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A STUDY OF CERTAIN BLUE-FLUORESCENT POLYPHENOLS IN TOBACCO LEAVES, FLOWERS AND CIGARETTE SMOKE

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A STUDY OF CERTAIN BLUE-FLUORESCENT POLYPHENOLS IN TOBACCO LEAVES, FLOWERS AND CIGARETTE SMOKE

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C. H. Y.

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CHAPTER I

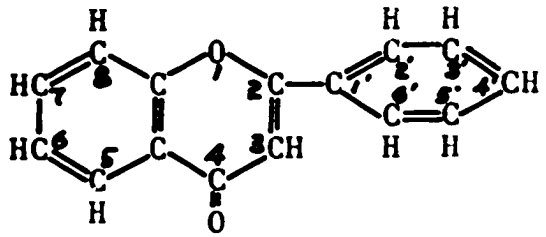
INTRODUCTION

The "flavonoid" compounds of plants are so called because they possess the carbon skeleton of flavone (2-phenyl-1.4-benzopyrone) (Figure 1). All of these compounds are widely distributed, as a class, in the higher plants, and probably in all parts of the plant: roots, bark, wood, stems, leaves, flowers, fruits, and seed. This distribution suggests that they owe their formation to certain processes fundamental to most higher plants.

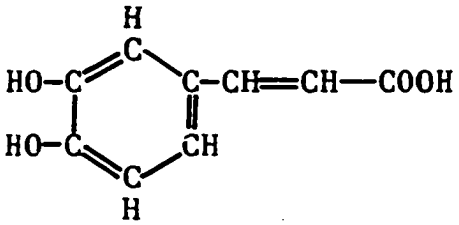
The literature (1, 2, 3, 4, 5) contains extensive information on the occurrence of flavonoids and related polyphenolic compounds in the plant kingdom. Hypotheses concerning the biogenesis of flavonoid compounds have been postulated (4). Although these hypotheses have not been sufficiently elaborated or substantiated by scientific evidence to allow their assessment at the present time, it has been noted recently that the most likely route to the biosynthesis of flavonoids is from a cinnamic acid and three acetic acid

FIGURE 1

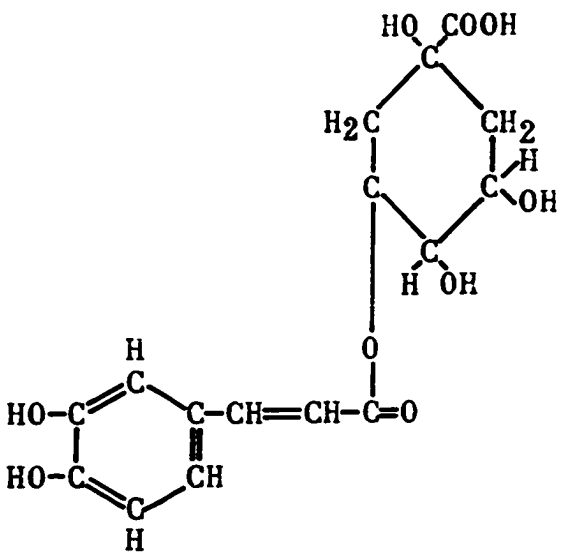
STRUCTURAL FORMULAE OF FLAVONE, CHLOROGENIC ACID, CAFFEIC ACID, SCOPOLETIN, ESCULIN, AND ESCULETIN



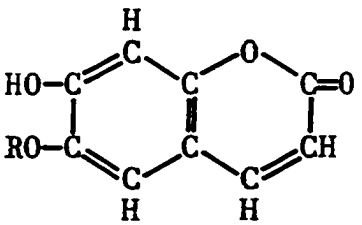
Flavone



Caffeic Acid



Chlorogenic Acid



Scopoletin: R-Methyl

Esculin: R-Glucosyl

Esculetin: R-H

units (6, 7).

The biological role of the flavonoid pigments as well as of their closely related polyphenols in higher plants, however, is for the most part unknown. A cooperative program was therefore established in 1954 with a group of scientists at the Argonne National Laboratory, Lemont, Illinois, to study the metabolism and interrelationship of these polyphenolic compounds in One-Sucker tobacco, Nicotiana tabacum, using radioisotope techniques. This program included infiltration studies in tobacco of C^{14}-labeled radioactive flavonoid compounds and closely related polyphenols to determine or trace their metabolic changes in the plant. Each C^{14}-labeled radioactive compound to be used for plant metabolism study was isolated and purified by chromatographic and partition techniques. Tobacco plants were grown in an atmosphere of radioactive carbon dioxide, $C^{14}O_2$, in the growth chambers of the Argonne National Laboratory, to serve as a source for the isolation of the radioactive compounds.

A preliminary study of the polyphenols in tobacco was made at the Argonne National Laboratory in the summer of 1954. This work indicated the presence of many flavonoid compounds and a group of compounds of polyphenolic nature which were blue-fluorescent under ultraviolet light. The blue-fluorescent compounds present in tobacco have previously received relatively little attention. Prior to 1954, the only blue-fluorescent compounds which had been isolated from

tobacco and identified were caffeic acid (8, 9) and chlorogenic acid (8, 9, 10) (Figure 1). A blue-fluorescent substance accumulating in the roots of decapitated tobacco plants infected with virus of spotted tomato wilt had been isolated and identified as scopoletin (11) (Figure 1).

As background information for the planned tobacco plant infiltration studies using radioactive polyphenols, it was necessary to isolate and identify as many as possible of specific polyphenolic compounds. This program, of course, is so vast, with so many ramifications, that the investigations of one individual must be limited, for a time, to one phase of the subject. For this dissertation research special emphasis has been placed on an effort to separate from tobacco, purify, and possibly identify phenolic constituents which fluoresce a blue color under ultraviolet light. The corresponding study on the flavonoid type compounds was carried out in parallel with this work by Edwin L. Murphy in this laboratory (12). Experimental details of the isolation and identification of these blue-fluorescent polyphenols in the tobacco leaves are presented in Chapter II.

The flowers of One-Sucker tobacco, Nicotiana tabacum, contain a relatively larger amount of polyphenolic constituents than do the leaves. All blue-fluorescent compounds found in the leaves and described in Chapter II were also found in the flowers. In addition to these, several unknown polyphenolic compounds were isolated. The presence of these

compounds in tobacco has never been previously reported in the literature. These new constituents include polyglycosides of flavonoids and of blue-fluorescent compounds. The details of the experimental studies undertaken on the isolation, identification, and characterisation of certain of these phenolic compounds in tobacco flowers are presented in Chapter III.

Extracts of the radioactive tobacco plants grown in an atmosphere of radioactive carbon dioxide, $C^{14}O_2$, in the growth chambers of the Argonne National Laboratory, were sent to this laboratory. The isolation of the radioactive blue-fluorescent polyphenols was carried out by applying and extending the techniques learned in the studies described in Chapters II and III. The experimental results and the procedures used for the isolation of the radioactive blue-fluorescent compounds are described in Chapter IV.

A general method for the quantitative determination of tobacco phenols has long been needed. For example, the total amounts of phenolic compounds in tobacco leaves are still uncertain. According to Frankenburg (13):

> "The main difficulty with the entire class of phenolic compounds in tobacco is the lack of satisfactory and dependable analytical methods that could permit exact determination of their total quantity and their individual composition. A fully satisfactory analytical procedure would have to be based on a quantitative separation of phenols, polyphenols, and flavonoids from the other leaf components, and the determination of individual compounds in this fraction."

A spectrophotometric determination has the advantage of speed and simplicity when a reliable quantitative separation of each individual phenolic component has been established. Such a method has been attempted by making use of extended mass paper chromatographic separation. The details of the procedure of this method and the results obtained are presented in Chapter V.

Because of the possible effects of tobacco smoking on health, many investigations have been carried out on tobacco smoke condensate (14). These studies, however, have been centered on carcinogenic polycyclic hydrocarbons or tobacco tar and nicotine. No systematic work whatsoever has been reported in the literature on the fate of the tobacco polyphenols during smoking. A preliminary study of the isolation and identification of polyphenols and their possible decomposition products persisting in the mainstream cigarette smoke was carried out. Cigarettes were smoked using a smoking machine designed to simulate conditions of human smoking, and the smoke condensates were collected. The experimental details and the procedure used for the identification of a blue-fluorescent compound in the cigarette tobacco and smoke are described in Chapter VI.

CHAPTER II

ISOLATION AND IDENTIFICATION OF CERTAIN BLUE-FLUORESCENT COMPOUNDS IN TOBACCO LEAVES

Isolation Process

Anhydrous Isopropyl Alcohol Extraction. Thirteen hundred grams of dried ground leaves of the One-Sucker tobacco plant, Nicotiana tabacum, grown in the greenhouse of the Argonne National Laboratory were divided into three portions (500 g., 500 g., and 300 g.); each was extracted separately with about 1 liter of anhydrous isopropyl alcohol. The mixture of the leaves and isopropyl alcohol was heated on a water bath, and the alcohol solution was filtered. The residue was subjected to further extraction repeatedly until the total volume of isopropyl alcohol solution amounted to approximately 20 liters. The solution was then concentrated to 2 liters under reduced pressure at a temperature less than 55^{o} C.

With the purpose of separating the pentane-insoluble flavonoid compounds from the original extracts, a pentane extraction was undertaken. Each time, approximately 200 ml. of the concentrate was placed in a 5 liter flask and about

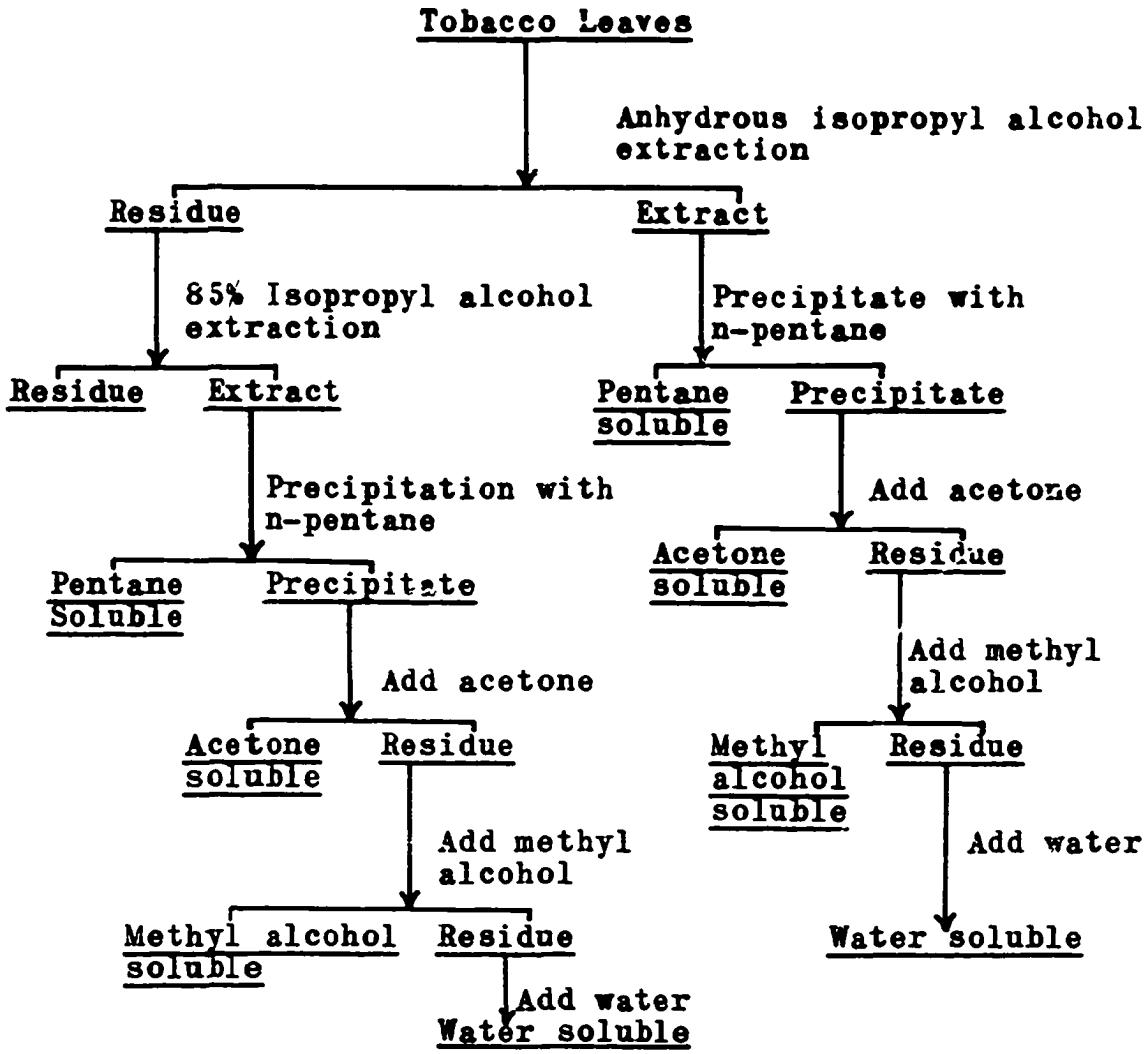
4 liters of dry n-pentane was added to it. The materials which precipitated out were then washed several times with fresh dry pentane and further fractionated in dry acetone, then anhydrous methyl alcohol, and finally water.

85% Isopropyl Alcohol Extraction. The tobacco residues remaining from the previous extraction with anhydrous isopropyl alcohol were subjected to further extraction with 85% aqueous isopropyl alcohol. The extracts were treated in exactly the same way as described above and were concentrated. About 6 liters of aqueous concentrates were obtained. After this large amount of water was co-distilled with isoamyl alcohol, the residue was again treated with dry n-pentane. The precipitates thus obtained were worked as before and were extracted by acetone, methyl alcohol, and water. Figure 2 illustrates the isolation process.

In order to ascertain which fractions contained blue-fluorescent compounds, a chromatogram was prepared by spotting approximately 0.2 ml. of each fraction 3 cm. apart on a line 12 cm. below the top of a sheet of chromatography paper, 22 by 58 cm., Schleicher and Schuell, No. 589, Red Ribbon. Other chromatograms were prepared as described above and each developed in a chromatographic tank with one of a series of several different solvent systems by one-dimensional descending chromatography. The solvent systems included in this study were 15% aqueous acetic acid, 60% aqueous acetic acid, n-butyl alcohol-acetic acid-water (6:

FIGURE 2

FLOW-SHEET OF THE OPERATIONS PERFORMED IN THE EXTRACTION OF TOBACCO LEAVES



1:2, v/v), and n-butyl alcohol-pyridine-benzene-water (5:3:1:3, v/v). After drying, each chromatogram was observed under long wave ultraviolet light, 3660 Angstroms.

Two acetone fractions and two methyl alcohol fractions, obtained in the previous extraction, were found to contain most of the blue-fluorescent compounds. It was also found that the n-butyl alcohol-acetic acid-water system was the best for initial separation of each individual blue compound.

Purification by Mass Paper Chromatography. A preliminary one-dimensional paper chromatographic analysis of the four fractions mentioned above indicated that the blue-fluorescent compounds contained in each fraction were similar. The main difference between the acetone fraction and methyl alcohol fraction was that the former contained more aglycone type blue compounds and the latter more glycoside type blue compounds. The latter have smaller R_f values in the n-butyl alcohol-acetic acid-water system. Therefore, after the preliminary mass paper chromatographic separation in the n-butyl alcohol-acetic acid-water system, the corresponding blue-fluorescent zones from the four fractions were added together and subjected to further purification.

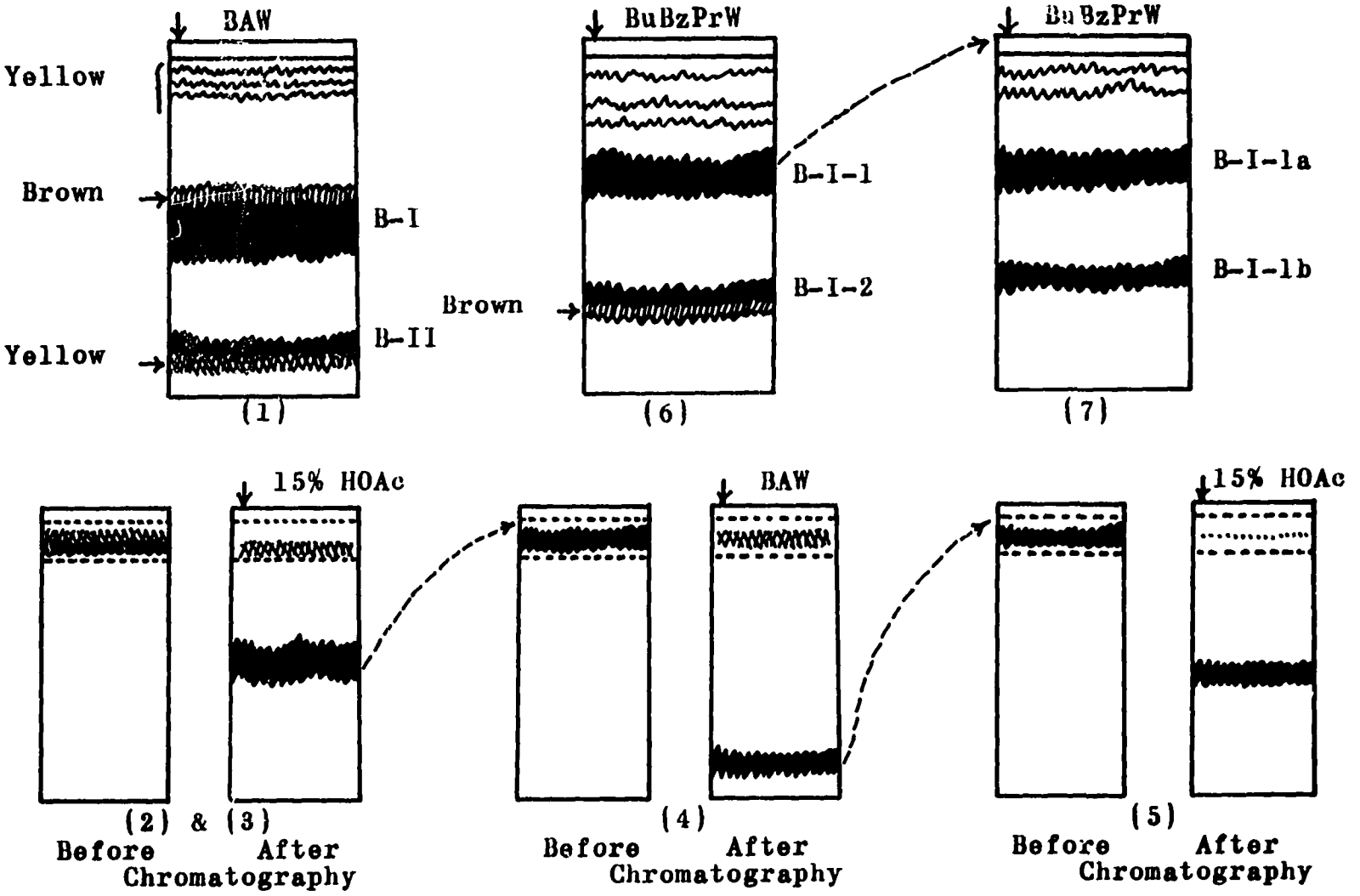
For mass paper chromatography, each fraction was streaked continuously along a line 8 cm. from one end of separate sheets of chromatographic paper, Whatman No. 3 MM,

19 cm. x 46 cm., by means of a pipette. After the spots had air dried, this group of chromatograms was placed in a chromatographic chamber containing n-butyl alcohol-acetic acid-water as the solvent system and allowed to develop until the solvent front had moved 35 or 40 cm. The chromatograms were then removed and air dried. Observation under long wave ultraviolet light showed that the original material of each fraction had separated into two main blue zones, B-I and B-II (Figure 3).

Purification of B-II. The brilliant blue fluoresing zone B-II running far down the paper with the n-butyl alcohol-acetic acid-water system, was not yet free of some chlorophyll-like green material. The latter has a very small R_f value in the 15% acetic acid system. This enabled one to carry out the purification as follows: The bottom zone, which contained B-II and green material, was cut off from each chromatogram and sewed onto a new sheet of Whatman 3 MM paper in such a direction that the B-II zone was now facing the bottom. The papers thus prepared were run in a 15% acetic acid system, which moved the B-II blue far away from the green materials. The B-II zone was cut off from the chromatograms, sewed onto another new sheet of paper and run in the 15% acetic acid system again. After this chromatographing, the B-II zone was again cut off, sewed onto a new sheet and run in the n-butyl alcohol-acetic acid-water system. The same procedure was repeated on a new

FIGURE 3

DIAGRAMS ILLUSTRATING MASS PAPER CHROMATOGRAPHIC SEPARATION OF SCOPOLETIN



sheet in the 15% acetic acid system. The pure B-II was finally cut off from the chromatograms and eluted with 95% ethyl alcohol. The alcohol solution was filtered and the solvent evaporated under reduced pressure.

Various organic techniques were tried on the residues to obtain the pure crystalline B-II. None of them was successful in yielding crystals. Tried were fractional recrystallization of the compound from the different concentrations of the aqueous alcohols and of some other organic solvents. These procedures failed to remove the paper impurities which interfere with crystallization (15, 16). Nor did B-II crystallize out from the solution. The solubility of the paper impurities is just so close to that of the B-II and other polyphenolic compounds that one was unable to apply the technique of fractional recrystallization to this problem.

One other method tried was adsorption chromatography using a Magnesol column (17). The impurities from paper were found to be only slightly soluble in absolutely dry acetone, while the B-II is quite soluble in dry acetone. A Magnesol column was made by packing the Magnesol powder as a slurry in dry acetone in a glass column. After washing with dry acetone, the mixture of B-II and paper impurities in dry acetone was poured on top of the Magnesol and the B-II was moved down the column by the dry acetone. Elution of B-II was continued until the blue fluorescence of B-II disappeared

from the column. Meanwhile, the dry acetone eluates were collected in several fractions. Evaporation of the acetone from the eluted fraction left the residue in a fairly clean form. It was then fractionally recrystallized to remove the trace amount of paper impurities remaining. The B-II from tobacco finally began to form a crystalline-like compound. However, it still was not good enough to give the sharp melting point desired.

Recovery of B-II from the Pentane Solution. In the process developed for the isolation of polyphenolic compounds from tobacco leaves, quite a large amount of pentane was used for fractionations. The pentane solution still contained some B-II, together with some black tar-like decomposition products. The recovery of B-II from this dark black solution was effected by using the cellulose column technique. Concentrated solutions of pentane were poured over cellulose powder (Whatman Standard Grade, ashless) which then was allowed to stand in a draft for a few days to obtain complete dryness. This cellulose containing the pentane solubles was then packed in a glass column by hand on top of some fresh cellulose powder and the B-II was developed and eluted out from the column with 15% acetic acid solution. The black tar-like impurities remained on the cellulose. The acetic acid eluates were concentrated under reduced pressure at room temperature in a flash evaporator. Further purification of B-II was

carried out by the method which was described in the mass paper chromatographic separation of B-II.

Further Separation of B-I. Another blue-fluorescent zone (B-I) of smaller R_f value in the n-butyl alcohol-acetic acid-water system (Figure 3) was carefully cut off from the chromatograms so that the zone would be separated from the brown-fluorescent flavonoid zone which ran just above it. The compounds contained in the zone were eluted from the paper with 85% isopropyl alcohol and re-chromatographed on Whatman 3 MM paper in the organic layer of n-butyl alcohol-benzene-pyridine-water (5:1:3:3 v/v) system. The paper chromatogram developed in this system is shown in section 6 of Figure 3.

The lower zone which was still poorly separated from the brown-fluorescent flavonoid zone was called B-I-2, and the wide upper zone B-I-1. The numbers I-1 and I-2 correspond to the order in which the various zones of B-I eluates appeared as they moved downward from the origin on the n-butyl alcohol-benzene-pyridine-water chromatogram.

The zone containing B-I-1 and the zone containing B-I-2 were each cut from the chromatograms and eluted separately with 85% isopropyl alcohol. The eluates of B-I-1 and B-I-2 were individually concentrated and subjected to further purification by mass paper chromatography on S. & S., No. 589, Red Ribbon filter paper.

Purification of B-I-2. The concentrate of B-I-2

was placed on 60 sheets of S. & S., No. 589, Red Ribbon filter paper (19 cm. x 58 cm.) and each paper was developed in the 15% acetic acid system. This procedure moved the B-I-2 blue far away from the brown-fluorescent flavonoid compounds. The zone containing B-I-2 was cut off from the chromatograms, sewed onto another new sheet of S. & S. paper and run in the n-butyl alcohol-acetic acid-water system again. After this chromatography, it was again cut off, sewed onto a new sheet and run in the 15% acetic acid system. The same procedure was repeated again on a new sheet in the n-butyl alcohol-acetic acid-water system. The pure B-I-2 was finally cut off from the chromatograms and eluted with 50% ethyl alcohol. The alcoholic extract was reduced in volume to approximately 50 ml. and placed in a refrigerator to await further examination for purity and identity.

Separation of B-I-1 into B-I-1a and B-I-1b and the Purification of Each New Compound. The concentrate of B-I-1 was placed on separate sheets of S. & S., No. 589, Red Ribbon paper (19 cm. x 58 cm.) by streaking, and each paper was developed for 20 to 26 hours in the n-butyl alcohol-benzene-acetic acid-water system. When the developed chromatograms of the B-I-1 zone were studied, it was observed that this zone could be resolved into two zones by development in the n-butyl alcohol-benzene-pyridine-water system above. These two zones were called B-I-1a (lower R_f value)

and B-I-1b (higher R_f value), respectively (Figure 3). Further purification of each zone was achieved by cutting out the zone and sewing it onto a fresh S. & S., No. 589 paper separately, using the techniques of mass paper chromatography.

Solvents used were a combination of the n-butyl alcohol-acetic acid-water and 15% acetic acid systems for B-I-1a and the n-butyl alcohol-benzene-pyridine-water and 15% acetic acid systems for B-I-1b. Each purified compound thus obtained was finally eluted from the respective paper by diluted alcohols. Both pure B-I-1a and B-I-1b were eluted with difficulty from the paper with cold 95% ethyl alcohol and various dilutions of cold ethyl alcohol. The alcoholic extracts from each compound were combined and placed in the refrigerator to await further tests for purity and identity.

The Identification of the Blue-Fluorescent Compounds from Tobacco Leaves

At this point, each of the zones isolated from the alcoholic extract of tobacco leaves could be considered a chromatographically pure compound in the solvent systems employed for their separation. In order to test their purity further as well as to characterize each isolated blue-fluorescent compound for comparison with the available literature for possible identification, the R_f value (Table 1) was obtained for each blue-fluorescent compound isolated

TABLE 1

CHROMATOGRAPHIC R_f VALUES*

Compound	Solvent Systems			
	15% Acetic Acid	60% Acetic Acid	n-butyl alcohol-acetic acid-water (6:1:2)	n-butyl alcohol benzene-pyridine-water (5:1:3:3)
B-II	0.32	0.75	0.84	0.86
Oat-Root Scopoletin	0.32	0.75	0.84	0.86
Synthetic Scopoletin	0.33	0.75	0.84	0.87
Esculetin	0.31	0.70	0.81	0.86
B-I-2 (B-3)	0.42	0.77	0.42	0.63
Esculin	0.40	0.71	0.45	0.63
B-I-1a (B-4)	0.13	0.56	0.23	0.35
B-I-1b (B-5)	0.81	0.83	0.60	0.61
Chlorogenic Acid	0.31	0.60	0.38	0.47
Isochlorogenic Acid	0.12	0.27	0.24	0.70
Neochlorogenic Acid	0.32	0.61	0.30	0.45

* Paper-Schleicher and Schuell, No. 589 Red Ribbon; Temperature 28° - 30° C.

from tobacco leaves in a series of solvent systems by the methods of Gage, Douglass and Wender (18) and of Swain (19). Further, the color was recorded for each untreated compound in visible and long wave ultraviolet light and with a series of chromogenic sprays which produce characteristic colors that can be used for qualitative identification of the blue-fluorescent compounds (Table 2).

Studies on Compound B-II. Corresponding data found in the literature (19) for (6-methoxy-7-hydroxy-coumarin) (Figure 1) were found to be very similar to those obtained with this compound.

In that the compound B-II appeared to be scopoletin, some authentic scopoletin was sought for comparison in final identification studies. Goodwin and co-workers (20) have reported the isolation of scopoletin and its glycoside from roots from oat seedlings, Avena sativa. Therefore, such an isolation to obtain scopoletin for comparison with the compound B-II from tobacco was undertaken.

Scopoletin from Oat Roots. Oat seeds were obtained from the Argonne National Laboratory and treated as described below. The extraction method finally used was based on a modification of Goodwin's work as suggested by Mr. Will Chorney and Mr. Ronald Watanabe of the Argonne National Laboratory.

Dry oat seeds were soaked in tap water for about 3 hours, then spread on a moisture-saturated thick filter

TABLE 2

COLORS PRODUCED WITH CHROMOGENIC SPRAYS

Compound	Untreated		NH_3 vapor		1% sodium nitrite + 1 N sodium hydroxide		Saturated ammonium molybdate		1% ethanolic aluminum chloride	
	V	UV	V	UV	V	UV	V	UV	V	UV
B-II	C	dB	C	dB	C	dB	C	dB	C	dB
Oat-Root Scopoletin	C	dB	C	dB	C	dB	C	dB	C	dB
Synthetic Scopoletin	C	dB	C	dB	C	dB	C	dB	C	dB
Esculetin	C	B	Y	Y	R*	Y	Y	pY	C	dB
B-I-2 (B-3)	C	dB	C	dB	C	dB	C	dB	C	dB
Esculin	C	B	C	B	C	B	C	B	C	B
B-I-1a	C	B	Y	gY	R	Bk	brY	Bk	C	B
B-I-1b	C	B	Y	gY	R	Bk	brY	Bk	C	B
Chlorogenic Acid	C	B	Y	gY	R	Bk	brY	Bk	C	B
Isochlorogenic Acid	C	B	Y	gY	R	Bk	brY	Bk	C	B
Neochlorogenic Acid	C	B	Y	gY	R	Bk	brY	Bk	C	B

V - visible light, UV - ultraviolet light, C - colorless, B - blue, Y - yellow, Bk - black, R - red, d - deep, g - greenish, p - pale, br - brownish.

* on standing, changed to orange.

paper. Fresh tap water was supplied to the seeds from time to time to keep them from becoming dry. After 2 to 3 days, the roots started to sprout. As soon as the roots started to grow, one could observe in the dark room a beautiful violet-blue fluorescence under the ultraviolet light. After 5 days, all of the roots were cut away from the seeds, and extraction of the roots with 85% isopropyl alcohol was started by placing the roots in the solvent and setting them aside at room temperature for about two weeks. With the aid of ultraviolet light, one could observe that by this time all of the fluorescence had left the roots and entered the alcohol solution. The resulting fluorescent solution was separated from the root residue by filtration, and its volume reduced at room temperature.

One-dimensional paper chromatography of the concentrated oat-roots extract and the scopoletin-like compound from tobacco (B-II) revealed that one spot of the oat-roots extract gave the same R_f value and similar blue fluorescence as did the compound B-II. This was the case in three different developing solvents used, namely, 15% acetic acid, 60% acetic acid and n-butyl alcohol-acetic acid-water (6:1:2 v/v). Of these solvents tested for the first step in the separation of the blue-fluorescent compounds present in the oat-roots extract, distilled water proved to be the best developing system. With water, the extract was separated on one phase paper chromatograms into four blue-fluorescent

zones. That zone which corresponded with the tobacco scopoletin was the third zone from the solvent head. This zone was cut out from the paper, extracted with acetone, and re-chromatographed in the 15% acetic acid system. The resulting purified zone was again extracted from the paper, and its R_f values compared, through co-chromatography, with those of scopoletin from tobacco in three solvent systems. They were the same in the 15% acetic acid (R_f - 0.32), 60% acetic acid (0.75) and in the n-butyl alcohol-acetic acid-water (6:1:2) (0.84). S. & S., filter paper No. 589, Red Ribbon was used at a temperature of 28.6° C. An attempt to obtain pure crystalline scopoletin from oat-roots was not successful because the amount of roots started with was too small. Oat-roots contain more than a dozen blue-fluorescent compounds. Some paper-chromatographically pure scopoletin in alcohol solution was obtained from oat-roots.

Ultraviolet Absorption Spectra of Tobacco Scopoletin, Oat-root Scopoletin, Esculin, and Esculetin. As soon as the sample of pure scopoletin was available from oat-roots, a comparison of its ultraviolet absorption spectrum with that of scopoletin from tobacco leaves was undertaken. In addition, absorption spectra of esculin and esculetin were also studied, since their structures are closely related to that of scopoletin.

Pure esculetin was obtained by the hydrolysis of esculin with 7% aqueous sulfuric acid solution. Both the

parent glycoside, esculin, and the aglycone, esculetin, were purified by mass paper chromatography in a combination of 15% acetic acid and n-butyl alcohol-acetic acid-water (6:1:2) systems.

A pure sample of each compound whose ultraviolet absorption spectrum was to be studied, namely the scopoletin from oat-roots and scopoletin from tobacco leaves, esculin, and esculetin, was streaked across Whatman No. 1 paper (19 cm. x 46 cm.) for mass paper chromatography. The amount of material put on each sheet was judged by the blue fluorescence under ultraviolet light and adjusted so as to be of nearly equal concentration. After the material streaked on paper was dried, the chromatograms were developed in a system of 15% acetic acid together with a blank sheet at the same time. Each blue-fluorescent compound developed as a narrow sharp zone of about the same width. The R_f values of scopoletin from oat-roots and scopoletin from tobacco leaves were the same. Now each zone of the blue-fluorescent compound was cut off from the respective chromatograms. The blank sheet was cut off in such a way that the width and location of paper was exactly the same as that of the zone containing scopoletin from oat-roots. These strips were then eluted in an elution chamber with 80% ethyl alcohol solution at the same time, until the blue fluorescence disappeared from the paper.

The absorption spectrum of each compound was taken

with the Beckman DU spectrophotometer in 80% ethyl alcohol solution between the wave lengths of 220-400 millimicrons with the eluate of the blank sheet as blank. The close structural relationship of oat-roots scopoletin, esculetin, and esculin is shown in their absorption spectra in Figure 4. The absorption spectrum of scopoletin from tobacco leaves compared very well with that of scopoletin from oat-roots in Figure 5.

Synthesis of Scopoletin

In order to confirm the identification of tobacco scopoletin, the synthesis of scopoletin by the method of Aghoramurthy and Seshadri (21) was undertaken. Figure 6 illustrates the reaction scheme for this preparation.

Esculetin (II) from Esculin (I). Three and two-fifths grams (0.01 mole) esculin (L. Light & Co. Ltd., England) was suspended in 120 ml. of 7% aqueous sulfuric acid. A 500 ml. round-bottom flask equipped with an air condenser and containing the mixture was heated on a steam bath for 3 hours. The glycoside, esculin, went into solution as soon as the mixture was heated. As the reaction proceeded, the solution became yellow and the aglycone, esculetin, precipitated out as fine needles. The reaction products were placed in an ice box overnight, then collected on a glass filter and were finally dried in a desiccator under reduced pressure. About 1.9 g. of fine yellow prisms

FIGURE 4

ABSORPTION SPECTRA OF OAT-ROOT SCOPOLETIN, ESCULETIN, AND ESCULIN

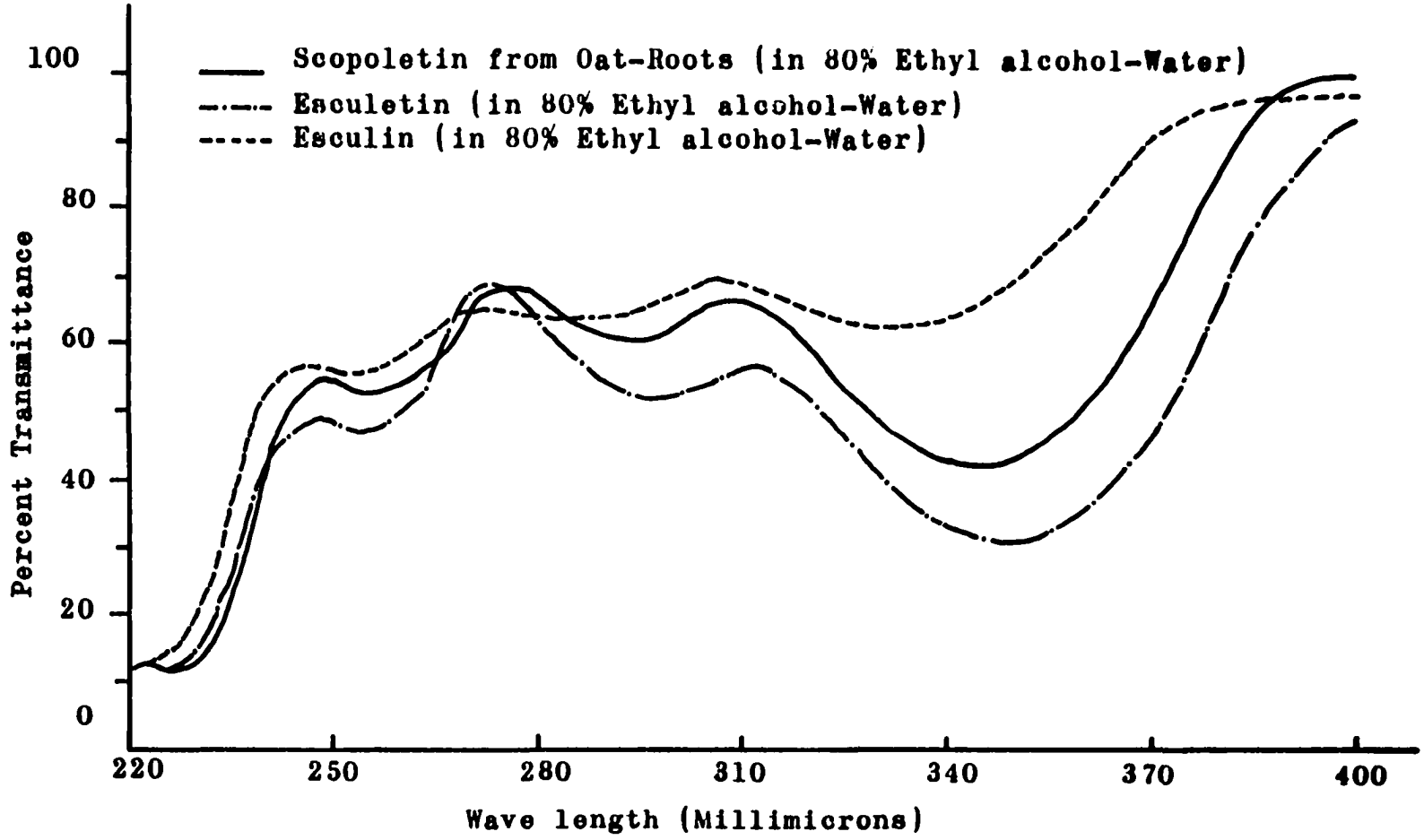


FIGURE 5

ABSORPTION SPECTRA OF TOBACCO-LEAF SCOPOLETIN AND OAT-ROOT SCOPOLETIN

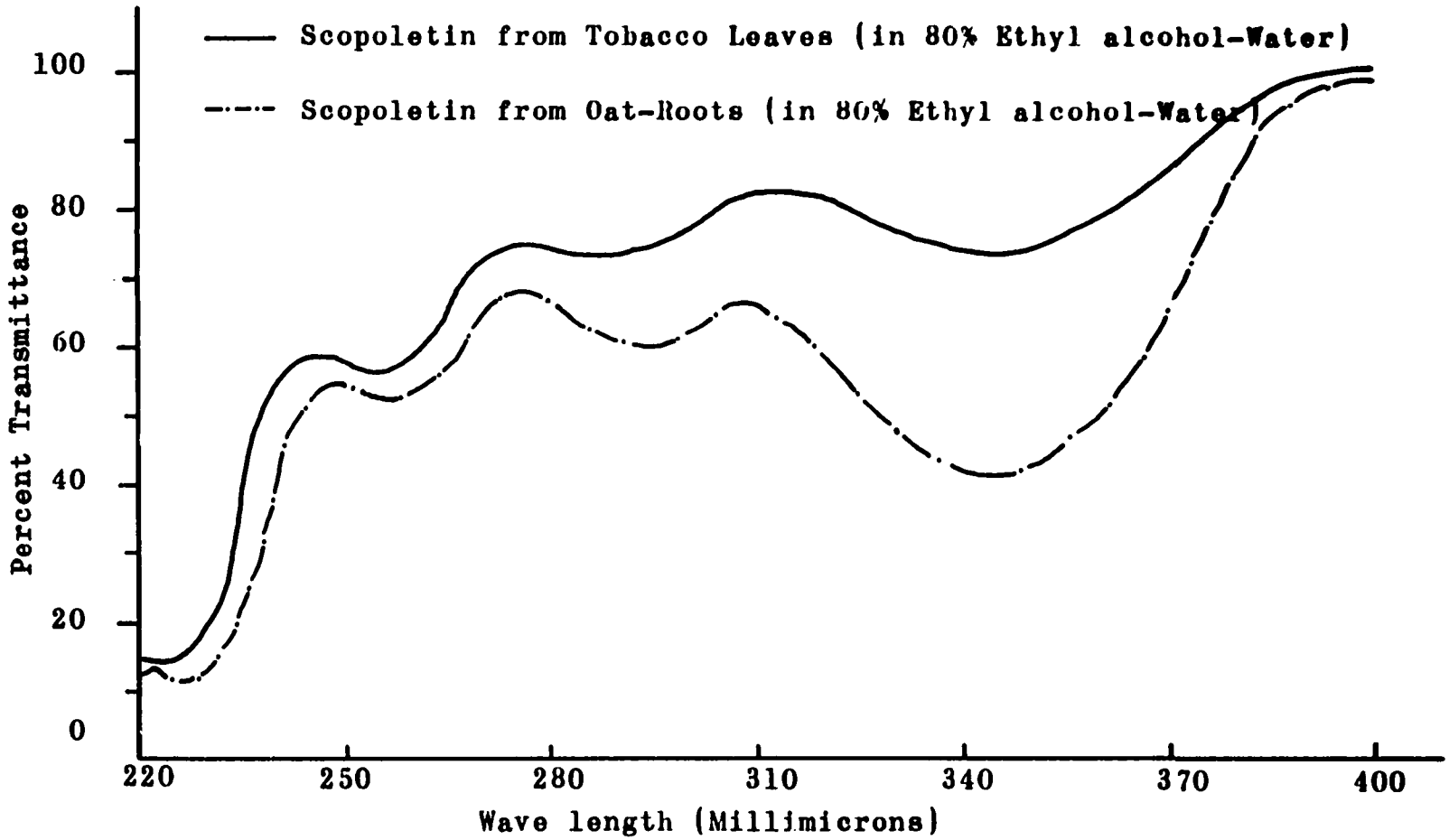
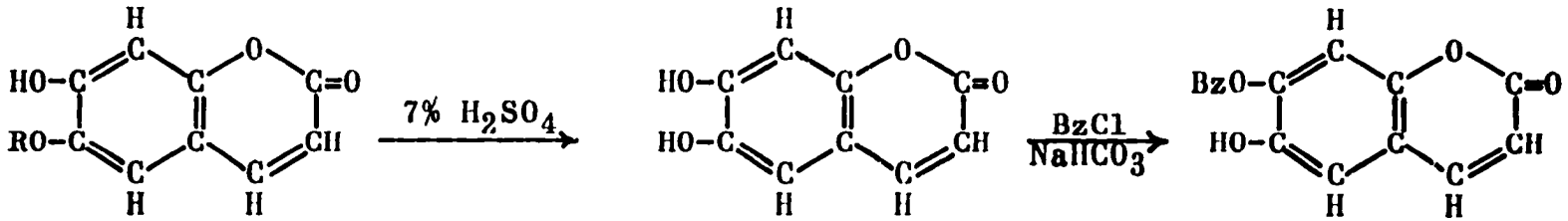


FIGURE 6

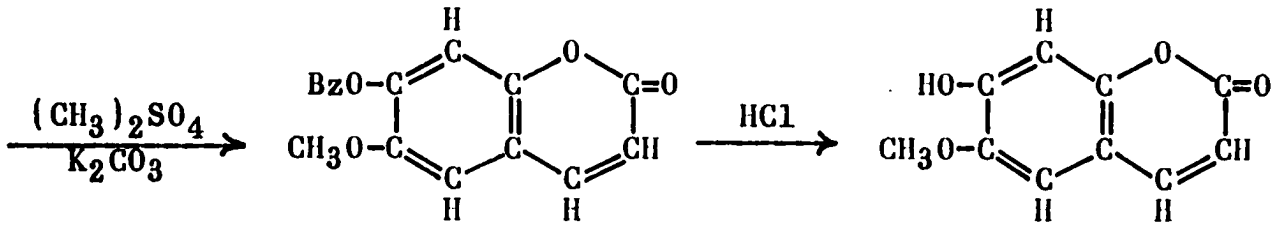
REACTIONS ILLUSTRATING THE SYNTHESIS OF SCOPOLETIN



Esculin (I)
R-Glucosyl

Esculetin (II)

7-Benzylesculetin (III)



Scopoletin-benzylether (IV)

Scopoletin (V)

were obtained. These crystals sintered at 268° C. and decomposed at 270° C. (uncorrected).

7-Benzyl-Esculetin (III). A solution of 1.8 g. esculetin (0.01 mole) and 2.6 g. benzyl chloride in 70 ml. of ethyl alcohol was treated with 1.9 g. solid sodium bicarbonate and refluxed for 12 hours on a steam bath. The reaction products finally became orange yellow in color. The alcohol was removed under reduced pressure, and the residue was treated with a small amount of chloroform. Chloroform was now removed under reduced pressure so as to remove the trace amount of alcohol completely. After this, 200 ml. of chloroform was added to the residue. The unreacted esculetin remained insoluble in the chloroform and was separated from the solution by filtration. The chloroform solution was then shaken with 100 ml. of 10% aqueous potassium carbonate solution, followed by shaking with 200 ml. of 1N sodium hydroxide solution. The 7-benzyl-esculetin precipitated out from the sodium hydroxide solution on acidification with 1N hydrochloric acid and was left to stand overnight in an ice box. The by-product, dibenzyl-esculetin, remained in the chloroform solution. The 7-benzyl-esculetin was collected on a glass filter and dissolved in acetone. The acetone solution was treated with active charcoal for several times and then the acetone was removed in vacuo. The products were finally recrystallized from 1, 2-dichloroethane as colorless prisms, which melted at 292-93° C.

(uncorrected). The yield was about 0.325 g.

Scopoletin-benzylether (IV). A mixture consisting of 0.325 g. of the 7-benzyl-esculetin (III) and 0.3 ml. of dimethyl sulfate in 20 ml. dry acetone was treated with 0.5 g. of anhydrous potassium carbonate and refluxed for 6 hours on a water bath. The potassium salts were filtered off and washed with hot acetone. Evaporation of the acetone solution left a residue which crystallized from alcohol as colorless prisms melting at 123-27 ^{o} C. (uncorrected).

Scopoletin (V). The foregoing benzylether (IV), 0.02 g. was dissolved in 1 ml. glacial acetic acid, and 0.5 ml. of concentrated hydrochloric acid was added to it. The mixture was heated on a steam bath for 30 minutes. The acetic acid, benzyl chloride, and hydrochloric acid were completely removed under reduced pressure, and the residue was washed with a small amount of petroleum ether to remove the last traces of benzyl chloride. The solid on recrystallization from 95% ethyl alcohol came out as colorless needles melting at 206-07^{o} C. (uncorrected).

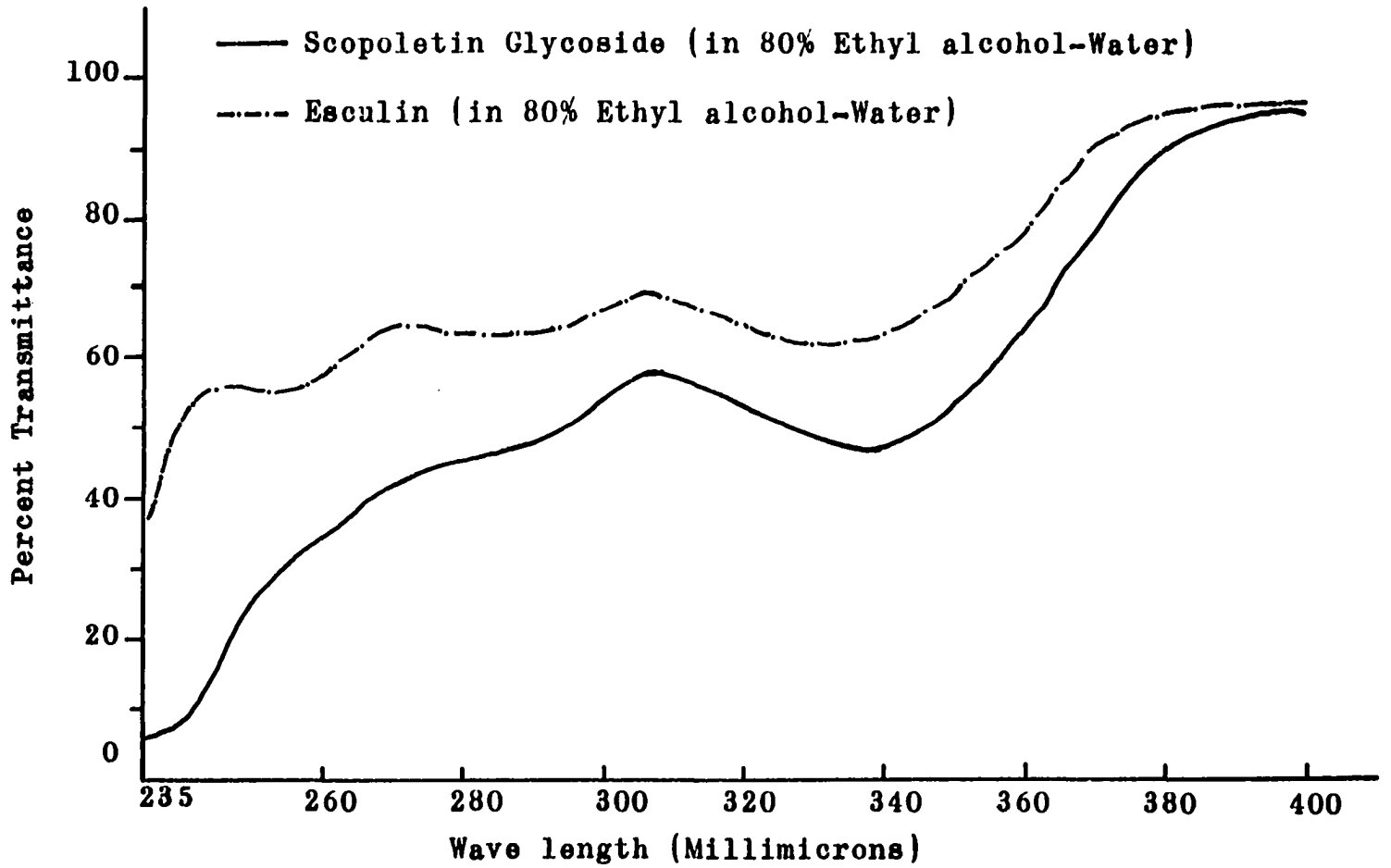
This synthetic scopoletin, when co-chromatographed with authentic scopoletin, had the same R_f values in 15% acetic acid, 60% acetic acid, and n-butyl alcohol-acetic acid-water (6:1:2) systems. (Table 1). The blue fluorescence of the synthetic scopoletin as well as the colors developed with several chromogenic sprays were the same as those of the tobacco scopoletin. (Table 2).

Studies on Compound B-I-2. Compound B-I-2 fluoresced a bright blue under ultraviolet light, and the fluorescence seemed to be intensified by exposure to ammonia vapors, but without changing color. No change in color was observed after spraying with 1% alcoholic aluminum chloride solution (18); with a saturated aqueous solution of ammonium molybdate (22); or with 1% sodium nitrite in 10% acetic acid solution followed by 1N sodium hydroxide solution (23). The "B-I-2" was colorless in visible light. The failure of compound B-I-2 to give a positive color test with aluminum chloride, ammonium molybdate, and sodium nitrite followed by sodium hydroxide indicated that "B-I-2" is neither a flavonoid type compound, nor a caffeic acid type compound with two adjacent hydroxyl groups on the aromatic ring, nor a chlorogenic acid type compound. It appeared most likely to be a coumarin type compound. This tentative conclusion was further supported when the ultraviolet absorption spectrum of "B-I-2" was taken. The ultraviolet absorption spectrum of "B-I-2" exhibited a close relationship to that of esculin. (Figure 7).

The R_f values of "B-I-2" on Whatman, No. 1 paper are: 0.77 in 15% acetic acid; 0.78 in 60% acetic acid; 0.40 in n-butyl alcohol-acetic acid-water (6:1:2) system; and 0.60 in n-butyl alcohol-benzene-pyridine-water (5:1:3:3) system. A very interesting relationship of R_f values was observed when this compound was co-chromatographed with

FIGURE 7

ABSORPTION SPECTRA OF TOBACCO LEAF SCOPOLETIN GLYCOSIDE AND ESCULIN



scopoletin, esculin and esculetin. The R_f values of the glycoside, esculin and its aglycone, esculetin, respectively are: (1) 0.73:0.57 in 15% acetic acid; (2) 0.72:0.71 in 60% acetic acid; (3) 0.43:0.78 in n-butyl alcohol-acetic acid-water (6:1:2) system. The R_f values of the "B-I-2" compared to those of scopoletin are: (1) 0.77:0.59 in 15% acetic acid; (2) 0.78:0.76 in 60% acetic acid; (3) 0.40: 0.81 in the n-butyl alcohol-acetic acid-water (6:1:2) system.

Hydrolysis of a portion of the "B-I-2" concentrate was carried out for approximately four hours with 2N sulfuric acid over a hot water bath. The resulting reaction mixture was extracted several times with ethyl acetate. The ethyl acetate extracts were combined, filtered, and concentrated for subsequent study. The aglycone obtained on hydrolysis of "B-I-2" fluoresced bright blue under ultraviolet light. Paper chromatographic comparison of this unknown aglycone with esculetin and scopoletin in several solvent systems resulted in a tentative identification of the aglycone as scopoletin. This was further confirmed by the identical ultraviolet absorption given by the unknown aglycone and scopoletin.

Before carrying out the paper chromatographic identification of the sugars present in the hydrolysate of B-I-2, this acidic solution was treated with an ion exchange resin, Amberlite IR-45, to remove the sulfate ion and neu-

tralize the solution. The resin itself was first converted into the basic form by treatment with a 2N sodium hydroxide solution, followed by thorough washing with distilled water until a neutral reaction was obtained. The resin was then added in small portions to the flask containing the filtrate, and the contents were well mixed. When the solution gave a neutral reaction to pH paper (Hydrion paper), a slight excess of resin was added, and the mixture filtered to remove the resin from the solution. The resin was washed ten times with distilled water, and the washings added to the filtrate, to remove as completely as possible any sugar which might have been trapped in the resin. The filtrate was then reduced in volume to 5 ml. or less in a rotating evaporator, in vacuo, over a warm water bath.

A portion of this concentrate was then spotted on Whatman No. 1 filter paper, along with known sugars for comparison, and developed in the n-butyl alcohol-benzene-pyridine-water (5:1:3:3) solvent system. After drying, the chromatogram was sprayed with aniline hydrogen oxalate reagent (24), and heated in a drying oven at 110° C. for approximately 15 minutes. The unknown solution contained two sugar spots with R_f values 0.28 and 0.52. The latter two spots were identical with the authentic glucose and rhamnose, respectively, run as comparison standards. After the identity of the hydrolysate sugars had been established to be glucose and rhamnose, a standard solution of glucose and

rhamnose mixture in equal molar ratio was made. The concentration of this solution was so adjusted that each microliter of the solution contained 1 microgram each of glucose and rhamnose. This standard sugar solution was spotted on Whatman No. 1 filter paper along with the concentrate of the hydrolysate, developed in the n-butyl alcohol-benzene-pyridine-water system and sprayed with aniline hydrogen oxalate reagent. Observation of the size of the spots and the intensity of the colors developed with aniline hydrogen oxalate reagent indicated that the ratio of glucose to rhamnose in the unknown solution is similar to that of the standard solution. Since more precise quantitative analysis was needed for structural determination, further study on quantitative estimation of the molar ratios of scopoletin, glucose, and rhamnose was attempted by applying and modifying the existing methods of sugar analysis reported by Timell, Glaudemans, and Currie (25). This work is not complete.

The following structure, however, is tentatively proposed for tobacco leaf compound "B-I-2": scopoletin-7-rhamnoglucoside.

Studies on Compound "B-I-1a" and Compound "B-I-1b." Both compound "B-I-1a" and compound "B-I-1b" when developed on paper chromatograms have properties similar to those observed with standard chlorogenic acid. Such properties are: (1) temporary change of blue fluorescence to greenish-yellow

fluorescence under ultraviolet light when these compounds are exposed to ammonia vapor; (2) a pink color is developed on successive treatment of these compounds with sodium nitrite and sodium hydroxide; and (3) they give a green color with ferric chloride spray. A saturated solution of ammonium molybdate produced brownish-yellow colors with both "B-I-1a" and "B-I-1b." These colors in visible light are characteristic of chlorogenic acid. (Figure 1). The R_f values of neither "B-I-1a" or "B-I-1b," however, check with that of standard chlorogenic acid. Typical R_f values of "B-I-1a," "B-I-1b" and standard chlorogenic acid on S. & S., No. 589, Red Ribbon paper are: (1) 0.13, 0.81 and 0.31 in the 15% acetic acid system; (2) 0.23, 0.60 and 0.38 in the n-butyl alcohol-acetic acid-water (6:1:2) system; and (3) 0.35, 0.61, and 0.47 in the n-butyl alcohol-benzene-pyridine-water (5:1:3:3) system, respectively. The R_f values of "B-I-1a" are quite low compared to those of the chlorogenic acid standard, while the corresponding values of "B-I-1b" are always higher than those of the chlorogenic acid standard. The "B-I-1a" was relatively more soluble in dilute ethyl alcohol, but "B-I-1b" seemed to be relatively more soluble in a mixture of ethyl acetate-ethyl alcohol (1:1). In solubility, "B-I-1a" resembled chlorogenic acid more than did "B-I-1b."

The commercially available samples of chlorogenic acid on hand (Delta Chemical Works, New York, and L. Light

& Co., Ltd. England) are very impure. One of the major impurities is caffeic acid (Figure 1), which is believed to be derived from the parent chlorogenic acid, and the other impurities are unknown compounds with smaller R_f values which fluoresce yellow or green under ultraviolet light. The purification of chlorogenic acid from a commercially available sample was, therefore, undertaken following the extended mass paper chromatography described in the purification of "B-I-1a." This involved the separation of chlorogenic acid on S. & S., No. 589 Red Ribbon paper in the n-butyl alcohol-acetic acid-water, 15% acetic acid, and n-butyl alcohol-benzene-pyridine-water systems. The chlorogenic acid, thus purified, was eluted from the paper with 50% ethyl alcohol. When this was spotted on S. & S., No. 589 Red Ribbon paper along with "B-I-1a" and the original impure chlorogenic acid before purification, and all were developed in the several solvent systems described above, it was found that the R_f values of the purified chlorogenic acid checked favorably with those of "B-I-1a" in all the systems used. This rather surprising discovery indicated that apparently some change had happened to chlorogenic acid to slow down its R_f values when it was purified by extended mass paper chromatography.

In order to clarify the findings mentioned above, a pure sample of chlorogenic acid was sought and finally purchased from Fluka AG Chemische Fabrik Buchs ISG in

Switzerland. This particular sample probably is the purest chlorogenic acid commercially available today.

A portion of this chlorogenic acid was dissolved in 50% ethyl alcohol and then spotted on S. & S., No. 589 Red Ribbon filter paper, along with the Light & Co. impure chlorogenic acid, the Oklahoma purified chlorogenic acid, and "B-I-1a" and developed in several solvent systems separately. Observation indicated that the R_f values of pure chlorogenic acid checked with those of the main spot of impure chlorogenic acid, but not with the purified chlorogenic acid and "B-I-1a." The R_f values of the latter two are far small compared to those of pure chlorogenic acid.

Careful observation of the developed chromatograms revealed that pure chlorogenic acid gave another blue-fluorescent spot of higher R_f value in the 15% acetic acid and 60% acetic acid systems. This additional spot was not observed when the paper was developed in the n-butyl alcohol-acetic acid-water and n-butyl alcohol-benzene-pyridine-water systems. Another portion of pure chlorogenic acid was streaked on S. & S., No. 589 Red Ribbon filter paper and subjected to procedures similar to those mentioned in the purification of "B-I-1a" and impure chlorogenic acid. When this was done, it was found that pure chlorogenic acid had been changed to give two blue-fluorescent zones by development in the 15% acetic acid system. The new zone again has a higher R_f value than the parent zone in the 15% acetic

acid system. The chlorogenic acid zone thus treated was eluted from the paper and again compared with "B-I-1a" and the purified chlorogenic acid. Then R_f values checked favorably in all the systems used.

The original alcohol extract of tobacco leaf was then spotted on S. & S., No. 589 Red Ribbon filter paper, along with pure chlorogenic acid and developed in the several solvent systems mentioned above. In the two systems, n-butyl alcohol-acetic acid-water and n-butyl alcohol-benzene-pyridine-water, the R_f value of pure chlorogenic acid checked favorably with a blue spot from which B-I-1a had been derived.

A solution of purified "B-I-1a" was obtained by elution from five sheets of S. & S., No. 589 Red Ribbon filter paper (19 x 58 cm.) with 50% ethyl alcohol, and the solution was heated on a hot water bath in 3 ml. of 2N sodium hydroxide for 30 minutes. After acidification with 3.2 ml. of 2N sulfuric acid, ether extraction was carried out several times with a total of 50 ml. ether. The resulting solid material, after filtration and evaporation of the ether, was dissolved in 95% ethyl alcohol. This was co-chromatographed with known caffeic acid. A blue spot was obtained. The R_f values and fluorescence of this compound correspond to those of caffeic acid. Thus, tobacco leaf compound "B-I-1a" has now been identified as chlorogenic acid.

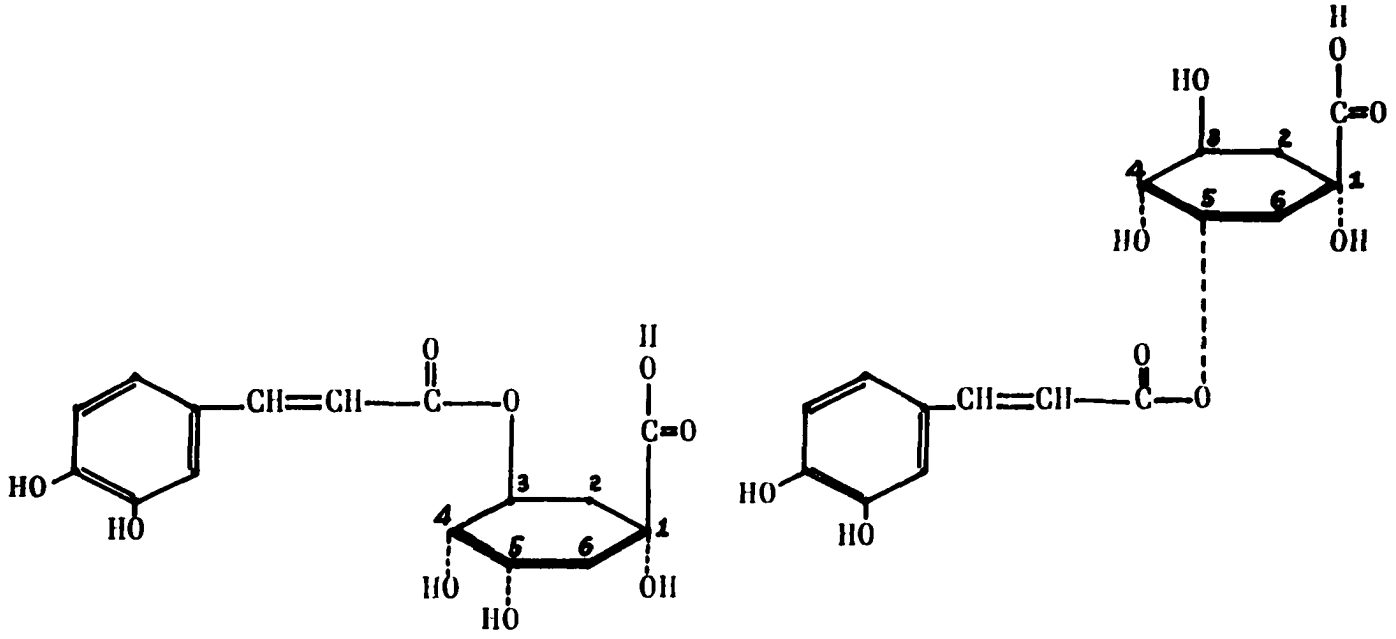
Two other known compounds closely related to chlorogenic acid are isochlorogenic acid and neochlorogenic acid. Isochlorogenic acid, a position isomer of chlorogenic acid, was first isolated from coffee together with chlorogenic acid by Barnes, Feldman and White (26) who proposed a structure for this compound as shown in Figure 8. The structure of neochlorogenic acid has not yet been completely clarified.

Authentic samples of isochlorogenic and neochlorogenic acid were received from Dr. H. W. Siegelman of the U. S. Department of Agriculture, Beltsville, Maryland, and Dr. J. Corse of the Western Regional Research Laboratory, Albany, California, respectively. When these two samples were co-chromatographed with purified "B-I-1b" in the several solvent systems mentioned above, it was found that the R_f values of neither isochlorogenic acid nor neochlorogenic acid checked with those of "B-I-1b." (Table 1). Similar treatment of "B-I-1b" with sodium hydroxide, as mentioned in the saponification of "B-I-1a" above, gave caffeic acid by one dimensional paper chromatographic examination.

All attempts to isolate compound "B-I-1b" in pure crystalline state have so far failed. It appears to isomerize or to decompose very rapidly, giving rise to two blue fluorescing and two green fluorescing materials when its mass paper chromatographic separation was carried out on S. & S., No. 589 Red Ribbon filter paper in 5% acetic acid

FIGURE 8

STRUCTURAL FORMULAE OF CHLOROGENIC ACID AND ISOCHLOROGENIC ACID



Chlorogenic Acid

Isochlorogenic Acid

system. It behaved, however, as a pure single zone on the same paper in the n-butyl alcohol-acetic acid-water and the n-butyl alcohol-benzene-pyridine-water systems. Further work on identification of "B-I-1b" was not completed. Tobacco leaf compound "B-I-1b" appears to be a new substance closely related to chlorogenic acid.

Isomerization of chlorogenic acid, isochlorogenic acid, and neochlorogenic acid by dilute hydrochloric acid was reported by Dickinson and Gawler (27). According to these workers, when the solutions of the three acids in n-propyl alcohol containing 1% hydrochloric acid were concentrated by distillation and co-chromatographed in a n-butyl alcohol-acetic acid-water (63:10:27) system, they all gave rise to a new fourth spot with a R_f value of 0.01. They reported that the normal R_f values for chlorogenic acid, isochlorogenic acid, and neochlorogenic acid in this system are 0.71, 0.85, and 0.62, respectively. Similar isomerization was also observed by the same workers when the three chlorogenic acids were chromatographed on paper using the Forestal solvent (28), hydrochloric acid-acetic acid-water (3:30:10 v/v).

Mass paper chromatographic separation of "B-I-1a" and "B-I-1b" on S. & S., No. 589 Red Ribbon paper in aqueous acetic acid systems seemed to effect isomerization or decomposition of chlorogenic acid and "chlorogenic-acid-like" compounds. This has been reported in the above paragraphs.

The isochlorogenic and neochlorogenic acid samples sent to this laboratory were found to isomerize under the same conditions. The isomerization product of each acid, however, was not the same, but differs depending on the parent acid. The evidence available at present is not sufficient to permit a completely satisfactory explanation of these phenomena. It has thus become evident that one should avoid the use of acidic solvent systems in paper chromatography of these chlorogenic acids. Likewise, Whatman No. 1 rather than S. & S., No. 589 paper is definitely recommended for use with the chlorogenic acids. A new solvent system, namely 15% tert-butyl alcohol was found to effect no isomerization of chlorogenic acids. It has been used in more recent work to replace the 15% acetic acid system during mass paper chromatographic separations.

CHAPTER III

ISOLATION AND IDENTIFICATION OF CERTAIN BLUE-FLUORESCENT COMPOUNDS AND FLAVONOID GLYCOSIDES IN TOBACCO FLOWERS

Isolation from Tobacco Flowers

Extraction. The sample of tobacco flowers used was from One-Sucker tobacco grown in the Argonne National Laboratory greenhouse. Immediately following their collection, the flowers were dried in an oven at 110° C., and ground to a powder. A total of 1.5 kg. of the powdered tobacco flowers was extracted with 85% aqueous isopropyl alcohol in Soxhlet extractors until the sample gave no coloration with fresh solvent. For each Soxhlet extractor, 50 G. of the tobacco flowers was used, and a total of approximately 1650 ml. of 85% isopropyl alcohol was necessary over a period of about 12 hours. A hot water bath was used. The combined alcoholic extracts were concentrated, under reduced pressure, to a volume of approximately 7 liters.

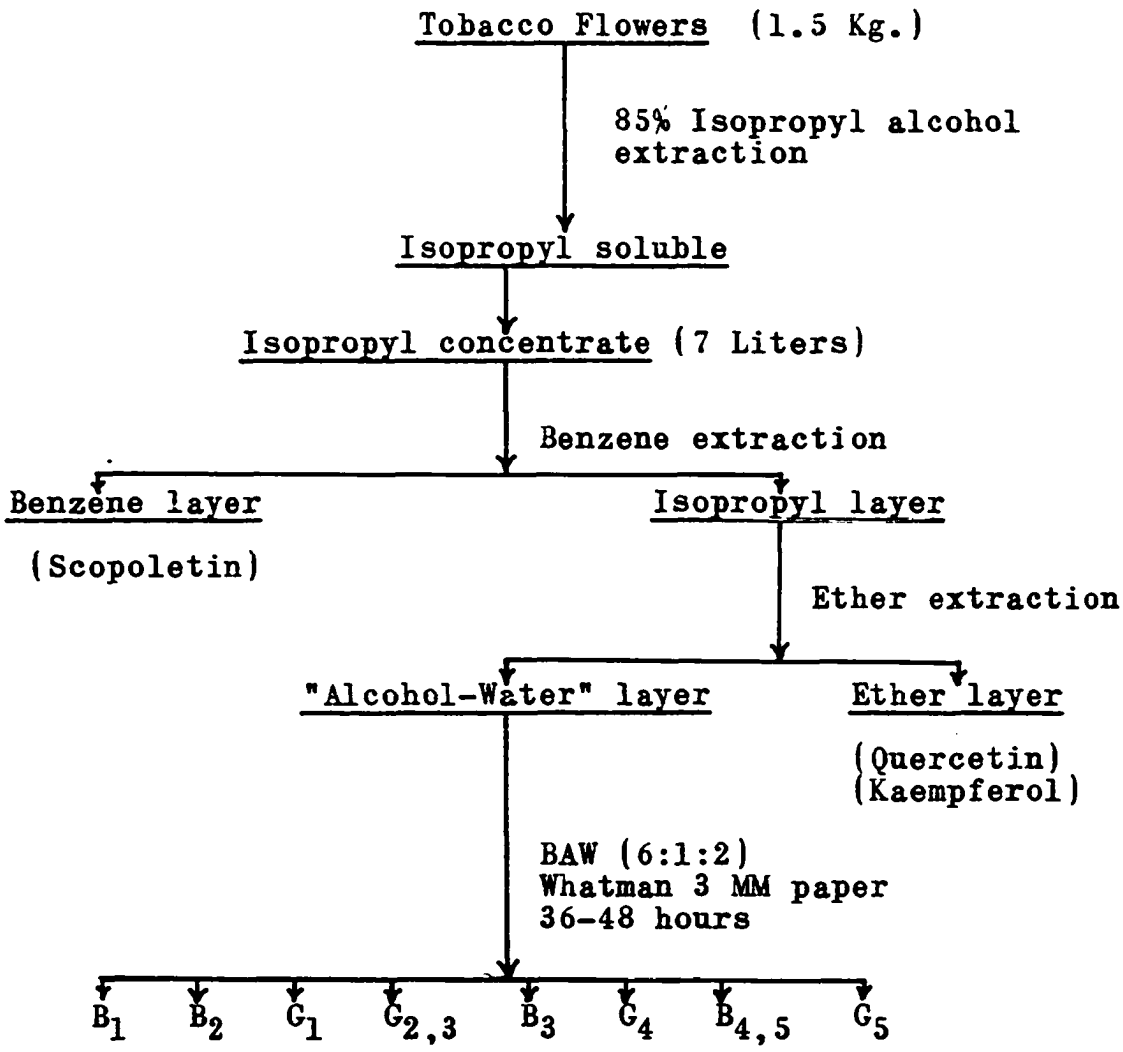
Solvent Separation. The alcoholic extracts of the tobacco flowers exhibited some red fluorescence under ultraviolet light. This was due possibly to "chlorophyll-like"

substances plus resinous materials. It is advisable to remove these from the alcoholic extracts before the flavonoid glycosides and related polyphenolic compounds are subjected to mass paper chromatographic separation, lest they interfere with sharp separation on paper chromatograms. The concentrates of the alcoholic extracts were, therefore, subjected to extraction with benzene (Figure 9). For each operation, a 250 ml. aliquot of the concentrate was used with 200 ml. benzene in a one-liter separatory funnel. The upper layer of benzene was then re-extracted with 35% isopropyl alcohol to recover some of the glycoside-like material that had gone into that layer. The alcohol-water layer and the alcohol solution washings were combined and concentrated *in vacuo* over a hot water bath to a volume of approximately 6.5 liters.

Scopoletin. The benzene fraction obtained after the extractions was subjected to mass paper chromatography in the 15% acetic acid system. The organic resinous materials, as well as the "chlorophyll-like" substances, have zero or very small R_f values in this solvent system, and therefore were readily separated by this procedure from the blue-fluorescent material, scopoletin. The blue zone, which contained scopoletin, was cut off from the paper chromatogram, sewed onto a new sheet of Whatman 3 MM paper and run in the n-butyl alcohol-acetic acid-water system to achieve further separation. Each purified, blue-fluorescent zone

FIGURE 9

FLOW-SHEET OF THE OPERATIONS PERFORMED IN THE EXTRACTION OF TOBACCO FLOWERS AND THE SEPARATION OF PHENOLIC CONSTITUENTS



was finally eluted with 95% ethyl alcohol. The scopoletin zone thus obtained was found to be identical, in all systems used, with scopoletin from tobacco leaves, scopoletin from oat-roots, and synthetic scopoletin.

Paper Chromatographic Separations. The concentrate of alcohol extracts remaining after the benzene extraction (Figure 9) was found to contain flavonol aglycone compounds on analysis with paper chromatography and chromogenic sprays. These were removed by extraction with ethyl ether. For each operation, a 600 ml. aliquot of alcohol concentrate was extracted five times with 250 ml. of ether and then twice with 200 ml. portions of ether. The combined ether layers were concentrated and subjected to mass paper chromatography. Two flavonol aglycones, namely quercetin and kaempferol, were purified and identified in this ether layer by Edwin L. Murphy (12) in this laboratory.

The alcohol, water-rich layers remaining after the ether extractions, were combined. The total volume of this concentrate was brought to 1 liter with n-propyl alcohol, and it was designated as the "Alcohol-Water" fraction (Figure 9). By developing paper chromatograms of this "alcohol-water" fraction on a large sheet of Whatman 3 MM paper (46.5 x 57 cm.) for 36 to 48 hours in the n-butyl alcohol-acetic acid-water system, sufficient resolution could be obtained to observe a minimum of four brown fluorescing zones and four blue fluorescing zones under ultraviolet

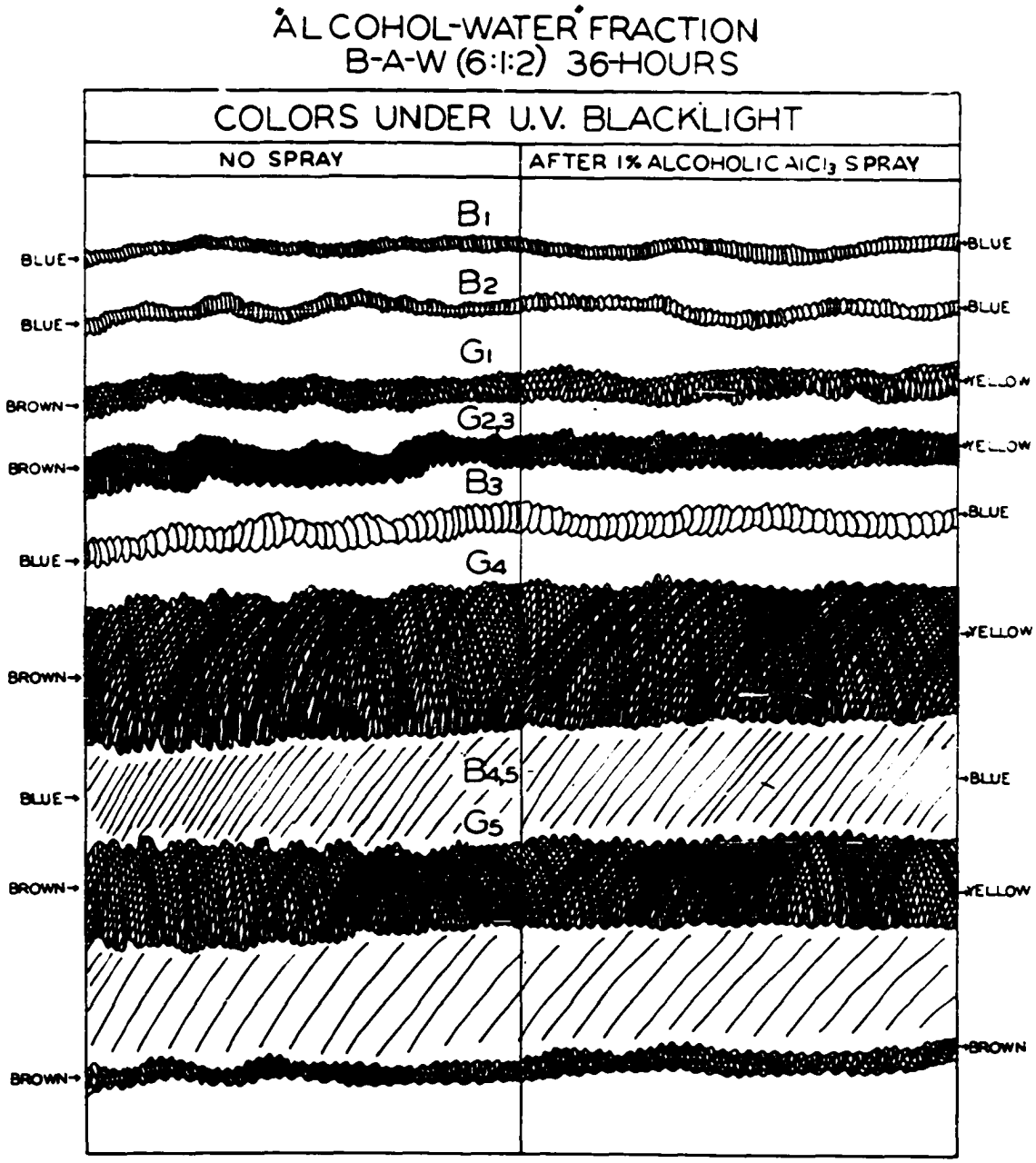
light (Figures 9 and 10). Figure 10 is somewhat idealized to emphasize the individual zones. Actually, there is some overlapping of compounds throughout, although the outlined separation, in general, can be observed. In this figure, the chromatogram has been cut down the middle. The left side represents the chromatogram portion which was not sprayed; the right side represents the portion which had been sprayed with 1% aluminum chloride in 95% ethyl alcohol.

Compounds "B-1" and "B-2." Both "B-1" and "B-2" developed in very narrow zones with the n-butyl alcohol-acetic acid-water system. These two blue-fluorescent compounds did not change their color under ultraviolet light after spraying with the aluminum chloride solution; with 1% potassium hydroxide solution; or with 1% sodium nitrite in 10% acetic acid solution followed by 1N sodium hydroxide solution. It seems unlikely, therefore, that either "B-1" or "B-2" could possibly be a flavonoid glycoside or a chlorogenic acid-like compound. Further characterization and identification of these two compounds were not completed partly due to insufficient amount of sample isolated from the flower extracts. The amounts of "B-1" and "B-2" present in tobacco flowers are very small.

Compound "G-1." The zone containing "G-1" was cut off from the paper chromatograms and eluted with 50% ethyl alcohol-water in an elution chamber. The combined eluates were concentrated under reduced pressure and again

FIGURE 10

CHROMATOGRAM OF THE "ALCOHOL-WATER" FRACTION



developed in the n-butyl alcohol-acetic acid-water system, this time for 14 hours. After this second chromatography, the remaining traces of "B-1" and "B-2" had been removed, but some "B-3" still contaminated the "G-1" zone. Therefore, the "G-1" zones were cut off from the paper chromatograms and extracted with the 50% ethyl alcohol in an elution chamber. The concentrated eluates were again streaked on Whatman 3 MM paper and this time developed in the 15% acetic acid system. In this system, the "B-3" moves considerably farther than "G-1," and separation of the two was almost complete. The "G-1" zones were again cut off, sewed onto a new sheet of Whatman 3 MM paper, and developed once more in the 15% acetic acid system to remove the last trace of the "B-3" blue. The "G-1" was eluted from the final sheets with 50% ethyl alcohol and concentrated in vacuo.

From its behavior during paper chromatography and from its color on paper under ultraviolet light, with and without sprays, "G-1" appeared to be a flavonoid-glycoside containing at least two units of sugar. For example, "G-1" fluoresces brown under ultraviolet light, and it gave a yellow fluorescence under ultraviolet on exposure to ammonia vapors. After being sprayed with 1% alcoholic aluminum chloride solution, it gave a golden yellow color under ultraviolet and in visible light. Its R_f values on S. & S., No. 589 Red Ribbon paper were: 0.08 in n-butyl alcohol-acetic

acid-water (6:1:2); 0.72 in 60% acetic acid-water; and 0.74 in 15% acetic acid-water. The lower R_f value in n-butyl alcohol-acetic acid-water and the higher R_f value in 15% acetic acid systems are typical of flavonoid glycosides on paper. The extremely low R_f value of "B-1" in n-butyl alcohol-acetic acid-water system and its R_f values in all other systems also indicated that this compound could not have been one of the three known flavonol (3-hydroxy-flavone) glycosides previously isolated and identified from leaf extracts of the same tobacco. These three flavonol glycosides are: isoquercitrin (quercetin-3-glucoside) (29); rutin (quercetin-3-rhamnoglucoside) (30, 12); and kaempferol-3-rhamnoglucoside (31, 12). "G-1," therefore, is a flavonoid glycoside isolated from the tobacco flowers for the first time in these studies.

A portion of the purified "G-1" fraction was hydrolyzed for 12 hours with 2N sulfuric acid on a hot water bath. The resulting yellow aglycone was extracted with ethyl acetate. The ethyl acetate extracts were combined, filtered, and concentrated for paper chromatography studies. The aqueous portions were concentrated, neutralized by Amberlite IR-45 anion exchange resin, as described in later paragraphs, and analyzed qualitatively for sugar present.

The aglycone obtained on hydrolysis of "G-1" fluoresced yellow under ultraviolet light. The yellow fluorescence was intensified on exposure to ammonia vapors. The

aglycone gave a greenish-yellow color after being sprayed with the alcoholic aluminum chloride solution. The fluorescence color of "G-1" aglycone and its change by the chromogenic reagents seemed to indicate that it is a flavonol. The brightly fluorescent greenish-yellow color of the flavonols is attributed to the presence of a free hydroxyl group at the 3 position of the flavone nucleus (5). If this group is substituted, as in the case of the flavonol-3-glycosides, the compound then absorbs ultraviolet radiation and appears as a dull brownish spot on a chromatogram. The flavones, lacking this hydroxyl group in the 3 position, also fluoresce brown in ultraviolet light. Substitution of a hydroxyl group in other positions on the quercetin nucleus seems not to produce this alteration of the fluorescence. The quercetin-7-glycosides, such as quercimeritrin (quercetin-7-glucoside), fluoresce yellow in ultraviolet light. The methyl ethers of quercetin such as rhamnetin, isorhamnetin and rhamnazin which are substituted in the 3' and 7 positions are yellow under ultraviolet light. According to the theory discussed above, it seemed to be logical to assume that "G-1" is a flavonol-3-glycoside.

The R_f values for the "G-1" aglycone using S. & S., No. 589 Red Ribbon paper were: 0.66 in n-butyl alcohol-acetic acid-water (6:1:2); 0.21 in 60% acetic acid-water; 0.02 in 15% acetic acid-water; and 0.04 in benzene-nitromethane-water system (3:2:5). The low movement of the

aglycone in the benzene-nitromethane-water system indicates that it probably has no methoxyl group present on the nucleus (32). Co-chromatography of "G-1" aglycone in several solvent systems with authentic kaempferol, quercetin, robinetin (3, 3', 4', 5', 7-pentahydroxy-flavone), and myricetin (3, 3', 4', 5', 5, 7-hexahydroxy-flavone), revealed that it was not identical with any of them. The R_f value of "G-1" aglycone in the n-butyl alcohol-acetic acid-water system using S. & S., No. 589 Red Ribbon paper was compared with those of the four authentic flavonols mentioned above. These values were: 0.89 for kaempferol; 0.79 for quercetin; 0.52 for robinetin; and 0.56 for myricetin. Flavonoid aglycones having the same number of hydroxyl groups have approximately the same R_f values, varying as a rule between one-third and two-thirds of the increase in R_f value which would result from the loss of this hydroxyl group (33). Applying this general approximation to the data obtained above, "G-1" aglycone would possibly be a hexahydroxy-flavone, or pentahydroxy-flavonol. Further paper chromatographic comparison of "G-1" aglycone with a known pentahydroxy-flavonol, gossypetin (3, 3', 4', 5, 7, 8-hexahydroxy-flavone) gave an inconclusive result due to the fact that the authentic gossypetin received from Dr. T. R. Seshadri of India had been oxidized or decomposed to give several spots on chromatograms. Studies on the identity of the "G-1" aglycone are being continued.

For neutralization of the hydrolysate, as mentioned above, Amberlite IR-45 was used. The resin was first treated with 2N sodium hydroxide solution. The resin was washed with distilled water until the pH was approximately 5, then washed several times with water-saturated-ethyl acetate, then with methyl alcohol and isopropyl alcohol, and finally with distilled water. The concentrated hydrolysate was added onto the resin, and the resulting filtrate collected in an Erlenmyer flask. This filtrate was again poured on top of the resin, and this process was repeated until the filtrate was about pH 5 (Hydrion paper). After this, the resin was washed several times with 5 ml. of distilled water to remove trace amounts of sugar which might be adsorbed on the surface of the resin, and the water washings were combined with the neutralized filtrate. The combined solution was treated several times with Norit and was finally reduced in volume in vacuo for identification studies of sugars.

For sugar identification, the organic layer of the system n-butyl alcohol-pyridine-benzene-water (5:3:1:3) was used, as was the aniline hydrogen oxalate spray. The sugars obtained from the hydrolysis of "G-1" were glucose and rhamnose, by mixed paper chromatography (R_f 0.28 glucose and 0.52 rhamnose). Quantitative studies to determine the ratio of the sugars to each other and to the aglycone have not been performed.

Compounds "G-2" and "G-3." The zones which contained "G-3" were cut off from the chromatograms and eluted with 50% ethyl alcohol-water in an elution chamber. After this elution, an examination of the paper under ultraviolet light revealed a yellow fluorescing compound still present. This compound, designated "G-2," appears to be different from the main compound "G-3." The "G-2" was easily eluted from the paper with 15% acetic acid-water or with distilled water made barely acidic with acetic acid. The "G-2" appears to be flavonoid, but as yet no definite facts have been obtained on its identity.

After elution of "G-3," further purification of "G-3" was effected by mass paper chromatography in the various solvent systems mentioned above. Its separation was tedious and quite difficult. The interfering impurity which could not be easily separated from "G-3" was a blue fluorescing compound which had R_f values very close to those of "G-3" in all solvent systems tried. It could not be completely removed from "G-3" on either Whatman No. 1 or Whatman No. 3 MM paper in these solvent systems. The use of S. & S., No. 589 Red Ribbon chromatography paper and 15% acetic acid-water, however, gave somewhat better separation to achieve final purification. The pure "G-3" was obtained by cutting the zone containing the "G-3" into two portions. The upper portion appeared to be free of the blue impurity.

The purified "G-3" was hydrolyzed by the procedure described above for "G-1." When spotted on Whatman No. 1 paper, the aglycone fluoresced brown and was not changed to yellow on exposure to ammonia vapor. This brown spot did not move in 15% acetic acid-water. When developed in 60% acetic acid-water, however, some yellow fluorescing material moved down the paper to a R_f value of approximately 0.60. When developed in the n-butyl alcohol-acetic acid-water system, all the spotted material moved to a spot with a R_f value about 0.83. The aglycone appeared to be flavonoid, but appeared somewhat unstable with our usual chromatographic treatment. It is now being studied for possible identification. The sugars need to be investigated further, although preliminary results indicate the presence of glucose and rhamnose. The presence of these flavonoid glycoside-like compounds, "G-2" and "G-3," in tobacco flowers have not yet been reported in the chemical literature.

Compounds "G-4" and "G-5." Both "G-4" and "G-5" were purified by extended mass paper chromatography, alternating the 15% acetic acid-water and the n-butyl alcohol-acetic acid-water systems. The purified zone of each compound was cut off, sewed onto new sheets of Whatman No. 3 MM paper and developed alternately in the systems mentioned in the above sentence. On paper, under ultraviolet light, both pure "G-4" and "G-5" fluoresced brown. This color changed to yellow and greenish yellow, respectively on

exposure to ammonia vapor and after spraying with 1% ethanolic aluminum chloride. The R_f values of "G-4" and its color change by chromogenic spray reagents agreed with those reported in the literature for the flavonol glycoside, rutin. Rutin has been isolated and identified from all species of tobacco by numerous investigators and also from Nicotiana tabacum in this laboratory by Edwin L. Murphy. Co-chromatography of "G-4" with authentic rutin in 15% acetic acid-water, 60% acetic acid-water, and n-butyl alcohol-acetic acid-water systems confirmed that "G-4" is rutin. By similar studies, "G-5" was proved to be identical with kaempferol-3-rhamnoglucoside, which has previously been isolated and identified from the same tobacco by Reagan H. Bradford (31) in this laboratory.

Compound "B-3." The "B-3" was purified by repeated mass paper chromatography, alternating the systems 15% acetic acid-water, n-butyl alcohol-acetic acid-water, and n-butyl alcohol-pyridine-benzene-water. It fluoresced a bright blue under ultraviolet light, and the fluorescence seemed to be intensified by exposure to ammonia vapors without changing color. No change in color was observed after spraying with 1% ethanolic aluminum chloride, with a saturated aqueous solution of ammonium molybdate, or with 1% sodium nitrite in 10% acetic acid-water followed by 1N sodium hydroxide solution. After hydrolysis of "B-3," the aglycone was identified as scopoletin and the sugars as

glucose and rhamnose. Therefore, "B-3" was chromatographed with the tobacco leaf compound "B-I-2," described in the previous chapter, in 15% and 60% acetic acid-water as well as in the n-butyl alcohol-acetic acid-water systems. The "B-3" compared chromatographically in these three solvent systems with compound "B-I-2." Tobacco flower "B-3" has, therefore, been identified as scopoletin-7-rhamnoglucoside.

Compounds "B-4" and "B-5." The zone running immediately ahead of "G-4" was a blue fluorescing zone that was wider than the other zones on the paper chromatogram (Figure 10). It overlapped "G-5" and extended even further. Separation of "B-4" and "B-5" was achieved using mass paper chromatography and the n-butyl alcohol-pyridine-benzene-water system.

The strongly blue fluorescence of both "B-4" and "B-5" changed temporarily to a greenish-yellow color when exposed to ammonia vapors. When sprayed with 1% sodium nitrite in 10% acetic acid solution followed by 1N sodium hydroxide solution, both "B-4" and "B-5" produced a reddish coloration. They both developed a green color with 0.1% aqueous ferric chloride solution. A saturated solution of ammonium molybdate produced brownish-yellow colors with both "B-4" and "B-5." The "B-4" apparently was present in larger amount than "B-5" in tobacco flowers and produced deeper coloration with the chromogenic sprays just mentioned.

Co-chromatographic studies of "B-4" and "B-5"

with tobacco leaf "B-I-1a" and "B-I-1b" in all systems tried revealed that "B-4" was identical with "B-I-1a" and "B-5" with "B-I-1b." The tobacco flower compound, "B-4" has, therefore, been identified as chlorogenic acid. The identification of "B-I-1b" has yet to be accomplished. Isomerization and decomposition of both "B-4" and "B-5" on S. & S., No. 589 Red Ribbon chromatography paper in aqueous acetic acid systems were found to occur in exactly the same manner as mentioned previously for tobacco leaf compounds "B-I-1a" and "B-I-1b." They each gave identical isomerization products. In the case of "B-5," the numbers and fluorescence colors of the decomposition products were found to be identical with those derived from tobacco leaf "B-I-1b."

CHAPTER IV

ISOLATION OF RADIOACTIVE BLUE-FLUORESCENT COMPOUNDS FROM THE LEAVES OF TOBACCO GROWN IN AN ATMOSPHERE OF CARBON-14 DIOXIDE

Initial Processing of Radioactive Tobacco Leaves

In connection with the plant metabolism studies, One-Sucker tobacco plants, Nicotiana tabacum, were grown in an atmosphere of carbon-14 labeled carbon dioxide in the growth chamber of the Argonne National Laboratory under the supervision of Dr. Nobert J. Scully and Mr. William Chorney.

Immediately after harvesting, the plants were placed in a deep freeze. The plants were harvested and stored in separate packages, according to the number of days of growing time in the radioactive atmosphere, and according to whether the tissues were those of leaves, roots, stems or flowers. The leaves were further subdivided according to their size and relative position on the plant. Each package was processed separately.

The general procedure for the leaves was as follows. The frozen leaves were partially crushed in their plastic bags and dropped into a beaker of boiling 85% isopropyl

alcohol and the solution was allowed to boil for three minutes to inactivate the enzymes. The alcoholic mixture was then reduced to fine particle size in a Waring Blendor. This tobacco leaf blend was decanted into a Soxhlet thimble. By means of a Soxhlet extractor the leaves were extracted with 85% isopropyl alcohol until no visible color appeared in the extract. This alcoholic extract was then added to the filtered alcoholic extract obtained by decanting through the Soxhlet thimble. The whole extract was then reduced in volume on a hot plate to approximately 100 ml. This concentrated alcoholic tobacco leaf extract was poured into a pint wide-mouthed Mason jar, No. 66, and Whatman standard grade ashless cellulose powder added, while stirring, until the particles no longer coalesced upon standing. The jar was placed in an oven and the tobacco leaf extract-cellulose powder mixture dried at 50° C. until the powder would flow readily. When all packages had been processed in this manner, they were stored for further investigation. Further fractionation of each batch on a cellulose column was carried out as described in later paragraphs.

Isolation of the Radioactive Tobacco Leaf Blue-Fluorescent Compounds

Preliminary Separation by Cellulose Column Chromatography. The jar containing the dry tobacco leaf extract-cellulose powder mixture, which represented 478 g.

of main stem tobacco leaves from plants exposed to $C^{14}O_2$ for 43 days, package No. 12, was opened and the whole contents were used for this separation.

A glass chromatographic column, 5 cm. in diameter and 58 cm. long, was packed to a depth of 40 cm. with Whatman standard grade cellulose powder by adding approximately 10 g. at a time and tamping it firmly with a ramrod tipped with a plastic head. The dried cellulose powder-alcoholic mixture of the package No. 12 was then packed to a depth of approximately 10 cm. at the top of the column in a similar manner. Additional fresh cellulose powder was packed on top of the cellulose mixture to a depth of 6 cm. A piece of clean glass wool was placed over the cellulose powder. The column thus prepared was now ready for development and elution.

Benzene was placed on the column from a separatory funnel as the first developing solvent and a dark zone was observed to move rapidly down the column with the solvent front. This dark zone contained "chlorophyll-like" compounds and black tar-like materials. The eluate was successively collected in 500 ml. fractions. Benzene development was continued until the dark materials had been eluted from the column (Fractions 1, 2, and 3). The developing solvent was then changed to anhydrous ethyl acetate in preparation for the addition of the water-saturated ethyl acetate as the major developing solvent (Fractions 4, 5, and 6).

After nineteen 500 ml. water-saturated ethyl acetate fractions had been collected, the column was washed with 500 ml. of 95% ethyl alcohol (Fraction 26), then twice with 500 ml. of 50% ethyl alcohol-water (Fractions 27 and 28), and finally with two 500 ml. of distilled water (Fraction 29 and 30). The fractions were separately concentrated, placed in 50 ml. volumetric flasks and stored in a refrigerator. A chromatogram was prepared by spotting 50 microliters of each fraction 3 cm. apart on a line ruled 8 cm. below the top of a sheet of S. & S., No. 589 Red Ribbon paper (58 x 58 cm.). The chromatogram was developed in the solvent system 15% acetic acid-water, dried and observed under ultraviolet light for the desired blue-fluorescent compounds. For detection of the flavonoid compounds, the developed chromatogram was sprayed with the 1% alcoholic aluminum chloride and the yellow fluorescence of flavonoid-aluminum chloride complexes was observed in a similar manner. Since none of the blue-fluorescent compounds was changed by the aluminum chloride, the spray reagent did not interfere with the location of blue-fluorescent compounds under ultraviolet light.

Isolation by Mass Paper Chromatography. Observation of the 15% acetic acid chromatogram of the column fractions indicated that: (1) scopoletin mainly appeared in column fractions 17, 18, and 19, although some scopoletin could be detected in fraction 5 and all the way through

fraction 22; (2) scopoletin-7-rhamnoglucoside was present in fractions 17, 18, 19, 20, 21 and 20 together with rutin and kaempferol-3-rhamnoglucoside; (3) chlorogenic acid was found in fractions 11, 12, and 13: (4) the "chlorogenic acid-like" depside was found in fractions 14, 15, and 16. Early eluates of the column, namely fractions 1, 2, 3, and 4, contained chlorophylls, tars, and flavonoid aglycone-type compounds. Later eluates, after fraction 23, contained possibly some alkaloids and sugars.

Scopoletin. Fractions 17, 18, 19, and 20 were added together and their combined volume reduced to 50 ml. The combined fraction was then streaked continuously across 8 sheets of Whatman No. 3 MM chromatographic paper (46.5 x 57 cm.) at a distance of 8 cm. from the top. These chromatograms were then developed in the n-butyl alcohol-acetic acid-water system. After drying, a sharp blue fluorescing zone could be seen under ultraviolet light, near the bottom on these chromatograms. A narrow strip was cut from one side of the developed chromatogram and run through an actigraph to indicate the presence of activity in the bottom blue zone. The bottom blue zones were cut off from each chromatogram and eluted with 80% ethyl alcohol-water. Co-chromatography of an ethyl alcohol solution from this blue zone and of pure authentic scopoletin showed the two to be identical in the solvent systems, 15% and 60% acetic acid-water, and the n-butyl alcohol-acetic acid-water. The

eluates were then streaked on new sheets of Whatman No. 3 MM paper and again developed in the n-butyl alcohol-acetic acid-water system. The blue fluorescing zone which contained scopoletin was cut off from the chromatograms and sewed onto other new sheets of Whatman No. 3 MM paper and this time developed in the 15% acetic acid system. The zone containing scopoletin was cut off and the scopoletin was eluted from the paper with 95% ethyl alcohol. The eluates were again streaked on Whatman No. 3 MM paper and developed yet again in 15% acetic acid to remove the last trace amount of yellow impurity. The pure scopoletin was eluted with absolute methyl alcohol in an elution chamber. All methyl alcohol eluates were combined and the volume was adjusted to 10 ml. in a volumetric flask. Five hundred microliters of this purified scopoletin solution were pipetted into an aluminum planchet and the radioactivity determined, in the Nuclear Model D47 gas flow counter. The 500 microliter aliquot registered 2,333 counts per minute (background: 17 counts per minute). The pure solution was sealed in a pyrex tube and sent to Argonne National Laboratory for infiltration study.

Scopoletin-glycoside. Fractions 21 and 22 were combined, concentrated and placed on 4 sheets of Whatman 3 MM paper and then developed in the n-butyl alcohol-acetic acid-water system. When observed under ultraviolet light, these chromatograms were found to be very similar to those

of combined fractions 17, 18, 19, and 20. The scopoletin concentration, however, was found to be very low in fractions 21 and 20. A second blue fluorescing zone, which developed in the middle of the paper and was partly obscured by an overlapping brown zone, was found to be present in all original fractions 17 through 22. This blue-fluorescent zone was cut off from each chromatogram and developed in the 15% acetic acid system. After drying, the bottom blue-fluorescent zone which had been separated from the brown zone in this solvent system, was cut off from the chromatogram and eluted with 50% ethyl alcohol-water. Co-chromatography of an ethyl alcohol solution from this blue zone and of the tobacco leaf compound B-I-2 indicated that the two were identical in all solvent systems tried. Compound B-I-2 is the tobacco leaf compound for which the structure, scopoletin-7-rhamnoglucoside, was proposed in Chapter II. Further purification of this compound was achieved by the same techniques of extended mass paper chromatography alternating the n-butyl alcohol-acetic acid-water and the 15% acetic acid systems twice. The pure scopoletin glycoside thus obtained was finally eluted from the paper with 50% ethyl alcohol-water in an elution chamber. All ethyl alcohol eluates were combined and quantitatively transferred into a 100 ml. volumetric flask. The volume was then adjusted to the mark with 50% ethyl alcohol.

A 0.5 ml. aliquot of this purified scopoletin

glycoside solution was taken from the 100 ml. volumetric flask and the activity measured in a gas flow counter. The aliquot gave 46,657 counts per minute (background: 24 counts per minute). The pure solution was stored in a pyrex tube awaiting the infiltration studies.

Chlorogenic Acid and "Chlorogenic acid-like" Depside. The two tobacco leaf blue-fluorescent compounds described as compound B-I-1a and B-I-1b in Chapter I are designated here as chlorogenic acid and "chlorogenic acid-like" depside, respectively. These two compounds appeared in the middle of the n-butyl alcohol-acetic acid-water chromatograms of fractions 11, 12, 13, 14, 15, and 16. This middle, wide, blue-fluorescent zone of combined solution from the column fractions 14, 15 and 16 had been further purified by streaking on 6 sheets of Whatman 3 MM paper (46.5 x 57 cm.). This zone on each paper was cut off, sewed onto another sheet of Whatman 3 MM paper, and developed in the solvent system n-butyl alcohol-benzene-pyridine-water. This system pulled out a green-fluorescent zone from the "chlorogenic acid-like" blue, but failed to resolve the blue-fluorescent compound. The blue zone was, therefore, cut off from each chromatogram, sewed onto a fresh sheet of Whatman 3 MM paper and developed in the 15% acetic acid system. Three blue-fluorescent zones could now be located. The one with the shortest R_f value appeared to be identical with tobacco leaf compound B-I-1b by co-chromatographing with the latter

in several solvent systems. Each of the three blue-fluorescent zones was cut off from the paper chromatogram and separately eluted with dilute ethyl alcohol containing acetic acid (ethyl alcohol: 15% acetic acid-water; 2:5 v/v). Further purification of the "chlorogenic acid-like" depside zone was carried out in a 5% acetic acid-water system, repeating this procedure three times. The combined eluates (ethyl alcohol-15% acetic acid-water; 2:5) were concentrated under reduced pressure, transferred to a 10 ml. volumetric flask and made to the mark. The activity of 200 microliters of this solution was 225 counts per minute (background: 18 counts per minute). The pure solution was sealed in a pyrex tube and sent to Argonne. This work had been carried out before the isomerization of chlorogenic acid and "chlorogenic acid-like" depside was thoroughly studied. Mass paper chromatographic purification involving the use of 15% acetic acid and 5% acetic acid systems, as described above, is no longer a recommended procedure. These two systems, especially the latter, were found to cause isomerization and decomposition of the "chlorogenic acid-like" depside which has been reported in Chapter II. Apparently the two blue-fluorescent zones, which had been pulled out from the "chlorogenic acid-like" blue in the 5% acetic acid-water development, were decomposition products derived from the parent "chlorogenic acid-like" depside.

Isolation and purification of radioactive chloro-

genic acid were, therefore, carried out on Whatman 3 MM and Whatman No. 1 papers avoiding aqueous organic acid systems. The concentrated combined solution of column fractions 11, 12, and 13 was streaked on 2 sheets of Whatman 3 MM paper and first developed in the n-butyl alcohol-acetic acid-water system. The middle, wide, blue-fluorescent zone, which temporarily changed to a greenish yellow fluorescence under ultraviolet light, was cut off from each chromatogram, sewed onto a fresh sheet of Whatman 3 MM paper and developed in the n-butyl alcohol-benzene-pyridine-water system, repeating this procedure two times. The blue zone thus obtained was then sewed onto Whatman No. 1 paper and developed in the 15% tert-butyl alcohol-water system twice. The pure chlorogenic acid was eluted with 50% ethyl alcohol-water in an elution chamber and the combined eluates were transferred to a 10 ml. volumetric flask. The volume was then adjusted to the mark with 50% ethyl alcohol. The activity of 100 microliters of this solution was 16,708 counts per minute (background: 24 counts per minute). The pure solution was sealed and stored for future infiltration study.

CHAPTER V

A QUANTITATIVE METHOD FOR THE MICROANALYSIS OF RUTIN AND OF SCOPOLETIN IN THE LEAVES OF TOBACCO

Quantitative Determination of Rutin

Several methods have been published for the quantitative determination of rutin in plant materials. Naghski et al. (34) reported a gravimetric method which was used by Badgett et al. (35) to determine the rutin content of several varieties of Nicotiana rustica and Nicotiana glauca. Gage and Wender (36), and Turner (37) have published spectrophotometric methods for the quantitative determination of rutin which differ in experimental procedure, but utilize the color developed by the rutin-aluminum complex. The gravimetric method is not specific for rutin in the presence of other flavonoid compounds. The spectrophotometric methods have the advantage of speed and simplicity, but the use of aluminum chloride in a quantitative determination of rutin is not specific for rutin. Many other flavonoid compounds will give similar color under the above conditions. The success of a quantitative determination of rutin,

therefore, depends upon the successful separation and purification of the compound from other interfering flavonoids and polyphenols of natural products. When the need arose for a method to determine rutin in the dried tobacco leaves, an attempt was made to design a quantitative method which would combine the chromatographic techniques for producing a pure rutin sample and the spectrophotometric method for speed and simplicity. The following method has been found to be good for the determination of rutin in dried tobacco leaves, tobacco flowers, various cured tobaccos, as well as commercially used tobacco mixtures in cigarettes. It can be used for quantitative determination of other polyphenolic compounds.

Processing of Sample. The sample to be analyzed was first dried, cut into small pieces with a blender, and then ground into a powder (40 mesh) with a Wiley mill. The powders analyzed were stored in sealed bottles, from which approximately 2 g. of the sample was taken and accurately weighed on a semi-micro balance. For the analyses of various cured tobaccos, the stems were not separated, but were ground together with the cured leaves. For the analyses on commercial cigarette tobaccos, the tobacco was separated from the cigarette paper (and filter, if present) immediately after each new package of cigarettes was opened. The cigarette tobacco was not dried, but was used immediately after having ground to a powder.

Extraction. The weighed tobacco powder (usually 2 g.) was transferred into an extraction thimble (Whatman) and extracted by 85% isopropyl alcohol-water in a Soxhlet extractor. The first extraction was started with 250 ml. of 85% isopropyl alcohol-water for 3 hours, using a hot water bath (80 - 90° C.). After removal of this first extract, another 250 ml. of fresh 85% isopropyl alcohol-water was used for the second extraction for another 3 hours when the sample gave no coloration with fresh solvent. The two extracts were combined and then reduced in volume in vacuo to approximately 140 ml. on a warm water bath (40 - 50° C.). The cooled concentrate was filtered into a 200 ml. volumetric flask and the filter was washed thoroughly with 85% isopropyl alcohol-water. The volume of filtrate was then adjusted to the mark with 85% isopropyl alcohol-water. An aliquot of this solution was used for separation and purification of the individual polyphenol, such as rutin.

Isolation and Purification by Extended Mass Paper Chromatography. An exact volume of the concentrate was taken with a 1 ml. pipet each time and streaked onto a sheet of S. & S., No. 589 Red Ribbon filter paper (19 x 58 cm.) and air dried. The amount streaked depends on the concentration of polyphenol in the concentrate. The paper chromatogram was first developed in the n-butyl alcohol-acetic acid-water system (6:1:2). For analysis of each sample, three papers were usually prepared from the concentrate. After

development, the paper was air dried. The zone which contained the rutin ran about one-third of the distance from the origin and fluoresced a brownish color under ultraviolet light. In order to locate the rutin zone more readily, the area containing rutin was exposed to ammonia vapors to make sure that the brown-fluorescent zone changed to yellow. The first development of the paper chromatogram separated scopoletin and other non-glycosides from rutin as well as from certain unknown polar compounds. The rutin zone, however, was still overlapped with several other compounds such as chlorogenic acid, "chlorogenic acid-like" compound, scopoletin glycoside, kaempferol-3-rhamnoglucoside, and several unknown compounds.

The rutin zone was cut off from the chromatogram and sewed onto a new sheet of S. & S., No. 589 paper. By cutting off the paper behind the sewed rutin zone, the paper could be developed smoothly for further separation. The paper thus prepared was next developed in the 15% acetic acid-water system to remove the scopoletin glycoside. After development, the rutin zone was again cut off, sewed onto another fresh sheet, and developed this time in a system of n-butyl alcohol-benzene-pyridine-water (5:1:3:3). This third system separated the rutin from the chlorogenic acid and "chlorogenic acid-like" compounds to some extent, but not completely. In order to achieve complete separation, this procedure was carried out four times by alternating

between the 15% acetic acid-water and n-butyl alcohol-benzene-pyridine-water systems. The rutin zone thus obtained was then sewed onto yet another new sheet of paper and this time developed in the n-butyl alcohol-acetic acid-water system for 24 - 30 hours to separate the rutin from the kaempferol-3-rhamnoglucoside. In those cases where the latter compound was present in relatively large amounts, a repeat of chromatography with n-butyl alcohol-acetic acid-water and then 15% acetic acid was often necessary to achieve complete separation. Final development of the rutin zone was carried out in the n-butyl alcohol-benzene-pyridine-water system.

The Spectrophotometric Determination. The resulting pure rutin zone was sprayed thoroughly on both sides with 1% alcoholic aluminum chloride solution and then was hung overnight for complete development of the rutin-aluminum chloride complex. The paper was examined with ultraviolet light and the yellow complex was cut out for elution. Each such strip was then cut up into small pieces and transferred into a 25 ml. Erlenmeyer flask. Ten ml. of distilled water were added and the contents were shaken frequently. The flask was then placed on a warm steam bath and heated for 30 minutes, with frequent agitation of the contents. The eluate was filtered through a small funnel into a clean beaker. Two additional extractions were made with 5 ml. portions of hot distilled water; and finally the papers

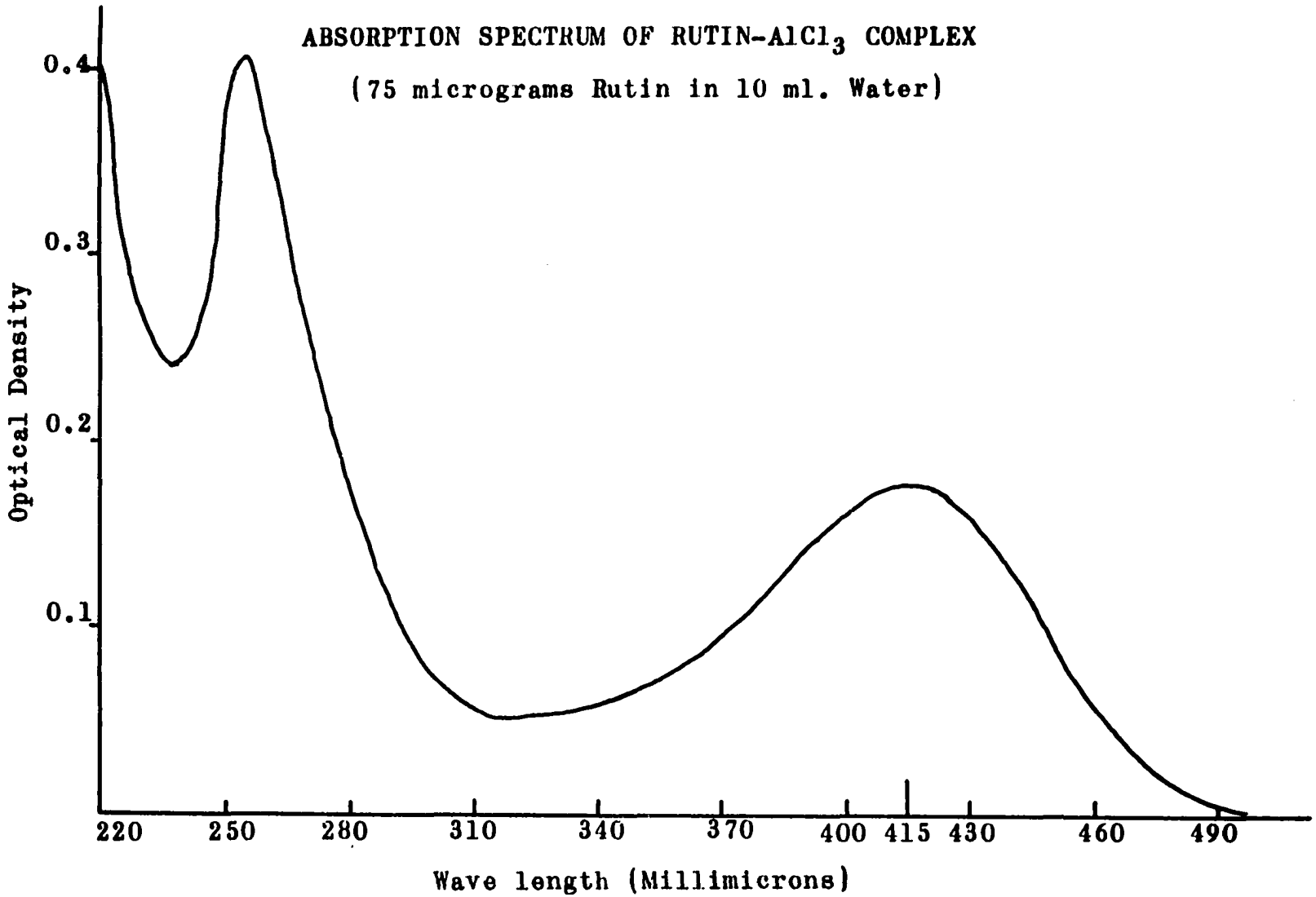
were placed on the funnel and washed with yet another 5 ml. of hot distilled water. The combined filtrate was transferred to a 25 ml. volumetric flask and adjusted to the mark. This solution was then poured into a 1 cm. silica cell and the optical density measured at a wave length of 415 millimicrons (Figure 11) using the Beckman model DU spectrophotometer. An eluate prepared by the same procedure as above, but using no tobacco, was used as blank. The present method has some advantages over the previous method reported by Gage and Wender (36). Such advantages are: (1) easier location of the rutin zone after spraying the purified rutin with aluminum chloride; (2) elution of the rutin-aluminum chloride complex with water rather than elution of free rutin with dilute alcohol. Filter paper impurities are least soluble in water.

Since the purification of rutin involved extended paper chromatographic separation, it was necessary to make a standard reference curve of rutin with known concentration. Exactly 10.00 mg. of pure authentic rutin was weighed on a semi-micro balance and quantitatively transferred into a 10 ml. volumetric flask to make a standard solution of rutin with a concentration of 1 microgram per ml. of solution. Four samples of rutin solution, namely 25, 50, 75 and 100 micrograms of rutin were taken from the above solution with micro-pipets. Each was directly streaked on a sheet of S. & S., No. 589 Red Ribbon filter paper (19 x 58 cm.)

FIGURE 11

ABSORPTION SPECTRUM OF RUTIN-$AlCl_3$ COMPLEX

(75 micrograms Rutin in 10 ml. Water)



together with a blank sheet which was streaked with 100 microliters of pure ethyl alcohol. The papers were then carried through the extended paper chromatographic procedures described previously for quantitative separation of rutin from dry tobacco samples. The rutin zone on the final paper chromatogram was sprayed with 1% alcoholic aluminum chloride and the developed yellow rutin-aluminum chloride complex eluted off from each paper with hot distilled water as described above. Each eluate was then quantitatively transferred into a 25 ml. volumetric flask, and made up to the mark with distilled water. The optical density was measured at 415 millimicrons. A standard reference curve (Figure 12) was made by plotting optical density against the micrograms of rutin originally spotted at the beginning of the chromatographic separation.

Analytical Results. Using the procedures described above, the tobacco from 24 brands of commercial cigarettes commonly used in the United States were analyzed for their rutin content. The results obtained are recorded in the following table (Table 3). Cigarettes included in this study were: (1) regular: Camel, Chesterfield, Lucky Strike, Old Gold, Philip Morris; (2) regular-denicotinized: Sano; (3) menthol: Kool (both regular and filter), Oasis, Salem, Spud; (4) king size: Cavalier, Chesterfield, Herbert Tareyton, Pall Mall, Philip Morris, Raleigh; (5) filter: Hit Parade, Kent, L.&M., Marlboro, Old Gold, Viceroy, and Winston.

FIGURE 12

STANDARD RUTIN CURVE

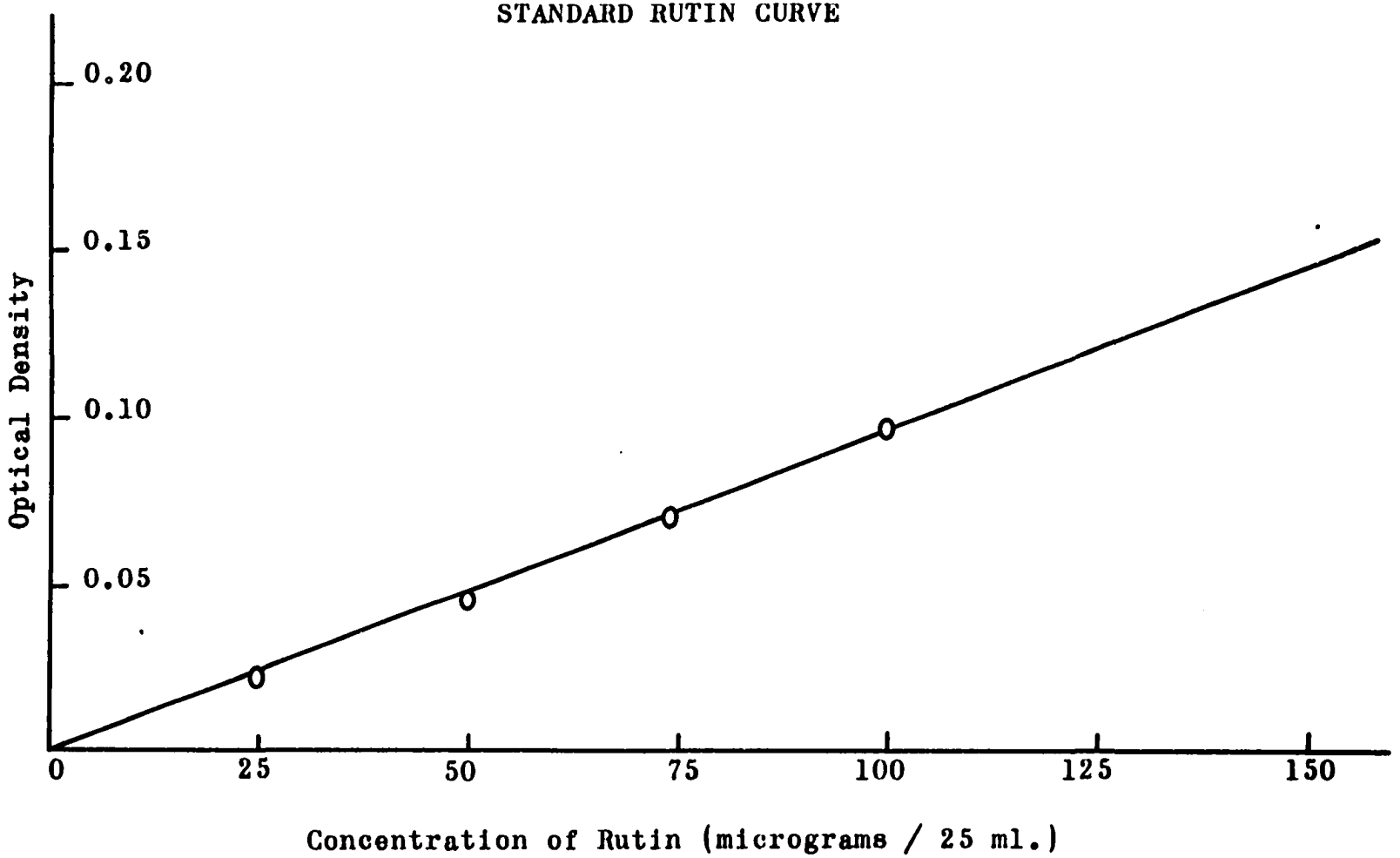


TABLE 3

RUTIN CONTENT IN TOBACCO OF 24 BRANDS OF CIGARETTES

Cigarette type and Brand	Tobacco Weight (g.)	Volume Extracted (ml.)	Volume Streaked (ml.)	Optical Density (25 ml.)	Rutin per aliquot (µg.)	Total Rutin (µg.)	% Rutin by Weight
REGULAR							
Cigarette A							
Sample 1	2.0009	200	15	0.087	92	1,226	0.061
Sample 2	2.1126	200	15	0.086	90	1,200	0.057
Cigarette B							
Sample 1	2.0005	200	15	0.114	120	1,600	0.080
Sample 2	2.0239	200	15	0.123	128	1,707	0.084
Cigarette C							
Sample 1	2.0004	200	15	0.109	116	1,547	0.077
Sample 2	2.0698	200	15	0.113	118	1,573	0.076
Cigarette D							
Sample 1	2.0008	200	15	0.159	168	2,240	0.11
Sample 2	2.0900	200	15	0.154	162	2,160	0.11
Cigarette E							
Sample 1	2.0010	200	15	0.100	105	1,400	0.070
Sample 2	1.8493	200	15	0.095	100	1,333	0.072
DENICOTINIZED							
Cigarette F							
Sample 1	2.0003	200	15	0.015	18	240	0.012
Sample 2	2.0007	200	15	0.014	16	213	0.012

TABLE 3 - Continued

Cigarette type and Brand	Tobacco Weight (g.)	Volume Extracted (ml.)	Volume Streaked (ml.)	Optical Density (25 ml.)	Rutin per aliquot (µg.)	Total Rutin (µg.)	% Rutin by Weight
MENTHOL							
Cigarette G							
Sample 1	2.0016	200	15	0.038	40	533	0.027
Sample 2	2.0006	200	15	0.031	33	440	0.022
Composite							
Sample 1	10.0000	500	10	0.046	48	2,400	0.024
Sample 2	10.0000	500	10	0.048	50	2,500	0.025
KING-SIZE							
Cigarette L							
Sample 1	1.9999	200	15	0.108	114	1,520	0.076
Sample 2	2.0003	200	15	---	---	---	---
Composite							
Sample 1	10.0000	500	10	0.090	95	4,750	0.048
Sample 2	10.0000	500	50	0.087	92	4,600	0.046
FILTER							
Cigarette R							
Sample 1	2.0004	200	15	0.076	80	1,067	0.053
Sample 2	2.0005	200	15	0.054	65	867	0.043
Cigarette S							
Sample 1	2.0011	200	15	0.055	58	773	0.039
Sample 2	2.0008	200	15	0.056	60	800	0.040
Composite							
Sample 1	10.0000	500	10	0.062	64	3,200	0.032
Sample 2	10.0000	500	10	0.069	72	3,600	0.036

In the analyses of the last three types of cigarettes, a composite was made up of five different brands of cigarettes belonging to the same type. Extraction of a composite was started with a total of 10 g. of mixed tobacco powder, each brand being represented by exactly 2 g. The weight of the powder from each brand was weighed on a semi-micro balance.

Four 250 ml. portions of 85% isopropyl alcohol-water were used for extraction of each composite and the volume of combined extracts concentrated to approximately 450 ml. This was filtered into a 500 ml. volumetric flask and the final volume adjusted to the mark of the volumetric flask. A measured volume (usually 10 ml.) of this concentrated extract was taken from the flask with a 1 ml. micropipet and streaked on each paper to start the extended chromatographic separation.

Quantitative Determination of Scopoletin

The following paragraphs describe a paper chromatographic and spectrophotometric procedure that has been successfully applied to the quantitative determination of micro amounts of scopoletin in various dry tobacco samples.

The dry tobacco sample was first ground to a powder and thoroughly mixed. The procedure is the same as described above for the quantitative determination of rutin. It was followed exactly down to the streaking of the extract on paper.

For analysis of scopoletin, the streaking of each extract solution was carried out on a 17 cm. long line ruled off 8 to 10 cm. below the top of a sheet of chromatographic paper, 19 x 58 cm., S. & S., No. 589 Red Ribbon. This leaves about 1 cm. of unstreaked blank on each side. When streaked entirely across the paper, the scopoletin often ran along both edges of the paper further than the main zone, and often produced some error in the quantitative determination. The papers thus prepared were then subjected to extended chromatographic separation.

The streaked papers were first developed in the n-butyl alcohol-acetic acid-water system. After drying, the zone which contained scopoletin was cut off from each of the developed chromatograms. Each scopoletin zone was separately sewed onto new sheets of S. & S., No. 589 paper (19 x 58 cm.). Sewing here was done in such a fashion that the paper containing the scopoletin zone was turned around so that the scopoletin zone would face the bottom of the paper. The extract solution of dry tobacco samples usually contained several compounds which ran in the range of the scopoletin R_f value when the first paper was developed in the n-butyl alcohol-acetic acid-water system. Some of these compounds were so close to the scopoletin zone on the developed chromatogram that their separation from scopoletin could not be achieved when the scopoletin zone was cut off from the paper. The interfering compounds ran just below

scopoletin in the n-butyl alcohol-acetic acid-water system and have zero or little movement in the second solvent system to be used, namely 15% acetic acid. Since scopoletin could not move smoothly through these interfering compounds, the paper containing the scopoletin zone was inverted and sewed onto a new sheet for development in the second solvent system. After development, the zone containing scopoletin was again cut off from the chromatogram and sewed onto another new sheet of S. & S., No. 589 paper. Here on the papers to be sewed onto new sheets are not inverted. The paper was again developed in the 15% acetic acid system. As soon as the blue fluorescing scopoletin zone had moved about 10 cm. beyond the position where the two papers were sewed together (this can be checked by the ultraviolet light even when the paper is still running in the development tank), the papers were taken out from the tank and air dried. These unfinished chromatograms were then put into the nitromethane-benzene-water system to finish the separation on the same sheet. Experimentation showed that it was better to use the 15% acetic acid (or the n-butyl alcohol-acetic acid-water) system to move scopoletin quantitatively across the position of sewing and then to subject the paper to development in the nitromethane system. The nitromethane system was found not to move the scopoletin zone across the sewed line smoothly and quantitatively. Once the zone has been quantitatively moved onto the new sheet by the 15%

acetic acid, where the paper is smooth, the nitromethane system can serve satisfactorily for the separation of scopoletin from its impurities in this system.

The scopoletin zones after development in the nitromethane system, were again cut off and sewed onto still other sheets of S. & S., No. 589 paper. These papers were again developed in the 15% acetic acid system for 12 to 18 hours until the scopoletin zone moved half way down the paper. At this stage, the scopoletin zone thus purified was usually spread out on the paper from 7 cm. to 15 cm., depending on the concentration. In order to bring the scopoletin zone into a narrow zone for elution, each zone containing scopoletin was again cut off from the paper and sewed onto a new sheet of paper and finally developed in the n-butyl alcohol-acetic acid-water system. Pre-washed Whatman No. 1 filter paper can be substituted in the last development of the scopoletin zone, since scopoletin can be eluted from this paper much faster than from the S. & S., No. 589 paper. The Whatman No. 1 papers used only in the last development were washed for approximately 24 hours in the n-butyl alcohol-acetic acid-water system to remove the filter paper impurities.

Elution of scopoletin was carried out in an elution chamber at room temperature with 50% ethyl alcohol-water, until each eluate amounted to a volume of 8 ml. As soon as the volume of the eluates reached this value,

elution was stopped and the eluates were quantitatively transferred into a 10 ml. volumetric flask. Each container of eluate was then washed three times with 50% ethyl alcohol and all washings added to the volumetric flask. The volume of each eluate was finally adjusted to the mark with 50% ethyl alcohol. Just before spectrophotometry, each eluate was transferred to a 15 ml. centrifuge tube and centrifuged. The eluate solution was then poured into a 1 cm. silica cell and the optical density of the solution measured with the Beckman Model DU spectrophotometer at wave length 344 millimicrons.

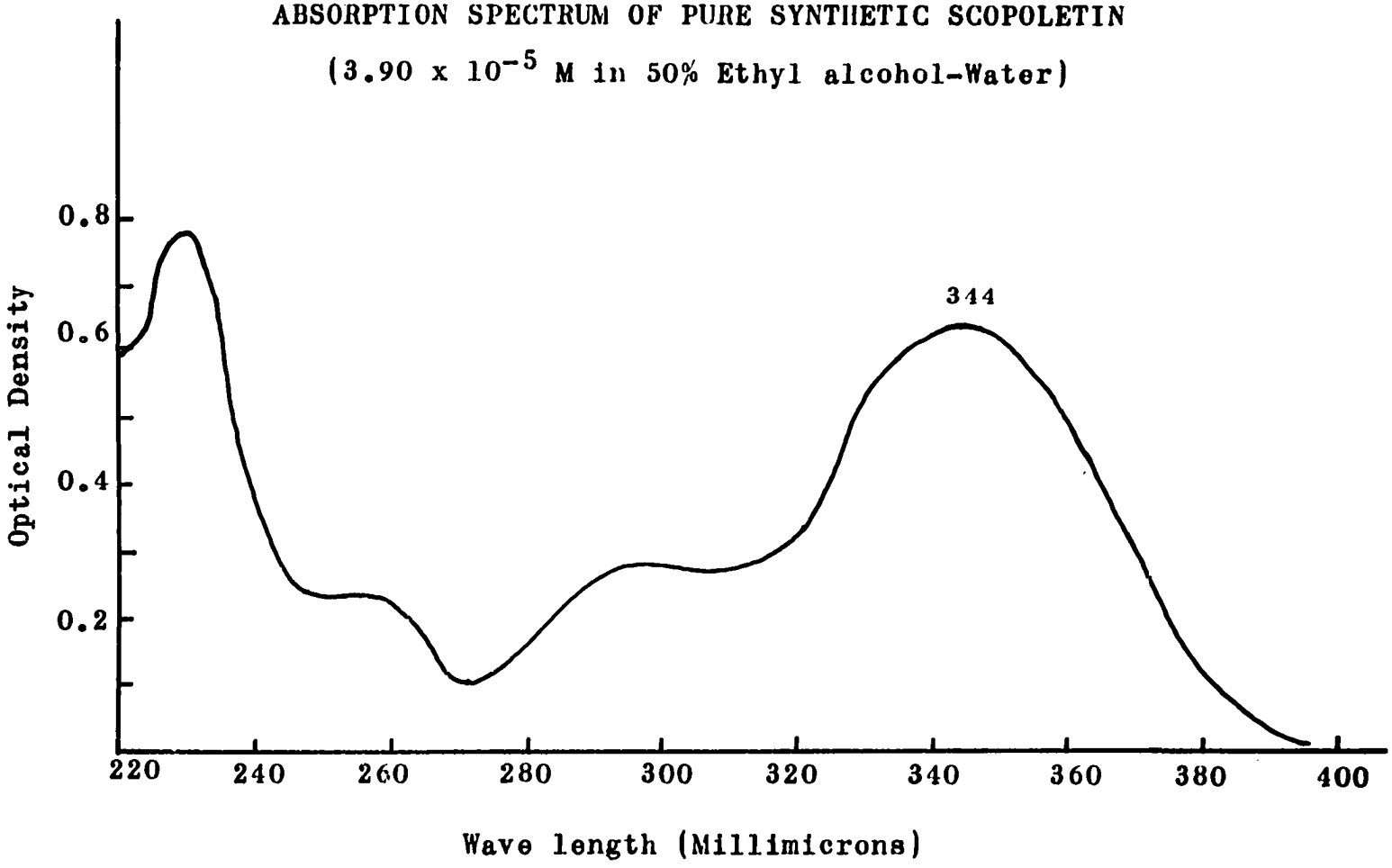
To determine the wave length for measurement of absorption, a 3.90×10^{-5} molar solution of scopoletin in 50% ethyl alcohol-water (7.5 mg./liter of 50% ethyl alcohol) was prepared from the synthetic scopoletin (Chapter II). Its absorption spectrum was taken from wave length 215 to 380 millimicrons in a 1 cm. silica cell with the Beckman DU spectrophotometer. Scopoletin exhibited a prominent absorption maximum at 344 millimicrons (Figure 13). This absorption maximum was, therefore, selected for the quantitative determination of the scopoletin.

A standard reference curve was prepared by using five aliquots of a scopoletin solution containing 0.5 microgram per 1 microliter. The five aliquots (10, 25, 50, 75, and 100 micrograms) were carried through the procedures described above. The standard curve was obtained by

FIGURE 13

ABSORPTION SPECTRUM OF PURE SYNTHETIC SCOPOLETIN

(3.90 x 10^{-5} M in 50% Ethyl alcohol-Water)



plotting optical density of the eluate solution against the micrograms of scopoletin originally spotted at the beginning of the chromatographic separation (Figure 14).

Analytical Results. The results obtained from the 24 brands of American cigarettes described in the previous section of this chapter are reported in the following table (Table 4). The cigarettes designated by the same alphabetical letter in both tables are the same brand cigarette. The result obtained here, however, was not from the same package of each brand but from the different packages purchased at different times.

FIGURE 14

STANDARD SCOPOLETIN CURVE

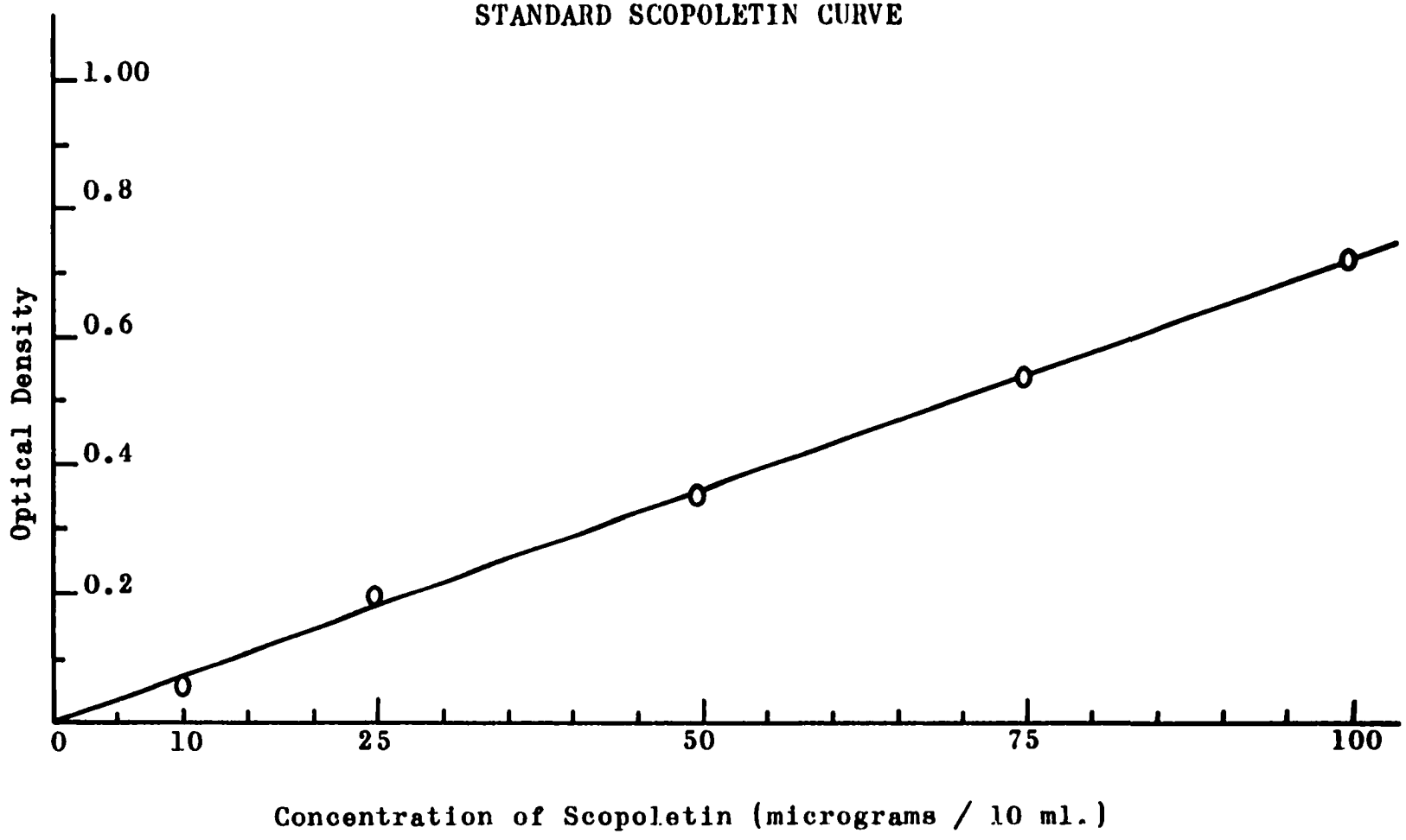


TABLE 4

SCOPOLETIN CONTENT IN TOBACCO OF 24 BRANDS OF CIGARETTES

Cigarette Type and Brand	Weight in g. cigarette tobacco extracted	Micrograms of scopoletin per aliquot (Average of 3 aliquots)	Micrograms of scopoletin per 1 g. of cigarette tobacco	Percentage of scopoletin in cigarette tobacco
REGULAR				
Cigarette A				
Sample 1	2.1542	19.8	123	0.012
Sample 2	2.1187	17.8	112	0.011
Cigarette B				
Sample 1	2.0634	16.5	107	0.011
Sample 2	2.0581	16.5	107	0.011
Cigarette C				
Sample 1	1.9996	16.3	109	0.011
Sample 2	2.0009	17.8	119	0.012
Cigarette D				
Sample 1	2.0121	12.9	86	0.0086
Sample 2	2.0203	12.7	84	0.0084
Cigarette E				
Sample 1	2.0546	10.4	68	0.0068
Sample 2	2.0692	10.2	66	0.0066
DENICOTINIZED				
Cigarette F				
Sample 1	2.0142	7.2	48	0.0048
Sample 2	2.3387	7.1	41	0.0041

TABLE 4 - Continued

Cigarette Type and Brand	Weight in g. cigarette tobacco extracted	Micrograms of scopoletin per aliquot (Average of 3 aliquots)	Micrograms of scopoletin per 1 g. of cigarette tobacco	Percentage of scopoletin in cigarette tobacco
MENTHOL				
Cigarette C				
Sample 1	2.1890	11.6	71	0.0071
Sample 2	2.3815	12.6	71	0.0071
Composite				
Sample 1	10.0000	18.4	92	0.0092
Sample 2	10.0000	19.1	96	0.0096
KING-SIZE				
Cigarette L				
Sample 1	2.0987	13.3	85	0.0085
Sample 2	2.1834	17.2	105	0.0105
Composite				
Sample 1	10.0000	19.2	96	0.0096
Sample 2	10.0000	18.9	95	0.0095
FILTER				
Cigarette R				
Sample 1	1.9995	10.8	72	0.0072
Sample 2	1.9996	12.6	84	0.0084
Cigarette S				
Sample 1	2.0025	17.4	116	0.012
Sample 2	2.0016	17.7	118	0.012
Composite				
Sample 1	10.0000	15.3	77	0.0077
Sample 2	10.0000	16.5	83	0.0083

CHAPTER VI

IDENTIFICATION OF SCOPOLETIN IN CIGARETTE TOBACCO AND SMOKE

In recent years, several papers involving studies on the chemical composition of tobacco smoke by partition chromatography have appeared in the literature. The compounds hitherto investigated in cigarette smoke are mainly low molecular weight aldehydes and ketones (38, 39, 40, 41, 42, 43, 44, 45, 46), organic acids (47, 48, 49), unsaturated and saturated hydrocarbons (44, 50), nicotine (49, 51, 52, 53, 54, 55, 56), and volatile phenols (57, 58).

Since cigarette tobacco is a special product of tobacco leaves manufactured through various processes, including curing, a preliminary survey has been undertaken to investigate whether or not each of the polyphenolic compounds that has been found in green One-Sucker tobacco leaves is also present in the cured tobaccos. This study covered the samples sent to this laboratory by Dr. Moseley of the American Tobacco Company of Richmond, Virginia, and which were labeled by him as "Maryland," "Burley," "Flue-cured," "One-Sucker," "Pennsylvania," and foreign "Turkish." It also included samples of "Burley" and domestic "Turkish"

tobacco sent by Mr. Luther Shaw of the Mountain Research Station, Waynesville, N.C. Qualitatively, though not quantitatively, there appeared to be fair similarity in the two-dimensional chromatograms of the various samples surveyed. Scopoletin glycoside could not be detected in any of these cured tobaccos. Scopoletin, however, was present in all samples studied.

In view of the above findings on the cured tobacco, it also appeared important to learn by experimentation whether or not scopoletin is present in tobacco after curing and incorporation into cigarettes, and, if so, whether or not any scopoletin, m.p. 204°, survives the smoking process to persist in the mainstream smoke from cigarettes.

This study covered tobacco in 29 brands of cigarettes commonly used in the United States, and it was found that every one tested contained scopoletin. These included regular size, filter, "denicotinized," menthol, and king size cigarettes. It was discovered also that the mainstream smoke from every cigarette sample tested contained scopoletin. This was the case under every different smoking condition that was used on the smoking machine. These different smoking conditions will be described in more detail in later sections of this chapter. The amounts of scopoletin present in the smoke, however, are apparently different, and quantitative studies have been undertaken in this laboratory.

Scopoletin from Cigarette Tobacco. Each qualitative analysis on the tobacco was performed separately on approximately 2 g. of cigarette tobacco obtained from cigarettes in a freshly opened pack or box, purchased locally on the open, retail market. The paper from each cigarette was removed before extraction of the tobacco. In the case of filter cigarettes, the tobacco was separated from both the filter and paper. Each 2 g. extraction was carried out in a separate Soxhlet extractor, using 200 ml. of 85% isopropyl alcohol for approximately 3 hours on a steam bath. A second extraction was made on each sample, using another 200 ml. of 85% isopropyl alcohol for another 3 hours. The two extracts of the 2 g. tobacco sample were combined, reduced to approximately 150 ml. in vacuo, and the volume was then adjusted with 85% isopropyl alcohol to the mark in a 200 ml. volumetric flask. Aliquots of this solution were then taken for one-dimensional and two-dimensional paper chromatographic analyses in comparison with authentic scopoletin, and also for additional paper chromatographic purification for further study on the scopoletin identification.

For the one-dimensional paper chromatograms, 0.5 ml. samples of each cigarette tobacco extract concentrate were spotted on S. & S., No. 589 Red Ribbon chromatographic paper next to a similar amount of an authentic sample of scopoletin. Solvent systems used were 15% acetic acid-water;

60% acetic acid-water; n-butyl alcohol-acetic acid-water (6:1:2 v/v); n-butyl alcohol-benzene-pyridine-water (5:1:3:3 v/v); and nitromethane-benzene-water (2:3:5 v/v). Typical R_f values for scopoletin in these solvent systems, respectively, using the S. & S., No. 589 Red Ribbon paper for chromatography at a temperature of $28^{\circ} \pm 3^{\circ}$ were: 0.47; 0.74; 0.82; 0.82; and 0.69. After chromatography, the papers were examined under ultraviolet "black light" (3660 Å). A bright blue fluorescence is exhibited by scopoletin.

Although a one-dimensional chromatogram of the various cigarette tobacco extract concentrates prepared as just described showed many spots when viewed by ultraviolet light, the scopoletin spot thereon could be readily detected and tentative identification made by co-chromatography with authentic scopoletin.

Two-dimensional paper chromatograms of the cigarette tobacco extract concentrates also were made, using in one group the n-butyl alcohol-acetic acid-water system in the first direction, then 15% acetic acid-water in the second direction.

In the second group of experiments on two-dimensional chromatograms, the nitromethane-benzene-water system was used in the first direction, then 15% acetic acid-water in the second direction. After chromatography, the scopoletin spot could be easily located in every case, even though

other spots could be seen on the chromatogram under the ultraviolet light.

By the methods described, the tobacco from 29 brands of cigarettes was examined, and every one was found to contain scopoletin. Cigarettes studied were: (1) regular: Camel, Chesterfield, Lucky Strike, Old Gold, Philip Morris; (2) regular denicotinized: Sano; (3) menthol: Kool (both regular and filter), Oasis, Salem, Spud; (4) king: Cavalier, Chesterfield, Dunhill, Herbert Tareyton, Pall Mall, Philip Morris, Raleigh; (5) filter: Encore, Hit Parade, Kent, L & M, Marlboro, Old Gold, Parliament, Regent, Tareyton, Viceroy, and Winston.

Scopoletin in Cigarette Smoke. Each cigarette was smoked on a standard smoking apparatus (Phipps & Bird, Inc., Richmond, Virginia) based on a design of the American Tobacco Company. This smoking machine consists of two units:

The first unit consists of a draft shield (housing for cigarettes, etc.) with a vent at the top and an ash drawer at the bottom for cleaning, and a door at one end for easy access. On the back are clamps to hold suitable collection flasks and the remainder of the absorption train.

The second unit consists of a puffing mechanism. This puffing mechanism is designed to give a constant volume puff on each of four cigarettes once each minute. The actual puff is produced by a falling column of water in a burette with a ground glass float valve. By means of a control

mechanism consisting of cams, timers, and switches, solenoid valves and a bank of rectifiers, the puff is taken consecutively on each of the four cigarettes at fifteen second intervals. The duration of the puff may be controlled between two and four seconds and the volume is adjustable from 0 to 60 ml.

The experiments on representative brands and types of cigarettes indicated that scopoletin was readily detectable in the smoke, when 10 individual cigarettes were smoked under all varying smoking machine conditions tested. Actually, now that it has been discovered that scopoletin is present in the smoke, one can easily locate it on a chromatogram of the smoke from one individual cigarette. Tried were a faster smoking rate (3.3 second duration; 54 $\pm$ 4 ml. volume; 60 second interval); a medium speed (2 second duration; 35 $\pm$ 4 ml. volume; 60 second interval); and a slower speed (1 second duration; 16 $\pm$ 1 ml. volume; 60 second interval). Also varied were the cigarette butt lengths; regular size cigarettes (2, 3.5, and 5 cm.) and king size and filters (3.4 and 6.5 cm.). (Table 5). All combinations of the above showed the presence of scopoletin in the smoke. Apparently, quantitative differences may have occurred, as estimated from gross observation of the size and intensity of the scopoletin on the paper chromatograms of the smoke obtained under different smoking conditions. The quantitative facts are to be determined by research on the

TABLE 5

VARIATIONS OF SMOKING RATE AND BUTT LENGTH ON REPRESENTATIVE BRANDS AND TYPES OF CIGARETTES

(1) Faster speed and medium butt length

Cigarette	Total length of cigarette	Butt length	Puffs	Duration	Interval	Volumes
Camel	7 cm.	3.5 cm.	6	3"	60"	52.8 ml.
Kool (regular)	8.5	4	7	3	60	53.4
Pall Mall	7	3.5	7	3	60	55.0
Winston	8.5	4	8	3	60	53.4

(2) Faster speed and longer butt length

Cigarette	Total length of cigarette	Butt length	Puffs	Duration	Interval	Volumes
Camel	7 cm.	5 cm.	4	3"	60"	51.3 ml.
Kool (regular)	7	5	4	3	60	53.0
Pall Mall	8.5	6.5	4	3	60	53.0
Winston	8.5	6.5	4	3	60	52.3

(3) Medium speed and shorter butt length

Cigarette	Total length of cigarette	Butt length	Puffs	Duration	Interval	Volumes
Camel	7 cm.	2 cm.	11	2"	60"	35.0 ml.
Kool (regular)	7	2	11	2	60	37.8
Pall Mall	8.5	3	11	2	60	37.0
Winston	8.5	3	11	2	60	37.6

TABLE 5 - Continued

(4) Medium speed and medium butt length

Cigarette	Total length of cigarette	Butt length	Puffs	Duration	Interval	Volumes
Camel	7 cm.	3.5 cm.	9	2"	60"	30.5 ml.
Kool (regular)	7	3.5	7	2	60	35.5
Pall Mall	8.5	4	7	2	60	32.5
Winston	8.5	4	9	2	60	38.5

(5) Medium speed and longer butt length

Cigarette	Total length of cigarette	Butt length	Puffs	Duration	Interval	Volumes
Camel	7 cm.	5 cm.	5	2"	60"	34.3 ml.
Kool (regular)	7	5	5	2	60	31.7
Pall Mall	8.5	6.5	5	2	60	34.3
Winston	8.5	6.5	5	2	60	37.0

(6) Slower speed and shorter butt length

Cigarette	Total length of cigarette	Butt length	Puffs	Duration	Interval	Volumes
Camel	7 cm.	2 cm.	13	1"	60"	16.0 ml.
Kool (regular)	7	2	14	1	60	16.0
Pall Mall	8.5	3	13	1	60	16.9
Winston	8.5	3	15	1	60	15.0

TABLE 5 - Continued

(7) Slower speed and medium butt length

Cigarette	Total length of cigarette	Butt length	Puffs	Duration	Interval	Volumes
Camel	7 cm.	3.5 cm.	10	1"	60"	16.2 ml.
Kool (regular)	7	3.5	9	1	60	15.6
Pall Mall	8.5	4	10	1	60	16.2
Winston	8.5	4	10	1	60	15.6

(8) Slower speed and longer butt length

Cigarette	Total length of cigarette	Butt length	Puffs	Duration	Interval	Volumes
Camel	7 cm.	5 cm.	6	1"	60"	16.3 ml.
Kool (regular)	7	5	6	1	60	17.0
Pall Mall	8.5	6.5	5	1	60	17.0
Winston	8.5	6.5	6	1	60	14.6

quantitative aspects now in progress in this laboratory. In that qualitatively, scopoletin was present under all the conditions tried for the selected, representative cigarettes, the following conditions were arbitrarily selected for smoking all 29 brands: butt length of 2 cm. for regular size cigarettes and 3 cm. for king size and filters; volume, 54 ± 4 ml.; puff duration, 3.3 ± 0.2 second at 60 second intervals; and one pack or box of 20 cigarettes per sample for study. (Table 6).

The smoke from each 20 cigarettes of one brand was trapped, in part, in a 300 ml. Kjeldahl flask, immersed in a salt-ice mixture (average temperature, -18° C.). Some scopoletin was found to escape into a second and into a third trap, even when a "dry-ice" acetone bath was used for cooling the first two traps. For the identification of scopoletin as described in this chapter, however, a sufficient amount was obtained in the first Kjeldahl flask, even with a salt-ice mixture, for clear-cut qualitative analysis.

The trapped smoke, in each case, was dissolved in dry acetone, To analyze for the presence of scopoletin, the acetone solution of the smoke was then subjected to both one-dimensional and two-dimensional chromatography according to the procedure already described for the extract from cigarette tobacco. In the case of every one of the 29 brands of cigarettes smoked, a bright blue fluorescent spot coinciding in color and R_f values with authentic scopoletin

TABLE 6

SMOKING RATE AND BUTT LENGTH OF 29 BRANDS CIGARETTES

Faster speed and shorter butt length

Cigarette	Total length	Butt length	Puffs	Duration	Interval	Volume
Regular						
Camel	7 cm.	2 cm.	8	3.3±0.2"	60"	54±4 ml.
Chesterfield	7	2	9	3.3±0.2	60	54±4
Lucky Strike	7	2	8	3.3±0.2	60	54±4
Old Gold	7	2	8	3.3±0.2	60	54±4
Philip Morris	7	2	10	3.3±0.2	60	54±4
Sano	7	2	8	3.3±0.2	60	54±4
Menthol						
Kool (regular)	7 cm.	2 cm.	8	3.3±0.2"	60"	54±2 ml.
Kool (filter)	8.5	3	9	3.3±0.2	60	54±2
Oasis	8.5	3	9	3.3±0.2	60	54±2
Salem	8.5	3	9	3.3±0.2	60	54±2
Spud	8.5	3	9	3.3±0.2	60	54±2
King						
Cavalier	8.5 cm.	3 cm.	13	3.3±0.3"	60"	56±2 ml.
Chesterfield	8.5	3	10	3.3±0.3	60	56±2
Dunhill	8.5	3	12	3.3±0.3	60	56±2
Herbert-Tareyton	8.5	3	12	3.3±0.3	60	56±2
Pall Mall	8.5	3	12	3.3±0.3	60	56±2
Philip Morris	8.5	3	11	3.3±0.3	60	56±2
Raleigh	8.5	3	12	3.3±0.3	60	56±2

TABLE 6 - Continued

Cigarette	Total length	Butt length	Puffs	Duration	Interval	Volume
Filter						
Encore	8.5 cm.	3 cm.	10	3.3±0.3"	60"	54±2 ml.
Hit Parade	8.5	3	7	3.3±0.3	60	54±2
Kent	8.5	3	6	3.3±0.3	60	54±2
L & M	8.5	3	10	3.3±0.3	60	54±2
Marlboro	8.5	3	8	3.3±0.3	60	54±2
Old Gold	8.5	3	9	3.3±0.3	60	54±2
Parliament	8.5	3	9	3.3±0.3	60	54±2
Regent	8.5	3	10	3.3±0.3	60	54±2
Tareyton	8.5	3	11	3.3±0.3	60	54±2
Viceroy	8.5	3	12	3.3±0.3	60	54±2
Winston	8.5	3	9	3.3±0.3	60	54±2

was observed. Additional spots, often 10 or more, usually could be found under the ultraviolet light. Two additional blue-fluorescent compounds present on the chromatograms from the smoke are now being investigated by other workers in this laboratory. In spite of the presence of the other compounds, and although the scopoletin spot itself was not completely free of other substances, the scopoletin could be readily detected on the chromatograms.

However, in order to obtain a pure sample of scopoletin from the smoke for further identification studies, the acetone solution of the smoke was subjected to extended paper chromatographic separation. For this purpose, the acetone solution of the smoke was streaked across a sheet of S. & S., No. 589 Red Ribbon chromatography paper, size 19 x 58 cm., and first developed in a 15% acetic acid-water system for 9 hours by descending chromatography. The developed chromatograms were taken from the developing tank and air dried. The zone which contained scopoletin and fluoresced a bright blue color under ultraviolet light was cut from the chromatogram and sewed onto a new sheet of the S. & S., No. 589 paper, 19 x 58 cm. The n-butyl alcohol-acetic acid-water system was used for 30 hours during this second chromatographic step. The resulting scopoletin zone (R_f of approximately 0.82) was cut from this chromatogram and sewed onto still another sheet of the S. & S., No. 589 paper. The third chromatographic run involved the use of

15% acetic acid-water as the developing solvent. The resulting scopoletin zone (R_f of about 0.47) was cut, sewed onto yet another new sheet of the chromatographic paper, and the n-butyl alcohol-acetic acid-water system was used for this fourth chromatographic step. After this extended chromatographic separation, the scopoletin zone appeared to be completely free of other compounds. It was, therefore, eluted off the paper with methyl alcohol in an elution chamber. This eluted scopoletin co-chromatographed with authentic scopoletin in the solvent systems already described.

CHAPTER VII

SUMMARY

Blue-fluorescent compounds present in tobacco have previously received relatively little attention. Many of them are phenolic in structure (possibly precursors of flavonoid compounds), and others are coumarins. To obtain fundamental, new information on these compounds, studies were undertaken involving the isolation, characterization, and identification of the blue-fluorescent compounds in the tobacco plant.

The existing methods of chromatographic and partition techniques have been applied, modified, and extended in the isolation and purification of the blue-fluorescent compounds. Four specific blue-fluorescent compounds were isolated in pure form from the alcoholic extracts of leaves and flowers of the One-Sucker tobacco plant. Two of them were identified as scopoletin and chlorogenic acid. The third blue-fluorescent compound was found to be a coumarin-glycoside, which upon hydrolysis with dilute mineral acid gave scopoletin, glucose and rhamnose. A tentative structure has been proposed for this glycoside: scopoletin-7-

rhamnoglucoside. The fourth blue-fluorescent compound has been characterized to be a "chlorogenic acid-like" depside by color tests, chromatographic R_f values, and by a saponification reaction. Its identity has, however, not been established. Also, three flavonoid glycosides in addition to isoquercitrin, rutin, and kaempferol-3-rhamnoglucoside were isolated from extract of flowers. The former have been partly characterized.

Tobacco plants grown at the Argonne National Laboratory in an atmosphere of radioactive carbon dioxide were extracted and the alcohol extracts fractionated on a cellulose powder column. The column fractions were purified further by mass paper chromatography to obtain the following radioactive compounds in pure form for planned metabolic studies: scopoletin, scopoletin-7-rhamnoglucoside, chlorogenic acid and the "chlorogenic acid-like" depside.

A method for the quantitative determination of polyphenols in dry tobacco sample was developed. Each pure individual polyphenol obtained after extended paper chromatography was eluted from the final chromatogram and spectrophotometrically measured. This quantitative method was then used to measure the rutin content and scopoletin content in tobacco of 24 brands of cigarettes commonly used in the United States.

Scopoletin has been identified in tobacco of 29

brands of cigarettes commonly used in the United States. By using a smoking machine designed to simulate conditions of human smoking, the mainstream smoke from every cigarette sample tested also was found, for the first time, to contain scopoletin.

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