Automated Affinity Liquid Chromatography System for On-Line Isolation, Separation, and Quantitation of Aflatoxins in Methanol-Water Extracts of Corn or Peanuts

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An automated liquid chromatography (LC) procedure has been developed for the determination of aflatoxins in corn and peanuts. The automated LC system consists of the following: two pumps, an autosampler, an isolation column (immunoaffinity or methacrylate copolymer), an automated switching valve, a C_{18} column, an electrochemical cell, a fluorescence detector, and a data system. Filtered methanol:water (75:25) extracts of the test portions are injected into the LC system. With the switch in position 1, water is pumped through the autosampler and isolation column and then discarded. With the switch in position 2, the mobile phase consisting of methanol:acetonitrile:water (26:18:56), 1 mM KBr, and 1 mM HNO_3 is pumped through the isolation column, C_{18} column, electrochemical cell, and detector. Aflatoxins B_1 and G_1 react with electrochemically generated bromine to form fluorescent derivatives. Both isolation columns can be used for >50 injections. Recovery of aflatoxins from both commodities spiked at 5-30 ng/g was about 85%.

INTRODUCTION

Many techniques have been used for the determination of aflatoxins in extracts of foods and feeds, including thin-layer chromatography (TLC) (AOAC, 1990) and liquid chromatography (LC) coupled with postcolumn iodination (Dorner and Cole, 1988; Beaver, 1989). These techniques are time consuming and usually require labor-intensive premeasurement cleanup procedures. Immunochemical procedures have been developed for the determination of aflatoxins; they include enzyme-linked immunosorbent assays (ELISA) (Trucksess et al., 1989; Koeltzow and Tanner, 1990) and immunoaffinity column separations (Groopman and Donahue, 1988). Since these procedures are simple, fast, and selective, they are an improvement over the techniques previously used. However, because immunoassays do not distinguish between individual aflatoxins, they are used primarily for screening. The immunoaffinity columns provide an improved cleanup for subsequent analysis by TLC or LC. They require manual operation and currently are not reusable.

Automated mycotoxin methods are being developed and used in several laboratories. Aflatoxin M_1 in milk has been determined by a robotic system consisting of solid-phase extraction, cleanup, dilution, filtration, and quantitation by LC (Gifford et al., 1990). Total aflatoxins in food and animal feeds were determined by automated LC using a disposable immunoaffinity cleanup column (Sharman and Gilbert, 1991). An on-line screening method for extracts of foods and feeds has been described which involves cleanup of multimycotoxins, including aflatoxins (Chamkasem et al., 1989). This system utilizes six solvents, four pumps, and two switching valves in addition to the LC equipment; the operating procedure is complicated.

We have developed a simplified automated LC system for the determination of aflatoxins in extracts. At the heart of the system is a reusable isolation column for separating aflatoxins from analytical interferences. We have investigated two alternatives for the isolation column. One is an epoxide-activated hydroxyethyl methacrylate/dimethacrylate support coupled with aflatoxin monoclonal antibodies; the other is a packing using the copolymer alone. The aflatoxins are separated on a C_{18} column and determined by measurement of fluorescence after on-line, postcolumn derivatization with electrochemically generated bromine (Traag et al., 1987).

MATERIALS AND METHODS

Chemicals. All chemicals were either analytical or LC grade unless otherwise specified. Aflatoxin standards (B_1, B_2, G_1, and G_2) were obtained from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies raised against aflatoxins were supplied by Vicam (Summerville, MA); rabbit antiserum raised against aflatoxin B_1 was obtained from Environmental Diagnostics, Inc. (Burlington, NC).

Column Support. Protein A-Sepharose CL-4B was obtained from Sigma Chemical Co. Durasphere-Epoxy, Affi-Prep 10, and Hema-Afc-Bio 1000 were obtained from Alltech Associates (Deerfield, IL). Protein-Pak (epoxy-activated) was obtained from Millipore-Waters (Milford, MA).

LC System. A schematic of the LC system is shown in Figure 1. The system consisted of the following: two pumps (Waters, Model 510), an autosampler (Perkin-Elmer, ISS-100), an automated switching valve (Waters) connected with an isolation column (4.6 × 30 mm), a reversed-phase C_{18} column (Waters, Resolve C_{18}, 5 μm, 3.9 × 300 mm), an electrochemical cell (Kobra cell, Free University, Amsterdam, The Netherlands), a fluorescence detector (Waters, Model 470), and a Waters 840 chromatography data station.

Preparation of Aflatoxin Standards. Stock standard solutions of aflatoxins B_1, B_2, G_1, and G_2 were prepared at 0.5, 0.15, 0.5, and 0.15 μg/mL in methanol, respectively (AOAC, 1990). The working standard solution was prepared by diluting the stock standard 1:100 with methanol:water (20:80).

Preparation and Extraction of the Test Sample. Corn or peanuts were ground with a Retsch mill (Brinkmann Instruments, Inc., Westbury, NY) and mixed to achieve homogeneity. The test portion (25 g) was placed in a Waring blender jar, 5 g of sodium chloride and 75 mL of methanol:water (75:25) were added, and the mixture was blended for 3 min at high speed. The resulting mixture was filtered through fluted filter paper; 3 mL of filtrate was pipetted into a 10-mL graduated cylinder and

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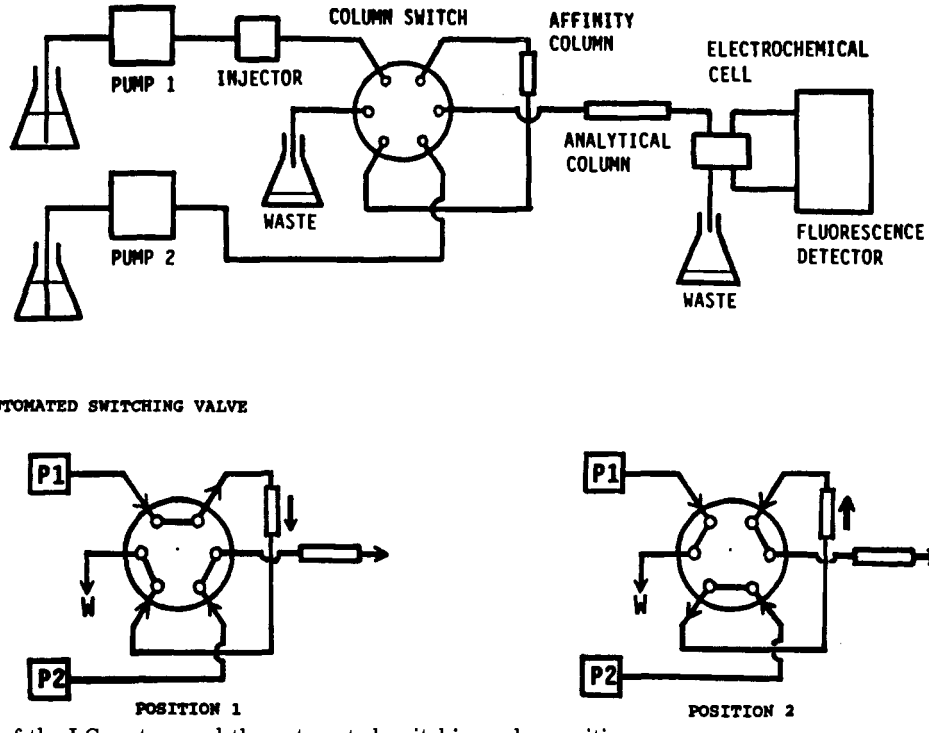

Figure 1. Diagram of the LC system and the automated switching valve positions.

diluted with water to 10 mL. The diluted extract was filtered through a 0.45-μm filter and transferred to an autosampler vial.

Purification of Aflatoxin Polyclonal Antibody. The antiserum was purified and isolated according to the method of Miller and Stone (1978), using a Protein A column. About 1.5 g of freeze-dried Protein A–Sepharose CL-4B (Sigma) was soaked in a 0.2 M phosphate–0.15 M NaCl buffer, pH 7.4, and packed in a 1- × 6-cm column. A 2-mL portion of rabbit antiserum raised against aflatoxin B_1 was diluted with 2 mL of the buffer and applied to the column. The column was washed with 25 mL of buffer at a flow rate of 0.5 mL/min. The bound antibody was eluted from the column with a 0.1 M glycine–HCl buffer, pH 3.5, at the same flow rate. Fractions (1 mL) were collected in small test tubes containing 0.5 mL of a 1 M Tris–HCl buffer, pH 8.0, to adjust the pH of the solution to near neutral. The elution of the antibody was monitored by measuring the UV absorbance of the fractions at 280 nm. The protein concentration was determined colorimetrically (Lowry et al., 1951).

Coupling the Monoclonal or Polyclonal Antibodies to Column Supports. One gram of Protein-Pak (epoxy-activated, silica-based, 40 μm, 500 Å; Millipore-Waters) was suspended in 3 mL of a phosphate–NaCl buffer in a screw-capped tube. A 5-mg portion of antibodies was added to the tube. The tube was then mechanically rotated for 24 h at 20 °C. The mixture was then centrifuged, and the concentration of the unbound antibodies was determined (Lowry et al., 1951). The unreacted epoxy groups of the packing were blocked overnight with 3 mL of 1 M ethanolamine, pH 9.5, at 20 °C. The packing was filtered through a fritted-disc funnel and washed with 5 mL of phosphate–NaCl buffer.

Preparation of the LC Immunoaffinity Column. The packing coupled with antibodies (in phosphate–NaCl buffer) was packed in an LC column (4.6 × 30 mm) and stored in a 0.1 M phosphate buffer, pH 7.4, containing 0.01% sodium azide.

Preparation of the LC Affinity Column. The column support (HEMA-Afc-Bio) was suspended in the phosphate–NaCl buffer and packed in the same way as the LC immunoaffinity column.

LC Conditions. Mobile phase 1 (water) was pumped with pump P1 through the autosampler set at a 1.5-mL injection volume. Mobile phase 2 (methanol:acetonitrile:water (26:18:56) with 1 mM KBr and 1 mM HNO_3) was pumped with pump P2. Outlets for the two pumps were connected to the automated switching valve. P1 and P2 were set at flow rates of 1.0 and 0.8 mL/min, respectively. The switching valve was set at position 1 (Figure 1); mobile phase 1 passed through the LC affinity column, and mobile phase 2 passed through the analytical column. Test extract was injected onto the affinity column, and the affinity column was washed with mobile phase 1 for 5 min. The switching valve was rotated to position 2 (Figure 1); mobile phase 1 passed directly to the waste, while mobile phase 2 passed through the affinity column and eluted the aflatoxins on-line to the analytical column. The valve stayed at position 2 for 3 min. The switching valve was then rotated to position 1 again. The affinity column was equilibrated with water while the aflatoxins were separated and eluted from the analytical column. Aflatoxins B_1 and G_1 were derivatized with bromine generated in an electrochemical cell connected to a voltage regulator set at 7 V. The derivatives were monitored with a fluorescence detector set at excitation and emission wavelengths of 360 and 440 nm, respectively.

RESULTS AND DISCUSSION

Four types of epoxy-activated affinity supports, Durasphere-Epoxy, Affi-Prep 10, Protein-Pak, and Hema-Afc-Bio, were used to couple separately with the IgG fraction of the monoclonal and polyclonal antibodies raised against B_1. The LC antibody-bound affinity columns (LCIACs) have the ability to recognize and bind to the aflatoxins in the test extract in a highly specific manner. The organic mobile phase was used to elute the toxins from the LCIAC and load them onto the analytical column. The LCIACs made with Durasphere-Epoxy and Affi-Prep 10 were unable to withstand the pressure of the system (500 psi). Subsequently, no aflatoxins were retained in the columns.

Protein-Pak (epoxy-activated) alone had no affinity toward the aflatoxins. Protein-Pak coupled with monoclonal antibodies gave decreasing peak areas for B_1 and G_1 after several injections. Aflatoxins B_2 and G_2 were not retained on the column after 10 injections, probably because of the gradual leaching of the antibody from the support by the organic solvents in the mobile phase.

Protein-Pak coupled with polyclonal antibodies gave consistent peak areas of the aflatoxin standards for more than 50 injections. The standard curves were linear for 0.2–25 ng of B_1 and G_1 and 0.06–15 ng of B_2 and G_2.

Table I. Percent Recoveries[a] of the Aflatoxins Added to Corn and Peanuts, Using the LC Immunoaffinity Column

aflatoxin added, ng/g	corn			peanuts		
	mean, %	SD	CV	mean, %	SD	CV
B_1, 1	96	3.1	3.2	95	7.9	8.3
B_1, 5	85	1.7	2.0	89	8.7	9.7
B_1, 10	86	3.6	4.2	85	6.1	7.2
B_2, 0.3	89	1.9	2.2	96	4.8	5.0
B_2, 1.5	92	1.9	2.1	91	8.8	9.7
B_2, 3	91	4.3	4.8	87	7.8	9.0
G_1, 1	89	1.7	2.0	93	4.4	4.7
G_1, 5	92	2.9	3.1	91	8.8	9.7
G_1, 10	90	4.3	4.8	87	7.8	9.0
G_2, 0.3	90	2.9	1.9	96	6.5	6.8
G_2, 1.5	95	2.4	2.5	93	9.0	9.7
G_2, 3	92	3.3	3.6	89	8.1	9.0

[a] Replicate analyses, n = 4.

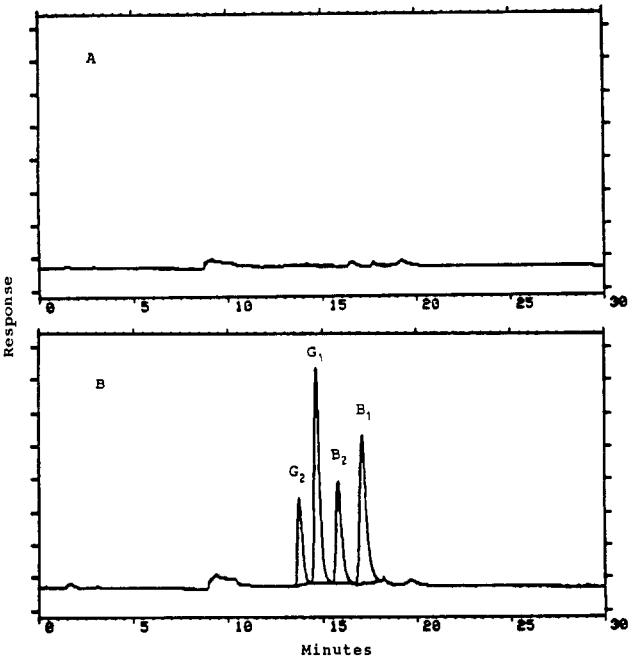

Figure 2. Chromatogram of (A) aflatoxin-free corn and (B) corn with B_1 and G_1 added at 10 ng/g and B_2 and G_2 added at 3 ng/g after LC immunoaffinity column cleanup.

Coefficients of variation were <3% (n = 7) when 1 ng of B_1 and G_1 and 0.3 ng of B_2 and G_2 were injected.

Table I presents the recoveries of the aflatoxins added as four-component mixtures to test samples of corn and peanuts at 3 levels: B_1 and G_1 at 1, 5, and 10 ng/g mixed with B_2 and G_2 at 0.3, 1.5, and 3 ng/g, respectively. Recoveries ranged from 85% to 96%, with coefficients of variation (CVs) of <5% and <10% for corn and peanuts, respectively. The limit of detection of the method described was calculated to be 0.5 ng/g for B_1 and G_1 and 0.15 ng/g for B_2 and G_2, based on a signal-to-noise ratio of >5. The limit was lowered by the following procedure. When a 5-fold volume of test extract was applied to the system (7.5 mL; 1 g of original test sample equivalent), the recoveries of aflatoxins were >80%. The limit of detection was then 0.1 ng/g for B_1 and G_1 and 0.03 ng/g for B_2 and G_2.

The limit of determination was about 1 ng of aflatoxin B_1/g. Figure 2 shows chromatograms of aflatoxin-free corn and corn containing added aflatoxins at 26 ng/g. Figure 3 illustrates LC chromatograms of blank peanuts and aflatoxin-spiked peanuts at 26 ng/g. The four aflatoxins were well resolved and free from interferences.

HEMA-Afc-Bio (epoxy-activated) has high affinity for aflatoxins, and coupling to antibodies is not necessary.

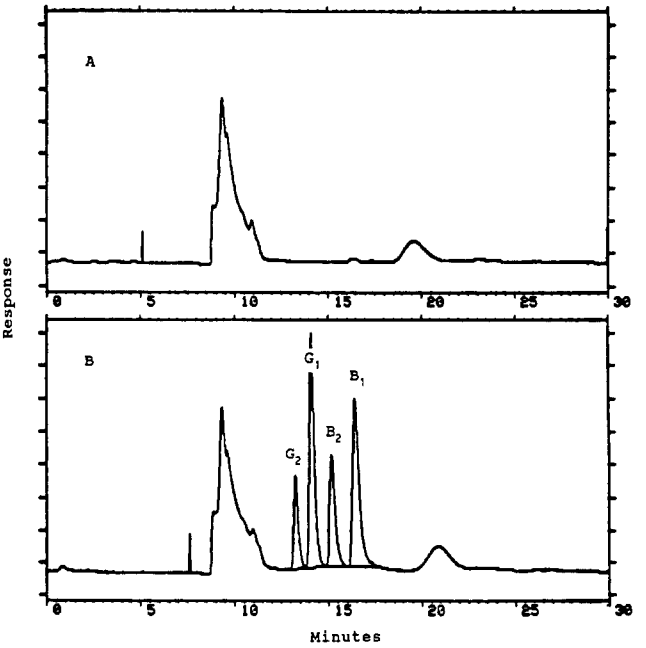

Figure 3. Chromatogram of (A) aflatoxin-free peanuts and (B) peanuts with B_1 and G_1 added at 10 ng/g and B_2 and G_2 added at 3 ng/g after LC immunoaffinity column cleanup.

Table II. Percent Recoveries[a] of the Aflatoxins Added to Corn and Peanuts, Using the LC Affinity Column

aflatoxin added, ng/g	corn			peanuts		
	mean, %	SD	CV	mean, %	SD	CV
B_1, 4	88	5.2	5.9	77	2.1	2.7
B_1, 8	81	5.2	6.4	86	7.9	9.2
B_1, 16	83	5.8	7.1	79	6.6	8.3
B_1, 24	88	9.7	11.0	83	2.5	3.0
B_2, 0.25	80	14	17.7	79	2.9	3.7
B_2, 0.5	89	6.0	6.8	89	5.8	6.6
B_2, 1.0	93	2.9	3.1	81	8.3	10.7
B_2, 1.5	94	7.7	8.1	84	5.5	6.5
G_1, 0.75	94	9.5	10.1	82	1.7	2.1
G_1, 1.5	89	12	13.3	83	3.8	4.6
G_1, 3.0	91	13	14.4	73	5.1	7.0
G_1, 4.5	89	8.6	9.7	72	5.5	7.6
G_2, 0.25				98	4.5	4.6
G_2, 0.5				83	4.6	5.6
G_2, 1.0				89	10	11.8
G_2, 1.5				84	8.1	9.7

[a] Replicate analyses, n = 4.

HEMA-Afc-Bio alone was used in the LC affinity column study (LCAC). This packing is a macroporous hydrophilic copolymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate. Aflatoxins are water soluble, and on the basis of their physical properties, they would be expected to bind to the packing by hydrophilic interactions. Consistent peak areas were obtained for the four aflatoxin standards even after 100 injections. The standard curves for the aflatoxins were linear over the range of 0.1–10 ng per injection for B_1 and G_1 and 0.3–3 ng per injection for B_2 and G_2. The CVs (n = 7) were <5%.

Table II presents the recoveries of aflatoxins added to test samples of corn and peanuts at four levels: 4, 8, 16, and 24 ng of B_1/g; 0.75, 1.5, 3.0, and 4.5 ng of G_1/g; and 0.25, 0.5, 1.0, and 1.5 ng of B_2/g and G_2/g. Recoveries ranged from 72% to 98%, with CVs of <18% and <12% for corn and peanuts, respectively. There was an interference peak near the aflatoxin G_2 retention time for the corn extract; however, G_2 is rarely found in corn naturally contaminated with aflatoxin. No attempt was made to quantitate G_2 in corn because of background interferences.

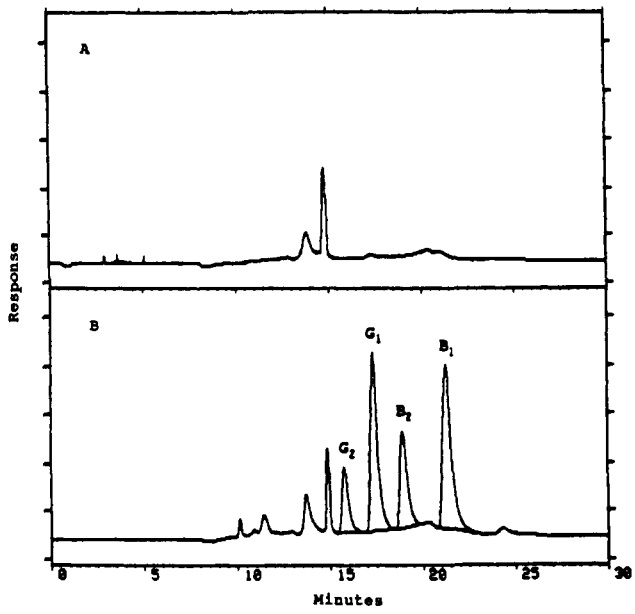

Figure 4. Chromatogram of (A) aflatoxin-free corn and (B) corn with B_1 and G_1 added at 5 ng/g and B_2 and G_2 added at 1.5 ng/g after LC affinity column cleanup.

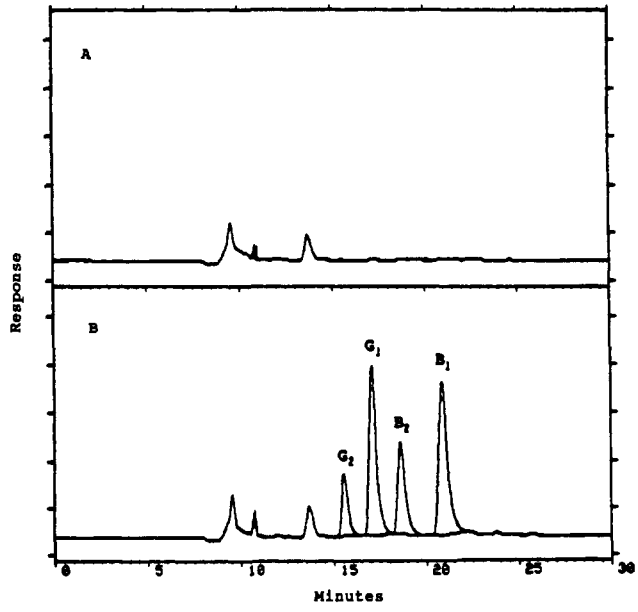

Figure 5. Chromatogram of (A) aflatoxin-free peanuts and (B) peanuts with B_1 and G_1 added at 5 ng/g and B_2 and G_2 added at 1.5 ng/g after LC affinity column cleanup.

Figure 4 shows the LC chromatograms of aflatoxin-free corn and aflatoxin added to corn at 13 ng/g. Figure 5 shows typical chromatograms of aflatoxin-free peanuts and aflatoxin added to peanuts at 13 ng/g. There were no interferences in the aflatoxin areas.

These methods were used to determine aflatoxins in 10 test samples of naturally contaminated corn and peanuts. The results obtained were similar to those obtained using other LC methods (Dorner and Cole, 1988).

In conclusion, both the epoxy-activated Protein-Pak support coupled with polyclonal antibody (LCIAC) and the Hema-Afc-Bio (LCAC) alone are chemically stable and can withstand the high pressure used in LC on-line purification of aflatoxins in corn and peanuts. The LCIAC eluate passing through the analytical column provided no interference peaks in the aflatoxin area. The potential interference in the G_2 area in corn when the LCAC was used is not a serious problem because the contamination levels of aflatoxin in corn are usually higher for B_1 than for G_1. Aflatoxin B_2 and occasionally G_2 were found in trace amounts (Wilson et al., 1988). LCAC is a better choice because LCIAC offers no advantages, whereas LCAC requires no antibody and is commercially available.

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