ml. volumes with ethyl acetate (0.001 ml. is equivalent to 1 γ of each substance). Store solutions in small glass-stoppered containers.

Procedure

On a line 1 inch from the bottom of an 8 × 8 inch sheet of filter paper, mark equal intervals, with a hard pencil, beginning about 1 inch from either side. By means of a capillary pipet, spot 0.001-ml. portions of solutions at the marked intervals. If use of a larger volume of sample is desired, allow spot to dry, and respot with a similar volume on the same location.

Add about a 0.5-inch layer (about 50 ml.) of mobile solvent (reagent 2) to each trough.

Clip top edge of paper to the rod, which will be used to suspend it in the tank; invert and clip bottom edge to an auxiliary glass rod suitably supported in a well ventilated hood. Impregnate the paper with immobile solvent (reagent 1) by spraying rapidly and uniformly in horizontal passes, beginning at the spotted base line and progressing down to the opposite edge of the paper. Immediately invert the sheet, unclip it from auxiliary glass rod, and transfer the paper to tank so that lower edge dips into the mobile solvent. Seal glass cover on the tank with cellophane tape. Allow it to stand while the mobile solvent front ascends the paper. Five minutes is approximately minimum, at which sensitivity is best (about 1 γ) but separation is poorest. One hour, or until solvent front closely approaches (but does not reach) the top of the sheet, is approximately maximum, at which separation is best but sensitivity is poorest—i.e., for the aldehyde, 3 to 4 γ; for the alcohol, 2 to 3 γ; and for the acid, 1 to 2 γ. From solutions of the pure compounds, separation is relatively good; this makes it practicable to take advantage of the better sensitivity of a comparatively short development period.

Remove the paper from the tank, mark solvent front, and hang it from a rod in the hood until dry (15 to 30 minutes). Spray the paper with mixed chromogenic agents (reagent 3). Allow it to stand for 3 to 4 minutes, place a pan underneath, and wash the paper with a gentle stream of water from a wash bottle to remove excess chromogenic agent.

Table I shows R_f values for the three compounds.

Table I. R_f Values for 2,3,4,5-Bis (Δ^2 - butenylene)tetrahydrofurfural, Its Alcohol, and Its Acid

Substance[a]	R_f Values Average	Range[b]
2,3,4,5-Bis(Δ^2-butenylene)tetrahydrofuroic acid	0.07	0.03–0.13
2,3,4,5-Bis(Δ^2 - butenylene) tetrahydrofurfuryl alcohol	0.44	0.25–0.57
2,3,4,5-Bis(Δ^2 - butenylene) tetrahydrofurfural	0.83	0.65–0.94

[a] 98 observations for each substance, temperature 23° C.

[b] Range of separation of substances in 14 chromatograms appears wide when tabulated or graphed; however, when chromatograms are viewed individually, range is not particularly noticeable. Average R_f values best illustrate order of separation and distance of substances from one another, as viewed in any chromatogram. Uncontrollable variables, which cause the high or low values of the range, affect all compounds in chromatogram similarly.

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REPELLENT RESIDUES

Determination of Small Quantities of 2,3,4,5-Bis(Δ^2-butenylene)tetrahydrofurfural (Repellent R-11) in Milk

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The insect repellent 2,3,4,5-bis(Δ^2-butenylene)tetrahydrofurfural, (R-11), was tested for its appearance in milk, when used as a spray for dairy cattle. It was administered daily for 5 weeks to dairy cows at a level 10 to 20 times that needed for effective fly control. Analysis of milk samples throughout the treatment period failed to show the presence of any of the repellent within the sensitivity of the method, which was 0.1 p.p.m. The analysis was carried out by separating the butterfat from the milk, reacting an extract of this fat with 2,4-dinitrophenylhydrazine, and measuring the resulting product spectrophotometrically. The validity of determinng total R-11 content of milk by analysis of the fat only was demonstrated by measurement of the partition of R-11 between butterfat and aqueous phases.

THE BUTADIENE-FURFURAL condensation product, 2,3,4,5-bis(Δ^2-butenylene)tetrahydrofural, hereafter referred to as R-11, has been shown to be an effective insect repellent providing protection against houseflies and stableflies (*3*), particularly when added to synergized pyrethrins (*5*), and offers promise as a repellent for use in dairy barns. Before R-11 could be used as an ingredient in dairy sprays, however, additional information was required on its possible appearance in milk following application to lactating animals. Milk samples from animals sprayed with R-11 were analyzed to determine if the repellent is excreted in the milk, and if so, to evaluate the amount which might be present.

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Testing Program

Treatment of Animals Five purebred Holstein cows were selected from a production herd, which was hand milked three times each 24 hours. The cows were kept on sudan grass and native grass pastures except for periods when they were brought into the barn for milking and supplemental feeding.

Beginning early in June and continuing until termination of the test period, each of the cows selected was sprayed daily, immediately following the 10:00 A.M. milking, with 1 ounce of spray containing 1 quart of Pyrocide 20 (a commercial product containing 2% pyrethrins), 190 ml. of *N*-octylbicyclo(2,2,1)-5-heptene-2,3-dicarboximide (MKG 264), and enough Soltrol 170 (Phillips Petroleum Co.) (*2*) to make 5 gallons. When the test for R-11 was conducted in October, the spray used on three of the animals was altered by the addition of 2.0% R-11. The use of the original formulation was continued on two of the animals which were considered control animals in the experiment. Sprays were applied with Idico Star Model hand sprayers, which provided uniform coverage of the animals with little waste. All applications were made in a barn to further reduce loss due to air movement. A small amount of spray was lost when the udders and parts of the flanks were washed just before each milking.

Collection and Analysis of Samples Six samples were obtained from each of the cows in a 2-week period before any R-11 was added to the spray. R-11 was then added to the spray used on three of the animals and milk collection was continued from all of the animals at the rate of three samples for the first 2 weeks, two samples in the third week,

Table I. 95% Confidence Intervals on Samples from Sprayed and Control Cows

Day of Experimental Period	Oklahoma A. and M. Data	Phillips Data
15	−.20 to .04	−.17 to .09
17	−.10 to .15	−.09 to .15
24	−.03 to .25	−.16 to .21
27	−.01 to .09	−.01 to .06
29	+.01 to .15	−.08 to .21
34	−.12 to .13	−.09 to .06
41	−.08 to .15	−.06 to .09
48	−.12 to .15	

Table II. Recovery of R-11 from Synthetic Samples

Sample	A_{338}	Recovery, %
R-11 added to fat phase		
Control (fat + R-11 not reconstituted into milk)	0.30	(100)
Fat + R-11 reconstituted into milk	0.21	70
	0.26	87
	0.31	103
	0.19	63
R-11 added to aqueous phase		
Control (fat + R-11 not reconstituted into milk)	0.30	(100)
R-11 added to whole milk	0.15	50
	0.23	77
	0.25	83

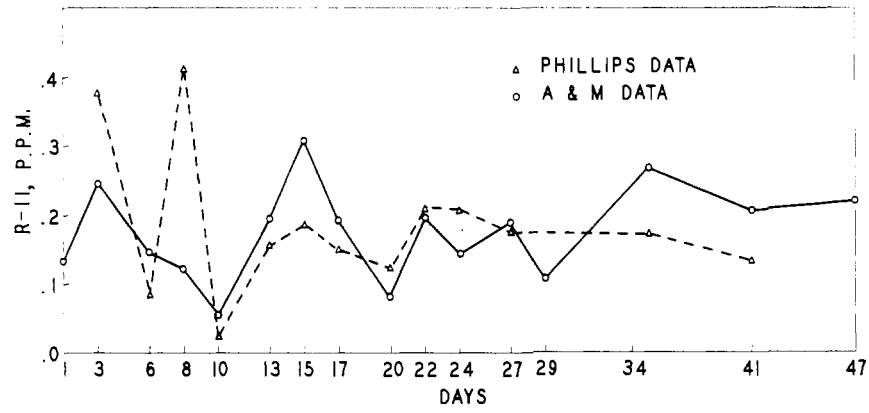

Figure 1. Carbonyl content of milk from unsprayed cows, calculated as p.p.m. of R-11

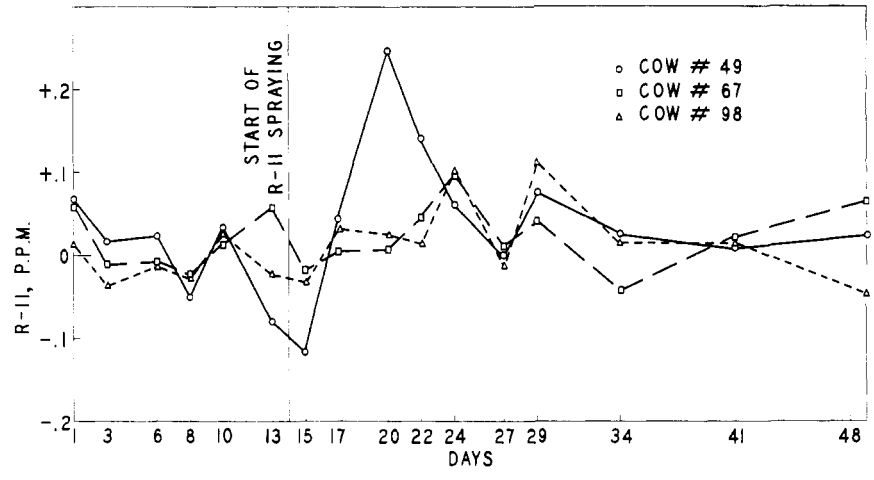

Figure 2. Carbonyl content of milk samples from treated cows, corrected by subtracting carbonyl content of samples from control cows

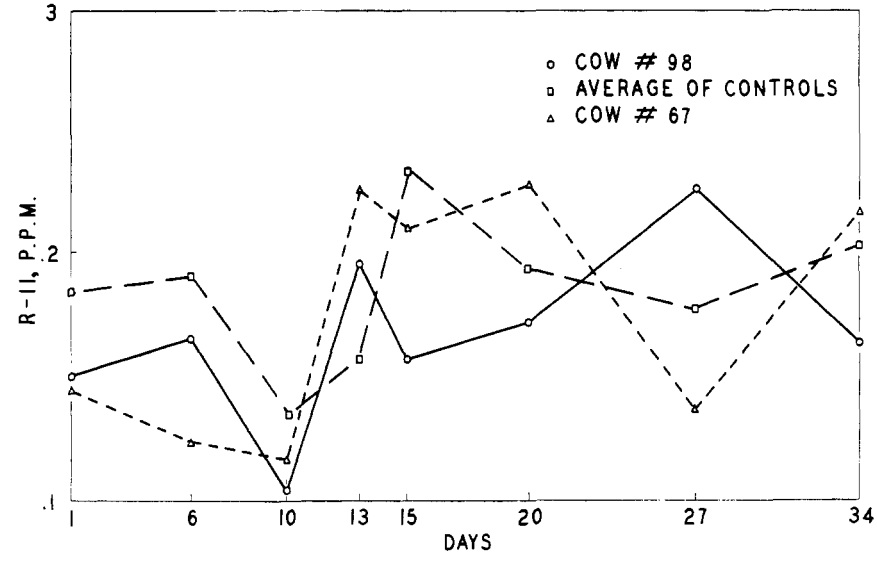

Figure 3. Comparative carbonyl content (as R-11) of samples from control and treated cows, analyzed as a group at the end of experimental period

and one sample each in the fourth and fifth weeks.

Duplicate samples of milk were obtained at each sampling period and stored at 2° C. One set of samples was delivered in glass containers to the Agricultural Experiment Station laboratory on the afternoon following the 10:00 A.M. milking. On the second day, the cream was churned to butter, the butter was stored in a freezer, and, in most cases, was analyzed for repellent on the third day. If a delay was encountered in analysis, the butter samples remained frozen until tested. The other set of samples was transported about 100 miles by automobile in refrigerated containers to the laboratories of the Phillips Petroleum Co. where the samples were analyzed for R-11. The two laboratories worked independently and results were not compared in detail until after termination of the experiment.

Unused portions of butterfat were stored in a deep freeze until the experimental period was terminated. At this time, a number of butter samples from a given cow were analyzed for R-11 in one single determination to show the absence of any day to day variation in analytical technique.

The method for the determination of R-11 in milk is described in detail later in this report. R-11 was extracted from butterfat and reacted with 2,4-dinitrophenylhydrazine and the product was measured photometrically. This procedure is limited in its sensitivity because of the presence of naturally occurring carbonyl compounds, which are not completely separated from the repellent by the extraction procedure. These compounds react with 2,4-dinitrophenylhydrazine to give a product which is not distinguished from the repellent by the method of analysis.

The analytical method determines the R-11 in milk only in terms of an increase in carbonyl content over a normal or reference value. One reference level was estimated by the analysis of milk samples from the two animals which did not receive the R-11. If the carbonyl content of milk was due to variation in feeding or management of the animals, samples from these animals would reflect the change. Secondly, after the determination of the carbonyl content of samples for a 2-week period preceding the application of R-11, the presence of R-11 should be indicated by a consistently higher carbonyl content during the treatment period.

Results and Discussion

The values from both laboratories for the carbonyl content of of the milk samples of the control animals are shown in Figure 1. This figure gives the carbonyl content of the milk samples, expressed in parts per million of R-11, for each day of analysis throughout the collection period. Each point represents the average carbonyl value for samples from the two control animals as calculated from the data from each laboratory. These samples showed some daily variation in carbonyl content with an average carbonyl level equivalent to about 0.2 p.p.m. expressed as R-11. The differences experienced in these analyses were due to differences in techniques in the two laboratories.

An over-all average value for the carbonyl content of milk samples from control animals for each day of sampling was calculated for each treated animal. The differences of these two values are plotted in Figure 2. When the naturally occurring carbonyl content of milk was thus corrected for, the increase in carbonyl content of samples from the cows receiving the R-11 did not exceed 0.1 p.p.m.

Animal No. 49 received an injury to her leg on the fifth or sixth day after R-11 spraying was started. She was put under surveillance and treatment. The milk obtained at the following two collection periods was of abnormal consistency as reflected by a difficulty experienced in collecting the cream. The increased carbonyl content of this milk—probably related to the effect of the injury—was reduced in a short time, even though R-11 application was continued.

Ninety-five per cent confidence intervals were established on the difference of the means of the carbonyl content of samples from the sprayed and control animals. These values are represented in Table I, for each day of analysis. The intervals are wide, partly because of the small number of animals in this test. They indicate a variation in carbonyl content—analyzed as R-11—in all the samples, therefore, the carbonyl content of samples from untreated animals may be expected, at times, to exceed the carbonyl content of samples from treated animals.

Analysis of selected butter samples stored in a deep freeze until the end of the collection period eliminated day to day variation in the analytical procedure. Samples from treated animals showed a carbonyl content of about the same magnitude as samples from the control animals (Figure 3). Differences between control samples was as great as the difference between a control sample and the sample from a treated animal.

There was also the possibility of metabolites occurring in the milk. The analytical procedure for R-11 based on the reactive carbonyl group would not apply if oxidation or reduction had taken place. Two likely metabolites are 2,3,4,5-bis(Δ^2-butenylene)tetrahydrofuroicacid and the corresponding tetrahydrofurfuryl alcohol. To determine whether R-11 was excreted in either of the above forms, samples of butterfat collected throughout the spray period were extracted with nitromethane to remove R-11 acid and alcohol. The extract was concentrated by evaporation, and the concentrate was analyzed by paper chromatography (*6*). This procedure would detect 0.2 p.p.m. of the acid, and 0.5 p.p.m. of the alcohol (based on a whole milk sample). Neither the acid nor the alcohol was detected by this procedure.

Values of the carbonyl content of duplicate milk samples should not differ by more than an amount corresponding to 0.11 p.p.m. R-11, at the 95% confidence level. The level of carbonyl in samples from treated cows is surprisingly constant as compared to the average value for the two controls. As the difference did not exceed, consistently, 0.1 p.p.m., it is reasonable to assume that no repellent was present within the limits of detection. The exception of this was the injured animal, whose carbonyl level returned to normal in a short period.

Analytical Method

In the testing program it was necessary to have an analytical method capable of detecting small concentrations of 2,3,4,5 - bis(Δ^2 - butenylene)tetrahydrofurfural in milk. A method capable of detecting 0.1 p.p.m. R-11 in milk would provide the necessary sensitivity for use in the program, which involved the spraying of 10 to 20 times the amount of R-11 recommended for effective fly control (*4*). If no R-11 were detected under these conditions, contamination would not result from normal applications of the repellent.

A method for the determination of 1 p.p.m. R-11 in hydrocarbons had been developed (*7*) so the problem remaining was to find a procedure for removing R-11 from milk, and concentrating it to ten times its original concentration to increase the sensitivity of the determination to 0.1 p.p.m. The most practical of the methods tried was that in use at the U. S. Department of Agriculture Experiment Station, Kerrville, Tex. (*1*). In this procedure, the milk is churned and the butterfat recovered is analyzed.

Partition of R-11 between Fat and Aqueous Phases in Milk

Before a method based on the analysis of fat removed from a milk sample could be adopted, it was necessary to show that an appreciable amount of the R-11 content of the sample is recovered in the separated butterfat. This was accomplished by measuring the partition coefficient of R-11 between butterfat and water, and by determining the fraction of a known amount of R-11 added to a sample of milk recovered in the separated fat. The partition was measured by extracting, with water, butterfat samples containing known concentrations of R-11 and determining the

R-11 content by reaction with 2,4-dinitrophenylhydrazine (7).

An average value for the partition coefficient, K (R-11 in fat/R-11 in water), of 110 was obtained by this method. Determination of the R-11 remaining in butterfat samples after five consecutive extractions with water gave an average partition coefficient of 100. From these and other results, a partition coefficient of 110 was assumed. Using this value of K and assuming whole milk to contain 4% butterfat, it was calculated that 82% of the total R-11 in a milk sample is concentrated in the fat phase.

This figure was verified by measuring the recovery from synthetic samples comparable to milk containing 0.1 p.p.m. R-11. This was done by separating butterfat from fresh milk, adding R-11 to the butterfat, and homogenizing the fat back into the skim milk. The reconstituted milk was agitated for 6 hours at room temperature and then analyzed for R-11 by the procedure described below. In other tests, R-11 was added directly to the milk (so that it was originally present in the aqueous phase). The milk was stirred for 6 hours and analyzed for R-11. Results of these tests, as well as of the control and blank determinations, are given in Table II. Although the precision of these results was not high, the agreement between these results and the value obtained from the fat-water partition measurements was considered satisfactory. Therefore, in the construction of the calibration curve, 80% of the R-11 was assumed to be present in the fat phase, and the measured R-11 content was corrected for the R-11 retained in the water phase.

Extraction of R-11 from Fat Preliminary experiments showed that butterfat itself contained substances which reacted with 2,4-dinitrophenylhydrazine and interfered with the determinations of R-11. The elimination of these interfering substances was complicated by the fact that the identity of these materials was not known, so that the search for a system which would separate the R-11 from interferences was a trial and error procedure. After a number of experiments with different chromatographic and liquid-liquid extraction systems, an extraction system—consisting of 25% fat and 75% iso-octane in one phase; and 6% water, 8% iso-octane, and 86% methanol in the other phase—was adopted. This system effectively retains the interferences in the fat, while extracting much of the R-11 into the alcohol phase. The extraction of the R-11 is not quantitative, and three 10-ml. extractions are required to remove 80% of the R-11 from the 20 ml. of the iso-octane-fat solution.

Reagents

Sodium sulfate, anhydrous.

Iso-octane. Spectro, ASTM reference fuel, or pure grade. (Phillips Petroleum Co., Bartlesville, Okla.)

Carbonyl-free methanol. Prepare by refluxing 600 to 700 ml. of reagent grade methanol for 2 hours with about 5 grams of 2,4-dinitrophenylhydrazine and a few milliliters of concentrated hydrochloric acid. Carefully distill the methanol—it should be colorless.

2,4-Dinitrophenylhydrazine (DNPH). Eastman Kodak grade.

Phosphoric acid, 85%.

Methanol extraction solvent. Add 15 ml. of water and 20 ml. of iso-octane to a 250-ml. volumetric flask or graduate, and fill with carbonyl-free methanol.

2,4-Dinitrophenylhydrazine reagent. Prepare a mixture of equal volumes of 85% phosphoric acid, water, and carbonyl-free methanol, and cool in an ice bath or refrigerator. Add a small amount of 2,4-dinitrophenylhydrazine (about 20 to 50 mg.) to the cold solution and mix for about 15 minutes. Allow the mixture to stand in a refrigerator until the excess has settled. Decant the clear yellow liquid into another vessel, filtering it through glass wool if necessary to remove all solid 2,4-dinitrophenylhydrazine. This solution should be prepared fresh each day and kept cold until used.

Procedure Allow a 2-liter sample of fresh milk to stand overnight in a refrigerator. Transfer as much of the cream as possible to a Waring Blendor and run at low speed until butter is formed. This may be done by connecting the Blendor to a Variac set at 40 to 50 volts. After the butter has formed, reduce the speed of the Blendor and stir the mixture slowly to collect the butter into a single mass. Transfer the butter to a 400-ml. beaker and remove as much liquid as possible by working the butter with a spatula. Wash the butter twice with small portions of water, working the butter with a spatula after each addition of water. Place the butter in a small beaker in a boiling water bath for 30 to 45 minutes to solidify the nonfat materials contained in the butter. Decant the melted fat into a 100-ml. centrifuge tube and add to this about 15% by volume of anhydrous sodium sulfate. Heat the mixture in boiling water, shake well, and centrifuge at 1500 r.p.m. for 10 minutes. Decant about 30 ml. of the fat into a 40-ml. screw-cap vial. Wash the fat with 3 small portions of hot water, shaking the mixture, and centrifuging before removing each portion of water with a suction tube. Store the washed fat in a closed bottle or vial in a refrigerator (7).

Weigh 5.0 ± 0.2 gram of purified fat into a 40-ml. screw-cap vial. Add 15 ml. of iso-octane and shake; warm slightly until the fat is dissolved. Add 10 ml. of the methanol solvent and mix for 15 minutes. Allow the solutions to stand until the lower phase is clear, centrifuging for about 1 minute, if necessary, and transfer the alcohol (lower phase) to a 50-ml. Erlenmeyer flask. Care should be taken that a minimum amount of the fat phase is transferred into the flask along with the alcohol phase.

Extract twice more with 10-ml. portions of the methanol extraction solvent for 10 instead of 15 minutes.

Add to the combined extracts in the flask a few small silicon carbide boiling chips and 5 ml. of water. Evaporate the solution to a volume of about 5 ml. on a hot plate. Care must be taken that the evaporation is carried to the same point with each sample. The samples should be removed when the temperature of the vapor in the flask reaches 79° C., as measured by inserting a thermometer into the vapor phase within the flask.

After the solution in the flask has cooled to room temperature, add 15-ml. of iso-octane and pour the contents of the flask into a 40-ml. screw-cap vial. Rinse the flask with a 5-ml. portion of the 2,4-dinitrophenylhydrazine reagent, adding this rinse to the vial also, and repeat the rinse with another 5-ml. portion. Close the vial with a cap containing a polyethylene gasket, and rotate the vial and its contents end over end for 30 minutes. A blank should be run along with the sample by evaporating and reacting 30 ml. of the methanol solvent in the same manner as the sample extract.

Withdraw portions of the iso-octane phases and measure their absorbance at 338 mμ. Subtract the absorbance of the blank from the absorbance of the sample solution to obtain the quantity ΔA_{338}, which is proportional to the carbonyl content of the sample.

Calibration Curve A straight-line calibration curve for this determination was prepared by analyzing synthetic samples of butterfat containing known amounts of R-11, and correcting for background by subtracting the absorbance of the sample containing no R-11 from each result. The plotted R-11 values were corrected for the partition between the fat and aqueous phases in milk by dividing them by 0.8.

The calibration was plotted in terms of Δ (R-11) and ΔA_{338} because it is usually necessary to measure actual samples in comparison with samples of similar milk known to be free of R-11, and determine R-11 content by the difference between the results.

Precision

On the basis of the analysis of 60 samples (five groups, each consisting of one sample from each of six animals, analyzed in parallel by two laboratories), the precision of the method was determined. Duplicate determinations of the same sample, using the same reagent solution, should not differ by more than an amount corresponding to 0.11 r.p.m. R-11, at the 95% confidence level. The true precision of the method is probably

better than this, because this value was calculated without considering the variation in the samples themselves—i.e., milk samples collected from different animals on the same day were assumed to be identical.

Acknowledgment

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INSECTICIDE ANALYSIS

Polarographic Determination of *O,O*-Dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate (Bayer L 13/59)

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A polarographic procedure has been developed for the analysis of technical grades and formulations of O,O-dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate, currently known as Bayer L 13/59. The reduction is carried out at 25° ± 0.5° C. in an aqueous solution containing 0.02*N* potassium chloride as the supporting electrolyte and 0.002% gelatin as the maximum suppressor. An accuracy of 2% is obtained under the specified conditions, and the half-wave potential against the saturated calomel electrode is −0.68 volt. Several commercial products have been analyzed with this method.

THE ORGANIC PHOSPHORUS COMPOUND currently known as Bayer L 13/59 (*O,O* - dimethyl 2,2,2 - trichloro - 1 - hydroxyethylphosphonate), as well as by the trade name Dipterex, was synthesized by Lorenz, Henglein, and Schrader (*10*). It is a condensation product of chloral and dimethyl hydrogen phosphite (*1*):

$$(CH_3O)_2POH + Cl_3CCHO \rightarrow (CH_3O)_2\overset{\overset{O}{\|}}{P}CHOHCCl_3$$

This compound has recently attracted wide interest in its use as an insecticide in various forms of fly baits (*5, 7, 8*) and possibly for the control of many other household and crop insects (*2*). However, the only reported analytical procedure for its estimation is the one described by Giang and Hall (*6*), which is based upon pyrolysis to split off chloroform and the development of a red color in aqueous pyridine by warming with alkali. This procedure may possibly be applied in the determination of Bayer L 13/59 in the small quantities present in plant residues, milk, or animal tissue extractives, but it is not suitable for use in the assay of technical materials. Consequently, reliable and sensitive methods of analysis are greatly needed for this new material in insecticide formulations. In view of the successful polarographic determination of chloral hydrate (*3, 4*) and other chlorinated aldehydes (*3, 9*), it was considered possible that Bayer L 13/59 could be determined by polarographic means.

Apparatus

The measurements were made with the Sargent pen-recording Model XXI polarograph. An H-cell with a saturated calomel electrode in the anode compartment was used, suspended in a water bath maintained at 25° ± 0.5° C. The capillary characteristics were: m = 3.1 mg. per second, t = 2.65 seconds per drop, and $m^{2/3}t^{1/6}$ = 2.50 (at 0.0 volt).

Reagents

Potassium chloride, 0.1*M*. Dissolve 7.456 grams of the salt (C.P. grade) in a liter of distilled water.

Gelatin, 0.1%. Dissolve 100 mg. of gelatin in water by heating, cool to room temperature, and make to 100 ml. with additional water. Make a fresh solution each day.

Nitrogen. Bubble through a portion of the test solution in a glass cylinder before passing through the sample solution.

Purified Bayer L 13/59. Recrystallize a technical material from petroleum ether containing a little benzene. Melting point, 78–80° C. (*1*).

Preparation of Standard Curves

Dissolve 1 gram of purified Bayer L 13/59 in water and make to 500 ml. Pipet aliquots of 3, 5, 7, 10, 15, and 20 ml. into a series of 100-ml. volumetric flasks. To each flask add 20 ml. of the 0.1*M* potassium chloride and 2 ml. of the 0.1% gelatin and make to volume.

Transfer a portion of each of the test solutions to the sample compartment of the H-cell and deaerate with nitrogen gas for 10 minutes. Record the polarogram for each solution from 0 to −2.0 volts at sensitivities of 0.04, 0.06, and 0.08 μa. per mm. with maximum damping. Plot the standard curves of wave height against milligrams of Bayer L 13/59 in 100 ml. of solution for each sensitivity.

Analysis of Fly Bait Formulations

For the analysis of fly bait formulations, weigh a sufficient amount of the sample and dissolve in water so that 1 ml. of the solution will contain approximately 1 mg. of Bayer L 13/59. Shake the solution intermittently for 1 hour and then centrifuge, if necessary. Pipet 20 ml. (or more) of the clear solution to a 100-ml. volumetric flask and proceed with the determination as described in the construction of the standard curves.

Results of Analysis

Four samples of technical Bayer L 13/59 were analyzed by the procedure above, employing samples of 20 to 30 mg. per 100 ml. The results are given in Table I. Baits and dusts of known