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High Performance Thin Layer Chromatographic Screening for Aflatoxins in Poultry Feed by Using Silica Sep-Paks

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Aflatoxins are a potent carcinogenic and poisonous class of naturally occurring compounds found in grains infected by some members of the Aspergillus genus of fungi. The high rate of primary liver cancer in Africa and Asia has been associated with high levels of aflatoxin in the diet (Krough 1977). Outbreaks of animal disease resulting from the ingestion of moldy meal are well documented (Campbell 1977). The need for a convenient, sensitive and highly selective method to test for aflatoxins in poultry feed samples led to the development of a method based on the CB procedure (AOAC 1980). The CB procedure requires large volumes of solvents, the preparation of silica gel of a specified moisture content and the use of large TLC plates, which have long development times. The desirability of running fortified control samples to monitor aflatoxin recovery and to serve as reference standards doubles the already high volume of solvents required.

To overcome these difficulties, a number of changes in the CB method were explored. Sample size reduction was used in an attempt to decrease the volume of solvents required. The practicality of using commercially available silica Sep-Paps was investigated. The use of high performance thin layer chromatographic (HPTLC) plates with preadsorbent zones was tested to decrease spotting and development time. The capability of readily detecting aflatoxins B_1, G_1, B_2 and G_2 at 10, 10, 5 and 5 ppb (10^{-9}), respectively, was set as the goal.

MATERIALS AND METHODS

Ethanol-free chloroform was prepared by slowly passing reagent grade chloroform (0.75% ethanol preservative) through a fritted glass column containing 4 Å molecular sieve. The chloroform should be freshly prepared and stored in a dark bottle away from light to decrease the rate of phosgene formation. Aflatoxins should remain on the silica gel base line when developed with the purified solvent. The absolute diethyl ether used in the Sep-Pak separation should also be dry enough so that aflatoxins do not go beyond the silica gel base line when developed on HPTLC plates. Dryness can be restored by slowly passing the ether through a

fritted glass column containing 4 Å molecular sieve. Care should be taken to protect the dried ether from excessive moisture by covering the space between the receiver and column tip with aluminium foil and wiping the tip before filling the next vial.

Aflatoxins were obtained from Aldrich Chemicals, Milwaukee, Wisconsin. Solutions of the aflatoxins in benzene-acetonitrile (98+2) were prepared at a concentration of 10 ug/mL. The solutions of aflatoxins B_1, G_1, B_2, and G_2 were combined in a 2:2:1:1 ratio for fortifying and TLC spotting. All the remaining chemicals were of reagent grade.

Poultry feed samples were extracted by stirring a 5-g subsample of finely ground feed, 2.5 mL of distilled water, 25 mL of ethanol-free chloroform, and 2.5 g of Hyflo Super-Cel (Fisher, Springfield, NJ) for 30 min. The mixture was pressure filtered ($\leq$ 5 psi) through a medium porosity 30-mL sintered glass funnel (Corning, Corning, NY) with a 5-mm layer of Hyflo Super-Cel. The filtrate was dried over 2.5 g of anhydrous sodium sulfate.

The aflatoxins were isolated from the extract by a silica Sep-Pak (Waters Assoc., Milford, MA) connected to a 10-mL syringe. A 10-mL volume of the filtrate was passed through the Sep-Pak by gravity flow. A 10-mL volume of hexane was then added. After the first 2 mL of hexane had entered the Sep-Pak, the next 5 mL were rapidly pushed through with a rubber bulb. Just before the last of the haxane left the syringe, 10 mL of absolute diethyl ether was carefully added so as to avoid unnecessary exposure to the air. The aflatoxins were eluted from the Sep-Pak with 10 mL of chloroform (0.75% ethanol)-methanol (97+3). The collected fraction was evaporated by gentle heating on a water bath under a stream of nitrogen.

The extract was redissolved in 200 uL of benzene-acetonitrile (98+2) for TLC spotting. The 20 x 10 cm LHP-K silica gel HPTLC plates (Whatman, Clifton, NJ) with preadsorbent zones were cut into 5-cm sections. A 10-uL volume of the extract was "spotted" with a 10-uL microsyringe in a 1-cm vertical line 0.5 cm from the bottom of the plate and 1 cm from the left side. The fortified control which had been treated the same as the sample was spotted in a similar manner 1 cm from the right side of the plate. The pure aflatoxin standard was spotted in the center of the preadsorbent zone. The plate was developed in a 400-mL beaker, after wetting the sides of the beaker with the chloroform (0.75% ethanol)-acetone-isopropanol (92+5+2.5) elution mixture to saturate the air in the beaker with solvent vapor. The plate was removed when the solvent front advanced 5.5 cm on the silica gel. For the development in the second dimension, the preadsorbent zone was scraped off. To serve as aflatoxin R_f standards for the second dimension development, the aflatoxin standard mixture was spotted above and below the regions where the aflatoxins in the sample and fortified control were located. The size of the aflatoxin spots was decreased by eluting first

with methanol (Felton 1980). One side of the plate was placed in a 125-mm wide crystallizing dish with a shallow layer of methanol. The plate was removed when the methanol front had almost completely moved across the aflatoxin spots and was rapidly dried by blowing a stream of air across the plate in the direction opposite to the flow of the solvent. The same technique was then applied to the remaining side. After a thorough drying, each side of the plate was then developed with diethyl ether-methanol-water (88+9+3) to a distance of 1.1 cm. The presence or absence of the aflatoxins was then determined with a long wave ultraviolet lamp (366 nm).

RESULTS AND DISCUSSION

For a sensitivity of 10 ppb for aflatoxins B_1 and G_1 and 5 ppb for B_2 and G_2, a sample size of 5 g, a final volume of 200 uL and spotting volume of 10 uL, 10 mL of extract must be purified in order to spot 1 ng of B_1 and G_1 and 0.5 ng of B_2 and G_2. A volume of extract this large on less than 0.7 g of silica gel, along with the use in the Sep-Paks of a silica gel with a moisture content much greater than the 1% recommended in the CB method, results in a poor recovery of the earlier eluting aflatoxins when chloroform-containing ethanol preservative is used. By using ethanol-free chloroform, the eluting power of the extract was found to be greatly decreased. Gravity flow is used in the Sep-Pak purification step since applying pressure to increase the flow rate through the Sep-Pak was found to smear the aflatoxins on the Sep-Pak. A forced flow of hexane to remove pockets of air is necessary, however, or else the air pockets would expand and stop the flow when ether was added to the Sep-Pac.

When filtering the crude aflatoxin extract, excessive flow of air through the filter cake can be detected by attaching a hose to the side arm of the filter flask and submerging the hose in water. After the filter cake begins to allow air through, a further amount of filtrate can be obtained by tamping down the filter cake and reapplying pressure. If difficulty is encountered in recovering sufficient extract, the amount of Hyflo Super-Cel may be reduced. When using pressure filtration, clogging of the frit eventually occurs. Simple backflushing with solvent is ineffective in restoring the flow. Treatment with chromic acid cleaning solution is necessary to restore the original flow. A ferrous sulfate solution acidified with sulfuric acid is used to destroy any residual dichromate adsorbed on the frit.

The presence of aflatoxins can sometimes be determined by the first development with chloroform-acetone-isopropanol. The development in the second dimension with ether-methanol-water, however, completely removes any background. The elution with methanol before the development in the second dimension increases the intensity of the aflatoxins by decreasing the size of the aflatoxin spots. The R_fs of the aflatoxins on the side of the

TLC plate may be found to be slightly greater than the R_fs of the aflatoxins on the center of the plate. This is attributed to the greater degree of evaporation of the elution solvent at the edges of the plate resulting in a concentration of the more polar components of the mixture. Because of this, The R_fs of the aflatoxins used in the control should be used for comparison to the extract being tested, and the aflatoxins on the center of the plate used only as a rough R_f standard. Lining the sides of the beaker used for the development with filter paper avoids this problem, but the aflatoxin spots are not as sharp.

The proposed method has also been found to be effective in analyzing corn and urine samples for aflatoxins. In adapting this method to other more complex substrates, more resolving power on the development in the second dimension may be necessary. By cutting the HPTLC plates into wider widths more distance will be available for the development in the second dimension. A smearing of the aflatoxins may occur on the HPTLC plates if more than 10 uL of the final extract from high lipid content samples is placed on the plate. A preliminary development with anhydrous diethyl ether sharpens the aflatoxin spots by moving the lipids and leaving the aflatoxins at the base of the silica gel (Kozloski 1981). Alternative eluting mixtures (AOAC 1984) for aflatoxins may be modified for use on HPTLC plates by changing the proportions of the solvents. Experience with this method has shown that aflatoxins B_1 and G_1 at 10 ppb and B_2 and G_2 at 5 ppb are readily detected in poultry feed samples.

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