NEMATOCIDE RESIDUES

Determination of Residues of *O*-2,4-Dichlorophenyl *O,O*-Diethyl Phosphorothioate (V-C 13 Nemacide) by Cholinesterase Inhibition

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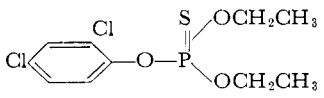
The effectiveness of O-2,4-dichlorophenyl O,O-diethyl phosphorothioate (V-C 13 Nemacide) in the control of nematodes, chinch bug, and other soil-borne insects necessitated the development of a method for the determination of residues on food crops. The cholinesterase inhibition procedure of Giang and Hall has been modified to facilitate routine application to a large number of samples. Sixteen fruits and vegetables have been analyzed with satisfactory precision and accuracy.

THE COMPOUND *O*-2,4-dichlorophenyl *O,O*-diethyl phosphorothioate (compound 1–13) has been shown (*6*, *7*) to give excellent control of nematodes, lawn chinch bugs, and certain other soil-borne insects without the phytotoxicity exhibited by nematocides now commercially available. It is a colorless, odorless, slightly viscous material, insoluble in water but soluble in most organic solvents. It has a melting point of 13° C. and an LD_{50} to rats of 270 mg. per kg. For actual application, this material is formulated with 20% of an emulsifier and the resulting solution is designated as V-C 13 Nemacide. In this paper "1–13" refers to the pure active component, *O*-2,4-dichlorophenyl *O,O*-diethyl phosphorothioate, and "V-C 13" to the emulsifiable concentrate.



O-2,4-Dichlorophenyl *O,O*-diethyl phosphorothioate (1–13)

A colorimetric method for the determination of residues of V-C 13 on several crops (*1*) was based on hydrolysis of the nematocide to 2,4-dichlorophenol and determination of the phenol by its reaction with 4-aminoantipyrine. Although this procedure has proved valuable for analyses of soil and commercial formulations of 1–13, it lacks the sensitivity necessary for residue data required by state and federal agencies.

The structure of 1–13 suggested that it or its oxidation product, 2,4-dichlorophenyl diethyl phosphate, would be an active inhibitor of acetylcholinesterase and that a sensitive analytical method might be based upon this property.

Preliminary experiments showed that 1–13 itself was a very poor inhibitor of cholinesterase, but that its oxygen analog was much more active in this respect.

Although it had been known for a long time that phosphate esters containing an acid anhydride group were strong inhibitors of acetylcholesterase, the classic paper of Giang and Hall (*4*) in 1951 described the first attempt to utilize this property as an analytical method. Since its publication several investigators have applied the inhibition method with more or less modification to the determination of small amounts of several phosphate esters (*2*, *3*, *5*).

To make this analytical principle more suitable for the analysis of a large number of samples on a routine basis, certain changes in previously published procedures were made.

Although the reactions and processes carried out in the analysis are basically simple, minute attention to detail and meticulous technique are required to obtain consistent and reproducible results.

The extraction of samples and the cleanup of the extract have been described (*1*).

Special Reagents

Ultrawet K, Atlantic Refining Co., 7% aqueous solution.

Oxidizing acid, 50% (by volume) fuming nitric acid, specific gravity 1.50, and 50% concentrated nitric acid, specific gravity 1.42.

Sodium bicarbonate solution, 10% in water.

Saline solution, 9 grams of sodium chloride per liter of water. Sterilize by boiling just before use.

Cholinesterase solution. With a sterilized syringe, inject 10 ml. of sterile saline solution into a 20,000-unit vial of cholinesterase (Bovine erythrocyte acetylcholinesterase, Winthrop Laboratories). Withdraw about 1.2 ml. with a sterile 2-ml. syringe, discharge 1.0 ml. into a 200-ml. volumetric flask, and dilute to the mark with saline solution. This solution should give a Δ pH of 1.9 to 2.1 by the described procedure.

Buffer solution. Dissolve 3.71 grams of barbital sodium in 450 ml. of water, add 44.75 grams of potassium dihydrogen phosphate, and stir until dissolved. Add 0.1*N* hydrochloric acid to pH 8.0.

Acetylcholine chloride, 12% in water.

Store ether and the cholinesterase, buffer, and acetylcholine chloride solutions in a refrigerator at 0° C. until immediately before use.

Procedure

Wash the entire vegetable sample under cold running water to remove all adhering soil and debris and select 200 grams of plant material which is representative of the entire sample. Place this in a Waring Blendor jar and add exactly 200 ml. of chloroform. Blend 3 minutes or until the mixture is uniform. Then add 10 ml. of Ultrawet K solution, stir in thoroughly with a glass rod, and allow the suspension to settle.

Transfer about 50 ml. of the chloroform layer to a 250-ml. beaker, add 20 grams of anhydrous sodium sulfate, and stir well. Filter by gravity through a fluted Whatman No. 12 paper. Pipet 10 ml. of the dried chloroform extract into a 30-ml. beaker. Add 1 gram of Attaclay, stir vigorously, and filter through a fluted paper.

Pipet 5 ml. of the clarified solution into a 250-ml. glass-stoppered Erlenmeyer flask and evaporate to dryness at room temperature in a stream of air. (The flask may be immersed in a water bath set at 25° C., to prevent excessive cooling and hasten evaporation.) Add 5.0 ml.

Table I. Recovery of 1-13 Added to Vegetable Extracts

Vegetable	Recovery, % Range	Av.
Beans	96–108	103
Cabbage	96–124	114
Carrots	99–116	105
Corn	72–116	104
Cucumbers	100–112	106
Lettuce	88–102	98
Oranges	92–112	105
Peppers	80–102	96
Potatoes	96–114	108
Radishes	92–116	103
Squash	96–109	104
Strawberries	76–116	105
Tomatoes	94–118	106
All samples	72–124	104.4

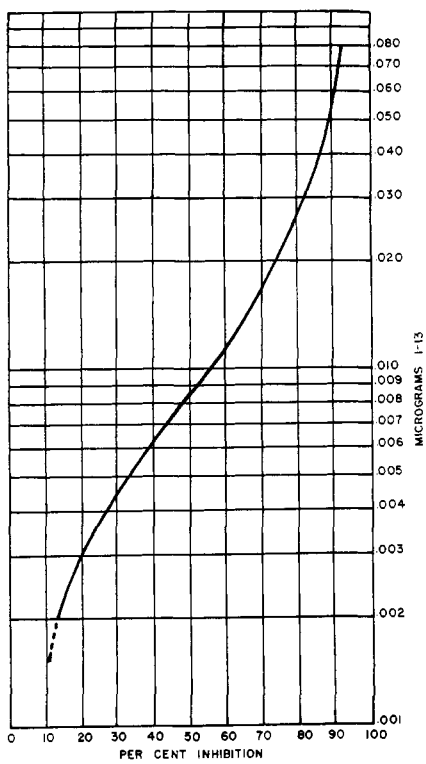


Figure 1. Cholinesterase inhibition vs. concentration of 1-13

of oxidizing acid to the dry residue, swirl to wet the walls of the flask, and allow the mixture to stand for exactly 10 minutes. Then add 50 ml. of water and cool in an ice bath. Slowly add about 100 ml. of sodium bicarbonate solution, being careful to avoid loss by frothing. Cool again in the ice bath and add exactly 50 ml. of cold ether from a pipet. Shake vigorously for three successive periods of 15 seconds each, then return the flask to the ice bath and allow the two layers to separate.

Pipet 0.50 ml. of the ether layer into a 5-ml. beaker and evaporate to dryness, using air from an electric fan under a hood. Add exactly 1.0 ml. of cholinesterase solution and exactly 1.0 ml. of buffer solution and mix well by swirling.

Table II. Residues of 1-13 on Treated and Untreated Crops

(P.P.M.)

Vegetable	Treatment, Gallon per Acre 0	5	7.5	10	20	30
			Root Crops			
Carrots	0.56	...	...	0.45	0.45	...
Onions	0.10	0.10	...	...	...	...
Potatoes	0.12	0.45	1.06	0.84	0.94	0.99
Radishes	0.15	0.30	0.86	0.51	0.69	0.74
			Leaf Crops			
Cabbage	0.43	...	...	0.24	0.34	0.28
Celery	0.19	...	...	...	...	...
Lettuce	0.27	0.01	0.01	0.20	0.48	0.51
Spinach	0.29	0.05	0.11	0.62	...	...
			Fruit Crops			
Beans	0.12	0.13	0.11	0.14	0.26	0.48
Corn	0.12	0.00	0.00	0.00	0.00	0.00
Cucumbers	0.17	0.02	0.08	0.04	0.01	0.03
Oranges	0.10	...	...	0.03	0.00	0.00
Peppers	0.10	0.03	0.04	0.04	0.03	0.05
Squash	0.07	0.04	0.04	0.02	0.02	0.03
Strawberries	0.07	...	...	0.08	0.08	...
Tomatoes	0.10	0.06	0.06	0.03	0.00	0.24

Place the beaker in a rack in a water bath maintained at 37.5° C. for 70 minutes, then remove it from the bath, allow to cool for 10 minutes, and measure the pH of the solution as accurately as possible with a Beckman Model G meter. Add 0.20 ml. of acetylcholine chloride solution, mix well, and return the beaker to the water bath for 120 minutes. Then remove from the bath, allow to cool for 10 minutes, and measure the pH again.

Calculate the change in pH during this period.

Calculations

Carry a blank solution—in a beaker containing no sample—through the procedure to determine the activity of the enzyme.

Calculate the per cent inhibition as follows:

$$\% \text{ inhibition} = \frac{\Delta \text{ pH (blank)} - \Delta \text{ pH (sample)}}{\Delta \text{ pH (blank)}} \times 100$$

From a calibration curve prepared by analyzing solutions of known amounts of 1-13 by the above procedure, find the micrograms of 1-13 present.

P.p.m. = micrograms in aliquot × 20

The calibration curve used in these analyses is shown in Figure 1.

Proof of Method

To estimate the reliability of this procedure, 16 aliquots of a solution of purified 1-13 were analyzed on different days. The average value obtained was 0.0075 γ per ml., the mean deviation 0.00085 γ per ml., and the standard deviation 0.0011 γ per ml. This standard deviation corresponds to about 0.02 p.p.m. on the size of sample specified.

The method was next applied to the analysis of samples of crops grown under known conditions in untreated soils. Each showed some inhibition of cholinesterase. These "blank" readings were generally small and varied from crop to crop (Table II).

As a final test of the method, known amounts of 1-13 were added to the chloroform extracts of various vegetables and these extracts were analyzed (Table I). Each entry is the average of ten determinations; 0.25 p.p.m. was added in three determinations, 0.50 p.p.m. in four determinations, and 1.00 p.p.m. in three determinations.

Application of Method

Samples of vegetables grown in soils treated with varying amounts of V-C 13 were then analyzed for residues (Table II). These vegetables can be divided into three groups: root crops, leaf crops, and fruit crops. In each case the appropriate blank value shown in the first column has been subtracted from the total apparent 1-13 found.

Discussion

Because V-C 13 Nemacide is applied to the soil, it is not surprising that the highest residues are found in root crops. The material appears to be translocated through the plants very slowly, as the residues found in leaves are much less than those found in roots. Residues in the true fruits are so small that they are insignificant.

Acknowledgment

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HERBICIDE RESIDUES

Determining Micro Amounts of Isopropyl *N*-Phenylcarbamate

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A new analytical method for determining microgram quantities of isopropyl *N*-phenylcarbamate is based upon alkaline hydrolysis and measurement of the resulting aniline, using the dye *N*-1-naphthylethylenediamine dihydrochloride. The method will determine concentrations of IPC as low as 0.1 p.p.m. in plant tissue. Direct hydrolysis of the plant tissue has been carried out with strawberries, thus eliminating extraction procedures. It is suggested that this method can also be used for the determination of isopropyl *N*-(3-chlorophenyl)-carbamates (CIPC).

A DIRECT and simplified analytical procedure has been found for microgram quantities of the selective herbicide isopropyl *N*-phenylcarbamate (IPC). Two methods are currently employed: The first (*3*) involves acid hydrolysis and measurement of the carbon dioxide liberated. The second (*1, 5*), which is more applicable to analyses of plant tissue, employs acid hydrolysis of the IPC. Either a phosphoric-hydrochloric-acetic acid mixture or a dilute (1 to 1) sulfuric acid mixture is used. Following the hydrolysis, the mixture is made basic and the aniline is removed from the mixture by steam distillation. It is then determined by hypochlorite-phenol-ammonia reagent. Determination of IPC in plant tissue requires that it be extracted prior to hydrolysis (*1*), because the aniline appears to combine with some product of hydrolyzed lettuce and cannot be recovered by steam distillation (*2*).

Although previous attempts to use basic hydrolysis failed because hydrolysis was incomplete (*1*), satisfactory hydrolysis was obtained using more concentrated basic solution and a longer hydrolysis period. In addition to this modification, aniline was determined by coupling with naphthylethylenediamine dihydrochloride (*2*).

Reagents

Sodium nitrite, 2%, prepared fresh daily
Sulfamic acid, 10%, prepared fresh every 3 days
N-1-Naphthylethylenediamine dihydrochloride, 2%, prepared fresh daily
Aniline, Eastman White Label redistilled
Isopropyl *N*-phenylcarbamate, recrystallized twice from Skellysolve B, 99.2% pure

Procedure

Preparation of Standard Curve. To prepare a standard solution of aniline, dissolve 0.1 gram in 100 ml. of 1*M* hydrochloric acid. Dilute this solution 100-fold with 1*M* hydrochloric acid (10 γ per ml.). Place 1-, 2-, and 3-ml. aliquots of this solution in 50-ml. volumetric flasks and add 1*M* hydrochloric acid until the volume is about 40 ml. Add 1 ml. of 2% sodium nitrite and allow 20 minutes for diazotization. Add 1 ml. of 10% sulfamic acid. Allow 15 minutes for complete destruction of excess nitrite; swirl flasks intermittently. After the decomposition of nitrite is complete, add 5 ml. of 2% *N*-1-naphthylethylenediamine dihydrochloride and make to volume with 1*M* hydrochloric acid. After 90 minutes determine the absorbance at 560 mμ. A plot of absorbance *vs.* aniline concentration data obeys Beer's law in the 0.1- to 0.8-p.p.m. range tested, representing 0.19 to 1.54 p.p.m. of IPC, respectively.

Method. Place 1 ml. of an acetone solution containing 20 to 200 γ in a 500-ml. round-bottomed boiling flask. Add 100 ml. of 20% aqueous sodium hydroxide and 30 to 40 ml. of water to simulate conditions encountered in analyzing plant tissue. Add 20 to 30 crystals of Norton 14X Alundum to prevent bumping. Connect a Liebig condenser to the flask and reflux 4 hours on a hot plate. After the hydrolysis period, set up a second Liebig condenser and join it to the first with connecting U-tubes. Stop the water flow in the first condenser and start the flow in the second condenser. Distill the aniline from the mixture with a Bunsen burner and use a 100-ml. beaker containing 20 ml. of 3*M* hydrochloric acid as the receiver. Raise the beaker to the tip of the condenser while the distillate is being received. Carry out the distillation at the rate of about 10 ml. per minute until 50 ml. of distillate has been received. When the distillation is complete, disconnect the second condenser and rinse the condenser tube into the distillate with a few milliliters of 1*M* hydrochloric acid. Transfer the distillate and washings to a 100-ml. volumetric flask and dilute to volume with 1*M* hydrochloric acid. Transfer a 40-ml. aliquot to a 50-ml. volumetric flask and determine aniline as previously described. Take smaller aliquots of the distillate when large amounts of IPC