INSECTICIDE RESIDUES

A Procedure for the Microdetermination of 1-Butoxy-2-(2-Thiocyanoethoxy)-ethane (Lethane 384) with Applications for Determination of Residues in Milk and Animal Tissues

C. F. GORDON, L. D. HAINES, and A. L. WOLFE

Rohm & Haas Co., Philadelphia, Pa.

A procedure has been developed that will determine micro amounts of organic thiocyanate using a colorimetric procedure. This has been applied to the determination of Lethane 384, a commercial pesticide. The method is based on the known cyanide procedure of Aldridge as modified by Bruce, Howard, and Hanzal. However, it was necessary to find suitable conditions for hydrolysis of Lethane to inorganic cyanide either alone or in the presence of interfering substrate, to permit detection at a level of 1 μg. The method was then extended to determination of such thiocyanates in the presence of milk and animal tissue extractives. A method for extraction and handling of milk and tissues is described which eliminates interferences.

LETHANE 384 [1-butoxy-2-(2-thiocyanoethoxy)ethane, Rohm & Haas Co.] has been extensively used as an insecticide for application on cattle for over 30 years. A precise study of the excretion of Lethane in milk following topical application to dairy cattle was of prime importance to ensure that proper use of Lethane on dairy cows does not produce a significant residue level. The analytical method developed for this study is based on the known cyanide procedure of Aldridge (*1*, *2*) as modified by Bruce, Howard, and Hanzal (*3*). However, it was necessary to find a suitable condition for hydrolysis of Lethane to inorganic cyanide in the presence of interfering milk fat, to allow for microsensitive detection. A method for milk extraction (*4*) is given which eliminates interferences due to naturally occurring thiocyanates.

Experimental

Apparatus. Any spectrophotometer capable of performing well in the 500-mμ region.

The Lethane aeration apparatus consists of three tubes connected in series as shown in Figure 1. Compressed nitrogen enters the gas washing tube containing 25 ml. of 20% sodium hydroxide.

Blender used to prepare tissue samples for analysis. (Since flammable solvents are used for blending medium, care should be exercised in the use of blenders. In this laboratory the explosive-proof type only is used.)

Reagents. Dimethylformamide. A technical grade is distilled and chromatographed over alumina. Degas the reagent with nitrogen prior to use.

n-Hexane. Technical grade chromatographed over alumina.

Sodium Ethylate (alcoholate). 0.1*N* solution.

Bromine Water. A saturated solution of bromine in deionized water is used.

Arsenous Acid. A 2% solution of arsenous acid is prepared by refluxing 2.0 grams of arsenous acid with 100 ml. of deionized water until solution is complete.

Trichloroacetic Acid. A solution of 20% trichloroacetic acid (w./v.) in distilled water is prepared.

Benzidine Hydrochloride Solution. A 4.0% solution of benzidine hydrochloride in water is prepared daily.

Pyridine Solution. Add 100 ml. of concentrated hydrochloric acid to 1 liter of 60% pyridine in water (v./v.). Store in a brown bottle.

Pyridine-Benzidine Solution. Immediately before use, mix 1 part by volume of benzidine hydrochloride solution with 5 parts by volume of pyridine solution.

Procedure. PREPARATION OF STANDARD CURVES. Prepare standard calibration curves for Lethane by carrying aliquots containing known amounts through the following procedure: In a 60-ml. separatory funnel place 5 ml. of dimethylformamide, 15 ml. of reagent *n*-hexane, 0.5 ml. of 0.1*N* sodium ethylate, and an aliquot of Lethane solution, usually 1.0 ml. Agitate the contents of the funnel by degassing the solvents for 10 minutes with a gentle stream of nitrogen passing through a filter stick inserted to the bottom of the funnel. Add 2 ml. of 0.1*N* sodium hydroxide and continue agitation with nitrogen for another 2 minutes. Allow the phases to separate clearly and drain off the lower aqueous layer into the Lethane reaction tube (Figure 1). In the receiving tube place 1 ml. of 0.1*N* sodium hydroxide. Stopper the tubes and connect in series with the gas washing tube (Figure 1). Syringe 3 ml. of 20% trichloroacetic acid through the rubber cap of the reaction tube and allow nitrogen to aerate through the system at a rate of 80 ml. per minute for a 30-minute period. At the end of this time, disconnect the apparatus and analyze the content of the receiver tube for cyanide.

COLOR DEVELOPMENT AND MEASUREMENT. With a gentle stream of nitrogen blow out the contents of the capillary inlet tubing of the trap and then wash it down with 0.5 ml. of 20% trichloroacetic acid. Immediately add 1 drop of saturated bromine water to the contents of the trap. Swirl and mix thoroughly. Remove the excess bromine by adding 0.2 ml. of a 2% arsenous acid solution. Blow off the vapors of bromine above the solution with a stream of air or nitrogen. Next add 3.6 ml. of pyridine benzidine reagent and allow the color to develop for 15 minutes. Measure the absorbance of the red color produced at 532 mμ.

Analysis of Milk

General. Lethane is extracted from milk with *n*-hexane and a detergent, Triton X-45, for milk fat separation (*4*). After concentration of the hexane extract, Lethane is hydrolyzed to cyanide using a dimethylformamide solution of sodium ethylate. The sodium cyanide formed is water-extracted from the mixture and the cyanide content in aqueous solution is then determined by acidifying and aerating the generated hydrogen cyanide for subsequent introduction into the standard cyanide color reaction.

Milk Extraction. Place a 100-ml. portion of milk in a 250-ml. centrifuge bottle. Add 3 ml. of 10% potassium oxalate solution to the bottle and warm to about 45° C. in a water bath. Then add 10 ml. of a 10% (v./v.) aqueous mixture of Triton X-45. Mix in thor-

oughly and maintain at 45° C. for 20 minutes. Allow sample to cool to room temperature. Centrifuge briefly to obtain a clear fat layer, then add 100 ml. of reagent *n*-hexane. Stopper tightly with airtight (plastic-lined) metal lid. Shake gently to gather the fat. Centrifuge until the hexane layer is clear. By means of a siphon arrangement, remove the hexane layer remaining on the milk. Slowly, so as not to disturb the milky layer, add 50 ml. of hexane and again remove the hexane into the Kuderna flask containing the hexane from the first extract. Perform a second extraction by adding another 100 ml. of hexane, stoppering, shaking, centrifuging, and blowing off the hexane as before. Wash with 50 ml. of hexane, combining the washing with previous extracts and washings. Evaporate off the hexane down to the fatty residue (about 5 ml.) in the Kuderna apparatus.

Hydrolysis. In a 125-ml. separatory funnel put 5 ml. of dimethylformamide and 0.5 ml. of 0.1*N* sodium ethylate. Transfer the concentrated extract to this separatory funnel by rinsing the tube three times with 4 ml. of hexane, adding all the washings to the funnel. Shake the funnel and its contents for 3 minutes and then continue agitation by degassing the solvents for 10 minutes with a gentle stream of nitrogen passing through a filter stick inserted to the bottom of the funnel. Add 2 ml. of 0.1*N* sodium hydroxide and continue agitation with nitrogen for another 2 minutes. Allow the phases to separate clearly and drain off the lower aqueous layer into the Lethane reaction tube (Figure 1). Attach the reaction tube to the system. Check and be sure all equipment is tightly assembled. With the aid of a syringe add 5 ml. of 20% trichloroacetic acid, while bubbling nitrogen through the system at a rate of 80 ml. per minute. The hydrogen cyanide generated will be carried over into the receiver tube containing 1 ml. of 0.1*N* sodium hydroxide. Continue the degassing for a 30-minute period. At the end of this time, disconnect the receiver tube and carry out the color development and measurement of its contents exactly as described in the standard procedure.

Calibration. A reagent control is run each time treated samples or fortified controls are analyzed. All readings are made against the pyridine solution. This will give a check on the quality and variation of reagent blanks from day to day. Fortified controls are run by adding known concentrations of sodium cyanide into the separatory funnel before hydrolysis. The concentration range should be from 0.1 to 10 μg. The calibration curve for Lethane is developed by adding known concentrations of Lethane (1 to 16 μg.) to the milk fat extract and then running it through the procedure. The over-all recovery is measured by making the additions of Lethane to the whole milk prior to extraction with benzene.

Analysis of Animal Tissue

Extraction. Weigh out up to 5 grams of ground up tissue into a Waring Blendor jar. Blend at high speed, for 5 minutes, with 100 ml. of chromatographed *n*-hexane. Add 100 ml. of hexane and blend for an additional 5 minutes at low speed. Transfer to the centrifuge bottle and centrifuge to separate solvent from solid material. By means of a siphon arrangement, remove the hexane layer into a Kuderna flask. Wash the solids remaining in the bottle by adding 100 ml. of hexane and again centrifuging and blowing off hexane in the Kuderna as before. Evaporate the hexane to about 5 ml. in the Kuderna apparatus and continue the analysis by carrying out hydrolysis of Lethane as instructed for milk analysis.

Discussion

Standard Curves. A primary problem centered around incorporating suitable conditions for hydrolysis of organic thiocyanate to inorganic cyanide. Literature sources (*5*, *7*) report organic thiocyanates as undergoing hydrolysis in alcoholic base:

$$2\ RSCN + 2\ NAOH \longrightarrow NAOCN + NACN + RSSR + H_2O$$

However, when the reaction was carried out on a microsensitive level, little or no recovery occurred. Various types of hydrolytic reagents were investigated, and it was found that a dimethylformamide solution of sodium ethylate produces quantitative yields of sodium cyanide from micro amounts of Lethane. However, there was some difficulty, at first, from inconsistent and high reagent blank values produced in the procedure. This was due mainly to dimethylformamide. A careful cleanup of this solvent was found necessary to attain acceptable blank values. A technical grade of dimethylformamide is distilled and then chromatographed over alumina. Just prior to analysis, the reagent is degassed with dry nitrogen to remove any traces of interfering ammonia. Dimethylformamide prepared in this manner is suitable for a 3-week duration.

The time required for completion of hydrolysis was investigated by conducting a series of determinations in which the

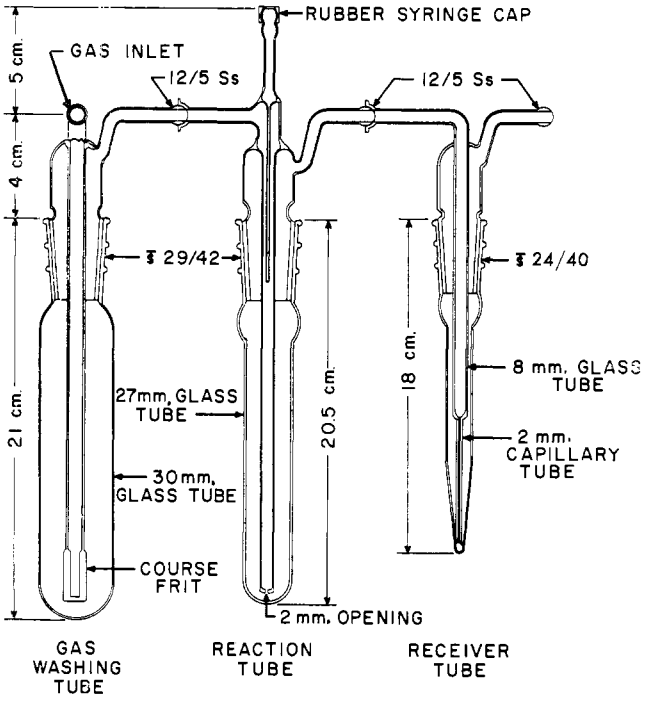

Figure 1. Lethane aeration apparatus

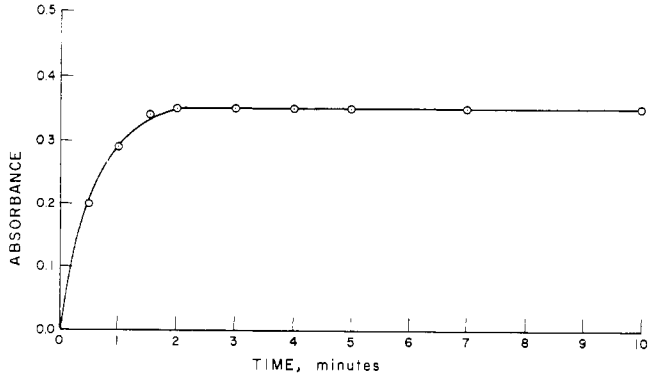

Figure 2. Effect of time of hydrolysis of Lethane on amount of color formed

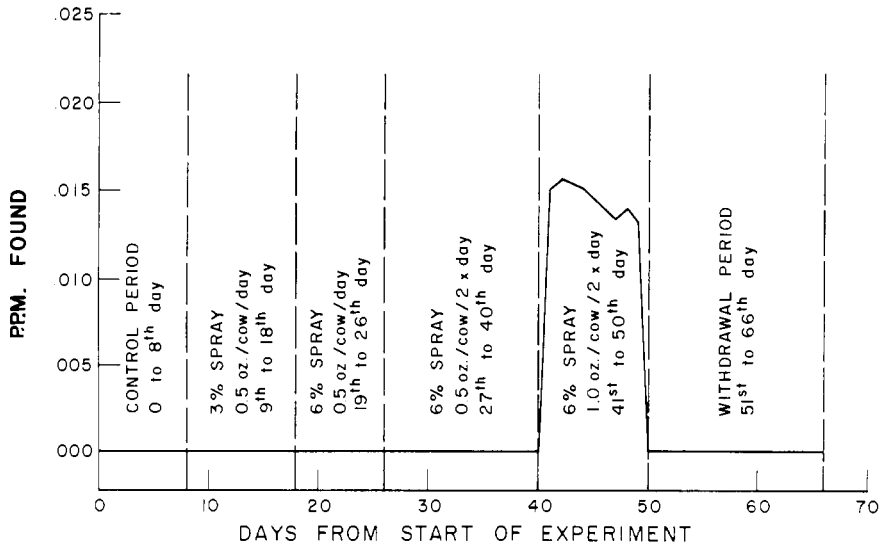

Fig. 3. Lethane excreted in milk following topical application

hydrolysis time was varied from 1 to 10 minutes. In this study, 8-μg. samples of Lethane were hydrolyzed with sodium ethylate in dimethylformamide. The results are shown in Figure 2. The hydrolysis is practically complete in 2 minutes, at room temperature. To ensure completion of hydrolysis a 10-minute period is routinely employed.

Table I indicates recovery of hydrolyzed cyanide from Lethane; average recovery is 86%.

Analysis of Milk. One of the problems complicating the method, when applied to milk, was interference from color-rendering milk extractives. Interference of various compounds in the basic cyanide method has been discussed by Aldridge (*1*, *2*). In general, any compound that can react to produce cyanogen bromide will interfere. Thus, the problem with milk was complicated not only by the various proteins in milk which will interfere, but also by various cyanides and thiocyanates reportedly occurring naturally in milk (*8*). Early in the work it was noted that high interfering control values were obtained when freeze-dried milk solids were analyzed without any preliminary extraction or cleanup. Also when extraction attempts were made using various solvents such as diethyl ether, petroleum ether, and various chlorinated compounds to extract milk fat in which Lethane is concentrated, high control values were experienced in the procedure. If the extraction is performed with chromatographed *n*-hexane, control values remain close to reagent blank values. In the method finally adopted, *n*-hexane is used along with a detergent, Triton X-45, to aid in separating milk fat from the aqueous phase of whole milk. Two extractions are carried out with *n*-hexane, allowing an efficiency of 85%. A third extraction has no effect on over-all recovery.

Various techniques were tried for isolating Lethane from milk fat in order to apply the standard procedure. Such methods as saponification and acid treatment of the milk fat failed to permit cyanide detection at a low enough sensitivity. However, micro quantities of Lethane are hydrolyzed in the presence of milk fat without saponification, if the reaction is carried out at room temperature using sodium ethylate and dimethylformamide. Following this reaction, a water extraction removes hydrolyzed cyanide from the fatty substrate.

At first, erratic and low recoveries were encountered with this procedure when the water extract was analyzed for its cyanide content. To improve this, the extract is thoroughly degassed with nitrogen to rid the solution of volatile interferences prior to generation and sweeping of the hydrogen cyanide.

It is possible to determine cyanide directly in the water extract without isolation of hydrogen cyanide into another tube. However, this requires added cleanup of the extract to permit quantitative absorbance readings. However, for qualitative work this method is suitable without elaboration. In cross-checking for possible Lethane metabolites, the red color developed in the reaction tube containing the water extract can be indicative of certain classes of compounds. Color in this tube is positive for cyanides (iso- and normal) and thiocyanates (iso- and normal). Although cyanides and normal thiocyanates which are hydrolyzed to cyanide can be degassed from the reaction tube under acid conditions, isothiocyanates do not hydrolyze to the cyanide; however, they do undergo the Aldridge color test. This test for isothiocyanate was tried during the Lethane cow spray study when no Lethane residue could be detected in the milk. The reaction tube with water wash was

Table I. Recovery of Cyanide from Lethane

No. of Detns.	Lethane Added, μg.	Cyanide Found, μg.	Av. Recovery, %	Std. Dev., %
8	1.0	0.05	85	4.0
5	2.0	0.11	86	5.0
8	4.0	0.23	87	2.2
6	8.0	0.44	86	2.1
5	10.0	0.54	85	2.5
5	16.0	0.87	85	2.5

Table II. Stability of Lethane in Whole Milk

Treatment	Recovery, %[a] Hexane fortifying solution	Ethanol fortifying solution
Prompt analysis	70	70
1-day storage	72	70
3-day storage	69	71
5-day storage	70	71

[a] Average recovery on duplicate determinations.

Table III. Recovery of Known Amounts of Lethane Added to 100 Grams of Whole Milk

Type of Milk Sample	Lethane Added, P.P.M.	Lethane Recovered P.p.m.	%
Control	0	0.010[a]	..
	0	0.011[a]	..
	0	0.010[a]	..
	0	0.011[a]	..
Fortified	0.01	0.007	70
	0.01	0.007	70
	0.02	0.013	69
	0.02	0.014	70
	0.04	0.029	73
	0.04	0.028	71
	0.08	0.056	70
	0.08	0.057	72
	0.16	0.109	68
	0.16	0.110	69

[a] Control values expressed in apparent parts per million.

Table IV. Duplicate Determinations on Milk Samples from Lethane-Sprayed Cow

Type of Milk Sample	Found, P.P.M. 1st detn.	2nd detn.
Control (milk obtained before spray period)		
1	0.011[a]	0.012[a]
2	0.011[a]	0.011[a]
From 41st day[b]	0.015	0.014
From 42nd day	0.016	0.015
From 44th day	0.015	0.015
From 45th day	0.014	0.015
From 46th day	0.013	0.013
From 47th day	0.012	0.012
From 48th day	0.013	0.013

[a] Control values expressed in apparent parts per million.

[b] Of spray schedule, for all data in this column.

checked for isothiocyanate. No color was produced at an estimated sensitivity of 1 μg. of isothiocyanate ion.

The possibility of Lethane degrading in the milk while stored was investigated. Table II shows the constancy of Lethane recovery from fortified milk stored under refrigeration over various periods. Two types of fortification were employed: Lethane added from a hexane solution and Lethane added via ethanol. Recoveries in each case proved constant over various periods.

Table III indicates over-all recovery on 100-gram milk samples fortified with varying amounts of Lethane. Recovery is based on the theoretical amount of cyanide produced from hydrolysis of Lethane. In the experimental cow spray study, the absorbance of the milk controls averaged 0.060 ± 0.007 measured in 2-cm. path length cells. With a 100-gram milk control, a fortification of 1 μg. of Lethane gives an absorbance of 0.115 ± 0.005 in 2-cm. cells.

As a measure of reproducibility, Table IV shows data for duplicate determinations on milk samples from Lethane-sprayed cows. Control values are expressed in apparent parts per million denoting amount of interference of controls as compared to the pyridine reagent used in the reference absorption cell.

Results

Detailed results of the cow spray experiment with respect to topical application, animal treatment, and excretion of the insecticide have been reported (*6*). Figure 3 summarizes results obtained during the four treatment stages of the investigation. No detectable residues of Lethane were present in any pre- or post-treatment samples or in any samples taken following application of the first three treatment formulations. Lethane was detected in milk the first day following application with a 6% 1-ounce spraying twice daily. This is a spray level equal to four times that which is recommended. This period showed a residue level ranging between 0.012 and 0.016 p.p.m. The residue disappeared the day following withdrawal from treatment.

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INSECTICIDE RESIDUES IN MILK

DDT Residues in Milk from Dairy Cows Fed Low Levels of DDT in Their Daily Rations

GUNTER ZWEIG, L. M. SMITH, S. A. PEOPLES, and RENATE COX

Pesticide Residue Research, Department of Food Science and Technology, and Department of Pharmacology, Physiology, and Biochemistry, University of California, Davis, Calif.

A feeding experiment with 12 dairy cows was conducted in which pairs of animals were fed 0 to 5.0 p.p.m. of DDT added to their daily rations for 31 days. In another experiment, six cows were fed 1 p.p.m. of added DDT over a period of 8 weeks. Milk was analyzed periodically for fat and DDT content. A maximum level of 0.5 p.p.m. of added DDT in the feed did not produce a residue of 0.01 p.p.m. or greater in the milk. At levels of 1, 2, 3, and 5 p.p.m. of added DDT, detectable residues were found in the milk of all animals. DDT concentration in milk was proportional to DDT level in feed. There was a correlation between DDT residue and fat concentration in milk at each feeding level but not between total DDT excretion and total fat production.

The excretion pattern of various chlorinated pesticides in the milk of dairy cows has been established by Gannon, Link, and Decker (*3*). The animals were placed on rations to which these pesticides were added daily at different levels. Ten parts per million was the lowest level for DDT which produced a detectable residue 7 days after feeding was started. The residue in the milk, however, did not seem to reach a plateau even after 14 to 16 weeks of feeding at this level of DDT in the feed. The maximum dose of DDT in daily rations that would not result in a detectable residue in the milk was not established by these workers (*3*).

Present work was undertaken to attempt to define a "safe" level of DDT in feeds that would produce milk with undetectable amounts of this insecticide. Twelve dairy cows, including producers of both high- and low-fat milks, were fed daily rations containing 0 to 5 p.p.m. of DDT, based on their feed intake. Milk samples were analyzed for DDT by colorimetric and paper chromatographic methods.

After the maximum safe level of DDT in the feed was determined, results were corroborated by placing another group of 10 animals on a ration