethanol and methanol were not surprising.

For each extractant the mean value for the marc and for the extract were added. These sums were 90.4, 73.9 and 70.5 milligrams per 100 grams of pear for 70% acetone, for 95% ethanol and for methanol, respectively. These values are comparable to the leucoanthocyanin contents as determined on the slurries which were 89.9, 71.9 and 71.1, respectively. Greater variation was found when the values for sub-samples on individual pears were compared as indicated by the standard deviations.

Acetone (70%), 40% aqueous acetone and 0.1 N HCl in 40% aqueous acetone. Because 70% acetone was the best solvent of the three tested, further comparisons were made with this solvent. Seventy percent acetone (alcoholic), 40% aqueous acetone and 0.1 N HCl in 40%

aqueous acetone were used in the second series. The results using 40% aqueous acetone as a solvent for leucoanthocyanin in pears were eagerly awaited because both Forsyth (12, p. 25) and Roux and Sykes (48, p. 19) had recommended aqueous acetone as the best solvent for flavanols. However, the use of dilute HCl in combination with the aqueous acetone was perhaps questionable. Hydrochloric acid is known to react with the ketone group of acetone (17, p. 82). Furthermore, no reference from the literature could be found as a precedent for its use as a solvent for leucoanthocyanin.

The procedures used for preparing the slurries, marcs and extracts and for measuring their leucoanthocyanin content were the same as those previously described. For developing the anthocyanidin a normality of 0.025 HCl in n-butanol and a heating period of 20 minutes were used.

Table VII shows the average leucoanthocyanin content, based on eight determinations, of the slurry, the extract and the marc of each pear in each solvent. The data are expressed as milligrams of cyanidin chloride per 100 grams of fresh pear. For the slurries in 70% acetone, the average leucoanthocyanin content expressed as milligrams of anthocyanidin per 100 grams of pear was 67.7, 151.4, 64.8 and 92.6 for pears 5, 6, 7 and 8, respectively. The mean for the four pears was 94.1 and the standard deviation was 17.96. In 40% aqueous acetone the values were 50.2, 73.2, 53.3 and 67.5, respectively,

TABLE VII. LEUCOANTHOCYANIN CONTENT OF PEAR SLURRIES, EXTRACTS AND MARCS IN ACETONE (as mg. of cyanidin chloride per 100 g. of fresh pear developed in 0.025 N HCl in n-butanol)

	70% Acetone		40% Aqueous Acetone		0.1 N HCl in 40% Aqueous Acetone	
Pear	mg/100g	Extraction %*	mg/100g	Extraction %*	mg/100g	Extraction %*
5 Slurry	67.7		50.2		67.5	
Extract	36.2	53.5	30.4	60.6	43.8	64.9
Marc	28.4		14.3		18.6	
6 Slurry	151.4		73.2		58.5	
Extract	74.9	49.5	38.7	52.9	28.2	48.2
Marc	78.3		27.7		17.4	
7 Slurry	64.8		53.3		49.8	
Extract	43.3	66.8	34.7	65.1	25.7	51.6
Marc	21.5		13.2		17.3	
8 Slurry	92.6		67.5		56.2	
Extract	63.7	68.8	49.5	73.4	33.4	59.4
Marc	37.5		18.6		14.3	
Mean of Slurry	94.1		61.1		58.0	
Mean of Extract	54.5		38.3		32.8	
Mean of Marc	41.4		18.5		16.9	
Sum of Extract and Marc (means)	95.9		56.4		49.8	
Mean %* in Extract		59.7		62.7		56.0

*Based on amount in slurry.

with a mean of 61.1 and a standard deviation of 6.24. In 0.1 N HCl in 40% aqueous acetone the values were 67.5, 58.5, 49.8 and 56.2, respectively, with a mean of 58.0 and a standard deviation of 18.47. Thus a greater amount of anthocyanidin developed in the slurries in acetone compared to those in aqueous acetone or in acidic aqueous acetone. Presumably the conversion of leucoanthocyanin to anthocyanidin was greater under more nearly anhydrous conditions.

For those extracts made with 70% acetone, the average leucoanthocyanin content expressed as milligrams of anthocyanidin developed per 100 grams of pear was 36.2, 74.9, 43.3 and 63.7 for pears 5, 6, 7 and 8, respectively. The mean for the four pears was 54.5 and the standard deviation was 8.15. For the extracts in 40% aqueous acetone the values were 30.4, 38.7, 34.7 and 49.5, respectively, with a mean of 38.3 and a standard deviation of 4.49. For 0.1 N HCl in 40% aqueous acetone the values were 43.8, 28.2, 25.7 and 33.4, respectively, with a mean of 32.8 and a standard deviation of 4.3. A greater amount of anthocyanidin was developed in the extracts made with 70% acetone than in extracts made with 40% aqueous acetone or with 0.1 N HCl in 40% aqueous acetone.

Also included in Table VII is the amount of leucoanthocyanin in each extract expressed as percent of the amount in the corresponding slurry. The percent extracted by the 70% acetone was 53.5, 49.5, 66.8 and 68.8 for pears 5, 6, 7 and 8, respectively, and the mean

for the four pears was 59.7. The percent extracted by 40% aqueous acetone was 60.6, 52.9, 65.1 and 73.4, respectively, with a mean of 62.7 and by 0.1 N HCl in 40% aqueous acetone was 64.9, 48.2, 51.6 and 59.4, respectively, with a mean of 56.0. Thus the percent of leucoanthocyanin extracted was similar for all three solvents. These values were analyzed for variance by the randomized block method (30, p. 207) and no significant differences were found. (The calculated F value was .98 with 2 and 6 degrees of freedom.)

In the marc extracted with 70% acetone, the average leucoanthocyanin content expressed as milligrams of anthocyanidin developed per 100 grams of pear was 28.4, 78.3, 21.5 and 37.5 for pears 5, 6, 7 and 8, respectively. The mean for the four pears was 41.4 and the standard deviation was 15.11. In 40% aqueous acetone the values were 14.3, 27.7, 13.2 and 18.6, respectively, with a mean of 18.5 and a standard deviation of 10.21. In 0.1 N HCl in 40% aqueous acetone the values were 18.6, 17.4, 17.3 and 14.3, respectively, with a mean of 16.9 and a standard deviation of 1.81. Thus more anthocyanidin developed in the marcs extracted with 70% acetone than in those extracted with 40% acetone or with 0.1 N HCl in 40% aqueous acetone.

The sum of the mean values for the marc and the extract was 95.9, 56.4 and 49.8 for 70% acetone, for 40% aqueous acetone and for 0.1 N HCl in 40% aqueous acetone, respectively. The leucoanthocyanin contents of the slurries in these same solvents were 94.1, 61.1,

and 58. 0, respectively. The totals of the marc plus extract for 40% aqueous acetone and for 0. 1 N HCl in 40% aqueous acetone were somewhat lower than the values determined on the slurries.

Browning of the slurries in 0. 1 N HCl aqueous acetone was noted. A possible source of the difficulty was that the extracts had been prepared the third week of December but the leucoanthocyanin content was determined during the first two weeks in January. To determine what effect this holding period might have had on the measurement of leucoanthocyanin in the acidic slurries, the following test was performed. The leucoanthocyanin contents of four aliquots of each of two slurries in 0. 1 N HCl in 40% aqueous acetone and their respective marcs and extracts were determined. Four weeks later the leucoanthocyanin contents of these marcs and extracts were remeasured. Table VIII shows the average leucoanthocyanin content of the slurry, the extract and the marc of each sample determined on the day of preparation. Also included are those values for the extracts and the marcs determined one month later. Each value is the average of four determinations and is expressed as milligrams of cyanidin chloride developed per 100 grams of pear tissue.

The average leucoanthocyanin content expressed as milligrams of anthocyanidin chloride developed per 100 grams of pear for the slurry, extract and marc of sample one was 36. 0, 22. 0 and 12. 2, respectively. One month later the value for the extract was 15. 9

TABLE VIII. LEUCOANTHOCYANIN CONTENT OF MARCS AND EXTRACTS STORED IN ACIDIC AQUEOUS ACETONE (as mg. of cyanidin chloride per 100 g. of fresh pear developed in 0.025 N HCl in n-butanol)

Sample	Fresh Preparation	Stored One Month	Percent Lost
1 Slurry	36.0		
Extract	22.0	15.9	30
Marc	12.2	6.1	50
*% in Extract	61.0		
2 Slurry	35.4		
Extract	28.1	6.1	60
Marc	11.4	7.3	30
*% in Extract	80.0		

*Based on amount in slurry.

showing a loss of 30% and that for the marc was 6.1 showing a loss of 50%. For sample 2, the original values were 35.4, 28.1 and 11.4 for the slurry, extract and marc, respectively. One month later the value for the extract was 6.1 showing a loss of 60% and that for the marc was 7.3 showing a loss of 30%. The extract of the second sample showing the loss of 60% had become quite brown whereas there appeared to be little change of color in the other extract and the two marcs. Thus, in Table VII, the data for the leucoanthocyanin content of the pears treated with 0.1 N HCl in 40% aqueous acetone is invalid. As the preliminary work had indicated that extracts of acetone were stable for over a month, it was assumed that the data for the

70% acetone was not affected by the holding period. The stability of the slurries, extracts and marcs in 40% aqueous acetone was not studied. However, browning did not occur in this solvent.

Conversion of a synthetic leucocyanidin to anthocyanidin. To examine further the factors influencing the measurement of leucoanthocyanin, a synthetic polymeric leucocyanidin[4] was converted to anthocyanidin under the conditions that had been used for the conversion to anthocyanidin of the leucoanthocyanin extracted from the pears. In each of two 10-milliliter volumetric flasks, 100 milligrams of the leucocyanidin were dispersed and made to volume in 90% ethanol. From each flask one-milliliter aliquots were pipetted into five 25-milliliter volumetric flasks and made to volume with 70% acetone (alcoholic), 95% ethanol, methanol, 40% aqueous acetone and 0.1 N HCl in 40% aqueous acetone. Thus the concentration of leucocyanidin in each solvent was 400 micrograms per milliliter. The leucocyanidin was converted to anthocyanidin using both 0.025 N and 0.6 N HCl in n-butanol. Four aliquots were heated with each of the normalities, those in 70% acetone for 20 minutes and those in the other media for 40 minutes. Two replications were made giving a total of eight determinations for each normality and solvent. Thus

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[4]Fluka, A. G., Buchs (SG), Switzerland.

the average of both weighings involved 16 determinations.

Table IX shows the average yield of anthocyanidin expressed as micrograms of cyanidin chloride developed from 400 micrograms of leucocyanidin in the different solvents with both 0.025 N and 0.6 N HCl in n-butanol. With 0.025 N HCl in n-butanol the yields were 441, 235, 235, 173 and 185 micrograms for the solvents 70% acetone,

TABLE IX. YIELD OF ANTHOCYANIDIN FROM 400 MICROGRAMS OF SYNTHETIC LEUCOCYANIDIN (expressed as cyanidin chloride)

Solvent	Normality of HCl in n-butanol	Micrograms	Conversion %
70% Acetone	0.025	441	110.1
	0.6	363	91.5
95% Ethanol	0.025	235	58.8
	0.6	106	26.5
Methanol	0.025	235	58.8
	0.6	101	25.0
40% Aqueous Acetone	0.025	173	43.3
	0.6	156	39.0
0.1 N HCl in 40% Aqueous Acetone	0.025	185	46.0
	0.6	168	41.8

95% ethanol, methanol, 40% aqueous acetone and 0.1 N HCl in 40% aqueous acetone, respectively. The corresponding percentages of conversions were 110.1, 58.8, 58.8, 43.3 and 46.0. With 0.6 N HCl

in n-butanol as the developer the yields were 363, 106, 101, 156 and 168, respectively, for the above solvents. The corresponding percentages of conversion were 91.5, 26.5, 25.0, 39.0 and 41.8. Analysis of variance of the data by the random block method (30, p. 207) showed that there was a significant difference between the conversions in the different solvents. (The calculated F value was 158.7 with 9 and 150 degrees of freedom.) The new multiple range test (30, p. 238) further revealed that the amount of anthocyanidin developed from the leucocyanidin dispersed in 70% acetone with 0.025 N HCl in n-butanol was significantly greater than the amount converted with any other combination of solvent and normality. The second best conversion was with 70% acetone and 0.6 N HCl in n-butanol followed by either ethanol or methanol and a normality of 0.025. The conversion in aqueous acetone was lower than that in 70% acetone or in alcohol treated with 0.025 N HCl in n-butanol. Whether 0.6 N or 0.025 N HCl in n-butanol was used to develop the anthocyanidin made no difference in the conversion. The lowest conversion was given with either alcohol as a solvent and a normality of 0.6.

The 26.5 and 25.0 % conversions to anthocyanidin of leucocyanidin when dispersed in 95% ethanol and in methanol and treated with 0.6 N HCl in n-butanol compare very well to the 25% reported by Swain and Hillis (51, p. 65) for similar conditions. The 58.8% conversion of the leucocyanidin dispersed in ethanol and in methanol when

treated with 0.025 N HCl in n-butanol was higher than the 37-42% reported by Roux and Bill (43, p. 42) for leucorobinetinidin under simlar conditions.

There are other interesting comparisons that can be made between the figures in this table and results cited previously. The conversion in 0.025 N HCl in n-butanol was twice as great as it was in 0.6 N HCl in n-butanol for both the synthetic leucocyanidin (Table IX) and the marc from the pears (Table III), both being dispersed in ethanol. The conversion of synthetic leucocyanidin was one-and-a-half times greater in 70% acetone treated with 0.6 N HCl in n-butanol than it was in ethanol and treated with 0.025 N HCl in n-butanol (Table IX). The same was true for the marc (Table V).

Puzzling discrepancies, however, are apparent in the conversions to anthocyanidin of leucoanthocyanin from various sources (marc, slurry or synthetic leucocyanidin) dispersed in acetone and treated with 0.025 N HCl in n-butanol. Under these conditions conversion in the marc in acetone was one-half that in ethanol (Table V), that in the slurries was 25% greater in acetone than in ethanol (Table VI) and conversion of synthetic leucocyanidin in acetone was almost twice that in ethanol (Table IX).

Chromatographic study of developed anthocyanidins. The high conversion (110%) to anthocyanidin of synthetic leucocyanidin in 70%

acetone when developed in 0. 025 N HCl in n-butanol indicated that perhaps the red pigment developed was not exclusively cyanidin chloride. In 1937 Robinson (35, p. 1138) had observed that acetone increased the yield of cyanidin and stated that it appeared modified.

The pigments obtained from both synthetic and pear leucoanthocyanin were analyzed by paper chromatography. The butanol containing the pigment was washed with distilled water, dried with Na_2SO_4, and made 0. 1 N with HCl. Along with pure cyanidin chloride, the anthocyanidins converted with 0. 025 N HCl in n-butanol from the synthetic leucocyanidin dispersed in 70% acetone and in 95% ethanol were spotted on strips of No. 1 Whatman filter paper. Chromatograms were developed with Forestal reagent (water, acetic acid, 12 N HCl; 10:30:3 v/v). The average R_f values for three runs are shown in Table X. A second chromatogram was developed with formic acid solvent (formic acid, 12 N HCl, water; 5:2:3 v/v). For this, anthocyanidins from the above two sources were spotted on filter paper along with anthocyanidins from synthetic leucocyanidin in 95% ethanol converted with a normality of 0. 6 and from pear marc in 70% acetone and in 70% ethanol converted with normalities of 0. 6 and 0. 025, respectively. Their R_f values are also shown in Table X.

Spots with R_f values corresponding to the cyanidin chloride standard appeared on the chromatograms from all the sources tested. In Forestal developer the cyanidin chloride standard had a R_f of 0. 24

TABLE X. R_f VALUES OF ANTHOCYANIDINS DEVELOPED WITH TWO SOLVENTS

Source of Anthocyanidin	Normality of HCl in n-butanol	Forestal		Formic Acid	
		R_f1	R_f2	R_f1	R_f2
Standard Cyanidin		0.24		0.20	
Leucocyanidin in 70% Acetone	0.025	0.24	0.67	0.20	0.44
Leucocyanidin in 95% Ethanol	0.025	0.24	0.65	0.20	0.39
	0.6			0.20	0.41
Pear Marc in 70% Acetone	0.6			0.19	0.38
Pear Marc in 70% Ethanol	0.025			0.18	0.38

and in formic acid an R_f of 0.20. None of the spots on the chromatograms showed any brown streaking due to the formation of phlobaphenes, but present from each preparation was a faster moving anthocyanidin which appeared to make up most of the pigment. The R_f for this second spot with Forestal reagent was approximately 0.66 whereas in formic acid it was approximately 0.40.

Roux and Bill (43, p. 42) have reported similar results with the anthocyanidins developed in 0.025 N HCl in propan-2-ol from leucorobinetinidin. They suggested that the fast moving spot was a 3-isopropyl ether derivative of robinetinidin. Possibly the appearance

of the second spot was due to the presence of a butyl-ether derivative of cyanidin.

SUMMARY

This study explored some of the difficulties involved in measuring the leucoanthocyanin content in Bartlett pears by a currently used method which involves extracting the leucoanthocyanin from the plant tissue, converting it to anthocyanidin and measuring the resulting pigment colorimetrically. Both the extraction and the conversion of leucoanthocyanin were investigated.

The effectiveness of five solvents, 70% acetone, 95% ethanol, methanol, 40% aqueous acetone and 0.1 N HCl in 40% aqueous acetone, for extracting the leucoanthocyanin from pears was studied. The leucoanthocyanin content was measured on the slurries, the marcs and the extracts. Approximately 60% of the leucoanthocyanin was extracted by acetone, approximately one-fourth by 95% ethanol and one-third by methanol. When the mean value for the marc and the extract was compared to the mean value for the slurry in the same solvent, the values agreed for all solvents except for the aqueous acetone.

The conversion to anthocyanidin of the leucoanthocyanin in Bartlett pears was examined by varying the normality of the HCl in n-butanol. Marc from pear previously extracted with 70% ethanol was used as the source of leucoanthocyanin. When this marc was dispersed in 70% ethanol and heated in 0.6, 0.1, 0.05 and 0.025 N HCl

in n-butanol, the normality of 0.025 gave the greatest conversion. When the marc was dispersed in 70% acetone and heated in 0.6, 0.1 and 0.025 N HCl in n-butanol, the normality of 0.6 gave the greatest conversion.

The heating period was also varied. The longer the heating period (up to 40 minutes) the greater the conversion for the marc dispersed in 70% acetone and treated with 0.6 N HCl in n-butanol, although 94% of the conversion had occurred in ten minutes. For marc dispersed in 70% ethanol and treated with a normality of 0.025, the longer the heating period (up to 50 minutes) the greater the conversion. However, only 92% of the anthocyanidin had developed in 40 minutes.

When the marc was dispersed in 70% ethanol and treated with 0.025 N HCl in n-butanol the amount of anthocyanidin developed was approximately two-thirds of that when the marc was dispersed in 70% acetone and treated with a normality of 0.6.

Further study of the effect of the dispersing medium was made with a polymeric synthetic leucocyanidin. The percentage conversion of leucocyanidin to anthocyanidin for each combination of solvent and normality of developing medium was as follows: 110% for 70% acetone and 0.025 N; 91.5% for 70% acetone and 0.6 N; 58.8% for either ethanol or methanol and 0.025 N; approximately 43% for 40% aqueous acetone and either 0.025 or 0.6 N; and approximately

26% for either ethanol or methanol and 0. 6 N.

By paper chromatography it was shown that two anthocyanidins were formed from both the pear and synthetic leucocyanidin. One had the same R_f as cyanidin and the other (and the major one) had a higher R_f value.

The conversion of the synthetic leucocyanidin to anthocyanidin was compared to that of the marc under similar conditions. The conversion in 0. 025 N HCl in n-butanol was twice as great as it was in 0. 6 N HCl in n-butanol for both the synthetic leucocyanidin and the marc, both being dispersed in ethanol. The conversion of synthetic leucocyanidin was one-and-a-half times greater in 70% acetone treated with 0. 6 N HCl in n-butanol than it was in ethanol and treated with 0. 025 N HCl in n-butanol. The same was true for the marc.

Puzzling discrepancies, however, were apparent in the conversions to anthocyanidin of leucoanthocyanin from various sources (marc, slurry or synthetic leucocyanidin) dispersed in acetone and treated with 0. 025 N HCl in n-butanol. Under these conditions conversion in the marc in acetone was one-half that in ethanol, that in the slurries was 25% greater in acetone than in ethanol and the conversion of synthetic leucocyanidin in acetone was almost twice that in ethanol.

Due to the above inconsistencies, it would appear that until more is known about the nature of the pear leucoanthocyanin, the method of measuring leucoanthocyanins will give only a rough estimate. On

the basis of this study it is recommended that procedures be used which gave the highest values for the leucoanthocyanin content. To this end the measurement would be made on the slurry as opposed to the extract, as the extracts contained only 25% to 70% of the leucoanthocyanin found in the slurries. For the dispersing medium the conversion was greater in 70% acetone (ethanolic) than in either ethanol, methanol or aqueous acetone. Furthermore, it is advised that 0.025 N HCl in n-butanol be used to convert the leucoanthocyanin to anthocyanidin as there was browning due to phlobaphene formation when higher normalities were used.

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