Table VI. Recovery from the Column

Reading	Column 1	Column 2	Column 3	Unchromatographed
1	0.6128	0.6138	0.6138	0.6208
2	0.6173	0.6128	0.6143	0.6213
3	0.6178	0.6143	0.6128	0.6218
Av.	0.6159	0.6136	0.6136	0.6213

Grand av. = 0.6144

$$\frac{0.6144 \times 100}{0.6213} = 98.9\% \text{ recovery}$$

Numbers expressed as absorbance.

Table VII. Analysis of a Barban Mixture

Technical material	72.14%
Recrystallized material	98.46%
Mixture	92.76%

Theoretical value

Technical

1.362 grams × 0.7214 = 0.9825 gram

Recrystallized

$$\frac{4.432 \text{ grams} \times 0.9846}{5.794 \text{ grams}} = \frac{4.3664 \text{ grams}}{5.3489 \text{ grams}}$$

$$\frac{5.3489 \times 100}{5.794 \text{ grams}} = 92.32\%$$

spectrum of the material is shown in Figure 7.

To evaluate the recovery from the column, the absorbance of a solution of purified barban was measured before and after chromatography. The loss of absorbance is then a direct indication of the extent of loss of barban on the column. The results of this evaluation are shown in Table VI. Three aliquots of a solution of purified barban were chromatographed in separate columns and three photometric readings taken in each case. A recovery of 98.9% was realized. The barban was recovered from the eluting solvent and rechromatographed. On the second pass through the column 98.0% recovery was attained.

The accuracy of the method was evaluated by analyzing a synthetic mixture. The results are shown in Table VII. The sample was prepared by adding 1.362 grams of technical material, which analyzed 72.17%, to 4.432 grams of recrystallized material which analyzed 98.46%. The material was mixed in solution and the solvent was evaporated. The sample analyzed 92.76% as compared to a theoretical value of 92.32%.

Interferences. Several impurities potentially present in the technical material were investigated as interferences in the method. The biscarbamate and the hydroxycarbamate are too strongly adsorbed to be eluted with barban. Bisurea is eluted after barban and has an ultraviolet spectrum sufficiently different from barban as to be recognizable as an impurity in the eluent. It is unlikely that 3-chlorophenylisocyanate would be present, but if so it would be converted to a 3-chloroaniline type compound on the chromatographic column. Toluene, present as residual solvent, is eluted before barban and has a discernably different ultraviolet spectrum.

Acknowledgment

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INSECTICIDE ASSAY

Chromatographic Separation of Active Components of Natural Pyrethrins and Their Characterizations

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IN THE COURSE of a study on the metabolic fate of different pyrethroids in insects, methods for isolation of pyrethrins I and II (PI and PII) and cinerins I and II (CI and CII) were developed. Established synthetic procedures, such as synthesis of pyrethroids from appropriate cyclopentadienolone and acids or by conversion of purified semicarbazones (*23*), are tedious and characterized by low yields and other uncertainties. A purely physical process of separation of the four components has been attempted by various workers. Enriched "PI" (mixture of PI and CI) and "PII" (mixture of PII and CII) have been obtained by repeated liquid–liquid partitions (*8, 20, 27*). "PI" and "PII" have also been separated by paper (*19, 29*) and column chromatography (*6, 10, 17, 18, 22*). Ward (*25*) and later Brown and coworkers (*2, 3, 4*) have achieved partial separation of four active components by displacement chromatography. The same method has been tried in our laboratory and gave only partial resolution at best. Results of these workers suggested that by use of a combination or modification of some of these procedures, a more direct separation and purification of the four pyrethroids could be accomplished.

Three colorimetric tests were used for identification and estimation of purity of individual pyrethroids. The colorimetric test developed by Williams and Sweeney (*24, 28*) was used to determine esters of pyrethrolone. PI and PII

The constituents of concentrated pyrethrum extracts were fractionated on a preparative scale by chromatography on Celite, silicic acid, and charcoal columns. Ultraviolet and infrared spectra of purified cinerins and pyrethrins and their physical properties are presented together with colorimetric and biological tests used to identify these materials.

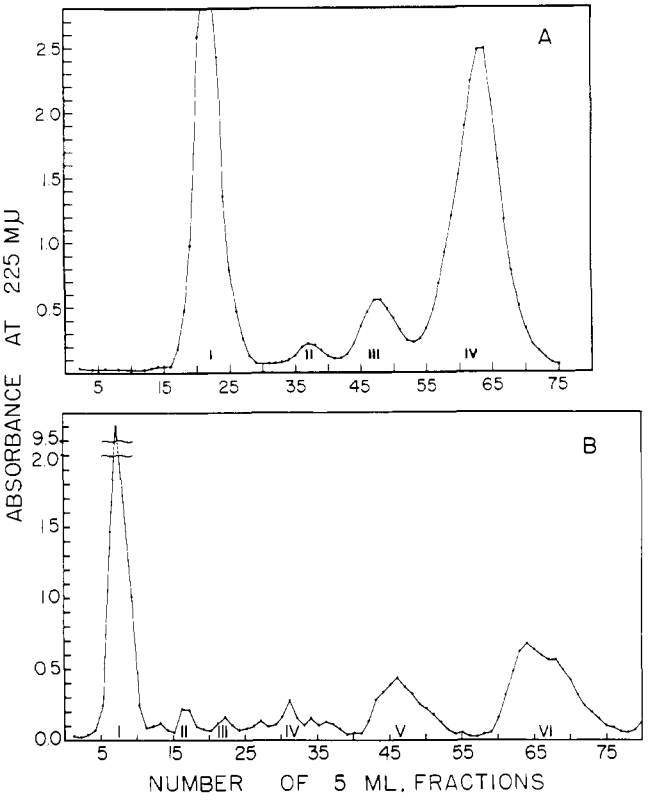

Figure 1. Chromatographic separation of pyrethrins

A. Celite 545 partition column; cyclohexane (mobile phase) saturated with acetonitrile (stationary phase) used as developing solvent. I, "PI"; II, unknown; III, CII; IV, PII

B. Silicic acid partition column; Skelly B (mobile phase) saturated with nitromethane (stationary phase) used as developing solvent. I, "PI"; II, III; IV, unknown; V, CII; VI, PII

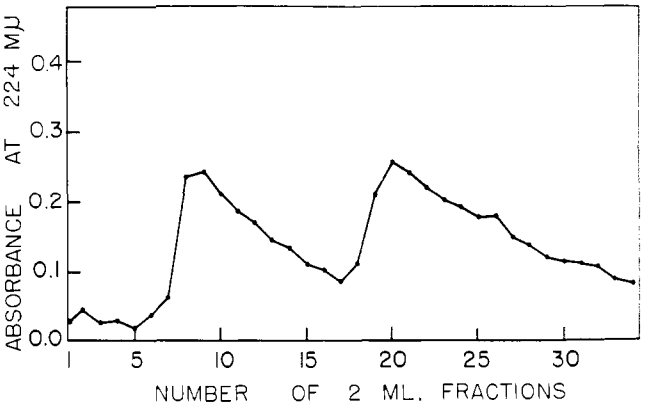

Figure 2. Chromatographic separation of "pyrethrin I" on charcoal column

Acidified ethyl ether used as developing solvent; I, CI; II, PI

developed a rose-red color whereas CI and CII were negative to this test. The colorimetric test described by Schreiber and McClellan (*21*) which involves use of the Denigès reagent was used to determine chrysanthemic acid. After saponification, clarification, filtration, and steam distillation (*7*) PI and CI yielded chrysanthemic acid which gave a purple color with the Denigès reagent, whereas pyrethric acid from PII and CII was not steam distillable. The colorimetric test for PII and CII was described by Levy and Molina (*16*). The methoxyl group in PII or CII was hydrolytically cleaved to methanol, the latter separated by steam distillation, and oxidized to formaldehyde which gave a lavender color after condensation with chromotropic acid.

Experimental

Purification of Pyrethroids. A kerosine extract containing approximately 20% of pyrethroids served as source of these compounds. One hundred milliliters of this material was extracted with 150 ml. of acetonitrile which had previously been purified by treatment with K_2CO_3 and distilled over P_2O_5. The lower acetonitrile layer was drawn off, and the kerosine layer was extracted twice with 100 ml. of acetonitrile. In the second and third extractions, the phases were reversed. The combined acetonitrile extract contains most of the pyrethroids as indicated by the negative reaction of the kerosine layer to the Sweeney-Williams test. The acetonitrile extract was decolorized by passage through a column of activated carbon (Nuchar C-190N). After decolorization, solvent was removed under reduced pressure at 60° C. The resulting concentrate mixture of pyrethroids was a light brown viscous oil.

Separation of "PI" from CII and PII on Celite Column. Separation of "PI" from CII and PII was achieved by use of Celite 545 and silicic acid column chromatography; the latter gave a sharper separation of CII from PII. However, for operational convenience, Celite column is far superior because flow rate is not critical and air pressure is not necessary to speed flow through the column. The column can be used several times without affecting resolution. The Celite column was prepared by grinding 40 grams of Celite 545 in a mortar with 25 ml. of acetonitrile, and 160 ml. of cyclohexane saturated with acetonitrile. The resulting slurry was transferred to a chromatographic tube 2.2 cm. in diameter and 60 cm. long, packed by air pressure. After packing, the length of the column was approximately 32 cm. A sample of 500 mg. of purified pyrethroids was dissolved in 4 ml. of cyclohexane saturated with acetonitrile and introduced on the column. The same solvent was used for development and elution of the chromatogram. An automatic fraction collector was used to obtain 5-ml. fractions at the rate of one fraction per minute.

From each fraction, an aliquot of 20 μl. was taken and the solvent removed in an air stream. The sample was dissolved in 3 ml. of 95% ethanol and its absorbance was determined at 225 mμ in a Beckman DU spectrophotometer. Since all of the known pyrethroids have maximum wave length in this range, a convenient means was available to eliminate fractions having little or no pyrethroids and to approximate the degree of separation of the pyrethroids. An elution curve (Figure 1, *A*) prepared from these data shows three predominant and one minor peak. By use of the

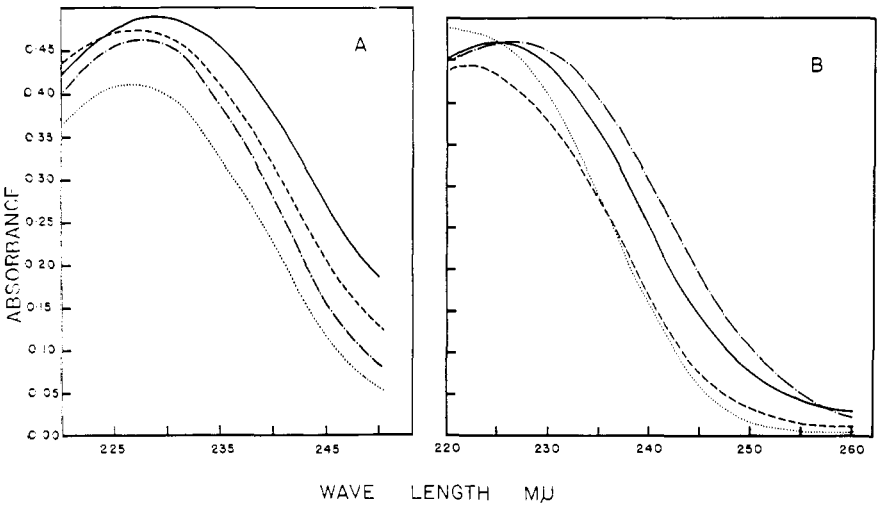

Figure 3. Ultraviolet absorption spectra

A ---------- CI
..... CI
——— PI
— — — PI

B CII in 95% ethanol
CII in *n*-hexane
PII in 95% ethanol
PII in *n*-hexane

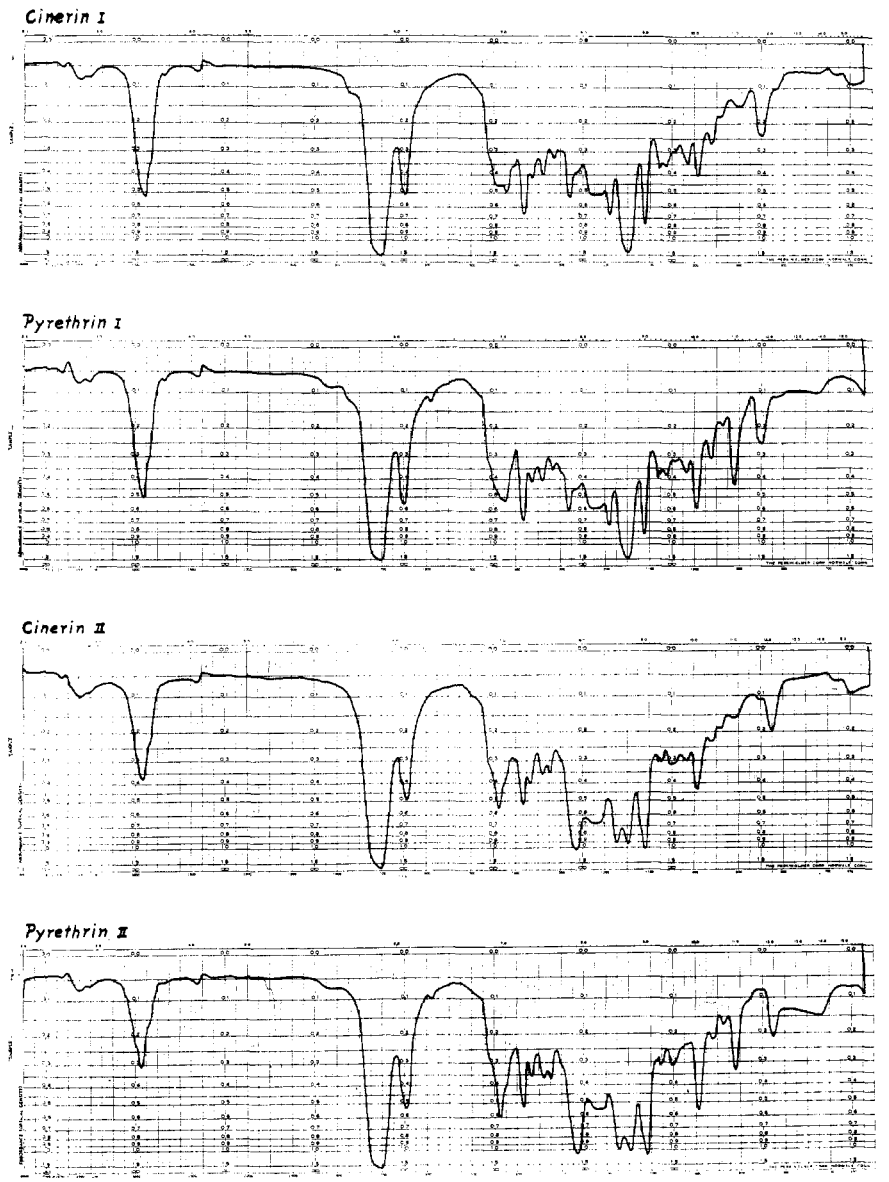

Figure 4. Infrared spectra

Williams and Sweeney test, it was found that part of the combined eluates constituting peak I was composed of esters containing pyrethrolone. Hydrolysis of esters followed by steam distillation showed the presence of chrysanthemum monocarboxylic acid in the distillate as determined by the use of the Denigès reagent. The methoxyl test of Levy and Molina for the methyl ester of chrysanthemum dicarboxylic acid was negative, indicating absence of PII and CII. Since the amount of chrysanthemum monocarboxylic acid was in excess of that which could be accounted for as the amount of pyrethrin I, it was tentatively concluded that fraction I consisted of a mixture of CI and PI.

Peak II did not respond to any of the tests used to characterize the pyrethroids and is of unknown composition. Peak III consisted largely of CII, but exhibited a weak positive test for pyrethrolone, a negative test for chrysanthemic acid, and a positive test for methoxyl. Peak IV produced a strong positive test for pyrethrolone and methoxyl, but a negative test for chrysanthemic acid indicating that this fraction was composed largely of PII. Celite column chromatography provided a quick and easy means for isolation of "PI" which is apparently free of other contaminating materials such as CII and PII. Subsequent separation of CI and PI was accomplished on a charcoal column.

Separation of "PI," CII, and PII on Silicic Acid Column. A column of silicic acid (Mallinckrodt, 100-mesh analytical reagent for chromatographic analysis) was prepared by grinding, in a Waring Blendor, 20 grams of silicic acid in 60 ml. of Skelly B for 15 seconds. Eleven milliliters of nitromethane was then added and the mixture was homogenized for an additional 15 seconds. The slurry was then poured into a chromatographic tube 1.6 cm. in diameter and the column packed under air pressure. A 200-mg. sample of purified pyrethrins was dissolved in 3 ml. of Skelly B and applied to the column. Skelly B saturated with nitromethane was used to elute the column. The rate of flow from the column was adjusted to yield 5-ml. fractions at 8-minute intervals. A series of 80 fractions was collected and each fraction was scanned for its absorbance at 225 mμ. From these data, an elution curve (Figure 1,*B*) showed six distinct components. The pooled fractions corresponding to peaks I to VI were tentatively characterized (Table I), on the basis of results of three colorimetric tests. Thus, peak I would appear to be a mixture of CI and PI; V, CII; and VI, PII. Peaks II, III, and IV are of unknown composition. The total of these three unknown peaks con-

stitutes approximately 1% of the original material and no attempt has been made to determine their structure.

Separation of PI and CI on Charcoal Column. Differential solubilities of PI and CI apparently are not great enough to permit their separation by partition chromatography. However, their polarities are such that they can be separated upon a strong adsorbent such as charcoal. A chromatographic tube 0.6 cm. in diameter was packed with 2 grams of Nuchar C-190N, which had previously been acid-washed (*15*). The column was constructed to allow for application of air pressure and continuous addition of solvent as described by Bulen, Varner, and Burrell (*5*). All glass joints were lubricated with an ether-insoluble grease prepared from starch and glycerol (*11*). Approximately 20 mg. of a mixture of PI and CI from the Celite column were dissolved in 2 ml. of Skelly B and the solution was added to the column and washed with 2-ml. portions of Skelly B. Acidified ethyl ether (5 drops of glacial acetic acid per 100 ml. of freshly distilled ethyl ether) was used as the developing agent. Two-milliliter fractions were collected automatically at a rate of 2 ml. per 10 minutes. The elution curve is shown in Figure 2. CI was found in the first peak and PI in the second. However, intermediate fractions 14 to 18 undoubtedly contained both CI and PI. Contamination of CI by PI can readily be revealed by the colorimetric test of Williams and Sweeney; however, contamination of PI by CI can only be determined from intensity of the red color developed from a given amount of material. In order to obtain pure CI and PI, intermediate fractions were usually not combined with the two major peaks.

Characterization of Individual Pyrethroids

Ultraviolet and Infrared Absorption Spectra. To avoid any possible contamination, only peak fractions giving maximum absorbance were used for spectra determinations. CI and PI were obtained from the charcoal column and CII and PII from the silicic acid column. Ethanol (95%) and *n*-hexane were used as solvents for measurement of ultraviolet absorption spectra. Data are shown in Table II and absorption curves in Figure 3,*A* and 3,*B*. It is evident that ultraviolet absorption shows a slight hypsochromic effect in the nonpolar solvent. Since the selective absorption band of the pyrethrins does not show fine structure, the $\lambda_{max.}$ cannot be determined with a high degree of accuracy. Generally "PI" showed maximum absorption about 2 to 3 mμ lower than "PII." However, this method can hardly be depended upon to identify CI from PI or CII from PII even with pure samples.

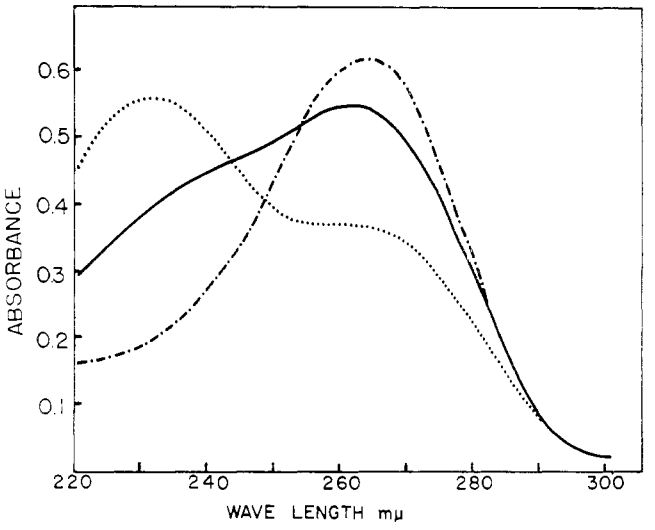

Figure 5. Ultraviolet absorption spectrum of CII- and PII-semicarbazone and cinerolone in 95% ethanol

——CII semicarbazone •—•— cinerolone semicarbazone
.....PII semicarbazone

A 10% solution (w./v.) of individual pyrethrins in chloroform was prepared and examined by accepted infrared techniques. Spectrograms with pertinent data are shown in Figure 4. CI and PI showed a single absorption peak at 8.7 microns, whereas CII and PII showed two peaks, of the same intensity, at 8.5 and 8.7 microns. Furthermore, PI and PII differ from CI and CII by possessing an additional sharp peak at 11.0 microns.

Preparation of CII and PII Semicarbazone. The CII semicarbazone was prepared according to the method of Haller and LaForge (*9*) from material separated on the silicic acid column. After repeated recrystallizations from methanol, the product gave clusters of fine needles, m.p. 154° C. The ultraviolet absorption spectra of CII semicarbazone in 95% ethanol is shown in Figure 5 with $\lambda_{max.}$ at 260 mμ.

Elemental Analysis. Calculated for $C_{22}H_{31}O_5N_3$: C, 63.29; H, 7.48; N, 10.06; O, 12.16. Found: C, 63.13; H, 7.08; N, 9.93; O, 19.92.

Cinerolone semicarbazone was prepared by alkaline hydrolysis of CII semicarbazone (*8*). After repeated recrystallizations from methanol, the final product melted at 202° to 203° C. Ultraviolet absorption spectra were shown in Figure 5 with $\lambda_{max.}$ at 264 mμ.

Elemental Analysis. Calculated for $C_{11}H_{17}O_2N_3$: C, 59.17; H, 7.6. Found: C, 59.88; H, 7.36.

PII semicarbazone was prepared by the method of LaForge and Barthel (*13*) from material separated on the silicic acid column. After repeated recrystallizations in methanol, the final product had a melting point of 266° to 268° C. Ultraviolet absorption spectra were shown in Figure 5 with $\lambda_{max.}$ at 232 and 258 mμ.

Elemental Analysis. Calculated for $C_{23}H_{31}O_5N_3$: C, 64.32; H, 7.27. Found: C, 64.32; H, 7.12.

Toxicity Tests on Houseflies. CSMA (Chemical Specialties Manufacturers Association) flies, 2 to 3 days old, were used in toxicity tests. Male and female flies were separated 24 hours before the test and sugar water was provided *ad libitum* before and after application of toxicants. Pyrethroids were dissolved in Skelly B and a volume of 0.612 μl. was applied topically to the dorsum of the thorax. Per cent mortality data were recorded at the end of 24 hours. The LD_{50} values of PI and CII were 0.28 and 1.08 μg. for male flies and 0.54 and 1.96 μg. for female flies, respectively. Results indicate that the toxicity of CII is approximately one fourth that of PI for both male and female flies. This is in agreement with the results of Gersdorff (*7*), LaForge and Green (*14*), and Incho and Greenberg (*12*).

The toxicity of three unknown compounds separated from the silicic acid column (peaks II, III, and IV) was tested on female CSMA flies by the method described above and the 24-hour mortalities were 15%, 0%, and 55%, respectively, at the level of 2 μg. per fly. Results indicate that the compound in peak III is relatively nontoxic. The toxicity of the compound in peak IV is approximately equal to that of CII. The compound in peak II possesses remarkable knockdown property, but lacks considerable killing power.

Discussion

For separation of "PI," CII, and PII partition chromatography has definite

Table I. Identification of Six Components Separated on Silicic Acid Column Peak

Conditions	Peak I	II	III	IV	V	VI
$\lambda_{max.}$, mμ	225	227	225	234	227–9	229
Pyrethrolone test (*29*)	Red	None	None	None	None	Red
Chrysanthemic acid test (*26*)	Purple				None	None
Pyrethric acid test (*20*)	None				Lavender	Lavender
Weight, mg.	115.6				24.2	58.2
Identified as	PI, CI	Unknown	Unknown	Unknown	CII	PII

Table II. Selective Absorption of Pyrethroids

Pyrethroids	$\lambda_{max.}$, mμ[a] This study	Ward (25)	ε Molar[a] This study	Ward (25)	$\lambda_{max.}$, mμ[b]
Cinerin I	226	222	17,720	18,200	220
Pyrethrin I	225	223	36,420	38,500	222
Cinerin II	227–9	229	28,946	29,400	226–8
Pyrethrin II	229	227	45,850	47,200	227–9

[a] In 95% ethanol.
[b] In *n*-hexane.

advantages over adsorption chromatography. Although CII and PII can be separated partially on Celite or entirely on silicic acid partition columns, separation of CI and PI requires adsorption chromatography on a strong adsorbent. On a fuller's earth column, there is some evidence of separation of CI from PI. Further study with various solvent systems might yield a more convenient method with greater resolution than the charcoal column. It must be emphasized that to obtain better separation of CI from PI, the charcoal column must be long and narrow, the amount of "PI" to be separated must be small relative to the amount of charcoal packed in the column, and the flow rate should be exceedingly slow. Chromatographic separation of the mixed semicarbazones of CI and PI has not been successful.

West (*26*), by fractional distillation and acetylation prior to conversion of the fractions into semicarbazones, obtained the semicarbazone of the *d*-form of cinerolone with a melting point at 202° to 203° C. and a $\lambda_{max.}$ at 266 mμ. Cinerolone semicarbazone prepared by the method reported here has a melting point of 202° to 203° C. and $\lambda_{max.}$ at 264 mμ. However, a spectrophotometric examination of the material showed that the ratio of absorbance at 266 mμ to that at 232 mμ was 3.02, whereas the ratio of absorptivities at these two wave lengths reported by West was only 1.67. This could be due to end absorption caused by impurities in the West preparation.

The amount of three unknown compounds separated on a silicic acid column is only 1% of the purified pyrethrins. Ease of operation of the Celite column suggests the possibility of obtaining large quantities of these unknown compounds from purified pyrethrins for precise chemical identification by group isolation on a Celite column (peak II) and further resolution on a silicic acid column.

Study of the metabolic fate of PI will be reported elsewhere.

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