strain M1 is taxonomically related, but has additional requirements for growth (Bryant *et al.*, 1971). These include an exogenous source of Cofactor M, a recently identified cofactor required for methane formation (McBride and Wolfe, 1971a). This surprising difference in the susceptibility of rumen microbes and pure cultures of nonrumen methanogens to inhibition by DDT may be due to differences in cell membrane permeability, preferential uptake by nonmethanogenic rumen microbes, or nonspecific absorption by other components of crude rumen fluid. Cellular uptake of DDT in rumen fluid is reported to be rapid and greater than 95% (Kutches and Church, 1971).

Some polychlorinated compounds are potent inhibitors of rumen methanogenesis and alter the composition of rumen fermentation metabolites. However, a single exposure to relatively high concentrations of DDT and several other polychlorinated insecticides does not appear to have a major direct effect upon *in vitro* rumen fermentations. The effect of long-term exposure *in vivo* is unknown.

LITERATURE CITED

Babers, F. H., *J. Amer. Chem. Soc.* **77**, 4666 (1955).
Bryant, M. P., Tzeng, S. F., Robinson, I. M., Joyner, A. E., Jr., *Advan. Chem. Ser.* **105**, 23 (1971).
Czerkawski, J. W., *World Rev. Nutr. Diet.* **11**, 240 (1969).
Fries, G. R., Marrow, G. S., Gordon, C. H., J. Agr. Food Chem. **17**, 860 (1969).
Haller, H. L., Bartlett, P. D., Drake, N. L., Newman, M. S., Cristol, S. J., Eaker, C. M., Hayes, R. A., Kilmer, G. W., Magerlein, B., Mueller, G. P., Schneider, A., Wheatley, W., *J. Amer. Chem. Soc.* **67**, 1591 (1945).
Kutches, A. J., Church, D. C., Duryee, F. L., J. Agr. Food Chem. **18**, 430 (1970).
Kutches, A. J., Church, D. C., *J. Dairy Sci.* **54**, 540 (1971).
McBride, B. C., Ph.D. Thesis, Department of Microbiology, University of Illinois, 1970.
McBride, B. C., Wolfe, R. S., *Biochemistry* **10**, 2317 (1971a).
McBride, B. C., Wolfe, R. S., *Nature (London)* **234**, 551 (1971b).
Miskus, R. P., Blair, D. P., Casida, J. E., J. Agr. Food Chem. **13**, 481 (1965).
Rufener, W. H., Jr., Wolin, M. J., *Appl. Microbiol.* **16**, 1955 (1968).
Sink, J. D., Varela-Alverez, H., Hess, C., J. Agr. Food Chem. **20**, 7 (1972).
Trei, J. E., Olson, W. A., *J. Anim. Sci.* **29**, 173 (1969).
Trei, J. E., Parish, R. C., Scott, G. C., *J. Anim. Sci.* **33**, 1171 (1971a).
Trei, J. E., Parish, R. C., Singh, Y. K., Scott, G. C., *J. Dairy Sci.* **54**, 536 (1971b).
Trei, J. E., Scott, G. C., Parish, R. C., *J. Anim. Sci.* **34**, 510 (1972).
Trei, J. E., Singh, Y. K., Scott, G. C., *J. Anim. Sci.* **31**, 256 (1970).
Van Nevel, C. J., Hendrix, H. K., DeMeyer, D. I., Martin, J., *Appl. Microbiol.* **117**, 695 (1969).

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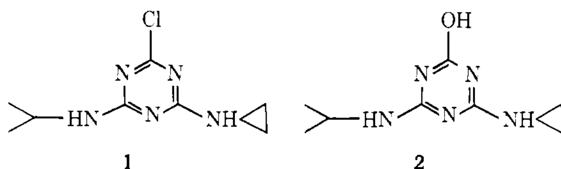
Analytical Method for Determination of Residue Levels of Hydroxycyprazine (2-Hydroxy-4-cyclopropylamino-6-isopropylamino-*s*-triazine) in Corn

An analytical procedure for determination of residue levels of hydroxycyprazine (2-hydroxy-4-cyclopropylamino-6-isopropylamino-*s*-triazine) in corn silage and grain has been developed. The procedure is based on extraction and separation of hydroxycyprazine, followed by its conversion to cyprazine (a chlorotriazine), which subsequently is determined gas chromatographically.

Chloro-*s*-triazines are widely used as herbicides, primarily on corn, *Zea mays*. Cyprazine (2-chloro-4-cyclopropylamino-6-isopropylamino-*s*-triazine, **1**) is a recently introduced member of this class of compounds. The metabolism of cyprazine in corn plants was investigated using ^{14}C ring-labeled cyprazine applied both foliarly and to the soil. Cyprazine, upon foliar application to corn plants in the 3–6 leaf stage (the recommended time of treatment), is converted rapidly and nearly quantitatively to a polar metabolite(s) (Riden and Asbell, 1969), later found to be analogous to the glutathione conjugate reported for atrazine (Lamoureux *et al.*, 1970). Time studies showed that this conjugate hydrolyzes sequentially through the γ-glutamylcysteine conju-



gate to hydroxycyprazine (2-hydroxy-4-cyclopropylamino-6-isopropylamino-*s*-triazine, **2**) and then to dealkylated products (Riden *et al.*, 1970). These metabolic products, containing the *s*-triazine ring, do not translocate from the treated leaves and are lost from the corn plant when these leaves naturally senesce and are shed. A field study on the uptake of soil-applied ^{14}C ring-labeled cyprazine into young corn plants showed the possibility of **2** being present in the leaves. The difficulties inherent in low level radioassay techniques prevented determination of an exact level of **2**. However, this study indicated that no more than 0.05 ppm could be present in the corn leaves with none found in the stems or ears. It was, therefore, necessary to develop an analytical procedure to determine the levels of **2**, if any, which might appear in the grain and silage of corn treated with **1**.

Identification techniques for hydroxy-*s*-triazines have been reported, including various types of chromatography (Fishbein, 1970; Flint and Aue, 1970; Harris, 1967; Montgomery and Freed, 1964), and mass spectrometry (Montgomery *et al.*, 1969). None of these methods were found to be suitable to residue analysis, due either to lack of sensitivity or interference by naturally-occurring materials.

Hydroxycyprazine exists as a tautomeric mixture involving ring-protonated structures similar to those reported for other hydroxy-*s*-triazines (Chen, 1967). Its chemistry, therefore,

is neither like an alcohol (or phenol) nor an amide. Almost all of the reagents we investigated which are purported to react with alcohols or amides failed to react satisfactorily with **2**.

A chlorination reaction was ultimately developed which gives excellent results and is the basis for the method described below. Separation of **2** from other compounds was a significant problem which was overcome by the use of two chromatographic columns for preliminary cleanup.

EXPERIMENTAL SECTION

Sample Preparation. A 300-g aliquot of a composite corn grain or silage sample is ground in 900 ml (some grain samples may require more) of methanol for 3–5 min in a Waring Blendor. The brei is squeezed through cheesecloth, with the resultant liquid introduced into a clinical basket centrifuge and filtered through Whatman 54SFC filter paper lining the basket of the centrifuge. The plant tissue is reground in a Waring Blendor with 300–500 ml of methanol. The brei is again squeezed through cheesecloth, and the liquid is filtered through the centrifuge. The blender is rinsed with 50 ml of methanol, which is used to wash the solids remaining in the centrifuge. The filtrates are combined in a 2-l. graduated cylinder.

Extraction with Toluene. An aliquot of the combined filtrates representing 50 g of crop sample is placed in a separatory funnel, and 0.15 times its volume of water is added. This solution is extracted twice with 0.4 times its volume of toluene. The toluene phases, which contain any cyprazine residues, are discarded (or separately analyzed for cyprazine). The aqueous methanolic phase is concentrated on a rotary evaporator at 50°, under aspirator vacuum, until the residual toluene has been removed and the solution is homogeneous.

Cation Exchange Column. A 25-g portion of AG50Wx4 cation exchange resin is prewashed by slurrying and decanting with the following series of washes: 50 ml of 7.5 *N* ammonium hydroxide, 50 ml of water, 50 ml of 3 *N* hydrochloric acid, and 50 ml of water. The resin is placed in a 20 mm i.d. × 150 mm chromatographic column, allowed to settle, and held in place with sea sand. The methanolic plant extract is acidified with 6 ml of 6 *N* hydrochloric acid and placed on the column. The flask is rinsed once with 50 ml of 50% aqueous methanol and once with 50 ml of water, both washes being sequentially added to the column. A 30-ml wash of 5% aqueous diethylamine solution is added to the column and the eluent is discarded. The hydroxycyprazine on the column is eluted with 100 ml of the 5% diethylamine solution and collected in a 300-ml round-bottomed flask. This eluent is evaporated to dryness (Caution: initial foaming) on a rotary evaporator at 50°, under aspirator vacuum. The resulting residue is dissolved in 5 ml of 0.5 *N* hydrochloric acid and evaporated *to dryness* on a rotary evaporator at 50° at less than 5 mm pressure.

Silica Gel Column. This column is prepared from SilicAR CC-7 (200–325 mesh) which has been suspended with methanol, decanted, and washed with three portions of chloroform. A plug of glass wool is placed in the bottom of an 18 mm i.d. × 100 mm chromatographic column with a 26 mm i.d. × 100 mm reservoir, and the slurry of SilicAR in chloroform is poured into the column to yield a column of silica gel about 60 ± 5 mm in length. This is gently settled by tapping and held in place with sea sand. The column is washed with 25 ml of chloroform. The dry, acidified residue from the cation exchange column is partially suspended in 10 ml of chloroform by swirling the flask in an ultrasonic cleaner for about 2 min. The suspension is then transferred with a pipet to the top of the silica column.

This suspension and transfer process is repeated on the residue in the flask with a second 10-ml portion of chloroform and then an 8-ml portion of a 25% methanol in chloroform (v/v) solution. The hydroxycyprazine on the column is eluted with a 20-ml portion of 25% methanol in chloroform, which is collected directly in a 50-ml round-bottomed flask. This eluent is evaporated to dryness on a rotary evaporator at 40°, under aspirator vacuum. The flask is then stoppered until the chlorination step is to be carried out.

Chlorination. To the flask containing the dried hydroxycyprazine residues eluted from the silica gel column is added 2 ml of acetonitrile, 2 ml of dimethylformamide, and 250 ± 10 mg of phosphorus pentachloride and the flask is restoppered. The flask is gently swirled until all of the residues appear to have dissolved, placed in a 40° water bath for 10 min, removed from the bath, and cooled in ice. One-and-one half milliliters of concentrated (15 *N*) ammonium hydroxide is quickly added. The flask is gently swirled in the ice for a minute; then it is removed from the ice and its sides are washed with about 4 ml of carbon tetrachloride and 5 ml of water. The resultant mixture is transferred with a disposable pipet to a 60-ml separatory funnel containing 15 ml of water. The flask is rinsed with 5 ml of water and then 3 ml of carbon tetrachloride, which are also transferred to the separatory funnel. The cloudy carbon tetrachloride phase is collected in a 125-ml Erlenmeyer flask, and the aqueous phase is extracted twice with fresh 10-ml portions of carbon tetrachloride. The combined carbon tetrachloride phases, containing the cyprazine generated from hydroxycycprazine residues, are dried over anhydrous sodium sulfate and filtered through a coarse glass frit. The sodium sulfate is rinsed with three 5-ml portions of carbon tetrachloride. The filtrates are collected in a 100-ml round-bottomed flask and evaporated to dryness on a rotary evaporator at 40°, under aspirator vacuum.

Aluminum Oxide Column. A 15-g sample of Woelm Basic aluminum oxide, activity grade IV, is added to a 20 mm i.d. × 150 mm chromatographic column, gently settled by tapping, and held in place with a layer of sea sand. The cyprazine remaining in the 100-ml flask after evaporation of the carbon tetrachloride is dissolved in 10 ml of toluene and placed on the column. The flask is given two additional 10-ml rinses of toluene, which are also placed on the column. Each portion of toluene is allowed to elute to the top of the sand before the next is added. The column is developed with a 50-ml portion of hexane followed by 100 ml of a 3% solution of ethyl acetate in hexane. Cyprazine is eluted from the column with 150 ml of a 5% solution of ethyl acetate in hexane. This eluent is taken to dryness on the rotary evaporator at 40°, under aspirator vacuum. The cyprazine residues in the flask are dissolved in 0.5 ml of toluene and transferred to a 2.5-ml graduated stoppered centrifuge tube. Additional small washes with toluene are made in order to bring the total volume in the tube to 1.0 ml.

Gas Chromatography. A 4-μl injection of the cyprazine solution is made into a Hewlett-Packard Model 5751B gas chromatograph equipped with a Model 15161A nitrogen detector. A 6 ft × 1/4 in. glass column containing 5% UCW-98 on 80–100 mesh Diatoport S is used to separate the cyprazine. The temperature of the column is maintained at 235°, with the injection port at 250° and the detector at 400°C. Helium is used as the carrier gas at a flow of 70 ml per minute. The hydrogen and air flow rates are adjusted for maximum

sensitivity for cyprazine. After an appropriate warm-up time, with the range set at 10^2 and the sensitivity set at 16, a 21.72-ng (4 μl of a 5.43 μg/ml solution) injection of cyprazine (equivalent to 20 ng of hydroxycyprazine) gives a peak height of 50–70 mm in slightly over 3 min.

Preparation of a Standard Curve. Standard solutions of cyprazine in toluene are prepared. To adjust for the difference in molecular weights of hydroxycyprazine (mol wt 209) and cyprazine (mol wt 227), concentrations of **2** are multiplied by 1.086 to yield the concentrations of **1** which are used as standards. A 4-μl volume from each solution of cyprazine is injected into the gas chromatograph under conditions described above. A plot of the logarithm of the concentration of cyprazine (1.086 × hydroxycyprazine) *vs.* the logarithm of the recorder response yields a straight-line correlation.

The response of the nitrogen-sensitive detector is highly dependent upon detector temperature and flow rates of carrier, hydrogen, and air, causing changes in response magnitude over a period of time; therefore the standard should be reinjected frequently.

RESULTS AND DISCUSSION

Known amounts of **2** were added to corn silage or grain prior to extraction to determine the efficiency of the procedure. Determinations of **2** from 50-g samples averaged 73% (range 62–88%, standard deviation ±7%) for added levels representing 0.01, 0.02, 0.05, 0.10, and 0.20 μg/g (ppm).

Interfering plant background arising from untreated corn silage or grain is usually nonexistent in this procedure. However, a peak at the retention time of cyprazine corresponding to not more than an apparent 0.015 μg of cyprazine/g corn is occasionally seen. A level of twice the natural background is arbitrarily chosen as significant, which results in a sensitivity of 0.03 ppm for this method. No hydroxycyprazine residues (*i.e.*, above 0.03 ppm, the sensitivity of the analytical procedure) were found in any harvested field samples which had been treated with cyprazine.

The chlorination reaction is not a simple exchange but involves attack of a tautomeric amide form of **2** by a Vilsmeier-type complex of the phosphorus pentachloride and dimethyl formamide. The completeness of this reaction and workup is the yield-limiting step in the analytical procedure. From 75 to 85% conversion in this step is routine in the range of 1–50 μg of **2**.

There appears to be no portion of this method which would preclude its application to any other 2-hydroxy-4,6-dialkylamino-*s*-triazines, save a slight adjustment in the elution volumes for the final alumina column.

LITERATURE CITED

Chen, J-Y. T., *J. Ass. Offic. Anal. Chem.* **50,** 595 (1967).
Fishbein, L., *Chromatogr. Rev.* **12,** 167 (1970).
Flint, G. T., Aue, W. A., *J. Chromatogr.* **52,** 487 (1970).
Harris, C. I., J. AGR. FOOD CHEM. **15,** 157 (1967).
Lamoureux, G. L., Shimabukuro, R. H., Swanson, H. R., Frear, D. S., J. AGR. FOOD CHEM. **18,** 81 (1970).
Montgomery, M. L., Botsford, D. L., Freed, V. H., J. AGR. FOOD CHEM. **17,** 1241 (1969).
Montgomery, M. L., Freed, V. H., J. AGR. FOOD CHEM. **12,** 11 (1964).
Riden, J. R., Asbell, W. J., Midwest Regional Meeting of the American Chemical Society, Kansas City, Missouri, 1969.
Riden, J. R., Asbell, W. J., Schroeder, R. S., Abstracts of Papers, 160th National Meeting, of the American Chemical Society, Chicago, Illinois, 1970, PEST 7.

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Anomalous Effects of Humidity Control

Oxidation of methyl linoleate supported on microcrystalline cellulose showed anomalous effects when humidified to $A_w = 0.62$. The highly catalytic effect was shown to be due to diffusion of dinitrogen trioxide formed in the saturated $NaNO_2$ solution through the vapor space into the samples.

Saturated salt solutions have been a convenient and reliable means of producing static constant relative humidity (water activity) conditions for study of experimental systems (Carr and Harris, 1949; Rockland, 1960). In most cases no interaction is expected between the solution and the system suspended in the vapor space above the solution unless some halide salt is used, such as KBr, which can give off bromine vapors.

In studying the phenomenon of moisture sorption hysteresis and its effect on metal-catalyzed lipid oxidation, we expected that a system mixed dry (cellulose powder–glycerol–methyl linoleate) and humidified to a specific water activity would oxidize at a much slower rate than that of systems which were mixed directly with all ingredients including water to that specific activity (Labuza *et al.*, 1972). This is due to the fact that sorption hysteresis occurs with the direct mixed system having a higher equilibrium moisture content and thus a lower viscosity aqueous phase for transport of catalysts. This phenomenon occurred at all water activities studied [0.52 $Mg(NO_3)_2$, 0.68 $SrCl_2$, 0.75 NaCl, 0.84 K_2CrO_4, and 0.89 KNO_3] except at 0.62. The latter water activity was obtained by using a saturated $NaNO_2$ solution and holding under vacuum for 24 hr. As shown in Figure 1, the humidified sample at 0.62 (DH) oxidized faster than any other sample and did not fit in the above hypothesis.

In order to determine if it was some factor contributed by the salt solution, the experiment was repeated but all directly mixed samples with water were held also in the desiccators simultaneously during rehumidification of the other sample set (24 hr). Oxygen uptake by the Warburg manometric