Burke, J. A.; Porter, M. L.; Young, S. J. V. *J. Assoc. Off. Anal. Chem.* **1971**, *54*, 142.
Dorough, H. W. In "Bound and Conjugated Pesticide Residues"; Kaufman, D. D.; Still, G. G.; Paulson, G. D.; Bandel, S. K., Eds.; American Chemical Society: Washington, DC, 1976; Chapter 2.
Lichtenstein, E. P.; Fuhreman, T. W.; Shulz, K. R.; Liang, T. T. *J. Agric. Food Chem.* **1973**, *66*, 863.
Saha, M.; Sumner, A. K.; Saha, J. G. *J. Assoc. Off. Anal. Chem.* **1973**, *56*, 45.
Sonobe, H.; Carver, R. A.; Krause, R. T.; Kamps, L. R., submitted for publication in *J. Agric. Food Chem.*, 1982.
Talekar, N. S.; Lee, E. M.; Sun, L. T. *J. Econ. Entomol.* **1977**, *70*, 685.
Walter, W. M., Jr.; Purcell, A. E. *Anal. Biochem.* **1966**, *16*, 466.
Watts, R. L. *J. Assoc. Off. Anal. Chem.* **1971**, *54*, 953.
Wheeler, W. B.; Thompson, N. P.; Andrade, P.; Krause, R. T. *J. Agric. Food Chem.* **1978**, *26*, 1333.
Wheeler, W. B.; Thompson, N. P.; Andrade, P.; Krause, R. T. *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 1286.
White, E. R.; Al-Adil, K. M.; Winterlin, W. L.; Kilgore, W. W. *Bull. Environ. Contam. Toxicol.* **1973**, *10*, 140.

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Determination of Chlorobutanol in Milk, Serum, and Tissues by a One-Step Cleanup and Gas–Liquid Chromatography

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A quantitative procedure for the determination of chlorobutanol in milk, serum, and tissues is described. A special distillation/extraction apparatus was designed to isolate the chlorobutanol in a single step. The chlorobutanol was quantitated on a 5-ft, 5% XE-60 column by electron capture detection with a 2-mCi ^{63}Ni source. The average percent recovery ± the standard deviation for chlorobutanol in milk over the 10–60-ppb range was 95.2 ± 2.7%. The average recovery for all tissues and serum at the 10-ppb level was 86.6 ± 3.8%. No interfering peaks were observed at the retention time of chlorobutanol. The analysis time for a single assay was 2 h.

Chlorobutanol is used as a preservative in products for the treatment of acute and chronic mastitis in dry cows. Since the route of administration is via the udder, an analytical method was needed to determine whether residual amounts of chlorobutanol were present in milk posttreatment and tissues upon slaughter. One published method for milk described a steam distillation technique (Wiskerchen and Weishaar, 1972). Because of the slow step with the Kurderna-Danish concentrator in their method, an all-glass distillation/extraction apparatus was designed, which carried out simultaneous steam distillation and transfer of chlorobutanol into a small volume of isooctane, therby eliminating the Kurderna-Danish concentrator.

The special steam distillation/extraction apparatus is a modification of the Bleidner-Heizler type described in the literature by Geissbuhler et al. (1971). However, several changes in design were necessary to ensure that the separation chamber remained cool throughout the distillation step. A temperature rise in the separation chamber of the Bleinder-Heizler design led to viscosity and density changes which resulted in phase boundary changes that were detrimental to a successful distillation/extraction.

This paper describes the design and use of the distillation/extraction apparatus for the assay of chlorobutanol in milk, serum, and tissues with a lower limit of detection of 10 ppb.

EXPERIMENTAL SECTION

Apparatus. The apparatus used were the following: special steam distillation/extraction apparatus (Figure 1); Waring Blendor, Model 702B, 1-L glass bowl refitted with gaskets cut from polyethylene; Beckman Zeromatic pH meter, Model SS-3; boiling chips, Fisher Scientific Co.; glass beads, No. 3000, 3 mm, chemically resistant, Fisher Scientific Co.; gas chromatograph, Hewlett-Packard, Model 402, equipped with a 2-mCi ^{63}Ni electron capture detector, a 5 ft × 3 mm i.d. glass column packed with 5% XE-60 on 60–80-mesh Diatoport S; Model 5A Kitchen Aid grinder.

Reagents. The reagents used were the following: chlorobutanol standard (1,1,1-trichloro-2-methyl-2-propanol, Matheson Coleman and Bell (mp 76–78 °C); silicotungstic acid, Fisher Scientific Co.; solvents, "distilled in glass" grade, Burdick & Jackson Laboratories; glassware cleaner, Orbit, Dubois Chemicals; silicotungstic acid solution (100 g/L).

Stock Standard Solutions. Chlorobutanol was prepared in isooctane, stock standard A (100 ppm) and stock standard B (1.0 ppm).

Fortification Standards. Aliquots of stock A (1.0, 2.0, and 3.0 mL) were pipetted into 100-mL volumetric flasks, and aliquots of stock A (2.0 and 3.0 mL) were pipetted into 50-mL volumetric flasks. All flasks were brought to volume with isooctane. This series of standards contained 1.0, 2.0, 3.0, 4.0, and 6.0 ppm of chlorobutanol, respectively.

GLC Standards. Aliquots of stock solution B (0.5, 1.0, 1.5, 2.0, and 3.0 mL) were pipetted into separate 100-mL volumetric flasks and brought to volume with isooctane. This series of GLC standards contained 5, 10, 15, 20, and 30 ng/mL and were equivalent to 10, 20, 30, 40, and 60 ppb of chlorobutanol, respectively, in a 50-g sample.

Sample Preparation. Fifty-milliliter aliquots of milk and serum from control and treated cows were placed in plastic containers and stored at −20 °C. The samples were

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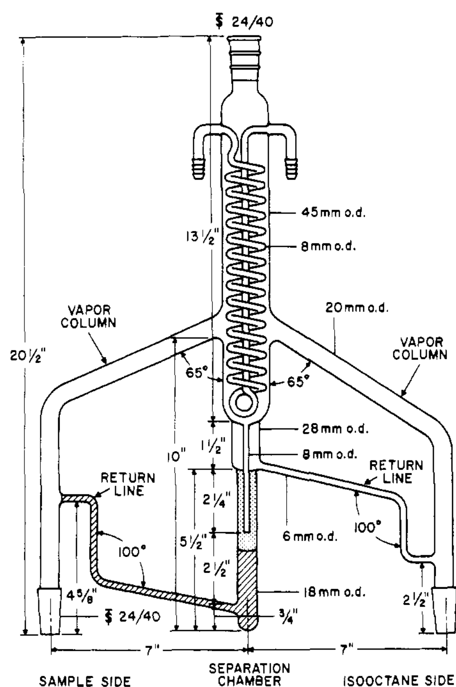

Figure 1. Dimensions (in millimeters and inches) of the distillation/extraction apparatus. The vapor columns were insulated with foam-rubber pipe insulation. The return lines should be positioned so that the phase boundary in the separating chamber is at the midpoint and below the delivery tube.

thawed to refrigerator temperatures (+4 °C) prior to assay.

Muscle, liver, kidney, fat, and udder (approximately 2 lbs) were ground twice through a 3-mm sieve plate attached to the grinder. The 50-g aliquots of control and treated tissues were stored in plastic containers at −20 °C. All tissues were thawed to +4 °C prior to assay.

Fortified samples were prepared by the addition of 0.5 mL of the appropriate fortifying standard to the *thawed control* milk, serum, and tissues.

Stability samples were prepared by pipetting 0.5 mL of the fortifying standard prior to freezing. Milk was fortified at the 0-, 10-, and 60-ppb level; tissues and serum were fortified at 0 and 10 ppb. For tissues a small depression was made with a test tube to contain the fortifying solution. The samples were placed in a hood for 30 min to evaporate the solvent prior to freezing.

In Vivo Fortified Milk. Samples were obtained from two lactating cows infused in each quarter with a penicillin–novobiocin oil suspenion [52.5 mg of chlorobutanol, anhydrous; 220 000 units of procaine penicillin; 440 mg of sodium novobiocin; 2% aluminum monostearate (peanut oil gel)]. Milk samples were collected every 12 h, aliquoted, and frozen until assayed.

Distillation/Extraction of Chlorobutanol. The thawed milk samples were transferred from the plastic containers to a 500-mL round-bottom flask. Then 40 mL of silicotungstic acid solution was added to the container, swirled to wash the sides, and transferred to the round-bottom flask. Three to four boiling chips and 100 mL of deionized water were added. Into a 250-mL round-bottom receiving flask, 50 mL of isooctane, 5 mL of toluene, and four to five glass beads were added (**caution:** use *glass* beads only). Both flasks were attached to the distillation/extraction apparatus (Figure 1) and then deionized water was added via the condenser until it siphoned into the sample flask. Next, isooctane was added until it flowed out the upper arm and into the isooctane receiver flask.

A 50-mL conical centrifuge tube containing approximately 25 mL of water was placed on top of the condenser. The condenser water and heating mantles were turned on. The heating mantles were controlled with variable transformers adjusted so that the isooctane pot began to boil first (65 units for the sample pot; 70 units for the isooctane receiver pot). The distillation was timed from the appearance of the first drops of distillate in the separation chamber and terminated after 45 min, but the condenser water was left flowing. The heating mantles were lowered and the isooctane receiver flask allowed to cool to room temperature. The condenser was rinsed with 15–20 mL of isooctane, and the apparatus was tilted to drain most of the isooctane (upper phase in the separating chamber) into the 250-mL round-bottom receiver flask. The isooctane was quantitatively transferred to a 100-mL volumetric flask and brought to volume with isooctane.

Serum samples were prepared for distillation in the same manner except that they were adjusted to a pH of 3.5 with concentrated sulfuric acid after the silicotungstic acid solution and water were added to the sample.

Musclě, liver, kidney, and udder were transferred to a Waring Blendor. The plastic containers were rinsed with 40 mL of silicotungstic acid solution and 100 mL of deionized water. Each rinse was added to the blender jar and the mixture blended at low speed for 3 min. The mixture was transferred to a 1-L round-bottom flask and the pH adjusted to 3.5 with concentrated sulfuric acid. then six to eight boiling chips were added, and the distillation/extraction was carried out as described for milk except that the distillation time was increased to 60 min.

Fat samples were placed into a 500-mL round-bottom flask with 40 mL of silicotungstic acid solution. The distillation/extraction procedure was the same except that the distillation time was increased to 75 min and the apparatus rinsed with isooctane before and after the cooling period.

The chlorobutanol in 100 mL of isooctane was quantitated by the injection of 5 μL onto an XE-60 column under the following conditions: carrier gas, helium (60–65 mL/min); purge gas, 90:10 argon–methane (80–85 mL/min); column temperature, 105 °C; detector temperature, 200 °C; chart speed, 0.25 in./min.

Calculations of the amount of chlorobutanol were based on the peak height response of known concentrations of pure standards in isooctane measured to the nearest 0.5 mm by using the base-line technique.

RESULTS AND DISCUSSION

Distillation/Extraction Apparatus. The apparatus was constructed from standard size Pyrex glass tubing (Figure 1). The dimensions for the attchment of the return lines from the separation chamber to the vapor column were critical. Some adjustment, by trial and error, was necessary to position the phase boundary at the midpoint or below the delivery tube or the separation chamber. However, a 1-cm change in the position of the side arm did not produce a 1-cm change in the phase boundary because of the density and viscosity differences of the two solvents.

Recovery Studies, in Vitro. Recovery in milk was based on the amount of chlorobutanol added to control milk (10, 20, 30, 40, and 60 ppb) carried out in triplicate (Table I). Calculations showed the average recovery over the entire fortification level was 95 ± 2.7% with a range of 90.9 −100%. No interfering peaks at the retention time of chlorobutanol were observed (Figure 2).

The recovery of chlorobutanol in tissues was determined at the 10-ppb level since preliminary data suggested no residues greater than 10 ppb were expected. Results showed overall recoveries were 86.6 ± 3.8% for the tissues

Table I. Percent Recovery of Chlorobutanol from Fortified Samples of Milk[a]

sample[b]	fortification level						
	0	10	20	30	40	60	av % ± SD[c]
milk 1	0	97.6	96.3	91.8	93.5	96.4	95.1 ± 2.4
milk 2	0	95.2	96.3	93.3	93.0	99.1	95.4 ± 2.5
milk 3	0	97.6	94.0	90.9	92.9	100.0	95.1 ± 3.7
av		96.8	95.5	92.0	93.1	98.5	95.2 ± 2.7

[a] Fortified by adding 0.5 mL of a 1.0, 2.0, 3.0, 4.0, and 6.0 ppm standard solution to 50 mL of milk. [b] Samples were assayed randomly. [c] Regression equation: $y = -0.894 \pm 0.983x$; $r = 0.9982$; $n = 15$ (zero excluded).

Table II. Percent Recovery of Chlorobutanol from Fortified Tissues at the 10-ppb Level[a]

sample	% found rep. 1	% found rep. 2	av %
muscle	93.2	84.1	88.6
liver	81.8	86.4	84.1
kidney	86.4	86.4	86.4
fat	90.5	95.2	92.8
udder	81.8	81.8	81.8
serum	85.7	85.7	85.7
av	86.6 ± 4.6	86.6 ± 4.6	86.6 ± 3.8

[a] Duplicate assays on separate weighed portions of tissues or fluid.

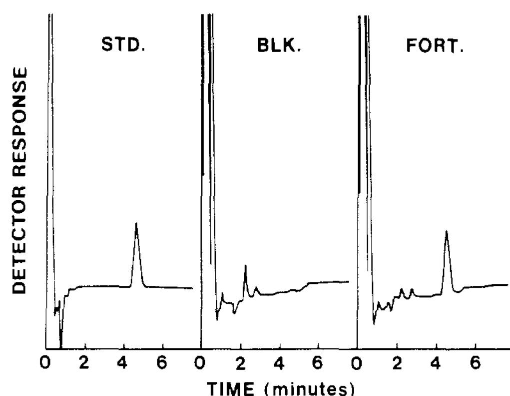

Figure 2. GLC–electron capture chromatograms of a standard, blank, and fortified milk sample chromatographed on a 5-ft, 5% XE-60 column; attenuation, 1 × 32 on an F & M 402 chromatograph; detector temperature, 200 °C, column temperature, 105 °C; standard, 25 pg of chlorobutanol; blank, 2.5 mg of control milk; fortified, 2.5 mg of milk fortified with 25 pg of chlorobutanol (10 ppb); sample size, 50; final volume, 100 mL; injection size, 5 μL.

and serum with a range of 81.8–95.2% (Table II).

Recovery Study, in Vivo. To provide milk samples with measurable levels of chlorobutanol, two lactating cows were infused with the penicillin–novobiocin suspension containing chlorobutanol and milk collection was started 12 h later, whereas under recommended treatment, cows are infused 30 days prior to their expected calving date, i.e., during their dry period. The results showed that under these conditions, levels of 10 ppb were present but decreased rapidly to <10 ppb in 60–96 h (Table III).

Method. For those who utilize this procedure for the first time, a solvent blank and a fortified solvent blank should be run to ensure that the procedure is free from contaminants and to demonstrate the level of recovery.

The phase boundary of the isooctane–water in the separation chamber should be at the midpoint. However, the accumulation of organic materials from the sample caused the height of the phase boundary to vary after approximately 10 distillation/extractions. This required that the apparatus be cleaned with the Orbit acid cleaning solution.

Table III. ppb Level for in Vivo Fortified Milk[a]

hours posttreatment	ppb found[b] cow A	ppb found[b] cow B
control[c]	0	0
control ± 10 ppb	10.0	
control ± 20 ppb		20.0
12	104.4	107.5
24	47.4	58.5
36	30.0	31.0
48	25.2	18.6
60	18.8	<10.0
72	16.0	<10.0
84	12.7	<10.0
96	<10.0	<10.0
108	<10.0	<10.0
120	<10.0	<10.0

[a] Fortification obtained by the treatment of a lactating cow. [b] Values below 20-mm peak response are reported as <10 ppb, i.e., the lowest limit of reliable detection. [c] Obtained from the same cow prior to treatment.

Table IV. Stability of Fortified Samples Stored at −20 °C

sample[a]	days stored	% found	% lost
fat	65	100	0.0
milk	117	101	0.0
serum	65	97	3.0
kidney	65	85	15.0
muscle	65	79	21.0
liver	65	65	35.0

[a] Average of milk aliquots fortified at 10 and 60 ppb. Tissues and serum fortified at 10 ppb.

A number of steps were taken to improve recovery during the developmental stage of the method: glass beads were substituted for boiling chips in the isooctane receiver flask because the boiling chips were found to absorb chlorobutanol from the solution; care was exercised when adding water to the apparatus to prevent it from getting into the isooctane receiver flask since the presence of water in the isooctane caused low recoveries. Although the additional steps were not fully investigated, it was found that recovery was improved by the addition of toluene to the receiver flask and the placement of a conical centrifuge tube containing water over the open end of the condenser.

Some lots of isooctane contained an interfering material with almost the same retention time as chlorobutanol. This interference was removed by passing the isooctane through a 19 × 380 mm column of Florisil (1250 °C activated grade, held at 130 °C). Approximately 15–20 L could be cleaned up on this size column.

Twenty minutes was allowed between injections on the gas chromatograph of serum, milk, and fat, and 30 min for all other tissues to permit peaks which occurred later in the chromatogram from interfering with the subsequent injection.

Because all tissues were assayed after several months of storage, the stability of chlorobutanol in frozen samples was determined. Results showed that the recovery of chlorobutanol decreased with storage time at −20 °C for serum, kidney, muscle, and liver while fat and milk showed no loss (Table IV). Samples were generally analyzed in our laboratory within 1–2 months.

Animal Study. No detectable residues of chlorobutanol (>10 ppb) were found in milk (colostrum), serum, and tissues of cows treated in all four quarters of the udder with

intramammary infusion of the penicillin–novobiocin oil suspension containing chlorobutanol during their prepartum nonlactating period approximately 30 days before her expected calving date.

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LITERATURE CITED

Geissbuhler, H.; Kossmann, K.; Baunok, I.; Boyd, V. F. *J. Agric. Food Chem.* **1971**, *19*, 365.

Wiskerchen, J. E.; Weishaar, J. *J. Assoc. Off. Anal. Chem.* **1972**, *55*, 948.

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Carotene and Chlorophyll Bleaching by Soybeans with and without Seed Lipoxygenase-1

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Carotene and chlorophyll bleaching activities of whole mature seed extracts of two soybean genotypes that lack lipoxygenase-1 (L-1) activity were compared with two soybean genotypes with normal L-1 activity. Assays were conducted with three substrates: (1) methyl linoleate, pH 7.0; (2) linoleic acid, pH 7.0; (3) linoleic acid, pH 9.0. No carotene or chlorophyll bleaching occurred with substrate 1. The two soybean genotypes without L-1 activity showed no conjugated diene formation with substrate 2 or 3 although carotene and chlorophyll were bleached with substrate 2. The presence of L-1 in seed extracts stimulated carotene and chlorophyll bleaching some at pH 7.0 and much at pH 9.0. The presence of L-1 stimulated chlorophyll cooxidation more than carotene cooxidation. L-1 purified from soybean seeds by ammonium sulfate fractionation and ion-exchange chromatography bleached both carotene and chlorophyll. However, the cooxidation of carotene and chlorophyll relative to the peroxidation of linoleic acid (cooxidation potential) is lower for purified L-1 than for whole mature seed extracts. The relevance of this information to food processing is discussed.

It has long been known that soybean seed lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) can cause coupled oxidation of carotene and unsaturated lipids (Sumner and Sumner, 1940). A number of investigators (Weber et al., 1974; Grosch et al., 1977) have presented evidence that soybean lipoxygenase type 2 [lipoxygenase isozymes 2 (L-2) and 3 (L-3) of Christopher et al. (1972)] are responsible for the coupled oxidation of carotene and that lipoxygenase type 1 (L-1) exhibits only slight carotene cooxidation activity. Ramadoss et al. (1978) demonstrated that purified soybean L-2 and L-3 in the presence of methyl linoleate or linoleic acid and oxygen were not effective catalysts of carotene bleaching. However, combinations of either L-1 and L-3 or L-2 and L-3 had a synergistic effect on carotene bleaching. Also, L-3 readily bleached carotene when L-1 or L-2 was replaced with the 13-hydroperoxide isomer from the peroxidation of linoleic acid.

The results of Grosch and Laskawy (1979) indicate that the lipoxygenase isozymes do not act synergistically in carotene oxidation. They also found that the 13-hydroperoxide generated from the action of L-1 and L-2 on linoleic acid effectively stimulated the oxidation of linoleic acid as well as the bleaching of polyenes such as carotene. Ikediobi and Snyder (1977) found that L-1 effectively cooxidized β-carotene while catalyzing the oxidation of linoleic acid.

Holden (1965) reported that lipoxygenase from soybean seeds also was involved in the bleaching of chlorophyll. However, another heat-labile factor involved in the destruction of the lipid hydroperoxide appeared to be necessary for chlorophyll bleaching.

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Additional studies confirmed that the lipid hydroperoxides resulting from lipoxygenase action on linoleic acid accelerate the bleaching of chlorophyll, but other enzymes which are possibly involved have yet to be identified (Imamura and Shimizu, 1974; Peiser and Yang, 1978).

Hildebrand and Hymowitz (1981) screened the U.S. Department of Agriculture soybean germ plasm collection for genotypes with greatly reduced or missing L-1, L-2, or L-3 activity. They found two soybean genotypes [Plant Introduction (P.I.) 133226 and P.I. 408251] that lacked detectable L-1 activity. The study reported herein was undertaken to determine if the lack of L-1 activty has an effect on the carotene and chlorophyll bleaching activities from seed extracts of these L-1 variants relative to "normal" soybeans and if purified L-1 is effective in carotene and chlorophyll bleaching.

EXPERIMENTAL SECTION

Materials and Reagents. Seeds analyzed were harvested from four soybean genotypes (P.I. 133226, P.I. 408251, cv. Amsoy 71, and cv. Williams) grown in the greenhouse in 1980 at Urbana, IL. Lysozyme, Folin reagent, β-carotene, chlorophyll *a*, Tween 20, Tween 80, linoleic acid, and methyl linoleate were obtained from Sigma Chemical Co. (St. Louis, MO). Chromatography equipment and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals (Piscatawa, NJ). All elutions were collected by an ISCO Model 328 fraction collector (Instrument Specialties Co., Lincoln, NE).

Carotene and Chlorophyll Solutions. Aqueous solutions of carotene and chlorophyll were prepared by using the method of Ben-Aziz et al. (1971) as modified by Reynolds (1981). The carotene solution was prepared by mixing 2 mg of β-carotene with 0.09 g of Tween 80 and dissolving in 2 mL of chloroform, which was then evaporated under a stream of nitrogen. The resulting residue

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