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INSECTICIDE MEASUREMENT

Determination of Toxaphene by a Spectrophotometric Diphenylamine Procedure

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A confirmatory method for the positive identification of toxaphene in small residues has not previously been reported. A sensitive method for the qualitative and quantitative determination of toxaphene in residues and in formulations is based on fusion with diphenylamine in the presence of zinc chloride. The greenish blue reaction product, dissolved in acetone or glacial acetic acid, has an absorbance maximum at 640 mμ. Toxaphene in the range of 20 to 700 γ can be readily determined by spectrophotometric measurement. When combined with cleanup procedures, the method has been applied to extracts of crops and foods and to various formulations, including mixtures with sulfur or DDT.

THE need for an accurate method for the specific determination of micro quantities of toxaphene in food and forage crops and in milk and beef has become increasingly apparent with the more widespread use of this insecticide. Toxaphene is chlorinated camphene containing 67 to 69% chlorine. Microdetermination by a sensitive method for organic chlorine (*5*) has been widely used for determining toxaphene residues, but this method lacks specificity. Macrodetermination by an infrared method (*4*) is specific, but has not been employed as a residue method because of low sensitivity. A qualitative colorimetric test with pyridine and methanolic potassium hydroxide has been reported (*3*). A spectrophotometric method based upon the reaction of toxaphene with thiourea in the presence of alkali, developed by Hornstein (*2*), is quantitative in the 0.5- to 5.0-mg. range. However, there is need for a more sensitive method.

The reaction of DDT with diphenylamine in the presence of zinc chloride has been reported to give a reddish orange color with an absorbance maximum at 490 mμ (*1*). Toxaphene reacts with these reagents at high temperature and in the absence of solvents to give a greenish blue complex which is soluble in acetone or glacial acetic acid and exhibits an absorbance maximum at 640 mμ; the absorbance–wave length curve is shown in Figure 1 and the spectrophotometric constants are given in Table I. This reaction has been made the basis of a sensitive quantitative method for both residue and assay analyses. Toxaphene in the range of 100 to 700 γ can be readily determined with an accuracy and precision to ±2%; in the 20- to 100-γ range, ±7% is usual. Naturally occurring organic chlorine and most chlorinated insecticides do not interfere; however,

Table I. Sensitivity Constants for Toxaphene Spectrophotometric Procedure

Absorptivity, a, liter/gram cm.	17.0
Molar absorptivity, ϵ, liter/mole cm.	7.0×10^3
Absorbance concentration, γ/ml. (γ/ml. required to give absorbance of 1.00 in 1-cm. cell)	58.8

Measurements made at 640 mμ

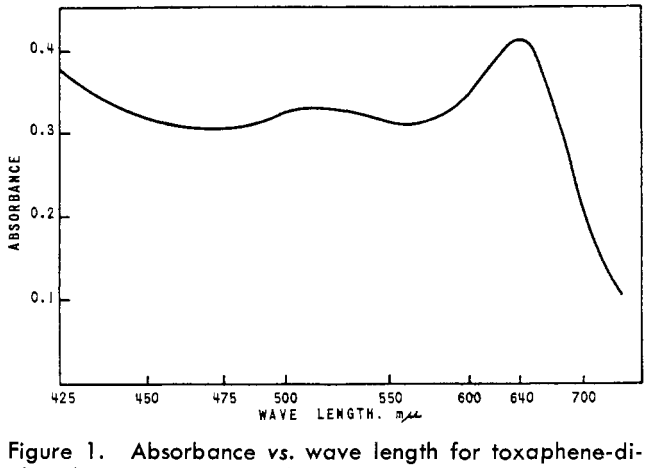

Figure 1. Absorbance *vs.* wave length for toxaphene-diphenylamine reaction product

Table II. Recovery of Toxaphene from Fortified Extracts[a]

Extract	Fortification Level, P.P.M.	Av. Control, P.P.M.[b]	Cleanup Procedure	Detns.	Av. Recovery, %	Std. Dev. of % Recovery
Alfalfa, green	1–5	0.2	A	2	69	..
	10–50	0.3	A	5	91	5
Barley, grain[c]	1–7	0.5	A	7	74	6
Beef fat	7	1.3	CA	5	72	10
Milk[d]	0–1	0.1	B	11	74	10
Milk	0–1	0.06	CA	7	78	7
Oats, green	7	0.6	A	2	75	..
Pears	1–4	0.2	BA	3	98	8
Rice, grain	1–7	0.4	A	7	76	7
Rice, straw	7–20	3.5	A	5	93	12
Tobacco, cured	10–1000	3.1	A	19	78	12
Tomatoes	3–10	0.5	A	6	100	6
Wheat, grain	0–3	0.2	A	6	83	1

[a] Range for colorimetric determination 0 to 700 γ generally.
[b] Not corrected for solvent blank.
[c] 0 to 100 γ. [d] Both ranges.

plant and animal extractives must be eliminated when the method is applied to residue analysis. For assay of toxaphene formulations, an aliquot of a methanol solution of the active ingredients may be used directly.

Experimental

Reagents. Diphenylamine, recrystallized from *n*-hexane, 0.5% solution in acetone.

Zinc chloride, reagent grade, 0.5% solution in acetone, filtered, prepared fresh daily.

Acetone, reagent grade.

Methanol, absolute.

n-Hexane (Skellysolve B, Solvents Division, Skelly Oil Co., St. Louis, Mo., or redistilled).

n-Hexane, acid-treated with fuming sulfuric acid supported on dry Celite 545, stirred for an hour, filtered through glass wool, washed with cold water, dried over anhydrous sodium sulfate, and redistilled.

Methylene chloride, redistilled.

Sulfuric acid, concentrated, 96%.

Sulfuric acid, fuming, 20 to 23% SO_3.

Florisil, 60 to 100 mesh (Floridin Co., Tallahassee, Fla.), conditioned by heating at 125° to 130° C. for 24 hours.

Celite 545 (Johns-Manville Corp., 4th and Chestnut Sts., Philadelphia 6, Pa.), dried at 110° C. for at least 2 hours.

Sodium sulfate, anhydrous, granular, reagent grade.

Apparatus. Chromatographic tubes, 28 mm. (inside diameter) × 340 mm., tapered sharply to a stem 8 mm. (outside diameter) × 50 mm.

Chromatographic tubes, No. 2 (20 mm.), Scientific Glass Co., Rahway, N. J., or equivalent.

Glass packing rod, 8 × 500 mm. glass rod, with flattened disk at one end 20 mm. in diameter.

Fritted-glass crucibles, medium porosity, and crucible holders.

Fisher Filtrator or other vacuum filtering apparatus.

Test tubes, 150 × 16 mm.

Oil bath, 205° C.

Spectrophotometer, Beckman Model B with red phototube or equivalent, and 1-cm. covered cells.

Preparation of Standard Curves

0- to 700-γ Range. An acetone or *n*-hexane solution of toxaphene containing 100 γ per ml. is prepared. Aliquots (1, 3, 5, and 7 ml.) of the solution are pipetted into separate test tubes, and 1 ml. each of the diphenylamine and zinc chloride solutions are added. A reagent blank is also prepared. The solutions in the test tubes are evaporated to dryness in a 60° to 70° C. water bath; a rapid stream of dry air is used to speed evaporation. The residue should be deposited in a thin film along the bottom inch of the test tube; rinsing down the side walls with a little acetone near the end of the evaporation may be necessary.

The test tubes are then immersed in a 205° C. oil bath for 3 minutes. After cooling, the greenish blue complex is dissolved in a little acetone and transferred quantitatively to a 25-ml. volumetric flask. The volume is adjusted to 25 ml. with acetone. This color is stable after a 15-minute interval for about 1 hour, then gradually fades. The fading is not due to a wave length shift of the peak.

The absorbance is measured at 640 mμ in covered 1-cm. cells; acetone is used to zero the instrument. The standard curve for this range is obtained by plotting absorbance *vs.* micrograms of toxaphene; Beer's law is obeyed.

0- to 100-γ Range. The same procedure is used, except that 0.2-, 0.4-, 0.6-, 0.8-, and 1.0-ml. aliquots of the standard solution are measured (a more dilute standard solution appears to degenerate on standing), and the colored complex is diluted to 5 ml. with acetone.

Removal of Interfering Substances

As with nearly all pesticide residue methods, the plant pigments and waxes and animal fats extracted with the pesticide interfere in its subsequent determination, and suitable cleanup procedures must be applied. The presence of more than 5 mg. of extraneous material affects the fused reaction mixture significantly and toxaphene recoveries are low. Three separation techniques have been used. Either alone or in combination they have been adequate for the preliminary treatment of all the crop extracts and fats shown in Table II.

Procedure A. Florisil Column. This procedure is used for the isolation of toxaphene from crop extracts which contain relatively small amounts of waxes, fats, or oils. It serves as the final cleanup step for extracts treated initially with sulfuric acid to remove large amounts of waxes, fats, or oils. Toxaphene is adsorbed weakly by Florisil, and is held just long enough to permit elution of most waxy or oily materials in a preliminary *n*-hexane cut.

A large tube (28-mm. inside diameter) is plugged with glass wool and packed with conditioned Florisil to a height of 15 cm. A circle of coarse filter paper prevents disturbance of the packing when solvent is added. An aliquot of *n*-hexane extract of a control sample is concentrated to 5 to 10 ml. and fortified with a known amount of toxaphene. Usually 300 to 500 γ of toxaphene in an extract from 50 to 100 grams of sample is used. The fortified extract is rinsed onto the dry column with small amounts of *n*-hexane and elution with this solvent is continued until 20 ml. of eluate has been collected. This cut, which contains the bulk of the wax, is discarded. Then the toxaphene is eluted with 125 ml. of 1 to 1 methylene chloride–*n*-hexane. This cut is evaporated to a small volume and transferred to a test tube with *n*-hexane, and the insecticide is determined by the spectrophotometric method. As the adsorptive capacity of Florisil seems to vary slightly from batch to batch and the interfering materials in crop extracts vary, a check on the recovery from a fortified control is necessary. If recovery is low, variations of the wax-cut volume must be tried—wax cuts from 10 to 30 ml. have been used in this laboratory. When the Florisil column is used in conjunction with Procedure B or C, a 10-ml. wax cut is generally satisfactory.

Procedure B. Sulfuric Acid Column. Toxaphene is stable in concentrated sulfuric acid–fuming sulfuric acid mixtures. Procedure B is a partitioning system between sulfuric acid and *n*-hexane for use with small fat samples (2 to 5 grams) or *n*-hexane crop extracts containing acid-sensitive material.

For fat samples, the large (28-mm. inside diameter) tube is used. After insertion of a glass wool plug, 10 grams of dry Celite 545 is packed in the bottom of the tube and tamped down firmly with the glass tamping rod. Then 18 ml. of 3 to 1 concentrated sulfuric acid–fuming sulfuric acid is triturated with 30 grams of dry Celite 545 in a mortar

Table III. Spectrophotometric and Organic Chlorine Analyses on Grain Extracts[a]

	Toxaphene, P.P.M.	
	Org. Cl	Spectro
Controls		
Barley 1	2.6, 2.8	0.6, 0.4
Barley 1, whole	0.3	0.4
Barley 2	2.5, 3.1	0.5
Barley 2, whole	0.7	0.4
Wheat 1	0.6	0.2
Rice 1, whole	1.2	0.4
Samples, corrected for control		
Barley 3	1.1	0.7, 0.9
Barley 4	1.7	1.4
Barley 5	6.7	7.1
Barley 6	13.0, 16.0	14.2
Wheat 2	0.3	0.3
Wheat 3	0.5	0.3
Wheat 4	0.3	0.5
Wheat 5	1.8	1.4
Wheat 6	1.4	1.6
Rice 2, whole	0.6	0.5
Rice 3, whole	2.3	1.6
Rice 4, whole	4.4	5.8
Rice 5	1.3	2.1
Rice 5, whole	1.2	1.6, 2.8

[a] Grain mealed in coffee grinder before extraction, except when designated "whole."

and packed above the dry Celite in at least three portions. The column is covered with a circle of coarse filter paper and acid-purified *n*-hexane is added until the column is saturated. An *n*-hexane solution of the fat sample, concentrated to 5 to 10 ml., is forced into the column with the aid of approximately 2 pounds of nitrogen or air pressure. The column is developed with the acid-purified *n*-hexane; the first 50 ml. is discarded and the next 150-ml. cut which contains the toxaphene is collected.

For crop extract samples, a smaller tube is adequate. Using a No. 2 chromatographic tube, the column is prepared as described above; 2 grams of dry Celite 545 are used in the bottom and 10 grams of dry Celite mixed with 6 ml. of the acid mixture on the top. The column is eluted with *n*-hexane; the first 15 ml. is discarded. The toxaphene is eluted in the following 60 ml.

Procedure C. Sulfuric Acid–Methylene Chloride Extraction. For samples containing large amounts of fat, Procedure B is inadequate. A separatory funnel extraction procedure, similar to Hornstein's (*2*), with subsequent column cleanup has proved satisfactory.

An aliquot containing approximately 30 grams of fat is concentrated by evaporation, and the residue transferred to a separatory funnel with 200 ml. of methylene chloride. The fat solution is extracted with three 50-ml. portions of concentrated sulfuric acid. A little sodium sulfate may be added to reduce emulsions. The acid layers are combined and back-washed with 100 ml. of methylene chloride. After 15 minutes have been allowed for the layers to separate, the acid is drained into a large beaker of crushed ice and discarded. The methylene chloride extracts are combined and concentrated to about 100 ml. Traces of acid are removed by passing through a chromatographic tube 28 mm. in inside diameter packed to a height of 15 cm. with 1 to 1 sodium sulfate–Celite 545, tamped firmly. The column is rinsed with 100 ml. of methylene chloride, and the solution is again concentrated to 100 ml.

The toxaphene and waxes must be transferred (without taking to dryness) to *n*-hexane solution, so that the final Florisil column cleanup described in Procedure A may be used to remove the waxes. This is done by adding an equal volume of *n*-hexane, evaporating to about 25 ml., again adding an equal volume of *n*-hexane, and finally concentrating to about 10 ml. All of the methylene chloride is thereby removed, and the final *n*-hexane solution is chromatographed according to Procedure A; a 10-ml. wax cut is discarded.

Isolation and Determination of Toxaphene Residues

Toxaphene-treated crop samples are appropriately fragmented and subsampled. The subsample, usually 0.5 to 1 kg., is extracted by rolling or shaking for at least 1 hour with *n*-hexane, or 2 to 1 *n*-hexane–isopropyl alcohol if the moisture content is high. The extract is water-washed several times with portions at least one third of the organic solvent volume, and then dried over anhydrous granular sodium sulfate.

Any toxaphene in a sample of milk or meat will be concentrated in the fat. Butterfat may be isolated by precipitating the milk proteins and fat at a low pH, drying, and extracting the fat with *n*-hexane, or by freeze-drying the whole milk and extracting the fat from the solids. Meat samples are generally ground with sodium sulfate before extraction. The *n*-hexane extracts from milk or meat are then washed and dried as for crop extracts.

The recovered volumes of the extracts are measured after filtration from granular sodium sulfate, and the proportion of sample represented by the recovered extract is calculated. The extracts are concentrated without loss of insecticide in Kuderna Danish evaporative concentrators equipped with a simple spray trap. The concentrated extract is then transferred to a volumetric flask and diluted quantitatively to a known volume with *n*-hexane. Aliquots containing an estimated 0 to 700 γ of toxaphene are taken for purification by Procedure A, B, or C. The resultant toxaphene cut, freed of all pigments and fats and all but a few milligrams of waxes, is evaporated to a low volume (but never to dryness) and transferred with acetone and *n*-hexane to a test tube for the diphenylamine reaction. The procedure given under Preparation of Standard Curves is followed, and the absorbance of the colored toxaphene complex is measured at 640 mμ. If a small amount of wax is present, it may inhibit the dissolving of the colored complex in acetone or cause a slight cloudiness in this solvent. Up to 40% *n*-hexane may be used in the final dilution to overcome this.

It is advisable to run several standards along with the samples in the spectrophotometric procedure to check the standard curve. By doing this, the likelihood of gross errors caused by unrealized variations in reagents and procedure is greatly reduced. The absorbance of the sample solution is translated to micrograms of toxaphene by reference to the standard curve.

Assay of Toxaphene Formulations

Dusts and wettable powders are weighed in fritted glass crucibles; at least 1-gram portions should be taken to ensure a representative sample. Each sample is extracted with a total of 35 ml. of boiling methanol in several increments; vacuum is used to speed the filtration. The methanol extract is cooled to room temperature and diluted to 50 ml., with filtering before dilution to volume if a sulfur precipitate appears. An aliquot of this solution is further diluted with acetone, so that the final solution contains about 500 to 700 γ of toxaphene per 5 ml. Emulsifiable concentrates are weighed and diluted with acetone to this concentration. A 5-ml. aliquot if sulfur was absent, or a 3-ml. aliquot from sulfur-containing formulations, is then pipetted into a test tube and the procedure given under Preparation of Standard Curves is followed. For the reasons noted in the residue determination section, a few standards should be run with the samples in the fusion reactions. Micrograms of of toxaphene in the aliquot are obtained graphically from the standard curve and the per cent toxaphene in the sample may then be calculated.

Discussion

Recovery from Fortified Extracts. Recovery data for various extracts to which known amounts of toxaphene have been added are shown in Table II. All are in the range of 70 to 100%.

Separation Procedure A yields a blank value equivalent to 10 to 30 γ of toxaphene, and the average control values are a reflection of this blank with

Table IV. Reaction Products of Other Chlorinated Insecticides and Interference in Toxaphene Method

Insecticide	Product Color	Abs. Max., Mμ	% Apparent Toxaphene Recovery from Mixture[a]
Rhothane	Yellow	<420	96
DDT	Orange-red	490	102
Lindane	Colorless	None	102
Endrin, 1-hr. soln.	Lt. green	General	104
Heptachlor	Lt. green	General	107
Methoxychlor	Yellow-green	<420	113
Aldrin	Lt. purple	525, >700	129
Endrin, 72-hr. soln.	...	...	130
Dieldrin	Lt. purple	490, 680	132
Chlordan, 72-hr. soln.	...	...	138
Chlordan, 4-hr. soln.	Blue	650	175

[a] Approximately 300 γ of each insecticide combined with 300 γ of toxaphene just before analysis.

but one exception. Rice straw control extract was contaminated with toxaphene, or contained an impurity which was incompletely removed by Procedure A and reacted with diphenylamine. Recovery of 0 to 100 γ of toxaphene in the absence of plant extractives was 98 ± 10% from Procedure A after correction for the separation procedure blank.

Comparison of Spectrophotometric and Organic Chlorine Analyses. Grain extract analyses by spectrophotometric and organic chlorine (*5*) methods, corrected for the appropriate control, are in good agreement, as shown in Table III. Comparison of data obtained from the whole grain and mealed grain extracts of the same sample shows clearly that all of the toxaphene is at or near the surface. The barley contains about 2 p.p.m. of naturally occurring organic chlorine, which is extracted from the mealed samples only.

The organic chlorine method was not applied to many of the other samples because they had been treated with other chlorinated materials in addition to toxaphene or because they contained high and variable amounts of naturally occurring organic chlorine.

Interference from Solvents and Other Insecticides. Interferences in the spectrophotometric method for toxaphene occur, but can usually be detected and eliminated. A trace impurity in carbon tetrachloride and chloroform (possibly phosgene) gives an azure blue color with an absorbance maximum at 600 mμ. Redistilled methylene chloride is free of such impurities. Nitromethane gives the same type of interference and is not recommended for preliminary separations or cleanup. Sulfur, which may be present in dust formulations, gives an emerald green color with toxaphene in the diphenylamine method, and hydrogen sulfide is evolved during the fusion reaction. Sulfur is difficult to separate from toxaphene at the residue level, and greatly enhanced absorbance results if the amount present is more than 10% of the toxaphene residue. Interference in the assay procedure is eliminated by using a limited volume of methanol, in which sulfur has a very low solubility, as the extracting solvent. Pentachlorophenol, which may be sprayed just prior to harvest to reduce moisture in rice, does not interfere.

A number of other chlorinated insecticides have been tested alone and in 1 to 1 mixtures with toxaphene. All except lindane give some color, but absorbance at 640 mμ is weak for most. Aldrin, dieldrin, and chlordan have absorbance peaks at other wave lengths which overlap the 640-mμ band. Toxaphene recoveries from mixtures (300 γ of each insecticide) are shown in Table IV. A freshly prepared solution of endrin in acetone did not interfere, but upon aging the reactivity approached that of dieldrin and aldrin. Chlordan solutions in acetone showed a decrease in reactivity after aging, but interference in toxaphene determinations was still serious. In practice, no serious interference problem from other chlorinated organic insecticides in this method is anticipated, as 1 to 1 residue mixtures of toxaphene with dieldrin, aldrin, or chlordan are unlikely. They are seldom, if ever, applied together and dieldrin and aldrin are used at much lower dosage levels than toxaphene. There is no problem in distinguishing between chlordan and toxaphene, because methods specific for chlordan are known.

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INSECTICIDE RESIDUES

Toxaphene Residues on Pangolagrass

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THERE are approximately one-half million acres of improved pangolagrass in Florida, and toxaphene is one of the leading commercial insecticides recommended for the control of caterpillars on this pasture grass. One of the primary needs of all cattlemen at this time is definite information on a safe interval between pasture grass insecticide treatment and turning in beef cattle for indefinite periods of grazing.

This paper summarizes data involving toxaphene residues on pangolagrass exposed to the varying weather conditions of north, central, and south Florida

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