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A STUDY OF SIMPLE PLANT PHENOLIC COMPOUNDS IN TOBACCO FLOWERS AND LEAVES

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A STUDY OF SIMPLE PLANT PHENOLIC COMPOUNDS IN TOBACCO FLOWERS AND LEAVES

CHAPTER I

INTRODUCTION

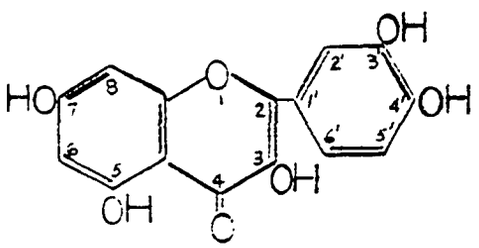
The simple plant phenolic compounds represent a heterogenous group of substances which may be classified according to structure as flavonoids, coumarins, depsides, and phenolic acids. A typical representative of each class is illustrated in Figure 1. Examination of their structure suggests a possible interrelationship among the various classes of compounds and their biosynthesis in the plant. With exception of the depsides, all these compounds probably exist in the plant cells as glycosides.

The functions of these compounds in plant cells are not known exactly, but they may be precursors to lignin (1) and tannins (2) and may be involved in oxidation-reduction reactions (3). Recently, it has been shown that the depside quinyl-p-coumarate (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(4-hydroxycinnamate)) (4) and certain flavonoids (5,6) are cofactors for the enzyme indoleacetic acid oxidase.

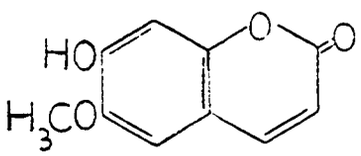
With few exceptions these compounds on paper and column chromatograms possess the property of fluorescence upon irradiation by ultraviolet light. This fluorescent property was used as the initial

FIGURE 1

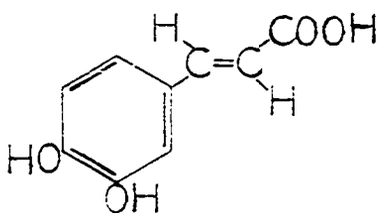
SIMPLE POLYPHENOLIC COMPOUNDS IN TOBACCO



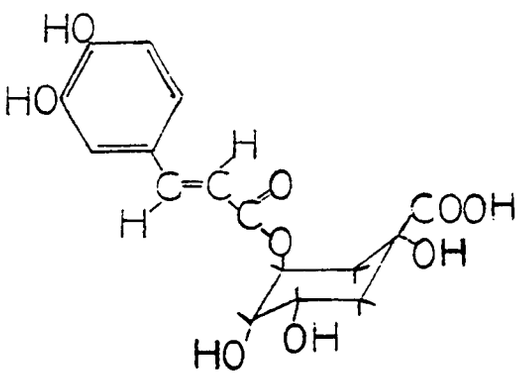
Quercetin (a Flavonoid)



Scopoletin (a Coumarin)



Caffeic Acid (a Phenolic Acid)



Chlorogenic Acid (a Depside)

basis for the isolation and identification of the simple phenols to be described in subsequent chapters.

A comprehensive review of the known phenolic compounds isolated from green, flue-cured, or dry tobacco leaves was given by Stedman (7). His list of phenolic substances, identified by various workers, were rutin (3,3',4',5,7-pentahydroxyflavone-3-rhamnoglucoside or quercetin-3-rhamnoglucoside), isoquercitrin (quercetin-3-monoglucoside), scopoletin (7-hydroxy-6-methoxycoumarin), scopolin (scopoletin-7-monoglucoside), and chlorogenic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)) (8). Subsequently nictoflorin (3,4',5,7-tetrahydroxyflavone-3-rhamnoglucoside or kaempferol-3-rhamnoglucoside) (9), quinyl-p-coumarate (3) and methyl ethers of quercetin (10) have been reported to be present in tobacco leaves. Investigations on tobacco flowers have been less extensive, and prior to 1956 only the flavonoids rutin (11), isoquercitrin (12) and nictoflorin (13) had been isolated.

In order to obtain a more complete compilation of plant phenolic substances present in tobacco tissues, an extensive study on the isolation and identification of these substances in tobacco flowers has been undertaken in this laboratory. In addition to the flavonoids rutin, nictoflorin, and isoquercitrin, the following compounds have been identified previously: esculetin (6,7-dihydroxycoumarin) (14), scopoletin (15), methyl ethers of quercetin (16), and chlorogenic acid (15). Utilizing the above knowledge of the occurrence of these compounds, Murphy (10) made an initial survey by paper chromatography of the relative distribution of these substances in the four major parts of the flower: calyx, corolla, stamen and pistil (Figure 2). A tobacco flower consists

of five stamens and one of each of the following: corolla, calyx, pistil, receptacle and pedicel.

Information on the distribution of phenolic compounds in each floral part could aid in understanding biochemically the differences

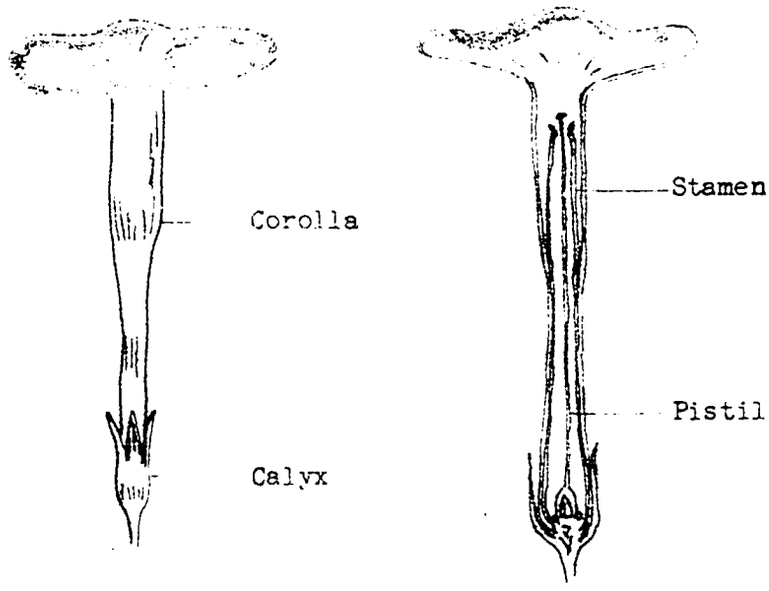


FIGURE 2. A SKETCH OF A TOBACCO FLOWER

between the classes of organs, and differences between species of plants. The inability of two varieties of Forsythia to cross-pollinate has been attributed by Kuhn and Low (17) to the fact that the pollen of one variety contained rutin and the other quercitrin. The extension of this initial study by Murphy on the floral parts of tobacco is described for stamens, corollas, calyxes and pistils in Chapters III, IV, V, VI and VII, respectively.

The indispensable requirement of all plants for small quantities of the element, boron, is well known (18). The exact physiological and biochemical role of this element in plant cells is not understood. It

has been suggested that boron may be involved in the translocation of carbohydrates in the plant (19). In 1958, investigators at Argonne National Laboratory observed on paper chromatograms that extracts of tobacco tissues from plants grown in boron free nutrient medium contained a greater amount of an unknown blue-fluorescing substance than corresponding tissues from plants cultured in normal nutrient medium. A cooperative program between these scientists and this laboratory was established to determine the nature of this compound. The isolation and identification of this blue fluorescent compound is described in Chapter VIII. A preliminary report of this work has been published (20).

CHAPTER II

GENERAL EXPERIMENTAL PROCEDURES

Source of Plant Material

All the plant material used in the present study was cultured in a controlled environment room or in the greenhouse at Argonne National Laboratory. At harvest, the plant tissues were macerated in hot isopropyl alcohol and the slurry cooled to room temperature and put into plastic containers. The plastic bottles containing the homogenized tissues were shipped to this laboratory. For the floral parts study, each fully opened flower was dissected manually into four major parts, and each part was placed into separate containers containing isopropyl alcohol. The receptacles and pedicels were not included in this study. Since the stamens were epipetalous, portions of the filament were included in the corolla tissues. For the current studies, careful selection for uniformity in floral development was not made.

Extraction of Plant Material

Upon receipt of the tissue homogenate, the material was stored in the refrigerator until ready for use. All tissues were exhaustively extracted at room temperature by percolating fresh solvent through the ground tissue in a glass funnel. Initially 50% isopropyl alcohol-water solvent was used, followed with solvents which progressively increased

in alcohol content to 85% isopropyl alcohol. The material was finally extracted with a water-saturated solvent mixture containing equal volumes of isopropyl alcohol, n-butyl alcohol, benzene, chloroform, ethyl acetate and methyl alcohol. The extraction was terminated when the final eluate and the ground tissue were nearly colorless. Approximately 3 liters of solvent were used for each extraction.

The combined extract was concentrated in vacuo at 25-30° C. to remove most of the organic solvents. The apparatus used for the concentration is briefly described in the following section. The aqueous concentrate was extracted with several small volumes of redistilled benzene to remove pigments and lipid material. After benzene extraction, the aqueous phase was extracted with several small volumes of chloroform. The final aqueous phase was adjusted in volume with methyl alcohol such that 1 ml. of concentrate represented 300-500 mg. of fresh weight of tissue. Since the fresh weight of the flower parts could not be determined, each extract of the flower parts was made up to 200 ml. The subsequent treatment of the various extracts is described in the following chapters.

Liquid Concentrator

The concentration of all extracts and eluates described in this study was carried out in the apparatus illustrated in Figure 3. The concentrator consists of two round-bottom flasks, A and B, which have ground-glass, standard tapered joints and a "U" tube, which has ground-glass, standard tapered joints at each end and an evacuation outlet. Flask B is twice the volume of flask A. The ground joints were fitted

with polytetrafluorethylene standard-tapered sleeves to aid in holding vacuum and to prevent "freezing" of the joints.

In operation, the material to be concentrated is placed in flask A; both flasks, A and B, are attached to the "U" tube; and the entire system is evacuated by connecting the vacuum outlet to a water aspirator.

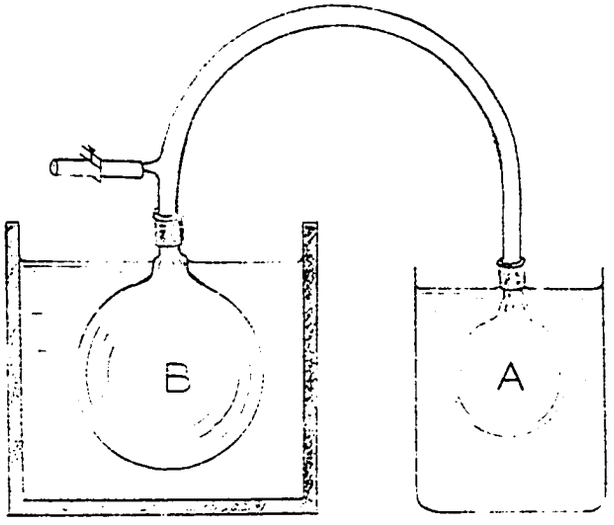


FIGURE 3. LIQUID CONCENTRATOR

Initially the system is pumped down carefully to prevent bumping of the liquid during the degassing stage. After all the trapped gases in the solution in flask A have been removed, flask B is heated in a steam bath. The pinchclamp on the rubber outlet is closed and the system disconnected from the aspirator. Flask B is cooled under tap water. The evacuated concentrator is placed into two baths; flask B is clamped into a 0-5° C. refrigerated water bath, and flask A is set into a thermostatically regulated 25-30° C. water bath. The concentrator is left in the baths until the solvent in flask A has distilled into flask B. In the case of aqueous solutions, isopropyl alcohol is added to a 30-50% concentration to facil-

itate the distillation.

This method of concentration of plant extracts has many advantages over other conventional methods. The greatest advantage of the method is that the material in flask A is never heated above 25-30° C. throughout the distillation. In other techniques, the material left in the distillation flask after removal of the solvent may become overheated unless the process is closely watched. The current method has additional advantages in that it requires no further attention during distillation; it conserves water, since the aspirator is used for only a short time; and virtually all the solvent may be recovered. The method has the disadvantage of being slow in concentrating, but this creates very little difficulty since the distillation can be left overnight.

Paper Chromatography Techniques

Whatman No. 1 chromatography paper was used in most of the present studies and will be referred to hereafter as chromatography paper or paper strips, unless indicated otherwise. Descending development of the paper was used unless specified otherwise. The irrigating solvents used in development of the papers will be indicated with the following abbreviations or notations:

BAW -- n-butyl alcohol-acetic acid-water (6:1:2 v/v/v). The irrigating and equilibration solvents were the same.

IFW -- isopropyl alcohol-formic acid-water (5:0.1:95 v/v/v). The irrigating and equilibration solvents were the same.

Phenol -- water-saturated phenol solution. The equilibration solvent was water saturated with phenol.

CIW -- chloroform-isobutyl alcohol-water (20:50:5 v/v/v).

The equilibration solvent was water saturated with isobutyl alcohol.

BAAW -- n-butyl acetate-acetic acid-water (4:1:5 v/v/v). The irrigating solvent was the upper phase. The paper must be equilibrated with the lower phase for at least 40 hours prior to the addition of the irrigating solvent.

CAW -- chloroform-acetic acid-water (2:1:1 v/v/v). The lower phase was used as the irrigating solvent.

EFW -- ethyl acetate-formic acid-water (10:2:3 v/v/v). The irrigating and equilibration solvent were the same.

BPBW -- n-butyl alcohol-pyridine-benzene-water (5:3:1:3 v/v/v/v). The upper phase was used for irrigation, and the lower phase was used as equilibration solvent.

WAB -- water-acetic acid-benzene (3:7:6 v/v/v). The upper phase was used for irrigation, and the lower phase was used as equilibration solvent.

BPS -- n-butyl alcohol-pyridine-water saturated with NaCl (1:1:2 v/v/v). The organic phase was used as irrigating solvent, and the lower phase was used as equilibration solvent.

BAm -- n-butyl alcohol-2 N ammonium hydroxide (1:1 v/v). The irrigating and equilibration solvents were the same.

2%, 15%, 30%, 40% and 60% acetic acid solutions. The number represents the volume of glacial acetic acid diluted with water to make 100 ml. of solution.

Mass Paper Chromatographic Separation

The separation of the various polyphenolic substances present in each extract was carried out by extensive paper partition chromatography using different irrigating solvents. Initially a small aliquot of each concentrated extract was streaked as a 1 inch band across the top of 9 inch wide chromatographic paper. The amount of extract applied to each paper must be determined by trial but, in general, it has been found that

a volume representing 300-500 mg. of fresh weight tissue will give reasonable resolution of the various desired compounds.

Each paper was developed in the BAW solvent system until the solvent front reached within 1 inch from the bottom of the paper. This process of streaking and developing of papers was continued until all the individual extract was used. In most cases, several hundred 9 inch paper strips were required for each extract. The various fluorescent zones on the chromatograms were observed with the aid of U.V. light (3660 Å, Blak-Ray, Ultra-Violet Prod. Inc., San Gabriel, California). Corresponding zones from each of the chromatograms were cut out and combined. The strips of paper were eluted in an elution chamber with 50% isopropyl alcohol-water solvent or other appropriate solvents. The eluates from each strip were combined and reduced to dryness in vacuo. The resulting residue was extracted with several small volumes of methyl alcohol, and the alcohol extracts were combined and rechromatographed on paper using different solvent systems. The alcohol insoluble residue was dissolved with a small volume of 50% acetic acid solution and rechromatographed on paper to separate additional amounts of desired compounds. The specific treatment of each alcohol and acetic acid extract will be described under appropriate sections in subsequent chapters.

The colors used to describe zones or spots present on chromatograms will refer hereafter to the appearance of the compounds viewed under 3660 Å U.V. light, unless specified otherwise. The fluorescent zones or bands which were present in each group of chromatograms were numbered arbitrarily. A few selected zones from each group of chromatograms were used in the current study. The remaining zones were omitted since they

appeared to be present in small amounts.

Hydrolysis and Identification Procedure for Unknown Compounds

Since the quantity of plant tissues used in the current studies was limited, a pure crystalline sample of each isolated polyphenolic compound could not be prepared. In order to hydrolyze the unknown compound, an aliquot of a solution containing the purified substance was reduced to dryness *in vacuo* and the residue refluxed for a short time in 1 ml. of hot 1 N HCl solution. The acid solution was cooled to room temperature and extracted with ethyl acetate or n-butyl acetate solvent by mixing the two solutions and then separating the two phases by freezing the aqueous layer in a deepfreeze chest. The aqueous solution was extracted at least twice by this procedure. The extracted aqueous phase was refluxed for an additional 1/2 hour to hydrolyze completely any disaccharide which may have been present.

The aqueous and organic solutions were reduced separately to dryness *in vacuo*. Each residue was dissolved in aqueous alcohol solution, spotted on separate chromatography paper alongside pure known compounds and developed in various solvent systems. For the identification of the unknown aglycone portion of a flavonoid, quercetin and kaempferol were used as reference standards. The chromatograms containing the unknown and reference standards were examined under U.V. light with and without ammonia fumes to determine the relative positions of the fluorescing substances and to distinguish differences and similarities in fluorescence between the compounds present. Pure glucose and rhamnose were used as reference standards for the identification of the monosaccharides. To

determine the position of the sugars, the chromatogram was sprayed with o-aminobiphenyl reagent (21), allowed to dry in air for a short time, and finally heated in an oven at 110° C. for a few minutes to develop the characteristic colors for each sugar. Pure standards of ferulic, isoferulic, caffeic, sinapic, p-coumaric and o-coumaric acids were used to aid in identifying cinnamic acid derivatives. The chromatograms containing the cinnamic acids were viewed under U.V. light to note R_f values and the characteristic fluorescence of known and unknown compounds, and each was then sprayed with diazotized sulfanilic acid reagent (22) to distinguish between cinnamic acids which had similar R_f values.

Similarities between the unknown hydrolysis product and a pure reference standard with respect to R_f values, appearance under U.V. light and color reactions with chromogenic sprays were used as criteria for identification of the unknown hydrolysis product. In certain instances, ultraviolet absorption spectrum was used to aid in the identification.

Absorption Measurements

All ultraviolet absorption spectra were determined over the range 210-420 mμ on a Beckmann Model DK-1 Recording Spectrophotometer or on a Beckmann Model DU Spectrophotometer, using 1 cm. silica cells. For the structural determination of flavonoid glycosides, empirical methods developed by Jurd and Horowitz (23) and by Jurd (24) based on spectral shifts induced by the addition of various chemical reagents to flavonoid solutions were used. Examination of the structure of quercetin (3,3',4', 5,7-pentahydroxyflavone) indicates 5 possible hydroxyl positions where substitutions might occur.

Addition of anhydrous, fused sodium acetate to an ethanolic solution of a flavonoid which is not substituted in the 7-hydroxyl position will produce a bathochromic shift in the short wave-length band by 8-20 mμ ; otherwise, there will be no change or an hypsochromic shift will occur. Compounds which possess free ortho-dihydroxy groups complex with borate ions to produce a bathochromic shift in the long wave-length band by 16-30 mμ. In the case of quercetin derivatives, for example, a bathochromic shift of 16 mμ in the long wave-length band upon the addition of boric acid and sodium acetate would indicate that the 3'- and 4'-hydroxyl positions were unsubstituted.

The reaction of flavonoid compounds with aluminum chloride (25, 26) indicates that the 5-hydroxyl position was unsubstituted.

The appearance of the flavonoid derivative on paper chromatograms irradiated with U.V. light distinguishes, in many cases, whether the 3-hydroxyl position was substituted or not. For instance, derivatives of quercetin and kaempferol, which have free 5-hydroxyl groups, usually appear brown if the 3-hydroxyl position is substituted, otherwise they fluoresce bright yellow.

CHAPTER III

ISOLATION AND IDENTIFICATION OF POLYPHENOLIC COMPOUNDS IN TOBACCO FLOWER STAMENS

Separation of Fluorescent Compounds in Stamen Extract

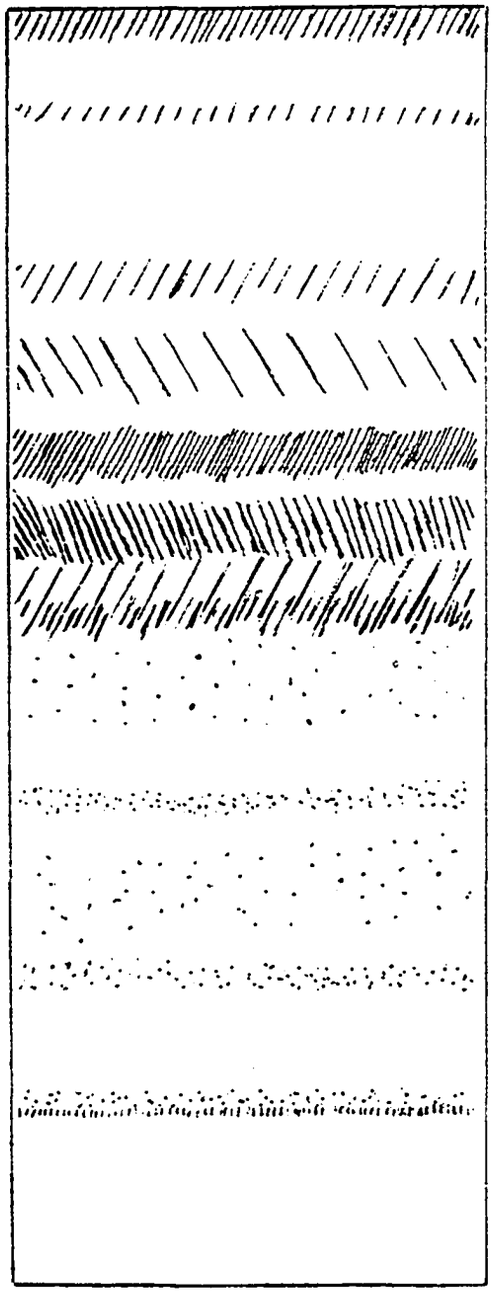
A diagrammatic sketch illustrating the appearance, under U.V. light, of a one-dimensional chromatogram of stamen extract developed in the BAW solvent system is shown in Figure 4. This solvent system separated the extract into 11 fluorescent bands which were not separated completely from each other or represented a single substance. Beginning at the bottom of the chromatogram, the fluorescent zones were labeled consecutively from ST-1 to ST-11.

Studies on Zone ST-1

The substance of main interest in this zone fluoresced blue only in the presence of ammonia vapors. All strips from the original chromatograms containing zone ST-1 were eluted with ethyl acetate-isopropyl alcohol-water (8:1:1 v/v/v) solution in elution chambers. The eluate was reduced to dryness *in vacuo*, and the residue was dissolved with a solvent mixture of ethyl acetate, chloroform and water. The aqueous and organic phases were separated, rechromatographed separately on several paper strips and developed in the 15% acetic acid solvent system. This solvent caused the blue fluorescent substance to spread over the full length of

FIGURE 4

ONE-DIMENSIONAL CHROMATOGRAM OF EXTRACT
FROM TOBACCO FLOWER STAMENS

Zone Number	Descending Development with the BAW Solvent System	Appearance under Ultraviolet light
		Origin
ST-11		Blue
ST-10		Dark Yellow
ST-9		Blue-white
ST-8		Light Green
ST-7		Dark Blue
ST-6		Blue
ST-5		Blue
ST-4		Dark Yellow
ST-3		Blue
ST-2		Light Yellow
ST-1		Blue with Ammonia Vapors
		Solvent Front

the paper and left a mixture of the blue fluorescing compound and a brown impurity at the origin. Each chromatogram was cut into two parts: one part contained the origin and the other contained the wide blue fluorescing zone. Each part was sewed onto fresh chromatography paper and developed in the BAW solvent system. Each blue zone was cut out from the chromatograms and eluted with 50% isopropyl alcohol solution. The eluate was reduced to a small volume, streaked on fresh paper and developed in the BAm solvent system. The blue fluorescing zone was cut from each chromatogram and eluted with 50% isopropyl alcohol. The eluate was reduced to dryness, and the residue was extracted with small portions of methyl alcohol.

A small aliquot of the alcohol solution was hydrolyzed by the method described in a previous section. A small aliquot of the concentrated organic phase was spotted on Schleicher and Schuell No. 589 red ribbon 11 by 11 inch chromatography paper alongside of known cinnamic acid standards. The paper was stapled into a cylinder and developed by the ascending method in the CAW solvent system. The unknown hydrolysis product had an R_f of 0.1 in this solvent. It fluoresced a faint blue, which enhanced in ammonia fumes, and did not react with diazotized sulfanilic acid reagent. It did not correspond to any substance on which data have been found in the literature. The aqueous concentrate was spotted on chromatography paper alongside of glucose, rhamnose and quinic acid and the chromatogram was developed in the BAW solvent system. Upon spraying the developed chromatogram with quinic acid reagent (27) and with the sugar reagent, no visible spot was observed for the unknown substance.

The ultraviolet absorption spectrum of a methanolic solution of the unknown substance had a maximum at 268 mμ and a minimum at 240 mμ. A bathochromic shift occurred with a maximum at 295 mμ and a minimum at 260 mμ when the solution was made alkaline with ammonium hydroxide. Neutralization with HCl restored the original absorption spectrum.

The unknown substance had R_f values of 0.00 in the IFW, 0.95 in the CIW, 0.92 in the BAW, 0.72 in the BAm and 0.95 in the BPS solvent systems. With 15% acetic acid-water solvent, the unknown substance had R_f values of 0.32, 0.58, 0.73, and 0.79. Each of these zones gave an identical ultraviolet absorption spectrum.

From the results of the above studies, this unknown substance does not correspond with known substances previously reported from natural sources according to the literature surveyed.

Studies on Zone ST-3

This zone contained a blue fluorescing substance which had a relatively high R_f value in the BAW solvent system. Strips from the original chromatograms were eluted with 50% isopropyl alcohol-water solvent, and the eluate was concentrated to dryness *in vacuo*. The residue turned brown upon exposure to the atmosphere. Methyl alcohol extracts of the brown residue were rechromatographed on paper using the BAW solvent system. The blue fluorescing zone was cut out and eluted with 50% isopropyl alcohol-water solvent. Attempts to obtain an ultraviolet absorption spectrum of the eluate resulted in a poorly defined spectrum, indicating that the material was not pure. Since the material seemed to be unstable upon chromatographing on paper, further work was discontinued.

Studies on Zone ST-4

Zone ST-4 appeared dark yellow under U.V. light and contained several phenolic substances. One compound of prime interest fluoresced soft blue only in the presence of ammonia vapors. All strips containing this zone were eluted with 50% isopropyl alcohol-water solvent. The eluate was reduced to dryness *in vacuo*, and the residue was extracted with several small volumes of methyl alcohol. The methanolic extract was applied as a series of spots on several paper strips and developed in the CIW solvent system. Examination of these chromatograms under U.V. light revealed the presence of 7 fluorescent zones. Beginning at the bottom of the chromatogram, the bands were labeled consecutively from ST-40 to ST-46.

Studies on Zones ST-42 and ST-43

The two closely moving zones were cut out together and eluted with 50% isopropyl alcohol-water solvent. The eluate was concentrated *in vacuo*; streaked on paper strips; and developed in the CIW solvent system. The eluate was resolved into two separate zones which were labeled ST-421 and ST-422. The two zones were cut out and eluted separately. The eluates were concentrated, streaked on separate paper strips and developed in the BAAW solvent system. Zone ST-421 separated into 6 fluorescent bands and zone ST-422 separated into 4 bands. Since the desired blue fluorescent zone on each paper had an identical R_f value in this solvent, they were considered to be the same chemically but probably were *cis* and *trans* isomers. The desired blue fluorescing zone was cut from each chromatogram and eluted with IFW solvent. The eluate was

reduced to dryness; the residue was extracted with methyl alcohol; and the extract was stored in a vial. The tentative identification of the blue fluorescing substance will be discussed in a subsequent section.

Studies on Zone ST-5

The strips of paper containing this zone were treated in similar manner as were the strips containing ST-4. Chromatographing the eluate from this zone with CIW solvent yielded 7 fluorescent bands which were labeled consecutively, beginning from the bottom of the chromatograms, as ST-50 to ST-56.

Studies on Zone ST-51 and ST-52

These two zones were treated in similar fashion as for zones ST-42 and ST-43 which were described previously. Paper chromatographic and ultraviolet absorption studies indicated that the unknown compound in these zones corresponded to substances from zone ST-4. The materials from the two zones were combined for identification studies.

Isolation and Tentative Identification of Glucosyl-ferulate in Zone ST-53

The strips of paper containing this blue fluorescent zone were eluted, the eluate concentrated, applied to new paper strips, and developed in the BAW solvent system. The desired band was cut out and eluted with 50% isopropyl alcohol-water solvent. The eluate was streaked on paper strips and developed in the CIW solvent system. The blue fluorescing zone was cut out and then eluted. The eluate was concentrated to dryness, and the residue was extracted with methyl alcohol.

The unknown substance had R_f values of 0.58 in the BAW, 0.68,

0.76 in the IFW and 0.65 in the CIW solvent systems (Table 1). Harborne and Corner (28) reported R_f values of 0.57 in BAW and 0.64, 0.73 in water for the hydroxycinnamic acid-sugar derivative, 1-feruloyl-glucose. An absorption spectrum of the unknown substance gave maxima at 250 and 326 mμ and a minima at 240 and 288 mμ. Addition of alkali produced a bathochromic shift with maxima at 304 and 380 mμ and minima at 274 and 328 mμ. Harborne and Corner (28) reported maxima at 240, 300 and 330 mμ for 1-feruloyl-glucose in ethyl alcohol and maxima at 250 and 382 mμ in the presence of sodium ethoxide. The unknown substance fluoresced blue and, in the presence of ammonia vapors, it fluoresced green. Hydrolysis of the unknown compound in acid solution and subsequent examination of the hydrolysis product by paper chromatography revealed that ferulic acid and glucose were the principal products.

The results indicate that the ST-53 is likely a glucoside of ferulic acid.

Identification of p-Coumaroyl-Glucose

An unknown substance from zones ST-42, ST-43, ST-51 and ST-52 had R_f values of 0.73, 0.81 in the IFW, 0.64 in the CIW, 0.68 in the BAW, 0.30 in the BAm and 0.83 in the BPS solvent systems (Table 1). The reported (28) R_f values for 1-p-coumaroyl-glucose in BAW, water and BAm solvents are 0.66, 0.73, 0.81 and 0.30, respectively. An ultraviolet absorption spectrum of the unknown substance gave a maximum at 316 mμ and a minimum at 254 mμ. Harborne and Corner (28) reported two maxima, 235 and 313 mμ, for p-coumaroyl-glucose. Hydrolysis of a small aliquot of the unknown substance with acid and subsequent examination of the

TABLE I

R_f VALUES OF REFERENCE DEPSIDES AND HYDROXY-CINNAMIC ACID COMPOUNDS FROM TOBACCO FLOWER STAMENS

Compounds	Solvent Systems				
	CIW	BAW	BAm	BPS	IFW
Chlorogenic Acid	0.15	0.59	0.01		0.67, 0.84
Quinyl-p-coumarate*	0.16	0.71	0.10	0.58	
ST-53	0.65	0.58			0.68, 0.76
ST-422	0.64	0.68	0.30	0.83	0.73, 0.81

*A pure sample of this compound was kindly supplied by Dr. A. H. Williams, Long Ashton Research Station, Bristol, England.

hydrolysis mixture by paper chromatography indicated that the unknown compound consisted of p-coumaric acid and glucose.

From the results of the current study, it is not possible to determine precisely how glucose and p-coumaric acid were attached since sufficient material was not available to give unequivocal proof of structure. On the basis of the hydrolysis products, R_f values and ultraviolet absorption spectrum, unknown ST-422 is probably 1-p-coumaroyl-glucose.

Studies on Zone ST-7

This zone fluoresced dark blue on chromatograms of the original stamen extract developed in the BAW solvent system. All the strips containing this zone were eluted with 50% isopropyl alcohol-water solvent; the eluate was concentrated *in vacuo*, and the concentrate was streaked on several new paper strips, which were developed in the IFW solvent system. This solvent system resolved the eluate into 9 additional fluorescent zones, which were numbered consecutively from ST-71 to ST-79 beginning from the bottom of the chromatograms.

Studies on Zones ST-77 and ST-78

Zone ST-77 fluoresced brown and zone ST-78 fluoresced blue with some brown fluorescing material mixed in it. The two zones were cut out together since they were not completely separated. The strips of paper containing the two zones were eluted with dilute alcohol; the eluate was concentrated, streaked on several paper strips, and developed with the 15% acetic acid-water solvent system. This solvent produced 5 additional fluorescent bands. The brown fluorescing band was cut out from each chromatogram, and was eluted with dilute alcohol. The eluate was concentrated

to dryness *in vacuo*, and the residue was extracted with methyl alcohol. The alcohol insoluble residue was dissolved with dilute acetic acid solution and rechromatographed in the BAW solvent system. The brown fluorescing bands from these chromatograms were cut out and eluted with dilute alcohol. The eluate was concentrated and rechromatographed in the 15% acetic acid solvent system. The brown fluorescent zone was eluted and concentrated. Then it was extracted with methyl alcohol and combined with the other extract.

Since the methyl alcohol extract contained more than one substance, it was subjected to further purification by rechromatographing with the BAW, the CIW, and the 15% acetic acid solvent systems until a sharp ultraviolet absorption spectrum was obtained. The final brown fluorescing substance was labeled ST-78531. The identification of the brown fluorescing substance will be described in a subsequent section.

Studies on Zone ST-8

This zone fluoresced light green on paper chromatograms irradiated with U.V. light. The paper strips containing this zone were cut out from the stamen extract chromatograms, and were eluted with 50% isopropyl alcohol-water solvent. The eluate was concentrated. Then it was streaked on several paper strips and developed in the IFW solvent system. This solvent resolved the eluate into 7 fluorescent zones which were numbered consecutively ST-80 to ST-87.

Studies on Zones ST-84, ST-85 and ST-86

Since a wide brown fluorescing zone was contaminated with blue fluorescing substances, it was considered expedient to cut this zone into

3 parts to facilitate subsequent purification. Each part was treated essentially in the same manner.

Since the blue and brown fluorescing zones always ran close together in all the solvent systems used for the isolation, the brown zone was carefully cut away from the blue zones and rechromatographed to remove additional amounts of the blue contaminant. The blue zones were rechromatographed several times to recover additional amounts of the brown pigment. By repeating this procedure several times, a brown zone, relatively free of blue contaminant, was obtained. The solvent systems and procedure described for zones ST-77 and ST-78 were used.

The final brown zone was rechromatographed in the CIW solvent system which resolved this zone into two brown zones. The faster moving zone was labeled ST-78531 since its R_f corresponded to the brown pigment from zone 7. The slower moving zone was labeled ST-78532. The tentative identification of the two brown pigments will be described in subsequent sections.

Studies on Zones ST-9 and ST-10

The two fluorescent zones were cut out together and eluted with 50% isopropyl alcohol-water solvent. The eluate was concentrated *in vacuo*, streaked on several paper strips and developed in the IFW solvent system. This solvent separated the two zones into 10 fluorescent bands, which were numbered consecutively ST-101 to ST-109.

Studies on Zone ST-106

Zone ST-106 was a brown fluorescing zone which was between two blue fluorescing bands. The brown zone was cut out and eluted with

dilute alcohol. The eluate was concentrated, streaked on several paper strips, and developed in the 15% acetic acid solvent system. This solvent separated the concentrate into 6 fluorescing zones. The brown zone was cut out along with a small amount of an unknown blue substance and eluted with 50% isopropyl alcohol-water solvent. The eluate was concentrated, streaked on several paper strips and developed in the CIW solvent system. This solvent separated the brown zone into two brown bands, which were labeled ST-78531 and ST-78532.

Identification of Rutin in Tobacco Flower Stamens

The brown pigment, which was isolated from zones 8, 9, and 10 and designated ST-78532, was chromatographed along with reference rutin (quercetin-3-rhamnoglucoside) and nictoflorin (kaempferol-3-rhamnoglucoside) in several solvent systems. The unknown substance had R_f values corresponding closely with rutin (Table 2). Its ultraviolet absorption spectra in ethyl alcohol, in ethyl alcohol with sodium acetate, in ethyl alcohol with boric acid and sodium acetate, and in ethyl alcohol with aluminum chloride corresponded with spectra of rutin under the same conditions. It reacted with ammonium molybdate spray reagent (10) in similar manner as rutin to produce a yellow color in visible light and a brown color in U.V. light which did not change upon exposure to ammonia fumes. Hydrolysis of the unknown pigment in acid solution and subsequent examination of the hydrolysis products yielded only quercetin as the aglycone and the sugars, glucose and rhamnose.

From the results of this study, it was concluded that ST-78532 was rutin.

TABLE 2

R_f VALUES OF REFERENCE FLAVONOID GLYCOSIDES AND FLAVONOID GLYCOSIDES FROM TOBACCO FLOWER STAMENS

Flavonoid Glycosides	Solvent Systems						
	15% Acetic Acid	Water	1FW	CIW	BAAW	Phenol	BAW
Rutin	0.59	0.32	0.33	0.41	0.24	0.55	0.27
ST-78532	0.57	0.30	0.30	0.41	0.25	0.52	0.27
Nictoflorin	0.65	0.32	0.37	0.68	0.46	0.71	0.40
ST-78531	0.67	0.35	0.35	0.52	0.22	0.72	0.29

Tentative Identification of ST-78531 in Tobacco Flower Stamens

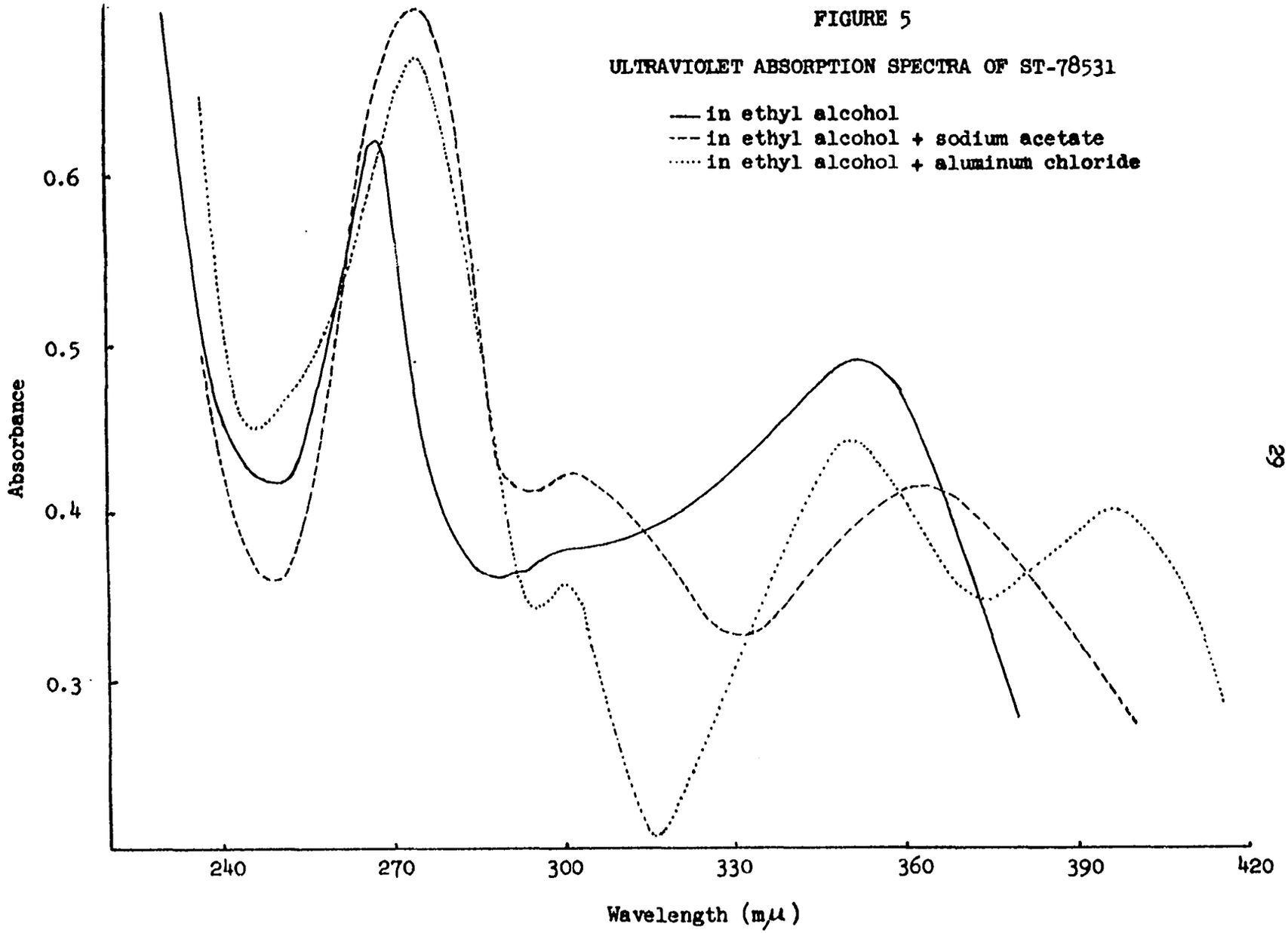
The brown pigment, which was isolated from zones 7 and 8 and designated ST-78531, was chromatographed along with reference rutin and nictoflorin in several solvent systems. The R_f values of this unknown pigment did not correspond exactly with either of the two flavonoid standards in all the solvent systems used (Table 2). The ultraviolet absorption spectra of the unknown pigment in ethyl alcohol (maxima: 268 and 352 mμ) and in ethyl alcohol with aluminum chloride (maxima: 275, 302, 348 and 398 mμ) are shown in Figure 5. Harborne and Sherratt (29) reported the ultraviolet absorption spectra maxima for kaempferol-3-diglucoside from the flowers of Primula sinensis as 267, 298, and 349 mμ in ethyl alcohol and 351 and 395 mμ in ethyl alcohol with aluminum chloride. The double maxima (348 and 398 mμ) produced by the addition of aluminum chloride may be characteristic of kaempferol derivatives. Addition of anhydrous sodium acetate to an alcoholic solution of the unknown pigment produced a bathochromic shift of 8 mμ in the short wave-length band. This indicated that the 7-hydroxyl position of kaempferol was unsubstituted.

Since its R_f values in certain solvent systems were similar to nictoflorin, it was suspected that the sugar attached to kaempferol may be a disaccharide. In order to demonstrate this possibility, partial hydrolysis of the unknown flavonoid was performed by reducing the time for hydrolysis to about 10 to 15 seconds. Examination of a small aliquot of the hydrolysis products by paper chromatography revealed only kaempferol as the aglycone and the sugars, glucose and a substance which reacted

FIGURE 5

ULTRAVIOLET ABSORPTION SPECTRA OF ST-78531

— in ethyl alcohol
--- in ethyl alcohol + sodium acetate
····· in ethyl alcohol + aluminum chloride



typically as a carbohydrate with o-aminobiphenyl reagent and had a R_f value slightly less than rutinose, 6-(β-1-L-rhamnosido)-D-glucose (kindly supplied by Mr. Richard Hagen). The remainder of the aqueous acid solution was heated for an additional 1/2 hour. Subsequent examination of this solution by paper chromatography revealed only the sugar, glucose.

On the basis of the above data, the exact nature of the brown pigment cannot be determined, but from the available information it is suggested that it may be a kaempferol-3-diglucoside. Attempts are currently underway to accumulate sufficient amount of this material to make a quantitative determination of the aglycone:sugar ratio.

Isolation and Identification of Scopolin (scopoletin-7-monoglucoside) in Zones ST-7 and ST-8

The strips of paper containing the zones ST-72, ST-73, ST-81, ST-82 and ST-83 were eluted individually with IFW solvent. Each eluate was concentrated, streaked separately on several chromatographic papers, and developed in the BAW solvent system. This solvent resolved each eluate into at least two major blue bands; one band corresponded in R_f value with esculetin, which will be discussed in a following section, and the other band had an R_f value of about 0.45. This latter band was cut out from each chromatogram and was eluted separately with IFW solvent. Each eluate was concentrated, streaked separately on several paper strips, and developed in the CIW solvent system. Corresponding blue bands in each chromatogram were cut out and eluted with 50% isopropyl alcohol.

An aliquot of the eluate was spotted on a sheet of chromatographic paper and subjected to two-dimensional chromatography using BAW solvent in the first direction and IFW solvent in the second direction. The un-

known substance gave only one bright blue fluorescing spot with R_f values of 0.42 in the BAW and 0.75 in the IFW solvent systems. Another aliquot of the eluate was spotted alongside synthetic scopolin (scopoletin-7-monoglucoside, kindly supplied by Mr. Alexis Zane) on a paper strip and sprayed with a 1% aqueous solution of emulsin. The sprayed paper was incubated in a moist chamber for 1 hour. The paper strip was dried in air and then developed in the BAW solvent system. A blue fluorescent spot (R_f = 0.89) corresponding to scopoletin was produced by the enzyme hydrolysis.

From the result of this study, it is concluded that tobacco flower stamens contain scopolin.

Isolation and Identification of Esculetin (6,7-dihydroxycoumarin) in Zones ST-4, ST-5, ST-6, and ST-7

An aliquot of each eluate from zones 4, 5, 6 and 7 (Figure 4) was streaked separately on several chromatographic papers and developed in the 15% acetic acid solvent system. This solvent resolved the eluates into at least 4 major fluorescent zones. A blue zone (R_f about 0.58) was cut out from each chromatogram and eluted with 50% isopropyl alcohol. The eluate was concentrated, streaked on several paper strips and developed in the BAAW solvent system. The blue fluorescent zone was cut out and eluted with 95% ethyl alcohol.

Small aliquots of the eluate were chromatographed alongside reference scopoletin (7-hydroxy-6-methoxycoumarin, kindly supplied by Dr. H. D. Braymer), esculetin, and caffeic acid (3,4-dihydroxycinnamic acid, purified by Mr. Y. Nakagawa) in various solvent systems. The unknown substance had R_f values of 0.61 in the 15% acetic acid, 0.79 in the BAW,

0.34 in the IFW, 0.77 in the BAAW, and 0.18 in the CAW solvent systems. These R_f values correspond closely to reference esculetin and caffeic acid listed in Table 3. In order to determine whether the unknown substance may be esculetin or caffeic acid, the observation reported by Dieterman and coworkers (14) was utilized. They noted that the fluorescence of caffeic acid was quenched when the chromatogram, still wet with BPBW solvent, was irradiated with long wave-length U.V. light (3660 Å), whereas esculetin fluoresced bluish-yellow. Under the above conditions, the unknown fluoresced similar to reference esculetin. In addition, esculetin fluoresces blue with a faint pink coloration whereas caffeic acid fluoresces with a light-whitish blue color. In aqueous acid solvent systems, caffeic acid undergoes cis-trans isomerization to produce two fluorescent spots (30) whereas esculetin yields only one spot (Table 3). Caffeic acid reacts typically with the ammonium molybdate spray reagent of Kallianos (31) to produce a yellow color in a visible light and a brown color in U.V. light which turns to a green color in ammonia fumes. Esculetin reacts only weakly with the ammonium molybdate to produce a faint yellow color in visible light and a faint blue fluorescence in U.V. light, which indicates that the reagent quenched the fluorescence of esculetin. The unknown substance reacted similar to esculetin with the ammonium molybdate reagent.

It is concluded from the above studies that tobacco flower stamens contain esculetin.

Isolation and Identification of Chlorogenic Acid in Zones ST-5, ST-6 and ST-7

A blue fluorescing band (R_f about 0.73) which was isolated from

TABLE 3

R_f VALUES OF REFERENCE COUMARINS, CHLOROGENIC ACID AND CAFFEIC ACID

Compounds	Solvent Systems				
	15% Acetic Acid	BAW	IFW	BAAW	CAW*
Esculetin	0.63	0.81	0.35	0.80	0.18
Scopoletin	0.69	0.87	0.41	0.89	0.92
Chlorogenic Acid	0.76, 0.83	0.63	0.61, 0.81	0.35	
Scopolin	0.88	0.49	0.77	0.07	
Esculin	0.81	0.53	0.65	0.10	
Caffeic Acid	0.58, 0.76	0.84	0.37	0.75, 0.83	0.14

*The reference compounds were spotted on Schleicher and Schuell No. 589 red ribbon 11 by 11 inch chromatography paper and developed by the ascending method in this solvent system.

the eluates of zones 5, 6 and 7 on chromatograms developed in 15% acetic acid solvent system, was cut out from each chromatogram and eluted with 50% isopropyl alcohol. The eluate was concentrated, streaked on several paper strips and developed in the BAAW solvent system. The blue band was cut out from each chromatogram and eluted with 50% ethyl alcohol.

Small aliquots of the eluate were chromatographed alongside reference chlorogenic acid in various solvent systems. The unknown blue fluorescent substance had R_f values of 0.75, 0.81 in the 15% acetic acid, 0.65 in the BAW, 0.61 in the IFW, and 0.32 in the BAAW solvent systems. These R_f values corresponded with R_f values for reference chlorogrenic acid in Table 3. The unknown substance and reference chlorogenic acid fluoresced blue which changed to yellow-green upon exposure to ammonia fumes. The reference and unknown compounds underwent cis-trans isomerization when they were chromatographed in aqueous acid solvent systems such as 15% acetic acid and IFW solvents. The two isomers are not present in equal quantities so that one isomer may not be readily seen unless exposed to ammonia fumes. The unknown and reference compounds reacted identically with ammonium molybdate spray reagent to give a yellow color in visible light and brown fluorescence in U.V. light which changed to green upon exposure to ammonia fumes.

On the basis of similarity in R_f values, fluorescence and reaction with ammonium molybdate spray reagent between the unknown substance and reference chlorogenic acid, it is concluded that tobacco flower stamens contain chlorogenic acid.

CHAPTER IV

ISOLATION AND IDENTIFICATION OF POLYPHENOLIC COMPOUNDS IN TOBACCO FLOWER COROLLAS

Separation of Fluorescent Compounds in Corolla Extract

A diagrammatic sketch illustrating the appearance, under U.V. light, of a one-dimensional chromatogram of corolla extract developed in the BAW solvent system is shown in Figure 6. This solvent system separated the extract into 12 fluorescent bands which were not separated completely from each other or represented a single substance. Beginning at the bottom of the chromatogram, the fluorescent zones were numbered consecutively from PE-1 to PE-12.

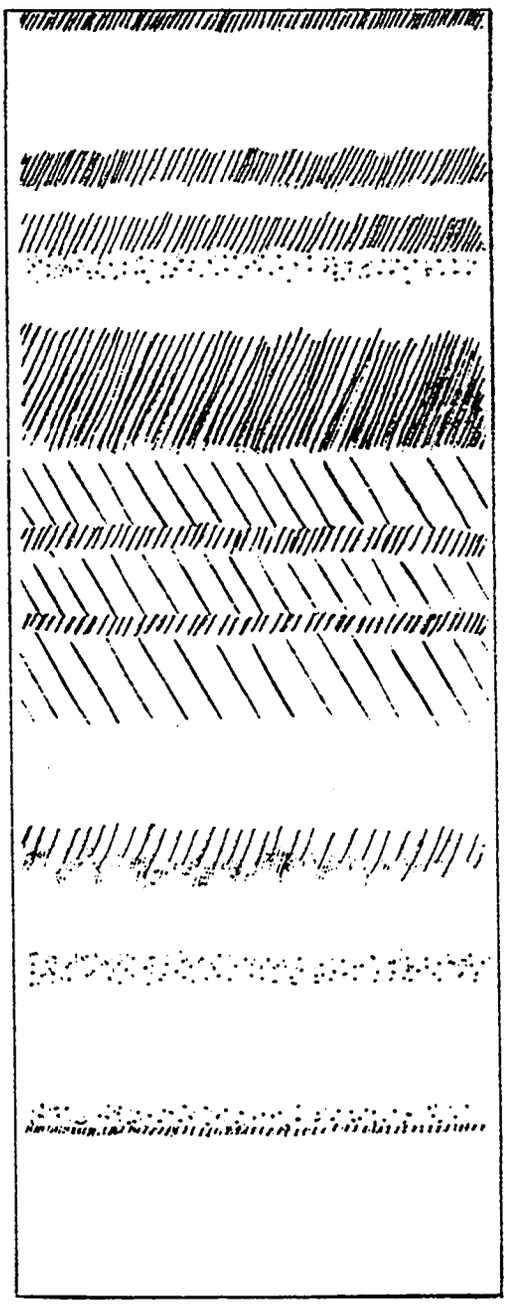
Isolation and Identification of Esculetin (6,7-dihydroxycoumarin) in Zone PE-2

The strips of paper containing the blue fluorescent Zone PE-2 were eluted with 50% isopropyl alcohol. The eluate was concentrated under reduced pressure to dryness. The residue was dissolved in water, and the aqueous solution was extracted with a small volume of ethyl acetate. The ethyl acetate extract was streaked on several paper strips and developed in the BAAW solvent system. The blue zone was cut out from each chromatogram and eluted with 95% ethyl alcohol.

Small aliquots of the eluate were chromatographed alongside refer-

FIGURE 6

ONE-DIMENSIONAL CHROMATOGRAM OF EXTRACT
FROM TOBACCO FLOWER COROLLAS (PETALS)

Zone Number	Descending Development with the BAW Solvent System	Appearance under Ultraviolet light
		Origin
PE-12		
PE-11		Brown
PE-10		Brown
PE-8 & 9		Yellow
PE-7		Brown
PE-6		Blue
PE-5		Brown Blue
PE-4		Brown
PE-3		Blue
PE-2		Violet
PE-1		Yellow
		Solvent Front

ence scopoletin, esculetin and caffeic acid in several solvent systems. The unknown substance had R_f values of 0.59 in the 15% acetic acid, 0.33 in the IFW, 0.78 in the BAAW and 0.81 in the BAW solvent system. These values correspond with values for reference esculetin (Table 3). The unknown fluoresced and reacted with ammonium molybdate spray reagent on paper chromatograms similarly to reference esculetin.

On the basis of R_f studies, fluorescence and reaction with ammonium molybdate, it is concluded that Zone PE-2 contained esculetin.

Isolation and Identification of Astragalin (kaempferol-3-monoglucoside) in Zone PE-3

Zone 3 was cut out from each of the original chromatograms of the corolla extract and eluted with 50% isopropyl alcohol-water solvent. The eluate was concentrated to dryness *in vacuo*, and the residue was extracted with a water saturated solvent containing 1 part isopropyl alcohol and 9 parts ethyl acetate. The ethyl acetate extract was streaked on several paper strips and developed in the BAW solvent system. This solvent resolved the extract into 3 closely moving fluorescent bands. The faint brown zone was cut out from between two blue zones and eluted with dilute alcohol solvent. The ethyl acetate insoluble residue was dissolved with a minimum amount of water, the aqueous solution extracted with chloroform, the two phases separated, and the aqueous phase streaked on several paper strips, which were developed in the BAW solvent system. The faint brown zone was cut out from each paper and eluted with dilute alcohol. The eluates were combined, reduced to a small volume, streaked on several paper strips and developed in the CIW solvent system. This solvent resolved the eluate into 5 fluorescent bands. The brown zone was cut out

and eluted with 85% isopropyl alcohol-water solvent. The eluate was concentrated, streaked on paper strips, and developed in the 15% acetic acid solvent system. The brown zone was cut out, eluted, and rechromatographed in the BAAW solvent system. The brown pigment was eluted and the eluate was concentrated and stored in a small vial.

The R_f values of the unknown pigment compared favorably with values for astragalin (kaempferol-3-monoglucoside, kindly supplied by Dr. Y. Oshima, Japan) (Table 3). The ultraviolet absorption spectrum of the unknown pigment had two maxima, 266 and 353 mμ in ethyl alcohol (the reference astragalin had maxima at 268 and 350 mμ). Addition of sodium acetate to an alcohol solution of the unknown pigment produced a bathochromic shift of 7 mμ in the short wave-length band. This indicated that the 7-hydroxy position of the flavonoid aglycone was unsubstituted. Hydrolysis of the unknown flavonoid in acid solution and subsequent examination of the hydrolysis products by paper chromatography yielded the aglycone, kaempferol, and the sugar, glucose.

From the results of this study, it is concluded that one of the polyphenols present in Zone PE-3 was astragalin.

Isolation and Identification of Isoquercitrin (quercetin-3-monoglucoside) in Zone PE-4

The narrow, brown-fluorescing zone labeled PE-4 was cut out from each original corolla extract chromatogram and eluted with dilute alcohol solvent. The eluate was concentrated, streaked on several paper strips and developed in the IFW solvent system. This solvent system resolved the original zone into 8 closely moving fluorescent bands, which were numbered consecutively from PE-41 to PE-46.

A brown zone labeled PE-43 was cut out from each chromatogram to be used for isolation of nictoflorin. The experimental details will be described in a subsequent section.

A yellow band, labeled PE-45, was cut out from each chromatogram and eluted with dilute alcohol. The eluate was concentrated to dryness in vacuo; the residue was extracted with methyl alcohol; and the alcohol extract was streaked on several paper strips and developed in the CIW solvent system. The alcohol insoluble residue was dissolved in water and rechromatographed in the BAW solvent system to recover an additional amount of the desired brown compound. The brown zone was cut out, eluted, and rechromatographed in the BAW solvent system. The brown band was still contaminated with an unknown blue substance, so it was rechromatographed in the n-butyl alcohol:acetic acid:water (4:1:5 v/v/v, upper phase) solvent system. The brown zone was eluted with methyl alcohol and stored in a vial.

The unknown compound (brown zone from PE-45) was chromatographed alongside reference isoquercitrin in numerous solvent systems and the correspondence of R_f values is shown in Table 4. The ultraviolet absorption spectra of the unknown pigment had 258 and 359 mμ maxima in ethyl alcohol, and 275 and 428 mμ maxima in ethyl alcohol with aluminum chloride. The spectra of reference isoquercitrin had similar maxima of 258 and 357 mμ in ethyl alcohol and 275 and 428 mμ in ethyl alcohol with aluminum chloride. Hydrolysis of the unknown in acid solution and subsequent examination of the hydrolysis products by paper chromatography yielded quercetin as the aglycone and glucose as the only sugar.

From the results of this study, it is concluded that PE-45 was

TABLE 4

R_f VALUES OF REFERENCE FLAVONOID GLYCOSIDES AND AGLYCONES AND FLAVONOID GLYCOSIDES FROM TOBACCO FLOWER COROLLAS (PETALS)

	Solvent Systems*						
Flavonoids	1	2	3	4	5	6	7
Astragalin	0.57	0.20	0.21	0.83	0.74	0.78	0.60
PE-3			0.20	0.82	0.75		0.60
Isoquercitrin	0.47	0.15	0.16	0.67	0.57	0.60	0.44
PE-45	0.47	0.15	0.17	0.64	0.57	0.60	0.43
Nictoflorin	0.65	0.32	0.37	0.68	0.46	0.71	0.40
PE-5642			0.37	0.68	0.46		0.40
Rutin	0.59	0.32	0.33	0.41	0.24	0.55	0.27
PE-7			0.33	0.41	0.24		0.27
Robinin	0.78	0.60	0.59	0.34	0.08	0.76	0.19
PE-10	0.78	0.60	0.71	0.0	0.0	0.61	0.07
Hydrolysis Product of PE-10	0.20			0.35	0.24	0.77	0.58
PE-11	0.73	0.49	0.61	0.0	0.0	0.42	0.04
Quercimeritrin	0.51**					0.39	0.25
Hydrolysis Product of PE-11	0.49**					0.38	0.24
Kaempferol	0.54**					0.62	0.84
Quercetin	0.37**					0.39	0.64

*Solvent Systems:
1. 15% Acetic Acid-Water
2. Distilled Water.
3. Isopropyl Alcohol-Formic Acid-Water (5:0.1:95).
4. Chloroform-Isobutyl Alcohol-Water (20:50:5).
5. n-Butyl Acetate-Acetic Acid-Water (4:1:5).
6. Water-saturated Phenol.
7. n-Butyl Alcohol-Acetic Acid-Water (6:1:2).

**60% Acetic Acid-Water.

isoquercitrin. This compound has been reported previously to be present in tobacco flowers (12).

Isolation and Identification of Nictoflorin (kaempferol-3-rhamnoglucoside) in Zones PE-4, PE-5 and PE-6

The two zones, 5 and 6, were cut out together since they were not separated sufficiently in the initial chromatograms of the petal extracts. The strips of paper containing the two zones were eluted with dilute alcohol. The eluate was concentrated *in vacuo*, streaked on several chromatographic papers and developed in the IFW solvent system. This solvent system resolved the two original bands into 6 fluorescent zones, which were numbered consecutively from PE-561 to PE-566.

The brown zone, number PE-564, was cut out from each chromatogram and eluted with dilute alcohol. The brown band PE-45, which was isolated from zone 4 in the previous section, was eluted with dilute alcohol. The eluates were combined, reduced to a small volume, streaked on fresh paper and developed in the CIW solvent system. This solvent system resolved the eluate into 1 blue and 2 brown bands. The slower moving brown band, PE-5643, was cut out to use in the identification of rutin, which will be described in the following section. The remaining brown band, PE-5642, was cut out of each chromatogram and eluted with 85% isopropyl alcohol. The eluate was concentrated, streaked on several paper strips, and developed in the BAAW solvent system. The brown zone was cut out, eluted, and the eluate rechromatographed in the CIW solvent system. The brown zone was cut out from each chromatogram and eluted with methyl alcohol.

The brown pigment was chromatographed alongside reference nicto-

florin (kaempferol-3-rhamnoglucoside, kindly supplied by Dr. William Dunlap) in several solvent systems, and the correspondence in R_f values is listed in Table 4. Hydrolysis of this pigment in acid solution yielded the aglycone, kaempferol, and the sugars, glucose and rhamnose.

From the results of this study, it is concluded that compound PE-5642 was nictoflorin. This compound has been isolated previously from flowers of tobacco (9, 13).

Isolation and Identification of Rutin (quercetin-3-rhamnoglucoside) in Zones PE-6 and PE-7

The wide brown zone, PE-7, was cut out from each chromatogram of the corolla extract and eluted with 85% isopropyl alcohol-water solvent. Also the brown band, PE-5643, which was isolated in the previous section, was eluted with 85% isopropyl alcohol-water solvent and combined with the eluate from Zone PE-7. Upon concentrating the eluate to a small volume, a yellow precipitate was produced. The precipitate was collected on a sintered glass funnel (medium porosity), washed with a small volume of 5% isopropyl alcohol-water solvent and dried with a small amount of acetone. The filtrate and washings were combined, concentrated to a small volume, and stored in a vial for future studies.

The yellow precipitate was dissolved in methyl alcohol and chromatographed alongside reference rutin in several solvent systems. The unknown pigment had R_f values similar to those of rutin (Table 4). Hydrolysis of the unknown in acid solution yielded quercetin and the sugars, glucose and rhamnose, as the principal hydrolysis products.

It is concluded from the above results, that the brown pigment, PE-7, was rutin. It has also been isolated previously from tobacco

flowers (12).

Isolation and Identification of Nictoflorin-7-glucoside (kaempferol-3-rhamnoglucosido-7-glucoside) in Zone PE-10

Zone PE-10 appeared reddish-brown on the original chromatograms of corolla extracts under U.V. light. This zone was cut out of each chromatogram, eluted with 50% isopropyl alcohol-water solvent, and the eluate was concentrated to dryness in vacuo. The residue was dissolved by addition of small amounts of water, and methyl alcohol was added to effect precipitation of extraneous material. The mother liquor was carefully withdrawn from the precipitate with a pipette and rechromatographed on paper with the IFW solvent. The precipitate was dissolved in water and rechromatographed to recover an additional amount of the brown pigment. The IFW solvent system resolved the mother liquor into 3 fluorescent bands. The brown zone was cut out, eluted, and rechromatographed on paper using distilled water as the developing solvent. This process of cutting out, eluting and rechromatographing of the brown pigment was repeated several times using the 15% acetic acid, BAW and IFW solvent systems until the pigment was reasonably pure as indicated by the general appearance of the ultraviolet absorption spectrum. Since this pigment had a relatively low R_f value in BAW solvent, it was suspected that it was a glycoside with at least 3 sugar molecules attached to the aglycone. This fact hindered its purification by paper chromatography, since the choice of solvent systems were limited to those which contained greater proportion of polar solvents.

The R_f values of this pigment in numerous solvent systems are

listed in Table 4. Harborne (32) has reported the following R_f values for a kaempferol-3-rhamnoglucosido-7-glucoside: 0.40 in the BAW, 0.54 in the water, 0.74 in the 15% acetic acid and 0.52 in the phenol solvent systems.

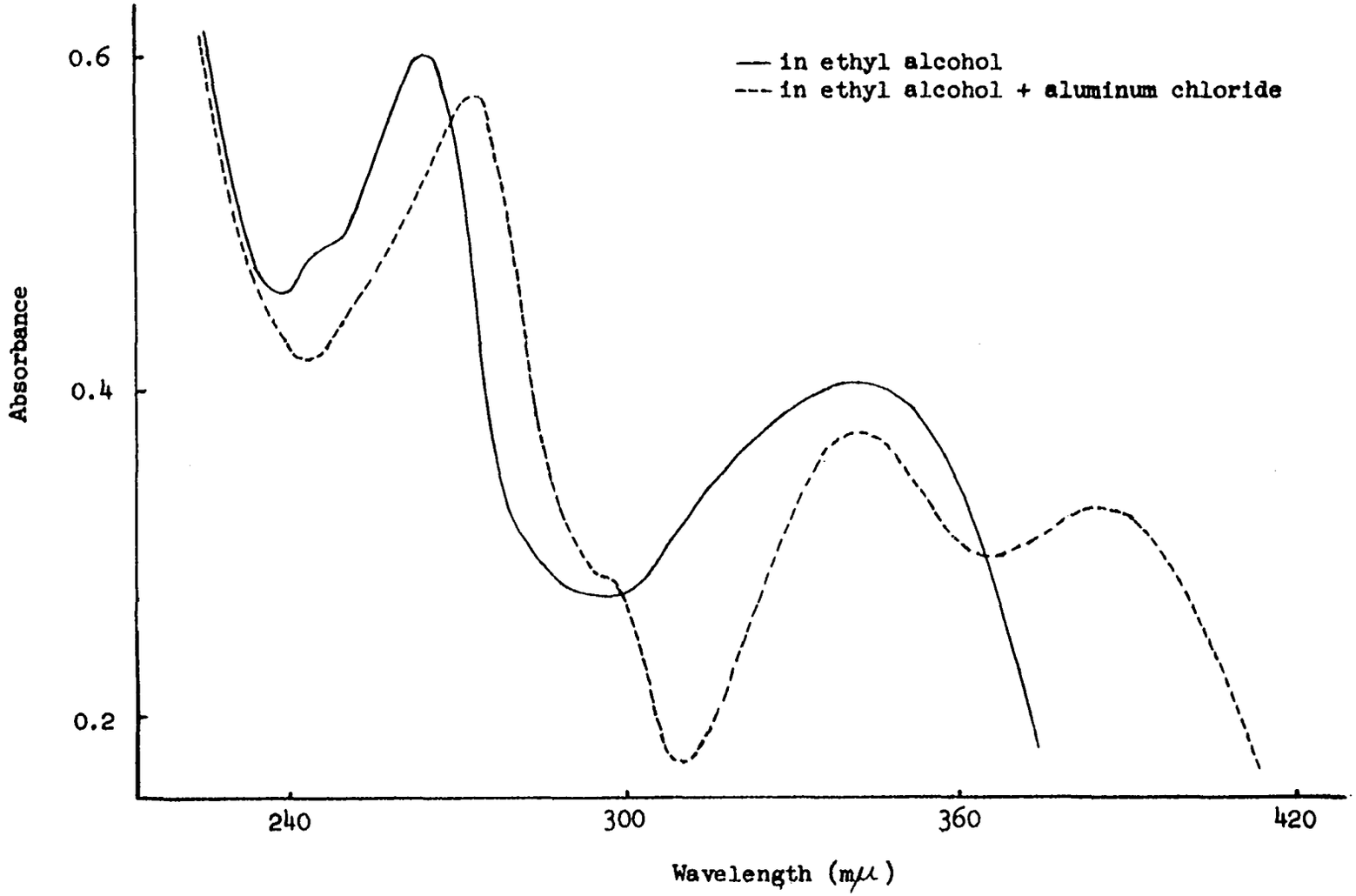
The ultraviolet absorption spectra maxima of the unknown substance in ethyl alcohol were 267 and 349 mμ and in ethyl alcohol with aluminum chloride, 276, 350 and 398 mμ (Figure 7). The two maxima, 350 and 398 mμ, produced by the addition of the aluminum chloride indicated that the unknown may be a kaempferol derivative.

The unknown was partially hydrolyzed by bringing the acid solution quickly to boiling, cooling to room temperature and extracting with ethyl acetate. This process of heating, cooling, and extracting was repeated three times. The ethyl acetate extract was reduced to a small volume and chromatographed alongside reference kaempferol and quercetin in several solvent systems. The partially hydrolyzed product had R_f values of 0.20 in the 15% acetic acid, 0.35 in the CIW, 0.24 in the BAAW, 0.77 in the phenol and 0.58 in the BAW solvent systems, (Table 4). These R_f values did not correspond with either quercetin or kaempferol, indicating that it was possibly a flavonoid glycoside. Harborne and Sherratt (29) reported the R_f values for kaempferol-7-glucoside as 0.54, 0.02 and 0.76 in BAW, water and phenol, respectively. It fluoresced yellow under U.V. light indicating that the number 3-hydroxyl position was unsubstituted. The ultraviolet absorption spectrum of this product in ethyl alcohol had maxima at 266 and 365 mμ. The reported (29) ultraviolet absorption maxima for kaempferol-7-glucoside were 265, 325, and 366 mμ.

The extracted acid solution was refluxed at boiling temperature

FIGURE 7

ULTRAVIOLET ABSORPTION SPECTRA OF
COMPOUND PE-10



for an additional 1/2 hour to hydrolyze completely the flavonoid and any possible disaccharide that may have been present. The acid solution was cooled to room temperature and extracted twice with small volumes of ethyl acetate. Both solutions were reduced in volume and chromatographed alongside known reference compounds. The aqueous phase contained the sugars, glucose and rhamnose. The ethyl acetate extract contained the aglycone, kaempferol, and the partially hydrolyzed product which was observed in the first ethyl acetate extract.

An enzymatic hydrolysis of the original unknown was performed with glucosidase (kindly supplied by Mr. Richard Hagen) by the techniques developed in this laboratory (33). The unknown substance was incubated with glucosidase in pH 4.5 citrate-buffer solution at 50° C. for 3 hours. The solution was cooled to room temperature and extracted with a small volume of ethyl acetate, which was discarded. The aqueous phase was concentrated to a small volume and isopropyl alcohol was added to precipitate the enzyme. The alcohol solution was carefully removed from the precipitate with a pipette and reduced to dryness in vacuo. The residue was extracted with methyl alcohol. The methyl alcohol extract was streaked on chromatographic paper and developed in the BAW solvent system. The brown band was cut out of the chromatogram, eluted with alcohol and rechromatographed alongside reference nictoflorin and rutin in various solvent systems. The R_f values of the unknown hydrolysis product corresponded with those of nictoflorin in all solvent systems tested. Treatment of the unknown and nictoflorin with ammonium molybdate spray reagent produced a yellowish-brown fluorescent spot in U.V. light which turned bright yellow on exposure to ammonia fumes. This appearance

under U.V. light and no observable color in visible light are typical for kaempferol derivatives.

The results of the R_f studies, ultraviolet absorption spectra, and chemical and enzymatic hydrolysis studies strongly suggests that the brown pigment, PE-10, is nictoflorin-7-glucoside.

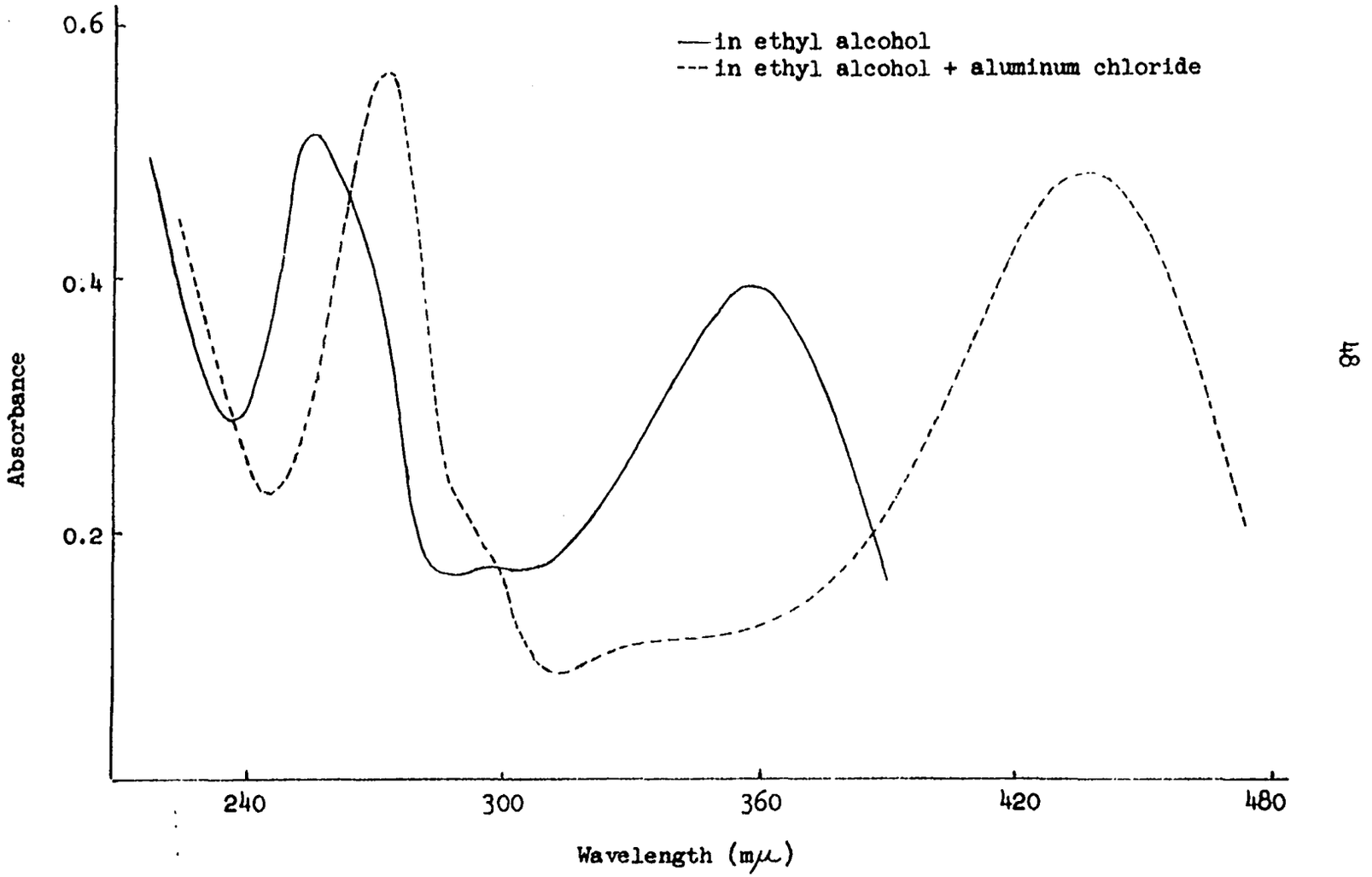
Isolation and Identification of Rutin-7-glucoside (quercetin-3-rhamnoglucosido-7-glucoside) in Zone PE-11

Zone PE-11 was another brown fluorescent band which had a relatively low R_f value in the BAW solvent system. This zone was treated in essentially the same manner as used for Zone PE-10, which was described in the preceding section. This pigment was somewhat more difficult to purify than the preceding compound in that it required extensive rechromatographing in the BAW, the IFW and the 15% acetic acid solvent systems.

This compound appeared brown in U.V. light and turned yellow in ammonia fumes. Upon spraying the chromatogram containing this compound with ammonium molybdate reagent, a yellow color was observed in visible light and a brown color in U.V. light which did not change upon exposure to ammonia fumes. This reaction of ammonium molybdate is typical for compounds possessing ortho-dihydroxy groupings, such as in quercetin derivatives. The unknown substance had a typical flavonoid ultraviolet absorption spectrum with maxima at 257 and 359 mμ in ethyl alcohol and maxima at 276 and 412 mμ in ethyl alcohol with aluminum chloride (Figure 8). The bathochromic shift of the long wave-length band upon the addition of aluminum chloride to produce a single maximum is characteristic

FIGURE 8

ULTRAVIOLET ABSORPTION SPECTRA OF
COMPOUND PE-11



of quercetin derivatives. The R_f values of the unknown pigment in numerous solvent systems are tabulated in Table 4. Harborne (32) has reported the following R_f values for a quercetin-3-rhamnoglucosido-7-glucoside: 0.36 in the BAW, 0.46 in the water, 0.71 in the 15% acetic acid and 0.31 in the phenol solvent systems.

Using the partial hydrolysis technique described in the preceding section, a product was obtained which corresponded with reference quercimeritrin (quercetin-7-monoglucoside) in R_f values, ultraviolet absorption spectrum, hydrolysis products, and appearance on chromatograms. A complete hydrolysis of the unknown compound produced the aglycone, quercetin, and the sugars, glucose and rhamnose. Enzymatic hydrolysis with glucosidase by the technique described in the previous section and subsequent examination of the hydrolysis mixture yielded rutin.

On the basis of the R_f studies, ultraviolet absorption spectra, chemical and enzymatic hydrolysis studies and reaction with ammonium molybdate spray reagent, it is concluded that the unknown, PE-11, is rutin-7-glucoside.

Isolation and Identification of Esculetin, Chlorogenic Acid and Scopolin in Zones PE-4, PE-5, PE-6 and PE-7

The isolation and identification of esculetin, chlorogenic acid and scopolin are discussed together in a single section in order to facilitate and simplify the discussion of the experimental work. Zone 4 and the lower portion of zone 5 were cut out together as one group and the remainder of zone 5, all of zone 6 and a small portion of zone 7 were cut out together as another group. Each group was processed

individually. Simplified diagrams illustrating the various zones which were separated from each group are shown in Figures 9 and 10. The numbers in the first column refer to the zones into which the IFW solvent resolved each group. The numbers in the second column refer to the zones into which the BAW solvent system resolved each zone from the chromatograms developed in the IFW solvent system. The fluorescence of each zone on the final chromatogram is indicated by (Brn) for brown, (Y) for yellow and (Bl) for blue. Corresponding zones from each chromatogram developed in the BAW solvent system were combined whenever it was practical. Tie lines are used in the diagrams to illustrate the combination of the various zones. The final column lists the principal compound or compounds identified in each combination.

The two groups of fluorescent bands were cut out from the chromatograms of the corolla extract and eluted separately with 50% isopropyl alcohol. Each eluate was reduced in volume under reduced pressure, streaked individually on several 9 inch paper strips and developed in the IFW solvent system. This solvent system resolved each eluate into 6 major fluorescent zones. Each zone was cut out from the chromatograms and eluted individually with 50% isopropyl alcohol. Each eluate was concentrated and chromatographed in the BAW solvent system. Zones which had similar R_f values and fluorescence were combined. Each combination of zones was eluted with 50% isopropyl alcohol. The eluates were reduced in volume and stored in individual vials.

Small aliquots of each concentrated eluate were chromatographed alongside reference compounds in several solvent systems. The R_f values, fluorescence and reaction with chromogenic spray reagents were noted for

FIGURE 9

NUMERICAL KEY TO THE ISOLATED ZONES
AND COMPOUNDS FROM ZONES PE-4 AND PE-5

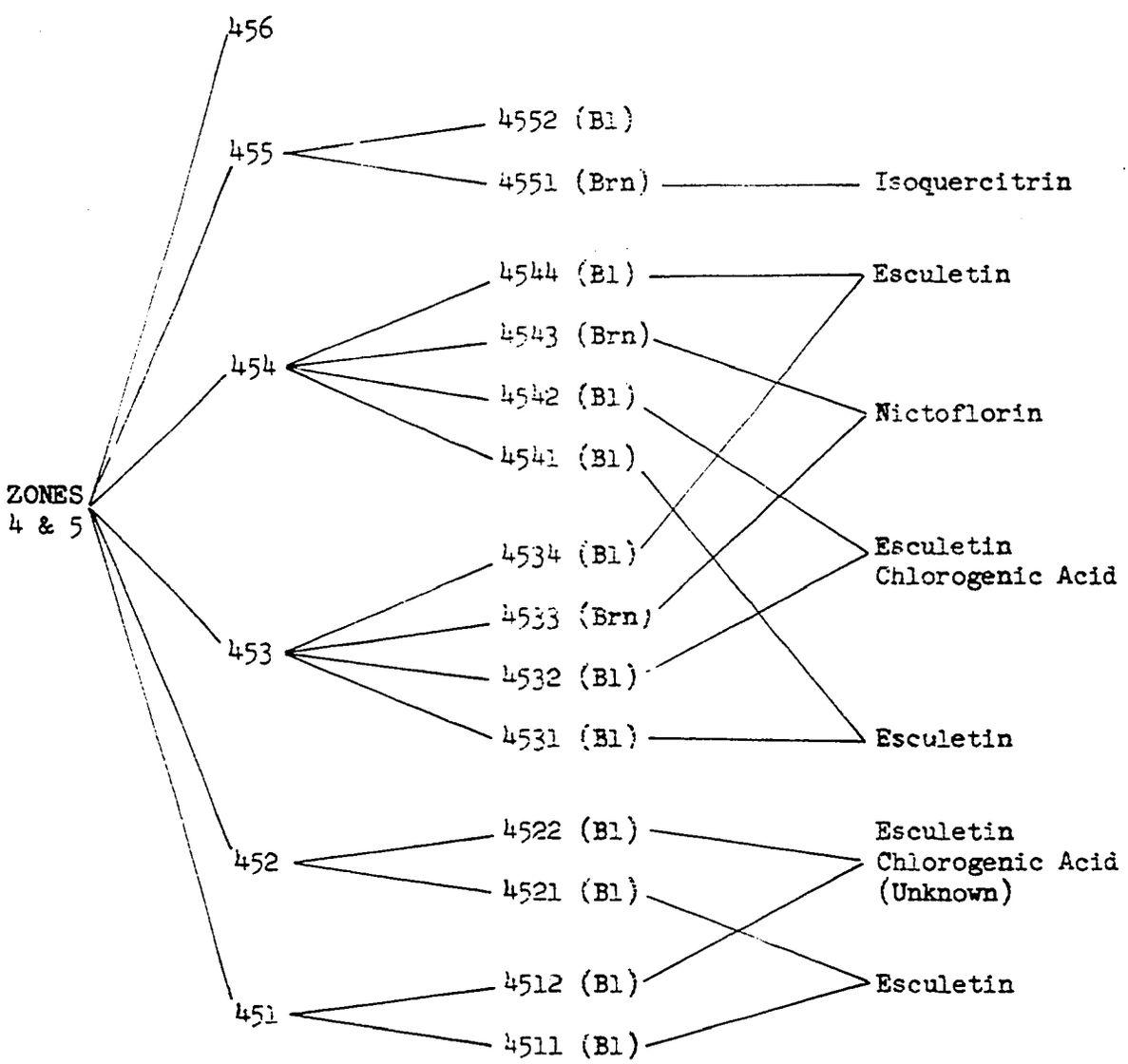
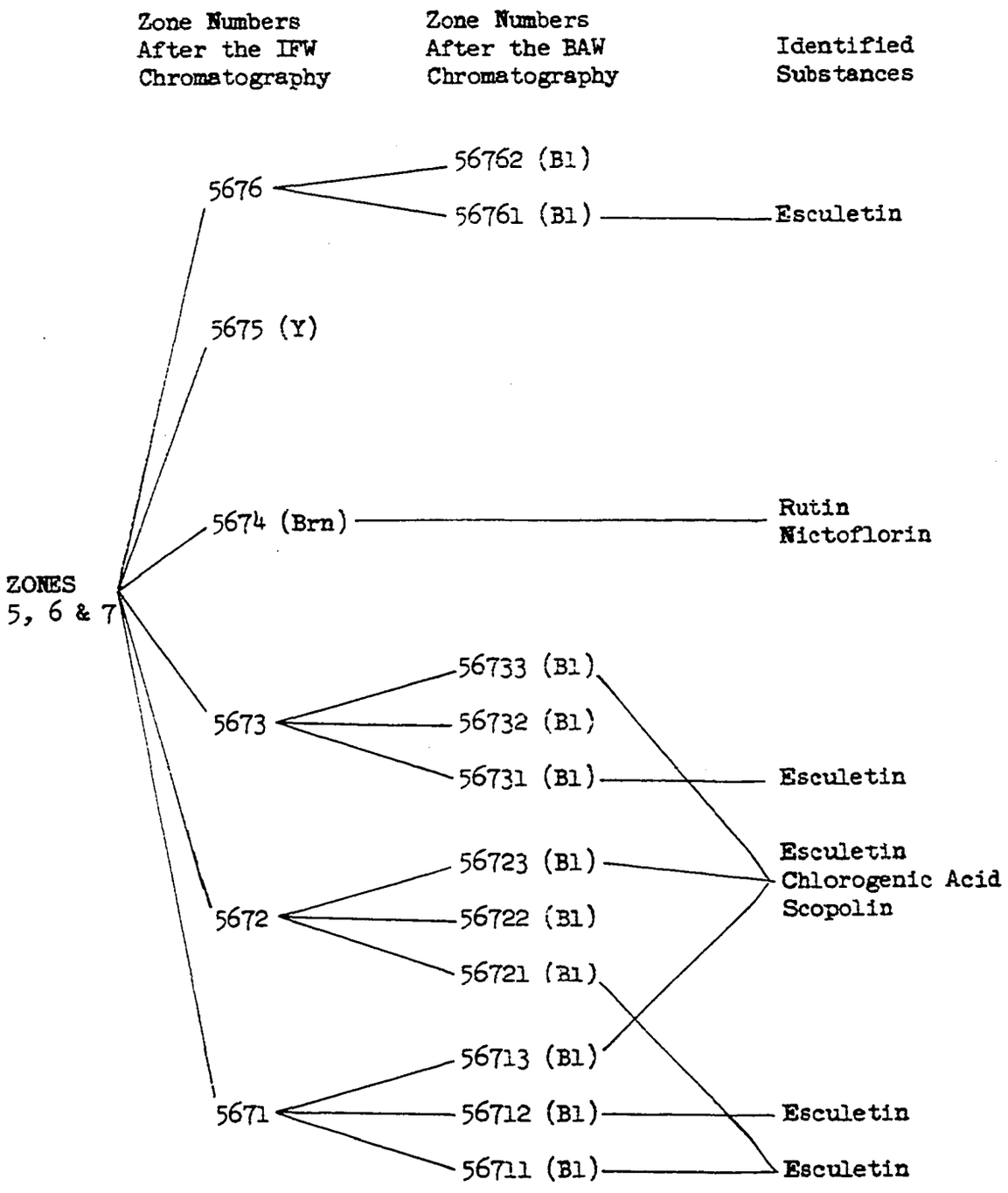


FIGURE 10

NUMERICAL KEY TO THE ISOLATED ZONES
AND COMPOUNDS FROM ZONES PE-5, PE-6 AND PE-7



each constituent present in every eluate. The eluates containing isoquercitrin, rutin and nictoflorin were combined with corresponding eluates isolated in previous sections.

Esculetin was identified by its similarity to reference esculetin in R_f values (Table 3), fluorescence and reaction with ammonium molybdate spray reagent as discussed in Chapter III. The criteria for identification of chlorogenic acid were the same as for esculetin except that reference chlorogenic acid was used. Scopolin was identified by its similarity to reference scopolin in fluorescence, R_f values in various solvents (Table 3) and hydrolysis with emulsin on paper chromatograms by the technique described in Chapter III to yield scopoletin.

CHAPTER V

ISOLATION AND IDENTIFICATION OF POLYPHENOLIC COMPOUNDS IN TOBACCO FLOWER CALYXES

Separation of Fluorescent Compounds in Benzene Extract of Calyxes

The original calyx extract, which had been concentrated *in vacuo* to remove most of the organic solvents, was extracted three times with small volumes of redistilled benzene. The study of the aqueous phase will be described in subsequent sections. In order to determine which of the acetic acid solvent systems would resolve the benzene extract into well-defined fluorescent zones, small aliquots of the extract were streaked on several chromatographic papers and each group of four papers was developed in the 15%, 30%, 40%, 50%, and 60% acetic acid solvent systems. The 40% acetic acid solvent system was selected for the mass paper chromatographic separation since it resolved the benzene extract into 3 major fluorescent zones. The blue band (R_f of 0.75) was labeled CAB-1; the faint brown band (R_f of 0.63) was labeled CAB-3; and another faint brown band (R_f of 0.53) was labeled CAB-4.

The Isolation and Identification of Esculetin (6,7-dihydroxycoumarin) in Zone CAB-1

The blue band was cut out of each chromatogram and eluted with 85% isopropyl alcohol-water solvent. The eluate was concentrated to dry-

ness; the residue was dissolved with a small volume of water and extracted with ethyl acetate. The ethyl acetate extract was streaked on several paper chromatograms and developed in the BAAW solvent system. The blue zone was cut out and eluted with 95% ethyl alcohol.

Small aliquots of the eluate were chromatographed alongside reference compounds in several solvent systems. The unknown blue fluorescent substance had R_f values of 0.62 in the 15% acetic acid, 0.75 in the BAW, 0.31 in the IFW, 0.72 in the BAAW, and 0.19 in the CAW solvent systems. Upon spraying the paper with ammonium molybdate spray reagent, the fluorescence of the unknown substance was quenched. On the basis of similarity in R_f values with reference esculetin (Table 3) and the reaction with chromogenic spray reagent, it is concluded that the unknown substance is esculetin.

Isolation and Identification of Quercetin -3,3'-dimethyl ether in Zone CAB-3

The paper strips containing the faint brown zone CAB-3 were eluted with methyl alcohol. The eluate was concentrated to dryness, and the residue was extracted with several small volumes of ethyl acetate. The ethyl acetate extract was streaked on chromatography paper and developed in the 15% acetic acid solvent system. This solvent system resolved the zone into a major brown band, which was CAB-3, and a slightly slower moving faint brown band, which was combined with CAB-4. The major brown band was eluted with methyl alcohol, and the eluate was concentrated to dryness. The residue was extracted with ethyl acetate, and the ethyl acetate extract was chromatographed in the 40% acetic acid solvent system. The brown zone was cut out and eluted with methyl alcohol.

Small aliquots of the alcohol eluate were chromatographed alongside reference synthetic quercetin-3,3'-dimethyl ether, quercetin-3,7-dimethyl ether and quercetin-3-methyl ether (the synthetic ether derivatives were kindly supplied by Dr. L. Jurd) in several solvent systems (Table 5). Examination of the table indicates that the R_f values of the unknown substance corresponds with values of the two dimethyl ether derivatives.

The ultraviolet absorption spectra of the unknown compound had maxima at 254 and 356 mμ in ethyl alcohol and at 271, 318, and 365 mμ in ethyl alcohol saturated with sodium acetate. The bathochromic shift of 17 mμ in the short wave-length band indicated that the 7-hydroxyl position of quercetin was unsubstituted. The ultraviolet absorption spectra of reference quercetin-3,3'-dimethyl ether had maxima at 254 and 355 mμ in ethyl alcohol and a short wave-length maximum at 276 mμ in ethyl alcohol saturated with sodium acetate.

Upon spraying a chromatogram with ammonium molybdate spray reagent, the unknown substance and reference quercetin-3,3'-dimethyl ether were colorless in visible light and reddish brown under U.V. light. They turned yellow upon exposure to ammonia fumes. These color reactions indicate that the unknown substance does not have a free ortho-dihydroxy group.

From the results of the R_f studies, ultraviolet absorption spectra, and color reaction with ammonium molybdate, it is concluded that compound CAB-3 is quercetin-3,3'-dimethyl ether. This compound has been isolated previously from tobacco flowers (16).

TABLE 5

R_f VALUES OF REFERENCE QUERCETIN-METHYL ETHERS
AND QUERCETIN-METHYL ETHERS FROM TOBACCO FLOWER CALYXES

Compounds	Solvent Systems			
	15% Acetic Acid	40% Acetic Acid	60% Acetic Acid	BAW
Quercetin-3,3'-dimethyl Ether	0.24	0.66	0.74	0.92
Quercetin-3,7-dimethyl Ether	0.21	0.67	0.74	0.91
Quercetin-3-methyl Ether	0.23	0.59	0.68	0.89
CAB-3	0.25	0.65	0.74	0.92
CAB-4	0.22	0.57	0.67	0.88

Isolation and Identification of Quercetin-3-methyl ether in Zone CAB-4

The second faint brown zone on the chromatograms of the benzene extract was processed in a similar manner to that for zone CAB-3. Examination of Table 5 indicates that the R_f values of CAB-4 correspond with values for reference quercetin-3-methyl ether. Spraying the chromatograms with ammonium molybdate spray reagent produced, for both the unknown and reference standards, yellow spots in visible light and dark brown spots under U.V. light which did not change to yellow color in ammonia fumes.

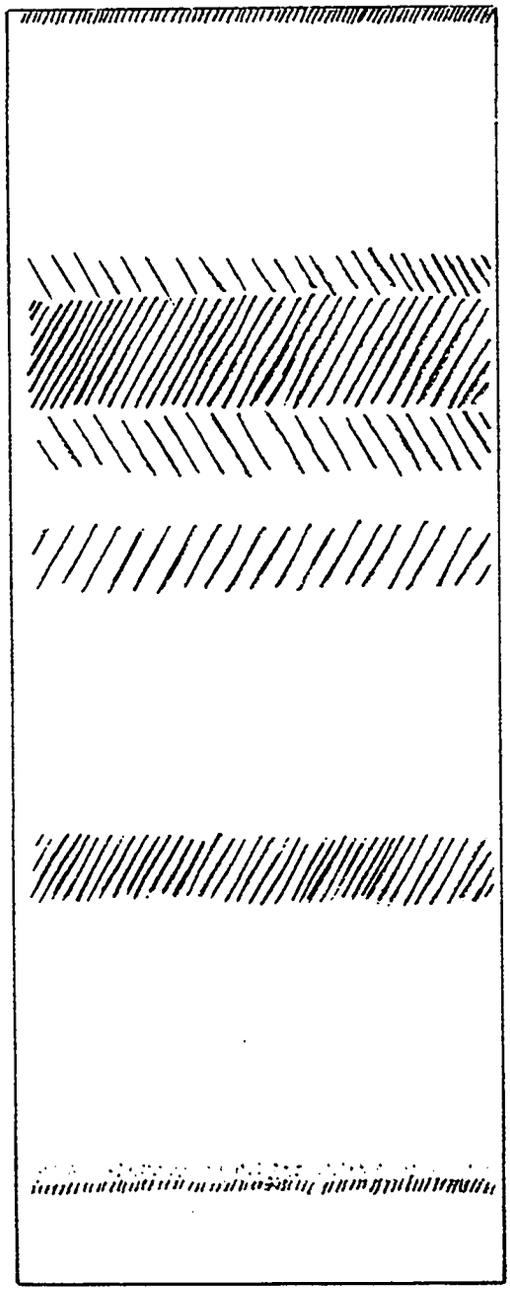
From the results of the R_f studies and the reaction with chromogenic spray reagent, it is concluded that CAB-4 is quercetin-3-methyl ether. This derivative of quercetin has been isolated previously from tobacco (10).

Separation of Fluorescent Compounds in the Aqueous Extract of Calyxes

The aqueous phase of the original calyx extract, which had been extracted with benzene, was streaked on several paper strips and developed in the BAW solvent system. A diagrammatic sketch illustrating the appearance, under U.V. light, of a representative one-dimensional chromatogram of this extract is shown in Figure 11. This solvent system separated the extract into 7 fluorescent bands which were not separated completely from each other or represented a single substance. Beginning at the bottom of the chromatogram, the fluorescent zones were numbered consecutively from CA-1 to CA-7.

FIGURE 11

ONE-DIMENSIONAL CHROMATOGRAM OF EXTRACT
FROM TOBACCO FLOWER CALYXES

Zone Number	Descending Development with the BAW Solvent System	Appearance under Ultraviolet Light
		Origin
CA-7		
CA-6		Light Tan
CA-5		Brown
CA-4		Blue
CA-3		Blue
CA-2		
CA-1		Blue
		Solvent Front

Isolation and Identification of Esculetin (6,7-dihydroxycoumarin) in Zone CA-1

Zone CA-1 was cut out from each of the original chromatograms of the calyx extract and eluted with 50% isopropyl alcohol-water solvent. The eluate was concentrated to dryness under reduced pressure, and the residue was dissolved with a small volume of water. The aqueous solution was extracted with several small volumes of ethyl acetate. The ethyl acetate extract was streaked on several chromatographic papers and developed in the BAAW solvent system. The blue band was cut out from each paper and eluted with 95% ethyl alcohol.

Small aliquots of the final eluate were chromatographed alongside reference esculetin, scopoletin and caffeic acid in numerous solvent systems. The R_f values and fluorescence of the unknown substance corresponded with reference esculetin and compound CAB-1. It is concluded that the unknown compound CA-1 is esculetin.

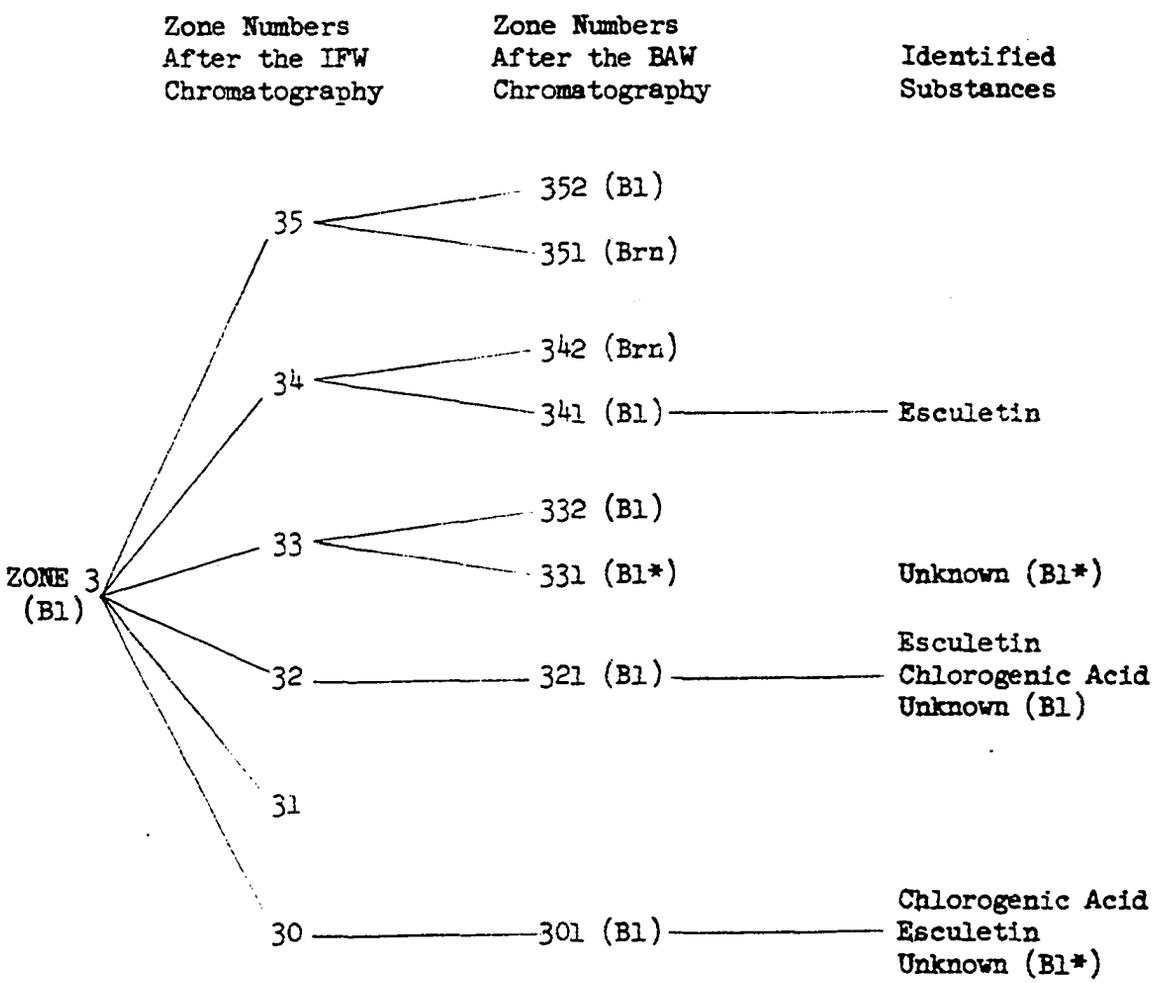
Studies on Zone CA-3

The paper strips containing the blue zone CA-3 were cut out from the original chromatograms and eluted with 50% isopropyl alcohol-water solvent. The eluate was reduced to a small volume, streaked on several chromatographic papers and developed in the IFW solvent system. This solvent resolved zone 3 into 10 fluorescent bands which were labeled consecutively from CA-30 to CA-35, beginning from the bottom of the chromatogram.

A simplified numerical key of the different zones resolved by the IFW and the BAW solvent systems are tabulated in Figure 12. The numbers in the first column refer to the zones into which IFW resolved the

FIGURE 12

NUMERICAL KEY TO THE ISOLATED ZONES
AND COMPOUNDS FROM ZONE CA-3



eluate of zone 3. The numbers in the second column refer to the major fluorescent zones into which the BAW solvent system resolved each previous zone. The final column lists a few of the compounds identified in each of the final zones. The fluorescence of the zones are indicated by (Bl) for blue, (Bl*) for blue only in ammonia fumes, (Brn) for brown, (P) for pink and (BlV) for blue-violet.

Each zone from the IFW chromatograms was eluted separately with 50% isopropyl alcohol. Each eluate was concentrated, streaked individually on several paper strips and developed in the BAW solvent system. Each major fluorescent zone was cut out from the BAW chromatograms and eluted individually with dilute alcohol. The eluates were spotted individually on four wide sheets of chromatography paper and developed in the IFW, BAW, BAAW and 15% acetic acid solvent systems. Reference standards were spotted alongside the unknowns in order to aid in their identification.

The identification of esculetin and chlorogenic acid was based on their similarity to respective reference standards in R_f values in various solvent systems, in fluorescence, and in their reaction with ammonium molybdate spray reagent.

Zones 301 and 331 contained a substance which fluoresced blue only in the presence of ammonia vapors. This unknown substance had R_f values of about 0.52 in BAW and two values of about 0.55, 0.85 in the IFW solvent systems. These observations suggest the possibility that the unknown substance may be a derivative of p-coumaric acid. Attempts to identify this material are currently underway.

Zone 321 contained, in addition to chlorogenic acid and escule-

tin, a blue fluorescent substance which reacted with ammonium molybdate spray reagent typically as do compounds with an ortho-dihydroxy group. It had R_f values of 0.51 in the BAW, 0.75, 0.83 in the 15% acetic acid, 0.59 in the IFW and 0.21 in the BAAW solvent systems.

The two brown zones 342 and 351 were combined with corresponding brown material isolated in zone 5.

Studies on Zone CA-4

This blue fluorescent zone was processed in similar fashion as had been used for zone 3. A diagram of the various zones which had been isolated is illustrated in Figure 13. Esculetin was identified by methods previously described. Scopolin was identified by methods described in Chapter III.

Zone 421 contained a pink fluorescent substance which had R_f values of 0.07 in the BAAW, 0.65 in the IFW, 0.81 in the 15% acetic acid and 0.45 in the BAW solvent systems. This substance is present in very small amounts.

Zones 432 and 441 contained a blue fluorescent substance which had R_f values of 0.44 in the IFW and 0.45 in the BAW solvent systems. The two brown zones 452 and 435 were combined with material isolated in zone 5.

Studies on Zone CA-5

This zone was processed in the same manner as the two preceding zones. All the blue zones isolated in the final BAW chromatograms were in low concentration as evidenced by their weak fluorescence. The major components in this zone were brown fluorescent. Figure 14 illustrates

FIGURE 13

NUMERICAL KEY TO THE ISOLATED ZONES
AND COMPOUNDS FROM ZONE CA-4

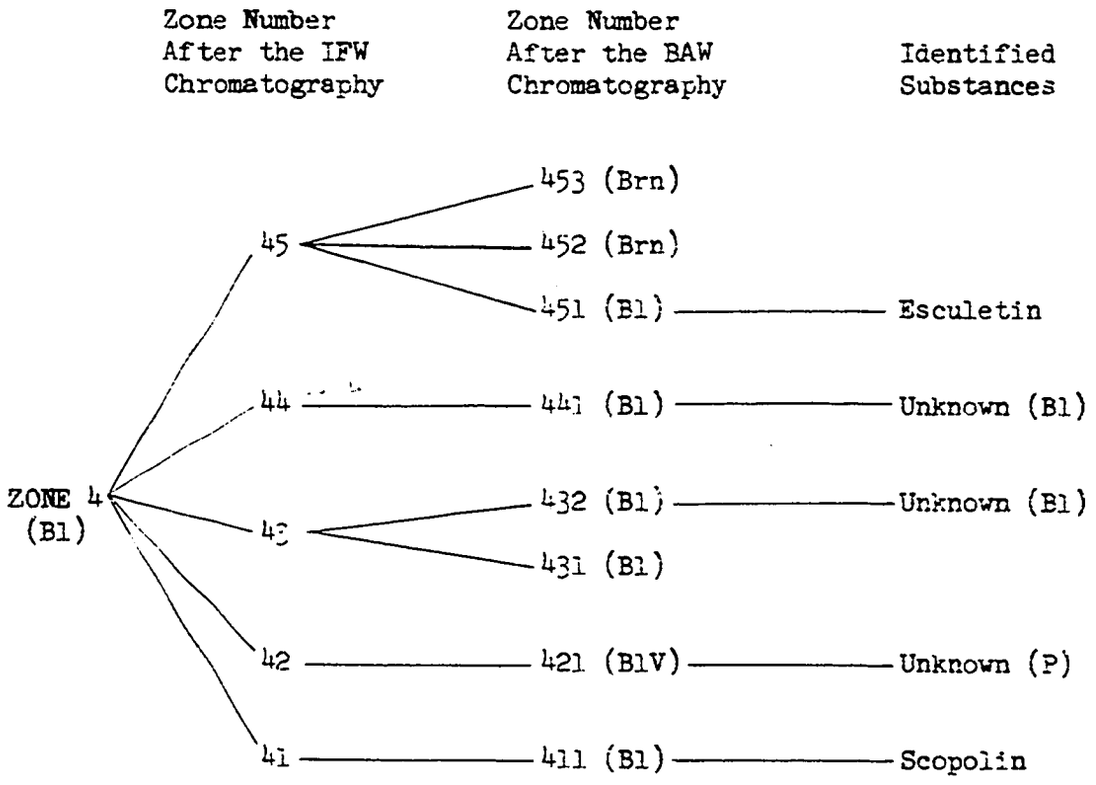
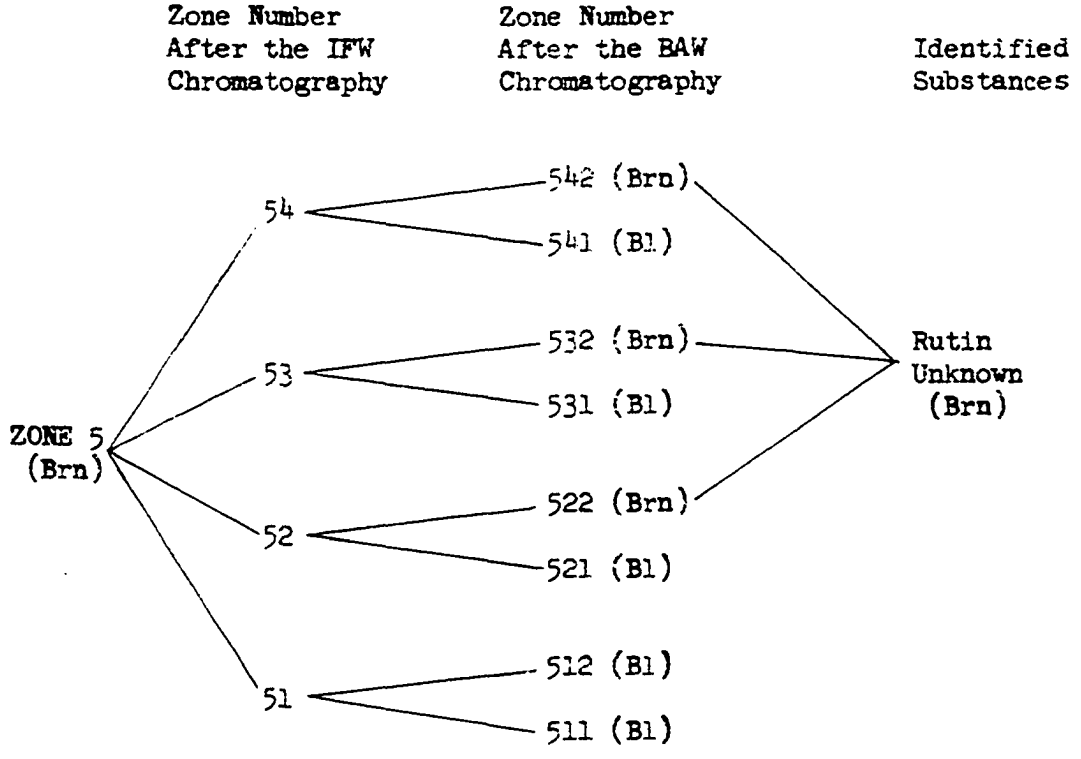


FIGURE 14

NUMERICAL KEY TO THE ISOLATED ZONES
AND COMPOUNDS FROM ZONE CA-5



the various zones isolated from zone 5.

The three brown fluorescent zones 522, 532, and 542 were treated in an identical manner. Each zone was eluted from the paper with IFW solvent and rechromatographed separately in the BAW solvent system. The brown zone was cut out from each chromatogram and eluted with 50% isopropyl alcohol. The brown zones isolated from zones 3 and 4 were eluted, and the eluates were combined with the above eluate. The combined eluate was concentrated to dryness under reduced pressure, and the residue was extracted with methyl alcohol. Acetone was added to the alcohol extract to effect precipitation of the flavonoid material. The precipitate was collected on a filter and washed with a small volume of acetone. The precipitate was dissolved with methyl alcohol and chromatographed in the 15% acetic acid solvent system. The filtrate and washings were combined, reduced to a small volume, and chromatographed in the same solvent system. This solvent system resolved the original material into two brown bands which were labeled 52341 (R_f about 0.70) and 52342 (R_f about 0.60). Each band was eluted seperately with 50% isopropyl alcohol and rechromatographed in the IFW, CIW and BAW solvent systems.

The methyl alcohol insoluble residue of the combined eluates of zones 522, 532 and 542 was dissolved with dilute acetic acid and rechromatographed in various solvent systems to recover additional amounts of the two brown pigments. Further study of each pigment will be discussed in the following sections.

Identification of Rutin in Zone CA-52342

The brown zone labeled 52342 was cut out from each of the final

chromatograms and eluted with 50% isopropyl alcohol. The eluate was concentrated to dryness under reduced pressure, and the residue was extracted with methyl alcohol. The alcohol extract was filtered through glass wool and stored in a vial.

Small aliquots of the solution containing the unknown substance were chromatographed alongside reference rutin, nictoflorin, xanthorhamnin (3,3',4',5-tetrahydroxy-7-methoxyflavone-3-rhamninoside), narcissin (3,4',5,7-tetrahydroxy-3'-methoxyflavone-3-rhamnoglucoside, kindly supplied by Dr. W. J. Dunlap), and robinin (kaempferol-3-rhamnogalactosido-7-rhamnoside, kindly supplied by Dr. E. M. Bickoff) in various solvent systems. The correspondence in R_f values between the unknown substance and rutin in all the solvent systems used is indicated in Table 6. Hydrolysis of the unknown substance in acid solution yielded the aglycone, quercetin, and the sugars, glucose and rhamnose. It is concluded from the results of this study that CA-52342 is rutin.

Tentative Identification of Quercetin-3-glucoside in Zone CA-52341

The brown zone labeled 52341 was cut out from each of the final chromatograms and eluted with 50% isopropyl alcohol. The eluate was concentrated to dryness under reduced pressure, and the residue was extracted with methyl alcohol. The alcohol extract was filtered through glass wool and stored in a vial.

Small aliquots of the solution containing the unknown substance were chromatographed alongside reference flavonoids in various solvent systems. The R_f values of the unknown substance did not correspond in all solvent systems with a single reference flavonoid (Table 6).

TABLE 6

R_f VALUES OF REFERENCE FLAVONOID GLYCOSIDES
AND FLAVONOID GLYCOSIDES FROM TOBACCO FLOWER CALYXES

	Solvent Systems						
Flavonoid Glycosides	15% Acetic Acid	Water	IFW	CIW	BAAW	Phenol	BAW
Rutin	0.59	0.32	0.33	0.41	0.24	0.55	0.27
CA-52342	0.59	0.32	0.32	0.42	0.23	0.55	0.26
Narcissin	0.58	0.33	0.30	0.59	0.37	0.76	0.33
CA-52341	0.68	0.40	0.40	0.37	0.14	0.48	0.21
Xanthorhamnin	0.70	0.39	0.41	0.29	0.17	0.74	0.20
Nictoflorin	0.65	0.32	0.37	0.68	0.46	0.71	0.40
Robinin	0.78	0.60	0.59	0.34	0.08	0.76	0.19

The ultraviolet absorption spectra of the unknown pigment are shown in Figure 15. The absorption maxima occurred at the following wave-lengths: 257 and 358 mμ in ethyl alcohol alone, 268 and 370 mμ in sodium acetate saturated ethyl alcohol, 262 and 380 mμ in ethyl alcohol with sodium acetate and boric acid, and 268, 296, 360 and 397 mμ in ethyl alcohol with aluminum chloride. The bathochromic shift of 11 mμ in the short wave-length band upon addition of sodium acetate indicated that the 7-hydroxyl position was unsubstituted. The bathochromic shift of 22 mμ in the long wave-length band upon the addition of boric acid and sodium acetate indicated that both the 3'-and 4'-hydroxyls were unsubstituted. The single major maximum at 397 mμ upon addition of aluminum chloride would indicate that the unknown substance may be a quercetin derivative.

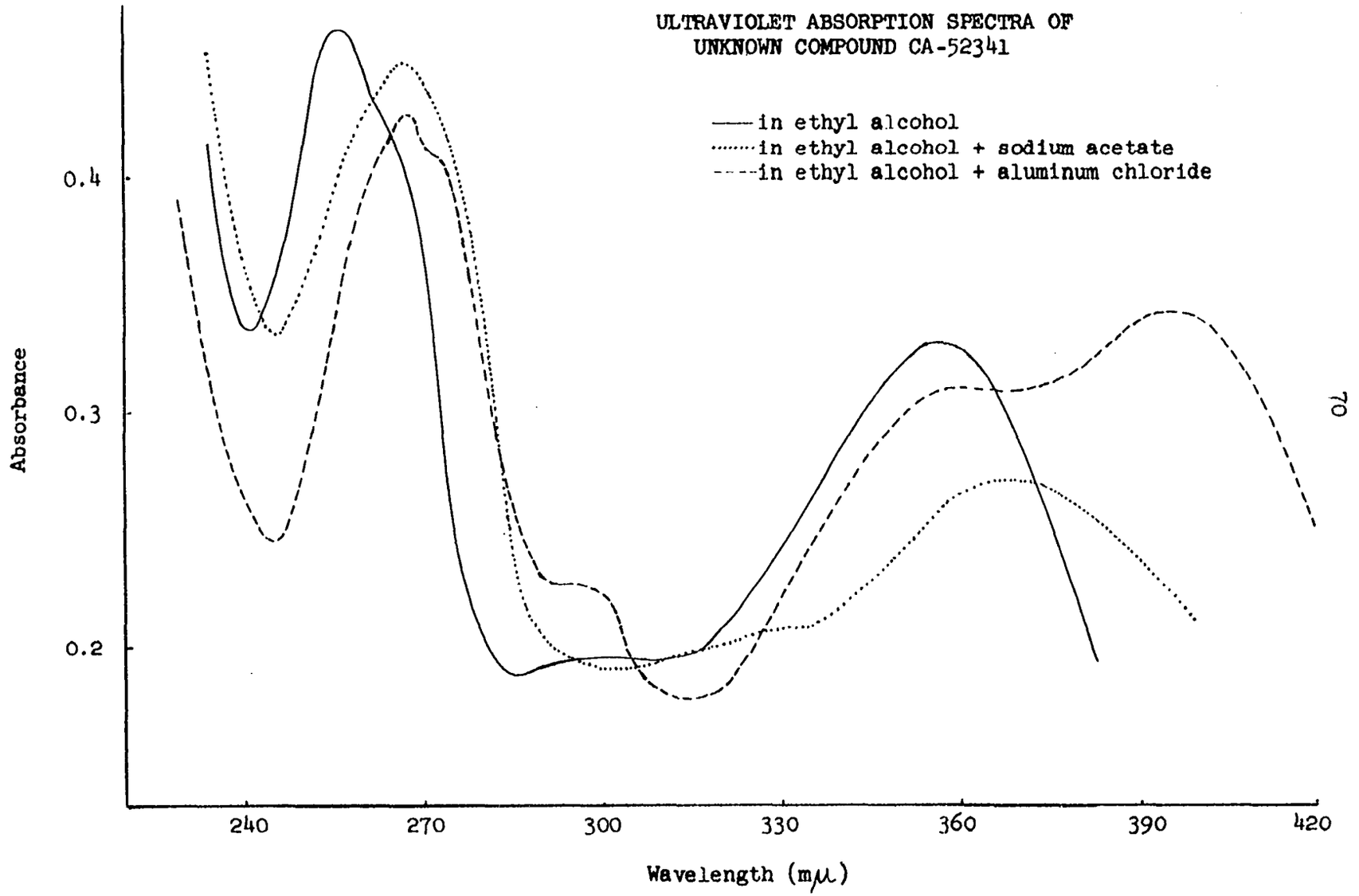
The unknown substance fluoresced brown on paper chromatograms irradiated with U.V. light indicating that the 3-hydroxyl position was substituted. Upon spraying a chromatogram with ammonium molybdate spray reagent, the unknown compound appeared yellow in visible light and brown under U.V. light. These colors did not change in ammonia fumes. This reaction indicated that the unknown pigment had an ortho-dihydroxy grouping present.

Acid hydrolysis of the unknown flavonoid yielded the aglycone, quercetin, and only the sugar, glucose. Attempts to accumulate sufficient amount of the pure material are underway in order to determine the aglycone:sugar ratio.

On the basis of its R_f values, ultraviolet absorption spectra, reaction with chromogenic spray reagent and hydrolysis products, it is possible that CA-52341 is a quercetin-3-di- or triglucoside.

FIGURE 15

ULTRAVIOLET ABSORPTION SPECTRA OF
UNKNOWN COMPOUND CA-52341



CHAPTER VI

ISOLATION AND IDENTIFICATION OF POLYPHENOLIC COMPOUNDS IN TOBACCO FLOWER PISTILS

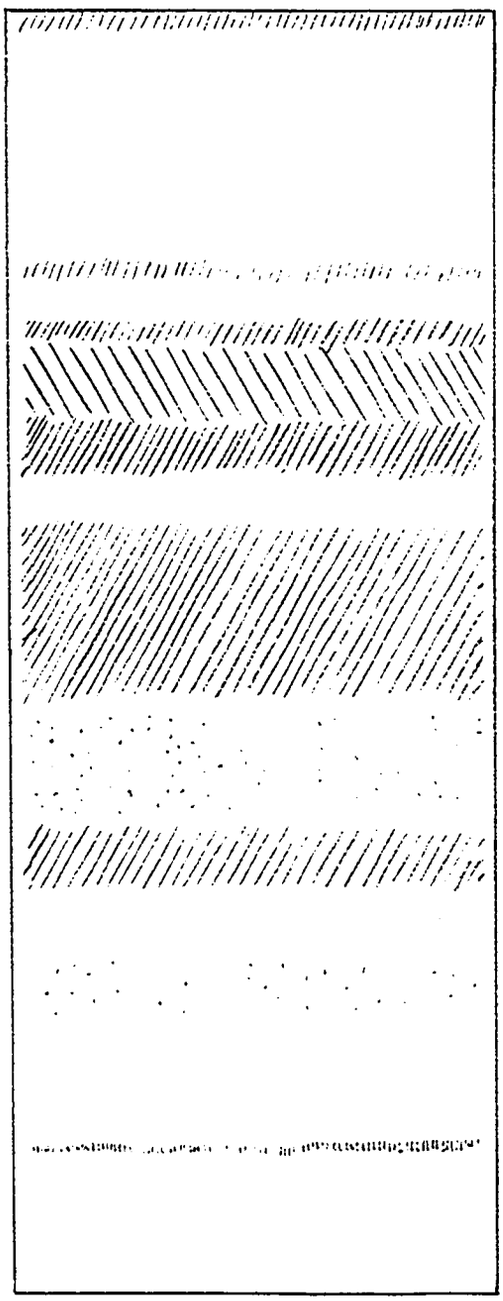
Separation of Fluorescent Compounds in Pistil Extract

The initial extract of the pistil tissues was reduced in volume *in vacuo* and extracted with small volumes of redistilled benzene. The aqueous layer was then extracted with chloroform. Small aliquots of each organic extract were streaked separately on paper strips and developed in the BAW and 60% acetic acid solvent systems. The organic extracts contained esculetin, and a yellow fluorescing substance, and another substance which fluoresced blue only in the presence of ammonia vapors. The latter material reacted with ammonium molybdate spray reagent, indicating the presence of an ortho-dihydroxy group. No attempt was made in this study to identify these substances.

The final aqueous extract was streaked on several paper strips and developed in the BAW solvent system. A diagrammatic sketch illustrating the appearance, under U.V. light, of a representive chromatogram is shown in Figure 16. This solvent system separated the extract into 8 fluorescent bands which were not separated completely from each other or represented a single substance. Beginning at the bottom of the chromatogram, the fluorescent zones were numbered consecutively from PI-1 to

FIGURE 16

ONE-DIMENSIONAL CHROMATOGRAM OF EXTRACT
FROM TOBACCO FLOWER PISTILS

Zone Number	Descending Development with the BAW Solvent System	Appearance under Ultraviolet Light
		Origin
PI-7		Light Tan
PI-6		Light Tan
PI-5		Light Tan
PI-4		Blue
PI-3		Blue
PI-2		
PI-1		Blue
		Solvent Front

PI-7. Examination of this one-dimensional chromatogram under U.V. light indicated that tobacco flower pistils probably do not contain the usual flavonoid compounds associated with tobacco tissues.

Isolation and Identification of Esculetin in Zone PI-1

The blue fluorescent zone PI-1 was cut out from each original chromatogram and eluted with 50% isopropyl alcohol. The eluate was concentrated to dryness under reduced pressure, and the residue was extracted with methyl alcohol. The alcohol extract was streaked on several paper strips and developed in the BAAW solvent system. The methyl alcohol insoluble residue was dissolved in a small volume of dilute acetic acid. This solution was streaked on paper and developed in the same solvent system. This solvent system resolved the extracts into a single blue zone (R_f about 0.75) and a yellow fluorescent material at the origin.

The blue zone was cut out from each chromatogram and eluted with 50% isopropyl alcohol. The eluate was reduced to dryness under reduced pressure, and the residue was extracted with methyl alcohol. Small aliquots of the alcohol extract were chromatographed alongside reference compounds in several solvent systems. The unknown substance had R_f values of 0.61 in the 15% acetic acid, 0.77 in the BAW, 0.35 in the IFW, 0.81 in the BAAW, and 0.18 in the CAW solvent systems. These R_f values correspond with values for reference esculetin (Table 3). Upon spraying a chromatogram with ammonium molybdate spray reagent, the fluorescence of the unknown substance was quenched.

From the results of the R_f studies and reaction with spray reagent, it is concluded that PI-1 is esculetin.

Isolation and Identification of Chlorogenic Acid in Zone PI-3

The blue fluorescent zone PI-3 was cut out from each of the original chromatograms and eluted with 50% isopropyl alcohol. The eluate was concentrated, streaked on several chromatographic papers, and developed in the BAAW solvent system. This solvent system resolved the original blue zone into 3 blue bands. One blue band (R_f about 0.79) was recognized to be esculetin; the second blue band (R_f about 0.32) was labeled PI-32; and the third blue band, labeled PI-33, was at the origin.

The blue zone labeled PI-32 was cut out from each chromatogram, eluted with dilute alcohol, and rechromatographed in the IFW solvent system. This solvent system resolved zone 32 into 5 blue bands. A blue band (R_f about 0.92) turned a yellow-green color upon exposure to ammonia fumes and gave a positive ammonium molybdate spray test. A second blue band (R_f about 0.77) was very similar to the first band. These two bands are probably similar to chlorogenic acid. The next blue band had an R_f value of about 0.65 and it was the most prominent of all the bands present. The major band was cut out and eluted with dilute alcohol. Small aliquots of this eluate were chromatographed alongside reference compounds in the 15% acetic acid, IFW, BAW and BAAW solvent systems. Chlorogenic acid and esculetin were identified as the major constituents on the basis of their R_f values, fluorescence and reaction with ammonium molybdate spray reagent. In addition, this zone contained an unknown blue fluorescent substance which had R_f values of 0.49 in the 15% acetic acid, 0.80 in the BAW, 0.26 in the IFW, and 0.86 in the BAAW solvent systems. Since this substance reacted typically with ammonium molybdate spray reagent for

compounds possessing an ortho-dihydroxy group, it is speculated that this may be caffeic acid. Further studies are currently underway to identify this substance.

The blue zone labeled PI-33 was cut out from each chromatogram, eluted with dilute alcohol, and rechromatographed in the BAW solvent system. This solvent system resolved zone 33 into one major blue band (R_f about 0.63) and several faint minor fluorescent bands. The major band was cut out from each chromatogram and eluted with dilute alcohol. Small aliquots of this eluate were chromatographed alongside reference compounds in the 4 solvent systems used in the previous paragraph. The major blue fluorescent compound identified in zone 33 was chlorogenic acid.

Studies on Zone PI-4

The dark blue fluorescent zone PI-4 was cut out from each chromatogram and eluted with 50% isopropyl alcohol. The eluate was concentrated to dryness under reduced pressure, and the residue was extracted with methyl alcohol. The alcohol insoluble residue was dissolved with a small volume of dilute acetic acid. The alcohol and acetic acid solutions were chromatographed separately in the BAW solvent system. This solvent system resolved both solutions into 5 fluorescent bands. One band was recognized to be esculetin. A weak fluorescent band (R_f about 0.62), which fluoresced more brightly in ammonia fumes and reacted positively with ammonium molybdate spray reagent, was similar to an unknown substance present in the benzene and chloroform extracts. The next band (R_f about 0.45) was the major blue fluorescent zone. Also two bands

(R_f about 0.28 and 0.38) which appeared yellow in visible light and yellowish-green under U.V. light were separated from zone 4.

The major blue band which had an R_f value of about 0.45 in the BAW solvent system was cut out from each chromatogram and eluted with 50% isopropyl alcohol. The eluate was concentrated to dryness under reduced pressure. The residue was dissolved in a small volume of dilute acetic acid and methyl alcohol solution. Small aliquots of this solution were chromatographed alongside reference compounds in various solvent systems. Examination of each chromatogram under U.V. light indicated that this solution contained esculetin, also a substance similar to chlorogenic acid, and several unidentified fluorescent substances.

Studies on Zones PI-5, PI-6 and PI-7

The three zones appeared yellow in visible light and fluoresced light-tan under U.V. light. Zones 5 and 6 were cut out together from each chromatogram and eluted with 50% isopropyl alcohol. Zone 7 was cut out from each chromatogram and eluted separately with 50% isopropyl alcohol. The eluates seemed to have a yellowish-green fluorescence in visible light. Each eluate was concentrated to dryness under reduced pressure. The residues were extracted initially with methyl alcohol and dissolved with dilute acetic acid solution. Both solutions were streaked separately on several paper strips and developed in the BAW solvent system. This solvent system resolved the solutions into a blue fluorescent band (esculetin) and a slower moving yellow-green band.

The yellow-green band was cut out from each chromatogram and eluted with dilute alcohol. Small aliquots of the eluate were chromato-

graphed alongside reference compounds in the IFW, 15% acetic acid, BAW and BAAW solvent systems. The only fluorescent substance present on the chromatograms developed in the first three solvent systems was esculetin. The chromatogram developed in the BAAW solvent system had, in addition to esculetin, a substance at the origin which appeared yellow both in the visible and U.V. lights. Until this yellow substance is identified, the above study merely indicates that esculetin was probably present in some form of a complex.

CHAPTER VII

DISCUSSION OF THE RESULTS ON THE STUDY OF POLYPHENOLS IN FOUR KINDS OF APPENDAGES OF THE TOBACCO FLOWER

The objectives of this study have been to identify as many as possible of certain polyphenolic compounds present in each of four classes of organs or parts of the tobacco flower; to determine the relative distribution of each substance in each part; and to note differences and similarities between these four classes of organs. A compilation of the various compounds identified in each floral part is illustrated in Table 7. The presence of a compound in a floral part is indicated with an "X", the absence of a compound is indicated with a dash, and the word "trace" is used to indicate a small but recognizable amount of a substance.

Examination of Table 7 indicates that the pistil does not contain flavonoid compounds usually associated with tobacco tissues; the corolla or petals contain the greatest number of flavonoid compounds; and only the calyxes or sepals contain the methylated derivatives of quercetin. The flavonoid, rutin, is distributed in all of the organs except the pistil. The coumarin, esculetin, and the depside, chlorogenic acid, are present in all parts of the flower. The glucoside of scopoletin, scopolin, is present in all of the organs except the pistil. The glucoside of the phenolic acids, ferulic and p-coumaric acids,

TABLE 7

DISTRIBUTION OF CERTAIN POLYPHENOLIC COMPOUNDS
IN FOUR CLASSES OF ORGANS OF THE TOBACCO FLOWER

Compounds	Classes of Organs			
	Stamen	Corolla	Calyx	Pistil
FLAVONOIDS				
Rutin	X	X	X	-
Nictoflorin	-	X	-	-
Isoquercitrin	-	X	-	-
Astragalin	-	X	-	-
Rutin-7-glucoside	-	X	-	-
Nictoflorin-7-glucoside	-	X	-	-
Quercetin-3,3'-dimethyl ether	-	-	X	-
Quercetin-3-methyl ether	-	-	X	-
Kaempferol-3-glycoside	X	-	-	-
Quercetin-3-glycoside	-	-	X	-
COUMARINS				
Esculetin	X	X	X	X
Scopoletin	-	-	trace	-
Scopolin	X	X	X	-
DEPSIDE				
Chlorogenic Acid	X	X	X	X
PHENOLIC ACID GLYCOSIDES				
Glucoside of ferulic acid	X	-	-	-
Glucoside of p-coumaric acid	X	-	-	-

were recognized only in the stamens.

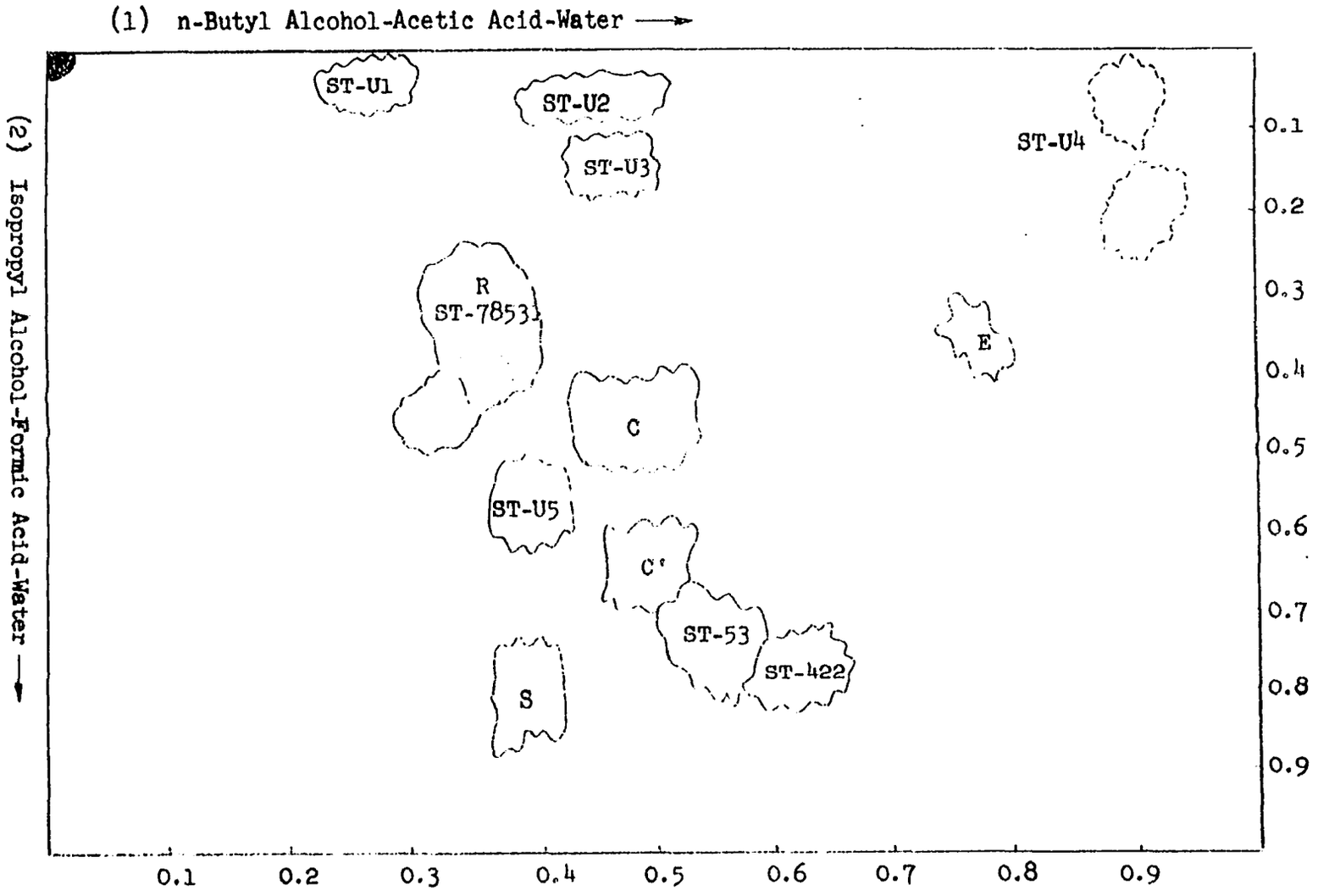
In contrast to the above results, Hattori and Shimokoriyama reported (34) that the two flavonoids, poncirin (5,7-dihydroxy-4'-methoxyflavanone-7-rhamnoglucoside) and naringin (4',5,7-trihydroxy-flavanone-7-rhamnoglucoside), were present in all parts of the flowers of Poncirus trifoliata, and that the pistil contained the highest, and the petals and stamens the lowest, concentration of poncirin.

Summary of the Results on the Study of Tobacco Flower Stamens

A diagrammatic sketch illustrating the appearance, under U.V. light, of a two-dimensional chromatogram of stamen extract (75μl) is shown in Figure 17. The chromatogram was developed in the BAW solvent system for the first direction and in the IFW solvent system for the second direction. The following notations are used to identify the major fluorescent areas in Figure 17. The capital letter E is used to represent esculetin, the letters C and C' for chlorogenic acid and its isomer, the letter S for scopolin and R for rutin. The numerical notations are the same as those used in Chapter III for each substance isolated and identified. For the unknown fluorescent spots, the capital letter "U" precedes each number.

Spots ST-U1 and ST-U2 were yellow fluorescent areas which were not identified in this study. Spot ST-U3 was a faint blue area which was not identified. The two areas marked ST-U4 represent the unknown substance which fluoresced blue only upon exposure of the chromatogram to ammonia fumes. It was isolated from zone ST-1. The large spot R and

FIGURE 17. TWO-DIMENSIONAL CHROMATOGRAM OF EXTRACT FROM TOBACCO FLOWER STAMENS



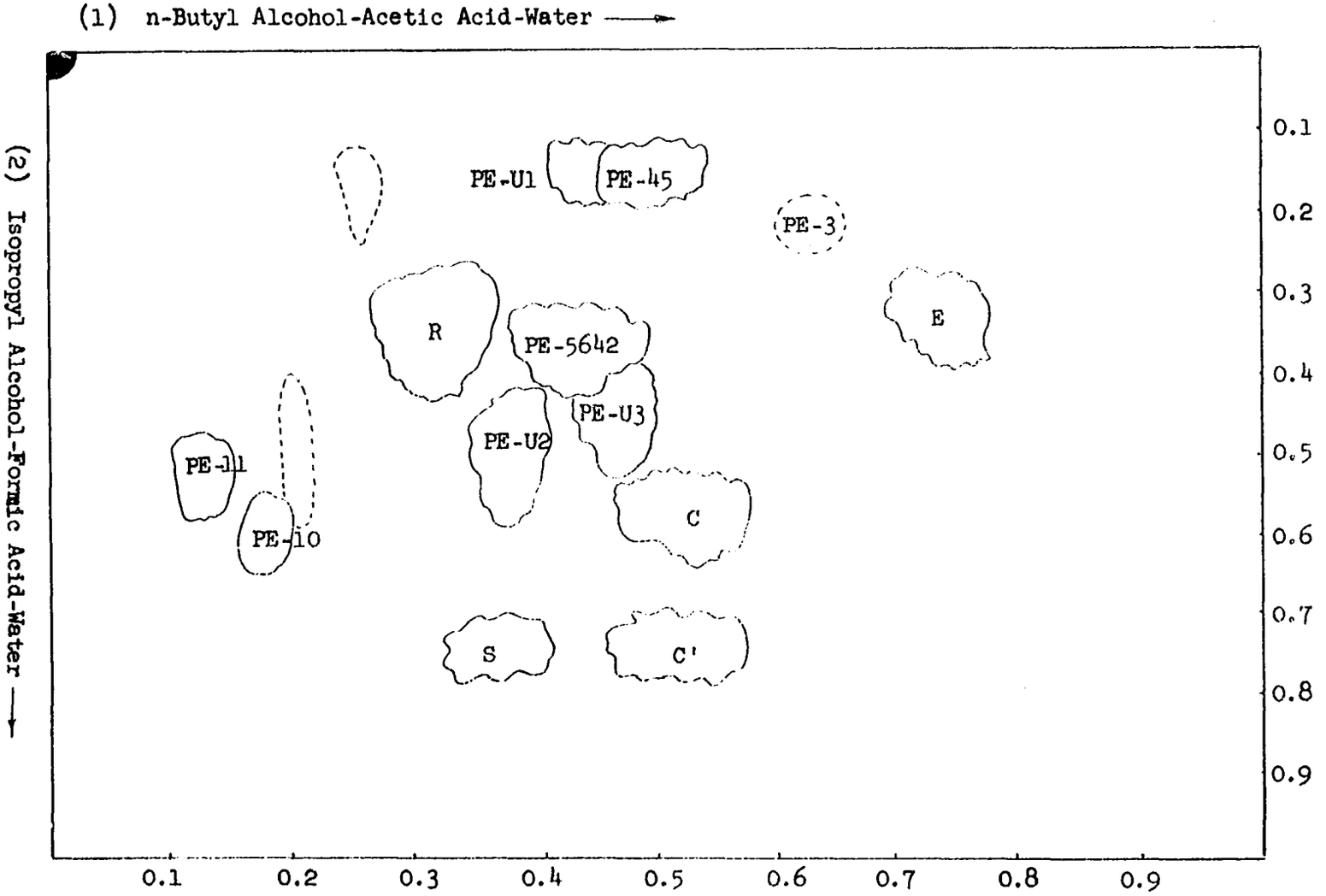
ST-78531 were overlapping brown fluorescent areas which correspond to rutin and a kaempferol-3-glucoside. They were isolated from zones ST-7, ST-8, ST-9 and ST-10. Spot ST-U5 was a blue fluorescing area which was not identified. Spot S corresponds to scopolin, which was isolated from zones ST-7 and ST-8. Spots C and C' correspond to chlorogenic acid and its isomer, which were isolated from zones ST-5, ST-6 and ST-7. Spot ST-53 was seen only upon exposing the chromatogram to ammonia fumes. This area contained the glucoside of ferulic acid which was isolated from zone ST-5. Spot ST-422 was another area which was seen only upon exposing the chromatogram to ammonia fumes. This area corresponds to the glucoside of p-coumaric acid which was isolated from zones ST-4 and ST-5. Spot E was a blue fluorescent spot which represents the area where esculetin normally migrates in the two solvent systems used.

In this preliminary study, the following compounds were found only in the stamens of the tobacco flower: glucosides of ferulic and p-coumaric acids, a kaempferol-3-glucoside, and the 3 unknown areas ST-U1, ST-U2 and ST-U4.

Summary of the Results on the Study of Corollas (Petals)

A diagrammatic sketch illustrating the appearance, under U.V. light, of a two-dimensional chromatogram of corolla extract (75 l.) is shown in Figure 18. The chromatogram was developed with the same solvent systems used for stamen extract. The capital letters, E, C, C', S and R are used to represent esculetin, chlorogenic acid and its isomer, scopolin and rutin, respectively. The numerical notations are the same as those used in Chapter IV for each substance isolated and identified.

FIGURE 18. TWO-DIMENSIONAL CHROMATOGRAM OF EXTRACT FROM TOBACCO FLOWER COROLLAS (PETALS)



For the unknown spots, the capital letter "U" precedes each number.

The spot PE-U1 was a faint blue fluorescent area which was not identified in this study. Spot PE-45 fluoresced brown and contains the flavonoid, isoquercitrin, which was isolated from zones PE-4 and PE-5. Spot R represents rutin which was isolated from zone PE-7. Spot PE-5642 fluoresced brown and contains the flavonoid, nictoflorin, which was isolated from zones PE-4, PE-5 and PE-6. Spot PE-3 could not be seen on the two dimensional chromatogram but represents the approximate area where astragalin would migrate in the two solvent systems used in developing the chromatogram. Spot PE-11 fluoresced brown and contained the flavonoid, rutin-7-monoglucoside, which was isolated from zone PE-11. Spot PE-10 also fluoresced brown and contained the flavonoid, nictoflorin-7-monoglucoside, which was isolated from zone PE-10. Spots PE-U2 and PE-U3 are blue fluorescent areas which were not identified in this study. Spots C and C' represent chlorogenic acid and its isomer which were isolated from zones PE-4, PE-5, PE-6 and PE-7. Spot S represents scopolin which was isolated from zones PE-5, PE-6 and PE-7. Spot E represents esculetin which was isolated mainly from zone PE-2.

On the basis of size and fluorescent intensity of spots or zones, the 6 flavonoids isolated from the petals may be listed in the following apparent decreasing order of concentration: rutin, nictoflorin, rutin-7-glucoside, nictoflorin-7-glucoside, isoquercitrin and astragalin.

In this preliminary study, the following compounds or substances were found only in the corollas or petals: rutin-7-glucoside, nictoflorin-7-glucoside, nictoflorin, isoquercitrin and astragalin.

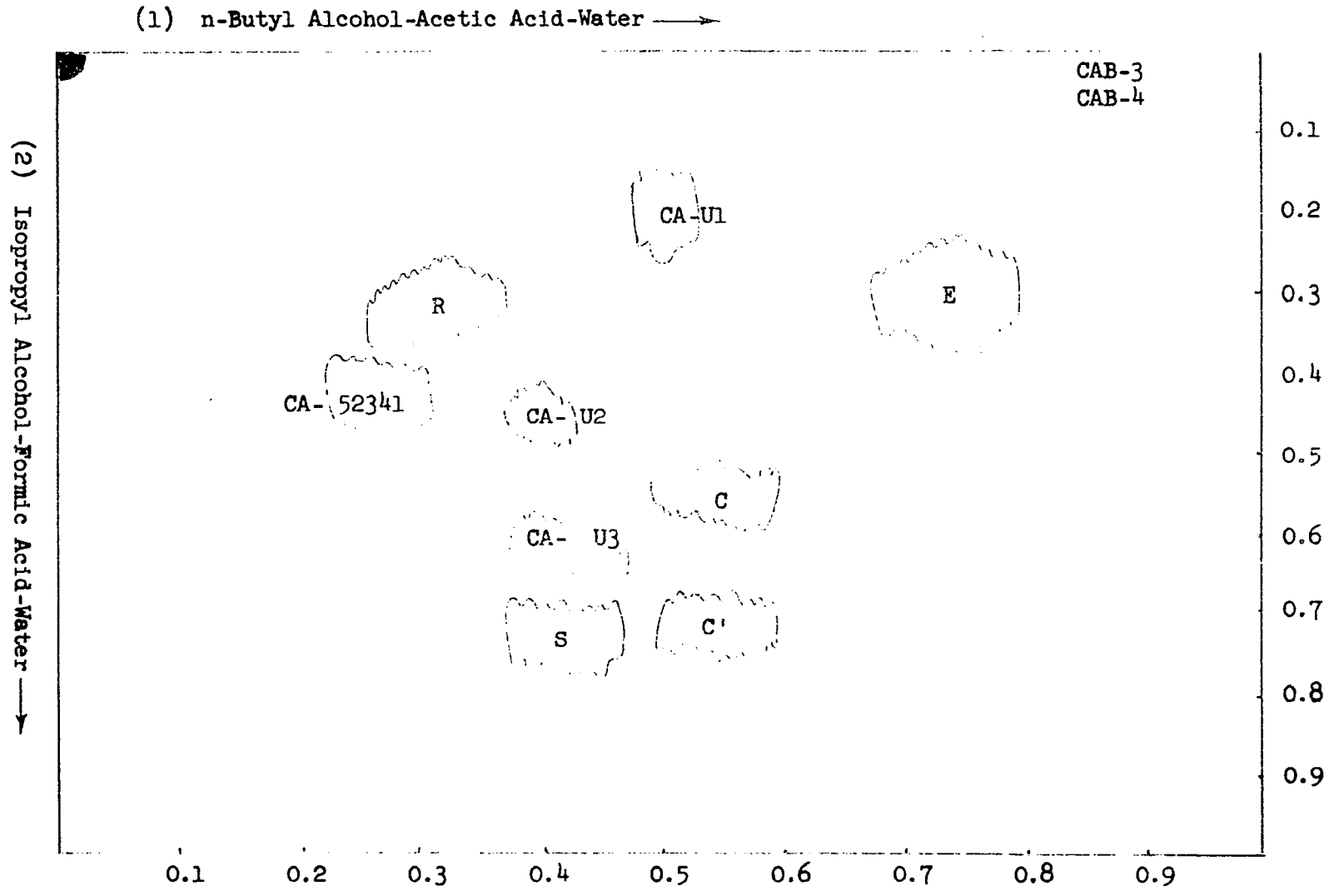
Summary on the Study of the Tobacco Flower Calyxes

A diagrammatic sketch illustrating the appearance, under U.V. light, of a two-dimensional chromatogram of calyx extract (75 μl.) is shown in Figure 19. The solvent systems used in the preceding sections were used. The capital letters E, C, C', S and R refer to the four compounds noted previously. The same system of notation was used as in the previous sections except that the numbers refer to compounds identified in Chapter V.

The area labeled CAB-3 and CAB-4 was not observed on the two-dimensional chromatogram since the compounds were present in small amounts. The area marked is the approximate position where these compounds would have migrated on the two-dimensional chromatogram with the two solvent systems used to develop the chromatogram. CAB-3 refers to quercetin-3,3'-dimethyl ether, and CAB-4 refers to quercetin-3-methyl ether. Both substances were isolated from the benzene soluble portion of the original calyx extract. The spot CA-U1 was a blue fluorescent area which was not identified in this study. The spot labeled R represents rutin, which was isolated mainly from zone CA-5. The area labeled CA-52341 refers to a quercetin-3-glucoside which was isolated mainly from zone CA-5. The blue fluorescent spots CA-U2 and CA-U3 were not identified. Spot S corresponds to scopolin which was isolated from zone CA-4. The two areas C and C' represent chlorogenic acid and its isomer which were isolated from zone CA-3. Spot E refers to esculetin which was isolated from zone CA-1.

From this preliminary study, the compounds quercetin-3,3'-

FIGURE 19. TWO-DIMENSIONAL CHROMATOGRAM OF EXTRACT FROM TOBACCO FLOWER CALYXES



dimethyl ether, quercetin-3-methyl ether and a quercetin-3-glucoside were found to be present only in the calyxes.

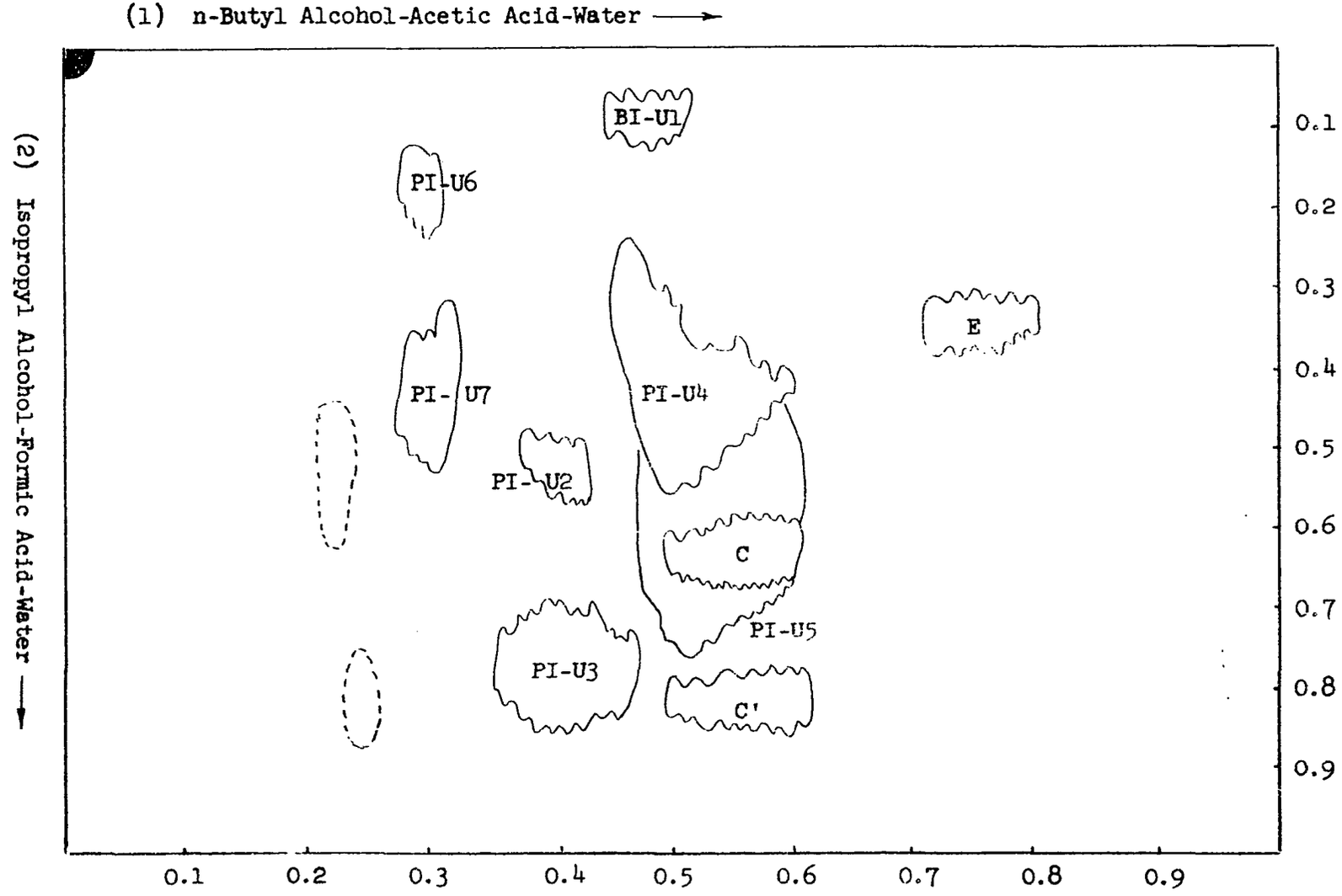
Summary of the Study of the Tobacco Flower Pistils

A diagrammatic sketch illustrating the appearance, under U.V. light, of a two-dimensional chromatogram of pistil extract (75 μl) is shown in Figure 20. The same solvent systems and notations are used as for the preceding 3 sections except that the numbers refer to those used in Chapter VI.

The 3 spots PI-U1, PI-U2 and PI-U3 were blue fluorescent areas which were not identified. The two large areas PI-U4 and PI-U5 fluoresced blue, which changed to yellow-green upon exposure to ammonia fumes, and reacted with ammonium molybdate spray reagent. They were not identified. Spots C and C' correspond to chlorogenic acid and its isomer, which were isolated from zone PI-3. Spot E corresponds to esculetin which was isolated from zone PI-1. Spots PI-6 and PI-7 were light tan fluorescing areas which contained esculetin and an unknown yellow fluorescing substance.

From this preliminary study, it appears that the pistil contains mainly blue fluorescent substances and does not have flavonoid or glycoside compounds. The pistil contains a large amount of benzene and chloroform soluble substances which prevented other compounds from migrating into well-defined spots or zones. This is apparent in Figure 20 where the large spots PI-U4 and PI-U5 are shown.

FIGURE 20. TWO-DIMENSIONAL CHROMATOGRAM OF EXTRACT FROM TOBACCO FLOWER PISTILS



CHAPTER VIII

A STUDY OF POLYPHENOLIC COMPOUNDS IN BORON-DEFICIENT TOBACCO LEAVES

Tobacco plants, Nicotiana tabacum L., var. One Sucker, were grown in a greenhouse at Argonne National Laboratory. The seedlings were cultured hydroponically on quartz sand and supplied with complete nutrient solution (20) until they were 40 days old. At this time, one group of plants was given minus-boron solution and the second group was continued on the complete plus-boron solution for an additional period of 38 days. At the end of this 38-day period, the plants were harvested and leaves from the 10-12th nodes were collected separately from each group. These young leaves from the minus-boron plants all showed marked boron deficiency symptoms. Leaves below the 10th node were also harvested from each group.

Leaves from each treatment were weighed and then macerated in 50% isopropyl alcohol-water solution with a Waring blendor. The slurry was brought to boiling, cooled and bottled. The bottles were shipped to this laboratory for further study.

Each slurry was exhaustively extracted by the method described in Chapter II. The extracts were made up to 2 liters with methyl alcohol in a volumetric flask. Aliquots from each extract were examined individually by one- and two-dimensional chromatography. Each aliquot repre-

sented approximately an equivalent amount of fresh weight of tissue. Examination of each chromatogram under U.V. light indicated that the extracts from leaves below the 10th node for both plus- and minus-boron cultured plants appeared to contain the same amounts of each recognizable compound. In the case of the younger leaves, the extract from minus-boron leaves contained a greater amount of a bright blue fluorescent substance, a lower amount of chlorogenic acid, a greater amount of an unknown blue fluorescent substance, a lesser amount of rutin and a greater amount of a kaempferol glycoside than extracts from corresponding plus-boron leaves. The isolation and identification of this bright blue fluorescent substance and the determination of relative approximate amounts of this substance in each group of young leaves are discussed in the following sections.

Isolation and Identification of Scopolin (scopoletin-7-glucoside) in Boron-Deficient Tobacco Leaves

A one liter aliquot of the extract from minus-boron leaves was concentrated under reduced pressure to remove most of the alcohol. The aqueous solution was extracted with redistilled benzene. The extracted aqueous layer was concentrated to a heavy syrup, which was fractionated by column chromatography, using Magnesol (Food Machinery and Chemical Corp., Westvaco Chemical Division, New York) as adsorbent. A 4.5 x 10 cm. Magnesol column was prepared by packing, under 10 lb. air pressure, an aqueous slurry of dried, methyl alcohol washed, Magnesol. The packed column was washed with 2 liters of distilled water under 5 lb. air pressure. The concentrated extract was carefully applied to the top of the

packed column. The extract was adsorbed on the adsorbent under 5 lb. air pressure. After all the extract was adsorbed, distilled water was added to the top of the column as the first developing solvent. The position of the bands on the columns was observed with the aid of U.V. light. The developing solvent was allowed to percolate down the column by gravity. After the initial blue bands had passed out of the column, the developing solvent was changed to methyl alcohol-water (1:9 v/v). This solvent separated a bright blue band and a closely moving brown band from the origin. After these two bands were midway down the column, the solvent was changed to acetone-water (1:9 v/v). The latter solvent moved the bright blue band away from the slower moving brown band. The bright blue band was collected in three fractions, designated 9a, 9b and 9c.

Fraction 9a was concentrated in vacuo, and the blue-fluorescent zone was purified by paper chromatography using BAW as the first developing solvent. This solvent resolved the zone into a fast moving bright blue band, which appeared to be scopoletin, and a slower moving bright blue-fluorescing band. The latter zone was cut out, sewed on fresh paper, and developed in IFW solvent system. The blue zone was cut out from the chromatogram and eluted with water. The absorption spectrum of the aqueous eluate showed minima at 262 and 304 mμ and maxima at 282 and 333 mμ. Published maxima for scopolin in ethyl alcohol are 227, 250, 288 and 339 mμ (35). The remainder of the eluate was concentrated to dryness in vacuo and hydrolyzed with 1 N HCl. After removal of HCl and addition of more water, the hydrolyzate was studied by paper chromatography, using the BAW solvent system. The sugar was located by spraying the

dried chromatograms with o-aminobiphenyl reagent (21). The sugar was found to be identical with reference glucose, and the aglycone was identical with pure, synthetic scopoletin (36).

Determination of Scopolin Concentration in the Boron-Deficient and in the Boron-Sufficient Leaves

To determine the approximate relative amounts of scopoletin glucoside present in the boron-deficient tobacco leaves as compared with boron-sufficient tobacco leaves, known volumes of extracts of each, representing about 300 mg. fresh wt. of leaf tissue, were subjected to extended paper chromatography. Solvents used were the IFW and then BAAW for 34 hrs. The blue zone was cut out from each chromatogram and eluted with water. Each water eluate of scopoletin glucoside was collected individually in a 10-ml. volumetric flask, and 1 ml. of 1 N HCl added. Each fraction was hydrolyzed, neutralized with NH_4OH to pH 10, and diluted to the mark with distilled water. Every step for both boron-deficient and control leaves was performed as quantitatively as possible. The intensity of fluorescence of each scopoletin sample was determined immediately with the Klett fluorimeter (filters No. 5860 and No. 3389). A standard reference curve was prepared using known quantities of pure scopoletin. A blank paper was carried throughout the procedure. Typical analyses indicated that three aliquots, each representing 315 mg. fresh weight of control tissue, contained 0.97, 1.24, and 1.17 μg. scopoletin or an average of 7.4 μg. scopoletin glucoside per gram of tissue. For the minus-boron tissue, three aliquots, each representing 283 mg. fresh weight, contained 22.6, 21.0, and 23.4 μg. scopoletin or an average of 155 μg. scopoletin glucoside per gram of tissue. Although these values

do not necessarily represent absolute concentrations of glycoside present in a given tissue, they indicate that the minus-boron tissue had about a 20-fold increase in glycoside concentration over comparable boron-sufficient tissues.

CHAPTER IX

SUMMARY

A preliminary study has been made on the identification of certain polyphenolic compounds present in four kinds of floral appendages of the one-sucker tobacco, Nicotiana tabacum L. The knowledge on the distribution of these compounds in each floral part could aid future biochemical, physiological and genetical investigations of tobacco. Paper partition chromatography techniques were used to isolate the polyphenolic compounds. The characteristic fluorescence of these compounds on paper chromatograms irradiated with 3660 Å ultraviolet light was used to locate and to differentiate the polyphenolic compounds. The isolated compounds were identified by two or more of the following methods: (1) comparison of R_f values and fluorescence with known reference compounds, (2) determination of the ultraviolet absorption spectra with and without the addition of certain chemicals, (3) chemical and/or enzymatic hydrolysis and subsequent identification of the hydrolysis products, and (4) reaction on chromatograms with chromogenic spray reagents.

The compounds identified in the stamens of the flower were: rutin (3-rhamnoglucoside of quercetin, 3,3',4',5,7-pentahydroxyflavone), scopolin (7-glucoside of scopoletin, 7-hydroxy-6-methoxycoumarin), esculetin (6,7-dihydroxycoumarin), chlorogenic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)), a 3-glucoside of

kaempferol (3,4',5,7-tetrahydroxyflavone), a glucoside of ferulic acid (4-hydroxy-3-methoxycinnamic acid) and a glucoside of p-coumaric acid (4-hydroxycinnamic acid). The last 3 compounds were found only in the stamens.

The corollas (petals) contained scopolin, esculetin, chlorogenic acid, rutin, nictoflorin (kaempferol-3-rhamnoglucoside), isoquercitrin (quercetin-3-monoglucoside), astragalin (kaempferol-3-monoglucoside), rutin-7-glucoside, and nictoflorin-7-glucoside. The last 5 compounds were found only in the petals.

The calyxes or sepals contained esculetin, chlorogenic acid, scopolin, rutin, a quercetin-3-glucoside, quercetin-3,3'-dimethyl ether and quercetin-3-methyl ether. The last 3 compounds were present only in the calyxes.

The compounds identified in the pistils were chlorogenic acid and esculetin. This study has shown that this part of the flower did not contain any flavonoids or glycosides usually associated with this species of tobacco.

The results of this study indicated that esculetin and chlorogenic acid were present in four parts of the flower. Rutin and scopolin were present in the stamen, corolla and calyx tissues but not in the pistils. The calyx was the only floral appendage of the 4 types studied which contained methyl ether derivatives of quercetin.

The compounds rutin-7-glucoside, nictoflorin-7-glucoside, astragalin, a kaempferol-3-glucoside, a quercetin-3-glucoside, a glucoside of ferulic acid and a glucoside of p-coumaric acid are reported to be present in tobacco tissue for the first time.

The biochemical role of the micronutrient element, boron, in higher plants is not known. To gain some insight as to its possible function, chromatographic comparison between extracts from normal tissue and boron deficient tissue were made. The low-boron tissue accumulated approximately 20 times more of a blue fluorescing compound than corresponding boron-sufficient tissue. By column and paper chromatographic techniques, this compound was isolated and identified as scopolin.

BIBLIOGRAPHY

1. Dieterman, L. J., "A Preliminary Study of the Metabolic Fate of Quercetin and Rutin in Nicotiana Tabacum," Ph.D. Dissertation, University of Oklahoma, 1961.

2. Griffiths, L. A., Nature, 180, 286 (1957).

3. Weaving, A. S., Tobacco Science, 2, 1 (1958).

4. Gortner, W. A. and Kent, M. J., J. Biol. Chem., 233, 731 (1958).

5. Furuya, M., Galston, A. W., and Stowe, B. B., Plant Physiol., 36, xlvii (1961).

6. Mumford, F. E., Smith, D. H., and Castle, J. E., Plant Physiol., 36, 752 (1961).

7. Stedman, R. L., Tobacco Science, 1, 155 (1957).

8. Reid, W. W., J. Soc. Leather Trades Chem., 40, 117 (1956).

9. Wender, S. H., Bradford, R. H., Chorney, W., Murphy, E. L., Scully, N. J., and Yang, C. H., Abstracts, A.C.S. 131st Meeting, 14D (April 1957).

10. Murphy, E. L., "A Study of Certain Flavonoid Compounds in the Leaves and Flowers of Tobacco," Ph.D. Dissertation, University of Oklahoma, 1957.

11. Charaux, C., Bull, soc. chim. biol., 6, 641 (1924). Chem. Abst. 19, 92 (1925).

12. Nio, S. and Wada, E., J. Agr. Chem. Soc. Japan, 24, 485 (1951). Chem. Abst. 46, 10551 (1952).

13. Wada, E., J. Agr. Chem. Soc. Japan, 26, 159 (1952). Chem. Abst. 48, 9482 (1954).

14. Dieterman, L. J., Yang, C. H., Nakagawa, Y., and Wender, S. H., J. Org. Chem., 24, 1134 (1959).

15. Yang, C. H., "A Study of Certain Blue-Fluorescent Polyphenols in Tobacco Leaves, Flowers and Cigarette Smoke," Ph.D. Disserta-

tion, University of Oklahoma, 1958.

16. Yang, C. H., Braymer, H. D., Murphy, E. L., Chorney, W., Scully, N., and Wender, S. H., J. Org. Chem., 25, 2063 (1960).

17. Kuhn, R., and Low, I., Chem. Ber., 81, 474 (1949).

18. Skok, J., "The Role of Boron in the Plant Cell," in Trace Elements, edited by C. M. Lamb, O. G. Bentley, and J. M. Beattie, Academic Press Inc., New York, 1958, pp. 227-243.

19. Gauch, H. G., and Dugger, W. M., Jr., Plant Physiol., 28, 457 (1953).

20. Watanabe, R., McIlrath, W. J., Skok, J., Chorney, W., and Wender, S. H., Arch. Biochem. Biophys., 94, 241 (1961).

21. Timell, T. E., Glaudenmans, C. P. J., and Currie, A. L., Anal. Chem., 28, 1916 (1956).

22. Albright, E. C., Larson, F. C., and Deiss, W. P., Proc. Soc. Exptl. Biol. Med., 84, 240 (1953).

23. Jurd, L. and Horowitz, R. M., J. Org. Chem., 22, 1618 (1957).

24. Jurd, L., Arch. Biochem. Biophys., 63, 376 (1956).

25. Harborne, J. B., Chem. & Ind., 1142 (1954).

26. Swain, T., Chem. & Ind., 1480 (1954).

27. Cartwright, R. A. and Roberts, E. A. H., Chem. & Ind., 230 (1955).

28. Harborne, J. B. and Corner, J. J., Biochem. J., 81, 242 (1961).

29. Harborne, J. B. and Sherratt, H. S. A., Biochem. J., 78, 298 (1961).

30. Yang, C. H., Nakagawa, Y., and Wender, S. H., J. Org. Chem., 25, 658 (1960).

31. Kallianos, A. G., "Synthesis and Chromatographic Characterization of Some Polyphenolic Compounds," M.S. Dissertation, University of Oklahoma, 1956.

32. Harborne, J. B., J. Chromatography, 2, 581 (1959).

33. Dunlap, W. J., and Hagen, R. E., Private Communications.

34. Hattori, S. and Shimokoriyama, M., Proc. Roy. Dublin Soc., 27, 139 (1956).

35. Harborne, J. B., Biochem. J., 74, 270 (1960).

36. Braymer, H. D., Shetlar, M., and Wender, S. H., Biochim. et Biophys. Acta, 44, 163 (1960).