centration of 4% Cr_2O_3 in the diet was satisfactory since it was not toxic to feeding insects and did not appear to affect consumption (unpublished data). This concentration was high enough, however, to provide measurable quantities of Cr_2O_3 in insect food and excreta.

Acknowledgment

The technical assistance of A. G. Hewitt is gratefully acknowledged.

Literature Cited

(1) Blundy, P. D., *Analyst* **83,** 555 (1958).
(2) Bolin, D. W., King, R. P., Klosterman, E. W., *Science* **116,** 634 (1952).
(3) Brisson, G. J., *Can. J. Agr. Sci.* **36,** 210 (1956).
(4) Currier, A., Kitts, W. D., Cowan, I. McT., *Can. J. Zool.* **38,** 1109 (1960).
(5) Czarnocki, J., Sibbald, I. R., Evans, E. V., *Can. J. Animal Sci.* **41,** 167 (1961).
(6) Dansky, L. M., Hill, F. W., *J. Nutr.* **47,** 449 (1952).
(7) Hill, F. W., Anderson, D. L., *Ibid.*, **64,** 587 (1958).
(8) Kasting, R., McGinnis, A. J., *Can. J. Zool.* **37,** 713 (1959).
(9) McGinnis, A. J., Kasting, R., *Can. J. Plant Sci.* **41,** 469 (1961).
(10) McGinnis, A. J., Kasting, R., *Entomol. Exptl. Appl.* **5,** 313 (1962).
(11) Moore, J. H., *Brit. J. Nutr.* **11,** 273 (1957).
(12) *Ibid.*, **12,** 24 (1958).
(13) Smith, D. S., *Ann. Entomol. Soc. Am.* **52,** 674 (1959).
(14) Troelson, J. E., M.Sc. thesis, University of Saskatchewan, 1960.
(15) Urone, P. F., Anders, H. K., *Anal. Chem.* **22,** 1317 (1950).

Received for review July 1, 1963. Accepted September 18, 1963.

HERBICIDE RESIDUE IN SEAFOOD

Determination of Butoxyethanol Ester of 2,4-Dichlorophenoxyacetic Acid in Shellfish and Fish

J. E. COAKLEY,[1] J. E. CAMPBELL and E. F. McFARREN

Milk and Food Research, Robert A. Taft Sanitary Engineering Center, Public Health Service, Cincinnati, Ohio

In view of the extensive shellfish industry operating in the Chesapeake Bay, information on the uptake and retention of the butoxyethanol ester of 2,4-dichlorophenoxyacetic acid by commercially important species of shellfish became a prerequisite to the consideration of the widespread use of this herbicide to control Eurasian watermilfoil. A colorimetric method is described which showed that oysters and clams contained between 3.5 and 3.7 p.p.m. of esters when exposed for 3 days at the center of a 1-acre plot treated at a dosage rate of 30 pounds acid equivalent per acre. The method as verified by gas chromatography can be used to determine either the free acid or ester content of shellfish.

Because of the rapid invasion and explosive growth of Eurasian watermilfoil, *Myriophyllum spicatum*, in the Chesapeake Bay Region, joint field studies were conducted by the Natural Resources Institute of the University of Maryland, the Virginia Institute of Marine Science, the Maryland Game and Inland Fish Commission, and the U. S. Fish and Wildlife Service to find a method for controlling this aquatic plant. These studies revealed that esters of 2,4-dichlorophenoxyacetic acid (2,4-D) impregnated on attaclay granules were more effective than other phenoxy compounds for the control of Eurasian watermilfoil (*11*).

In view of the extensive shellfish industry operating in those waters, information concerning the uptake and retention of these esters by commercially important species of shellfish became a prerequisite to a consideration of the widespread use of this herbicide. Accordingly, a plot containing watermilfoil was experimentally treated with the butoxyethanol ester of 2,4-D at a dosage rate of 30 pounds of acid equivalent per acre, and oysters, clams, fish, and crab were held at the center of this plot. Three days after herbicide treatment, samples of each of the above-mentioned species were collected by the Chesapeake Biological Laboratory (*1*), Natural Resources Institute of the University of Maryland, Solomons, Md., and sent to the Robert A. Taft Sanitary Engineering Center for analysis. As a result, it became necessary to develop a method of analysis for 2,4-D and its esters in shellfish and fish.

Freed (*4*) found that a characteristic wine-purple color was produced when 2,4-D was heated in concentrated sulfuric acid with 4,5 dihydroxy-2,7-naphthalenedisulfonic acid (chromotropic acid). Marquardt and Luce (*8*) developed a quantitative colorimetric application of this reaction to the determination of 2,4-D in milk. In part, their method has been applied to the determination of 2,4-D in shellfish and fish.

The method described here for determining the butoxyethanol ester or 2,4-D content of shellfish and fish involves hydrolysis of the ester to 2,4-D by shaking with a dilute base, acidification and extraction of the 2,4-D with benzene, extraction of the benzene with buffer, extraction of the buffer with carbon tetrachloride, chromatography on Florisil (*9, 10*) to remove some of the fats and pigments, elution of the 2,4-D from the Florisil with methanol, evaporation of the methanol, reaction of the 2,4-D with chromotropic acid, and spectrophotometric measurement of the color.

Reagents

2,4-Dichlorophenoxyacetic acid (2,4-D) recrystallized from benzene (Matheson, Coleman and Bell). Dissolve 0.1000 gram of purified 2,4-D in absolute methanol and dilute to volume in a 100-ml. volumetric flask. Store in amber-colored bottle with an aluminum-lined screw cap. Prepare from this stock solution 2,4-D standards containing 5, 10, 20, 40, 60, and 80 μg. per ml.

Butoxyethanol ester of 2,4-D (Technical, Amchem Products, Inc., Ambler, Pa.). Dissolve 0.4000 gram of butoxyethanol ester of 2,4-D in methanol and dilute to volume in a 100-ml. volumetric flask. Prepare from this stock solution 2,4-D ester standards containing 1000, 2000, and 3000 μg. per ml.

Phosphate buffer, pH 6.7. Dissolve 25 grams of reagent-grade dibasic sodium phosphate and 10 grams of monobasic sodium phosphate in 1 liter of distilled water. Adjust to pH 6.7 by adding monobasic sodium phosphate.

4,5 - Dihydroxy - 2,7 - naphthalenedisulfonic acid, practical (chromotropic acid) (Matheson, Coleman and Bell).

[1] Present address: Food and Drug Administration, Washington 25, D. C.

Dissolve 0.1500 gram of chromotropic acid (recrystallized from ethanol) in concentrated sulfuric acid and dilute to 100 ml. with concentrated sulfuric acid. Prepare fresh daily.

Florisil, 60- to 200-mesh (Chromatographic adsorbent) (Matheson, Coleman, and Bell). Activate Florisil by heating at 650° C. for 2 hours, cooling in an oven at 130° C. for 2 hours, and finally cooling to room temperature in a desiccator. Place activated Florisil in an amber-colored bottle with a screw cap and store in a dessicator until used.

Polyester, cross-linked diethylene glycol adipate (F and M Scientific Corp., New Castle, Del.).

Glass Beads, Class IV, Type 507 (Microbeads, Inc., Jackson, Miss.).

Apparatus

Bottles polyethylene, 250-ml. with screw caps.

Platform shaker.

Centrifuge with head and cups for 250-ml. glass bottles.

Wrist-action shaker.

Beckman Model B spectrophotometer with 5-cm. cuvettes.

Gas chromatograph, Jarrell Ash, Universal Model 700, fitted with an electron affinity detector.

Water bath, Army Medical School Model boiling water bath (Fisher Scientific Co.).

Experimental Procedure

Preparation of Standard Curve for 2,4-D. One milliliter each of methanol solutions of 2,4-D containing 5, 10, 20, 40, 60, and 80 μg. per ml. was placed in lipless test tubes (19 × 105 mm.) and evaporated to dryness in a boiling water bath, having only about $1^1/_2$ inches or less of the tube extending above the hot water bath.

After evaporation of the methanol, the residue was heated for an additional 10 minutes to ensure complete volatilization of the methanol. The residue was taken up in 5 ml. of chromotropic acid and heated in a boiling water bath for 30 minutes with an occasional twirling. The resulting wine-purple solutions were cooled in ice water and transferred with rinsings to 25-ml. volumetric flasks containing 13 ml. of chilled water. The samples were diluted almost to volume with water and were allowed to reach room temperature before final volume adjustment. One milliliter of methanol was similarly treated and used as a blank for zeroing the spectrophotometer.

The absorbances were measured in 5-cm. cuvettes with a Beckman Model B spectrophotometer at 570 mμ. With the sensitivity set at 3, the slit width fell between 0.03 and 0.06 mμ when the instrument was zeroed on the blank.

Determination of 2,4-D Content of Technical-Grade Ester. One milliliter each of methanol solutions containing 1000, 2000, 3000, and 4000 μg. of ester were pipetted into 50-ml. volumetric flasks. Twenty-five milliliters of water was added and the solution adjusted to pH 12 with 6 ml. of 0.1*N* NaOH.

The solutions were shaken on a platform shaker for $1^1/_2$ hours to hydrolyze the ester. The samples were diluted almost to volume with water, and 2 ml. of concentrated HCl was added to adjust the pH to 2. These solutions were then made up to volume with water. One-milliliter aliquots of each of these, representing 20, 40, 60, and 80 μg. of ester, were placed in test tubes, evaporated to dryness, treated with chromotropic acid for color development, and their absorbances measured as before.

The absorbance of a given amount of hydrolyzed ester was related to the 2,4-D content, as read from the 2,4-D standard curve, to determine the acid equivalent of the technical butoxyethanol ester.

Efficiency of Solvent Extraction. A known amount of 2,4-D was added to 50 ml. of acidified water, extracted twice with 50 ml. of benzene each time, and the 2,4-D remaining in the water determined by evaporating of an aliquot to dryness and developing the color with chromotropic acid. From the amount of 2,4-D remaining, the distribution coefficient for 2,4-D in water extracted with benzene was calculated. Likewise, the distribution coefficients for 2,4-D in benzene solution extracted with buffer and for 2,4-D in buffer extracted with carbon tetrachloride were calculated.

From these distribution coefficients, it was then possible to calculate the amount of 2,4-D that theoretically could be recovered by using any combination of solvents, solvent volumes, or number of solvent extractions.

Hydrolysis of Ester. Frozen oyster, clam, crab, and fish samples were thawed and blended for 1 minute. Fifty-gram homogenates of each were weighed into 250-ml. polyethylene bottles. The pH was adjusted to 12 by adding 2.5 ml. of 6*N* NaOH. The mixture was shaken on a mechanical shaker for $1^1/_2$ hours to hydrolyze the ester to 2,4-D.

Extraction of 2,4-D. A small amount of water was used to transfer the samples to 250-ml. glass centrifuge bottles. Five grams of NaCl was added, and the pH was adjusted to 2 by the addition of 3 ml. of concentrated HCl. Fifty milliliters of benzene was added to the mixture; the bottles were stoppered with aluminum foil-covered rubber stoppers and vigorously shaken for 3 minutes. The samples were centrifuged for 10 minutes at 2400 r.p.m. The bottles were twirled gently to wash down any residue clinging to the walls of the bottle above the benzene. The samples were centrifuged for an additional 15 minutes, and the benzene was decanted into a 250-ml. graduated cylinder. This extraction procedure was repeated three more times; the extracts were pooled; and the total volume measured to obtain a solvent recovery factor. For example, although a total of 200 ml. of benzene was always added, in general, only about 170 ml. was recovered because of some remaining emulsions. In this case, the benzene recovery factor would be 170 divided by 200, or 85%. This factor was calculated for each determination, and applied, to correct for the resulting loss in 2,4-D.

The combined benzene extracts were filtered through Whatman No. 1 filter paper into 250-ml., pear-shaped separatory funnels. The filter was washed with 5 ml. of benzene. The filtered benzene was extracted twice with 24-ml. portions of the buffer. Both buffer extracts were filtered through Whatman No. 1 filter paper into a 250-ml. separatory funnel, and the filter paper was washed with 2 ml. of buffer.

The combined buffer extract and washings were adjusted to a pH of 2.0 with concentrated HCl, and extracted four times with 50-ml. portions of carbon tetrachloride. The CCl_4 extracts were pooled in 300-ml. Erlenmeyer flasks and evaporated to about 25 ml. on a hot water bath.

Chromatography on Florisil. A 25-ml. buret with an inside diameter of 1 cm. was cut off to give a column about 20 cm. in length, but any other glass tube of similar diameter fitted with a stopcock can be used. A glass-wool plug was placed in the bottom of the column, and the column was almost filled with carbon tetrachloride. About 2 grams of activated Florisil was added to the column, which was tapped gently with a ruler until the Florisil had slurried and settled to the bottom of the column. Additional Florisil was added in a like manner until it had reached a height of about 9 cm. The column was stoppered with an aluminum foil-covered cork, shaken, inverted, and uprighted until the Florisil had slurried with the carbon tetrachloride. The column was supported in an upright position and tapped gently from top to bottom until no more settling occurred when carbon tetrachloride was passed through at the rate of 1 ml. per minute. The height of the Florisil in the column was finally adjusted to 9.0 cm. by adding a little Florisil at a time, stirring with a glass rod, and allowing to settle as before. About 2 ml. of carbon tetrachloride was allowed to remain above the top of the Florisil. At no time in the process was the column allowed to run dry.

Anhydrous sodium sulfate was added to the top of the column and tapped down with a glass rod until a 1.25-cm. layer was firmly packed on the Florisil. The carbon tetrachloride was drained off until about 0.3 ml. remained above the top of the sodium sulfate. The

concentrated carbon tetrachloride extract of the sample was allowed to flow through the column at the rate of 1 ml. per minute. The flasks were rinsed 5 times with 10-ml. portions of carbon tetrachloride, which was subsequently passed through the column. The column was then washed with 15 ml. of ethyl ether, which removed most of the remaining fats and pigments.

As the last of the ether entered the top of the sodium sulfate layer, absolute methanol was added to elute the 2,4-D. Approximately 5 ml. more of ether was collected before the methanol appeared in the capillary of the buret. The emergence of methanol can be detected by a change in the appearance of the effluent in the buret tip when the first drop of methanol enters the capillary tip. The first 30 ml. of methanol eluate collected contained all of the 2,4-D. All or aliquots of the methanol eluate (depending on the concentration of 2,4-D) were evaporated to dryness, treated with chromotropic acid for color development, and the absorbances measured after the spectrophotometer was zeroed on a similarly prepared methanol eluate of a control sample. The amount of 2,4-D in the eluate was determined by relating the absorbances of these solutions to the equivalent amount of 2,4-D as read from the 2,4-D standard curve.

Differentiation between Ester and Free Acid. Essentially, the technique of Erickson and Heild (*2*) was used to separate 2,4-D from its ester; a nonhydrolyzed homogenized sample was acidified to a pH of 2 and extracted with benzene. The benzene solution containing both free acid and the ester was then extracted with buffer to remove the free acid (2,4-D). The buffer in turn was extracted with carbon tetrachloride; the carbon tetrachloride extract was chromatographed on Florisil; and the 2,4-D in the eluate measured by developing its color with chromotropic acid in the usual manner. The benzene solution remaining (from above) after extraction with buffer was evaporated to dryness, taken up in methanol, diluted with water, adjusted to pH 12 with sodium hydroxide; the ester was hydrolyzed by shaking for $1^1/_2$ hours, and the 2,4-D content was determined as before.

Verification of 2,4-D in Florisil Column Eluates by Gas Chromatography. A 1-foot, stainless steel column with an inside diameter of $^1/_4$ inch was packed with glass beads coated with 1% diethylene glycol adipate containing 0.4% phosphoric acid. A similar column has been used by Jowett and Harrocks (*5*) for separation of unesterified fatty acids. The column was conditioned in the gas chromatographic oven for 2 days at 180° C. With this column, maximum peak heights for a given concentration of 2,4-D could be obtained with a nitrogen carrier-gas flow rate of 200 ml. per minute, an oven temperature of 180° C., and an electron affinity detector potential of 24 volts.

Aliquots of methanol eluates from Florisil columns prepared as for 2,4-D determination with chromotropic acid were evaporated to dryness and made up to a known volume in a 0.01*N* acetic acid solution in benzene. Ten-microliter aliquots were run on the gas chromatograph.

Table I. Determination of Amount of 2,4-D in Technical-Grade Ester

Ester Added μg.	Absorbance[a] of Hydrolyzed Ester Reacted with Chromotropic Acid	Amount of 2,4-D from 2,4-D Standard Curve, μg.	Per Cent Acid Equivalent
20	0.165	13.7	68.50
40	0.328	27.4	68.50
60	0.490	40.9	68.17
80	0.654	54.6	68.25
		Av.	68.36

[a] Average of duplicate determinations.

Results

When a standard curve for 2,4-D was prepared as described in the experimental procedure, points were obtained which fell on a straight line. In repeated determinations, these points fell within a standard deviation range varying from 3.6% at 10 μg. to 7.6% at 80 μg.

The acid equivalent of the technical butoxyethanol ester averaged 68.36 (Table I), when the absorbance of a given amount of the hydrolyzed ester reacted with chromotropic acid was determined and the 2,4-D content was read from the 2,4-D standard curve.

The distribution coefficient for the extraction of 2,4-D from water with benzene was 0.95; for 2,4-D in benzene extracted with buffer, 0.03; and for 2,4-D in buffer extracted with carbon tetrachloride, 1.30. From these distribution coefficients, it was calculated that by four 50-ml. extractions with benzene, 93.5% of the 2,4-D could be recovered. Likewise, two 24-ml. buffer extractions of the pooled benzene extracts should recover 96.5%, and four equal-volume extractions of the pooled buffer extracts with carbon tetrachloride should recover 89.6%. Therefore, with a quantitative recovery of the solvent in each step and no change in the distribution coefficients due to the presence of other substances, the maximum amount of 2,4-D that could be expected to be recovered with such a procedure applied to shellfish or fish would be (93.5 × 96.5 × 89.6) or 80.7%.

The recovery of ester or free acid, when added to 50-gram samples of oysters, clams, and fish, and analyzed by the described method, was as indicated in Table II. The amount of 2,4-D recovered averaged 70.9% with a precision (Table III) of ±3.7%. On the basis of the maximum possible recovery calculated from distribution coefficients (see above), the actual recovery is 70.9 divided by 80.7, or 87.9%.

To test the applicability of this method for determining 2,4-D or ester residues in shellfish and fish, freshly shucked oysters, clams, crabs, and fish collected prior to herbicide treatment (control samples), and samples of these same species that had been held at the center of a 1-acre plot for 2 days after treatment with 2,4-D ester (30 pounds of acid equivalent per acre) were analyzed.

The results obtained (Table IV) indicate a residue of 3.5 to 3.7 p.p.m. of ester and/or 2,4-D in the exposed oysters and clams. However, when nonhydrolyzed extracts were analyzed, only traces of 2,4-D were found; whereas, 2.8 p.p.m. of ester determined as 2,4-D was found in hydrolyzed extracts. Thus in oysters and clams the residue is primarily ester rather than the acid. Whether this is true for fish and crabs is not known, since they were not similarly analyzed.

The previously described procedure for the determination of 2,4-D by gas chromatography was used to construct standard curves for both 2,4-D and its ester. Concentrations of 2,4-D used were in the range of 0.02 to 0.2 μg. per 10 μl. of a 0.01*N* acetic acid solution in benzene. With the ester, concentrations of 0.01 to 0.1 were used successfully. The retention time for 2,4-D was $6^1/_2$ minutes, and for the ester, $2^1/_4$ minutes. Mixtures of the ester and 2,4-D were also separated and compared with peak heights and retention times previously obtained with the individual compounds of the same concentration. Finally, eluates from Florisil columns prepared as for 2,4-D determination with chromotropic acid were analyzed by gas chromatography and peaks corresponding to the retention time of 2,4-D were found in both experimental and spiked samples.

Discussion

The method of Marquardt and Luce (*8*) described for milk was only partly satisfactory for shellfish. In the initial extraction with ether, emulsions were formed which were exceedingly difficult to break. Of other solvents tried, benzene proved the most satisfactory. Some difficulty was still experienced with emulsions, but this problem was overcome largely by the addition of sodium chloride, and the benzene extract was successfully extracted with buffer and the buffer in turn with carbon tetra-

Table II. Recovery of 2,4-D When Added to Shellfish or Fish as Either the Acid or the Ester

Ester[a] Added, μg.	2,4-D Content of Ester, μg.	2,4-D Found in Sample, μg.	Benzene Extract Recovery Factor	2,4-D in Sample Corrected, μg.	Recovery of 2,4-D,[b] %
644.80	441.30	255.0	0.835	305.39	69.2
866.10	592.76	320.25	0.845	378.99	63.9
...	520.0[c]	315.50	0.860	366.86	70.6
230.96	158.07	88.8	0.855	103.86	65.7
230.96	158.07	97.2	0.845	115.03	72.8
433.05	296.38	172.5	0.855	201.75	68.1
433.05	296.38	178.1	0.850	209.53	70.7
461.90	316.12	211.5	0.890	237.64	75.2
230.96	158.07	108.8	0.915	118.91	75.2
115.50[d]	79.05	53.85	0.915	58.85	74.4
57.75[d]	39.52	24.45	0.880	27.78	70.3
115.50[e]	79.05	55.05	0.935	58.88	74.5

[a] Butoxyethanol ester of 2,4-D added to oysters unless otherwise indicated. [b] Maximum theoretical recovery, calculated from distribution coefficients, is 80.70%. [c] Added as the acid. [d] Clams. [e] Fish.

Table III. Precision of 2,4-D Recovery

2,4-D Added, μg.	2,4-D Found, Grams	Recovery, %	Deviation from Mean	Deviation from Mean Squared
441.30	305.59	69.2	1.70	2.89
592.76	378.99	63.9	7.00	49.00
520.00	366.86	70.6	0.03	0.09
158.07	103.86	65.7	5.20	27.04
158.07	115.03	72.8	1.90	3.61
296.38	201.75	68.1	2.80	7.84
296.38	209.53	70.7	0.20	0.04
316.12	237.64	75.2	4.30	18.49
158.07	118.91	75.2	4.30	18.49
79.05	58.85	74.4	3.50	12.25
39.52	27.78	70.3	0.60	0.36
79.05	58.88	74.5	3.60	12.96
	Mean	70.9	Std. Dev.	±3.7

Table IV. Butoxyethanol Ester Content of Oyster, Clam, Crab, and Fish Homogenates from Species Exposed in Herbicide-Treated Areas

Sample No. (50 Grams Each)	2,4-D in Sample	Benzene Recovery Factor	2,4-D in Sample Corrected,[a] μg.	Ester[b] Content	P.P.M. of Ester
		Oysters (*Crassostrea virginica*)			
1	75.1	0.875	121.1	176.0	3.5
2	73.5	0.860	120.5	175.1	3.5
3	85.5	0.925	130.3	189.4	3.8
4	81.0	0.910	125.6	182.6	3.7
		Clams (*Mya arenaria*)			
1	77.1	0.910	119.5	173.7	3.5
2	80.3	0.930	121.9	177.2	3.5
3	80.7	0.920	123.8	179.9	3.6
4	81.3	0.920	124.7	181.3	3.6
		Fish (*Lepomis gibbus*)			
1	7.5	0.935	11.3	16.4	0.3
2		not detectable			
		Bluecrabs (*Callinectes sapidus*)			
1	18.0	0.960	26.4	38.4	<0.8

[a] Includes 2,4-D recovery factor of 0.709. [b] Butoxyethanol ester of 2,4-D with complete hydrolysis of ester and an acid equivalent of 68.8% for pure ester.

chloride, as per the method of Marquardt and Luce. However, when chromotropic acid was used to develop the color in the carbon tetrachloride extracts, fats and pigments from the shellfish still remained, giving high blanks and obscuring any color due to 2,4-D in the extracts. The modification of Marquardt and Luce (*7*), in which stannous chloride is used to bleach the amber color due to the reaction of acidic material from grain with chromotropic acid, was found to be inapplicable to oysters. Their (*6*) method of splitting the phenoxy acids and determining the resulting phenolics was not tried for it was felt that the problems of interference in the determination of phenols woud be equally great. Chromatography on Florisil was successfully applied to cleaning up the carbon tetrachloride extracts, but difficulty was still encountered with the chromotropic acid color test. When the chromotropic acid solution of 2,4-D was heated in a boiling water bath for 30 minutes instead of in an oven at 130° to 135° C. for 20 minutes, as was done by Marquardt and Luce, a more reproducible color development was obtained.

The color reaction of chromotropic acid in concentrated sulfuric acid, is not entirely specific for 2,4-D. According to Freed (*4*), phenoxyacetic acid and its various halogen derivatives also give a wine-purple color. According to Figel (*3*), formaldehyde or any substance that breaks down to give formaldehyde, such as monochlorobenzene, methanol, hexamethylene tetramine, formaldoxine, trimethylene, L-hammite, celose formalas, etc., also gives the characteristic color with chromotropic acid. In 2,4-D determinations, therefore, reagents and glassware must not be contaminated with these substances, and all of the methanol must be volatilized from 2,4-D solutions before the color is developed with chromotropic acid. In early experiments, some of the methanol condensed on the walls of the test tubes above the boiling water in the bath and was not completely volatilized. The authors found, however, that all of the methanol could be removed by using shorter test tubes so that only about $1^1/_2$ inches or less of the tube extended above the hot water bath. No 2,4-D was found in any of the control samples (oysters, clams, fish, and crabs collected from nontreated areas), but controls do contain a small amount of a brownish interfering material. Since this interfering material is also in samples containing 2,4-D but not in reagent blanks, it is most easily corrected for by zeroing the spectrophotometer on a similarly prepared control.

Acknowledgment

The authors wish to express their appreciation to G. Francis Beavens, who furnished shellfish and fish samples which had been exposed to the ester in areas experimentally treated to control watermilfoil.

Literature Cited

(1) Beaven, G. F., Rawls, C. K., Beckett, G. E., *Proc. Northeast. Weed Control Conf.* **16,** 449 (1962).
(2) Erickson, L. S., Heild, H. Z., J. Agr. Food Chem. **10,** 204 (1962).
(3) Figel, F., "Spot Tests in Organic Analysis," p. 374, Elsevier Publishing Co., New York, 1960.
(4) Freed, V. H., *Science* **107,** 98 (1948).
(5) Jowett, P., Harrocks, B. J., *Nature* **192,** 966 (1961).
(6) Marquardt, R. P., J. Agr. Food Chem. **9,** 266 (1961).
(7) Marquardt, R. P., Luce, E. N., *Ibid.*, **3,** 51 (1955).
(8) Marquardt, R. P., Luce, E. N., *Anal. Chem.* **23,** 1484 (1951).
(9) Mills, P. A., *J. Assoc. Offic. Agr. Chem.* **42,** 734 (1959).
(10) Moddes, R., *Ibid.*, **44,** 169 (1961).
(11) Steenis, J. H., Stotts, V. L., Gillette, C. R., *Proc. Northeast. Weed Control Conf.* **16,** 442 (1962).

Received for review March 18, 1963. Accepted November 18, 1963. The use of commercial product names in this paper should not be interpreted as endorsement by the Public Health Service.