2-*sec*-Butyl-4,6-dinitrophenol and 2-Amino-6-*sec*-butyl-4-nitrophenol in Milk and Cream from Cows Fed 2-*sec*-Butyl-4,6-dinitrophenol

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Holstein dairy cows were fed rations containing 2-*sec*-butyl-4,6-dinitrophenol (DNBP) at 1, 3, 10, 30, and 100 ppm levels. Milk samples were collected and analyzed for DNBP and a possible metabolite, 2-amino-6-*sec*-butyl-4-nitrophenol (2-ABNP). The methods used included an acid hydrolysis step to liberate DNBP and 2-ABNP if they were present as glucuronides. Both compounds were determined by gas chromatography employing electron capture detection, with DNBP as its methyl ether and 2-ABNP as its trimethylsilyl ether derivative. No residue of DNBP was found by the methods validated for 0.01 ppm in milk and 0.05 ppm in cream. No 2-ABNP was found using methods sensitive to 0.1 ppm in milk and 0.1 ppm in cream.

Premerge herbicide (a formulation of The Dow Chemical Company containing the ethanol and isopropanolamine salts of 2-*sec*-butyl-4,6-dinitrophenol (DNBP) Figure 1) is used to control many seedling weeds and grasses. It is used on such crops as peanuts, soybeans, and field beans. The green forage of some crops such as soybeans may be fed to dairy cows. Small residues of DNBP have been found to occur on green soybean forage and thus the chemical could be ingested by the dairy cow (Getzendaner, 1968). This study was undertaken to determine to what extent DNBP would be present in the milk of cows fed graduated levels of the chemical.

EXPERIMENTAL

Six Holstein cows weighing about 1000 lb each were housed in individual box stalls using wheat straw for bedding and having free access to water and feed. The animals were conditioned for 3 weeks to their environment, including the milking procedure. During the conditioning period it was determined that 10 lb of grain ration plus 10 lb of alfalfa hay were the maximum each animal would consume per day without leaving significant amounts.

After the conditioning period, the DNBP was incorporated in the grain ration in the following manner. The amount of DNBP needed for a total of 900 lb of feed was calculated. The volume of Premerge containing the amount of DNBP needed for a particular feeding level was premixed with 20 lb of feed. The premix was then blended with sufficient feed to make 900 lb, which was stored in a vertical blender and was blended 2 min before each feeding. A given batch of grain ration was prepared at twice the desired feeding level since the hay, constituting 0.5% of the total diet, contained no DNBP.

Three cows were continued on a DNBP-free diet while the other three were fed 1 ppm for 14 days, 3 ppm for 17 days, 10 ppm for 15 days, 30 ppm for 14 days, and 100 ppm for 21 days in succession.

All milking was done by machine according to the same daily time schedule. All control cows were milked with the same machine. The machine was washed thoroughly before milking the next cow. Each treated animal had an individual milking machine. Sampling was done by taking a 2-qt aliquot from both the morning and evening milking. Aliquots from the same cow were composited. The milk remaining from each cow was composited and separated with a DeLaval, Model 100, electric farm separator adjusted to give medium heavy cream. The milk fraction was discarded. The cream was collected and stored in tin cans with screw tops and immediately frozen. During the feeding period in which the chemical DNBP was ingested, milk samples were collected at the 1, 3, 10, 30, and 100 ppm levels. The milk samples were analyzed immediately for DNBP at all levels and for 2-ABNP at the 100 ppm level. After about 6 weeks the cream samples were analyzed for DNBP. In about 8 weeks aliquots from the same samples were analyzed for 2-ABNP. The average daily milk production for each cow was recorded throughout the test (Table I).

MATERIALS AND METHODS

Reagents. Mix 50 ml of 0.1 *M* potassium dihydrogen phosphate with 13.9 ml of 0.1 *M* sodium hydroxide. *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co., Inc., Milwaukee, Wis., *N,O*-bis(trimethylsilyl)acetamide, Pierce Chemical Co., Rockford, Ill., analytical grade 2-*sec*-butyl-4,6-dinitrophenol (DNBP), methyl ether of 2-*sec*-butyl-4,6-dinitrophenol (DNME), and 2-amino-6-*sec*-butyl-4-nitrophenol (2-ABNP) were used. All are obtainable from the Sampling Coordinator, Agricultural Department, The Dow Chemical Co., Midland, Mich.

DNBP Aqueous Solution. A 50-mg sample of DNBP is dissolved in 500 ml of diethyl ether to obtain a 100 μg/ml solution. For spiking, pipet an appropriate aliquot of the ether standard into a volumetric flask and evaporate the ether with a stream of air. Dilute to volume with water and mix well.

DNME in Benzene. (Gas chromatographic standard). A 52.9-mg sample of DNME is dissolved in 500 ml of benzene to give a concentration equivalent to 100 μg DNBP/ml. Appropriate volumetric dilutions of this solution are made with benzene to obtain standard solutions covering the concentration range from 0.01 to 0.05 μg DNBP equivalent/ml.

2-ABNP Ethereal Solution. A 50-mg sample of 2-ABNP is dissolved in 500 ml of diethyl ether to give a concentration of 100 μg/ml.

Gas Chromatography. Columns, 72 in. × 3 mm i.d., glass, U-shaped borosilicate packed with 5% DC200 on Gas-Chrom Z for DNBP, and 5% DC550 on Gas-Chrom Z for 2-ABNP. Column temperature, 185° C; injector block temperature, 230° C; detector bath temperature, 230° C; detector operating voltage, 10 V. Carrier gas, prepurified N_2 at 85

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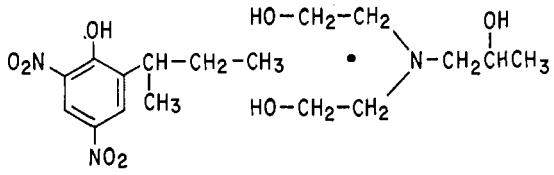

Figure 1. Ethanol and isopropanolamine salt of 2-*sec*-butyl-4,6-dinitrophenol

ml/min (40 psi inlet pressure), passed through a 4 Å molecular sieve. Recorder 0–5 mV. Electrometer sensitivity, 3.3 × 10^{-10} A. Chart speed, 20 in/hr. For 2-ABNP, column temperature is 211° C. Fill the needle of the 10-μl syringe with benzene, thereby eliminating all air bubbles from the syringe. Draw 4 μl of solution into the syringe and inject onto the chromatographic column for about 2 sec.

Preparation of Standard Curve. For DNBP inject 4-μl aliquots of DNME standard solutions, covering the range from 0.01 to 0.05 μg/ml DNBP equivalent, into the chromatograph and record the resulting peak heights. Plot the peak heights on the ordinate as percent full scale deflection and the corresponding concentrations of DNBP equivalent on the abscissa. For 2-ABNP pipet 1 ml of a 0.5 μg/ml benzene standard solution (obtained by diluting appropriate aliquots of the ethereal standard) into a 10-ml volumetric flask. Rinse the sides of the flask with about 0.5 ml of benzene. Add to the flask 75 μl of *N,O*-bis(trimethylsilyl)acetamide and react at room temperature for a minimum of 5 min. Dilute to volume of 10 ml with benzene. Use this as a standard of the trimethylsilyl derivative of 2-ABNP. Prepare other concentrations in the same way.

ANALYTICAL

DNBP in Milk. Pipet a 10-ml aliquot of raw milk into a Kimble No. 4, 4.25-in. tube. Add 0.2 ml of concentrated HCl to the tube, mix, and place in a 70° C oven for 1 hr. Cool, add 8 g of sodium chloride, and mix well. Add 20 ml of diethyl ether, cap, and shake the sample 15 min on a mechanical shaker. Centrifuge the sample for 2 min. Transfer a 10-ml aliquot of the ether to a 50-ml beaker. Add 0.5 ml of diazomethane (DeBoer and Backer, 1963). (CAUTION: **diazomethane is toxic and may be explosive under certain conditions.**) Cover with a watch glass and react at room temperature for 30 min. Evaporate the ether to just dryness and immediately add 1 ml of hexane to the beaker. During the ether evaporation do not overheat as this will cause a loss of the extremely volatile DNME. Prepare an anionic alumina column by placing 2.5 cm of anionic alumina in a 13 × 130 mm column. Add the hexane solution to the column. Rinse the column with 1 ml of hexane. Discard all eluate. Elute the column with 1 ml and then 5 ml of diethyl ether into a 50-ml beaker. Evaporate the ether to about 0.3 ml and immediately add about 1 ml of benzene. Transfer the benzene solution to a 5-ml volumetric flask and dilute to volume with benzene. Chromatograph 4 μl of this benzene solution as described under injection technique. Measure and record the resulting peak height. From the standard curve determine the concentration of the injected solution in μg per ml.

DNBP in Cream. Weigh a 10-g aliquot of cream into a 200-ml heavy-wall centrifuge tube. Add 1 ml of 5 *M* sulfuric acid solution and mix. Hydrolyze in an oven at 70° C for 1 hr. Cool and add 50 ml of diethyl ether. Cap the sample and shake 15 min. Centrifuge for 5 min. Pipet 5 ml of the ether phase into a beaker. Add diazomethane until yellow color persists. React at room temperature for 30 min. Evaporate the ether until residue becomes syrupy. Add 1 ml of hexane. Prepare an anionic alumina column topped with 1 cm of Na_2SO_4. Add the hexane solution to the column. Rinse the column with portions of hexane. Discard all eluate. Elute the compound into a volumetric flask with about 3 ml of ether. Dilute to 5 ml total volume with benzene. Chromatograph 4 μl of this benzene solution as described above for DNBP in milk.

2-ABNP in Milk. Pipet 10 ml of raw milk into a Kimble No. 4, 4.25-in. tube. Add 1 ml of a 5 *M* sulfuric acid solution. Hydrolyze in an oven at 70° C for 1 hr and cool. Partition with 20 ml and 7 ml of ether, shaking the sample 7 min each time. Combine ether phases and dilute to 25 ml with ether. Pipet 2.5 ml into a 10-ml volumetric flask. Add 75 μl of *N,O*-bis(trimethylsilyl)acetamide and react at room temperature for a minimum of 5 min. Dilute to 10 ml with benzene. Chromatograph 4 μl of this solution as described above for DNBP.

2-ABNP in Cream. Weigh 10 g of cream into a Kimble No. 4, 4.5-in. tube. Add 1 ml of 5 *M* sulfuric acid. Hydrolyze at 70° C for 15 min in an oven and cool. Add 10 ml of water. Adjust the pH to about 6.5 with concentrated ammonium hydroxide. Add 10 ml of buffer solution and 50 ml of benzene. Cap the sample and shake it 15 min. Centrifuge for 2 min. Pipet 2.5 ml of the benzene phase into a 5-ml volumetric flask. Rinse sides of flask with about 0.5 ml of benzene. Add to the flask 75 μl of *N,O*-bis(trimethylsilyl)-acetamide. React at room temperature for a minimum of 5 min. Dilute to 5 ml with benzene. Chromatograph 4 μl of this benzene solution as described above.

Table I. Milk Production and Weights

	Average Daily Milk Production (lb) Cow No.					
Week of	93[a]	89[a]	82[a]	78	49	32
8/23/68	33	35	41	38	39	35
9/23/68	35	35	43	42	39	35
10/23/68	30	36	43	41	37	27

Cow No.	Body Weight 8/23/68	11/5/68
93	955	1009
89	1012	1134
82	1089	1167
78	1052	1110
49	1098	1190
32	1089	1316

[a] Control.

VALIDATION OF METHODS

The efficiency of each method was determined by fortifying control milk and cream with known amounts of DNBP and 2-ABNP and applying the analytical procedures already described. Table II summarizes the results of these analyses. For DNBP, an average recovery of 80 and 98% for milk and cream, respectively, was found. For 2-ABNP an average recovery of 92% in milk and 92% in cream was found. A standard curve was used only in the analysis of DNBP in milk. All other data was quantified by injecting a standard before and after each sample and interpolating between the two points.

Table II. Recovery of DNBP and 2-ABNP from Milk and Cream Fortified with Known Amounts

ppm Added	No. of Determinations	Range		% Recovery
		DNBP		
Milk				
Blank[a]	14			
0.01	14	74–100		87
0.05	4	53–75		64
0.10	4	57–86.5		72
0.25	2	82–90		86
0.50	2	87–89		88
1.00	13	61–103		82
Cream			Average	80
Blank[a]	2			
0.05	3	100–110		103
0.10	3	90–105		98
0.50	3	90–98		94
			Average	98
		2-ABNP		
Milk				
Blank[a]	2			
0.10	3	90–103		98
0.25	3	88–100		93
0.50	3	82–88		86
			Average	92
Cream				
Blank[a]	1			
0.10	3	103–105		104
0.25	4	64–82		81
0.50	3	88–92		90
			Average	92

[a] Blank = 0.

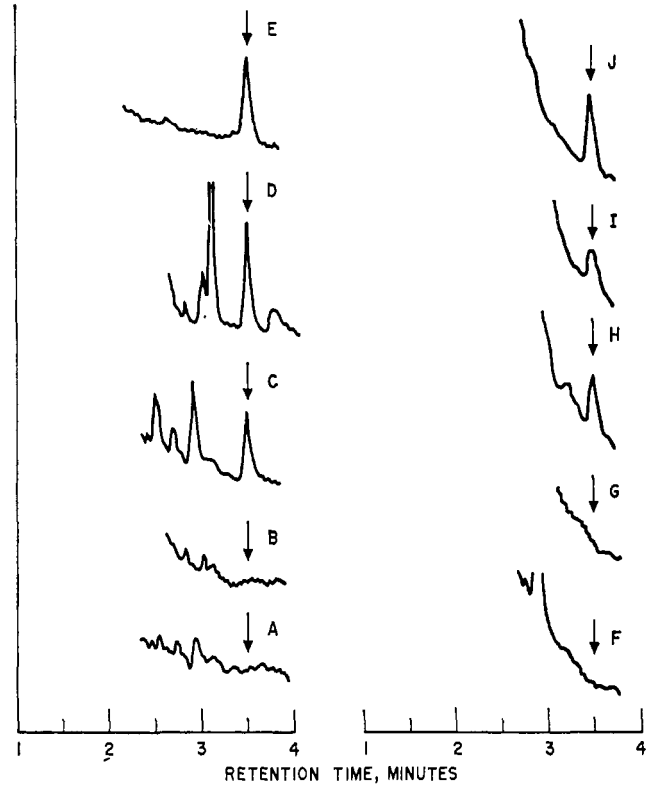

Figure 2. Typical chromatograms. A. Control milk. B. Control cream. C. Fortified milk, 0.01 ppm DNBP. D. Fortified cream, 0.05 ppm DNBP. E. DNBP standard, 0.01 μg/ml. F. Control milk. G. Control cream. H. Fortified milk, 0.1 ppm 2-ABNP. I. Fortified cream, 0.1 ppm 2-ABNP. J. 2-ABNP standard, 0.01 μg/ml

RESULTS

The data in Table I indicate that there were no ill effects due to DNBP as determined by milk production and body weight. There were no adverse effects in gross appearance or behavior.

No significant blanks occurred in any of the samples. In Figure 2 typical chromatograms of a milk control sample, a milk sample fortified with 0.01 ppm DNBP (0.01 ppm response = 10% peak height), and a 0.01 μg/ml DNBP equivalent standard are shown. Also shown in Figure 2 is a cream control sample and a cream sample fortified with 0.05 ppm DNBP. Again, in Figure 2, typical chromatograms of a milk and cream control sample and a milk and cream sample fortified with 0.1 ppm 2-ABNP are shown (0.1 ppm response = 10% peak height). No residues of DNBP were found in milk at any level or in cream at the 100 ppm feeding level. No residues of 2-ABNP were found in milk or cream at the 100 ppm level.

In the figures the peak heights are not directly comparable with each other, since response of the electron capture detector may change from one day to the next. They are, however, indicative of the magnitude of the measurement involved for a given level.

LITERATURE CITED

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