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STUDIES ON POLYPHENOLS IN COTTONSEED EXTRACTS

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The author wishes to thank his fellow graduate students for their assistance and fellowship during the research. He also wishes to show his gratitude to the members of the Chemistry Department for their instruction and fellowship during his stay at the University.

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CHAPTER I

INTRODUCTION

Polyphenols occur widely in the plant kingdom. Among them, many of the flavonoid compounds, coumarins, cinnamic-acid type compounds and chlorogenic-acid-type depsides have been identified by Wender and co-workers (1, 2, 3, 4, 5), and others (6, 7, 8, 9, 10). These compounds were considered likely to be present in cottonseed extracts but had not previously been investigated. The author of the research reported in this dissertation undertook a study of the blue-fluorescent compounds of cottonseed extracts. The last three types of compounds mentioned are among the blue-fluorescent compounds.

The cinnamic-acid type compounds have been identified in many plant sources (7, 8, 11). Caffeic, ferulic, p-coumaric, sinapic acids, as well as two coumarins--scopoletin and umbelliferone--have been identified by Bate-Smith (8) in the hydrolysates of 50 species of monocotyledonous and 80 species of dicotyledonous leaves. Ferulic acid was first identified by Bamberger (12) from the black Scots pine, Pinus sylvestrias.

In the research covered by this dissertation, the author has isolated and identified five cinnamic-acid type compounds from cottonseed extracts. They will be discussed in Chapter II, Section A.

Coumarins have also been isolated from various plants. The literature gives many thorough discussions on them including their occurrence (13, 14). Many coumarins, as well as some cinnamic-acid type compounds have been identified by Swain from the bark, root, rind, leaf and fruit of various plants (7). In this dissertation research, the author has tentatively proved the presence of scopoletin and other coumarins in cottonseed extracts. They will be discussed in Chapter II, Section B.

Chlorogenic acid itself has been identified by Gorter (9) from the coffee bean. It has been identified also from tobacco (3) and tea leaves (10) and other plants (8, 9, 10, 11, 12, 13, 14, 15). The other depsides of the chlorogenic-acid type involving the esterification of one of the hydroxyl groups in quinic acid and the carboxyl group of one of the cinnamic acids, have also been identified from various plant sources (16). The presence of chlorogenic acid in the cottonseed was suspected (17). The author failed to detect the presence of chlorogenic-acid type depsides and the presence of quinic acid. These negative results will not be discussed in this dissertation.

The flavonoid compounds have also been identified from

various plants (1, 6). The identification of the specific flavonol glycosides in cottonseed was first made by Wender and Pratt (5). There would be possibly some other flavonol glycosides present in cottonseed extracts; therefore, an attempt has been made to identify them. Their identification and the identification of their aglycones will be discussed in Chapter II, Section B.

Since quercetin is the most abundant aglycone of the flavonoid compounds, its biological action in animals is worth studying. The specific labeled quercetin would be desirable for such a study and for this reason the author has synthesized quercetin-3-C^{14} for animal tests. He modified the work of Fox (18) and Gutzke (19) on Kostanecki's (20) method which has been used similarly in the author's (21) synthesis of quercetin-4-C^{14} (discussed in his master's thesis). Details of this synthesis are given in Chapter II, Section C.

CHAPTER II

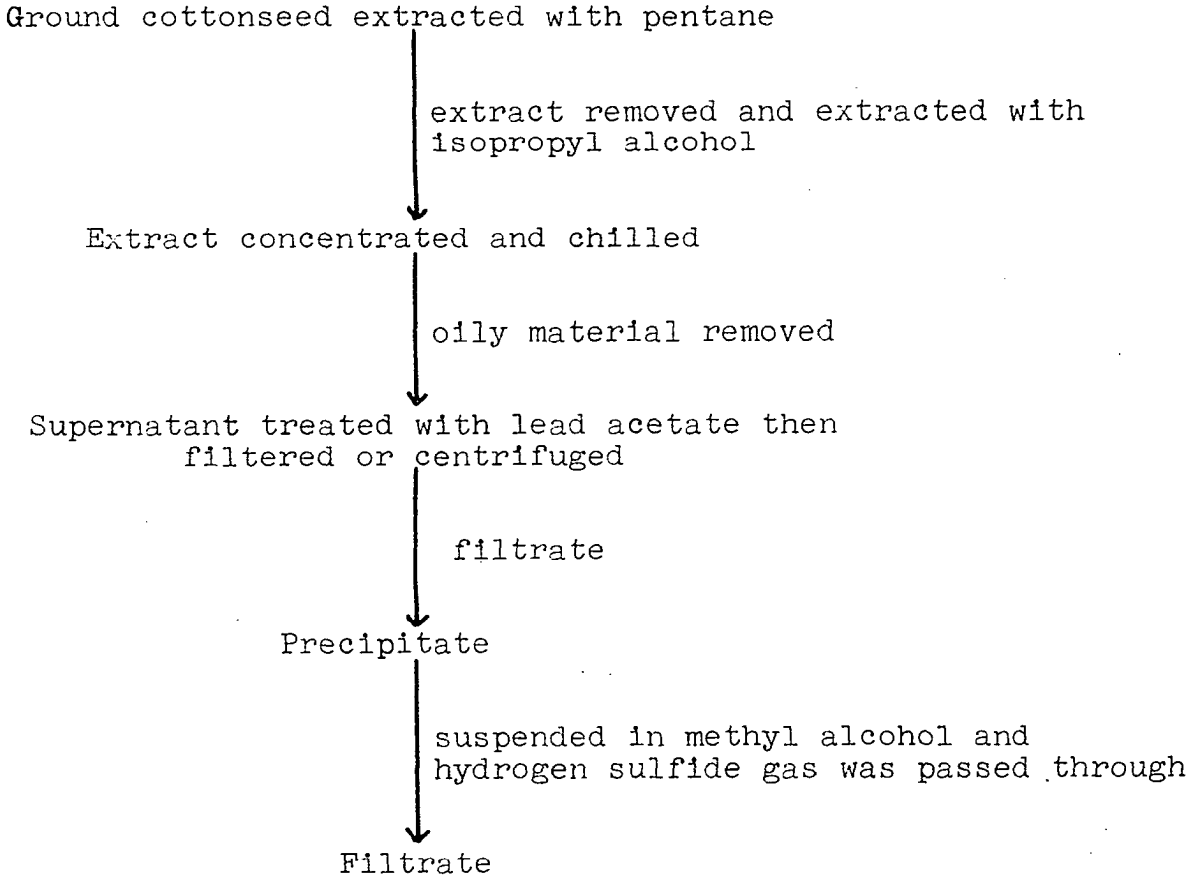
EXPERIMENTAL

A. Isolation and Identification of the Blue-Fluorescent Polyphenols in the Cottonseed Extract

Delinted cottonseed (1,200 gm.) was ground in a Waring blendor and was then extracted with pentane (3.0 liters) in a Bergmann extractor (22) for three to five days. The major portion of oils and lipids was removed by the solvent. Then 85% isopropyl alcohol was used as the extracting agent for three days. Fresh solvent was used each day. The three extracts (about six liters) were combined; concentrated to about 1.61 liters under reduced pressure; and then chilled in a cold bath for one to two days. The supernatant liquid was separated from an oily material by decantation. A saturated lead acetate solution was added with stirring to the supernatant (500 ml.) until no more precipitate was formed. The residue was separated from the mother liquor either by centrifugation or by filtration. It was then suspended in methyl alcohol (300 ml.) and deleaded by hydrogen sulfide gas. The lead sulfide formed was removed from the solution by centrifugation. The precipitate was washed with methyl alcohol (50 ml.,

Figure 1

Sketch Showing the Identification of the Blue-fluorescent Polyphenols in the Extracts of the Cottonseed



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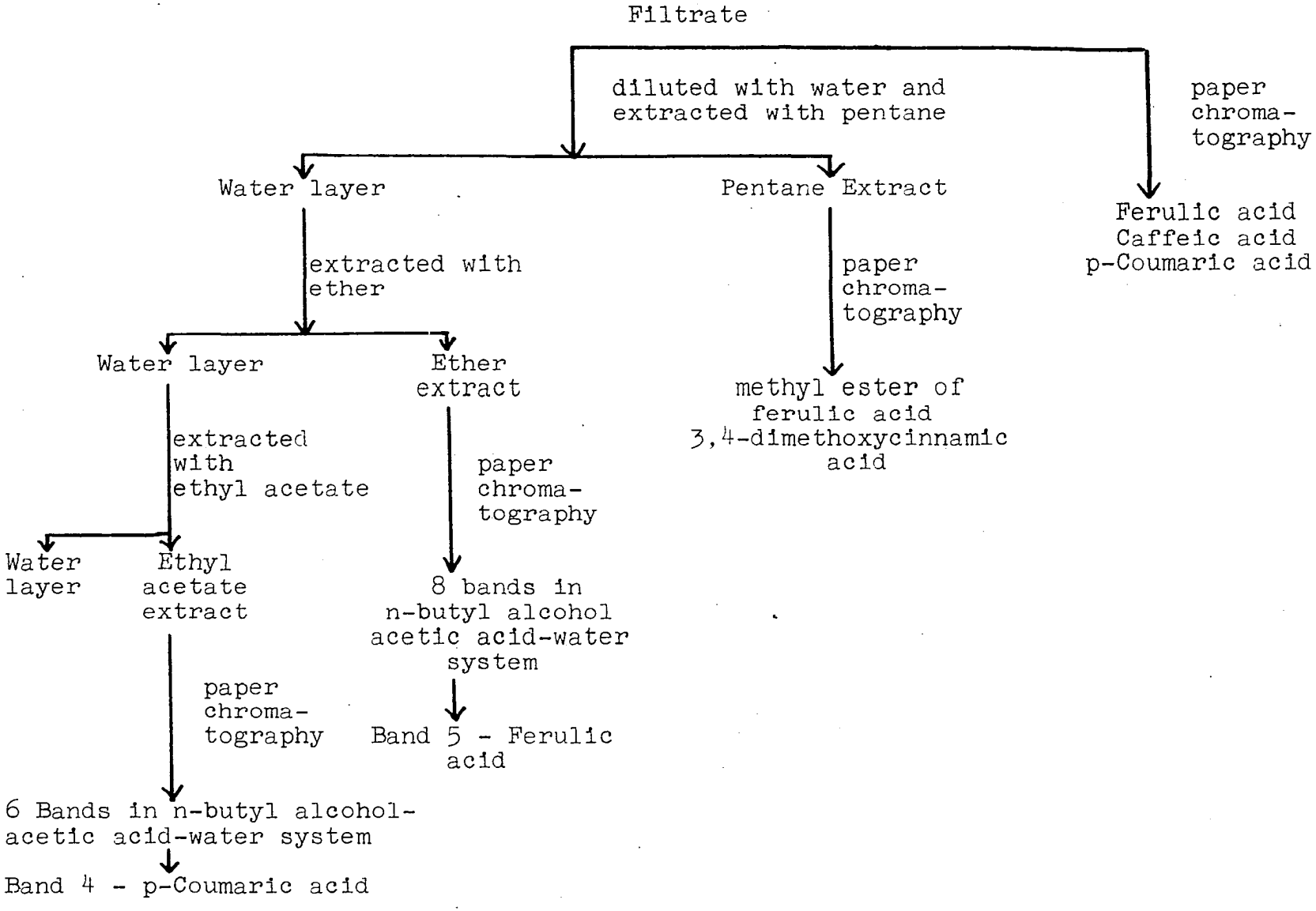

Filtrate
diluted with water and extracted with pentane
paper chroma-tography
Water layer
Pentane Extract
Ferulic acid
Caffeic acid
p-Coumaric acid
extracted with ether
paper chroma-tography
Water layer
Ether extract
methyl ester of ferulic acid
3,4-dimethoxycinnamic acid
extracted with ethyl acetate
paper chroma-tography
Water layer
Ethyl acetate extract
8 bands in n-butyl alcohol acetic acid-water system
paper chroma-tography
Band 5 - Ferulic acid
6 Bands in n-butyl alcohol-acetic acid-water system
Band 4 - p-Coumaric acid

4 times). The supernatant liquid was concentrated to about 200 ml. under reduced pressure. If any precipitate was formed in the concentration, it was removed by filtration. The concentrated methyl alcohol solution was used directly for mass paper chromatography. Ferulic, caffeic and p-coumaric acids were identified in this fraction. Details are given in succeeding sections.

Modifications in the isolation procedure were introduced in subsequent experiments. The concentrated methyl alcohol solution was further concentrated to about 100 ml., and 200 ml. of water were added. The resulting solution was extracted thoroughly and separately with n-pentane, ether, and ethyl acetate, successively. In each case, it was extracted with about 200 ml. of the solvent five times. The combined extract in each case was evaporated almost to dryness under reduced pressure, and the concentrated extract was dissolved in methyl alcohol (about 50-100 ml.). The resulting solutions were labeled as pentane extract, ether extract, and ethyl acetate extract, respectively. Each extract was then streaked on S. and S. No. 589 filter paper, red ribbon, and developed in the solvent system n-butyl alcohol-acetic acid-water (6:1:2 v/v). Chromatograms of each of the developed bands were studied separately.

The pentane extract, after having been streaked on filter paper and developed in the n-butyl alcohol-acetic acid-water system, gave three bands having approximate R_f values

of 0.80, 0.88, and 0.92. They were labeled as the first, second, and third bands of the pentane extract. The first one was identified chromatographically as the methyl ester of ferulic acid. The second band was probably the 3,4-dimethoxy-cinnamic acid. It gave R_f values and a fluorescence similar to those of the synthesized compound. Since both the unknown and the synthesized compounds were still very impure, the second band needed further study. The third band was neither blue under ultraviolet light, nor would it show blue fluorescence on exposure to ammonia vapor under ultraviolet light.

The ether extract, after having been streaked on filter paper and developed in the n-butyl alcohol-acetic acid-water system, gave six or eight bands on the chromatograms, depending on how heavily it was streaked. These bands exhibited varying degrees of blue fluorescence, especially on exposure to ammonia vapor while under ultraviolet light. Their approximate R_f values are as follows: 0.17, 0.59, 0.65, 0.72, 0.79, 0.85, 0.92, and 1.0. Band 5, which had an approximate R_f value of 0.79, proved to be ferulic acid.

Similarly, the ethyl acetate extract gave six blue-fluorescent bands. Their approximate R_f values were 0.24, 0.51, 0.69, 0.81, 0.85, and 0.96. The band having an approximate R_f value of 0.81 was chromatographically identified as p-coumaric acid. Details will be discussed in a section beginning on page 25.

Under ultraviolet light, one could see yellow or brown

bands overlapping the blue ones after the chromatograms of the three extracts were developed in the n-butyl alcohol-acetic acid-water system. The pentane extract produced very few or none, while the ether extract and the ethyl acetate extract produced many. The nature of all these yellow or brown bands has not yet been investigated.

All bands on each chromatogram which had been developed in the n-butyl alcohol-acetic acid-water system, were cut out and eluted with methyl alcohol. The eluates were concentrated under reduced pressure. Each one was streaked again on filter paper and developed in n-butyl alcohol-acetic acid-water. Once again each original band gave four or five bands. The identification of these latter compounds from the chromatograms was not achieved.

Identification of Ferulic Acid

Ferulic acid was present in the cottonseed extract in a relatively large quantity. It could be easily recognized even from the paper chromatogram made from the 85% isopropyl alcohol extract of the cottonseed. When this extract was streaked on filter paper and developed in the solvent systems listed in Table 1 alongside the known ferulic acid, the unknown and reference ferulic acid gave identical bands. However, other blue-fluorescent polyphenols were also present. A good method of separation had to be found; otherwise, it would be impossible to identify the other blue-fluorescent

TABLE 1

R_f VALUES OF THE IDENTIFIED AND KNOWN COMPOUNDS

Compound Name	Solvent Systems[a] 15% HOAc[b]		60% HOAc	BAW	Py-Bz	H_2O saturated BuOH	EFW
	1	2					
Ferulic Acid	0.54	0.75	0.77	0.87	0.60	0.74	0.92
Possibly Ferulic	0.54	0.75	0.80	0.87	0.61	0.73	0.92
Caffeic Acid	0.59	0.71	0.66, 0.72	0.83	0.59	0.69	0.84
Possibly Caffeic	0.60	0.72	0.66, 0.73	0.82	0.58	0.69	0.84
p-Coumaric Acid	0.60		0.75	0.94	0.73	0.87	0.91
Band 1 of Band 4 EtOAc Extract	0.60		0.75	0.94	0.72	0.87	0.91
Synthesized Methyl Ferulate	0.68[c]	0.79[c]	0.84	0.94	0.93[c]	0.88[c]	0.96
Band 1 Pentane Extract	0.67[c]	0.78[c]	0.84	0.94	0.93[c]	0.88[c]	0.96
[d]Ferulic Acid	0.33	0.45	0.71	0.81	0.67		0.83
[d]Hydrolysate of Band 1 Pentane Extract	0.32	0.44	0.69	0.81	0.67		0.83

[a]15% HOAc = 15% Acetic acid (v/v).
60% HOAc = 60% Acetic acid (v/v).
BAW = n-butyl alcohol-acetic acid-water (6:2:1 v/v).
Py-Bz = n-butyl alcohol-pyridine-benzene-water (5:3:1:3 v/v).
EFW = ethyl acetate-formic acid-water (10:2:3 v/v).

[b]Each acid studied, except p-coumaric, gave two spots (cis-trans isomers) with 15% acetic acid.

[c]Approximate values.

[d]S. & S. filter paper, No. 589; Whatman No. 1 used for all others.

polyphenols. It was found that a new solvent system, (water-acetic acid-n-butyl alcohol; 90:3:5) would not move most of the yellow and brown compounds present, while the blue-fluorescent compounds under discussion would move. However, the approximate R_f values on S. and S. No. 589 filter paper did not show very much difference between the acids being investigated. Each acid had two spots, apparently because of cis-trans isomerism in this solvent system.

	Approximate R_f values		Approximate R_f values
p-coumaric acid	0.11, 0.20	ferulic acid	0.18, 0.27
caffeic acid	0.18, 0.25	sinapic acid	o.18, 0.22

The methyl alcohol solution, containing the deleaded mother liquor of the concentrated isopropyl alcohol extract of the cottonseed, was massively streaked on S. and S. No. 589 filter paper and developed by descending chromatography in the water-acetic acid-n-butyl alcohol system. Three or four bands appeared on the paper chromatogram under the ultra-violet light. The first band, simply labeled as possibly ferulic acid, close to the origin on the paper, was cut and eluted by methyl alcohol. The eluate was concentrated under reduced pressure. It was streaked on filter paper again and developed in the n-butyl alcohol-acetic acid-water system. The process of streaking, cutting, and developing was alternately repeated in n-butyl alcohol-acetic acid-water and ethyl acetate-formic acid-water (10:2:3) systems until the

ultraviolet absorption spectrum of the unknown was identical with that of the known ferulic acid (Figure 2). Ordinarily, the bands or spots of a paper chromatogram can be cut and sewed on another filter paper and then developed in a second solvent system. This method could not be applied here because, if streaked too thick, the bands would not move across the thread, causing much loss of the material; if too thin, it was difficult to locate the proper band on the paper chromatogram.

Chromatographically, the presence of ferulic acid in the cottonseed extract was proved because it gave R_f values identical to those of the known ferulic acid on co-chromatography in the solvent systems listed in Table 1.

During the identification of the ferulic acid, a minor band with whiter fluorescence than that of ferulic acid, a little above the main band, appeared. It was caffeic acid. Its identification will be described in the next section. It was also later found that p-coumaric acid was present as a minor band below that of ferulic acid. It was the same as the compound from the band IV, ethyl acetate extract.

Identification of Caffeic Acid

When the concentrated solution containing the suspected ferulic acid from the eluate described in the previous section was streaked on filter paper and developed in the n-butyl alcohol-acetic acid-water system, a bluish-white

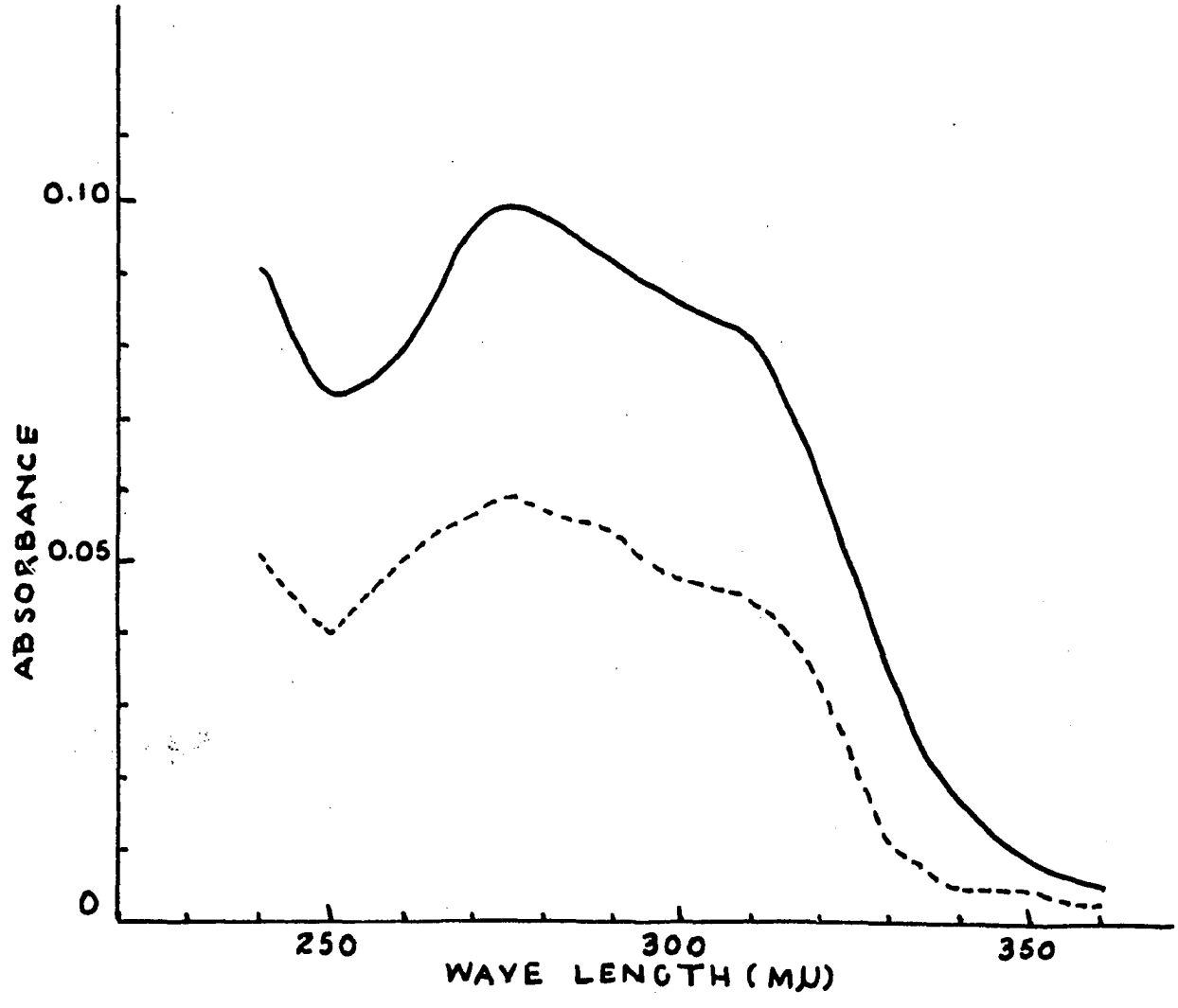
Figure 2

Ultraviolet Absorption Spectra of Reference Ferulic
and
Ferulic acid from Cottonseed

_____ Reference Ferulic acid

---- Cottonseed Ferulic acid

Ethyl alcohol was used as a solvent.



band appeared under ultraviolet light a little above the main band of ferulic acid. The compound in it was obtained by cutting the band from the filter paper and eluting it with methyl alcohol. The methyl alcohol eluate was streaked again and developed in the n-butyl alcohol-acetic acid system. The process of streaking, developing in the n-butyl alcohol-acetic acid-water system, cutting, and eluting was repeated ten times. The unknown was then streaked, developed, cut and eluted twice in the following solvent systems with the sequence of n-butyl alcohol-pyridine-benzene-water (5:3:1:3 v/v); ethyl acetate-formic acid-water; 15% acetic acid; n-butyl alcohol-acetic acid-water; and ethyl acetate-formic acid-water. This established the fact that the unknown was identical with the known caffeic acid chromatographically. The unknown which had been labeled as cottonseed caffeic acid, was further purified repeatedly in the above solvent systems. Finally, it was found to be identical with the known caffeic acid spectrophotometrically (Figure 3).

The ultraviolet absorption spectra of the reference caffeic acid from paper chromatograms using various solvent systems were determined (Fig. 4 to 8). The paper impurities of the chromatogram should be compensated for by using a blank. This was done by running a blank filter paper of the same size as that containing the paper chromatogram in a given solvent system. The paper was then cut and eluted in the same manner as that for the paper chromatogram. However,

Figure 3

Ultraviolet Absorption Spectra of the Reference Caffeic acid and Cottonseed Caffeic Acid

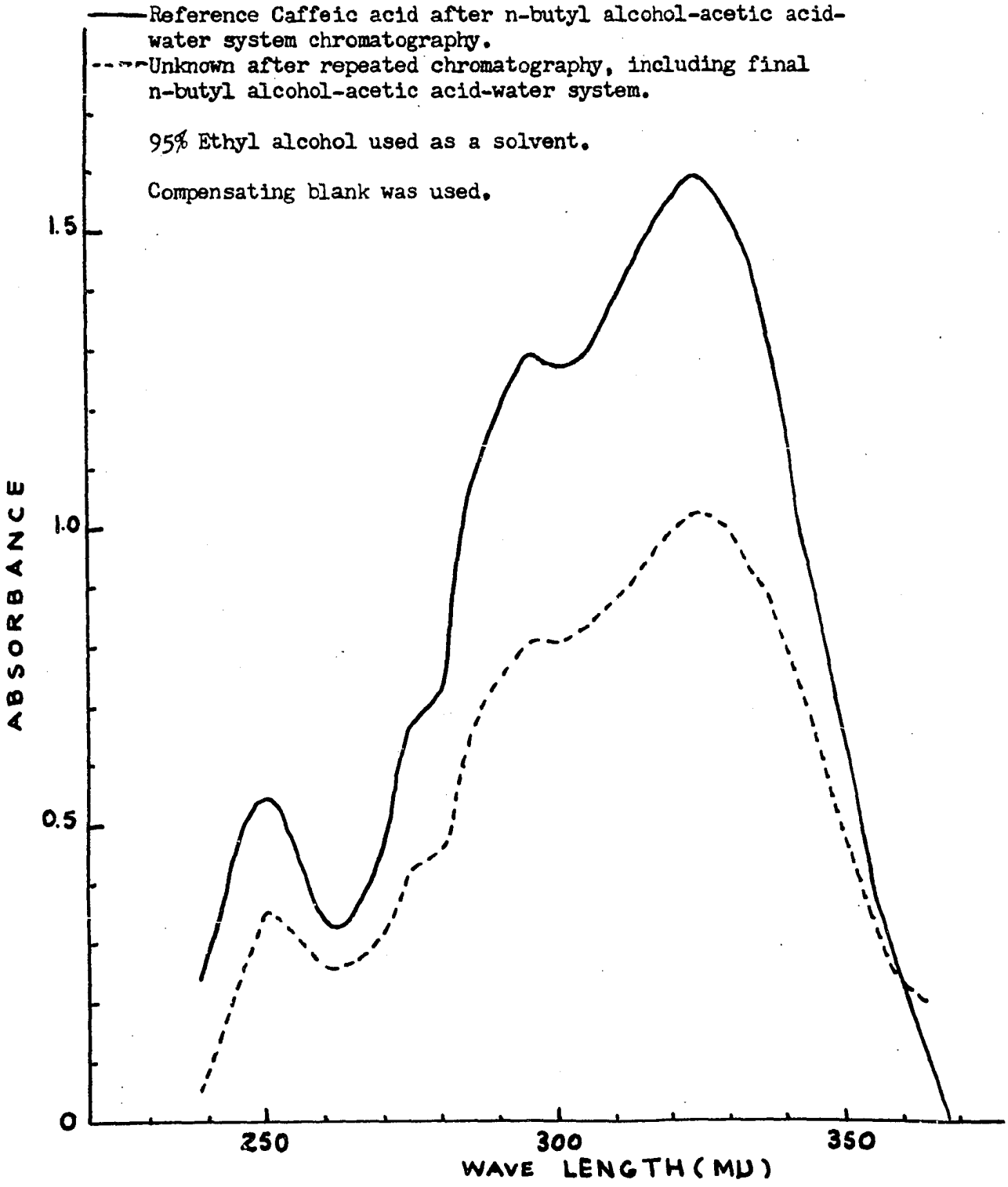


Figure 4

Ultraviolet Absorption Spectra of Reference Caffeic Acid after having been run in 15% Acetic acid and n-Butyl alcohol-Acetic acid-Water Solvent Systems

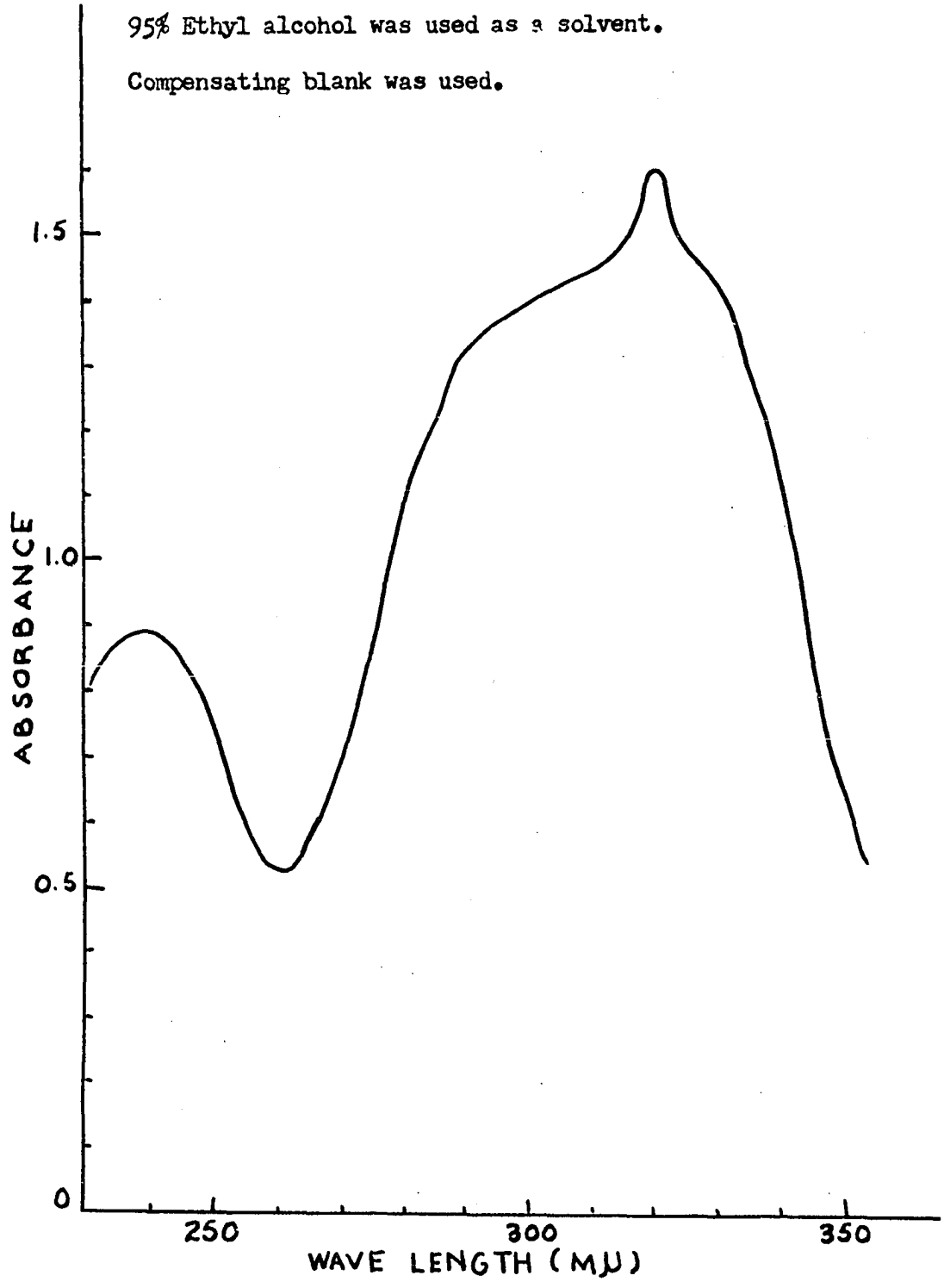


Figure 5

Ultraviolet Absorption Spectra of Reference Caffeic Acid

Its paper chromatogram has been developed in n-butyl alcohol-pyridine-benzene-water system.

———Top band
- - - - -Second band
-·-·-·Bottom band

95% Ethyl alcohol was used as a solvent.

No compensating blank was used.

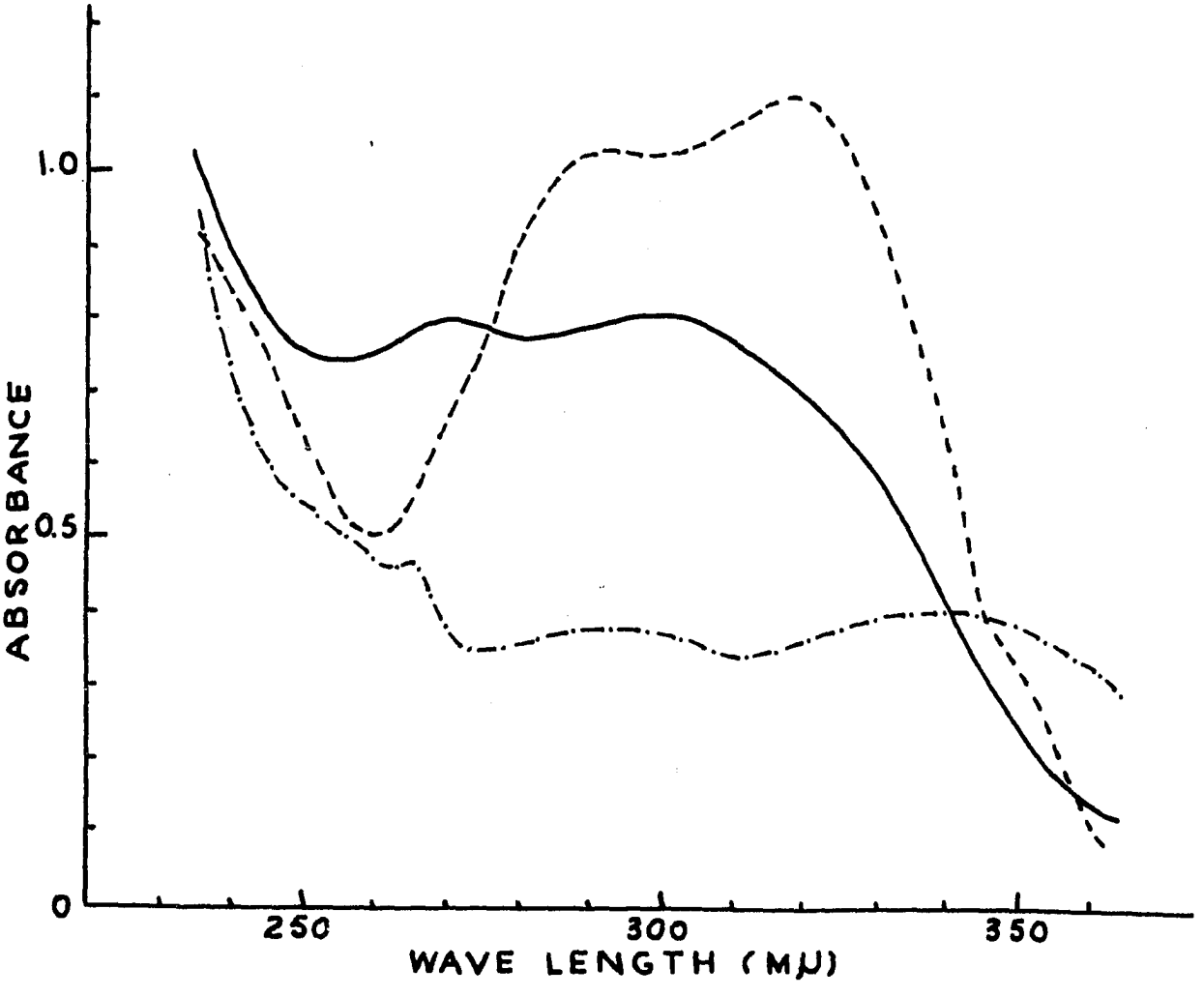


Figure 6

Ultraviolet Absorption Spectra of Reference Caffeic Acid

Its paper chromatogram has been developed in n-butyl alcohol-pyridine-benzene-water system.

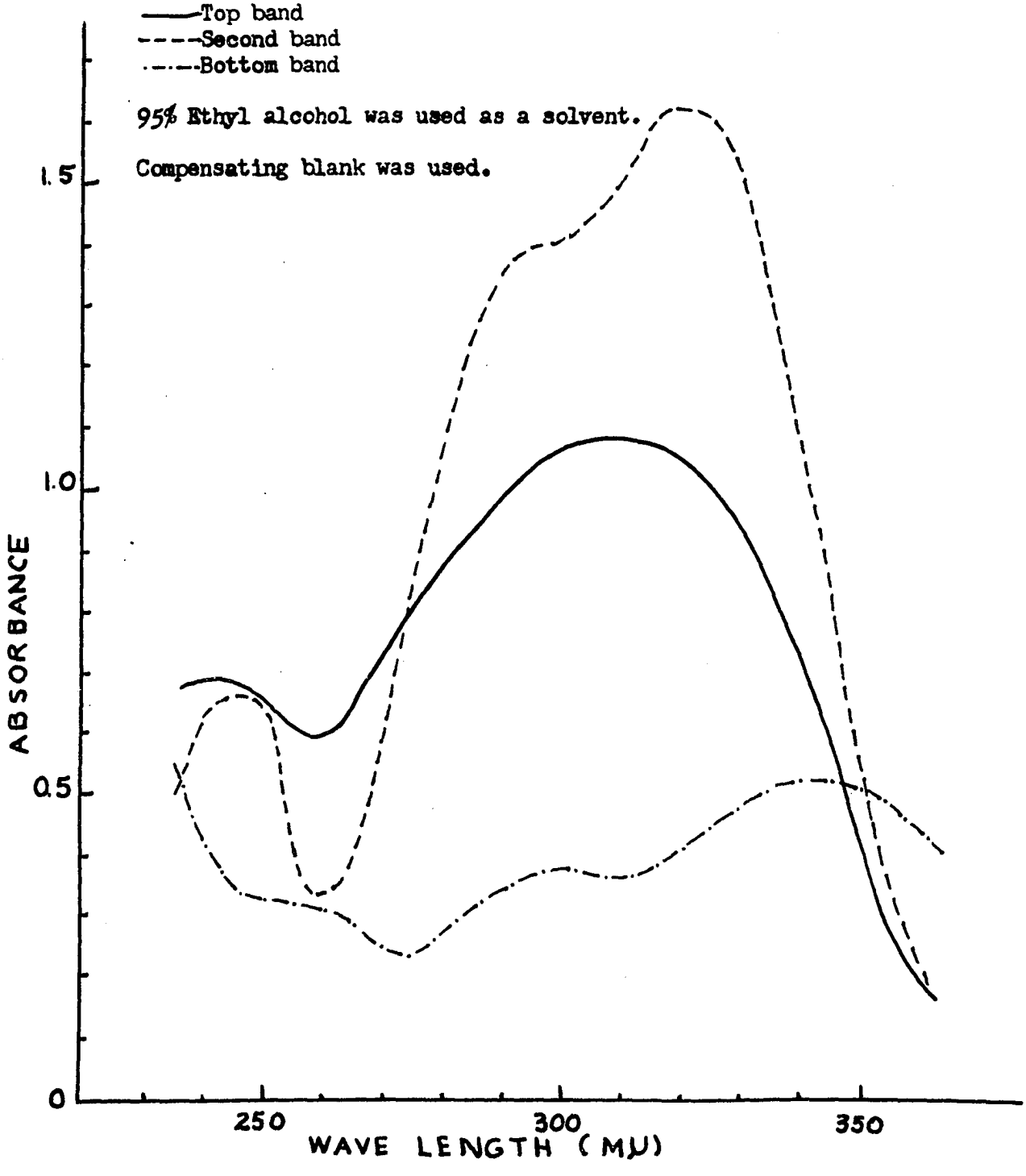


Figure 7

Ultraviolet Absorption Spectra of Reference Caffeic Acid

Its paper chromatogram has been developed in n-butyl alcohol-benzene-water system.

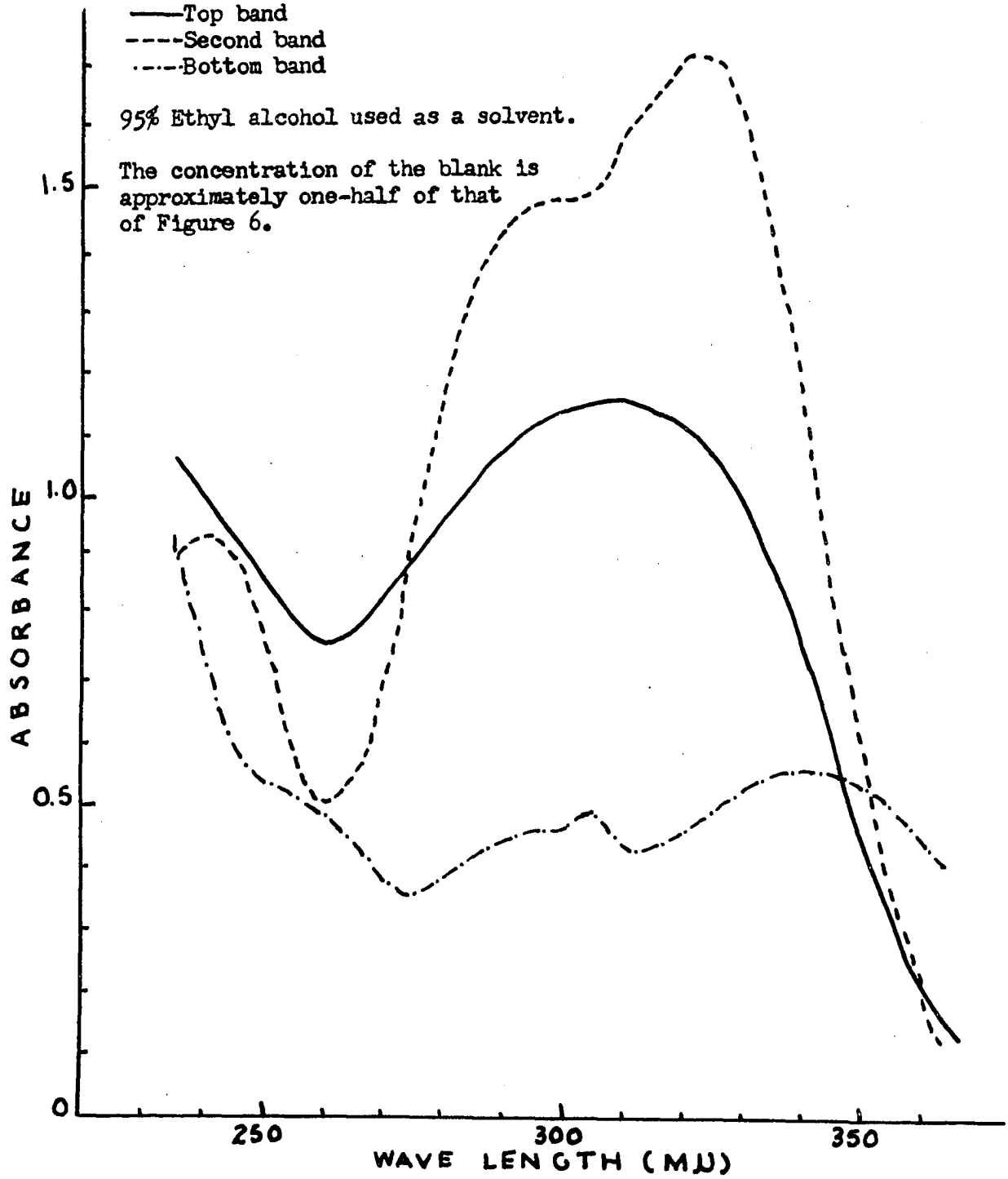
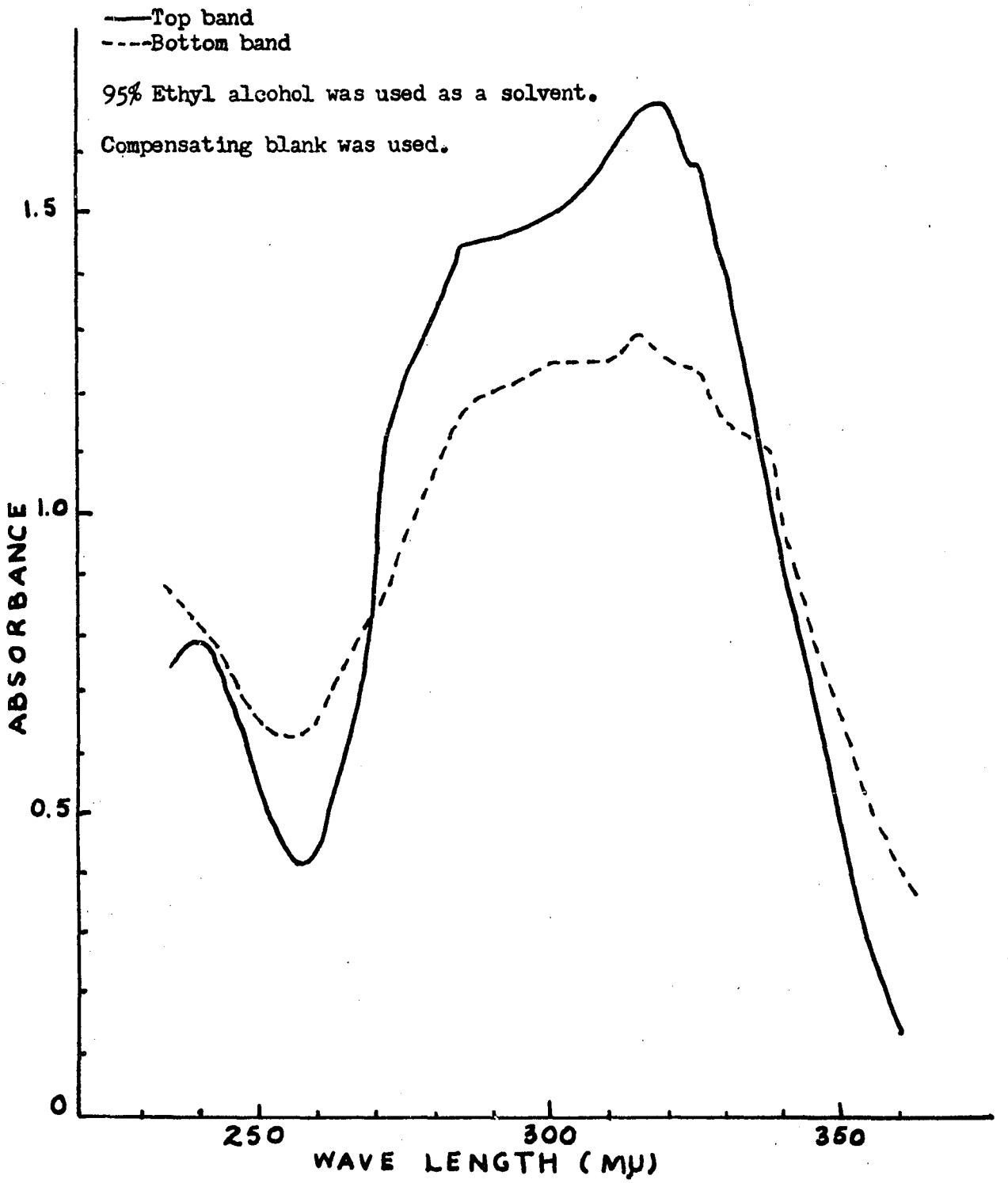


Figure 8

Ultraviolet Absorption Spectra of Reference Caffeic acid

Its paper chromatogram has been developed in 60% acetic acid.



the concentration of the compensated blank seemed not to be very important, because in one case, double dilution of the blank did not seem to affect the spectra very much (Fig.7).

Identification of p-Coumaric Acid

The eluate of the chromatogram of the band IV ethyl acetate extract (See Fig. 1) was streaked on S. and S. No. 589 filter paper and developed in the n-butyl alcohol-acetic acid-water system. Three bands appeared. The chromatogram of the first band (top) was eluted with methyl alcohol, streaked on S. and S. No. 589, and developed in several different solvent systems. The approximate R_f values obtained essentially agree with those of known p-coumaric acid. Actually, they were obtained on different sheets of filter paper and at different times.

	15% HOAc	60% HOAc	BAW	PyBz
p-coumaric acid	0.53	0.79	0.80	0.85
chromatograms of the first band of band IV ethyl acetate extract	0.49	0.79	0.82	0.84

They both gave the same blue fluorescence under ultraviolet light, and turned more blue on exposure to ammonia vapor.

This first band was purified by streaking its solution on filter paper, developing in various solvent systems, cutting the band off of the chromatogram, and eluting again with methyl alcohol. The solvent systems used were n-butyl alcohol-acetic acid-water and the pyridine-benzene system.

The R_f values in different solvent systems were finally obtained by co-chromatography with the known p-coumaric acid (Table 1). Its identification by spectrophotometry was not completed, because the first band was still too impure.

The same material (i.e., as present in the first band of the chromatogram) had also been obtained from the purification of ferulic acid in the lead precipitate, described in an earlier section on page 12. After this compound was found in band 1 in n-butyl alcohol-acetic acid-water of the band IV ethyl acetate extract, it was also obtained from some crude ferulic acid which had been isolated from the lead precipitate by separation on paper chromatograms, first using n-butyl alcohol-acetic acid-water, and then water-saturated-n-butyl alcohol. Ferulic acid gave an R_f value in water-saturated n-butyl alcohol of approximately 0.75, while p-coumaric acid gave 0.84.

Identification of Methyl Ester of Ferulic Acid

The concentrated pentane extract was streaked on S. and S. No. 589 filter paper and developed in the n-butyl alcohol-acetic acid-water system. Three bands were found, having approximate R_f values of 0.80, 0.88 and 0.92 respectively. The first band of the chromatogram, having an approximate R_f value of 0.80, was purified by streaking, developing, cutting and eluting repeatedly in different solvent systems, especially in the n-butyl alcohol-acetic acid-

water and 15% acetic acid systems. Its approximate R_f values were again compared with those of the synthesized methyl ester of ferulic acid. They are shown as follows:

	15% acetic acid*	60% acetic acid	n-butyl alcohol-acetic acid-water
Band 1 pentane extract	0.51; 0.64	0.83	0.93
Synthesized methyl ferulate	0.51; 0.63	0.87	0.93

*Two bands formed.

Both the known and unknown gave a pale blue fluorescence under ultraviolet light and turned to yellowish-blue-green on exposure to ammonia vapor. The purification was later improved by treating the pentane extract with 10% sodium bicarbonate aqueous solution. The band 1 of the pentane extract was not soluble, while its bands 2 and 3 were soluble. The sodium bicarbonate insoluble portion was further purified paper chromatographically as described above and subjected to hydrolysis. The methanolic eluate of the chromatogram of the sodium bicarbonate insoluble portion was evaporated under reduced pressure to dryness. About one milliliter of 1.0 N sodium hydroxide was added, and the resulting mixture was refluxed for about two hours. It was then acidified with dilute hydrochloric acid. It was finally extracted with ether (five times, each time about 5 ml.). The ether extract was concentrated and spotted on filter paper and co-chromatographed in different solvent systems with the known ferulic

acid. The resulting R_f values are shown in Table 1. The R_f values of the chromatogram of the band 1 pentane extract are also shown in Table 1.

From the R_f values of the unknown compared with those of the synthesized methyl ferulate, and from the R_f values of the hydrolysate of the unknown compared with those of the known ferulic acid, it can be concluded that the sodium bicarbonate insoluble portion of the pentane extract (Band 1 pentane extract) is an ester of ferulic acid. However, there was still a question whether Band 1 pentane extract is a methyl or ethyl ester of ferulic acid, because these would give very close R_f values.

Hence, the ethyl ester and also the methyl ester of ferulic acid were synthesized for comparison. Their synthesis will be discussed briefly beginning on page 25. The band 1 pentane extract was streaked on S. and S. No. 589 filter paper and co-chromatographed with the synthesized ethyl and methyl esters of ferulic acid. Their approximate R_f values are given below:

	15% acetic acid		60% acetic acid	n-butyl alcohol-water	ethyl acetate-formic acid-water
Synthesized Methyl ferulate	0.541	0.652	0.868	0.871	0.893
Band 1 pentane extract	0.539	0.650	0.856	0.873	0.893
Ethyl ferulate	0.559	0.613	0.884	0.921	0.929

From the comparison of approximate R_f values, it can be concluded that the band 1 pentane extract is the methyl ester rather than the ethyl ester of ferulic acid. In addition, the ethyl ester of ferulic acid, under ultraviolet light, gives a more greenish fluorescence and becomes more yellow on exposure to ammonia vapor than the unknown and methyl ester of ferulic acid. The latter two behaved identically under these conditions.

Spectrophotometrically, the identification of the sodium bicarbonate insoluble portions of the pentane extract was not yet completed because the material was still very impure, although it had been chromatographed many times. In dealing with the cottonseed extract, certain bands on the chromatogram seemed to be pure in 60% acetic acid, or n-butyl alcohol-acetic acid-water. But when these same bands were streaked on filter paper and developed in 15% acetic acid, a band of impurities, which had a white color under ultraviolet light, remained in its original position; however, the material being investigated moved down on the paper.

Synthesis of the methyl ester of ferulic acid. About 10 mg. of pure ferulic acid were dissolved in 10 ml. of anhydrous methanol. To the solution, one drop of concentrated sulfuric acid was added. The resulting solution was refluxed for three hours. The excess methyl alcohol was evaporated under reduced pressure. To the residue, a 10% sodium bicarbonate aqueous solution was added until it was distinctly

basic. The resulting alkaline solution was then extracted five times with 5 ml. ether. The water layer was discarded. The ether extract was washed with two one-milliliter portions of water, then evaporated to dryness and re-dissolved in about 10 ml. of 95% ethyl alcohol.

The product was then purified by streaking it on S. and S. No. 589 filter paper and developing the papers in the n-butyl alcohol-acetic acid-water system. Two bands close to each other were found. It was not understood why such a phenomenon occurred since the n-butyl alcohol-acetic acid-water system would usually not give two bands of cinnamic-acid type compounds. The top band gave the same fluorescence as the lower one--blue under ultraviolet light--except for some pink and brown color on the top edge. On exposure to ammonia vapor, both bands turned yellowish-blue as seen under ultraviolet light. In repeating the process of streaking and developing, the top band was converted to the lower. In the 15% acetic acid system, two bands resulted; the top one was more impure, having the pink or brown color as seen under ultraviolet light. The chromatogram of the lower band, developed in the 15% acetic acid system from the repeatedly purified top and bottom bands in the n-butyl alcohol-acetic acid-water system was subjected to spectrophotometric study. Its ultraviolet absorption spectrum is shown in Figure 9.

With a method similar to the one described, 15 gm. of ferulic acid were esterified. The product, the methyl ester

Figure 9

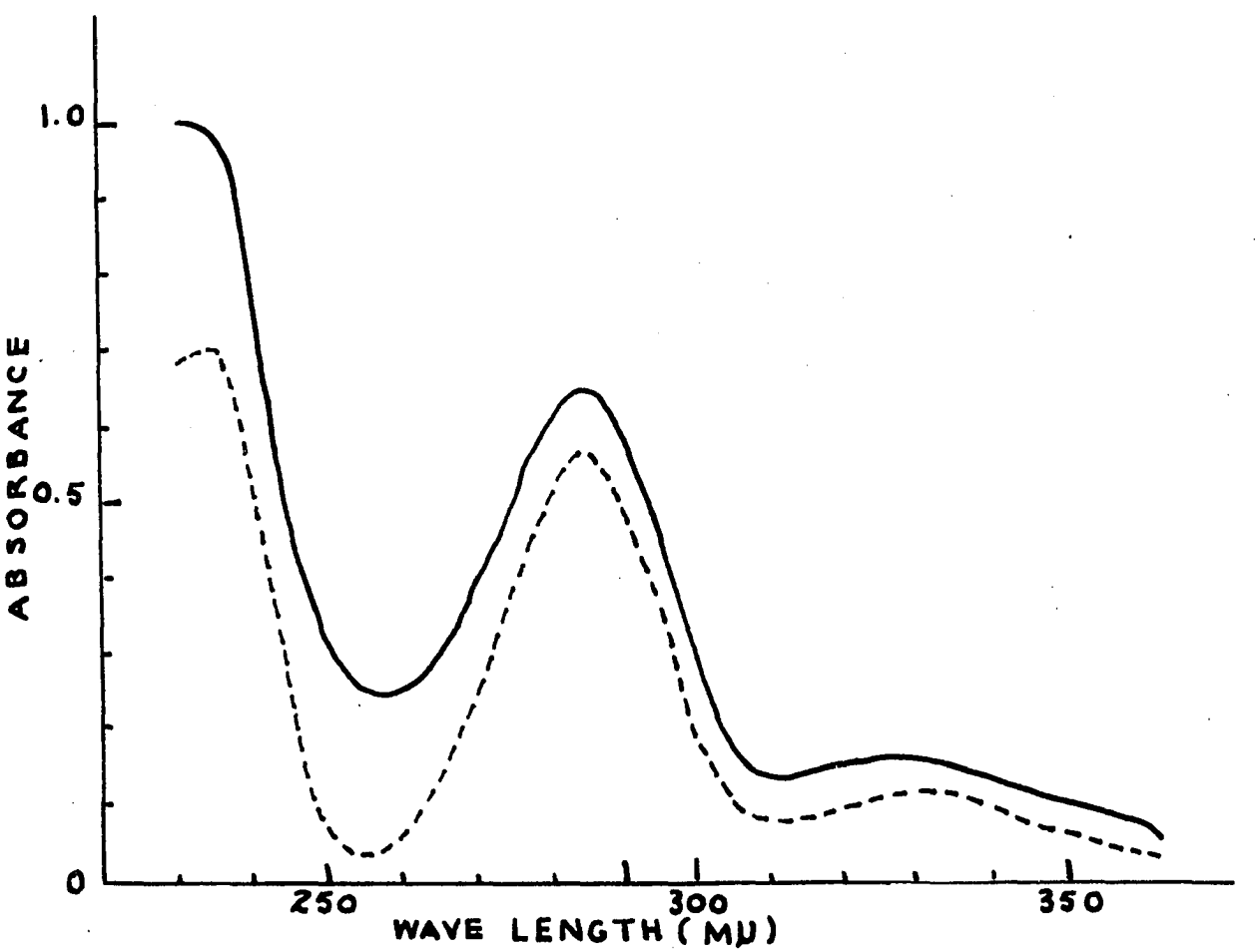
Ultraviolet Absorption Spectra of Synthesized Methylferulate

——The bottom band of the paper chromatogram in 15% Acetic acid after repeated purification of the top band from the n-butyl alcohol-acetic acid-water system.

- - - -Same as above except that of the bottom band in n-butyl alcohol-acetic acid-water system.

95% Ethyl alcohol was used as a solvent.

Compensating blank was used.



of ferulic acid, weighed 6.0 gm. with a yield of 37.2% based on the ferulic acid. It melted at 62°-64°C. (Lit. 63-64°) (23).

Synthesis of the ethyl ester of ferulic acid. Five grams of ferulic acid were dissolved in 50 ml. anhydrous ethyl alcohol. To the solution 1.0 ml. of concentrated sulfuric acid was added, and the resulting solution was heated with reflux for three hours. At the end of that time, the excess ethyl alcohol was evaporated under reduced pressure. To the resulting residue was added 10% sodium bicarbonate aqueous solution, until the solution was distinctly basic. The basic solution was then extracted with two portions of 15 ml. ether. The ether extract was evaporated to dryness under reduced pressure. It weighed 2.5 gm. with a yield of 44% based on ferulic acid. It was purified once by recrystallization in dilute ethyl alcohol solution. It melted at 76°C. (Lit. 75.5-76.5°C) (24).

Tentative Identification of 3,4-Dimethoxycinnamic Acid

The band 2 of the pentane extract was streaked on S. and S. No. 589 filter paper and developed in the n-butyl alcohol-acetic acid-water system. Three bands were seen. The first one corresponded to band 1 of the pentane extract. The third one corresponded to band 3 of the pentane extract. They were all compared chromatographically and then combined, respectively. The chromatogram of the second band was

collected by elution with methyl alcohol. The eluate was concentrated under reduced pressure. The concentrated eluate was streaked on S. and S. No. 589 filter paper and co-chromatographed in different solver systems with the synthesized 3,4-dimethoxycinnamic acid. Except in 15% acetic acid, all gave a chromatogram of the same approximate R_f values as the main bands produced by the synthesized compound. The chromatograms of the unknown using 15% acetic acid were so faint that the desired compound could not be located with certainty.

Synthesis of 3,4-dimethoxycinnamic acid. The 3,4-dimethoxycinnamic acid was synthesized by the Robinson (25) method. About 10 mg. of ferulic acid were dissolved in 5.0 ml. of 1 N sodium hydroxide. Dimethyl sulfate (2 ml.) was dropped into the alkaline solution drop by drop with shaking. When this process was completed, the reaction mixture was allowed to stand at room temperature for three hours. It was then acidified with dilute hydrochloric acid, and then extracted five times with 5 ml. portions of ether. The ether was evaporated. The residue was re-dissolved in 95% ethanol. The approximate R_f values of the product were determined and are shown as follows. In the 15% acetic acid system, there were two bands--0.45 (main) and 0.55. In 60% acetic acid there were three bands--0.65, 0.78 (main), 0.84. In n-butyl alcohol-acetic acid-water system there were two--0.79 and 0.89 (main). In the pyridine-benzene system there were two--0.78

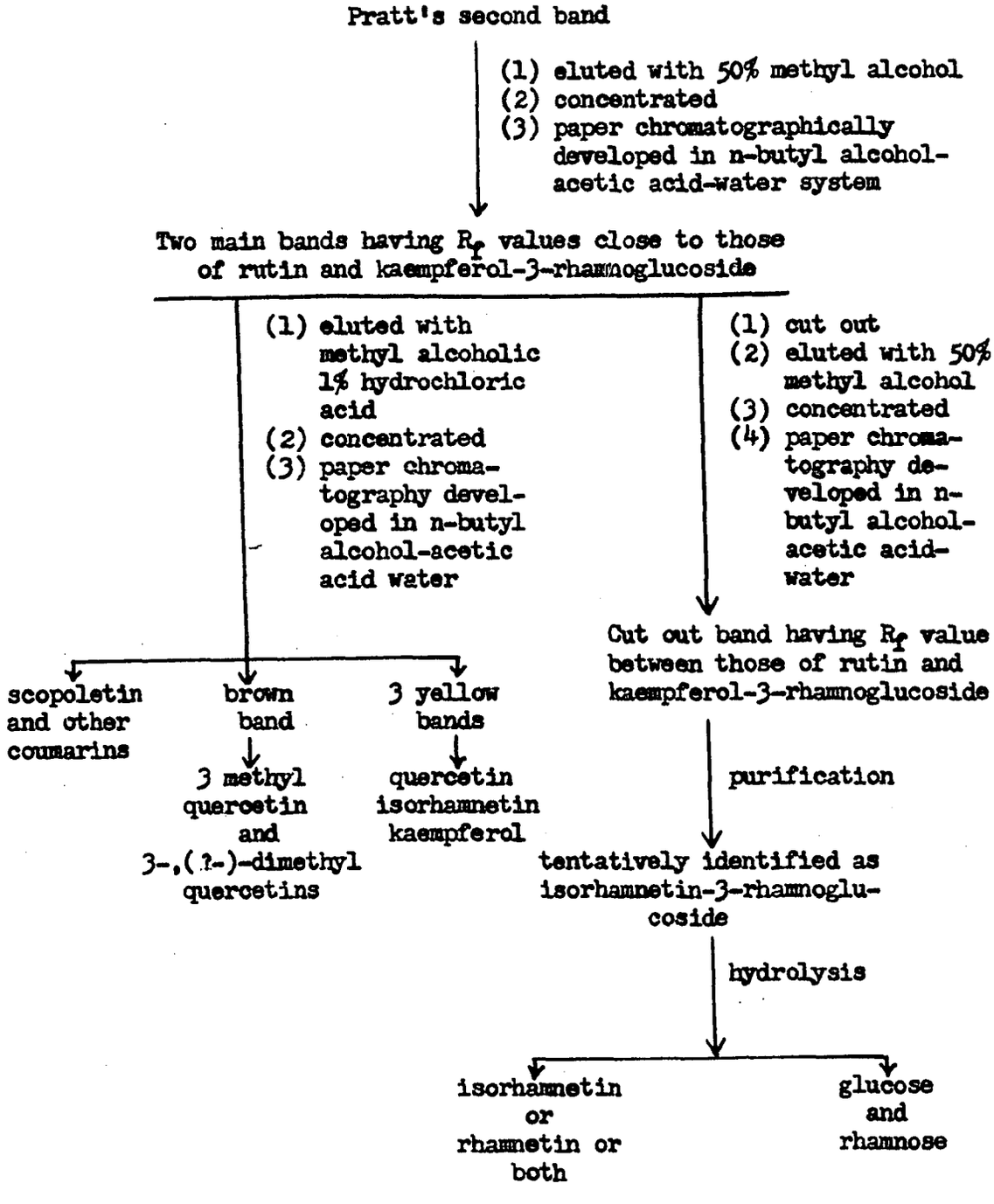
(main) and 0.87, and in water saturated n-butyl alcohol there was one--0.78. Obviously, the synthesized 3,4-dimethoxy-cinnamic acid was very impure.

B. Identification of Flavonoid Compounds and Coumarins in the Cottonseed Extracts

Rutin and isoquercitrin have been identified in the cotton seed extract by Pratt (5). His procedure is briefly summarized here. The concentrated solution of 85% isopropyl alcohol extract was diluted with water. The resulting solution was allowed to stand in a refrigerator overnight. The suspended frozen oil was separated. The solution was streaked on filter paper and developed in the n-butyl alcohol-acetic acid-water system. Three main bands were formed. From the second band, rutin was isolated and identified, and from the third (bottom), isoquercitrin was isolated and identified. In the first band (top), additional flavonol glycosides are present and have been investigated further by Pratt. In the second and third bands there are also some other possible flavonol glycosides present. The present author obtained the paper chromatogram of the second band from Pratt and tried to identify flavonol glycosides from it. The paper chromatogram of the second band was eluted with 50% methyl alcohol. The eluate was concentrated under reduced pressure and the water in it was removed as much as possible. Some precipitates were formed and were removed by filtration. The filtrate was passed through a large magnesol column

Figure 10

Procedure for Identification of the Flavonoid Compounds and Coumarins in the Extracts of Cottonseed



(diameter 2 1/4 inches, length 20 inches or more), and then eluted with water-saturated ethyl acetate, and fractions of the eluate were obtained. This process was repeated. Two fractions of eluate, one corresponding to rhamnetin (or isorhamnetin)-3-rhamnoglucoside, and the other corresponding to kaempferol-3-rhamnoglucoside, were obtained. Since there was only a trace of both, proof of identities has not yet been confirmed.

Identification of Aglycones

Mass paper chromatography was then tried. The concentrated, filtered methyl alcohol eluate of Pratt's second band was streaked on Whatman 3 MM filter paper and developed in the n-butyl alcohol-acetic acid-water system. Two main bands with brown color under ultraviolet light appeared. On comparison chromatography, they gave R_f values close to those of rutin and kaempferol-3-rhamnoglucoside. They were then cut out and eluted with 50% methyl alcohol. The resulting eluate was again streaked and compared chromatographically in the n-butyl alcohol-acetic acid-water system with rutin and kaempferol-3-rhamnoglucoside. Two main bands were found again. They were cut out separately and eluted with methyl alcoholic 1% hydrochloric acid. This was adapted from the method of Harborne (6). The processes of concentrating, streaking and developing in the n-butyl alcohol-acetic acid-water system were repeated. From the first band, three

yellow bands as viewed under the ultraviolet light, were now obtained. (The second band was later labeled as CIIc2a). The original brown color under ultraviolet light disappeared, and their R_f values were no longer close to those of rutin and kaempferol-3-rhamnoglucoside. Instead, they were close to the aglycones in R_f value and fluorescence under the ultraviolet light. They were cut out and eluted in methyl alcoholic 1% hydrochloric acid separately and labeled respectively as CIIY1, CIIY2, and CIIY3. They were streaked and developed in 15% acetic acid, 60% acetic acid and n-butyl alcohol-acetic acid-water systems. In each solvent system they all gave similar bands but with different concentrations according to the R_f values of the bands. By using the known aglycones--quercetin, isorhamnetin, and kaempferol--as reference compounds, the band CIIY1 was found to contain mainly quercetin, CIIY2 mainly isorhamnetin, while CIIY3 contained mainly kaempferol. By repeating the process of cutting, eluting, streaking, and developing, these aglycones were identified as quercetin, isorhamnetin (or rhamnetin, or both, because they cannot be separated chromatographically) and kaempferol. The R_f values of the known aglycones on S. and S. No. 589 filter paper are given below.

	15% acetic acid	60% acetic acid	n-butyl alcohol-acetic acid-water
Quercetin	0.023	0.27	0.66
Isorhamnetin	0.027	0.41	0.77
Kaempferol	0.036	0.44	0.81

In addition to these three compounds, the chromatogram developed in the n-butyl alcohol-acetic acid-water system from the CIIY1, CIIY2, and CIIY3 also showed a band of brown (below the three yellow bands discussed above), plus a band of blue followed by various shades of blue. On the basis of R_f values and fluorescence, the brown band might possibly be 3-methylquercetin and the dimethyl quercetins, in which position 3 has a methyl group. It was labeled as the brown band of CIIY1, CIIY2 and CIIY3, respectively. Since these bands were in minor quantity, they were combined into the brown band of CIIY1-3. The blue band right below the brown band was labeled as the first blue band of CIIY-1-3, and the rest was labeled as the bottom bands of CIIY-1-3. They gave phenomena similar to those of CIIc-2a, so they will not be discussed separately.

The second band, CIIc-2a, after eluting, concentrating, streaking and developing in the n-butyl alcohol-acetic acid-water system gave bands similar to those of CIIY1-3. After CIIc-2a was developed in various solvent systems, the yellow bands were found to be quercetin, rhamnetin (or isorhamnetin) and kaempferol, the same as those in CIIY1-3. The brown bands, compared with reference samples of 3-methyl quercetin and 3,7-dimethylquercetin were the same as these compounds. Additional experiments are needed, however, for proof of these identifications.

Tentative Detection of the Presence of Coumarins

The blue bands, developed from CIIc-2a were proved to be coumarins and cinnamic acid type compounds. On comparison in different solvent systems with scopoletin, CIIc-2a was found to contain scopoletin. The R_f values of the reference scopoletin on S. and S. No. 589 filter paper are given below.

	15% acetic acid	60% acetic acid	n-butyl alcohol-acetic acid-water	nitromethane-benzene-water	pyridine-benzene
Reference scopoletin	0.47	0.76	0.78	0.77	0.83

The known and unknown both gave blue fluorescence under the ultraviolet light. The fluorescence turned to yellowish-blue on exposure to ammonia vapor. However, in the n-butyl alcohol-acetic acid-water system, the unknown gave a more yellow coloration on exposure to ammonia vapor. This could be due to the presence of impurities, because the same coloration did not appear in the band corresponding to the scopoletin in the other solvent systems. In the nitromethane system (nit thane-benzene-water; 2:3:5, organic layer), some oth oumarins having larger R_f values than those of scopoletin were also present.

Identification of Flavonol Glycosides

The two main bands, developed in the n-butyl alcohol-acetic acid-water system by the concentrated filtered methyl alcohol eluate of Pratt's second band, were then cut into

three portions, one corresponding to rutin, one corresponding to kaempferol-3-rhamnoglucoside, and the other corresponding to the band in between. They were separately eluted, concentrated, streaked and developed in the n-butyl alcohol-acetic acid-water system. In each case, the band corresponding to that one in between rutin and kaempferol-3-rhamnoglucoside was cut out. The process of eluting, concentrating, streaking and developing in the n-butyl alcohol-acetic acid-water system was repeated until the band was quite pure and gave only one band. The process was again repeated, using S. and S. No. 589 filter paper. The band was cut out, sewed on to another sheet of filter paper, and developed in 15% acetic acid, 60% acetic acid and the n-butyl alcohol-acetic acid-water systems successively. Finally the band was cut out again, eluted with 50% methyl alcohol, and evaporated under reduced pressure to dryness. To the residue were added 5 ml. of 2% hydrochloric acid, and the resulting solution was refluxed for three hours in an oil bath. The refluxed solution was extracted with 2 ml. portions of ethyl acetate three times. The extract was twice washed with 1 ml. portions of water. The extract was concentrated to a minimum volume. The washings were combined with the water layer portion. The concentrated ethyl acetate extract was streaked on Whatman No. 1 filter paper and co-chromatographed in various solvent systems with known isorhamnetin. The known and unknown were identical. The unknown is possibly rhamnetin or

isorhamnetin or both rhamnetin and isorhamnetin. The R_f values on Whatman No. 1 filter paper of the reference isorhamnetin are given below:

	15% acetic acid	60% acetic acid	n-butyl alcohol-acetic acid water
isorhamnetin	0.065	0.46	0.86

The water layer portion was then put onto a mixed bed of Amberlite MB-1 cation and anion exchange resin. It was then eluted with distilled water. The eluate was concentrated under reduced pressure to the minimum volume and streaked on Whatman No. 1 paper about one inch long, and chromatographed in the pyridine-benzene system alongside known glucose and rhamnose. The unknown gave two spots identical to the known sugars after the paper chromatogram was sprayed with sugar spraying agent.

From the results described above, it can be tentatively concluded that rhamnetin (or isorhamnetin or both)-3-rhamnoglucoside is present in the cottonseed extract.

C. Synthesis of Quercetin-3-C^{14}

Modifications in the original synthesis of quercetin by Kostanecki (20) have been made by Fox (18) and Gutzke (19). Methods similar to those reported by these latter workers were used for the reactions in this research.

Figure 11 gives the numbering system of flavones. The over-all method for the synthesis of quercetin-3-C^{14} is briefly summarized in Figures 12 and 13.

Figure 11

Numbering System of Flavone

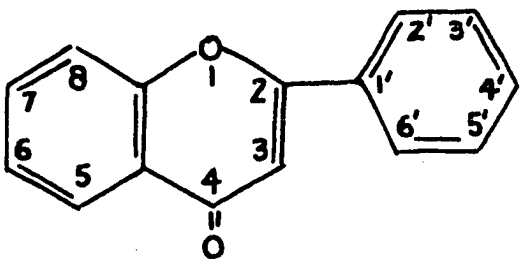
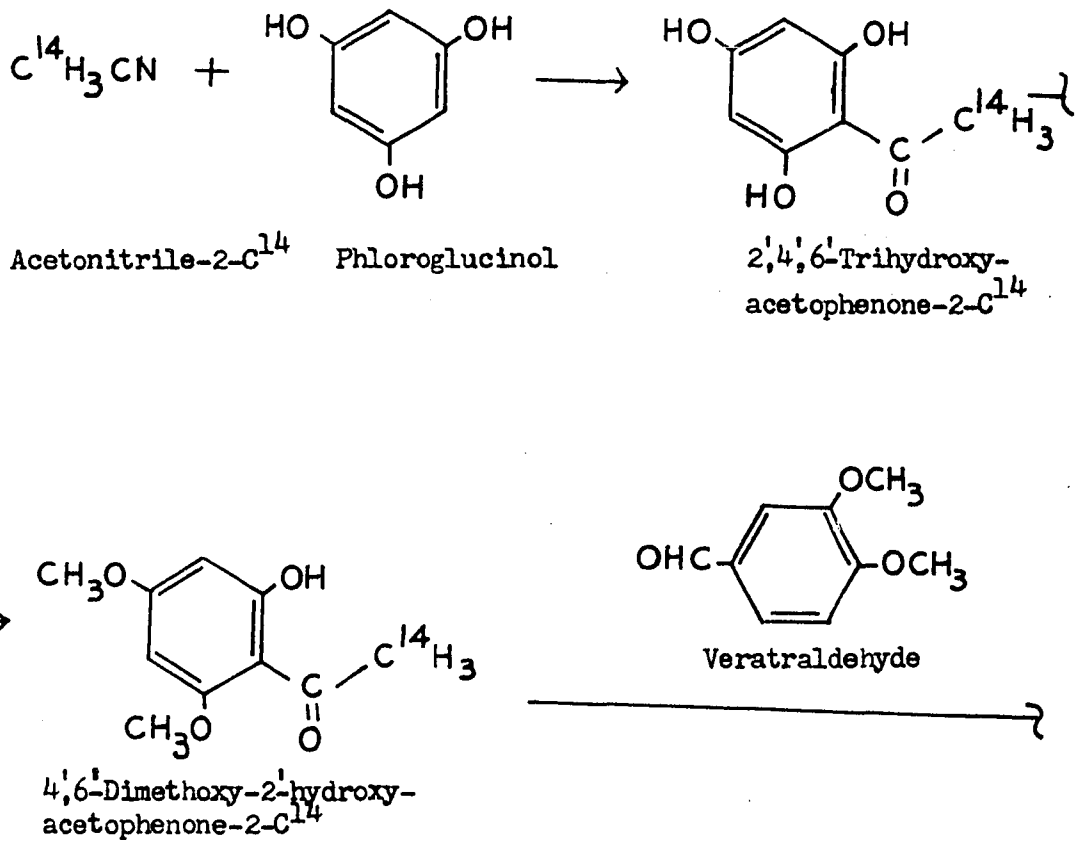


Figure 12

Synthetic Scheme for Quercetin-3-C^{14}



Acetonitrile-2-C^{14} Phloroglucinol

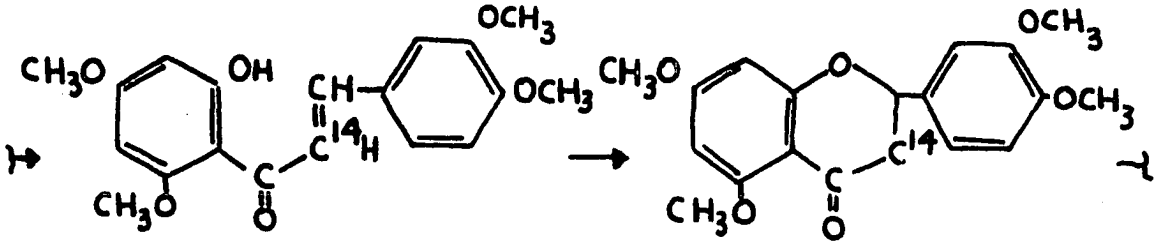
2',4',6'-Trihydroxy-acetophenone-2-C^{14}

Veratraldehyde

4',6'-Dimethoxy-2'-hydroxy-acetophenone-2-C^{14}

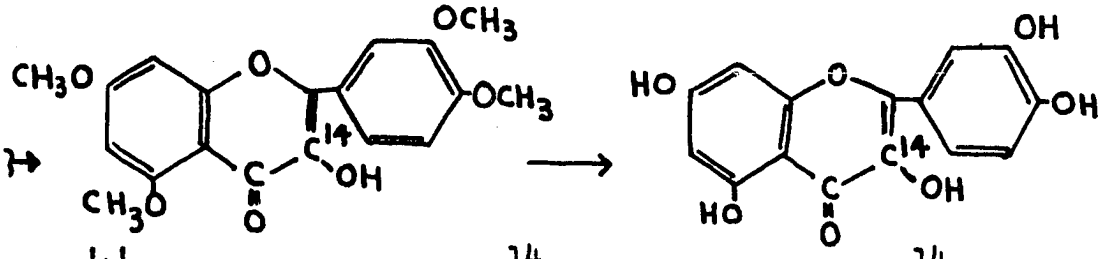
Figure 13

Synthetic Scheme for Quercetin-3-C^{14}
(Continued from Figure 12.)



Chalcone of
5,7,3',4'-tetramethyleriodictyol-3-C^{14}

5,7,3',4'-
Tetramethyleriodictyol-3-C^{14}



5,7,3',4'-Tetramethylquercetin-3-C^{14}

Quercetin-3-C^{14}

The acetonitrile-2-C^{14}, which had a specific activity of 7.84 mc/mM, was supplied by the Nuclear Instrument and Chemical Corporation, Chicago. Two ampoules, each of which contained one millicurie, were used. In the first case, the acetonitrile-2-C^{14} (1.0 mc.) was diluted with 3.0 gm. anhydrous purified acetonitrile and was converted into quercetin-3-C^{14}, step by step, using methods similar to those previously described by Wei (21). The yields were good up to the last step. However, in purification during the last step, the quercetin-3-C^{14} was completely destroyed. The ethyl acetate solution containing the quercetin-3-C^{14} was streaked on Whatman 3 MM filter paper and the chromatogram was developed in 60% acetic acid and then was eluted with 60% acetic acid. The eluate was evaporated under reduced pressure to almost dryness. Much fiber from the dissolved filter paper was left. The desired final product, quercetin-3-C^{14}, could not be recovered, apparently because it had been decomposed during the chromatography.

The other millicurie of acetonitrile-2-C^{14} was used for the second attempt at synthesis of quercetin-3-C^{14}. The synthetic procedures are described in the following sections.

Synthesis of 2',4',6'-Trihydroxyacetophenone-2-C^{14}

The method adopted for the synthesis of 2',4',6'-trihydroxyacetophenone-2-C^{14} was that of Gulati (26). The purified and dried phloroglucinol (5.0 gm.) was dissolved in dry ether

(20 ml.). To the ethereal solution was then added acetonitrile (1.5 gm.) and acetonitrile-2-C^{14} (1.0 millicurie). The acetonitrile-2-C^{14} was first dissolved with dry ether, and then the resulting solution was poured into the filtering flask containing the other reactants. The ampoule was then rinsed with ether several times, and the rinsings were added to the mixture. Anhydrous zinc chloride (0.5 gm.) was added to the solution. The flask containing the ethereal solution was surrounded with a freezing mixture throughout the experiment. A stream of dry hydrogen chloride gas was bubbled into the reaction mixture for three hours. The filtering flask, which was used for the reaction flask, was stoppered while its side arm was guarded with a drying tube. The reaction flask was then placed in the ice chest for three days. The ether layer in the reaction flask was then decanted off from the ketimine hydrochloride formed. The latter was washed three times with dry ether. The ketimine hydrogen chloride was then transferred to a round-bottom flask and dissolved in 20 ml. of water. The solution was heated with reflux for three hours. The refluxed solution was left in a refrigerator overnight. The resulting precipitate, the 2',4',6'trihydroxyacetophenone-2-C^{14} (phloroacetophenone), was filtered and washed three times with 5 ml. of water. It was then dried in a vacuum drying oven at about 70°C. It weighed 1.50 gm. and gave a yield of 24.43% on the basis of acetonitrile. It melted at 215-218°C. (Lit. 217-219°) (26).

It was later found that, when the stoppered filtering flask containing the reaction mixtures was in the ice chest, the hydrogen chloride gas escaped from its side arm, causing the low yield of the product.

The radioactivity of the products was not estimated in each step. For such estimations, it would have been necessary for the product to be purified to a greater extent for each reaction. However, a laboratory monitor with a mica end-window Geiger-Mueller tube as a probe was used to test the presence of radioactivity at all times.

Synthesis of 4',6'-Dimethoxy-2'-Hydroxyacetophenone-2-C^{14}

Since the yield of 2',4',6'-trihydroxyacetophenone-2-C^{14} was low, it was hoped that the use of the crude product described in the previous section for the partial methylation of phloroacetophenone would prevent further loss of radioactive compound. However, this was not the case, and the yield of the desired product was still lower.

Sastri and Seshadri's (27) method was used for the partial methylation of 2',4',6'-trihydroxyacetophenone-2-C^{14}. The dried crude 2',4',6'-trihydroxyacetophenone-2-C^{14} (1.5 gm.) was dissolved in 5.0 ml. dry acetone. To the solutions were successively added 15 ml. dry benzene, 4.0 gm. freshly ignited potassium carbonate, and 2.2 gm. dimethylsulfate. The reaction mixture was refluxed over a hot water bath for 12

hours, with occasional shaking. The inorganic salt, which remained as a solid in the bottom of the flask throughout the reaction, was then filtered and washed with hot benzene. The filtrate was washed twice with water (3 ml. each time) in a separatory funnel and then extracted with 5% aqueous sodium hydroxide four times (6 ml. each time). The combined alkaline extract was poured into ice-cold dilute hydrochloric acid. 4',6'-Dimethoxy-2'-hydroxyacetophenone-2-C^{14} precipitated as a pale crystalline mass. It was filtered and thoroughly mixed with 5% sodium carbonate solution to remove any monomethyl ether that might have been formed. The mixture was again filtered and washed with water. The resulting filtrate did not yield any precipitate on acidification. The yield was 0.8 gm. or 45.7% of the theoretical. On crystallization from dilute alcohol twice, the product obtained consisted of colorless, irregular prisms. It weighed 0.5 gm., melting at 82° (Lit. 81-83°C.) (28).

Preparation of the Chalcone of 5,7,3',4'-Tetramethyleriodictyol-3-C^{14}

4',6'-Dimethoxy-2-hydroxyacetophenone-2-C^{14} (0.5 gm.) (0.0026 mol.) and purified veratraldehyde (0.45 gm.) (0.0026 mol.) were dissolved in 40 ml. 50% ethyl alcohol. To the solution was added rapidly, with stirring, 1.0 ml. of 50% aqueous potassium hydroxide solution. The temperature of the mixture increased slightly, and the solution changed in color from amber to cherry-red within about 30 minutes. The

reaction flask was then stoppered and stored at 35-37°C. for 36 hours.

One hundred forty milliliters of water were added, and the mixture was acidified with cold dilute hydrochloric acid. This caused a very dense, flocculent, almost gelatinous precipitate to form. After standing in the refrigerator overnight, the chalcone, which was recovered as the residue on filtration, was washed with ice water and dried in a desiccator. The chalcone of 5,7,3',4'-tetramethyleriodictyol-3-C^{14} weighed 0.60 gm., a 69% yield.

The chalcone was purified chromatographically by dissolving it in benzene and passing the solution through a column containing magnesol. Three major zones developed. The impure zone in the lower portion of the column (which is the region below the chalcone zone, middle portion), gave a pale-yellow color in visible light, and a yellow color in ultraviolet light. The unreacted substances, which would have preceeded this first zone and would have been eluted easily, were not found.

The first zone was easily eluted by benzene. The second, or middle zone, which contained the chalcone, was yellowish-brown in visible light and brown in ultraviolet light. This zone consisted of almost pure chalcone, which could not be eluted completely by benzene. The third, or upper zone, which is above the chalcone zone, was light yellow in visible light and bright yellow in ultraviolet light. A

very small amount of dark, resinous impurities was present above the third zone. Before the first zone on the column had been completely eluted, the magnesol column was extruded from the glass chromatographic tube, using air pressure, onto a stainless steel plate. This was done by holding the glass tube containing the magnesol in a horizontal position and forcing the magnesol with air pressure out through the top of the tube onto the plate. The extruded magnesol was cut into three portions, corresponding to the three major zones. Each of the three portions was separately eluted with acetone, and the acetone was then evaporated. The residue from the second zone was pure chalcone. The residues from the first and third zones were separately passed through the new magnesol columns. The process of extrusion outlined above was repeated, and the additional chalcone thus obtained was combined with the main portion from the second zone of the original column. It weighed 0.5 gm. and melted at 157°C. (Lit. 157°) (29).

Formation of 5,7,3',4'-Tetramethyleriodictyol-3-C^{14}

To the purified chalcone of the 5,7,3',4'-tetramethyl-eriodictyol-3-C^{14} (0.5 gm.) were added 60 ml. of 95% ethyl alcohol. To this solution were added 3.0 ml. of concentrated hydrochloric acid in 8.0 ml. water. The resulting solution was refluxed for 20 hours. After the refluxing, 90 ml. of water were added, and this solution was extracted immediately with four 10-ml. portions of benzene. The benzene solution

was dried by evaporation. Thus, the very small amount of water present in the solution was removed. The residue from the evaporation of the benzene solution was again dissolved in anhydrous benzene, and the solution passed through a column containing magnesol. The benzene did not elute the unreacted chalcone completely. The column was extruded as before and cut into two portions according to the two zones present. The first or lower zone was chalcone, with a little flavanone as an impurity. The second zone was the radioactive flavanone (5,7,3',4'-tetramethyleriodictyol-3-C^{14}). While still adsorbed by the magnesol, the zone was lemon-yellow in visible light and gave almost no fluorescence in ultraviolet light.

The flavanone zone was eluted with acetone, and the eluate was evaporated to dryness. The residue weighed 0.25 gm. A little amount of the resinous material was formed on the top of the magnesol column, which was combined later with the chalcone of the first zone, after the latter had been recycled for making more flavanone.

The unconverted chalcone (0.22 gm.) was converted into flavanone by repeating the method described above. This time more resinous material was formed. The flavanone obtained this time weighed 0.1 gm. The combined radioactive flavanone weighed 0.35 gm. There was still some unreacted chalcone that had not recycled. The yield of the radioactive flavanone was 70% of the radioactive chalcone. It melted at 160° (Lit.

159-160°) (20)

Preparation of 5,7,3',4'-Tetramethylquercetin-3-C^{14}

5,7,3',4'-Tetramethyleriodictyol-3-C^{14} (0.35 gm.) was dissolved in 30 ml. of 95% ethyl alcohol. To the boiling solution were added 0.2 ml. of n-butyl nitrite, which had been purified by distilling the commercial product at 27°C., 88 mm., and 3.6 ml. of concentrated hydrochloric acid, alternately, in small quantities. The addition took about 5 minutes. Boiling of the solution was continued for 5 more minutes. The flask was stoppered and allowed to stand at 25° overnight. To this solution, 50 ml. of water were added. The resulting solution was then allowed to stand for an additional 12 hours. A gelatinous precipitate of almost pure 5,7,3',4'-tetramethylquercetin-3-C^{14} was formed. This precipitate was removed by filtration and washed with several small portions of 25% ethyl alcohol and finally, the fine, pale-yellow needles (0.09 gm.) were obtained with a melting point 195-197°C. (Lit. 197-198°C.) (20).

The filtrate resulting from removal of these crystals was extracted with ethyl acetate. After evaporating off the ethyl acetate, a resinous solid was obtained. The flavonol was obtained from this by crystallization from 50% ethyl alcohol. An additional 0.01 gm. of the radioactive tetramethyl quercetin was thus obtained.

An attempt was made to recover the unreacted

tetramethyleriodictyol-3-C^{14} from the alcohol filtrate of this crystallization. It was evaporated under reduced pressure to dryness, and then the residue was dissolved in benzene. The resulting benzene solution was passed through a column containing magnesol. Because there was too much resinous impurity present, no tetramethyleriodictyol-3-C^{14} was recovered.

Preparation of Quercetin-3-C^{14}

Tetramethylquercetin-3-C^{14} (0.10 gm.), without further purification, was made into a paste with about 0.5 ml. of acetic anhydride in a 50-ml. centrifuge tube, and 10 ml. of hydriodic acid (specific gravity 1.70) was added. The hydriodic acid used was obtained from Merck Company and contained hypophosphorous acid as the preservative. The centrifuge tube was fitted with a condenser. The tube was immersed in an oil bath and refluxed for 2.5 hours. A precipitate appeared in about 30 minutes. After refluxing, the heating was stopped, and the solution was diluted with sufficient water to cause the quercetin-3-C^{14} to precipitate. The precipitate and its mother liquor were let stand in a refrigerator overnight. The supernatant liquid was decanted off. This dilution and decantation were repeated 3 times. The precipitate, each time, was collected by filtration and was washed three times with 15 ml. ice-cooled water. The radioactive flavonol obtained was dried in a drying oven at 120°C.

for 4 hours. The dried quercetin weighed 0.04 gm. and melted at 314-315° (Lit. 313-314°) (20).

The quercetin-3-C^{14} was purified by dissolving it in acetone, and passing the resulting solution through a column containing magnesol. The quercetin was then eluted with water-saturated ethyl acetate. The chief zone on the column was yellow in visible light and greenish yellow in ultraviolet light. This was the quercetin-3-C^{14}. There were some dark, resinous impurities on the top of the column. No other zones were seen. While eluting with water-saturated ethyl acetate, three other zones developed behind the chief zone. Presumably these are undemethylated methyl quercetin compounds. Their exact nature, however, has not been investigated.

The eluate containing quercetin-3-C^{14} was evaporated to dryness under reduced pressure. It was further purified by repeating the procedure involving use of the magnesol column. This time, only one zone was shown throughout the process. The final eluate was evaporated to dryness under reduced pressure. It weighed 20 milligrams. It was spotted on Whatman No. 1 filter paper and run in 15% acetic acid, 60% acetic acid and n-butyl alcohol-acetic acid-water (n-butyl alcohol:acetic acid:water = 6:1:2) systems. Known quercetin was used for the reference standard. The R_f values are identical with the known quercetin.

R_f in	Known Quercetin	Synthesized Quercetin-3-C^{14}
15% Acetic acid	0.04	0.04
60% Acetic acid	0.30	0.30
n-Butyl alcohol-acetic acid-water	0.70	0.69

The fluorescence of quercetin-3-C^{14} on the paper chromatograms is the same before and after development in different solvent systems.

The paper chromatogram of the synthesized quercetin-3-C^{14}, after having been developed in 60% acetic acid, was subjected to radioautography for one month. At the end of that time, the x-ray film was fixed and developed. There was only one spot on the x-ray film negative, corresponding to the original chromatogram developed in the solvent system.

Estimation of the Radioactivity of Quercetin-3-C^{14}

The activity of the synthesized quercetin-3-C^{14} was also estimated. It is stated in the following sections.

Instrumentation. The Geiger-Mueller counter, used for the estimation of the radioactivity of the synthesized compound, was the Model 182 scaler with the Model 3031 B shield and manual charger. All were the products of the Nuclear Instrument and Chemical Corporation, Chicago.

Efficiency of the System. The efficiency of the system was determined by the following formula using a standard source:

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

This is the ratio of the number of counts the system will detect to the total number of particles emitted from the source.

The standard source used was a secondary standard, sodium carbonate-C^{14} solution having 997 dps/ml. or 59820 dpm/ml. or 5982 dpm/0.1 ml., because the primary standard was not at hand. One-tenth milliliter of this solution was evenly placed in a planchet from a micropipette. The planchet was placed over a hot plate set at a low temperature to stimulate the evaporation of the solvent of the secondary standard. The planchet was then put in the system to determine its radioactivity by the Geiger-Mueller counter. The background counts were determined to be 15.3 cts/min. The count of the secondary standard sodium carbonate was 115.9 cts/min. Therefore the efficiency was (115.9-15.3)/5982 x 100 or 1.7% (1.682%).

Determination of the Specific Radioactivity of Quercetin-3-C^{14}

One milligram of the quercetin-3-C^{14} was dissolved in 1 ml. of acetone. One-tenth milliliter of the resulting solution was measured by a micropipette and was carefully put into a planchet, and the manner employed to measure its radioactivity was similar to that used in the determination of the efficiency of the system. The background counts in both

cases were almost identical. One-tenth milliliter of the quercetin-3-C^{14} solution gave 260.4 cts/min. with a background count of 14.0 cts/min. One milliliter of that solution, then would give 2464 counts per minute. Since one millimore of quercetin is 302 mg., it would give 744,128 counts per millimole per minute. This number divided by $\frac{1.7}{100}$, the efficiency of the system, gives 43,772,235 true counts per minute. The resulting number divided by 60 gives 729,534 true counts per millimole per second. Since one millicurie equals 3.7×10^7 disintegrations per second, the quercetin-3-C^{14} gives the specific radioactivity $729,534/3.7 \times 10^7$ or 1.94×10^{-2} mC/mM.

CHAPTER III

SUMMARY

Although the occurrence of many groups of polyphenolic compounds is widespread in the plant kingdom, no detailed study had previously been successfully made for the isolation and identification of polyphenolic coumarins, flavonols, and cinnamic acid derivatives present in cottonseed. Some polyphenols, under certain conditions, darken on continued exposure to air, and hence, if present, might conceivably affect the color of oil obtained from cottonseed. Thus, it is of value to know the identity of as many of these compounds as possible. The present study was undertaken, therefore, in an effort to identify the polyphenolic flavonols, coumarins, and cinnamic acid derivatives present in cottonseed.

Methyl alcohol and 85% isopropyl alcohol extracts of cottonseed were studied by column and paper chromatography. Separated compounds were compared with authentic reference samples, where available, both by chromatographic and spectrophotometric procedures.

The following phenolic cinnamic acid derivatives were

separated from cottonseed and identified: caffeic acid (3,4-dihydroxycinnamic acid); ferulic acid (4-hydroxy-3-methoxycinnamic acid); p-coumaric acid (p-hydroxycinnamic acid); and the methyl ester of ferulic acid. Also, tentatively identified was 3,4-dimethoxycinnamic acid.

Flavonoid compounds separated and identified were kaempferol (3,4',5,7-tetrahydroxyflavone); quercetin (3,3',4',5,7-pentahydroxyflavone); rhamnetin (7-methyl ether of quercetin); and the 3-methyl ether of quercetin. Rhamnetin-3-rhamnoglucoside and a dimethyl ether of quercetin were tentatively identified in the cottonseed.

Scopoletin (6-methoxy-7-hydroxycoumarin) was also tentatively identified.

For biological testing, quercetin-3-C^{14} was synthesized, using acetonitrile-2-C^{14} as the initial radioactive compound. The acetonitrile was converted by a Hoesch reaction to 2',4',6'-trihydroxyacetophenone-2-C^{14}, which was then partially methylated to 2'-hydroxy-4',6'-dimethoxyacetophenone-2-C^{14}. The latter was condensed with veratraldehyde to form the chalcone of 3',4',5,7-tetramethyleriodictyol-3-C^{14}. After ring closure, the methylated eriodictyol was converted into 3',4',5,7-tetramethylquercetin-3-C^{14} by treatment with butyl nitrite and hydrochloric acid. The tetramethyl quercetin was demethylated with hydriodic acid to produce quercetin-3-C^{14}. The product was purified by adsorption chromatography.

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