procedure described under Experimental since otherwise natural inhibitors and masking factors caused extremely low recoveries, similar to the ones discussed above for sugar beet leaves. As may be seen from Table IV, the apparent residue values of these check samples of cottonseeds varied considerably. However, as in many residue analyses, it is important to obtain an untreated sample from the same general area as the sample under investigation, so that residue values may be corrected for background.

Sugar beet roots did not require any cleanup prior to analysis since cholinesterase inhibition of check samples were below 20% and recoveries were 81%.

Whereas a 2.5-gram sample of potatoes could be analyzed satisfactorily without cleanup (15.2% cholinesterase inhibition by check sample), interference from a 5.0-gram sample (36.4% inhibition) persisted even after the above described cleanup procedures. A 2.5-gram aliquot sample was, therefore, chosen for potatoes giving a sensitivity of 0.016 p.p.m. phorate (Table IV).

Residue Determinations. Actual residue determinations on treated crop samples are shown in Tables III and IV. In only one crop analyzed were detectable amounts of residues found.

Other crops which have also been analyzed for phorate residues by this procedure with minor cleanup modifications include apples, hops, lima beans and pods, and red kidney beans. Recoveries and sensitivity were in the same range as for cottonseeds (Table IV).

Comparison of Two Residue Methods. Table III contains data for phorate residues in sugar beet leaves obtained by two cholinesterase inhibition methods. The plant material was cleaned up as previously described. Samples were analyzed by two separate cholinesterase inhibition procedures, the first procedure presently described while the second procedure was that of Curry (*6*). In the latter procedure, phorate phosphorothiolate sulfone was used as a standard, and the crop extract was analyzed directly without chemical activation. Check material was fortified prior to cleanup with either phorate (Method 1) or phorate phosphorothiolate sulfone (Method 2), and recoveries were 87.5 and 95.0%, respectively (Table III). Since phorate (least polar) and the corresponding phosphorothiolate sulfone (most polar) resulted in high recoveries through the cleanup procedure, it was assumed, therefore, that all other intermediate metabolites would behave in a similar manner. As can be seen from the data in Table III, the residue results obtained by Method 2 were lower, indicating that not all the phorate present in the sugar beet leaves has been oxidized by the plant material.

Acknowledgment

The authors wish to thank the American Cyanamid Co., Stamford, Conn., for financial support and for supplying phorate and its metabolites for analytical compounds.

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Received for review January 10, 1962. Accepted May 11, 1962.

INSECTICIDE RESIDUES

Colorimetric Method for the Estimation of Dimethoate Residues

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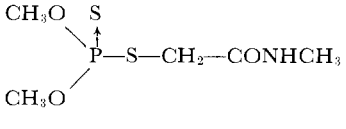
THE RECENT INTEREST in dimethoate [*O,O* - dimethyl *S* - (*N* - methyl carbamoylmethyl) phosphorodithioate], also known as Rogor in European countries, as a systemic insecticide has created the need for a simple but sensitive analytical method of detecting and estimating residues of this compound in milk and treated plants. Dimethoate is highly effective against some aphids, mites, and cattle grubs, and has low toxicity to warm-blooded animals and humans (*1, 3–5, 9*).

Dimethoate is a white, crystalline, solid material, m.p. 51–52° C. (*1, 3, 9*), with the following structural formula:



This compound is very soluble in most organic solvents except saturated hydrocarbon solvents, such as petroleum ether or *n*-hexane, in which it is only slightly soluble. Its solubility in water is about 1 to 2% at room temperature. Dimethoate is relatively stable in dilute acid and in water at room temperature, but it hydrolyzes rapidly in aqueous alkaline solutions to methylamine and thioglycolic acid in addition to the thiophosphate moiety.

Several methods for analysis of residues have been described. Santi and Bazzi (*12*) published a colorimetric method based on analysis for phosphorus for the determination of dimethoate residues present in olive oil. Chilwell and Beecham (*3*) adapted a similar phosphorus method for estimating residues in sprayed crops. In 1956, Bazzi, Pietri-Tonelli, and Santi (*2*) worked out a method for residues of this compound by oxidizing methylamine, which is formed by hydrolysis of dimethoate, to formaldehyde, and colorimetrically

A rapid and sensitive colorimetric method has been developed for the estimation of dimethoate residues in milk and crops. It determines both dimethoate and its P=O oxygen analog but does not distinguish between them. The method involves alkaline hydrolysis of dimethoate and colorimetric determination of the thioglycolic acid formed by reaction with sodium phospho-18-tungstate solution to form a blue color (absorbance maximum at 720 mμ). About 0.05 p.p.m. of dimethoate can be determined with 200 grams of treated plant sample or of milk.

determining the latter with chromotropic acid dissolved in concentrated sulfuric acid. Enos and Frear (*6*) have reported a semiquantitative method for residues in milk based on a paper chromatographic separation and development of a red spot with 2,6-dibromo-*N*-chloro-*p*-benzoquinone imine solution. Some preliminary work was done on a procedure to determine the methylamine by reaction with 1-chloro-2,4-dinitrobenzene to yield a bright yellow derivative, but interferences from reagents and check analyses were too high. These difficulties have recently been overcome by George (*10*).

The method described in this article is based on hydrolysis of dimethoate with alkali to yield thioglycolic acid. The presence of thioglycolic acid among the hydrolysis products was proved by preparation of the *p*-nitrobenzyl derivative and by comparison of the melting point (88–89° C.) and infrared curve with those of the *p*-nitrobenzyl derivative of pure thioglycolic acid. Also, equimolecular quantities of pure dimethoate and of thioglycolic acid gave the same analytical results in the method. Thioglycolic acid may be determined by several known methods (*11*, *13*, *15–17*). The method worked out in this study is based on the original Folin's uric acid method (*7*, *8*, *14*) using sodium phospho-18-tungstate solution as the color reagent. This method determines both dimethoate and its P=O oxygen analog but does not distinguish between them, nor does it determine any of the metabolites or breakdown products of the two compounds.

The plant sample is extracted with chloroform, the extract is chromatographed through a Nuchar-alumina-Celite column, and the eluate is evaporated to dryness. The residue is treated with an ammonium chloride coagulation solution, heated, then chilled, and filtered into a separatory funnel. The aqueous solution in the funnel is further cleaned up by extractions with *n*-hexane. Dimethoate in the aqueous solution is then extracted into chloroform, and the solvent is completely evaporated. The color is developed by hydrolysis of the residue with sodium hydroxide solution and reaction with sodium phospho-18-tungstate and cyanide-urea solutions. The intensity of the color is measured in a spectrophotometer at 720 mμ against a reagent blank carried through the same procedure.

Apparatus

Spectrophotometer, Beckman Model B or equivalent.

Colorimetric cuvettes, Corex D glass, matched, 1-cm. light path.

Rotating evaporator, Rinco or equivalent.

Reagents

Ether–*n*-hexane mixture. Two parts of redistilled diethyl ether and 1 part o redistilled *n*-hexane by volume.

Sodium phospho-18-tungstate solution. Dissolve 100 grams of sodium tungstate (Folin grade) in 500 ml. of water, add 75 ml. of 85% phosphoric acid, and reflux gently for 6 hours. Decolorize the resulting solution by adding a few drops of bromine and boiling for 30 minutes without a condenser. Dilute to 800 ml., filter, and store in a brown, stoppered bottle. The solution should be pale yellow.

Sodium hydroxide, 1.4*N*, prepared daily from 98% ACS grade in glass; avoid contact with rubber, cork, or metal.

Cyanide-urea solution. Dissolve 6.0 grams of sodium cyanide (reagent grade) and 40.0 grams of urea (U.S.P. grade) in 200 ml. of water. Add 1.0 gram of calcium oxide (reagent grade), shake for 20 minutes and filter. To the filtrate add 1.0 gram of lithium oxalate (purified grade), shake occasionally for 30 minutes, and filter through a highly retentive filter paper such as Whatman No. 42. If necessary, filter again; the filtrate must be perfectly clear. Keep this solution refrigerated when not in use. Prepare this solution every 3 days.

Nuchar-alumina-Celite mixture. Boil some Nuchar (C-190 N) or equivalent, in a large flask with concentrated hydrochloric acid for 45 minutes, wash several times by decantation with distilled water, filter, and continue washing until the filtrate is neutral to litmus. Then wash with two 200-ml. portions of 95% ethyl alcohol, dry in the hood, then at 100° C. overnight. Thoroughly mix 1 part of Nuchar and 2 parts of adsorption alumina (acid, Brockman activity 1, 80–200 mesh) with 1 part of Celite 545 or equivalent.

Coagulating solution. Dissolve 5.0 grams of ammonium chloride in 500 ml. of water containing 5.0 ml. of 85% phosphoric acid. Prepare this solution daily.

Cotton. Extract cotton in a Soxhlet extractor with acetone, dry in air, and then in a 100° C. oven.

Dimethoate. 99% purity, may be obtained from American Cyanamid Co., New York, N. Y.

Procedure

Preparation of Standard Curve. Transfer an accurately weighed sample of 100 mg. of dimethoate to a 500-ml. volumetric flask and make up to the mark with chloroform (technical grade, redistilled). After mixing, pipet 10.0 ml. of the solution into a 100-ml. volumetric flask. Make up to the mark with chloroform and mix well. One milliliter contains 20 μg. of dimethoate. Pipet aliquots of 0, 1.0, 2.0, 4.0, 6.0, and 8.0 ml. of the second solution into test tubes. Evaporate the solvent carefully in a 70° C. water bath in the hood, and remove the last traces of solvent with slight vacuum at room temperature.

Add 1.0 ml. of 1.4*N* sodium hydroxide to each tube, mix, and heat in a 50° C. water bath for 10 minutes. Pipet 1.0 ml. of the sodium phospho-18-tungstate reagent into each tube, mix 1 minute, then add 4.0 ml. of the urea-cyanide solution, mix well, and heat in the 50° C. water bath for 30 minutes. Remove the tubes from the bath, and cool at once in a beaker of water. If the colored solution shows some turbidity, transfer it into a 60-ml. separatory funnel, extract the solution with 5 ml. of *n*-butyl alcohol (reagent grade, redistilled), and filter the aqueous layer. Fill cuvettes with the solutions and measure the absorbance at 720 mμ against the blank solution carried through the same procedure. Prepare the standard curve by plotting the absorbance readings against micrograms of dimethoate. The curve follows Beer's law having a slope of 16.5 μg. of dimethoate per 0.100 unit of absorbance.

Analysis of Plant Samples. Cut the sample into small pieces, macerate with a measured volume of chloroform (about 2 ml. per gram of vegetable is usually sufficient) in a blender for about 2 minutes, and strain the extract through cheesecloth. If the chloroform and aqueous layers do not separate readily, centrifugation may be used. Filter

the chloroform layer through a Gooch crucible holder containing cotton and anhydrous sodium sulfate. If the extract contains large amounts of plant pigments, shake with 5 to 10 grams of Nuchar and filter again. Measure the volume recovered, and evaporate the solvent to approximately 20 ml. with a rotating evaporator on a warm water bath, or on a steam bath with the use of a Snyder column.

For chromatography, use 20 grams of the Nuchar-alumina-Celite mixture for each 100 grams of sample. Stir the mixture with chloroform, pour into a chromatographic tube (about 20 × 400 mm.), and pack the column with a little air pressure. Add approximately 2 inches of anhydrous sodium sulfate on top of the column followed by more chloroform.

When the chloroform level reaches the top of the sodium sulfate, quantitatively transfer the chloroform extract to the column, start collecting the eluate, and apply a little pressure if necessary so that the speed of percolation will be at least 60 drops per minute. When the level of the chloroform extract reaches the top of the column, carefully add more solvent and collect approximately 200 ml. of eluate. Evaporate the chloroform completely.

Heat the waxy residue with 50 ml. of the coagulating solution on the steam bath with occasional swirling until the wax is melted, then chill thoroughly in an ice bath. Filter through a filter-paper disk in a small Gooch crucible, and collect the filtrate in a 125-ml. separatory funnel. Extract the filtrate twice with cold, redistilled *n*-hexane, and discard the *n*-hexane layers.

Extract the aqueous layer with 75 ml. of chloroform by vigorously shaking for at least 1 minute in a clean separatory funnel. When the layers separate, transfer the chloroform layer into a second separatory funnel. Wash the chloroform in the second funnel by shaking with 50 ml. of water, and then again with another 50 ml. of water in a third separatory funnel. Filter the chloroform layer through anhydrous sodium sulfate into a flask. Extract the aqueous layer in the first funnel again with two successive 25-ml. portions of chloroform, wash each chloroform extract in the other two funnels, and filter through the same sodium sulfate.

Evaporate the chloroform on a steam bath in a flask fitted with a Snyder column, or with a rotating evaporator on a warm water bath until 5 to 10 ml. is left. Quantitatively transfer the extract to a test tube with two successive 5-ml. chloroform rinses. Evaporate the solution carefully almost to dryness in a hot water bath and remove the last traces of solvent with slight vacuum at room temperature.

Add 1.0 ml. of 1.4*N* sodium hydroxide solution to the residue in the tube, and finish the determination as described for preparation of the standard curve.

Analysis of Milk Samples. Extract 100 ml. of the milk in a large separatory funnel with three 150-ml. portions of the diethyl ether–*n*-hexane mixture, vigorously shaking for at least 1 minute each time. Filter the solvent layer through a plug of cotton. With the aid of a three-bulb Snyder column, concentrate the combined extracts on the steam bath to approximately 25 ml.

Transfer the concentrated extract to a 125-ml. separatory funnel using 75 ml. of *n*-hexane. Rinse the flask with 25 ml. of acetonitrile (reagent grade, redistilled), and add the rinse to the funnel. Shake the contents of the funnel vigorously for at least 1 minute. When the layers separate, transfer the acetonitrile layer into a second separatory funnel. Extract the acetonitrile in the second funnel with 50 ml. of fresh *n*-hexane by vigorous shaking. Finally, transfer the acetonitrile layer into a small Erlenmeyer flask. Extract the contents of the first and second funnels successively with two additional 15-ml. portions of acetonitrile, and finally drain the acetonitrile extracts into the same Erlenmeyer flask. Evaporate the combined acetonitrile extracts on the steam bath with the aid of a three-bulb Snyder column. Remove the last traces of solvent carefully with a little vacuum at room temperature. Add 20 ml. of chloroform to the residue, and finish the analysis as described for the plant sample, starting from chromatography with the Nuchar-alumina-Celite mixture column.

Tests Made with Method

Recovery from Plant Materials. One hundred grams of the sample was chopped and placed in a blender. An aliquot of a standard chloroform solution of dimethoate was added, and the sample was blended with 200 ml. of chloroform. Results obtained with cabbage, tomatoes, apples, and spinach are shown in Table I.

Recovery from Whole Milk and Butterfat. Recovery studies were run on dimethoate added to milk and butterfat samples. Each 100-ml. sample of milk was extracted with the mixture of diethyl ether and *n*-hexane, and each 25-gram butterfat sample dissolved in *n*-hexane was extracted with acetonitrile. Extracts were analyzed by the method described (Table II).

Table I. Recovery of Dimethoate from 100 Grams of Plant Material

Added, μg.	Recovered[a] μg.	Recovered[a] %
	Cabbage	
40.6	38.3	94.2
81.6	74.4	91.0
163.2	153.0	93.6
	Spinach	
42.3	37.2	87.8
84.6	76.3	90.2
169.2	149.9	88.6
	Apples	
40.6	37.7	90.0
81.6	77.0	94.4
163.2	147.3	90.2
	Tomatoes	
42.3	39.7	93.8
84.6	76.3	90.2
169.2	162.1	95.2

[a] Average of two or three analyses.

Table II. Recovery of Dimethoate from Milk and Butterfat

Added, μg.	Recovered[a] μg.	Recovered[a] %
	From 100 Ml. of Whole Milk	
44.4	40.0	90.2
88.8	78.5	88.4
177.6	167.3	94.2
	From 25 Grams of Butterfat	
44.4	41.0	92.4
88.8	84.5	95.2
177.6	167.3	94.2

[a] Average of two analyses.

Discussion

Experiments were carried out to determine the optimum proportions of reagents and the time and temperature for development of the color. The concentrations of reagents and the procedure outlined in Folin's uric acid method (7) did not produce sufficient sensitivity in the determination of the thioglycolic acid from dimethoate for residue analysis. A considerable increase in sensitivity was achieved, however, when the concentration of sodium phospho-18-tungstate solution was increased and when this color reagent was added to the hydrolyzed reaction mixture before adding the cyanide-urea solution. Furthermore, the same intensity of color was developed by keeping the reaction mixture at room temperature for $2^1/_2$ hours or by heating at 50° C. for 30 minutes.

The sodium phospho-18-tungstate reagent in the original Folin method is diluted to 1 liter, but to increase the sensitivity of the method, it is suggested that it be diluted to 800 ml. The reagent diluted to 1 liter gives about 20% less intensity of color development.

Under the specified experimental conditions, most of the known insecticides such as parathion, schradan, Trithion, Phosdrin, malathion, DDT, BHC, chlordan, aldrin, dieldrin, and Diazinon do not interfere with this colorimetric

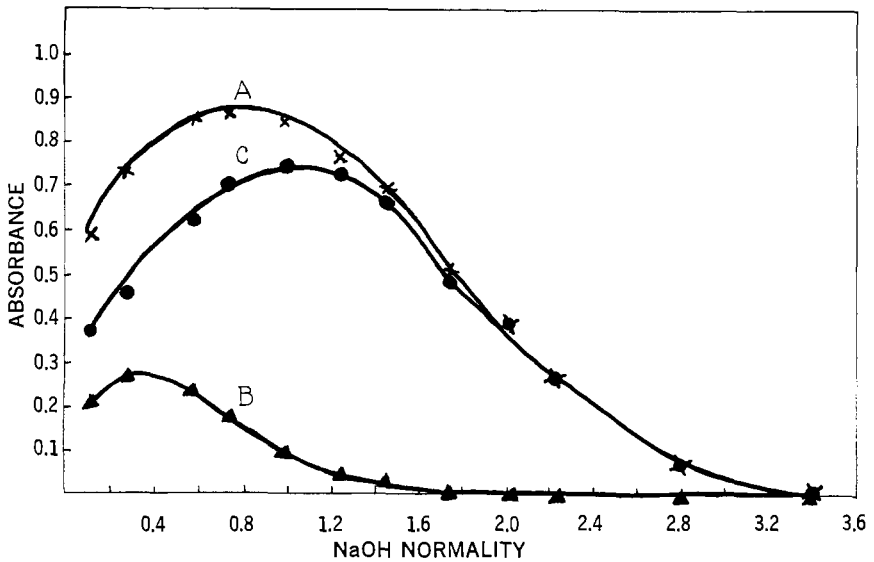


Figure 1. Influence of the normality of sodium hydroxide solution on the intensity of the developed color

(A) Spectrophotometric readings of dimethoate-treated sample solutions, 112.0 μg. of dimethoate in each solution
(B) Readings of blank solutions
(C) Sample solution readings after related blank solution readings were subtracted

analysis. However, demeton will interfere if present in amounts exceeding 0.5 mg. Phorate definitely causes interference, and this method, with certain modifications, may possibly be applied for the estimation of phorate residues.

The normality of sodium hydroxide solution used in the hydrolysis of dimethoate and its influence on the color development were carefully studied, and the results are shown in Figure 1. As indicated by the curves, when the normality of sodium hydroxide was below 1*N*, the intensity of the developed color from the hydrolyzed dimethoate solution was comparatively low, but that in the reagent blank solution was rather high. The highest sensitivity of the method was achieved when 1*N* sodium hydroxide solution was used. At this concentration, however, the color intensity of the blank solution was still too high for residue analysis. On the other hand, the intensity of the color produced in the sample solution gradually decreased when the normality of sodium hydroxide solution was increased beyone 1*N*, until it reached 3*N*, after which the color disappeared almost completely. The color intensity of the blank solution dropped almost to zero when the normality of the sodium hydroxide solution was increased beyond 1.5*N*. For the method described, the use of 1.4*N* sodium hydroxide solution is recommended. At this normality, the sensitivity of the method is still close to optimum, as shown by the curves in Figure 1, and the color in the reagent blank solution has decreased to a very low value.

Although very little interference has thus far been encountered in plant materials and milk not treated with dimethoate, it is necessary to run such control analyses. Some plants contain materials which if not completely removed in the cleanup will cause reduction of sodium phospho-18-tungstate solution with a resulting color in the control sample solution. The cleanup procedures in the method should be very carefully followed.

To avoid excessively high blanks, certain general precautions should be taken. All glassware should be cleaned and rinsed thoroughly with water. Contact of the sample solution and all the chemical reagents with metals, metallic foil, rubber, cork, and stopcock grease must be avoided, since these materials can cause high results.

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Received for review January 15, 1962. Accepted April 17, 1962.