Concentration of Sulfuric Acid. The effect of concentration of sulfuric acid upon the fluorescence was investigated with the maximum formed with about 85% sulfuric acid by weight as is shown in Table IV. The fluorescence values were the result of two opposite effects of the sulfuric acid concentration: one on the formation of the fluorogen, and the other on the intensity of the fluorescence of a given amount of fluorogen. Maximum fluorescence of the fluorogen occurred at some acid concentration between 20 and 70%. The formation of fluorogen by 70 and 96% acids was lower than with 85% acid. The solvent influenced the relative effects obtained from 85 and 96% sulfuric acid (Table II). The sample in methanol gave about twice as much fluorescence as the sample in water when development was by 96% acid in contrast to a 13% increase when 85% acid was used. Greater fluorescence was obtained from the 85% acid than the 96% regardless of the solvent in which gibberellic acid was dissolved.

Fluorogen. Two different principal substances were formed by the reaction between gibberellic acid and 85% sulfuric acid. The major product was a yellow, acidic substance with a golden fluorescence under ultraviolet on air-dried paper chromatograms and a faint pinkish fluorescence when 85% sulfuric acid was applied to the paper. The minor substance had a blue fluorescence on paper, both before and after wetting with 85% sulfuric acid. Presumably, the substance with the blue fluorescence was the one measured in assay. When the isolated fluorogenic mixture was chromatographed in the butanol-ammonia system used for gibberellic acid (*1*, *6*), about 10 spots appeared in addition to the two principle ones. Three of the spots were yellow with yellow fluorescence and behaved toward sulfuric acid exactly as did the principle yellow material (R_f = 0). One spot (R_f 0.63) had green fluorescence before and after the acid was added. The remainder of the spots had blue fluorescence which was intensified when acid was added. The largest of these had an R_f of 0.16. The two principal spots reduced permanganate just as gibberellic and gibberellenic acids did (*1*).

Temperature of Sulfuric Acid. The higher the temperature of the acid when it was added to the sample, the lower was the subsequent fluorescence. There was a limit to the increase obtained by lowering the temperature of the acid. Rapid addition of acid supercooled to minus 5° C. to a frozen sample increased the fluorescence very little over that obtained when acid at 12° C. was added equally as fast. Acid added at 25° C. gave a fluorescence only two thirds as great as that obtained with chilled acid.

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INSECTICIDE EVALUATION

Determination of Allethrin Residues in Milk and Meats

DONALD B. McCLELLAN and JOSEPH B. MOORE

McLaughlin Gormley King Co., Minneapolis, Minn.

A method accurate to 0.1 p.p.m. is presented to determine allethrin in the milk or meat tissues of domestic farm animals, which have been subjected to a concentrated allethrin spray. The determination involves solvent extraction of the tissues, concentration, and reaction with highly acidic mercuric oxide–sulfuric acid reagent to produce a red color. The color developed follows Beer's law and can be measured colorimetrically. Allethrin was not found in the milk of dairy cows, which had been sprayed daily for 3 weeks, or in the meat tissues of a female goat that had been sprayed daily for 5 weeks—all with a large overdose of the spray.

THE EXPERIMENTS presented determine whether or not allethrin (*dl*-2-allyl-4-hydroxy-3-methyl-2-cyclopentene-1-one ester of *cis-trans-dl*-chrysanthemummonocarboxylic acid) will appear in the milk of lactating cows or in the meat tissues of farm animals, which have been sprayed for extended periods with concentrated oil sprays of allethrin. For this determination, a modification of the method of Schreiber and McClellan (*3*) was used, which was suggested by the color reaction produced by the Deniges reagent in the determination of pyrethrins by the Association of Official Agricultural Chemists method (*1*) and by Fischer's method (*2*). The reagent used differs from that of AOAC and Fischer by its degree of concentration and acidity.

Chemical Analysis

The method of analysis, which was developed in the laboratory of McLaughlin Gormley King Co., involves the separation of the allethrin from the material in question by solvent extraction and the reaction of the concentrated extract with the mercuric oxide–sulfuric acid reagent. Allethrin will react with the reagent to give a pronounced red color. If the chrysanthemummonocarboxylic acid component of the allethrin is present in the free state, a pink to purple color will develop. The absence of these colors will show an absence of either of these compounds. The colors developed, which follow Beer's law, can be measured on a Klett-Summerson or other photoelectric colorimeter with suitable filters. The method is sensitive to as low as 10 γ of allethrin.

Table I. Sampling and Testing Schedule

Date Sampled	Wt. of Sample, G.	Allethrin Added	Cow A (1% Spray) Color reaction	Klett scale	Cow B (20% Spray) Color reaction	Klett scale	Cow C (10% Spray) Color reaction	Klett scale
11/12/55	200	0	Yellow tinge	30	Negative color	12	Negative	10
				34	Negative color	9	Negative	10
				27	Negative color	8	Negative	9
				26				
			Av.	29	Av.	10	Av.	10
	200	100 γ (0.5 p.p.m.)	Strong reddish	144			Strong reddish	90
		70 γ (0.35 p.p.m.)	Strong reddish	97			Strong reddish	72
		50 γ (0.25 p.p.m.)	Strong reddish	80			Strong reddish	64
		20 γ (0.10 p.p.m.)	Positive	55			Positive	31

Experimental Procedure

Three Guernsey cows of the dairy herd of Robert Colebank of Osseo, Minn., which are machine-milked twice daily, were used in these experiments. These animals were on winter feed and were held in the confines of the dairy barn and its exercise yard. The time of the year and the confines of housing eliminated any possibility of the animals being washed with rain.

The day before the spraying period began, the milk of each cow was sampled for use as control samples and for preparation of calibration data.

Starting in November 1955, each cow was sprayed daily at about 9:00 A.M. in the barn for a period of 3 weeks, with a spray concentrate using different percentages of allethrin in deobase—cow A, 1%; cow B, 20%; and cow C, 10%. The spray was applied to the animals with a Microsol sprayer from a distance of about 2 feet so as to cover the animal with 1.5 ounces of the spray concentrate.

On Monday, Wednesday, and Friday of each of the 3 weeks of the spray period, the milk from each cow was sampled and tested. Nine samples of milk from each animal were taken after the spray program began.

A female goat of about 80 pounds was sprayed daily for 5 weeks with a spray of 20% allethrin in deodorized kerosine with a hand spray in such a manner as to cover the animal with 1.5 ounces of the 20% allethrin spray. At the end of the 5-week period, the animal was slaughtered and the carcass, heart, liver, and kidneys were put into frozen storage. Samples were taken for testing as needed.

Methods of Analysis

Reagent. Mix 0.7 gram of yellow mercuric oxide with 80 ml. of water. While stirring the solution in a cold-water bath, slowly add 46 ml. of concentrated sulfuric acid. Stir until completely dissolved. Do not store in direct sunlight.

Special Apparatus. Klett-Summerson photoelectric colorimeter with adapter and tubes for 10-ml. colorimetric tubes and a No. 47 filter.

Procedure for Milk Samples. In a 500-ml. separatory funnel, shake 200 grams of the milk sample with 100 ml. of low-boiling petroleum ether, boiling range 20° to 40° C. Allow the layers to separate. The petroleum ether layer will be somewhat emulsified, but there will be a distinct separation of a light and heavy layer. Draw off the heavy layer into another flask, re-extract with 100 ml. of petroleum ether, allow to separate, and draw off lower layer into a third flask. To the petroleum ether layers in flasks 1 and 2, add about 5 drops of sulfuric acid and swirl to break emulsion. Draw off the lower layers into flask 3. Extract the material in flask 3 with an additional portion of petroleum ether. Combine the petroleum ether layers, concentrate to about 150 ml., and cool overnight at about 40° F. Decant the solvent into a 250-ml. Erlenmeyer flask but do not rinse the residue. Evaporate the solvent on a water bath. To the residue in the flask, add 7 ml. of the mercuric oxide–sulfuric acid reagent and mix by vigorous shaking for about 30 seconds. Allow the sample to stand an additional 60 seconds, then filter through a fine filter into a 10-ml. colorimetric tube. After about 4 ml. has been collected, centrifuge the filtrate at high speed for 45 seconds. Measure the color produced in the Klett-Summerson colorimeter. Maximum color is produced in 4 to 5 minutes. Do not carry out the reaction in the sunlight. If no red color is produced, allethrin is not present.

Procedure for Meat Samples. Mince portions of the meat to be tested at high speed in a Waring Blendor—drop small portions of the partially frozen meat into the whirling blades to be shattered rather than chopped. Extract a 100-gram portion of the minced sample three times with enough petroleum ether (boiling range 35° to 75° C.) to immerse the sample and allow stirring. Allow about one half hour for each extraction. Decant the solvent layers into a 250-ml. Erlenmeyer flask and remove the solvent by evaporation on a water bath. To the residue in the flask, add 7 ml. of the mercuric oxide–sulfuric acid reagent and mix by vigorous shaking for about 30 seconds. Allow the sample to stand an additional 60 seconds, then filter through a fine filter into a 10-ml. colorimetric tube. After about 4 ml. has been collected, centrifuge the filtrate at high speed for 45 seconds. Measure the color produced in the Klett-Summerson colorimeter. Do not carry out the reactions in the sunlight. If no red color is produced, allethrin is not present.

Table II. Reference Chart of Known Amounts of Allethrin

(100-gram samples of meat)

Allethrin Added, γ	P.P.M.	Klett Scale	Average
100	1.0	128	
		128	127
		128	
		124	
75	0.75	120	117
		124	
50	0.50	90	89
		91	
		86	
25	0.25	61	59
		60	
		57	
10	0.10	41	40
		43	
		36	
0	0	32	30
		32	
		32	
		27	
		28	
		30	
		30	

Results and Conclusions

All samples tested gave negative results for allethrin in the milk. Average Klett scale readings for 200-gram samples of milk were 29 for cow A, 11 for cow B, and 10 for cow C.

Samples of 100 grams each of the flank, rump, loin, liver, heart, kidney, and tallow of the goat all gave negative results. All except the tallow gave readings on the Klett scale ranging from 28 to 33. The tallow gave a negative color reaction but produced interference in the Klett.

Reference Curves. Known amounts

of allethrin were added to the milk samples taken prior to the start of the spray program in amounts ranging from 0.1 to 0.5 p.p.m. Samples of 200 grams were then analyzed as described under the heading of procedure. The zero point of the scale was set with the fresh reagent (Table I).

Known amounts of allethrin in a volatile solvent were added to 100-gram samples of meat. The solvent was allowed to vaporize and the meat extracted and tested as described under procedure (Table II).

Qualitatively, the method indicated that no allethrin or its component, chrysanthemummonocarboxylic acid, appeared in the milk or meat during the spray program.

Quantitatively, the method indicated that allethrin, if present, was less than 0.1 p.p.m.

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INSECTICIDE RESIDUES

Colorimetric Determination of Residual Perchloroethylene in Fumigated Wheat

J. H. BRUMBAUGH and D. E. STALLARD

Research Center, Diamond Alkali Co., Painesville, Ohio

The use of tetrachloroethylene in fumigants to control insect infestation in grains makes it necessary to determine the residual amount of this compound present after fumigation. A simple, sensitive colorimetric method for determining small amounts of perchloroethylene in wheat—utilizing a new-found color reaction of perchloroethylene with pyridine, aniline, and sodium methylate—is presented.

PUBLISHED ANALYTICAL METHODS for small amounts of perchloroethylene fall into three general classes. First, a direct spectrophotometric measurement in either the infrared or ultraviolet regions as reported by Bernstein, Semeluk, and Arends (*1*), Berton (*2*), and Hanson (*6*). Even assuming that sufficient sensitivity could be obtained by this method, the problem of "cleanup" of other absorbing materials present in the wheat prohibits its use. A second general method involves decomposition of the perchloroethylene by one of several methods with subsequent determination of the liberated chloride, generally on a micro scale. Such procedures are reported by Mapes and Shrader (*9*), Buscarons and Mir (*3*), Winteringham (*12*), Johnson (*7*), and Elliot (*4*). Although results are good the procedures are generally lengthy, always exacting, and highly susceptible to extraneous chloride pickup. The third method is an application of the Fujiwara (*5*) color reaction of halogenated compounds with pyridine and aqueous caustic as reported by Lugg and Wright (*8*) and Webb, Kay, and Nichol (*10*). While the Fujiwara reaction provides a simple and sensitive colorimetric method for most chlorinated methanes and ethanes, perchloroethylene does not enter into the reaction nearly so readily. The sensitivity is poor and the color fades rapidly.

When perchloroethylene is refluxed with a pyridine-aniline mixture for about 15 minutes, the sodium methylate solution is added, and the refluxing is continued for about 45 minutes, a sensitive and reproducible color reaction is obtained. This is the basis for determining residual perchloroethylene in wheat.

The sample of grain is digested in 0.15*N* sulfuric acid solution under reflux. The condenser is held at a temperature of approximately 65° C., which holds back most of the water but permits the released perchloroethylene to be swept over into pyridine absorbers when aided by a continuous sweep of aspirated air through the system. The perchloroethylene is then determined colorimetrically.

Procedure

The digestion-aeration train is shown in Figure 1. Absorption tube, *A*, is packed with activated carbon aimed at removing halogenated compounds that might be present in the air. The digestion flask, *B*, is heated by a heating mantle with Variac control. The temperature of the 8-inch, straight-tube condenser, *C*, is conveniently maintained at 65° C. by means of a flame-heated, 15-foot coil of $^1/_4$-inch copper tubing connected directly to the cold water tap. Tube *D* serves as a condensate trap; bulbs *E* and *F*, sulfuric acid scrubbers, remove some of the unwanted material which distills over from the wheat, and water vapor, which has the undesirable effect of decreasing the color intensity of the perchloroethylene reaction. Bulb *G* serves as a mist trap and *H* and *J* are pyridine absorbers for the perchloroethylene.

Ten milliliters of Karl Fischer grade pyridine, measured with a pipet, are divided—approximately 7 ml. and 3 ml.—between the first and second pyridine absorbers, 5 ml. of concentrated sulfuric acid are pipetted into each of the sulfuric acid scrubbers, and the absorbers are placed in the train. With the train completely assembled and 65° C. water flowing through the condenser, 200 ml. of 0.15*N* sulfuric acid are added to the digestion flask. Eighty grams of wheat sample are then quickly weighed and added to the flask, and the flask is stoppered. By means of a bleed control on the line going to the aspirator, a rate of sweep of about 150 ml. of air per minute is maintained through the system. The current applied to the heating mantle is adjusted so that the wheat suspension will boil in 10 to 15 minutes. Heating and sweeping are continued for an additional 45 minutes, the air sweep is then stopped by venting the bleed line, and the plug in the neck of the flask is removed. The contents of the first pyridine absorber are transferred to a 125-ml. flat-bottomed, standard-taper Soxhlet extraction flask. A rubber squeeze bulb is helpful in forcing the liquid through the frit.

The contents of the second pyridine absorber are used to rinse the first absorber before transfer to the flask. Both absorbers are finally rinsed with 5 ml. of pure pyridine, measured with a