Analysis of Aflatoxins (B_1, B_2, G_1, and G_2) in Rodent Feed by HPLC Using Postcolumn Derivatization and Fluorescence Detection

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Aflatoxins are cancer-causing toxins that are produced by the molds *Aspergillus flavus* and *Aspergillus parasiticus* when the ideal environmental conditions such as temperature and humidity are met. An existing method was modified for application to the analysis of aflatoxins in vitamin-fortified rodent feeds. The aflatoxins were extracted from the feeds with 70% methanol/water followed by cleanup on an affinity column. The aflatoxins were eluted from the affinity column with methanol and quantitated via HPLC using postcolumn derivatization with iodine followed by fluorescence detection. The minimum detectable limit was 0.25 ppb for B_1, B_2, and G_1 and 0.12 ppb for G_2. Recoveries for B_1 and G_1 averaged 85% over a concentration range of 0.5–50 ppb. Recoveries for B_2 averaged 77% over the same range, while recoveries for G_2 averaged 58% over a concentration range of 0.25–25 ppb. The method was also successfully used for analysis of aflatoxins in animal cage bedding material.

INTRODUCTION

Aflatoxins are secondary toxic metabolites produced by the molds *Aspergillus flavus* and *Aspergillus parasiticus*. These molds grow on corn, peanuts, milo, rice, and many other grains and nuts under appropriate conditions. The well-known aflatoxins B_1, B_2, G_1, and G_2 may be produced during growth, harvest, and storage of these crops. It is also known that aflatoxins, especially aflatoxin B_1, are both toxic and carcinogenic to many species of animals including man (Busby and Wogan, 1984).

For research purposes, the National Center for Toxicological Research (NCTR) maintains a colony of rodents. An ultrasensitive procedure for monitoring aflatoxins in rodent feed was needed when published reports indicated that as little as 2 ppb of aflatoxin exposure in the diet over the lifetime of a rat causes liver tumors (Busby and Wogan, 1984).

A review of the literature for available analytical procedures for analysis of aflatoxins in a variety of substrates revealed numerous methods.

Shepherd and Gilbert (1984) determined the optimum conditions needed for HPLC postcolumn iodination of an aflatoxin B_1 standard. Thiel et al. (1986) used postcolumn derivatization (PCD) with iodine after cleanup using CB procedure (AOAC, 1975) to determine aflatoxin B_1 in corn and peanuts with some success. Beaver (1989) reviewed various HPLC techniques for determining aflatoxins in corn and peanuts and concluded that more efficient cleanup procedures would have to be developed to substantially lower the detection limit below 1 ppb.

Goto et al. (1979) experimented with a cleanup column for aflatoxins prepared with alumina, silica gel, and sodium sulfate with limited success.

Tuinstra and Haasnoot (1983) used PCD with iodine to analyze aflatoxin B_1 in feedstuff. The cleanup was done with TLC after solvent extraction prior to HPLC. Using TLC to clean up extracts was very labor intensive and gave erratic accuracy and reproducibility.

Kok et al. (1986) determined aflatoxins in cattle feed using HPLC and PCD with electrochemically generated bromine prior to fluorescence detection. TLC was, however, the method used to clean up the extract prior to HPLC.

Traag et al. (1987) used PCD with iodine to determine aflatoxin B_1 in feedstuff. Both TLC and C_{18} Sep-Paks (Waters) were used to purify extracts. Injections of 250 μL or more into the HPLC were needed to attain a sensitivity of 20 ppb.

Groopman and Donahue (1988) determined aflatoxins in corn, peanut butter, and milk via HPLC using PCD with iodine after monoclonal antibody affinity column cleanup. Mortimer et al. (1987) also used affinity column cleanup in determining aflatoxin M_1 in powdered milk samples.

Chamkasem et al. (1989) used HPLC to analyze for aflatoxins, ochratoxin A, and zearalenone in different substrates by PCD with iodine utilizing an on-line sample cleanup which gave a sensitivity of 5 ppb with injections of 300 μL. Chamkasem et al. (1989) concluded that since finished feeds contain a variety of ingredients, the C_{18} precolumn cleanup was not entirely effective in removing interferences from the mycotoxins, especially aflatoxins.

Since none of these reported methods was entirely adequate for our application, we subsequently modified the method of Groopman and Donahue (1988) to enable analysis of aflatoxins in rodent feed. Our modification and its application are the subject of this paper.

EXPERIMENTAL PROCEDURES

Apparatus. The HPLC system consisted of two pumps (a Waters Model M 6000A and an Altex Model 100A) and a 250 × 4.6 mm 5-μm Supelco reversed-phase C_{18} column. A guard column with a 2-μm frit was placed between the injector (Rheodyne Model 7125 with a 50-μL loop) and the column. Saturated aqueous iodine was introduced postcolumn into a reactor coil via a low volume T and detection was achieved by utilizing a Shimadzu Model RF-535 fluorescence detector. A Shimadzu Model C-R3A integrator was also used.

The reactor coil was 5000 × 0.3 mm i.d. stainless tubing placed in a liquid chromatograph column oven where the temperature was regulated to within ±0.1 °C. A schematic of the system is shown in Figure 1.

Reagents. The iodine reagent was prepared by placing 1 g of iodine in 500 mL of deionized water (Millipore 18 megohm)

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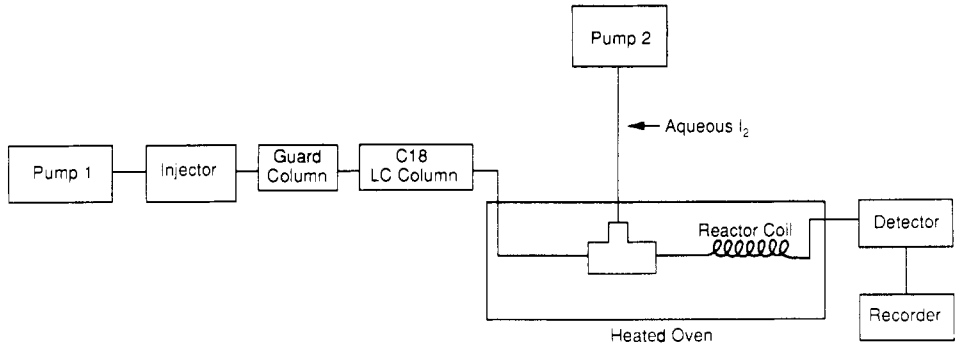

Figure 1. Schematic of HPLC system.

followed by shaking on a mechanical shaker for 2 h in a bottle wrapped in aluminum foil. The reagent was then filtered through a 0.45-μm filter prior to HPLC use.

The aflatoxin standards (B_1, B_2, G_1, and G_2) (Applied Science, Deerfield, IL 60015) were used as received. Stock standards of each aflatoxin were prepared at 0.01 mg/mL in methanol and stored in amber volumetric flasks in a refrigerator. Composited spiking standards were prepared daily to contain 100 ng/mL B_1, B_2, and G_1 and 50 ng/mL G_2 in methanol/water (50/50).

Aflatoxins are toxic and possibly carcinogenic and should be handled by using safety percautions listed in AOAC (1984). These safety guidelines indicate that mycotoxins, which includes aflatoxins, should only be handled with gloves and used only in properly ventilated hoods or gloveboxes.

The monoclonal antibody affinity columns (Aflatest P) used for cleanup of feed sample extracts were obtained from Vicam (Somerville, MA 02145).

The rodent feed (NIH-31 plus vitamins Type 5022-4) was obtained from Ralston Purina Co. (St. Louis, MO 63166) (see Table II).

Extraction of Vitamin-Fortified Rodent Feed. A 50-g feed sample and 5 g of sodium chloride were weighed into a 500-mL blender cup. One hundred forty milliliters of methanol/water (70/30) was added, and the mixture was blended at high speed for 2 min. The extract was filtered (Whatman No. 4), and 12 mL of the extract was diluted to 42 mL by adding 30 mL of deionized water (Millipore 18 megohm) to obtain a final methanol concentration of 20%. The diluted extract containing a 1 g-equiv of feed was passed through the affinity column to collect the aflatoxins. After the affinity column was washed one time with 10 mL of deionized water, the aflatoxins were washed off the column with 1 mL of methanol into a 10-mL graduated centrifuge tube. The methanol extract was evaporated to 0.5 mL under a stream of nitrogen and diluted to 1 mL with deionized water for injection into the HPLC.

HPLC and Postcolumn Derivatization. The mobile phase was water/methanol/acetonitrile (50/40/10) with a flow rate of 0.8 mL/min. The column was reverse-phase C_{18} having a 5-μm particle size and a length of 25 cm. A fluorescence detector was used with the excitation and emission wavelengths set at 365 and 440 nm, respectively.

Postcolumn derivatization was accomplished with a second pump introducing saturated aqueous iodine solution at a flow rate of 0.5 mL/min via a low dead volume T into a 5000 × 0.3 mm i.d. reactor coil located in a heated LC column oven to keep the coil temperature at 68 °C.

Recovery Experiments. Triplicate 50-g feed samples were spiked with 0.5, 5, and 50 ppb of B_1, B_2, and G_1 and 0.25, 2.5, and 25 ppb of G_2 by adding appropriate volumes of spiking standards to respective 50-g feed samples to give the desired concentrations. The feed samples were then taken through the analytical procedure.

RESULTS AND DISCUSSION

As indicated in the literature, the techniques indicating the most potential for success for the analysis of the aflatoxins were a monoclonal antibody affinity column for cleanup of the sample extract, reversed-phase HPLC, postcolumn derivatization, and fluorescence detection for quantitation. This combination was reported for aflatoxin analysis of corn, peanut butter, and milk by Groopman and Donahue (1988). After evaluating their procedures for our particular application—vitamin fortified rodent feed—we found that modifications would have to be made to their method to enable ultrasensitive analysis of vitamin-fortified rodent feed for aflatoxins B_1, B_2, G_1, and G_2. The modifications consisted of the following: (1) The volume of the extracting solvent was changed from 100 to 140 mL to achieve enough volume to thoroughly wet the fortified feed and to provide sufficient volume for filtration and subsequent workup. (2) The methanol concentration change of methanol/water (80/20) to 70/30 required less dilution, thus allowing application of approximately 1 g-equiv of feed to the affinity cleanup column in a minimum volume of about 10 mL or less, which aided in preventing aflatoxins, mainly G_2, from washing off the column. (3) The affinity column rinse volume was changed from two 10-mL rinses to only one 10-mL rinse. This change improved recoveries of G_2 from about 50% up to about 60% at all spike levels. (4) The HPLC mobile phase was changed from methanol/water (50/50) to water/methanol/acetonitrile (50/40/10) to achieve baseline separation of G_1 and G_2, which did not occur with methanol/water (50/50). (5) The mobile phase flow rate was changed from 1.0 to 0.8 mL/min, which improved resolution of all four aflatoxins in the rodent feed extracts.

Table I. Analysis of Vitamin-Fortified Feed Spiked at Various Levels with Aflatoxins

		recovered, $\bar{x} \pm SD^a$	
aflatoxin	ng/g added	ng/g (ppb)	%
B_1	0.5	0.42 ± 0.05	83 ■ 10
B_2	0.5	0.35 ± 0.02	69 ± 3.0
G_1	0.5	0.38 ± 0.03	75 ± 5.0
G_2	0.25	0.14 ± 0.01	54 ± 2.0
B_1	5.0	4.4 ± 0.10	88 ± 2.8
B_2	5.0	3.9 ± 0.10	78 ± 2.0
G_1	5.0	4.6 ± 0.10	92 ± 2.0
G_2	2.5	1.4 ± 0.06	55 ± 2.3
B_1[b]	50.0	41.5 ± 0.95	83 ± 2.0
B_2	50.0	41.6 ± 1.97	83 ± 3.8
G_1	50.0	43.3 ± 0.84	87 ± 1.2
G_2	25.0	16.4 ± 0.85	65 ± 3.0

[a] Mean and standard deviation from triplicate assays. [b] A half gram-equivalent of extract was passed through affinity column since total aflatoxins was more than 100 ng.

Recovery values for B_1 and G_1 each averaged 85%, while those for B_2 and G_2 averaged 77 and 58%, respectively, for all three spike levels. Recovery data are shown in Table I. The derivatization and the detector response were linear over the working concentration range 0.03–1 ng injected for B_1, B_2, and G_1 and 0.03–0.5 ng injected for G_2.

The affinity column cleanup of the feed extracts worked very well. The antibodies in the affinity column were

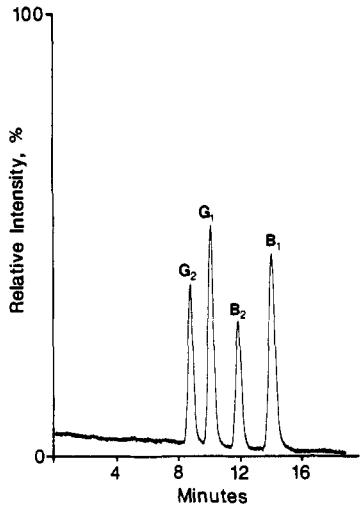

Figure 2. HPLC chromatogram of an aflatoxin standard containing 5 ppb of B_1, B_2, and G_1 and 2.5 ppb of G_2.

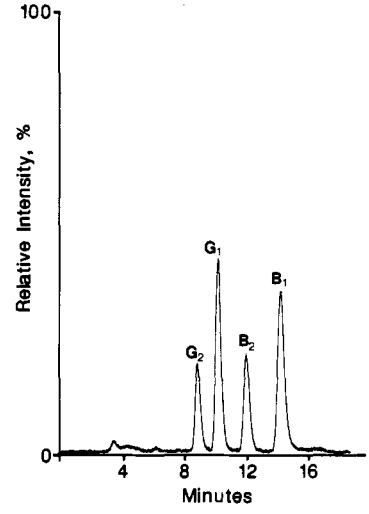

Figure 3. HPLC chromatogram of feed extract from feed spiked with 5 ppb of B_1, B_2, and G_1 and 2.5 ppb of G_2.

Table II. NIH-31 Rat and Mouse Ration

ingredients	% by wt
fish meal (60% protein)	9.0
soybean meal (48.5% protein)	5.0
alfalfa meal (17% protein)	2.0
corn gluten meal (60% protein)	2.0
ground whole hard wheat	35.5
ground no. 2 yellow shelled corn	21.0
ground whole oats	10.0
wheat middlings	10.0
brewers' dried yeast	1.0
soy oil	1.5
salt	0.5
dicalcium phosphate	1.5
ground limestone	0.5
premixes (vitamins and minerals)	0.5
total	100.0

designed to be specific for aflatoxins, and the HPLC chromatograms after affinity column cleanup were void of any interferences. Figure 2 shows the HPLC chromatogram of an aflatoxin standard containing 5 ppb of B_1, B_2, and G_1 and 2.5 ppb of G_2. Figure 3 shows the HPLC chromatogram of a cleaned up extract from a vitamin-fortified feed sample that had been spiked with 5 ppb of B_1, B_2, and G_1 and 2.5 ppb of G_2. Essentially no peaks are present other than the aflatoxins B_1, B_2, G_1, and G_2. Figure 4 shows an HPLC chromatogram of the cleaned up extract

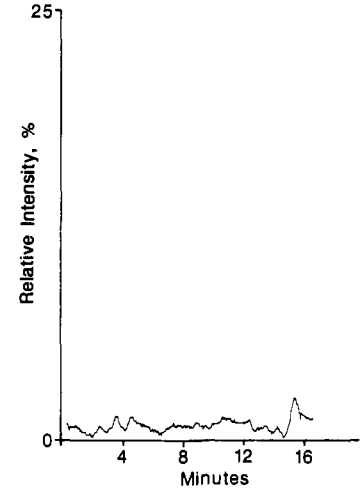

Figure 4. HPLC chromatogram of feed extract from unspiked feed using fluorescence detector on high sensitivity and integrator attenuation of 1 mV/full scale for maximum sensitivity.

(1 g-equiv) of vitamin-fortified rodent feed sample, as received from the supplier, indicating no aflatoxins except possibly a trace of B_1.

Since the aflatoxins are separated from any interferring materials by using the affinity column cleanup, the sensitivity of analysis can be changed simply by changing the amount of extract passed through the affinity column (realizing that this change could affect G_2 results) or by changing the amount injected into the HPLC. For this work a gram-equivalent of feed was passed through the affinity column, taking care not to exceed the affinity column load capacity of 100 ng of total aflatoxins. The injections made into the HPLC were 50 μL. The MDL for B_1, B_2, and G_1 was 0.25 ppb, and that for G_2 was 0.12 ppb, which was based on twice background.

This procedure was also used successfully to analyze aflatoxins in bedding (hardwood chips) used in rodent cages at the NCTR. The recoveries of aflatoxins B_1, B_2, and G_1 from the bedding spiked at 1 ppb each averaged above 90%. Recovery of aflatoxin G_2 from bedding spiked at 0.5 ppb was 52%.

It should be mentioned that in the postcolumn derivatization of aflatoxins only B_1 and G_1 react with iodine to enhance fluorescence. No change in fluorescence was noted with B_2 and G_2. Additional work is in progress to elucidate the structure of the iodine derivatives of aflatoxin B_1 and G_1, which will be the subject of another paper.

Our procedure provides the analyst with a rapid and sensitive procedure for analysis of aflatoxins in a grain-based vitamin-fortified rodent diet.

LITERATURE CITED

AOAC. *Official Methods of Analysis*, 12th ed.; Association of Official Analytical Chemists: Washington, DC, 1975; Sections 26.014–26.019.

AOAC. *Official Methods of Analysis*, 14th ed.; Williams, S., Ed.; Association of Official Analytical Chemists: Arlington, VA, 1984.

Beaver, R. W. Determination of Aflatoxins in Corn and Peanuts using High Performance Liquid Chromatography. *Arch. Environ. Contam. Toxicol.* **1989**, *18* (3), 315–318.

Busby, W. F., Jr.; Wogan, G. N. *Chemical Carcinogens*, 2nd ed.; Seale, C. E., Ed.; American Chemical Society: Washington, DC, 1984; pp 945–1136.

Chamkasem, N.; Cobb, W. Y.; Latimer, G. W.; Salinas, C.; Clement, B. A. Liquid Chromatographic Determination of Aflatoxins, Ochratoxin A, and Zearalenone in Grains, Oilseeds,

and Animal Feeds by Post-Column Derivatization and On Line Sample Clean-up. *J. Assoc. Off. Anal. Chem.* **1989**, *72* (2), 336–341.

Goto, T.; Manabe, M.; Matsuura, S. Application of HPLC to the Analysis of Aflatoxins in Foods and Feeds. *Agric. Biol. Chem.* **1979**, *43* (12), 2591–2592.

Groopman, J. D.; Donahue, K. F. Aflatoxin, a Human Carcinogen: Determination in Foods and Biological Samples by Monoclonal Antibody Affinity Chromatography. *J. Assoc. Off. Anal. Chem.* **1988**, *71* (5), 861–867.

Kok, W. T.; Van Neer, T. C. H.; Traag, W. A.; Tuinstra, L. G. M. T. Determination of Aflatoxins in Cattle Feed by Liquid Chromatography and Post-Column Derivatization with Electrochemically Generated Bromine. *J. Chromatogr.* **1987**, *367*, 231–236.

Mortimer, D. N.; Gilbert, J.; Shepherd, M. J. Rapid and Highly Sensitive Analysis of Aflatoxin M_1 in Liquid and Powdered Milks using an Affinity Column Cleanup. *J. Chromatogr.* **1987**, *407*, 393–398.

Shepherd, M. J.; Gilbert, J. An Investigation of HPLC Post-Column Iodination Conditions for Enhancement of Aflatoxin B_1 Fluorescence. *Food Addit. Contam.* **1984**, *1* (4), 325–335.

Thiel, P. G.; Stockenstrom, S.; Gathercole, P. S. Aflatoxin Analysis by Reversed-Phase HPLC using Post-Column Derivatization for Enhancement of Fluorescence. *J. Liq. Chromatogr.* **1986**, *9* (1), 103–112.

Traag, W. A.; Van Trijp, J. M. P.; Tuinstra, L. G. M. T.; Kok, W. T. Sample Clean-Up and Post-Column Derivatization for Determination of Aflatoxin B_1 in Feedstuffs by Liquid Chromatography. *J. Chromatogr.* **1987**, *396*, 389–394.

Tuinstra, L. G. M. T.; Haasnoot, W. Rapid Determination of Aflatoxin B_1 in Dutch Feeding Stuffs by High Performance Liquid Chromatography and Post-Column Derivatization. *J. Chromatogr.* **1983**, *282*, 457–462.

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Registry No. Aflatoxin B_1, 1162-65-8; aflatoxin B_2, 7220-81-7; aflatoxin G_1, 1165-39-5; aflatoxin G_2, 7241-98-7.