cal extraction of the gut contents may lead to the identification of the pesticide by physical methods such as chromatography, or by biochemical methods such as the addition of the extract to a standard tissue preparation before electrophoresis, and subsequent demonstration of inhibition in the resulting zymogram.

Acknowledgment

The authors wish to thank E. N. Wright and M. R. Boulton for technical assistance, and R. R. Page for photographing the gels. Gifts of pheasant tissue for control purposes from J. S. Ash, Game Research Association, Fordingbridge, Hants., and Thimet from British Cyanamide, Ltd., are also gratefully acknowledged.

Literature Cited

(1) Aldridge, W. N., *Biochem. J.* **53,** 110 (1953).
(2) Barron, K. D., Bernsohn, J., Hess, A. R., *J. Histochem. Cytochem.* **11,** 139 (1963).
(3) Bernsohn, J., Barron, K. D., Norgello, H., *Biochem. J.* **91,** 21C (1964).
(4) Braux, P., Dormal, S., Thomas, G., *Ann. Biol. Clin.* **22,** 375 (1964).
(5) Bunyan, P. J., *Analyst* **89,** 615 (1964).
(6) Burgen, A. S. V., Chipman, L. M., *Quart. J. Exptl. Physiol.* **37,** 61 (1952).
(7) Echobijon, D. J., Kalow, W., *Can. J. Biochem. Physiol.* **41,** 1537 (1963).
(8) Gomori, G., *J. Lab. Clin. Med.* **34,** 275 (1949).
(9) Hunter, R. L., Markert, C. L., *Science* **125,** 1294 (1957).
(10) Kaminski, M., *Bull. Soc. Chim. Biol.* **46,** 555 (1964).
(11) Karnovsky, M. J., Roots, L., *J. Histochem. Cytochem.* **12,** 219 (1964).
(12) Laws, E. Q., Webley, D. J., *Analyst* **86,** 249 (1961).
(13) McKinley, W. P., Read, S. I., *J. Assoc. Offic. Agr. Chemists* **44,** 726 (1961).
(14) Michel, H. O., *J. Lab. Clin. Med.* **34,** 1564 (1949).
(15) Nachmansohn, D., Feld, E. A., *J. Biol. Chem.* **171,** 715 (1947).
(16) Read, S. I., McKinley, W. P., *J. Assoc. Offic. Agr. Chemists* **46,** 869 (1963).
(17) Smithies, O., *Biochem. J.* **61,** 629 (1955).
(18) *Ibid.*, **71,** 585 (1959).
(19) Stransky, Z., Benes, S., *Sb. Cesk. Akad. Zemedel, Ved, Vet. Med.* **6,** 733 (1961); *CA* **62,** 7050 (1965).
(20) Taylor, A., *Analyst* **87,** 824 (1962).
(21) Taylor, A., Rea, R. E., Kirby, D. R., *Ibid.*, **89,** 497 (1964).
(22) Thompson, R. R., Cook, J. W., *J. Assoc. Offic. Agr. Chemists* **44,** 199 (1961).
(23) Turtle, E. E., Taylor, A., Wright, E. N., Thearle, R. J. P., Egan, H., Evans, W. H., Soutar, N. M., *J. Sci. Food Agr.* **14,** 567 (1963).
(24) Warburg, O., Christian, W., *Biochem. Z.* **310,** 384 (1941).

Received for review August 31, 1965. Accepted December 28, 1965.

RESIDUE RECOVERY

Determination of Translocated Tetramine in Foliage by Hydrogen-Flame Gas Chromatography

ROGER W. BULLARD

Wildlife Research Center, Bureau of Sport Fisheries and Wildlife, Denver, Colo.

An analytical method for the quantitative determination of translocated tetramine (tetramethylenedisulfotetramine) in plant foliage, techniques for sample preparation, extraction, cleanup, and analysis by hydrogen-flame gas chromatography are reported. Different quantities of tetramine were added to Douglas fir foliage and then recovered. When the values of tetramine added *vs.* tetramine recovered were plotted, a straight-line relationship with a slope of 1.20 was found. This relationship was applied to the analysis of translocated samples. The procedure was applicable to recovery of added tetramine from representative species of five plant genera.

Effective reforestation in many areas of the United States has been seriously limited because of animals' feeding on seed and young seedlings. Research directed at minimizing these losses through the development of chemical repellents or toxicants is being carried out by the Denver Wildlife Research Center (*3, 5*). A chemical, either repellent or toxicant that is also plant systemic, is especially sought. Tetramine (tetramethylenedisulfotetramine) is classified as a systemic toxicant (*3, 5, 6*).

The synthesis of tetramine from formaldehyde and sulfamide was reported by Hecht and Henecka in 1949 (*1*). In 1953, a U. S. patent for the procedure was issued to Hecht, Henecka, and Meisenheimer, who claimed their product as a rodenticide (*2*). In 1952, Spencer and Kverno found that plants growing from tetramine-treated seed were toxic to meadow mice (*Microtus* spp.) and concluded that the compound had been translocated (*6*). Subsequently, Kverno and Campbell found that foliage from Douglas fir [*Pseudotsuga menziesii* (Mirb) Franco] seedlings growing in soil treated once with tetramine was toxic to hare (*Lepus americanus*) for as long as 4 years (*4*).

An analytical method for the detection of tetramine in plant foliage was needed to facilitate study of the translocation characteristics of the compound. Previously, mouse bioassay was the only analytical technique available, and was considered inadequate because tetramine concentrations of less than 20 p.p.m. could not be detected in vegetation, and because of individual variation in the test animals.

This paper describes a method for the quantitative detection of translocated tetramine in plant foliage by a process of extraction, cleanup, and subsequent analysis by hydrogen-flame gas chromatography.

Methods and Materials

The analytical instrument was a Wilkens Aerograph Hy-Fi Model 600-C gas chromatograph, incorporating a hydrogen - flame ionization detector, equipped with a $^1/_8$-inch o.d. × 10-foot stainless steel column packed with 10% Dow 11 Silicone on Fluoropak 80. A Wilkens Aerograph Model 650 hydrogen generator served as the hydrogen source, and the recorder was a 1-mv. Honeywell Electronik 15 with Disc chart integrator.

The tetramine used to establish the analytical procedure was purified by recrystallization from an acetone solution, followed by washing with water. The solubility of tetramine in eight common solvents at room temperature was established by analyzing the filtrates of the respective saturated solutions by gas

Table I. Tetramine Solubility

Solvent	Solubility, Grams/100 Ml.
Petroleum ether (30°–60° C.)	0.0014
n-Hexane	0.0016
H_2O	0.0054
Chloroform	0.062
Ethyl alcohol	0.081
Acetone	0.284
Dimethyl sulfoxide	2.937
Dimethylformamide	7.098

Table II. Recovery of Tetramine from 5-Gram Translocated Samples

Plant	Extraction Period, Hours	X Amount Detected, Mg.	Y Calculated[a] Quantity Present, Mg.	Coefficient of Variation (C), %
Alfalfa	8	10.93	13.09	4.96
Douglas fir	72	73.66	88.24	1.11
	36	72.82	87.24	1.48
	8	73.56	88.12	1.03

[a] 1.20 $X = Y$. 1.20 = correction factor for slope of straight line obtained by plotting added *vs.* recovered tetramine from Table III.

chromatography (Table I). The low solubility of tetramine in common volatile solvents caused limitations in development of the procedure. It can be translocated in quantities as high as 1.76% dry-weight Douglas fir foliage (87.86 mg. per 5-gram sample average from Table II). To accommodate high concentrations of tetramine without substantial volume changes, the solvent was required to have appreciable solvency for tetramine. Dimethylformamide satisfied this criterion, and, although solvent tailing interference during gas chromatography analysis developed, this effect was minimized by a column temperature of 200° C. and a nitrogen carrier flow of 30 ml. per minute.

Sample Preparation. Foliage samples were collected from 2-year-old Douglas fir seedlings that had been growing approximately one year in soil to which tetramine (suspended in a 0.5% Carbopol solution) had been added in close proximity to the root systems at a rate of 200 mg. of tetramine per seedling. Plant material collected from this treatment will hereafter be referred to as translocated samples.

Needles were collected from Douglas fir seedlings, thoroughly washed, oven dried at 60° to 65° C. for 48 hours, and then ground to pass through a 40-mesh sieve. Five-gram aliquots of this preparation were removed for all sample analyses. Known quantities of purified tetramine were added to aliquots obtained from untreated seedlings to form the "pegged" samples.

Acetone was chosen as the extraction solvent because it most adequately fulfilled the requirement of having enough volatility and solvency for low-temperature Soxhlet extraction of tetramine (Table I). An extraction period of 8 hours was as effective as 72 hours (Table II); therefore, the samples were extracted with acetone for 6 hours, soaked 16 hours overnight in the extraction chamber, then extracted 2 hours before removal. The extract was concentrated in a 100-ml. beaker to approximately 15 ml. by heating at 45° to 50° C. combined with a flow of air over the surface of the liquid.

The column chromatography apparatus was designed for simultaneous concentration of the eluate as it collected. The 22 × 400 mm. borosilicate glass chromatographic columns were packed with 5 grams of 1:1 Merck activated charcoal N.F. powder (no additional activation was required) and Celite 545 and capped with a $^1/_2$-inch layer of the Celite to protect the column. Redistilled, reagent grade acetone (25 ml.) was used to prewash the columns prior to adding the concentrated plant extract. The extract was eluted with 100 ml. of acetone and flow rate of eluate into a 15 × 80 mm. test tube was adjusted to allow drop emergence every 5 to 8 seconds by applying air at 0.5 to 1 p.s.i. Partial immersion of the collecting tube in a 55° C. water bath, combined with an air stream down the inside of the test tube, provided accelerated evaporation of the acetone and subsequent concentration of the eluate. The plant residue was concentrated in the bottom of the test tube by rinsing the sides with acetone, which was then evaporated. This process removed most of the plant extractive interferences leaving tetramine and a clear, waxy residue consisting primarily of essential oils.

Addition of 0.2 ml. of 0.5*N* KOH to the test tube placed the extraneous residue in solution, leaving insoluble tetramine as a precipitate. The aqueous solution was removed with a filter stick made from a 2-mm. i.d. × 100-mm. glass capillary tube which, by applying vacuum at one end, held a 7-mm. Whatman No. 40 filter paper disk in place on the opposite end, through which the filtration took place. The recovered tetramine was washed twice with 0.5 ml. of distilled water.

Reagent grade dimethylformamide was added to the purified tetramine residue to form the analytical solution. Different volumes were required depending on the quantity of tetramine residue present (0.2, 1, or 5 ml.). With experience, an analyst could choose one of these volumes by visual estimation of the amount of residue present.

Sample Analysis. Optimum gas chromatograph operating conditions were established for tetramine analysis. The gas chromatograph column oven was operated at 200° C. The nitrogen carrier gas and hydrogen gas flow rates were 30 and 26 ml. per minute, respectively. Standard tetramine solutions were analyzed every third or fourth sample injection. Chromatogram peak areas were determined by Disc integration.

Solvent tailing interferences were held constant by injecting 5 μl. of solution for each sample, and allowing 10 minutes after each peak before a subsequent injection. The tailing interference from each injection, however, lasted in excess of 45 minutes. Injections at shorter time intervals caused an additive effect in upward baseline shift. Because a 45-minute time lapse between injections was impractical, the interference was overcome by maintaining constant baseline values for Disc integration. The zeroing potentiometer (bucking voltage) was used to place the leading edge of the peak at a given chart value (depending on the required attenuation), thereby permitting the trailing edge of the peak to reposition on the baseline. This was achieved by positioning on chart value 1 for the least sensitive setting required and increasing this value at the same rate as attenuation.

Results and Discussion

Efficiency of tetramine recovery from translocated samples was determined by analysis of pegged samples (Table III). These data of added *vs.* recovered quantities of tetramine resulted in a straight line with a slope of 1.20, indicating that a linear relationship existed. This relationship was utilized in the analysis of translocated samples. The evaluation of tetramine quantities originally present was obtained by extrapolation of recovery data.

Table II contains analysis data from translocated samples of both alfalfa and Douglas fir. Failure to remove added tetramine from translocated Douglas fir samples after extraction periods in excess of 8 hours lent support to the correction factor premise. If tetramine remained in the foliage, the quantity would be relatively constant between samples and a close correlation would still exist.

The reproducibility of the procedure was estimated by computing a coefficient of variation C for all observations in a given sample.

$$C = \frac{100S}{\bar{X}} \text{ where } S = \left[\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}{n-1}\right]^{1/2}$$

and $\bar{X} = \frac{\Sigma X}{n}$ for sample X of n replications.

Applicability of the procedure to other plant genera was tested by adding and recovering tetramine from the foliage of alfalfa, wheat, beans, and milo (Table III). These species contained much smaller quantities of essential oils than Douglas fir and it is probable that the KOH cleanup treatment would not

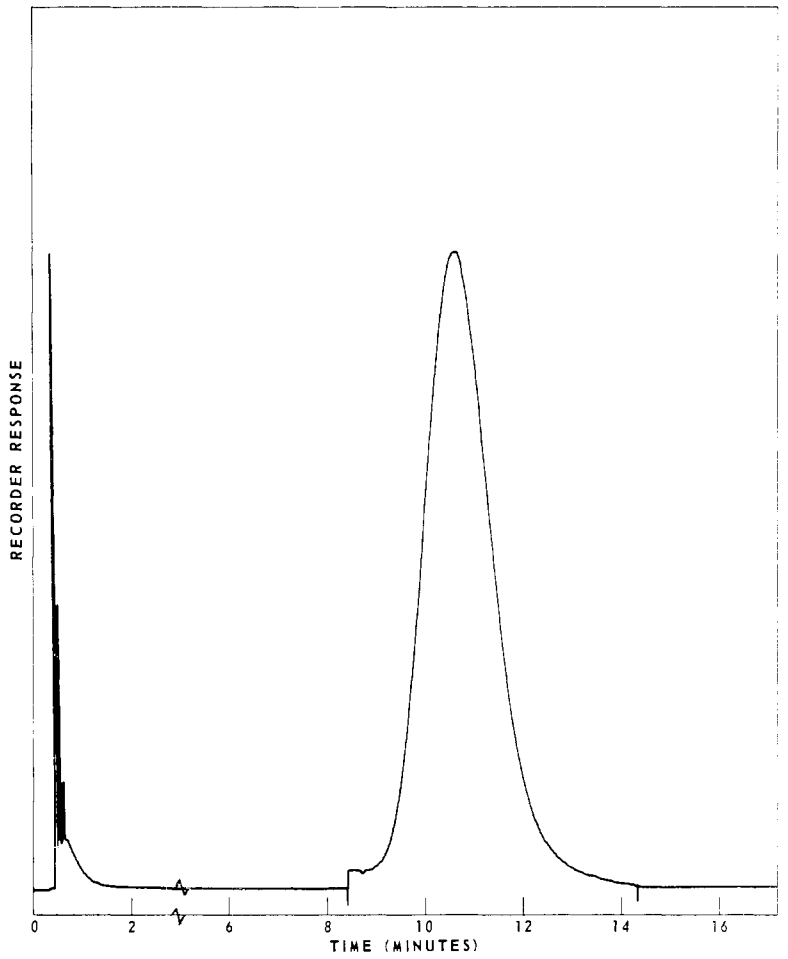

Figure 1. Typical gas chromatograph of extract from translocated Douglas fir sample containing 88.24 mg. per 5-gram sample

Table III. Recovery of Added Tetramine from 5-Gram Pegged Samples[a]

Plant	Added, Mg.	Recovered		Coefficient of Variation (C), %
		Mg.	%	
Douglas fir	100	81.98	81.98	2.54
	75	62.55	83.40	3.20
	50	43.33	86.66	5.75
	25	20.59	82.36	3.43
	10	8.45	84.50	2.97
	5	3.82	76.40	5.08
	1	0.69	69.00	1.66
	0.5	0.35	70.00	0.68
	0.2	0.13	65.00	1.27
Bean	50	44.37	88.74	2.82
Milo	50	43.22	86.44	0.34
Wheat	50	44.56	89.12	0.63
Alfalfa	50	44.12	88.24	0.23

[a] Samples from each species were run in triplicate.

be necessary; however, it was included for direct comparison of the analytical procedure with Douglas fir.

For analysis of coniferous foliage where quantities of essential oils are high, there is a significant loss owing to tetramine solubility in the 1.2 ml. of water used during the required KOH treatment and filtration step (Table I). The availability of foliage limited the sample size to 5 grams, which is adequate considering that the procedure is capable of detecting 25 p.p.m., and since tetramine is so readily translocated. Higher sensitivity could be attained by increasing sample size. A 25-gram sample, for example, would allow one to detect 5 p.p.m. For plant species of low essential oil content, where the KOH treatment would not be necessary, sensitivity of less than 1 p.p.m. may be achieved.

Acknowledgment

The author thanks Charles M. Loveless, biometrician, Denver Wildlife Research Center, for statistical assistance.

Literature Cited

(1) Hecht, G., Henecka, H., *Angew. Chem.* **61,** 365 (1959).
(2) Hecht, G., Henecka, H., Meisenheimer, M. (to Farben-Fabriken Bayer), U. S. Patent **2,650,186** (Aug. 25, 1953).
(3) Kverno, N. B., *Proc. Soc. Am. Foresters*, San Francisco, Calif., p. 97, 1959.
(4) Kverno, N. B., Campbell, D. L., Progress Report, Denver Wildlife Research Center, Denver, Colo., unpublished data, 1961.
(5) Spencer, D. A., *Proc. Soc. Am. Foresters*, Salt Lake City, Utah, pp. 183–5, 1958.
(6) Spencer, D. A., Kverno, N. B., Progress Report, Denver Wildlife Research Center, Denver, Colo., unpublished data, 1952.

Received for review March 25, 1965. Accepted October 8, 1965. Trade names referred to in this publication do not imply government endorsement of commercial products.