FLAVONOID PETAL CONSTITUENTS OF CHRYSANTHEMUM CORONARIUM L.

by

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ABSTRACT

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Methods for the isolation, purification and identification of naturally occurring flavonoids are discussed. The various constituent flavonoid glycosides of the Chrysanthemum coronarium L. petal are identified. These constituents are quercetagetin-7-glucoside, kaempferol-7-rhamnoside, quercemeritrin, luteolin-7-glucoside, chlorogenic and isochlorogenic acids.

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SIGNED: Tom Amyo

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Cornelius Steelink — August 13 1959

C. STEELINK
Assistant Professor of Chemistry

Date

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I. INTRODUCTION

Since the beginning of the systematic study of organic chemistry, considerable interest has been directed toward the processes and mechanisms by which the higher plants synthesize the great number of compounds found in their roots, bark, flowers and leaves.

Theories concerning these mechanisms in relation to the flavonoid constituent class of compounds have been forwarded by Geissman, [1] Seshadri, [2] Steelink, [3] Neish, et. al. [4] and others. It is the aim of these theories to explain the origin of the experimentally determined constituents of the leaves, flowers and stems of the higher plants by proposing suitable biosynthetic sequences, starting from simpler compounds.

The common feature of all theories of plant synthesis today, according to Geissman, [5] is found in their necessarily speculative nature. This indecisiveness in this field is brought about by the simple lack of adequate information on reaction intermediates in plant synthesis and the lack of enough data on plant constituents in many genuses and species.

The chemist is therefore faced with the problem of formulating a valid theory of biosynthesis without sufficient knowledge of either the precursors or intermediaries or final products in a biosynthetic scheme.

The approach to this problem has been threefold:

A. APPROACH TO PROBLEM

Experiments showing the in vitro interconvertability of some compounds which co-occur in vivo have been advanced as evidence that these transformations can account for the coexistance of two or more related compounds in a single plant or a single species. This coexistance along with the evident structural relationship suggests what is termed by some experimenters as a plausible mechanism of biosynthesis.

Seshadri[5] speculates on the biogenesis of these observed compounds utilizing the concept of the plant's in vivo addition and removal of the hydroxyl function. A correlation is then drawn from this process to the in vitro oxidation, as suggested by Seshadri, which occurs in the stage of the synthesis wherein one flavone acts as an immediate precursor of another.

Isotopic tracer work, using C^{14} labelled precursors fed to growing plants with the resultant plant constituents being extracted and analyzed for C^{14} content, has contributed much to the current knowledge of biosynthetic sequences. Isotopic dilution experiments by Watkins, Underhill and Neish[4] have helped to verify the origin of the A and B rings (see Discussion) of the flavonoid group of compounds.

Isolation and identification of the major constituents present in

all the plants of a specific genus or species is known as the statistical approach to the problem. Through these means of investigating a group of very closely related plants, a correlation can be presented between the precursors, the compounds present in the environment of the growing plant, and the final products, the compounds found in the plant itself. With this correlation the chemist can attempt to formulate a mechanism describing the biosynthetic reaction intermediates, thereby moving one step closer to a generalized biosynthetic concept.

For full utilization of this approach, consideration of a group of closely structurally related compounds is necessary. The co-occurrence of such related compounds in a particular genus or species suggests a genetic basis for their structural similarities. It would appear in these cases that a common precursor, having a characteristic, presumably genetically determined substitution pattern, may be modified along several different paths giving rise to compounds which have certain distinctive structural features in common.

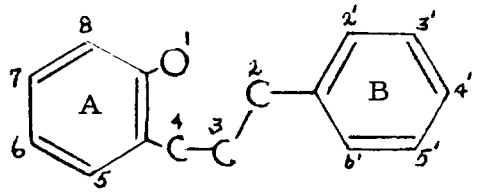
A group of compounds well suited for this consideration are the anthocyanin pigment and anthoxanthin constituent portions of the plant. Most of the red, blue and purple pigments are found in the anthocyanin pigments with the slightly colored and light yellow pigments found in the anthoxanthins.[6]

The flavonoid compounds, constituting a more specific portion

of this group, are therefore considered suitable for this work. These compounds are found widely distributed, as a class, in higher plants. This fact suggests that they owe their existance to processes fundamental in higher plants and makes these compounds extremely desirable for study.

B. CHARACTERISTICS OF FLAVONOID COMPOUNDS

1. The flavonoids are a class of compounds which can be seen to be derived from the basic $C_6C_3C_6$ carbon skeleton (I).



I

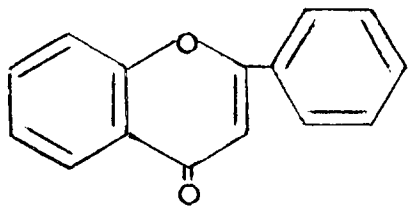
2. In naturally occurring flavonoids, the degree of oxidation of the C_3 fragment and the degree of hydroxylation of the A and B rings (I) determines the characteristics of the compound.

3. The A ring, depicted as the ring fused to the heterocycle and adjacent to the carbonyl group of the C_3 fragment, is typically substituted in a phloroglucinol hydroxylation pattern.

4. The B ring, the other benzene ring, is characteristically substituted in the same manner as the aromatic ring of the typical naturally occurring C_6C_3 compounds such as cinnamyl alcohol, coniferyl alcohol, cinnamic acid (the

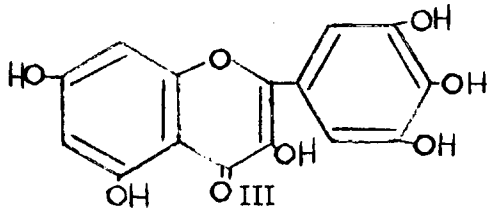
derivatives of C_6C_3 alcohols, aldehydes and acids), or is completely unsubstituted. The B ring of all but six of the many known naturally occurring flavonoid and isoflavonoid compounds are either unsubstituted or substituted with one, two or three hydroxyl functions in the 3', 4' or 3', 4', 5', positions respectively. The exceptions, naturally, are those compounds having a hydroxyl group in the 2' (ortho) position of the B ring.

5. The variation of the hydroxylation patterns in the flavonoid group range from the unhydroxylated parent compounds, flavone (II)



II

to the highly hydroxylated flavonol, myricetin (III).



III

6. The C_4 carbon is in most cases present as the carbonyl, but state of oxidation of this fragment may vary from the very slightly oxidized state (IV) to the highly oxidized "diketo" state (V).

A-CH_2CHOH-CHOH-B A-CO-CO-CHOH-B

IV V

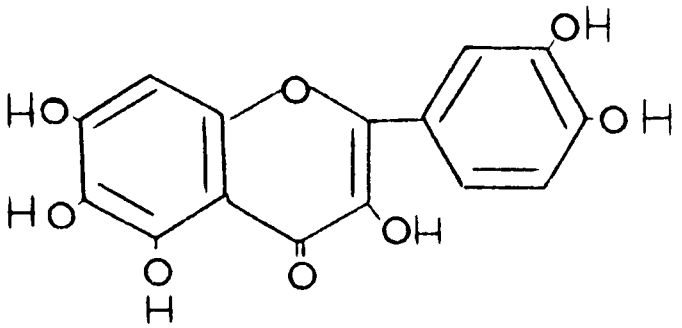
7. Hydroxylation is known to occur at all points on the A ring. Hydroxyl groups have been reported on the 5, 6, 7, 8-positions of the A ring.

8. Methylation and gylcosylation is restricted to hydroxyl groups in specific positions in the molecule, or at least occurs with much greater frequency in certain positions.

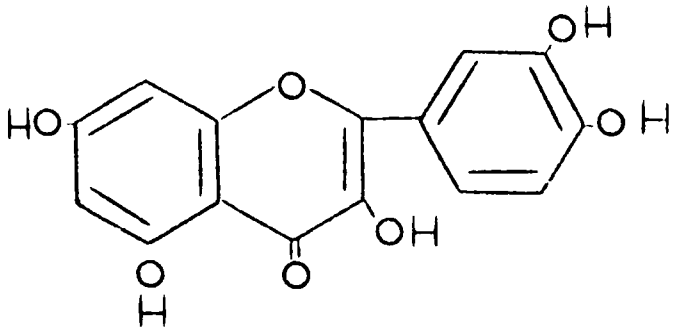
9. Flavonols are most commonly glycosated in the 3-position and the 7-position, with slightly less tendency toward the 3', 4'-positions are perhaps the 8-position.

10. Flavones and flavonols which have no 3-position usually occur as 7-glycosides. In very few flavones is the sugar residue attached to the 5-hydroxyl group.

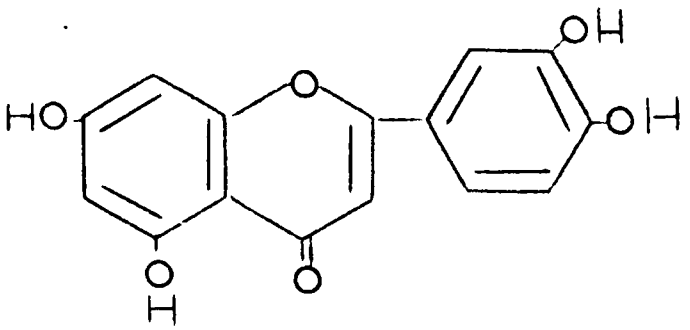
II. FORMULAS



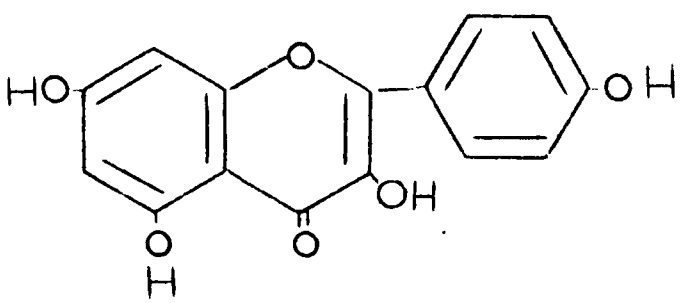
Quercetagetin



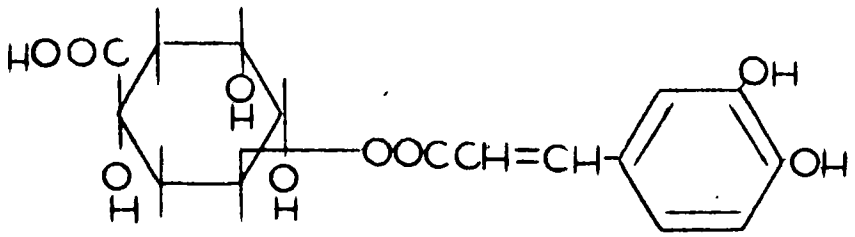
Quercetin



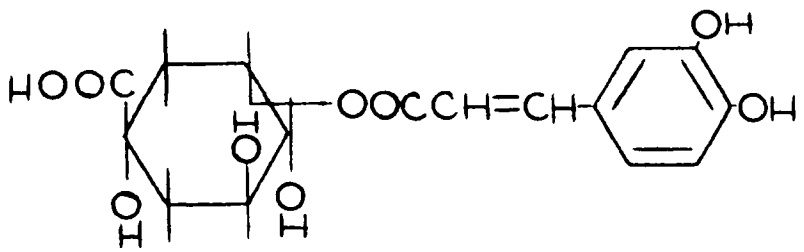
Luteolin



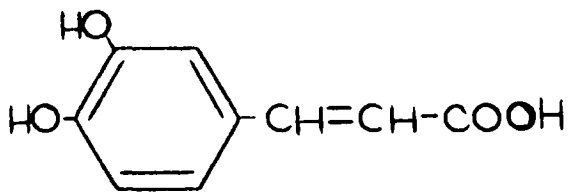
Kaempferol



Chlorogenic Acid



Isochlorogenic Acid



Caffeic Acid

III. DISCUSSION

The development of Partition Chromatography techniques and the increasing application of absorption spectroscopy have made powerful tools available for the detection, separation and structural elucidation of water soluble plant pigments. The data gained from these techniques on the chemical composition of the higher plants will increase the body of knowledge from which a valid theory of biosynthesis may be formulated. It is the aim of this work, utilizing these methods, to make a contribution toward this end.

A. EXTRACTION TECHNIQUES[1]

Since plant tissues usually contain either general or specific glycosidases as well as other kinds of enzymes capable of modifying cellular constituents, autolytic processes may ensue directly after collection of fresh plant material. These processes can result in either the hydrolysis of the glycosides present or the destructive oxidation of any sensitive compounds.

As can then be seen, autolysis of the fresh plant material, upon its storage, may result in the production of the aglycones corresponding to the glycosides originally present within the cells. Subsequent isolation and structural determination of these aglycones, would

thereby lead to an erroneous description of the constitution of the plant.

In order to circumvent this autolytic destruction, the plant material is immediately and rapidly dried after picking. This material is then preserved in a form which is now substantially equivalent to the fresh material and this thoroughly dried material may be stored for extended periods of time with no harmful effects.

Since most flavonoid glycosides are rather readily hydrolyzed by acid, care must be taken when using fresh material, to prevent the decomposition of glycosides during extractions with boiling solvents. Rapid exposure of the plant materials to boiling alcohol is effective in deactivating hydrolytic enzymes, but the materials in the extract are still exposed to the danger of hydrolysis by accompanying plant acids. Therefore, longer, milder treatment by use of a Soxhlet Extractor is customary.

B. CHROMATOGRAPHIC TECHNIQUES

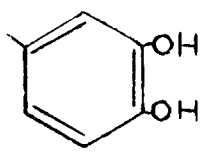
Isolation and purification of the constituents of the concentrated extract was carried out by using the standard paper chromatographic techniques. Identity of all the aglycones was proven by cochromatography with authentic samples, whenever possible, by use of these techniques. (See Table I.) Glycosidic sugars were identified by standard sugar chromatographic techniques using p-anisidine, trichloroacetic acid indicator spray.

C. SPECTROPHOTOMETRIC TECHNIQUES, EFFECT OF "SHIFT" REAGENTS

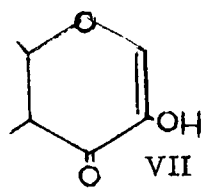
By the development of ultraviolet spectrophotometric techniques, a powerful tool of identification is made available to the chemist, as many of the flavonoids exhibit fluorescent colors under ultraviolet light and possess characteristic ultraviolet spectra.[7]

Individual flavonols, aside from having their own characteristic spectra also have their own individual spectral "shift" upon treatment with a reagent such as alcoholic aluminum chloride, sodium ethylate, sodium acetate and/or boric acid. These reagents are relatively specific for compounds having certain hydroxylation patterns, thereby allowing some descriptive speculation as to the identity of the compound under study.

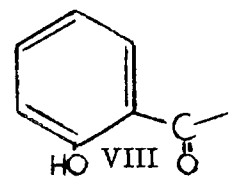
Most of the naturally occurring compounds of the flavonoid class are polyphenols that contain one or more of the following structural features, which present knowledge indicates are involved in complex formation with metal ions or complexing agents.[8]



VI



VII



VIII

These groups occur either singly or in combination in a single molecule. (i.e., quercetin, VI, VII, VIII; luteolin, VI, VIII, etc.) To illustrate.

the value of these reagents for the structural elucidation of these compounds, a discussion of their effect on the spectrum of the substances containing the above chelatogenic groups is necessary.

I. ALUMINUM CHLORIDE

ALUMINUM CHLORIDE - SODIUM ACETATE[8]

The addition of the aluminum chloride reagent to a flavonoid containing any available, highly chelatogenic group, will result in chelate formation involving the aluminum ion and that group with a definitive bathochromic shift in the long wave length of the flavonoid's spectrum.

Grouping VI shows little tendency to react with aluminum chloride alone. With addition of solid sodium acetate however, the spectrum of the compound is changed completely with the long wave length maximum (350 - 380 mμ) undergoing a 10 - 30 mμ bathochromic shift. This fact, that sodium acetate produces a large shift in the spectrum when aluminum chloride is present, indicates that the formation of the metal complex is associated with the ionization of at least one of the hydroxyl groups.

Luteolin, containing the weakly complexing 5-hydroxyl-

4-carbonyl system (VIII), forms a complex with aluminum chloride and upon addition of sodium acetate, a further large bathochromic shift (35 mμ) is observed. It is suggested that this complex now involves the 3'4'-dihydroxyl grouping and the 4-carbonyl-5-hydroxyl system. Stoichiometric evidence points to the involvement of two metal ions, a fact consistent with the experimental evidence obtained to date.

Other flavones, containing both the strongly chelating 3-hydroxyl-4-carbonyl system (VII) and the 3'4' dihydroxyl grouping (VI) and/or the 5-hydroxyl-4-carbonyl system (VIII) show complex formation with ethanolic aluminum chloride. The resultant bathochromic shifts range from 10 to 40 mμ.

The specificity of the aluminum chloride reagent has been questioned by Jurd[9] who feels that it reacts in such a general manner with such a large number of substances that it is not possible to establish, by its use alone, the presence of any one grouping. Its usefulness lies, however, in giving indication of the possible chelatogenic group present and in its high complex stability. The absorption spectra, therefore, of these complexes have been determined to be stable for several hours,[10] thereby even allowing the

flavonoid pigment to be leached from paper strips with aluminum chloride solution with no great loss found in solvent instability of complex.

2. SODIUM ETHYLATE[11]

Flavonols containing a free dihydroxyl grouping in the 3,4' positions are unstable in alcoholic sodium ethylate and can be distinguished spectrophotometrically by this instability. From tests with addition of base, it became evident by the stability of the λ_{max} in the complete ultraviolet spectrum (200 - 400 mμ) that all the compounds at which the C_3 hydroxyl was protected by glycosidation are stable in the alkaline medium and that this stability is not appreciably influenced by the number and location of the other hydroxyl groups in the molecule. Treatment with sodium ethylate will cause a stable bathochromic shift with compounds having a protected 3,4' dihydroxyl group, while a hypsochromic shift with subsequent destruction of the spectrum can be expected from a compound containing a free 3,4' dihydroxyl grouping or a pyrogallol or hydroquinone-type nucleus in the molecule. The latter type compounds are very susceptible to basic medium and decompose independently in base whether the hydroxyl group at position 3 is protected or not.

These sodium ethylate shifts have also been classified by Jurd[9] as being too general to establish, by their use alone, the presence of any one grouping. They also serve as indicators however, of the possible structures present.

3. SODIUM ACETATE[12]

Sodium ethylate, described above, ionizes all phenolic hydroxyl groups in the molecule unless sterically hindered. Sodium acetate, on the other hand, is capable of ionizing only those groups which are rather strongly acidic. Since theoretical and experimental evidence indicates that the 7-hydroxyl group of flavones and flavonols is probably more acidic than the other hydroxyl groups in the molecule[13], the possibility of using sodium acetate to differentiate this group was studied.

Results of the study showed that with compounds having the 7-hydroxyl group free, the short wave length band in their spectrum (usually 250 - 270 mμ) undergoes a bathochromic shift of 8 - 20 mμ upon addition of solid sodium acetate to a sample solution. If no free 7-hydroxyl group is present, no shift is observed.

The 250 - 270 mμ band in the flavonol spectrum is mainly associated with absorption in the A ring and the

heterocycle. Free hydroxyl groups at the 3, 5, 8, 3', 4' positions showed no interference and no effect upon the addition of the sodium acetate.

4. BORIC ACID

BORIC ACID - SODIUM ACETATE [9]

The ultraviolet spectra of phenolic compounds containing o-dihydroxyl groups are altered characteristically in the presence of a mixture of boric acid and sodium acetate. These compounds show that in the presence of boric acid and sodium acetate, a considerable bathochromic shift is observed, due to the interaction of the boric acid molecule with the available o-dihydroxyl groups.

According to Jurd, [9] the reliability and ease of interpretation of spectral changes in the presence of boric acid and sodium acetate makes this an ideal method for the detection of ortho-dihydroxyl groups. Compounds unstable in alkali are stable in this medium. Some classes of other compounds have been investigated and in every case Jurd reports a shift of the λ_{max} for the compounds possessing the o-dihydroxyl grouping.

This investigater feels, however, that the reliability of this reagent has been somewhat overstressed. Results

obtained with this reagent during the course of this investigation were poor even when checking the reagent against authentic samples of compounds known to contain o-dihydroxyl groupings. The poor results in this case are attributed to the low rate of solubility of the boric acid, sodium acetate in the ethanolic solution, thereby allowing no complex formation to take place within a reasonable length of time.

5. APPLICATION TO CHRYSANTHEMUM CORONARIUM L.

In applying these tests to the constituent flavonoids of Chrysanthemum coronarium L., their usefulness soon becomes evident. For instance, in the identification of Band 1 (see Table 1 and Figures 1, 2) a shift produced by aluminum chloride reagent and additional shift produced with the subsequent addition of sodium acetate indicated the presence of a strongly chelatogenic group (VIII) and also of a weaker dihydroxyl chelating group (VI).

The very rapid destruction of the band's characteristic spectrum upon addition of sodium ethylate indicated the presence of a hydroquinone or pyrogallol-type aromatic nucleus.

The addition of solid sodium acetate caused a distinct shift both in Band 1 and in the authentic sample of

Quercetagetin-7-Glucoside.* This would indicate that this position is extremely acidic in this molecule as hydrolysis of the sugar is apparently carried out by the addition of the sodium acetate.

The above spectral evidence indicated the identity of the band to be Quercetagetin-7-Glucoside. Identical spectral characteristics and cochromatography of the band with an authentic sample of Quercetagetin-7-Glucoside confirmed the identity of this band.

Similiarly, treatment of Band 2 (see Table 1 and Figure 5) indicated the identity of this band to be a Kaempferol-7-glycoside. Cochromatography and identity of spectral characteristics of the hydrolysate of this band with an authentic sample of Kaempferol proved the identity of this band.

Application of these tests to the other bands found in the extract, along with cochromatography and comparison of spectral data with an authentic sample, whenever possible, proved the identity of Band 3 (quercimeritrin, Table 1 and Figures 9, 10) of Band 4 (luteolin-7-glucoside, Table 1

*Kindly supplied to us by Professor T. R. Seshadri, Chairman, Department of Chemistry, University of Delhi, India.

and Figures 12, 13) and of Bands 5 and 6 (chlorogenic and isochlorogenic acids, Table I and Figures 15, 16). The latter two compounds, being structural isomers of each other (see Formulas) have identical spectral behavior and were conclusively identified on the basis of R_f values (see Table I).

All glycosidic sugars were later identified by use of the aforementioned standard sugar chromatographic techniques (see Part IV, Section D).

IV. EXPERIMENTAL

A. COLLECTION AND EXTRACTION

The plants used for this study were picked in Los Angeles, California, April 21, 1958. The petals were separated from the disc and were immediately immersed in methanol.

To effect extraction, the petals were placed in a clean cloth bag and were fitted into a large Soxhlet extractor. Extraction of the petals was then carried out for thirty-six hours. At the completion of this process, the petals had a bleached white coloration and the alcoholic solution exhibited a deep yellow color.

A straight glass condenser, in distilling position was then attached to the boiler and the extract was distilled at reduced pressure. Through these means the extract was concentrated to 1/15 of its original volume and now was dark brown in color and of slurry-like consistency.

This slurry was then transferred to a 250cc separatory funnel and extracted repeatedly with benzene. Benzene extraction was considered complete only when no further coloration was observed in the benzene layer.

A sample of the purified extract was cochromatographed with a sample of the benzene solution on a strip of Whatman #1 chromatographic filter paper. The paper was developed in a butanol:acetic acid:water

(11/3/8, v/v) tank for thirteen hours. It was then examined under visible and ultraviolet light for signs of flavonoid compounds in the benzene extract. Since the benzene spot remained stationary at the origin and the alcoholic extract revealed several distinct compounds, it was assumed that no flavonoids had been carried over into the benzene.

B. SEPARATION AND PURIFICATION

1. Development Techniques

The chromatographic tanks used for this study were 12" x 12" x 24" square chromatographic jars (101A, Research Specialities Corporation, Berkeley, California) fitted with a stainless steel tray support capable of supporting four 1" x 10" glass chromatographic troughs.

Standard descending chromatographic techniques were used throughout the course of this study with all R_f values being derived by these methods. The identification of the sugar moieties, as described in section D, was undertaken through standard sugar chromatographic techniques.[14]

2. Elution Methods[15]

In order to facilitate the investigation of a purified

constituent of the chrysanthemum extract, it was necessary to obtain a pure, concentrated alcoholic solution of that constituent. The most widely used and most accurate procedure for this is based on the simple expedient of cutting out the section of the developed chromatogram which contains the single pure substance and removing the substance from the paper by the proper eluting agent. The concentrated alcoholic solution was collected in a 20 ml beaker ready for further investigation.

3. Hydrolysis Techniques

The 10 ml alcoholic solution, eluted from a chromatogram as described in section 2 is placed in a 50 ml one-neck, round-bottom flask which is fitted with a short water condenser. To this is added 25 ml of 4N sulfuric acid and the system is brought to reflux for two and one-half hours and then allowed to stand overnight.

The hydrolysate is transferred to a separatory funnel and the flavonoid portion extracted with a solvent such as butyl acetate. The aqueous portion is concentrated and stored for subsequent chromatography in an ethyl acetate:acetic acid: water (9/2/2, v/v) system for the identification of the sugar groups.

The butyl acetate extraction is concentrated to 2-3 ml, spotted on Whatman #1 paper and developed in butanol:acetic acid:water. The hydrolysate is then isolated from the chromatograph by elution with the appropriate solvent.

4. Preliminary Investigation

The alcoholic chrysanthemum extract, dark brown in color, was spotted on several sheets of Whatman #1 paper. The sheets were placed in a number of different solvent systems for development. The systems used in the preliminary investigation include:

Butanol:Acetic Acid:Water (11/3/8, v/v)

m-Cresol:Acetic Acid:Water (50/2/48, v/v)

Phenol:Water (73/27, w/w)

Benzene:Methanol:Acetic Acid (1/1/1, v/v)

Benzene:Ethyl Acetate:Butyl Alcohol (1/1/1, v/v)

Ethyl Acetate (saturated with water)

Ethyl Acetate:Acetic Acid:Water (50/2/48, v/v)

Water:Acetic Acid:Hydrochloric Acid (10/30/3, v/v)

Ethyl Acetate:Water:Acetic Acid (9/2/2, v/v)

Development of the chrysanthemum extract was attempted in these solvents on both Whatman #1 and

Whatman #3 paper. Those solvent systems which proved most effective for the separation of this extract into its component parts were, butanol:acetic acid:water (BAW), phenol:water, and 5% acetic acid.

The extract was then concentrated in a stripe across the top of a number of sheets of Whatman #1 paper and these papers were placed in the abovementioned three solvent systems. The papers were removed after appropriate development and examined under ultraviolet light. The phenol:water system appeared to give the best separation of the extract into its constituent bands, therefore, more papers were striped for development in this system.

The papers were removed after eighteen hours and air dried. The constituent bands were examined and outlined, with pencil, under visible and ultraviolet light. The bands were then cut out and eluted with 70% ethanol. The eluates were concentrated to 2 ml and striped on separate sheets of Whatman #1 paper. These were placed in the butanol:acetic acid:water tank and allowed to develop for thirteen hours. They were then removed, air dried, the bands outlined under visible and ultraviolet light. The bands were then eluted; the eluates were concentrated

and respotted for chromatography in the 5% acetic acid tank. After five hours, the chromatograms were removed, dried, the well-defined bands eluted and concentrated to 5 ml and labelled for spectrophotometric analysis.

5. Spectral Methods

Spectrophotometric analyses in the course of this investigation were made on the Cary Recording Spectrophotometer, Model 11 (Applied Physic Corporation, Monrovia, California). The spectra of the purified flavonoids were determined in the 200 mμ to 500 mμ range; the last 100 mμ (400-500 mμ) being investigated only after addition of "shift" reagents. These "shift" reagents are added directly to the cuvette containing the sample and in equal amount, to the cuvette containing the solvent blank; the solvent blank being obtained by the elution of a blank portion of the chromatogram from which the sample was taken, thereby eliminating any spectral interference from the chromatogram paper itself. Aluminum chloride[8] and sodium ethylate[11] are added in alcoholic solution (10 drops 1M Aluminum chloride, 5 drops 1N sodium ethylate), while sodium acetate[12] and boric acid[9] are added in solid form. A time lag of approximately five minutes was allowed for the reaction of some of these reagents.

C. IDENTIFICATION OF CONSTITUENT BANDS

1. Identification of Band 1

The yellow (visible)-brown (ultraviolet) band was cut out from a phenol:water developed paper, eluted with 70% ethanol, concentrated and rechromatographed in butanol: acetic acid:water. Band 1 split into two distinct sub-bands (1a, 1b) upon chromatography in the BAW solvent system. The bands were separately eluted and investigated. Both bands were rechromatographed in BAW and after subsequent elution and concentration their ultraviolet absorption spectra were determined. Spectrophotometric analysis, including the previously described spectral shifts, tentatively identified Band 1a as a quercetagetin-7-glycoside (see Figures 1, 2). The spectrum of an authentic sample of Quercetagetin-7-glucoside was determined and was found to be identical with that of Band 1a (see Figures 3, 4).

Band 1a was cochromatographed in four different solvent systems with an authentic sample of quercetagetin-7-glucoside. The observed identity of R_f values proved the identity of this band (see Table 1).

The isolated band was hydrolyzed in 4N sulfuric acid and the hydrolysate analyzed spectrophotometrically.

Quercetagetin-7-glucoside was then hydrolyzed in identical manner and the aglycone was also analyzed spectrophotometrically. The two sets of spectral characteristics were found to be identical. In fact, the spectra of the aglycones were also found to be identical to the glycosides, indicating an extremely acidic 7-hydroxyl position.

Cochromatography of band 1a hydrolysate with quercetagetin in four different solvent systems yielded identical R_f values (see Table 1) and further proved the identity of this band.

Band 1b was further found to be identical with band 3 and is discussed under that heading.

2. Identification of Band 2

Band 2 was cut out from the phenol:water chromatogram and was eluted and rechromatographed as described in 1. The purified band's spectral characteristics and R_f values corresponded closely to those of a Kaempferol-7-glycoside (see Table 1).

This yellow-green band was then hydrolyzed in 4N sulfuric acid, extracted with butyl acetate, concentrated and rechromatographed in butanol:acetic acid:water. The resultant stripe was eluted and concentrated for spectral determinations.

An authentic sample of Kaempferol was obtained, purified by paper chromatography, and its ultraviolet absorption spectrum determined. The identity of the spectra of the authentic sample and that of the isolated band characterized Band 2 as a Kaempferol-7-glycoside (see Figures 5, 6, 7, 8).

The two aglycones were subsequently cochromatographed in four different solvent systems. Identical R_f values were observed (see Table 1) thereby confirming the tentative assignment of Kaempferol-7-glycoside to Band 2. Subsequent sugar chromatographic techniques proved the glycosidic portion of this molecule to be Rhamnose (see Section D).

3. Identification of Band 3

Band 3, a highly colored yellow band was isolated from a phenol:water chromatogram of the chrysanthemum extract and eluted with 70% ethanol to give a bright yellow solution. This band was purified chromatographically in the usual manner.

Spectrophotometric analysis of Band 3, along with R_f values from three different solvent systems led to the tentative assignment of Quercimeritrin (Quercetin-7-glucoside)

to this band (see Table 1).

The band was then hydrolyzed in 4N sulfuric acid and the aglycone was purified chromatographically. The spectrum of the aglycone was determined (see Figure 10).

An authentic commercial sample of quercetin was purified chromatographically in butanol:acetic acid:water and its spectrum was determined. The spectra of the isolated band and of the purified sample of quercetin were identical (see Figures 9, 10, 11). Identity of the band hydrolysate and quercetin was also shown by cochromatography in four different solvent systems (see Table 1). Final proof of the band's identity came upon identification of the sugar moiety as being glucose (Section D).

4. Identification of Band 4

Band 4 was isolated from the phenol:water chromatogram and eluted with 70% ethanol. The eluate was concentrated and rechromatographed in the usual manner. Spectrophotometric data and Rf values from three different solvent systems indicate the identity of this band to be a Luteolin-7-glycoside (see Table 1 and Figures 12, 13).

The light yellow (visible)-brown (ultraviolet) band was hydrolyzed in 4N sulfuric acid and the aglycone was

purified chromatographically. The spectrum of the hydrolysate was determined and comparison made with that of an authentic sample of luteolin, which had first been purified chromatographically. The spectral and R_f values of luteolin were identical with those of Band 4 (see Table 1 and Figure 14). Identical R_f values obtained upon cochromatography of the aglycones in four different solvents gave further indication of the identity of this band. Final proof of the identity of Band 4 came with the identification of the sugar moiety as glucose (Section D).

5. Identification of Band 5

This highly fluorescent blue band, which, when placed in the vapors of ammonia turns a bright blue-green, was isolated from a butanol:acetic acid:water developed chromatogram and examined spectrophotometrically. These data and R_f values from three different solvent systems indicated the identity of this band to be Chlorogenic Acid (see Table 1).

An authentic sample of chlorogenic acid was purified chromatographically and analyzed spectrophotometrically. The spectral characteristics of Band 5 and Chlorogenic acid were identical (see Figures 15, 16).

Band 5 was hydrolyzed and cochromatographed with an authentic sample of its hydrolysate, Caffeic Acid. Identical R_f values in four different solvent systems proved the identity of this band (see Table 1).

6. Identification of Band 6

This other highly fluorescent blue band was isolated from the phenol:water chromatogram and was purified chromatographically and its spectrum was determined. This band was found to have spectral characteristics identical with Band 5, with the only obvious differentiation between the two bands occurring in the R_f values (see Table 1).

Hydrolysis of this band yielded a compound with identical R_f values and spectral characteristics as that of the hydrolysate of Band 5 and also as those of caffeic acid (see Figures 17, 18). Cochromatography with an authentic sample of Caffeic Acid gave identical R_f values in four different solvents (see Table 1) showing identity of the hydrolysate.

Band 6 was confirmed as Isochlorogenic acid by comparison of the experimentally determined R_f values to those found in the literature.[1]

D. ISOLATION AND IDENTIFICATION OF THE SUGAR MOIETIES[14]

The aqueous phase of the luteolin, quercetagetin, kaempferol and quercetin hydrolysates were concentrated to 2-3 ml and spotted on Whatman #1 paper. The chromatogram was developed in ethyl acetate:acetic acid:water (9/2/2, v/v) for thirteen hours, air dried and then sprayed with a sugar indicator (0.8g p-anisidine, 4 g trichloroacetic acid in 100 ml 50% ethanol) and allowed to dry and develop color for nine hours. Identical R_g values of the glycosidic sugars with the authentic samples of glucose and rhamnose proved the identity of the sugars as follows:

Luteolin-7-	glucoside	(R_g = 1.00)
Quercetagetin-7-	glucoside	(R_g = 1.00)
Kaempferol-7-	rhamnoside	(R_g = 1.44)
Quercetin-7-	glucoside	(R_g = 1.00)

V. SUMMARY

The flavonoid petal constituents of Chrysanthemum coronarium L. have been identified by use of paper-chromatographic and spectrophotometric techniques. These constituents are quercetagetin-7-glucoside, kaempferol-7-rhamnoside, quercemeritrin, luteolin-7-glucoside and chlorogenic and isochlorogenic acids.

The flavonol glycosides found in Chrysanthemum coronarium L. have therefore all been shown to be 7-glycosides. This pattern seems to be common to the petal constituents of Chrysanthemum segetum, Coreopsis maritima L., Chrysanthemum coronarium L., and Gossypium herbaceum.[3]

The co-occurrence of a quercetagetin derivative with a quercetin derivative is reported. Since statistical surveys have shown that the co-occurrence of quercetin derivatives with gossypetin (3, 3', 4', 5, 7, 8 - hexahydroxy flavone) derivatives seems to be biogenetically preferred to those of quercetin and quercetagetin,[16] this report then sites one of the very rare co-occurrences of these two compounds.

The identification of a 7-rhamnoside derivative of Kaempferol is the first record of its occurrence in the field.

VI. Table I

Constituents of Chrysanthemum coronarium L.

Band	R_f BAW	R_f Phenol/H_2O	R_f 5% HAc	R_f m-Cresol HAc/H_2O	Color V	Color UV
1	0.34	0.24	0.15	0.11	Y	Br
Quercetagetin-7-Glucoside*	0.36	0.24	0.15	0.11		
2	0.47	0.36	0.05	....	Y	YGr
Kaempferol-7-Rhamnoside						
3	0.56	0.46	0.07	....	Y	Y
Quercimeritrin						
4	0.68	0.60	0.10	....	Y	Br
Luteolin-7-Glucoside						
5	0.72	0.39	0.64	0.47	..	Bl
Chlorogenic Acid*	0.72	0.39	0.64	0.47		
6	0.83	0.29	0.33	....	..	Bl
Isochlorogenic Acid						
		Acid Hydrolysates				
1	0.50	0.32	0.01	0.08		
Quercetagetin	0.50	0.32	0.01	0.08		
2	0.88	0.68	0.05	0.31		
Kaempferol*	0.88	0.68	0.05	0.31		
3	0.83	0.49	0.00	0.41		
Quercetin*	0.83	0.49	0.00	0.41		
4	0.85	0.75	0.00	0.65		
Luteolin*	0.85	0.75	0.00	0.65		
5	0.93	0.64	0.52	0.39		
Caffeic Acid*	0.93	0.64	0.52	0.39		
6	0.92	0.64	0.52	0.39		
Caffeic Acid*						

* Authentic sample

VII. FIGURES

Legend for all Spectra

———————— pure compound

°°°°°°°°°°°°°°°° with sodium ethylate

– – – – – – – – with aluminum chloride

°°°°°°°° with sodium acetate

—"—::—"—"— with aluminum chloride-sodium acetate

—•—•—•—:— with boric acid

—"":—:":—":":— with boric acid-sodium acetate

FIGURES 1, 2

SPECTRUM OF BAND 1

	λ_{max}
pure compound	260, 272, 362
sodium ethylate	destroys spectra
aluminum chloride	278, 388
sodium acetate	305, 378
aluminum chloride-sodium acetate	285, 430
boric acid	262, 372
boric acid-sodium acetate	262, 372

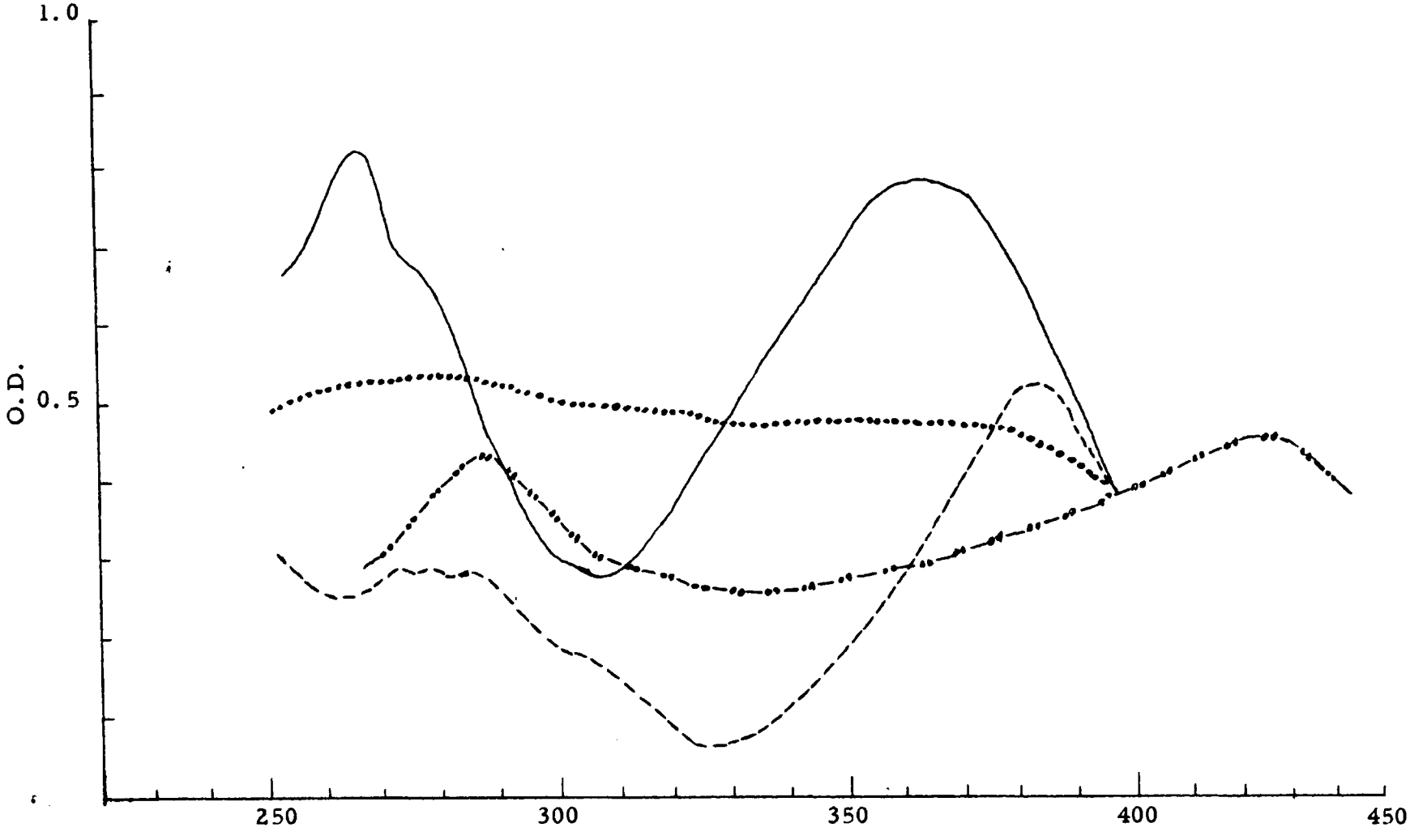


Figure 1. SPECTRUM OF BAND 1

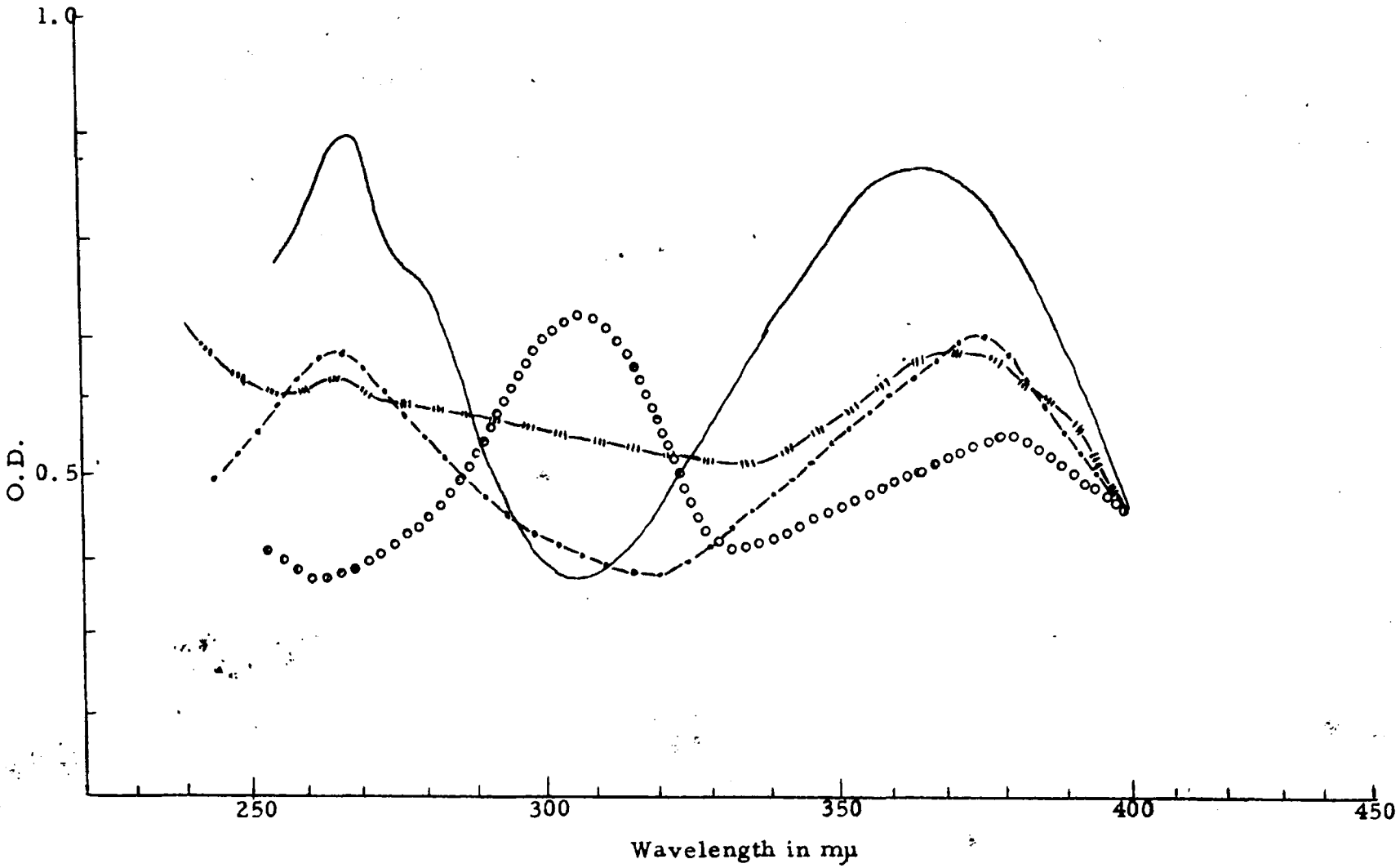
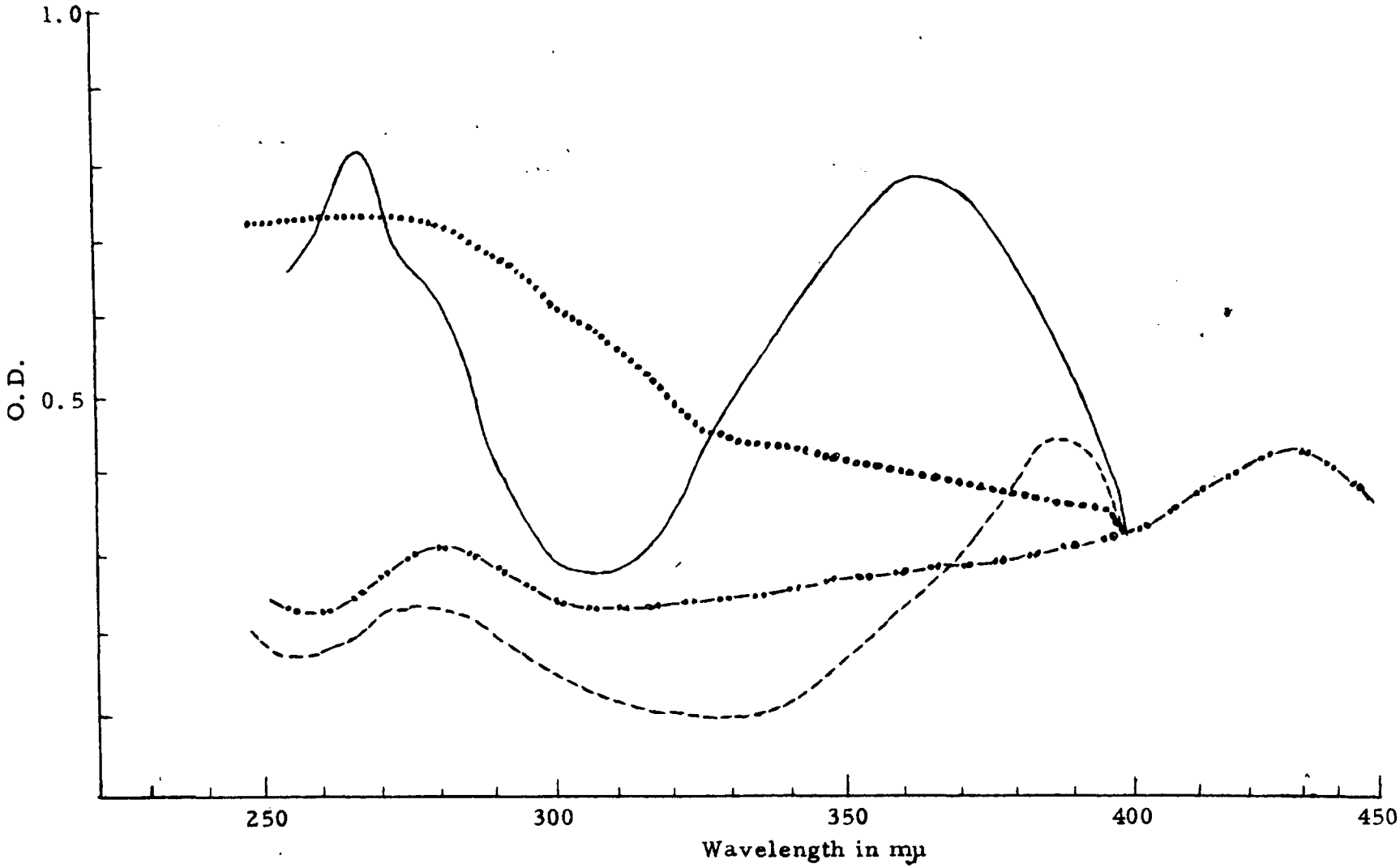


Figure 2. SPECTRUM OF BAND 1

FIGURES 3, 4

SPECTRUM OF QUERCETAGETIN-7-GLUCOSIDE

	λ_{max}
pure compound	260, 272, 362
sodium ethylate	destroys spectra
aluminum chloride	278, 388
sodium acetate	305, 378
aluminum chloride-sodium acetate	280, 430
boric acid	262, 372
boric acid-sodium acetate	262, 372



Figures 3. SPECTRUM OF QUERCETAGETIN-7-GLUCOSIDE

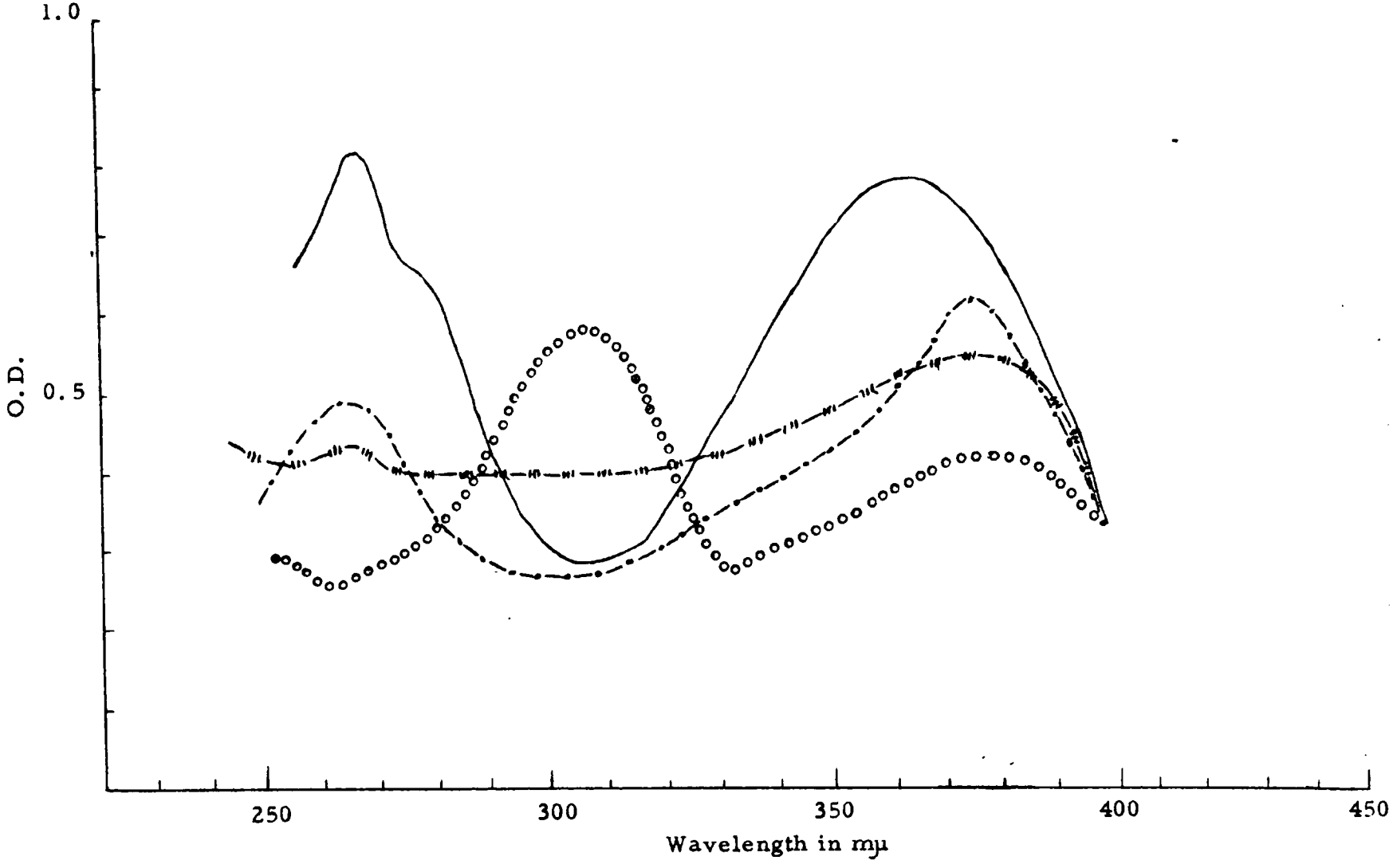


Figure 4. SPECTRUM OF QUERCETAGETIN-7-GLUCOSIDE

FIGURE 5

SPECTRUM OF BAND 2

	λ_{max}
pure compound	268, 370
sodium ethylate	destroys spectra
aluminum chloride	270 (305) 350, 425
sodium acetate	no shift
aluminum chloride-sodium acetate	270 (305) 350, 425
boric acid	no shift
boric acid-sodium acetate	no shift

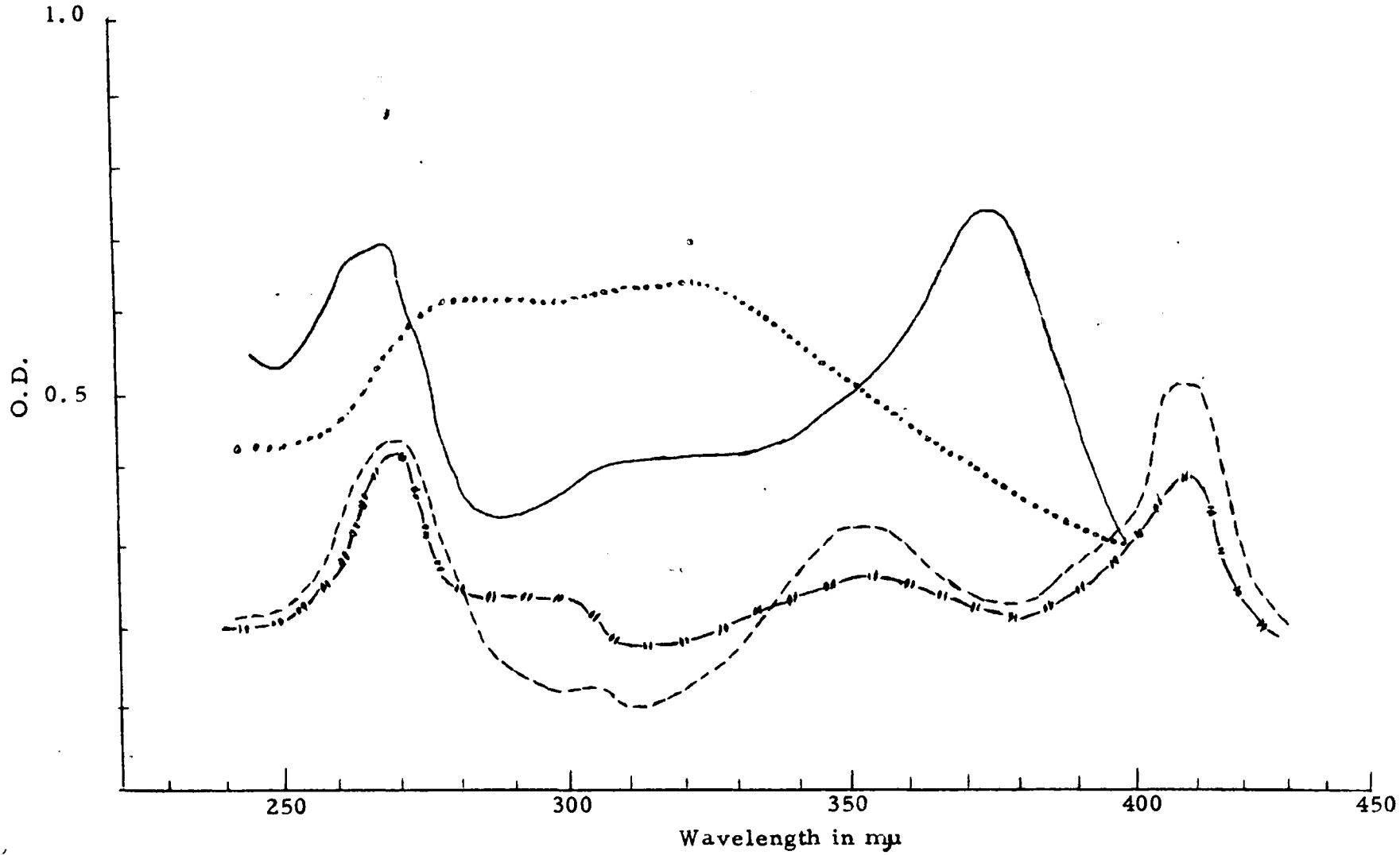


Figure 5. SPECTRUM OF BAND 2

FIGURE 6

SPECTRUM OF BAND 2 HYDROLYSATE

	λ_{max}
pure compound	268, 370
sodium acetate	273, 385*

* All other λ_{max} found to be identical with Figure 4.

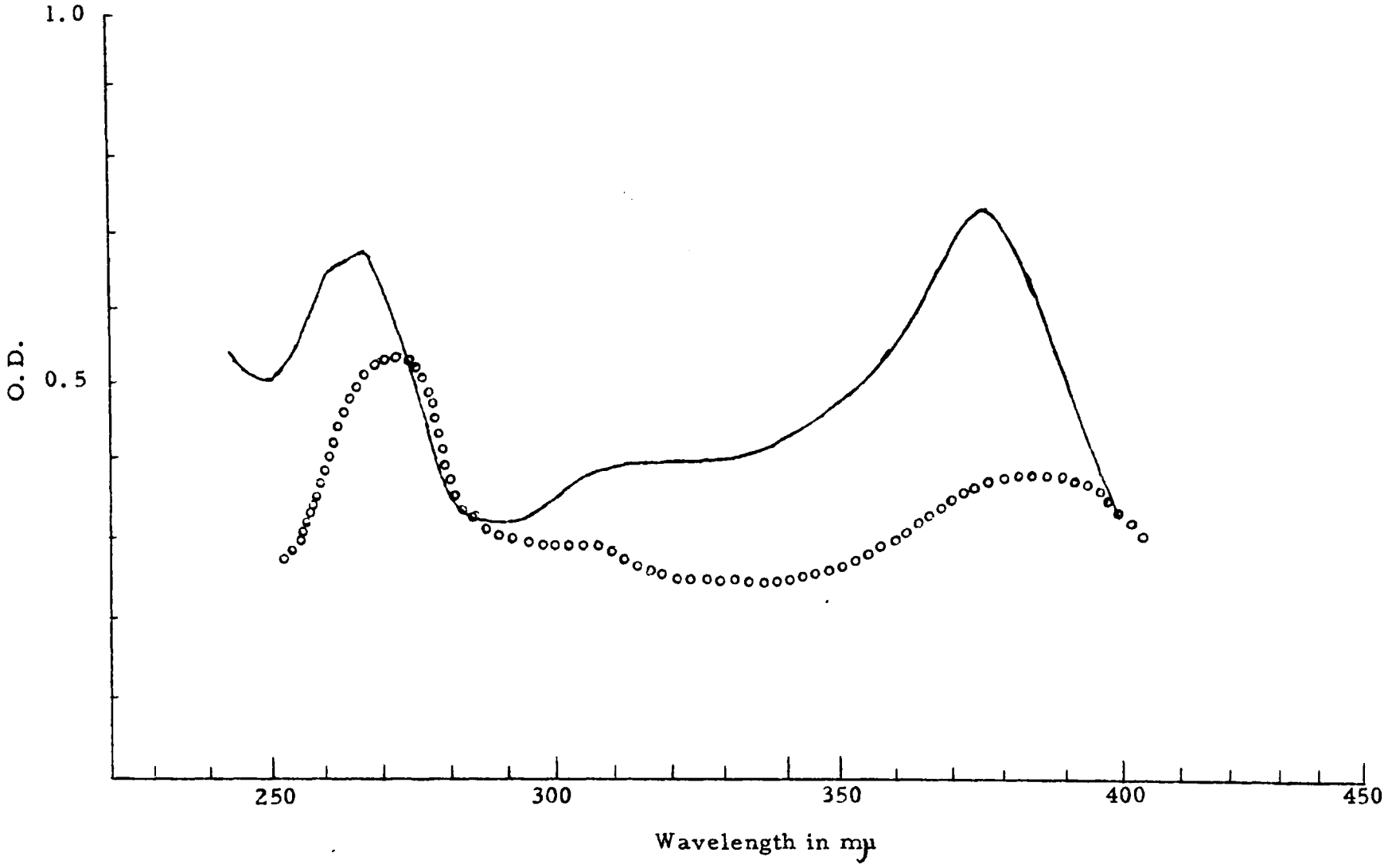


Figure 6. SPECTRUM OF BAND 2 HYDROLYSATE

FIGURES 7, 8

SPECTRUM OF KAEMPFEROL

	λ_{max}
pure compound	268, 370
sodium ethylate	destroys spectra
aluminum chloride	268, (303), 350, 425
sodium acetate	273, 385
aluminum chloride-sodium acetate	268, (303), 350, 425
boric acid	no shift
boric acid-sodium acetate	no shift

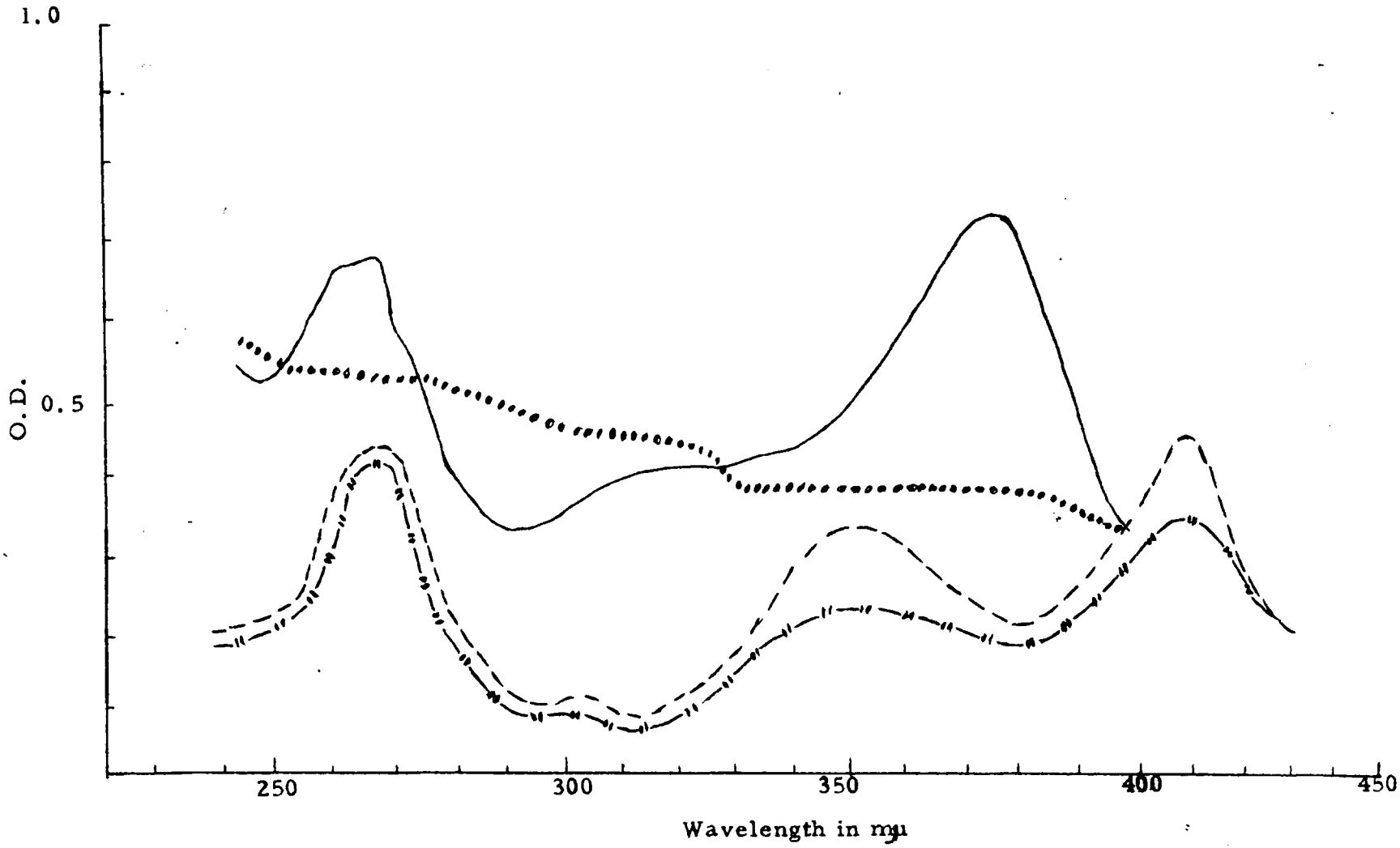


Figure 7. SPECTRUM OF KAEMPFEROL

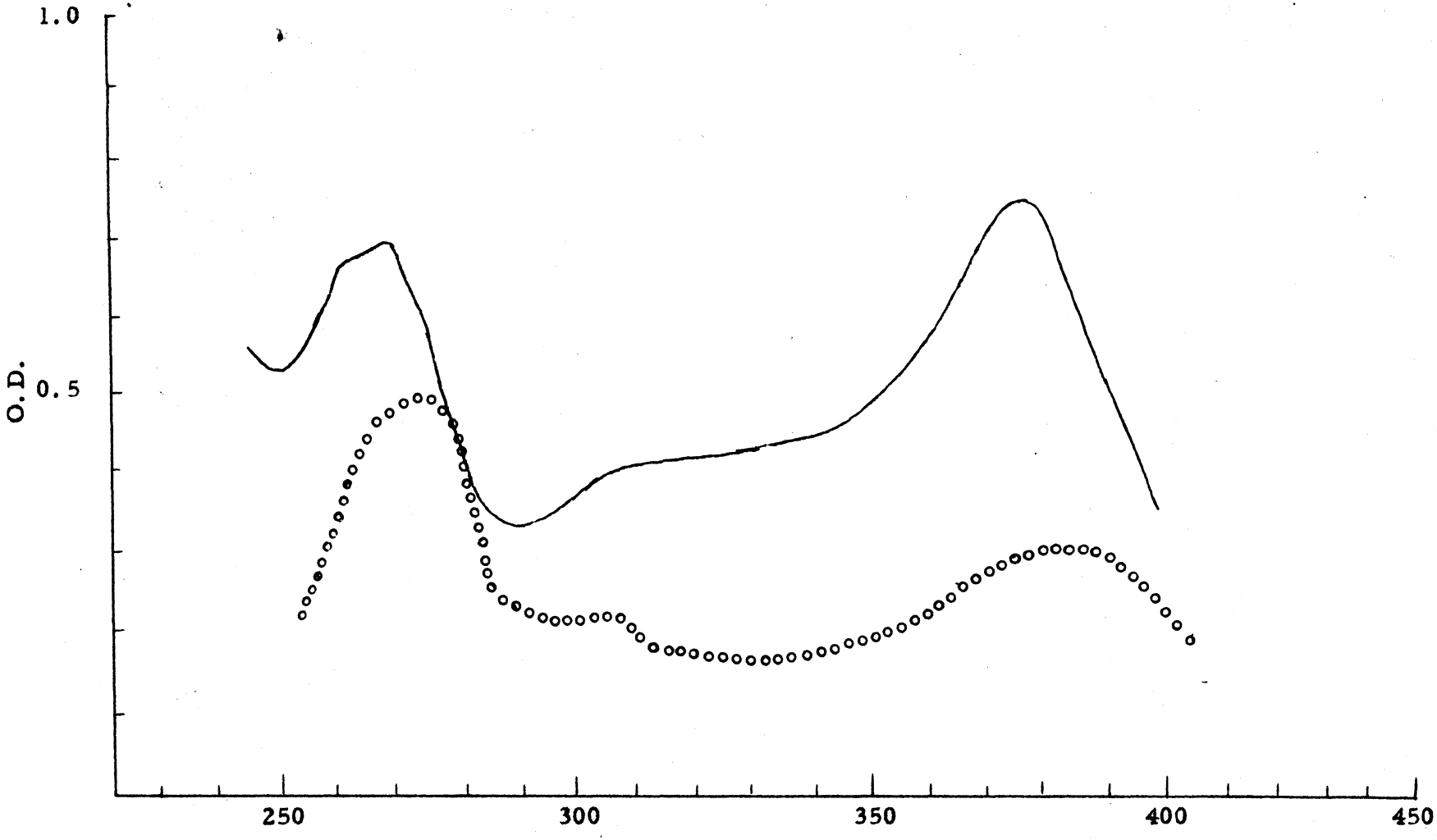


Figure 8. SPECTRUM OF KAEMPFEROL

FIGURES 9, 10

SPECTRUM OF BAND 3

	λ_{max}
pure compound	258, 300, 372
sodium ethylate	245 (i), 325
aluminum chloride	268, 355, 440
sodium acetate	no shift
aluminum chloride-sodium acetate	....
boric acid	260, 385
boric acid-sodium acetate	260, 385

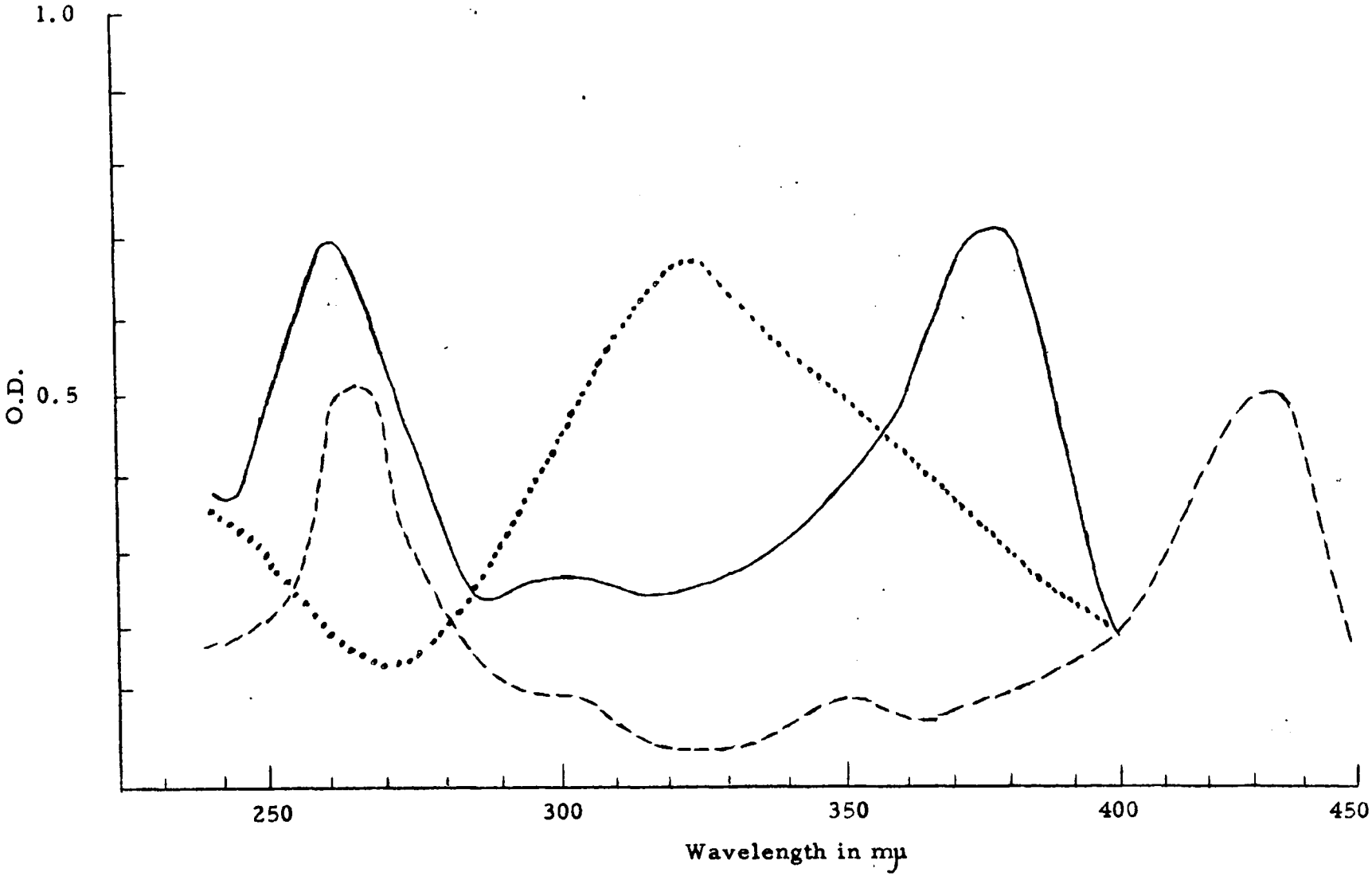


Figure 9. SPECTRUM OF BAND 3

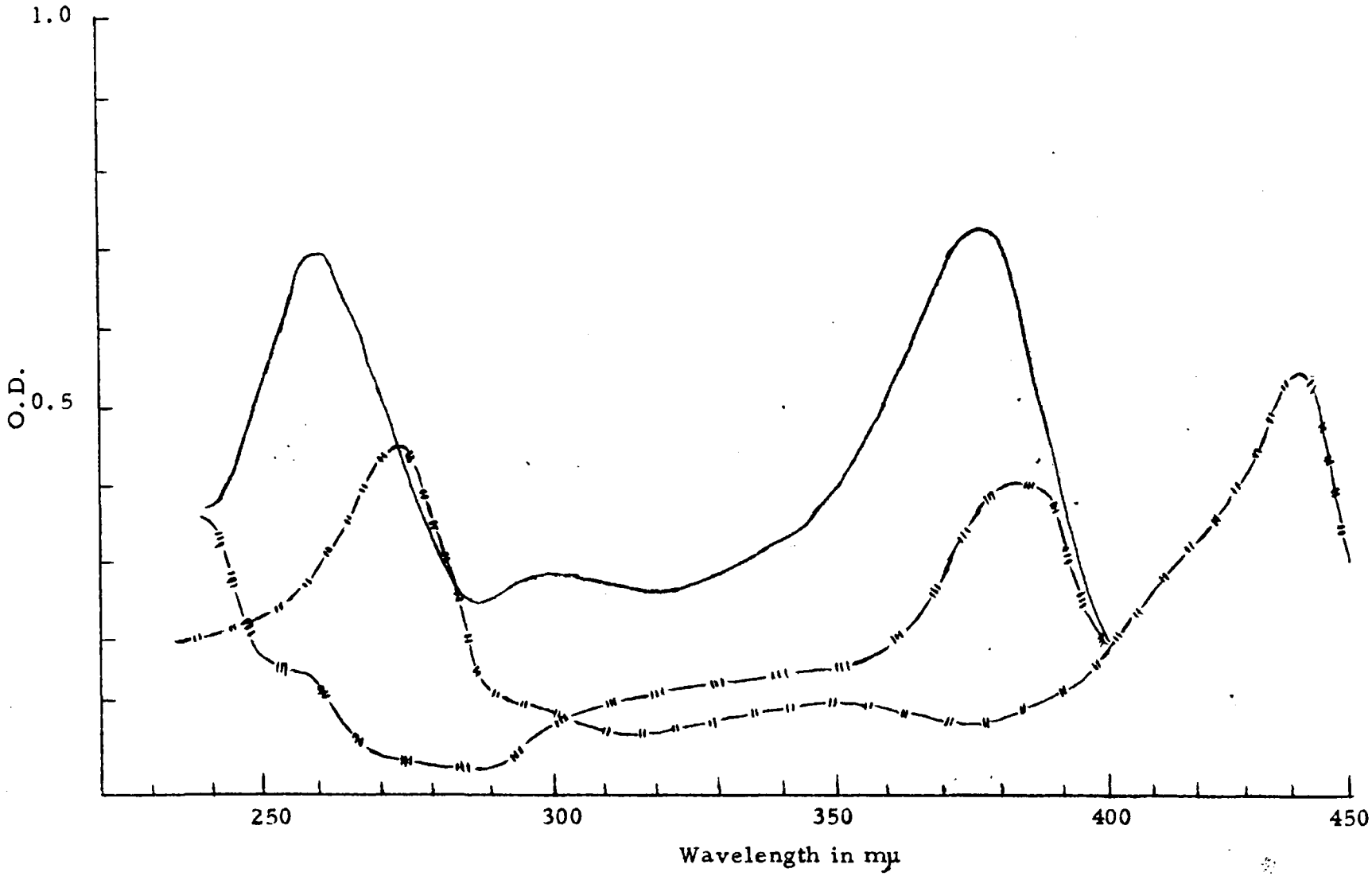


Figure 10. SPECTRUM OF BAND 3

FIGURE 11

SPECTRUM OF BAND 3 HYDROLYSATE

	λ_{max}
pure compound	258, 300, 372
sodium acetate	278, 325, 385*

*All other λ_{max} found to be identical with Figures 8, 9.

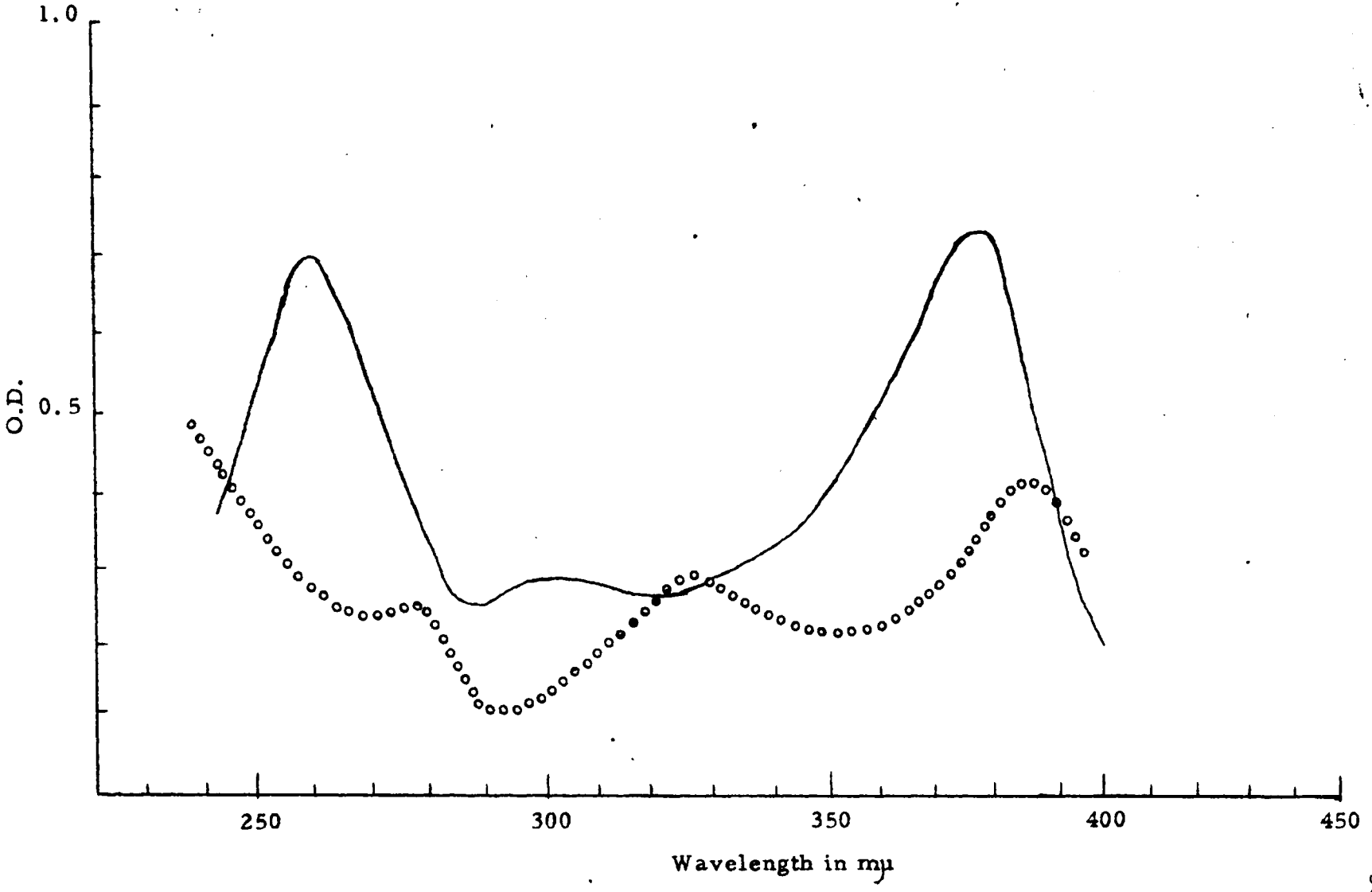


Figure 11. SPECTRUM OF BAND 3 HYDROLYSATE

FIGURES 12, 13

SPECTRUM OF BAND 4

	λ_{max}
pure compound	253, 268, 350
sodium ethylate	265, (330), 410
aluminum chloride	275, (295), 360, 390
sodium acetate	no shift
aluminum chloride-sodium acetate	....
boric acid	260, (300), 370
boric acid-sodium acetate	260, (300), 370

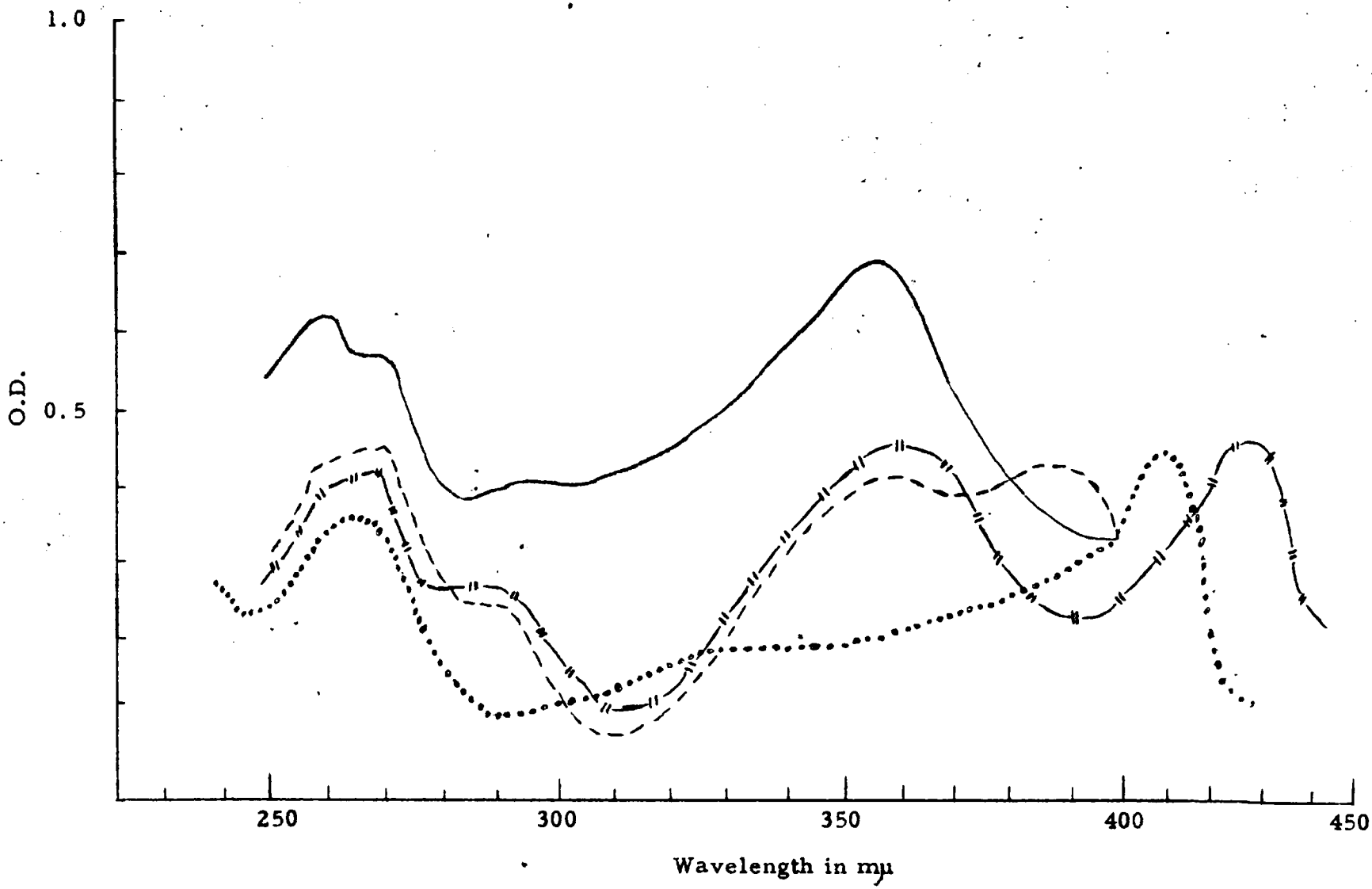


Figure 12. SPECTRUM OF BAND 4

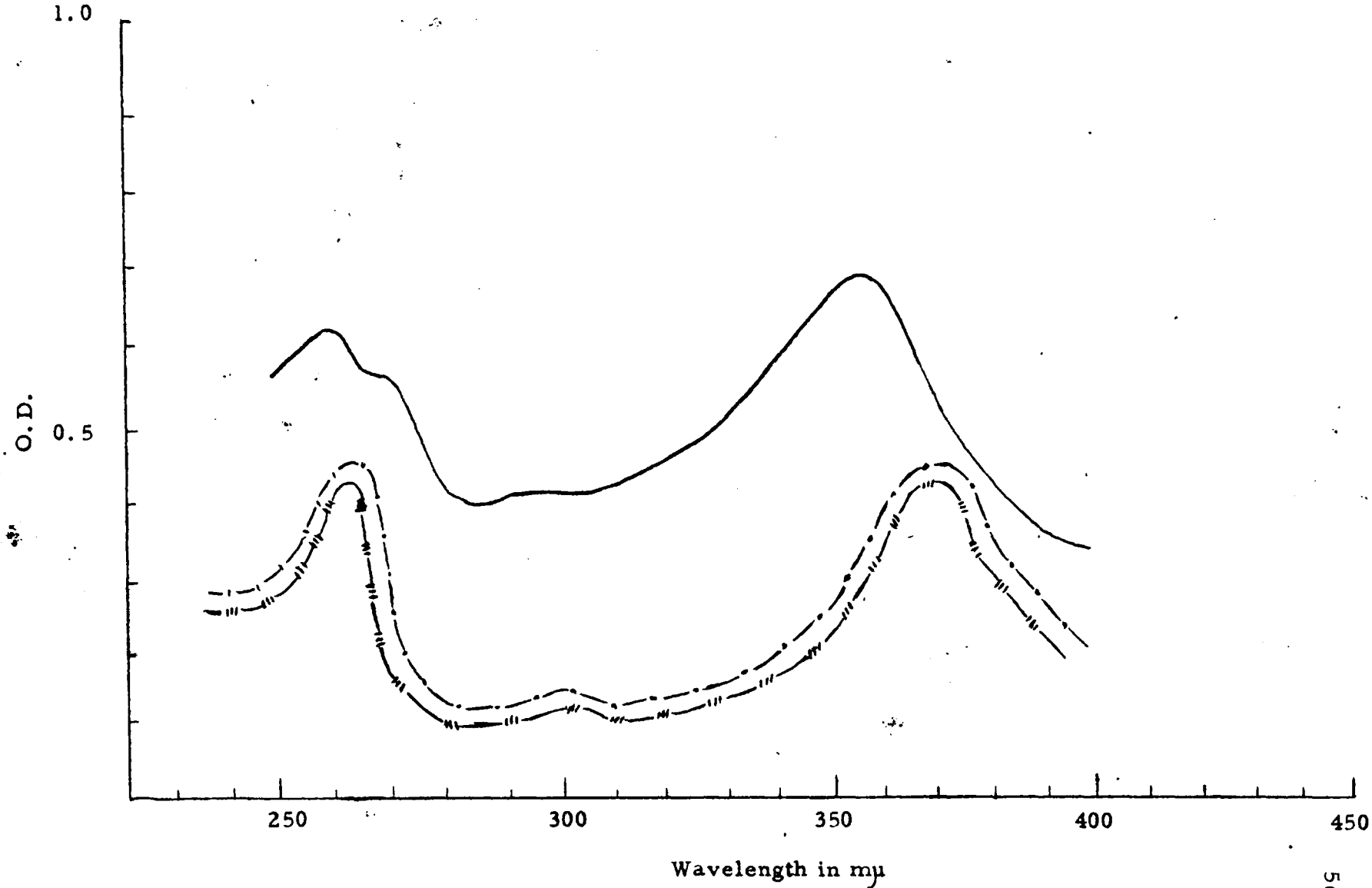


Figure 13. SPECTRUM OF BAND 4

FIGURE 14

SPECTRUM OF BAND 4 HYDROLYSATE

	λ_{max}
pure compound	253, 268, 350
sodium acetate	275, 320, 405*

*All other spectra found to be identical with Figures 11, 12.

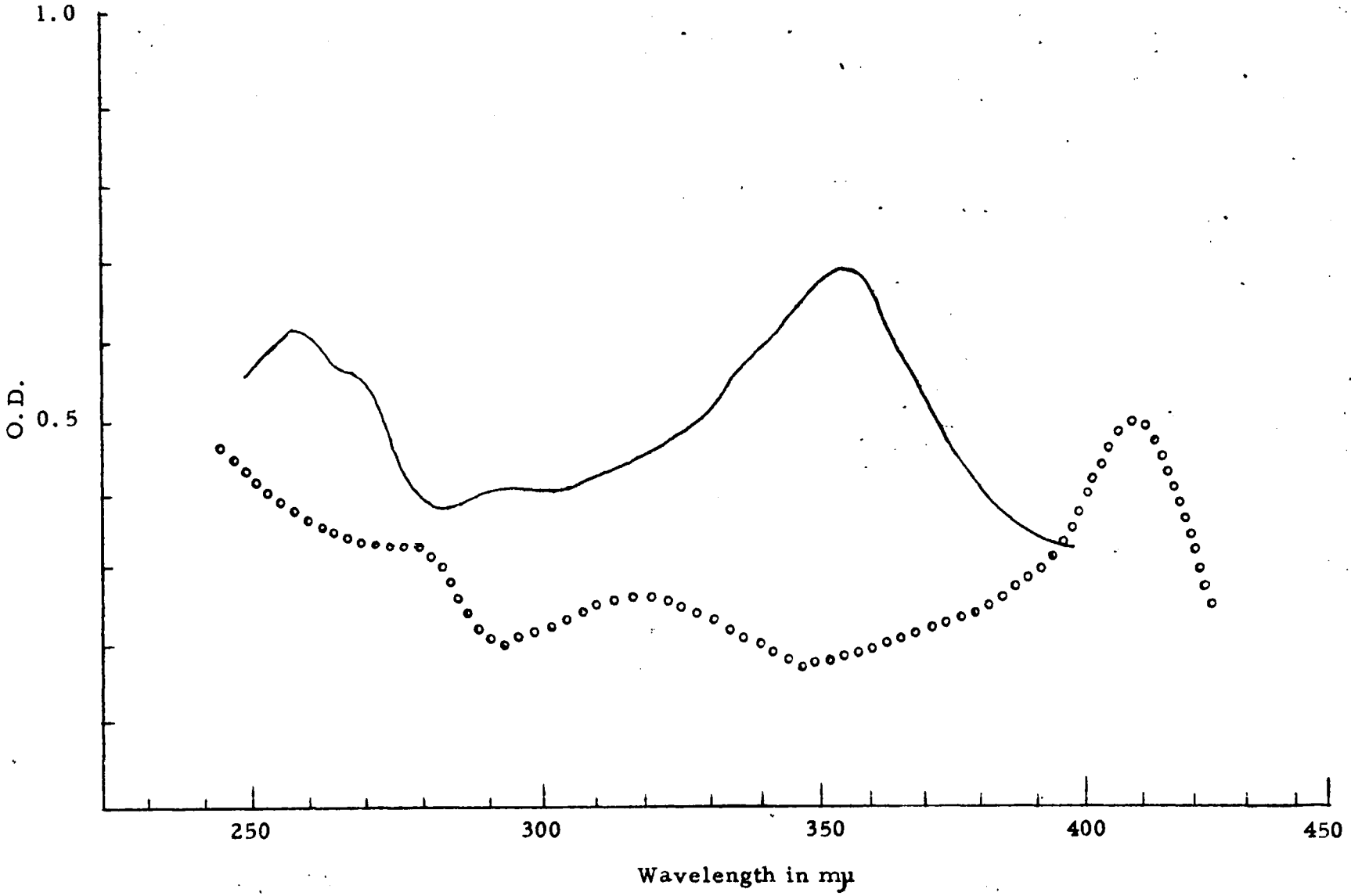


Figure 14. SPECTRUM OF BAND 4 HYDROLYSATE

FIGURES 15, 16

SPECTRUM OF BAND 5

	λ_{max}
pure compound	245, 300, 330
sodium ethylate	263, 310, 375
aluminum chloride	no shift
sodium acetate	360
aluminum chloride-sodium acetate	....
boric acid	270, 345
boric acid-sodium acetate	260, 362

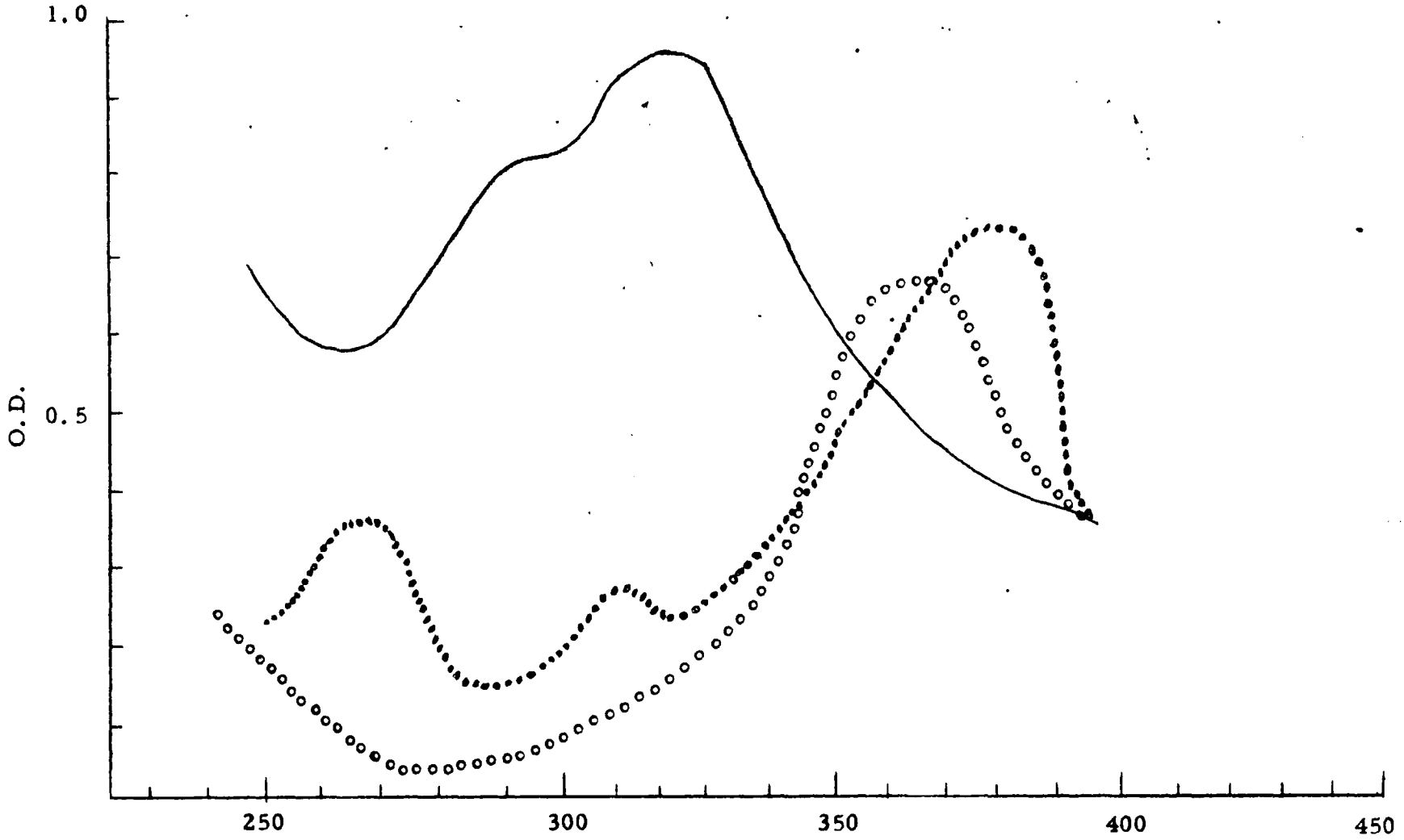


Figure 15. SPECTRUM OF BAND 5

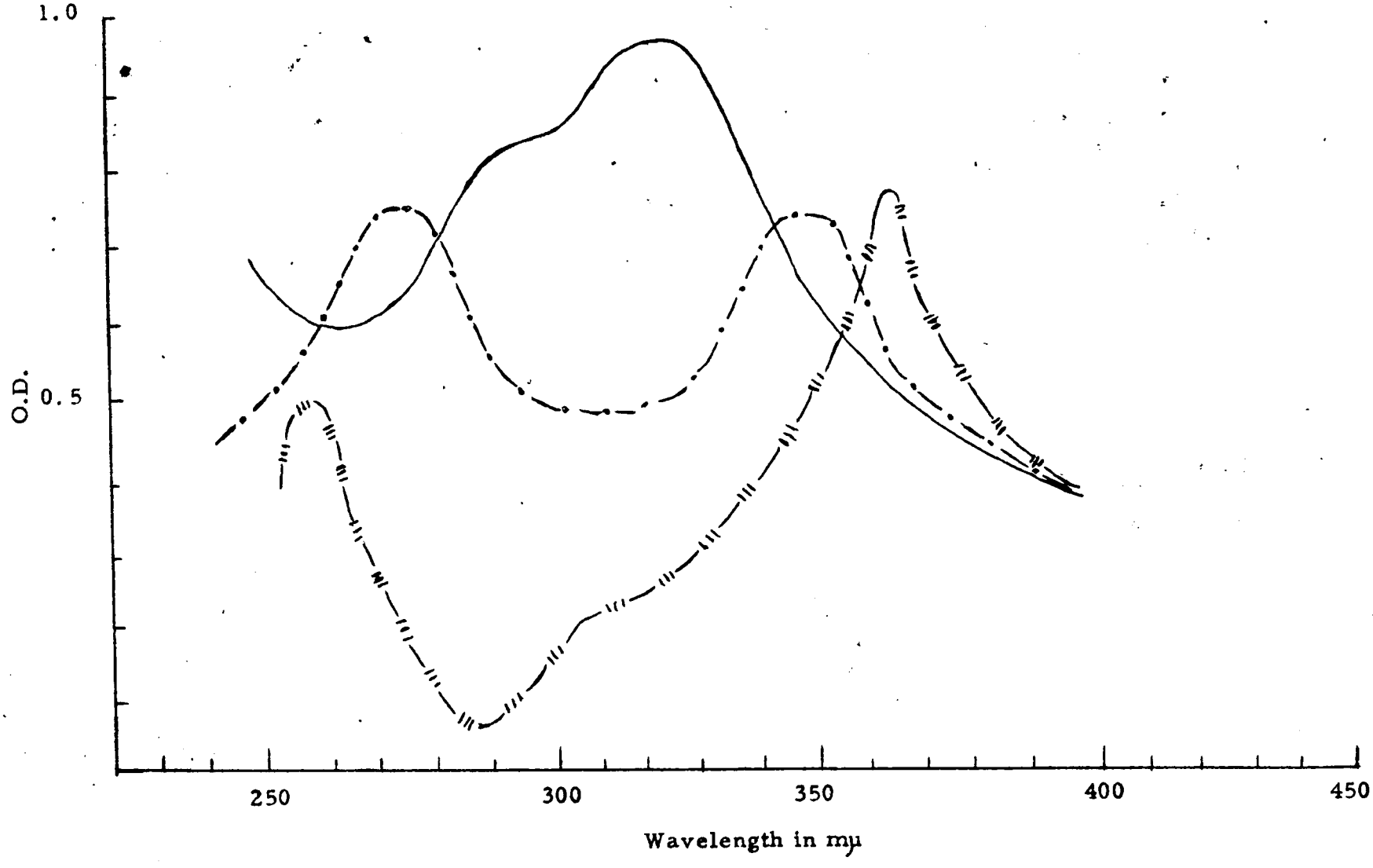


Figure 16. SPECTRUM OF BAND 5

FIGURES 17, 18

SPECTRUM OF CAFFEIC ACID

	λ_{max}
pure compound	300, 330
sodium ethylate	280, 325
aluminum chloride	300, 338
sodium acetate	300, 350
aluminum chloride-sodium acetate	300, 330
boric acid	.....
boric acid-sodium acetate	.....

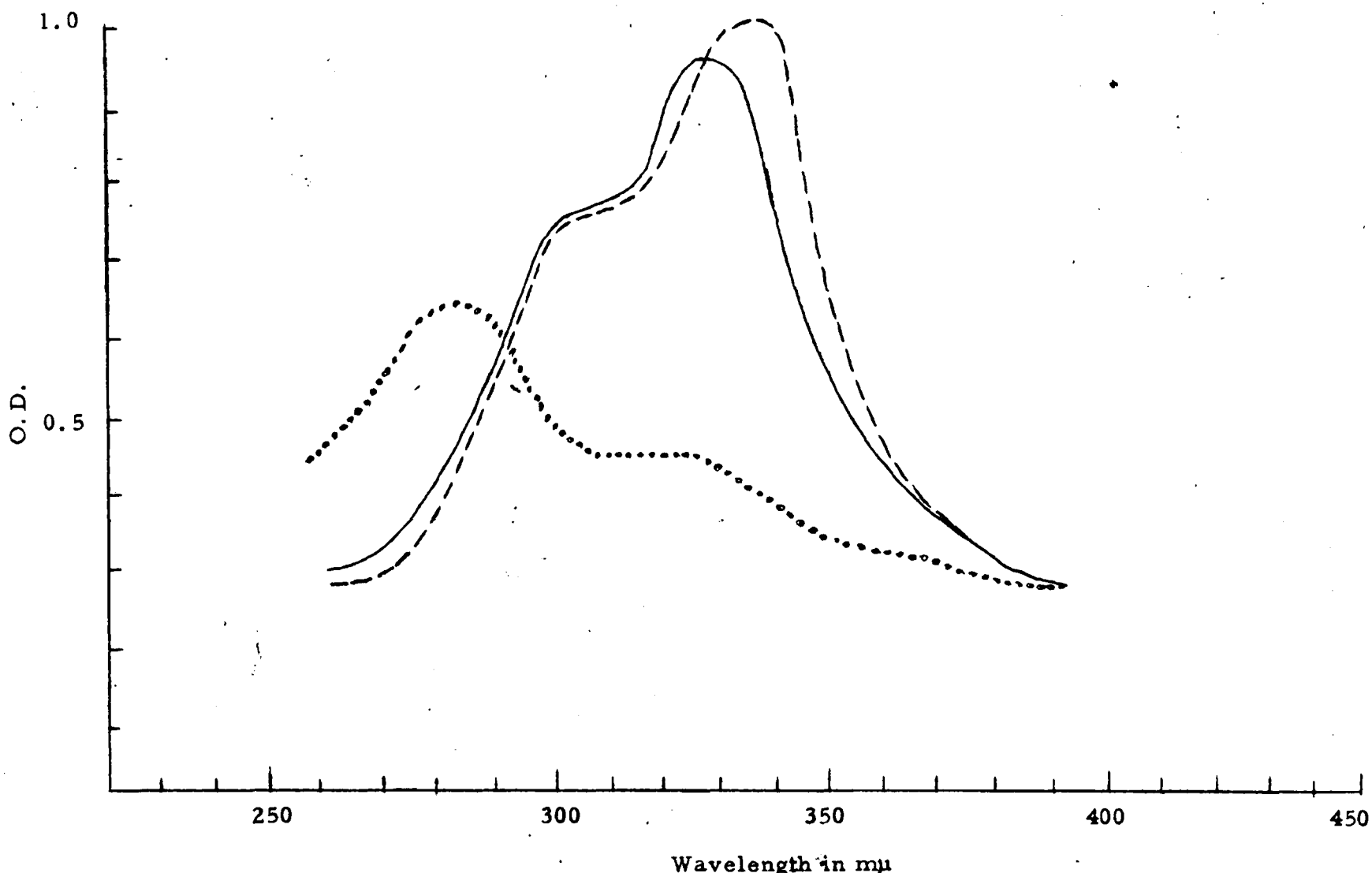


Figure 17. SPECTRUM OF CAFFEIC ACID

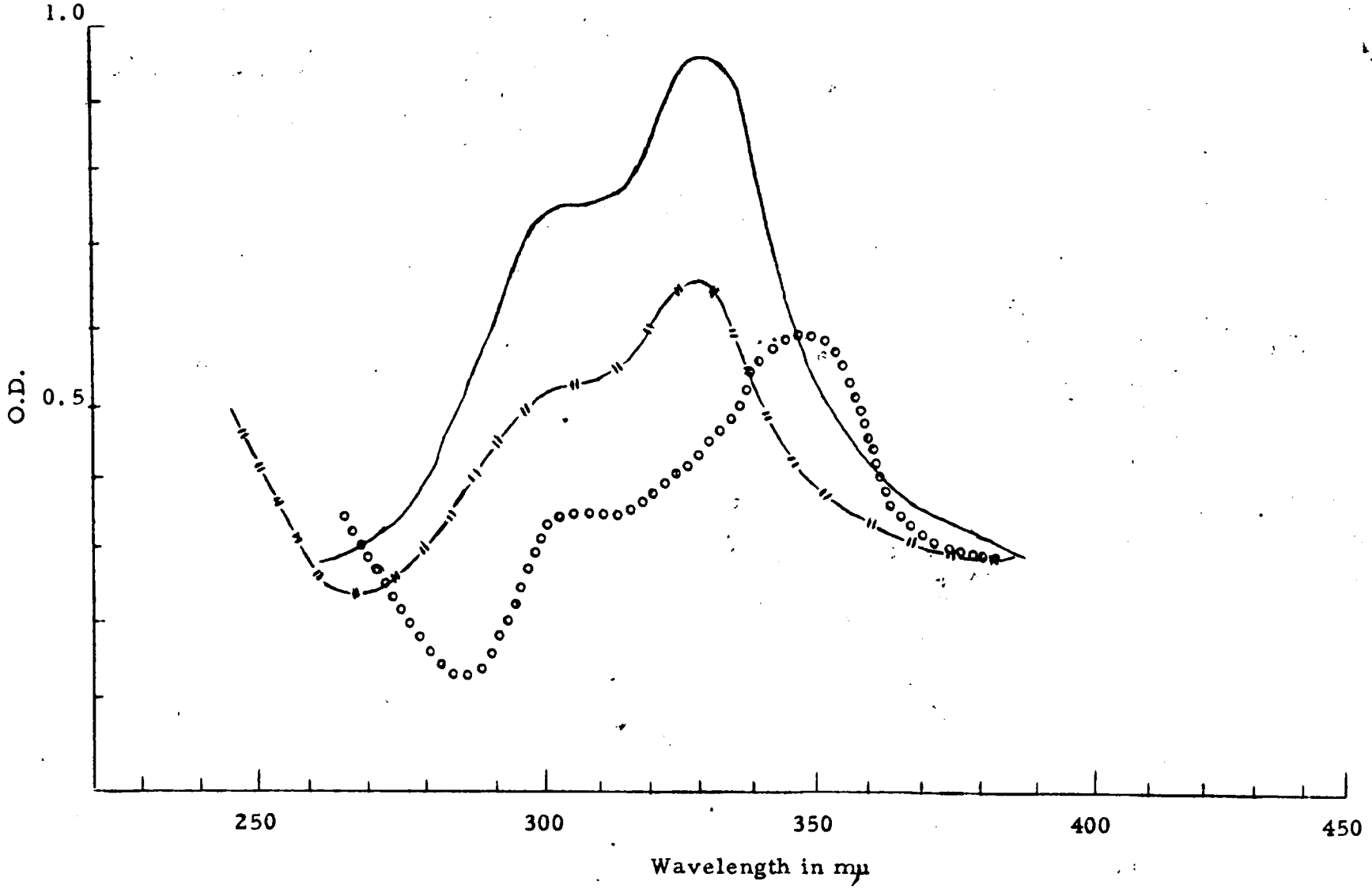


Figure 18. SPECTRUM OF CAFFEIC ACID

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