

1960

# A chromatographic study of the polyphenols in cured tobacco

Marvin Dennis Edmonds

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A CHROMATOGRAPHIC STUDY OF THE  
POLYPHENOLS  
IN CURED TOBACCO

By

MARVIN DENNIS EDMONDS

A THESIS  
SUBMITTED TO THE GRADUATE FACULTY  
OF THE UNIVERSITY OF RICHMOND  
IN CANDIDACY  
FOR THE DEGREE OF  
MASTER OF SCIENCE IN CHEMISTRY

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## DEDICATION

This thesis is dedicated to Dr. Lee S. Harrow, whose personal interest and encouragement have enabled me to complete this work.

## ACKNOWLEDGMENT

On completion of this project I wish to express my sincerest thanks to Dr. W. Allan Powell. His personal interest and guidance have made the solution of the problem possible.

I am deeply indebted to Dr. Robert B. Seligman for his assistance and many valuable suggestions, especially his interest in teaching me the fundamentals of chromatography.

I am grateful to Dr. H. Wakeham, Director of Research, at Philip Morris Incorporated, for his consent to allow me to present this work at the Virginia Academy of Science and to publish it at a later date.

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## INTRODUCTION

The object of this project was to isolate, separate, and identify the polyphenols in tobacco. Following this, the aim was to develop a method for the quantitative determination of the major polyphenols in tobacco.

Existing methods for the determination of polyphenols in tobacco involve lengthy procedures which are concerned with one or two of the polyphenols rather than the complete class of compounds. The methods consist of exhaustive extraction procedures, lengthy chromatographic procedures, and numerous methods of identification.

Extensive research on the determination of polyphenols by chromatography has been carried out in the field of tobacco and in other fields. It was the desire of the author to apply a combination of chromatography and spectroscopy to the determination of polyphenols in tobacco.

## HISTORICAL

Polyphenolic substances have been known to occur in tobacco for over 50 years. In 1884, Savery (77) detected an unidentified substance which he called "tobaccotannic acid" and which might have been chlorogenic acid. Later (1909), in a distribution survey of this caffeotannin in plants, Gorter (28) found that Nicotiana tabacum contained chlorogenic acid, although some doubt of the specificity of the color reaction employed was subsequently cited. In 1900, Loew (44) found that when the cells of the tobacco leaf die, the polyphenols produce an oxidation product which acquires a red-brown color and which is mainly responsible for the color changes taking place in the leaf during curing. It is known that the enzyme, polyphenolase, which occurs in green tobacco is able to oxidize certain polyphenols; this was shown by Mason (46) in 1955. Neuberg and Kobel (58-60) in

1935 and in 1936, suggested that the development of brown pigment in the tobacco leaf during curing is due to the enzymic oxidation of rutin. Frankenburg (21), in 1950, showed that similar reactions between oxidized polyphenols and amino acids occur during the curing process.

The importance of polyphenols in influencing tobacco quality was shown by Shmuk and Semenova (82), in 1927. They concluded that polyphenol and carbohydrate fractions contribute to color and quality of tobacco. Later, Koenig and Dorr (41), in 1933, isolated chlorogenic acid and concluded that it is responsible for the aroma in tobacco. In 1950, Frankenburg (21) concluded that polyphenols are important because they contribute towards the flavor of tobacco during smoking. Bruckner (7), in 1936, concluded from his studies that the amount of the polyphenols present in tobacco is proportional to the quality of the tobacco. In 1931, Shmuk (80) proposed a polyphenol index on phenol coefficient for use in the chemical evaluation of tobacco qualities. In 1954, Wilkinson, Phillips, and Bacot (94) showed that the chlorogenic acid content of flue-cured tobacco is directly related to quality as shown by U. S. D. A. grades.

The importance of fluorescent compounds in determining the quality of tobacco leaf was shown by Johanson (38), in 1953. He found that normal tobacco leaves, cured or uncured, fluoresced with a brilliant bluish white, tinged with green when exposed to ultraviolet

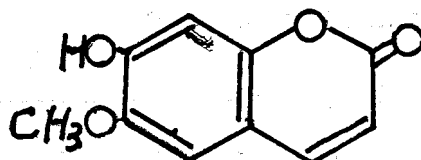


radiation. On the other hand, fully trashy leaves, whether cured or uncured, remained dull showing only a reddish brown color. Johanson (39), in 1951, showed that the fluorescence of tobacco leaf is the resultant of four major components, one of which was shown to be scopoletin. The amount of fluorescent material present was found to decrease with increasing trashiness and there were indications that the relative amounts of the four components change as the fluorescence diminished.

Although marked variations in composition among different types of tobacco exist, the major polyphenolic and related substances of pertinent Nicotiana species have been isolated and identified. Among these substances are coumarins, caffetannins, flavonoid, anthocyanins, and hydroxylated cycloalkanes occurring as free aglycones or combined with sugars as glycosides. The presence of simply hydroxylated benzene derivatives has been claimed, but few of these compounds have been identified. An array of minor polyphenolic substances have been detected, but their conclusive identification has been lacking in most instances (92).

#### Coumarins

To date, scopoletin (6-methoxy-7-hydroxy-coumarin) is the only coumarin which has been isolated from tobacco.



Scopoletin

In 1944, Best (5) isolated scopoletin from tobacco plants which had been infected with tomato spotted wilt virus; the same substance was isolated by Mizukami (53), in 1951, from tobacco roots. Tryon (88) reported that scopoletin was present in cultured tobacco tissue. Akaike (1), in 1955, isolated scopoletin from an ethanol extract of tobacco leaf. More recently, 1956 a and b, Reid (71, 72) established the presence of free scopoletin, scopolin, and unknown scopoletin-like substances in flue-cured South Carolina tobacco.

According to available data, it appears that free scopoletin, a glycoside of scopoletin (scopolin), and at least three unidentified coumarin-like substances are present as minor polyphenolic substances in flue-cured tobacco.

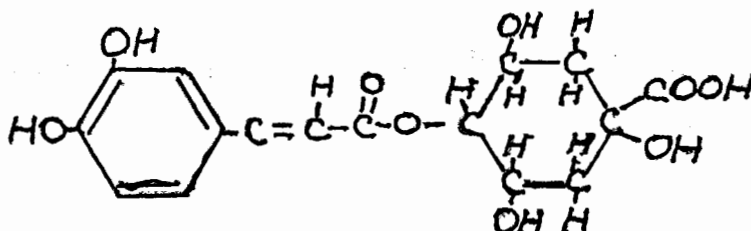
Reid (73), in 1958, showed that scopoletin is directly derived from phenylalanine or a  $C_6 - C_3$  unit produced from it. Findings confirm that phenylalanine is directly incorporated into caffeic acid. Both scopoletin and caffeic acid may derive from a common  $C_6 - C_3$

unit. The studies confirm that acetate is not utilized in either case, and that phenylacetic acid is a poor precursor. Yang *et al* (98), in 1958, presented a quantitative chromatographic procedure for determining scopoletin in cigarette smoke and in tobacco. Later, in 1958, Yang (99) isolated four specific blue-fluorescent compounds from an alcoholic extract of tobacco leaves. Two of these were identified as scopoletin and chlorogenic acid. The third was a coumarin glycoside, scopoletin-7-rhamnoglucoside, and the fourth was not identified.

Dieterman *et al* (19), in 1959, isolated esculetin from an extract of tobacco; esculetin has similar properties to those of scopoletin and it is very difficult to resolve the two compounds. Separation was accomplished by paper chromatography using a solvent system of nitro-methane-benzene-water (2:3:5 v/v).

#### Tannins and Their Hydrolytic Products

From the variety of tannins found in nature, only certain caffetannins have been isolated from tobacco. In all but one instance, the isolated substances have been shown to be chlorogenic acid, its isomers, and its hydrolytic products, caffeic and quinic acids.



Chlorogenic Acid (Caffeoylquinic Acid)

In 1930, Shmuk (80) and Shmuk and Piatnichl (81) isolated a depside of caffeic and quinic acid from Samsun tobacco which they presumed to be chlorogenic acid. Later, Koenig and Dorr (41) isolated free caffeic and quinic acids from a hydrolysis mixture and indicated that the acids were hydrolytic products from combinations of chlorogenic acid and terpene compounds. In 1951, Roberts and Wood (76) isolated three substances (positive Hoepfner test) from extracts of Pennsylvania Seed Filler tobacco by paper chromatography. One of these substances was identified as chlorogenic acid; the other two gave an atypical color reaction and were assumed to be isomers of chlorogenic acid. This same type of investigation was carried out on a South African tobacco by Pearse and Norvellie (65), in 1953; this work duplicated that of Roberts and Wood, and thus, the identity of chlorogenic acid was confirmed. In 1955, Phillips (68) developed a new isolation method for obtaining chlorogenic acid after the procedure of Koenig and Dorr was found unsatisfactory. Dawson and Wada (18), in 1957, studied the flavonoid and depside of shade-grown and sun-grown green cigar tobacco, and reported the qualitative and quantitative differences; chlorogenic acid was not isolated in their work.

It should be emphasized that at least three isomers of chloro-

genic acid are known. The most common isomer, chlorogenic acid, has a depside linkage at the 3-hydroxyl group of quinic acid which is cis to the carboxyl. Iso-chlorogenic acid has the linkage at the 5-position and is trans; this isomer has not been crystallized - Barnes et al (3). Neochlorogenic acid has been crystallized, but the position of the caffeoyl linkage is not known (Corse) (13). Isolation and identification studies by Williams (95), in 1955, and by Roberts (75), in 1956, showed that chlorogenic acid undergoes inversion on paper chromatograms and that lactonization or hydrogen bonding occurs during the analyses for chlorogenic acid.

Reid (72), in 1957, found chlorogenic acid and many related substances in both the lead acetate-precipitated material (major fraction) and in the supernatant of the extract after precipitation (minor fraction). He felt that some of these substances represented the products of the inversion of chlorogenic acid and its isomers which occur during a chromatographic separation. Mikhailou (47), in 1956, obtained the same type of results for his work with chlorogenic as those of Reid in his work on fermented Bulgarian tobacco; he felt that he obtained geometric isomers of chlorogenic acid.

Giovannozzi - Sermanni G. (27), in 1958, showed that the tobacco with the highest chlorogenic acid and caffeic acid contents

was their Italian "Bright." They also demonstrated that topping increases the caffeic acid content while chlorogenic acid remains unchanged. The hydrolyzing activity characterizing the curing processes also acts on chlorogenic acid, while caffeic acid is not subject to variations. In 1957, Weaving (91) found that rutin and related compounds interfere with the alkaline hypiodate determination of chlorogenic acid. Later, in 1958, Weaving (92) detected chlorogenic acid, neo-chlorogenic, rutin, scopoletin, and scopolin by two-dimensional paper chromatography. In air-cured and fire-cured tobaccos it was found that most of the major polyphenols except scopoletin and scopolin had been destroyed and there was an increase in the amount of a dark brown material.

Frey-Wyssling and Babler (23), in 1957, showed that tobacco, cultivated under glass, does not produce any rutin and only about a seventh of the normal amount of chlorogenic acid. Ultraviolet treatment increases the formation of chlorogenic acid up to the normal level, while the synthesis of rutin cannot be stimulated. In 1957, Wada et al (90) worked on the enzymic oxidation of chlorogenic acid alone and in the presence of nornicotine, nicotine, glycine, and proline under the influence of tobacco polyphenoloxidase. Nornicotine leads to the formation of a characteristic red color during the reaction, whereas nicotine and the two amino acids do not substantially influence

the color changes as observed with chlorogenic acid alone. Caffeic acid produced a red color on enzymic oxidation only in the presence of nornicotine. The chemical nature of the red pigment formed is still obscure.

In 1958, Nagasawa and Taguchi (55), studies of chlorogenic acid in the leaf during flue-curing showed that the acid increases about nine times as much as that of its content in the fresh leaf.

Stedman and Rusanivskyj (85), in 1958, observed altered  $R_F$  values for chlorogenic acid on 5" x 5.5" papers versus 18.25" x 22.50". This behavior is due to the presence of inorganic salts.

Sisler and Evans (83), in 1958, compared chlorogenic acid and catechol as substrates for the polyphenol oxidase from tobacco and mushroom. Evidence obtained from purification experiments indicate both mushroom and tobacco contain more than one enzyme capable of catalyzing the oxidation of polyphenolic compounds.

Zagoruiko and Asmaw (100), in 1956, showed that polyphenoloxidase activity is decreased most during sun curing due to enzyme decomposition as a direct effect of sun action, and in cellar curing due to inactivation of oxidation and accumulation of oxidation products.

Curing at higher temperatures usually causes inactivation or even destruction of the enzyme, which makes the tobacco incapable of fermentation. Martin (45), in 1958, made a comparative study of the

activity of polyphenol oxidase in healthy and virus-inoculated tobacco. He concluded that there was an accumulation of phenolic compounds in the infected plant.

Jean and Reid (37), in 1959, showed that chlorogenic acid plus three isomers were present in green leaves of tobacco. In 1959, Kataoka (40), showed a relationship between the degradation (by autoxidation or separation of its aqueous solution) products of chlorogenic acid and the browning process; over a period of one year, 48.6% of chlorogenic acid was involved in the browning process.

Yamada and Akaike (96), in 1959, isolated chlorogenic acid from fresh and flue-cured tobacco leaves. Also, they showed that flue-curing methods cause a decrease in the chlorogenic acid content of tobacco.

Mikhailou (49), in 1958, showed that tobacco polyphenol oxidase does not attack rutin, isoquercetin, or quercetin, but caffeic acid and chlorogenic acid are attacked and yield dark products. The unreactive polyphenols were attacked by  $H_2O_2$  and peroxidase preparation and gave dark products. Polyphenols extracted with ether from fresh tobacco do not undergo oxidative changes in the process.

In 1958, Zucker's (101) work showed that the oxidation of chlorogenic acid by polyphenol oxidase or peroxidase is in large part responsible for the formation of the brown color of the cured leaf.



This may involve a number of other leaf components not substrates for the enzymes themselves and produce major changes in the composition of the leaf. Chlorogenic acid has been implicated as a respiratory pigment in green leaves and as an inhibitor of indole acetic acid oxidase and oxidative phosphorylation.

Izard (36), in 1958, identified chlorogenic acid, rutin, and scopoletin in N. glauca. A polyphenolase showing high activity toward chlorogenic and caffeic acids was isolated.

Penn (66), in 1958, found that the major polyphenols present in fresh leaves of four flue-cured varieties were qualitatively similar (chlorogenic acid, scopolin, rutin, and scopoletin). Polyphenolic constituents were found to increase with maturity. Only minor contrasts in the polyphenols were noted between flue-cured and burley when both were cultured and cured alike. Similarly, air-cured leaves of either type were lower in polyphenols than their flue-cured counterparts.

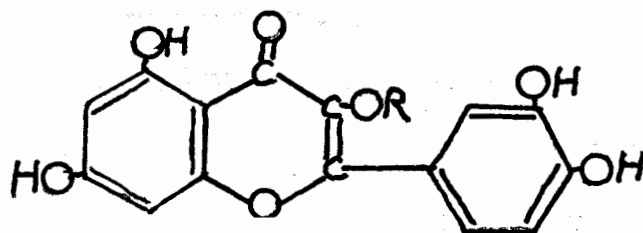
Clayton (10), 1959, observed oxidations of catechol, caffeic acid, and chlorogenic acid when catalyzed by polyphenol oxidase from tobacco leaf. A caffeic-chlorogenic acid specific oxidase was isolated from leaf extracts; this oxidase is strongly inhibited by catechol. Tobacco leaf polyphenol oxidase is at least two enzymes. Reid (74), in 1959, showed that two of the polyphenolase oxidation products of

chlorogenic acid occur in flue-cured tobacco. An enzyme preparation from Pectinol 100D hydrolyzed rutin, scopolin, and chlorogenic acid.

The presence of free caffeic acid in tobacco has been demonstrated, but the possible occurrence of free quinic acid has received less attention. Palmer (64), in 1956, identified free quinic acid in mature, fresh cigar leaves, and demonstrated that this compound was lost when the tobacco leaves were dried at 80°C. Failures to detect free quinic acid in various types of tobacco could have been caused by methods of laboratory analyses and by methods of curing the tobaccos. Nagasawa and Taguchi (56), 1958, reported the content of quinic acid in flue-cured tobacco as 0.23% of dry matter.

#### Flavonoids

Rutin - Several flavonoids have been isolated from various positions of the tobacco plant, such as root, stalk, leaves, flowers, etc. Glycosides and aglycones belonging to the flavonol (3-hydroxyflavone) type of structure have been detected. The most commonly occurring flavonoid in tobacco is rutin (quercetin 3-rhamnosidoglucoside; quercetin 3-rutinoside; 3-rutinosido-3', 4', 5, 7-tetrahydroxyflavone).



Rutin (R = rutinose (L-rhamnoside-D-glucose))

Charaux (8), 1924, isolated rutin from the flowers of tobacco, and, in 1931, Hasegawa (32) extracted rutin from tobacco leaves, using the lead salt precipitation method. Neuberg and Kobel (58), in 1953, duplicated the work of Hasegawa, with some minor analytical changes, and showed that the rutin content of Zichna cigarette tobacco decreased during the curing process. Later, in 1936, these same workers found that the decrease in rutin resulted from an enzyme-catalyzed oxidative process in which the rutin was transformed into slightly soluble brown pigments.

Griffith et al (29), in 1944, reported the value of rutin as a medicinal product (strengthen of capillarity associated with hypertension). Following this report, a series of articles appeared concerning the isolation of rutin for pharmaceutical purposes. Couch and Krewson (14), in 1944, reported the most effective isolation procedures for rutin, and Badgett et al (2), in 1949, determined the rutin content of several varieties of Nicotiana rustica and N. glauca. They found

that the rutin content fluctuated during the growth of Nicotiana rustica and that N. glauca contained levels of rutin up to 2% (moisture free basis). The quantity of rutin in tobacco was found to vary considerably with leaf quality and age, ranging from 0.008 - 0.61 per cent with an average of 0.4 per cent for good quality tobacco. It was observed that the decrease in rutin occurred during air curing, but not during flue-curing (2); inferring that the behavior may be a reflection of heat inactivation of oxidative enzymes.

Dussy (20), in 1947, analyzed ten species and varieties of Nicotiana for rutin; he concluded that N. tabacum was a hybrid species. In 1951, Nio and Wada (61) duplicated Charaux' work by isolating rutin from the flowers of a tobacco type designated "Bright Yellow."

Roberts and Wood (76), in 1951 reported the presence of rutin in fresh dried cigar (Pennsylvania Seed Filler) and Indian (Harrison Special) tobacco. The presence of rutin as well as chlorogenic and caffeic acids in Japanese cigarette tobacco was demonstrated, in 1955, by Skirova et al.

Reid (69, 70), in 1956 a and b, identified rutin in South Carolina flue-cured tobacco. In the same year, Mikhailov (47), reported that rutin is one of the important polyphenols in Bulgarian tobacco.

According to the aforementioned data on rutin, it is clear that

this flavonoid is one of the major polyphenols in tobacco, occurring in the flowers as well as the leaves of the tobacco plant and contributing, in some instances, to the brown coloration which occurs during air-curing. The role of rutin is apparently that of a substrate for enzymatic oxidative processes. The percentage of rutin found in tobacco fluctuates with species, variety, leaf position, age, handling (curing), and experimental methods used for analyses (85).

Ferguson and Weaving (22), in 1959, showed that the rutin content of tobacco grown in a greenhouse was 0.23 to 0.43%; whereas, that from field-grown flue-cured tobacco was 1.0 to 1.7%.

Mikhailou (52), in 1958, showed the presence of kaempferol (structurally related to the base structure of rutin-querceetin) in co. The substance is originally present as a glucoside.

Isoquercitrin. Isoquercitrin is a glucoside of quercetrin (3, 3', 4', 5, 7-pentahydroxyflavone) having the sugar substituent at the same position as rutin.

Kurilo (43), in 1935, isolated a glycoside from tobacco which upon hydrolysis gave quercetin and D-glucose. Two years later, in 1937, Kourilo (42) isolated and identified isoquercitrin from unfermented Tyk-Koulak tobacco. The flavonoid concentration varied from 0.25 - 1.7 per cent; the D-glucose was characterized by the melting point of the methylated derivative.

In 1950, Howard, Gage, and Wender (34) extracted isoquercitrin from low grade Kentucky Burley tobacco. This was the first time a flavonoid was extracted from air-cured tobacco.

Roberts and Wood (76), in 1951, were unable to detect isoquercitrin in cigar filler and Indian tobaccos. Reid (72), in 1956 b, isolated a very minute amount of the flavonoid from flue-cured tobacco; Nio and Wada (61), in 1951, reported that the flowers of tobacco contained traces of isoquercitrin. The flavonoid was detected by Mikhailou (47), in 1956, in four classes of fermented Bulgarian tobacco.

The current data indicate that isoquercitrin is a minor polyphenol in tobacco, and that it is present only in some types of tobacco.

#### Other Identified Flavonoids and Miscellaneous Phenolic Or Related Substances

Kaempferol (3-rhamnosidoglucoside) was isolated and identified from the tobacco plant, in 1952, by Wada. Reid (72), in 1956 b, isolated a glycoside from flue-cured; hydrolysis of the glycoside gave kaempferol, rhamnose, and glucoside. From this, he concluded that it was the same compound which had been reported by Wada. Howard et al, in 1950, reported traces of quercitrin in Burley tobacco.

Miscellaneous polyphenolic and related substances identified to date in tobacco are simple phenolic derivatives, a hydroxylated cyclohexane compound, and one anthocyanin. Naghski et al (57), in

1944, studied the volatile phenols obtained from various fire-cured and light air-cured tobaccos. Unfermented Pennsylvania cigar leaf and Maryland cigarette leaf tobaccos showed a level of 0.01 - 0.04 per cent simple phenols. Frankenburg (21), in 1950, duplicated the results of Naghski.

Fire-cured types, including Latakia, yielded larger but variable amounts of phenols - up to one per cent; they concluded that this was caused by the smoke (deposition of substances on leaf surface) used to fire-cure the tobacco.

Eugenol, phenol, and guaiacol were identified in Virginia tobacco by Oniski and co-workers (62), in 1955 and 1956.

The presence of inositol in tobacco was established originally by Shumk (81), in 1930 a. Phillips and Bacot (69), in 1950, isolated meso-inositol from flue-cured tobacco, U. S. Grade B4GF. Mellitic acid (2-hydroxyhydrocinnamic acid) was cited, in 1952, by Geissman (25) as a constituent of Nicotiana.

#### Unidentified Polyphenolic Substances

The application of chromatographic techniques has shown a wide diversity of polyphenolic composition among a vast array of minor polyphenol substances which are known to exist in tobacco.

Two rather unusual polyphenolic substances have been reported in the earlier literature. Shmuk (80), in 1930 b, isolated a material

from unfermented Tyk-Kulak tobacco which yielded rhamnose on acid hydrolysis; a depside of caffeic and quinic acids was isolated from the substances. Evidently, the fraction was a mixture rather than a single compound. Possibly the rhamnose was derived from rutin or quercitrin; the quercitrin point would be questionable since this has not been isolated from tobacco.

The other unusual polyphenol appearing in the older literature was described by Yamafuji (97), in 1932. The isolated substance, "tobacinin," was a glycoside having certain properties of rutin, but yielding glucuronic acid on hydrolysis.

Schoulties and Wender (78), in 1947, used adsorption techniques to separate the flavonoid substances of tobacco. By using alumina, Celite, and talc, alone and in combinations, four colored pigments were isolated from aqueous extracts of Burley lugs. These pigments were tentatively classified as flavones on the basis of color reactions and absorption spectra by Naff and Wender (54), in 1947.

Dussy (20), in 1947, isolated a glycoside differing from rutin and yielding rhamnose and a yellow, amorphous aglycone on hydrolysis. In 1953, Wegner (93) reported the appearance of four distinct spots on papergrams which were not identical with the chlorogenic isomers and rutin.

The remaining investigations have been more informative, and



represent the beginning of exhaustive attempts to complete the picture of the polyphenols in tobacco.

Roberts and Wood (76), in 1951, isolated seven constituents from extracts of filler by two-dimensional chromatography; two of these were rutin and chlorogenic acid, two were assumed to be isomers of the caffetannin, and the remainder were unidentified.

In 1956, Mikhailou (47) detected eleven flavonoid and caffetannin-like substances in fermented Class II Bulgarian tobacco by paper chromatography. Five of these were flavonols, including rutin and isoquercitrin. Two flavonol glycosides were characterized, but not identified. Neither flavone, isoflavone, nor flavonone derivatives was noted. The other six substances were coumarin-like derivatives.

Reid (71), in 1956, made the most comprehensive study of polyphenols in tobacco. Reid extracted South Carolina flue-cured tobacco with 80% ethanol (aqueous) and precipitated the bulk of the polyphenols as their lead salts with lead acetate. The solvent from the supernatant liquid was evaporated to a residue; the residue was extracted with diethyl ether, ethyl acetate, n-butanol, and acetone, respectively. Each fraction was chromatographed on paper and developed in several solvent systems. The major polyphenolic components were contained in the precipitate; in all, 18 components were detected in this fraction. Rutin, isoquercitrin, kaempferol rhamnoglucoside, four chlorogenic acid-like zones, and four zones closely related to chlorogenic acid

were detected. The remainder of the zones showed a distinct fluorescence. Fifteen substances were detected in the minor fraction (extracts of supernatant); free scopoletin, four spots that might have been glycosides of scopoletin, two spots with a flesh pink fluorescence and unknown structure, one zone which was typical of chlorogenic acid, one zone which resembled p-coumarylquinic acid but which failed to yield the correct hydrolysis product, and five unidentified zones were detected. Anthoxanthin-like compounds were indicated by the pale yellow color of some of the hydrolysis products.

The aforementioned isolation work was extended by Reid (73) in 1958. He prepared an enzymatic fraction having significant polyphenoloxidase activity from the tobacco. Using chlorogenic acid and caffeic acid as substrates, products of the action of this enzyme were identified on chromatograms as being similar to certain of the zones obtained in the isolation studies. The quercetin-type compounds showed very little oxidative activity with the enzyme. The enzymological portion of Reid's work is most important from a standpoint of biosynthetic studies and determination of the precursors or products of compounds.

Penn and Weybrew (67), in 1958, presented some factors affecting the content of the principal polyphenols in tobacco leaves. They concluded that when two types of tobacco are cultured and cured alike only minor differences in the principal polyphenols were noted; this difference was quantitative rather than qualitative.

Spondr (84), in 1957, showed that the polyphenols content of tobacco leaf decreased during fermentation.

Most workers have used various mixtures of ethanol-water for extracting polyphenols from plant materials (18, 22, 34, 47, 71, 72, 73 and 74). The ratios of the ethanol-water mixture were determined by the objectives of the respective workers. In the course of the previous discussion, many workers were looking for one or two polyphenols rather than the entire class of polyphenols. Therefore, from the aforementioned discussion, it is difficult to say that a particular ratio of ethanol-water is the proper one to extract the entire class of polyphenols.

The majority of the workers have employed various combinations of organic solvents for resolving the polyphenols by paper chromatography, and invariably, each worker concluded that the upper layer of a mixture of n-butanol-acetic acid-water (4:1:5) is the ideal solvent mixture.

Again, the developing solvents for paper chromatography were determined by the objectives of the respective workers. For example, if one was attempting to determine an individual polyphenol such as rutin, a solvent system of 2% HOAc would suffice.

In previous studies, workers have used Whatman No. 1 filter paper almost exclusively, and have varied the solvent; whereas, it

probably would have been more advantageous to have varied the type of paper.

## EXPERIMENTAL

### I. Reagents

Ferric Chloride, C. P. or equivalent

Sodium carbonate, C. P. or equivalent

Phosphomolybdic acid, C. P. or equivalent

Aluminum chloride, anhydrous

Acetic acid, glacial, C. P. or equivalent

Acetone, C. P. or equivalent

Ammonia vapors, from C. P. or equivalent ammonium hydroxide

Ammonia, anhydrous, Matheson Co., Inc.

n-Butyl alcohol, C. P. or equivalent

Ethyl alcohol, 95%

Methyl alcohol, C. P. or equivalent

Ethyl ether, anhydrous Mallinckrodt Chemical Works

Petroleum ether, b. p. 30-60° C.

Folin-Ciocalteu Reagent, C. P. or equivalent

Trimethylchlorosilane, Dow Chemical Co.

Magnesol, 2001, Westeraco Chemical Division of Food Machinery  
and Chemical Corp.

## II. Apparatus

Cary Spectrophotometer, Model 14

Chromatocab (62.2 cm x 61 cm x 41 cm)

Chromatographic Chambers (14 cm x 45 cm and 28 cm x 59 cm)

Chromatographic column (2.54 cm x 60 cm)

Coarse sintered glass filter

Glass sprayer or atomizer

Rapid evaporator

Soxhlet extractor (small, medium, and large sizes)

Two-ounce vials-screw cap

Ultraviolet Mineralight, Model SL 2537

Waring Blendor, capacity - 1 quart

Whatman filter paper, Nos. 1 and 2

### III. Development of Method for Extraction and Separation

#### A. Paper Chromatography

Prior to attempts to isolate and determine the polyphenols in tobacco leaves, it was desirable to learn the best methods for separating known polyphenols; especially, the ones which we anticipated as being present in tobacco.

Paper partition chromatography is a micro analytical technique for the separation of closely related compounds in a chemical family. This technique is based on the relative solubilities of compounds in two immiscible solvents, i. e., their partition coefficients.

In paper chromatography, the mixture of substances is placed as a spot on a strip of filter paper (stationary phase); the paper is placed in a sealed chamber with one end of the paper immersed in the solvent (mobile phase). As the advancing mobile solvent reaches the mixture of substances on the paper, the components of the mixture undergo partitioning between the two phases.

The position occupied by a component on paper, unless it is colored, must be located by aid of an appropriate chromogenic reagent. Under properly controlled conditions, the component can be identified by its " $R_F$  value" in the particular solvent system used. The  $R_F$  value is expressed as the ratio of the distance moved by the component to the

distance moved by the advancing front of the solvent. The absolute  $R_F$  values depend considerably on the conditions of the experiment; therefore it is necessary that unknown components be compared with known substances on the same chromatogram.

When some of the components of a complex mixture have very similar partition coefficients, as in the case of amino acids, overlapping of the spots may occur in the one-dimensional paper chromatography. In this case, after components have been partially separated in one direction, the paper can be dried, rotated  $90^\circ$ , and introduced into a different solvent system so that the boundary is made to move at a right angle to the original direction. The second solvent is chosen so that the partition coefficients of those components which overlapped are sufficiently different to permit better separation. This is called "two-dimensional" paper chromatography.

Another method that can be used sometimes in the case of components with very similar partition coefficients is based on the fact that the further the solvent moves, the greater will be the actual distances the individual components of the mixture move. Although it would be impractical to employ sheets of paper of great length to obtain a separation, the same result can be approximated with a comparatively short length of paper by allowing the solvent to run down the paper by gravity and then "overflow" for as long a period as necessary. This



is termed "descending" paper chromatography.

Since the solvent is allowed to overflow the end of the paper,  $R_F$  values cannot be calculated. Instead, the distance each component moves in a given period of time is measured and used as a means of identification; or, the unknown compounds are compared with known compounds on the same chromatogram.

In placing a compound on a paper chromatogram, the distance the spot is placed from the bottom of the paper is dependent on the volatility of the solvent system. The spot in a volatile solvent system is placed a greater distance from the bottom of the paper so that when the paper is inserted in a chamber the system will re-equilibrate before the solvent front reaches the material to be chromatographed.

The chromatographic chambers used in this experimental work were 24 inches high by 12 inches in diameter. The chambers were covered with ground glass plates to allow the solvent system to maintain equilibrium. The polyphenols were spotted on 18.25 inch by 22.50 inch sheets of Whatman Nos. 1 and 2 filter paper, 6 cm. from the bottom of the sheet in approximately 10 microgram quantities. The spots were confined to 0.5 cm. diameter and spaced 4 cm. apart. The chromatograms were developed until the solvent front was near the top of the sheet.

In initiating the work to separate polyphenols on paper chroma-

tograms, a chromatographic chamber was equilibrated by placing the organic phase from a mixture of BuOH-HOAc-H<sub>2</sub>O (40:10:50) in the base of the chamber and by placing the aqueous phase in a beaker inside the chamber. A strip of Whatman No. 1 filter paper was spotted with 10 microliters each of 1% methanolic solutions of caffeic acid, chlorogenic acid, scopoletin, catechin, epicatechin, rutin, kaempferol, quercetin, hydroquinone, resorcinol, phloroglucinol, and gallic acid.

The spotted paper was set in the base of the chromatographic chamber which had been equilibrated, previously. The paper was developed (overnight), and dried under a hood to remove the organic solvent. The paper was checked under an ultraviolet hand lamp for fluorescence, and sprayed with a 1% aqueous phosphomolybdic acid solution and exposed to ammonia vapors. The  $R_F$  values are tabulated below:

<u>Compound</u>	<u><math>R_F</math> Value</u>
Caffeic acid	0.79
Chlorogenic acid	0.54
Scopoletin	0.86
Catechin	0.57
Epicatechin	0.40
Rutin	0.21
Kaempferol	0.48
Quercetin	0.65
Hydroquinone	0.46
Phloroglucinol	0.75
Pyrogallol	0.68
Gallic acid	0.65

<u>Compound</u>	<u>2% Acetic Acid</u>	<u>tert. Amyl Alcohol Acetate buffer</u>	<u>Amyl Alcohol saturated with water</u>
Caffeic acid	0.32 (0.65)	0.47	0.28
Chlorogenic acid	0.60 (0.76)	0.01	0.00
Scopoletin	0.41	0.47	0.57
Catechin	0.41	0.25	0.38
Epicatechin	0.45	0.20	0.13
Rutin	0.35	0.04	0.07
Kaempferol	0.00	0.30	0.15
Quercetin	0.00	0.60	0.46
Hydroquinone	0.34	0.60	0.40
Phloroglucinol	0.58	0.61	0.47
Gallic acid	0.42	0.27	0.14

For quantitative analysis, the BuOH:HAc:H<sub>2</sub>O system is recommended. The 2% HAc system has the disadvantage of producing two zones each for two of the main tobacco polyphenols (caffeic and chlorogenic acids); this is probably due to hydrolysis. The amyl alcohol systems are good for grouping the compounds according to structure, but both systems gave poor resolution between the two major tobacco polyphenols (chlorogenic acid and rutin).

#### B. A Study of Methods of Extraction

Following the work on the establishment of the solvent systems for resolving known polyphenols on paper, it was necessary to isolate the polyphenols from tobacco quantitatively. The paper methods (Section III-A) were used to determine the feasibility of using a particular solvent.

### 1. Soxhlet-type Extraction of Tobacco with 95% Ethanol

To determine whether or not ethanol would remove all of the polyphenols from tobacco, a 40 gram sample of finely ground tobacco was extracted exhaustively with 95% ethanol. The extraction was continued until the liquid in the extraction thimble gave a negative test for polyphenols.

The residual tobacco from the ethanol extraction was extracted further with ethyl ether. Some colored material was removed with the ether, but as a spot test on undeveloped paper, this solution gave a negative test for polyphenols with a phosphomolybdic reagent.

### 2. Column Extraction of Tobacco with 95% Ethanol

To determine whether or not the heat involved in a Soxhlet-type extraction would cause a degradation of the polyphenols, a cold extraction was carried out.

A 10 gram sample of finely ground tobacco was blended with 95% ethanol in a Waring blender. The mixture was removed from the blender and Celite was added to form a slurry; the slurry was mixed thoroughly and poured into a column. The column was developed continuously with 95% ethanol until the eluate showed a negative spot test for polyphenols. Approximately three liters of ethanol were required to extract the polyphenols from the column. The volume of eluate was concentrated (reduced pressure) to 250 ml; this was done

to convert the 10 gram sample (10 g/250 ml) to the same weight ratio as for the corresponding hot extraction (40 g/1000 ml).

The residual tobacco-Celite slurry from the column was extracted further by the Soxhlet-type method with one liter of 95% ethanol. This gave a negative test for polyphenols, but upon concentrating the volume to 250 ml, the solution gave a positive test for polyphenols.

Paper chromatographic analyses show that the Soxhlet-type extraction (hot) removes the polyphenols quantitatively with less solvent than that which was required for the non-quantitative elution of the phenolic material from the tobacco-Celite column.

The paper work showed that there was a phenolic compound present in the hot extract which was absent in the cold extract. It was felt that the additional zone represented a degradation product of chlorogenic acid. To prove this point, a 100 ml aliquot of the cold extract was subjected to a hot-type extraction. Chromatographic analysis of the final extract showed that the additional zone which was obtained from the original hot extract was present.

### 3. Petroleum Ether Extraction of Tobacco

To determine if petroleum ether removed any of the phenolic material from tobacco, a residue obtained from a petroleum ether extract of tobacco was examined chromatographically.

The residue was dissolved in methanol and chromatographed two-dimensionally in a system of 2% HOAc. The compound and/or compounds were immobile in both directions. The immobile material was yellow in visible light and gave a blue color with phosphomolybdic acid plus ammonia vapors. This was repeated and the paper was tested by spraying with a solution of 0.1% FeCl<sub>3</sub> in water; a negative test for polyphenols was obtained.

The petroleum ether extract was chromatographed in n-BuOH;HOAc:H<sub>2</sub>O as the first direction, and in 2% HOAc as the second direction. In the first direction, the material migrated to an R<sub>F</sub> value of 0.94; this was yellow in visible light and possessed a pale yellow-like fluorescence. The material was immobile in the second direction. The zone had a blue-green coloration with phosphomolybdic acid and ammonia vapors; these are not the typical colors for polyphenols with this reagent.

Apparently, petroleum ether does not remove a measurable amount of polyphenols from ground tobacco.

#### 4. Sixty-three Per cent Ethanol Extract of Residual Tobacco

Since 63% ethanol was recommended by Dr W. W. Reid (72) as being a better solvent for all types of polyphenols than 95% ethanol, and since petroleum ether does not remove any of the phenolic material from tobacco, a 40 gram sample of dry residual tobacco (previously

extracted exhaustively with petroleum ether) was extracted exhaustively (Soxhlet) with 63% ethanol. The 63% ethanol was prepared by adding 60 ml. of 95% ethanol to 35% of water.

The extraction required slightly less than a liter of 63% ethanol; this was diluted to one liter with the extracting solvent. The extract was darker in color than the 95% ethanol extract mentioned earlier; this was due to the increased water content of the extracting solvent which removed the majority of the water soluble compounds.

A 100 ml aliquot of the 63% ethanol extract was reduced to a 10 ml volume to concentrate the minor components. A 15 microliter application of the 10 ml volume was chromatographed in BuOH:HOAc:H<sub>2</sub>O (overnight) ascending. The paper was dried and developed in 2% HOAc (descending). The extract contained 24 zones as was shown by a combination of fluorescence and phosphomolybdic acid plus ammonia vapors.

The aforementioned chromatographic work was repeated in which the first solvent ratio (40:10:50) was altered to a ratio of (60:10:20); the second solvent remained the same.

The papergram contained 13 zones. The n-BuOH-HOAc-H<sub>2</sub>O(6:1:2) system is good for a gross separation, but it does not reveal the minor components.

The n-BuOH-HOAc-H<sub>2</sub>O(40:10:50) system is quite good for resolving both minor and major components.

Although a Soxhlet-type extraction requires a smaller volume of solvent to extract the polyphenols quantitatively than does a column-type extraction, the heat involved in the hot extraction tends to degrade chlorogenic acid.

Petroleum ether does not remove a measurable amount of polyphenols from ground tobacco; therefore, this solvent can be used to remove some of the non-polar compounds from tobacco, prior to a final extraction with a mixture of ethanol-water.

### C. A Study of Additional Methods for Separation

#### 1. Column Chromatography of 63% Ethanol Extract

An attempt was made to resolve the phenolic content of the 63% ethanol extract on a column of Magnesol (magnesium silicate).

Magnesol was mixed with acetone to form a slurry; this was poured into a glass column and packed tightly with the aid of nitrogen pressure. The column was washed successively with acetone and ethyl acetate.

The elutriant for the column consisted of the organic phase of ethyl acetate-water; three per cent glacial acetic acid was incorporated in the organic phase.

A portion of the 63% ethanol extract of tobacco was evaporated to dryness (room temperature); the residue was dissolved in ethyl



acetate; the solution was applied to the Magnesol column. The solution was allowed to migrate down to the top of the column. The elutriant was added to the column and almost immediately there were three unresolved fluorescent bands just below the top of the column. The lower band was quite mobile with ethyl acetate and it moved rapidly down the column.

The first eluate from the column (non-fluorescent) was yellow in color; the second eluate was less colored than the first. The third eluate contained the lower portion of the first fluorescent band; eluate number four contained the main portion of the first fluorescent band, and the fifth eluate contained the remainder of the fluorescent band.

Eluate number six was an in-between cut; whereas, eluate seven and eight contained the second fluorescent band.

The top fluorescent band remained stationary during the collection of the first eight eluates. At this point, the band appeared to be moving quite slowly, but the fluorescence faded out; two additional cuts were taken (numbers nine and ten) with the acidic ethyl acetate solution.

Since there was no indication of the presence of rutin and of chlorogenic acid on preliminary papergrams, acetone was applied to elute the rutin. The acetone removed some of the yellow material from the top of the column. Petroleum ether was used on the column to desorb the porphyrin-type compounds, but this did not remove all of the colored material from the column. The 63% EtOH was used as the final solvent; this removed all of the visual colored material.

The fractions obtained from the Magnesol column were spotted on paper and examined by two-dimensional chromatography. The paper was developed in the first direction in BuOH-HoAc-H<sub>2</sub>O (40-10-50); the development in the second direction (descending) was in 2% HOAc.

Fraction number one was resolved into two zones ( $R_F$  0.71 and 0.90) in the first direction; the two resolved zones were immobile in the second direction. Both zones were indicative of quercetin or quercetin-type compounds.

Fraction number two showed two zones in the first direction, and this split into six zones in the second direction, three of which fluoresced and three of which stained blue with phosphomolybdic acid plus ammonia vapor, but did not fluoresce. Two of the zones were similar to caffeic acid.

Fractions three and four indicated the presence of scopoletin. This was, also, true when number four was chromatographed two-dimensionally in 2% HOAc.

Fraction number five was resolved into two zones in the first direction; the zone at  $R_F$  0.79 had a blue fluorescence and this was typical of scopoletin in the second direction. The zone at  $R_F$  0.90 had a rose colored fluorescence, but this was immobile in the second direction; again this zone was indicative of quercetin or a quercetin-type compound.

Fraction number six contained five unresolved zones; their fluorescence varied from blue to skin-pink and a combination of the two colors. All but one of these zones were immobile in 2% HOAc.

Fraction number seven contained five resolved zones in the first direction ( $R_F$  0.04, 0.12, 0.24, 0.74, and 0.92). There were three zones in the second direction; the zone at  $R_F$  0.04 changed to 0.92; the zone at 0.24 changed to 0.85, and the zone at 0.74 migrated to 0.09.

Fraction number eight contained one zone ( $R_F$  0.91) in the first direction; this changed to 0.94 in the second direction.

Fraction number nine showed one zone ( $R_F$  0.91) in the first direction; this migrated to 0.03 in the second direction.

Fraction number ten indicated one zone ( $R_F$  0.80) in the first direction, and one zone (0.1) in the second direction.

Fractions eleven and twelve showed the same results as for fraction number ten.

The aforementioned results indicate that the Magnesol column has two disadvantages; the individual components are not eluted quantitatively, and some of the compounds are changed on the column.

## 2. Column Chromatography of 2% HOAc Immobile Material

The 63% ethanol extract of tobacco was streaked on a large

number of filter sheets to obtain a workable size sample of the immobile material. Since the immobile material consists of a flavonol glycone (quercetin), and other unidentified material, such as flavone aglycones, flavanone aglycones, and flavone aglycones, it was felt that this would be a good source for these compounds.

The immobile material was eluted from the paper with methanol; this volume was reduced to a residue and the residue was dissolved in ethyl acetate for column chromatography.

The magnesol column and the elutriant were prepared as mentioned earlier; also, the sample was placed on the column as was shown earlier.

Since the immobile material had a skin-colored fluorescence on paper, it could not be followed on the column by either visual or fluorescent techniques. Nine cuts were taken from the column, each cut contained a visual yellowish color, except cut number nine which was water-clear. There was some colored material left at the top of the column. At this point, it seemed advantageous to wash the column with solvents ranging from non-polar to polar. Benzene was added to the column; two cuts were taken, the second cut being water-clear. The column was washed successively with chloroform, acetone, methanol, 63% ethanol, and water; one cut of each solvent was obtained.

All of the fractions were evaporated to dryness (room temperature) and each was dissolved in methanol for paper chromatography and

spectroscopic examination. Both methods of analyses showed that the fractions were mixtures. Magnesol columns cannot be used in quantitative analyses of polyphenols; this is because the polyphenols undergo degradation on the columns, and the polyphenols are not eluted quantitatively from the columns.

Since the polyphenols have a tendency to break up during column chromatography, an attempt was made to identify them by one-dimensional paper chromatography. The 63% ethanol extract of tobacco was chromatographed in a solvent system of BuOH-HOAc-H<sub>2</sub>O. Ten individual zones were detected and eluted from the papergrams. According to instrumental (ultraviolet) and paper chromatographic analyses, the extract contained fructose, nicotine, seven amino acids, rutin, two forms of chlorogenic acid, quercetin, scopoletin, and kaempferol.

### 3. Countercurrent Distribution (CCD)

To learn the techniques of CCD, a standard sample (Vitamin B<sub>12</sub>) was carried through a separational procedure using a micro Craig apparatus.

#### CCD Separation (Micro Scale) of 63% EtOH Extract of Tobacco

An unmeasured volume of a 63% ethanol extract of tobacco (aged) was carried through a separational procedure using a micro Craig apparatus. The extract was partitioned between petroleum ether and 90% methanol (aq).

The liquid in the zero tube was siphoned out; the extract was added and the contents were shaken thoroughly. The emulsion which formed between the two layers was removed by filtration; the two layers were re-injected into the zero tube.

The separation was carried through 24 tubes; tubes 0 through 6 contained a light yellow color; tubes 6 through 17 were water-clear, and tubes 18 through 24 contained gradient colors (yellow to red-orange).

The contents of each tube were evaporated in tared beakers, and the residual materials were weighed; only tubes 23 and 24 contained enough material to be weighed. A weight distribution of the sample was not obtained because a very small sample was used.

Paper chromatography (Whatman No. 1 paper - 2% HOAc) of the contents of tubes 22 through 24 showed six fluorescent zones. Three of the six zones displayed paper mobilities equal to those for rutin, chlorogenic acid, and caffeic acid; the remainder of the zones were not identified. Tubes 0 through 5 contained pigments, such as carotenoids.

The aforementioned work showed that the pigments were separated from more polar compounds, such as polyphenols. This work demonstrated the necessity for using the large Craig apparatus.

CCD Separation (Macro Scale) of 63% EtOH Extract of Tobacco

A measured volume (25 ml = 27.2555 g.) of a 63% ethanol

extract of tobacco was partitioned between petroleum ether and 90% methanol (macro scale).

The separation was carried through 22 tubes; the weight extracted into each tube and the per cent of total weight in each tube are tabulated below:

Tabulation of Weight vs. Tube for CCD

<u>Tube No.</u>	<u>Weight (gms.)</u>	<u>% Weight</u>
0	14.7938	54.20
1	0.6297	2.30
2	0.0658	0.24
3	0.0360	0.13
4	0.0250	0.09
5	0.0214	0.07
6	0.0199	0.07
7	0.0199	0.07
8	0.0240	0.08
9	0.0292	0.11
10	0.0365	0.13
11	0.0400	0.14
12	0.0337	0.12
13	0.0310	0.11
14	0.0368	0.13
15	0.0371	0.13
16	0.0403	0.14
17	0.0458	0.16
18	0.0563	0.23
19	0.0867	0.31
20	0.1437	0.52
21	0.2355	0.86
22	0.0767	0.28

Paper chromatographic analyses of the contents from each tube revealed that the 63% ethanol extract was separated into three main classes of compounds -- polar (phenolic, sugars, amino acids, etc.)

compounds, unsaturated hydrocarbons, and pigments plus saturated hydrocarbons. The phenolic material was confined to tubes #0 and a very minute amount was in tube #1.

### Countercurrent Distribution of Tube 0

The material from tube #0 was partitioned between BuOH-HOAc-H<sub>2</sub>O (40-10-50); the separation was carried through 24 tubes. The solvent from each tube was separated into top and bottom phases, and checked by the schemes shown below:

#### 1. Qualitative Paper Chromatography

The solutions were spotted on Whatman #1 filter paper (undeveloped) and each paper was tested for the presence of alkaloids, sugars, amino acids, and polyphenols. The alkaloids reached an equilibrium state (top  $\rightleftharpoons$  bottom phases) in tubes 0-12. The sugars reached an equilibrium state (top  $\rightleftharpoons$  bottom phases) in tubes 0-7. The amino acids were distributed throughout the phases with the greatest concentration in tubes 0-10. The phenolic material was distributed through tubes 10-24.

#### 2. Tube Number vs. Weight

The top and bottom phase of each tube were combined; the solvent was evaporated (steam bath), and the residual material from each tube was weighed.



The following tabulation shows the breakdown of tube #0 by weight with n-BuOH-HoAc-H<sub>2</sub>O:

Breakdown of Tube #0 by Weight

<u>Tube No.</u>	<u>Weight(grams)</u>
0	0.8260
1	1.2948
2	1.4897
3	1.2257
4	0.9724
5	0.7456
6	0.4798
7	0.4133
8	0.3357
9	0.3551
10	0.3575
11	0.3842
12	0.3642
13	0.3832
14	0.4097
15	0.3941
16	0.3894
17	0.3674
18	0.3511
19	0.3697
20	0.3387
21	0.3179
22	0.3388
23	0.3552
24	0.3431

3. Qualitative Ultraviolet Analyses

Each of the solutions (tubes 0 - 24) were checked by ultraviolet analyses in neutral, acidic, and basic media. Tubes 0 - 7 produced normal curves for nicotine-type alkaloids. The spectra for

tubes 8 - 9 contained an unidentified peak at 285 millimicrons. Tubes 10 - 24 produced spectra which were typical of phenolic material.

4. Paper Chromatography of Tubes 10 - 24

The solutions ( tubes 10 - 24) were spotted quantitatively (50 microliters each) on Whatman No. 1 filter paper and developed in BuOH-HOAc-H<sub>2</sub>O in the first direction followed by development in 2% HOAc or the second direction.

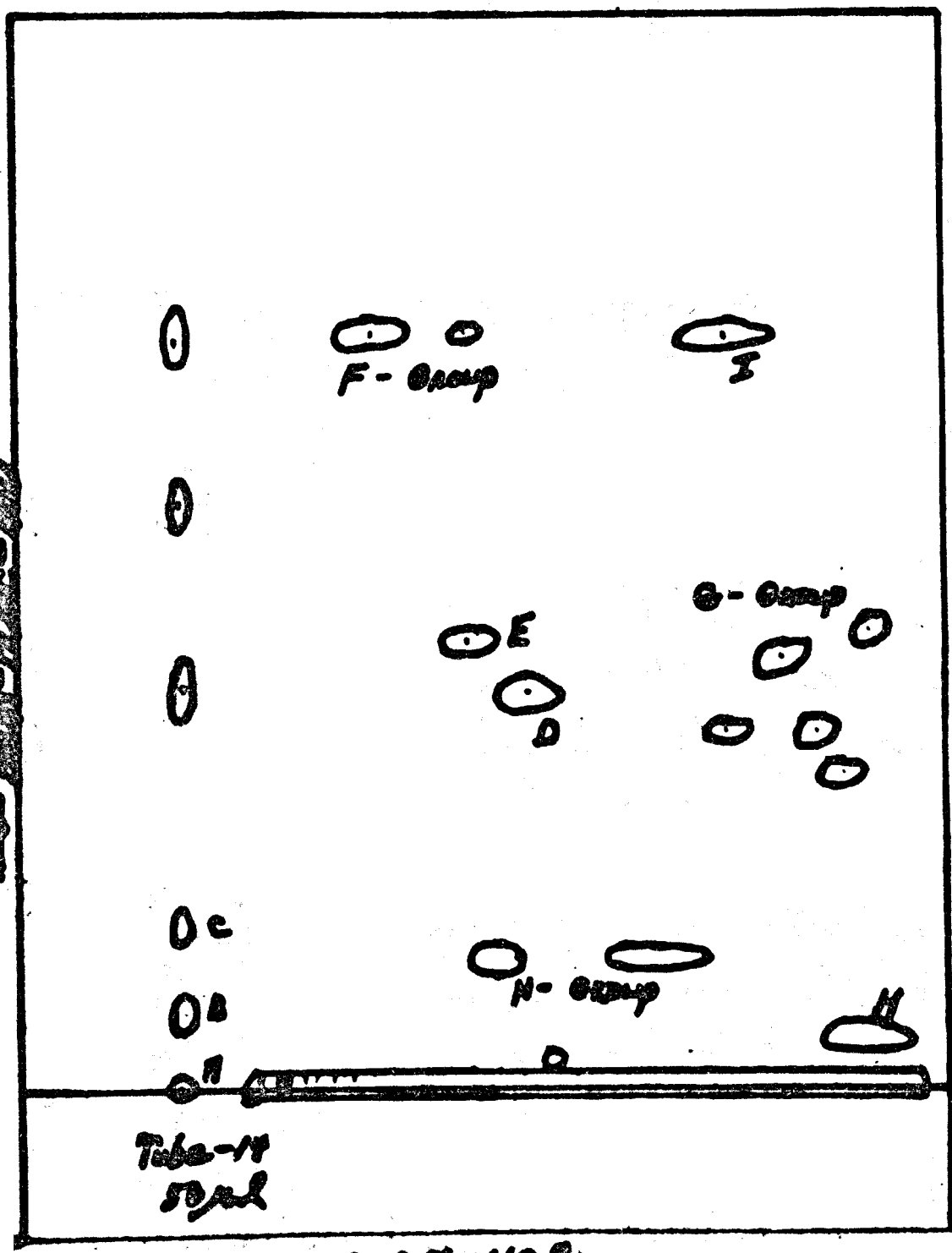
The materials in tubes 10 - 19 were alike with tube #14 being the best representative sample between tubes 10 - 19.

The materials in tubes 20 - 24 were alike with tube #23 being the most representative of this group.

5. Quantitative Paper Chromatography of Tube No. 14

Fifty-four papers (Whatman No. 1 filter paper) containing 50 microliters per paper or a total of 2.7 ml. of the original material from tube No. 14 were chromatographed two-dimensionally: firstly, in BuOH-HOAc-H<sub>2</sub>O, and secondly in 2% HOAc. Figure No. 1 shows the positions occupied by the resolved zones.

The fluorescent zones were cut out and eluted with methanol; the samples (codes), final volumes, and colors are summarized:



→ 201-11-11-11

→ 2% H<sub>2</sub>O<sub>2</sub>

<u>Sample</u>	<u>Volume</u>	<u>Color</u>
Blank	1 ml	clear
A	1 ml	yellow
B	1 ml	pink
C	1 ml	pink
D	lost-oxidation	
E	2 ml	light yellow
F	2 ml	light pink
G	3 ml	yellow
H	2 ml	light yellow
I	2 ml	pink
N	2 ml	light pink
O	2 ml	yellow

Samples E, F, G | I were rechromatographed in 2% HOAc.

The materials were detected on paper by their fluorescence, and as ferric cyanide complex. The samples underwent further oxidation as was shown by immobile materials at the baseline of the papergrams.

Compounds A, B, and C were immobile in 2% HOAc; the A zone was polymeric, and B and C showed a pink fluorescence; all three compounds are unknown. Compound D (unknown) had a skin color fluorescence; this compound and compound C (unknown) decreased gradually.

Compound E (rutin) decreased gradually, forming compound N. As compounds C, D and E decreased, compound O (polymeric) began to form and increased gradually until all of compounds C, D, E and N were used up. During the process, compound G (chlorogenic acid group) decreased in fluorescent intensity. Compounds F (caffeic acid group), H (unknown) and I (unknown) did not appear to have undergone any change.

### 6. Ultraviolet Analyses

The compounds from section 5 were concentrated and analyzed by ultraviolet analyses. The spectral work showed that the background absorbances contributed by the papers were greater than the absorbances of the respective polyphenols. Thus, no identification of the phenolic material could be made by ultraviolet analyses.

An attempt was made to clarify one of the phenolic materials by passing it through a column of cellulose. The eluted eluate contained more of the background material than it did prior to the column work; this indicated that the original background was caused by a cellulose-type material.

A further attempt was made to clarify one of the samples by passing it through a column of Magnesol. The solvent (ethyl acetate saturated with water) which was used to desorb the phenolic material desorbed the background material also.

The aforementioned analysis demonstrated the great need for identifying the materials on the filter paper which caused the high background absorbance.

Filter paper impurities do not hinder identification of phenolic components on paper. By paper chromatography, caffeic acid (cis-trans isomers), chlorogenic acid (plus isomers), rutin, quercetin, and quercetrin were identified in the No. 14 fraction (section 5).

7. Quantitative Paper Chromatography of Tube No. 23

Thirty-nine papers (Whatman No. 1) containing 50 microliters per paper or a total of 1.95 ml of the original material of tube No. 23 were chromatographed, using the procedure which was mentioned in Section 6. Figure 2 shows the arrangement of the zones on a papergram.

The fluorescent zones were sectioned out and eluted from the papers with methanol; the samples (codes), final volumes, and colors are tabulated:

<u>Sample</u>	<u>Volume</u>	<u>Color</u>
Blank	2 ml	pale yellow
J	3 ml	yellow
K	2 ml	pale yellow
L	2 ml	pale yellow
M	1 ml	pale yellow

Compounds J (quercetin), K (unknown), and L (unknown) were stable compounds. Compound M (unknown) was not present originally; this appeared during the early part of the procedure.

As before, because of the high background absorbance from the filter paper, no identifications could be made by ultraviolet analyses.

Conclusions on #14 and 23

To identify the phenolic constituents of tobacco (qualitatively or quantitatively) by paper chromatography combined with ultraviolet

FIGURE 2

Two Dimensional Chromatography  
of the Contents of Tube-23 from  
Countercurrent Distribution

41- NICH-HOAc-440

OJ

O



K



L

OM

Tube-23

50  $\mu$ l

→ 2% HOAc



spectroscopy, it is necessary that the absorbent material and/or materials on filter paper be identified. If the impurities are identified, they can be removed prior to chromatographic analyses.

#### 8. Conclusions

Countercurrent distribution offers an excellent means of separating polar compounds (polyphenols) from non-polar compounds and pigments. The disadvantage of this method is that the polyphenols are easily oxidized, even in mixtures, and when they are separated from non-polar compounds and pigments they are more easily oxidized. This method could be used in qualitative work, but not in quantitative measurements.

#### IV. Development of Method for Quantitative Determination of Polyphenols

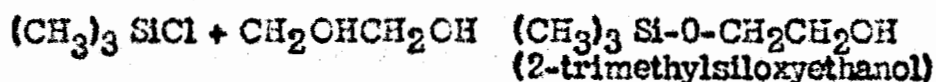
##### A. Preliminary Studies

##### 1. Trimethylsilyl Ether Derivatives

An interesting and advantageous approach to the polyphenol problem is the formation of trimethylsilyl derivatives of the polyphenols. The trimethylsilyl derivative protects the hydroxyl (OH) group; the derivatives are thermally stable, thereby permitting a fractional distillation without decomposition of the individual components. The derivatives have the same or lower boiling points as those of the

original hydroxylated compounds. The trimethylsilyl group is easily removed by hydrolysis at room temperature, giving the parent compound plus hexamethyldisiloxane. In the formation of the derivative, there is a mass increase of 72 for each hydroxyl group. The derivatives can be identified by the mass spectrometer, utilizing the heated inlet.

Two moles of ethylene glycol were dissolved in one mole of pyridine; the mixture was placed in a 2-liter, 3-neck flask which was immersed in an ice bath. To the rapidly stirred solution was added, from a separating funnel, 0.455 mole of trimethylchlorosilane during 40 minutes at 0° to 1.0° C. A stream of nitrogen was passed into the reaction vessel (during the reaction) to remove HCl vapors.



Pyridine was used as a catalyst in the reaction (acid acceptor). The reaction mixture was allowed to warm up to room temperature, and finally the mixture was heated to reflux for 10 minutes.

A short, water-cooled, condenser was attached to the center opening of the 3-neck flask (the other two openings were closed with



hydroxylated compounds to their trimethylsilyl derivatives. No large amount of precipitate is handled and the small amount of precipitate formed in the process of catalyzing the reaction is removed during the course of the reaction by sublimation into the reflux condenser. The reaction is easily followed by the change (generally a rise) in reflux temperature in the reaction flask or by the evolution of ammonia. The reagent requires few precautions (do not excessively expose to atmospheric moisture) and is not noxious. The hydroxylated compound need not be anhydrous, since water reacts with hexamethyldisilazane to give trimethylsilanol and hexamethyldisiloxane, both of which can be separated from trimethylsilyl ethers by distillation (99°C and 102°C, respectively).

#### Procedure

A 1-liter, 3-neck flask was fitted with a stirrer and a gas inlet tube (schematic drawing). A total of 375 ml of petroleum ether (b. p. 30-60°C) was added to the flask, followed by 600 grams of trimethylchlorosilane (Dow Chemical Co.). Anhydrous ammonia from a lecture bottle was passed through a flow meter into the reaction vessel. The reaction was spontaneous; the evolution of heat was so great that it caused the petroleum ether to boil; this was remedied by immersing the reaction vessel in an ice bath. The reaction was continued until the escaping vapors (HCl) from the oil trap became basic (NH<sub>3</sub>); at this

point, the evolution of heat had ceased. The reaction required approximately 7.5 hours.

After completion of the reaction, the reaction mixture was refluxed for one hour; ammonia was passed into the mixture during the reflux period.

The inside wall of the reaction flask was covered completely with ammonium chloride. The precipitate ( $\text{NH}_4\text{Cl}$ ) was collected on a Buchner funnel and this was washed with successive 200, 100, 100 and 100 ml portions of petroleum ether.

The petroleum ether was stripped from the filtrate by distillation. A Vigreux column was used in the distillation. Petroleum ether distilled at  $30^\circ$  to  $62^\circ\text{C}$ ; another fraction distilled at  $85^\circ\text{C}$  to  $94^\circ\text{C}$ . This fraction possessed an odor like gasoline. The third fraction distilled at  $122^\circ\text{C}$  to  $126^\circ\text{C}$ , and the final fraction came over at a constant temperature of  $126^\circ\text{C}$ . The final fraction was analyzed in the mass spectrometer and found to be hexamethyldisilazane.

#### Silyl Ether Derivatives

##### a. Materials

Hexamethyldisilazane was prepared from ammonia and trimethylchlorosilane.

##### b. Procedure

To 5 grams of resorcinol, 11.5905 gm. (15 ml) of hexamethyl-

disilazane was added. After one drop of trimethylchlorosilane had been added, a white precipitate ( $\text{NH}_4\text{Cl}$ ) formed immediately. The reaction flask (micro equipment) was fitted with a condenser and a drying tube (Drierite) and the reaction mixture was refluxed for nine hours. The evolution of ammonia began at  $43^\circ\text{C}$ . During the reflux period, the temperature in the reaction flask fluctuated from  $158^\circ\text{C}$  to  $161^\circ\text{C}$ . A white precipitate ( $\text{NH}_4\text{Cl}$ ) sublimed out of the reaction flask into the condenser, leaving a clear solution. Completion of the reaction was indicated by a constant reflux temperature taken at the thermometer well. A more sensitive indication of reaction termination is the cessation of decomposition of indicating Drierite (blue to pink color change) in the drying tube when no more ammonia is evolved.

The reaction requires an excess of reagent. Neither the reagent nor the hydroxylated compound needs to be measured accurately because the final product can be measured quantitatively by mass spectral analyses. Presence of excess reagent is assured if no further ammonia evolved on addition of a few drops of hexamethyldisilazane at completion of the reaction.

The aforementioned procedure was followed to prepare silyl ether derivatives of quinic acid, glucose, water, chlorogenic acid, and caffeic acid.

The silyl ether derivative of water required a longer reflux period than did the other compounds because in the presence of water, an intermediate product (alcohol) is formed which reacts further to form the corresponding ether derivative.

c. Distillation of Derivatives

The silyl ether derivative of resorcinol was subjected to distillation under vacuum. The micro distillation apparatus, as purchased, was used with one exception. Dr. Lee Harrow made a Vigreux column which contained more theoretical plates than did the original column which came with the micro apparatus.

No resolution was obtained through distillation. The distillation indicated that the derivative did not contain excess reagent because the reagent would have distilled at 65°C.

Silyl ether derivatives of quinic acid and glucose were subjected to the aforementioned distillation procedure. When working with individually known hydroxylated compounds it is necessary to remove only the excess reagent; this permits a short distillation period because the reagent distills at a temperature below that of the derivative.

Due to a change in vapor pressure, the derivatives of water, chlorogenic acid, and caffeic acid were freed of excess reagent by distillation at standard conditions (1 atmosphere).

#### d. Infrared Analyses

Silyl ether derivatives of resorcinol, quinic acid, glucose, chlorogenic acid, and water were analyzed as thin films. The individual spectrum did not reveal the presence of any of the parent compounds, but the reaction products (derivatives) were indicated. The reaction products could not be identified positively because of the lack of reference spectra of the derivatives. All of the derivatives exhibited the  $-\text{Si}(\text{CH}_3)_3$  characteristic band at  $756\text{ cm}^{-1}$ ,  $840\text{ cm}^{-1}$ , and  $1250\text{ cm}^{-1}$ . All of the spectra except the spectrum of reagent and the spectrum of resorcinol exhibited the strong characteristic Si-O band between  $1040\text{ cm}^{-1}$  and  $1090\text{ cm}^{-1}$ . In the spectrum of resorcinol, the band believed to be due to Si-O was at  $995\text{ cm}^{-1}$ ; the shift in the Si-O band is believed to be due to the phenyl group of resorcinol.

The spectrum of the derivative of chlorogenic acid exhibited the characteristic band at  $1730\text{ cm}^{-1}$  due to the (C = O) group. Bands due to unsaturation of the phenyl ring and (C = C) were present in the region  $1560\text{ cm}^{-1}$  to  $1650\text{ cm}^{-1}$ . The characteristic bands as mentioned for the other derivatives were present in the spectrum of the derivative of chlorogenic acid.

#### e. Gas Chromatography

Silyl ether derivatives of resorcinol, quinic acid, chlorogenic acid, glucose, and water were chromatographed on a column of poly-



glycol E-20,000 - firebrick at a helium flow rate of 40 ml/min. The temperature was varied; various retention times were recorded. The retention times were indicative of a change in vapor pressure of the present compounds versus the derivatives.

The gas chromatographic analyses were beneficial in that they showed that the structures of the parent compounds were changed.

#### Mass Spectrometric Analyses

The silyl ether derivative of resorcinol was chromatographed (gas) as mentioned previously. The peak at 34' 44" was trapped at liquid nitrogen temperature; this was analyzed by mass spectral analyses; a good spectrum of the derivative was obtained.

The silyl derivative of water, prior to distillation, was chromatographed; a peak at 7' 13" was trapped for mass spectral analysis.

The spectrum contained the intermediate product--silanol  $[(\text{CH}_3)_3 \text{Si OH}]$ , mass 90. This was a good reference spectrum.

#### g. Hydrolysis

The silyl ether derivative of resorcinol was hydrolyzed in mild acid solution (0.25 N HCl); the aqueous solution was removed by evaporation on a steam bath. The residue (solid) was analyzed as a KBr disc; the spectrum obtained for the residue was more correct for resorcinol than was the spectrum for the parent compound. This shows that the hydroxylated compounds can be derivatized; the derivatives can be

hydrolyzed to obtain the parent compound in a pure form.

At this point, it was concluded that a standard silyl ether derivative should be prepared from a known polyphenol, and the per cent conversion of the parent compound to the derivative should be established. Caffeic acid was chosen to be derivitized because of its chemical structure (hydroxyl groups in the ortho and meta positions, and an alkyl chain attached to a carboxyl group in the para position) - 1, 2-dihydroxycinnamic acid or caffeic acid. Also, caffeic acid combines with quinic acid in a ratio of 1:1 to form the major polyphenol of tobacco - chlorogenic acid.

The derivative of caffeic acid was formed by the procedure mentioned earlier.

The reaction mixture was subjected to distillation (S. T. P.); the still-pot temperature rose to 230°C, but no distillation occurred. The pressure was reduced to 320 mm; a small volume of distillate (approximately 0.5 ml) was obtained between 30-47°C; or standing, this material became a semi-solid. The pressure was taken down to 150 mm; no distillate was obtained. Finally, the pressure was reduced to zero (as measured on a manometer scale); there was a constant bumping in the still-pot; the addition of coal did not remedy the bumping. At this point, it was obvious that the derivative was non-distillable under the aforementioned conditions.

The silyl ether derivative of caffeic acid was analyzed as a thin film. The spectrum showed that none of the parent compound was present; if any of the reagent was present, it was there at an extremely low concentration. The carbonyl ( $-C=O-$ ) band was present at  $1695\text{ cm}^{-1}$ ; unsaturation ( $-C=C-$ ) was indicated in the  $1550-1670\text{ cm}^{-1}$  region. The characteristic  $-Si-(CH_3)_3$  bands were present in the 756, 840, and  $1250\text{ cm}^{-1}$  positions. A band at  $980\text{ cm}^{-1}$  was attributed to the  $\beta$ - $o$ - $Si(CH_3)_3$  linkage. Reference spectra of the silyl ether derivatives are not available, but based on the aforementioned information it is obvious that a reaction product was formed.

#### Gas Chromatography

A silyl ether derivative of caffeic acid was dissolved in chloroform and chromatographed on an 8-foot polyglycol-firebrick column at  $200^\circ\text{C}$  with a helium flow rate of 40 ml/min. No peaks (retention) were observed with the 8-foot column. When the column was shortened to one foot, chloroform (the solvent) and hexamethyldisilazane peaked immediately upon injection of the sample. The derivative peaked at 3 minutes at a temperature of  $200^\circ\text{C}$ . Several runs were made collecting (at liquid nitrogen temperatures) the 3 minute peak for mass spectral, for infra-red, and for ultra-violet analyses.

#### Mass Spectrometric Analyses

The silyl ether derivative of caffeic acid was confirmed by its

mass - 396.

#### Infrared Analyses

The derivative of caffeic acid was confirmed from the fraction which was obtained by gas chromatography.

#### Ultraviolet Analyses

In the ultraviolet region, the silyl ether derivative of caffeic acid exhibited an 18 millimicron hypsochromic shift in the long wavelength (325 millimicrons for caffeic acid). An 11 millimicron hypsochromic shift was observed in the short wavelength (302 millimicrons for caffeic acid). In addition to the hypsochromic shift, there was a change in the ratio of the absorbance at the long wavelength to the absorbance of the short wavelength; the ratio decreased from 1.34 for caffeic acid to 0.90 for the silyl ether derivative.

#### Hydrolysis

A portion of the silyl ether derivative of caffeic acid was hydrolyzed in concentrated hydrochloric acid (12N); the liquid was evaporated off on a steam bath, leaving a solid material. The residual material was dried; the melting point range was 190-192° C as versus 195° C for caffeic acid. The ultraviolet curve was intermediate between the silyl derivative and the caffeic acid; the ratio of the 320 millimicron absorbance to the

290 millimicron absorbance (0.430/0.342) was 1.26 ( $\Delta$  millimicrons = 30). Infrared analysis of the hydrolyzed product showed caffeic acid plus a compound like hexamethyldisiloxane. More than likely the additional compound was hexamethyldisiloxane since this compound should be obtained by hydrolyzing a silyl ether derivative to its parent compound.

#### Recrystallization

A portion of the silyl ether derivative of caffeic acid was recrystallized from hot water. The precipitate was filtered off and dried; the melting point was 198°C, versus 195°C for caffeic acid. The filtrate and the residue were analyzed by ultraviolet and by infrared analyses. The ultraviolet spectrum of the filtrate was similar to that of the derivative; whereas, the residue was identified as caffeic acid.

The infrared spectrum of the filtrate showed a mixture of derivative and caffeic acid; whereas the residue was identified as caffeic acid.

The aforementioned results show that the derivative was hydrolyzed rather than recrystallized. Since the crude derivative was slightly acidic and at a 100°C recrystallization temperature, it was likely for hydrolysis to occur.

The theoretical yield of the derivative of caffeic acid was roughly 73%.

### Mono, Di, and Tri Substitution

Silyl ether derivatives were prepared from phenol (mono-hydroxy) and phlorogucinol (tri-hydroxy). A derivative of resorcinol (di-hydroxy) was prepared, as mentioned previously.

Infrared studies demonstrated that by characteristic fingerprint regions, one can distinguish between mono-, di-, and tri-substituted silyl ether derivatives of hydroxylated aromatic compounds.

### Preparatory Work on Extracts of Tobacco

All of the work up to this point demonstrated that numerous preliminary separational schemes are necessary to resolve the phenolic materials of tobacco, prior to the formation of silyl derivatives.

A 200 ml aliquot of a 63% ethanol extract of tobacco was reduced to a residue by azeotroping off the ethanol and the water with chloroform. To see if the major portion of the phenolic material could be confined to one or possibly two solvents, the residue was extracted successively with benzene, chloroform, ether, acetone, and methanol. The chloroform, the ether, and the acetone solubles were chromatographed in a solvent system of BuOH-HOAc-H<sub>2</sub>O, and in a system of water. The major polyphenols were confined to the ether and the acetone solubles as was shown by paper chromatography.

Since chloroform and acetone form an azeotropic mixture (maximum type curve), it was advisable to extract the polyphenols

from tobacco with a mixture of chloroform and acetone plus a low percentage of ethyl ether.

Two 40 g. grams samples of Type I and Type II tobaccos were extracted exhaustively with chloroform-ether-acetone (25%-5%-70% VV). A 500 ml volume of extract was obtained from each type of tobacco. The residual tobaccos were extracted with 63% ethanol (aqueous); a volume of one liter was obtained for each sample.

The mixture of chloroform-ether-acetone did not extract the polyphenols quantitatively as was shown by paper chromatography.

The chloroform solubles, the ether solubles, and the acetone solubles were subjected to the procedure for the formation of silyl ether derivatives. No derivatives of the polyphenols were isolated because the fractions were not free of the original 63% ethanol-water mixture. If the derivatives were formed, they were not detected because of the derivatives of water and ethanol.

Although the preliminary work on the silyl ether derivatives showed excellent promise, this work was terminated because of the excessive work required on known compounds. Previously, these types of derivatives had not been prepared from polyphenols.

#### ULTRAVIOLET STUDIES

An attempt was made to measure polyphenols quantitatively by a combination of paper chromatography and ultraviolet analyses. This

method required recrystallization of the knowns, extinction coefficient data, chromatography, elution of the compounds from paper, and a final measurement by ultraviolet analyses.

Since rutin and chlorogenic acid are two of the major polyphenols in tobacco, these compounds were used in the ultraviolet studies.

A. Rutin

1. Recrystallization of Rutin

Rutin was recrystallized five times from hot ethanol and hot water. The product showed an impurity when it was rechromatographed in 2% HOAc. The filtrate was chilled and the second precipitate was filtered out. The second precipitate was free of impurities as was demonstrated by chromatography in 2% HOAc.

The purified rutin was dried over phosphorous pentoxide ( $P_2O_5$ ); an Abderholden drying pistol was used. The dried product melted at 191-193°C.

2. Specific Absorbance

The purified rutin was weighed and dissolved in methanol (4.21 mg/ml = stock solution). The stock solution was diluted to contain 0.021mg/ml, 0.042mg/ml, and 0.063mg/ml. The spectrophotometric analyses (large peak at 358 millimicrons) are tabulated below:



	<u>Conc. of Sample</u> <u>Mg/Ml</u>	<u>Abs. Max.</u>	<u>Abs. Avg.</u>	<u>A/Mg/Ml</u>
1.	0.021	0.65	0.66	31.1
1.	0.021	0.66		
2.	0.042	1.42	1.34	31.7
2.	0.042	1.32		
2.	0.042	1.27		
3.	0.063	1.91	1.93	30.5
3.	0.063	1.94		
			Avg.	31.1

The apparent pH (pH reading in methanol) of the samples varied between pH 6.93 - 7.03, but the variation in pH was not sufficient to cause a shift in the absorbance at 358 millimicrons.

### 3. Quantification of Rutin

The purified rutin was subjected to a quantitative paper chromatographic procedure.

Duplicate samples at three levels of concentration (same solution used for extinction data) were streaked over an 8-inch area on Whatman No. 1 filter paper. The concentrations of the final solutions (mg/ml after elution from paper) were calculated so that they would be in the same order of magnitude as those (solutions) used to establish the extinction coefficients. Location zones were spotted 5 cm from the outer edges of the papers.

After the development period, the papers were dried at room

temperature. The rutin band and location zones were detected by their visual yellow color, and by fluorescence ( $R_F$  0.37). The location strips were tested with phosphomolybdic acid plus ammonia vapor; these gave the typical color characteristics of rutin (grey-blue with a tinge of yellow-green).

The rutin bands and corresponding blanks were sectioned out and eluted with methanol. The elutions were carried out overnight; 7 to 9 ml of eluate was collected from each paper strip, and each was diluted to a 10 ml volume for spectroscopic examination.

The spectrophotometric work consisted of measuring rutin in methanol versus a methanolic eluate of rutin from paper. A blank was included for each determination. For example, the spectrum for a complete analysis consisted of a measurement of rutin in methanol (0.021mg/ml), a measurement of rutin eluted from paper with methanol (0.020mg/ml), and a measurement of the paper blank. This type of measurement was obtained, also, for the concentrations of 0.041 mg/ml and 0.061mg/ml, respectively.

The calculations were carried out as per the following sample calculation:

$$A \text{ max.} = 0.65$$

$$A \text{ min.} = \underline{0.04}$$

$$A \text{ net} = 0.61$$

$$\frac{\text{Net Absorbance}}{\text{Specific Absorbance}} = \text{mg/ml}$$

$$\frac{0.61}{31.1} = 0.020 \text{ mg/ml}$$

$$\frac{\text{mg/ml found} \times 100}{\text{mg/ml applied}} = \% \text{ recovery}$$

$$\frac{0.020}{0.021} \times 100 = 95.2\%$$

The following tabulation shows the per cent recovery for each individual run:

	<u>Amount Used</u>	<u>Amount Found</u>	<u>% Recovery</u>
1)	0.021mg/ml	0.020 mg/ml	95.2
2)	0.021 "	0.020 "	95.2
3)	0.041 "	0.039 "	95.1
4)	0.041 "	0.040 "	97.6
5)	0.061 "	0.059 "	96.7
6)	0.061 "	0.059 "	96.7

## B. Chlorogenic

### 1. Quantification of Chlorogenic Acid

Chlorogenic acid exists in several isomeric forms; therefore, all of the experimental studies will be directed toward the measurement of total chlorogenic acid (chlorogenic acid plus its many isomers).

Previous chromatographic data have shown that chlorogenic acid can be confined to one zone on paper when developing with either BuOH-HOAc-H<sub>2</sub>O or BuOAc-HOAc-H<sub>2</sub>O.

The stock solution of chlorogenic acid in 63% ethanol contained 0.74 mg/ml. A total of 340.36 microliters was applied to a paper strip; provided all of the acid was eluted from the paper with 10 ml. of 63% ethanol, the final solution would contain 0.030mg/ml. The paper was developed in a solvent system of BuOAc-HOAc-H<sub>2</sub>O (40-10-50); the paper was dried, and the fluorescent band was sectioned out and eluted with 63% ethanol. A blank was carried through the entire procedure.

A spectroscopic examination showed that the eluted blank versus 63% ethanol contained a large background absorbance. The background caused a displacement of the normal peak of chlorogenic acid. A normal curve for chlorogenic acid was obtained when the eluted chlorogenic acid was run versus the eluted blank in the reference cell; however, this method caused approximately 2% loss in total absorbance.

The aforementioned procedure was repeated exactly with two exceptions; the paper was developed in 2% HOAc, and the fluorescent band was eluted with methanol. The eluted chlorogenic acid was run versus methanol in the reference cell and also versus the eluted blank in the reference cell.

The recovery of chlorogenic acid for the two runs could not be determined because the known chlorogenic acid (spotting) solution was not in the same solvent media as that for the eluted material.

A stock solution of chlorogenic acid in methanol was prepared (1.32 mg/ml). This was chromatographed quantitatively to give a concentration from paper of 0.021mg/ml. During the development period, the chlorogenic acid broke up into two bands; each band (A and B) was sectioned out and eluted with methanol. A recovery of 52.4% was obtained from band "A". Band "B" was too dilute to obtain an accurate measurement.

The chlorogenic acid which was used in the aforementioned analyses was purchased from Delta Chemical Works. Chromatographically, the Delta sample has a tendency to break up into two compounds, or possibly it contains two forms of chlorogenic acid.

A sample of chlorogenic acid was obtained from Aldrich Chemical Co., Inc. This sample was subjected to the quantitative procedure. Chromatographically, the Aldrich sample produced one band. A recovery of 69.6% was obtained from the paper analysis.

Since there was a great variation between the specific absorbance of the old sample of Chlorogenic acid (Delta Chemical Works) and the new sample of Chlorogenic Acid (Aldrich Chemical Works), a new sample was obtained from Delta Chemical Works.

Two samples of the Delta Chlorogenic acid and two samples of the Aldrich Chlorogenic acid were weighed and dissolved in a mixture of isopropyl alcohol-water (1:1). (Chlorogenic acid is more soluble in this

mixture than in methanol). The dilutions of the stock solutions and the spectrophotometric analyses are tabulated below:

Delta Sample

a) Stock solution = 80.9 mg/50 ml = 1.62 mg/ml

b) " " = 91.4 mg/50 ml = 1.83 mg/ml

		<u>Conc. of Sample Mg/ml</u>	<u>Abs. Max.</u>	<u>A/mg/ml</u>
a)	1)	0.020	0.81	49.9
	2)	0.030	1.62	50.0
	3)	0.050	2.42	49.8
b)	1)	0.020	0.90	49.3
	2)	0.040	1.80	49.2
	3)	0.050	2.70	<u>49.3</u>
Avg.				49.6

Aldrich Sample

a) Stock solution = 45.9 mg/50 ml = 0.92 mg/ml

b) " " = 57.0 mg/50 ml = 1.14 mg/ml

		<u>Conc. of Sample Mg/ml</u>	<u>Abs. Max.</u>	<u>A/mg/ml</u>
a)	1)	0.030	1.45	52.6
	2)	0.040	1.92	52.3
	3)	0.020	0.97	52.9

		<u>Conc. of Sample Mg/MI</u>	<u>Abs. Max.</u>	<u>A/mg/ml</u>
b)	1)	0.020	1.21	52.9
	2)	0.030	1.79	52.7
	3)	0.010	0.61	<u>52.3</u>
			Avg.	52.6

To study the decomposition of chlorogenic acid in solution, one Delta sample was maintained at room temperature, and another Delta sample was left at refrigeration temperature. After a period of 22 days, the samples were re-analyzed; the results are tabulated below:

<u>Temperature</u>	<u>Specific Abs. 1/7/59</u>	<u>Specific Abs. 1/29/59</u>
Refrigeration	50.0 A/mg/ml	48.7 A/mg/ml
Room temperature	49.2     "	43.7     "

At refrigeration temperature the specific absorbance of the Delta chlorogenic acid in solution decreased significantly.

The decomposition of the Aldrich chlorogenic acid was determined by the same procedure mentioned for the Delta sample. The results are tabulated:

<u>Temperature</u>	<u>Specific Abs. 1/7/59</u>	<u>Specific Abs. 2/24/59</u>
Refrigeration	52.9 A/mg/ml	51.6 A/mg/ml
Room temperature	52.6     "	49.6     "

The chlorogenic acid from Aldrich Chemical Company was used in our work because the rate of decomposition was quite small.

A combination of paper chromatography and ultraviolet analyses could be used to measure rutin quantitatively, but this procedure produced low recovery values for chlorogenic acid. Since chlorogenic acid is the major polyphenol in tobacco and it could not be measured quantitatively by the aforementioned procedure, another method of measurement was investigated.

#### B. Study of Colorimetric Method

The method of Richard W. Keith, Duane Le Tourneau, and Dennis Maklum as published in The Journal of Chromatography, 1, 534-36 (1958) was studied for measuring individual polyphenols. The colorimetric procedure depends upon the reduction of compounds containing hexivalent tungsten and molybdenum to colored compounds.

Standard solutions of caffeic acid, chlorogenic acid, gallic acid, rutin, quercetin, quercitrin, and scopoletin were prepared to contain 50 mg/20 ml of ethanol, or 2.5 mg/ml.

The standard solutions of each compound were diluted as follows:

#### Stock Solutions

1. 1 Ml diluted to 100 Ml = .025 mg/Ml.
2. 2 Ml diluted to 100 Ml = .050 mg/Ml.
3. 3 Ml diluted to 100 Ml = .075 mg/Ml.
4. 4 Ml diluted to 100 Ml = .010 mg/Ml.



Twenty ml. (20.) of each stock solution was pipetted into a 25 ml volumetric flask; 0.5 ml. of Folin-Ciocalteu (diluted 1:1 with water) was added, followed with 2 ml of a 20%  $\text{Na}_2\text{CO}_3$  solution, and brought to a 25 ml volume with water. A blank was carried through the same procedure. This gave final concentrations of:

1. 500 micrograms/25 ml.
2. 1000           "       /25 ml.
3. 1500           "       /25 ml.
4. 2000           "       /25 ml.

The solutions were permitted to stand for two hours, prior to being read in the visible region of the Cary Spectrophotometer.

In the instrumental analysis, the blank was used in the reference compartment as well as in the sample compartment to zero the instrument. At 800 millimicrons, the curve began at an optical density greater than 0.5. The position in which the curve fell indicated that the origin of the curve was in the near infrared region. The samples were re-analyzed; the curve began at 1380 millimicrons and extended down to 380 millimicrons with a broad maximum peak at 660 millimicrons. The spectral work showed that the individual samples were not additive because of the excessively high concentrations.

Since the original article on the use of the Folin-Ciocalteu reagent included a standard curve for gallic acid, an attempt was made to reproduce the curve.

A standard solution of gallic acid (2.5 mg/ml) was diluted (1 ml to 100 ml) to contain .025 mg/ml. From this stock solution, samples were prepared to contain 25, 75, 125, 187, 250, and 375 micrograms/25 ml respectively. These samples were prepared by the aforementioned procedure. Instrumentally, at a wavelength of 660 millimicrons, the solutions from 25 micrograms/25 ml through 250 micrograms/25 ml followed the Beer's law relationship, but above this concentration, linearity was not obeyed.

The aforementioned solutions were read on the Bausch and Lomb "Spectronic 20" at 660 millimicrons; the same results were obtained as those mentioned for the Cary Spectrophotometer. At this point, it was decided that we could use the "Spectronic 20" advantageously in this work.

To study the time required for maximum color development, a new set of samples was prepared and readings were taken every 20 minutes over a three-hour period. This showed that one hour was a sufficient time for obtaining maximum color.

To determine the amount of solvent which could be added to the samples, prior to the addition of the Folin-Ciocalteu reagent and sodium carbonate, without affecting the color development, one set was made up with sample plus reagents and diluted to 25 ml; another set was made up with sample plus 20 ml of water, plus reagents, and diluted to 25 ml. The results showed that the water could be added at either

point mentioned above without affecting the color. It was found that the reagent must be added prior to the sodium carbonate to obtain the proper color.

By the aforementioned procedure, standard curves were obtained for caffeic acid, chlorogenic acid, gallic acid, rutin, quercetin, quercitrin, and scopoletin.

To adapt the method to samples obtained from paper chromatography, each of the standard solutions were chromatographed on paper in increments of 60.45, 120.90, and 181.35 microliters, respectively, and developed with the upper phase of BuOH-HOAc-H<sub>2</sub>O.

After freeing the papers of the organic solvent the bands were located by their fluorescence with a UV hand-lamp. The individual bands were sectioned out along with an equal size blank; these were cut into small pieces and placed in a coarse sintered glass filter. Each sample was washed with two 10-ml portions of hot water into a 25 ml volumetric flask; 0.5 ml of Folin-Ciocalteu reagent and 2 ml of 20% sodium carbonate were added to each flask. The solutions were read immediately, and at 20-minute intervals over a period of one hour. The materials were not removed quantitatively from the papers as was shown by the standard curves.

To increase the recovery of the compounds from filter paper,

the first part of the procedure was repeated, but the extraction method was changed. After cutting the bands of paper into small pieces, these were placed in screw-cap vials; 10 ml of hot 2% methanol was added to each vial, and these were allowed to stand for 30 minutes. At this point, 0.5 ml of Folin-Ciocalteu reagent was added to each vial, and these were allowed to stand 10 minutes prior to the addition of 2 ml of 20% sodium carbonate. Immediately after adding the reagents, the solutions were filtered through a coarse porosity sintered glass filter into a 25 ml volumetric flask. Small portions of hot 2% methanol were added to the vials to remove the materials quantitatively from the papers. This was continued until a total volume of 25 ml was obtained.

The results (average of three runs at each concentration) obtained for the various concentrations are tabulated:

<u>Compound</u>	<u>60.45 <math>\mu</math>l/25 ml</u>	<u>120.90 <math>\mu</math>l/25 ml</u>	<u>181.35 <math>\mu</math>l/25 ml</u>
Galic acid	93.73%	90.00%	96.68%
Scopoletin	98.33%	90.00%	93.30%
Rutin	93.00%	90.00%	75.00% *
Chlorogenic acid	101.00%	95.00%	95.30%
Caffeic acid	94.15%	99.00%	100.00%
Quercetin	95.00%	93.00%	91.00%

\* This result is low because at this concentration, a portion of the material remains at the baseline on the papergram.

At this point, an old extract of tobacco was carried through the aforementioned procedure and was found to work equally as well as it did with the known polyphenols.

A 3-gram sample of ground tobacco was placed in a one-inch diameter glass column; 75% ethanol was added in small portions, and the solvent was permitted to penetrate the tobacco prior to being forced through the column with a stream of nitrogen. This was continued until the eluate was almost colorless, giving a negative test for polyphenols. A final volume of 25 ml was obtained.

By paper chromatography, the aforementioned extract contained as many, if not more, polyphenols as did the previously mentioned methods of extractions.

The advantages of the above extraction are: 1.) small sample size, and 2.) small volume of solution.

#### Summary

From the data established in these preliminary studies, a quantitative method for polyphenols was established as shown in Section V.

In summary, the polyphenols can be measured spectrophotometrically as colored reaction products with Folin-Ciocalteu reagent.

## V. Method Developed

### Quantitative Determination of the Major Polyphenols in Tobacco

#### 1. Method

The proposed method, in brief, involves extraction of the polyphenols from tobacco, paper chromatography of the extract, elution of the samples from paper, and colorimetric determination. The colorimetric procedure depends upon the reduction of compounds containing sexivalent tungsten and molybdenum to colored compounds.

#### 2. Apparatus and Reagents

a) All spectrophotometric measurements were made at 25°C with a Cary Model 14 recording spectrophotometer using 1 cm fused quartz cells, or with a Bausch and Lomb manual "Spectronic 20" spectrophotometer.

b) Folin-Ciocalteu Reagent - purchased commercially. The reagent consists of a mixture of 100 grams of sodium tungstate ( $\text{Na}_2\text{WO}_4 - 2\text{H}_2\text{O}$ ), 25 grams of sodium molybdate ( $\text{Na}_2\text{MoO}_4 - 2\text{H}_2\text{O}$ ), 700 ml of water, 50 ml of 85% phosphoric acid ( $\text{H}_3\text{PO}_4$ ), and 100 ml of concentrated hydrochloric acid (HCl). For our use the commercial reagent was diluted 1:1 with water.

c) 20%  $\text{Na}_2\text{CO}_3$  solution (W/V)

d) Chromatography Papers - Whatman grades 1 and 2.

e) Solvent Systems - Organic phase of butanol-acetic acid-water (4-1-5 V/V/V).

### 3. Procedure

#### a) Extraction

Two grades of ground tobacco (3 gram samples of each) were extracted with 75% ethanol; another set of samples was extracted with 20% ethanol. Each tobacco sample was placed in a glass column; the solvent was added in small portions, and the solvent was permitted to penetrate the tobacco prior to being forced through the column with a stream of nitrogen. This was continued until the eluate was almost colorless. A final volume of 25 ml was obtained.

### 4. Paper Chromatography

#### a) Extracts

The 20% ethanol and the 75% ethanol extracts were chromatographed on Whatman No. 2 filter paper (175 microliters per paper). The papers were developed (ascending) in a solvent system of BuOH-HOAc-H<sub>2</sub>O for 15-1/2 hours.

#### b) Knowns

To prepare standard curves, 50 mg. of each of the known polyphenols were dissolved in 20 ml of 95% ethanol. Amounts varying from 25 to 500 micrograms of each substance were streaked (calibrated

microliter pipette) over a 2.5 inch length on the reference line of a strip of filter paper leaving an equal size area for a blank. The papers were developed by the ascending technique, at room temperature, for 15-1/2 hours in a solvent system of BuOH-HOAc-H<sub>2</sub>O (4-1-5, V/V/V).

c) Color Development

After development, the papers (containing the tobacco extracts and the knowns) were freed of the organic solvent in a ventilated hood. The individual bands were located by their fluorescence under an ultraviolet hand lamp. The bands were sectioned out along with an equal size band for the blank; each band was cut into small pieces and placed in a screw-cap vial. Ten milliliters of hot 2% methanol was added to each vial, and these were permitted to stand for 30 minutes. At this point, 0.5 ml of Folin-Ciocalteu reagent was added to each vial, and these were allowed to stand 10 minutes prior to the addition of 2 ml of 20% Na<sub>2</sub>CO<sub>3</sub>. It is necessary that the reagents be added in the order shown to obtain the proper color. Immediately after adding the reagents, the solutions were filtered through a coarse porosity sintered glass filter into a 25 ml volumetric flask. Small portions of hot 2% methanol were added to the vials to remove the materials quantitatively from the papers. This was continued until a volume of 25 ml was obtained.



FIGURE 3

Standard Curve of Caffeic  
Acid

## AUTOBIOGRAPHY

I, Marvin Dennis Edmonds, was born on March 23, 1921, in Kenbridge, Virginia, where I attended elementary school, graduating in 1934. I graduated from Kenbridge High School in 1938, and from Richmond Business College as a Junior Accountant in 1940. I worked as an accountant until 1943, at which time I served in the U. S. Navy (World War II) until 1945. After this, I worked as an accountant until 1949, and enrolled at the Richmond Professional Institute of the College of William and Mary where I graduated with a B. S. in Chemistry in 1952. I worked with the Virginia Department of Agriculture (Division of Chemistry) until 1953. Since then, I have been working with Philip Morris Inc. in Richmond, Virginia, as a research chemist.