Determination of Ciodrin from Fortified Animal Tissues by Oscillopolarography of Its Conversion Product Acetophenone

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Ciodrin (crotonic acid, 3-hydroxy-α-methyl-benzyl ester, dimethyl phosphate) is determined in extractives from animal tissues by oscillopolarography after its near-quantitative reaction cleanup conversion to acetophenone. Polarographic conditions for the determination of acetophenone are described with the lower limit of detection 0.2 μg. of acetophenone in 2 ml. Extraction and cleanup procedures for up to 100-gram samples are given for meat, fat, liver, eggs, and milk, and recovery data are presented with a lower limit of detection of 0.1 p.p.m. of Ciodrin in 14-gram samples of all tissues.

Ciodrin (crotonic acid, 3-hydroxy-α-methyl-benzyl ester, dimethyl phosphate) is promising for dermal application as sprays or in rubbing devices for the control of some external parasites such as flies, lice, and ticks on livestock. The development of an analytical method to determine possible residues in animal tissues, therefore, became necessary. Oscillopolarography was considered as a possible analytical technique, as Ciodrin could readily be converted to acetophenone, which has a reducible carbonyl group.

Although direct gas chromatography of Ciodrin is possible (Westlake, 1967), the conversion of Ciodrin to acetophenone (Westlake *et al.*, 1969) and oscillopolarographic determination of acetophenone proved to give very reproducible results with high sensitivity and minimum background interference. Extraction and cleanup procedures for eggs (Sawyer, 1966), animal tissues (Ivey *et al.*, 1967), and milk (Beroza and Bowman, 1966) have been adapted and modified for the quantitative determination of Ciodrin. The coagulation solution reported by Frehse and Niessen (1963), when applied to Ciodrin-containing extractives, results in extremely clean samples with good recoveries; it may be adaptable to other slightly water-soluble organophosphorus compounds.

EXPERIMENTAL

Materials and Methods. APPARATUS. Microdistillation apparatus was described by Westlake *et al.* (1969). A Davis Southern Analytical Differential Cathode Ray Polarotrace, Type A 1660A, with D.C.R.P. electrode stand (Western Scientific Associates, San Ramon, California) was used and a mercury-amalgamated No. 22 gauge silver wire reference electrode (anode) was fitted to a standard dropping mercury capillary tube and bung assembly of the above instrument. Gajan 10-ml. polarographic cells were from Western Scientific Associates, San Ramon, Calif.

REAGENTS. The electrolyte solution was 0.1*M* boric acid (A.C.S.) in 0.1*M* sodium hydroxide (A.C.S.), aqueous solution, having a pH of 10.25. The coagulation solution was made by dissolving 1.25 grams of ammonium chloride, analytical reagent grade, in distilled water, adding 2.5 ml. of concentrated phosphoric acid (85%, A.C.S.), and diluting to 1 liter with distilled water.

Extraction and Cleanup Procedures. MILK. Two-hundred milliliters of milk was fortified with from 20 to 200 μg. of Ciodrin in from 0.5 to 2 ml. of acetone and shaken for 1 minute. After half an hour this milk was shaken for 1 minute in a 1-liter separatory funnel with 200 ml. of a 1 to 1 hexane-ether mixture and the separated hexane-ether phase was filtered in a 28-mm. I.D. filter tube through a 2-inch layer of anhydrous sodium sulfate into a 1-liter round-bottomed flask. The milk was reextracted with an additional 200-ml. portion of the hexane-ether mixture which, after separation, was filtered through the same sodium sulfate. The combined extracts were evaporated to a few milliliters on a rotary evaporator (water bath approximately 55° C.) and retained for conversion and steam distillation.

MEAT. Beef sirloin, pork steak, and chicken flesh and skin were ground in a Hobart food grinder. One-hundred-gram samples were weighed into 1-quart Mason jars and fortified with from 10 to 200 μg. of Ciodrin in from 0.5 to 2 ml. of acetone and the mixtures were shaken manually for about 1 minute to disperse the insecticide. Two hundred milliliters of acetonitrile was added about 10 minutes later and the samples were blended for 5 minutes with an Omni-Mixer at medium speed (60-volt setting). The purees were filtered under vacuum through a Buchner funnel, with a Whatman No. 2 filter paper, and the Mason jar and the residues on the filter were rinsed three times with acetonitrile. The combined extracts and washings were placed in a 1-liter round-bottomed flask and the solvent was evaporated on the rotary evaporator (water bath temperature approximately 70° C.) to an aqueous residue (approximately 20 ml.). Fifteen milliliters of acetone were then added to the residues and possible flocculations were dissolved by swirling. Fifty milliliters of coagulation solution were then added with swirling and the samples were kept in the refrigerator for at least 30 minutes before light

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vacuum filtration of the strongly turbid solutions through a 3-inch Buchner funnel fitted with a Whatman No. 2 filter paper and a 5-mm. pad of Hyflo-Super-Cel. The flask and filter were rinsed with another 20 ml. of coagulation solution and the clear or slightly turbid combined filtrates extracted twice with 60-ml. portions of chloroform. The chloroform solutions were filtered through a 2-inch layer of anhydrous sodium sulfate in 28-mm. I.D. filter tubes into 500-ml. round-bottomed flasks and evaporated to dryness on a rotary evaporator (water bath approximately 50° C.). The residues were then redissolved in a few milliliters of acetone and retained for conversion distillation.

LIVER. Preliminary experimentation indicated a possible decomposition of the insecticide in contact with liver tissue and further investigation was designed to determine the rate and extent of such decomposition. Beef liver was cut into small pieces and six 100-gram samples were weighed into 1-quart Mason jars. Two hundred micrograms of Ciodrin in 1 ml. of acetone was added to each sample with careful shaking to effect distribution. Two-hundred-milliliter portions of acetonitrile were added at intervals of 0, 5, 10, 20, 30, and 60 minutes to the series of samples which were subsequently blended for 5 minutes with an Omni-Mixer at medium speed. Extracts were filtered, evaporated, resuspended in acetone, and treated with coagulation solution as described for meat. After filtration and extraction into chloroform, 5 ml. of glacial acetic acid was added to the chloroform in a separatory funnel and shaken for 2 minutes (see discussion). Forty-five milliliters of distilled water was then added and the samples were shaken for an additional 30 seconds. The aqueous layer was discarded and the chloroform fractions were washed three times with 20-ml. portions of distilled water, filtered through a 2-inch layer of anhydrous sodium sulfate in a 28-mm. filter tube into 500-ml. round-bottomed flasks, and evaporated on a rotary evaporator to dryness. The residues were then redissolved in a few milliliters of acetone and retained for conversion and steam distillation.

EGGS. Two whole eggs, weighing 111 to 115 grams, were placed in a 1-quart Mason jar, 20 to 200 μg. of Ciodrin in 0.5 to 2 ml. of acetone was added, and the samples were blended for 2 minutes with an Omni-Mixer at medium speed. Two milliliters of acetonitrile per gram of egg was added to each sample and the mixture blended another 2 minutes for extraction. The extract was vacuum filtered through a Buchner funnel, fitted with a Whatman No. 2 filter paper and a 5-mm. pad of Hyflo-Super-Cel, and the Mason jar and residue on the filter were rinsed three times with acetonitrile. The combined extract and washings were placed in a 1-liter round-bottomed flask and the acetonitrile was concentrated on a rotary evaporator to foaming. Antifoam A spray was added, and the sample was evaporated further to approximately 30 ml. of aqueous residue. Acetone was added, and the sample was treated with coagulation solution as described for meat. The chloroform extract was filtered through a 2-inch layer of anhydrous sodium sulfate in a 28-mm. filter tube, then evaporated on a rotary evaporator (50° C. water bath) to dryness, and redissolved in 10 ml. of ether. The partially cleaned-up sample was then passed through a Florisil column 16 × 80 mm. that had been pre-wet with ether. An additional 30 ml. of ether was passed through the column to elute the sample and the total eluate evaporated on a steam bath and retained for conversion and steam distillation.

FAT. Beef and pork fat were ground in a Hobart food grinder. One-hundred-gram samples were weighed into 1-quart Mason jars and fortified with 10 to 200 μg. of Ciodrin in 0.5 to 2 ml. of acetone by careful shaking. Three hundred milliliters of 2 to 1 hexane-acetone mixture and 50 grams of anhydrous sodium sulfate were added to each sample and the mixture was blended for 5 minutes with an Omni-Mixer at medium speed. The extracts were filtered through a Buchner funnel with Sharkskin filter paper, and the Mason jars and residues on the filter were rinsed three times with 20 to 30 ml. portions of hexane-acetone mixture. The combined extract and washings were placed into a 1-liter round-bottomed flask and evaporated on a rotary evaporator to a fatty residue of approximately 60 ml. (water bath temperature approximately 55° C.). The residues were then transferred to 1-liter separatory funnels with 200 ml. of hexane and 200 ml. of acetonitrile and shaken for 30 seconds. The initial acetonitrile extracts, and two additional acetonitrile extracts of 50 ml. each, were collected in 1-liter round-bottomed flasks. The acetonitrile was evaporated to dryness on a rotary evaporator at 70° C. and the residues were redissolved in 15 ml. of acetone. The samples were then treated with coagulation solution, kept under refrigeration, filtered, and extracted into chloroform as described for meat. The chloroform was evaporated to dryness; the residues were redissolved in a few milliliters of acetone and retained for conversion and steam distillation.

Conversion and Steam Distillation. Samples were transferred with acetone to 125-ml. Erlenmeyer flasks (which had been oven-dried for at least half an hour at 55 to 60° C.), evaporated almost to dryness on a steam bath and the remaining solvent removed with a gentle stream of air. After samples appeared to be dry, they were left about 3 minutes longer under ventilation to ensure complete removal of solvent.

Fifty milliliters of 2*N* sulfuric acid, 5 ml. of 10% sodium dichromate solution, and a boiling chip were added to the dry residues. The flasks were immediately connected to a microdistillation apparatus (Westlake *et al.*, 1969) and placed on a hot plate. Exactly 7 ml. of distillate was collected in a 10-ml. graduated cylinder, then carefully mixed to ensure a homogeneous solution for analytical sampling.

Oscillopolarography. One milliliter of distillate was pipetted into a Gajan-type polarographic cell, and 0.5 ml. of 95% ethanol and 0.5 ml. of electrolyte solution were added. Nitrogen was bubbled through the solution for 3 minutes to remove dissolved oxygen. With the initial voltage set at 1.45 V., the acetophenone peak appeared at -1.71 ± 0.02 V. and the concentration was quantitatively measured by peak height.

An acetophenone standard curve was prepared by pipetting known amounts of acetophenone standard solution in 95% ethanol into a series of Gajan cells; 95% ethanol was added to give a total volume of 0.5 ml., and 1 ml. of distilled water and 0.5 ml. of electrolyte solution were added. The standard samples were analyzed as above.

A Ciodrin working standard curve was prepared by simultaneous conversion and steam distillation of known amounts of Ciodrin, collecting exactly 7 ml. of distillate, as above. A 1-ml. aliquot of the distillate was transferred to the polarographic cell, 95% ethanol and electrolyte solution added, and the sample analyzed. Polarographic units for 1 ml. of distillate were plotted against the total amount of Ciodrin to give a working standard curve from which fortified samples could be read directly. The operation of the polarograph, especially proper maintenance of the electrodes, has been discussed in detail by Hearth *et al.* (1966). Note: A polarographic unit is the number of scale divisions (full scale = 100) times the amplification factor.

Table I. Recoveries of Ciodrin from Milk and Animal Tissue

Amount Added, p.p.m.	Recovery, %						
	Milk	Beef	Pork	Chicken	Eggs	Beef fat	Pork fat
0.1	109	62	73	52	119	40	52
0.2	108	74	71	71	109	69	58
0.3	107	...	...	...	...	...	...
0.5	104	86	83	84	88	73	72
1.0	108	72	83	78	78	62	66
2.0	...	87	...	...	72	65	72

RESULTS AND DISCUSSION

Recovery data from fortified milk and animal tissues are shown in Table I. Only in milk was there a slight contamination at the same potential as acetophenone, for which corrections were required. The procedure for milk was the only one for which no coagulation solution was necessary in the cleanup; despite this slight blank contamination and tendency toward high recovery (107.1% average), milk samples were quite clean and could easily be evaluated.

When no coagulation solution was used, all other tissues showed high background and interference peaks at −1.63 and −1.81 volts despite the steam distillation step. Aldehyde, or other carbonyl-containing compounds, is probably formed, as evidenced by a distinct odor in the distillate, and quantitative determination below 1 p.p.m. was difficult. Cleanup by other means as with charcoal, for example, somewhat reduced this background and reduced the minimum limit of detectability to approximately 0.5 p.p.m. With the addition of 1 ml. of 1% sodium bisulfite solution to the distillate, the interference which preceded the acetophenone peak was eliminated, but not the following interference. An 0.2M electrolyte buffer solution was then used to keep the pH approximately the same (pH 10.16) as that without sodium bisulfite and with 0.1M buffer electrolyte. In the presence of sodium bisulfite, the acetophenone peak shifted to −1.58 to −1.59 V. maximum with nearly the same sensitivity, but the following peak interference persisted and had also shifted to a lower voltage maximum, thus increasing the degree of interference.

The treatment with coagulation solution resulted in extremely clean samples. The ratio of a 15-to-50 ml. acetone-to-coagulation solution should not be exceeded. The addition of acetone helped to keep the pesticide in solution, but an excess prevented complete coagulation.

It was advisable to add the acid and sodium dichromate solution immediately before the conversion and steam distillation step. With longer standing in this oxidation solution, the tissue was gradually attacked and formed increasing amounts of interfering substances. The addition of the oxidation solution immediately before conversion and rapid heating for steam distillation allowed sufficient time to hydrolyze and oxidize Ciodrin for good recovery of acetophenone. Under these conditions only occasional trace interference at −1.63 V. was found in tissue distillates. Beef, pork, and chicken meat (see Table I) showed an average recovery of 77.2% above the 0.2 p.p.m. level.

The average recovery from fortified eggs (Table I) was 88.8% above 0.2 p.p.m. but tended to be higher at lower levels. A small but distinct peak occurred at −1.63 V., which slightly raised the background in the −1.71 V. region and might be responsible for the slightly higher apparent recovery. The use of coagulation solution as a cleanup step for eggs removed all emulsifying substances present and simplified the handling during sample processing.

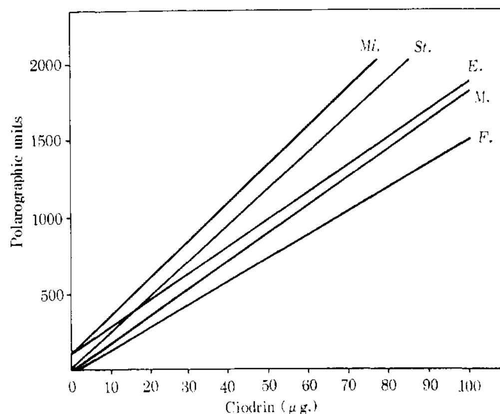

Figure 1. Recovery of Ciodrin from fortified samples, compared to Ciodrin standard
St = Ciodrin standard, Mi = milk, E = eggs, M = meat, and F = fat

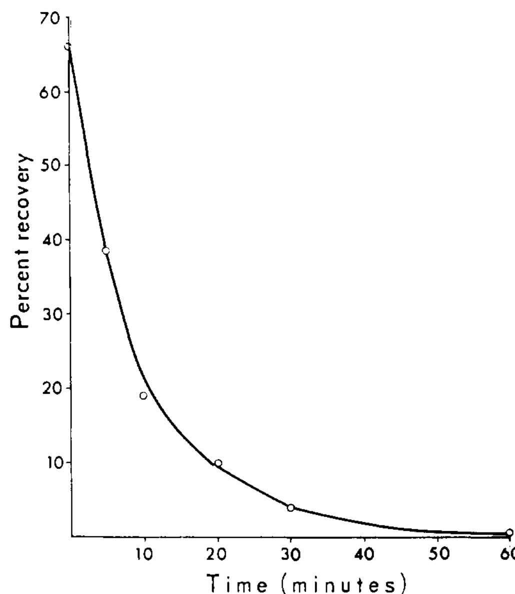

Figure 2. Breakdown of Ciodrin in liver (200 μg. per 100 grams of liver)

By processing larger samples of meat, fat, and eggs, it should be possible to decrease the minimum limit of detection. The lower limit of detection in 100-gram samples was limited by the response to acetophenone derived from Ciodrin, not by background interference, being 5 μg. for pure Ciodrin standard and approximately 10 μg. in fortified samples when 7 ml. of distillate was collected.

All recovery data are plotted in Figure 1 in comparison to a Ciodrin standard. These recovery curves should be used as working standards for actual residue determinations.

During the development of the extraction and cleanup procedure for liver which would be suitable for polarographic analysis, it was found that Ciodrin was decomposed very rapidly by enzyme action. Two hundred micrograms of Ciodrin in contact with chopped liver had, after 1 hour, almost completely disappeared (Figure 2), proving that no Ciodrin residues would be expected in liver. Liver also contained unidentified compound(s) that caused interference during polarography. Washing the chloroform solution of

extractives with acetic acid after the cleanup steps and before steam distillation effectively removed the interference.

According to Westlake *et al.* (1969), who investigated the conversion of Ciodrin to acetophenone, 10 ml. of distillate should be collected for complete recovery. With the more sensitive oscillopolarographic method, recoveries for 5, 7, and 10 ml. of distillate were compared and read from an actual acetophenone standard curve based upon the theoretical conversion value of 2.62 μg. of Ciodrin to 1 μg. of acetophenone. Percentages of 88.5, 89.9, and 100.5, respectively, for the three distillate volumes were recovered with a deviation of $\pm 6.0\%$ for the 7-ml. distillate and $\pm 7.3\%$ for the 10-ml. distillate. Although recovery from a 7-ml. distillate is not quite complete, the sensitivity of the analytical method is increased by using 7 ml. instead of 10 ml. The 5-ml. volume was not used because it was on the borderline for reproducibility, particularly for actual samples. The linear relationship between polarographic peak heights and micrograms of acetophenone produced holds up to 500 μg. of Ciodrin.

The procedure of conversion and steam distillation with subsequent polarographic determination required the use of oven-dried Erlenmeyer flasks. Those which had not been oven-dried gave a contamination peak at -1.82 V., which interfered seriously with the quantitative evaluation of acetophenone. Traces of adsorbed acetone, used to clean the flasks, that react with the oxidation solution may be responsible for this interference. Acetone is also one of the reaction products but is apparently distilled unchanged from the reaction mixture as it is formed. The presence of acetone in the distillate does not interfere with the polarographic analysis of acetophenone. It interferes only when attacked by the oxidizing solution. As much as 3 drops of acetone, added to the distillate, did not affect polarographic measurement.

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