with Squoxin-*t* as an indicator of potential residues in fish is confirmed by the field experiments (Tables V, VI, and VII). These samples were analyzed by the GC method of Kiigemagi et al. (1975) which is specific for Squoxin.

The results of the analyses of squawfish from the John Day and Mollala River treatments with Squoxin are shown in Tables V and VI. The fish were dead when collected so that neither time of exposure nor distance from the treatment site is accurately known.

Table VII summarizes the results of the analyses of rainbow trout captured during and immediately after completion of the treatment of the John Day River. The dead trout probably died from exposure to high concentrations of Squoxin at the treatment site. As shown in the table, there was little Squoxin in the edible tissues of these fish.

LITERATURE CITED

Johnston, J. M., *Prog. Fish Cult.* **34,** 122 (1972).
Keating, J. F., Kiigemagi, U., Terriere, L. C., Swan, R. L., Proceedings of the 52nd Annual Conference of the Western Association of State Game and Fish Commissioners, 1973, p 609.
Kiigemagi, U., Burnard, R. J., Terriere, L. C., *J. Agric. Food Chem.*, following paper in this issue.
MacPhee, C., Ruelle, R., *Trans. Am. Fish. Soc.* **98,** 676 (1969).
Mahin, D. T., Lofberg, R. T., *Anal. Chem.* **16,** 500 (1966).
Ogata, Y., Kawasaki, A., Goto, T., *Tetrahedron* **25,** 2589 (1969).

Received for review April 5, 1974. Accepted April 4, 1975. Oregon Agricultural Experiment Station Technical Paper No. 3791. This work was supported by grants from the National Oceanic and Atmospheric Administration, the Idaho Fish and Game Department, and the Oregon State Wildlife Commission.

Analytical Methods for the Detection of the Piscicide 1,1′-Methylenedi-2-naphthol (Squoxin) in Fish and Water

Ulo Kiigemagi, Robert J. Burnard, and Leon C. Terriere*

Procedures are given for the measurement of the piscicide 1,1′-methylenedi-2-naphthol (Squoxin) in fish tissue, using gas chromatography, and in water, using a colorimetric method. For the GC method, Squoxin is converted to its dimethyl ether and detected by electron capture. The method is sensitive to 0.1 ppm of Squoxin in fish tissue. The second method, which is designed for use in the field, involves coupling the piscicide with the chromogenic agent, tetrazotized *o*-dianisidine (Diazo Blue B). With the use of extraction and concentration steps this method is sensitive to 2 ppb of Squoxin in water. The two methods have been used in the analysis of fish and water samples from field tests of Squoxin.

The piscicide 1,1′-methylenedi-2-naphthol (Squoxin) is highly selective for the squawfish, a competitor and predator of trout and salmon in fresh water streams of the Pacific Northwest (MacPhee and Ruelle, 1969; Johnston, 1972). It has been used experimentally in Idaho, Oregon, Washington, and in Canada to gain information about its effectiveness in fish management and its impact on nontarget organisms (Keating et al., 1973).

These studies have required the development of analytical methods suitable for the monitoring of Squoxin in streams and for the measurement of its residues in various organisms. We have developed two methods for these purposes. A gas chromatographic procedure involving the formation of the dimethyl ether of the piscicide can be used to detect residues as low as 0.1 ppm in fish tissue. It has been used on fish collected from two field experiments conducted in 1973. The second method is a simple colorimetric procedure which can be used in the field for on-site measurements of Squoxin in water at concentrations as low as 2 ppb. Details of the analytical procedures are given in this report.

EXPERIMENTAL SECTION

Chemicals and Reagents. Squoxin (1,1′-methylenedi-2-naphthol) was obtained from Aldrich Chemical Co. (Milwaukee, Wisc.). Squoxin dimethyl ether for standards was prepared from Squoxin by reaction with dimethyl sulfate according to the method of Ogata et al. (1969). The melting point of the product obtained, 148.5–149.5°, did not agree well with that reported (Ogata et al., 1969), 144–145°, but we were able to confirm the structure by mass spectrometry. The preparation of Squoxin-*t* (1,1′-methylenedi-2-naphthol-*6,6′-t*) is described elsewhere (Terriere and Burnard, 1975). Diazomethane was prepared in ether solution from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co.) by the method of Fieser and Fieser (1968).

Reagent grade hexane was passed through a column of activated Florisil prior to use. Reagent grade benzene was refluxed over metallic sodium for a minimum of 12 hr and distilled. Reagent grade diethyl ether was refluxed over metallic sodium for 48 hr and distilled. Sodium sulfate was dried at 400–450° overnight and stored in a sealed bottle. Florisil was activated at 400–450° overnight and stored at 100°.

Diazo Blue B reagent was prepared by mixing 2 parts of 1% aqueous Diazo Blue B (*o*-dianisidine, terazotized, zinc chloride complex, Sigma Chemical Co., St. Louis, Mo.) with 5 parts of 5% aqueous sodium lauryl sulfate (Sigma Chemical Co.). This reagent must be prepared every 2 days and stored in a refrigerator. Phosphate buffer (pH 8) was prepared by mixing 5.3 ml of NaH_2PO_4 solution (27.8 g/l.) with 94.7 ml of $Na_2HPO_4 \cdot 7H_2O$ solution (53.65 g/l.) and diluted to 200 ml.

All other chemicals were reagent grade and were used as received.

Development of a GC Method. *Tissue Extraction and Cleanup.* Control fish were homogenized with Squoxin-*t* in a Waring Blendor at a tissue level of 5 ppm. The scintillation counter was used to measure the recovery of radioactivity on extraction of the homogenate with chloroform, methanol, diethyl ether, or benzene. The best recoveries

Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331.

Table I. Elution of Squoxin-*t* Dimethyl Ether from a Florisil Column

Fraction	Solvent	Vol, ml	% of total act.	Accumulative % of total act.
1	Hexane	100	4.3	4.3
2	Benzene	50	1.6	5.9
3	10% Ether–benzene	50	0.2	6.1
4	Ether	50	7.6	13.7
5	Ether	50	17.2	30.9
6	Ether	50	46.7	77.6
7	Ether	50	18.4	96.0
8	Ether	50	1.3	97.3
9	Ether	50	0.2	97.5
10	Ether	50	0.1	97.6

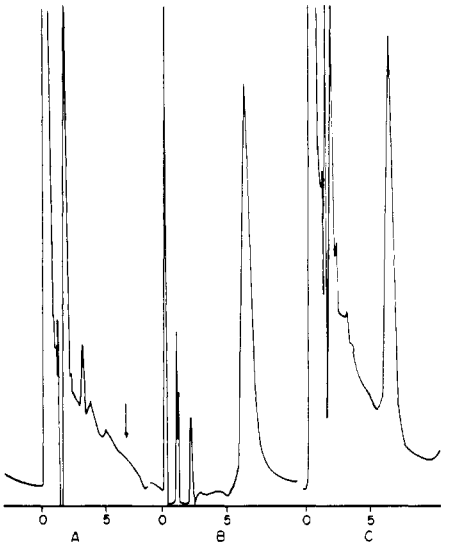

Figure 1. Typical chromatograms from Squoxin analysis as Squoxin dimethyl ether: (A) untreated rainbow trout; (B) 9.5 ng of Squoxin dimethyl ether standard; (C) treated rainbow trout containing 1.26 ppm of Squoxin measured as the dimethyl ether.

were obtained with benzene (average 95.0 ± 2.34%, in six tests). Methylation with diazomethane was performed at this point followed by a further purification of the sample by extraction with acetonitrile. The details of these steps are described later under the section Final Procedure for GC Analysis.

Florisil Column Cleanup. To remove neutral lipids still present, the extracts were passed through a Florisil column. The elution pattern of Squoxin dimethyl ether was determined by scintillation counting of aliquots of 50-ml portions of the eluate. The results of this study are shown in Table I where it can be seen that complete removal of the dimethyl ether is accomplished by elution with 250 ml of ether. The average recovery in this step from samples fortified with 5 ppm of Squoxin-*t* was 94.1 ± 3.02% (six replicates).

The procedures for preparing and storing Florisil for use in the column must be carefully followed. Otherwise, it will be necessary to use a more polar solvent to elute the Squoxin. This may result in inadequate cleanup.

In tests of the procedure to this point, control fish were homogenized with 5 ppm of Squoxin-*t* and the steps of extraction, dimethyl ether formation, acetonitrile transfer, and column cleanup were completed. The samples were then assayed for tritium as before. The average recovery, from six samples, was 84.8 ± 4.70%.

GC Conditions. Samples were analyzed with a Varian Aerograph Model 204-B gas chromatograph equipped with a ^3H foil electron capture detector. The 0.25 × 120 cm glass column was packed with 4% SE 30 on Chromosorb G, 80–100 mesh. Temperatures were: column, 235°; injector, 230°; and detector, 220°. The nitrogen flow rate was 60 ml/min.

After packing, the column was conditioned for 48 hr at 250°. Injection of Squoxin dimethyl ether standards at this point resulted in broad, badly tailing peaks of low sensitivity. Sensitivity was increased about 20- to 40-fold by massive injections of the standard. Further improvement of sensitivity and peak shape occurred as additional fish samples were injected during the course of analysis. The retention time for the dimethyl ether was 6.25 min and the response of the detector was linear in the range 0.5–15 ng. Typical chromatograms of a standard and untreated and fortified fish samples are shown in Figure 1. As little as 0.5 ng of the dimethyl ether can be detected by this method permitting the use of 5-g samples and an overall sensitivity of 0.1 ppm of Squoxin.

Final Procedure for GC Analysis. *Extraction.* Fish are homogenized by grinding with Dry Ice according to the method of Benville and Tindle (1970). The grinding is done in a 1-quart container adapted to fit a 1.5 hp Waring Blendor. The pulverized mixture is poured into a plastic bag which is lightly sealed and placed in a −10° freezer overnight to allow the carbon dioxide to sublime. The samples are stored in this condition prior to extraction.

Five-gram samples of the finely pulverized fish tissue from above are weighed into a 90-ml Sorvall Omnimixer cup and blended with 10 ml of distilled benzene. The benzene is then recovered by suction filtration and the extraction is repeated with two additional portions of benzene. The combined benzene filtrate plus approximately 15 ml of benzene used in the transfer are then evaporated to 3–5 ml.

Methylation. The benzene extract from above contained in a 50-ml beaker is treated with an excess (yellow color of diazomethane persists on swirling) of ethereal diazomethane. After standing at ambient temperature for about 3 min with occasional swirling, the mixture is heated gently on a steam bath until the yellow color disappears. Samples are stored at 0° until the cleanup procedures are begun.

Acetonitrile Transfer. The methylated material, volume less than 5 ml, is taken up in 100 ml of reagent grade acetonitrile and transferred to a 250-ml separatory funnel. The sample is extracted three times with 50-ml portions of purified hexane. Emulsions which result occasionally can be broken by allowing them to stand for about 16 hr. The acetonitrile layer is then flooded with 400 ml of distilled water and the mixture extracted with four 100-ml portions of purified diethyl ether. Addition of a small amount of NaCl (solid) aids in the delineation of layers.

Florisil Column Cleanup and GC Analysis. A 22-mm o.d. column containing 2.5 cm of anhydrous sodium sulfate above 5 cm of activated Florisil is washed with 50 ml of purified hexane. The sample from the previous step is evaporated to 1–2 ml and introduced into the column with 100 ml of purified hexane. The column is then eluted with 50 ml of benzene followed by 100 ml of 10% diethyl ether in benzene. These are discarded because they contain lipid material which interferes with subsequent analysis. The Squoxin dimethyl ether is eluted with 250 ml of diethyl ether. The ether eluate, evaporated to 3–6 ml, is ready for gas chromatographic analysis as described earlier.

Development of a Colorimetric Method. The main requirements of this method were for sensitivity to Squoxin concentrations in water in the parts per billion range and a simplicity which would permit its use in the field where laboratory facilities may be limited. It was possible to

Table II. Recovery of Added Squoxin from Water with the Colorimetric Method

Water source	Squoxin added, ppb	Time of extraction	Squoxin recovery, %
Distilled	100	Immediately	90
Distilled	10	Immediately	94
Distilled	5	Immediately	99
Distilled	2	Immediately	96
River	100	Immediately	80
River	10	Immediately	84
Creek	100	Immediately	85
Pond	100	Immediately	80
Pond	10	Immediately	81
Pond	130	90 min	52
Pond	130	90 min	54

Table III. Decline of Squoxin Concentration in Pond Water after Treatment at a Rate of 150 ppb

Time after treatment, hr	Squoxin concn, ppb[a]		
	Av	Range	SD
Pretreatment	0.45	0.4–0.5	
0.5	142	116–176	±25.77
6	17.6	16.0–19.1	±1.45
24	10.9	9.2–14.2	±2.31
48	9.6	8.2–12.0	±1.22
144	5.8	5.1–7.2	±0.81
366	3.6	2.8–4.4	±0.58

[a] Four samples collected each time.

Table IV. Concentration of Squoxin in the John Day River during Treatment at a Rate of 50 ppb

Distance from treatment site, miles	Time, after[a] beginning treatment, hr	Squoxin, ppb[b]	
		A	B
0.25	1.5	5.5	7.9
0.25	3	19.6	18.0
0.25	7.5	71.2	67.2
0.25	18	56.0	59.2
0.25	26	56.0	52.8
0.25	29	1.3	2.1
0.25	32	0.4	0.7
1.5	12	26.4	25.6
1.5	26	19.6	20.8
3	22	19.6	20.8
5	25	6.6	6.3
6	22	3.1	2.9
7	24	0.9	1.0

[a] Treatment continued for 26 hr. [b] Two samples collected at each site.

Table V. Standard Curve for the Colorimetric Method for Squoxin

μg of Squoxin	Absorbance		
	Av[a]	Range	SD
0	0.05	0.02–0.07	±0.017
2	0.21	0.18–0.24	±0.020
4	0.36	0.34–0.38	±0.013
8	0.68	0.64–0.71	±0.023
10	0.83	0.79–0.86	±0.023

[a] Average of seven determinations.

adapt the Diazo Blue method for 1-naphthol (Van Asperen, 1962) to meet these requirements. Applied directly to aqueous solutions of Squoxin, this method is sensitive to only 1 ppm. However, by extracting such samples with a volatile solvent followed by a concentration step, the desired sensitivity can be attained.

The solvents tested included benzene, dichloromethane, chloroform, and carbon tetrachloride. Dichloromethane and chloroform proved unsuitable because of their relatively high water solubility. Carbon tetrachloride was selected over benzene because of its ease of evaporation and its specific gravity.

It was found that 1500 ml of water containing Squoxin could be extracted with as little as 50 ml of this solvent with a resulting sensitivity of 2 ppb. Extraction of natural waters frequently resulted in the formation of emulsions which could be prevented by the addition of 2-propanol. Suspended matter sometimes caused cloudiness in the CCl_4 extract. This was removed by filtering before color development. It is evident from the data in Table II that this procedure is satisfactory for the extraction of Squoxin if the samples are processed within a few minutes. Recoveries from distilled water were consistently higher than those from natural waters, indicating some breakdown of the piscicide or its adsorption on suspended matter.

It is important that the water be acidic at the time of extraction. Experiments with water containing 24 ppb of Squoxin indicated that only about 10% was recovered from pH 8 water as compared to 96% recovery from pH 4 water.

The method finally developed has been found reliable during 3 years use in field experiments. Typical results from two of these experiments are shown in Tables III and IV. It should be realized in studying these data that they include sampling errors. These are particularly high in the results shown in Table III because the conditions, closed system, prevented rapid mixing of the Squoxin. In Table IV where the method was applied to water from a moving stream, mixing should have been rapid and complete. It can be seen that the precision of the method in this case was high.

Specificity of the Method. Squoxin rapidly breaks down in water to approximately a dozen products. The excellent correlation between fish mortality data and Squoxin concentration, as measured by the colorimetric method (Keating et al., 1973), indicates that none of these breakdown products interfere sufficiently with the method to hinder its usefulness in stream monitoring. The persistence of low level residues for as long as 15 days (Table IV) indicates that one or more of the breakdown products are detected. However, these account for only 1–2% of the Squoxin applied. Since the decline in Squoxin content of the closed system represented in Table III is nearly as rapid as that in a moving system, Table IV, we conclude that other breakdown products either do not react with the reagents used, they are not extracted by the solvents used, or they are adsorbed on suspended matter and kept from interfering.

None of the degradation products have been firmly identified but we have tested some of those suspected of being present. One of these, the dehydrogenation product, 2-oxo-2*H*,1′*H*-spiro[naphthalene-1,2′-naphthol-2,1-*b*]furan (Chatterjea, 1958; Rieker et al., 1966), which we call dehydrosquoxin, does not react to produce color. Three others,

2-naphthol, 2-hydroxy-1-naphthoic acid, and bis(2-hydroxy-1-naphthyl) ketone, all produce colors with the same absorption maximum as Squoxin. From the results presented in Table III, we conclude that the latter compounds are not produced in significant amounts during the field use of Squoxin.

Final Procedure for Colorimetric Analysis. A calibration curve is prepared from standards containing 2 to 10 μg of Squoxin in 1 ml of ethanol and 3.2 ml of pH 8 buffer and 0.8 ml of Diazo Blue B reagent are added. The absorbance at 552 nm is measured within 10 min. Data for a standard curve showing the precision of this step are presented in Table V.

The 1500-ml water sample in a 2000-ml separatory funnel is acidified with 1 ml of 6 *N* HCl and 50 ml of 2-propanol and 25 ml of CCl_4 are added. The mixture is shaken for 2 min, the layers allowed to separate, and the lower layer removed. The aqueous layer is re-extracted with 15- and 10-ml portions of the solvent and the extracts combined and filtered if necessary. A suitable aliquot (2 to 10 μg of Squoxin) is taken and the CCl_4 removed by evaporation. The residue is dissolved in 1 ml of ethanol, and 3.2 ml of pH 8 buffer added followed by 0.8 ml of the Diazo Blue B reagent. The absorbance is measured at 552 nm within 10 min.

LITERATURE CITED

Benville, P. E., Jr., Tindle, R. C., *J. Agric. Food Chem.* **18,** 948 (1970).
Chatterjea, J. N., *J. Indian Chem. Soc.* **35,** 37 (1958).
Fieser, L. F., Fieser, M., "Reagents for Organic Synthesis", Wiley, New York, N.Y., 1968, p 191.
Johnston, J. M., *Prog. Fish Cult.* **34,** 122 (1972).
Keating, J. F., Kiigemagi, U., Terriere, L. C., Swan, R. L., Proceedings of the 52nd Annual Conference of the Western Association of State Game and Fish Commissioners, 1973, p 609.
MacPhee, C., Ruelle, R., *Trans. Am. Fish. Soc.* **98,** 676 (1969).
Ogata, Y., Kawasaki, A., Goto, T., *Tetrahedron* **25,** 2589 (1969).
Rieker, A., Zeller, N., Schurr, K., Müller, E., *Justus Liebigs Ann. Chem.* **697,** 1 (1966).
Terriere, L. C., Burnard, R. J., *J. Agric. Food Chem.*, preceding paper in this issue.
Van Asperen, K., *J. Insect Physiol.* **8,** 401 (1962).

Received for review April 5, 1974. Accepted April 4, 1975. Oregon Agricultural Experiment Station Technical Paper No. 3792. This work was supported by grants from the National Oceanic and Atmospheric Administration, the Idaho Fish and Game Department, and the Oregon State Wildlife Commission.

Adsorption, Mobility, and Persistence of Thiabendazole and Methyl 2-Benzimidazolecarbamate in Soils

Nadav Aharonson* and Uzi Kafkafi

Adsorption of thiabendazole (TBZ) and 2-benzimidazolecarbamic acid methyl ester (MBC) to three soils was studied. With increasing acidity of the soil suspensions an increase in the adsorption of these fungicides to the soil occurred. Results indicated ionization of benzimidazole fungicides at lower pH values, and adsorption of these ionized molecules on the soil. Thiabendazole was adsorbed to the soil in much larger quantities than MBC, and therefore at equilibrium TBZ was detected in the free soil solution in much smaller concentrations. The mobility of TBZ in the soil was also much smaller than that of MBC. The persistence of these fungicides in soil samples, incubated at 25° in small glass vials, was also examined. Nine months after adsorption of these fungicides to the soil, 85–95% of the applied TBZ and 65–75% of the applied MBC were recovered from air dried soils. However, in moist soil after 9 months of incubation only 75–90% of the applied TBZ and 20–30% of the applied MBC were recovered.

Previous experiments have shown that uptake of systemic benzimidazole fungicides by plants from the soil is very inefficient. Erwin (1973) stated that control of vascular wilt diseases required extremely large dosages and was too limited to be of practical control for deep-rooted crops. Presumably this was the result of tight adsorption of these residues to soil (Baude et al., 1974). It has been shown that more benomyl was taken up from sandy soils than from clay soils (Fuchs et al., 1970), and that the rate of uptake of benomyl by plants was increased when they were grown on soils with low organic matter content, and with higher pH (Schreiber et al., 1971). The relative long term persistence of benzimidazole fungicides in the soil and their low water solubility and movement in soil have been demonstrated by Hine et al. (1969), Baude et al. (1974), and Rhodes et al. (1974).

Pesticide Chemistry and Residue Research Laboratory, and Division of Soil Chemistry and Plant Nutrition, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

Since adsorption of benzimidazole derivatives by the active surfaces of the soil may govern the effectiveness of the fungicide and may also affect their persistence and leaching properties, a study was undertaken to further examine these parameters. A mechanism of adsorption of benzimidazole derivatives on mineral clays has previously been suggested by Aharonson and Kafkafi (1975). In this investigation, further studies were conducted on adsorption mechanism, mobility, and persistence of these fungicides in various soils.

EXPERIMENTAL SECTION

Materials. *Soils.* Three soils having substantial differences in levels of clay, $CaCO_3$, organic matter, and cation capacity were used in this study (Table I).

Chemicals. Analytical grade and technical 2-benzimidazolecarbamic acid methyl ester (MBC) as well as analytical grade and technical thiabendazole (TBZ) were kindly provided by E. I. DuPont de Nemours & Co. Inc., E. Merck, and Agan Chemicals.

Methods. *Analytical Methods; Extraction from Soil and Determination of TBZ, MBC, and 2-Aminobenzimidazole.* Ten-gram subsamples, taken from air-dried and