A PHYTOCHEMICAL ANALYSIS OF *GALIUM TRIFLORUM*

by

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INTRODUCTION

The use of plants in the treatment of disease has been practiced for centuries. Considerable folklore and superstition, such as the Doctrine of Signatures, has been associated with the medicinal use of plants. Only a few of the plants used empirically prior to their scientific investigation proved to have a sound scientific rationale, examples being cinchona bark, foxglove, and chaulmoogra oil.

Many advancements in medicine today stem from the early pharmacognostical, chemical and pharmacological investigations of plant constituents. Because the plant structure includes a mixture of many principles, there has always been a definite need for better methods of separating, purifying and identifying these principles. With the gradual development of newer analytical methods, phytochemical analyses became more thorough and thus more revealing.

A phytochemical study may sometimes lead to the discovery of a new chemical principle which demonstrates some potential value in medicine. An extensive chemical and pharmacological study could reveal the chemical nature of this principle and more clearly elucidate its appropriate place in medicine.

With an understanding of the chemistry of the principle, attempts can be made to alter the structure for such purposes as enhancing the activity or lessening the incidence of side effects. Further studies

could determine that portion of the molecule necessary for activity and it then becomes possible to synthesize compounds with similar structures. Thus, a phytochemical analysis may reveal a new principle and biochemorphology may lead to the discovery of improved derivatives.

When considering a phytochemical analysis, several factors will affect the choice of plant for such an investigation. Perhaps the most important are the following:

1. The potential value of the plant in medicine.
2. The extent of previous investigations carried out on the plant.
3. The availability of the plant.

After consideration of these factors, it was decided that a phytochemical analysis of Galium triflorum would be valuable.

A. Potential Value of Galium triflorum in Medicine

In reference to the potential value of Galium triflorum in medicine, Meyer (34, p. 900-901) has summarized the early uses of this genus. A relative of the plant under consideration, Galium verum, was considered an antispasmodic and diaphoretic, and the roots were thought to be valuable as an aphrodisiac. Galium roduntifolium was also considered to be antispasmodic, while the fresh juice of Galium mollugo was once used to treat epilepsy.

Perhaps worthy of the closest consideration is the information available on Galium aparine, a species more closely related to

Galium triflorum. In early times the fresh juice of this plant was used to treat liver disease, goiter and vitamin C deficiencies. It was also thought to be useful as a diuretic in the reduction of edema, and the aqueous extract was used to heal wounds as well as in the treatment of skin disorders.

Recently Delas and co-workers (10, p. 61-65) have demonstrated the hypotensive effects of aqueous extracts of Galium aparine. Their studies indicated that injections of the aqueous extract administered by the intravenous route could reduce arterial pressure as much as 50 per cent below the normal. The mechanism of the hypotensive action was investigated to some extent, but no attempt was made to isolate the principle in the plant that was responsible for the fall in blood pressure.

The possibility that Galium triflorum may have some potential value in medicine was brought to the attention of Professor H. C. Forslund of Oregon State College School of Pharmacy by a physician residing in Oregon. A patient of the doctor had used the plant to treat a hypertrophic osteoarthritis, and upon subsequent examination it was found that this patient had lost most of his arthritic spurs. The physician forwarded a large quantity of Galium triflorum to the School of Pharmacy where it was identified.

As is apparent from the discussion thus far, many claims for the therapeutic rationale of a plant are made but few can be justified. Perhaps the most significant observation was the hypotensive effect of aqueous extracts of Galium aparine.

B. Literature Study of *Galium triflorum*

A survey of the available literature indicated that only one investigation dealt to some extent with the analysis of *Galium triflorum*. Von Cotzhausen in 1876 (55, p. 405-406) gave a brief reference to a species he called *Galium triflorum*. In this plant he found coumarin, an astringent principle, a yellow resin, a fatty and rather unpleasant oil and what he referred to as grape sugar. No experimental methods or other data were presented. In this reference, mention was made of the fact that *Galium triflorum* was often collected by the Germans under the supposition that it was the Waldmeister (*Asperula odorata*), which is also a member of the family Rubiaceae commonly used in Germany to make the pleasant "May Wine."

Many references have been found pertaining to work done on related species or on plants in the same family. An early reference deals with the composition and food value of the seeds of *Galium aparine*, *Galium vaillantii* and *Galium tricorne* (5, p. 675-687). The seeds of these plants were often found in wheat siftings used as food for livestock. The seeds were found to contain protein, carbohydrate and cellulose, and in addition, from one per cent to two per cent of an oil so finely divided in the tissues as to render its extraction and determination very difficult.

The glycosides in the roots of the genus *Galium* have long been known to yield red and yellow dyes of importance in the dye industry (2, p. 101-141). Of the glycosides isolated from the roots the one

containing the aglycon of most importance to the dye industry was ruberythric acid. This glycoside was first isolated from Rubia tinctorum, also a member of the family Rubiaceae, in 1851 (23, p. 547) and has been shown to be alizarin primveroside.

In 1934 Hill (20, p. 628) proved the existence of a glycoside of purpurin-3-carboxylic acid in the roots of the mild madder (Rubia peregrina) and Galium verum. Hill first assigned the name "galieide" to this glycoside because it was found in all the bedstraws (Galium species) examined, but the name was later changed to galiosin. Galiosin was found to be a primveroside of purpurin-3-carboxylic acid (21, p. 38).

There have been some five glycosides isolated from the roots of Rubia tinctorum and many species of Galium. These are ruberythric acid, rubiadin-3-glucoside, galiosin, rubiadin primveroside (22, p. 1714-1718), and the glucoside of purpurin-3-carboxylic acid. Rubiadin-3-glucoside was first isolated by Schunck and Marschlewski (46, p. 969-974) in 1893, and a subsequent study by Marschlewski (31, p. 1137-1142) gave a good indication of its structure. In 1930 Jones and Robertson (27, p. 1699-1709) synthesized this glucoside.

Hill and Richter (22, p. 1714-1718) isolated the relatively unstable rubiadin primveroside. This glycoside was readily converted into rubiadin-3-glucoside by boiling with dilute acid which removed the terminal pentose residue. The rubiadin is connected to the glucose portion of the primverose molecule (6-beta-d-xylosido-d-glucose).

The extraction procedures for the isolation of galiosin, rubiadin primveroside and ruberythric acid are outlined in "Modern Methods of Plant Analysis" (37, p. 308-309). The yield obtainable for galiosin from Rubia tinctorum was 0.06 per cent of the fresh plant. Extraction of rubiadin primveroside from Galium verum resulted in a yield of 0.14 per cent from the fresh roots. Ruberythric acid, when extracted from the fresh roots of Rubia tinctorum, gave a yield of 0.2 per cent.

Hill and Richter (23, p. 547-560) conducted a survey of the tribe Galieae with regards to the presence of hydroxyanthraquinone glycosides in order to determine if these plants were suitable for a study of chemical variation. This tribe includes Rubia tinctorum and the bedstraws. Their observations led to the conclusion that the amount of the coloring matter, alizarin, in different species was subject to great variation. Purpurin and its carboxylic acid, on the other hand, was subject to less variation in this tribe. Both purpurin-3-carboxylic acid and alizarin were determined colorimetrically after extraction from the fresh roots which were used to avoid decomposition of the glycosides by the drying process. Purpurin-3-carboxylic acid decomposes to purpurin on drying. The former was not found in any other genera of Rubiaceae. This investigation also includes a scheme useful for the extraction and separation of these glycosides from the fresh plants.

Hill's experiment showed that extracts of primveroside-containing plants, such as Galium verum, readily hydrolyzed these

glycosides. All the glycosides mentioned were readily hydrolyzed by enzymes present in species of Primula.

Trim (54, p. 101-125) in 1955 referred to galiosinase, a highly specific enzyme, which split the glycoside linkage of galiosin and the glucoside of purpurin-3-carboxylic acid, another glycoside isolated from Galium. This enzyme had little action on the other anthraquinone glycosides. Both ruberythric acid and rubiadin primveroside were hydrolyzed by the enzyme erythrozyme which occurred in the roots of all Rubiaceae investigated. Trim pointed out that in the majority of the plants in the tribe Stellateae, which includes the genus Galium, anthraquinone pigment accumulation is largely confined to the roots and in the short basal internodes of the shoot.

Other principles have been isolated from species related to Galium triflorum. Rutin (9, p. 641-647) was extracted from several plants including Galium cruciatum. The rutin was obtained from the flowering tops of the plants investigated.

A study of the distribution of hesperidin in genus Galium by Klein (29, p. 295-306) revealed another incidence of chemical variation in this genus. In the family Rubiaceae, hesperidin was found only in the genus Galium. Out of 34 species of this genus examined, hesperidin was found only in seven species. In four of these seven species hesperidin was present in all the samples examined. In the other three were some samples in which none was found. It was concluded that the variable occurrences of hesperidin in some species appeared to depend on climate, habitat and the age

of the plant.

Another example of chemical variation was shown by Roberg (44, p. 145-166). Many plants were tested for saponins using hemolytic tests. Galium aparine gave a negative test for this type of glycoside. Results of the investigation indicated that there was no rule for the distribution of saponins in plants. They may be everywhere or completely wanting. In 1941 Wagner (56, p. 35) reported Galium silvaticum as a source of saponin.

In 1851 Rochleder investigated the roots of madder (Rubia tinctorum) and discovered a principle he called rubichloric acid (17, p. 1208). An aqueous decoction of the dried roots of madder was prepared to which was added a solution of neutral lead acetate. The precipitate so formed removed such principles as alizarin, purpurin and citric acid. Basic lead acetate solution was then added to the filtrate in excess and another precipitate formed which was considered to be rich in rubichloric acid. The rubichloric acid was obtained in the free state by the reaction of the lead compound with hydrogen sulfide. When the rubichloric acid was heated with hydrochloric acid, the solution turned blue, gradually changed to green and finally a greenish-black precipitate was formed.

Years later in 1893 Perkin and Hummell (40, p. 1160-1184) studied Indian madder which is Oldenlandia umbellata, Lynn., Rubiaceae. The roots of this plant were found to contain rubichloric acid also. A later publication by these workers involving a study on the root-bark

of Morinda umbellata also gave this characteristic color test with acids (41, p. 855-857).

It wasn't until 1925, when Herissey began an extensive investigation of members of the family Rubiaceae, that information was uncovered as to the true nature of rubichloric acid (15, p. 1695-1697). From an investigation of Asperula odorata Herissey isolated from this plant a pure crystalline compound having well defined physical constants and showing the characteristic test with acids. Herissey named this compound asperuloside and considered it identical with rubichloric acid. Rubichloric acid was considered to be an impure form of asperuloside. The name asperuloside stems from the genus from which it was obtained.

Herissey recognized that the color test mentioned by Rochleder would be an excellent method for the qualitative detection of asperuloside in many other plants of this family. Treatment of extracts with dilute sulfuric acid and boiling led to the formation of a greenish solution and subsequently a greenish-black precipitate. It was pointed out in this publication that there was the possibility other glucosides still unknown might give the test. A positive test would be indicative of asperuloside but only subsequent extraction and identification of the glucoside would be absolute proof. A negative test, on the other hand, would strongly suggest its absence and obviate necessity of the long and tedious extraction procedure.

Herissey (16, p. 490-496) investigated some of the species of Galium and verified the presence of asperuloside in Galium cruciata,

verum, mollugo and aparine. Besides considering members of the genus Galium, Herissey (18, p. 1674-1675) investigated some other genera of the Rubiaceae family. Some members of the cinchona and coffee tribes gave positive tests for this glucoside while others gave a negative reaction.

In 1951, Trim and Hill (52, p. 310-319) made a comprehensive study of asperuloside and another glycoside, aucubin, which at the time was believed to be closely related chemically to asperuloside. They developed a colorimetric method for the estimation of asperuloside and carried out many chemical tests in order to more clearly elucidate its chemical structure. At the time the aglycon of asperuloside was thought to be related to a furan.

Still more recent work involved a chromatographic study of asperuloside by Janot et al. (25, p. 1101-1110). In 1954 Briggs and Cain (8, p. 4182-4193) isolated asperuloside from species of the Coprosma genus and postulated a chemical structure for this glucoside.

C. The Availability of the Plant

The third point of interest when considering a plant study is the availability of the plant. The plant can be obtained principally by three means. The first possibility is to gather specimens or seeds of the plant and cultivate a sufficient quantity for the investigation. The objections to such an approach would be the time factor, the availability of suitable space, and the type of plant

under consideration.

The second possibility is to have a collection made commercially in order to obtain a sufficient quantity of the plant material for investigation. This approach is limited because of expense and distance.

The third possibility, and the means by which the plant was obtained for this investigation, is the collection of the plant by the investigator. Galium triflorum is readily available in the Pacific Northwest but some difficulties were encountered in collection primarily due to the fact that the plant is a small herb that is quite scattered.

D. The Taxonomy of Galium triflorum

Galium triflorum belongs to the order Rubiales and the family Rubiaceae which is also referred to as the madder family (30, p. 712-713). It is one of the largest plant families including as many as 4,500 species (4, p. 291). Of the nearly 400 genera of Rubiaceae, most are found in the tropics and subtropics, but the genera of the tribes Galieae, Anthospermeae and Oldenlandieae, which are all predominantly herbaceous, extend into temperate zones. The rubiaceae are especially abundant in northern South America, while fifty genera are indigenous to Mexico and fourteen in the United States.

The tropical plants give the family Rubiaceae its greatest economic importance. For example, this family includes the genus

Coffea from which coffee is obtained, the genus Cinchona (quinine) and Cephaelis (ipecac).

The genus most prevalent in North America is Galium which includes some thirty species in eastern North America and many others in the west. This genus is a large one that contains as many as three hundred species. The name Galium comes from the Greek word "gala" meaning milk, derived from the use of Galium verum for curdling milk. Species of Galium are known as the bedstraws.

Galium triflorum, also known as fragrant bedstraw, has been found in many parts of Oregon. Hitchcock et al. (24, p. 444-453) describes the taxonomy of this plant as follows: it is a "perennial from creeping rhizomes; stems 2 to 8 dm. long, usually retrorsely hooked-scabrous on the angles, at least below; prostrate or sometimes ascending or scrambling on other vegetation, often forming a loose rosette; leaves vanilla scented, mostly five to six in a whorl or only four on the smaller branches, narrowly elliptic to somewhat oblanceolate, cuspidate, commonly 1.5 to 4.5 cm. long, one-nerved, generally antrorsely scabrous-ciliate on the margins and retrorsely hooked-scabrous on the midrib beneath, otherwise mostly glabrous; peduncles axillary, elongate, generally three flowered at the end, with divergent pedicels, sometimes branched and several flowered, occasionally the uppermost several whorls of the leaves reduced so that something of a terminal inflorescence is formed; corolla four parted, 2 to 3 mm. wide, whitish; fruit 1.5 to 2 mm. high, the segments approximate, covered with hooked bristles mostly 0.5 to 1 mm. long."

Galium triflorum is most commonly found in moist shady woods from near sea level to moderate elevations in the mountains. The plant can usually be found from May through October. Descriptions of the taxonomy of fragrant bedstraw can also be found in "A Manual of the Higher Plants of Oregon" (39, p. 684-685) and "Gray's Manual of Botany" (13, p. 1318-1321).

E. Differentiation from Galium aparine

During collection Galium aparine was easily confused with Galium triflorum because of its marked similarity. Both these plants are commonly found in Oregon and differentiation of them from a taxonomical standpoint is essential. Among the points of difference between the two species are the following:

1. Galium triflorum is a perennial from creeping rhizomes. The roots are large with extensively branched underground systems. There is much branching at the base of the plant. Slender rhizomes, also branching, are evident.
   The roots of Galium aparine are generally small and slender since this plant is an annual. There is some branching but it is not extensive. Most of the branching is evident at the base of the plant.
2. The stem of Galium triflorum is covered with small retrorsely hooked scabrous bristles. Such bristles are also found on the midrib beneath the leaf.

The stems of Galium aparine are also covered with retrorsely hooked scabrous bristles, as are the undersides of the leaves. Microscopic examinations revealed that these bristles, however, are much larger and stiffer. For this reason, the plant will readily cling to clothing.

3. The fruits of both plants are covered with hooked hairs, but those of Galium aparine are generally hooked to a greater extent. This observation may be made with a magnifying lens in the process of gathering the plant as an aid in the differentiation. Because this difference was so minor, little emphasis was placed upon it.

4. The leaves of Galium aparine are usually in whorls of six, being narrow and cuspidate. The leaves of Galium triflorum are vanilla scented and usually six in a whorl. On the younger plants, the leaves are five to six in a whorl. They are narrowly elliptic to somewhat oblanceolate as well as being cuspidate. These differences in the leaves are clearly shown in Figures 1 and 2.

PLANTS OF OREGON

FIGURE 1

GALIUM TRIFLORUM MICHX.

BENTON COUNTY: CORVALLIS

MAY, 1960

COLLECTED BY ROBERT KNOTT

DETERMINED BY THE OREGON STATE COLLEGE HERBARIUM

PLANTS OF OREGON

FIGURE 2

GALIUM APARINE L.

BENTON COUNTY: CORVALLIS

MAY, 1960

COLLECTED BY ROBERT KNOTT

DETERMINED BY THE OREGON STATE COLLEGE HERBARIUM

EXPERIMENTAL

A. The Collection and Handling of *Galium triflorum*

Samples of *Galium triflorum* that were collected in many different counties of Oregon have been forwarded to the Oregon State College herbarium where specimens were preserved for future reference. The herbarium was an aid in finding sources of the plant. The plant collected for this study was gathered in and around Benton County. Most of the plant was obtained from Mary's Peak, Avery Park and Helmick Park.

The first collection of the plant was made in September and early October, 1959. All large collections were examined by Dr. Albert Newton Steward of the Oregon State College herbarium in order to verify that the plant was *Galium triflorum*.

Although the plant is found in many areas of the Pacific Northwest, some difficulty was encountered in collecting a large quantity of the plant because it was sparsely distributed over large areas. In the two month period in which the first collection of the plant was made, approximately 60 pounds of the aerial portion of fresh *Galium triflorum* was obtained. After air drying, about 12 pounds of the plant remained available for study. This amount of plant was sufficient for an analysis of the ash and a partial proximate analysis.

The aerial parts of the plant were separated from the roots and were spread out on table tops. The plant was allowed to dry at room

temperature for at least five days prior to pulverization.

The dried aerial parts of the plant were pulverized using a Wiley mill. The pulverized plant so obtained was approximately a number 40 powder (42, p. 935). The powder was thoroughly mixed by hand to insure uniformity, and was stored at room temperature in tightly sealed cannisters that were further sealed with cellulose tape. Since the roots of the plant were relatively small in comparison to the tops, the small amount of dried root obtained was insufficient for investigation.

Unless otherwise specified, all the determinations to be discussed were carried out using samples of the dried, pulverized aerial parts of the plant. Such determinations were done in duplicate or triplicate and repeated until checks were obtained.

B. Histological Study of the Powdered Plant

A small amount of the powdered plant was placed on a slide and moistened with distilled water. A microscopic examination disclosed the presence of needle-like crystals ranging in length from 7 to 105 microns (100 diameters) as well as some raphides. These crystals were quite abundant and were found to dissolve in diluted hydrochloric acid without effervesence but were insoluble in acetic acid, which suggested they were calcium oxalate crystals. Figure 3 depicts the raphide observed in the powdered plant.

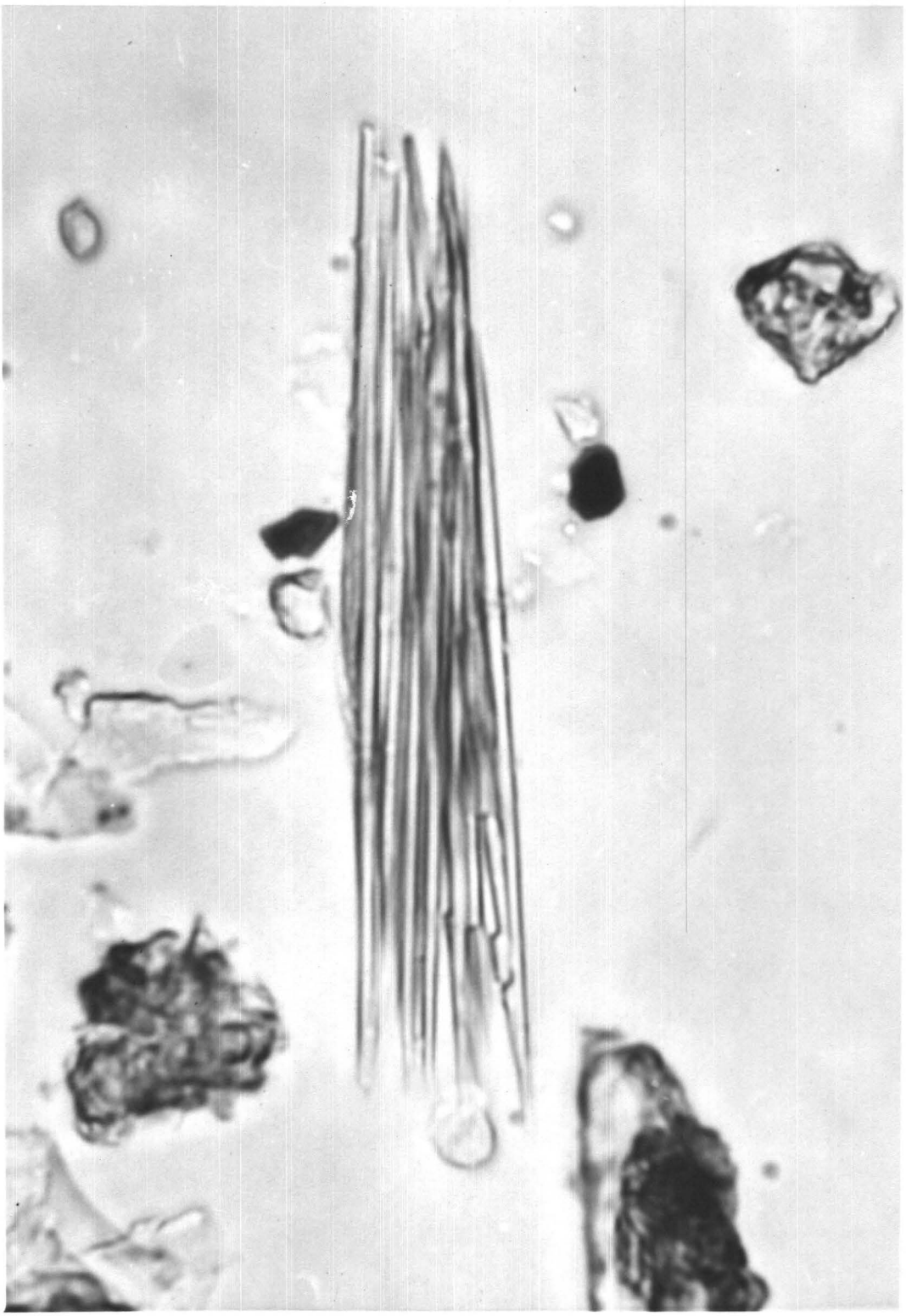


Figure 3.
Raphide of Galium triflorum (1500X)

Besides the crystals, the other two tissues found most abundantly in the plant were unicellular non-glandular hairs and stomata. The hairs ranged in size from 140 to 525 microns (100 diameters) and are shown in Figure 4. The stomata were of the rubiaceous type, in which the stomate is surrounded usually by two subsidiary cells having their long axes parallel to the stomatal pore (57, p. 82). An example of the stomata that were observed is shown in Figure 5.

Also observed were large epidermal cells with wavy walls and some small tracheae. Starch grains were absent.

C. Moisture Determinations

Moisture determination of the dried plant.

This determination was carried out using the U.S.P. XV gravimetric method (42, p. 945). According to the procedure for vegetable drugs, an accurately weighed sample of the dried plant was placed in a tared evaporating dish and dried to constant weight at 105° C. The samples were allowed to cool for five minutes in the dessicator and weighed immediately. Under these conditions, reproducible results were obtained and the water content of the dried plant was calculated to be 8.41 per cent. This figure was the mean of four such determinations.

Moisture determination of the fresh plant.

The procedure followed for this determination was that of the U.S.P. XV with some modifications. Twenty-five gram samples of the fresh plant were weighed one hour after gathering and allowed to dry

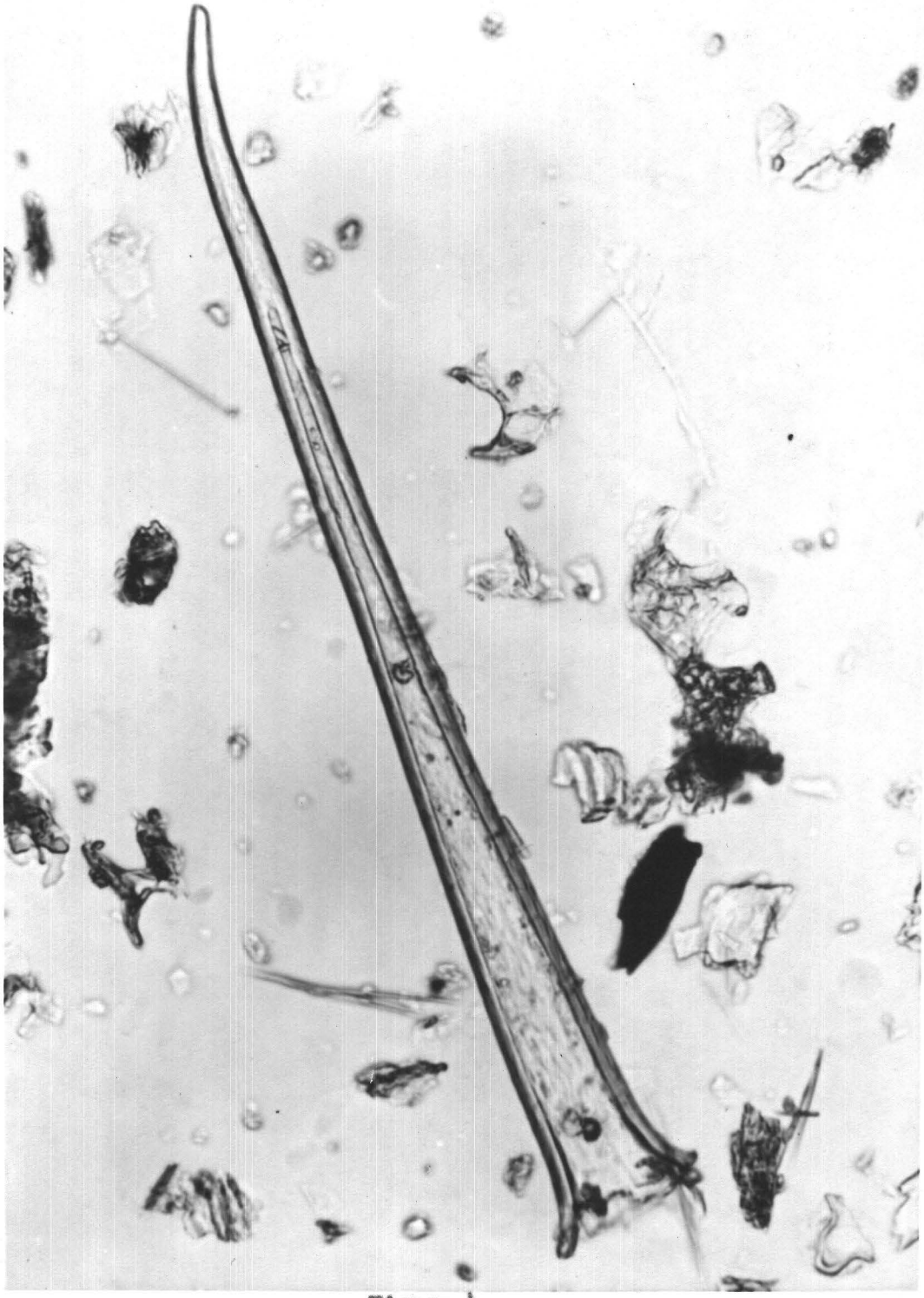


Figure 4.
Unicellular non-glandular hair of *Galium triflorum* (330X)

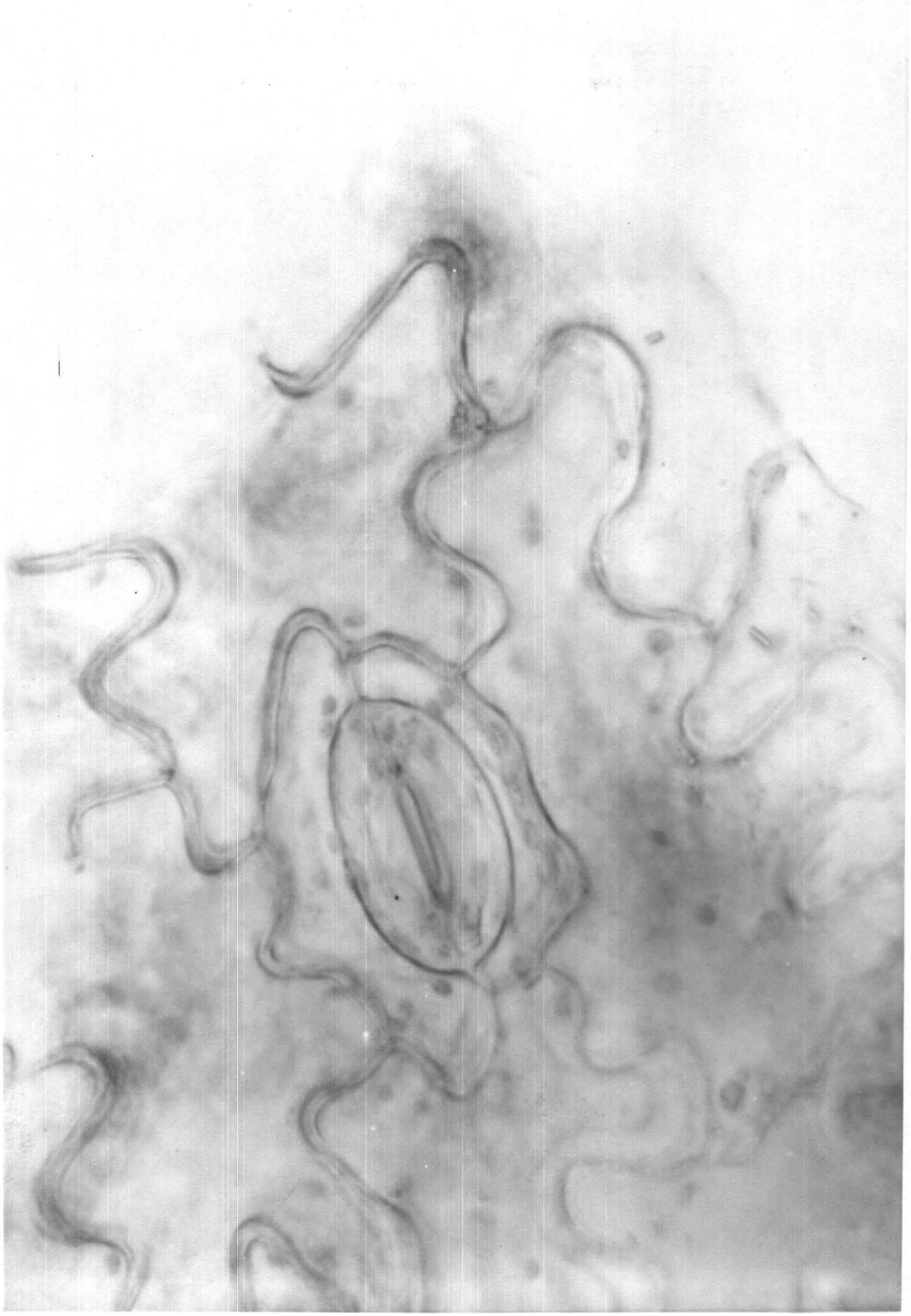


Figure 5.
Stomate (rubiaceous type) of Galium triflorum (1100X)

at room temperature for five days. The unground plant was then cut into pieces so that the component parts were 1 cm. or less in any dimension. The samples were then dried as described under the moisture determination of the dried plant.

The moisture content of the fresh plant, as calculated by using samples of the mature fresh plant, was found to be 82.9 per cent. This represents the mean of three determinations.

Table I.
Results of the Moisture Determinations

Condition of the Plant	Per cent Moisture
Dried Plant	8.41%
Fresh Plant	82.95%

D. Ash Determinations

Total Ash.

The ash determinations were first attempted using the U.S.P. XV method (42, p. 835). Accurately weighed samples of the dried plant were placed in a tared porcelain crucible and incinerated at a temperature not exceeding very dull redness. The charred mass was extracted with hot water and the residue was collected on ashless filter paper. The residue and ashless filter paper were then incinerated, the filtrate added, evaporated, and the whole heated to dull redness. The mean of a total of six determinations was found

to be 16.46 per cent total ash, but the variation from the mean was greater than for other methods to be discussed.

Total ash determinations were also carried out using Method 2 as outlined in "Official Methods of Analysis" (1, p. 535). An accurately weighed sample of the dried plant was placed in a platinum dish and incinerated at 538° C. Treatment with hot water as previously mentioned was sufficient to obtain a satisfactory ash. The average total ash obtained from four determinations was 16.46 per cent.

The third procedure utilized for total ash determinations was one very similar to the "Official Methods of Analysis" procedure, the principal difference being that the incineration temperature was 477° C. Four determinations using this procedure gave an average total ash of 17.37 per cent. The lower temperature was employed to prevent the loss of potassium.

Table II.
Results of the Total Ash Determinations

Procedure	Per cent total ash
U.S.P. Method	16.46%
"Official Methods of Analysis"	16.46%
Modified "Official Methods of Analysis"	17.37%

Acid Insoluble Ash Determinations.

The acid insoluble ash determinations were first carried out using the U.S.P. XV method (42, p. 835). The ash obtained under total ash was boiled with 25 ml. of dilute hydrochloric acid for five minutes and the insoluble matter collected on ashless filter paper. This residue was washed with hot water, ignited and weighed. The results obtained were very inconsistent and the procedure was discarded.

Acid insoluble ash determinations were next attempted using the total ash obtained from the "Official Methods of Analysis" procedure. The ash was washed with three 25 ml. portions of hot dilute hydrochloric acid and the insoluble matter collected on ashless filter paper. This residue was washed with hot water, ignited at 538° C. and weighed. The results obtained were consistent and determinations gave an average acid insoluble ash of 6.22 per cent.

The third procedure used for the determination of the acid insoluble ash was done using the total ash obtained by the modified "Official Methods of Analysis" procedure. This procedure was identical to the aforementioned procedure, the only difference being the incineration temperature which was maintained at 477° C. The results obtained using this procedure were very consistent and four determinations gave an average value of 6.63 per cent for the acid insoluble ash.

Table III.
Results of the Acid Insoluble Ash Determinations

Procedure	Per cent acid insoluble ash
Using incineration temperature of 538° C.	6.22%
Using incineration temperature of 477° C.	6.63%

Water Soluble Ash Determinations.

Water soluble ash determinations were first carried out using the total ash obtained from the U.S.P. method. The total ash was washed with three 25 ml. portions of boiling water and the insoluble matter collected on ashless filter paper. The residue was ignited at very dull redness, cooled and weighed. The water soluble ash was calculated giving results that were very inconsistent. The procedure was discarded.

Water soluble ash determinations were then done using the procedure outlined in "Official Methods of Analysis" (1, p. 535). The total ash obtained by using the "Official Methods of Analysis" procedure was boiled with water, filtered through ashless filter paper and the residue washed thoroughly with hot water. The residue was ignited to constant weight at 538° C. The weight of the residue was subtracted from the weight of the total ash to give the water soluble ash figure. Four determinations gave a mean of 2.66 per cent water soluble ash.

The third procedure used for the determination of the water soluble ash was very similar to the two methods already described,

the only difference being that the incineration temperature was maintained at 477° C. throughout. Four determinations gave an average water soluble ash of 3.30 per cent.

Table IV.
Results of the Water Soluble Ash Determinations

Procedure	Per cent water soluble ash
"Official Methods of Analysis"	2.66%
Using incineration temperature of 477° C.	3.30%

E. Composition of the Ash of Galium triflorum

Examination of the acid soluble ash using the qualitative scheme of Engelder et al. (12, p. 252-269) revealed the presence of cations in the ammonium hydroxide group, the ammonium carbonate group (alkaline earth metals) and the soluble cation group (magnesium, potassium and sodium).

A spectrographic analysis was carried out on the ash to further determine the cations qualitatively and to obtain a rough quantitative estimation of them. The results of this analysis showed that aluminum, calcium, magnesium and silicon were present in concentrations greater than 0.1 per cent of the ash. Only sodium and iron were present in concentrations greater than 0.01 per cent of the ash but less than 0.1 per cent.

Analytical procedures were chosen for the quantitative determination of most of those elements present in a concentration greater than 0.01 per cent. Also present in concentrations less than 0.01 per cent were boron, copper, manganese, lead and tin. Strontium, chromium, silver, gold, barium, molybdenum, nickel and vanadium were shown to be absent. The elements present in concentrations less than 0.01 per cent were not determined quantitatively since the amounts present were considered trace quantities.

Aluminum and Iron Determination.

The iron and aluminum determinations were done using the method outlined in "Official Methods of Analysis" (1, p. 100) with some modifications. The procedure employed for the preparation of the sample was somewhat modified as follows: A 10 to 50 gram sample of the dried pulverized plant was ignited in a platinum dish at a temperature of 477° C. in a muffle furnace until the residue was white, or nearly so. The ash was moistened with 5 to 10 ml. of dilute hydrochloric acid, brought to a boil and heated on a steam bath about three hours to render the silicon dioxide insoluble. The residue was then extracted with three 25 ml. portions of hot dilute hydrochloric acid, in each case the mixture being allowed to heat on a steam bath for about 30 minutes. After each extraction the mixture was filtered and the filtrates combined. The presence of iron in the extract in the form of ferric ion helped serve as an indicator for the purpose of determining when the extraction was complete. The residue was washed with hot water that was added to the combined filtrates.

The filtrate was then diluted to 500 ml. with water and the resulting dilution designated as solution A.

A 100 ml. aliquot of solution A was taken for each iron and aluminum determination. The iron was oxidized with nitric acid and an excess of ammonium acid phosphate was added to the solution. After neutralization with ammonium hydroxide, 0.5 ml. of hydrochloric acid and 50 ml. of 25 per cent ammonium acetate solution were added. The precipitate of iron and aluminum phosphate so obtained was ignited and weighed.

The iron and aluminum phosphates were then fused with a mixture of sodium and potassium carbonates. Sulfuric acid was added to the cooled crucible and heat applied until copious fumes of SO_3 were given off. The mixture was diluted with water and the iron reduced with zinc. The solution was then titrated with 0.1 N potassium permanganate, a blank determination also being run, and the iron calculated as per cent ferric phosphate. This percentage was subtracted from the total iron and aluminum phosphate figure to obtain the percentage of aluminum phosphate present.

This method gave an average of 2.68 per cent for the combined aluminum and iron phosphates. The iron determination described gave very inconsistent results. When titrations were carried out using 0.1 N potassium permanganate a volume of less than one ml. of the reagent gave the endpoint. The determination was repeated using 0.01 N potassium permanganate and the results obtained were more satisfactory.

This determination gave an average figure of 0.24 per cent ferric phosphate which indicated that the concentration of aluminum phosphate present was 2.44 per cent. Both iron and aluminum percentages were expressed in terms of their oxides (Table V).

Calcium Determination.

The calcium determination was done using the macro method of the "Official Methods of Analysis" (1, p. 101). Fifty ml. aliquots of solution A were used for each determination. Essentially, the procedure involved the precipitation of calcium as the oxalate and subsequent titration with 0.5 N potassium permanganate. The average result of two such determinations was 2.74 per cent calcium expressed as the oxide.

Magnesium Determination.

According to the spectrographic analysis, the magnesium content was between 0.1 to 1 per cent. For a more accurate estimation of the magnesium content, determinations were performed using the method outlined in "Official Methods of Analysis" (1, p. 102). A gravimetric procedure was used involving the precipitation of magnesium as the pyrophosphate. Three determinations gave an average value of 0.42 per cent magnesium.

Potassium and Sodium Determinations.

The sodium and potassium determination of "Official Methods of Analysis" (1, p. 103), a gravimetric procedure, was used with some modifications. An accurately weighed sample of the dried plant was moistened with a dilute sulfuric acid, dried and ignited in a muffle

at 477° C. to destroy the organic matter. The residue was dissolved with the aid of water acidulated with hydrochloric acid and aluminum and iron were removed by precipitation with ammonium hydroxide. Ammonium salts were expelled by ignition at 477° C., the residue dissolved in hot water, and a saturated solution of barium hydroxide was added to precipitate any sulfate present. Diluted ammonium hydroxide and 10 per cent ammonium carbonate were added to the heated filtrate to bring about the precipitation of the alkaline earth metals. The potassium and sodium were then determined as total chlorides gravimetrically. In order to determine the concentration of the individual cations, the perchloric acid method of "Official Methods of Analysis" (1, p. 103-104) was performed. The residue of the chlorides was treated with 60 per cent perchloric acid to form the perchlorates. The sodium salt was removed from the mixture by washing with a mixture of anhydrous ethyl acetate and n-butanol (1+1). The potassium perchlorate was then determined gravimetrically and the amount of sodium present was calculated by difference.

Results of the determinations for potassium and sodium showed the presence of 2.73 per cent potassium. The figures obtained for sodium were quite small and inconsistent. The concentration of sodium was approximately 0.02 per cent.

Anion Analysis.

In order to determine the anions present in the ash, the systematic scheme for anion analysis of Engelder et al. (12, p. 270-277) was employed. Group one anions which include carbonates, oxalates,

Table V.
Results of the Cation Analysis of the Ash of Galium triflorum

Cation	Per cent of dried plant
Aluminum oxide	1.02%
Ferric oxide	0.13%
Calcium oxide	2.74%
Magnesium	0.42%
Potassium	2.73%
Sodium	about 0.02%

borates, sulfites, arsenites, arsenates and phosphates has a group reagent of 1 M calcium nitrate. No test was obtained with the group reagent when applied to acid soluble extracts of the dried plant. An additional test for phosphate, the ammonium molybdate test, was also carried out on the acid soluble ash and proved negative.

Analysis of group two, which includes sulfates and chromates, using barium nitrate as the group reagent, gave a white precipitate indicative of either sulfate or chromate. Since the precipitate formed in the acid medium (hydrochloric acid), it was concluded that sulfate was present since barium chromate is soluble in acid solutions. Chromate ion was not detected.

Preliminary studies indicated that the sulfate concentration was low, necessitating the use of large samples of the dried plant. Accurately weighed samples approximating 40 grams of the dried plant were placed in platinum crucibles and ignited in a muffle at 477° C.

The ash so obtained was extracted with three 50 ml. portions of dilute hydrochloric acid and a method similar to the official procedure of "Official Methods of Analysis" (1, p. 580) was used for the quantitative determination. The acid extract was evaporated to dryness in a platinum dish and the silicon dioxide was removed by two additional evaporations. The filtrate so obtained was heated to boiling and a slight excess of barium chloride solution was added. The precipitate of barium sulfate so obtained was determined gravimetrically. The result of two such determinations gave an average figure of 0.21 per cent sulfate. This procedure would not necessarily account for any volatile sulfur that might be present.

Group three included the sulfides and cyanides. When the acid soluble ash was tested with the group reagent, which was 0.5 M zinc nitrate, a negative test was obtained.

Present in group four are the halides, the group reagent being a 5 per cent solution of silver nitrate. A positive test was obtained with this group reagent which was indicative of one or more of the halides. All tests were carried out on the water soluble extract of the ash since no hydrochloric acid could be used. This seemed plausible since the salts of the halides are reasonably water soluble (12, p. 290).

Since thiosulfates and thiocyanates, also members of this group, gave negative tests, it remained to determine which one or more of the halides was present. About 5 ml. of the aqueous extract was acidified with sulfuric acid and 1 ml. of chloroform was added.

To this mixture were then added a few drops of freshly prepared chlorine water. The test tube was stoppered and shaken thoroughly and the chloroform layer inspected for color. No color was observed which strongly suggested the absence of bromide and iodide. A few drops of silver nitrate test solution was added to another aliquot of the aqueous extract that was acidified with nitric acid and a white precipitate formed at once. This precipitate dissolved in ammoniacal silver nitrate solution, which further pointed to the fact that the halide present was chloride.

The chloride content of the dried plant was determined quantitatively using the residual titration procedure outlined in "Official Methods of Analysis" (1, p. 116). An accurately weighed sample of the dried plant was placed in a platinum crucible and moistened with 5 per cent sodium carbonate solution. The mixture was evaporated to dryness and ignited at 477° C. The ash was thoroughly extracted with hot water and the filtrates were combined and diluted to a specific volume with water. Aliquots of this solution were used for the chloride determinations.

The chloride determination involved the precipitation of silver chloride with standardized silver nitrate solution, the excess silver nitrate solution being titrated with standardized ammonium thiocyanate solution. Three such determinations gave an average result of 0.63 per cent chloride.

Group five contains the ions chlorate, acetate, nitrite and nitrate. Since these ions are all water soluble, no group reagent

is available and no group test could be made. The water soluble extract of the ash gave a faint ring test for nitrate ion.

Table VI.
Results of the Anion Analysis

Anion	Per cent of dried plant
Sulfate	0.21%
Chloride	0.63%

F. Partial Proximate Analysis

Total Nitrogen Determinations.

Total nitrogen was determined according to the micro-Kjeldahl method as described in "Official Methods of Analysis" (1, p. 805). The procedure was adopted to account for nitrate nitrogen by the addition of salicylic acid and sodium thiosulfate (1, p. 12). Accurately weighed samples of the dried plant were digested for a period of two hours and the ammonium sulfate thus formed distilled from the mixture using the Kjeldahl distillation apparatus into a boric acid solution, which was subsequently titrated with standardized hydrochloric acid solution. Results of the determinations gave an average total nitrogen content of 1.58 per cent.

Reducing Sugars.

The initial separation of the sugars from other plant principles was accomplished by employing the "Official Methods of Analysis"

method (1, p. 373-374) for grain and stock feeds. An accurately weighed sample of the plant material was boiled on a steam bath with 50 per cent alcohol to extract the reducing sugars and sucrose. Upon evaporation of the alcohol, the aqueous solution was cleared with neutral lead acetate, and aliquots of this solution were used for both the reducing sugar and sucrose determinations. To obviate repetition, this solution will be referred to as solution B.

The reducing sugar determination was carried out using the Munson-Walker general method (1, p. 545-546). Aliquots of solution B were mixed with copper sulfate and alkaline tartrate solutions and the cuprous oxide precipitated in the prescribed manner.

The cuprous oxide was determined by direct weighing as described in "Official Methods of Analysis" (1, p. 546). When Gooch crucibles using a specially treated asbestos were employed, results were erratic because the crucibles were losing weight probably due to the hot alkaline tartrate solution. In order to correct this difficulty fine fritted glass crucibles were substituted for the asbestos mats.

The modified procedure can be summarized as follows: Filter the hot alkaline solution at once through an accurately weighed fine fritted glass crucible using suction. Wash the precipitate thoroughly with hot water at about 60° C., then with 10 ml. of alcohol, and finally with 10 ml. of ether. Dry the precipitate for 30 minutes in an oven at 100° C., cool and weigh. Wash the crucible with nitric acid and rinse thoroughly. Dry the clean crucible in an oven at 100° C. for 30 minutes and weigh. From this data the weight of the

cuprous oxide formed was calculated. Known dextrose solutions were analyzed using this modification and the results verified its accuracy.

The amount of invert sugar equivalent to the weight of cuprous oxide formed was determined by reference to the Munson-Walker tables (1, p. 890). These determinations gave an average percentage reducing sugar of 1.57 per cent.

Sucrose Determination.

The sucrose determination was carried out using a procedure similar to that employed for the reducing sugars (1, p. 374). An aliquot of the prepared solution (solution B) was placed in a volumetric flask with a specified amount of hydrochloric acid and inversion was allowed to proceed at room temperature for a period of 24 hours. The solution was neutralized with sodium carbonate and aliquots were analyzed for invert sugar by the procedure mentioned under reducing sugars. The percentage sucrose was calculated by subtracting the percentage reducing sugar before inversion from the total sugar after inversion. Sucrose determinations gave an average figure of 3.44 per cent sucrose.

Starch.

The histological study indicated the absence of starch granules. Further, a qualitative test for the presence of starch was performed. One gram samples of the dried plant were ground in a mortar with water and allowed to macerate for one hour. The mixture was brought to a boil, strained and filtered. Portions of the filtrate were tested with iodine T.S. and no coloration was observed. This data

suggests the absence of any significant amount of starch.

Polysaccharide.

This determination was adopted from the procedure outlined in "Official Methods of Analysis" (1, p. 374). The procedure employed was a direct acid hydrolysis utilized for starch determinations. An accurately weighed sample of the dried plant was stirred in cold water for one hour, filtered and washed with a large volume of cold water. The insoluble residue was refluxed with a hydrochloric acid solution, neutralized and diluted to a specific volume. Aliquots of this solution were analyzed for the dextrose content by the procedure described under reducing sugars. Determinations, which were consistent, gave an average figure of 10.56 per cent of what will be referred to as total polysaccharide that could be hydrolyzed under these conditions.

Crude Fiber.

The crude fiber determination was done by the procedure outlined in "Official Methods of Analysis" (1, p. 372-373). Two gram samples of the dried plant, that was previously extracted with ether, were transferred to digestion flasks containing a small amount of asbestos and refluxed with 200 ml. of 1.25 per cent sulfuric acid for 30 minutes. The residue was washed free of the acid and 200 ml. of 1.25 per cent sodium hydroxide solution was added. The mixture was again refluxed for 30 minutes and the residue transferred to a weighed Gooch crucible, washed with water and alcohol, dried at 100° C., cooled and weighed. The contents of the crucible was then incinerated in a

muffle furnace until all the carbonaceous matter had been removed. The crucible was cooled, weighed and the loss in weight taken as the crude fiber. Determinations gave an average figure of 21.93 per cent of the ether insoluble residue.

Table VII.
Results of the Partial Proximate Analysis of Galium triflorum

Determination	Result
Total Nitrogen	1.58% of the dried plant
Reducing Sugar	1.57% of the dried plant
Sucrose	3.44% of the dried plant
Starch	Negative
Polysaccharide	10.56% of the dried plant
Crude Fiber	21.93% of the ether insoluble residue

G. Selective Extraction of Galium triflorum

Dragendorff (11, p. 8-94) has presented a scheme for the selective extraction of plants which included information regarding the chemistry of the various fractions. The Dragendorff procedure recommended extraction with solvents in a sequence of increasing polarity. The extraction procedure utilized in this investigation was adopted from the work of Stuart (49, p. 6-7) and the sequence used was petroleum ether, benzene, chloroform, alcohol and water. This procedure was similar to that recommended by Dragendorff.

Twenty gram samples of the air dried plant were extracted selectively in a Soxhlet apparatus by the continuous extraction method for a period of 24 hours. The extracts were evaporated, dried to constant weight at 100° C. and the average percentage extractive calculated. After each extraction the residue was air dried in the thimble before extraction with a new solvent was attempted. The results of the selective extraction procedure are tabulated in Table VIII.

Table VIII.
Selective Extraction of Galium triflorum

Extract	Per cent of Dried Plant
Petroleum Ether Extract	2.02%
Benzene Extract	2.99%
Chloroform Extract	0.81%
Alcohol Extract	11.39%
Water Extract	15.24%

Examination of the Water Soluble Extract.

Dragendorff (11, p. 65-66) has discussed a procedure for the isolation of mucilaginous substances from the aqueous extract of plant material. One hundred grams of dried Galium triflorum was macerated for 24 hours with water at room temperature, strained and filtered. An aliquot of the aqueous extract so prepared was mixed with two volumes of absolute alcohol and allowed to stand overnight

in a stoppered container. The precipitate so obtained was collected on filter paper and washed with 95 per cent alcohol. It was a dark brown amorphous mass having the characteristics of a mucilage. The precipitate was redissolved in water and a portion of this aqueous solution was boiled in the presence of a few ml. of dilute hydrochloric acid for about two hours. The aqueous solution that had not been boiled in the presence of acid caused no more reduction of Fehling's solution than did a blank. That portion of the aqueous solution that had been boiled in the presence of the acid showed a far greater reduction of Fehling's solution.

Another portion of the aqueous solution was treated with a few drops of basic lead acetate solution, and a precipitate formed at once. These tests are indicative of the presence of a mucilaginous substance.

To the alcoholic solution from which the mucilaginous substance was precipitated was added a large additional volume of absolute alcohol. A small amount of precipitate formed which was removed by filtration. The filtrate was evaporated at room temperature until most of the alcohol was removed. The aqueous solution so obtained was treated with a few drops of neutral lead acetate solution. A precipitate formed which was indicative of the presence of organic acids.

Fifty grams of the dried plant material was macerated with 2 per cent hydrochloric acid for 48 hours at room temperature. The aqueous

extract so obtained gave negative tests with Mayer's and Wagner's reagents which indicated the absence of alkaloids.

Examination of the Alcoholic Extract for Tannins.

Twenty grams of the dried plant was macerated for 24 hours with absolute alcohol. The mixture was filtered and the alcoholic filtrate was evaporated at room temperature. The residue was extracted with ether at room temperature to remove most of the chlorophyll and other soluble principles and then dissolved in water. A subsequent test with ferric chloride test solution gave a negative reaction for tannins. Aqueous extracts of the dried plant also gave a negative ferric chloride test for tannins.

H. Coumarin Extraction

The characteristic odor of Galium triflorum was suggestive of coumarin which has been reported to be present in some species of Galium but never substantiated with physical or chemical data (55, p. 405-406). Coumarin is soluble 1 to 400 in water at room temperature and soluble 1 to 50 in boiling water. Also, coumarin is freely soluble in chloroform and other organic solvents.

On the basis of the solubility properties of coumarin a method of extraction was developed. A 20 gram sample of the dried plant was mixed with 150 ml. of water and the mixture boiled for 15 minutes. The hot solution was immediately strained and the residue rinsed twice with about 50 ml. portions of hot water. The aqueous solution

was then extracted with three equal volumes of chloroform, the chloroform extractions being combined and evaporated on a steam bath. When only a few ml. of the chloroform remained, the beaker was taken from the steam bath and the remaining chloroform was evaporated with the aid of a jet of cool air resulting in the crystallization of crude coumarin. Using this procedure about 0.28 per cent of crude coumarin was isolated from Galium triflorum.

Coumarin was also isolated from the dried plant by another procedure involving a direct extraction of the plant with acetone, subsequent evaporation to dryness and extraction of the residue with boiling water. The aqueous solution was then extracted with chloroform which yielded the coumarin. No quantitative estimation was made using this procedure.

Water was found to be a satisfactory solvent for the recrystallization of the crude coumarin. A sufficient amount of boiling water was used to dissolve all the extract and after filtering the hot aqueous solution it was immediately placed in a cooler at 5° C. The coumarin readily crystallized in the form of white needle-like crystals. Repeated recrystallization from boiling water yielded a product having a reproducible melting point.

The white needle-like crystals had the characteristic odor of coumarin which may be described as being a vanilla-like odor. The melting point of the crystals was 68-69° C. which corresponded to that of coumarin as noted in the "Merck Index" (33, p. 280). Some of the crystals obtained from Galium triflorum were mixed with an

equal amount of known coumarin. The mixed melting point was also observed to be 68-69° C. which strongly suggested that the isolated principle was coumarin.

I. Vitamin Determinations

Ascorbic Acid.

The "Official Methods of Analysis" procedure (1, p. 838-839) was used for this determination. About 180 grams of the fresh plant collected in the spring was thoroughly pulped in a Waring blender with the aid of water. About 500 ml. of water was added and the mixture boiled for one hour. The mixture was cooled, transferred to a 1 liter volumetric flask and diluted to volume. The mixture was filtered and a 100 ml. aliquot of the clear filtrate was added to an equal volume of the metaphosphoric acid stabilizing solution. Ten ml. aliquots of this solution, which were equivalent to the extract from approximately one gram of the fresh plant, were titrated with the standardized indophenol reagent. Further titrations were carried out using 20 and 40 ml. aliquots of this solution.

All titrations required no more than 0.1 ml. of the indophenol reagent, each ml. of which was equivalent to 0.13 mg. of ascorbic acid. Blank determinations required approximately 0.05 ml. of the indophenol reagent. These results indicate that ascorbic acid is either absent from the fresh plant or the plant content of this vitamin is too low to be detected by the method employed.

Nicotinic Acid.

The nicotinic acid determinations were carried out according to the method of "Official Methods of Analysis" (1, p. 828-829) which is the official chemical method. The procedure for the preparation of the sample was altered because of the difficulty encountered when the aqueous extract of the plant was heated with sulfuric acid. Fifty grams of the fresh plant was pulverized in a Waring blender with the aid of water and the suspension was transferred quantitatively to a 500 ml. volumetric flask. The suspension was allowed to macerate one hour, brought to volume with water, and filtered. Aliquots of this solution were used for the determinations.

This method of extraction leads to results that would account for all the nicotinic acid present but only half the nicotinamide content of the plant, since the latter was not hydrolyzed with acid (50, p. 983-986).

The procedure involved the development of a color upon the addition of sulfanilic acid and cyanogen bromide to solutions containing nicotinic acid. The unknown solution was compared with a standard solution prepared with U.S.P. nicotinic acid reference standard. Blanks were prepared for both the unknown and standard solutions. The determination was done colorimetrically by measuring the absorbance of both standard and unknown solutions at 450 mu. with a Beckman model DU spectrophotometer.

The unknown solutions showed no absorption at the specified wave length, which indicated the absence of nicotinic acid and

nicotinamide in the fresh plant or the possible presence of such small amounts that they could not be detected with the procedure employed.

Carotene Determination.

The extraction involved the removal of the carotene from the dried plant by maceration with an acetone-commercial hexane mixture (3+7) (1, p. 816-817). The carotene was separated from other plant principles by the use of column chromatography using a mixture (1+1) of activated magnesia and diatomaceous earth. Carotenes passed rapidly through the column while the xanthophylls and chlorophylls remained in the column. After eluting the column with an acetone-hexane mixture (1+9) the eluate was brought to volume and the carotene content was determined spectrophotometrically by measuring the absorbance of the solution at 436 mu. with the Beckman model DU spectrophotometer. The results indicated the presence of 27 mg. of beta carotene per pound of the dried Galium triflorum.

Table IX.
Results of the Vitamin Determinations

Vitamin	Result
Ascorbic Acid	Negative
Nicotinic Acid	Negative
Carotene	27 mg./pound dried plant

J. Extraction of Asperuloside from Galium triflorum

Aqueous extracts of the dried plant gave the color test that is indicative of asperuloside. A few drops of dilute sulfuric acid added to 5 ml. of the aqueous extract, which was then boiled, caused the formation of a deep green coloration and eventually a green-black precipitate that was insoluble in water (16, p. 490-491).

For the purpose of obtaining asperuloside the dried plant was first extracted using the procedure of Herissey (15, p. 1695-1697). Approximately 500 grams of the dried plant was boiled one hour with a sufficient quantity of alcohol, strained and filtered. The residue was washed with two portions of hot alcohol which were added to the filtrate. The filtrate was evaporated to dryness at room temperature with the aid of a stream of cold air. The residue was dissolved in hot water and the aqueous solution was cooled and filtered. This aqueous solution was evaporated to dryness at room temperature in a similar way, and the residue was extracted with three portions of boiling ethyl acetate. The ethyl acetate fractions were combined and concentrated. Two volumes of ether were added and the solution was cooled to about 5° C.

After standing over 48 hours, no crystals could be obtained from this solution. When the solution was evaporated, a brownish amorphous residue containing a very few crystals was obtained. This residue gave the sulfuric acid color reaction indicative of asperuloside. Attempts at recrystallization failed because of the small amount of residue.

The method of Trim and Hill (52, p. 310-311) for the extraction of asperuloside from the young shoot tips of Rubia tinctorum and related plants was then employed. The dried plant was extracted with 0.33 N hydrochloric acid and filtered with the aid of kieselguhr. The filtrate was stirred 10 minutes with 25 grams of activated charcoal, filtered, and the filtrate stirred with a further 100 grams of activated charcoal for 10 minutes. A large bulk of kieselguhr was added and the suspension filtered through a Buchner funnel, washed with 300 ml. of distilled water and the glucoside eluted with three liters of 50 per cent ethanol. The eluate was reduced to 150 ml. in vacuo in the presence of 2 grams of calcium carbonate. Then an equal volume of ethanol was added and the light gelatinous precipitate was removed by filtration with the aid of kieselguhr. The filtrate was reduced to a syrup at room temperature and dried in a desiccator over calcium chloride. The residue was then extracted with hot ethanol and the solution was filtered and reduced to a small volume. The concentrated solution was diluted with two volumes of ether and kept at 5° C. for over 48 hours. No crystals were obtained.

Plouvier's method (43, p. 1643-1645) for the extraction of asperuloside was then attempted. The dried plant was extracted with acetone in a large Soxhlet apparatus and the acetone solution was filtered. The acetone was evaporated at room temperature and the residue was dissolved in hot water. The aqueous solution was filtered and cleared with a slight excess of normal lead acetate solution. The solution was again filtered and the excess lead was

removed by saturating the solution with hydrogen sulfide. The filtrate was filtered and concentrated in the presence of calcium carbonate. The residue so obtained was extracted with hot ethanol and the ethanol solution was reduced in volume. Five volumes of ether were added to the ethanol solution and the whole allowed to stand at 5° C. for over 48 hours. No crystals could be obtained by use of this procedure.

In all the procedures it was observed that when the solutions were concentrated, whether by vacuum distillation or air drying, they gradually turned from colorless to a light yellow to a light brown color as the evaporation progressed. The residue usually obtained was a dark brown amorphous mass which was insoluble in ethyl acetate or alcohol, but soluble in water.

The alcoholic extracts of the residue obtained by evaporating the aqueous solution yielded a yellow precipitate when ether was added. At first this precipitate was thought to be asperuloside, but further observations revealed that it immediately changed into a brownish amorphous mass when exposed to the air. The identity of this precipitate was not determined and attempts to purify this substance yielded only the same brown amorphous mass.

Spring collections made in May and June of 1959 were handled differently than the original collections that were made in the fall of 1958. Within two hours after collection the aerial parts of the fresh plant were placed in a drying oven and dried at a temperature

of 49° C. for 48 hours. The dried plant was then reduced to a moderately coarse powder (42, p. 935) in a Wiley mill.

The procedure of Plouvier was repeated using the spring collections of Galium triflorum. This procedure was considered the most desirable of those employed. The dried plant was extracted with acetone and water as previously described. The aqueous residue was taken up with boiling ethanol which was concentrated and 5 volumes of ether were added. Upon the addition of the ether a large amount of yellow precipitate formed that readily changed into a brownish amorphous mass on standing. The ethanol-ether solution was filtered to remove this substance and kept at 5° C. for 48 hours. Within 24 hours colorless needle-like crystals were obtained which were later proven to be asperuloside. The yield, which was reproducible, was approximately 0.12 per cent of the dried plant.

The total asperuloside obtained by this procedure amounted to less than a gram. The crystals, which were stable in air, were recrystallized three times from ethanol, dried in a desiccator over calcium chloride, and kept in a tightly sealed screw-cap amber bottle at room temperature.

K. Chemical and Physical Properties of the Isolated Asperuloside

The purified asperuloside gave a melting point between 127 and 129° C. Melting points were taken by employing the procedure outlined in Shriner and Fuson (47, p. 26).

The specific rotation of the asperuloside obtained from Galium triflorum was calculated to be -194.2 at a temperature of 23° C. This figure pertains to the hydrated glucoside. Readings were taken using the Bausch and Lomb saccharimeter and were converted to angular degrees with the aid of the conversion factor 0.3468.

The asperuloside obtained from Galium triflorum, having been recrystallized from ethanol and dried over sulfuric acid in a desiccator, was found to show a maximum ultra violet light absorption at 238 mu. in aqueous solution. A concentration of 5×10^{-2} grams/liter was found suitable for this determination. Figure 6 shows the ultra violet absorption curve of a freshly prepared aqueous solution of asperuloside. Readings were taken with the Beckman model DU spectrophotometer. The molecular extinction coefficient was calculated to be 6800 pointing to the possibility of a diene system.

An infrared analysis of the isolated asperuloside that was dried over sulfuric acid in vacuo gave bands in the following regions when the glucoside was prepared in a nujol mull: 3350s, 3570m, 3660w, 1750s, 1710m, 1672s, 1290m, 1270s, 1230w, 1220m, 1200w, 1188m, 1170w, 1090s, 1079s, 1063m, 1047s, 1032s, 1020m, 990s, 955m, 916m, 862m, 815m, and 768m (s = strong, m = medium, w = weak). Readings were taken using a Beckman infrared spectrophotometer. Figure 7 shows a picture of the absorption bands reported.

Bands between 1300 and 1500 cm^{-1} and in the region of 3000 cm^{-1} were obscured by the absorption of the nujol. In order to observe any possible absorption in these ranges, readings were taken in a

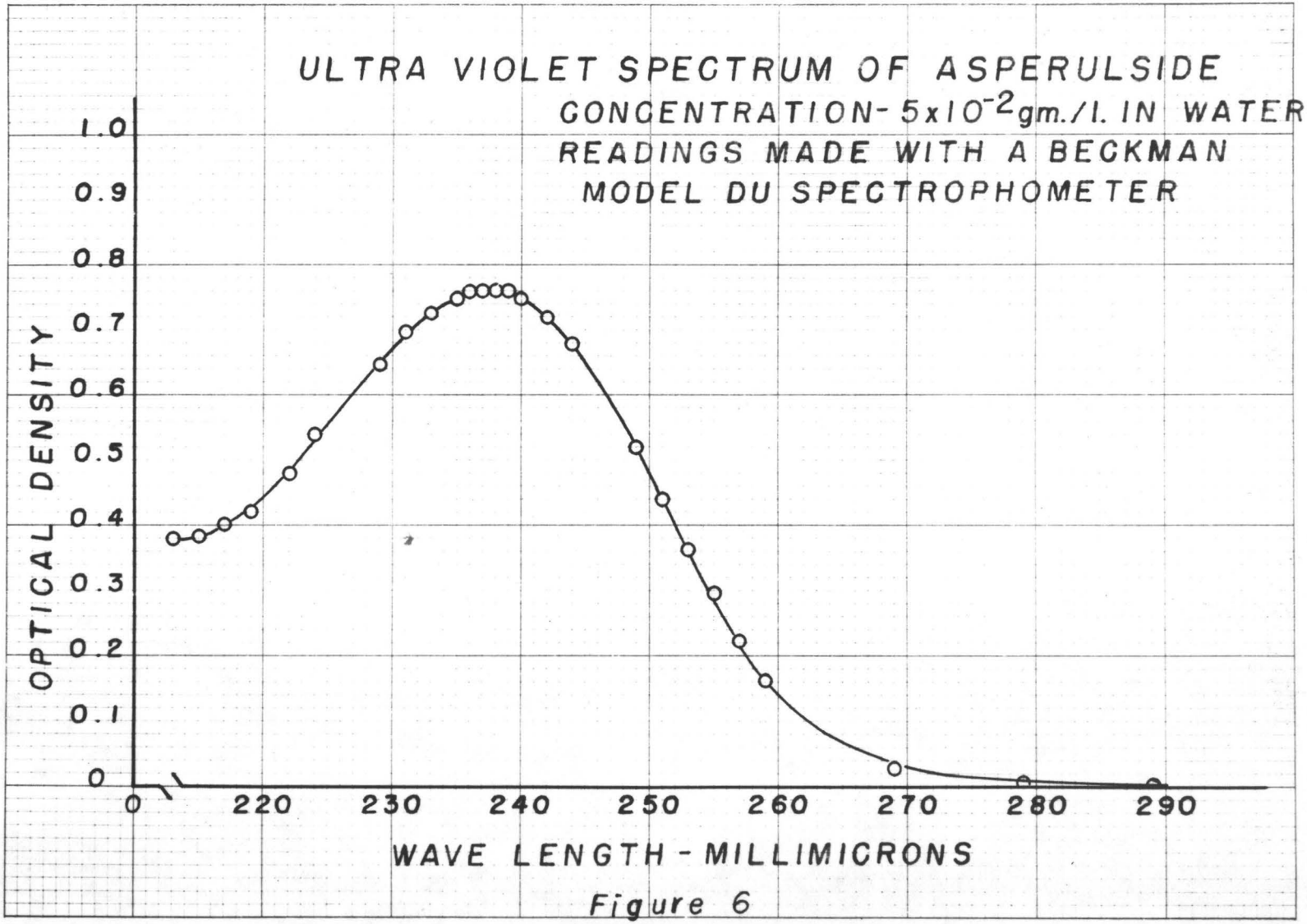

ULTRA VIOLET SPECTRUM OF ASPERULSIDE
CONCENTRATION- 5x10⁻² gm./l. IN WATER
READINGS MADE WITH A BECKMAN
MODEL DU SPECTROPHOMETER
OPTICAL DENSITY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
220
230
240
250
260
270
280
290
WAVE LENGTH - MILLIMICRONS

Figure 6

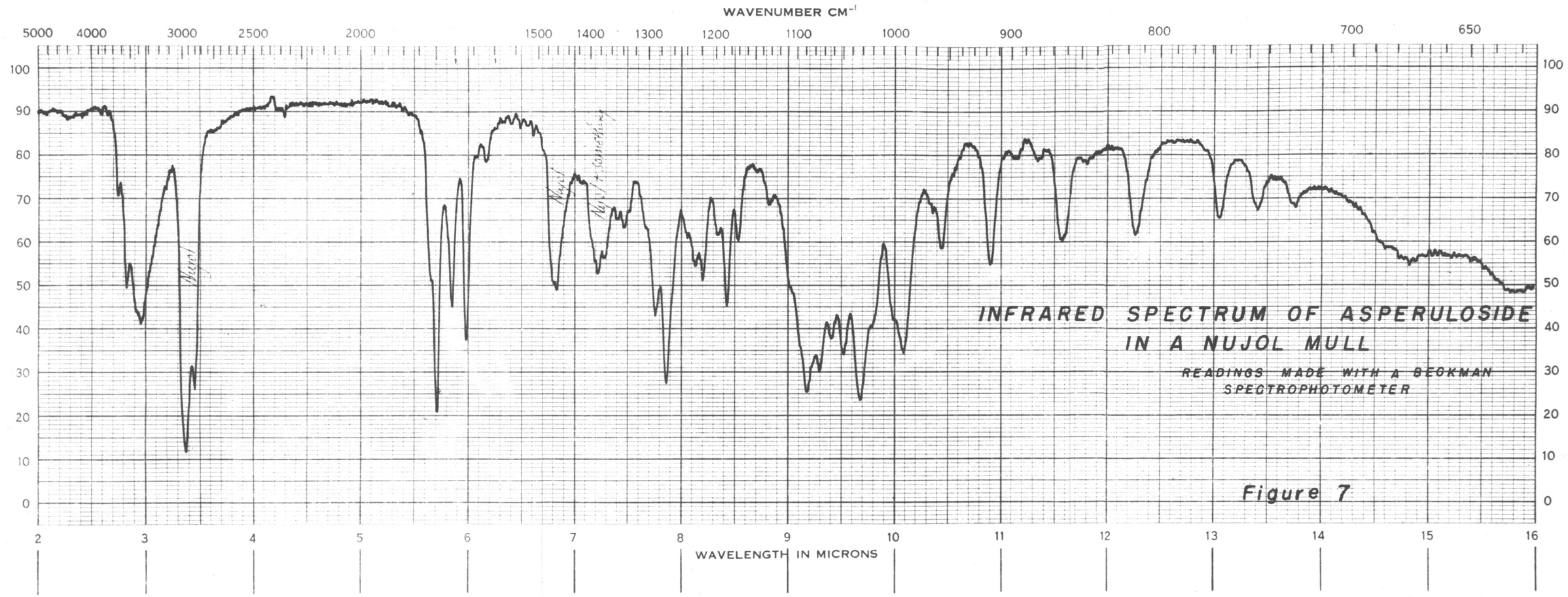

WAVENUMBER CM⁻¹
5000 4000 3000 2500 2000 1500 1400 1300 1200 1100 1000 900 800 700 650
100 90 80 70 60 50 40 30 20 10 0
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
WAVELENGTH IN MICRONS
INFRARED SPECTRUM OF ASPERULOSIDE
IN A NUJOL MULL
READINGS MADE WITH A BECKMAN
SPECTROPHOTOMETER

Figure 7

potassium chloride pellet. The following bands were observed: 3350s, 3000s, 2940s, 2885s, 1742s, 1700s, 1660s, 1382s, 1365s, 1340s, 1262s, 1174s, 958s, 915s, 860s, 811m, 764w, 740w, 725w, and 675w. Figure 8 shows the actual recording obtained using the potassium chloride pellet.

Thirty mg. of the asperuloside obtained from Galium triflorum was dissolved in 5 ml. of water and the solution made acidic by the addition of a drop of concentrated sulfuric acid. This solution was allowed to stand at room temperature for 48 hours to allow for the formation of the greenish-black precipitate. The precipitate was washed with distilled water until washings no longer were acid to litmus. The water was decanted off and the precipitate was dried at room temperature. The solid residue obtained was pulverized and dried in vacuo over phosphorus pentachloride.

A potassium bromide pellet of the solid residue, representing the decomposition product of asperuloside, was prepared and an infrared spectrum was obtained using the Beckman infrared spectrophotometer. Bands at 1710m, 2920m, 3080w, and 3450s were observed.

A carbon and hydrogen determination on the asperuloside extracted from Galium triflorum showed 49.91 per cent carbon and 5.61 per cent hydrogen. An elemental analysis indicated the absence of nitrogen, the halides and sulfur. The remainder of the asperuloside molecule was considered to be oxygen, amounting to 44.48 per cent. The glucoside was dried over sulfuric acid in a desiccator prior to this

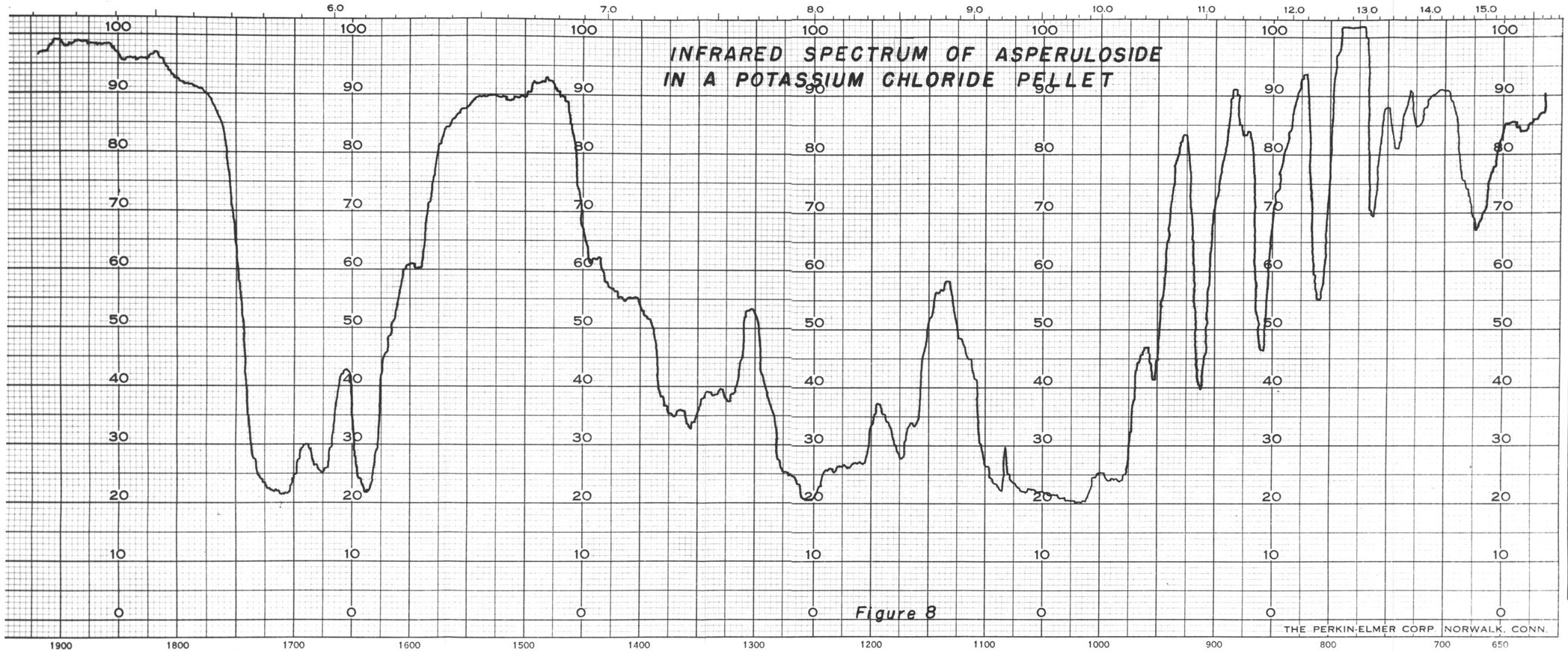


Figure 8

SPECTRUM NO. 9-9-59(4)
SAMPLE ASPERULOSIDE

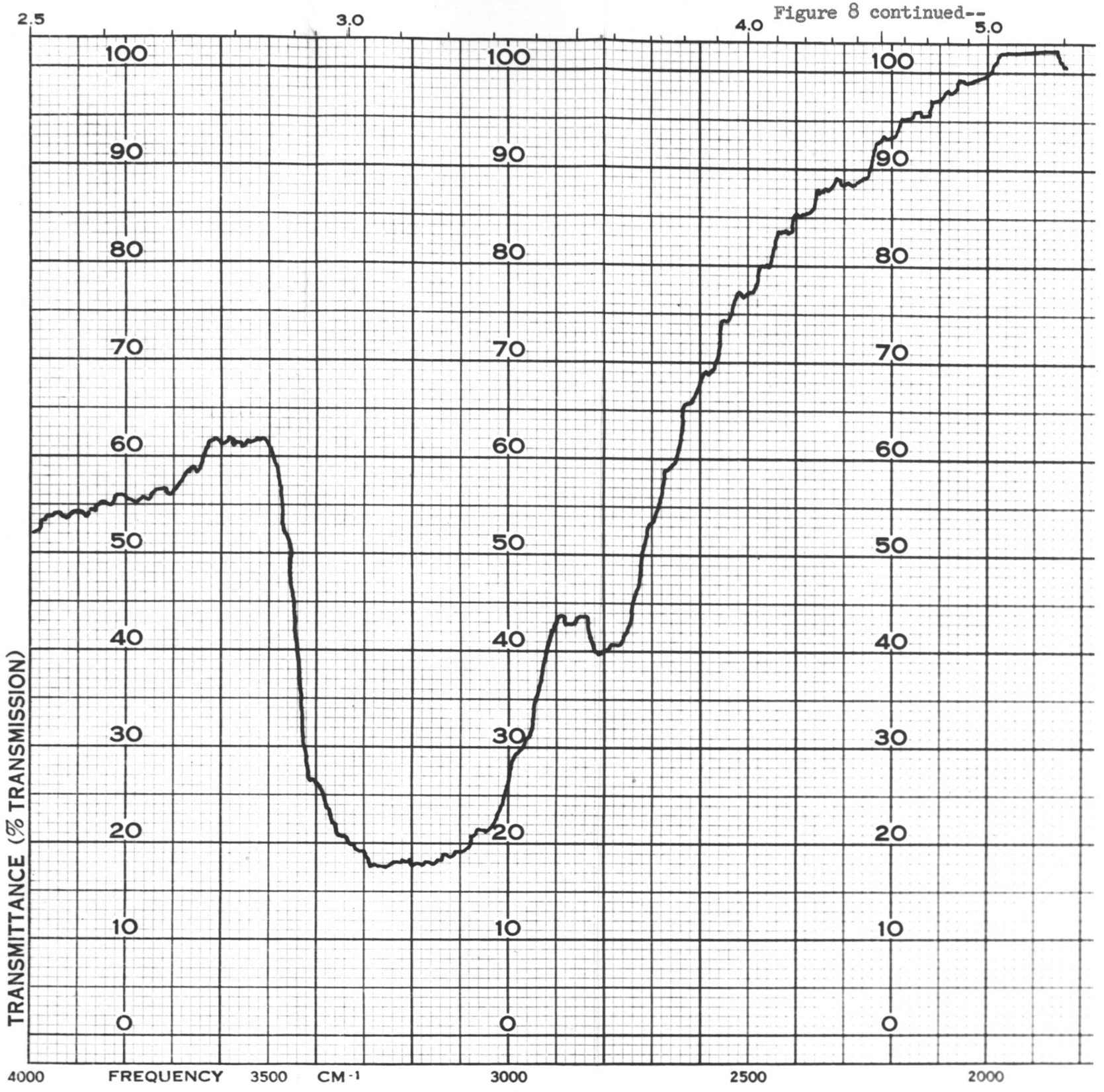
ORIGIN GALIUM TRIFLORUM
PURITY

PHASE
THICKNESS
1.
2.
3.
DATE 9-9-59
OPERATOR
REMARKS
27% SCREEN USED

PRISM Na Cl
RESOLUTION 927
RESPONSE 1
GAIN 6-1/2
SPEED 10
SUPPRESSION 6
SCALE NORMAL

NO. 021-6312

Figure 8 continued--



determination. Because of its insolubility in camphor the molecular weight of asperuloside cannot be determined by the Rask method. An acetyl determination indicated the presence of 9.04 per cent CH_3CO.

A one per cent solution of asperuloside gave a negative test with Fehling's solution but did reduce Tollen's reagent. This aqueous solution also gave a negative reaction with ferric chloride test solution, readily decolorized 0.1 N permanganate at room temperature and did not decolorize bromine in carbon tetrachloride at room temperature.

L. The Effect of Asperuloside on Blood Pressure

A preliminary study using aqueous extracts of the air dried Galium triflorum gathered in the fall was carried out in order to see the effects on blood pressure. An aqueous extract was injected intravenously into the femoral vein of a dog. The extract was prepared by macerating about two grams of the plant with 20 ml. of distilled water. Results revealed a rather rapid drop in the blood pressure and a relatively graded response. The blood pressure returned to normal in about 5 to 10 minutes. Two ml. of the aqueous extract produced a drop of about 15 mm. in the blood pressure while 4 ml. of the extract dropped the pressure some 27 mm. The experiment was repeated again using a dog, and a similar drop in the blood pressure was observed. This depressor effect of aqueous extracts of the dried plant was reproducible.

Studies were continued using aqueous extracts of the dried plant in order to obtain a better insight into the cause of the fall in blood pressure. A solution of coumarin (approximately one per cent) showed no drop in the blood pressure when 2 ml. was injected into the femoral vein of a dog. Further, an aqueous extract of the plant that was freed of coumarin by extraction with chloroform gave a pronounced drop in the blood pressure.

An acetone extract of the dried plant was prepared which would be mostly free of some principles soluble in water such as the sugars. The acetone solution was concentrated and the residue taken up with water and filtered. Injections of these water soluble principles of the acetone extract also produced a fall in the blood pressure.

Blood pressure studies were carried out using the asperuloside isolated from Galium triflorum. A one per cent solution of asperuloside was prepared using distilled water and stored at 0° C. Asperuloside solutions stored at 0° C. did not change color upon standing. Solutions kept at room temperature gradually turned yellow and finally brownish in color. This change began to come about within 24 hours after the preparation of the solution. The pH of the freshly prepared solutions was found to be 5.1.

A female albino rabbit weighing 4.5 kilograms and anesthetized with nembutal was given doses of the asperuloside solution ranging from 1 to 8 mg. per kilogram. Injections were given rapidly into the femoral vein. The first dose administered was 1 mg. per kilogram. The second dose was 2 mg. per kilogram, the third was 4 mg. per

kilogram, and the fourth was 8 mg. per kilogram. As is apparent, the dose was doubled until a significant response was obtained. The 4 mg. per kilogram dose of asperuloside produced a drop in blood pressure that lasted over thirty minutes before returning to normal. The initial drop in the blood pressure was some 12 mm. of mercury. The 8 mg. per kilogram dose produced an initial drop of some 26 mm. and also produced a rather sustained action that lasted over 30 minutes. Both asperuloside and aqueous extracts of the dried plant gave the same typical blood pressure response.

A further study relating to the effect of asperuloside on blood pressure was conducted using a dog. The carotid artery of a male dog weighing 12.7 kilograms was cannulated after the dog was anesthetized with nembutal. One per cent solutions of asperuloside were prepared in distilled water and in a phosphate buffer solution of pH 7 (42, p. 932). Injections of the buffered asperuloside solution approximating a dose of 4 mg. per kilogram were made. A similar dose of the aqueous solution of asperuloside was injected into the femoral vein. No response was obtained after any of the injections at this dose level.

M. A Preliminary Study of the Toxicity of Asperuloside

A preliminary investigation of the acute toxicity of asperuloside was carried out using mice. Doses of 50, 100, 200 and 400 mg. per kilogram of a one per cent aqueous solution of asperuloside were

given to mice by IP injection. There was no immediate adverse effect upon injection of any of the above-mentioned doses and observation for 12 hours after the injections indicated that the test animals differed in no way from the control animals. Each control animal received a volume of distilled water comparable to that of the test dose.

N. Test for the Antibacterial Potency of Asperuloside

The antibacterial potency of asperuloside was tested against Staphylococcus aureus by a method similar to that described in Suter (50, p. 223-224). Agar plates inoculated with Staphylococcus aureus were prepared and filter paper discs having a diameter of about one cm., which were previously saturated with either a one per cent solution of asperuloside or a solution of distilled water, were placed in the plates. Two plates were prepared containing in each two discs saturated with asperuloside solution and one plate was prepared containing two discs saturated with sterile distilled water. Another plate containing one disc saturated with the asperuloside solution as well as one disc saturated with sterile distilled water was also prepared. The plates were incubated at 37° C. for a total of 72 hours. They were examined once every 24 hours for zones of inhibition. No inhibition of growth was observed in any of the plates over this period of time.

DISCUSSION

The ash determinations were first carried out using the U.S.P. XV method which requires incineration to a very dull redness. By definition (26, p. 240) very dull redness infers a temperature between 500 and 550° C. The determinations gave results that showed considerable variation.

The procedure of "Official Methods of Analysis" was then resorted to. This method, as previously pointed out, employed a constant incineration temperature. Consistent results were obtained for total ash and acid insoluble ash determinations, while the figures obtained for the water soluble ash determinations, although more consistent than the results of the U.S.P. method, showed a variation from the mean by as much as 7 per cent. This variation in the water soluble ash determinations performed by both methods could not be attributed to poor sampling since total ash figures were in very close agreement. It was apparent, however, that by employing a constant temperature, more consistent results could be obtained.

It has been shown conclusively that with high incineration temperatures potassium will be lost, and a temperature of 480° C. should not be exceeded if all the potassium is to be retained (36, p. 470). With this information in mind, the third method was adopted in which the incineration temperature was held constant at about 477° C. This third procedure was identical to the method of "Official Methods of Analysis" with the exception of the lower incineration temperature.

The results obtained using this third method were consistent for total ash, acid insoluble ash and water soluble ash. The average total ash was considerably higher than the average figures obtained using the first two methods discussed. The total ash averaged 17.39 per cent which was approximately 0.91 per cent higher than the average percentage obtained by the other methods. Similarly, the acid insoluble ash and the water soluble ash figures were higher. The fact that the plant contains a high percentage of potassium may account in part for the larger figures obtained at the lower incineration temperature.

The data strongly indicated that the incineration temperature is a critical factor when doing ash determinations. Not only must the incineration temperature be low enough to prevent the loss of certain cations and anions, but it must be held constant. To further clarify the data obtained, a statistical study was performed, the results of which are shown in Table X. The null hypothesis, which stated that no difference in the results existed at different incineration temperatures, was rejected for each of the three determinations considered (48, p. 80-81).

The only reference found in the literature relating to the elemental composition of the ash of Galium was the work of Bertrand and Silberstein (6, p. 2386-2387). This study was concerned with the manganese content of phanerogams including Galium mollugo. Most common values for the manganese content of Galium mollugo were from 0.02 to 0.06 grams per kilogram of the fresh plant. The manganese

Table X.
A Statistical Study of the Effect of Incineration Temperature on Total Ash, Acid Insoluble Ash, and Water Soluble Ash of *Galium triflorum*

Determination	Incineration Temperature	Results		Number of Determinations	Degrees of Freedom	Mean Total Ash	T Test
Total Ash	477	17.35 17.39	17.37 17.38	4	3	17.37	
	538	16.42 16.46	16.49 16.46	4	3	16.46	P<0.01
Acid Insoluble Ash	477	6.62 6.65	6.64 6.60	4	3	6.63	
	538	6.20 6.16	6.31	3	2	6.22	P<0.01
Water Soluble Ash	477	3.30 3.39	3.24 3.26	4	3	3.30	
	538	2.60 2.52	2.81 2.70	4	3	2.66	P<0.01

content of the Galium triflorum ash was estimated to be within the range of 0.001 to 0.01 per cent, which would approximate the most common figures quoted by Bertrand and Silberstein.

Nitrogen is considered to be the most important element taken in by the plant root. It is part of the chlorophyll structure and is necessary for the formation of amino acids, alkaloids and proteins. The nitrogen content of the aerial parts of Galium triflorum (1.58 per cent) was lower than that usually found in plant material. The figure is commonly between 4 and 7 per cent. It has been pointed out, however, that the percentage will vary with the age, type of tissue, and kind of plant (14, p. 11-13). The fact that the plant was gathered in the fall may partly explain this low nitrogen content.

The selective extraction of Galium triflorum gave some indication as to the principal constituents of the plant. Petroleum ether is a good solvent for ethereal oils, fatty oils, waxes and related compounds. The 2.02 per cent petroleum ether extract would probably represent such principles. It is possible that volatile oils would be lost due to the drying process and thus would not be included in this figure. Some work has been reported by Neu (35, p. 239-240) relating to the wax constituents of Galium verum. A petroleum ether extract was reported to be 3.12 per cent.

The large alcohol extract, amounting to 11.39 per cent of the dried plant, would be indicative of such principles as resins, tannins and glycosides. Tannins are apparently absent, so it is possible that a major part of this extract is composed of resins.

This can to a small extent be supported by Von Cotzhausen's report (55, p. 405-406) of a yellow resin in Galium triflorum.

The aqueous extract revealed the presence of a mucilaginous principle, the chemical nature of which was not determined. The presence of such a principle was further verified by the high polysaccharide content of the dried plant.

The fact that no glucoside was isolated from the air dried plant collected in the fall by any of the extraction procedures discussed could possibly be explained by a consideration of principally three points. One possibility is that the glucosidal content of the mature plant collected in the fall is considerably smaller than the amount found in the young plant. Trim and Hill's observation (53, p. 326) that the amount of asperuloside in the young shoot tops of Galium aparine increases with the growth of the plant and begins to decrease as the plant matures would tend to support this consideration. Herissey's work with Galium aparine (16, p. 496) brought him to the conclusion that asperuloside is present in the very young plant, and as the plant grows the amount of glucoside decreases, possibly due to enzymatic reduction.

A second consideration was that Galium triflorum did not contain asperuloside. Briggs (7, p. 3940-3943) has shown that Galium umbrosum did not contain the glucoside. The fact that extracts of Galium triflorum did give a positive sulfuric acid test for asperuloside, however, strongly indicated that such a possibility was remote. Also, as is evident from Table XI, the yield from different plants will vary considerably.

Table XI.
Yields of Asperuloside Obtained from Different Plant Sources

Source	Part Used	Yield
Coprosma tenuifolia (7, p. 3940-3943)	air dried bark	2.9 gms/ 100 gms
Coprosma baueriana (19, p. 793-795)	leafy branches	0.45gms/ 500 gms
Galium verum (18, p. 1674-1675)	dried aerial parts	0.19gms/ 400 gms
Asperula odorata (15, p. 1695-1697)	dried aerial parts	0.5 gms/1000 gms
Rubia tinctorum (52, p. 311-312)	fresh young shoot tops	10 gms /1730 gms
Daphniphyllum macropodum (52, p. 311-312)	fresh mature leaves	2 gms / 315 gms
Galium aparine (52, p. 311-312)	dried seedlings	4.5 gms/8980 gms
Galium aparine (52, p. 311-312)	fresh young shoot tops	6.5 gms/ 600 gms
Crucianella maritima (28, p. 56-62)	young leafy branches	0.11gms/1000 gms
Escallonia philippiana (43, p. 1643-1645)	dried leaves	6-10gms/ 100 gms
Galium triflorum	dried aerial parts	0.56gms/ 480 gms

A third possibility was the enzymatic hydrolysis of the glucoside during the drying process. In support of this consideration was Plouvier's observation (43, p. 1643-1645) that the leaves and stems of Escallonia contained a beta-glucosidase which hydrolyzed asperuloside. During the drying process, however, the plant tissue was not ruptured and therefore there appears to be no good explanation as to why hydrolytic breakdown of the glucoside would occur under these conditions.

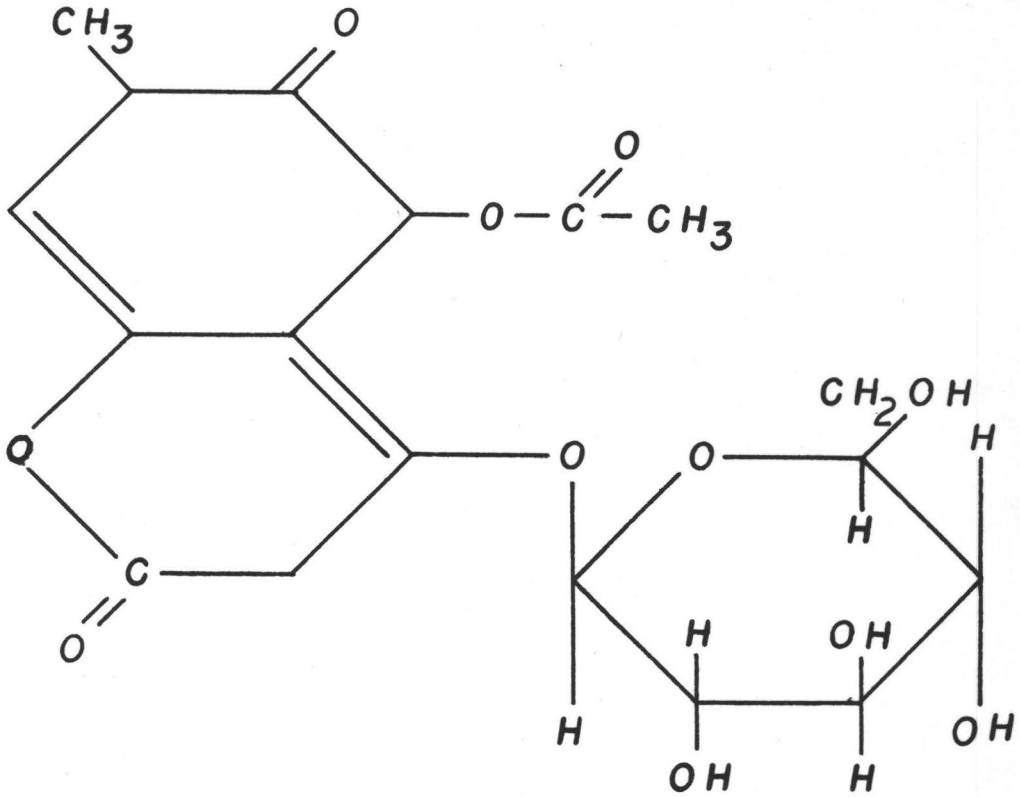
Worthy of mention are two other considerations. The possibility of translocation of the glucoside cannot be obviated in this work since the roots of the plant were not employed. Also, one might question the methods used for the isolation of the glucoside. The methods employed, however, were utilized for the extraction of this glucoside from other plants and there is little doubt that they are satisfactory procedures.

A spring collection of Galium triflorum was made and because of the problem of obtaining a sufficient bulk of the plant a large percentage of the collection was composed of the mature plant. In order to account for the possibility that the asperuloside was destroyed by enzymes during the process of drying, the fresh plant was dried at 49° C. in a drying oven for 48 hours. Analysis of this plant material using the Plouvier procedure yielded 0.12 per cent asperuloside. Another collection was made and treated similarly, giving the same yield.

These results would indicate that the asperuloside content of Galium triflorum is primarily influenced by the time of year the collection is made, which would be related to the age of the plant. Possibly the method of drying the plant would be a factor but indications are that the former explanation would weigh most heavily for reasons already discussed. Also, asperuloside has been extracted from the air dried Galium aparine by Herissey even though the yield was very small. Table XI summarizes the yields of asperuloside obtained by different investigators from a variety of plants. As is evident from this table, the young fresh plant yielded the highest percentage of the glucoside.

The physical and chemical properties of the isolated asperuloside were studied and Table XII offers a comparison of the melting points and specific rotations obtained by a number of investigators who isolated asperuloside from a variety of plants. Perhaps the most radical report was that of Briggs, who isolated asperuloside from several species of Coprosma.

Asperuloside is derived from an aglycon which to date has not been isolated in the free state. Trim and Hill (52, p. 318) proposed that the aglycon was a furan derivative on the basis of some chemical tests, and Briggs (8, p. 4182-4193) later performed a rather thorough study of the constitution of asperuloside. On the basis of his observations, Briggs proposed a chemical structure for asperuloside which is shown in Figure 9. Many of the chemical and physical properties reported by Briggs vary somewhat from those obtained in this



PROPOSED STRUCTURE FOR
ASPERULOSIDE
Figure 9

investigation or those obtained by other investigators. As is evident from Table XII, the melting point and specific rotation were higher than the figures obtained by other investigators. Trim and Hill have suggested the molecular formula $C_{17}H_{24}O_{11}$ for asperuloside while that proposed by Briggs was $C_{18}H_{24}O_{12}$.

Briggs has reported that asperuloside has an absorption peak at 234.5 mu. in the ultra violet while results obtained for asperuloside isolated from Galium triflorum indicate an absorption maximum at 238 mu.

Table XII.
A Comparison of the Specific Rotations and Melting Points for Asperuloside Obtained from Different Sources

Source	Specific Rotation (Hydrate)	Melting Point (Degrees C.)
Galium triflorum	-194.2 (23°)	127-129
Daphniphyllum macropodum (52, p. 311-312)	-196 (18°)	126-129
Rubia tinctorum (52, p. 311-312)		125-127
Coprosma tenuifolia (7, p. 3940-3943)	-198.6 (25°)	131-132
Asperula odorata (15, p. 1695-1697)	-195.5	126-127
Escallonia philippiana (43, p. 1643)	-195	125-129
Coprosma baueriana (19, p. 794)	-194.47	125-126

In this investigation the infrared spectrum of the asperuloside isolated from Galium triflorum was obtained using a nujol mull and later a potassium chloride pellet. A study of the spectrum showed that it was comparable to that obtained by Briggs (8, p. 4191). The absorption maxima reported by Briggs were as follows: 3497w, 3300m, 3165m, 1786m, 1748s, 1701s, 1661s, 1534w, 1508w, 1330w, 1282s, 1217m, 1185m, 1081s, 1059s, 1025s, 990s, 954m, 913m, 862m, 815m, 765w, 745m, and 727w (s = strong, m = medium, and w = weak).

Table XIII gives an interpretation of the infrared data obtained for the asperuloside isolated from Galium triflorum (3). The peaks reported in this table were those obtained using the potassium chloride pellet. From Table XIII it is apparent that two major points of difference from the work of Briggs is the possible presence of a terminal CH_2=CH_2 and the possibility of an aromatic ring. It must be remembered, however, that only a complete chemical study supplemented by infrared data would offer sufficient information to establish the presence of any specific functional group or to postulate a structure for the glucoside.

An infrared spectrum of the black polymer obtained when asperuloside is treated with acid was also obtained. The band at 1710 was also reported by Briggs, who collected infrared data on the polymer he obtained from the asperuloside isolated from Coprosma. The asperuloside spectrum showed three bands indicative of the carbonyl, namely 1742, 1700 and 1660. The polymer had only the 1700 band, which indicated that carbonyl was lost after treatment with acid.

Table XIII.
Interpretation of Infrared Data (Potassium Chloride Pellet)

Frequency	Indication
3350 Region	Hydroxyl Groups
3000	= C-H
2940	
2885	C-H, CH_3 or CH_2
1742	Possibly Lactone
1700	Carbonyl
1660	Perhaps C = C-C = O
1620	C = C Conjugated
1382 to	
1340	$C-CH_3$
1262	Possibly C = C-O(R) or (H)
1174	Hydroxyl Group
958	HC = CH Trans
915	CH = CH_2 Terminal
860 to	
675	Suggestive of the Aromatic Ring

It appears that the lactone group was lost, which would coincide with Brigg's observation.

The medium band at 2920, which was not reported by Briggs, may possibly be due to CH stretching of the CH_3 group in the polymer. Another band not reported previously was the weak 3080 band. This

may possibly be due to CH stretching on the aromatic unit in the polymer. The strong band at 3450 was also found by Briggs and is probably indicative of a hydroxyl group with hydrogen bonding.

To summarize, the infrared data for both asperuloside and its polymer are in reasonably close agreement with the observations of Briggs, but a more thorough study of the asperuloside obtained from Galium triflorum would be warranted. Because of differences noted in the physical constants, such as the melting point and specific rotation obtained by different investigations, it appears plausible to consider the possibility that more than one glycoside of the asperuloside type exists in the family Rubiaceae. Perhaps the differences in these physical constants could be explained by the fact that what is called asperuloside, is essentially a mixture of two or more glucosides closely related chemically, or that the various genera contain glucosides of the asperuloside type that differ to some degree chemically.

In support of the consideration that more than one glycoside of the asperuloside type exists was the observations of Trim and Hill (52, p. 316). They report the presence of a glycoside in the pulp of the unripe fruit of Genipa americana (family Rubiaceae) that gave a blue-violet reaction with primary amines which, according to their observations, was characteristic for asperuloside. This information, although quite inconclusive, does suggest the possibility that other glycosides chemically similar to asperuloside may exist.

The preliminary study concerning the effect of plant extracts on the blood pressure indicated that these extracts do significantly drop the blood pressure and that asperuloside could well have been the principle that initiated this response. Studies using the pure glucoside isolated from Galium triflorum showed that asperuloside did exert a depressor effect on the blood pressure of the rabbit. A similar effect has been obtained on cats (38). The fact that no depressor response could be obtained using the dog could very possibly be attributed to the small dose employed.

The depressor response observed with aqueous extracts of the dried plant gathered in the fall was much more pronounced in some cases than that obtained with the pure glucoside. Since it has been established that the asperuloside content of the plant gathered in the fall was nil, it is logical to assume that asperuloside is not the major principle to which this depressor effect can be attributed.

The limited supply of asperuloside prevented further studies relating to the pharmacology of this compound. Indications are, however, that it is a relatively non-toxic compound and is worthy of further consideration. It would be interesting to note the effects of asperuloside on hypertensive animals and to undertake a more complete pharmacological study of this glucoside.

SUMMARY

A histochemical study of the powdered aerial parts of Galium triflorum showed the presence of a non-glandular unicellular hair, crystals and raphides of calcium oxalate, and a characteristic stoma.

The composition of the ash of Galium triflorum was determined. The total ash, acid insoluble ash and water soluble ash figures were obtained using an incineration temperature of 477° C., which was employed throughout the ash analysis. Observations indicated that, when doing ash determinations with the intent of carrying out an ash analysis, incineration temperature is a critical factor. This temperature should be constant and maintained below 480° C. for plants high in potassium.

A partial proximate analysis was performed which showed a low total nitrogen (1.58 per cent). The fact that the material used for this determination was the aerial part of the fall gathered plant may in part explain the low nitrogen content. The sugar content and crude fiber were found to be high. The high crude fiber could possibly be explained by the fact that the plant was very stemy.

A selective extraction of Galium triflorum showed high alcohol and water extracts, which were 11.39 per cent and 15.24 per cent respectively. Examination of the water soluble extract indicated the presence of a mucilaginous principle and organic acids.

Coumarin was extracted from the dried plant by two methods and identified by melting point and mixed melting point. Vitamin

determinations indicated the presence of carotene in the dried plant and the absence of ascorbic acid and nicotinic acid in the fresh plant or amounts too small to be detected by the chemical procedure employed.

Asperuloside was extracted from the aerial parts of the spring gathered plant that was oven dried at 49° C., giving a yield of 0.12 per cent. Asperuloside could not be obtained from the fall gathered plant that was air dried. Indications are that the asperuloside content varies with the age of the plant and possibly the method of drying. Apparently the fresh young plant contains the highest percentage of this glucoside.

A study of the chemical and physical properties of asperuloside showed them to be in reasonable agreement with those reported by other investigators. Ultra violet and infrared data suggest that asperuloside is a highly unsaturated compound mainly in agreement with the proposed structure of Briggs. Enough difference in the physical properties exists to warrant consideration of the possibility that the Rubiaceae family could contain more than one glucoside of what may be called the asperuloside type.

Aqueous extracts of the dried aerial parts of the plant were shown to produce a depressor effect on the blood pressure of dogs. No definite effect on the blood pressure could be established for asperuloside but indications were that it is not the principal constituent of the plant responsible for the depressor effect.

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