Microdetermination of the Fungicide Dinitrocaprylphenylcrotonate in Food Crops and Animal Tissues

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With the commercial development of Karathane, dinitrocaprylphenylcrotonate, a method of analysis was developed for microdetermination of the fungicide in extracts of fruits and vegetables. The method is based on a separation by steam distillation of the dinitrocaprylphenylcrotonate, together with any free dinitrophenol. The distillate is extracted with chloroform, evaporated to dryness, and taken up in pyridine-water. After 1 hour, the color developed is read at 442 mμ. Reproducible results are obtained at a sensitivity as low as 10 γ of Karathane in a 200-gram fruit or vegetable sample. Recovery experiments from actual samples are presented and compared to standards. Equipment for semiautomatic steam distillation is described.

DINITROCAPRYLPHENYLCROTONATE (Karathane, Rohm & Haas trade name) is used as an economic poison on a wide variety of food crops. Analytical data on residues on these crops are needed along with the analysis of Karathane residues in animal tissues. The method chosen for analysis must be not only a measure of the compound itself, but also, if possible, of certain closely related decomposition products that might be formed. With Karathane, the most likely decomposition route is hydrolysis, which yields crotonic acid and dinitrocaprylphenol. With this in mind, it was decided to base the method on determination of the total dinitrocaprylphenol content of the residue samples.

While the development of an intense color by nitrophenols upon base addition is well known, only one reference could be found in the primary literature to the analysis of dinitrophenolics as residues on natural products (*1*), and then the reference was not applicable, owing to high control values. Examination of the transcript of the 1950 Federal Security Administration (*2*) hearings revealed, in brief outline, that the Dow Chemical Co. had developed a method for dinitrophenolics which was based on steam distillation, extraction with chloroform, evaporation, and colorimetric determination in pyridine. The details of this procedure were obtained through private communication (*6*). This was not suitable for Karathane residues, but was the starting point for the method described in this paper.

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General Procedure

The sample is suitably extracted, carefully evaporated, and then steam distilled semiautomatically under rigorous conditions. The distillate is extracted in chloroform, evaporated gently, and taken up in a pyridine-water reagent. The pyridine-water solution is allowed to stand for 1 hour to assure complete hydrolysis of the Karathane to the phenol and finally the absorbance is measured at 442 mμ.

Reagents

All chemicals should be analytical reagent grade, except where specifically stated otherwise.

Benzene, thiophene-free.

Chloroform. Pass through alumina to remove interferences. Use 1 pound of alumina to 2 gallons of solvent. If the chloroform is received in cans, be sure that the top of the can is cleaned well with solvent to remove lacquers, solder flux, and the like before opening.

Karathane, purified. Purify technical Karathane, Rohm & Haas Co., by dissolving it in 40 volumes of pentane and filtering it until clear. Wash the filtrate with an equal volume, in five portions, of a 50% methanol and 50% water (containing 10% sodium hydroxide) solution. Follow this by three rinses of water. At the time of the last rinse, add concentrated hydrochloric acid until the water layer remains acid (pH 2 to 3) after shaking with the pentane solution. Finally, rinse with portions of water until the pH is around 7. Dry the pentane solution over 0.1 weight by volume of anhydrous sodium sulfate for 30 minutes with agitation, filter it and evaporate the solvent on a steam bath under nitrogen.

Pyridine-water. Add one part of distilled water to pyridine and store in a brown bottle in a cool place.

Sulfuric acid, 50%. Mix equal volumes of concentrated sulfuric acid and distilled water.

2B Alcohol. Ethyl alcohol (95%) may be substituted.

Alumina, any material suitable for chromatographic absorption—e.g., Merck No. 71707.

Sodium sulfate, anhydrous.

Nitrogen, water pump grade.

The following reagents are needed only for animal tissue residue analysis:

Diethyl ether.

n-Hexane, technical grade, 95 mole % minimum. Distill and pass through alumina (2 gallons of solvent to 1 pound of alumina).

Acetonitrile, technical grade. Distill and pass through alumina (2 gallons of nitrile to 1 pound of alumina).

Ottawa sand. Wash with distilled water and then with acetone, and dry.

Apparatus

Separatory funnels, 2-liter.

Erlenmeyer flasks, 1-liter and 125-ml.

Steam distillation equipment. The proper dimensions for most of the parts are given in Figures 1 and 2. Those requiring additional information are:

Variable transformers, two—one to control the steam generator and one to

control the steam distillation flask. Any type capable of controlling from 0 to 120 volts alternating current is satisfactory.

Electronic relay, 115 volts, 60 cycles, or any type which can handle or be modified to handle an inductive load. The type used in this study was a Fisher-Serfass electronic relay.

Thermoregulator. A fixed point thermoregulator, 130° ± 1° C., with a $\frac{10}{30}$ joint and a 2-inch immersion stem.

Distillation flask and head. This part of the apparatus has important dimensions and is described in detail in Figure 2. The flask and head are supplied by the Kontes Glass Co., Vineland, N. J. (drawing reference RH-1, No. 3233-1). The head is furnished without the splash trap, which is added in the laboratory.

Spectrophotometer. Use any type that performs satisfactorily at 425 to 450 mμ. A Beckman Model B was used in this study.

Blender. Any type capable of mincing samples well.

The following pieces of equipment are used only for the analysis of animal tissues residue:

Mortar and pestle.

Soxhlet extraction apparatus.

Separatory funnels, 125- and 250-ml.

Procedure

Preparation of Samples **Surface Extraction of Vegetable Matter.** Place a representative sample (about 1000 grams) in a 1-gallon paint can and add benzene in the ratio of 1 ml. of benzene to 2 grams of sample. In the case of certain exceptionally dry materials—e.g., tea or alfalfa—the solvent ratio must be increased. Tumble the can for 45 minutes. For this purpose a ball-mill tumbler was adapted to hold ten paint cans. After the can is tumbled, decant the extract into a glass jar and filter, if necessary, through glass wool or cheesecloth. Seal the bottle tightly with an aluminum foil-lined cap and store at 10° C. until analyzed.

In the case of soft, fragile fruits, such as tomatoes, cherries, or strawberries, put the sample into a wide-mouthed jar with an aluminum foil-lined cap and add the proper amount of solvent. Agitate the jar by hand in a rolling motion for 10 minutes. Allow the sample to remain in contact with the solvent for another 15 minutes, then again hand tumble for 10 minutes, and finally allow to stand for 10 minutes before separating. Various pieces of apparatus for extractions are described by Gunther and Blinn (*3*).

Animal Tissue Samples. Using a mortar and pestle, macerate the thawed tissues with Ottawa sand—5 grams of sand to 1 gram of sample. Transfer this mixture to an extraction thimble and place it in the Soxhlet extractor. Wash the mortar and pestle with two 10-ml. portions of diethyl ether, and pour it slowly into the extraction thimble after the thimble is in place in the apparatus. Pour the remaining 55 ml., of the 75 ml. of diethyl ether used for extraction, into the flask and extract the sample for 4 hours. After carefully taking the ether extract just to dryness on the steam bath, dissolve the residue in 30 ml. of *n*-hexane and extract it four times with acetonitrile, with 30-ml. portions. Back-extract the combined acetonitrile layers with 30 ml. of *n*-hexane and then transfer them to a special distilling flask and evaporate carefully, just to dryness on the steam bath under a gentle nitrogen stream. From this step on, follow the general procedure.

Grinding and Extraction of Vegetable Matter Cut up 500 grams of sample and feed into a blender at such a rate that fluidity is maintained. After 5 minutes of grinding, add 10 ml. of 50% sulfuric acid, continue mixing for 2 minutes, and add 400 ml. of benzene. Allow the mixture to blend for 5 minutes more. Finally, add 100 grams of anhydrous sodium sulfate and continue mixing for 2 minutes more. Place the pulp emulsion from the blending in suitable jars and centrifuge at 2400 r.p.m. for 20 minutes. Collect the supernatant benzene layer, redisperse the solids, and centrifuge again. Add any additional supernatant liquid which separates to the first decanting. Tightly cap the extract and store in a cool place until analyzed. (Take care to prevent loss of solvent during the grinding. This is discussed later in the section dealing with solvent extraction.)

Evaporation of Solvents Transfer an aliquot of the sample containing up to 130 γ of Karathane to a steam distillation flask and carefully evaporate to 1 or 2 ml. on a steam bath under a gentle nitrogen stream. With the flask removed from the steam bath (*5*), but while still hot, drive off gently the remaining few milliliters of solvent with the nitrogen stream.

Steam Distillation Bring the steam generator to a boil and then set the Powerstat so that 800 ml. of distillate are collected in 1 hour (about 110 volts). Add 15 ml. of 50% sulfuric acid to the distillation flask containing the residue and set the flask in its mantle and insert the thermoregulator into the $\frac{10}{30}$ joint. Connect the dis-

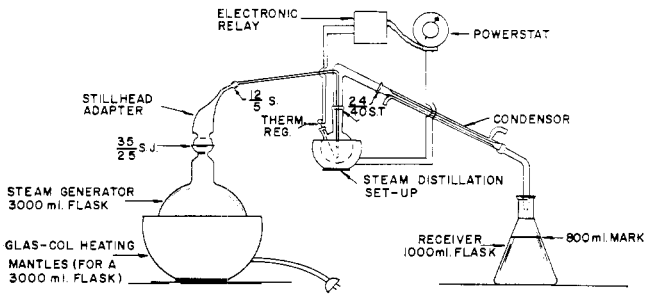

Figure 1. Steam distillation apparatus

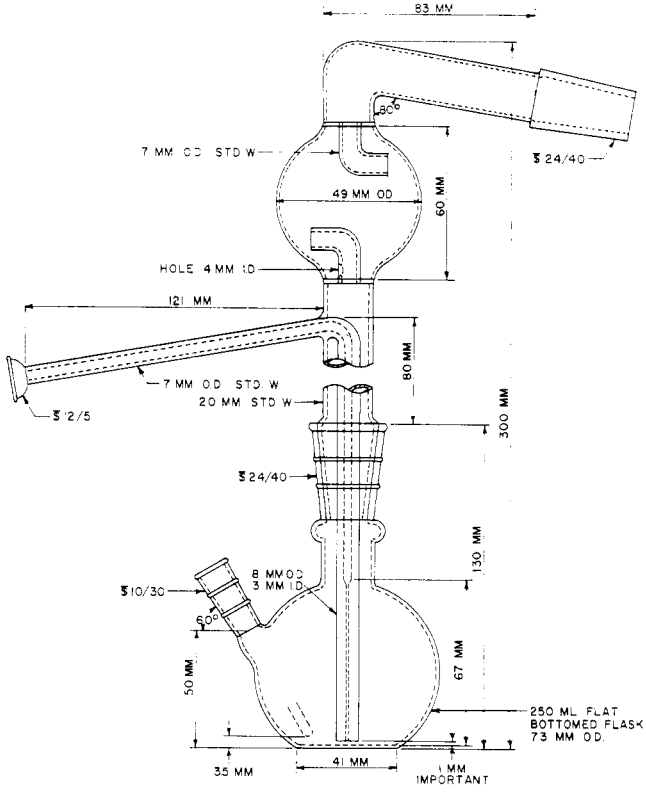

Figure 2. Distillation flask and head

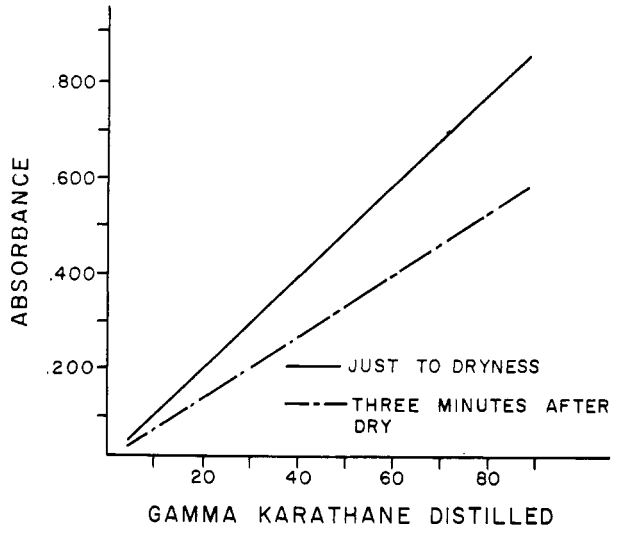

Figure 3. Effect of overheating on the recovery of Karathane (442 mμ)

tillation unit to the apparatus, attach the clip to the thermoregulator, turn on the preset transformer (the setting used in this laboratory is 90), and wrap glass wool around the distillation flask. The setup will now operate automatically at 130° ± 1° C. until shut down. When approximately 775 ml. of distillate have been collected, allow the condenser to steam down to flush out any waxes or fats that may have solidified in the condenser and collect the final 25 ml. Remove the receiver and lower the distillation flask before the steam is shut off to prevent suck-back.

Colorimetric Determination Transfer the steam distillate to a 2-liter ungreased separatory funnel. Rinse the receiver with 10 ml. of 2B alcohol and then with two 50-ml. portions of chloroform, and add all the rinses to the separatory funnel. Equilibrate the two phases (about 2 minutes of shaking) and remove the chloroform phase into a 125-ml. Erlenmeyer flask. Evaporate the chloroform to dryness, taking the same precautions as with the original extract. Add 3 ml. of pyridine-water reagent in such a way as to rinse down the sides. Stopper the flask and swirl the contents occasionally to ensure a pick-up of all the Karathane and phenol. After 1 hour, transfer the resulting yellow solution (*4*) to a 1-cm. cell and read against a reagent blank at 442 mμ.

Correct the value obtained by a control sample blank prepared from untreated samples subjected to the same procedure as the sample. Use the corrected absorbance value to obtain the micrograms of Karathane from a calibration curve.

Calibration Prepare a standard recovery curve from purified Karathane, but until interferences from each substrate have been checked, it is better to prepare a calibration curve for each type of sample. From a stock solution, add Karathane—amounting to 10, 20, 40, and 80 γ—to aliquots of untreated control samples. Run these fortified solutions in the same manner as samples and use the values obtained to prepare a calibration curve. Prepare a standard curve by running aliquots of the same stock solution, without any substrate, through the procedure.

Discussion

Solvent Extraction. Benzene was selected because of its high solubility for Karathane, low boiling point, and particularly its low level of interference. The time of surface stripping was set at 45 minutes to ensure a complete stripping of the residue. Duplicate natural samples analyzed after varying periods of extractions showed little difference between the 20-minute and the 1-hour extraction. Because the amount of Karathane on normally sprayed and harvested apples is low, the extraction time was checked at a higher level of Karathane residue where smaller trends would be more reliably detected. To do this, a large group of apples was sprayed in the laboratory using a hand sprayer of the type used in paper chromatography. A spray containing 0.0768 gram of 25% wettable dust per ml. of water was used. Sufficient solution was used to give an average residue of 80 γ per 200 grams of apples or about 0.4 p.p.m. The samples were then allowed to dry thoroughly before being used and were divided into 10 sets. The sets of apples were then extracted in duplicate for periods of 10, 20, 30, 45, and 60 minutes. Such a procedure is only a first approximation to normal spraying under growing conditions, but it was considered worth while as a gross check on prior observations with natural samples. After 20 minutes, no significant differences in completeness of extraction were found. A period of 45 minutes was selected as convenient and, also, as long enough to ensure a margin of safety.

During the grinding of the pulp, care must be taken to keep the temperature of the mix below 30° C. Above this temperature, high blanks are produced or there is a significant loss of solvent. This is the case, particularly, with apples and pears. Heat is generated by the addition of the acid solution and by the action of the cutter. With these types of samples, it is necessary to add 250 ml. of ice-cold water before the initial blending and to cool all of the reagents. If, during the grinding, the mix goes above 30° C., the blender is jacketed with a plastic film into which dry ice is placed.

The hexane-acetonitrile extraction step is used in the basic procedure when analyzing animal tissues to remove the major portion of the fats before steam distillation. If more than 0.5 gram of fat is allowed to come through to the steam distillation, the Karathane remains dissolved in the fat giving poor recovery.

Evaporation of Solvents. Care must be exercised in the evaporation of solvents in the analysis of residues. A study was made of several factors in the evaporation of sample aliquots and the final chloroform extracts. Prolonged heating leads to significant losses of Karathane or its decomposition products. On the other hand, there was the practical problem of reasonable evaporation rates. The data on these experiments are given in Table I and Figure 3. A standard solution containing 87 γ of dinitrocaprylphenol in benzene was used in each experiment. The conditions chosen are the maximum and minimum ones for the influence of heat and nitrogen. The importance of the care exercised in this particular step cannot be overemphasized. Too much heat and too strong a current of nitrogen will give results with poor reproducibility and which are low.

The final conditions chosen for use in this procedure involve heating on top of (not in) a steam bath under a gentle current of nitrogen; do not let the sample go to dryness. When about 2 to 3 ml. of solvent remain, the flask is removed from the steam bath and the last traces of solvent are shown off as the sample is cooling. The stream of nitrogen should be removed just as the sample goes to dryness. This gives values slightly below evapora-

Table I. Factors Affecting Losses of Residue during Evaporation

Solvent Evaporated by	Absorbance
Using heat[a] alone	0.572, 0.545
Using N_2[b] and heat	0.580, 0.570
Exposing to room atmosphere (17 hours)	0.650, 0.655
Using N_2 alone	0.680, 0.685

[a] Samples on a steam bath to dryness.
[b] About 3 standard cu. ft./hr.

tion at room temperature under nitrogen; but the increase in rate warrants this.

Steam Distillation. The efficiency of steam distillation is dependent on the relative vapor pressure of the components to be volatilized to that of water at 100° C. In the steam distillation of a plant extract, a compromise must be made between the volume of steam collected and the amount of desired constituent and interferences distilled over. Generally the steam distillation is not run under equilibrium conditions, so that in order to get reproducibility one must rigidly set the conditions of distillation. The variables that must be fixed are: the dimensions of the equipment, the rate of distillation, the temperature of distillation, and the constancy of sample volume throughout the distillation. All of these conditions are fulfilled in the setup used in these experiments. Instead of attempting to maintain a constant sample concentration in the distillation flask by visual estimation of volume and manual application or removal of heat, a system is used which automatically maintains the pot temperature at 130 ± 1° C. Constant temperature means constant volume in a binary system such as this.

The rate is fixed by using a standard heating mantle and a 3-liter boiler flask, controlled by a variable transformer at a calibrated setting, and by wrapping the equipment in glass wool. Because of the relatively low vapor pressure of Karathane, a higher distillation temperature than 100° C. is desirable. A temperature of 130° C. is attained by the use of 50% sulfuric acid in the distillation flask. The sulfuric acid, also, keeps any free phenol in an undissociated state and therefore increases its vapor pressure over that of the salts.

To maintain the dimensions, standard equipment is carefully constructed. The most important dimension is the spacing between the bottom of the steam ejector and the bottom of the flask. Variations from 1.0 to 1.5 mm. are without noticeable effect. If the tube is too high from the bottom, recovery values start to drop, and if too low, occasionally the apparatus will seal, resulting in a higher temperature in the steam boiler, and a slower distillation rate. At 140° C., the values for control samples are much higher than those obtained at 130° C. At lower temperatures, the recoveries of added Karathane were erratic and the distillation time increased by as much as a full hour. A trap is used in the distillation head to prevent a physical carryover of substrate or acid. During the steam distillation the solution is under violent agitation and the continuous splashing leaves undistilled material in the gooseneck. This residue is then carried through to the receiver, causing high control values or erroneous results.

As the amount of sample is relatively small compared to the volume of 50% sulfuric acid, the system can be considered essentially a two-component one. As the volume of sulfuric acid must remain constant during the distillation, this means that at constant temperature the amount of water, and, therefore, the total volume in the distillation flask, is also fixed. Repeated measurements of the volume of liquid remaining in the flask after distillation showed that it is between 16 and 17 ml.

The controlling system consists of a fixed-point thermoregulator connected through an electronic relay to a heating mantle on the distillation flask. When steam condenses in the distillation flask, the sulfuric acid concentration falls and the boiling temperature is lowered, causing the regulator to switch on the heating mantle. The additional heat increases the boiling rate and more water distills out than is condensed, raising the sulfuric acid concentration and therefore the boiling point. At the preset temperature, the regulator shuts off the mantle and the temperature starts down again. By means of a simple system such as this, the temperature can be kept well within ±1.0° C., resulting in much better control than was possible manually and also saving up to 1.5 hours of operator time.

Table II gives recovery values at different distillation times.

Table II. Recovery Values at Different Distillation Times

Time, Min.	Volume Collected, Ml.	Absorbance, 442 Mμ[a]
16	200	0.300
35	400	0.295
70	800	0.315
145	1600	0.305

[a] Reagent blank used as a reference.

Table III. Effect of Addition of Water to Karathane Color Development in Pyridine

Water Added, %	Absorbance, 442 Mμ[a]
0.00	0.540
0.99	0.643
1.96	0.666
2.91	0.680
3.84	0.686
4.76	0.677
5.66	0.670
6.54	0.673
7.40	0.666

[a] Reagent blank used as a reference.

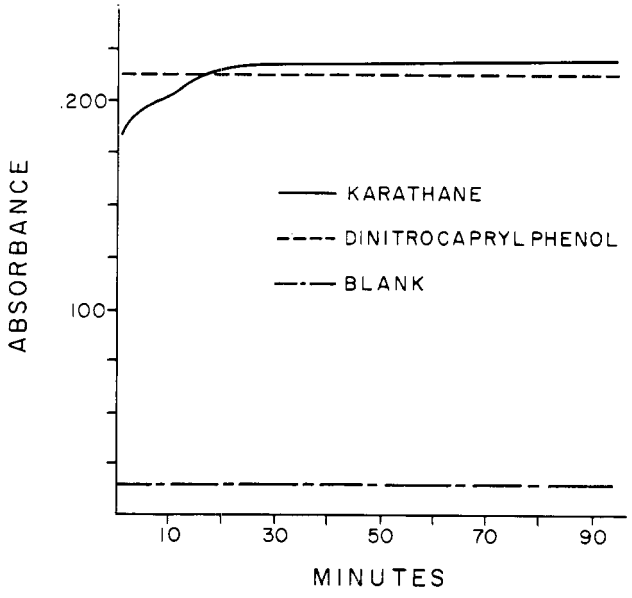

Figure 4. Stability of the color developed in pyridine-water reagent (442 mμ)

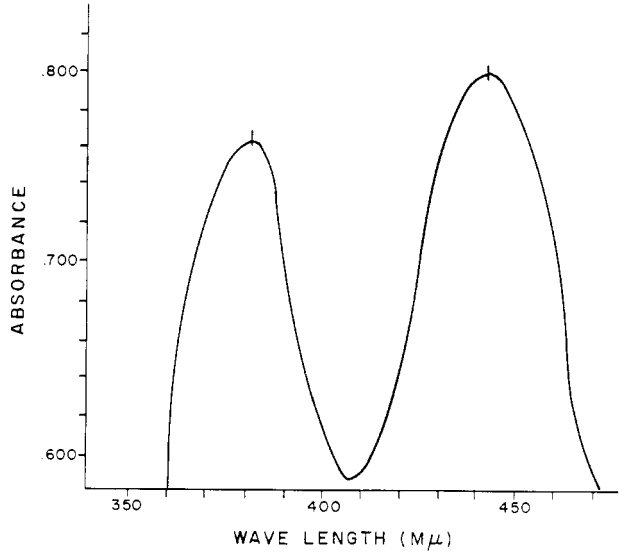

Figure 5. Absorption curve characteristics of dinitrocaprylphenol in pyridine-water reagent

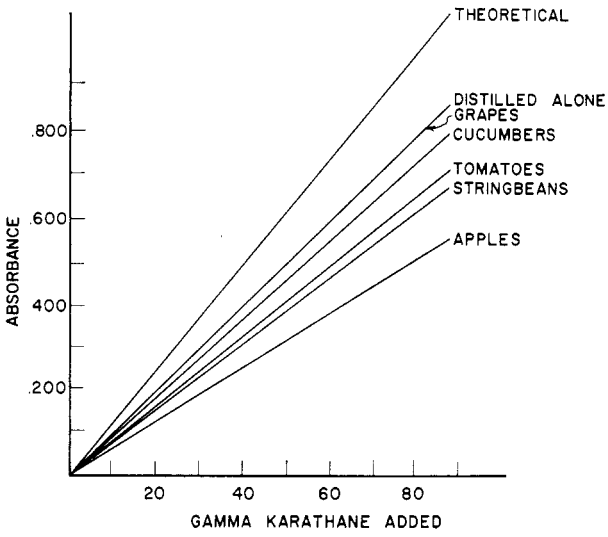

Figure 6. Recovery results compared to theoretical

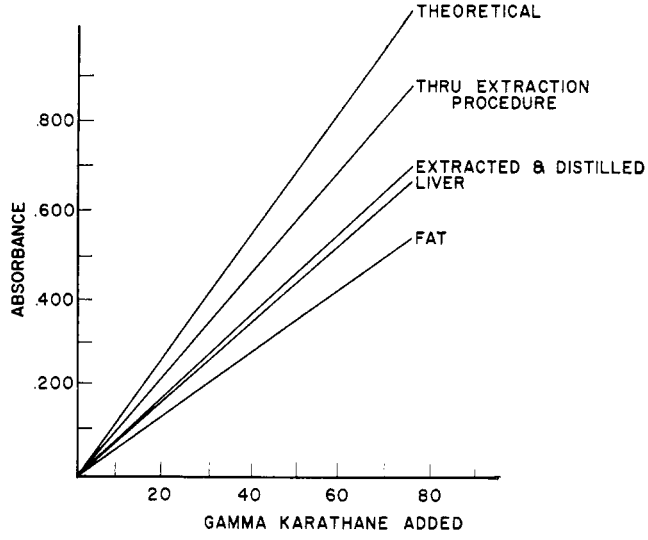

Figure 7. Recovery values of Karathane from animal tissues

Color Development and Spectrophotometric Determination. Addition of dinitrocaprylphenol to pyridine gives a color which is relatively stable (Figure 4) with a broad maximum around 442 mμ (Figure 5).

The absorption band at 442 mμ is due to the dinitrocaprylphenylate ion. Karathane shows very little absorption at this wave length. If the absorbance of a freshly prepared solution of Karathane in pyridine is read *vs.* time, the absorbance rises over a period of 45 minutes (Figure 4) and then levels out at close to the theoretical value for the dinitrocaprylphenol content of the Karathane. The spectrum obtained is similar to that obtained by starting with the dinitrocaprylphenol. The Karathane slowly hydrolyzes in the pyridine solution and, under the conditions used, this is complete after 45 minutes. Karathane samples that are steam distilled show a similar behavior, indicating that the Karathane itself is the principal steam distillation product and that hydrolysis is rather slow in the acid conditions used in the distillation flask.

Variations in the water content of the pyridine cause variations in the color intensity as shown in Table III.

There is an appreciable increase in sensitivity of about 20% followed by a gradual decrease with the use of a water-pyridine solvent. Aside from the increase in sensitivity, however, there is also less chance for error due to the accidental variation of water content, as the maximum of the absorbance water concentration curve is relatively flat while at the zero water level the slope is appreciable.

Because of solubility difficulties with waxy samples such as apples, use of the optimum concentration of water (3 to 4%) is not always practical. The solubility of this type of sample is, however, satisfactory at the 1% water level if, after adding the pyridine or pyridine-water to the flask containing the residue, the flask is put on a steam bath for just a few seconds to warm the bottom of the flask and loosen the waxy or fatty deposits. The final procedure adapted uses the 1% water-in-pyridine solution. This allows nearly maximum sensitivity and eliminates solubility difficulties.

Precision. Replicate determinations on three sets of apparatus with different operators over a period of 2 months gave a standard deviation of ±7.7% at the 40 γ level (12 runs) and ±8.7% at the 20 γ level (9 runs).

Recovery. As long as the absolute recovery figure is reproducible and large enough to give the required sensitivity, that is all that is of importance. Actually, by making the steam distillation volume larger and defining conditions more critically, larger absolute recoveries should be achieved. The present methods and equipment are found to be very satisfactory from the reproducibility standpoint.

Figure 6 illustrates over-all recovery results compared to the theoretical. Recovery from a 200-gram sample extract averages 55 to 100% of that obtained in the absence of substrate. The values obtained by distilling Karathane in the absence of crop extracts are 83% of the theoretical obtained by directly reading Karathane in 3 ml. of pyridine-water. In the case of animal tissues, the modifications in procedure necessary to eliminate interferences lead to losses in the extraction step. This is shown in Figure 7, along with some recovery values.

Blank Values. In general, surface residues on untreated samples are equal to or slightly greater than the solvent or procedure blanks. The solvent blank is 0.02 to 0.03 absorbance, depending on the source of the chloroform, pyridine, and sulfuric acid; and the sample blanks have varied from 0.02 to 0.10 absorbance. With the sample size used for surface residues, this corresponds to about 0.01 to 0.04 p.p.m. on an absolute basis. In a sense, then, it is not necessary to have a check sample to analyze for significant amounts of Karathane surface residues. In this study, check samples were used for all substrates analyzed.

Bulk or pulp samples present another problem. The blank values vary from one class of vegetable to another and there are even variations between types of the same fruit. In studying cucurbits, for example, there is a variation in cucumbers from 0.04 to 0.06 absorbance, and in cantaloupes, from 0.06 to 0.09 absorbance. This corresponds on the extremes to about 0.1 p.p.m. Of course, in correcting for blanks by using fruit from the same field or vicinity, the error is reduced to less than this. In any one determination, however, it is not certain that the check fruits are comparable to the sprayed samples and, therefore, on any sample this blank uncertainty is about 0.1 p.p.m.

Interferences. Parathion, carbamates, and chlorinated insecticides do not interfere.

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