Assay for Residues in Milk and Cream from Cows Fed 6-Chloropicolinic Acid

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Six dairy cows were fed rations containing 6-chloropicolinic acid, the principal metabolite of the nitrification inhibitor 2-chloro-6-(trichloromethyl)-pyridine (Dowco 163). Samples of milk and cream were assayed for 6-chloropicolinic acid by a procedure in which the sample was hydrolyzed with sulfuric acid to liberate the acid from possible conjugates. The 6-chloropicolinic acid was then extracted with ether, followed by cleanup on a Woelm neutral alumina 200 column. The acid was esterified with diazomethane and quantitatively determined by gas chromatography of this ester employing a LAC-446-H_3PO_4 column and electron capture detection. Recoveries were 89% from milk and 83% from cream. Assay results reveal that no measurable residues (<0.025 ppm) appeared in milk or cream from cows ingesting 6-chloropicolinic acid at levels up to 100 ppm in the diet.

Minimizing nitrogen loss is an important goal in good soil management. Nitrification of ammonia can lead to significant nitrogen loss by leaching or can lead to nitrite accumulation in plants and toxicity to plants and animals (Goring, 1962). Prevention of nitrification thus stabilizes soil ammonia, thereby eliminating or reducing the need for multiple fertilizer applications to obtain optimum crop growth and yield. A developmental product called N-SERVE 24 nutrient stabilizer (The Dow Chemical Co.) reduces nitrification. This product contains 2-chloro-6-(trichloromethyl)-pyridine (Dowco 163), which prevents nitrification by killing the primary soil microorganisms responsible for the oxidation of ammonia to nitrite nitrogen (Goring, 1962; Turner and Goring, 1966).

The recommended use of Dowco 163 applied to soil can result in residues of its principal metabolite, 6-chloropicolinic acid (Figure 1), of 1 ppm or less in corn and cotton (Getzendaner and Daun, 1968; Kutschinski, 1964; Meikle and Redemann, 1966; Redemann *et al.*, 1965). Thus dairy feed could contain small residues of the metabolite. This study was undertaken to determine the extent to which 6-chloropicolinic acid is secreted into milk and cream by cows fed various graduated amounts of the chemical.

EXPERIMENTAL

Six Holstein dairy cows, weighing from 433 to 537 kg, were confined to pens and conditioned for 2 weeks on a complete dairy ration. This was given twice daily in equal 8.15-kg amounts. Medicated feed was prepared by dissolving analytical grade 6-chloropicolinic acid (99%) in acetone, mixing with silica gel to give 10 to 25% active ingredient in the dry powder, and this powder was blended with sufficient complete cattle feed to make a ration containing 1, 10, or 100 ppm of 6-chloropicolinic acid.

Following a 2-week conditioning period, three cows were continued on control rations while three were fed 6-chloropicolinic acid at 1 ppm for 14 days, then at 10 ppm for 14 days, and 100 ppm for 21 days in succession. The chemical was then withdrawn from the diets and the cows maintained on basal rations for 5 days.

Throughout this test, milk samples were obtained at periodic intervals by combining one-half pint subsamples of the thoroughly mixed entire milk sample from the evening milking with an equal amount collected in the same way the following morning. Samples were then frozen until analyzed 3 months later.

Cream samples were collected from morning milk only. Milk from the three control cows was combined before processing for cream and another pooled milk sample was obtained in the same manner from the treated cows. The cream processed from these samples was stored frozen until analyzed 8 months later.

ANALYTICAL PROCEDURES

Apparatus. Gas chromatograph, Tracor Model MT 220, was equipped with Ni^{63} electron capture detector. The detector operating voltage was supplied by Tracor Pulse Power Supply (Tracor Part No. 830310).

Reagents. 6-Chloropicolinic acid, 99% pure. Methyl-6-chloropicolinate, 99% pure. Both of these reagents are obtainable from Sampling Coordinator, Agricultural Department, The Dow Chemical Co., Midland, Mich. 48640

Procedure. GAS CHROMATOGRAPHIC OPERATING CONDITIONS. The gas chromatograph equipped with a 3-mm i.d. × 74-in. column packed with 4% LAC 446 and 0.5% phosphoric acid on 80 to 100 mesh Chromosorb WAW was operated at a column temperature of 148° C with the flash heater set at 215° C and the detector set at 350° C. The nitrogen carrier gas flow rate was 60 ml per min and the detector operating voltage was 54 V pulsed with a 270 μsec pulse rate and a 10 μsec pulse width. The electrometer sensitivity was 1.3×10^{-9} A full scale.

SUBSAMPLING FROZEN SAMPLES. Place the pint can of frozen milk or cream in 70° C water for 0.5 hr with occasional shaking. This should make the can hot to the touch. Cool to body temperature and shake vigorously just before removing the subsample.

Milk Assay. Step 1. Weigh 10 g of well-mixed milk into a 12-dram vial with polyseal cap. Step 2. Add 1 ml of concentrated H_2SO_4, swirl to disperse the H_2SO_4, and place on top of a steam bath for 30 min, swirling occasionally. Step 3. Cool and add about 2 g of NaCl and 15 ml of ether. Cap the tube, shake vigorously for 5 min, and separate the phases by centrifugation for 3 min. Step 4. Add 1 g of alumina stored at 110° C to a 1-cm i.d. × 15-cm long chromatography column and wash with 10 ml of hexane. Step 5. Employing a 15-ml pipet and a pipettor, withdraw the ether layer (step 3) and transfer it to the alumina column allowing it to run through. Step 6. Reextract the milk with another 15 ml of ether, transferring that extract to the column and allowing it to run through. Wash the column with 10 ml of ether. Discard the effluent. Step 7. Elute the column

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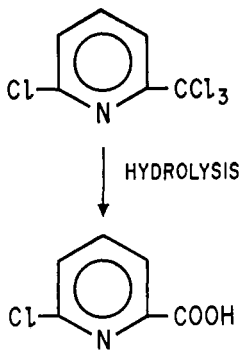

6-CHLOROPICOLINIC ACID

Figure 1. Conversion of Dowco 163 to 6-chloropicolinic acid

Table I. Animal Weights and Milk Production of Cows Eating 16.3 Kg of Feed Daily

Date	Cow number: Controls 1	2	3	Treated 4	5	6
	Body weights, kg					
3/28/69	493	497	470	492	537	433
6/6/69	498	536	490	505	557	432
Average	495	516	480	498	547	432
	Average daily milk production, kg					
4/7–11/69	18.6	20.4	20.4	25.4	23.1	23.6
4/21–25/69	16.8	17.2	18.6	24.0	20.9	24.5
5/5–9/69	16.8	18.1	19.5	24.5	22.7	24.0
5/26–30/69	15.4	16.3	19.1	20.9	20.0	21.8

with 15 ml of 0.03 *M* aqueous $NaHCO_3$ solution, collecting the eluate in a clean 12-dram vial. Step 8. Acidify the eluate with 0.5 ml of 85% H_3PO_4 and dissolve about 2 g of NaCl in the solution. Step 9. Successively extract this solution with 15 ml and 10 ml of ether, combining the extracts in a clean 12-dram vial. Step 10. Add 0.1 ml of MeOH, 2 ml of benzene, and 0.5 ml of diazomethane reagent and allow to stand for 30 sec. Add a boiling chip and boil off the ether on a steam bath. Cool to room temperature. Step 11. Quantitatively transfer this solution to a 10-ml volumetric flask employing a disposable pipet. Rinse the tube with 1-ml portions of benzene and transfer to the volumetric flask. Repeat the washing until the flask volume is about 9 ml. Dilute to volume with benzene. Step 12. Chromatograph 1 μl of the benzene solution and determine its concentration by referring the peak height of the peak obtained to a calibration curve.

Cream Assay. Step 1. Weigh 10 g of well-mixed cream into a 12-dram vial. Step 2. Add 3 ml of water, 1 ml of concentrated H_2SO_4, swirl to disperse the H_2SO_4, and place on top of a steam bath for 30 min, swirling occasionally. Step 3. Cool and add about 2 g of NaCl and 15 ml of ether. Cap the tube, shake vigorously for 5 min, and separate the phases by centrifugation for 3 min. Step 4. Employing a 20-ml pipet and a pipettor, withdraw the ether layer and transfer it to a clean 12-dram vial. Step 5. Add 15 ml of 0.1 *M* sodium bicarbonate and 2 g of NaCl. Shake vigorously for 3 min, and separate phases by centrifugation for 3 min. Discard ether layer. Step 6. Add 1 g of alumina stored at 110° C to the chromatography column (a 1-ml beaker full is just right) and wash with 10 ml of hexane. Step 7. Reextract the cream with another 15 ml of ether and transfer

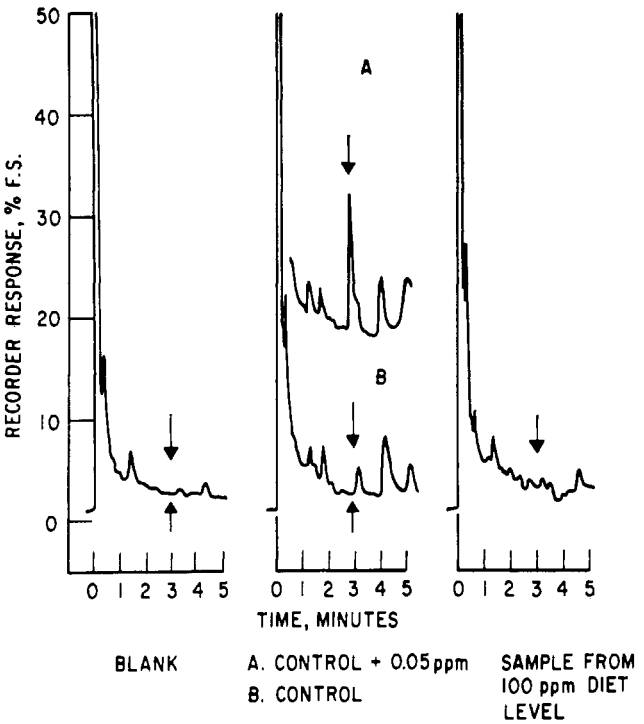

Figure 2. Representative chromatograms of milk

that extract to the column (step 6) allowing it to run through. Step 8. Add 0.5 ml of concentrated H_3PO_4 to the bicarbonate solution (step 5), 15 ml of ether, and 2 g of NaCl. Shake vigorously for 3 min and separate layers by centrifugation for 3 min. Transfer the ether extract to the alumina column, allowing it to run through. Step 9. Reextract the aqueous layer (step 8) with another 15 ml of ether and transfer that extract to the column, allowing it to run through. Wash the column with 10 ml of ether. Discard the effluent. Step 10. Follow steps 7–12 of milk assay procedure.

Standard Curve. Inject standard benzene solutions of methyl 6-chloropicolinate covering the concentration range equivalent to 10 to 100 ng of acid per ml. Establish a standard curve by plotting peak heights *vs.* corresponding acid concentrations in nanograms per milliliter. This same scale can be read in parts-per-billion residue directly.

Calculations. The final 10 ml of solution represents 10 g of sample. Therefore, the residue found is the concentration of 6-chloropicolinic acid found in this final solution expressed as parts-per-billion obtained by referring to the standard curve.

Recovery Determination. Fortify 10-g subsamples of untreated control milk or cream at concentration levels from 25 to 500 ppb by adding appropriate amounts of aqueous solutions of 6-chloropicolinic acid before extraction. Then assay these fortified samples by the appropriate method used for treated samples and determine recovery efficiency. Water was assayed the same way for a reagent blank.

Storage Stability. Fortified 10-g subsamples of milk and cream in 12-dram glass vials with polyseal screw caps were stored in a 0° C freezer. Milk was stored for 12 weeks before assay and cream was stored 23 weeks and assayed.

RESULTS AND DISCUSSION

No adverse effects due to feeding dairy cows 6-chloropicolinic acid were observed as evidenced by milk production, body weight, or gross appearance and behavior (Table I).

Table II. Recovery of 6-Chloropicolinic Acid from Fortified Milk and Cream

6-Chloropicolinic acid added, ppm	Number of determinations	Recovery, % Range	Recovery, % Average
Milk			
0 (Control)	10	0–0.005 (ppm)	
0.025	6	72–104	91
0.050	7	81–90	84
0.10	4	77–100	89
0.50	1	100	100
1.0	2	77–82	80
			Average 89 ± 17[a]
Cream			
0 (Control)	6	0–0.003 (ppm)	
0.025	8	72–100	88
0.050	7	64–90	77
0.10	4	80–93	87
			Average 83 ± 20[a]

[a] 95% confidence limits.

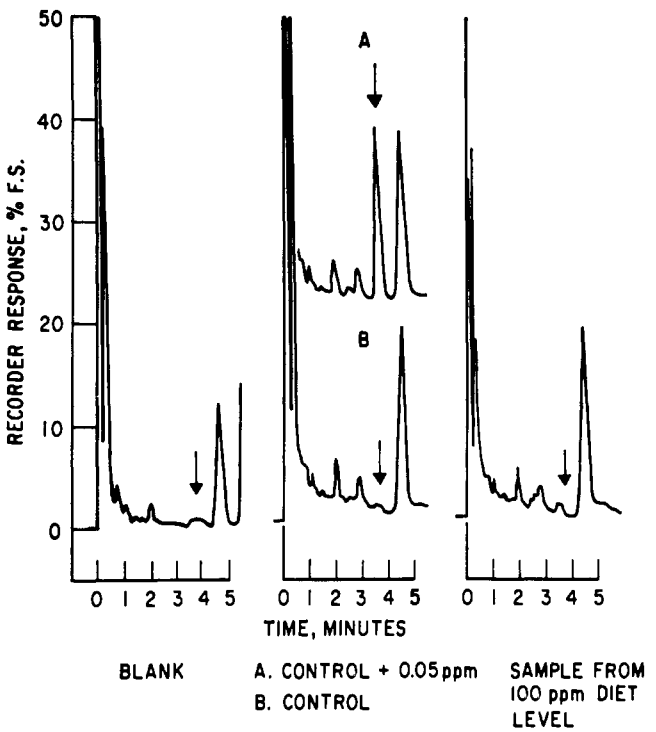

Figure 3. Representative chromatograms of cream

The cows ate all of the 16.3 kg of feed presented to them daily throughout the test, which represents an average consumption by cows four, five, and six of 3.4 mg of 6-chloropicolinic acid per kilogram average body weight per day when fed at the 100-ppm diet level.

Results of the recovery studies are summarized in Table II and typical chromatograms are given in Figures 2 and 3. These data show that the recovery from milk was 89 ± 17% and from cream it was 83 ± 20%. It was demonstrated that

Table III. Recovery of 6-Chloropicolinic Acid from Fortified Samples after Frozen Storage

6-Chloropicolinic acid added, ppm Added	Found	Recovery, %
Milk after 12 weeks		
0		
0.025	0.023	92
0.025	0.025	100
0.050	0.041	82
0.050	0.036	72
0.10	0.095	95
0.10	0.088	88
0.50	0.42	84
0.50	0.43	86
		Average recovery 89 ± 19[a]
Cream after 23 weeks		
0		
0.025	0.019	78
0.025	0.018	72
0.050	0.039	78
0.050	0.039	78
0.10	0.079	79
0.10	0.079	79
		Average recovery 77 ± 5[a]

[a] At 95% confidence level.

there was no degradation of 6-chloropicolinic acid in milk or cream samples during frozen storage. Subsamples of milk fortified with 25 to 500 ppb of acid and stored for 12 weeks had an average recovery of 89 ± 19% (Table III), which is the same as that from milk assayed immediately after fortification. Assays of cream subsamples fortified from 25 to 100 ppb of acid and stored for 23 weeks had an average recovery of 77 ± 5% (Table III), which is not significantly different from the average recovery observed immediately after fortification.

All assays of individual milk samples and cream composites gave results less than 0.025 ppm the validated sensitivity of the assay procedure. Thus, no significant residues (>0.02 ppm) appeared in milk or cream from cows ingesting 6-chloropicolinic acid at levels up to 100 ppm in the diet.

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