nolic hydroxy group appears to be a factor influencing the toxicity. In this series of compounds, only those having the properties of aromatic phenols were satisfactory fungicides. This is in agreement with Sexton (*20*), who attributed the activity of oxine to its phenolic nature. The hydroxyl group in the 8-position on quinoline is an important toxiphore. Only those chelating agents which readily form stable lipophilic complexes with copper are strongly toxic. This finding is in agreement with Albert and associates (*2*, *3*) and Block (*6*, *7*), who suggest that the 2 to 1 chelate is necessary for penetration, and that the site of action is inside the cell. Zentmeyer (*22*), assuming that 8-quinolinol precipitated trace metals from the medium, found that the toxicity could be reversed by adding excess metal ions. If the mechanism were simply one of precipitation, any strong chelating agent would be toxic. Zentmeyer did find that ammonium nitrosophenylhydroxylamine (cupferron) had considerable fungistatic value. Quinaldic acid and 8-quinolinecarboxylic acid are well known for their ability to precipitate heavy metals through chelation and have been used by analytical chemists for this purpose, but these tests with *M. fructicola* show that these chelators were unsatisfactory toxicants regardless of their complexing ability.

From the limited number of derivatives tested, a combination of lipoid solubility and chelation ability appears to be necessary for toxicity, perhaps for penetration of the cell wall only. The phenolic 8-hydroxyl group on quinoline must play an important role in spore inhibition at a site of action inside the cell.

Acknowledgment

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INSECTICIDE RESIDUES

Determination of Endrin in Agricultural Products and Animal Tissues

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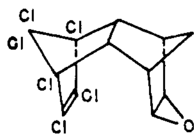
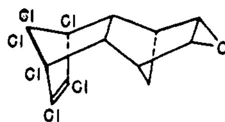
ENDRIN (1,2,3,4,10,10-hexachloro-6,7-epoxy - 1,4,4*a*,5,6,7,8,8*a*-octahydro-1,4, - *endo* - *endo* - 5,8 - dimethanonaphthalene) is an insecticide having the structure:



It is a diastereoisomer of dieldrin (1,-2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4*a*, 5,6,7,8,8*a*-octahydro-1,4-*endo-exo*-5,8-dimethanonaphthalene):



but differs from dieldrin in its insecticidal activity, chemical properties, and residual persistence. Endrin is being used in ever-increasing amounts in agriculture because of its ability to control a wide variety of economically important insects. Its use on crops that subsequently may be consumed by man or domestic animals necessitated the development of methods capable of accurately determining residues of this material in concentrations in excess of 0.1 p.p.m. in crops and animal products.

Bioassay techniques that employ insects have been used extensively for assaying insecticide residues and for screening compounds for insecticidal properties. Techniques such as those of Sun and Sun (*7*), Laug (*4*), and Klein and coworkers (*3*), have been applied successfully to the determination of endrin residues in crops. Davidow and Schwartzman (*2*) also have found a method using goldfish capable of determining endrin at the 5-γ level. While bioassay methods have been useful in laboratories possessing the necessary facilities, chemical methods were desired because of their relative specificity.

Combustion chlorine methods (*1*, *5*, *6*),

The use of endrin on crops that subsequently may be consumed by man or domestic animals necessitated the development of methods capable of accurately determining residues of this material in concentrations in excess of 0.1 p.p.m. in crops and animal products. A sensitive method for the colorimetric determination of endrin, with phenyl azide and diazotized sulfanilic acid as the analytical reagents, is described. As little as 5 γ of endrin can be measured. Suitable procedures are given for isolation of endrin from agricultural products and animal tissues in a form satisfactory for application of the method. Thirty different crops or biological materials have been analyzed successfully by the method. Apparent endrin values on untreated samples range from 0.01 to 0.1 p.p.m.

while applicable, were considered only partially satisfactory, as they would provide an accurate measure of endrin only if no other chlorine-containing materials were present.

This paper describes a sensitive method for the colorimetric determination of endrin, with phenyl azide and diazotized sulfanilic acid as the analytical reagents. As little as 5 γ of endrin can be measured.

Apparatus

The apparatus described in the phenyl azide method for aldrin (*6*), with the exception of the filter apparatus, is required in addition to that described below. The remarks pertaining to interferences caused by rubber, cited in the dieldrin paper (*5*), also apply here.

Air condenser as shown in Figure 1.

Concentration assembly as shown in Figure 2.

Dechlorination tube as shown in Figure 1.

Oil bath assembly capable of maintaining a temperature of 85° ± 1° C. throughout the bath. The bath should be fitted with a rack constructed to hold the reaction tubes free of the sides and bottom.

Water bath capable of maintaining a temperature of 35° ± 1° C.

Reagents

The reagents used in the phenyl azide colorimetric method for dieldrin (*5*) are required, with the exception of the acid mixture and the diazotized 2,4-dinitroaniline. The following additional reagents are needed:

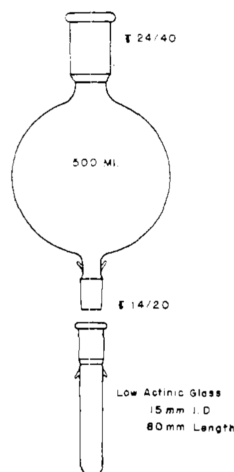


Figure 1. Dechlorination tube and air condenser

Chloroform, reagent grade.

Endrin. Use endrin of at least 99.9% purity (obtainable from Agricultural Chemical Sales Division, Shell Chemical Corp., 460 Park Ave., New York 22, N. Y.), dissolved in purified isopropyl alcohol to make a solution containing 10 γ per ml.

Methanol, absolute, reagent grade.

Purified isopropyl alcohol. Heat 2600 ml. of isopropyl alcohol (99%) with 10 grams of sodium cut into slices, at 75° C. until the sodium is dissolved. Distill in all-glass apparatus protected from water vapor, discarding the first 50-ml. cut and collecting the next 2000 ml. of distillate.

Purified petroleum ether. Distill 2600 ml. of reagent grade, 30° to 60° C. petroleum ether, discarding the first 200 ml. and collecting next 2000 ml. The residue from evaporation of 100 ml. of the distillate to dryness should not give more than 2 γ of apparent endrin when analyzed by the method.

Florisil, 200-mesh. A synthetic magnesia-silica adsorbent, manufactured by the Floridin Co., Tallahassee, Fla.

Purified Skellysolve B. This solvent is a commercial C_6-petroleum fraction produced by the Skelly Oil Co. and available at most chemical supply houses. Purify by distillation over sodium metal in an all-glass apparatus, discarding a 5% forecut and 15% bottoms.

Purified phenyl azide reagent. Prepared as described in the method for aldrin (*5*), but use either purified Skellysolve B or purified petroleum ether as the diluent. In addition to the caustic and water washes, this reagent is purified further immediately before use by passage through a 2 by 2 cm. column of the 2 to 1 magnesia-Celite adsorbent mixture (*5*).

Sodium metal, reagent grade. Store under purified Skellysolve B in an airtight container.

Sodium nitrite solution, reagent grade, 0.05%, aqueous.

Sulfanilic acid, 0.5%. Prepared by dissolving, with warming to 50° C., 0.5 gram of reagent grade sulfanilic acid in 100 ml. of a solution containing equal volumes of glacial acetic acid and distilled water.

Procedure

Extraction of Endrin from Crops

Use this extraction procedure with all crops except oils (or animal products). Reduce 1200 grams of a representative sample to a fine particle size using a food chopper, blender, laboratory mill, or the equivalent. Transfer a weighed sample of the macerated material to an extraction container and add 2 ml. of purified Skellysolve B and 0.5 ml. of purified isopropyl alcohol per gram of sample. (Low-density materials such as tobacco, dried forage, and grass, may require a higher solvent ratio to get good solvent mobility. Soil samples should be extracted with Skellysolve B solution containing 10% acetone.) Tumble the extraction container end over end for 1 hour at 15 to 30 r.p.m.

Allow the solids to settle and decant through a filter paper into a separatory funnel. Wash the extract with a volume of distilled water equivalent to the amount of alcohol or acetone present. Discard the water wash and repeat the washing two additional times. Measure the volume of recovered hydrocarbon extract and store over anhydrous sodium sulfate in a suitable container.

Extraction of Endrin from Glycerides or Animal Products

When the extract from the above procedure contains glycerides, transfer an aliquot of the extract obtained above, representing no more than 50 grams of glyceride, to a conical flask and concentrate under a spray trap until only oil and approximately 10 ml. of solvent remain. Complete the evaporation of the solvent with a gentle stream of air.

When analyzing oils (corn, cottonseed, olive, and peanut), milk, eggs,

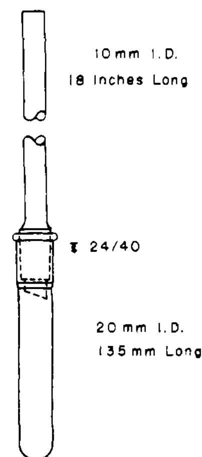


Figure 2. Concentration assembly

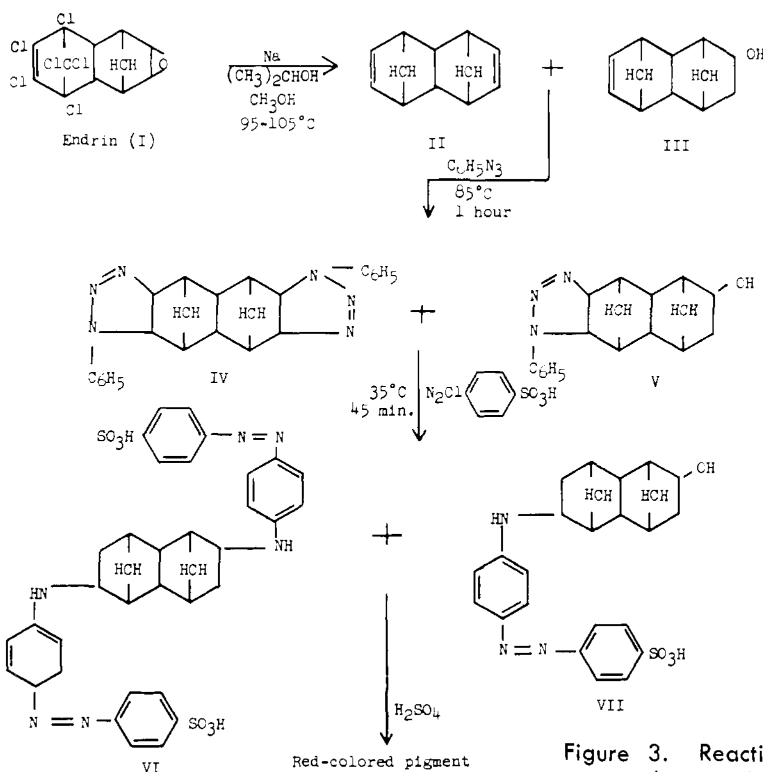


Figure 3. Reactions in the spectrophotometric method for endrin

blood, butter, or animal tissues, weigh 50 grams of the material directly into an Erlenmeyer flask. (Best results are obtained if animal tissues are diced while still frozen. Milk samples should be thoroughly homogenized prior to sampling.)

To the flask, add 0.5 ml. of 50% aqueous potassium hydroxide and 3 ml. of 95% ethyl alcohol for each gram of sample or extractive oily residue, with a minimum of 3 ml. of potassium hydroxide and 18 ml. of ethyl alcohol. Attach the flask to a reflux condenser and reflux gently on a steam bath for 1 hour or until no oily layer is discernible. Allow the solution to cool and add, through the top of the condenser, a volume of distilled water equal to the volume of solution in the flask.

Transfer the mixture to a separatory funnel and add 150 ml. of purified Skellysolve B. Shake vigorously for a minute, allow the layers to separate, and drain off the lower water phase. Transfer the Skellysolve B phase to a 1-liter separatory funnel. Return the water phase to the original separatory funnel and re-extract 5 times with 50-ml. portions of Skellysolve B—each time combining the Skellysolve B portions with the total extract.

Wash the combined Skellysolve B extract with 300-ml. portions of distilled water until the water layer is colorless to phenolphthalein indicator. Retain the interfacial "cuff" in the hydrocarbon phase during the first washes. Store the extract over anhydrous sodium sulfate in a suitable container.

Chromatographic Separation of Endrin from Extractive Interferences

Calibration of Chromatographic Column. Attach a 250-ml. Erlenmeyer flask with a 24/40 standard-taper joint to the bottom of the chromatographic column. Add anhydrous sodium sulfate to give a 0.5-cm. layer in the bottom of the column, and then add 200-mesh Florisil to a height of 10 cm. Level the top of the Florisil layer by tapping the column gently, and then add 5 cm. of a 2 to 1 mixture of magnesia-Celite or a 10-cm. column of a 1 to 1 mixture of magnesia-Celite. Apply a vacuum of approximately 200 mm. of mercury to the side arm and lightly press the surface of the adsorbent using a flat-ended wooden dowel. Add a top layer of 3 cm. of anhydrous sodium sulfate and a pad of glass wool. Add 100 ml. of purified Skellysolve B to the column and allow it to pass through the column until the liquid level falls to the top of the sodium sulfate layer. Release vacuum, remove flask from column, and replace with a clean 100-ml. flask.

Pour into the reservoir of the column a solution of 200 γ of endrin in 20 ml. of purified Skellysolve B. Complete the transfer and wash down the sides of the reservoir with a stream of Skellysolve B. Draw the surface of the solution down to the top of the sodium sulfate layer, rinse down the reservoir, and draw the rinse into the column.

Add and draw down 50 ml. of purified Skellysolve B. Release the vacuum, remove the flask from the column, and repeat the addition of 50-ml. portions 16 additional times. Analyze each effluent fraction for endrin content according to the procedure given below. Assuming that fractions registering less than 0.125 absorbance unit (1-cm. light path) are free of endrin, note the first fraction which contains endrin. The accumulative volume of solvent through this fraction less 100 ml. should be taken as the forecut volume which can be discarded without losing any of the endrin added to the column.

Best results are obtained in crop analysis if a 250-ml. forecut can be taken prior to elution of endrin from the column. To achieve the desired volume of forecut, adjustment of the magnesia column length may be necessary because of difference in the adsorptive qualities of magnesia from batch to batch.

Separation of Endrin from Sample Interferences. Prepare a column as described under Calibration of Chromatographic Column and prewash with 100 ml. of purified Skellysolve B.

Concentrate an amount of sample extract equivalent to 100 grams of sample in an Erlenmeyer flask fitted with a spray trap to a volume of 20 ml. Quantitatively transfer the concentrated extract to the reservoir of the column using a few milliliters of purified Skellysolve B to complete the transfer. Apply a light vacuum to the column (about 200 mm. of mercury), and allow the solution to pass through the column until the liquid level drops just below the top of the sodium sulfate. Wash down the sides of the reservoir with a few

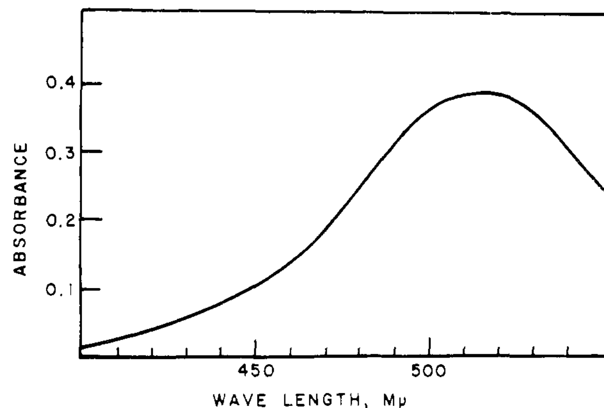


Figure 4. Spectral absorbance curve for endrin product

Using Beckman Model DU spectrophotometer; 1-cm. light path; reference, water; and product from 20 γ of endrin

milliliters of purified Skellysolve B, and draw the liquid level just below the top of the sodium sulfate. Repeat the washing and add purified Skellysolve B until the safe forecut volume established in the preceding section has been reached. Draw it into the column, release the vacuum, and replace the flask. Discard the eluate obtained up to this point.

Add to the reservoir a volume of Skellysolve B sufficient to elute all the endrin from the column. Draw it into the column and then wash the column with an additional 50 ml. of purified Skellysolve B. Collect all of the eluate for subsequent analysis. Extracts containing large amounts of extractive residues may cause a slight displacement of the endrin chromatogram. In such cases a calibration of the column using endrin and the crop extractives is recommended to determine the volume of forecut which can be discarded without losing any endrin.

Prepare a standard solution of endrin in purified isopropyl alcohol having a concentration of 10 γ per ml. Using a pipet, transfer aliquots representative of 0, 10, 20, 30, and 40 γ of endrin into separate dechlorination tubes. Prepare three replicated samples at each concentration. Adjust the volume in each tube to 7.0 $\pm$ 0.2 ml. with purified isopropyl alcohol.

Cut a 1-cm. ($\pm$10%) cube of sodium so that all the surfaces are clean and rinse with purified petroleum ether. Slice the cube into six approximately equal pieces and add the slices to the dechlorination tube. Attach an air condenser to the dechlorination tube and immerse the tube to the solution level in an oil bath at 104° $\pm$ 1° C. Heat the tube and contents and add 2-ml. portions of methanol 7, 10, and 25 minutes after heating has commenced.

When the sodium has been completely dissipated, remove the tubes without further delay from the oil bath and allow them to cool at room temperature for 2 minutes. Slowly add 10-ml. portions of purified petroleum ether through each condenser tube. Pour the contents of a reaction tube into a 500-ml. separatory funnel containing 50 ml. of purified petroleum ether. Fill the tube once with petroleum ether and twice with water and add to the funnel. In a similar manner, transfer the contents of the other tubes to separate funnels before proceeding further with the extraction of the sample. Add an additional 200 ml. of water to each separatory funnel. Stopper the funnels and shake vigorously for 30 seconds. Allow the layers to separate. Withdraw and discard the lower layers. Wash each petroleum ether solution with one 50-ml. portion and three 10-ml. portions of cold water and discard the washings. Discard the water wash each time.

Quantitatively transfer the petroleum ether solution to a concentration assembly having a low-actinic reaction tube attached securely. Attach an air condenser to the flask and concentrate on a steam bath until 3 ml. of solvent remains when the assembly has cooled to room temperature. (Do not evaporate solutions of dechlorinated endrin to dryness, as this would lead to loss by volatilization.)

Pipet 0.5 ml. of phenyl azide solution into the tube, immerse the tube 2 inches in the oil bath at 85° $\pm$ 2° C., and heat for 1 hour. Remove the tube and attach to a glass manifold connected to a vacuum pump. Immerse the lower end of the tube in a 60° C. water bath and evacuate the system below 0.5 mm. of mercury for 5 minutes.

Mix equal volumes of the 0.05% sodium nitrite solution and the 0.5% sulfanilic acid solution and add 7 ml. of the mixture to each reaction tube and shake thoroughly. Heat in a constant-temperature water bath at 35° $\pm$ 1° C. for 1 hour. Shake the tubes briefly at 15-minute intervals during the heating period. Cool the tubes to room temperature and add 0.5 ml. of 2 to 1 sulfuric acid. Measure the absorbance at 515 mμ relative to distilled water using a spectrophotometer. Subtract the average absorbance of the zero endrin standards from each of the other endrin standards. Plot net absorbances as ordinate against micrograms of endrin as abscissa and draw the straight line which best fits the points.

The average absorbance of the zero endrin standards should not exceed 0.100, and the slope of the calibration curve should be approximately 0.026 absorbance unit per microgram of endrin when measurements are made using a 1-cm. light path.

Determination of Endrin in Sample Attach a distillation trap to the Erlenmeyer flask containing the endrin effluent fraction from the chromatograph column and concentrate on a steam bath to a volume of approximately 10 ml. Quantitatively transfer the concentrate to a dechlorination tube. Evaporate the solvent almost to dryness in the dechlorination tube under a stream of dry air while immersing the tube to two thirds its length in a warm water bath (50° to 60° C.). Rinse down the sides of the tube with a few milliliters of purified Skellysolve B several times during the air evaporation step. Add 7.0 $\pm$ 0.2 ml. of purified isopropyl alcohol to the dechlorination tube and continue the procedure as for the preparation of the calibration curve.

After the color is developed, add 3 ml. of purified Skellysolve B to the solution and stir vigorously for 1 minute. Allow the layers to separate and, using a hypodermic syringe, remove and discard the upper layer. Centrifuge for 10 minutes at 2500 r.p.m. Measure the absorbance of the colored solution against distilled water at 515 mμ using a spectrophotometer. Using a previously prepared standard curve, determine the weight of endrin equivalent to the absorbance of the solution. Correct the endrin content of the sample by subtracting the apparent endrin content of the control crop and calculate the endrin content of the sample with the following equation:

$$\text{Endrin content, p.p.m.} = \frac{W}{S}$$

where W is weight of endrin found, micrograms, and S is weight of sample represented by aliquot of crop extractive taken for analysis, grams.

Development of the Dechlorination Phenyl Azide–Sulfanilic Acid Procedure

Chemistry of the Method The principal chemical reactions involved in the method are shown in Figure 3. When the endrin is heated with sodium metal and isopropyl and methyl alcohols, a mixture of 1,4,4*a*,5,8,8*a*-hexahydro-1,4-*endo*, *endo*-5,8-dimethanonaphthalene (II), and 1,2,3,4,4*a*,5,8,8*a*-octahydro-1,4-*endo*, *endo*-5,8-dimethanonaphthalene-2-ol (III) is obtained. These compounds react with 2 and 1 moles of phenyl azide, respectively, to form the corresponding phenyldihydrotriazoles (IV and V). Compounds IV and V react with diazotized sulfanilic acid in an acidic medium to form an intense red pigment having an absorbance maximum at 515 mμ. These intermediates were identified by elemental functional group and infrared analysis and by absorptivity measurements. Calculations from the absorptivities obtained from compounds II and III, supplemented by infrared data, place the percentage yield of II and III from endrin as 24 and 62%, respectively.

Investigation of Method Variables **Temperature and Kind of Alcohol for Dechlorination.** A water-soluble alcohol was desired for use in the sodium reduction step to permit subsequent separation of the reduced endrin products from alcohol and alkali by solvent partition using water and a nonpolar solvent. Three water-soluble alcohols were tested: isopropyl alcohol, boiling point 82° C.; isobutyl alcohol, boiling point 108° C.; and Carbitol (diethyl glycol monoethyl ether), boiling point 202° C. Isopropyl alcohol was superior to the others. With Carbitol, only 50% of the chlorine in endrin was removed. Furthermore, the absorptivity of endrin in the phenyl azide–spectrophotometric procedure was only half of that obtained when isopropyl alcohol was used under the same con-

ditions. Isobutyl alcohol gave complete removal of chlorine from endrin, and the absorptivity for endrin in the spectrophotometric procedure was approximately 10% better than that for endrin using isopropyl alcohol under the optimum conditions. However, the reagent blanks with isobutyl alcohol were 50% higher than those obtained using isopropyl alcohol.

While superior to the other two alcohols tested, isopropyl alcohol alone still was not satisfactory. The time required to consume the sodium varied widely (25 minutes to 2 hours) and these conditions were potentially hazardous because of formation of solid cakes of alkoxide which trapped hydrogen. These difficulties were overcome by using 7 ml. of isopropyl alcohol and adding three 2-ml. portions of methanol during the reaction. The best temperature range for the initial stage of the procedure was fixed at 95° to 105° C. Higher temperatures melted the sodium in occasional samples whereas lower temperatures usually delayed the reaction.

Amount of Sodium. One gram of sodium and 7 ml. of alcohol were used in the temperature tests previously described and are specified in the method. Equally good recovery was obtained when the amount of sodium was doubled or halved. However, the 2-gram test required 30 minutes more than usual to dissolve the excess sodium and the reagent blanks were 40% higher than in the other two tests where the blanks were identical.

Amount of Endrin. Triplicate samples containing 20 and 40 γ and 10, 30, and 60 mg. of endrin were dechlorinated and aliquots from each sample representing 20 γ of endrin were analyzed by the remainder of the method. There were no significant differences among the absorptivities for endrin in any of the samples.

Time of Dechlorination. No significant difference in absorptivity of endrin or the magnitude of blanks was found with reaction times of 20 to 60 minutes, provided the heating was discontinued within 10 minutes after the sodium was all dissolved. In a group of samples where the sodium dissolved in 35 minutes and heating was continued for an additional 30 minutes, recoveries were 30% low although blanks were unaffected. The procedure of the method is designed so that the sodium will dissolve within a few minutes after the last addition of methanol. This permits the analyst to continue heating all the samples of a group until the sodium in the slowest sample has dissolved, and then to extract them without regard for the order in which the sodium was consumed.

Promptness of Transfer of Dechlorinated Endrin. Prompt addition of petroleum ether to the warm dechlorination mixture and transfer to the funnel containing additional solvent and dilution with water were necessary for obtaining low blanks. Tests were made with 10 and 20 γ of endrin where the petroleum ether was added after either 1 or 5 minutes. Absorptivity was the same for both sets, but reagent blanks were 75% higher in the 5-minute tests. The alkoxides had started to precipitate in most samples in the 5-minute tests before the petroleum ether was added. The higher blanks may be due to contact of the dechlorination product with air drawn into the apparatus during the longer cooling period. Once the samples are transferred to the separatory funnels and shaken with water, blanks are not affected by delays of up to 15 hours in completing the separation and washing of the extracts.

Extraction of Dechlorinated Endrin. Water and petroleum ether are the solvent pair used for separating dechlorinated endrin from the other products of the dechlorination reaction. Maximum absorptivity was obtained when the warm dechlorination product was diluted with hydrocarbon solvent before adding water. When the water was added first, absorptivities were low by as much as 20%. A full 30 seconds of vigorous shaking is necessary to complete hydrolysis and to remove alkoxides. The alcohol was diluted with a relatively large amount of water to provide a better distribution of dechlorinated endrin products into the hydrocarbon phase. The single extraction specified in the method is 99% as effective as three such extractions; as measured by the amount of reactive materials taken into the hydrocarbon phase. The effect of washing the hydrocarbon extract with water was evaluated by comparing blanks for samples where the extracts were not washed with those washed once or twice with 50 ml. of water. All blanks were uniformly low.

Concentration of Extract for Triazole Formation. The extract is concentrated to a few milliliters by evaporation of the petroleum ether on a steam bath. Phenyl azide is then added and the solution heated to remove solvent and effect reaction. Removal of the residual solvent by blowing with air prior to heating, as in the aldrin and dieldrin methods (*5*, *6*), gave erratic recovery with dechlorinated endrin, presumably because of the loss of volatile components.

Amount of Phenyl Azide. Experience has shown that 0.3 to 1.0 ml. of 30% phenyl azide reagent brings about consistent and essentially complete reaction with the dechlorination products of endrin. To work at a safe operating margin, 0.5 ml. of phenyl azide reagent per test has been found satisfactory.

Time and Temperature for Triazole Formation. To establish the conditions necessary for uniform and quantitative conversion of a given amount of dechlorinated endrin into its phenyldihydrotriazole derivatives, 1-ml. aliquots of a dechlorinated endrin solution in Skellysolve B were mixed with 0.5 ml. of purified phenyl azide reagent. These mixtures were heated in an oil bath at different temperatures and varying periods of time, after which the endrin-triazole was measured by the colorimetric procedure. Reaction at 70° and 80° C. is incomplete after 45 minutes, but essentially complete at 80° or 90° C. after 60 minutes. Subsequent experience showed that with the reaction mixture dissolved in 2 to 3 ml. of petroleum ether, reproducible and essentially complete reaction may be achieved by heating at 85° C. for 1 hour. Should the volume of the reaction mixture (in petroleum ether) be larger than 2 to 3 ml., the mixture should be heated an extra 5 minutes for each milliliter of excess solvent.

Removal of Excess Phenyl Azide. The excess phenyl azide is removed from the dechlorinated endrin phenyldihydrotriazole by immersing the tubes in a water bath at 60° C. and evacuating the system to less than 0.5 mm. of mercury for 5 minutes. No loss of dechlorinated endrin phenyldihydrotriazole occurred under these conditions for periods as long as 15 minutes. While diazotized sulfanilic acid does not react with phenyl azide, the decomposition products of phenyl azide, if not removed, could possibly react with the diazonium reagent to give interfering colors.

Preparation of Diazotized Sulfanilic Acid Reagent. Results of tests varying the concentrations of sulfanilic acid and sodium nitrite indicate that optimum conditions exist when equal volumes of 0.5% sulfanilic acid in 1 to 1 acetic acid and an aqueous 0.05% sodium nitrite solution are used. Unless the diazonium reagent is prepared with the sulfanilic acid in excess of the sodium nitrite, the final red-colored dye will not be stable. When sodium nitrite is in molar excess over sulfanilic acid, no color is produced. Variations in the acetic acid concentration of the sulfanilic acid solution from 25 to 100% had no effect on the intensity of the final color.

Conditions for Forming Dye. When diazotized sulfanilic acid reacts with endrin phenyldihydrotriazole, a yellow-orange color develops which reaches a peak intensity after approximately 45 minutes. The rate at which this color forms is dependent on the temperature of the reaction. While only a maximum 4% error will result from failure to control the temperature between 25° and 35° C., a standardization of the

method at 35° C. is recommended.

Choice of Acid for Color Intensifying. The dye formed can be reversibly changed to a yellow basic form or a red acid form—the red form has a greater absorptivity. There is considerable flexibility on the amount and type of acid that may be used for the final development of maximum color. A study of various acids used for converting the indicator to the red acid form has shown the following to be equally effective: perchloric, hydrochloric, hydrobromic, nitric, sulfuric, and the dilute sulfuric (2 to 1) prescribed.

Results with Pure Endrin. The constants for a typical curve for the range of 0 to 30 γ of endrin are (using a Coleman, Jr., spectrophotometer equipped with 7-mm. cylindrical cells): slope, 0.018 absorbance unit per microgram; absorptivity, 92 liters per gram-centimeter; average blank, 0.10 absorbance unit; and standard deviation, 2.0 γ. The standard deviation was calculated from a straight line determined by the method of least squares. Table I summarizes the standard deviations obtained at different endrin levels. Essentially identical curves were obtained by two operators who used different batches of reagent and performed their work at various times over a period of several months.

The spectral absorbance curve of the endrin color product is shown in Figure 4.

Purity of Reagents

Solvents. All solvents must be purified as directed under Reagents. Unless proper precautions are taken blanks of the order specified above will not be attainable.

Phenyl Azide Reagent. Preliminary washing of the hydrocarbon solution of phenyl azide with caustic and water, as in the aldrin and dieldrin methods, failed to remove all impurities which give interfering colors in the endrin test. A satisfactory reagent was obtained if, in addition to washing with caustic, the reagent were passed through a 2-cm. column of a 2 to 1 magnesia-Celite absorptive mixture. This procedure should be carried out within an hour before the reagent is to be used.

Results with Endrin in Presence of a Nonreactive Diluent

Neither reagent blanks nor endrin standards were affected in any way by the presence of as much as 15 mg. of paraffin wax.

Development of Separation Procedures

Extraction of Endrin from Crops

The solubility of endrin is similar to that of dieldrin; hence, the extraction procedures used for dieldrin were adopted for the endrin method without modification. Numerous tests on a variety of crops have shown the described technique to be completely adequate for the quantitative removal of endrin from the crop into the solvent phase.

Separation of Endrin from Crop Extractives

Extraction of the crop or tissue with a hydrocarbon solvent mixture not only removes the endrin from the sample, but also substantial quantities of chlorophyll, colored pigments, waxes, and oils. The magnitude of these extractives is dependent on the type of material being extracted, its freshness, its degree of subdivision, and preliminary handling (canning, cooking, dehydration, and peeling).

These extractive interferences must be removed from the crop extract prior to its analysis by the colorimetric procedure. A number of separation techniques have been employed for extractive removal with varying degrees of

Table I. Standard Deviations of Endrin Calibration Curve

Endrin Level, γ	Standard Deviation γ	%
0	2.13	...
10	1.57	15.7
20	2.54	12.7
30	1.94	6.5
Over-all standard deviation of points from least square line	1.98	...

Table II. Typical Results from Determination and Recovery of Added Endrin from Crops and Materials

Material	Endrin, P.P.M. Added[a]	Determined	Recovered[b]
Alfalfa	0.00	0.03, 0.09, 0.08	...
	0.10	0.14, 0.18, 0.15	0.11, 0.09, 0.07
	0.30	0.31, 0.39, 0.30	0.28, 0.30, 0.22
Blood	0.00	0.02	...
	0.10	0.11	0.09
Broccoli	0.00	0.08, 0.09	...
	0.10	0.17, 0.17, 0.16	0.09, 0.09, 0.08
	0.30	0.37, 0.35	0.29, 0.24
Cabbage	0.00	0.02, 0.03, 0.05	...
	0.10	0.11, 0.14, 0.13	0.09, 0.11, 0.08
	0.30	0.29, 0.29, 0.30	0.27, 0.26, 0.25
Cottonseed oil	0.00	0.01, 0.05, 0.08	...
	0.10	0.15	0.10
	0.30	0.25, 0.27, 0.30	0.24, 0.22, 0.22
	1.00	1.20	1.10
Cucumbers	0.00	0.03, 0.07, 0.05	...
	0.10	0.13, 0.15, 0.14	0.10, 0.08, 0.09
	0.30	0.33, 0.39, 0.32	0.30, 0.32, 0.27
Kale	0.00	0.05, 0.06, 0.08	...
	0.10	0.13, 0.17, 0.18	0.08, 0.11, 0.10
	0.30	0.29, 0.37, 0.41	0.24, 0.31, 0.33
Lettuce	0.00	0.04, 0.02, 0.04	...
	0.10	0.14, 0.14, 0.13	0.10, 0.12, 0.09
	0.30	0.35, 0.28, 0.25	0.31, 0.26, 0.21
Liver	0.00	0.09	...
	1.0	1.2	1.1
	2.5	2.2	2.1
Squash	0.00	0.04	...
	0.10	0.13	0.09
	0.30	0.32	0.28
Tomatoes	0.00	0.01, 0.01	...
	0.10	0.08, 0.09	0.07, 0.08
	0.30	0.30, 0.23	0.29, 0.22
Wheat	0.00	0.01, 0.04, 0.05	...
	0.10	0.10, 0.12, 0.14	0.09, 0.08, 0.09
	0.30	0.29, 0.31, 0.33	0.28, 0.27, 0.28

[a] Toxicant added to macerated sample prior to extraction.
[b] Corrected for apparent toxicant value obtained on untreated check sample.

Table III. Interference of Various Insecticides in Endrin Photometric Method

Toxicant	γ of Chemical Giving Interference Equivalent to 1 γ of Endrin	
	Without chromatography	With chromatography
Aldrin	2.6	0
Chlordan	<0.5	0.6
DDT	143	0
Dieldrin	1.8	2.1
γ-Benzene hexachloride	0	0
Isodrin	0.7	0
Parathion	200	0
Toxaphene	14.3	200

success. In general, techniques involving solvent partition, batch treatment with adsorbents, and steam distillation of dechlorinated endrin proved far less successful than simple chromatography.

From tests made during development of the dieldrin methods, endrin was known to have approximately the same absorption characteristics as dieldrin on magnesia. Therefore, as a first test, endrin was chromatographed according to the procedure of the dieldrin method, which consisted of adding a hydrocarbon solution of the insecticide to a column containing 50 grams of a 2 to 1 mixture of Westvaco magnesia No. 2642 and Celite 545 and developing the column with a purified hydrocarbon solvent. This column proved successful on a relatively small number of crops yielding minor amounts of extractive interferences, but was too restricted to be recommended as a standard technique. Endrin was eluted from the column somewhat more rapidly than dieldrin, and its advanced elution in the presence of crop extractives thereby limited greatly the volume of endrin-free forecut which could be discarded safely. Either increasing the ratio of magnesia to Celite or increasing the amount of adsorbent mixture was impractical because the flow rate was thereby decreased to an unsatisfactory level.

Florisil best fulfilled the needs of the method, and when used with magnesia in the manner described in the method provided a versatile column capable of separating endrin from a wide variety of crop extractives. Its use in combination with saponification techniques enabled use of the method on high glyceride-containing materials such as oils and animal products.

In the early development work, the first 200 ml. of Skellysolve B developer washed out most of the extraneous extractives (except pigments) causing interferences, yet did not elute added endrin; more than 95% of added endrin was eluted in the 300- to 600-ml. fraction of Skellysolve B wash; and 70 ml. of a 1 to 19 chloroform–Skellysolve B mixture could be substituted for the 300 ml. of Skellysolve B required to wash out the endrin. However, this 5% chloroform eluent sometimes displaced adsorbed plant pigments from the column in addition to endrin. Because the pigments caused interferences, the 5% chloroform solvent is not recommended although this solvent, when applicable, would reduce the time needed for chromatography.

Applicability of the Method to Determination of Endrin Residues

Subsequent to the development of the method, the techniques described herein have been applied to the determination of endrin residues on a number of crops or materials. Known amounts of the insecticide were added directly to the untreated macerated material and determined by the method. These "recovery" samples were generally prepared to have an endrin concentration of 0.1 to 0.3 p.p.m. based on a 100-gram original sample. Thus far, the method has been applied successfully to 30 different crops or materials. Results representative of those obtained have been summarized on 12 typical crops or materials in Table II. Apparent endrin values in the untreated check samples in excess of 0.09 p.p.m. are rarely obtained. Duplicate results should not differ by more than 4 γ on aliquots of the same extract or by more than 5 γ on identical initial unextracted samples.

Results in Table II indicate that to obtain most reliable values, the endrin value for the sample should be corrected for the apparent endrin value for an insecticide-free control sample determined by concurrent analyses. Recovery samples in the range of the expected residue level should be determined concurrently with the treated samples.

Specificity of the Method

The method is based on the reduction with sodium of endrin to give olefinic unsaturated products, which react with phenyl azide to form phenyl dihydrotriazole derivatives and the subsequent conversion of these products to colored materials with diazotized sulfanilic acid. The dechlorination step would be expected to remove all the chlorine from a chlorine-substituted olefinic group in most organic compounds. Phenyl azide readily reacts with an unsubstituted double bond in a bicycloheptene ring structure. Simple olefins in which the double bond is not subject to strain react less readily. Tests have shown that phenyldihydrotriazoles of bicycloheptic compounds, whether of the *exo-endo* or *endo-endo* configuration, form a color under the conditions of the method if (but only if) they are chlorine-free. However, diazotized sulfanilic acid forms a more intense color with the phenyldihydrotriazoles of *endo-endo* compounds.

In the light of the foregoing, toxicants such as aldrin, chlordan, dieldrin, heptachlor, isodrin, and toxaphene might be expected to interfere in the dechlorination-spectrophotometric method. Eight common insecticides in 100-γ amounts were tested for interference as apparent endrin, both with and without the recommended chromatographic procedure (Table III).

These data show that aldrin, chlordan, dieldrin, isodrin, and toxaphene all interfere to a substantial degree when analyzed, omitting the chromatographic step. In combination with chromatography, however, only chlordan and dieldrin gave any significant interference.

Applicability of Method to Determination of Other Pesticides with Bicycloheptene Structure

In view of the data presented in Table III, the sodium reduction–phenyl azide–sulfanilic acid method should be applicable to the determination of microgram amounts of chlordan, isodrin and, probably, heptachlor. With appropriate chromatographic cleanup, the method probably could be used for the determination of these insecticides in crops, but it is not recommended for replacement of the existing methods for aldrin (*5*) and dieldrin (*6*), as these latter methods are more specific and sensitive.

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