[CONTRIBUTION FROM THE RESEARCH LABORATORY OF MCLAUGHLIN GORMLEY KING COMPANY]

STUDIES ON PYRETHRUM FLOWERS. I. THE QUANTITATIVE DETERMINATION OF THE ACTIVE PRINCIPLES

BY C. B. GNADINGER AND C. S. CORL

RECEIVED APRIL 26, 1929 PUBLISHED OCTOBER 5, 1929

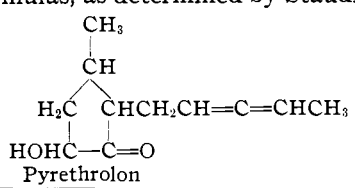
Although the insecticidal properties of *Pyrethrum* flowers have been known for more than a century, no satisfactory method has existed for the determination of the toxic principles. During the last five years the use of *Pyrethrum* has grown enormously, because of the development of new types of liquid insecticides, and the need for a method for evaluating the flowers has increased correspondingly. Of the methods in use prior to 1927,[1] only the physiological tests on insects give results that indicate the comparative value of different samples. These tests are difficult to conduct under constant conditions and are, at best, inaccurate. The chemical methods, involving the determination of ash constituents, ether-extract, nitrogen, etc., afford no idea of the amount of toxic principles present.

Staudinger and Harder[2] have published a method that is an adaptation of the process originally used by Staudinger and Ruzicka,[3] for the isolation of the active principles of *Pyrethrum*. This method is long, tedious and difficult to conduct quantitatively, requiring 500 g. of sample for a single determination.

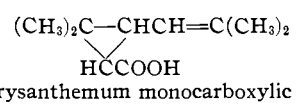
The purpose of this paper is to describe an accurate and comparatively rapid chemical method for determining the percentage of active principles in *Pyrethrum* flowers.

Experimental Part

Preparation of the Pure Active Principles.—In 1916 Staudinger and Ruzicka[4] isolated the toxic principles of *Pyrethrum* and definitely established their composition and structure. The two toxic substances, which they named pyrethrin I and pyrethrin II, were shown to be esters of the ketone-alcohol pyrethrolon with two acids, chrysanthemum monocarboxylic acid and chrysanthemum dicarboxylic acid methyl ester. Since these compounds will be referred to frequently throughout this paper, their structural formulas, as determined by Staudinger and Ruzicka, are given herewith



Pyrethrolon



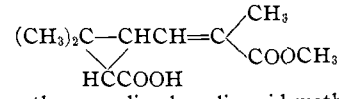
Chrysanthemum monocarboxylic acid

[1] "Insect Powder," U. S. Department of Agriculture Bulletin No. 824 revised.

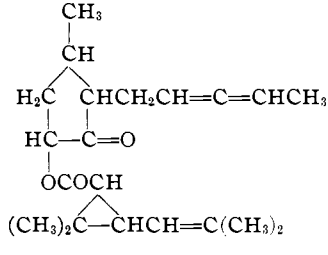
[2] Staudinger and Harder, *Ann. Acad. Sci. Fennicae*, **29A**, 1–14 (1927).

[3] Staudinger and Ruzicka, *Helv. Chim. Acta*, **7**, 177–201 (1924).

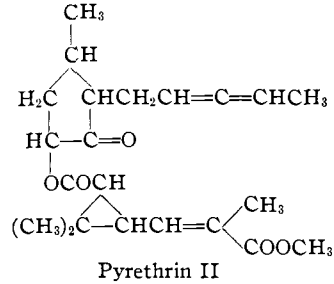
[4] Staudinger and Ruzicka, *ibid.*, **7**, 177–259, 377–458 (1924).



Chrysanthemum dicarboxylic acid methyl ester



Pyrethrin I



Pyrethrin II

By using a modification of the method of Staudinger and Ruzicka, quantities of nearly pure pyrethrin I and pyrethrin II were prepared from the best quality of Japanese *Pyrethrum* (*Chrysanthemum cinerariaefolium* Bocc.). For this purpose 680 kg. of the flowers was ground to 30-mesh and extracted with 3800 liters of acetone. The acetone was removed by distillation *in vacuo*, and there remained 90 kg. of oleo-resin of *Pyrethrum*. This material was worked up in small portions because of the difficulty of handling the large volumes of solvents required in the process. For example, the last portion was treated in the following manner: 6.5 kg. of the oleo-resin (corresponding to 49 1 kg. of flowers) was dissolved in 10 liters of acetone and mixed with 15 liters of low-boiling petroleum ether. A large amount of gummy material separated and it was washed with 8 liters of ether and then with 8 liters of benzene. The insoluble residue, which weighed 3120 g., contained only 2.96 g. of pyrethrins and was discarded. The mixture of solvents, containing the main portion of pyrethrins, was distilled *in vacuo* to 4 liters and was poured into 16 liters of petroleum ether. A further separation of green, semi-solid material occurred; it weighed 1680 g., contained 77.76 g. of pyrethrins and was discarded. The petroleum ether solution was distilled *in vacuo* and the residue was poured into 4 liters of methanol. The fatty material that separated was washed four times with 1-liter portions of methanol. The methanol-insoluble fat weighed 640 g. and contained 5.60 g. of pyrethrins. The methanol solution was chilled at $-7°$ until no further precipitation took place and the fatty precipitate weighing 90 g. was filtered off; it contained 9.74 g. of pyrethrins. The methanol solution was distilled *in vacuo* to a volume of 2500 cc. and sufficient water was added to make the alcohol content 88%. The solution was chilled overnight and the fatty material that separated was washed three times with 1-liter portions of 90% methanol. This insoluble fat weighed 260 g. and contained 13.31 g. of pyrethrins. All of the fatty residues were discarded. The methanol solution was distilled *in vacuo* and the residue was dissolved in petroleum ether. The petroleum ether solution was filtered from the 149 g. of insoluble material that contained only a trace of pyrethrins and was distilled *in vacuo*. The oily residue from the petroleum ether distillation weighed 548 g. and contained 304 g. of pyrethrins. In addition to the 304 g. of pyrethrins recovered in impure condition, 109 g. was lost in the process. Thus the total pyrethrin content of the 49.1 kg. of flowers was 413 g., or 0.84%. Subsequently, analysis of the same lot of flowers showed a pyrethrin content of 0.87%.

The oil containing 55.5% of pyrethrins was further purified by treatment with sodium carbonate solution and basic lead acetate. The pyrethrins were then converted into their semicarbazones by the method described by Staudinger and Ruzicka;[5] 288

[5] *Cf.* ref. 4, p. 190.

g. of the oil yielded 220 g. of the impure, mixed semicarbazones of pyrethrin I and pyrethrin II.

The semicarbazone of pyrethrin I was separated in nearly pure condition (m. p. 117–119°) by repeated crystallization of the crude mixture from benzene and alcohol. The pure semicarbazone of pyrethrin II could not be isolated, but after fifty-one crystallizations from benzene, alcohol and mixtures of benzene and petroleum ether, a semicarbazone was obtained that melted at 56–59°. Both semicarbazones crystallized from benzene in white needles. A large amount of mixed pyrethrin semicarbazones melting at 70–90° was also obtained.

By heating the semicarbazones with oxalic acid solution,[6] they were converted into the corresponding pyrethrins. The crude pyrethrins were extracted from the oxalic acid solution with petroleum ether and washed free from acids with 1% sodium hydroxide solution. Further purification was effected by washing with 3% chromic acid solution and 3% potassium permanganate solution, which did not attack the pyrethrins appreciably. The petroleum ether was then removed *in vacuo* and the residue was dissolved in alcohol, whereby a small amount of insoluble matter was precipitated. The alcohol solution was filtered and distilled *in vacuo*, the residue was dissolved in petroleum ether and the solution was filtered and distilled *in vacuo* at a maximum temperature of 55° until constant weight was attained. Ten grams of semicarbazone of pyrethrin I (m. p. 117–119°) yielded 3.53 g. of pure pyrethrin I; 10 g. of semicarbazone of pyrethrin II (m. p. 56–59°) yielded 2.76 g. of nearly pure pyrethrin II.

Anal. Calcd. for $C_{21}H_{30}O_3$ (pyrethrin I): C, 76.31; H, 9.16. Found: C, 75.95, 76.36; H, 9.32, 9.06. Calcd. for $C_{22}H_{30}O_5$ (pyrethrin II): C, 70.54; H, 8.08. Found: C, 72.71; H, 8.18.

In the course of this work it was noticed that when a drop of solution containing the pyrethrins was applied to the tongue, a very marked numbing effect was produced. Eventually it was proved that the sensation was caused by pyrethrin I and pyrethrin II. The numbing or tingling sensation was much the same as that produced by rubbing the tongue with aconite root. With pyrethrins the numbness developed five or ten minutes after placing on the tongue, and the effect persisted for one to three hours.

Toxicity of the Pyrethrins to Insects.—The insecticidal activity of the isolated pyrethrins was determined by experiments on cockroaches (*Blatta germanica*). One of us had observed that an acetone or alcohol

TABLE I

TOXICITY OF THE PYRETHRINS TO COCKROACHES

Composition of solution tested				
Pyrethrin, mg.		Water, cc.	Ratio pyrethrin:water	Cockroaches dead in 24 hours, %
I	33	1000	1:30,300	100
I	20	1500	1:75,000	100
I	12.5	1000	1:80,000	100
I	20	2000	1:100,000	50 Balance disabled
I	20	3000	1:150,000	None dead; all disabled
II	33	1000	1:30,000	100
II	20	1500	1:75,000	100
II	12.5	1000	1:80,000	50 Balance disabled
II	20	2000	1:100,000	50 Balance partly disabled
II	20	3000	1:150,000	None dead; all recovered

[6] *Cf.* ref. 4, p. 194.

solution of pyrethrins, when diluted with water, forms a stable colloidal suspension of great insecticidal power.[7] For the experiments with cockroaches the pyrethrins were dissolved in alcohol and then diluted with water to the desired strength. The amount of alcohol in the solution applied to the cockroaches was less than 0.5%. The dilute solutions were freshly prepared before each experiment and the pyrethrins remained uniformly distributed without settling or floating. The results of the experiments are given in Table I.

Pyrethrin I was slightly more active than pyrethrin II, as Staudinger and Ruzicka have noted. The pure pyrethrins were extremely toxic to cockroaches.

Reducing Action of the Pyrethrins on Alkaline Copper Solution.—Staudinger and Ruzicka have mentioned that pyrethrolon reduces alkaline copper solution. It was found that pyrethrin I and pyrethrin II have the same property and the idea of using the reaction for determining the pyrethrins at once suggested itself. The copper reducing power of the pyrethrins was found to be considerably less than that of dextrose and the other reducing sugars, and for this reason the gravimetric sugar methods could not be applied satisfactorily. Of the other sugar methods, the colorimetric method of Folin,[8] for determining dextrose in blood, seemed to be the most promising. In this method a measured amount of specially treated blood is heated with an alkaline copper solution, in a tube designed to prevent oxidation of the precipitated cuprous oxide. A tube containing a known amount of dextrose is treated in precisely the same manner. When the heating is finished, the Folin phosphomolybdate reagent is added to the tubes and a deep blue color is developed by the action of the cuprous oxide on the phosphomolybdate solution. The intensities of the colors are compared and the amount of dextrose in the blood is calculated in the usual way.

In a long series of experiments this method was adapted to the determination of pure pyrethrins in alcoholic solution. A special size of Folin sugar tube was used because oxidation of the reduced copper took place rapidly when test-tubes were used as suggested by Benedict.[9] Several modifications of Folin's reagent were tried and a large number of alkaline copper solutions were investigated. The effects of varying the temperature and time of the reaction were noted. More than 700 experiments were made before the best procedure for conducting the test was determined.

Comparisons of the colors produced by known amounts of the pyrethrins with those obtained from known amounts of dextrose, indicated that dextrose could be used as a standard, as in the Folin method. The ratio of the pyrethrin used to the copper reduced was not a constant. A number

[7] Patent applied for by C. B. Gnadinger, July 6, 1926.

[8] Folin, *J. Biol. Chem.*, **67**, 357–370 (1926).

[9] Benedict, *ibid.*, **64**, 207–213 (1925).

of comparisons were made between different quantities of pyrethrins and a constant amount of dextrose; the results of these experiments were plotted. The curves were not linear functions, but more nearly conformed to the parabolic equation used by Allihn[10] in his work on dextrose. In Table II the amounts of dextrose equivalent in copper reducing power to different quantities of pyrethrin I and pyrethrin II are given. These ratios were obtained by comparing the color produced by 2 mg. of dextrose with the colors yielded by the different weights of pyrethrins; the method by which these comparisons were made will be described later.

TABLE II

COMPARISON OF COPPER REDUCING POWER OF DEXTROSE AND PYRETHRINS

Pyrethrin I, mg.	Weights having equivalent copper reducing power: Dextrose, mg.				Av., mg.	Pyrethrin II, mg.	Dextrose mg.		Av., mg.
5.0	0.735	0.767	0.787	0.797	0.772	..	...	...	...
7.5	1.166	1.175	1.228	1.246	1.204	7.5	0.973	1.003	0.988
12.5	2.020	2.036	2.046	2.061	2.041	12.5	1.636	1.642	1.639
15.0	2.222	2.222	2.253	2.292	2.247	15.0	1.956	1.980	1.968
17.5	2.469	2.500	2.500		2.489	17.5	2.253	2.272	2.263
20.0	2.730	2.826	2.836		2.797	20.0	2.402	2.539	2.471

By substituting the values in Table II in the equation $y = a + bx + cx^2$, (where y = mg. of dextrose, x = mg. of pyrethrin, and a, b and c are constants) and applying the method of least squares, two equations were obtained

Eq. I (for pyrethrin I): $y = -0.2536 + 0.2237x - 0.00365x^2$

Eq. II (for pyrethrin II): $y = -0.0101 + 0.1437x - 0.0009x^2$

From these equations the dextrose equivalent to the quantities of pyrethrins in Table II was calculated; the observed and calculated values are compared in Table III, where the ratio of the reducing power of pyrethrin I to that of pyrethrin II also appears.

TABLE III

OBSERVED AND CALCULATED RESULTS AND COMPARATIVE REDUCING POWER OF PYRETHRINS I AND II

Pyrethrin, mg.	Dextrose equiv. to Py. I: Obs., mg.	Calcd., mg.	Diff., mg.	Dextrose equiv. to Py. II: Obs., mg.	Calcd., mg.	Diff., mg.	Ratio[a] Py. I:Py. II
5.0	0.772	0.773	0.001	...	...	...	...
7.5	1.204	1.218	.014	0.988	1.017	0.029	1.22
12.5	2.041	1.972	− .069	1.639	1.645	.006	1.24
15.0	2.247	2.280	.033	1.968	1.943	− .025	1.14
17.5	2.489	2.543	.054	2.263	2.229	− .034	1.10
20.0	2.797	2.760	− .037	2.471	2.504	.033	1.13
Av.			− .0007			.0018	1.166

[a] Calculated from dextrose equivalent to given weight of pyrethrin I and pyrethrin II; theoretical ratio, 1.133 (374/330).

[10] Allihn, *J. prakt. Chem.*, **22**, 46 (1880).

It was expected that the amounts of dextrose equivalent to a given weight of the two pyrethrins would be inversely proportional to the molecular weights of the pyrethrins. This is shown to be approximately the case in Table III. Direct colorimetric comparisons were made between equal weights of pyrethrin I and pyrethrin II. The colorimeter readings were almost directly proportional to the molecular weights of the pyrethrins; the ratio of the pyrethrin II readings to the pyrethrin I readings was 1.152, compared with the theoretical ratio 1.133.

By substituting different values for y in Equation I, the weights of pyrethrin I equivalent to amounts of dextrose from 0.750 to 2.875 mg. were calculated. These calculations were made at intervals of 0.125 mg. of dextrose and other values were interpolated at intervals of 0.025 mg. In this way Col. II of Table IV was formed.

TABLE IV

COPPER REDUCING POWER OF DEXTROSE AND PYRETHRINS[a]

Dextrose, mg.	Pyrethrin I, mg.	Pyrethrin I and II, mg.	Dextrose, mg.	Pyrethrin I, mg.	Pyrethrin I and II, mg.	Dextrose, mg.	Pyrethrin I, mg.	Pyrethrin I and II, mg.
0.750	4.87	5.19	1.475	9.07	9.68	2.200	14.31	15.26
.775	5.01	5.34	1.500	9.23	9.85	2.225	14.52	15.49
.800	5.14	5.48	1.525	9.39	10.02	2.250	14.73	15.71
.825	5.28	5.63	1.550	9.55	10.19	2.275	14.95	15.94
.850	5.41	5.77	1.575	9.72	10.37	2.300	15.17	16.18
.875	5.55	5.92	1.600	9.88	10.54	2.325	15.40	16.42
.900	5.69	6.07	1.625	10.04	10.71	2.350	15.62	16.66
.925	5.82	6.21	1.650	10.21	10.89	2.375	15.85	16.91
.950	5.96	6.36	1.675	10.38	11.07	2.400	16.09	17.16
.975	6.10	6.51	1.700	10.55	11.25	2.425	16.33	17.42
1.000	6.24	6.66	1.725	10.72	11.43	2.450	16.57	17.68
1.025	6.38	6.81	1.750	10.89	11.62	2.475	16.81	17.93
1.050	6.52	6.95	1.775	11.07	11.81	2.500	17.05	18.19
1.075	6.67	7.11	1.800	11.24	11.99	2.525	17.31	18.46
1.100	6.81	7.26	1.825	11.42	12.18	2.550	17.58	18.75
1.125	6.95	7.41	1.850	11.60	12.37	2.575	17.85	19.04
1.150	7.10	7.57	1.875	11.78	12.57	2.600	18.11	19.32
1.175	7.25	7.73	1.900	11.96	12.76	2.625	18.38	19.61
1.200	7.39	7.88	1.925	12.15	12.96	2.650	18.67	19.91
1.225	7.54	8.04	1.950	12.33	13.15	2.675	18.95	20.21
1.250	7.69	8.20	1.975	12.52	13.35	2.700	19.25	20.53
1.275	7.84	8.36	2.000	12.71	13.56	2.725	19.56	20.86
1.300	7.99	8.52	2.025	12.90	13.76	2.750	19.86	21.18
1.325	8.14	8.68	2.050	13.10	13.97	2.775	20.20	21.55
1.350	8.29	8.84	2.075	13.30	14.19	2.800	20.55	21.92
1.375	8.44	9.00	2.100	13.49	14.39	2.825	20.89	22.28
1.400	8.60	9.17	2.125	13.69	14.60	2.850	21.24	22.65
1.425	8.76	9.34	2.150	13.90	14.82	2.875	21.59	23.03
1.450	8.92	9.51	2.175	14.11	15.05			

[a] Third column calculated for a mixture of equal parts of pyrethrin I and II.

Staudinger and Ruzicka found that the active material, in the flowers they used, consisted of about 40% of pyrethrin I and 60% of pyrethrin II. The proportion of the two pyrethrins in the flowers probably varies.[2] It was assumed that the pyrethrins occur in equal amounts, and from the values for pyrethrin I, in Table IV, the corresponding values for a 1:1 mixture of pyrethrin I and II were calculated, using the average molecular weight 352 and multiplying the figures for pyrethrin I by the factor 1.06666 (third column, Table IV).

The semicarbazones of the pyrethrins had little or no copper reducing action. The by-products of the semicarbazone conversion, with oxalic acid, were carefully examined for copper reducing compounds; none was found that resisted the purification with sodium hydroxide, chromic acid and permanganate.

The pyrethrins were altered by prolonged heating at 90°; they became insoluble in petroleum ether and lost their activity but the copper reducing power was increased slightly. Saponification of pyrethrin I with alcoholic sodium hydroxide solution lowered the copper reducing power.

The semicarbazone of pyrethrolon was prepared from the mixed semicarbazones of pyrethrin I and II by saponification at 0°, with a methanol solution of sodium hydroxide.[11] The pyrethrolon semicarbazone melted at 203°; it was converted into the ketone-alcohol by prolonged shaking with benzene and potassium bisulfate solution.

The copper reducing power of pyrethrolon was slightly less than that of pyrethrin I. Pyrethrolon was dissolved in petroleum ether and washed with 3% potassium permanganate solution; 98.5% of the pyrethrolon was oxidized and removed from the petroleum ether. The residue from the evaporation of the petroleum ether solution had very little copper reducing action.

Application of the Copper Reduction Method to Pyrethrum Flowers.—Having found that the amount of pure pyrethrins in alcoholic solution could be determined accurately, it remained to apply the method to pyrethrum flowers and to eliminate, by appropriate means, any other copper reducing material that might be present.

Preliminary tests with petroleum ether extracts yielded percentages of pyrethrins of about the magnitude expected. Washing the petroleum ether extracts with 1% sodium hydroxide or with concentrated sodium bisulfite solution did not remove any material that reduced alkaline copper solution.

Staudinger and Ruzicka found that the pyrethrins in petroleum ether solution were not oxidized by chromic acid solution. Washing the petroleum ether extracts of *Pyrethrum* flowers with chromic acid solution did not decrease the amount of copper reducing material. No petroleum ether

[11] Staudinger and Ruzicka, *cf.* ref. 4, pp. 196 and 216.

soluble compounds that reduced copper could be found, excepting the pyrethrins.

The residues from the purification of the semicarbazones of the pyrethrins were carefully examined for petroleum ether soluble copper reducing compounds but none was found.

No other compounds that formed semicarbazones could be isolated from the petroleum ether extract excepting the pyrethrins; this agrees with the findings of Staudinger and Ruzicka.

Application of the method to daisy flowers (*Chrysanthemum leucanthemum*) showed no pyrethrins present and tests on *Pyrethrum* stems yielded mere traces of pyrethrins. Finally, the pyrethrins were isolated from 2500 g. of *Pyrethrum* flowers and weighed as semicarbazones. The same flowers were analyzed by the copper reduction method; the gravimetric method yielded 0.66% of pyrethrins; the copper reduction method, 0.88%. In view of the unavoidable losses in separating the semicarbazones, the agreement is good.

It is believed, therefore, that the following method determines only the toxic principles in *Pyrethrum* flowers.

Method for the Evaluation of Pyrethrum Flowers

Reagents. (a) **Petroleum ether,** 90–99% distilling between 20 and 40°; maximum boiling point, 60°.

(b) **Aldehyde-Free Alcohol.**[12]—Allow 95% alcohol, containing 5 g. of *m*-phenylenediamine hydrochloride per liter, to stand for twenty-four hours with frequent shaking. Boil under a reflux condenser for at least eight hours, allow to stand overnight and distil, rejecting the first 10 and the last 5% of distillate. Store in a dark place in well-filled bottles.

(c) **Basic Lead Acetate Solution.**—Dissolve 20 g. of Horne's basic lead acetate in sufficient recently boiled water to make 1 liter.

(d) **Alkaline Copper Solution.**—Dissolve 2.5 g. of purest copper sulfate, $CuSO_4.5H_2O$, in about 100 cc. of water, warming gently; cool when dissolved. Dissolve 5 g. of highest purity sodium potassium tartrate and 7.5 g. of purest sodium hydroxide separately in about 100 cc. of cold water. Transfer the solutions to a 500-cc. volumetric flask, mix and dilute to the mark. This solution should not be used after it is three days old.

(e) **Folin's Reagent.**[13]—Dissolve 150 g. of sodium molybdate, $Na_2MoO_4{\cdot}2H_2O$, in 300 cc. of water. Filter through a 15-cm. quantitative filter paper into a 1-liter flask and wash with 75 cc. of water. Add 0.1 to 0.2 cc. of bromine and shake until the bromine is dissolved. Let stand for one hour, then add with shaking 225 cc. of 85% phosphoric acid. Add 150 cc. of sulfuric acid (1 vol. of concentrated acid mixed with 3 vols. of water and cooled). Remove the liberated bromine by means of a moderately rapid current of air; the aëration requires about half an hour. Finally add 75 cc. of 99% acetic acid, mix and dilute to a volume of 1 liter.

(f) **Standard Dextrose Solution.**—Dissolve exactly 1 g. of pure anhydrous dextrose

[12] "Methods of Analysis," Association of Official Agricultural Chemists, Washington, D. C., **1925,** 2d. ed., p. 353.

[13] Folin, *J. Biol. Chem.*, **67,** 357–370 (1926).

in water and transfer to a 200-cc. volumetric flask. Add 40 cc. of aldehyde-free alcohol, mix and dilute to 200 cc. with water. Transfer 10 cc. of this solution to a 250-cc. volumetric flask by means of a pipet, add 210 cc. of aldehyde-free alcohol and dilute to 250 cc. with water. Ten cc. of this dilute solution contains 2 mg. of dextrose. The strong solution is stable for months; the dilute solution should be made fresh each week.

Apparatus. (a) **Constant-temperature water bath,** set at 78°, corrected, and controlled within ±0.2°.

(b) **Colorimeter** of Duboscq or Klett type, with artificial illuminator.

(c) **Folin sugar tubes,**[14] blown to contain 15.5 cc. to base of constriction. When heated to 78° the surface of the liquid must fall between points A and B, Fig. 1. The internal diameter of the constricted portion should be the same for all tubes in a set.

Fig. 1.—Modified Folin tube. Bulb contains 15.5 cc. to Point B.

Determination.—Extract 20 g. of ground *Pyrethrum* flowers (about 30-mesh) for five hours with petroleum ether in a Soxhlet extractor. Cool the petroleum ether solution to about 20° and let stand for at least half an hour. Filter through a quantitative filter paper into a 400-cc. beaker, add a few grains of ignited sand and evaporate at a temperature not exceeding 75°. As soon as the last traces of petroleum ether are driven off, transfer the residue with five or six portions of boiling 95% aldehyde-free alcohol to a 100-cc. volumetric flask (previously marked at the 80-cc. point), using sufficient boiling alcohol to make the volume 80–85 cc. To the hot solution add from a pipet 15 cc. of basic lead acetate solution and make to the mark with hot alcohol. Shake vigorously, cool at once to 20° and again make to the mark with alcohol. Filter and to the filtrate add about 1 g. of anhydrous sodium carbonate. Let stand for ten to fifteen minutes, shaking frequently, and filter. Immediately pipet 10 cc. of the clear filtrate into a Folin tube and add, also from a pipet, 6 cc. of alkaline copper solution. Mix thoroughly, keeping the solution in the bulb of the tube. Measure 10 cc. of standard dextrose solution (2 mg. of dextrose) with a pipet into a second tube and add 6 cc. of copper solution. Place the tubes upright in the constant-temperature bath, set at 78° corrected, and heat for exactly forty-five minutes. Remove from the bath and place in water at 20° for three minutes. Add 10 cc. of Folin's reagent from a pipet and let stand for three minutes; then stopper the tubes, mix thoroughly, transfer to 100 cc. volumetric flasks and make to the mark with water. Filter through a Gooch crucible fitted with a heavy asbestos pad, using gentle suction. Do not use filter paper. The dextrose solution need not be filtered. Compare the solutions at once in the colorimeter and from the readings calculate the dextrose equivalent to the unknown solution in the usual way. Reference to Col. 3 of Table IV will give the amount of pyrethrins, in milligrams, equivalent to the dextrose found in the unknown solution, or milligrams of pyrethrins in the 2 g. aliquot of flowers taken.

Discussion of Method

Blank determinations were run several times each day, or with nearly every set of tests. No difficulty was experienced in obtaining blanks equivalent to 0.05 mg. of dextrose, and for this reason no correction for the blank was made in calculating Table IV. Reagents that yield a blank equivalent to more than 0.10 mg. of dextrose should be rejected. High blanks may be due to impurities in any of the reagents, but the sodium potassium tartrate,

[14] Folin, *J. Biol. Chem.*, **41**, 372 (1920).

sodium molybdate and aldehyde-free alcohol especially should be carefully tested. If the blank is between 0.05 and 0.10 mg. of dextrose, when compared with the standard dextrose solution, the error due to the blank is negligible.

The petroleum ether extract should not be heated longer than necessary to drive off the solvent. Generally only five or ten minutes' heating above 60° will be required if the petroleum ether meets the specifications given. High-boiling petroleum ether should not be used.

The proportion of alcohol and water in the contents of the Folin tubes is carefully adjusted; if more alcohol is used the copper salts will be precipitated; if the percentage of water is increased the pyrethrins will be thrown out of solution. In either case incorrect results will be obtained. The Folin tubes should be dried before using and the measurements should be made with pipets. Smaller aliquots than 10 cc. can be used for a determination, but sufficient aldehyde-free alcohol (80.5%) must be added to the tube to make the total volume of the pyrethrin solution 10 cc. Larger aliquots than 10 cc. cannot be used.

It is essential that the bath be maintained at 78° corrected, ±0.2°, and a stirrer should be provided to insure even temperature and circulation around the tubes, which should be immersed to a depth of 8 to 10 cm. Variations in time of heating or in temperature will yield results that are not comparable with Table IV. After removing the tubes from the bath they should be treated as nearly alike as possible; therefore it is inadvisable to run more than 3 or 4 tubes at a time.

In making the color comparisons the standard dextrose is set at 20 mm. The unknown will then read between 14 and 50 mm., for quantities between 23 and 5 mg. of pyrethrins. If the reading is less than 12 mm., the entire amount of copper may have been reduced, and the determination should be repeated using a smaller aliquot. It is also desirable, if the reading is more than 40 mm., to run a duplicate determination using 40 g. of flowers instead of 20.

The pyrethrins, in petroleum ether solution, can be washed with dilute sodium hydroxide solution, concentrated sodium bisulfite solution and 3% chromic acid solution without appreciable loss. These methods of purification were not necessary for the analysis of the flowers, but may be used in the examination of *Pyrethrum* sprays.

The dextrose used as standard in this work was of the highest purity obtainable and was dried for thirty days over sulfuric acid. All volumetric glassware, weights and thermometers used were Bureau of Standards certified. Colorimeter readings were made with artificial light and a blue glass screen.

Percentage of Toxic Principles in Pyrethrum Flowers.—The foregoing method was applied to *Pyrethrum* flowers and stems and to daisy flowers. The analyses are shown in Table V.

TABLE V

DETERMINATION OF PYRETHRIN CONTENT OF FLOWERS AND STEMS

No.	Description of sample	Pyrethrins, %	No.	Description of sample	Pyrethrins, %
7	Pyrethrum flowers, 1928 crop	0.40	2	Pyrethrum flowers, 1926 crop	0.87
6	Pyrethrum flowers, 1927 crop	.43	20	Pyrethrum flowers, 1928 crop	.94
13	Pyrethrum flowers, 1925 crop	.44	19	Pyrethrum flowers, 1928 crop	1.10
21	Pyrethrum flowers, 1925 crop	.45	17	Pyrethrum flowers, 1928 crop	1.17
16	Pyrethrum flowers, 1925 crop	.47	18	Pyrethrum flowers, 1928 crop	1.20
14	Pyrethrum flowers, 1925 crop	.53	1	Pyrethrum flowers, 1928 crop	1.21
4	Pyrethrum flowers, 1927 crop	.56	8	Dalmatian stems, 6 years old	0.00
11	Pyrethrum flowers, 1925 crop	.57	9	Spanish stems, 1927 crop	.04
5	Pyrethrum flowers	.59	15	Dalmatian stems	.04
3	Pyrethrum flowers, 1928 crop	.80	22	Daisy flowers, 3 years old	.00

The pyrethrin content varied, in the samples examined, from 0.40 to 1.21%; duplicate determinations agreed within 0.03% or less. The range of the pyrethrin content found is greater than reported by Staudinger and Harder (0.4 to 0.6%); Staudinger and Ruzicka found only 0.3% of pyre thrins in the flowers they examined.

Further work is in progress to determine the comparative value of the different varieties and grades of *Pyrethrum*, and to determine the distribution of the active principles in the different parts of the flower. The method is also being adapted to the examination of oleo-resin of *Pyrethrum* and to the analysis of *Pyrethrum* insecticidal sprays for household and garden use.

Summary

1. Pyrethrin I and II have been isolated from Japanese *Pyrethrum* flowers, and their action on alkaline copper solution has been investigated. The copper reducing power of the pyrethrins has been compared with that of dextrose.

2. A method has been described for determining the percentage of active principles in *Pyrethrum* flowers.

3. The percentage of pyrethrins ranged from 0.40 to 1.21%, in the sixteen samples of *Pyrethrum* flowers examined.

4. *Pyrethrum* stems contain about one-tenth the amount of pyrethrins found in the poorest flowers.

5. Daisy flowers contain no pyrethrins.

6. The active principles of Japanese *Pyrethrum* are the same as those of Dalmatian *Pyrethrum* flowers.

7. The toxicity of the pyrethrins to cockroaches has been determined.

MINNEAPOLIS, MINNESOTA