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A PHYTOCHEMICAL STUDY OF MONTANA-GROWN
PYRETHRUM FLOWERS

by

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B.S., Montana State University, 1941

Presented in partial fulfillment of the
requirements for the degree of Master
of Science.

Montana State University

1947

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INTRODUCTION

Pyrethrum (Pyrethrum Flowers, Insect Flowers) "is the dried flower-head of Chrysanthemum cinerariaefolium (Trev.) Bocc., Chrysanthemum coccineum Willdenow (Chrysanthemum roseum Web, et Mohr) or of Chrysanthemum Marshallii Aschers (Fam. Compositae)."¹ In addition to the official definition, the National Formulary requires that Pyrethrum yield not less than 0.5 per cent of total pyrethrins (Pyrethrin I and Pyrethrin II).

The genus Chrysanthemum contains more than one hundred species, of which only a few in the section Pyrethrum are toxic to insects. Chrysanthemum (Pyrethrum) cinerariaefolium is the only species that is commercially important, the quantities of Chrysanthemum (Pyrethrum) roseum and Chrysanthemum Marshallii imported into this country being negligible.

To the casual observer, P. cinerariaefolium resembles the ordinary field daisy, C. leucanthemum; the two plants are, however, readily distinguishable.

P. cinerariaefolium is a glaucous perennial 18 to 24 inches high. The stems are unbranched and slightly hairy. The leaves are petioled and finely cut. The dried flower heads are hemispherical, each consisting of a short rounded receptacle; a straw-colored involucre composed of three rows of scales; a disk composed of numerous yellow flowers; a circle of white or cream-colored ray flowers. The outer involucre scales are lanceolate and have a pronounced keel their outer surface is light brown and hairy; the inner surface is smooth and lighter in color. The inner involucre scales are spatulate and longer than the outer ones. They are also lighter in color and have membranous margins. The disk florets are yellow, tubular, perfect and have a

five-lobed corolla born on the achene, which has five ribs and a toothed crown. The ray florets are ligulate, pistillate and the corolla is cream-colored or white; it is delicately veined and has three teeth at the tip. Commercial flowers vary from 6 - 24 mm in width and from 0.070 to 0.300 gram in weight.²

Powdered Pyrethrum cinerariaefolium from newly harvested flowers properly dried and ground has a bright yellow color; after standing for some time, or when ground from old or poorly cured flowers, it has a dull and browner color. The powder has a pleasant, characteristic odor which is more pronounced in the freshly prepared material; it is slightly sternutatory. The taste is at first acrid and bitter, followed by a numbing sensation on the tongue and lips. This numbness is caused by the active principles of the plant and is similar to that caused by aconite root, although less intense.³

The powder shows numerous T-shaped, non-glandular hairs from the involucrel bracts, the hairs usually broken but when entire consisting of a 2-celled stalk and a curved or twisted horizontal end cell, the latter tapering to a point at either end; numerous spherical, spinose pollen grains, up to about 30 microns in diameter and fragments of the outer epidermis of the involucrel scales with polygonal or wavy-walled epidermal cells and broadly elliptical stomata, the latter with three to four neighboring cells. These fragments also show the T-shaped, non-glandular hairs as well as sessile glandular hairs with 2-8-celled glandular heads. Fragments of achene and pistil tissue are numerous and show club-shaped sessile, glandular hairs, brownish resin canals, and small prisms of calcium oxalate. Fragments of achene tissue also show cells of the pericarp with thin, nearly colorless walls, rectangular cells of the seed-coat with thick, porous and strongly lignified walls and elongated resin secretion cells having a brownish amorphous content. Portions of the corolla show outer epidermal cells with a striated cuticle, occasional stomata, the latter surrounded by four to five neighboring cells and an inner epidermis with cells having the form of striated papillae and appearing polygonal in surface view. Fragments of the teeth of the tubular florets show rows of longitudinally elongated cells, many of which contain a rosette aggregate of calcium oxalate.

Fragments of vascular tissues from the scales and stem portions showing lignified sclerenchymatous cells and fibers are also present.⁴

Pyrethrum roseum, commonly known as "painted daisy,"⁵ is widely grown for its ornamental flowers. Its ray florets are pink, carmine, rose, crimson or white. This species blossoms earlier and less profusely than P. cinerariaefolium and is somewhat more resistant to disease and injury. Its dried flowers are easily distinguished from P. cinerariaefolium by the purple color of the ray florets, the ten-ribbed achenes and the brown margins of the involucreal scales. C. Marschallii resembles P. roseum. It is seldom seen in this country. These two species yield a powder darker than P. cinerariaefolium, which is the most important commercially of the three pyrethrums and is the species referred to throughout this work when the term pyrethrum, unmodified, is used.

The use of pyrethrum flowers for insecticidal purposes apparently originated in Persia.⁶ Great secrecy is said to have surrounded the early use and preparation of the material which was made from P. roseum and P. carneum, and this, no doubt, accounts in part for the difficulty of fixing the date of discovery of its activity. The powder was introduced into Europe early in the nineteenth century by an Armenian merchant named Juntikof,⁷ who discovered the secret of its preparation while traveling in the Caucasus. In 1840, a new species, P. cinerariaefolium, was produced in Dalmatia and rapidly superseded the Persian species in Europe. An interesting story traces the discovery of the effect of P. cinerariaefolium on insects to a

German woman of Dubrovnik, Dalmatia, who picked a bouquet of the flowers for their beauty. When they withered she threw them into a corner where, several weeks later, they were found surrounded by dead insects. She associated the death of the insects with the insecticidal property of the flowers and embarked in the business of manufacturing pyrethrum powder.

Pyrethrum powder was introduced into the United States about 1860; for some time no information was available as to its botanical source. Later, importations of the powder were almost entirely replaced by the whole flowers, which were then powdered in this country, thus preventing the addition of powdered stems -- which were added as an adulterant in the country of its origin. Consumption of pyrethrum increased from 600,000 pounds in 1885 to a peak of 16,126,000 pounds in 1935. In 1916 kerosene extracts of pyrethrum began replacing the powder for household purposes; nine years later, the use of the powder had nearly ceased.⁸

Until 1914 nearly all of the pyrethrum used in this country was imported from Dalmatia. World War I eliminated this source and enabled Japan to seize the market which she held until 1939, when Kenya became the principal supplier of pyrethrum to the United States.⁹

Pyrethrum has also been grown in France, India, Brazil; in the United States in California, Colorado,¹⁰ and Nebraska.¹¹

CHEMISTRY OF PYRETHRUM

The search for the active principle of pyrethrum began about the middle of the nineteenth century. A large number of investigators attacked the problem, but discovered but little more than was known about the toxic constituents in 1909 than had been discovered sixty years earlier. The toxicity was believed variously to be due to ethereal oil, santonin, a glycoside, an amine, a resin, a free volatile acid, or its esters.¹²

When every other attempt to solve the problem had failed, Staudinger and Ruzicka in 1924¹³ published a series of papers describing the isolation of the two active principles of pyrethrum and the determination of their chemical nature. These two active principles were shown to be esters. Staudinger and Ruzicka also isolated a ketone alcohol—the alcoholic portion of the active esters—to which they assigned the name pyrethrolone (Formula A). (Formulas are on page 8.) Pyrethrolone is a colorless, viscous, levorotatory, oily liquid, insoluble in water, but miscible with alcohol, ether and benzene.¹⁴

The acids of the ester, as isolated, were chrysanthemum monocarboxylic acid (B), and chrysanthemum dicarboxylic acid. Pyrethrolone, when esterified with chrysanthemum monocarboxylic acid, yielded an active ester, called by Staudinger and Ruzicka, Pyrethrin I (C). Pyrethrolone, when esterified with chrysanthemum dicarboxylic acid, did not yield an active ester, but when the monomethyl ester of chrysanthemum dicarboxylic was combined with the alcohol, the resulting compound proved to be almost as active as Pyrethrin I. This compound

was called Pyrethrin II (Z). Pyrethrin I is a viscous liquid, boiling at about 150° C. in a nearly absolute vacuum. Pyrethrin II, also a heavy liquid, decomposes when distilled under a low vacuum.

LaForge and Barthel¹⁵ have recently shown (1945) that pyretholone, formerly considered to be a homogeneous substance, is in reality a mixture of two structurally related compounds. The predominant (80%) constituent for which the name "pyretholone" has been retained is represented by Formula F. This compound exists as a mixture of the dextro and racemic forms.

The other constituent, containing one carbon atom and one double bond less and having one terminal methyl group more than does pyretholone, has been named "cinerolone" (G). From its chemical and physical properties and from its similarity to pyretholone, it has been assigned Formula G. This compound, present in lesser amount, also exists in the dextro and racemic forms. The formulas (H and I) of the two pyrethrins, as based on the structure suggested by LaForge and Barthel, are given on page 8.

According to these researchers, the proportions of the different pyrethrolones combined with chrysanthemum acids are unknown. The terms, "Pyrethrin I" and "Pyrethrin II," however, must henceforth be regarded as defining, not compounds, but groups characterized only according to the acid component, which are, in each case, esterified with more than one and probably with several pyrethrolones.

In addition to the chrysanthemum acids, Ripert has isolated the following acids from pyrethrum flowers: protocatechuic, isovaleric, caproic, lauric, palmitic, oleic and linoleic.¹⁶ Most of these compounds

occur in both the free and combined states.

Saturation of the alcohol side chain causes a marked drop in insecticidal effects,¹⁷ the deterioration during storage being due to this saturation.

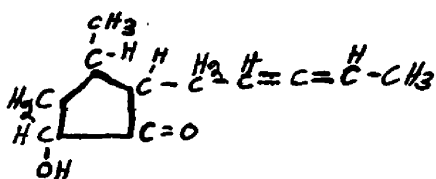
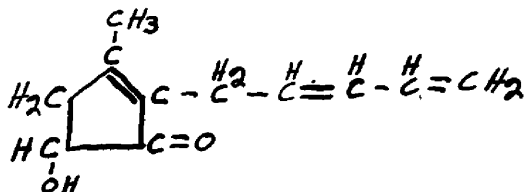
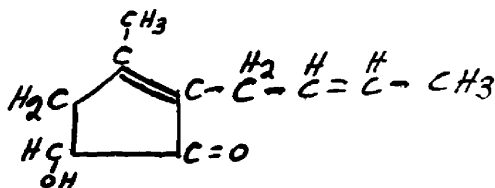
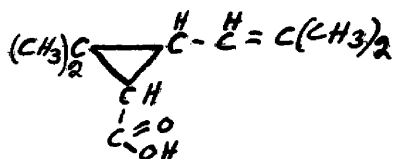
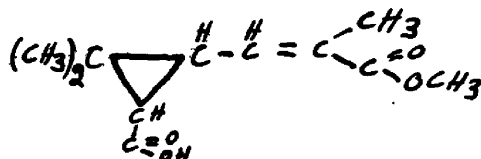
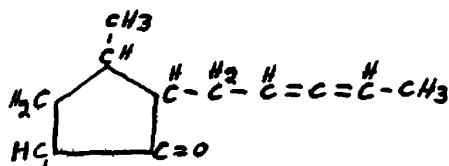
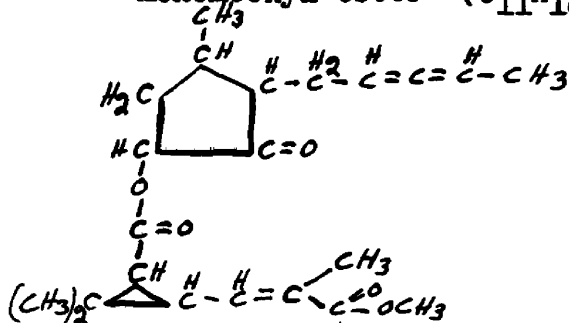
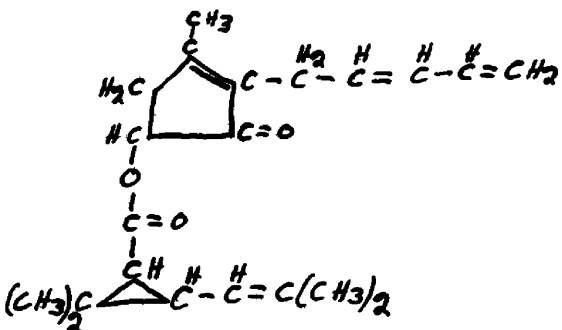
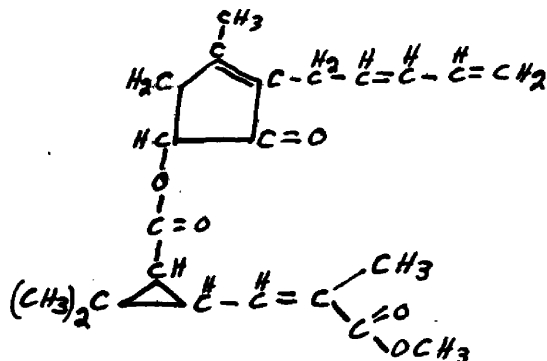
The pyrethrins are highly unsaturated, both in the acid and the alcohol parts of the molecule. The toxicity depends, not only on the composition, but also on the spatial arrangement of the atoms.

Synthetic derivatives of the pyrethrins have been prepared. Pyrethrolone has been esterified with 32 acids, including saturated aliphatic acids, unsaturated aliphatic acids, aromatic acids, acids of the terpene series, and other trimethylene carboxylic acids. Chrysanthemum monocarboxylic acid has been combined with 82 compounds, including alcohols, thio-alcohol, amines, phenols, aliphatic-aromatic alcohols, alicyclic compounds, terpenes, ketone-alcohols, cyclopentanone and cyclopentenolone derivatives.

A few of the 104 compounds synthesized have been found to be slightly active; none, however, approaches the pyrethrins in toxicity. Small changes either in the alcohol or in the acid part of the pyrethrin molecule lower the toxicity greatly.

STRUCTURAL FORMULAS OF PYRETHRINS AND RELATED COMPOUNDS

Figure A.

(A.) Pyrethrolone ($C_{11}H_{16}O_2$)
Staudinger and Ruzicka(F.) Pyrethrolone ($C_{11}H_{14}O_2$)
LaForge and Barthel(G.) Cinerolone ($C_{10}H_{14}O_2$)
LaForge and Barthel(B.) Chrysanthemum mono-
carboxylic acid ($C_{10}H_{16}O_2$)(D.) Chrysanthemum dicarboxylic acid
monomethyl ester ($C_{11}H_{16}O_4$)(C.) Pyrethrin I ($C_{21}H_{30}O_3 = 330.45$)
Staudinger and Ruzicka(E.) Pyrethrin II ($C_{22}H_{30}O_5 = 374.46$)
Staudinger and Ruzicka(H.) Pyrethrin I ($C_{21}H_{28}O_3 = 328.43$)
LaForge and Barthel(L.) Pyrethrin II ($C_{22}H_{28}O_5 = 372.44$)
LaForge and Barthel

CHEMICAL ASSAY OF PYRETHRUM

Great variation of insecticidal powers in samples of pyrethrum grown in various regions, as well as variations due to deterioration in storage, demand an efficient chemical method of evaluation.

Until 1929 no satisfactory method for determining the toxic principle was available. Earlier chemical methods of analysis were used solely for detection of adulterants or foreign materials. Physiological tests on insects, at that time, were inaccurate and unsatisfactory.

The identification of the pyrethrins by Staudinger and Ruzicka in 1924 opened the way for the development of chemical methods for determining the potency of the flowers.

The first chemical assay methods were based on the fact that the pyrethrins are esters and form insoluble semicarbazones. This led to the development of the acid and semicarbazone methods of analysis.¹⁸

The acid method involved extracting pyrethrum powder with petroleum ether, evaporating the petroleum ether, and extracting with methanol, which eliminated the resin and fat. The solution was then saponified and the chrysanthemum monocarboxylic acid was volatilized by distilling with steam. The acid in the distillate was then determined by titration with 0.1 N sodium hydroxide solution. The chrysanthemum dicarboxylic acid remained in the residue from the steam distillation. After extraction with ether, the acid was titrated with 0.1 N sodium hydroxide solution.

The semicarbazone analysis followed the above procedure up to the methanol extraction; here, instead of the saponification of the residue, the solvent was removed and the crude oil obtained was treated with a solution of semicarbazide hydrochloride. The nitrogen in the semicarbazones formed was determined by the Kjeldahl method. From the nitrogen content of the semicarbazones, the pyrethrin content was then calculated.

The semicarbazone method gave only total pyrethrins, a factor which was not as valuable an index to insecticidal effects as was the acid method, which gave the values for both Pyrethrin I and Pyrethrin II. Discordant results, obtained in assaying the same sample, proved the two methods unsatisfactory.

Tattersfield and associates¹⁹ slightly modified Staudinger's and Harder's acid method and also developed their own semicarbazone method. Tattersfield²⁰ also adapted Staudinger's and Harder's method as a micro-analysis.

Gnadinger and Corl²¹ perfected a method of analysis based on the fact that pyrethrins have the property of reducing alkaline cupric solutions. This is due to the presence of the ketone group in the alcoholic component of the esters. With the addition of Folin's phosphomolybdate reagent, a deep blue color is developed. Comparisons of the colors produced by known amounts of pyrethrins with the colors obtained with known amounts of dextrose, indicated that dextrose could be used as a standard.

Seil²² has modified the acid method, making it shorter and less cumbersome.

Haller and Acree²³ have established an assay for Pyrethrin II which makes use of the property that methyl esters yield methyl iodide quantitatively when treated with hydriodic acid. The methyl iodide is absorbed in a solution containing acetic acid, bromine and potassium acetate, which converts it into methyl bromide and iodic acid. The iodic acid is then treated with potassium iodide and the liberated iodine is titrated with 0.05 N sodium thiosulfate solution. (1 mol. pyrethrin II \rightarrow CH₃I \rightarrow HIO₃ \rightarrow 6I)

Pyrethrin II content, as determined by this methoxyl method, is significantly lower than the Pyrethrum II content determined by the Tattersfield and Seil acid methods.

Wilcoxon,²⁴ in a study of the determination of Pyrethrin I by the Seil method, prepared pure monocarboxylic acid. Weighed portions of the pure monocarboxylic acid were distilled with steam in the presence of sulfuric acid and the distillate was extracted with petroleum ether as in the Seil method. Titration of the petroleum ether extract showed only 69-71 per cent recovery of the weight taken. Wilcoxon concluded that the monocarboxylic acid is "either not entirely volatilized by steam or it is partly decomposed by heating in the presence of the sulfuric acid."

Pantsios²⁵ found that the destructive effect of steam distillation on the chrysanthemum monocarboxylic acid renders the acid methods

for determination of Pyrethrin I inaccurate and unreliable.

Holaday²⁶ found that there are two sources of error in the Seil method: (1) that there is incomplete recovery of the monocarboxylic acid in the steam distillation, and (2) that an appreciable portion of the acids titrated in the Seil method is not the monocarboxylic acid but a contaminating acid material soluble in petroleum ether. The error caused by the contaminating acids is directly opposite to that caused by the incomplete recovery of the monocarboxylic acid. The net result is a low Pyrethrin I report.

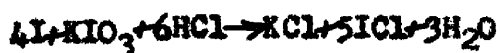
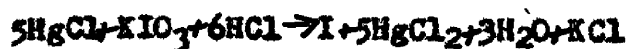
Wilcoxon next investigated the reaction of Denigès' reagent²⁷ (an acidic solution of mercuric sulfate) with chrysanthemum monocarboxylic acid.

The reaction is characterized by the formation of a series of striking colors, beginning with phenolphthalein red which gradually changes to purple, then to blue, and finally to bluish green.²⁸ The color changes are believed to be the result of the formation of a colloidal dispersion of metallic mercury, or of some mercury compound, which, on standing, undergoes spontaneous, successive increase in particle size, until a coarse blue suspension is formed.²⁹

This reaction was utilized by Wilcoxon as a means of determining the monocarboxylic acid, and thus, indirectly, Pyrethrin I.

The chrysanthemum monocarboxylic acid reduces the mercuric sulfate to mercurous mercury, which is precipitated as mercurous chloride by the addition of a saturated solution of sodium chloride.

The mercurous chloride is titrated with 0.01M potassium iodate solution and the iodine released in the reaction is titrated with the potassium iodate solution. The nature of the reaction is indicated by the following equations:



Holaday³⁰ obtained satisfactory results with Wilcoxon's mercury reduction method when applied to pyrethrum flowers, but the products of saponification in insecticidal sprays containing mineral oil, perfumes, and other substances interfered with the iodate titration by absorbing iodine, thus causing high results. Holaday removed these interfering substances (largely unsaturated organic compounds) by washing the precipitate of mercurous chloride with acetone and chloroform. This modification made the mercury reduction method satisfactory when applied to pyrethrum powder mixtures and to mineral oil pyrethrum extracts.

Another modification of the Wilcoxon mercury reduction method for Pyrethrin I determination was the addition of a temperature control. The revised method provides for a reduction temperature of $25^\circ\text{C} \pm 2^\circ$ for a period of one hour.

Green and Carter³¹ observed that the usual color changes obtained by the reaction of chrysanthemum monocarboxylic acid do not take place when this reaction occurs in bright sunlight. The solution becomes reddish purple, as usual, but the color immediately begins to fade and, in about 5 minutes, becomes pale yellow, and finally colorless.

A small amount of tan precipitate then is formed. Some restoration of color is obtained by taking the solution from the direct sunlight and leaving it in subdued light. Other sources of light, such as a mercury-vapor lamp, a fluorescent lamp, and an incandescent bulb, all caused fading. By analysis, an increased amount of mercury reduced to the mercurous state was found.

LaForge and Acree³² devised a method for the quantitative determination of pyrethrins based on cleavage on hydrogenation. On cleavage, Pyrethrin I yields hexahydropyrethronone and the dihydro derivative of chrysanthemum monocarboxylic acid; Pyrethrin II yields hexahydropyrethronone and chrysanthemum dicarboxylic acid monomethyl ester. Dihydro chrysanthemum monocarboxylic acid is volatile with steam and is thus separated from the practically non-volatile chrysanthemum dicarboxylic acid monomethyl ester, which is insoluble in water and soluble in ether or organic solvents.

The Association of Official Agricultural Chemists³³ adopted the Wilcoxon-Holaday mercury reduction method in 1939 as an "Official Method" for the determination of Pyrethrin I in pyrethrum flowers and as a "Tentative Method" for Pyrethrin I in mineral oil extracts. A slight modification of Seil's method was adopted as a "Tentative Method" for Pyrethrin II in pyrethrum flowers, but no method was adopted for Pyrethrin II in mineral oil extracts.

Since the National Formulary uses the mercury reduction method for Pyrethrin I and a modification of the Seil method for determination of Pyrethrin II, it will be quoted directly.

Extract about 15 Gm. of Pyrethrum in fine powder, accurately weighed, in a Soxhlet or other continuous extraction apparatus for 7 hours with petroleum benzin. Evaporate the petroleum benzin on a water bath, heating no longer than is necessary to remove the solvent. Do not pass a current of air through the flask during evaporation. Add 20 cc. of 0.5 N alcoholic sodium hydroxide to the flask containing the extract; connect it to a reflux condenser and boil gently for 1 hour to 1 hour and 30 minutes. Transfer the contents of the flask to a 600 cc. beaker and add sufficient water to bring the volume to 200 cc. Add a few glass beads and boil gently until the volume is about 150 cc. Transfer to a 250-cc. volumetric flask and add 1 Gm. of purified siliceous earth and 10 cc. of barium chloride solution (1 in 10). Do not shake before diluting to volume. Add sufficient water to make the volume 250 cc., mix thoroughly and filter, collecting exactly 200 cc. of the filtrate. Neutralize the filtrate with dilute sulfuric acid (1 in 5) using 1 drop of phenolphthalein T. S. as the indicator, and add 1 cc. of the acid in excess. (If it is necessary to have the solution stand overnight at this point, it should be kept in the alkaline condition.) Filter through a 7 cm. filter paper that has been coated lightly with a suspension of purified siliceous earth in water, on a Buchner funnel and wash several times with water. Transfer the filtrate and washings to a 500-cc. separatory funnel and extract with two 50-cc. portions of petroleum benzin. Wash the combined petroleum extracts with two or three 10-cc. portions of water, retaining the washings. Filter the petroleum benzin extract through cotton into a 250-cc. separatory funnel, washing the cotton with 5 cc. of petroleum benzin. Combine the aqueous washings with the acid-aqueous solution and retain for the assay of Pyrethrin II. Extract the combined petroleum benzin solution with 5 cc. of 0.1 N sodium hydroxide, shaking vigorously. Draw off the aqueous layer into a 100-cc. beaker; wash the petroleum benzin with 5 cc. of water or with an additional 5-cc. portion of 0.1 N sodium hydroxide and add this to the beaker. Add 10 cc. of mercuric sulfate T.S. and allow to stand for 1 hour at $25^{\circ} \pm 2^{\circ}$. Add 20 cc. of alcohol and 3 cc. of a saturated solution of sodium chloride. Warm to 60° and filter through a small filter paper, transferring all of the precipitate to the filter paper, and wash with two 10-cc. portions of hot alcohol. Wash with two 10-cc. portions of hot chloroform and transfer the filter paper and contents to a 250-cc. glass-stoppered Erlenmeyer flask. Add 30 cc. of hydrochloric acid and 20 cc. of water to the flask and allow to cool. Add 6 cc. of chloroform or carbon tetrachloride and 1 cc. of iodine monochloride T. S. and titrate with 0.01 M potassium iodate, shaking vigorously after each addition, until there is no iodine color in the chloroform (or carbon

tetrachloride) layer. Each cc. of 0.01 M potassium iodate is equivalent to 0.0057 Gm. of Pyrethrin I. If necessary, filter the aqueous residue from the petroleum benzine extraction above through a Gooch crucible and concentrate the filtrate to about 50 cc. Transfer to a separatory funnel and neutralize with sodium bicarbonate. Extract twice with chloroform, washing the chloroform extract with about 15-cc. portions of water in each of 2 separatory funnels. Combine the aqueous solution and washings, acidify strongly with hydrochloric acid (about 8cc.), saturate with sodium chloride adding cautiously at first to prevent excessive ebullition of carbon dioxide and extract with 50 cc. of ethyl ether. Draw off the aqueous layer into a second separatory funnel and extract with 50 cc. of ether. Continue this extraction and drawing off of the aqueous layer using 35 cc. of ether for the third and fourth extractions. Wash the 4 ether extracts successively with 10-cc. portions of water, and repeat with a second successive washing with another 10-cc. portion of water. Combine the ether extracts, draw off any water that separates and filter through cotton into a 500-cc. Erlenmeyer flask. Evaporate the ether on a water bath and dry the residue for 10 minutes at 100°. Add 2 cc. of neutralized alcohol and 20 cc. of water and heat to dissolve the residue. Cool, filter through a Gooch crucible and add 1 or 2 drops of phenolphthalein T. S. and titrate with 0.02 N sodium hydroxide. Each cc. of 0.02 N sodium hydroxide is equivalent to 0.00374 Gm. of Pyrethrin II.³⁴

The results of the chemical determination of pyrethrin content by different methods do not check very well, although improvements have been made rather rapidly in the last decade. Also, since the recent discovery of cinerolone, there appear to be, not two, but four pyrethrins corresponding to the four combinations of two acids and two alcohols.³⁵ This complicates the present chemical assays which are based on the presence of only two pyrethrins. The present chemical methods of determining pyrethrin content are relatively long and complicated with many chances for error.

For these reasons, there exists a need for further investigation of pyrethrum assay methods.

BIOLOGICAL METHODS FOR EVALUATING PYRETHRUM

The first biological tests with pyrethrum were made by the ~~method~~ of its toxicity to insects. Early workers used flies, tadpoles, roaches, aphids, mosquito larvae, goldfish, bees, con-weevils, frogs, silkworms, ticks, and the blunt-nosed leaf-hopper.³⁶ A method, based on the action of pyrethrins on isolated rabbit intestine, has been used. Most of these methods involve the per cent of kill or the time required to paralyze the insect.

When kerosene extracts of pyrethrum were introduced commercially as household insecticides in 1919, methods were sought for testing their efficiency. The most obvious test consisted in releasing a few flies in a small room and then spraying a definite amount of kerosene extract. This method is known as the Peet-Grady Method and was adopted as an official method by the National Association of Insecticide and Disinfectant Manufacturers in 1932. The Peet-Grady method more nearly approximates the actual conditions under which household insecticides are used than any of the other biological methods. There are, however, many sources of error, such as the varied resistance of the fly and the quantity of spray actually reaching the fly.

The biological method of assaying pyrethrum is inherently less accurate than the chemical assay due to the fact that in conducting biological tests, the operator is subject not only to the errors in manipulation, but also to the variability of the materials

with which he works. In quantitative chemical analyses, the ability of the analyst to obtain concordant results is largely determined by his skill in avoiding errors in following the procedure.

Despite the fact that the value of biological assay is inherently less accurate than that of the chemical assay, the value of any chemical method for assaying pyrethrum must be based on comparison with toxicity tests on insects.

While insecticides containing only pyrethrins as active principles can be rather accurately assayed chemically, mixtures of pyrethrum with other active principles or with synergists can usually be evaluated only by means of biological tests.

TOXICOLOGY OF PYRETHRUM

The action of pyrethrum upon insects seems to be that of a neuromuscular poison and paralytic agent.³⁷ Hartzall and Scudder³⁸

clumping of the chromatin of the nucleus in the central nervous system of the housefly, Musca domestica.

Pyrethrum is both a contact and a stomach poison, but the contact effect is so dominant that insects are often incapacitated before they are able to eat it.

Pyrethrum powder was tried in the laboratory on flies which were imprisoned beneath a beaker. Approximately a gram of powder was introduced into the beaker. The flies rubbed the mandibles with their antenna, collapsed shortly afterward on their back and died within 10 minutes.

STATEMENT OF THE PROBLEM

This investigation of Montana-grown pyrethrum is divided into two parts.

The first phase involves a chemical study with an accurate determination of the pyrethrin content of the flowers using the National Formulary Method of analysis.

An extensive review of the literature indicated that nothing had been published regarding the adsorption of pyrethrum extracts on a chromatographic column. This, therefore, constitutes the second phase of the problem. This phase is concerned with the possibility of the separation of a pyrethrum extract mixture on a chromatographic column into different colored bands, with the subsequent identification, estimation, and isolation of the component parts (especially pyrethrins) of the extract.

The usual chromatographic procedure³⁹ consists of the following operations:

1. Preparing the column by compression of the adsorbing medium.
2. Pouring on the solution under investigation.
3. Developing the finished chromatograph by addition of a solvent.
4. Expressing the column from the tube.
5. Cutting up the column according to the layers formed.
6. Eluting the separate portions of the column.
7. Separating by filtration of the empty adsorbent, now in powder form.

Chromatography is, theoretically, a matter of selection based on the adsorption affinities of several substances showing different degrees of activity to the same adsorbent in a common solution.⁴⁰

The individual components form layers in a descending order, corresponding with the diminution in their surface activities.

According to Martin,⁴¹ chromatography bears the same relationship to simple methods of separation by adsorption in which the adsorbent is merely stirred with the solution, as distillation with a fractionating column does to simple distillation.

EXPERIMENTAL

The material used was the flowers of Chrysanthemum roseum and Chrysanthemum cinerariaefolium, grown in the drug garden of the School of Pharmacy, Montana State University. The flowers from the 1946 and 1947 crops were used.

The flowers, harvested in June, were hand-picked. Since the then available literature indicated the pyrethrin content to be highest just before the opening of the flowers, the 1946 crop was picked with this objective in view. It was not until 1947, with the receipt of later information that the open flowers have the highest pyrethrin content,⁴² that a proper harvest of the flowers was made. Accordingly, the open flowers were gathered.

The flowers, in each case, were air dried in the shade, the 1946 flowers being dried about three months before being powdered. The 1947 flowers were dried ten days before being powdered.

These flowers were reduced to coarse powders by grinding them in an electrical mill, care being taken that the temperature did not rise enough to cause any deterioration of the pyrethrins. The powdered flowers were passed through a #40 standard mesh sieve. Approximately twenty per cent did not pass through the sieve and was incorporated with the finer powder as such. The powder was then thoroughly mixed and stored in tightly stoppered bottles in a cool, dark place.

The 1947 powder was used for the determination of the various values as listed in the following tables. All determinations were made with original samples of the air-dried powder.



**Fig. 1. A field of Pyrethrum growing in the Drug Garden,
School of Pharmacy, Montana State University.**

TABLE I

Ash and Extractive Determinations of C. cinerariaefolium and C. roseum

	Sample	P. Cinerariaefolium	P. Roseum
Moisture	Sample 1.	8.76%	7.40%
	Sample 2.	8.70%	7.73%
Total Ash	Sample 1.	6.33%	7.91%
	Sample 2.	6.39%	7.81%
Acid-insoluble ash	Sample 1.	0.52%	0.43%
	Sample 2.	0.58%	0.82%
Petroleum Benzinsoluble Extractive	Sample 1.	6.42%	2.53%
	Sample 2.	6.04%	2.74%
Non-volatile Ethersoluble Extractive	Sample 1.	8.20%	4.54%
	Sample 2.	8.23%	4.70%
Volatile Ethersoluble Extractive	Sample 1.	2.66%	1.33%
	Sample 2.	2.25%	1.24%
Total Ether-soluble Extractive	Sample 1.	10.86%	5.87%
	Sample 2.	10.48%	5.94%

The pyrethrin content was determined by using the method official in the National Formulary. The values obtained are shown in Table II on the following page.

TABLE II
Pyrethrin Content of C. cinerariaefolium and C. roseum

Species	Year	Sample	% Pyrethrin I	% Pyrethrin II	% Total Pyrethrins
C. Cinerariaefolium	1946	1.	0.28%	0.35%	0.63%
		2.	0.28%	0.37%	0.65%
		3.	0.28%	0.34%	0.62%
		Average	0.28%	0.35%	0.63
C. Cinerariaefolium	1947	1.	0.80%	0.52%	1.32%
		2.	0.86%	0.56%	1.42%
		3.	0.81%	0.63%	1.44%
		4.	0.76%	0.60%	1.36%
		Average	0.81%	0.58%	1.39%
C. Roseum	1946	1.	0.04%	0.18%	0.22%
		2.	0.04%	0.17%	0.21%
		Average	0.04%	0.175%	0.215%

The pyrethrin content of the 1947 crop of C. cinerariaefolium was much higher than the other year, a fact which was probably due to the 1947 crop being assayed within three weeks after harvesting. The 1946 C. cinerariaefolium was assayed one year after harvesting. The low assay results of C. roseum agree with the literature.

It is evident that considerable deterioration takes place in pyrethrum flowers which have been in storage for a considerable time.

Another factor which may partly account for the low pyrethrin content of the 1946 cinerariaefolium may be the fact that the plants were only recently transplanted to their present location.

Chromatographic Experimental Work

Preliminary trials, using many different substances as adsorbents, were run. In theory, all substances in powder or finely divided form can act as adsorbents, as also can fibres, provided they are not soluble in the solvent used and that they have no destructive action on the compound to be adsorbed. In practice, however, there is generally a much narrower choice since many organic compounds have small quantities of soluble substances which are difficult to remove and since most organic compounds are unsuitable because they can only be dried with care and cannot be regenerated with ignition. Strongly acid or strongly basic inorganic adsorbents can be ruled out; dark or highly colored substances can not usually be used, for the sequence of layers on them would be difficult to establish.

A petroleum ether extract of C. cinerariaefolium was made by percolating 420 grams of the flowers with petroleum ether (boiling point 30° - 60°C.), as the menstruum. The extract was slowly evaporated to a volume of 200 cc.

The chromatographic apparatus used was very simple, the column consisting of a glass tube with a rubber stopper fitted around its base

to make it possible to insert the tube in a filter flask and to apply gentle suction with a water pump. A cork was bored and inserted in the neck of the tube. A layer of glass wool was then packed on the cork.

The tube was now ready for packing with the adsorbent, the most important item of the procedure. Twenty to thirty minutes were required to properly fill the tube. The adsorbent was divided into about twenty portions, the first portion about twice the size of the other portions. After each addition of powder, the column was pressed down by tapping, a procedure which was done by using a glass rod. This glass rod had one end flattened to about three-fourths the area of the tube. Each addition of adsorbent required about fifteen to twenty taps from a height of 3 to 6 cm. From one-third to one-fifth of the tube volume was left free for the solution. A circle of filter paper was put on the column to protect the surface when the solution was added. Just before the experiment this was moistened with the solvent.

The size of the tube was not selected according to any pre-conceived plan. When the formation of separate zones is of no consequence, a short wide tube is usually recommended. When the material to be worked up has many components, of which some are present only in small proportions, it may be necessary to use a narrow tube, since the zones may be too thin to be separated from one another. On some occasions, a first separation may be carried out in an apparatus of large capacity and a resulting fraction further split up in a narrow

tube for the final analysis. The tubes used in this experiment varied in diameter from $1\frac{1}{2}$ to 4 cm. and in length from 30 to 60 cm.

Powdered sucrose proved unsatisfactory as an adsorbent giving no well-defined bands. Fisher's adsorption alumina for chromatographic analysis proved too active. All the constituents of the petroleum ether extract remained in an orange-colored zone located near the top of the column. Addition of pure petroleum ether did not develop the column, the colored band remaining in the same position. Lloyd's Reagent acted similarly. Sargent's activated alumina was also tried and found unsatisfactory.

Celite No. 505 (a hydrated aluminum silicate product of John's-Marville), did not give distinctive bands. Calcium oxide, calcium hydroxide, calcium carbonate, and magnesium carbonate were tried and found unsatisfactory.

Heavy magnesium oxide of the ordinary commercial grade was found to furnish the most well-defined bands. Three definite zones were observed when a concentrated petroleum ether extract of C. cinerariaefolium of approximately two and one-half times native plant strength was used. The top band, which contained the components with highest adsorption-affinity, was greenish-yellow in color. The next lower zone was orange-yellow in color and contained the components with the next highest adsorption-affinity. In a few cases a very small, pinkish-colored band was observed. This band always disappeared within 30 minutes and was never present in the extruded column. The third zone was the colorless bottom band.

The moist column was extruded by holding the tube horizontally over plain paper and inserting into the tube an empty wooden thermometer case. One end of the case was pressed against the body and pressure exerted on the tube. Smaller columns were loosened by letting the tube fall several times from a height of 1 to 2 cm. onto a cloth. The column usually moved slowly toward the end of the tube and was then easily pushed out.

Cutting the column required careful procedure. A spatula was used and unloaded portions of the column were first removed. The separated large portions were scraped free of the white adsorbent. The scraping was done at an acute angle to the axis.

The individual homogeneous portions of the column were coarsely broken up with the spatula and then immediately dropped into the eluent, which was at hand. The eluent, in this case, was petroleum ether. The mixture was stirred and filtered, and the filter-cake washed.

The next part of the problem was to determine in which of these three layers the pyrethrins were located. In order to do this, a qualitative test for Pyrethrin I, using the color reaction with Denigès' reagent, was employed. This test involved the evaporation of a petroleum ether extract of pyrethrins to dryness, refluxing the residue with alcoholic sodium hydroxide solution to hydrolyze the esters into the acids and alcohols. Interfering plant principles were removed with barium chloride solution and the chrysanthemum monocarboxylic acid was eliminated by extraction with petroleum ether. A dilute sodium hydroxide

solution was used to extract the acid into water solution. Deniges' reagent was then added and, if as little as one milligram of monocarboxylic acid was present, a color would be seen. With five milligrams a definite color reaction took place, starting with a pink and progressing to bluish-green.

By use of this chromatographic procedure and the Deniges' qualitative test for Pyrethrin I, a slight pink color was observed in the eluents obtained from the yellow and the white colored bands. The pink color faded in each case within three minutes, a reaction which indicated the presence of only traces of chrysanthemum monocarboxylic acid. No reaction was obtained in the eluent from the top green layer. This was repeated with similar results, except that the temporary color reaction in this case was only obtained from the orange yellow band. This color persisted for eight minutes.

There could be only two reasons for the non-recovery of the pyrethrins from the column. One possibility was that the pyrethrins could have been decomposed by the action of the magnesium oxide. The other possibility was that the pyrethrins were so strongly adsorbed by the heavy magnesium oxide that the simple process of elution was not sufficient to extract them from the adsorbent.

To eliminate the latter reason, the three different color zones were put into different thimbles and subjected to continuous extraction in a Soxhlet extractor for a period of seven hours with petroleum ether as a solvent. The qualitative test was then again performed. This test

now showed a very highly colored reaction with the orange yellow zone, with the formation of large quantities of a bluish-green precipitate. The other two bands showed a slight color reaction indicating only traces of pyrethrins.

Next, a quantitative determination of the amount of pyrethrins in each color band was undertaken.

An extract was prepared by subjecting 492.5 grams of P. cinerariae-folium (1946 crop), to twenty hours of continuous extraction in a Fredericks Extractor with petroleum ether, as a solvent (boiling range 30° to 60° C.). The resulting solution was carefully evaporated to exactly 200 cc. This extract assayed 0.007360 grams of Pyrethrin I per cc. and 0.006614 grams of Pyrethrin II per cc.

A chromatographic column of heavy magnesium oxide was prepared in the manner described and, by the use of a volumetric pipette, exactly thirty cc. of the extract was introduced onto the adsorbent column. The column was developed, extruded, and the three different color zones divided. Each of these powdered zones were now subjected to the National Formulary method of assay for pyrethrum.

Another column was prepared using 27.1 cc. of the extract and the three zones in this were in turn analyzed. The results are given in the following table:

TABLE III

Analysis for Pyrethrins of Different Color Chromatographic Bands

Color band		% of Pyrethrin I present in original sample	% of Pyrethrin II present in original sample
Green band (Top)	No. 1 Chromatograph	4.91%	9.38%
	No. 2 Chromatograph	2.60%	9.13%
Orange-Yellow Band (Middle)	No. 1 Chromatograph	47.50%	58.50%
	No. 2 Chromatograph	53.36%	59.44%
White band (Bottom)	No. 1 Chromatograph	12.13%	1.65%
	No. 2 Chromatograph	10.07%	2.22%
% of Recovery of total Pyrethrins	No. 1 Chromatograph	64.54%	69.53%
	No. 2 Chromatograph	66.03%	70.69%

Another extract was prepared by the continuous extraction of 800 grams of *P. cinerariaefolium* (1947 crop) in a Fredericks Extractor. The resultant solution was evaporated to a volume of 500 cc. and stored in the refrigerator. The resultant extract assayed 0.013744 grams Pyrethrin I per cc. and 0.007658 grams Pyrethrin II per cc.

Two magnesium oxide chromatographic columns, using 30 cc. of this new extract, were set up and run. The magnesium oxide was dried for four hours at 110°C. before use. The color bands obtained were assayed as before, the only difference being that the extraction from the adsorbent was lengthened from seven hours in a Soxhlet to 20 hours in the continuous extraction apparatus.

The results obtained are given in the following table.

TABLE IV

Analysis for Pyrethrins of Different Color Chromatographic Bands Using 20 Hours of Continuous Extraction

Color Band		% of Pyrethrin I present in original sample	% of Pyrethrin II present in original sample
Green Band (Top)	No. 1 Chromatograph	20.47%	23.46%
	No. 2 Chromatograph	21.05%	21.39%
Orange-Yellow Band (Middle)	No. 1 Chromatograph	65.19%	70.31%
	No. 2 Chromatograph	66.30%	64.57%
White Band (Bottom)	No. 1 Chromatograph	0.85%	0.79%
	No. 2 Chromatograph	0.92%	0.53%
% of Recovery of total Pyrethrins	No. 1 Chromatograph	86.43%	94.56%
	No. 2 Chromatograph	88.27%	86.49%

These tables apparently show the bulk of the pyrethrins to be concentrated in the orange-yellow band. Pyrethrin I was present in the white band in a higher concentration than is Pyrethrin II. The greenish colored band contained a higher percentage of Pyrethrin II than Pyrethrin I. The recovery from the column of the total pyrethrins present in the original solution was approximately 67 per cent. Lengthening the extraction of the adsorbent from 7 to 20 hours yielded about 20% higher recovery of the pyrethrins.

Perfect separation of the different zones was difficult to obtain, due to the fact that the bands were shaped in the form of a meniscus. The colors were deeper on the outside of the column and extended into the

next zone.

The chromatographic columns were examined under ultraviolet light, but showed no distinctive bands.

An adsorbent column composed of one per cent mercuric sulfate and 99 per cent heavy magnesium oxide was prepared and run, using the 1947 P. cinerariaefolium extract. It was hoped that possibly Deniges' color reaction would take place in the column. The column was developed with 0.5 per cent alcoholic sodium hydroxide in an effort to hydrolyze the ester and form the chrysanthemum monocarboxylic acid. The resulting column differed in no way from the others.

In an effort to attain further concentration the orange-yellow band containing the bulk of the pyrethrins was extracted and run through another chromatographic column. No distinctive layers were obtained, the entire column assuming a homogeneous bright yellow color.

SUMMARY AND CONCLUSIONS

Montana-grown P. cinerariaefolium was found to contain a high percentage of total pyrethrins. The Pyrethrin I to Pyrethrin II ratio was approximately 1.3 : 1, a favorable ratio.

Pyrethrum which was stored one year assayed one-half the pyrethrin content of the freshly harvested flowers.

The pyrethrin content of P. roseum was approximately one-third that of the cinerariaefolium.

Pyrethrum extracts were found to distribute themselves on a chromatographic column into three distinct zones. The pyrethrins are largely concentrated in the highly-colored orange-yellow zone.

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