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# ISOLATION, IDENTIFICATION, SYNTHESIS AND PYROLYSIS OF PLANT PHENOLIC COMPOUNDS

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ISOLATION, IDENTIFICATION, SYNTHESIS AND

PYROLYSIS OF PLANT PHENOLIC COMPOUNDS

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# ISOLATION, IDENTIFICATION, SYNTHESIS AND PYROLYSIS OF PLANT PHENOLIC COMPOUNDS

## CHAPTER I

## INTRODUCTION

There has been no previous report in the literature on the identification of polyphenolic compounds present in spinach and in boron-deficient sunflower leaves. Consequently, a study has been made on the isolation and determination of the major polyphenolic compounds present in these plants. During the course of these investigations, it was found necessary to synthesize several compounds for use as chromatographic standards. These include gentisic acid glucosides, scopolin and scopoletin-4-$C^{14}$.

Rutin and chlorogenic acid are among the principal polyphenols present in cigarette tobacco, but neither has been found in the smoke from cigarettes. It was, therefore, of interest to investigate the products of pyrolysis of these two compounds, as well as those of quercetin, the aglycone of rutin.

# CHAPTER II

# FLAVONOLS IN SPINACH LEAVES

## Introduction

There has been no previous report of the identification of individual flavonol compounds in spinach leaves, although a colorimetric, quantitative determination of the gross, flavonoid-like compound content of spinach (Spinacia oleracea) has been reported by Weatherby and Cheng (1). Williams (2) has isolated a flavonoid compound in pure form from spinach leaves. Its exact identity, however, was not determined. This work describes the extraction from spinach and identification of the flavonol, patuletin, and also of a quercetagetin dimethyl ether. The latter compound has not been found previously in nature, and the name "spinacetin" is proposed for it. Spinacetin has been tentatively identified as quercetagetin-3',6-dimethyl ether. Quercetagetin is 3,3',4',5,6,7-hexahydroxyflavone. Patuletin was first isolated by Rao and Seshadri from the flowers of Tagetes patula

(3), and later proven to be quercetagetin-6-monomethyl ether (4).

## Extraction of Spinach Leaves

Fifty pounds of packaged, frozen fresh spinach (Safeway Stores, Inc., "Belair" brand) was ground in an ice crusher, then loaded into 4 1. containers with 1.5 1. of water, and pressure-cooked at 115°C for 45 min. The juice was squeezed out, and filtered through cotton cloth and then through a bed of Super Cel (Fisher Scientific). The clear, yellow-brown filtrate was adsorbed under pressure on wet Magnesol (Food Machinery and Chemical Corp., New York, N. Y.) packed on three glass funnels (22 cm. diam., 18 cm. deep). A yellow zone, 6 cm. deep was formed on the Magnesol in each funnel. The adsorbent was washed with 1 1. of water, and the yellow zone was eluted with 70% ethyl alcohol-water. The dark-brown eluate (4.5 1.) was concentrated *in vacuo* to 200 ml. and then "freeze-dried" to yield a black-appearing solid. Pulverization and then extraction with hot methyl alcohol (12 x 100 ml.) gave a reddish-brown extract which was poured onto an 8 cm. column previously filled to a depth of 50 cm. with Magnesol in methyl alcohol. Development under 5 lb. pressure, with ethyl acetate saturated with water, readily

moved a broad, bright-yellow zone. The yellow eluate (1600 ml.) was taken to dryness *in vacuo*. Crystallization from acetone-water gave 0.205 g. of a yellow powder. Paper chromatography revealed the presence of at least two compounds. Separation into individual compounds was achieved by silicic acid chromatography. A column (6 cm. diam.) was packed to a depth of 38 cm. with silicic acid (Mallinckrodt No. 2847) in benzene-acetone (84:16 v/v), under 5 lb. pressure. The yellow powder (0.205 g.) was dissolved in acetone (18 ml.) and diluted with benzene (102 ml.), and then chromatographed. On development with the benzene-acetone, two major zones formed, and they were eluted separately. After removal of the solvent *in vacuo*, the eluate from the faster moving zone yielded 75 mg. of a yellow powder, called "Compound A-2." From the eluate of the slower moving zone, 76 mg. of fine yellow needles, called "Compound A-1," were obtained.

## Identification of Compound A-1

The yellow product containing compound A-1 was chromatographed on a silicic acid column using benzene-acetone (84:16 v/v), and then crystallized from ethyl alcohol-water to give yellow needles. These were dried at 110° *in vacuo*, yield 59 mg., m. p. 261-263°C (all melting points are

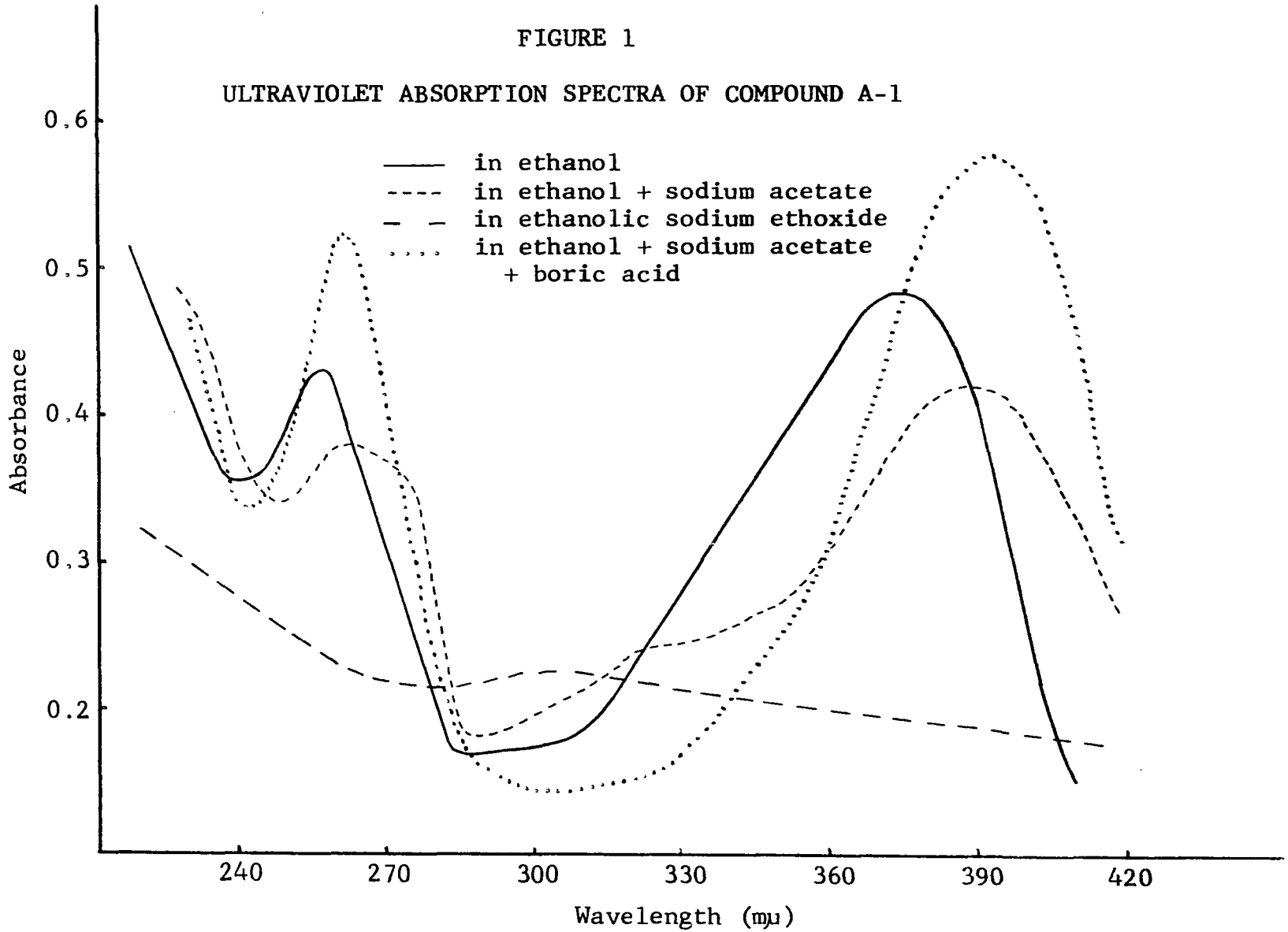
uncorrected). $R_f$ values in 60% acetic acid, n-butyl alcohol-acetic acid-water (6:1:2 v/v/v), and phenol-water (3:1 w/w), using Whatman No. 1 chromatography paper and descending chromatography, were 0.47, 0.75, and 0.63 respectively. The ultraviolet absorption spectrum showed maxima at 257 and 375 mμ and minima at 240 and 285 mμ (Figure 1).

Anal. Calcd. for $C_{16}H_{12}O_8$: C, 57.83; H, 3.64; $OCH_3$, 9.34. Found: C, 58.05; H, 3.98; $OCH_3$, 9.28.

Compound A-1 (10 mg.) was refluxed for 6 hr. with 1 ml. dimethyl sulfate in 8 ml. anhydrous acetone and potassium carbonate (2.5 g.) to give colorless needles, m.p. 143-144°C. No depression occurred on mixed melting point determination with synthetic quercetagetin hexamethyl ether. The melting point of authentic patuletin was not depressed by the addition of compound A-1. The ultraviolet and infrared spectra of compound A-1 and the reference patuletin were identical, respectively. With spectral measurements by the method of Jurd and Horowitz (5), both the reference patuletin and compound A-1 behaved exactly alike. Each exhibited a 4 mμ shift of the short wavelength band, after 5 minutes, from 257 mμ to 261 mμ in the presence of sodium acetate. With boric acid-sodium acetate mixture (6), there was a shift of 18 mμ for each in the higher wavelength peak, indicating the

FIGURE 1

ULTRAVIOLET ABSORPTION SPECTRA OF COMPOUND A-1



presence of the o-dihydroxy group in both. In sodium ethoxide, however, the higher wavelength peak disappeared in each case after 5 minutes (Figure 1). Thus, patuletin and compound A-1 are identical by every test used.

## Identification of Compound A-2

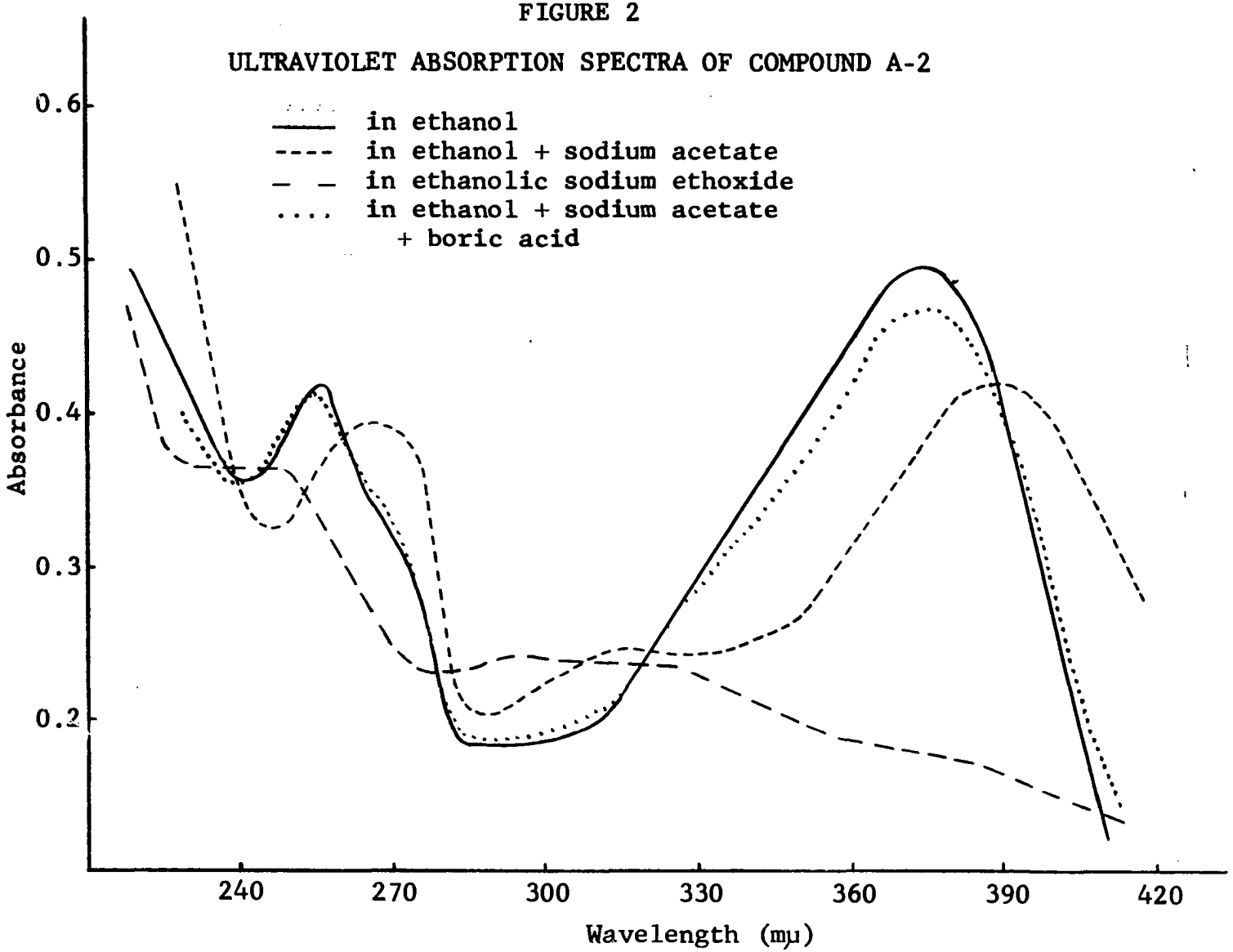
The yellow powder (75 mg.), containing compound A-2 obtained from the faster moving eluate of the silicic acid column, was crystallized twice from benzene-methyl alcohol to yield fine yellow needles. These were dried at 110°C, *in vacuo*; m.p. 235-236°C. $R_f$ values in 60% acetic acid; the n-butyl alcohol-acetic acid-water; and the phenol-water solvent systems were 0.56, 0.85, and 0.88, respectively. The ultraviolet absorption spectrum showed maxima at 257 and 373 mμ and minima at 241 and 287 mμ (Figure 2).

*Anal.* Calcd. for $C_{17}H_{14}O_8$ (346.28): C, 58.96; H, 4.08; $OCH_3$, 17.92. Found: C, 59.12; H, 4.03; $OCH_3$, 18.57.

Compound A-2 (10 mg.) was methylated with 1 ml. dimethyl sulfate in 8 ml. anhydrous acetone and 2.5 g. potassium carbonate to give colorless needles, m.p. 143-144°C, not depressed by authentic quercetagetin hexamethyl ether. Demethylation of compound A-2 with hydriodic acid, sp. gr. 1.7, and acetic anhydride yielded quercetagetin. The latter

FIGURE 2

ULTRAVIOLET ABSORPTION SPECTRA OF COMPOUND A-2



had $R_f$ values of 0.27, 0.45, and 0.20 respectively, in the 60% acetic acid, n-butyl alcohol-acetic acid-water, and phenol-water systems.

Degradation with potassium hydroxide by a procedure used previously by Yang et al. (7) produced vanillic acid. Identification of vanillic acid was achieved by the method of Hergert and Goldschmid (8). Thus, compound A-2 contained the 3'-methoxy-4'-hydroxy grouping. Compound A-2 on paper chromatograms gave a greenish-yellow color under long wavelength ultraviolet light, indicating that its 3-hydroxy group is not substituted. Spectral shift measurements (5,6) indicated that a free 7-hydroxy group is present, as the short wave length band shifts from 257 mμ to 269 mμ with sodium acetate. Boric acid-sodium acetate addition produced no shift for the higher wavelength peak, indicating the absence of an o-dihydroxy group. In 0.002N sodium ethoxide, the longer wavelength peak was partially suppressed after 5 minutes, and disappeared after 1 hr. (Figure 2). These data indicate that compound A-2 is the 3',6-dimethyl ether of quercetagetin.

Both patuletin and quercetagetin-3',6-dimethyl ether have also been isolated from fresh spinach leaves obtained from a wholesale produce company.

# CHAPTER III

# A STUDY OF POLYPHENOLIC COMPOUNDS IN BORON - DEFICIENT SUNFLOWER LEAVES

## Introduction

The biochemical role of the micronutrient element, boron, in higher plants is not known. It has been pointed out that the role of boron in plant growth appears to be related to the differentiation and maturation of the cell (9, 10), and that liquification of the cell, a specific phase of cellular differentiation, may be involved (10). Certain compounds that have been considered as possible lignin precursors are often found to accumulate in tissues of boron-deficient plants. Hillis (11) and Reeve (12) have shown relationship between phenolic compounds and lignification. Reed (13) has demonstrated that boron-deficient plants are high in phenolic compounds. Spurr (14) noted that boron-deficient celery leaves and petioles fluoresced in ultraviolet light, while Perkins and Aronoff (15) identified the

blue fluorescent materials in boron-deficient lettuce, radish, tomato and sunflower plants as chlorogenic acid and caffeic acid.

Sunflower leaves were previously investigated by Urban (16) who has reported the presence of scopolin, chlorogenic acid, caffeic acid and an unknown flavonoid. Watanabe (17) has found scopolin, chlorogenic acid, isoquercitrin, scopoletin, esculin and a gentisic acid glucoside in sunflower leaves.

In order to obtain additional information on the accumulation of polyphenolic compounds in the boron-deficient tissues, leaves of the sunflower, Helianthus annus, Russian mammoth variety, were investigated. Comparison of chromatograms under ultraviolet light (3660 Å) between extracts from normal tissue and boron-deficient tissue clearly indicated a marked increase in the overall blue fluorescent materials in the boron-deficient extracts. This report describes the extraction from sunflower leaves and identification of three caffeic acid esters, chlorogenic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3 - (3,4-dihydroxycinnamate)), isochlorogenic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 5 - (3,4-dihydroxycinnamate)) and neochlorogenic acid.

Chlorogenic acid was first obtained in crystalline form by Gorter in 1909 (18) from green coffee beans. Its structure was established later by Fischer and Dangschat (19). In 1950, Barnes, Feldman and White (20) isolated an isomer of chlorogenic acid from green coffee and named it "isochlorogenic acid." They showed that the most probable structure for isochlorogenic acid is that of 5-caffeoylquinic acid. Neochlorogenic acid was first extracted from peaches by Corse (21) in 1953 and shown to be also an isomer of chlorogenic acid. The position of attachment of the caffeoyl group to the quinic acid was not determined.

## Extraction of Sunflower Leaves

The plant materials used in the present study were grown hydroponically in the greenhouse at Argonne National Laboratory. One group of plants was given minus-boron solution and the second group was grown on plus-boron solution. At harvest time, the leaves at the third and fourth node were collected separately from each group, frozen in liquid nitrogen and put into plastic containers. These samples were then packed in dry ice and shipped to this laboratory.

Equal amounts (194 g.) of minus-boron and plus-boron leaves were extracted twice by boiling the mixture with 1 l.

of 85% isopropyl alcohol-water for 5 min. The extracts were filtered, concentrated in vacuo at 37°C to a low volume, and then made up to 250 ml. with isopropyl alcohol in a volumetric flask. Aliquots (100 λ) from each extract were examined individually by two-dimensional chromatography on Whatman No. 1 paper. The chromatograms were developed first in butyl alcohol-acetic acid-water (6:1:2 v/v/v), and then in isopropyl alcohol-formic acid-water (50:1:950 v/v/v). Twenty fluorescent spots were observed on the final chromatogram with the aid of ultraviolet light (3660 Å, Blak Ray, Ultraviolet Prod. Inc., San Gabriel, Calif.) (Figure 3). The extracts were then concentrated in vacuo at 35°C to remove most of the organic solvents, and the aqueous concentrates were extracted several times with benzene to remove green pigments and lipid material. The final aqueous phase was made up to 250 ml. with isopropyl alcohol in a volumetric flask. Aliquots from each extract were reexamined individually by two-dimensional chromatography as before. The boron-deficient extract showed a much higher intensity of blue fluorescing compounds under ultraviolet (3660 Å) than did the plus-boron extract (Figure 4).

The minus-boron fraction was poured on a (4.5 x 10 cm.) magnesol column which had been previously packed under

FIGURE 3. TWO-DIMENSIONAL CHROMATOGRAM OF EXTRACT FROM SUNFLOWER LEAVES

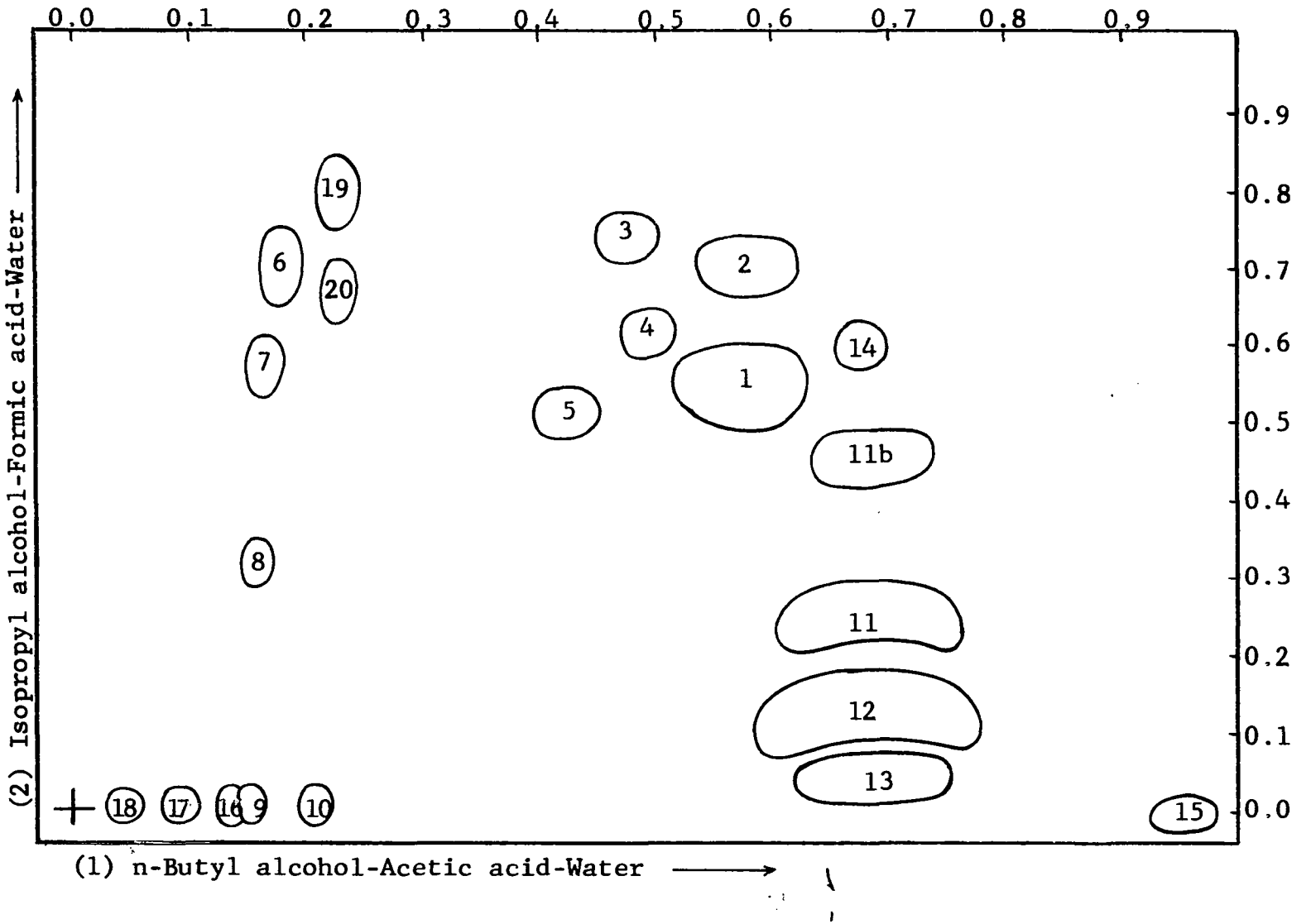
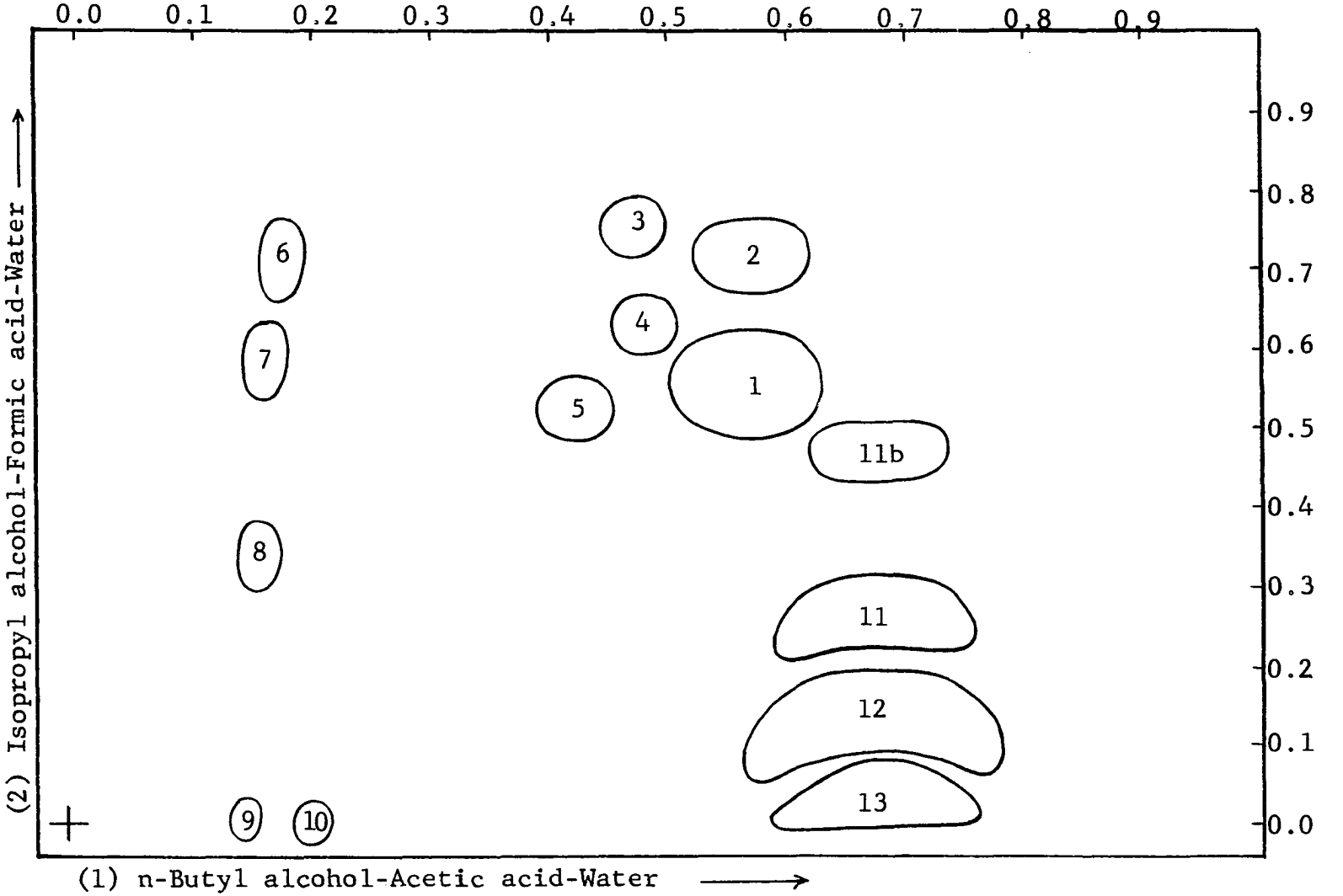


FIGURE 4. TWO DIMENSIONAL CHROMATOGRAM OF LIPID-FREE EXTRACT FROM SUNFLOWER LEAVES



5 lb. pressure with distilled water. The yellow band was developed with distilled water to produce two yellow zones, fraction A and fraction B, which were eluted off separately. Two-dimensional chromatography of fraction A showed essentially the presence of 13 spots under ultraviolet light, while fraction B showed 6 spots.

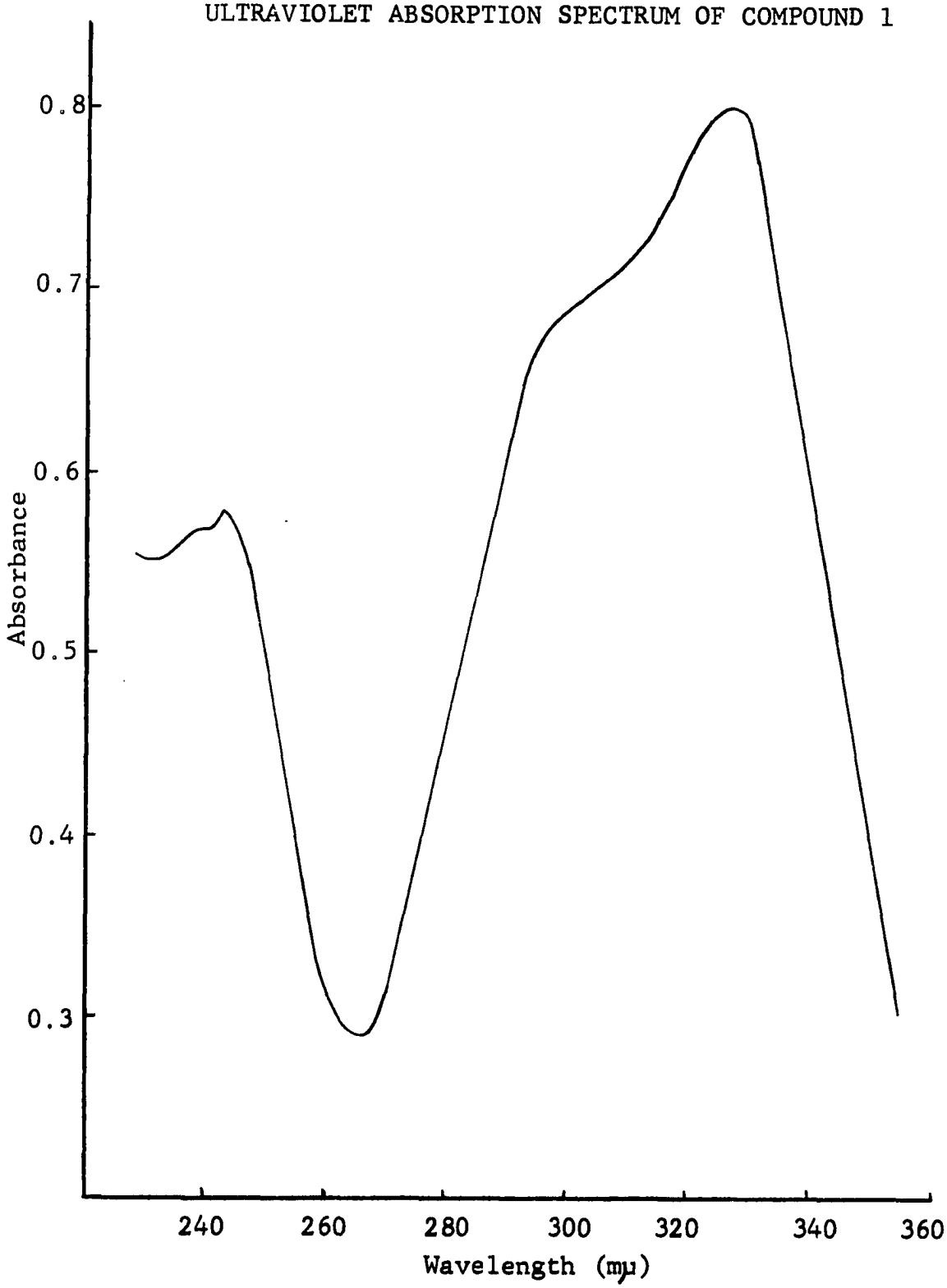
## Isolation of Chlorogenic Acid

Fraction A, the faster moving band from the magnesol column, was streaked on 10 sheets of Whatman 3 MM paper and developed in n-butyl alcohol-acetic acid-water (6:1:2 v/v/v). The broad band at $R_f$ 0.75 was cut out and eluted with methyl alcohol-water (1:1 v/v). The extracts were concentrated and streaked on 10 sheets of Whatman 3 MM paper and developed in butyl acetate-acetic acid-water (4:1:5 v/v/v) for 20 hr. The broad band at $R_f$ 0.70 (called "Compound 1") was separated from a narrow one at $R_f$ 0.25 (called "Compounds 3 and 4"). "Compound 1" was eluted off the paper with 70% isopropyl alcohol and the extract was taken to dryness *in vacuo* at 35°C. The resulting solid was dissolved in a small amount of methyl alcohol and poured on top of a (2.5 x 25 cm.) magnesol column previously packed under 5 lb. pressure with distilled methyl alcohol. The column was developed with one column length of

methyl alcohol followed by methyl alcohol-water (1:1 v/v). A broad yellow band which fluoresced greenish-blue under ultraviolet light (3660 Å) was collected. The absorption spectrum of the aqueous methanol eluate showed maxima at 242 and 326 mμ, a shoulder at 300 mμ, and a minimum at 265 mμ (Figure 5). This spectrum was identical to that of chlorogenic acid (CHR grade, Fluka, Switzerland) in methyl alcohol-water (1:1 v/v). Hulme (22) reported maxima at 220, 244, and 328 to 330 mμ, and a shoulder at 300 mμ in ethyl alcohol solution. The $R_f$ values of purified "Compound 1" and reference chlorogenic acid were identical in the solvent systems tested (Table 1). "Compound 1" was hydrolyzed with 10 ml. of 5% sodium hydroxide for 20 min. at room temperature. The yellow solution was then acidified with concentrated hydrochloric acid and extracted three times with ethyl acetate. The ethyl acetate extract was concentrated and studied chromatographically on Whatman No. 1 paper. After the evaporation of the aqueous phase, the dry crystalline residue was extracted with absolute ethyl alcohol (5 ml.). The extract was filtered, concentrated and studied chromatographically. The $R_f$ values of the ethyl acetate extract and reference caffeic acid, and those of the ethyl alcohol extract and reference quinic acid and quinide, were identical in the solvent systems tried

FIGURE 5

ULTRAVIOLET ABSORPTION SPECTRUM OF COMPOUND 1



(Table 1). On the basis of the hydrolysis products, $R_f$ values and ultraviolet spectrum, "Compound 1" is concluded to be chlorogenic acid.

## Isolation of Neochlorogenic Acid

"Compounds 3 and 4" were further purified by streaking on 7 sheets of Whatman 3 MM paper and developing the chromatograms in butyl alcohol-acetic acid-water (6:1:2 v/v/v). This procedure was repeated on 9 sheets of Whatman 3 MM paper, and then on 8 sheets of Whatman No. 1 paper in isopropyl alcohol-formic acid-water (50:1:950 v/v/v). "Compound 4," running at $R_f$ 0.62, was cut out and eluted off with alcohol. The extract was taken to dryness in vacuo, dissolved in a small amount of methyl alcohol and purified on a magnesol column by the procedure previously described for "Compound 1." The absorption spectrum of the eluate showed a maximum at 326 mμ, shoulders at 242 and 300 mμ, and a minimum at 265 mμ (Figure 6). An absorption spectrum of neochlorogenic acid in methyl alcohol-water (1:1 v/v) gave maxima at 242 and 326 mμ, a shoulder at 300 mμ, and a minimum at 265 mμ (Figure 7). The $R_f$ values of purified "Compound 4" and reference neochlorogenic acid were identical in the solvent systems tested (Table 1). On the basis of $R_f$ values

FIGURE 6

ULTRAVIOLET ABSORPTION SPECTRUM OF COMPOUND 4

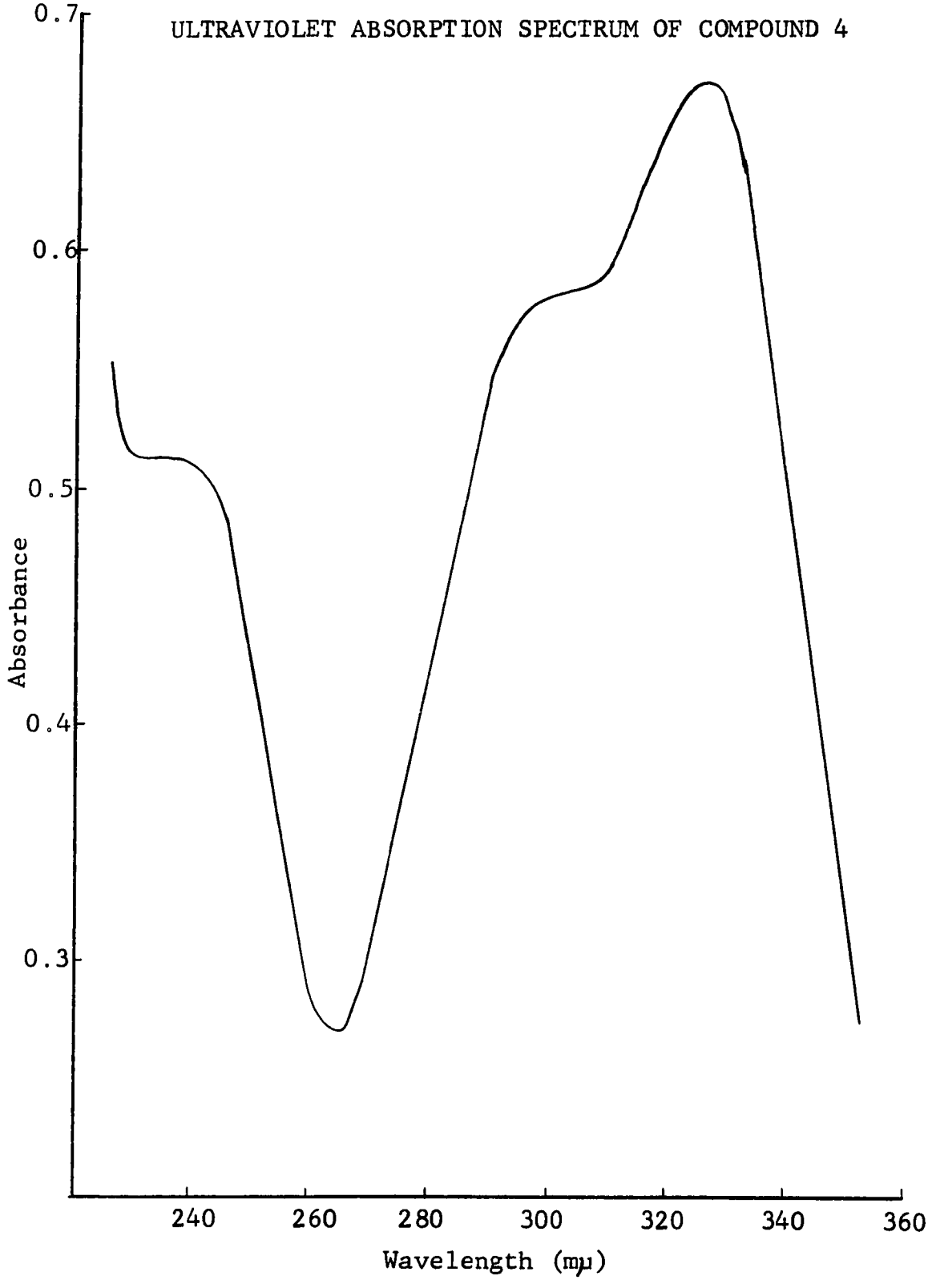
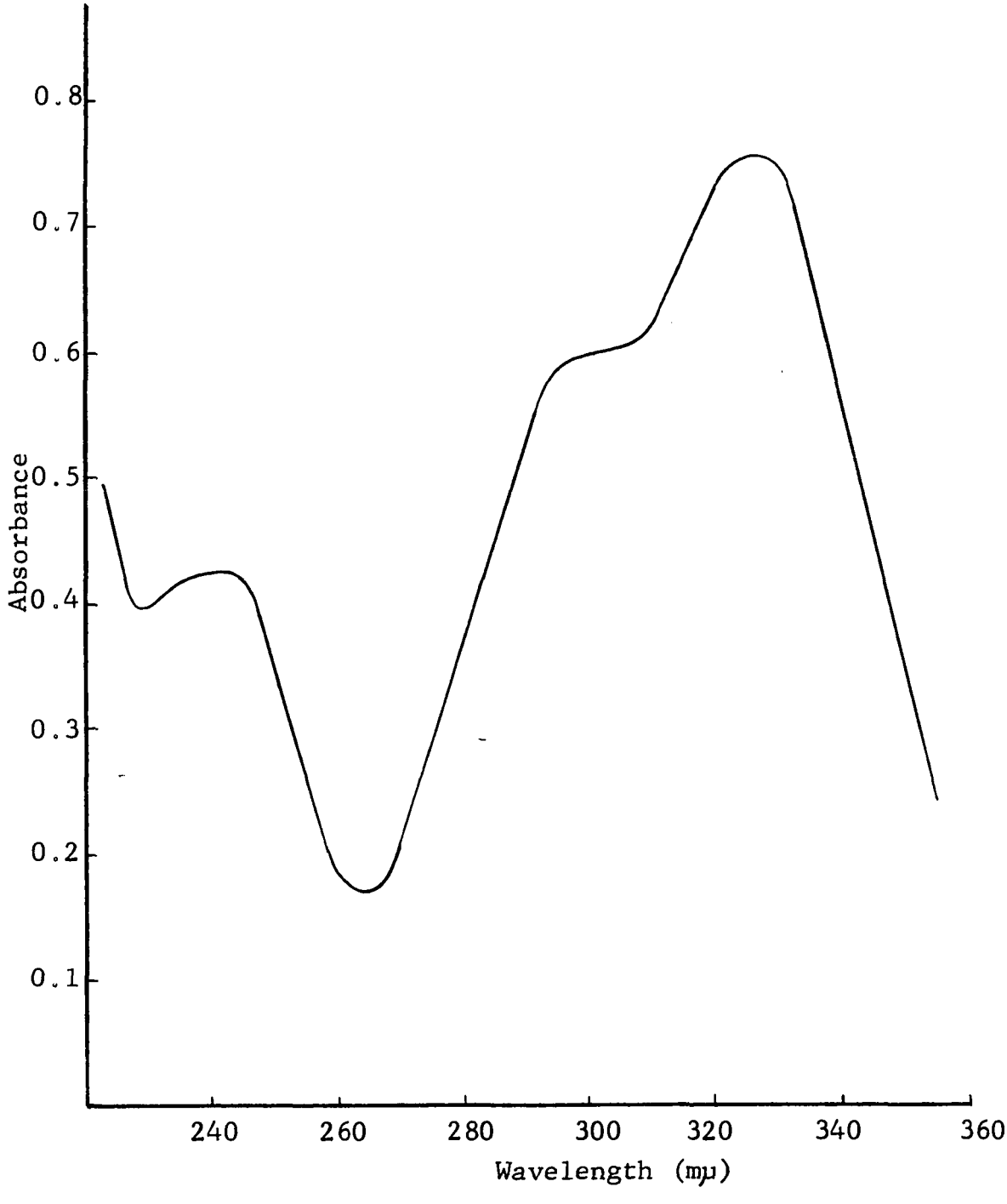


FIGURE 7

ULTRAVIOLET ABSORPTION SPECTRUM OF NEOCHLOROGENIC ACID



and ultraviolet spectrum, "Compound 4" has been tentatively identified as neochlorogenic acid.

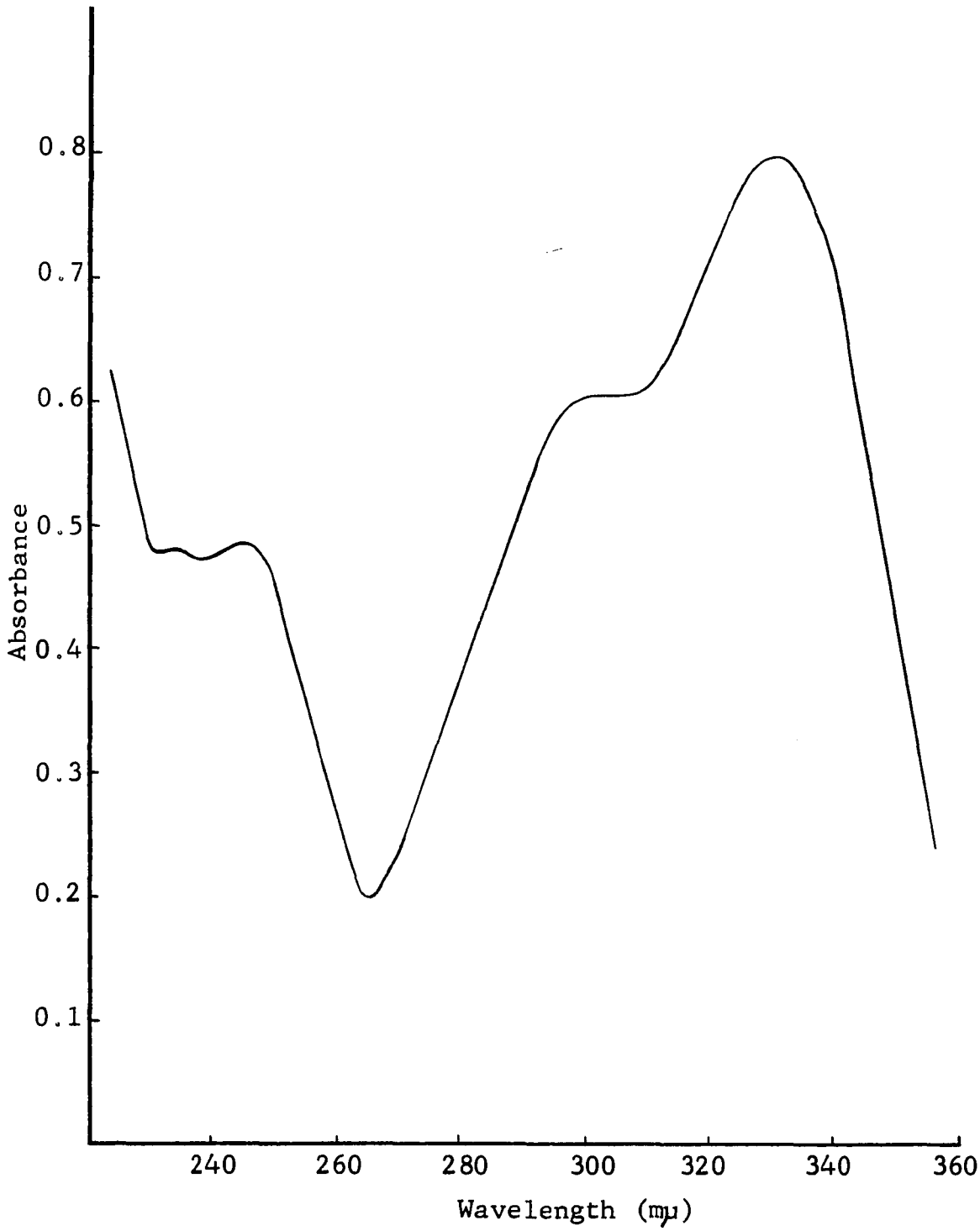
## Isolation of Isochlorogenic Acid

Fraction B, the slower moving band from the magnesol column, was concentrated *in vacuo* and streaked on 20 sheets of Whatman 3 MM paper and developed in isopropyl alcohol-formic acid-water (50:1:950 v/v/v) to give three bands. The broadest and most intense band under ultraviolet light at $R_f$ 0.22 (called "Compound 12") was cut out and eluted off with 70% isopropyl alcohol. The eluate was concentrated to a low volume and extracted twice with ethyl acetate. The extracts were dried over anhydrous sodium sulfate and taken to dryness *in vacuo*. The absorption spectrum of an alcoholic extract of "Compound 12" showed maxima at 245 and 350 mμ, a shoulder at 300 mμ, and a minimum at 265 mμ (Figure 8).

Hydrolysis of "Compound 12" was carried out by the procedure previously described for "Compound 1." The $R_f$ values of the ethyl acetate extract and reference caffeic acid, and those of the ethyl alcohol extract and reference quinic acid and quinide, were identical in the solvent systems tried (Table 1). On the basis of hydrolysis products, $R_f$ values and ultraviolet spectrum, "Compound 12" is

FIGURE 8

ULTRAVIOLET ABSORPTION SPECTRUM OF COMPOUND 12



concluded to be isochlorogenic acid.

## Isolation of "Compound 5"

Another compound was found in both the minus-boron and plus-boron extracts. It was isolated from the plus-boron extract by paper chromatography. Twenty sheets of Whatman 3 MM paper were streaked with plus-boron extract of sunflower leaves and the chromatograms were developed in isopropyl alcohol-formic acid-water (50:1:950 v/v/v). The broad chlorogenic acid band at $R_f$ 0.75 was cut out and eluted with 70% isopropyl alcohol. The concentrated eluate was reapplied on 20 sheets of Whatman 3 MM paper and developed in n-butyl alcohol-acetic acid-water (6:1:2 v/v/v). Three bands were formed. The middle band at $R_f$ 0.42 was cut out, eluted with 70% isopropyl alcohol, reapplied on 10 sheets of Whatman No. 1 paper, and developed in isopropyl alcohol-formic acid-water (50:1:950 v/v/v). The major fraction at $R_f$ 0.48 (called "Compound 5") was cut out and eluted with 70% isopropyl alcohol. The eluate was taken to dryness *in vacuo* at 35°C and the pale yellow residue dissolved in some methyl alcohol and purified on a magnesol column by the procedure previously described for "Compound 1." The absorption spectrum of the aqueous methanol eluate showed maxima at 242 and

and 330 mμ, a shoulder at 300 mμ, and a minimum at 270 mμ (Figure 9).

"Compound 5," like chlorogenic acid, isochlorogenic acid and neochlorogenic acid, fluoresces blue in ultraviolet light (3660 Å) and in the presence of ammonia vapor, greenish-blue. No further work has been done on this material.

FIGURE 9

ULTRAVIOLET ABSORPTION SPECTRUM OF COMPOUND 5

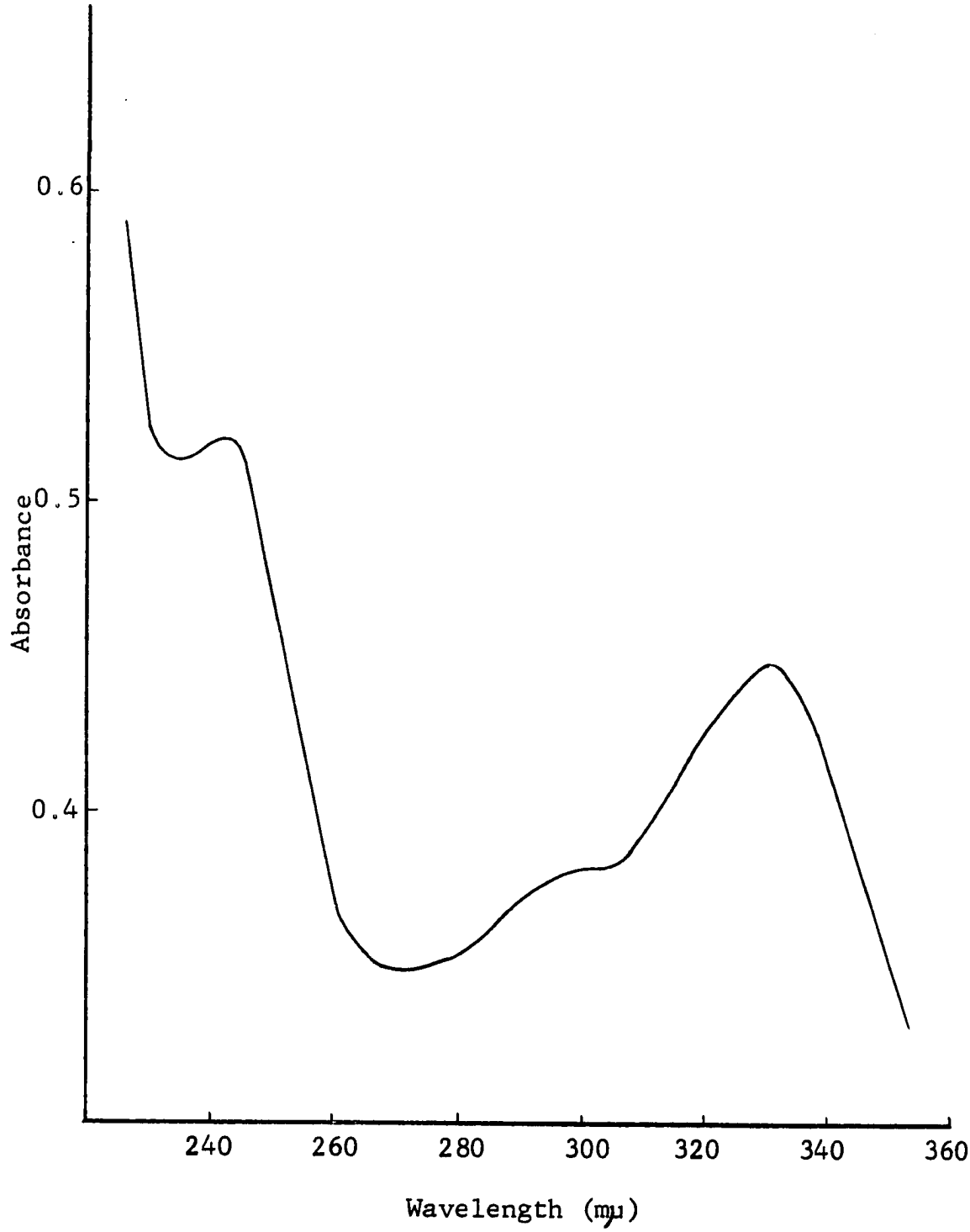


TABLE 1

$R_f$ VALUES OF REFERENCE PHENOLIC COMPOUNDS

| Compounds | Solvent Systems[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Chlorogenic acid | 0.68 | 0.57<br>0.78 | 0.75<br>0.82 | 0.42 | 0.19 | 0.54 | 0.49 |
| Isochlorogenic acid | 0.80 | 0.06<br>0.12 | 0.42<br>0.56 | 0.78 | 0.55 | 0.65 | 0.74 |
| Neochlorogenic acid | 0.57 | 0.62<br>0.80 | 0.78<br>0.81 | 0.22 | 0.08 | 0.47 | 0.49 |
| Caffeic acid | 0.84 | 0.36 | 0.54<br>0.61 | 0.86 | 0.75 | 0.76 | 0.57 |
| Quinic acid | 0.24 | 0.90 | 0.89 | 0.06 | 0.00 | 0.50 | 0.08 |
| Quinide | 0.51 | 0.70 | 0.89 | 0.24 | 0.16 | 0.70 | 0.70 |

[a]Solvent systems: (1) butyl alcohol-acetic acid-water (6:1:2 v/v/v); (2) isopropyl alcohol-formic acid-water (50:1:950 v/v/v); (3) 15% acetic acid; (4) isobutylmethyl ketone-formic acid-water (3:1:2 v/v/v); (5) butyl acetate-acetic acid-water (4:1:5 v/v/v); (6) isopropyl alcohol-acetic acid-water (6:1:2 v/v/v); (7) butyl alcohol-pyridine-benzene-water (5:3:1:3 v/v/v/v).

# CHAPTER IV

# PYROLYSIS PRODUCTS OF RUTIN, QUERCETIN AND CHLOROGENIC ACID

## Introduction

Rutin (quercetin-3-rutinoside) and chlorogenic acid (3-caffeoyl quinic acid) are among the principal polyphenols present in cigarette tobacco, but neither has been found in the smoke from cigarettes. It was, therefore, of interest to investigate the products of pyrolysis of these two compounds, as well as those of quercetin (3,3',4',5,7-pentahydroxyflavone), the aglycone of rutin.

Rapid, dry distillation of rutin produced catechol and smaller amounts of 4-methylcatechol, resorcinol, furfural, 5-hydroxymethylfurfural, and 5-methylfuran-2-aldehyde. Under similar conditions, quercetin produced catechol, 4-methylcatechol, resorcinol, and phloroglucinol. No phloroglucinol, however, has been found among the products of pyrolysis of rutin. Chlorogenic acid distillation yielded catechol,

4-methylcatechol, 4-ethylcatechol, benzoic acid, and quinide (quinic acid-$\gamma$-lactone). In addition, paper chromatographic analysis of the pyrolysis products of each compound indicated the presence of other, as yet unidentified, components.

Of the compounds listed above, catechol, resorcinol, furfural, and benzoic acid are included in a summary of compounds previously identified in cigarette smoke (23). Quinide (24) and 5-hydroxymethylfurfural (25) have also been found previously in cigarette smoke. The others have not previously been reported in cigarette smoke.

## Pyrolysis of Quercetin

Quercetin (10 g.; Nutritional Biochemicals Corp., Cleveland, Ohio) was heated at atmospheric pressure in a 50 ml. flask with a Bunsen burner in an all glass distillation unit (Metro Industries, ME 523), which had an 8 cm. distilling head and an 8 cm. water-cooled jacket. As quercetin melted, foamed, and charred, yellow fumes were evolved. After about 5 min., all of the original quercetin had blackened on decomposition, and the distillation had ceased. When a thermometer was inserted into the midst of the molten mixture in the flask, readings up to 600°C were registered before the heating was discontinued. The yellow distillate

(2 ml.) was extracted with ether, and the extracts were dried over anhydrous sodium sulfate. On removal of the ether *in vacuo*, a semi-crystalline orange-colored residue resulted. Extraction of this residue with three 10 ml. portions of benzene gave, on slow evaporation in air, a brown crystalline solid (350 mg., called "Q-1"). The material that had not dissolved in benzene was now extracted with 20 ml. water. The aqueous yellow solution was then extracted five times with 15 ml. portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate and taken to dryness *in vacuo* to yield 20 mg. of a yellow solid, called "Q-2".

Fraction "Q-1" was further fractionated on a 41 cm. x 6 cm. diameter column of silicic acid (Mallinckrodt No. 2847) prepared from a slurry of 2:1 (w/w) silicic acid-water in benzene under 4 lb. pressure. 200 mg. "Q-1", dissolved in 4 ml. ether and diluted to 80 ml. with benzene, was added to the column and the resulting pale yellow band developed under pressure with benzene-ether (95:5 v/v) saturated with water. Two pale yellow bands moved down the column. The faster band gave a fraction (380 ml.) which on evaporation *in vacuo* yielded a small amount of an orange-red oil, called "Q-1-A". The slower moving band (600 ml.) produced grayish-

white crystals (130 mg., called "Q-1-B"), after removal of the solvent. Further elution of the column gave a very small amount of a white solid, called "Q-1-C".

"Q-1-A" was sublimed in vacuo to produce a white solid which has been identified as 4-methylcatechol. Its ultraviolet absorption spectrum showed a maximum at 283 mμ in ethyl alcohol, as did the reference 4-methylcatechol. The $R_f$ values of "Q-1-A" and authentic 4-methylcatechol were identical when chromatographed together in the solvent systems studied (Table 2). The detecting spray reagent used in all these experiments was the stabilized diazo salt of p-nitroaniline (Fast Red Salt GG) (26). The color produced was a reddish-orange with a bluish-purple rim.

"Q-1-B" was sublimed in vacuo to yield a white crystalline solid, m. p. 104-106°C. A mixed melting point determination with authentic sublimed catechol gave no depression. All melting points listed in this work are uncorrected. $R_f$ values of "Q-1-B" and of authentic reference catechol were identical (Table 2) on the same paper chromatographs.

The $R_f$ values of "Q-1-C" (Table 2) and the yellow color produced with the spray reagent corresponded to those of reference resorcinol.

Fraction "Q-2" contained several compounds, including

catechol and 4-methylcatechol. To this fraction was added 2.4 ml. ether, followed by 50 ml. benzene. The solution was concentrated to 40 ml., and the precipitate which formed was filtered off and washed with benzene. The precipitate was dissolved in acetone and streaked on 8 sheets of Whatman 3 MM chromatography paper, and the chromatograms were developed in 2% acetic acid. The main band fluorescing blue with ammonia vapor as observed with ultraviolet light (3660 Å) was cut out and extracted with ether in a Soxhlet extractor. After removal of ether, the pale yellow residue was placed on a column packed with silicic acid-water (2:1 v/v) as before. After a blue fluorescent band was removed, the column was eluted with ether. A small amount of white solid, now pure "Q-2", was obtained from the ether eluate. Pure "Q-2" was identified as phloroglucinol. Its ultraviolet absorption spectrum in ethyl alcohol exhibited a maximum at 268 mμ, corresponding to that obtained with authentic, sublimed phloroglucinol. The $R_f$ values of "Q-2" and reference phloroglucinol when chromatographed together were identical in the solvent systems tested (Table 2), as was also the orange color produced in each case by the spray reagent.

## Pyrolysis of Rutin

Rutin (10 g.: Penick and Co., New York) was pyrolyzed by the same procedure described for quercetin. The distillate (3 ml.) was diluted with water and extracted several times with ether. The ether extract was dried over anhydrous sodium sulfate. The ether was removed *in vacuo* to leave an orange oil (1 g., called "R-1").

Fraction "R-1" was dissolved in 20 ml. ethyl alcohol, and a freshly prepared 2,4-dinitrophenylhydrazine solution was added to give a brick-red precipitate immediately. After remaining 15 min. at room temperature, the precipitate was filtered off, washed with aqueous ethyl alcohol, and dried to a brick-red powder (0.28 g., called "R-2").

The 2,4-dinitrophenylhydrazone fraction, "R-2", was fractionated on a 36 x 6 cm. diam. column of 2:1 (w/w) silicic acid-"Super Cel" (Fisher) in hexane under 5 lb. pressure. "R-2" was dissolved in 100 ml. warm benzene and diluted with 100 ml. hexane. A small amount of precipitate was filtered off, and the filtrate was added to the top of the adsorbent to form an orange band. On development with hexane-ether (96:4 v/v) under 5 lb. pressure, several colored bands appeared and were eluted separately. After removal of the solvent *in vacuo* from the eluate containing the fastest moving band

(yellow), an orange powder was obtained (19 mg., called "R-2-1"). After evaporation of the solvent from the eluate containing the next eluted band (a broad orange band), dark red needles resulted (48 mg., "R-2-2"). When the "R-2-2" was chromatographed still another time on a fresh column, two compounds were separated. The first one from the column produced a dark red, crystalline material called "R-2-2-1" (9 mg.) and the other gave 10 mg. of a red solid called "R-2-2-2".

Compound "R-2-1" was crystallized from ethyl alcohol-ethyl acetate to produce fine orange needles, m. p. 216-217°C. No depression resulted on a mixed melting point determination with the 2,4-dinitrophenylhydrazone of 5-methylfuran-2-aldehyde.

Compound "R-2-2-1" was crystallized twice from ethyl alcohol-ethyl acetate to give fine, short, black needles (5 mg.) m. p. 199-200°C. This 2,4-dinitrophenylhydrazone derivative has not yet been identified.

Compound "R-2-2-2" gave a red crystalline product (5 mg.), m. p. 225-227°C. On mixed melting point determination with the synthetic 2,4-dinitrophenylhydrazone of furfural, no depression resulted.

When the original "R-1" was chromatographed two-

dimensionally, first in benzene-acetic acid-water (6:7:3 v/v/v) and then in 2% acetic acid-water and subsequently sprayed with Fast Red Salt GG, no phloroglucinol was found.

Fraction "R-1" was streaked on chromatography paper and developed in chloroform-formic acid-water-methyl alcohol (500:2:48:50 v/v/v/v). The zone at $R_f$ 0.74 was cut out and eluted first with acetone and then with methyl alcohol. The eluate was concentrated and analyzed. $R_f$ values (Table 2) on the same paper chromatograms, and color produced with 2,4-dinitrophenylhydrazine spray (27) corresponded to those exhibited by authentic 5-hydroxymethylfurfural.

An aliquot (1 g.) of fraction "R-1" was chromatographed on a silicic acid column by the procedure previously described for quercetin. Pale yellow bands were formed and eluted separately. Each eluate was studied by paper chromatography, using the benzene-acetic acid-water solvent system. The zone at approximately $R_f$ 0.50 yielded an orange oil (45 mg., called "R-1-A"). Sublimation of "R-1-A" gave a product identical with the reference 4-methylcatechol in $R_f$ values (Table 2), ultraviolet spectrum, and color developed with spray used. A zone with an $R_f$ value 0.38 was chromatographed on another silicic acid column to give an eluate from which a semi-crystalline product (90 mg., called "R-1-B") was obtained.

Sublimation at 50°C *in vacuo* yielded a white solid, m. p. 104-106°C. On mixed melting point determination with reference sublimed catechol, no depression was observed. $R_f$ values and color produced on the same chromatograms also corresponded to those of authentic catechol.

The zone with $R_f$ value of approximately 0.16 yielded a small amount of a yellow oil (called "R-1-C"). The $R_f$ values (Table 2), and also the yellow color produced with the spray reagent agreed with those of reference resorcinol.

## Pyrolysis of Chlorogenic Acid

Chlorogenic acid (4 g. CHR grade, Fluka, Switzerland) was pyrolyzed in a 20 ml. flask by the same procedure as described for quercetin. The orange distillate (1.3 ml.) was diluted with water and extracted five times with 15 ml. portions of ether. The ether solution was dried over anhydrous sodium sulfate and the solvent was removed *in vacuo* to produce a reddish-orange oil (called "C-1"). The aqueous fraction was allowed to evaporate in air to yield a pale yellow semi-crystalline solid (called "C-2").

Fraction "C-1" was chromatographed using silicic acid by the procedure described previously for the fraction "Q-1". The first fraction collected from the column, after

evaporation *in vacuo*, yielded an orange oil, "C-1-A". The next fraction produced a yellow solid "C-1-B". This was followed by two fractions which gave, after evaporation, yellow, oily compounds "C-1-C" and "C-1-D", respectively.

After rechromatography for greater purification on another column containing silicic acid, "C-1-A" produced a pale yellow crystalline solid (19 mg., "C-1-A-1"). Sublimation of "C-1-A-1" *in vacuo* at 60°C produced a white crystalline solid (15 mg.), m. p. 121-122.5°C. On mixed melting point determination with authentic sublimed benzoic acid, no depression resulted.

Rechromatography of "C-1-B" on a silicic acid column gave a yellow oil, which on analysis by paper chromatography, gave an approximate $R_f$ value of 0.64 in the benzene-acetic acid-water system. This oil was purified by streaking it on five sheets of Whatman 3 MM paper and developing these chromatograms in 2% acetic acid-water. The dull purple band ($R_f$ 0.75) as seen under short wavelength ultraviolet light (2537 Å) was cut out and extracted with ether in a Soxhlet extractor. The pale yellow oil was further purified by column chromatography using silicic acid as described previously. The eluate with $R_f$ 0.64 in benzene-acetic acid-water system was taken to dryness *in vacuo* to produce a pale yellow oil,

which proved to be identical with an authentic preparation of 4-ethylcatechol in $R_f$ values (Table 2), color of spot (orange-red with bluish-purple rim) produced with the Fast Red Salt GG reagent, and in ultraviolet absorption spectrum (max. 283 mμ and min. 250 mμ).

Fraction "C-1-C" was further purified by silicic acid chromatography. The eluate was taken to dryness in vacuo to yield a yellowish orange oil (10 mg.). Sublimation at 45°C in vacuo gave a product identical with 4-methylcatechol in $R_f$ values when chromatographed together, in color, and ultraviolet spectra.

After removal of solvents from fraction "C-1-D", a yellow crystalline product (117 mg.) resulted. Sublimation at 60°C in vacuo gave white crystals, m. p. 104-106°C. A mixed melting point with authentic catechol was not depressed. Also, $R_f$ values (Table 2) on the same paper chromatograms and color reactions were identical to those of reference catechol.

$R_f$ values (Table 2) of compound "C-2" were identical with those of authentic quinide when chromatographed together. Both gave a positive Cartwright-Roberts test (28).

TABLE 2

$R_f$ VALUES OF SOME PYROLYSIS PRODUCTS

| Compounds | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Catechol | 0.38 | 0.79 | 0.50 | 0.89 |
| 4-Ethylcatechol | 0.60 | 0.75 | 0.74 | 0.94 |
| 5-Hydroxymethylfurfural | 0.34 | 0.85 | 0.74 | |
| 4-Methylcatechol | 0.49 | 0.77 | 0.60 | 0.92 |
| Phloroglucinol | 0.03 | 0.65 | 0.00 | 0,75 |
| Quinide | 0.00 | 0.88 | 0.10 | 0.53 |
| Resorcinol | 0.15 | 0.76 | 0.19 | 0.90 |

[a]Solvent systems: (1) benzene--acetic acid--water (6:7:3 v/v/v); (2) 2% acetic acid--water; (3) chloroform--acetic acid--water (2:1:1 v/v/v); and (4) n-butyl alcohol--acetic acid--water (6:1:2 v/v/v).

# CHAPTER V

# PREPARATION OF SCOPOLETIN-4-$C^{14}$ AND SCOPOLIN

## Introduction

One of the most important isotopes that is used in the biological fields today is $C^{14}$. Two of the inherent advantages of this isotope have to do with its long half-life and with its comparatively soft radiation. The half-life of $C^{14}$ is approximately 5,600 years (29) and therefore requires no decay corrections in assay procedures. The average range of the negative beta radiation from $C^{14}$ is about 4-6 cm. in air with a maximum energy of approximately 0.15 million-electron-volts (30). This soft radiation keeps the radiation damage of living tissue at a very low level, and gives the scientist a chance to follow the specific atom or grouping throughout a metabolic pathway.

In this work the synthesis of labelled scopoletin was undertaken as part of a long range program of studying the metabolic fate of coumarins in biological systems.

Scopoletin (7-hydroxy-6-methoxycoumarin) is widely found in higher plants, usually in the form of the 7-glucoside, scopolin. It was decided to have the labelled carbon atom in the 4 position. If the molecule is metabolized by a biological system, there should be a good chance that the labelled atom will follow a fragment that might stay intact and that will be readily identified.

Scopolin, the 7-glucoside of scopoletin, was also prepared for biological and assay studies.

## Dilution of Potassium Cyanide-$C^{14}$

Potassium cyanide-$C^{14}$ (11.55 mg., 0.17 mM., 16.8 mc./mM.; Volk Radiochemical Co., Skokie, Illinois) was dissolved in distilled water (2.5 ml.) which contained non-radioactive potassium cyanide (1.56 g., 24 mM.; Baker 96.9%). The colorless solution was used in the preparation of zinc cyanide-$C^{14}$.

## Preparation of Zinc Cyanide-$C^{14}$

To the potassium cyanide-$C^{14}$ solution were added 3 drops of a saturated magnesium chloride solution. A small amount of a white precipitate that formed was filtered off immediately, and the filtrate was added to a solution

containing 1.8 g. (0.013m) of zinc chloride (Merck, Reagent Grade) dissolved in 6 ml. of 50% ethyl alcohol. The white precipitate of zinc cyanide-$C^{14}$ formed immediately and was filtered off. After washing the precipitate with ethyl alcohol and ether, and drying in a desiccator over phosphorus pentoxide, a white powder (1.27 g., 90%) was obtained.

## Preparation of 5-Hydroxyguaiacol

5-Hydroxyguaiacol (1,3-dihydroxy-4-methoxybenzene) was prepared by the method of Drake et al. (31). A yield of 20-30% of a white crystalline product with m. p. 71.5-72.5°C (lit. 72°C) was obtained.

## Preparation of 2,4-Dihydroxy-5-methoxy-benzaldehyde-1-$C^{14}$

Dry hydrogen chloride was passed through a mixture of 5-hydroxyguaiacol (1.0 g., 0.007 m.), zinc cyanide-$C^{14}$ (1.27 g., 0.011 m.) and dry ether (20 ml.) for 1.5 hr. The reaction mixture was worked up according to the procedure of Head and Robertson (32). Short yellow needles (1.07 g., 89%) with m. p. 152-153°C (lit. 152°C) were obtained.

## Preparation of 6-methoxy-7-acetoxycoumarin-4-$C^{14}$

2,4-Dihydroxy-5-methoxybenzaldehyde-1-$C^{14}$ (1 g., 0.06 m.) was condensed with acetic anhydride (5 ml., 0.06 m.) in the presence of fused sodium acetate (2 g., 0.02 m.) at 180°C. The reaction mixture was worked up by the procedure of Head and Robertson (32). A yield of 0.50 g. (36%) of a pale yellow crystalline product was obtained with m. p. 177-178°C (lit. 177°C).

## Preparation of Scopoletin-4-$C^{14}$ (6-methoxy-7-hydroxycoumarin-4-$C^{14}$)

A mixture of 6-methoxy-7-acetoxycoumarin (0.50 g., 0.002 m.), methyl alcohol (10.7 ml.) and concentrated hydrochloric acid (7.2 ml.) was refluxed for 15 min. The reaction mixture was then processed by the method of Head and Robertson (32). A greenish-yellow crystalline product (0.290 g., 71%) was obtained.

## Purification of Scopoletin-4-$C^{14}$

A 2.5 cm. diameter column was packed to a depth of 25 cm. with magnesol in methyl alcohol under 5 lb. pressure. Crude scopoletin-4-$C^{14}$ (0.14 g.), dissolved in 20 ml. of

methyl alcohol, was added to the column and developed under pressure with ethyl acetate saturated with water. A major yellow band was eluted off to yield, after the removal of the solvent *in vacuo*, a pale yellow product (0.120 g.) with m. p. 203-206°C (lit. 204°C). This product was further purified on a similar column using silicic acid as absorbent. The column was packed 25 cm. deep with silicic acid in benzene under 5 lb. pressure. One hundred mg. of scopoletin-4-$C^{14}$, dissolved in 4 ml. acetone and diluted to 25 ml. with benzene, was added to the column and the resulting pale yellow band developed under pressure with benzene-acetone (84:16 v/v). Four bands, visible even under ultraviolet light, moved down the column. The fastest moving band gave a fraction which, on evaporation *in vacuo*, yielded a very pale yellow crystalline product (75 mg.).

Column-purified scopoletin-4-$C^{14}$ (140 mg.) was then crystallized from 5 ml. hot acetic acid. The white crystalline product (100 mg.) showed m. p. 205-206°C (lit. 204°C).

## Radioactive Assays and Procedures

The instrumentation and assay procedures used in this work are described below.

## Instrumentation

The instrumentation used in assaying for radioactivity consisted of a mica end-window Geiger-Mueller tube as the counter, mounted in a lead shield over a suitable sample holder, and an automatic scaling unit.

## Efficiency of the System

The efficiency of the counter was determined using the following formula:

$$\frac{\text{Recorded counts per minute - Background}}{\text{Disintegration per min. of the standard source}}$$

The standard source was a sample of barium carbonate-$C^{14}$ obtained from Nuclear Instrument and Chemical Corp. (Chicago) with specific activity of 18.6 counts per sec. per mg.

All samples were run on similar planchets, machined stainless steel cups, with a measured surface area of 4.7 $cm^2$. One mg. of the standard barium carbonate-$C^{14}$ was weighed out on a planchet and spread as uniformly as possible with some ethyl alcohol. After the evaporation of the alcohol, the sample was counted over a period of 20 min. and 646 counts were obtained. The background count was 365 counts in 20 min. By subtracting the background count from the sample count a value of 14 counts per min. per mg. was

obtained for the standard. The efficiency of this system was then calculated to be 1.26%.

## Assay of Scopoletin-4-$C^{14}$

Scopoletin-4-$C^{14}$ (1 mg.) was made up to 1 ml. with ethyl alcohol in a volumetric flask. One hundred λ (0.1 ml.) of the solution was pipetted into a planchet and allowed to evaporate to dryness at room temperature. Nine hundred and thirty-six counts per minute were recorded to give a total of $9.36 \times 10^5$ counts per min. per 100 mg. of sample. The absolute activity of the synthetic scopoletin-4-$C^{14}$ was calculated to be $7.43 \times 10^7$ counts per min. when using the following formula:

$$\frac{\text{Counter reading (c.p.m.)} \times 100}{\text{Percentage counter efficiency}}$$

As one microcurie is defined as $2.22 \times 10^6$ disintegrations per min. then the total amount of activity could be calculated from the following formula:

$$\frac{\text{Absolute activity (c.p.m.)}}{2.22 \times 10^6 \text{ (d.p.m. per 1 } \mu\text{c.)}}$$

A total activity of 33.47 μc. was obtained with a specific activity of 0.33 μc. per mg. scopoletin-4-$C^{14}$.

## Preparation of Scopolin

Scopoletin was prepared from esculin (6,7-dihydroxy-coumarin-6-$\beta$-D-glucoside) by the method of Braymer (33) with an average overall yield of 33% and m. p. 207°C. Scopolin was then synthesized from scopoletin by the procedure of Chaudhury (34) with an overall yield of 35% and m. p. 214.5-216°C.

# CHAPTER VI

# PREPARATION OF GENTISIC ACID MONOGLUCOSIDES

## Introduction

A gentisic acid glucoside has been reported by Watanabe (17) to be present in sunflower leaves extract. The position of attachment of the glucose unit in the molecule was not determined. Efforts have therefore been made to synthesize the hereto unknown glucosides of gentisic acid (2,5-dihydroxybenzoic acid) for use as chromatographic standards.

The three possible mono-β-D-glucosides of gentisic acid are gentisic acid-5-β-D-glucoside, gentisic acid-2-β-D-glucoside and gentisic acid-β-D-glucose ester.

Gentisic acid-5-β-D-glucoside and gentisic acid-2-β-D-glucoside were prepared by the deesterification of 2-hydroxy-5-β-D-glucopyranosyloxybenzoic acid and 5-hydroxy-2-β-D-glucopyranosyloxybenzoic acid methyl esters, respectively. The two methyl esters have been previously prepared by Wagner

(37) from gentisic acid. Gentisic acid-β-D-glucose ester has not been prepared.

## Preparation of 2-Hydroxy-5-β-D-glucopyranosyloxybenzoic acid methyl ester

2-Hydroxy-5-β-D-glucopyranosyloxybenzoic acid methyl ester was prepared from gentisic acid by the method of Wagner (35). In this procedure, 2,5-diacetylgentisic acid was first prepared in 70% yield by the acetylation of gentisic acid with acetic anhydride in the presence of sulfuric acid. The diacetate was partially hydrolyzed at 0°C by dilute aqueous ammonium hydroxide solution to give a 14-22% yield of 2-acetylgentisic acid, which was then methylated at 5°C with diazomethane in ether to produce methyl 2-acetylgentisate in 53-63% yield. α-Acetobromoglucose and methyl 2-acetylgentisate were condensed by the Koenigs-Knorr method to produce 2-acetoxy-5-(tetraacetyl-β-D-glucopyranosyloxy)benzoic acid methyl ester in 35-40% yield. Deacetylation of the pentaacetate in 0.1 N methanolic sodium methoxide solution produced a 50-60% yield of 2-hydroxy-5-β-D-glucopyranosyloxybenzoic acid methyl ester. Recrystallization of the product from water yielded a white granular crystalline product with m. p. 175-177°C (lit. 177-178.5°C).

## Preparation of 2-Hydroxy-5-β-D-glucopyranosyloxybenzoic acid

2-Hydroxy-5-β-D-glucopyranosyloxybenzoic acid methyl ester (4.3 g., 0.013 m.) was dissolved in barium hydroxide (0.43 N, 210 ml., 0.18 m.) solution, and allowed to stand for 5 hr. at room temperature. The reaction mixture was then neutralized with an equivalent amount of oxalic acid solution. The precipitate was filtered off after 1 hr., and the clear pale yellow filtrate was concentrated in vacuo at 38°C until crystallization occurred. The mixture was cooled overnight at 5°C. On the next day, the white needle-like crystalline product was filtered off and washed with a small amount of ice cold water and dried at room temperature; yield, 3.8 g. (92.7%), with m. p. 97-100°C. The product was dissolved in ethyl alcohol (30 ml.) and the solution was filtered. The filtrate was taken to dryness in vacuo and the resulting white product was crystallized twice from hot water (30 ml.) to produce white needles (1.9 g.), with m. p. 98-100°C.

Anal. Calcd. for $C_{13}H_{16}O_9 \cdot 2H_2O$ (352.29): C, 44.32%; H, 5.72%. Found: C, 44.51%; H, 5.69%.

Drying a sample (27.91 mg.) for 6 hr. over phosphorus pentoxide at 100°C in vacuo produced a white opaque material (24.98 mg.). This material rapidly absorbed moisture from

the air to give a white product (27.01 mg.) within 15 min. and a m. p. 126-128°C. No increase in weight and melting point was experienced after 24 hr.

Anal. Calcd. for $C_{13}H_{16}O_9 \cdot 1\frac{1}{2}H_2O$ (343.28): C, 45.48%; H, 5.58%. Found: C, 45.21%; H, 5.60%.

Paper chromatography in various solvent systems showed the presence of only one compound which fluoresced blue under ultraviolet light. The product dissolved readily in 5% sodium bicarbonate solution with effervescence. Acid hydrolysis produced gentisic acid and glucose.

## Preparation of 5-Hydroxy-2-β-D-glucopyranosyloxybenzoic acid methyl ester

5-Hydroxy-2-β-D-glucopyranosyloxybenzoic acid methyl ester was prepared from gentisic acid by the method of Wagner (35). In this procedure, gentisic acid was acetylated in alkaline medium to produce a 90% yield of 5-acetylgentisic acid. Methylation of the product with diazomethane in ether at 5°C gave a 67-75% yield of methyl 5-acetylgentisate. Condensation of methyl 5-acetylgentisate with α-acetobromoglucose by the Koenigs-Knorr method produced a 45-50% yield of 5-acetoxy-2-(tetraacetyl-β-D-glucopyranosyloxy)benzoic acid methyl ester. Deacetylation of this pentaacetate in 0.1 N

methanolic sodium methoxide solution resulted in a 50% yield of 5-hydroxy-2-β-D-glucopyranosyloxybenzoic acid methyl ester. Crystallization of the product from methyl alcohol yielded a white crystalline material with m. p. 175-176°C (lit. 178-180°C).

## Preparation of 5-Hydroxy-2-β-D-glucopyranosyloxybenzoic acid

5-Hydroxy-2-β-D-glucopyranosyloxybenzoic acid methyl ester (10 g., 0.03 m.) was dissolved in barium hydroxide (0.43 N, 500 ml., 0.43 m.) solution and allowed to stand for 5 hr. at room temperature. The reaction mixture was then neutralized with an equivalent amount of oxalic acid solution. The precipitate was filtered off after 1 hr., and when the clear pale yellow filtrate was concentrated *in vacuo* to 200 ml. volume, crystallization occurred. The mixture was cooled at 5°C overnight. On the next day, the white crystals were filtered off and discarded. The filtrate was taken to dryness *in vacuo* and the white solid was dissolved in warm ethyl alcohol (120 ml.) and filtered. The filtrate was concentrated to half volume and added to an excess of ethyl acetate (1500 ml.). The resulting solution was concentrated to half volume and the white precipitate was filtered off, washed

with ethyl acetate and air-dried to yield 3.3 g. (35%) of a product with m. p. 155-156°C. On crystallization from hot water (50 ml.), very fine short white needles (1.0 g.) were produced with m. p. 129-131°C. On concentrating the filtrate to 35 ml. volume, another crop of product (0.8 g.) was obtained with m. p. 129-131°C. Recrystallization from hot water gave a product with m. p. 129-131°C.

Anal. Calcd. for $C_{13}H_9O_{16}\cdot H_2O$ (334.27): C, 46.71%; H, 5.43%. Found: C, 46.72%; H, 5.42%.

Acid hydrolysis of the product produced gentisic acid and glucose. It dissolved readily in 5% sodium bicarbonate solution with effervescence. Paper chromatography in various solvent systems showed the presence of only one purplish-blue fluorescing compound under ultraviolet light.

The water content of the product was obtained by a coulometric Karl Fischer titration method (36), courtesy of Mr. Ron Grigsby. A sample (8 mg.) of the product showed 6.4% water content.

## Preparation of Gentisic Acid Diacetate

Gentisic acid diacetate (2,5-diacetoxybenzoic acid) was prepared by the procedure of Wagner (35). A yield of 73% of a white crystalline product was obtained with m. p.

119-122°C (lit. 118-119°C).

## Preparation of 1-(2,5-Diacetoxygentisoyl)-β-D-glucose-2,3,4,6-tetraacetate

Gentisic acid diacetate (19 g. 0.08 m.) and α-acetobromoglucose (32.8 g., 0.08 m.) were dissolved in warm quinoline (55 ml.). The temperature of the mixture rose from 40°C to 60°C after silver oxide (10 g.) was added and the mixture stirred manually for 5 min. After 2 hr., the viscous material was extracted with 200 ml. hot acetic acid and filtered. The dark red filtrate was quenched with stirring in 2.5 l. cold water to give a brown precipitate. After filtration and air drying, the brown product (28.1 g.) was crystallized from methanol and charcoal to produce 20 g. (44%) of a white crystalline product with m. p. 156.5-157.5°C. Recrystallization from methyl alcohol gave a m. p. 158-159°C.

Anal. Calcd. for $C_{25}H_{28}O_{15}$(568.50): C, 52.82%; H, 4.96%. Found: C, 52.99%; H, 4.97%.

This compound has not been reported in the literature.

## Attempted Preparation of 1-Gentisoyl-β-D-glucose

β-D-Tetraacetylglucopyranosyl-2,5-diacetoxybenzoate (10 g.) dissolved rapidly in sodium methoxide (500 ml.,

0.1 N) to give a bright yellow solution. After 2 min. the solution turned orange. It was neutralized with concentrated sulfuric acid, and the precipitate was filtered off. Methyl alcohol was removed in vacuo to produce a brown oil (6 g.). Addition of a small amount of cold water, and also rubbing induced crystallization of the oil. The product was filtered off and washed with a small amount of cold water to give 2.2 g. (39.6%) of a crystalline cream-colored material. Crystallization from hot water (55 ml.) produced pale yellow crystals (1.8 g.) with m. p. 84-85.5°C. Recrystallization from hot water produced fine white needles with m. p. 86-87°C.

Anal. Calcd. for $C_{13}H_{16}O_9$ (316.27): C, 49.37%; H, 5.10%. Found: C, 56.85%; H, 4.58%.

The product did not dissolve in 5% sodium bicarbonate solution. Acid hydrolysis yielded no glucose. Mixed melting point with authentic synthetic methyl gentisate was undepressed and the carbon and hydrogen analysis agreed with those of methyl gentisate. The product obtained is believed to be the methyl ester of gentisic acid.

All the carbon and hydrogen determinations in this investigation were performed by Galbraith Laboratories, Knoxville, Tenn.

The unknown gentisic acid glucoside from sunflower

leaves was chromatographed against synthetic gentisic acid-5-β-D-glucoside and gentisic acid-2-β-D-glucoside on Whatman No. 1 paper in several solvent systems (Table 3). The blue fluorescence of the unknown glucoside and that of the gentisic acid-5-β-D-glucoside were the same on the developed chromatograms under ultraviolet light (3660 Å). Ammonia vapor intensified the blue fluorescence. However, the $R_f$ values of the unknown glucoside did not compare exactly with those of the two synthetic glucosides (Table 3).

The ultraviolet spectrum of the unknown gentisic acid glucoside from sunflower leaves showed a maximum at 312 mμ. Addition of excess solid anhydrous sodium acetate produced no shift. Likewise, the addition of one drop of 1% ethanolic aluminum chloride gave no shift of the maximum.

Synthetic gentisic acid-5-β-D-glucoside showed a maximum at 318 mμ. Addition of anhydrous sodium acetate produced a shift of the maximum to 312 mμ, whereas one drop of 1% ethanolic aluminum chloride solution shifted the maximum to 327 mμ.

Hence, on the basis of $R_f$ values and ultraviolet absorption spectra, the unknown gentisic acid glucoside does not correspond with synthetic gentisic acid-5-β-D-glucoside and gentisic acid-2-β-D-glucoside.

TABLE 3

$R_f$ VALUES OF GENTISIC ACID GLUCOSIDE

| Compounds | Solvent Systems[a] 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Gentisic acid-5-β-D-glucoside | 0.45 | 0.24 | 0.38 | 0.83 | 0.85 | 0.08 | 0.52 |
| Gentisic acid-2-β-D-glucoside | 0.45 | 0.15 | 0.38 | 0.83 | 0.83 | 0.04 | 0.54 |
| Unknown Sunflower Gentisic acid glucoside | 0.42 | 0.24 | 0.38 | 0.83 | 0.85 | 0.08 | 0.47 |

[a]Solvent systems: (1) butyl alcohol-acetic acid-water (6:1:2 v/v/v); (2) isobutylmethyl ketone-formic acid-water (3:1:2 v/v/v); (3) butyl alcohol-pyridine-benzene-water (5:3:1:3 v/v/v/v); (4) 15% acetic acid; (5) isopropyl alcohol-formic acid-water (50:1:950 v/v/v); (6) butyl alcohol-2N ammonium hydroxide saturated; (7) isopropyl alcohol-acetic acid-water (6:1:2 v/v/v).

# CHAPTER VII

## SUMMARY

A study has been made on the identification of flavonoids present in spinach leaves. The flavonols extracted and identified were patuletin (quercetagetin-6-monomethyl ether) and a quercetagetin dimethyl ether. The latter compound has not been found previously in nature and the name "spinacetin" has been proposed for it. Spinacetin has been tentatively identified as quercetagetin-3',6-dimethyl ether (3,4',5,7-tetrahydroxy-3',6-dimethoxyflavone).

A preliminary column and paper chromatographic study has been made on the polyphenolic compounds present in boron-deficient and boron-normal sunflower leaves. A marked increase of blue fluorescent compounds was observed under ultraviolet light (3660 Å) in the boron-deficient extracts. The compounds isolated from sunflower leaves proved to be chlorogenic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)), isochlorogenic acid (1,3,4,5-

tetrahydroxycyclohexanecarboxylic acid 5-(3,4-dihydroxycinnamate)) and neochlorogenic acid (a caffeoylquinic acid).

The pyrolysis products of the naturally-occurring polyphenolic compounds rutin (quercetin-3-rutinoside), chlorogenic acid (3-caffeoyl quinic acid), and quercetin were studied by column and paper chromatographic techniques. The pyrolysis of rutin produced catechol, 4-methylcatechol, resorcinol, furfural, 5-hydroxymethylfurfural and 5-methylfuran-2-aldehyde. Under similar conditions quercetin produced catechol, 4-methylcatechol, resorcinol and phloroglucinol. Chlorogenic acid pyrolysis yielded catechol, 4-methylcatechol, 4-ethylcatechol, benzoic acid and quinide (quinic acid-$\gamma$-lactone). In addition, paper chromatographic analysis of the pyrolysis products of each compound indicated the presence of other, as yet unidentified, components.

Scopoletin-4-$C^{14}$ (6-methoxy-7-hydroxycoumarin-4-$C^{14}$) was prepared by the method of Head and Robertson. The starting material was potassium cyanide-$C^{14}$. The product was column-purified and its specific activity determined by radioassay. Scopolin was also prepared for chromatographic purposes by the procedure of Chaudbury.

Gentisic acid-5-$\beta$-D-glucoside and gentisic acid-2-$\beta$-D-glucoside were synthesized for the first time from gentisic

acid (2,5-dihydroxybenzoic acid). Synthesis of 1-gentisoyl-β-D-glucose was unsuccessful. The unknown sunflower gentisic acid glucoside did not correspond in ultraviolet spectra and $R_f$ values to the two synthetic glucosides.

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