A PHYTOCHEMICAL INVESTIGATION OF ACACIA ANGUSTISSIMA (MILL) KUNTZE. FAMILY LEGUMINOSAE

by

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After inspection of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

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INTRODUCTION

Numerous studies have been made on different acacia species, although Acacia angustissima (Mill) Kuntze, has not been the subject of either phytochemical or pharmacological investigations.

The acacia plants in the past have served primarily as a source of the dried gummy exudate from the stems and branches which has been used as a demulcent, emulsifying agent and vehicle in the preparation of emulsions and troches. It has also been used intravenously in the treatment of post-hemorrhagic shock. Acacia mearnsii (formerly A. mollisima) and other acacia heartwoods are also utilized as a source of condensed tannins for the tanning industry (1). Pollia (2) in 1937, tested various concentrations of acacia against sarcoma tumors in white rats, but observed no inhibition of the tumor growth.

The most frequently occurring class of compounds found in the acacia species has been the flavonoid pigments. The pharmacology of the flavonoids is quite variable (3). According to Deeds (4), who has reviewed the physiology of the flavonoids, many beneficial clinical uses have been claimed for these compounds. Some of these beneficial effects are: (1) capillary fragility, (2) retinal hemorrhage in hypertension, (3) diabetic retinopathy, (4) purpura, rheumatic fever, arthritis, radiation disease, habitual abortion,

frostbite, histamine and anaphylactic shock and experimentally induced cancer. Other properties attributable to the flavonoids are their antioxidant effect on adrenalin and ascorbic acid, smooth muscle constriction, and inhibition of enzyme systems. The flavonoid metabolite also stimulates the pituitary-adrenal axis, which causes an increased production of the adrenocorticotropic hormone.

The phytochemical exploration of the acacia plants for the flavonoid pigments has usually been done by examining the water or alcohol soluble extracts of the plant. Petrie (5) in 1923, isolated the flavonol glycoside kaempferol (I*) from the water soluble extracts of Acacia discolor, A. linifolia, A. decurrens, and A. longifolia. He also identified tannins, carotene and xanthophylls in the plants.

Marini-bettolo and Falco (6) extracted the fresh flowers of A. longifolia with boiling water, precipitated the pigments with basic lead subacetate, and identified the flavanone naringenin (II).

Three flavonols, fisetin (III), quercetagetin (IV), quercetin (V), and phlobatannins were isolated by Hathway and Seakins (7) from the heartwood of A. catechu. These flavonols were characterized by paper chromatography.

Flavonoids have been obtained from the pollen of A. dealbata

*The structures discussed throughout this dissertation will be designated by Roman Numerals, and illustrations will be found in the appendix, pages 111-114.

by the procedure of Spada and Carmeroni (8). They extracted the pollen with benzene, then methanol, and ethanol. Then the ethanol extract, after purification on a column of cellulose, was precipitated with neutral and basic lead subaceatate to yield the flavanone naringenin (II) and the flavonol quercetin (V).

Paris (3, 9) found the flavonoid pigments very difficult to purify by conventional methods. He employed a soxhlet apparatus and extracted the plant material with solvents of different polarity. He then removed the sugars of the ethyl alcohol extracts by yeast fermentation. He found that the highest concentration of flavonols usually existed in the leaves of Acacia cavenia, A. farnesiana, and A. cyanophylla. A. longifolia leaves yielded the flavanone naringenin (II), as had the flowers previously (6).

Other compounds recently found in certain acacia species in combination with the flavones and flavonols are the leuco-anthocyanidins. Clark-Lewis (1) has stated that three or more of the different classes of flavan* compounds usually occur together. Bate-Smith (10) also stated that flavonols and leuco-anthocyanidins occur together and predominate in woody families; and flavones and flavanones usually predominate in herbaceous families, from which leuco-anthocyanidins are rarely found. Roux (11), in a recent review, indicates that as many as nine different compounds of a flavan or flavonoid nature have been isolated from Acacia mearnsii.

*Flavan compounds are considered to be flavonols, flavones, leuco-anthocyanidins, catechins, dihydroflavonols and flavan.

Difficulty has also been encountered in the purification of leuco-anthocyanidins, obtained from the heartwood of various Acacia species. King and Bottomley (12, 13) extracted Acacia melanoxylon heartwood with ethyl ether, which after partial removal of the solvent, deposited a crude tan leuco-anthocyanidin called melacacidin (VI). They were able to characterize the structure by its tetra methyl ether derivative and by synthesizing the racemic compound (14).

Keppler (15), in 1956, obtained a crystalline leuco-anthocyanidin from the heartwood of Acacia mollisima (recently renamed A. mearnsii) by extracting it with acetone and purifying the extract on a column. It was not positively identified, but was believed to be one of three possible isomers. In 1957, Keppler (16) positively identified the second leuco-anthocyanidin as (+)-mollisacacidin (VII)*. The enantiomorphous isomer of mollisacacidin called leuco-fisetinidin (VIII) was isolated by Roux in 1958 from Schinopsis quebracho-colorado (17).

Roux and Freudenberg (18), in 1958, were able to identify a new leuco-anthocyanidin, occurring in Acacia mearnsii, appropriately called leuco-robinetinidin (IX). Subsequently, in 1959, Roux (19)

*The absolute configuration of (+)-mollisacacidin (VII) has recently been revised by Lillya, Drewes and Roux (94), with the aid of nuclear magnetic resonance spectra. The corrected structure is represented by (XXVI) in the appendix.

identified a fourth new leuco-anthocyanidin, occurring in Guibourtia Coleosperma, G. tessmannii and G. demeusei, which was identified chemically as 7, 4'-dihydroxy-flavan-3,4-diol (X).

Clark-Lewis and Mortimer (1, 20) were able to separate melacacidin (VI) from isomelacacidin (XI) and isolate 4-hydroxypipecolic acid (XII) (21) from A. excelsia. They were able to achieve this by using an elaborate extraction scheme employing a counter current purification step, and converting the 4-hydroxy-isomelacacidin into the ethyl ether derivative, thereby enabling them to separate it from melacacidin.

Clark-Lewis, et al (22) in 1961, isolated and identified the fifth leuco anthocyanidin, teracacidin (XIII) from A. intertexta. They found that teracacidin occurred with isoteracacidin (XIV) as had malacacidin (VI) with isomelacacidin (XI) previously.

Recently, the 5-hydroxy leuco-anthocyanidin analogues have been isolated in nature, although not from acacia species. Ganguly and Seshadri (23) isolated (+)-leuco-cyanidin (XV) from the gum of Butea frondosa, and leuco-pelargonidin (XVI) from Eucalyptus calophylla; and Row and Rao (24) found leuco-dephinidin (XVII) occurring in Terminalia arjuna.

Miscellaneous substances that have been reported as occurring in acacia plants are shown in Table 1.

The investigation of Acacia angustissima emanates from the anti-tumor properties recently located in the aqueous and ethanol extracts of the plant. The anti-tumor activity of the initial and subsequent extracts was determined by the Cancer Chemotherapy National Service Center in Washington, D. C.

The preliminary extracts of A. angustissima were prepared and submitted to the screening center by Dr. Mary Caldwell, et al, of the University of Arizona College of Pharmacy. This has been done as part of a project to screen plants indigenous to Arizona and the Southwest for their possible anti-tumor properties.

Due to the positive confirmation received from the testing center on Acacia angustissima, further fractionation studies were pursued, both from a purely phytochemical viewpoint, and if possible, to isolate the active anti-tumor constituent present in the plant. This investigation was undertaken with these objectives in mind.

Table 1

MISCELLANEOUS SUBSTANCES OCCURRING IN THE ACACIA SPECIES

Substance	Species	Reference Number
Saponins	Acacia albicorticata, A. itsia	25, 26
Alkaloids	--	27, 28
Ethyl Gallate	A. adonsonii, A. seyal	29
Cyanogenetic glycosides	A. giraffae, A. lasiopetala, A. litakunensis, A. robusta, A. stolonifera	30
Maclurin	A. catechu, A. catechuoides, A. sundra	31
Leucomaclurin glycol ether, acacatechol, catechol	A. catechu	32, 33
Tannins	Numerous species	4, 34
Sterols	Numerous species	35, 36, 37, 38
Phlobatannins	Numerous species	6, 11
Carotenes, xanthophylls	Numerous species	4, 39, 40

Description of Acacia angustissima (Mill) Kuntze

Acacia angustissima grows on dry rocky slopes, usually in chaparral, May to September, at elevations of 3000 to 6500 feet. In Arizona, the plant can be found in Greenlee County to eastern Yavapai County, south to Cochise, Santa Cruz and Pima Counties. It also can be found in Missouri, Texas, southern Florida and as far south as Guatemala (41).

> The plants are low shrubs or bushes, scarcely woody, usually attaining a height of 1-1.5 meters high. The twigs are deeply grooved, often hispid or hirsute (with long hairs); primary leaflets usually 6-14 pairs, acute at the apexes; secondary leaflets in usually 20-33 pairs; each leaflet 3-6 millimeters long, normally 1 millimeter or less wide; stipules scale-like, brown, 2-3 millimeters long, ciliate; panicled round heads of cream colored flowers; calyx green, 0.7 millimeters long; petals green, separate, 2.5 millimeters long; stamens 100 or more, the filaments white or tinged with pink or lavender, 6.8 millimeters long; pod brown, linear oblong, 4-7.5 centimeters long, 7-9 or 10 centimeters broad, glabrous; seeds mottled, gray with brown, dark brown or black, oblong, 3.5-4 millimeters long, 2.5-3 millimeters broad (42).

Collection and Storage

The acacia plants used in this investigation grow near Mount Lemmon, located about 35 miles from Tucson, and in Sabino Canyon, located approximately ten miles from the city. The leaves, stems and flowering parts were collected during September, 1961, immediately placed in polyethylene bags, and stored in a freezer at -10°C until April 1962, at which time the extraction was undertaken.

Figure 1. Acacia angustissima growing in lower Sabino Canyon, Pima County, Arizona.

GENERAL EXTRACTION PROCEDURE

The fresh frozen leaves, stems and flowering parts were ground in a Wiley Mill, equipped with a 4 millimeter size screen, to a fine material which had the appearance of freshly cut grass. A 0.722 kilogram sample of this plant material was placed inside an improvised extraction thimble of a one liter soxhlet apparatus, and extracted with 2.5 liters of petroleum ether (Bakers Analyzed Reagent, B.P. 30-60°C) for a period of 51 hours. The first few returns of solvent to the soxhlet flask were dark green and subsequent ones were yellow. The extraction was continued until the extracts were pale yellow to colorless.

During this time, it was found necessary to add an additional 1.20 liters of petroleum ether to the system in order to maintain the original solvent volume. A double condenser or ice water circulated through a single condenser prevented excessive solvent loss.

An additional small quantity of the ground plant material, which would not fit into the soxhlet (0.178 kilograms), was placed in a gallon jar and layered with 0.50 liters petroleum ether. The mixture was also allowed to stand for 51 hours with frequent shaking, then filtered through Pratt Dumas No. 33 filter paper. The filtrate was then added to the soxhlet petroleum ether extract.

The combined olive colored petroleum ether extracts were placed in a large evaporating dish and the solvent was evaporated

overnight with the aid of a fan. The crude pasty residue weighing 7.67 grams was set aside for saponification.

The plant marc from the petroleum ether extraction was removed from the soxhlet, air dried for 30 minutes, and re-extracted in the soxhlet with 2.50 liters of ethanol, USP, for 42 consecutive hours. The first few extractions were of a viscous nature and had a dark green color; subsequent extracts became progressively lighter in color until they were yellow. After 42 hours of extraction, the soxhlet was recharged with 2.5 liters of fresh ethanol, and the plant was extracted a second time for 48 hours until the solvent extracts were pale yellow.

Ethanol (0.50 liters) was also added to the 0.178 kilograms of plant marc in the gallon jar and it was macerated with frequent shaking for 42 hours. The mixture was then filtered as above and added to the soxhlet ethanol extract.

Some ethanol was also lost to the atmosphere, therefore, an additional 0.90 liter of solvent was added to maintain the original solvent volume. This extraction again required the use of a double condenser with circulating ice water or tap water to minimize excessive loss of ethanol. The greatest loss of solvent occurred during the night when tap water was circulated through the condensers.

The combined ethanol extracts were concentrated under house vacuum to a thick, olive-colored, syrupy material which weighed 147.8 grams. The original chloroform-ethanol extraction performed by

Dr. Caldwell* gave the first anti-tumor activity and subsequent defatted ethanol and chloroform extracts showed the activity to reside in the ethanol fraction. Therefore, the defatted ethanol soluble extract from Acacia angustissima was the principle extract used in this investigation.

The marc from the petroleum ether and ethanol extractions was mixed with 2.5 liters of distilled water and macerated for a period of 48 hours. The mixture was then filtered through gauze and the aqueous extract was lyophilized. The yield was 10.5 grams of tan powder. The marc was discarded. The lyophilized tan powder was submitted to the Cancer Chemotherapy National Service Center for anti-tumor screening. The results were negative. See Figure 2 for the general extraction scheme.

* Private communication.

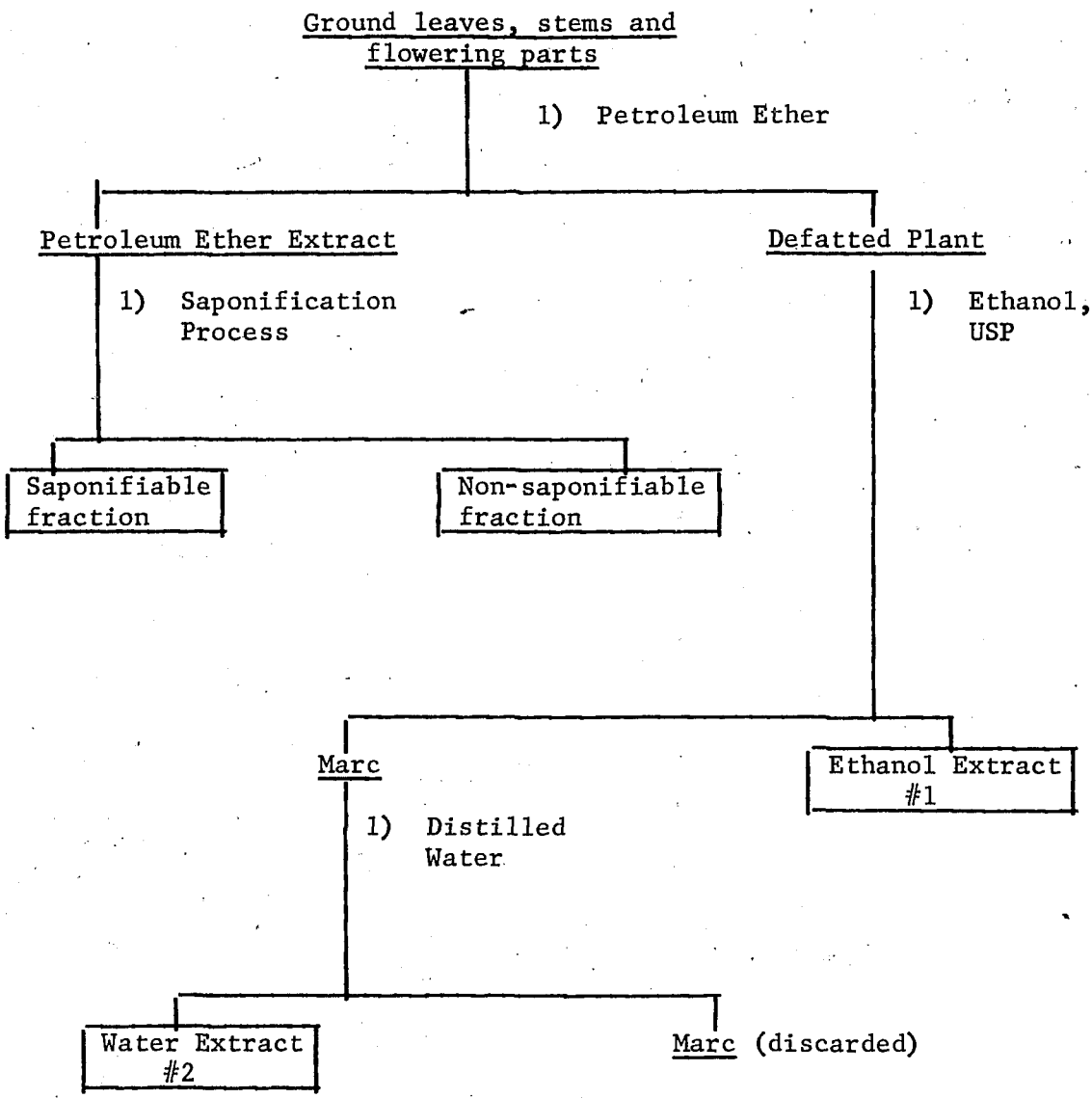


Figure 2. General extraction of Acacia angustissima.

PURIFICATION OF THE ETHANOL EXTRACT

Preliminary Purification Method

The syrupy ethanol extract (7.0 grams) was triturated in a mortar with ethyl ether (100 ml x 4) until the final decanted extract was practically colorless. The syrupy thick residual extract was gummy and had a shredded appearance after the ether extractions. The gummy substance was further extracted with chloroform (100 ml x 2) which left a brown wax-like material in the mortar. The residual wax-like gum was then layered with a 100 ml volume of acetone and triturated into the gum. The gum immediately turned to a brown powdery substance on the bottom of the mortar. The acetone was decanted from the brown powder and filtered. The brown powder dried rapidly in the air and left a tan substance which was powdered with the mortar. The weight of the substance was 2.0 grams. See Figure 3 for the purification scheme.

At this point, preliminary qualitative physical and chemical tests were run on the tan fraction (#3) in order to determine, if possible, what particular class of compounds was present in the plant. The primary basis for choosing the following color tests was the flavonoid or flavan compounds that have occurred repeatedly in the acacia species. The chemical tests were used throughout this investigation to follow the fractionation of the ethanol extract. The reagents and the procedures of the tests were as follows:

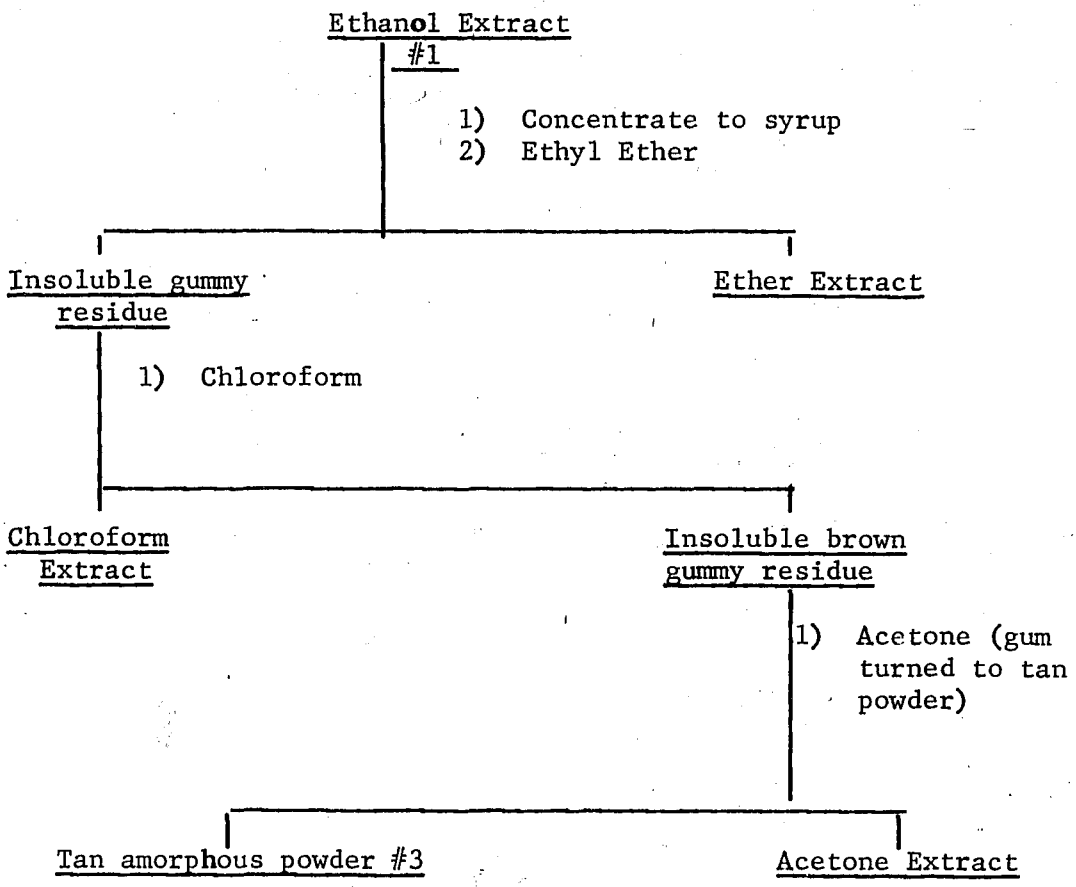


Figure 3. Preliminary Fractionation of the Ethanol Soluble Fraction.

Qualitative Chemical Test Procedures

1. Wilson's Boric Acid Test (43, 44)

0.5 mgm sample was dissolved in 1 ml acetone and the solution divided into equal parts. To one part was added 2 ml of boric acid-citric acid-acetone reagent and the other portion was diluted to an equal volume with a mixture of equal parts of citric acid-acetone solution and acetone. The colors of the two tubes were compared at the end of a few minutes and any more definitely intense color in the boric acid containing tube was indicated as a positive test reaction for flavones or flavonols.

Reagents

(a) Absolute acetone saturated with boric acid.

(b) Absolute acetone containing 10% anhydrous citric acid.

2. Flavonol Test (45)

1 ml alcoholic extract (25-50 milligrams of dry plant material) was treated with 0.5 ml. HCL (10%) and magnesium turnings. The color, after allowing the reaction to proceed spontaneously for several minutes, varied from pale to deep red depending on the quantity of flavonols. For solubility purposes, a methanol-water solvent system was used for certain plant fractions.

3. Ferric Chloride Test Solution (46)

1 drop of ferric chloride solution (9%) was added to the plant fraction dissolved in methanol or methanol-water. A color produced with ferric chloride is typical of phenols and enols, although oximes also give a color.

4. Fehling's Test (47)

Reagents

(a) Copper sulfate solution (34.6 grams of hydrated copper sulfate in 500 ml of water.)

(b) Sodium potassium tartrate (173 gm) and sodium hydroxide (70 grams) diluted to 500 ml of water.

To an aqueous solution of the material was added 5 ml of Fehling's solution (mix the two above solutions in equal parts just before using) and the mixture was heated to boiling. A positive color test may be red or yellow depending on the amount of reducing sugar or aliphatic aldehyde present.

5. Gelatin Test (45)

1 ml of an aqueous gelatin solution (1%) was added to the plant material dissolved in water or methanol-water media. The presence of tannins yields a milky precipitate.

6. Mayers Test (48) (Mercuric-potassium Iodide Test Solution)

Reagents

(a) Mercuric chloride (1.358 grams) in 60 ml of water.

(b) Potassium Iodide (5.0 grams) in 10 ml of water.

The two solutions were mixed and water was added to make 100 ml of solution. The test solution was added to both an aqueous and 1.5% hydrochloric acid extraction of the ethanolic syrupy plant extract. An immediate precipitate was indicative of a positive test for alkaloids or other nitrogen compounds. A negative test does not completely exclude alkaloids.

7. Lieberman-Burchard Test (49)

A solution of a small amount of steroid in acetic anhydride (with or without added chloroform) was treated with a few drops of concentrated sulfuric acid. The production of a series of transient colors constitutes a positive test for unsaturated steroids.

8. Salkowski (49)

A small sample of material discolved in chloroform was treated with an equal volum of concentrated sulfuric acid. Various colors will appear in the upper and lower phases. The production of a series of transient colors, similar to the Lieberman-Burchard test, constitutes a positive test for unsaturated steroids.

9. Vanillin Test (50)

The sample was dissolved in 2 ml of methanol and divided into 2 tubes. To both tubes were added 3-5 drops of concentrated hydrochloric acid and to one was added 3-5 drops of a saturated solution of vanillin in ethanol. A positive reaction varies from an immediate deep cherry red coloration to one which is only slightly darker in color than the control.

10. Methanolic-Hydrochloric Acid Test (50)

A small sample dissolved in methanolic - HCl (5%) was heated to boiling for 10-15 minutes. The production of a pink to red color indicated the presence of antho-cyanidins which were produced by the oxidation of leuco-anthocyanidins in this medium.

Qualitative Test Results For The Preliminary Tan Powder #3

The results were as follows: (1) No distinctive melting point was observed. The substance began to swell around 100°C and decomposed at 175-185°C, gradually changing to a darker color. (2) It was insoluble in water, acetone, ethyl acetate, and chloroform, slightly soluble in ethanol, and freely soluble in methanol. (3) The powder gave a positive Wilson's Boric Acid Test, a green color with ferric chloride solution (9% aqueous), positive Fehling's Test for reducing sugars, pink-violet color when heated in methanolic-hydrochloric acid (5%) solution (leuco-anthocyanidins), negative Mayer's alkaloid test, positive gelatin, questionable Lieberman-Burchard Test (sterols), and positive pink-red color upon reduction with magnesium and hydrochloric acid (43, 49).

The preliminary physical and qualitative tests indicated the presence of tannins, flavan compounds, reducing sugars, and possibly sterols, while negative for alkaloids. All attempts to recrystallize the powder using methanol, methanol-water, and ethanol combinations ended in failure. The material was classified as a tannin or flavan like amorphous powder due to its positive gelatin, Wilson's, and

magnesium and hydrochloric acid tests. It was also noted that foaming occurred whenever water was added to the substance dissolved in methanol. This is not uncommon, however, when tannins or saponins are present in a plant.

Due to the small quantity of amorphous powder isolated in the above fractionation and to the presence of impurities, a more elaborate extraction procedure was devised.

Final Purification Method

The olive colored syrupy ethanol extract (See Figure 2) (147.8g) was extracted with 1.6 liters of hot anhydrous methanol, (AR) (200 ml x 8). 1.7 liters of distilled water was added to the combined methanol extracts and the mixture was extracted in a 4 liter separatory funnel with chloroform (400 ml x 2, 200 ml x 5). Addition of the distilled water enabled the separation of the two immiscible layers to occur. The dark green chloroform extracts were combined and evaporated on a steam bath leaving a residue weighing 8.0 grams. This fraction was set aside for saponification. The rust brown methanol-water phase, after extraction with chloroform, was distilled under vacuum to remove the methanol-water. A total of 0.4 liters of distilled water was added periodically during the distillation. Considerable foaming took place while removing the methanol-water. The hot mixture was filtered, allowed to stand overnight, and filtered a second time. The combined amorphous

brown precipitates filtered from the mixture was dried in an oven at 50°C. The amorphous dried powder weighed 12.1 grams and was submitted for anti-tumor screening. The powder, when tested, exhibited some anti-tumor activity. (National Institute of Health fraction number F-017.)

The brown aqueous filtrate was extracted with 2.5 liters of a n-butyl alcohol, AR (400 ml x 1, 300 ml x 1, 200 ml x 9) in a separatory funnel until the extractions were only a pale yellow color. The combined n-butyl alcohol extractions were a cherry red color, and the aqueous phase was a pale yellow. Distillation of the combined n-butyl alcohol extracts under vacuum at 45-48°C left a dark viscous substance on the bottom of the flask with a tan residue adhering to the sides.

The dark n-butyl alcohol residue was extracted in the distillation flask with hot distilled water (200 ml x 8) to give a turbid brown aqueous mixture. Not all of the residue was soluble in hot water and a gummy substance remained in the distillation flask upon completion of the aqueous extraction. The combined aqueous extracts were filtered through Pratt Dumas No. 33 filter paper and the orange-brown aqueous filtrate was set aside for a future extraction with ethyl acetate. The insoluble residue in the distillation flask and the filtered precipitate were dried, combined, and triturated thoroughly in a mortar to a brown amorphous powder. This powder was retained for characterization and also submitted to the anti-tumor

screening center. This fraction (NIH fraction number F-021) was found to actively inhibit tumor growth.

The aqueous filtrate was extracted with ethyl acetate (500 ml x 1, 300 ml x 5) until the extracts were practically colorless. The combined organic ethyl acetate extracts were anorange brown and the aqueous layer pale yellow. The ethyl acetate was removed under vacuum leaving a tan residue in the flask weighing 3.70 g.

The isolation of the ethyl acetate tan residue, given in the procedure above, was first obtained on a smaller scale by purifying only 23.2 grams of the ethanol extract. An attempt was made to hydrolyze the ethyl acetate residue with sulfuric acid which would have split any plant glycosides into the respective sugar and aglycone components. However, the solution turned a deep red color upon hydrolysis and yielded a red insoluble material which was probably a phlobatannin brought about by condensation of catechol tannins, catechins or leuco-anthocyanidins. This result led to the second large purification procedure as enumerated above and the subsequent countercurrent distribution step. See Figure 4 for the final purification scheme of the ethanol soluble plant extract.

Qualitative Test Results of the Final Purification Fractions

During the general extraction of the plant (Figure 2) and the final purification procedure adapted for the ethanol extract, certain qualitative chemical tests were used to follow the flavan compounds or tannin constituents. The tests chosen for this

characterization were Wilson's Boric Acid Test, magnesium and hydrochloric acid, ferric chloride solution, Fehlings, and the gelatin test.

Fraction 1 (ethanol extract) was positive for both tannins and flavan compounds. The aqueous phase, after the n-butyl alcohol and ethyl acetate extractions, (fractions 4 and 6) gave in both cases negative tests for tannins and flavan compounds. The qualitative chemical tests (Table 2) indicate that the tannin and flavan compound components were carried through the purification procedure. This was shown by the positive tests fractions 5 and 7 gave with Wilson's Boric Acid, magnesium and hydrochloric acid and 1% gelatin.

Both water insoluble brown powders (See pp. 20-21), that inhibited tumor-growth, gave positive Wilson's Boric Acid tests, and negative flavonol tests, similar to fractions 5 and 7 (Table 2). The ferric chloride test produced an olive green color in both cases. The vanillin tests were positive for both powders, producing pink to red colors immediately, compared to brown standards. These test results are not listed in Table 2.

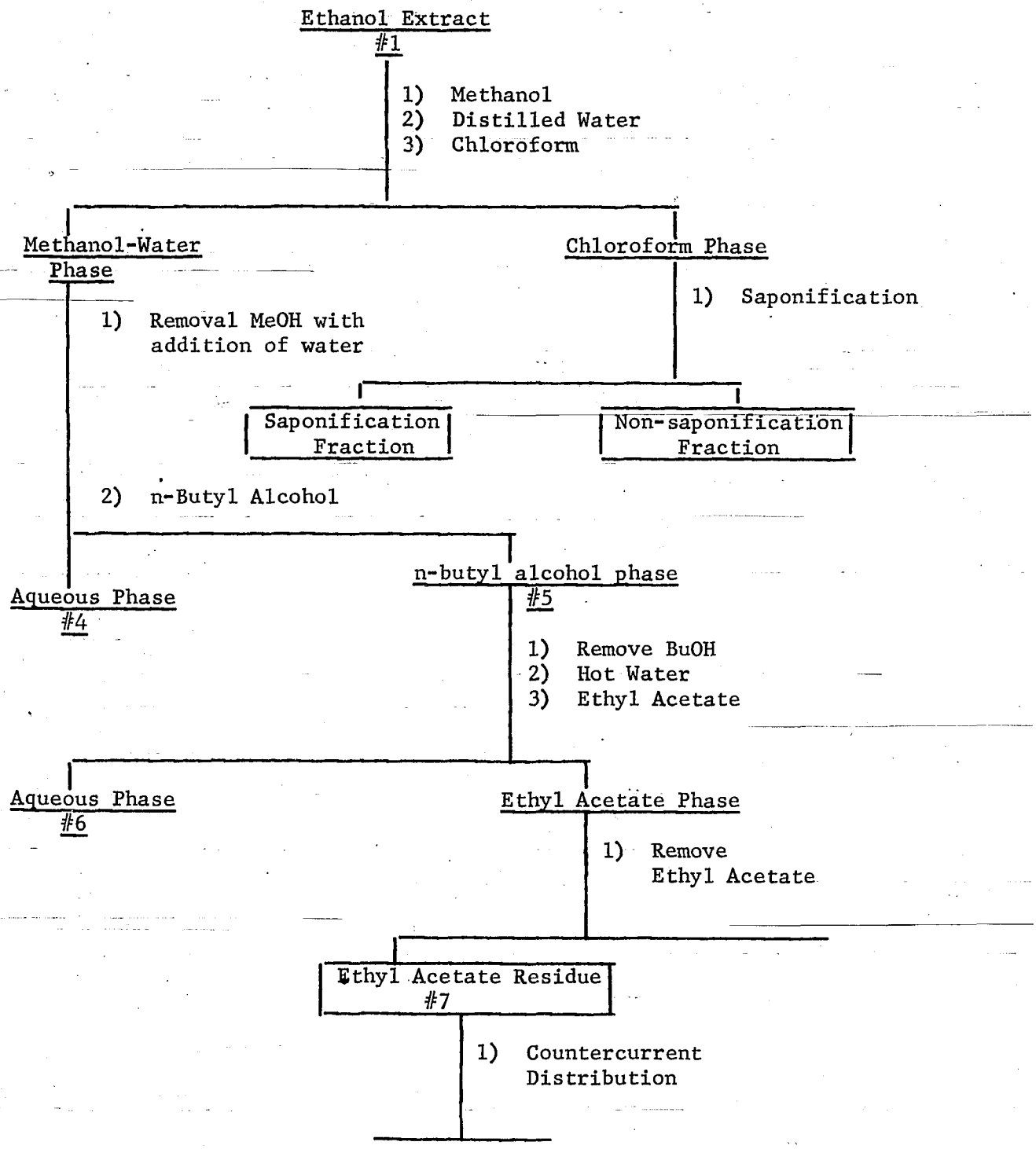


Figure 4. Final Fractionation Procedure of the Ethanol Extract.

TABLE II

QUALITATIVE CHEMICAL TESTS OF THE SAMPLES OBTAINED DURING THE FRACTIONATION OF THE ETHANOL EXTRACT

Fraction Number [a]	Wilson Boric Acid Test	Flavonol Test	Ferric Chloride	Fehlings	Gelatin
1	b	+	+ (O)	+	+
2	b	-	-	b	b
3 [c]					
4	b	-	+	+	-
5	+ (Y)	+ (PO)	+ (O)	d	+
6	b	d	+ (O)	+	-
7	+ (Y)	+	+ (G)	d	+

a. Refer to Figures 2 and 4 for the fraction identification.
b. Designates that a test was not run.
c. Fraction number 3 refers to the tan powder isolated in the preliminary purification and the chemical tests for this fraction are given on page 19.
d. Questionable.

Color Code:
(Y) Yellow (O) Olive (PO) Pale Orange (G) Green

(+) Positive Test (-) Negative Test

Saponification Procedure

Saponification of the Petroleum Ether Extract

Prior to saponification of the petroleum ether extract, a preliminary Lieberman-Burchard (49) test for sterols was performed on the ethanolic extract #1 (Figure 2, Page 13), according to the method of Wall (45, 51), by dissolving a portion of the extract in chloroform, and treating the mixture with acetic anhydride and sulfuric acid. The results of this test were an immediate moss green color in the top layer and a yellow bottom phase compared to a cholesterol, U.S.P. standard, which turned blue first, then moss green. This reaction could be from carotenes, xanthopylls, or sterols.

The test was then performed on the petroleum ether extract of the plant. It also gave a questionable test similar to the results obtained from the ethanol extract. Due to the unreliability of one test, a second sterol test (Salkowski) (49) was performed on the petroleum ether fraction. The Salkowski test consisted of dissolving the material in chloroform and shaking the solution with concentrated sulfuric acid. The color turned yellow-green immediately upon addition of the acid, then the top layer turned blue green and the bottom layer yellow green.

The Lieberman-Burchard and Salkowski chemical tests for sterols were both inconclusive for the petroleum ether extract. However, due to the frequent occurrence in the acacia species of sterols and terpenes (4, 35, 36, 37, 38, 39, 40), the petroleum ether fraction was subjected to a saponification procedure to isolate any possible sterols or terpenes present.

The saponification procedure followed was one used by Nicholas (52) for determining the sterol and triterpene content of Ocimum basilicum and Salvia officinalis at various stages of growth. The petroleum ether extract (7.67 grams) was dissolved in 1500 ml of 10% alcoholic potassium hydroxide, refluxed for one hour, and acidified with concentrated hydrochloric acid. The mixture, upon acidification, became turbid and a gelatinous, and a yellow precipitate was observed. The entire yellow mixture was mixed in a 4 liter separatory funnel with 1250 ml of ethyl ether, 150 ml of petroleum ether, and 500 ml of distilled water. The aqueous layer was discarded after the two layers had separated and the organic layer was extracted with 5% sodium bicarbonate (250 ml x 4). The aqueous sodium bicarbonate layer was discarded, and the organic layer was extracted with 1% potassium hydroxide (250 ml x 4). The orange-brown organic layer was washed with distilled water and allowed to evaporate spontaneously in a hood for 72 hours. The yield was 3.20 grams. The brown aqueous potassium hydroxide extracts were combined and neutralized with concentrated hydrochloric acid (solution became yellow), and

extracted with ethyl ether (200 ml x 6). The yellow-brown ether soluble extracts were combined and evaporated spontaneously in a hood overnight leaving 1.05 grams of a pasty substance. This saponifiable residue normally would contain the terpene materials of the plant. Both of these residues were dried and submitted to the National Institute of Health for anti-tumor testing. The results were negative.

The non-saponified residue (sterol fraction) was subjected to the Lieberman-Burchard and Salkowski tests for sterols. The Lieberman-Burchard test gave an immediate moss green color compared to an initial pale blue, slowly darkening to a blue-green color with a beta-sitosterol standard; the Salkowski test produced an immediate dark brown color in both layers, compared to an initial orange color, slowly changing to an orange upper layer, and red lower layer with beta-sitosterol.

The tests for sterols were of a questionable nature and did not give the transient colors of an unsaturated sterol. The moss green color could be indicative of other unsaturated entities commonly found in plants such as carotenes or xanthophylls. Further test results would have to be obtained to confirm the presence of sterols. Due to the inconclusive nature of these saponification residues, and the fact that the anti-tumor activity resides in the ethanol extracts of the plant, further investigation was deemed unnecessary. The fractions were stored in a freezer at -10°C for an indefinite period.

Saponification of Chloroform Extract

In order to thoroughly search the plant for the presence of sterols, the petroleum ether fraction was subjected to a saponification procedure as mentioned above. For a complete screening of the plant for these constituents, it was also necessary to examine the chloroform extraction of the methanol-water layer (see Figure 3, Page 15). The chloroform extracts obtained in the purification of the ethanol extract of the plant were combined and evaporated on a steam bath. The procedure of Nicholas (52) was followed again and the dark residue was taken up in 100 ml of 10% potassium hydroxide and refluxed for one hour. The same procedure was followed as before except 1500 ml of ethyl ether instead of 1250 ml was used. The olive colored potassium hydroxide saponified layer weighed 3.20 grams and the non-saponified orange fraction 3.40 grams. Samples of both fractions were dried and submitted for anti-tumor screening. Negative results were obtained.

The non-saponified residue gave questionable results when tested for sterols. Lieberman-Burchard test gave an immediate moss green color, while the Salkowski test produced a dark brown color in both layers. These fractions were placed in a freezer at -10°C and saved for future investigation as were the petroleum ether saponification fractions.

Countercurrent Distribution of The Purified Ethanol Extract

The Craig countercurrent instrument used in this investigation was a 100 stage model manufactured by H. O. Post Scientific Instrument Company, Inc. The machine was completely automatic and was set for a predetermined number of rotations for thorough mixing, number of cycles desired, length of settling period of the two phases, and emptying of the upper phases into a fraction collector. The lower phase capacity was 100 ml and the upper phase was 100, 50, or 25 ml. The upper phase was either removed or recycled after 100 transfers, thereby making it possible to obtain any number of transfers desirable. See Figure 5.

The countercurrent method has recently become a useful tool for separating and resolving intractable complex plant fractions into its different components. Craig, a pioneer in the field of countercurrent distribution, has two reviews (53, 54) on the applications, and solvent systems used for countercurrent procedures. The application of countercurrent procedures, specifically, to plant analysis, has been covered in a review by Horhammer (55). A few of the plant products which have been purified by a countercurrent procedure in the past are tannins (56, 57), condensed tannins and leuco-anthocyanidins (58, 59), flavonoids (20, 60, 61), and alkaloids (62, 63).

One of the most variable factors of a countercurrent procedure is the solvent system employed. Craig (53) summarized and compiled tables of the many solvent systems used for different classes of organic and inorganic compounds. However, the choice of a suitable solvent system is usually made on an experimental basis with the following factors considered: (1) availability of solvents, (2) ease of purification, (3) adequate solubility of the solutes in the solvent system, (4) boiling points of the solvents in a range which permits easy isolation of solute, and (5) relatively constant phase composition during several hundred transfers.

Many different solvents have been used. For example, a group from Ciba Pharmaceutical Laboratories (62) used a chloroform-menthanol-water system for separating an oleoresin into two components, while Svoboda (63) used a chloroform-water-phosphate buffered system to determine the quantitative amounts of alkaloids in commercial Veratrine. Roux and Paulus (58, 59) applied a countercurrent procedure to amorphous wattle wood tannins (Acacia mearnsii) and found a butan-2-ol-water-petroleum ether solvent system, followed by adsorptive separation on cellulose, was effective for separating and isolating related flavan compounds, and tannins of different molecular weights. Clark-Lewis and Mortimer (20) used a solvent system of ethyl acetate-water which separated isomelacacidin (XI) from melacacidin leuco-anthocyanidin (VI). Horhammer and Wagner (60)

made a study of many different solvent systems for the separation of flavones and their aglycones. They came to the conclusion that the following four solvent systems were all effective for separation: (1) ethyl acetate-n-butanol-water in varying concentrations; (2) ethyl acetate-water or ethyl acetate-phosphate buffer (1-1.5:1); (3) ethyl acetate-ether-water (1:5:5); and (4) ethyl acetate-n-heptane-methanol-water (3:12:4:6).

A solvent system of ethyl acetate-methanol-water (6:1:5) was chosen for the purpose of purifying the complex ethyl acetate residue (3.7g) (Figure 4) isolated from the ethanol extract of Acacia angustissima. The factors previously enumerated which influenced this choice of solvent system were 1, 3 and 4. It was first attempted to use ethyl acetate-water alone, but the residue was only soluble in the lower phase whenever a small volume of methanol was present. The partition coefficient was 3.0. It was felt that this particular solvent system would give an effective separation, as similar systems had been employed effectively, in the past, for flavan compounds.

The upper and lower phases were mixed and saturated with respect to one another in six liter separatory funnels. The two phases were then allowed to stand for twenty-four hours for complete saturation. The lower phase was removed and added manually to the Craig tubes through small attachable funnels. The upper

phase solvent was added (10.0 liters) to the large tank, which supplied 100 ml aliquots to tube 0 before each cycle. Additional upper phase system was added during the distribution. A small amount of the lower phase solvent was added to the auxilliary tank to replenish the small amounts which might be extracted into the upper phase during the distribution.

The ethyl acetate residue was dissolved in the lower phase of the first six tubes, and the settling rate was determined by trial and error of the first few cycles to be fifteen minutes. The number of rotations was set at twenty. The lead tube tended to form a slight emulsion and took the longest to separate, but this was remedied by a fifteen minute settling time. However, the time required for the two phases to separate out was set for forty-two minutes per cycle to prevent any unforseen complications overnight. The next morning after sixty-five cycles had been completed, the lead upper phase had diminished in volume and had to be replenished by more of the upper phase solvent. This difficulty of phase composition could possibly be corrected by using a solvent system containing n-heptane in addition to ethyl acetate-methanol-water combination, or by purifying a smaller sample of plant material with the instrument.

When the leading upper phase reached tube 99, the automatic fraction collector was set for one upper phase per collection tube and the upper phases were withdrawn. At this point, there were two

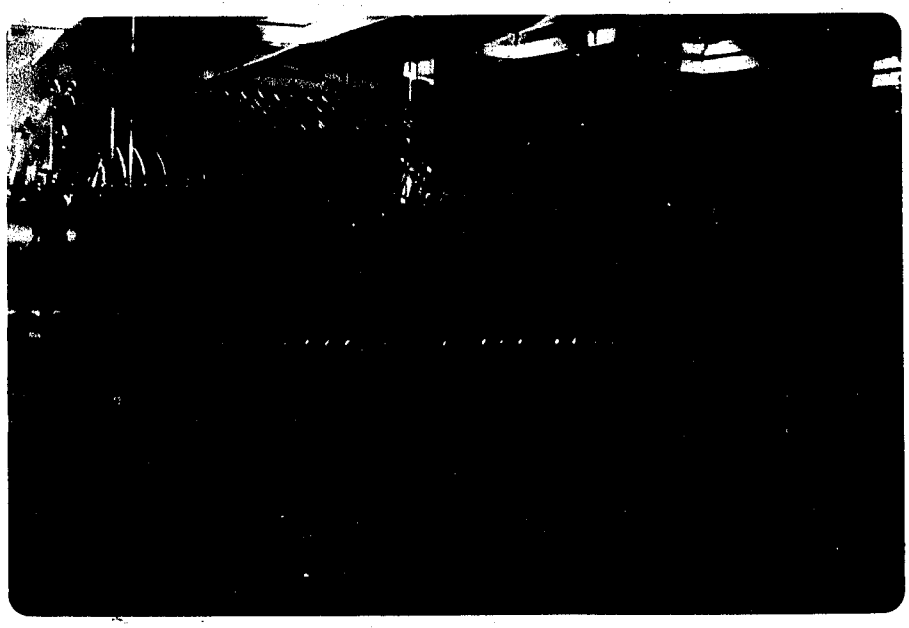


Figure 5. 100 Stage Craig Countercurrent Instrument.

distinct colored fractions visible. One was present in the lower phase from tubes 2-10. The other was in the upper phase, tubes 0-25. Both were a brown color with the upper phase band of the greatest intensity. The collection was continued until one-hundred upper phase layers had been collected in the corresponding numbered tube in the fraction collector. The upper phase layers were then transferred into four ounce bottles numbered from 0-99 inclusive. The lower phase layers were then transferred into four ounce bottles also by physically rotating the machine to the proper position to drain the contents of the tube, and numbered from 0-99.

Spectrophotometric Analysis of The Countercurrent Upper Phase Tubes

The use of ultraviolet absorption for determining the presence of different substances and their location after a countercurrent distribution, was used by Craig (64) in 1947, and Titus, et al (65) in 1948. The latter group were able to locate degradative products from the urine of patients given an anti-malarial drug, after a 24 stage countercurrent procedure, by plotting the absorbancy in the ultraviolet versus the tube number. Other methods that have been used for analyzing countercurrent bands are titration and direct weight.

Flavonols and flavones generally absorb at approximately 240, 310 and 360 millimicrons respectively, while leuco-anthocyanidins and condensed tannins around 275. This will vary according to the number of hydroxyl groups and whether the glycoside or aglycone is

present. Due to the solvent system containing ethyl acetate, the lowest wavelength possible, without interference in the ultraviolet region was 260 millimicrons. The wavelengths chosen for analyzing the upper phase were therefore 260, 300, and 360 millimicrons. By measuring the absorbancy of each tube at the given wavelength, and plotting the optical density versus the tube number examined, it was possible to determine the number of components and which tubes to combine to isolate the respective bands.

The absorbancy or optical density of each upper phase was measured on a Beckman Model DU Spectrophotometer with a photomultiplier attachment, and a hydrogen lamp as the light source. Silica cuvettes were used with three unknowns and a standard of the upper phase solvent run simultaneously. After each reading, the cuvettes were washed with upper or lower phase solvent system before adding another sample.

Figure 6 shows the results of plotting the absorbancy versus the tube or phase number. Due to the high concentration of material in tubes 1-6, the optical density was greater than 3.0 and was not obtained. Tubes 17-20 show a small inflection at both 260 and 300 millimicrons. Tubes 60-62 also show a component at all three wavelengths with the greatest absorption at 260 millimicrons. Tubes 17-20 were combined and labeled fraction A; tubes 60-62 fraction B; tubes 0-16, 21-30 which contained the major portion of the separated material called fraction C; and the remaining tubes 31-59, 63-99 were

called fraction D. (See Figure 7 for a summary of the countercurrent distribution.)

In order to obtain some qualitative characteristics of these fractions, ultraviolet absorption curves were obtained for tubes 10, 19 and 61, which represented a sample from each respective fraction. (See Figure 8 for the absorption spectra of the three upper phase bands.)

The upper phase tube (tube 10) used to determine the spectrum of fraction C was too concentrated and absorbed above 3.0 from 260 to 305 millimicrons. The tube should have been diluted, and rerun to obtain a proper curve, if this curve was going to be used for diagnostic purposes; however, the spectrum was obtained, primarily, to acquire some idea of the degree of separation produced by the countercurrent procedure, relative to one another. Consequently fractions A and C, which both had some absorption above 3.0, were not diluted and rerun.

Fraction B appeared to be a purer band, or due to its dilute concentration, gave a better resolution than fractions A and C. Fraction C was practically a straight line which indicated that the fraction probably contained more than one component. Fraction A resembled the curve of fraction B and appeared to be a purer band than fraction C also. The curves also had a small broad absorption at 340 millimicrons, which may be indicative of flavones or flavonols mixed with the fractions. Fraction B had

a good peak at 280 millimicrons, which is the general area of absorption for catechins (66), dihydroflavonols, and leuco-anthocyanidins (67), condensed tannins (68), and hydrolyzable tannins (69).

It was concluded from the ultraviolet absorption spectra that tubes 19 and 61 representing fractions A and B were relatively pure and very similar in nature. Tube 10 representing fraction C appeared impure in relation to A and B.

Spectrophotometric Analysis of The Countercurrent Lower Phase Tubes

The lower phase tubes were analyzed using the same procedure as was used for the upper phases except that 275, instead of the 260 millimicrons was used. It was believed that a better resolution of the bands would be obtained at the 275 wavelength if the structures were similar to the upper phase components, which absorbed strongly at 280 millimicrons.

Figure 9 shows that two components were present in the lower phase distribution. The first one was visible, but the second one was not detected except through the ultraviolet analysis. The first band, tubes 0-20, was labeled fraction E; the second band, tubes 60-99, fraction F, and the third, which included tubes 21-59, fraction G.

Ultraviolet absorption curves were obtained (Figure 10) for tubes 7 and 82, representing fractions E and F respectively. Fraction E curve closely resembles fractions A and B curves. It has a

maximum absorption at about 278 millimicrons. Fraction F shows a broad absorption band at $\sim$350 and around 270 millimicrons. This curve does not resemble any of the other fractions, but more closely resembles a flavonol or flavone spectra (70). Figure 7 summarizes the countercurrent distribution and isolation of seven fractions.

Distillation of the Upper and Lower Phase Solvents and Isolation of the Respective Bands

Fraction A

Fraction A, tubes 17-20 (400 ml), was placed in a 500 ml, 3-necked round bottom flask, and distilled under reduced pressure with a Cenco Hyvac vacuum pump. The color of the combined phases before distillation was a yellow-orange, but after removal of the solvent at 23-35°C, a brown pungent residue remained. The residue was extracted with 45 ml acetone and the extract filtered. Only a small quantity of residue was insoluble in acetone. It was then evaporated on a steam bath leaving a caramel colored residue that was again extracted with acetone, filtered, and evaporated. The second time the residue was more homogenous in nature. The sample was dried in an oven for several hours at 50°C. and saved for further analysis. The dried sample weighed 0.123 grams.

Fraction B

Fraction B, tubes 60-62 (300 ml), was concentrated under vacuum using the same procedure as given for fraction A. The band prior to distillation was a pale yellow and upon concentration

gave a yellow residue with visible brown particles dispersed throughout it. The residue was extracted with 50 ml of acetone, filtered, and evaporated on a steam bath which gave a pale yellow residue. The sample was dried in an oven at 50°C for several hours and placed in a vacuum dessicator. The residue weighed 0.037 grams. Future testing with this fraction was very limited due to the lack of material.

Fraction C

Fraction C, tubes 0-16, 21-30 (2.7 liters), was concentrated under vacuum as previously described. This solution was a brown-orange color and contained the largest quantity of material of all the fractions. Due to the large volume of upper phase being distilled, a considerable amount of water was present from the countercurrent distribution, which required a slightly higher temperature (35-40°C) to remove the solvent under vacuum. The distillation was stopped when approximately 10 ml remained. A brown residue was adhering to the sides of the flask at this stage. The aqueous residue was extracted with 125 ml acetone, filtered and dehydrated with 10.0 g of anhydrous sodium sulfate. Fraction C was completely soluble in acetone. The dehydrated acetone solution was evaporated on a steam bath leaving a brown residue, which was dried in an oven at 50°C for twelve hours. The dried residue weighed 1.574 grams. It was stored in a vacuum dessicator for future testing. 0.75 grams was

submitted to the National Institute of Health for anti-tumor screening. The results were inconclusive due to a lack of material preventing the proper dosage adjustment in the animals.

Fraction D

Fraction D, tubes 31-59, 63-99 (6.6 liters), which consists of the balance of the upper phase fractions, was concentrated under vacuum as previously described. The solution was a pale yellow color and after distillation, a brown resinous appearing substance remained on the bottom of the flask in about 10 ml of aqueous solvent similar to fraction C. The aqueous residue was extracted with 50 ml of acetone, filtered, and evaporated on a steam bath which left a rust-brown residue and a yellow oily substance that separated out from the residue. The oil would not solidify on cooling. The oil and residue dried in an oven at 50°C for several hours. The dried residue and oil weighed 0.472 grams.

Fraction D containing the yellow oil was next extracted with petroleum ether (15 ml x 3) to remove the oil if possible. The extracts were yellow and upon evaporation left a yellow oily substance. Due to the presence of this yellow oil in fraction D, it was also suspected that fractions A, B and C would also contain this substance. They were extracted in a similar manner with petroleum ether until the petroleum ether extracts were colorless. All the petroleum ether extracts were combined and evaporated on a

steam bath leaving behind a yellow oil as had fraction D. Fraction D yellow oil was combined with the other fractions, labeled fraction O, and stored in a vacuum dessicator for future testing. The substance weighed 0.075 grams. After several weeks, the substance (fraction O) changed to a dark brown color.

Fraction E

Fraction E, lower phase tubes 0-20 (2.1 liters), was concentrated under vacuum as previously described for the upper phase bands. The distillation of the yellow-brown methanol-water solution proceeded at a much slower rate than did the ethyl acetate. The distillation range varied between 35-40°C. The solvent removal left a dark brown residue in the flask which was extracted (130 ml x 2) with a polar solvent mixture (50 ml ethanol, USP, 50 ml methanol, 30 ml water), filtered, and concentrated under vacuum leaving a brown residue with a pungent odor. The substance that was insoluble in the polar solvent extractions was discarded. The fraction E residue was dried in an oven at 50°C for three hours leaving a residue weighing 0.854 grams. This residue was stored in a vacuum dessicator.

Fraction F

Fraction F, lower phase tubes 60-99 (4.0 liters), was concentrated under vacuum as previously described. This band was practically colorless until it had been concentrated to about two

liters, when it became a yellow-orange color. The distillation was continued until about 5 ml of solvent remained in the flask. There was a small olive colored residue deposited on the sides of the flask and in the orange colored solvent. The mixture, after standing overnight, was layered with 150 ml of methanol, heated on a steam bath and filtered. The filtrate was yellow and the filter paper contained a dark brown substance, insoluble in methanol. The yellow filtrate was concentrated under vacuum leaving a brown-yellow residue with a slight acetic acid odor. It was dried in an oven at 50°C for twelve hours and weighed 0.174 grams. The residue was stored in a vacuum dessicator.

Fraction G

Fraction G, tubes 21-59 (3.9 liters), which consisted of the balance of the lower phase fractions, was concentrated to dryness under vacuum as previously described. It was dried in an oven and weighed 0.2245 grams.

Preliminary Characterization of The Countercurrent Bands

The following color tests were run on the fractions isolated from the Craig countercurrent distribution: Wilson's Boric acid test, magnesium and hydrochloric acid, and ferric chloride test. The procedures for these tests have been discussed previously. See Table 3 for a summary of the results.

Qualitative Chemical Test Results

The Wilson boric acid test was run on fractions A, B, C and D. The test was not run on fractions E, F, and G due to their lack of solubility in acetone. Fractions B and D were both highly positive. They were a bright green-yellow color compared to the almost colorless standard, and resembled closely the color intensity of the kaempferol and quercetin authentic samples. Fractions A and C were both positive, but due to the deeper color of the standard, the differential was not as great as fractions B and D. A and C had a deeper yellow color than the standard.

The reduction reaction using magnesium ribbon and 10% hydrochloric acid with the material dissolved in either methanol-water or ethanol-water combinations gave a positive test for fraction F only. The color change noted was from a yellow to orange color. This test is positive if a color is produced ranging from orange to red. A positive test here indicated a possible flavonol, flavone, or similar flavonoid compound.

The ferric chloride test was positive for all fractions except E and G, which were questionable. A positive test indicated the presence of free phenolic groups.

The positive Wilson's Boric Acid and negative magnesium and hydrochloric acid tests for fractions A, B, C and D indicated that some structure was present other than a flavonol (I), flavone (XVIII), or dihydroflavonol (XIX), which all give a positive test

with magnesium and hydrochloric acid.

Wilson (43) stated that the test was positive for chalcones and all flavones and flavonols with a 5-hydroxy OH group, and negative for flavanones (II) tannic acid, comarin, comarinic acid, sucrose, dextrose, salycylic acid and phloroglucinol. Wolfram et al (44) enumerated the limitations of the Wilson Boric Acid Test and listed the systems (XX, XXI) as necessary for a positive test. According to their group, methylation of position (a) of both systems destroyed the color reaction with Wilson's Boric Acid. He extended the test to cover also isoflavones with a free hydroxy OH group at position (a) which also give a positive test.

The Wilson test was run on fisetin (III), which does not have the 5-hydroxy OH group and according to Wilson, should not give a positive test. However, a positive test was obtained for this commercial grade of fisetin, contrary to Wilson's results. The affirmative result obtained for fisetin cannot be explained if a 5-hydroxy OH group is a prerequisite for a positive test. The test was rerun several times to verify the first positive reaction, and in all cases, the result was positive. The commercial fisetin was investigated by paper chromatography in BAW and 5% acetic acid solvent systems to determine, if possible, the state of purity. Two spots were observed in visible light, and three under ultraviolet light with BAW. This established the impurity of the commercial grade of fisetin and would disqualify the positive Wilson Boric Acid test. Positive results were also

obtained for quercetin (V), dihydroquercetin (XXII) and leucocyanidin (XV) (both synthesized from quercetin), and kaempferol (I).

Fractions A, B, C, and D would no doubt give a positive Wilson's Boric Acid Test if they were leuco-anthocyanidins, regardless of whether there was a 5-hydroxy OH group (VI) present or not. If the leuco-anthocyanidin present contained a 3, 4 diol system, a complex would perhaps be formed with boric acid, which could give the color change of a positive test. If the leuco-anthocyanidin had a 5-hydroxy OH group, however, it would possibly fit into Wolfram's system shown by comparing leucocyanidin (XV) to Structure (XXI).

A study of the Wilson's Boric Acid test on different optical isomers of leuco-anthocyanidins (eight optically active forms possible) and condensed tannins would be informative as to the application of the test for this class of compounds, and the necessary structure for a positive test. Other compounds which would prove interesting for a future study, to compare with the above results by testing with Wilson's Boric Acid, would be 1-leucomaclurin glycol ether (XXIII) from Acacia catechu (32); and maclurin (XXIV) found in A. catechu, A. catechuoides, and A. sundra (31).

The Wilson Boric Acid Test does not appear very selective for a particular flavan structure and does not differentiate compounds in this class, but it does appear to give a qualitative test, except for catechin and flavanone structures, for the class of flavan compounds.

TABLE 3

PRELIMINARY QUALITATIVE EXAMINATION
OF THE COUNTERCURRENT FRACTIONS

Fraction	Wilson's Test	Magnesium, Hydrochloric Acid (a)	$FeCL_3$
A	+ (Y)	- (YO to PO)	+ (OL)
B	+ (YG)	(b)	(b)
C	+ (Y)	- (YO to PO)	+ (DB)
D	+ (YG)	- (YO to PO)	+ (DB)
E	(c)	- (PY to C)	(d)
F	(c)	+ (Y to O)	+ (OL)
G	(c)	-	(d)
fisetin	+ (YG)	+ (Y to O)	+ (OL)
quercetin	+ (YG)	+ (Y to R)	(b)
dihydro-quercetin	+ (YG)	+ (Y to R)	+ (B)
leuco-cyanidin	+ (YG)	- (slight O)	+ (OL)
D - Catechin	-	(b)	(b)
kaempferol	+ (YG)	(b)	(b)

(a) The colors are given before and after the tests were obtained.
(b) Test was not run.
(c) The material was not soluble in acetone.
(d) Questionable.

Color Code:

Y - Yellow	OL - Olive
G - Green	O - Orange
PY - Pale Yellow	C - Colorless
PO - Pale Orange	DB - Dark Brown
	R - Red

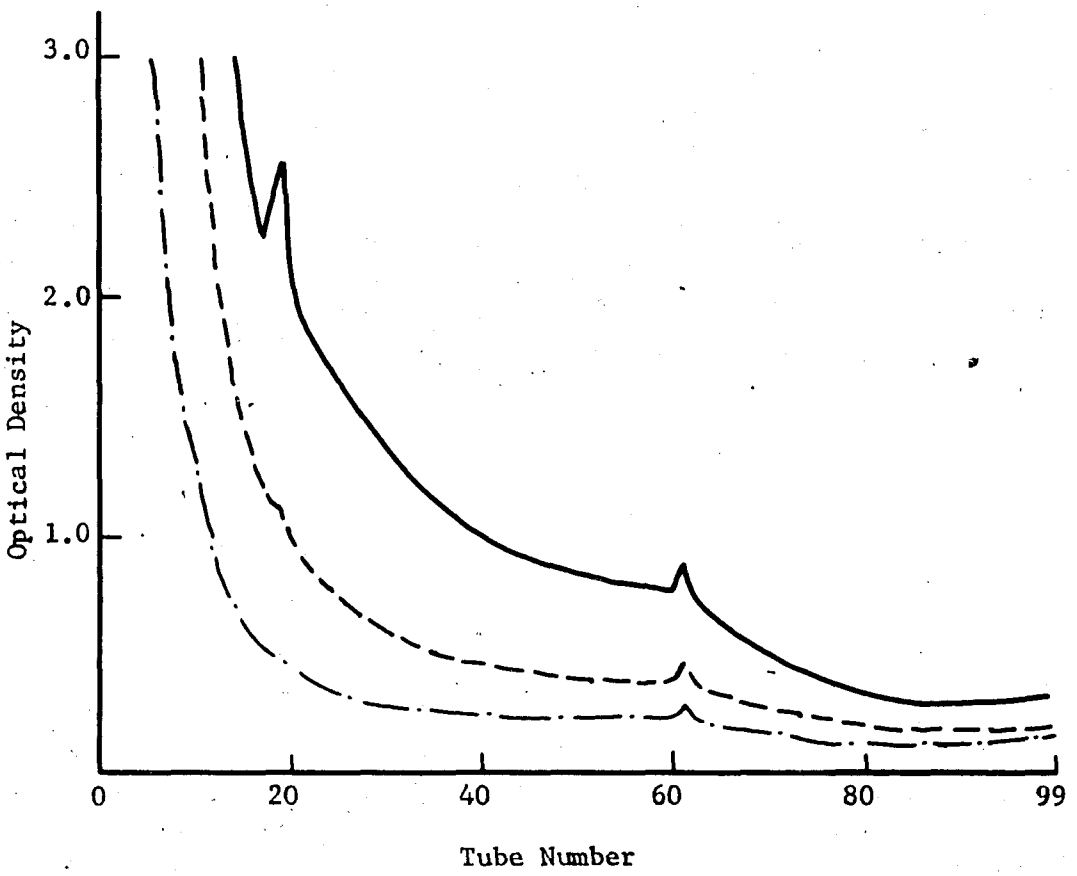


Figure 6. Ultraviolet Absorption of the Upper Phase Countercurrent Tubes at 260, 300, and 360 Millimicrons.
(——) 260 Millimicrons. (---) 300 Millimicrons.
(-.-) 360 Millimicrons.

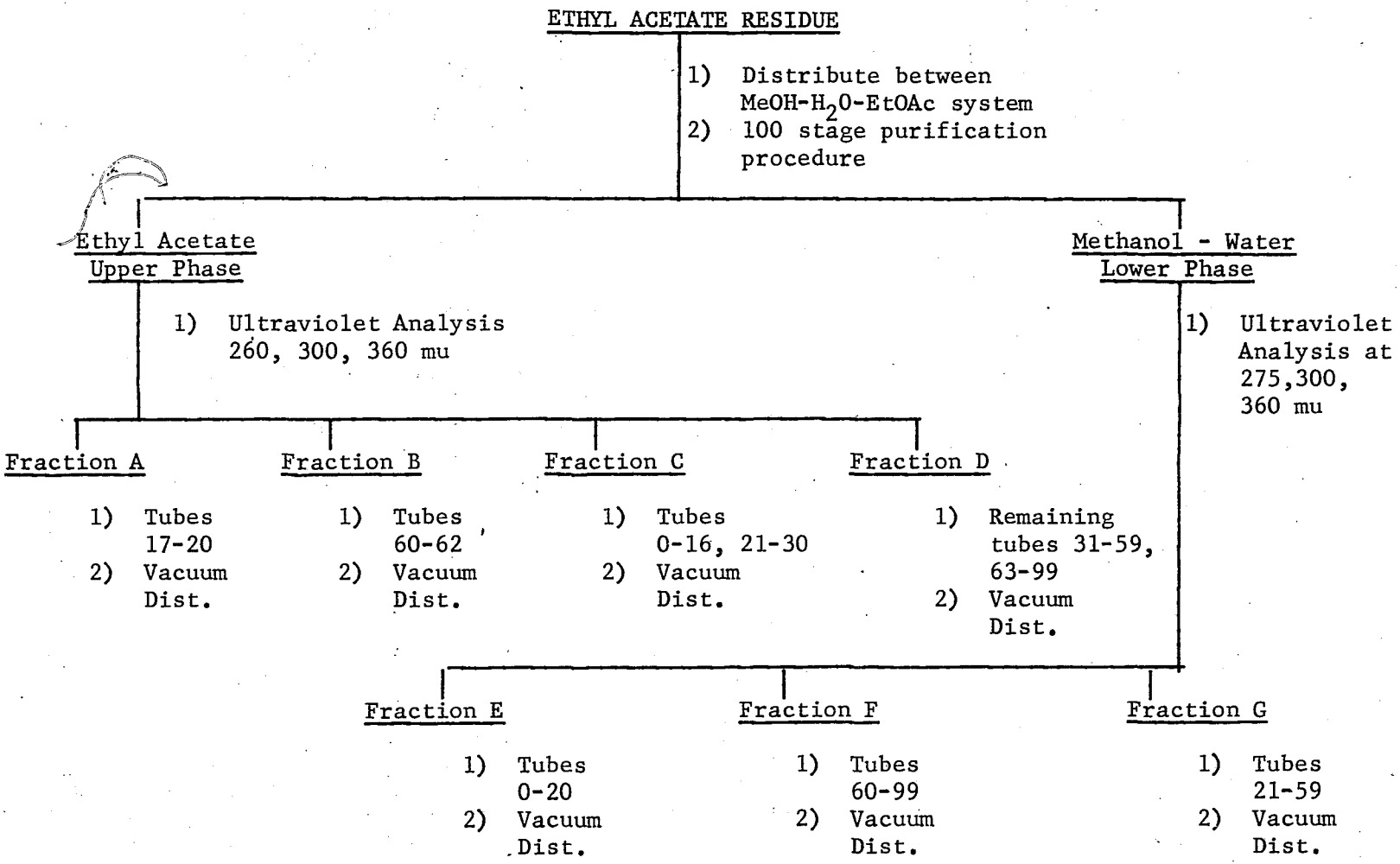


Figure 7. 100 stage Craig Countercurrent Purification Procedure. Solvent system: Methanol-Water-Ethyl Acetate (1:5:6).

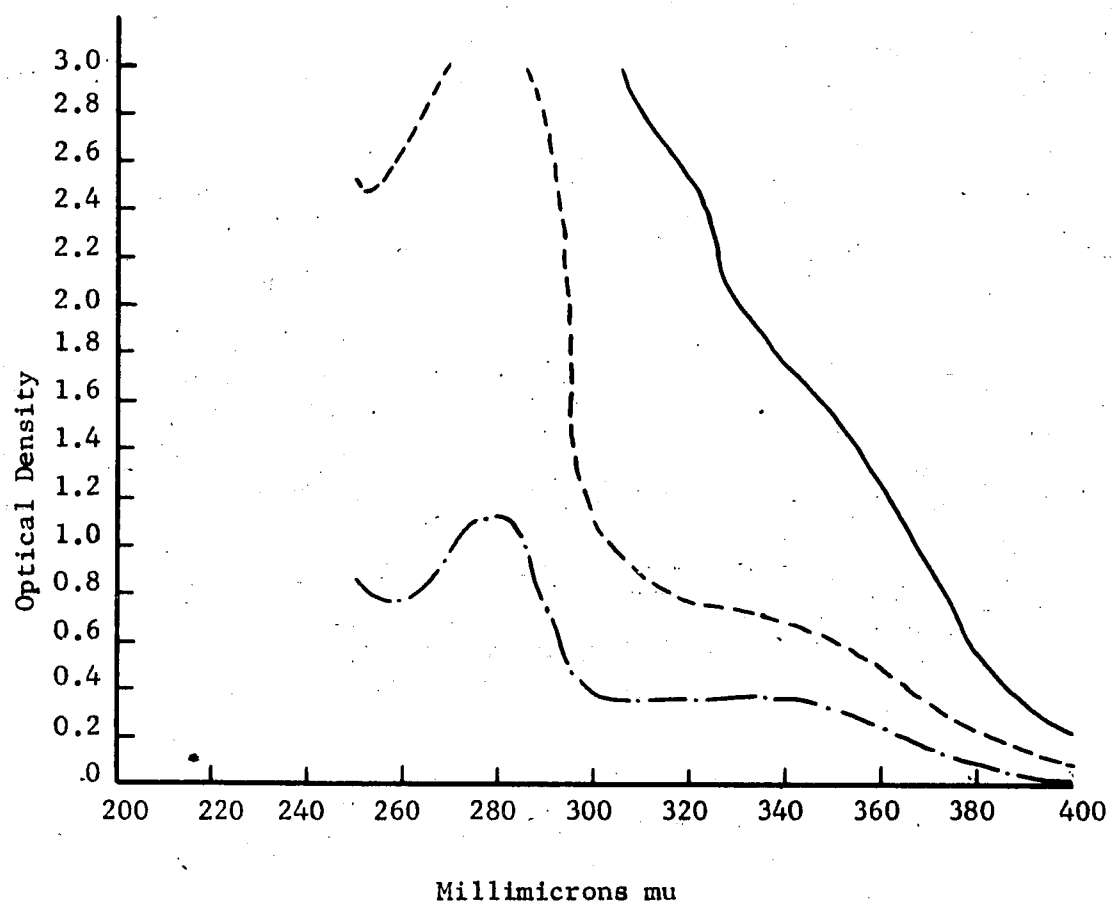


Figure 8. Ultraviolet Spectra of Tubes 10, 19, and 61 of the Upper Phase. Tubes 10 (——), 19 (---) and 61 (-.-)

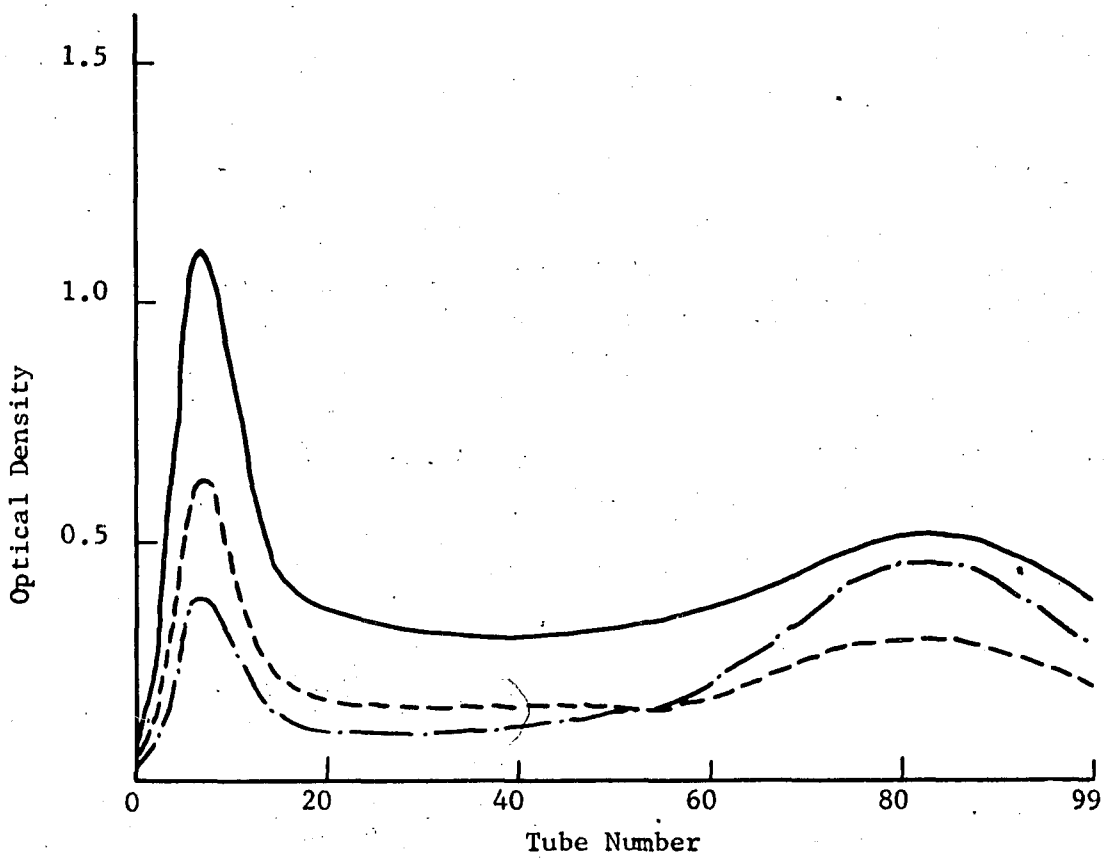


Figure 9. Ultraviolet Absorption of the Lower Phase Countercurrent Tubes at 275, 300, and 360 Millimicrons.
(——) 275 Millimicrons. (---) 300 Millimicrons
(-.-) 360 Millimicrons.

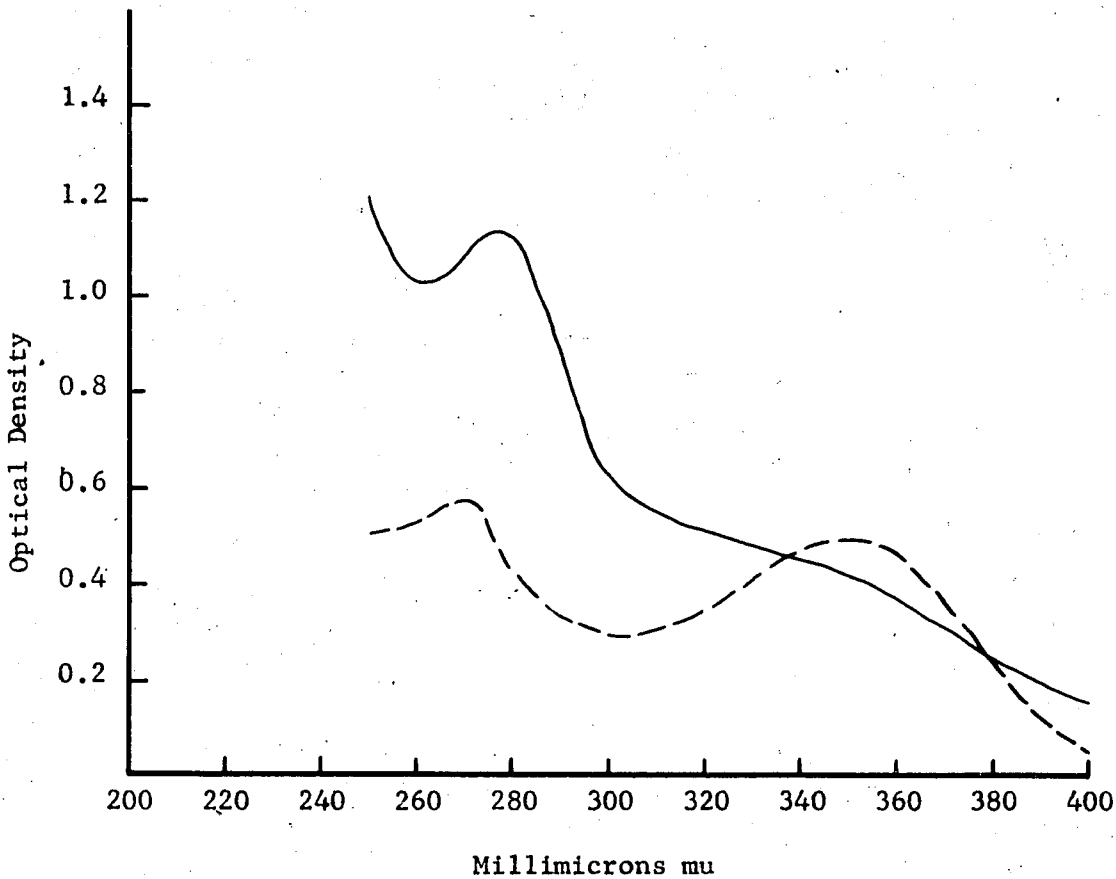


Figure 10. Ultraviolet Spectra of Tubes 7 and 82 of the Lower Phase. Tubes 7 (—) and 82 (---)

Countercurrent Distribution of Fraction F

The solvent system proposed by Horhammer and Wagner (60) for the distribution of flavones was used for the purification of fraction F obtained from the countercurrent distribution. This solvent system consisted of three of the solvents used in the first distribution, with the addition of n-heptane. The system consisted of ethyl acetate-n-heptane-methanol-water in the concentration ratio 3:12:4:6 respectively.

The solvents were mixed in 6-liter separatory funnel (ethyl acetate 900 ml, n-heptane 3600 ml, methanol 1200 ml, distilled water 1800 ml) and allowed to become thoroughly saturated for 24 hours. Tubes 3-25 were filled with 100 ml each of lower phase solvent. Fraction F (0.118 grams) was dissolved in 200 ml of the lower phase solvent mixture, stoppered, and allowed to stand overnight. In the morning a brown substance had deposited in the bottom of the flask. The mixture was filtered, and the yellow colored filtrate was placed in tubes 1 and 2 of the instrument. The filter paper with the fine precipitate was saved for further testing. Tube 0 was filled with lower phase solvent and served as a blank to keep the upper phase saturated with respect to the lower phase. A 25-stage distribution of the fraction was made. A slight emulsion tended to form in the lead upper phase, thereby making it necessary to lengthen the settling time from 18 minutes to 45 minutes.

After 25 cycles were completed, the upper phase was withdrawn after each cycle until all the upper phases had been removed. The lower phases were removed as before.

Spectrophotometric Analysis of the Upper and Lower Countercurrent Phases

The lower and upper phase absorption was determined for the contents of each tube at the ultraviolet wavelengths 275 and 360 millimicrons. The 300 millimicron wavelength was not used for this distribution as before. The previous curves of the tubes obtained in the first countercurrent distribution (Figures 6 and 9) indicated that the 300 millimicron wavelength absorption was repetitious of the 360 and 275 wavelengths; it also was a low point of absorbancy as shown by the ultraviolet spectra of fraction F (Figure 10). The absorption at 300 millimicrons was therefore deleted. The absorption was obtained for both the upper and lower tubes which indicated that only one component was present in the lower phase and none in the upper phase.

The graph of the lower phase optical density plotted against the tube number indicated that the substance was present in tubes 1-5, which was labeled fraction F-1.

Fraction F-1, tubes 1-5 (500 ml), was concentrated under vacuum as previously described for the first countercurrent distribution and isolation. The yellow solution, upon removal of the solvent, left a yellow-brown colored residue. The residue

was extracted with methanol (25 ml x 6), filtered and distilled under vacuum. The precipitate and the filter paper were both saved for further testing. The yellow residue, obtained from the distillation, was dried in an oven at 50°C, and stored in a dessicator for future testing. The dried residue weighed 0.123 grams.

FINAL CHARACTERIZATION AND IDENTIFICATION OF THE COUNTERCURRENT BANDS

Preliminary Paper Chromatography

Harborne (71) found that paper chromatography was a good method for rapidly screening plant extracts for flavonoids on a small scale. He used the solvent system BAW (n-butanol-glacial acetic acid-water, 4:1:5) with success and compiled an extensive list of R_f values for flavonoid glycosides and aglycones. He also tested the different classes of flavonoids with several developing sprays and compared their colors in visible and ultraviolet light. Bate-Smith (72) established certain standards for obtaining consistent R_f values, which he found to differ considerably by modifying the temperature at which the chromatogram was run, saturating the solvents for varying lengths of time, and equilibrating the paper with the aqueous phase for different times before irrigation with the organic phase. Gage, et al (73) tested eleven different solvent systems and developing sprays with different flavonols and flavones, and tabulated their respective R_f values. The work of these investigators enable paper chromatography to be used as an identification method when compared with authentic samples.

Paper chromatography has also been applied to leucoanthocyanidins and condensed tannins. Roux and Evelyn (74)

used BAW and 2% acetic acid plus a toluene-p-sulfonic acid spray to identify several leuco-anthocyanidins and condensed tannins.

Procedure

The procedure followed was essentially that of Gage (75). Whatman Number 1 paper, 46 x 57 centimeters, was cut into strips 22 x 57 centimeters, unless otherwise indicated. The spots were applied with an improvised glass dropper to a lightly penciled line 5.0 centimeters from one end of the strip, and dried with a hair dryer. The amount of material spotted on the paper varied, but ample material was applied for location of the spots on the developed chromatogram. The United States Pharmacopeia XVI (76) recommended that the spots be made 6.0 - 10.0 millimeters in diameter and at least 3.0 centimeters apart. Two pyrex glass chambers were used, one being circular (25 x 46 centimeters), and the other rectangular (31 x 31 x 62). Both chambers were equipped with stainless steel accessories.

Fractions A, C, D, E, F, F-1, fiestin (III), and leucocyanidin (XV), (synthesized from quercetin) were spotted and run in one to four different solvent systems. The developed chromatograms were observed in visible and ultraviolet light and the spots were circled lightly in pencil. They were next sprayed with 3% toluene-p-sulfonic acid, and heated in an oven at 80°C for 10-15 minutes (74). If leuco-anthocyanidins or condensed tannins (polymers of leuco-anthocyanidins) were present, a pink spot or streak was observed.

Solvent Systems

Included below are the solvent systems and developing sprays used throughout this investigation.

1. BAW

n-butanol	4 Parts
glacial acetic acid	1 part
distilled water	5 parts

2. Forestal Solvent System

glacial acetic acid	30 parts
concentrated hydrochloric acid	3 parts
distilled water	10 parts

3. 5% Acetic Acid

4. PW (Liquefied Phenol)

phenol	9 parts
distilled water	1 part

5. Formic Acid (90%) 1 part
   3N-Hydrochloric Acid 1 part

Developing Sprays

1. Ferric Chloride 2% in distilled water (77).

2. Toluene-p-sulfonic acid 3-5% in absolute alcohol (74).

Results

The solvent system BAW seemed to give the best resolution of the complex fractions. All of the unknown fractions except E and F tended to streak on the paper, especially in 5% acetic acid. The spots of the unknown fractions were not seen in visible light; however, under ultraviolet light, fractions A, C and D in the BAW

solvent system each revealed three spots. Fraction A spots were at R_f 0.79 (yellow), 0.88 (bright blue), and 0.91 (light blue); fraction C spots at R_f 0.80 (yellow), 0.86 (light blue), and 0.92 (pale yellow); and fraction D spots at R_f 0.27 (light blue), 0.65 (pale yellow), and 0.91 (light blue). The toluene-p-sulfonic acid spray revealed a pink streak for fraction C at 0.80-0.95 in BAW, and a pink streak from the starting line to R_f 0.33 in 5% acetic acid solvent system. A pink streak such as fraction C revealed in 5% acetic acid, usually is indicative of condensed tannins comprised of leuco-anthocyanidin units (74). Fraction D gave a discrete pink spot at R_f 0.66 in BAW and 0.49 in 5% acetic acid. Small, elongated pink spots confirm the presence of the monomer leuco-anthocyanidins or may indicate a condensed tannin containing three leuco-anthocyanidin units (68, 74). The only known leuco-anthocyanidin available for comparison was leuco-cyanidin, which did not give a pink color with TSA spray as expected. It did give a pink streak from the starting line to R_f 0.14 which may indicate that the leuco-cyanidin was not applied heavily enough to the paper before irrigation for proper development. Fraction A was negative for leuco-anthocyanidins and condensed tannins.

Fraction E showed a bright yellow spot under ultraviolet light at R_f 0.77 in BAW, and a light blue streak from the starting line to 0.76 in 5% acetic acid. This fraction was negative for leuco-anthocyanidins and condensed tannins.

A comparison between fractions F and F-1 was of special interest as the F fraction had previously shown a flavone or flavonol component before the band was subjected to a second countercurrent distribution (see Figure 10 and related discussion). This was also shown by the dull brown spot at R_f 0.79 under ultra-violet light, which disappeared when the product isolated from the second countercurrent procedure (F-1) was subjected to paper chromatographic examination. Therefore, as stated earlier, it appears that the flavone or flavonol component present in fraction F was the residue which was not soluble in the lower phase solvent system for the second distribution. This substance should be investigated for the flavone component that was lost in the transition from fraction F to F-1.

The commercial fisetin revealed two spots (R_f 0.85 dull brown, 0.88 yellow) in visible light, and three spots (R_f 0.85 fluorescent yellow, 0.88 fluorescent green, 0.95 yellow) under ultraviolet light with BAW solvent system. The positive Wilson's Boric Acid Test obtained previously with this grade of fisetin (See Table 3) must be re-evaluated due to the impurities, and considered a questionable positive test.

The leuco-cyanidin was synthesized by the investigator (described later) from quercetin. It gave a pale yellow spot at 0.85 with visible light, and a green-yellow spot at 0.86 under ultra-violet light with BAW solvent system. The known gave one

pale green spot at R_f 0.65 under ultraviolet light with 5% acetic acid. This spot was probably dihydroquercetin (intermediate of synthesis).

The above results (Table 4) indicate that fraction C has as one component, a condensed tannin, and fraction D has a leuco-anthocyanidin, or a condensed tannin also, comprised of leuco-anthocyanidin units.

Spectrophotometric Analysis of Selected Countercurrent Procedures

Ultraviolet Analysis

The ultraviolet spectra were obtained for fractions A, C, D, F-1, and dihydroquercetin (XXII), leuco-cyanidin (XV), and D-catechin (XXV). All of the samples were run in methanol. The results are summarized in Table 5.

Results

Fractions A, C, and D had almost identical curves in the ultraviolet region (see Figure 11 for the ultraviolet spectrum of fraction D). The unknown spectra also resembled the curve for synthetic leuco-cyanidin (XV), which showed a maximum absorption at 208 and 282 millimicrons. The D-catechin spectra was similar to the unknown fractions, but based on previous qualitative tests on fractions A, C and D (positive Wilson's Boric Acid Test), a catechin-like structure was not likely, unless it occurred in very small quantities concurrently with the other components of

TABLE 4

R_f VALUES OF COUNTERCURRENT FRACTIONS
AND AUTHENTIC SAMPLES IN THREE SOLVENT SYSTEMS

	BAW			Forestal			5% Acetic Acid		
Fraction	Visible	UV	TSA	Visible	UV	TSA	Visible	UV	TSA
A	T-LBr	T-Y 0.79Y 0.88B 0.91LB	(b)	T-LBr	T-DBr 0.98B	(b)	T-LBr	T-PG 0.43B 0.54LB	(b)
C	T-LBr	T-Y 0.80Y 0.86LB 0.92PY	0.80-0.95 PS	T-Br,	T-DBr 0.87Y 0.92Y	(b)	T-LBr	T-PG 0.43LB	S-0.33P
D	T-LBr	T-Y 0.27LB 0.65PY 0.91LB	0.66P	T-LBr	(a)	(b)	T-LBr	(b)	0.49P
E	(b)	T-LB 0.77Y	(b)	---	---	---	S-Br	S-FG T-0.76LB	(b)
F	(b)	0.79DBr	(b)	0.91PY	0.91DBr	(b)	---	---	---
F-1	(b)	T-LB	(b)	---	---	---	S-Br	S-DBr T-0.74LB	(b)
Fisetin	0.85DBr 0.88Y	T-GY 0.85YF 0.88GF 0.95Y	(b)	---	---	---	S-Y	S-FG	(b)
leuco- cyanidin	T-LBr 0.85PY	T-Y 0.86GY	S-o.14P	0.81Y	0.81GY	(b)	(b)	0.65PG	(b)

TABLE 4

R_f VALUES OF COUNTERCURRENT FRACTIONS
AND AUTHENTIC SAMPLES IN THREE SOLVENT SYSTEMS
(Continued)

Abbreviations:

B - Blue
G - Green
Y - Yellow
LB - Light Blue
PY - Pale Yellow
DBr - Dull Brown
LBr - Light Brown
YF - Yellow Fluorescent
GF - Green Fluorescent
PG - Pale Green
PS - Pink streak
P - Pink
Br - Brown
T - Tailing
S - Starting Line
TSA - Toluene-p-sulfonic acid
BAW - n-butanol-acetic Acid-Water

(a) There were no spots observed except a dull brown area on the yellow solvent front.

(b) No spots were observed.

fractions A, C, and D.

Due to the many flavan compounds that possess similar ultraviolet spectra (leuco-anthocyanidins, condensed tannins, dihydro-flavonols, catechins, and hydrolyzable tannins), the results were limited for differentiating between the possible compounds present in the plant fractions.

Fraction F-1 did not resemble fractions A, C, or D spectrums as it had absorption peaks at 202 and 270 millimicrons. It was interesting to note that the F-1 spectrum differed markedly from the spectrum obtained from tube 82 (see Figure 10) of the first countercurrent distribution. Fraction F spectrum (Figure 10) resembled a flavone or flavonol, and had a broad absorption band at 340-350 millimicrons. The F-1 spectrum (See Table 5), however, completely lacked this broad absorption band. This further substantiates the information obtained by comparing fractions F and F-1 by paper chromatography (Table 4). As mentioned in the discussion related to the preliminary paper chromatography results, the flavone component must have been the insoluble precipitate recovered from the lower phase solvent system whenever fraction F was being prepared for the second countercurrent distribution. Secondly, the ultraviolet spectrum was obtained for tube 82, which was the tube of maximum flavone concentration. The isolation of fraction F encompassed tubes 60-99, which as can be seen from Figure 9, included too many

lower phase tubes. The fraction, instead, should have been tubes 70-90, for a maximum concentration of the flavone or flavonol component. Inclusion of a wider band for fraction F probably contaminated the flavone fraction.

Fraction F-1 was saved for further characterization. The insoluble precipitate from the second countercurrent distribution should be analyzed for the missing flavone or flavonol components, although it was not attempted during this study.

Infrared Analysis

In an effort to further elucidate the structures of the countercurrent fractions, infrared spectra were made using a Perkin-Elmer Infracord Spectrophotometer. The recordings were made by distributing 1.2 to 1.8 milligrams of sample in 0.4 grams of potassium bromide, and pressing 0.3 grams of this material, under vacuum, into a clear disc with a hydraulic press.

Infrared spectrums were determined for fractions A, C, D, and F-1. See Figures 12, 13, 14 and 15 for the individual infrared spectra of the respective fractions.

Results

Fractions A, C, and D were identical except A and C had an absorption peak at approximately 9.80 microns that fraction D did not have. Fraction F-1 had one common absorption peak, at 2.92 microns, with fractions A, C, and D; but the remainder of the spectrum was completely different.

The evidence obtained from the infrared (Table 6) and ultraviolet spectra (Table 5) of fractions A, C, and D, qualitative chemical tests, paper chromatography, and the amorphous tannin-like properties, indicate that a skeletal flavan structure (XXVI) is present.

Fractions A, C, and D (Table 6) all have a common hydroxyl group absorption peak at 2.94 microns, and the peaks at 7.40, 7.90, and 9.05 microns, all indicate secondary alcohols. Secondary alcohols could only be located at positions 3 and 4 of the flavan structure (XXVI); therefore, a 3-OH, 4-OH, or 3,4-diol could be possible. The 7.40 peak also may indicate phenols, along with 8.52 and 8.72 micron peaks, which could be located at positions 5, 6, 7, 8, 2', 3', 4', 5', or 6'. The most common phenolic OH positions of flavan compounds, found in nature, are 5, 7, 8, 3', 4', 5', and various combinations of these positions. The 12.0 micron peak may be characteristic of two adjacent, aromatic CH absorption frequencies. The 5.95 micron peak is usually characteristic of carbonyl absorption. This may be from the presence of two components in the fractions, as shown previously by paper chromatography (Table 4). One possible combination would be a dihydroflavonol with a leuco-anthocyanidin, condensed tannin or both. An attempt to methylate fraction C, extraction with ethyl ether, and the infrared spectrum of the resultant product, showed almost complete

TABLE 5

ULTRAVIOLET ABSORPTION OF SELECTED COUNTERCURRENT FRACTIONS

Fraction	Wavelength of Maximum Absorption in Millimicrons	Wavelength of Minimum Absorption in Millimicrons	Concentration G/ml
A	209, 283 mu	258 mu	1.86×10^{-5} g/ml
C	210, 282 mu	260 mu	1.80×10^{-5} g/ml
D	210, 283 mu	260 mu	2.32×10^{-5} g/ml
F-1	202, 270 mu	259 mu	4.80×10^{-5} g/ml (d)
(a)	216, 292 mu	251 mu	1.49×10^{-5} g/ml
(b)	208, 287 mu	260 mu	1.88×10^{-5} g/ml
(c)	210, 282 mu	252 mu	8.08×10^{-6} g/ml

Note: (a) Dihydroquercetin

(b) Leuco-cyanidin

(c) D-Catechin

(d) The designated quantity was not completely soluble in methanol.

removal of the carbonyl absorption. The remainder of the curve was identical to the previous infrared spectrum of fraction C before the methylation attempt.

Previous investigations have disclosed similar infrared spectra for condensed tannins and leuco-anthocyanidins (68). Roux and Paulus (78) obtained infrared curves for (+) -7, 3', 4', 5'-tetrahydroxyflavan-3,4-diol, a leuco-anthocyanidin occurring in the heartwood of Acacia mearnsii. This curve was similar to fractions A, C, and D, although not identical. Putnam, et al (79) made a study of the infrared spectra of condensed tannins and catechins, acetylated, and methylated tannins. The tannins from different sources (wattle wood, quebracho wood, gambier and mangrove wood) all had similar infrared spectra. Infrared spectra have been obtained for all of the leuco-anthocyanidins occurring in nature, but have not been published along with the other data; therefore, infrared curves of the known leuco-anthocyanidins are not always available for comparison.

Vanillin Color Test

The vanillin chemical test was obtained for fractions A,C,D, F-1, leuco-cyanidin, dihydroquercetin and tannic acid, USP. The procedure followed was that of Bate-Smith (50) which was given on page 18.

TABLE 6

INFRARED WAVELENGTH ABSORPTION PEAKS IN MICRONS OF COUNTERCURRENT FRACTIONS A, C AND D

A		C		D		Indicated chemical group assigned to the peak (80)
2.94	(s)	2.95	(s)	2.95	(s)	Hydroxyl OH
3.45	(w)	3.44	(w)	3.44	(m)	Intermolecular polymeric association
5.94	(w)	5.96	(w)	5.90	(w)	Carbonyl C=O
6.26	(s)	6.28	(s)	6.28	(s)	Phenyl ring
6.70	(s)	6.70	(m)	6.70	(m)	Phenyl ring
7.00	(s)	7.00	(m)	7.00	(m)	Phenyl ring
7.40	(w)	7.40	(w)	7.40	(m)	Secondary alcohol, phenol
7.90	(m)	7.85	(w)	7.90	(m)	Secondary alcohol, pyran
---		8.50	(w)	---		Phenol, 1, 2 and 1, 4 disubstituted phenyl
8.70	(w)	8.72	(w)	8.70	(w)	Phenol, 1, 2, 4 trisubstituted phenyl
9.05	(m)	9.05	(w)	9.05	(m)	Secondary alcohol, 1,2 and 1,4 disubstituted phenyl
9.35	(m)	---		9.45	(m)	1,2 and 1,4 disubstituted phenyl
9.78	(m)	9.80	(m)	---		1,2 and 1,4 disubstituted phenyl
10.32	(w)	10.34	(m)	10.32	(w)	1,2 and 1,4 disubstituted phenyl
---		---		11.15	(w)	No interpretation
11.95	(w)	12.00	(m)	12.00	(w)	2 adjacent free aromatic hydrogens
---		12.35	(w)	12.35	(w)	No interpretation
---		12.90	(w)	12.90	(w)	No interpretation

Code: (s) Strong intensity of absorption
(m) Medium intensity of absorption
(w) Weak intensity of absorption

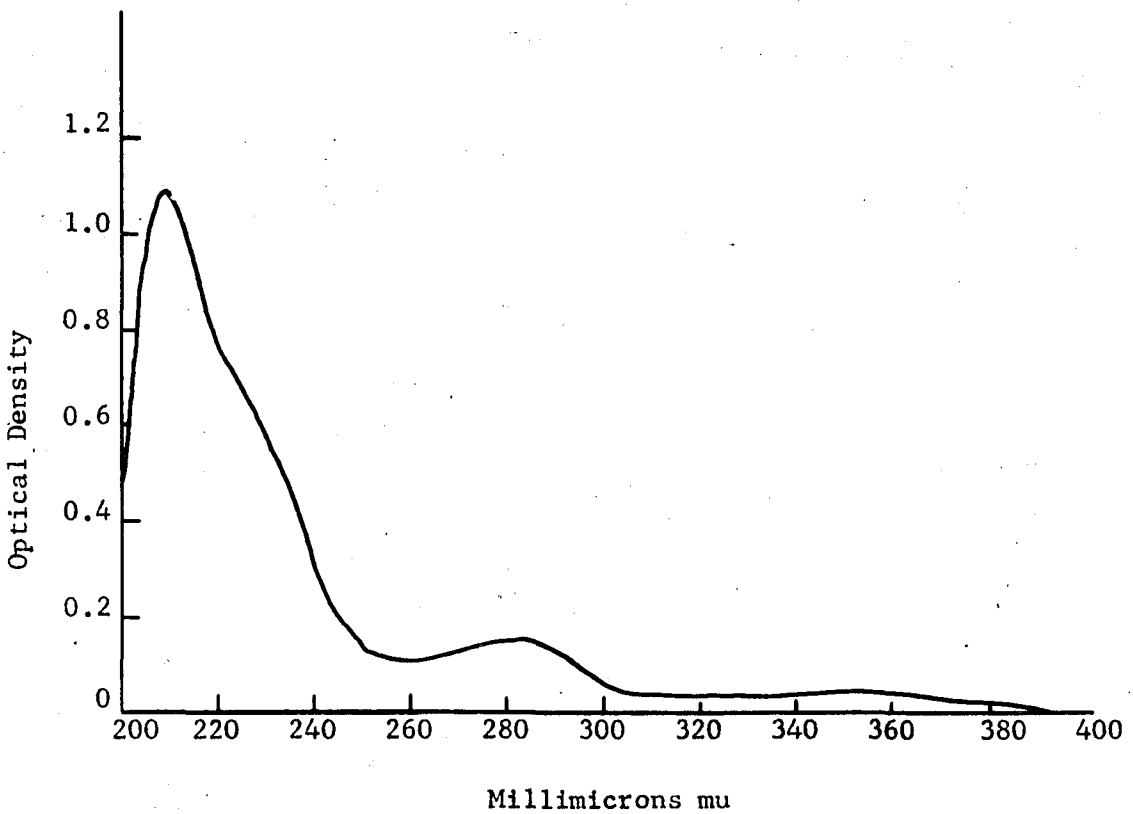


Figure 11. Ultraviolet Spectrum of Fraction D.

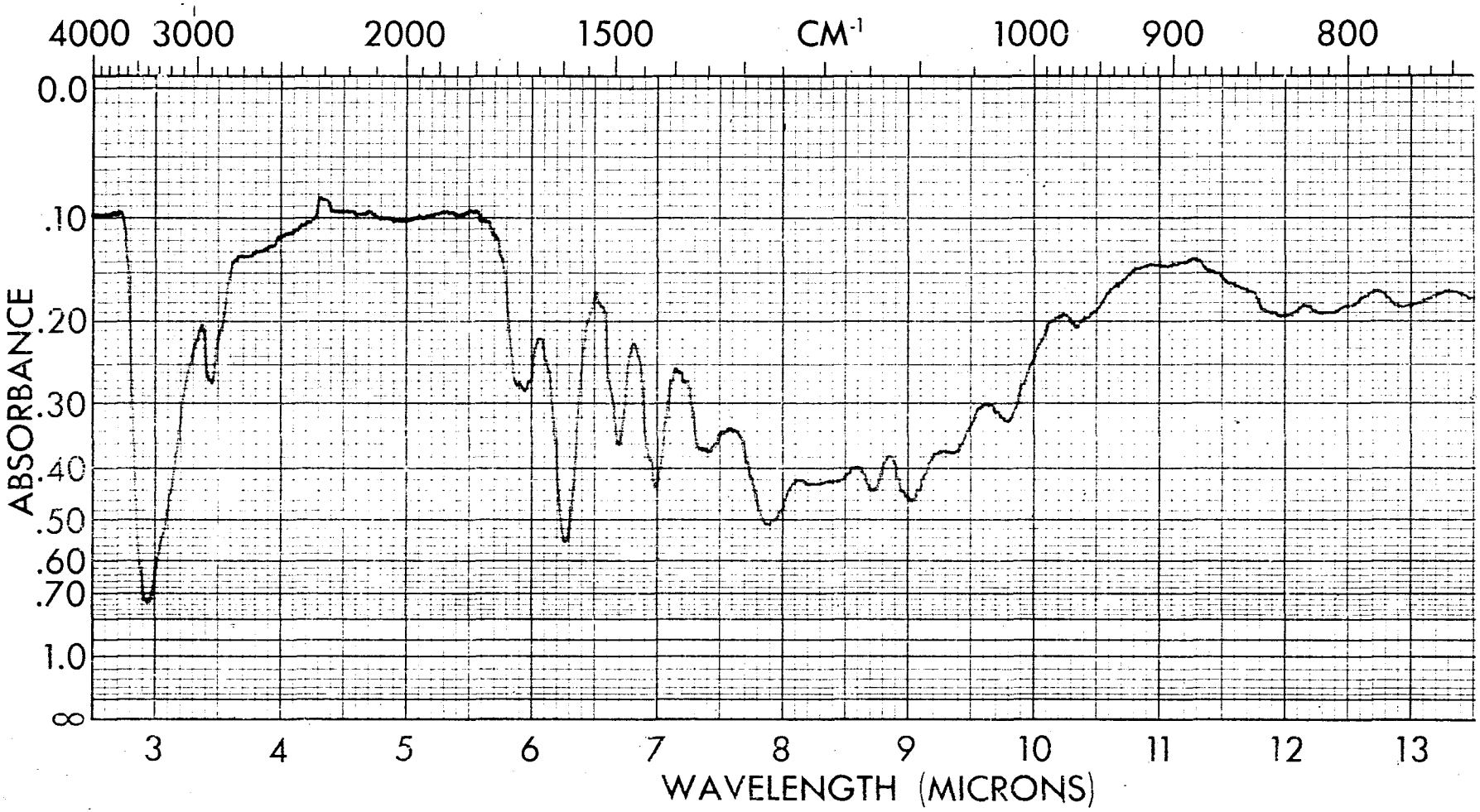


Figure 12. Infrared Spectrum of Fraction A.

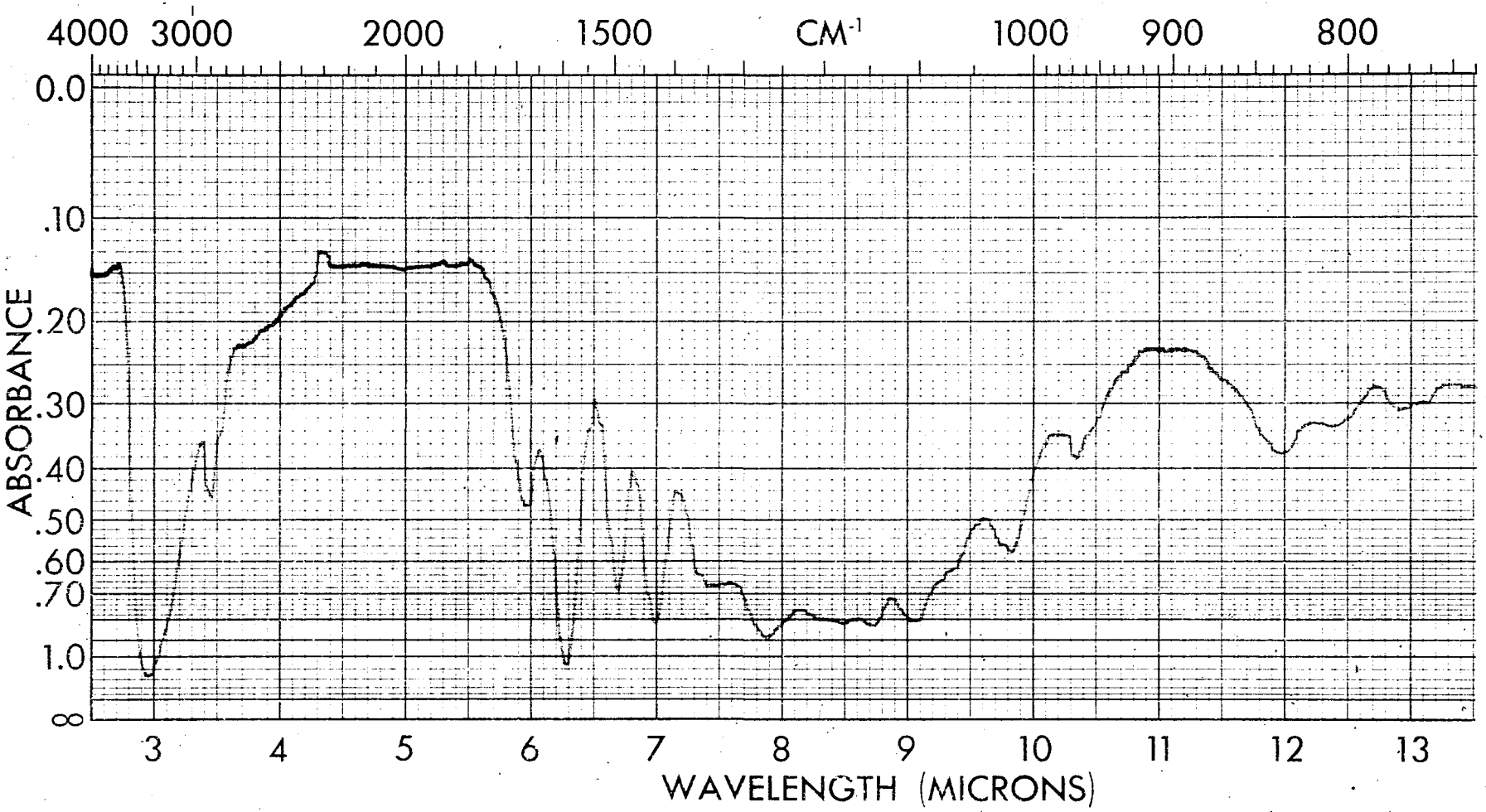


Figure 13. Infrared Spectrum of Fraction C.

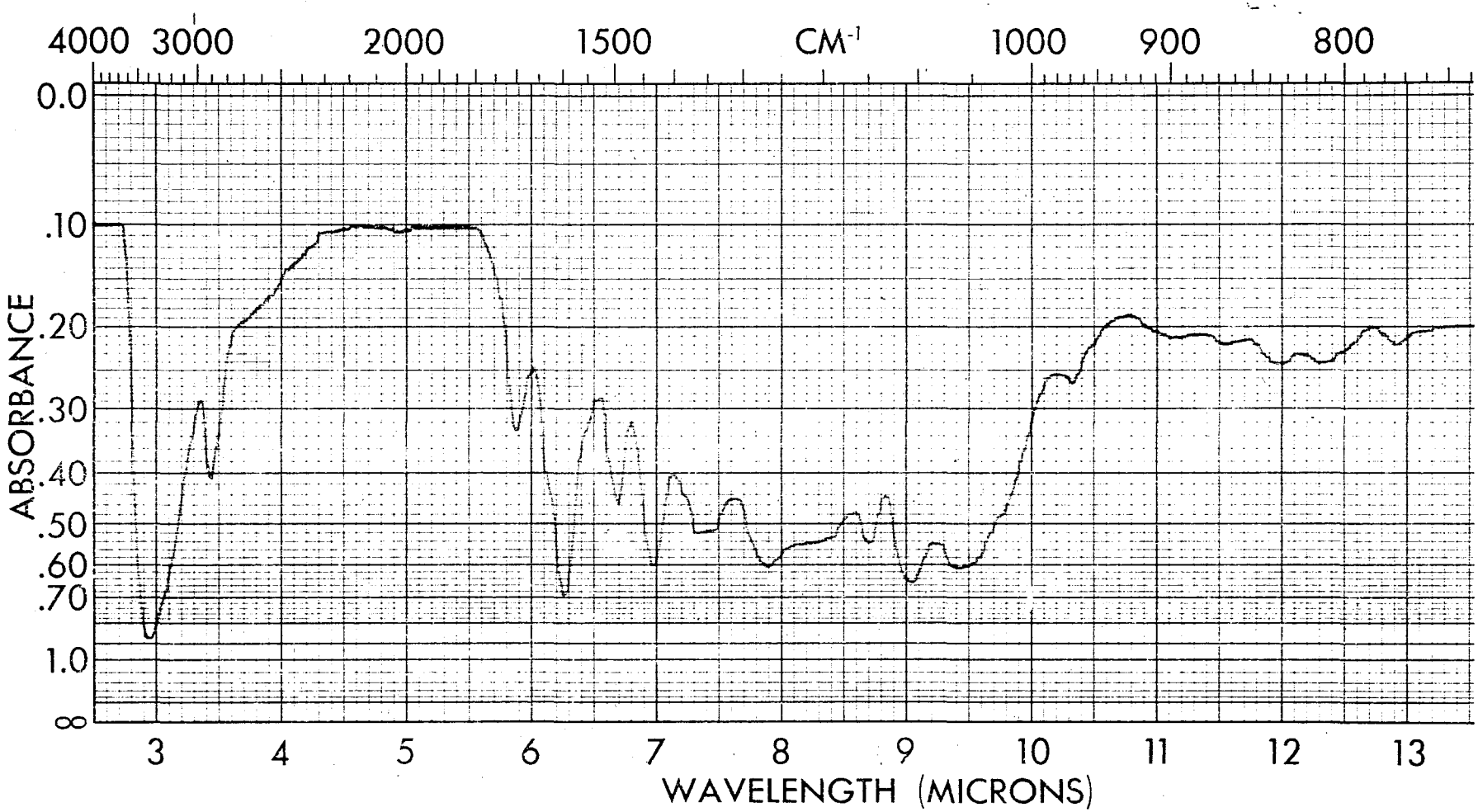


Figure 14. Infrared Spectrum of Fraction D.

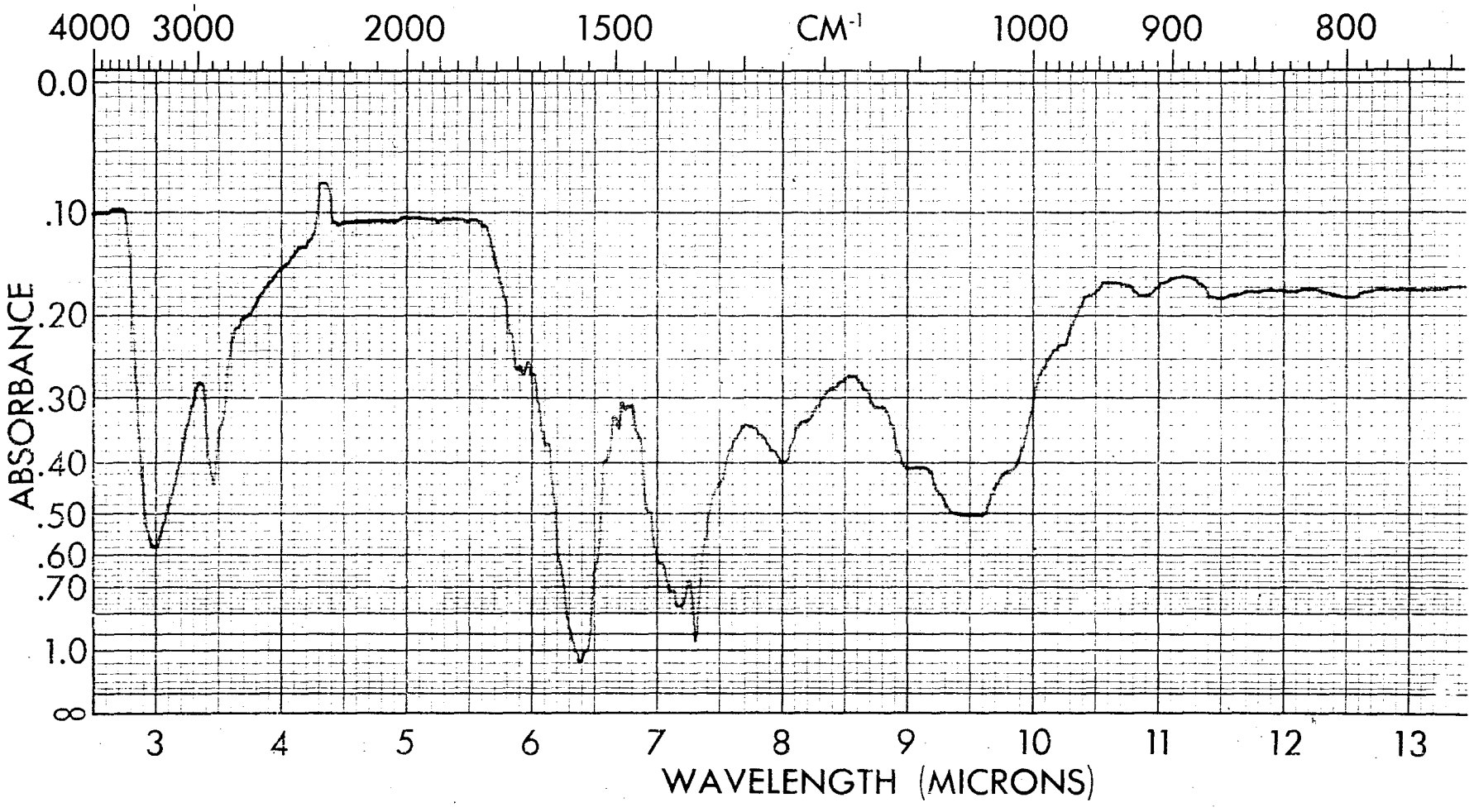


Figure 15. Infrared Spectrum of Fraction F-1.

TABLE 7

VANILLIN COLOR TEST ON SELECTED COUNTERCURRENT FRACTIONS

Fraction	Color
A	Positive. Bright pink color immediately.
C	Positive. Dark cherry red color immediately.
D	Positive. Bright pink color immediately.
F-1	Negative. A slight orange color after 15 minutes.
Leuco-cyanidin (a)	Positive. Dark red immediately.
Dihydroquercetin	Negative.
Tannic Acid, USP (a)	Negative. A slight orange color after 15 minutes.

Note: (a) The leuco-cyanidin and dihydroquercetin used for tests were products obtained in the synthesis of leuco-cyanidin from quercetin.

Results

See Table 7 for a summary of the test results. A positive color test indicates the presence of leuco-anthocyanidins, catechins, or condensed tannins which are polymers comprised of the first two classes of compounds (50, 81). Fractions A, C, and D, all had positive color reactions to the vanillin reagent. Fraction F-1 was negative, although a slight color developed after fifteen minutes. Leuco-cyanidin gave a red color similar to fractions A, C, and D, while dihydroquercetin and tannic acid were both negative. The positive results further substantiate the previous tentative conclusion that fractions A, C, and D contain leuco-anthocyanidins and condensed tannins.

Attempted Methylation of Fraction C

Due to the amorphous nature of fraction C and other countercurrent fractions, all attempts at recrystallization were met with failure; consequently, an attempt was made to prepare a crystalline derivative by methylating the free phenolic hydroxy groups. The methods generally used for methylation are dimethyl sulfate or diazomethane. The dimethyl sulfate procedure was utilized due to the availability of the chemicals required for the reaction, although diazomethane generally would give a more quantitative yield.

The procedure of King and Bottomley (13) was utilized for the reaction. They had successfully methylated melacacidin (VI),

a leuco-anthocyanidin obtained from several Acacia species (1, 20), by using dimethyl sulfate and potassium carbonate.

Fraction C (0.150 grams) was dissolved in 50 ml of acetone in a 200 ml round bottom flask. Anhydrous potassium carbonate (1.05 grams) and purified dimethyl sulfate (0.3 grams, 0.23 ml) were added and the mixture was refluxed for 2 hours, filtered, and distilled under a vacuum to a residue. The residue was partially dissolved in 75 ml of dilute ammonia water, allowed to digest for 3 hours, and acidified with hydrochloric acid. A brown floculent precipitate immediately settled out which was then extracted in a continuous liquid-liquid extractor with ethyl ether. After extraction of the brown mixture for 32 hours, the ether solution was evaporated in a hood leaving a yellow pungent substance (0.050 grams) that was set aside for future investigation. The brown residue that was not soluble in ether was filtered leaving 0.075 grams of an amorphous residue. An infrared curve of this material was obtained, as described previously, and the curve was of the same intensity at 2.94 microns as fraction C (figure 13). This would indicate that no reaction has occurred at the phenolic OH groups. The brown substance had a similar spectrum as fraction C and only differed by a lack of absorption at 5.95, 9.80 and 10.32 microns respectively, of fraction C. Ether extraction of the reaction mixture had apparently removed the substance giving a carbonyl peak in the infrared region at 5.95 microns.

This attempt to methylate fraction C with dimethyl sulfate was unsuccessful, as shown by the presence of an infrared absorption peak at 2.95 microns. Alteration of the reaction conditions might possibly give a derivative with this reagent, but a lack of material (fraction C) prevented any further experimentation with this or the diazomethane reagent.

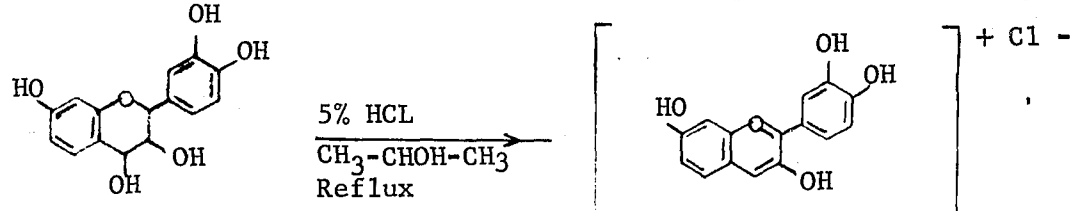
Conversion of Possible Leuco-anthocyanidins to Anthocyanidins

Procedure for Conversion

The evidence accumulated thus far indicates the presence of leuco-anthocyanidins and condensed tannins in fractions C, D and possibly A (although negative results were obtained with paper chromatography for A). If leuco-anthocyanidins or condensed tannins are present, their conversion to anthocyanidins should be accomplished by heating the leuco-anthocyanidin in methanolic-hydrochloric acid. Bate-Smith (50, 81) used this conversion to antho-cyanidins in conjunction with paper chromatography, and the vanillin test, to screen numerous species of plants for leuco-anthocyanidins, catechins or condensed tannins. Catechins are not converted to anthocyanidins, yet give a positive vanillin test along with leuco-anthocyanidins and condensed tannins. These two tests, therefore, provide a way to differentiate between catechins and leuco-anthocyanidins. Bate-Smith heated the plant tissue in aqueous 2N hydrochloric acid or methanolic hydrochloric acid, spotted the substance on paper, and irrigated it with (1:1)

n-butanol-HCl (2N), Forestal system, and m-cresol-acetic acid-HCl mixture. The best results were obtained with the highly acidic Forestal system, as it prevented the loss of color on the paper.

Bottomley (82),and King and Clark-Lewis (83) also converted leuco-anthocyanidins to anthocyanidins, in order to identify the unknown leuco-anthocyanidins, or establish their presence. Bottomley (82) employed a slightly different method by heating the tentative leuco-anthocyanidin in 2-propanol containing 5% hydrochloric acid, and separating the resultant mixture of phlobatannins and anthocyanidins before spotting the paper. King and Clark-Lewis (83) did the conversion in n-butanol-water with hydrochloric acid and spotted this mixture directly on the paper. The method of Bottomley (82) was adopted for this study.



7, 3',4'-trihydroxyflavan-3,4-diol

7,3',4',-trihydroxy-flavylium Chloride

Fraction A

Fraction A was not tentatively converted to a anthocyanidin due to the negative results obtained for leuco-anthocyanidins and condensed tannins with paper chromatography. Secondly, only a small quantity of fraction A remains and as the characteristics of this fraction were very similar to fraction C, the results

obtained from the conversion of it and the other fractions to anthocyanidins may be applied later to fraction A.

Fraction B

Prior to this tentative conversion to an anthocyanidin, Fraction B was only used sparingly for several qualitative tests (Table 3), due to the small quantity (0.037 grams) that was isolated from the countercurrent distribution. The fraction was considered to be relatively free of impurities compared to fractions A and C, as was shown by the ultraviolet spectra of the three fractions (Figure 8). The detection of leuco-anthocyanidins in fraction D by paper chromatography (Table 4) and other tests confirming leuco-anthocyanidins, all made it possible to assume the presence of a leuco-anthocyanidin in fraction B also. First, the preliminary qualitative test results (Table 3) of fraction B resemble those of fraction D; and secondly, fraction B was taken from the middle of fraction D of the countercurrent distribution upper phase (Figures 6 and 7). This similarity of fraction B to fraction D prompted the use of it for conversion to an anthocyanidin, for comparison to the other fractions reputedly containing leuco-anthocyanidins or condensed tannins, and possible identification.

The total quantity of fraction B was refluxed for 1 hour with 100 ml of isopropyl alcohol which contained 5% hydrochloric acid. The solution turned from a yellow to pink color even before

refluxing had commenced. The solution was evaporated on a hot plate to about 15 ml and diluted with 100 ml 2N hydrochloric acid. There was a slight floculent precipitate noted after 15 minutes. The aqueous mixture was then extracted with ethyl acetate (50 ml x 1, 25 ml x 4) until the extracts were colorless. The ethyl acetate extracts were a golden brown color. The aqueous layer was next extracted with n-butanol (25 ml x 2), which removed all the color from the aqueous phase. The pink to red alcohol phase was distilled under vacuum until it was concentrated enough to spot the paper chromatograms.

Fraction C

Fraction C (0.030 g) was treated with 100 ml of isopropyl alcohol which contained 5% hydrochloric acid, and the same purification procedure was followed as described for fraction B. The significant difference noted with fraction C, compared to fraction B, was the brown-pink color of the final n-butanol organic extractions. Also, the original refluxing gave at first a lavender color which finally turned to a red brown color. There was a larger quantity of brown material in this reaction that fraction B did not have. Fraction C was shown previously by paper chromatography to be a condensed tannin, and therefore, the yield of anthocyanidin produced was less than fraction B, which may be a pure leuco-anthocyanidin. Roux and Evelyn (67) found that both condensed tannins and leuco-anthocyanidins give anthocyanidins upon heating with hydrochloric acid, although

the yield of anthocyanidin is related to the extent of polymerization of the tannin. The monomer leuco-anthocyanidin would give a higher percent yield of anthocyanidin than the condensed tannins.

Fraction D

Fraction D (0.020 gram) was refluxed for 1 hour as previously described, giving a red-orange colored mixture. The final n-butanol phase was a pink colored solution of the order of fraction B.

Synthetic Leuco-cyanidin

Synthetic leuco-cyanidin (0.030 g) (synthesis described later) was refluxed for 1 hour as previously described, giving a red colored solution. The final n-butanol phase was a pink-lavender color.

Standard leuco-cyanidin

In order to compare the authenticity of the leuco-cyanidin synthesized by the investigator, a commercial leuco-cyanidin (0.030 g) was also converted to the anthocyanidin as previously described. This substance was not as soluble in the isopropyl alcohol as the previous fractions and the synthetic leuco-cyanidin. It also was a pink-lavender color as was the previous leuco-cyanidin.

Procedure For Paper Chromatography

Whatmans No. 1 paper was cut into strips 15 x 46 cm and spotted with a micro-dropper on a finely penciled line 5 cm from

one end. Fractions B, C, D, synthetic and commercial leuco-cyanidin were applied heavily in order that the spots would be seen on the irrigated chromatogram.

The first solvent system that was used was n-butanol-glacial acetic acid (1:1). This solvent system apparently was not polar enough to move the ionic anthocyanidins, as the spots remained on the starting line. The solvent system employed with the most success was the Forestal system (glacial acetic acid-concentrated hydrochloric acid-distilled water, 30:3:10).

The paper was saturated in the tank for 18 hours before the ascending irrigation was begun. The paper was irrigated for 24 hours and solvent front had traveled 24.0 cm when the paper was removed from the tank.

Results

Fractions B, C, and D each gave an orange-red spot (R_f 0.94, 0.94, 0.93), and slight yellow spots near the solvent front that were bright yellow in ultraviolet light. The orange-red spots were a dull orange color under ultraviolet. Fraction C had an additional faint violet spot at R_f 0.78, which was similar to the violet spots of synthetic and commercial leuco-cyanidin (R_f 0.79, 0.78). The latter two spots were a dull red color under ultraviolet light. The results are summarized in Table 8.

The identical orange-red anthocyanidin spots of fractions B, C, and D indicate that identical leuco-anthocyanidins, or

condensed tannins comprised of identical leuco-anthocyanidin units, are present. Fraction D has previously shown the presence of a monomer leuco-anthocyanidin (Table 4) when developed with toluene-p-sulfonic acid, while fraction C showed the characteristic streak of a condensed tannin with the same developing reagent. Therefore, fraction C must be a condensed tannin, or polymer of the same leuco-anthocyanidin which is present in fraction D. Fraction C also revealed a second anthocyanidin spot similar to anthocyanidin itself. This indicates that fraction C may be a condensed tannin comprised of two different leuco-anthocyanidins, or two different condensed tannins. Fraction B, although it has not been investigated previously by paper chromatography and developed with toluene-p-sulfonic acid, revealed an identical anthocyanidin spot; therefore, the same leuco-anthocyanidin or condensed tannin comprised of flavan-3,4 diols must be present in fraction B as with fractions C and D.

The second anthocyanidin spot of fraction C (R_f 0.78) appears to be identical to leuco-cyanidin. However, without degrading the structures, and identifying the fragments, the same R_f values are not conclusive for identification of a leuco-anthocyanidin structure. Roux (84) found that pelargonidin and fisetinidin both gave nearly the same R_f values in the Forestal system; and melacacidin and leuco-cyanidin, converted to anthocyanidins, both gave similar R_f values in Forestal and a formic acid-water solvent system. It is possible then, that two

leuco-anthocyanidins with the same number of hydroxy OH groups, regardless of position, will give the same R_f values with certain solvent systems. It is not possible to ascertain, therefore, the identity of the second leuco-anthocyanidin present in fraction C, without degrading the molecule and identifying the fragments.

The R_f values obtained for synthetic and commercial cyanidin were unusually high compared to literature values (82, 84). This fact can be explained by the high temperatures used (27°C) in this investigation compared to lower temperatures (22°C) used by other investigators. The R_f values obtained in this investigation were significant for identification only when compared to authentic samples.

Micro-Fusion of Fraction D

The fusion of condensed tannins, catechins, leuco-anthocyanidins, and other flavonoids with potassium hydroxide has been used in the past to identify a particular compound, by isolating and identifying its fragments. Freudenberg and Maitland (85) used alkali fusion on the tannins of commercial quebracho extracts, which yielded resorcinol and protacatechuic acid. Upon analyzing the fragments from the fusion process, they accordingly synthesized (±)-eip-7, 3', 4' - trihydroxy-flavan-3-ol by catalytic hydrogenation of fisetinidin chloride. Roux (86) improved the techniques of identification of the fragments, by fusing black wattle wood (A. mearnsii) and other

TABLE 8

PAPER CHROMATOGRAPHICAL RESULTS OF SELECTED COUNTERCURRENT FRACTIONS AFTER CONVERSION TO ANTHOCYANIDINS

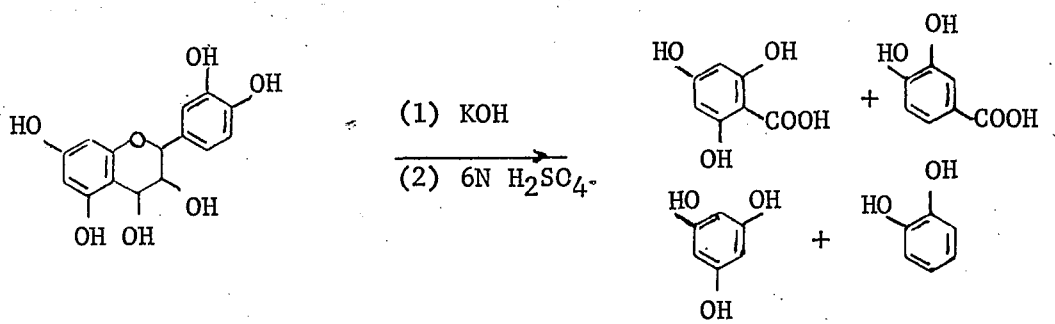
Sample	R_f	Forestal Solvent System	
		Visible Light	Ultraviolet Light (a)
Fraction B	0.94	1 orange-red spot	1 dull orange spot 1 bright yellow near solvent front
Fraction C	0.78	1 faint lavender	(b)
	0.94	1 orange-red	1 dull orange,
		1 faint yellow near solvent front	1 bright yellow near solvent front
Fraction D	0.93	1 orange-red spot	1 dull orange 1 bright yellow spot near solvent front
Synthetic leuco-cyanidin	0.79	1 violet spot	1 dull rose spot
Commercial leuco-cyanidin	0.78	1 violet spot	1 dull rose spot

Note: (a) The chromatogram was examined under a black raymaster lamp (356-365 millimicrons)
(b) There was no spot observed at R_f 0.78

tannins. Roux (77) later adapted the technique to micro amounts of plant material (2-10 milligrams). He used the solvent system BAW and various developing sprays to identify the fragments. The latter procedure (77) was employed for this study due to the inherent advantage of being able to use micro amounts of fraction D.

Fraction D was chosen for the degradation procedure as it contains only one leuco-anthocyanidin, while fraction C possibly contains two leuco-anthocyanidins (see Table 8). The identification of the fragments would consequently be simplified by using fraction D for the fusion, and subsequent identification of the leuco-anthocyanidin present in fractions B, C, and D.

Many products can be produced by fusing a flavan compound as illustrated below in the fusion of leuco-cyanidin.



The phenols and phenolic acids were separated and identified on paper chromatography by comparing the unknowns to authentic samples of resorcinol, catechol, pyrogallol, phloroglucinol, protocatechuic acid, B-resorcylic acid, gallic acid, 2,4,6-trihydroxybenzoic acid, and p-hydroxybenzoic acid.

Procedure for Fusion and Separation of Degradation Products With Sodium Bicarbonate.

Fraction D (10.0 mgm) was placed in the bottom of a 15 x 2 cm test tube and five potassium hydroxide pellets were placed midway to the bottom of the tube, and heated to a molten consistency over a small flame, being careful not to heat fraction D. When the potassium hydroxide had reached a molten consistency, the tube was removed from the flame and the potassium hydroxide was allowed to flow onto fraction D. The material partially dissolved in the molten potassium hydroxide and was then heated over a flame for 1-1/2 minutes in such a manner that the solution did not boil. The mixture was then cooled in ice water, and 6N sulfuric acid was added until the mixture was acidic. The mixture was extracted with ethyl ether (25 ml x 1, 10 ml x 5), concentrated to about 25 ml on a steam bath and extracted with 5% sodium bicarbonate (25 ml x 3). The aqueous phase was then extracted with ethyl ether (15 ml x 4), concentrated to about 5 ml, and dehydrated with anhydrous sodium sulfate for 24 hours. The pale yellow solution contained the phenolic degradation products. The aqueous sodium bicarbonate phase after the ether extraction was acidified with 6N sulfuric acid, extracted with ethyl ether (15 ml x 4), concentrated on a steam bath to about 5 ml and dehydrated over anhydrous sodium sulfate for 24 hours. This solution was a darker yellow than the

phenolic fraction and contained the phenolic acid degradation products.

Identification of Degradation Products by Paper Chromatography

The phenols and phenolic acid degradation products were spotted on separate paper strips, 22 x 57 cm. On one paper were placed the unknown phenols, and the authenticated samples, resorcinol, phloroglucinol, catechol, and pyrogallol. The other paper contained unknown phenolic acids, B-resorcylic acid, protocatechuic acid, gallic acid, 2,4,6-trihydroxybenzoic acid, and p-hydroxybenzoic acid. They were irrigated at 27°C in four different solvent systems: (1) BAW, (2) Forestal system, (3) phenol-water, and (4) 5% acetic acid. The resultant papers were air-dried for twelve hours, and sprayed with 2% ferric chloride. See Table 9.

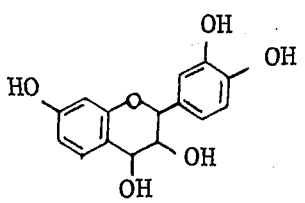
The unknown phenolic acid sample was resolved into two spots with each solvent system except 5% acetic acid. The latter system gave only one blue-green spot at R_f 0.54. The other three solvent systems resolved the sample into two spots each. In each case, the developed spots were almost identical to B-resorcylic and protocatechuic acids, both in color and R_f values.

The unknown phenol degradation products could not be identified when compared to the authentic samples. The unknown sample, irrigated with BAW, formed an elliptical (R_f 0.35) spot,

which turned pale yellow with ferric chloride, and extended back to the starting line. The unknown was similar with phenol-water solvent, and gave a pale yellow streak to R_f 0.42. It is conceivable that the phenols produced during the fusion of the micro-sample were very small compared to the phenolic acids. This may be due to technique, reaction conditions, or the micro quantity used for the fusion.

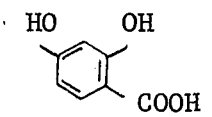
Proposed Structure of the Leuco-anthocyanidin

The identification of B-resorcylic and protocatechuic acids as degradative fragments of fraction D establishes the tentative structure of the leuco-anthocyanidin as 7, 3', 4'-trihydroxyflavan-3,4-diol. This structure could be theoretically degraded to B-resorcylic and protocatechuic acids as given below. Roux and Evelyn (67) fused leuco-fisetinidin (VIII) with potassium hydroxide and identified B-resorcylic and protocatechuic acid as the degradation products.

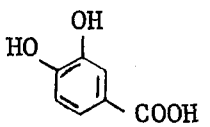


7,3'4'-trihydroxy-flavan-3,4-diol

KOH Fusion



B-resorcylic acid



Protocatechuic acid

In order to obtain the degradation acids, B-resorcylic and protocatechuic acids, the leuco-anthocyanidin would have to

TABLE 9

R_f VALUES OF THE MICROFUSION PRODUCTS OF FRACTION D AND THE AUTHENTICATED SAMPLES IN FOUR SOLVENT SYSTEMS:

Sample	BAW	Forestal	PW	5% Acetic Acid
Unknown Phenolic Acids	0.86G 0.94V	0.94G 0.96V	0.49G 0.53V	0.54 BG (a)
Unknown Phenols	0.35Y(b)	--	0.42 PY(c)	--
2,4 Dihydroxybenzoic Acid (B-Resorcylic Acid)	0.95V	0.97V	0.57V	0.59V
3,4 Dihydroxybenzoic Acid (Protocatechuic Acid)	0.89G	0.94G	0.52G	0.61G
3,4,5-trihydroxybenzoic Acid (Gallic Acid)	0.73B	0.79B	0.13B(c)	0.47GR (c)
4-Hydroxybenzoic Acid	0.95Y	0.94Y	0.47Y(c) 0.65B	0.58Y (c)
2,4,6-Trihydroxybenzoic Acid	0.83V	0.84BV	0.27V(c)	0.60V (c)
2-Hydroxyphenol (Catechol)	0.96BV	0.95BV	0.74B	0.74OL
3-Hydroxyphenol (Resorcinol)	0.92PY	0.92V	0.42PY	0.66V
3,5-Dihydroxyphenol (Phloroglucinol)	0.77PY	0.72Y	0.21V(c)	0.58V (c)
2,3-Dihydroxyphenol	0.84Y	0.90Y	0.42V	0.68Y

Color Code: V - Violet, B - Blue, G - Green, Y - Yellow, PY - Pale Yellow, OL - Olive, GR - Grey

(a) One spot only was observed with 5% acetic acid.

(b) A large elliptical spot extended from the starting line to the designated R_f value.

(c) A continuous streak from the starting line to the designated R_f value.

contain the structure illustrated on page 90. Fraction D must be one of the four possible racemates or one of the eight possible optical isomers of 7, 3', 4'-trihydroxyflavan-3,4-diol. Fractions B and C must also contain the same leuco-anthocyanidin (fraction C may be a polymer) as seen by the identical anthocyanidins on paper chromatography (Table 8).

Further proof of structure will be undertaken by synthesizing the above leuco-anthocyanidin, conversion to an anthocyanidin, and comparing it to the unknown anthocyanidin.

Four racemates or eight optically active isomers are possible due to the three asymmetrical centers at 2, 3, and 4-positions of the leuco-anthocyanidin structure. Two enantiomorphous isomers have been isolated in nature: (+) - mollisacacidin (VII) from Acacia mearnsii, and (-) - mollisacacidin (leuco-fisetinidin) (VIII) from quebracho wood (16, 17, 18). Therefore, three racemates or six optically active isomers have yet to be found in nature. It was not possible to obtain the optical rotation of the countercurrent fractions due to the highly colored solutions in methanol.

FINAL PROOF OF STRUCTURE

Synthesis of Leuco-cyanidin

During the latter half of this investigation, leuco-cyanidin (XV) was used for comparison to the unknown plant fractions. Synthesis of leuco-cyanidin supplied a known leuco-anthocyanidin to compare with the other fractions, and provided the investigator a one-step reduction method with which to rapidly prepare the 7, 3', 4'-trihydroxyflavan-3,4-diol structure for comparison to the unknowns which was shown to be present in fractions B, C, and D, and for conversion to the respective anthocyanidin.

Leuco-anthocyanidins have been synthesized by many different methods, depending on the particular racemate desired. Four possible racemates are possible, as stated earlier, and each one requires different synthetic methods. King and Clark-Lewis (14), and Clark-Lewis, Kateckar, and Mortimer (22) obtained the 2,3,4-cis conformation by reducing the flavonol with hydrogen and Raney Nickel at 100°C/100 atmospheres for 24 hours. The 2,3-trans, 3,4-cis conformation has been synthesized by reducing the dihydroflavonol with sodium borohydride and lithium aluminum hydride (14, 22, 87, 88, 89). According to Kulkarni et al (87), lithium aluminum hydride produces mixtures of two possible

racemates. Weinges (90) has also prepared the 2,3-trans, 3,4-cis conformation by reducing the proper dihydroflavonol with platinum and hydrogen in methanol. Kulkarni et al (87) also reported the synthesis of the fourth possible racemate by reducing the 3-bromoflavanone with lithium aluminum hydride and replacing the bromo group by boiling the reduction product with alkali in dioxane.

The simplest method possible was chosen for preparing leuco-cyanidin and 7,3'4'-trihydroxyflavan-3,4-diol as the identification of the unknown plant components depends on converting the leuco-anthocyanidins to anthocyanidins. The particular conformation resulting from the chosen synthesis was not important due to the loss of optical activity upon converting the leuco-anthocyanidins to anthocyanidins. Further studies should be made on these plant fractions, however, to determine the conformation at the three asymmetric centers of the active components.

Synthesis of leuco-cyanidin entailed a two step reduction. First, quercetin was reduced with sodium hydrosulfite, according to the method of Geissman and Lischner (91), to obtain dihydroquercetin. Secondly, the dihydroquercetin was reduced with sodium borohydride to give the leuco-cyanidin (22, 88). The yield was very low from the first step and was consequently repeated twice. The second step proceeded almost quantitatively.

Procedure

Quercetin (10.0 grams) and sodium carbonate (85.0 grams) were placed in a 3-necked 2-liter round bottom flask. One liter of boiling distilled water was added to form a dark brown mixture to which 200.0 grams of sodium hydrosulfite was added slowly with heating and stirring maintained by a hot plate-magnetic stirrer combination. The solution gradually changed from a brown to a yellow orange color after 30 minutes. The mixture was cooled, and acidified with concentrated hydrochloric acid, which produced a heavy yellow precipitate. The large yellow precipitate (unreacted quercetin) was filtered through Whatman No. 52 filter paper and reacted again with 200.0 grams of sodium hydrosulfite as indicated above. The yellow filtrate was extracted in two 500 ml capacity liquid-liquid extractors with ethyl acetate for 26 hours. The combined extracts were distilled under a vacuum to a residue, and 75 ml of hot water was added, heated on a steam bath, and filtered. The filtrate was treated with 100 milligrams of Norite (activated charcoal), heated on a steam bath and filtered a second time. The filtrate was placed in a ice bath for 4 hours giving a tan crystalline appearing substance on the bottom of the beaker. The crystalline material was filtered off and the filtrate was evaporated further on the steam bath which yielded, upon cooling, a second crop of crystals. The melting point of the first crop was 228-232°C dec., and the second 223-225°C dec. The two crops were combined, dried

in an oven at 50°C, and weighed 0.21 grams. The second reduction of quercetin gave a higher yield of dihydroquercetin and when combined with the first run, the total yield of dihydroquercetin was 1.26 grams, and the melting point range of the crystalline crops was 225-229°C dec. The crude product gave a positive red color with magnesium and hydrochloric acid.

Crude dihydroquercetin (1.20 grams) was dissolved in 100 ml of methanol (AR), in a 250 ml beaker. The brown solution, after being cooled in an ice-salt bath, was treated with 0.36 grams of sodium borohydride (98%), and allowed to stand for 24 hours. Upon the initial addition of sodium borohydride, a vigorous effervescence occurred and the solution turned yellow at once. After 2-3 minutes the effervescence subsided. The mixture, after 24 hours, was acidified with 12 ml of 10% acetic acid, and distilled under vacuum (26°C) leaving a yellow-brown residue, which was dried in a dessicator for 24 hours. The crude yellow-brown powder weighed 1.50 grams. The crude leuco-anthocyanidin was partially purified by dissolving in hot water and cooling in an ice bath, which deposited a tan crystalline (0.10 grams) substance (dihydroquercetin). The tan filtrate was distilled under vacuum leaving 1.42 grams of tan amorphous substance. This substance was used throughout this investigation as a control leuco-anthocyanidin for comparison to the unknown plant fractions. It was also converted to cyanidin and proved to be identical to a commercially obtained

leuco-cyanidin, which was also converted to cyanidin.

Synthesis of Leuco-robinetinidin

Leuco-robinetinidin (7,3',4',5'-tetrahydroxyflavan-3,4-diol) was prepared from commercially obtained dihydrorobinetin (XXVII) (m.p.dec.) by reduction with sodium borohydride in methanol. The procedure was adopted from the reduction of dihydroquercetin to leuco-cyanidin with sodium borohydride.

Dihydrorobinetin (0.070 g) was dissolved in methanol (AR) (10 ml), in a 50 ml beaker and cooled in an ice-salt mixture. Sodium borohydride (98%) (0.030 g) was added slowly and the reaction proceeded for 24 hours. The mixture was acidified with 10% acetic acid (1.0 ml). 2.0 ml of this mixture was concentrated under vacuum yielding a crude tan residue. The balance of the reaction mixture was saved for conversion to the anthocyanidin in isopropyl alcohol-hydrochloric acid. The crude tan leuco-robinetinidin mixture was saved for paper chromatography characterization.

Synthesis of Racemic
(±)-7, 3', 4'-trihydroxyflavan-3,4-diol

The reduction method used for preparing leuco-fisetinidin (±)-7,3',4'-trihydroxyflavan-3,4-diol) from commercial dihydrofisetin (XIX) (m. p.222-225°C, uncorrected) was identical to the one used for reducing dihydroquercetin and dihydrorobinetin.

Dihydrofisetin (0.070 g) was treated with sodium borohydride (0.030 g) in 15 ml of methanol (AR), for 24 hours. At the end of this period, the mixture was acidified with 10% acetic acid (1.0 ml),

and 2.5 ml of this mixture was concentrated under vacuum leaving a pale yellow residue which was saved for paper chromatography characterization. The remainder of the mixture (10.5 ml) was saved for conversion to fisetinidin by refluxing in isopropyl alcohol-hydrochloric acid.

Conversion of the Synthetic Leuco-anthocyanidins to Anthocyanidins

The crude methanol mixtures set aside from the reduction of dihydrorobinetin and dihydrofisetin for conversion to robinetinidin and fisetinidin were both refluxed for 1-1/2 hours in 100 ml of isopropyl alcohol containing 5% hydrochloric acid. Leuco-robinetinidin turned gradually to a red color, while leuco-fisetinidin immediately turned to a lavender color, gradually becoming pink-red, after refluxing for one-half hour. The reaction mixture was then treated as previously described for the plant fractions (page 78). The final n-butanol extracts were red and pink for the robinetinidin and fisetinidin respectively.

Comparison By Paper Chromatography of the Synthetic to the Unknown Anthocyanidins

Fractions B, C, and D which had previously been converted to anthocyanidins and examined on paper chromatography (Table 8), were now examined in two solvent systems and compared to robinetinidin, fisetinidin, and cyanidin. Fraction A was not tested previously for anthocyanidins due to negative results on paper chromatography

for leuco-anthocyanidins. However, in order to completely cover the upper phase countercurrent fractions, fraction A was subjected to the anthocyanidin coversion and paper chromatography procedures at this time.

The procedures followed were the same as before except the paper was not saturated in the tank for 24 hours before solvent irrigation. The paper chromatograms were determined in Forestal and formic acid-hydrochloric acid solvent systems. 2N-hydrochloric acid system was also employed, but met with little success due to the immobility of the anthocyanidins in this system.

Fractions B, C, and D (R_f values 0.89, 0.88, 0.88) spots were identical to fisetinidin (R_f 0.89) in the Forestal solvent system. The spots were pink to red in visible light and a dull orange color under ultraviolet light. Robinetinidin and cyanidin were both red in visible light and could not be seen under ultraviolet light. Fraction A did not show any spots in visible light, but ultraviolet light revealed a pale yellow spot (R_f 0.73); a faint dull orange spot (R_f 0.90) resembling fisetinidin, although barely visible compared to fraction B, C, and D; and a yellow spot (R_f0.97). See Table 10.

In the formic acid-hydrochloric acid solvent system, fractions B, C, and D (R_f values 0.45, 0.44, 0.45) were also identical to fisetinidin (R_f 0.45) as they were in the Forestal system. The spots were again pink in visible light and dull orange in ultra-

violet light. The robinetinidin and cyanidin spots (R_f 0.31, 0.23) were red in visible light and a faint rose color under ultraviolet light.

Visible Spectrophotometric Analysis of the Anthocyanidin Spots Before and After the Addition of Aluminum Sulfate.

Final proof of structure may also be established comparing the unknown anthocyanidins to the synthetic anthocyanidins, by using the method of Bradfield and Flood (92) for analyzing spots on paper chromatograms without eluting the spot first. They were able to analyze flavonoid spots directly on the paper in ultraviolet light with accuracy to about 3-5 millimicrons. Roux (93) employed this procedure to anthocyanidin spots obtained from leucoanthocyanidins in visible light, and then obtained the absorption again after the addition of 5% aluminum sulfate to the spot on the paper. Anthocyanidins absorb at different wavelengths in visible light and some also shift to longer wavelengths with aluminum sulfate, although of different increments.

The anthocyanidin spots were cut from the paper in thin strips so that they would fit into the silica cuvettes. A blank strip was cut and inserted in a cuvette for the blank sample. The spectra were obtained on a Perkin-Elmer Model 202 Recording Ultraviolet-Visible Spectrophotometer. After obtaining the visible spectrum of each spot, several drops of 5% aluminum sulfate solution were added and the strip was air-dried and the visible

TABLE 10

R_f VALUES OF SELECTED SYNTHETIC ANTHOCYANIDINS COMPARED TO FRACTIONS A, B, C, AND D

Fraction	Forestal		Formic Acid-HCl	
	Visible	UV	Visible	UV
A	(a)	0.73PY 0.90DO (b) 0.97Y	(c)	
B	0.89P	0.89DO 0.95FY	0.45P	0.45DO 0.58PY
C	0.88P	0.88DO 0.96FY	0.44P	0.44DO
D	0.88P	0.88DO 0.95FY	0.44P	0.44DO
Fisetinidin	0.89P	0.89DO 0.96FY	0.45P	0.13PY 0.45DO
Robinetinidin	0.80R	(a)	0.31R	0.31R FRo
Cyanidin	0.76R	(a)	0.23R	0.23R FRo

Abbreviations:

P - Pink
R - Red
Y - Yellow
PY - Pale Yellow
DO - Dull Orange
FY - Fluorescent Yellow
FRo - Faint Rose

(a) There were not any spots observed.

(b) The dull orange color was only faintly observed under UV.

(c) R_f value was not obtained in this solvent system.

absorption was obtained a second time. Fraction B, C, D, and fisetinidin all had a similar absorption peak, while robinetinidin and cyanidin differed slightly. See Table 11. However, the peak absorption did not shift towards a longer wavelength upon the addition of aluminum sulfate, but remained the same. The identical absorption curves in visible light of fractions B, C, D and fisetinidin establishes further the presence of 7,3'4'-trihydroxyflavan-3,4-diol in Acacia angustissima.

TABLE 11

VISIBLE ABSORPTION MAXIMUM OF THE ANTHOCYANIDIN SPOTS

Fraction	Absorption Maximum (millimicrons)
B	530
C	529
D	530
Fisetinidin	529
Robinetinidin	540
Cyanidin	549

SUMMARY AND CONCLUSIONS

The pulverized leaves, stems, and flowering parts of Acacia angustissima have been the subject of a phytochemical investigation for possible anti-tumor constituents. The petroleum ether extract was saponified and separated into saponified and non-saponified fractions. The original petroleum ether extract and the non-saponified fraction contained possible sterols, or carotenes and xanthophylls.

The defatted ethanol extract was partially purified with a solvent fractionation procedure that yielded an amorphous condensed tannin residue. The amorphous fraction was resolved into seven fractions by employing a 100 stage countercurrent distribution procedure. These fractions were examined in ultraviolet and infrared light, and by paper chromatography to determine the chemical qualitative characteristics.

One of the seven fractions (fraction D) was degraded by fusing with potassium hydroxide, and the degradation products, B-resorcylic and protocatechuic acids, were isolated and identified by paper chromatography in four solvent systems. The two acids, along with the leuco-anthocyanidin properties demonstrated by paper chromatography, indicated a tentative structure of 7, 3', 4'-trihydroxyflavan-3,4-diol for fraction D. Assuming that leuco-

anthocyanidins were present in fractions C and D, and possibly in fractions A and B, the four countercurrent upper phase fractions (A, B, C, D) were converted to anthocyanidins and compared by paper chromatography in two solvent systems to the synthetic anthocyanidins, fisetinidin, robinetinidin, and cyanidin. Fraction B, C, and D were found to be identical to fisetinidin. The anthocyanidin spots were examined on the paper in visible light with a spectrophotometer. Fractions B, C, and D and fisetinidin all had identical absorption peaks at 529-530 millimicrons. Therefore, the degradative studies, the identical R_f values compared to fisetinidin in two solvent systems, and the identical absorption peaks in visible light compared to fisetinidin, established the presence of 7, 3', 4'-trihydroxyflavan-3,4-diol in fractions B, C, and D.

Further studies are being conducted to determine the extent of polymerization of the leuco-anthocyanidin unit 7, 3', 4'-trihydroxyflavan-3,4-diol in fractions B, C, and D, and to isolate, if possible, the monomer leuco-anthocyanidin. A difference was observed when comparing the three fractions by paper chromatography, which indicates that the three fractions are all polymerized to a different degree.

A fourth countercurrent fraction exhibited different flavonoid characteristics in ultraviolet light from the other three condensed tannin fractions by giving a broad absorption

band at approximately 350 millimicrons. The ultraviolet spectrum, paper chromatography, and qualitative characteristics of this fraction indicate a flavonol or flavone. A more extensive study is now being conducted to determine the identity of this component.

Pharmocological screening, by the anti-tumor division of the Cancer Chemotherapy National Service Center, of the aqueous and ethanol extracts of the plant, revealed, in addition to the anti-tumor properties, a sedative and hypnotic effect in animals. The plant is currently under investigation to determine what subsequent plant fractions and components possess this hypnotic and sedative effect.

The anti-tumor testing of the plant fractions has been done by the Cancer Chemotherapy National Service Center. The anti-tumor activity resides in the defatted ethanol extract of the plant. Subsequent fractionation of the ethanol extract has yielded active fractions, although the activity has not been conferred on any single component. Further isolation of the larger quantities of the various fractions and further anti-tumor studies are now being undertaken to determine if the activity resides in the condensed tannin-leuco-anthocyanidin fractions, or in other flavan or flavonoid compounds in Acacia angustissima.

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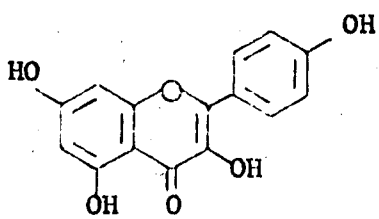
APPENDIX



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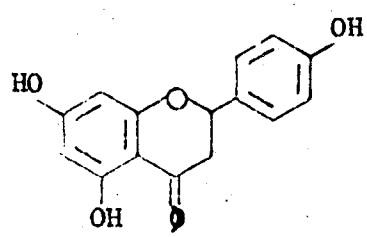
Kaempferol



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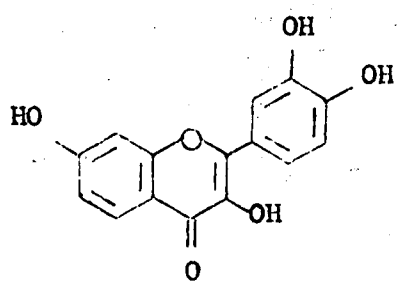
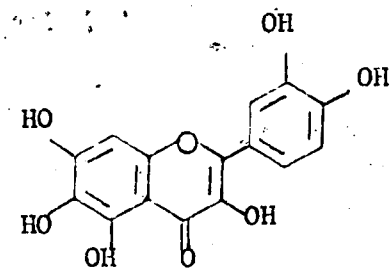
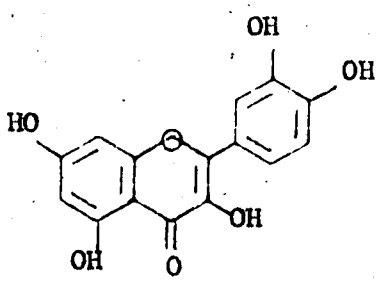


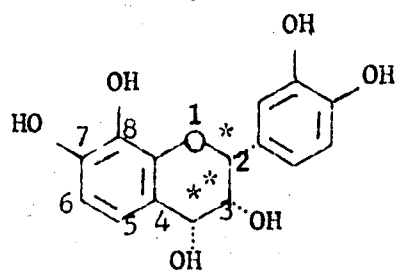
III
Fisetin



IV
Quercetagetin



V
Quercetin



VI
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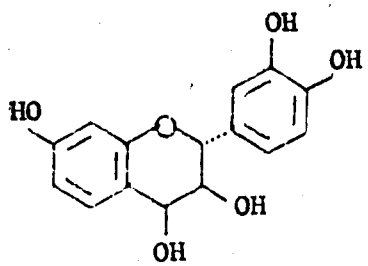
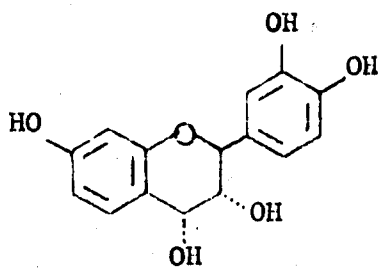
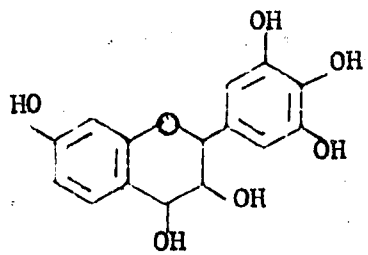


VII
(+) - Mollisacacidin



VIII
(-) - Mollisacacidin
(leuco-fisetinidin)



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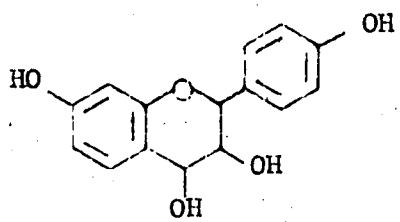
leuco-robinetinidin



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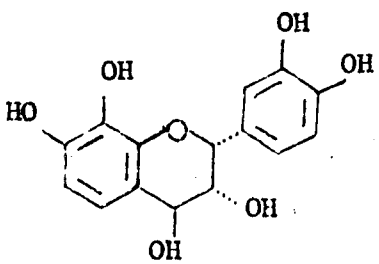
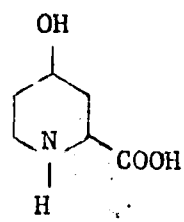


XI
Isomelacacidin



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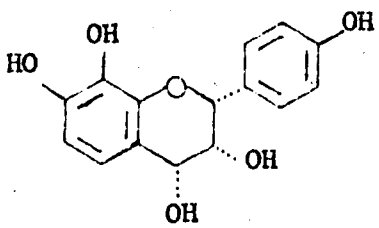
4-Hydroxypipecolic Acid



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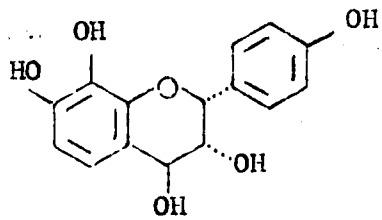
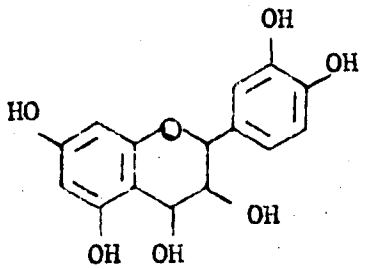
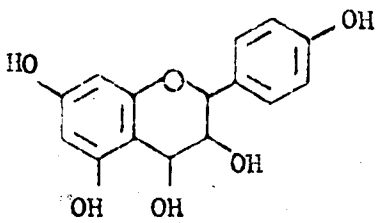
(-) -Teracacidin



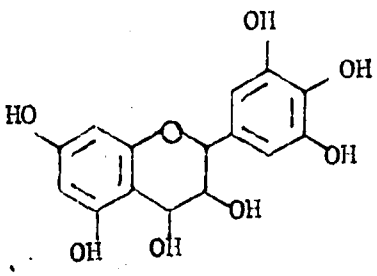
XIV
Isoteracacidin



XV
(+) Leuco-cyanidin



XVI
Leuco-Pelargonidin



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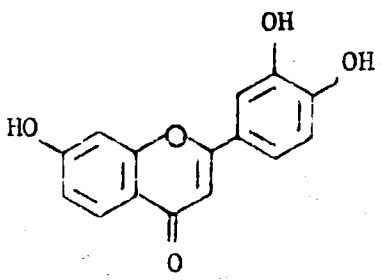
Leuco-delphinidin



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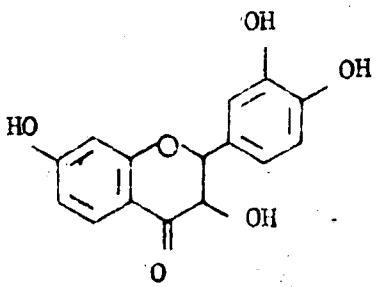
Flavone



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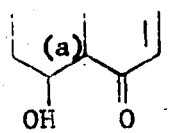

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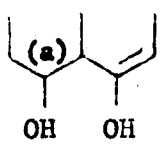
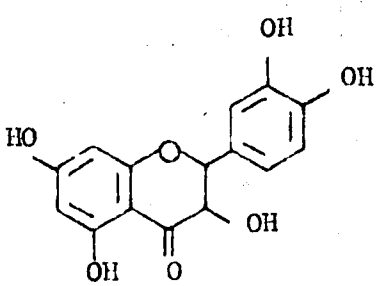


XXI



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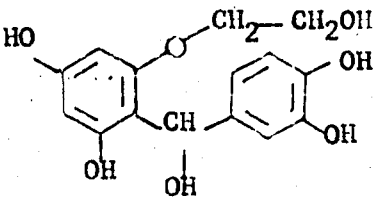

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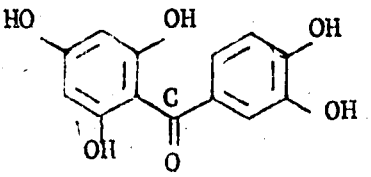
1-Leucomaclurin-glycol ether



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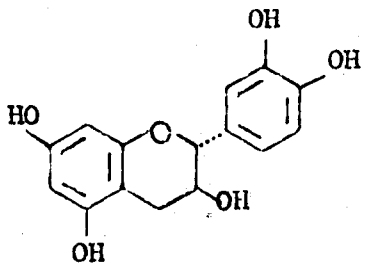

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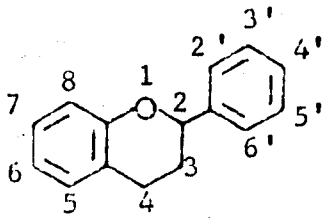

XXVI
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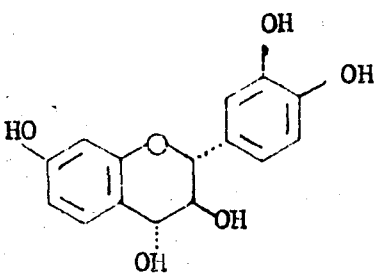

XXVI
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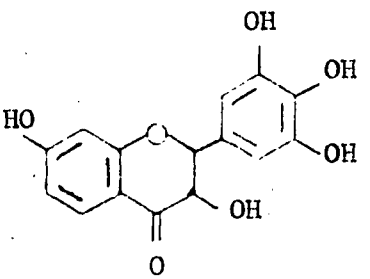

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