# Decontamination of Aflatoxin-Contaminated Peanut Meal Using Monomethylamine:$Ca(OH)_2$

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## ABSTRACT

Radiolabeled aflatoxin $B_1$ was added to naturally contaminated peanut meal, and the fate of aflatoxin-related by-products after decontamination by monomethylamine:$Ca(OH)_2$ was monitored. The decontamination process resulted in a 94-100% reduction in aflatoxin levels, depending on the level of contamination and the chemical structure of the aflatoxin. Following air drying and extractions with chloroform/water, methanol, acetic acid and water, the majority (69-86%) of the decontamination by-products remained with the residue. Significant amounts of aflatoxin $B_1$ (12%) and aflatoxin-related compounds (27%) were liberated from the residue after enzymic digestion. Isolation and identification studies revealed a wide variety of aflatoxin-related decontamination by-products. High performance liquid and thin layer chromatographic separation of chloroform-soluble compounds revealed compounds that exhibited some degree of toxicity. Comparative toxicity tests showed that, although some decontamination by-products exhibited elevated responses to specific toxicity tests, the relative toxicity was inferior to aflatoxin $B_1$. The moisture, protein and nitrogen levels did not change significantly. There was a significant change in the mold flora following the decontamination process, and the product was susceptible to recontamination.

## INTRODUCTION

Since the realization that mycotoxin contamination in foods and feeds can result from fungal contamination before harvest, as well as during harvesting and storage operations, interest in finding suitable methods for decontamination has increased. Unquestionably, prevention is the best method for controlling mycotoxin contamination; however, if contamination occurs, the hazard associated with the toxin must be removed if one hopes to use the product for food or feed. In general, decontamination techniques fall into 2 classifications: (a) removal—physical, chemical or biological, and (b) inactivation—physical or chemical (1). The decontamination technique most widely used by the food industry is physical removal, in which discolored, damaged or inadequately developed kernels or nuts are segregated from the apparently noncontaminated product. However, as mycotoxins diffuse away from the mycelia, physical removal may not effectively detoxify the material.

The absence of adequate decontamination procedures could lead to increased human exposure through direct consumption of the contaminated product or the feeding of contaminated feed to food-producing animals, as well as to a potential for economic loss and reduced interest in low-cost feed material (2). Physical separation techniques have been widely used, and recently, several chemical inactivation decontamination methods using ammonia have been studied and put to limited industrial use (1). A petition for the use of ammonia-detoxified cottonseed meal for feed for cattle and laying chickens is currently under review by the U.S. Food and Drug Administration. Research efforts designed to provide information on the safety aspects of the process are underway (D.L. Park, unpublished data; 3,4). Other methods with potential industrial application have been studied (5,6). Giddey et al. (7) reported on a pilot industrial application of the monomethylamine:$Ca(OH)_2$ (lime) method for decontaminating peanut meal at a rate of 10 MT/hr. This method has been put into limited industrial operation in Senegal. Feeding trials and toxicological studies are underway and preliminary results appear promising (8).

The objective of this study was to provide additional information on the safety and efficacy of the monomethylamine:$Ca(OH)_2$ decontamination process.

## EXPERIMENTAL PROCEDURES

### Preparation of Samples

Radiolabeled aflatoxin $B_1$ ($^{14}C$; 53 mCi/mmol sp act and >97% purity) was purchased from Moravek Biochemicals (City of Industry, CA). Radiopurity and sp act were confirmed by thin layer (TLC) and high performance liquid chromatographic (HPLC) methods. Radioactivity concentrations were determined by liquid scintillation spectrography and autoradiographic analysis. Peanut meal pellets, naturally contaminated with aflatoxin, were ground with a 140 Microjet 10 ultracentrifugal mill (Micro Materials Corp., Westbury, NY). Ground peanut meals with various aflatoxin concentrations were mixed to obtain 5-kg samples containing 50, 400, 800, 2,000 and 5,500 μg aflatoxin $B_1$/kg. Total aflatoxin concentration ($B_1$, $B_2$, $G_1$ and $G_2$) was determined for each sample. Radiolabeled aflatoxin $B_1$ in chloroform was added to each sample, and the chloroform was evaporated at ambient temperature in the dark. The final sp act was 1 μCi/kg.

### Aflatoxin Determination

Aflatoxin ($B_1$, $B_2$, $G_1$ and $G_2$) levels in samples before and after decontamination treatment were determined by Association of Official Analytical Chemists' (AOAC) method 26.026-26.031 (9), except that ether/methanol/water (96:3:1) was used as the developing solvent. The presence of aflatoxin $B_1$ was confirmed by derivatization with trifluoroacetic acid to aflatoxin $B_{2a}$ or by spraying the plate with sulfuric acid.

The HPLC method described by Pons and Franz (10) was also used for aflatoxin and aflatoxin-related decontamination by-product determinations except that chloroform/cyclohexane/acetonitrile (750:225:30) plus 2% ethanol was used as the solvent. Ultraviolet (UV) and fluorescent (equipped with a silica-gel-packed flow cell) detectors were used.

### Monomethylamine:$Ca(OH)_2$ Decontamination Process

Samples were subjected to the monomethylamine:$Ca(OH)_2$ process under pilot conditions. The decontamination process consisted of adding $Ca(OH)_2$ to the dry sample at a concentration of 2% of the sample weight and mixing for 5 min (Gabrader/Lodige paderden mixer). Monomethylamine, at a concentration of 0.5%, and water, necessary to adjust the meal to 24% moisture, were mixed together and subsequently mixed with the sample. The sample was then transferred to a mixer equipped with a steam jacket (Draiswerke) and mixed in a closed environment (0 psi) at 100 C for 1 hr. Following treatment, the samples were dried in a convection oven at 40 C to remove volatile compounds. Radioactivity concentrations in samples before and after decontamination treatment were determined.

### Toxicity Studies

The toxicological profile of isolated fractions before and after decontamination treatment was determined using bacterial (*Bacillus megaterium* [NRRL B1368] and *Bacillus subtilis* [Difco]) and animal (LF-spontaneous hepatoma) cell cultures, and chick embryo bioassay. *B. megaterium* was maintained on tryptone-glucose-yeast extract (TGY) agar supplemented with $K_2HPO_4$ and $KH_2PO_4$. The spore suspension was prepared by washing the spores with 3 mL sterile phosphate buffer and transferring to 300 mL sterile seed agar (1 g meat extract, 1 g NaCl and 17.5 g agar in 1,000 mL water) in a Roux flask. Each flask was incubated at 35 C until 99% sporulation was observed through microscopic examination (6-16 days). The spores were harvested by centrifugation at 7,000 rpm for 30 min and washed repeatedly with sterile phosphate buffer until the supernatant was clear. Vegetative cells were killed by heating at 70 C for 15 min. The final vol of the spore suspension was 30 mL.

Bacterial cell testing plates were prepared by adding 1 mL spore suspension to 99 mL TGY agar and mixing. Five mL of this mixture was added to each petri dish and allowed to harden on a horizontal surface. The dishes were prepared on the day they were to be used. Test samples were evaporated to dryness under nitrogen and dissolved in acetonitrile. Each sample, aflatoxin $B_1$ standard and negative control (acetonitrile) was applied to separate sterile filter discs and allowed to dry (ca. 15 min). The discs were inverted and placed on the agar, leaving at least 2-cm spacings. The plates were incubated at 37 C for 24 hr.

For the animal cell culture tests, the samples were evaporated to dryness under nitrogen and dissolved in dimethyl sulfoxide. Two $\mu$L of each test solution was added to 2.0 x $10^4$ LF-hepatoma cells (Falcon 3040 microplates) and incubated for 24 hr at 37 C in an atmosphere of air/$CO_2$ (95:5). Following incubation, 1 mCi [$^3$H] thymidine (TNM 199B, sp act 25 Ci/mmol [C.E.A. France]) was added to each well and incubated for 2 hr. The cultures were harvested on glass filters (Reeve Angel) using a multiple automated sample harvester and placed in Unisolve, and the radioactivity concentrations were determined. The results are expressed as percentage inhibition of thymidine incorporation compared to controls.

The chick embryo bioassay was performed as outlined in AOAC method 26.084-26.089 (9).

### Radioactivity Determination

All samples were counted using a liquid scintillation spectrometer (Intertechnique Plaisir, France) and conversion to disintegrations/min (dpm) was computed using appropriate internal standards and quench curves. Lypolima (Limac, Switzerland) was used as the scintillation fluid for all except aqueous samples, for which Aquasol (Limac, Switzerland) was used. For autoradiographic analysis, developed TLC plates were exposed against X-ray film (Kodak-Royalomatic). Following exposure, the emulsion was developed and fixed in X-ray film developer and fixer (Kodak).

### Protein and Moisture Determination

The Kjeldahl method (AOAC method 7.015, 10) for nitrogen was used for the determination of protein content (5.7 was used as the nitrogen/protein conversion factor). Moisture content was determined by gravimetric analysis.

### Reversibility Test

Decontaminated samples were slurried with water and the pH was adjusted to 2.0 with 1 N HCl. The samples were incubated at 37 C and the aflatoxin concentration was determined periodically.

### Mycological Examination

Czapek-Dox agar (Difco), containing penicillin (0.25 mg/

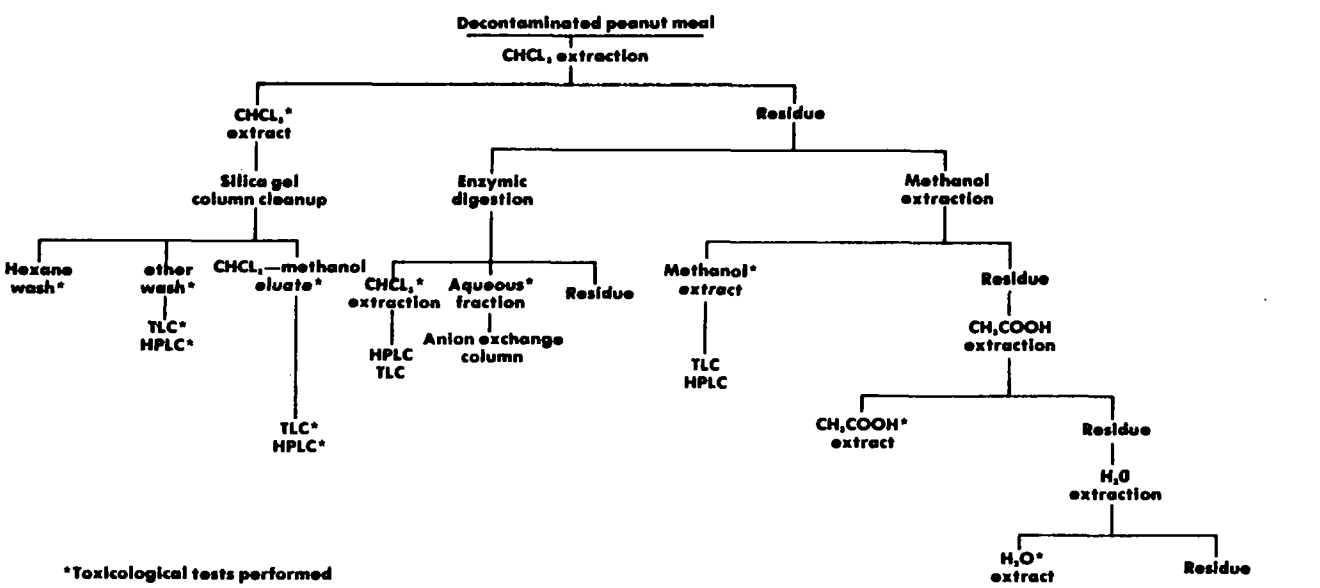


FIG. 1. Separation of aflatoxin-related decontamination by-products in defatted peanut meal following monomethylamine:$Ca(OH)_2$ treatment.

TABLE I

Concentration (μg/kg) of Aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ in Defatted Peanut Meal before and after Treatment with Monomethylamine:$Ca(OH)_2$

| Sample | Aflatoxin $B_1$ | | | | Aflatoxin $B_2$ | | | | Aflatoxin $G_1$ | | | | Aflatoxin $G_2$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Before[a] | After | | | Before | After | | | Before | After | | | Before | After | | |
| | | 46 days[b] | Acid extr.[c] | %[d] | | 46 days[b] | Acid extr.[c] | %[d] | | 46 days[b] | Acid extr.[c] | %[d] | | 46 days[b] | Acid extr.[c] | %[d] |
| 1 | 28 | 1[a] | tr | –[e] | 96 | 9 | 0[a] | tr | – | 100 | 11 | 0[a] | 0 | 100 | 4 | 0[a] | 0 | – | 100 |
| 2 | 400 | 11 | 12 | – | 97 | 90 | 4 | 4 | – | 96 | 50 | 0 | 0 | 100 | 20 | 0 | 0 | – | 100 |
| 3 | 825 | 50 | 46 | – | 94 | 186 | 15 | 15 | – | 92 | 100 | 0 | 0 | 100 | 50 | 0 | 0 | – | 100 |
| 4 | 2000 | 100 | 75 | – | 95 | 450 | 30 | 25 | – | 93 | 250 | 0 | 0 | 100 | 100 | 0 | 0 | – | 100 |
| 5 | 5500 | 310 | 244 | 266 | 94 | 1275 | 82 | 76 | 71 | 94 | 700 | 0 | 0 | 100 | 300 | 0 | 0 | 0 | 100 |

[a]Average of duplicate analyses, standard deviation (SD) ± 3%.
[b]Aflatoxin concentration in decontaminated peanut meal following 46 days' incubation at 21 C. Average of duplicate analyses, SD ± 3%.
[c]Aflatoxin concentration in decontaminated peanut meal following 5-hr incubation at 37 C at pH 2.0. Single determination.
[d]Percentage reduction in aflatoxin concentration following decontamination treatment.
[e]Not tested.

plate) and streptomycin (0.75 mg/plate) to suppress bacterial growth, was used to determine total mold counts for peanut meal samples before and after decontamination treatment.

### Separation of Decontamination By-Products

The procedures used for separating decontamination by-products are presented in Figure 1. Extraction solvents included chloroform/water, methanol, glacial acetic acid (pH 1.5-2.0) and water. Following each extraction, the residue was evaporated to dryness at room temperature to remove traces of the preceding solvent. The final extracts, except for aqueous extracts, were evaporated to dryness under nitrogen and stored in the dark at -10 C until analyzed. A silica gel column, described in AOAC method 26.030 (9), was used for fractionating the chloroform/water extract.

TLC of the various extracts was performed on 0.25-mm-thick, commercially prepared Silica Gel 25 HR plates containing gypsum (Macherey, Nagel & Co., Duren, West Germany). Developing solvents used included ether/methanol/water (96:3:1), chloroform/acetone (9:1) and toluene/ethyl acetate/formic acid (6:3:1). The distribution of the radioactivity on the developed TLC plates was determined by autoradiography and by scraping increments of the absorbent from the plate directly into scintillation vials and counting, using Lypolima (Limac, Switzerland) scintillation counting fluid. Preparative TLC plates were used for collection of isolated decontamination by-products from the chloroform/methanol eluate.

HPLC of the various extracts was performed as outlined by Pons and Franz (10), except that chloroform/cyclohexane/acetonitrile (750:225:30) plus 2% ethanol was used as the solvent.

### Enzymic Digestion

Treated and nontreated peanut meal samples were subjected to enzymic digestion before and after chloroform extraction. A 10-g sample was slurried with 100 mL water (pH adjusted to 7.0). Pronase (0.1 mg/mL, Calbiochem, San Diego, CA) was added to each sample and the solution incubated for 24 hr at 37 C with periodic shaking. The Pronase solution was predigested for 2 hr at 37 C in 25 mL Tris buffer solution (pH 7.0) prior to addition to the sample.

Following digestion, the samples were extracted with chloroform, and the aflatoxin and radioactivity concentrations were determined. The aqueous fractions of the control and experimental digestates were freeze-dried, reconstituted, applied to separate anion exchange columns and eluted stepwise with 0.0625, 0.125, 0.25, 0.5 and 1.0 M NaCl. The flow rate was maintained at 0.5 mL/min, and 10-mL fractions were collected. Each fraction was assayed for radioactivity. The column was prepared by slurrying 25 g Amberlyst A-26 anion exchange resin (Mallinckrodt Chemical Works) in distilled water and packing it in a 1.5 × 25 cm Pyrex chromatographic column. The column was back-washed with distilled water, using a slow upflow rate. The column was conditioned with 200 mL 1 N HCl at a flow rate of 0.5 mL/min. The column was then rinsed with distilled water until the eluate was neutral.

## RESULTS

The monomethylamine:$Ca(OH)_2$ decontamination process resulted in a significant reduction in aflatoxin levels (Table I). The levels did not change significantly following incubation of the peanut meal for 46 days at 21 C or treatment under acidic conditions (pH 2.0) for 5 hr at 37 C.

**TABLE II**

**Percentage of Added Radioactivity Found in [$^{14}$C] Aflatoxin Decontamination-Related By-Products in Defatted Peanut Meal Following Monomethylamine:Ca(OH)$_2$ Decontamination Treatment**

| Sample | Total[a] | $CHCl_3$ extraction[b] | | | | Methanol extraction[b] | $CH_3COOH$ extraction[c] | Water extraction[d] | Residue |
|---|---|---|---|---|---|---|---|---|---|
| | | Hexane wash | Ether wash | $CHCl_3$/methanol eluate | Total | | | | |
| 1 | 97.8 | 0 | 4.1 | 9.6 | 13.7 | 3.8 | 0.0 | 0.0 | 80.3 |
| 2 | 94.6 | 0 | 1.4 | 6.0 | 7.4 | 1.5 | 0.0 | 0.0 | 85.7 |
| 3 | 100.0 | 0 | 1.8 | 9.7 | 11.5 | 3.4 | 0.4 | 0.0 | 84.7 |
| 4 | 95.5 | 0 | 2.6 | 8.4 | 11.0 | 5.0 | 2.5 | 0.0 | 77.0 |
| 5 | 90.1 | 0 | 3.7 | 8.1 | 12.8 | 4.4 | 3.5 | 0.0 | 69.4 |

[a]Radioactivity remaining after initial air drying.
[b]Average of triplicate extractions, SD ± 0.5.
[c]Average of duplicate extractions, SD ± 0.5.
[d]Single determination.

**TABLE III**

**Percentage of Added Radioactivity Found in [$^{14}$C] Aflatoxin Decontamination-Related By-Products in Defatted Peanut Meal before and after Enzymic Digestion**

| Sample | Aflatoxin $B_1$ conc (μg/kg) | Chloroform extraction | After enzymic digestion | | |
|---|---|---|---|---|---|
| | | | Chloroform extraction | Aqueous fraction | Residue |
| Untreated[a] | 5500 | 75.6 | 3.2 | 19.9 | 1.3 |
| Treated[b] | 310 | 11.7 | 12.5[c] | 27.6 | 38.4 |

[a]Aflatoxin $B_1$ concentration, 5,500 μg/kg.
[b]Decontaminated using the monomethylamine:Ca(OH)$_2$ procedure; aflatoxin $B_1$ concentration = 310 μg/kg. 90.1% of the radioactivity added was detected in the peanut meal after the initial air drying.
[c]Aflatoxin $B_1$ concentration = 375 μg/kg.

Between 90 and 100% (Table II) of the radioactivity added to the peanut meal was detected in the peanut meal after the initial air drying. An additional 7-14% was extractable with chloroform. Subsequent extractions with methanol, acetic acid and water showed that the majority of the decontamination by-products remained with the residue (69-86%). Lee et al. (3) reported that 45-50% radioactivity remained with the meal residue following air drying and that 33% was chloroform-soluble in peanut meal following ammoniation. Enzymic digestion (Pronase) of the treated peanut meal increased the amount of chloroform-extractable compounds, including liberated aflatoxin (Table III). However, even following enzymic digestion, over 38% of the aflatoxin-related compounds remained with the residue; for the nontreated sample, 1% remained. A significant concentration of aflatoxin $B_1$ (375 ppb) was liberated by Pronase and over 27% of the radioactivity was found in the aqueous fraction. Lee et al. (3) reported that 4-6% of the radioactivity was associated with the protein portions of peanut meal following ammoniation. Almost 20% of the added radioactivity was found in the aqueous fraction of the nontreated sample. Anion exchange chromatography of the aqueous fraction from the treated sample showed a single major peak eluting with 0.0625 M NaCl.

There was a slight increase in the moisture content of the product following the decontamination process (Table IV). The change in protein and nitrogen levels was not significant.

Mold flora of the peanut meal changed significantly following the decontamination treatment (Table V). Our studies, although limited in scope, indicated recontamination by *A. flavus* and *Penicillium* species.

HPLC separation of aflatoxin-related by-products in the ether wash and the chloroform/methanol eluate is shown in Figures 2 and 3, respectively. Figure 4 shows the high performance liquid chromatogram for aflatoxins in peanut meal before treatment. Figure 5 shows the changes in thin layer chromatograms of the purified chloroform extract following the decontamination process; chloroform/acetone (9:1) was used as the developing solvent. Figure 6 shows the preparative thin layer chromatogram when ether/methanol/water (96:3:1) was used as the developing solvent. HPLC and TLC separation of aflatoxin-related decontamination by-products is presented in Tables VI and VII. With the exception of compounds in the chloroform/methanol eluate having $R_f$ values of 0.32, 0.50 and 0.81 (Fig. 5), all compounds had fluorescent properties. Compound(s) at $R_f$ 0.61 of the ether wash did not fluoresce. Using the same methodology as Lee et al. (3), the gray fluorescent spot at $R_f$ 0.3-0.4, aflatoxin $D_1$, reported by them was not appar-

**TABLE IV**

**Moisture, Protein and Nitrogen Content (%) of Peanut Meal before and after Decontamination Treatment with Monomethylamine:Ca(OH)$_2$**

| Sample | Moisture | | Protein[a] | | Nitrogen | |
|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After |
| 1 | 8.69 | 11.06 | – | – | – | – |
| 2 | 9.08 | 10.74 | – | – | – | – |
| 3 | 8.58 | 12.81 | 45.1 | 45.4 | 7.91 | 7.96 |
| 4 | 8.46 | 11.11 | – | – | – | – |
| 5 | 8.45 | 11.56 | – | – | – | – |

[a]5.7 used as nitrogen/protein conversion factor.

**TABLE V**

**Levels of Mold Contamination (Organisms/g) in Peanut Meal before and after Decontamination Treatment with Monomethylamine:$Ca(OH)_2$**

| Sample | Before treatment[a] | | | After treatment | | |
|---|---|---|---|---|---|---|
| | Total ($\times 10^3$) | *A. flavus* | *Penicillium* | Total ($\times 10^3$) | *A. flavus* | *Penicillium* |
| 1 | 0.92[b] | 0 | 59 | 0.39 | 109 | 92 |
| 2 | 8.1 | 31 | 0 | 0.45 | 50 | 75 |
| 3 | 3.0 | 26 | 0 | 4.0 | 281 | 352 |
| 4 | 1.5 | 0 | 0 | 0.63 | 150 | 36 |
| 5 | 0.52 | 39 | 236 | 0.31 | 79 | 39 |

[a]*Mucorales* showed overgrowth.
[b]Includes 496 *Cladosporium* and 17 *Fusarium*.

ent in the decontamination product; however, additional studies are necessary to fully evaluate the potential formation of aflatoxin $D_1$ during the monomethylamine:$Ca(OH)_2$ treatment.

The comparative toxicological properties of these fractions are presented in Tables VI and VII. For the chloroform/methanol eluate, the compounds with $R_f$ values of 0.81, 0.90 and 1.0 show greater than 25% inhibition. One fraction ($R_f$ 0.68) showed an inhibitory effect on *B. megaterium* (Fig. 7) with no effect on hepatoma cells. Four chloroform-soluble isolated compounds exhibited simultaneous positive responses to the 2 toxicity tests employed, *B. megaterium* and LF-hepatoma cells. Compounds exhibiting greater than 99% inhibition were subsequently diluted to determine the level of maximal inhibition. *B. subtilis* was positive only for compound(s) at $R_f$ 1.0 in the chloroform/methanol eluate. Two $\mu$g aflatoxin $B_1$ showed no inhibitory effect on *B. subtilis*. Two fractions ($R_f$ values 0.90 and 1.0) showing high inhibitory effects on either bacterial and/or LF-hepatoma cells had no effect on chick embryo development.

## DISCUSSION

For the products tested, the monomethylamine:$Ca(OH)_2$ decontamination process is effective in significantly reducing aflatoxin contamination levels which appear stable under ambient conditions.

Reversibility studies designed to simulate upper gastrointestinal tract conditions (pH 2.0 and 37 C) also reflect a permanency of the conversion by-products.

Determination of chloroform-extractable decontamination-related compounds showed a substantial shift to ether solubility, including detectable levels of aflatoxin in that

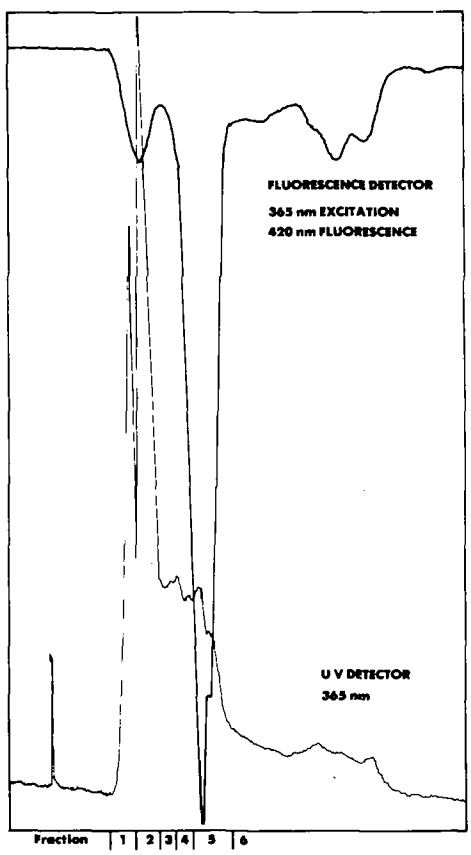


FIG. 2. High performance liquid chromatogram of ether wash fraction of silica gel column cleanup of chloroform extraction of decontaminated peanut meal (see Table VI).

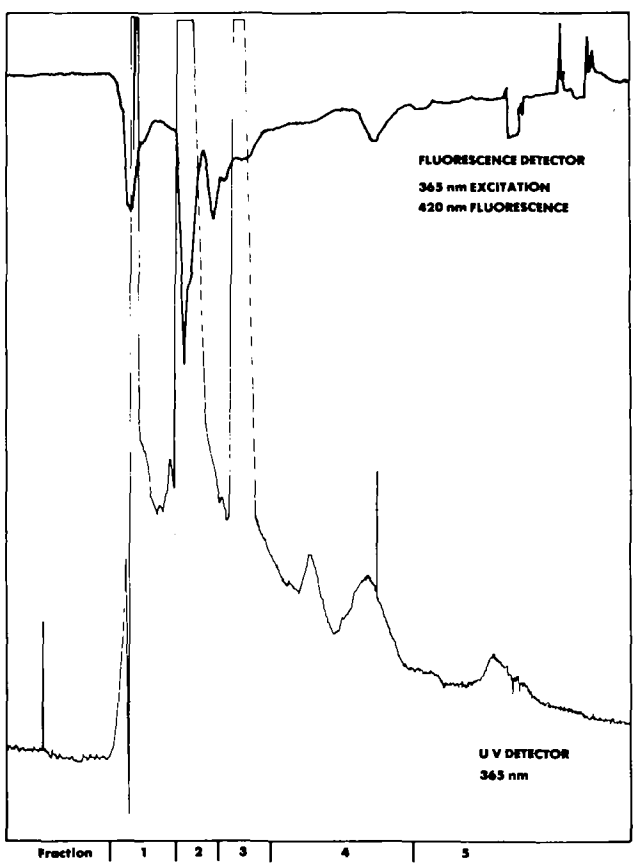


FIG. 3. High performance liquid chromatogram of purified chloroform extract of decontaminated peanut meal (see Table VI).

fraction (0.5% original aflatoxin concentration). Subsequent solvent extractions showed that aflatoxins have interacted with the matrix of the peanut meal, making it and/or decontamination by-products not readily extractable. Enzymic digestion studies further indicated the binding properties of the decontamination by-products (38% of the original aflatoxin concentration remained with the residue following chloroform extraction and enzymic digestion). Also, additional aflatoxin was extractable following enzymic digestion (375 ng/kg), indicating a potential for increased aflatoxin exposure during mammalian digestion of the decontaminated product. Additional studies are necessary to characterize the compound(s) associated with the aqueous fraction.

Our studies show that the decontamination process and peanut meal matrix may have an adverse effect on the efficacy of the analytical method in addition to the formation of aflatoxin-related decontamination by-products. Aflatoxin was detected in the ether wash fraction of the silica gel column clean-up of the chloroform extraction following the decontamination process. About 24% of the [$^{14}$C] aflatoxin was not extractable using the outlined test procedures; 83% of this was found in the aqueous fraction following enzymic digestion. A large number of aflatoxin-related decontamination by-products were formed as a result of the process. Many of these were chloroform-extractable (Tables VI and VII, Figs. 2, 3, 5 and 6). Relative radioactivity and by-product(s) concentrations indicate that several of these compounds should be studied further as possible decontamination process indicator compounds. TLC of the various HPLC fractions showed that both methods are sufficiently sensitive to detect these compounds. Additional studies are necessary to identify the structural characteristics.

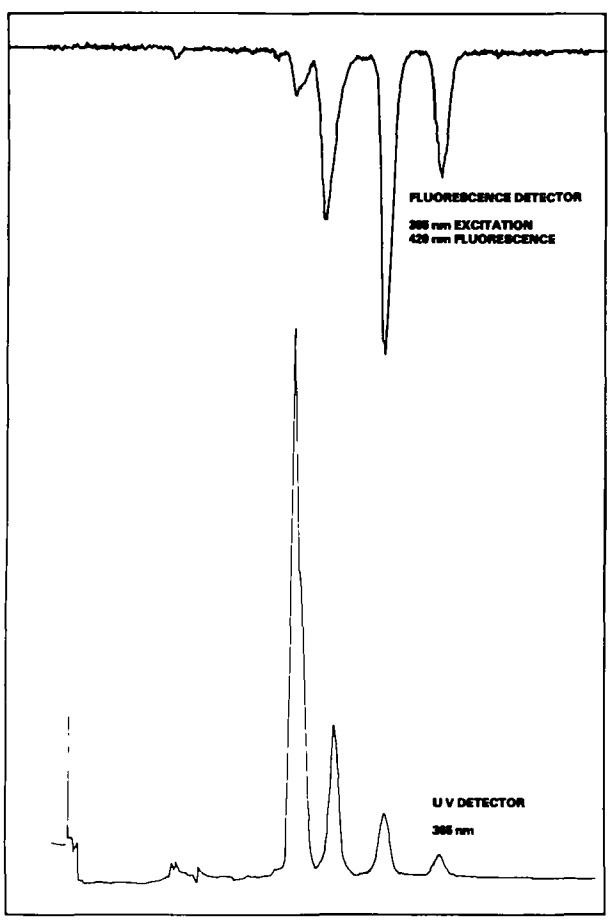


FIG. 4. High performance liquid chromatogram of aflatoxin-contaminated peanut meal.

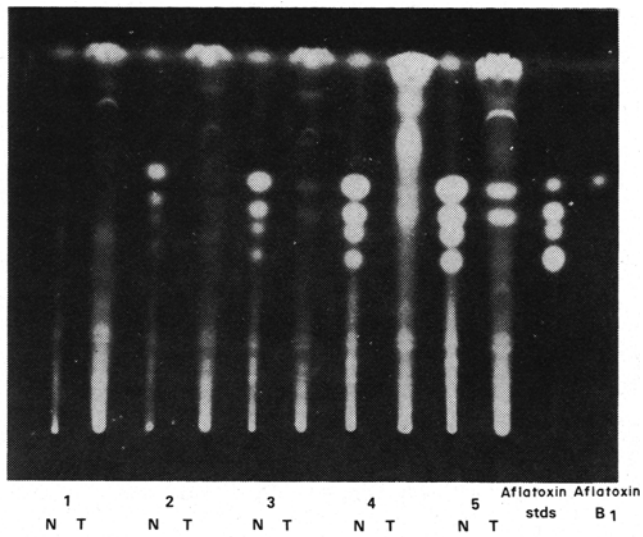


FIG. 5. Thin layer chromatogram of purified chloroform extract of peanut meal before and following decontamination treatment. Developing solvent was chloroform/acetone (9:1). (Samples 1-5; N = nontreated; T = treated; STD = aflatoxin standards $B_1$, $B_2$, $G_1$, $G_2$; $B_1$ = aflatoxin $B_1$).

UV (365 nm) and fluorescence (365 nm excitation and 420 nm fluorescence) detectors were used for HPLC. The 2-detector system proved to be of particular value when analyzing the decontaminated peanut meal for unreacted aflatoxin and decontamination-related by-products. One reaction by-product was originally thought to be unreacted aflatoxin based on the $R_f$ and UV detection responses; however, the magnitude of the fluorescent response showed that other compound(s) were involved.

Decontamination by-product concentration levels and comparative toxicological results of isolated compounds indicate that the process significantly reduced the acute toxicological risk posed by aflatoxin contamination. Animal cell, bacterial cell and whole animal test systems were used to evaluate isolated decontamination-related

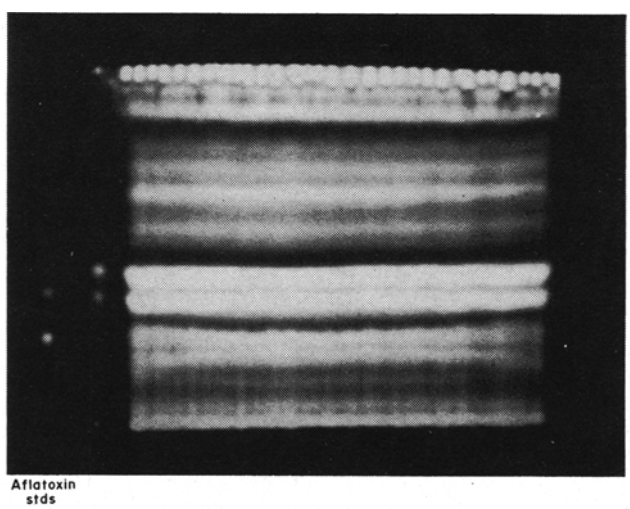


FIG. 6. Preparative thin layer chromatogram of purified chloroform extract of peanut meal following decontamination treatment. Developing solvent was ether/methanol/water (96:3:1) (see Table VII) (STD = aflatoxin standards $B_1$, $B_2$, $G_1$, $G_2$).

**TABLE VI**

**Relative Radioactivity and Decontamination By-Product Concentrations and Comparative Toxicological Properties of Fractions Separated by High Performance Liquid Chromatography**

| Peak | Relative concentration | | Comparative toxicity | |
|---|---|---|---|---|
| | Radioactivity | By-product (ng)[a] | LF-Hepatoma cells[b] | *B. megaterium*[c] |
| Ether wash (Fig. 2) | | | | |
| 1 | 4.3 | 10 | 25 | Neg. |
| 2 | 27.4 | 62 | 14 | Neg. |
| 3 | 28.3 | 64 | 37 | Neg. |
| 4[d] | 1.3 | 3 | 2 | Neg. |
| 5 | 4.8 | 11 | 67.5 | Neg. |
| 6 | 33.9 | 76 | 37.5 | Neg. |
| Chloroform/methanol eluate (Fig. 3) | | | | |
| 1 | 5.8 | 9 | 0 | Neg. |
| 2[d] | 16.9 | 310[e] | 19.5 | Neg. |
| 3 | 8.9 | 15 | 9.5 | Neg. |
| 4 | 25.6 | 42 | 59 | Neg. |
| 5 | 42.8 | 70 | 48 | Pos.+ |

[a]Calculations based on equivalent [$^{14}$C] aflatoxin radioactivity concentrations added to peanut meal before decontamination treatment (1 μCi [$^{14}$C] aflatoxin/kg).

[b]Percentage inhibition at concentrations equivalent to 0.032 and 0.017 g peanut meal for chloroform/methanol eluate and ether wash, respectively.

[c]Equivalent to 1.6 and 0.85 g peanut meal for chloroform/methanol eluate and ether wash, respectively.

[d]Aflatoxin $B_1$ present.

[e]Confirmed by TLC.

**TABLE VII**

**Relative Radioactivity and Decontamination By-Product Concentrations and Comparative Toxicological Properties of Fractions Separated by Thin Layer Chromatography**

| Fraction ($R_f$) | Relative concentration | | Comparative toxicity | | Chick embryo |
|---|---|---|---|---|---|
| | Radioactivity (%) | By-product (ng)[a] | LF-Hepatoma cells[b] | *B. megaterium*[c] | |
| Ether wash | | | | | |
| 0.05 | 2.5 | 8 | 49 | Pos.+ | –[d] |
| 0.19 | 0.0 | 0 | –[d] | Neg. | – |
| 0.28 | 0.0 | 0 | – | Neg. | – |
| 0.61 | 3.4 | 9 | 0 | Neg. | – |
| 0.69 | 2.5 | 8 | 27 | Neg. | – |
| 0.77 | 0.8 | 2 | 68.5 | Pos.± | – |
| 0.85[e] | 21.0 | 53 | – | Neg. | – |
| 0.89 | 24.4 | 61 | 94 | Neg. | – |
| 0.93 | 21.8 | 55 | 46 | Neg. | – |
| 1.0 | 23.5 | 59 | >99[f] | Pos.++ | – |
| Chloroform/methanol eluate | | | | | |
| 0.03 | 1.8 | 13 | – | Neg. | – |
| 0.08 | 1.1 | 8 | – | Neg. | – |
| 0.14 | 1.8 | 13 | – | Neg. | – |
| 0.21 | 1.8 | 13 | – | Neg. | – |
| 0.29 | 1.8 | 13 | – | Neg. | – |
| 0.32 | 2.2 | 15 | 6.5 | Neg. | – |
| 0.38 | 1.8 | 13 | – | Neg. | – |
| 0.46[e] | 35.1 | 245 | 6 | Neg. | – |
| 0.50 | 2.9 | 20 | 17.5 | Neg. | – |
| 0.60 | 1.8 | 13 | – | Neg. | – |
| 0.63 | 0.0 | 0 | – | Neg. | – |
| 0.68 | 5.4 | 38 | 0 | Pos.+ | – |
| 0.74 | 14.6 | 103 | 17 | Neg. | – |
| 0.81 | 20.0 | 140 | 27 | Neg. | – |
| 0.90 | 3.6 | 25 | 71.5 | Neg. | Neg.[g] |
| 0.94 | 1.7 | 10 | – | Neg. | – |
| 1.0 | 2.9 | 20 | >99[h] | Pos.++ | Neg.[i] |

[a]Calculations based on equivalent [$^{14}$C] aflatoxin radioactivity concentrations added to peanut meal before decontamination treatment (1 μCi [$^{14}$C] aflatoxin/kg).

[b]Percentage inhibition at concentration equivalent to 0.3 g peanut meal.

[c]Equivalent to 15 g peanut meal.

[d]Not tested.

[e]Aflatoxin $B_1$ present.

[f]73% inhibition at concentration of 0.06 g peanut meal.

[g]Equivalent to 7.35 g peanut meal.

[h]56.5% inhibition at concentration of 0.06 g peanut meal.

[i]Equivalent to 3.68 g peanut meal.

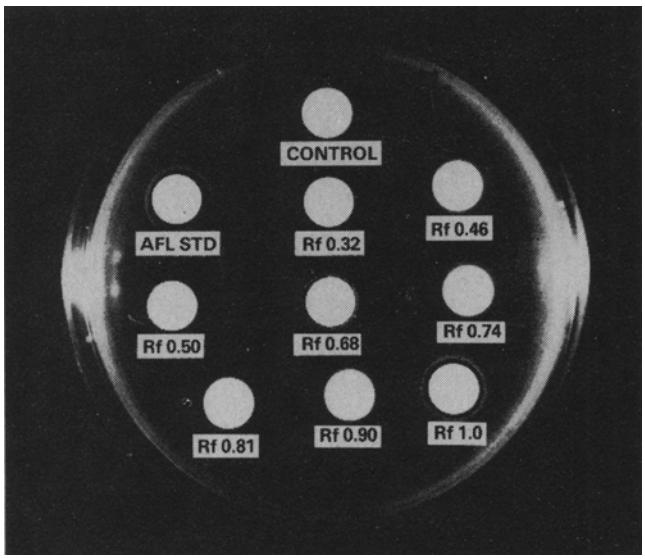


**FIG. 7. Inhibitory effect of selected isolated decontamination by-products and aflatoxin $B_1$ on *B. megaterium* (see Table VII) (negative control—acetonitrile; aflatoxin $B_1$ standard 0.3 ng; $R_f$ values of tested isolated component indicated for each disc).**

by-products. Some compound(s) showed toxic responses to one test but were negative in others, emphasizing the need for battery testing when performing safety evaluations. Also, the level of sensitivity varied significantly with the test system: 5 ng, 1 μg and 50 ng for the animal cell, bacterial cell and chick embryo test systems, respectively. Compared to aflatoxin $B_1$, the relative concentrations and toxicities of the isolated decontamination-related compounds are inferior to unchanged aflatoxin remaining after the decontamination process by several orders of magnitude. Although some decontamination by-products may exhibit elevated responses to specific toxicity tests, the process significantly reduces the risk associated with the original contamination.

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# Isolation and Purification of Deoxynivalenol and a New Trichothecene by High Pressure Liquid Chromatography

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## ABSTRACT

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothec-9-ene-8-one) was extracted from corn with methanol/water (80:20, v/v) and purified by liquid:liquid partitioning and by preparative high pressure liquid chromatography (HPLC). This procedure was used to prepare mg quantities of toxin from field-inoculated corn for reference standards. Analysis of the isolated deoxynivalenol by analytical HPLC, gas liquid chromatography (GLC) and gas liquid chromatography/mass spectroscopy (GLC/MS) indicated the presence of a second compound similar to deoxynivalenol. This compound comigrates with deoxynivalenol on thin layer chromatography plates in chloroform/methanol (90:10, v/v), but can be separated by HPLC on a reverse-phase $C_8$ column with methanol/water (10:90, v/v). GC/MS of the compound and the trimethylsilyl ether derivative gave parent ions of m/e 280 and 424, respectively. These data and NMR data indicate that the compound is 3,15-dihydroxy-12,13-epoxytrichothec-9-ene-8-one, a previously unreported trichothecene.

## INTRODUCTION

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothec-9-ene-8-one), also known as vomitoxin (1), is an important mycotoxin produced by certain species of Fusaria that invade grains in the field and in storage. This toxin is one of 4 known naturally occurring trichothecenes (the others are T-2, diacetoxyscirpenol and nivalenol) which singly or in combination are implicated as being responsible for animal disorders known as fusariotoxicoses (2-4). Recent documentation on the frequency of occurrence of deoxynivalenol (5,6) suggests that this toxin and other trichothecenes may cause greater health problems than aflatoxin in animals, especially in the midwest Corn Belt. Analytical methodology for trichothecenes is difficult due to the physicochemical properties of these toxins. Extensive clean-