deviation). As a result of these studies, acetic and dichloroacetic acids are recommended as lubricants for the nitration apparatus. Good seals of the joints were obtained, and the reproducibility of absorbance values of standards, when these lubricants were used, was good—the percentage deviation being about one third to one fifth of the deviation found when phosphoric acid was used as the lubricant.

These findings emphasize the limitations and some of the critical points of the present colorimetric method for lindane. As a result of further research, especially on the removal of interfering materials, the Schechter-Hornstein method can probably be modified to include a wider variety of plant and animal products.

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INSECTICIDE RESIDUES

Colorimetric Determination of Toxaphene

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A method has been developed for determining as little as 0.5 mg. of toxaphene or 0.25 mg. of Strobane in 5 ml. of solution, and has been applied to alfalfa and butterfat extract. In tests with other chlorinated insecticides, interferences were encountered only with chlordan and heptachlor.

RECENT FEDERAL LEGISLATION has made it necessary to establish tolerances for pesticide residues. The establishment of a tolerance implies that analytical methods are or will be available for accurately estimating residues. Analytical data obtained by nonspecific tests such as bioassay techniques, total chlorine determination, or cholinesterase inhibition are considerably strengthened if a reasonably specific chemical method is also available for determining the pesticide in question.

A tolerance of 7 p.p.m. has been established for toxaphene on many agricultural crops. Although bioassay and total-chlorine methods can be used in obtaining residue data, no specific method for toxaphene is at present available. Kenyon (*2*) described an infrared procedure of qualitative value, and Johnson (*1*) described a test for the detection of toxaphene in formulations in which pyridine and methanolic potassium hydroxide are used to give a Fujiwara-type test.

In general, toxaphene and Strobane respond to the same reagents in similar fashion, as they are closely related materials. Toxaphene is a mixture of chlorinated camphenes, Strobane is a mixture of chlorinated terpenes, both approximately 68% chlorine.

In this paper, a method is described for determining a minimum of 0.5 mg. of toxaphene or 0.25 mg. of Strobane in a final volume of 5 ml. of solution. Although this is by no means as high a sensitivity as might be desired, it should prove useful in view of the 7 p.p.m. tolerance. For example, a 500-gram sample containing 4 p.p.m. of toxaphene would yield 2 mg. of the insecticide, an amount readily determined by this procedure.

The method is based on the reaction of the several related compounds in toxaphene or Strobane with thiourea in the presence of alkali to give a yellow color that may be measured photometrically. Although thiourea reacts with acyl, alkyl, and heterocyclic halides in inert solvents to give pseudothioureas, this reaction does not take place when toxaphene or Strobane is refluxed with thiourea in isopropyl alcohol or isopropyl alcohol plus water. However, in the presence of a small amount of strong alkali the development of a yellow color starts almost immediately and is enhanced by heating. The reaction appears to be catalyzed by a strong base and does not take place in a medium that is only slightly alkaline.

Experimental

Reagents

Isopropyl alcohol, 99%.

Thiourea, recrystallized from methanol, 2% w./v. solution in 2% w./v. aqueous potassium hydroxide.

Magnesium silicate (chromatographic grade Florisil), obtainable from Floridin Co., Tallahassee, Fla.

n-Hexane, redistilled.

Methylene chloride, redistilled.

Sulfuric acid, concentrated.

Preparation of Standard Curve. An isopropyl alcohol solution of toxaphene containing 1 mg. of toxaphene per ml. is prepared. The solution, 1, 2, 3, and 4 ml., respectively, is pipetted into borosilicate glass test tubes, which can be glass-stoppered and which are calibrated to the 5-ml. mark. Each solution is made up to 4 ml. with isopropyl alcohol, and 1 ml. of the thiourea–potassium hydroxide solution is pipetted into each test tube. The test tubes are stoppered and heated for 1 hour in a constant-temperature bath at 70° C. after which, they are removed from the bath, and the solution is allowed to come to room temperature. If necessary, the volume is adjusted to 5 ml. with isopropyl alcohol. The yellow color is stable for several hours.

The absorbance is read at 400 mμ, using a Beckman Model DU spectrophotometer and 1-cm. absorption cells. To set the instrument at zero absorbance, isopropyl alcohol is used. The standard curve is prepared by plotting absorbance against milligrams of toxaphene. A standard curve for Strobane can be prepared in a similar manner.

Preparation and Analysis of Samples. The absorption of the thiourea reaction products is measured at a wave length where considerable interference from naturally occurring products may be expected. Extraneous yellow colors are often encountered and, to apply this method successfully, one must use adequate cleanup procedures, particularly as large samples must be processed.

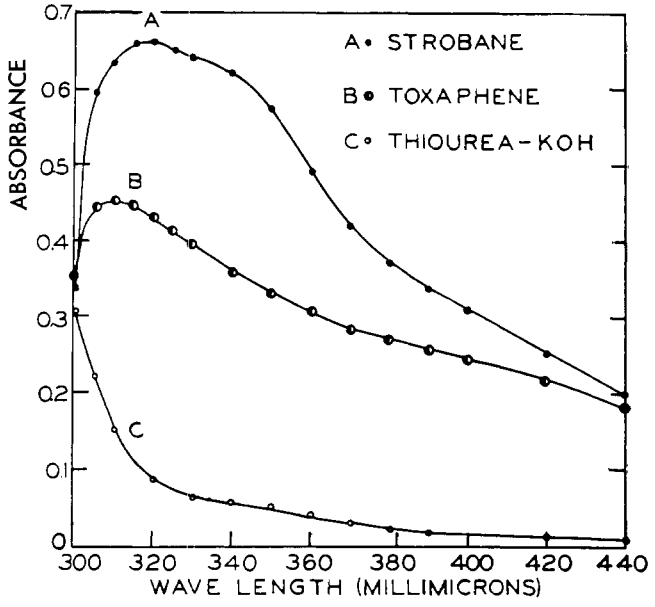

Figure 1. Absorption curves for toxaphene, Strobane, and reagent reaction products

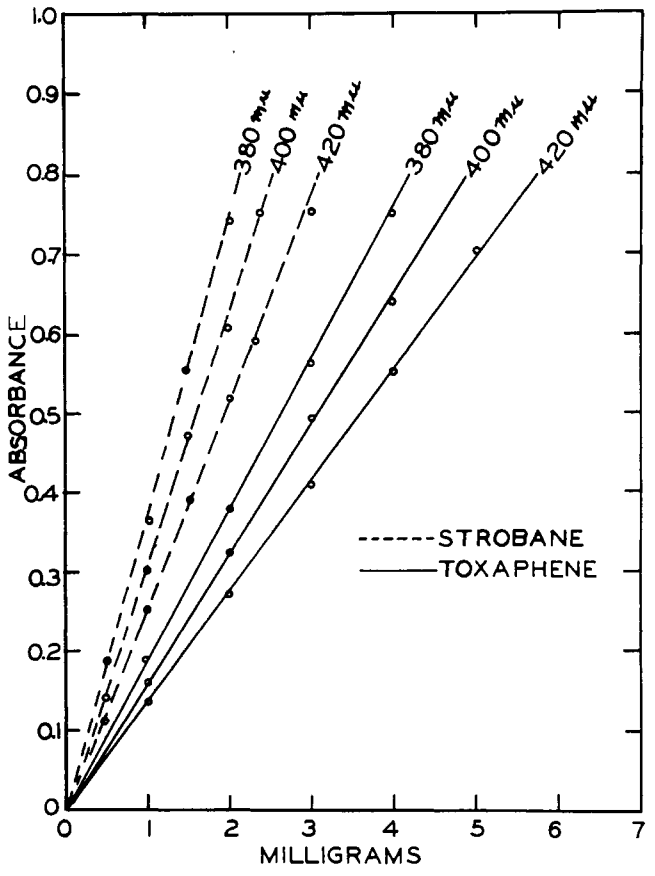

Figure 2. Absorbance of toxaphene and Strobane in 5 ml. of solution

Two related properties of toxaphene have proved useful in developing these procedures: its resistance to attack by concentrated sulfuric acid and its relative nonpolarity, which makes it easy to elute from an adsorbent when utilizing adsorption chromatographic separations. Experimental procedures that proved satisfactory for the recovery of toxaphene from plant material and from butterfat are described below.

Recovery of Toxaphene from an Alfalfa Extract. Dried alfalfa, 350 grams, was extracted with 1 liter of redistilled *n*-hexane by tumbling for 1 hour. The hexane extract was decanted through a dry filter paper, and 1 mg. of toxaphene was added to an aliquot representing 250 grams of alfalfa. The solution was concentrated on a steam bath in a Kuderna-Danish evaporative concentrator to about 50 ml. The concentrated extract was chromatographed in a 25 × 375 mm. column on 25 grams of magnesium silicate that had been dried overnight at 125° C. After the extract entered the column, the chromatogram was developed with an additional 150 ml. of redistilled *n*-hexane followed by 200 ml. of a 1 to 1, by volume, *n*-hexane–methylene chloride mixture. The two fractions were retained separately, and concentrated to dryness in a manner similar to that used for the solvent removal. A test tube with a ground-glass joint formed the lower portion of the evaporator. Four milliliters of isopropyl alcohol and 1 ml. of the thiourea-potassium hydroxide solution were added to the residue in each tube, the tubes were stoppered, and the color was developed as described under Preparation of Standard Curve.

When an alfalfa extract without toxaphene was carried through the procedure, some slight color was present at this point. However, this color did not interfere, since it was preferentially soluble in the oily droplets that quickly solidified when the isopropyl alcohol solution was cooled to room temperature. These small amounts of solid material settled out and were removed by filtering through a 10-mm. filter stick in a microfiltration setup. To facilitate filtration a little diatomaceous earth was added. Absorbance readings were made directly on the filtrates.

The hexane fraction was kept separate because it contained most of the waxes and only a small amount of color. As absorbance is additive, the toxaphene present can be estimated directly from the standard curve by adding the absorbance of the two fractions (corrected for the reagent blanks). In the example described, 0.94 mg. of toxaphene was recovered—i.e., 94%. Recoveries from five samples of alfalfa containing 1 to 5 mg. of toxaphene ranged from 90 to 102%.

Recovery of Toxaphene Added to Butterfat. Forty-five grams of butterfat was dissolved in 250 ml. of redistilled methylene chloride and 1.5 mg. of toxaphene was added. This was equivalent to 1.5 p.p.m. of toxaphene in 1 liter of milk, containing 4.5% of butterfat. The solution was extracted in a 500-ml. separatory funnel with three 50-ml. portions of concentrated sulfuric acid containing 10% of sodium sulfate to minimize emulsification. This cleanup procedure is similar to that of Schechter, Pogorelskin, and Haller (*3*) for determining DDT in milk.

After each extraction, the sulfuric acid layer was drained into a second separatory funnel containing 100 ml. of methylene chloride, which was used to extract any methylene chloride entrapped in the sulfuric acid. The combined methylene chloride extracts were washed with successive 50-ml. portions of 10% sodium sulfate, 5% sodium bicarbonate, and 10% sodium sulfate. The methylene chloride was dried by filtering through a pad of oven-dried cotton, covered with anhydrous sodium sulfate, and evaporated on a steam bath until only a small amount of a yellow viscous liquid remained. This liquid was dissolved in 25 ml. of a 1 to 1 mixture of *n*-hexane and methylene chloride, and chromatographed in a 25 × 375 mm. column on 25 grams of magnesium silicate as the adsorbent. Two hundred milliliters of the *n*-hexane–methylene chloride solution was used to elute the toxaphene from the column. The solvent was removed from the eluate containing toxaphene, as described in the previous section, the color developed in the standard way,

and 1.6 mg. of toxaphene–i.e., 106%–was recovered. A 45-gram butterfat sample, to which no toxaphene was added, was carried through the same procedure and no measurable interferences were encountered.

Discussion

Figure 1 shows the absorption curve measured against isopropyl alcohol for thiourea–potassium hydroxide alone, and the absorption curves for toxaphene and Strobane corrected for the absorption of thiourea–potassium hydroxide. The peak absorption for toxaphene is in the vicinity of 310 mμ and for Strobane at about 320 mμ. The absorption of the thiourea reagent becomes appreciable in this region and contributes a considerable fraction to the total absorption; however, above 380 mμ the absorbance due to thiourea is less than 0.025. Errors due to this reagent blank can be eliminated either by correcting for the blank or by reading absorbance against the reagent itself. As the chances of introducing errors due to the much higher readings of the blank are greater at the lower wave length, in actual practice, readings were made at 400 mμ even though some sacrifice in sensitivity was entailed. The absorbance, corrected for the reagents, at three wave lengths for several concentrations of toxaphene and Strobane are plotted (Figure 2). Beer's law is followed at each wave length, but the sensitivity is somewhat lower at the higher wave lengths. As a matter of convenience 400 mμ was selected for preparing the working curves.

The constitutents of toxaphene do not all have the same absorptivity. For example, recrystallization of toxaphene from methanol yields approximately 15% of a white noncrystalline solid fraction whose absorptivity is about 40% less than that of the mixture itself. However the technical commercial samples seem to be uniform since four separate samples gave approximately the same working curves. The standard sample used in establishing the final curves was one of a series of standard insecticide samples sponsored by the Entomological Society of America and distributed through the Nutritional Biochemicals Corp., 21010 Miles Ave., Cleveland 28, Ohio.

To see what interference might be expected from other chlorinated insecticides, 20 mg. each of aldrin, dieldrin, methoxychlor, DDT, lindane, BHC, TDE, chlordan, and heptachlor were carried through the reaction. Interferences were encountered only from heptachlor and chlordan. The color produced by both these insecticides was considerably greener than that due to toxaphene or Strobane, but of the same magnitude. This interference may be minimized in practice by either the sulfuric acid treatment or proper choice of condition for carrying out the chromatographic separation.

In the cleanup procedures for determining toxaphene in butterfat, concentrated sulfuric acid, rather than mixtures of concentrated and fuming sulfuric acid, proved satisfactory. Toxaphene is stable in the presence of fuming sulfuric acid and, for other vegetable or animal extracts, treatment with this may be preferred.

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ENZYMATIC ANALYSIS

Quantitative Determination of L- Glutamic Acid by L- Glutamic Acid Decarboxylase (from *E. coli*)

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An accurate method adaptable for the routine determination of L-glutamic acid in foods and pharmaceuticals, raw materials used in manufacturing monosodium glutamate, and plant process streams was needed. A modification of the method of Umbreit and Gunsalus, using L-glutamic acid decarboxylase prepared from *Escherichia coli* ATCC strain 4157 gives values, which are accurate and reproducible within narrow limits, and is well adapted to routine analytical work.

THE ENZYMATIC DECARBOXYLATION OF AMINO ACIDS and particularly glutamic acid has been investigated. The preparation of bacterial enzymes specific for the decarboxylation of L(+) glutamic acid, L(+) lysine, L(−) histidine, L(+) arginine, L(−) tyrosine, and L(+) ornithine has been described (*4, 5, 7–9, 21*). The application of these preparations to the rapid analysis of amino acids in protein hydrolyzates has also been discussed. A suspension of *Clostridium perfringens* strain SR 12 (NCTC No. 6784) has been described (*7*) and has been claimed to be specific for decarboxylation of L-glutamic acid. However, Meister, Sober, and Tice (*14*) found that this suspension of *Clostridium perfringens* SR 12 yielded equivalent amounts of carbon dioxide from L-aspartic acid at about 10% of the rate for L-glutamic acid decarboxylation. Methods were described for the separate determination of aspartic and glutamic acids by decarboxylation. Krebs (*13*) found that this same suspension decarboxylates L-glutamic acid and hydrolyzes L-glutamine. Schales, Mims, and Schales (*17*) reported the preparation of clear solutions of an enzyme from higher plants which specifically decarboxylated L-glutamic acid. Schales and Schales (*18*) described the enzyme kinetics of L-glutamic acid decarboxylase from carrots, and the quantitative determination of L-glutamic acid by L-glutamic acid decarboxylase prepared from squash (*19*). Umbreit and Gunsalus (*24*) prepared enzyme suspensions of *Escherichia coli* ATCC strain 4157, which specifically decarboxylated L-glutamic acid, and hydrolyzed L-glutamine.

The purpose of the work described herein was to develop an accurate method adaptable for routine determination of L-glutamic acid in foods, raw materials used in manufacturing monosodium glutamate, and plant proc-

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