Improved Colorimetric Determination of Phorate Residues in Plant Tissues

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Improvements to the recently published oxidative colorimetric method for the determination of THIMET phorate in plant tissue, based on the chromotropic acid–formaldehyde reaction, have alleviated the high, erratic absorbance values previously experienced. Modifications include the preparation of solvents by purification with acid alumina and the elimination of the time-consuming Nuchar C-190-N–acid treatment and Nuchar–Celite column. The use of anhydrous sodium sulfate and untreated Nuchar in slurry form, followed by percolating the oxidized phorate through an acid alumina column, then partitioning the phorate from water into benzene proved beneficial to the determination of phorate in plant tissue and the resulting color development. Satisfactory recoveries were obtained from phorate-treated potato and tomato tissues while maintaining low, reproducible reagent and control-blank absorbance values. The sensitivity to detection was not altered appreciably from that of the original method. The method appears to be applicable to a variety of agricultural crops.

THE OXIDATIVE colorimetric method developed by Giang and Schechter (*5*) for the determination of phorate [*O,O*-diethyl *S*-(ethylthio)methyl phosphorodithioate] has met with limited success in the authors' laboratory in its application to the measurement of phorate residues in plant tissues. The absorbance values for reagent and control blanks were generally quite high and erratic, sometimes exceeding the values for treated plant samples which, in turn, were not always reproducible.

Of the limited number of methods available for the determination of phorate residues in plant tissues, the colorimetric method is the most specific, because only those organophosphates having a methylene linkage that will produce formaldehyde upon hydrolysis [Guthion, Trithion, ethion, and Thimet phorate (American Cyanamid Co.)] are sensitive to this color reaction (*5*). The oxidative cholinesterase method (*2*) is much more sensitive for detecting phorate residues, but is much less specific because it is used for the determination of many other organophosphorus insecticides. The results obtained by this latter method are quite difficult to interpret unless the phorate residue is oxidized (*6*) and the degree of anticholinesterase activity of each oxidation product determined. Thus, within its limits of sensitivity, the colorimetric method has definite advantages for phorate residue analysis.

The purpose of the study was to modify the chromotropic acid procedure for formaldehyde developed by Giang and Schechter (*4*, *5*) to provide a reliable, reproducible measurement of the phorate residues possibly present in harvested agricultural crops.

Apparatus

Rinco evaporators equipped with 250-ml., round-bottomed flasks, 100-ml. acetylation flasks, and 20 × 200 mm. evaporation test tubes with ⅌ 24/40 or appropriate ground glass joints.

Separatory funnels with Teflon (polytetrafluoroethylene) stopcocks.

Gooch crucibles with perforated bottom, small No. 2A and No. 4.

Beckman DU spectrophotometer and 1.0-cm. cells.

Waring Blendor with jars.

Reagents

Chloroform, A.C.S. Grade. Purify by passing 1 to 2 liters of solvent through a 20 × 400 mm. absorption column containing 30 to 35 grams of WOELM acid alumina. Prepare either immediately prior to use, or the preceding afternoon, and store overnight in a freezer.

Benzene, A.C.S. Grade. Purify by passing the solvent through an absorption column containing 20 grams of WOELM acid alumina. Although relatively stable, the solvent can be easily prepared immediately before use. Be sure the colored components that separate on the alumina column are not eluted with the pure solvent.

Perbenzoic Acid in Benzene. Prepare and analyze as outlined by Swern (*7*). Pipet 5 ml. of solution into separate 50-ml. volumetric flasks and store in the freezer. Thaw the solution and dilute to volume with pure chloroform before using. Do not use the sample of diluted perbenzoic acid more than 1 day.

Nuchar C-190-N (Activated Vegetable Carbon), Unwashed. Industrial Chemical Sales, Division West Virginia Pulp and Paper Co., Covington, Va.

Sodium Metabisulfite ($Na_2S_2O_5$). 0.5% Solution, freshly prepared.

Chromotropic Acid Reagent. (4,5-Dihydroxy - 2,7 - naphthalenedisulfonic acid, disodium salt), P230 (Distillation Products Industries, Rochester, N. Y.). Recrystallize the salt according to the method of Bricker and Johnson (*1*). Prepare a 0.2% chromotropic acid solution by dissolving 50 mg. of crystallized compound in 25 ml. of concentrated sulfuric acid. Shake vigorously at room temperature until completely dissolved. Prepare fresh daily.

Aluminum Oxide. WOELM, acid (anionotropic), activity grade 1 for chromatography. Alupharm Chemicals, New Orleans, La.

Standard Phorate. 99.+% Pure, American Cyanamid Co., Princeton, N. J.

Procedure

Preparation of Standard Curve. Weigh by difference one drop (approx. 25 mg.) of standard phorate or phorate oxygen analog sulfone into a 50-ml. volumetric flask and dilute to volume with purified chloroform. Pipet 5 ml. of the stock solution into a 100-ml. volumetric flask and dilute to volume with chloroform (1 ml. = 25 μg). Pipet 0, 1, 2, 3, and 4 ml. of this solution into separate 250-ml., round-bottomed flasks and make to 115-ml. volume with purified chloroform. Concentrate the solution to approximately 15 ml. on a Rinco evaporator. Pipet 1 ml. of dilute perbenzoic acid into the sample flask, shake, and incubate the stoppered flasks for 15 minutes in a 45° to 50° C. water bath. Incubate only two samples at a

time to prevent delay in the subsequent removal of excess perbenzoic acid.

After the sample has been incubated 15 minutes, immediately transfer it to a 60-ml. separatory funnel along with two subsequent 5-ml. chloroform washes of the flask. Wash the chloroform solution in the separatory funnel, in order, with 15 ml. of 0.5% sodium metabisulfite; and twice with 15 ml. of saturated sodium chloride. Discard the aqueous layer after each washing. Transfer the chloroform layer to a 100-ml. round-bottomed or acetylation flask and add 20 ml. of distilled water and a few glass beads. Evaporate the solution in the Rinco evaporator at a bath temperature of less than 60° C., until the odor of chloroform is completely removed. Transfer the water solution to a 125-ml. separatory funnel along with three additional 15-ml. distilled water washings of the evaporation flask. Extract the phorate from the water by vigorous shaking with two 20-ml. portions of purified benzene. Separate the benzene and water layers as cleanly as possible each time, and filter the benzene solution into a 100-ml., acetylation flask through a perforated Gooch crucible containing a plug of acetone-cleaned cotton covered with anhydrous sodium sulfate. After the second extraction, wash the separatory funnel with 10 ml. of benzene, and pass this wash also through the sodium sulfate. Evaporate the solvent carefully on the Rinco evaporator. When the volume is concentrated to 5 to 10 ml., pour the solution into an evaporation test tube, rinse the acetylation flask twice with 5 ml. of benzene, add the washings to the tube, and continue the evaporation to dryness.

The color is developed according to the procedures outlined by Giang and Schechter (5). Hydrolyze the residue remaining after evaporation with 3 ml. of 0.5*N* NaOH for 1 minute in a boiling water bath and then cool the solution in a pan of crushed dry ice. Slowly add 3 ml. of chromotropic acid. Develop the color for 30 minutes in a boiling water bath. After cooling the solution to room temperature, read the absorbance in a 1.0-cm. cell with a Beckman DU spectrophotometer at a wavelength of 570 mμ. The curve follows Beer's law and has a slope of approximately 17 μg. of phorate or 20.5 μg. of phorate oxygen analog sulfone per 0.100 unit of absorbance.

Determination of Phorate Residue in Plant Tissue. Macerate 100 grams of washed, cut plant sample with 200 ml. of purified chloroform for about 3 minutes or until the solution is well blended. Strain the extract through four layers of cheesecloth and collect the filtrate in a beaker. Gently squeeze the sample in the cheesecloth to collect any remaining filtrate. Transfer the filtrate to a 250-ml. separatory funnel. Separate the two layers, collect the chloroform phase in a beaker, and discard the water phase. Vigorously stir a little anhydrous sodium sulfate into the chloroform solution until the liquid is free of suspensions. Add 4 grams of Nuchar C-190-N to the solution, and stir vigorously for several minutes. Filter the chloroform solution through a perforated Gooch crucible fitted with a cotton plug covered with anhydrous sodium sulfate, and collect the clear liquid in a graduated cylinder. Record the volume collected. A reagent blank consisting of an equal amount of chloroform should also be introduced as a sample at this point. Evaporate the solution to 25-ml. volume on the Rinco evaporator. Transfer the chloroform solution to a 125-ml. Erlenmeyer flask equipped with a ground glass stopper; retain the evaporation flask for subsequent use. Add 1 ml. of dilute perbenzoic acid and incubate at 45° to 50° C. for 15 minutes. Proceed as with the standard curve to and including the evaporation of chloroform from the water solution.

Prepare an alumina column by placing 3 grams of WOELM acid alumina in a 10 × 300 mm. absorption column equipped with a sintered glass filter. Wash the alumina with 10 to 15 ml. of distilled water. Percolate the water solution of oxidized phorate through the alumina column. Slight pressure may be necessary, but the flow rate should not exceed 60 drops per minute. Rinse the evaporation flask three times with 15-ml. portions of water and add these washings to the column when the previous solution level reaches the surface of the alumina. Transfer the eluate to a 125-ml. separatory funnel. Extract the phorate with benzene and proceed with the color development as outlined in the procedure for the standard curve. A control sample of untreated plant material should be analyzed along with the treated samples as a basis for determining the intensity of the color attributed to the phorate residue in the sample. The amount of phorate present in the sample can then be calculated from the following formula:

$$\text{p.p.m. phorate} = \frac{\mu\text{g. read from standard curve}}{\text{ml. of chloroform recovered from sample}} \times \frac{200 \text{ ml. } CHCl_3}{100 \text{ grams tissue}}$$

Results

The procedure as outlined in the previous section was applied to the recovery of phorate from potato tubers, green tomatoes, and red tomatoes. Known amounts of phorate in methylene chloride were added to a series of samples in Waring Blendor jars prior to maceration. Standard solutions of known phorate content without the presence of plant tissue were also analyzed by the entire procedure to substantiate the efficiency and reproducibility of the method. The

Table I. Recovery of Phorate from Plant Tissues

Plant Tissue	No. of Samples	Absorbonce Range for Reagent Blanks	Phorate Added to Plant Tissue, P.p.m.	Phorate Recovered: Apparent phorate in control blanks, P.p.m.	Phorate Recovered: Range of Recovery, P.p.m.	Phorate Recovered: Range of Recovery, %	Phorate Recovered: Average recovery, %
Green tomatoes	10	0.132–0.171	0.5	0.00	0.426–0.558	84.9–111.6	94.76
	3	0.153	0.2	...	0.162–0.214	80.7–106.5	90.8
	10	0.103–0.133	0.5 (Oxygen analog sulfone)	0.01	0.376–0.628	78.0–130.2	100.57
Red tomatoes	8	0.126–0.196	0.5	0.01	0.204–0.385	40.8–76.8	62.5
Potatoes	12	0.074–0.104	0.5	0.02	0.311–0.472	57.9–94.4	72.5
	10	0.148–0.152	0.5 (Oxygen analog sulfone)	0.00	0.556–0.647	95.4–111.1	102.31
Standard solutions[a]	4	0.132–0.171	0.5	...	0.375–0.585	74.8–117.0	94.13
	4	0.103–0.133	0.5 (Oxygen analog sulfone)	...	0.512–0.624	104.8–129.3	113.1

[a] Standard solutions without the influence of plant material were analyzed with most sample series as an added check on the efficiency and reproducibility of the method.

ecoveries of phorate are shown in Table I. In most cases, the recoveries were fairly close to the average recovery reported, although the range, including the extremes, is also recorded.

Discussion

Several solvents were investigated to find one that would satisfactorily extract phorate from plant tissue and at the same time produce a low reagent blank. Benzene produced the lowest and most reproducible reagent blank, but apparently extracted only 50% of the phorate. Chloroform, on the other hand, was the most efficient extractor, but yielded somewhat high and erratic reagent-blank values. Purification of the solvents by percolation through the alumina column not only proved to be faster than the conventional method of washing with water and redistilling, but also tended to control the erratic behavior of the chloroform reagent blank. However, the absorbance remained high. It was found advantageous to use both solvents—chloroform for extracting the phorate from the plant tissue and dry benzene as the final extractor prior to the development of color. It was important that both solvents be free of impurities and that good analytical conditions be maintained in order to obtain valid and reproducible results.

There are two other causes of variation in the color reaction that should be considered. These are the presence of formaldehyde in the laboratory atmosphere and the presence of phosgene in the chloroform. Thornberg (*8*) found that formaldehyde was not only a common air-borne contaminant in industrial areas, but was also produced by laboratory burners and by heating and air conditioning equipment. He found that phorate standard curves went up tremendously on still, humid, warm days and also on winter days when all heating equipment was operating constantly. On a winter day when the room had not been heated for 12 hours or more, the results were constant. Excessive variation from day to day in the reagent blanks and the recoveries could thus very likely be caused by the climatic fluctuations and the subsequent means of laboratory temperature regulation.

Phosgene, which is a normally occurring oxidation product of chloroform, attacks most phosphate esters very readily (*8*). It forms in distilled chloroform quite rapidly, although low temperatures reduce the rate considerably. Unexplainable, low, erratic recoveries of phorate could be due possibly to an occurrence of phosgene in the chloroform. However, in this laboratory, variations were minimized by using alumina-purified chloroform in preference to the redistilled.

To study the effect of phosgene on the color reaction of chromotropic acid and hydrolyzed phorate, a series of standards was prepared in chloroform solutions that had been treated with phosgene. The phosgene gas was bubbled into pure chloroform, and then aliquots were diluted to obtain the desired phosgene concentration. As little as $1.13 \times 10^{-5}\%$ phosgene produced slight inhibition; approximately $7.0 \times 10^{-5}\%$ caused 50% inhibition; $1.6 \times 10^{-4}\%$ caused 75% inhibition; and $5 \times 10^{-4}\%$ and greater completely inhibited the chromotropic acid–formaldehyde color developed from 50 μg. of phorate.

The effect of phosgene on phorate, itself, was studied by infared analysis. There was no observed difference in the spectra of 99.+% pure phorate and that into which an excess of phosgene had been bubbled, except that, in the latter sample, the phosgene peak at 5.57 microns gradually disappeared over a period of several days. However, the phorate quite rapidly became dark yellow in color after being treated with phosgene. When a standard of phorate in phosgene-treated chloroform was compared with a nontreated standard, a noticeable difference in the spectra attributed to the S—CH_2 functional group of phorate was found in the region of 8.2 to 8.9 microns. The significance of this change was not determined, and there were no further changes in the spectrum after several days of sample storage.

Infrared analysis, gas chromatography, and a phenylhydrazine–cinnamate spot test (*3*) were investigated as means for detecting minute traces of phosgene in chloroform. Under the conditions of this study, none of these methods had the sensitivity required to detect the concentrations of phosgene which had previously proved to be detrimental to the color reaction. Infrared analysis was not sensitive enough to detect the build-up of phosgene during storage of pure and contaminated solvent. It did, however, show that a reaction was taking place, because an additional absorption peak appeared at 6.27 microns which almost equaled the magnitude of the phosgene peak at 5.57 microns in a 0.05% solution.

The concentration of chromotropic acid used in the original colorimetric procedure (*5*) was 1.6%. This is equivalent to a weight ratio of 4288 to 1 of chromotropic acid to formaldehyde, based on the amount of formaldehyde hydrolyzed from 50 μg. of phorate in 100 grams of plant tissue. Investigation of the color reaction of 5 μg. of formaldehyde (approx. 50 μg. of phorate) with various concentrations of chromotropic acid verified the weight ratio of 500 to 1, previously indicated by Bricker and Johnson (*1*), as giving the optimum color. This ratio corresponded approximately to a 0.2% chromotropic acid solution, which in turn resulted in a more easily dissolved, lighter colored reagent, and subsequently a much lower absorbance in the reagent blank.

The use of anhydrous sodium sulfate in the cleanup procedure removed water and suspended material that had been retained in the extract, and eliminated the need for centrifugation. Nuchar was important in removing plant pigments which previously caused the formation of emulsions during the washing steps following oxidation of the phorate. Acid alumina was found to be a very efficient final cleanup medium. The color of the resulting chromotropic acid–formaldehyde reaction product was very clear and distinct. However, the phorate must be oxidized prior to its percolation through the column in order to obtain satisfactory recoveries. Neither phorate nor its oxidation products were satisfactorily eluted from the acid alumina column by chloroform, benzene, acetonitrile, or petroleum ether; but approximately 100% recovery was obtained when the oxidized phorate was applied to the column in a water solution and eluted with water.

Extreme care must be exercised during the course of analysis. Contamination from glassware, the Rinco evaporator, and other sources, and condensation of the steam from the boiling water bath had definite effects on the color reaction. It is sometimes advisable to include a standard sample with the treated plant samples to test the validity of the results. Occasionally, the recoveries on a particular day may appear to be completely erroneous. In this study, one day's analysis of oxygen analog sulfone gave recoveries of 126.0 to 130.2%. A standard sample analyzed at the same time had a value of 129.3% recovery (Table I). Thus, a good relationship still existed, and the recoveries were considered valid.

Although recovery data are presently available for only tomatoes and potatoes, the method appears applicable to the analysis of other vegetable crops of similar composition. The cleanup procedure has been used successfully in this laboratory for the preparation of green bean samples analyzed by the cholinesterase inhibition method. However, oil-bearing crops, such as peanuts, peanut foliage, and possibly other forages, may require additional or alternate cleanup techniques before the phorate residue can be measured. It was noted in this laboratory that, although the phorate extract of peanut foliage appeared to be clear and free from contamination following the cleanup procedure, a substance was retained which imparted an orange pigment to the pink-violet color of the chromotropic acid–formaldehyde reaction. This phenomenon made it difficult to interpret the recovery data, especially at low concentrations. This problem has not yet been resolved.

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INSECTICIDE RESIDUES

Chemical Residues in the Milk of Cows Grazed on Chlordan-Treated Pasture

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Dairy cows were grazed on plots of pasture grass that had been treated with granular chlordan at 0.25, 0.50, or 1.0 pound per acre. Analysis of milk samples from the cows revealed the presence of small amounts of heptachlor epoxide (less than 0.1 p.p.m.) that persisted to the end of the experiment, 8 weeks after treatment. Chlordan was also detected in the milk of cows on the 0.5- and 1.0-pound plots, but levels of chlordan plus heptachlor epoxide were generally below 0.1 p.p.m. The identity of the heptachlor epoxide was confirmed by gas chromatographic and spectrophotometric procedures.

CHLORDAN has been proposed for use on pasture land to control the imported fire ant (*Solenopsis saevissima richteri* Forel) provided such use will not result in undesirable chemical residues in the milk of dairy cows grazing thereon. The experiment reported here was designed to furnish data upon which to base a decision on the feasibility of using chlordan.

A farm was selected near Gulfport, Miss., and four plots of good grassland were laid out for the experiment. The soil was light, sandy loam, well covered with a mixture of bahia grass and lespedeza. The forage was not as succulent as desirable for the best feed, but the cattle foraged readily and ate considerable quantities of the grass. On September 25, 1961, three of the plots were treated with granular formulations at estimated rates of 0.25, 0.5, or 1.0 pound of technical chlordan per acre. Exact calculations based upon plot areas and weights of formulation applied revealed that the plots actually received 0.24, 0.43, or 0.96 pound per acre. The fourth plot was left as an untreated check. Treatment was made under ideal conditions, but rainfall occurred about 30 minutes later.

On September 26, two cows were placed on each treated plot and the untreated check plot. Six additional cows were also placed on the check plot to be used on treated pasture at later dates. On October 2, 1 week after treatment, two additional cows were placed on the 0.5-pound plot, and, on October 9 and 24, 2 and 4 weeks after treatment, two more cows were placed on this plot on each date. The experiment was terminated on November 21, 8 weeks after application of the insecticide.

The cattle were predominantly Jersey, but were mixed with Holstein and beef breeds. They were very thin.

Milk samples were taken from all of the cows before the experiment was begun and analyzed to determine whether or not there was any contamination. The first posttreatment samples were collected from each cow 24 hours after exposure, and daily milk samples were taken for 2 weeks. Samples were then taken on a weekly basis during the remainder of the exposure period. The milk samples were processed to extract the butterfat in the laboratory of the Methods Improvement Operations, Plant Pest Control Division, Gulfport, Miss., and the butterfat was shipped by air to the pesticide chemists of the Entomology Research Division at Beltsville, Md., for analysis.

Samples of grass and soil from the treated plots were collected and analyzed in the Gulfport laboratory.

Analytical Procedure

Milk Samples. Milk samples were first put through a cream separator to separate most of the butterfat in heavy cream. Approximately 150 ml. of the cream was placed in a 1-quart fruit jar equipped with a rubber-sealed lid, granular anhydrous sodium sulfate added, and the contents mixed until the cream was dehydrated. About 300 ml. of *n*-pentane was added, the lid sealed, and the jar shaken vigorously for several minutes. The butterfat and insecticide were extracted by the pentane under these conditions. The pentane solution was then decanted into an Erlenmeyer flask equipped with a three-ball Snyder column and the solvent removed on a steam bath. The last traces of pentane were removed at room temperature, under vacuum. The

Table I. Recovery of Chlordan and Heptachlor Epoxide from 10 Grams of Butterfat

μG. Added	% Recovered
CHLORDAN[a]	
5	80
10	75
20	79
HEPTACHLOR EPOXIDE[b]	
5	90
10	97
20	98

[a] Corrected for check sample reading of 3 μg.
[b] Check sample reading was zero.