A NEW METHOD FOR QUANTITATION OF AFLATOXIN IN CORN[1]

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ABSTRACT

A new method for quantitation of aflatoxin in corn is described which is faster, easier, about one-seventh as expensive, and requires less toxic and/or flammable solvents than AOAC Method 1 (CB method). The new method is an extension of a simple screening test previously described. Aflatoxin was removed from the corn by double extraction with methanol-water (75/25, v/v). Ammonium sulfate solution was added to a portion of the initial extract, and the resulting aqueous phase was partitioned once with hexane to remove oil and pigments and then twice with methylene chloride to extract aflatoxin. Aspiration was used to remove the hexane (top) layer from the separatory funnel without draining off the aqueous (bottom) layer. Transfer of final extract from one container to another was avoided. Standard thin-layer chromatography was used for final detection and quantitation. When seven lots of yellow corn "naturally" contaminated with aflatoxin B_1 were analyzed by the new method and by the CB method, results were similar.

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The method validated for official quantitation of aflatoxin in corn is the AOAC Method 1 (Sections 26.037 and 26.039), known as the "CB method" (1). It is long and requires large quantities of expensive, flammable, and/or toxic solvents. Our method differs in the initial extraction and cleanup steps; both methods use thin-layer chromatography (tlc). Certain portions of the new method are similar to a simple screening test for aflatoxin in corn (2).

MATERIALS AND METHODS

Although our method reduces danger of toxicity and flammability, a well-ventilated laboratory equipped with a fume hood is advisable. A steam bath and a source of nitrogen should be available in the hood.

Care should be taken to ensure that each sample extracted is representative of the lot being analyzed. Sampling and preparation of sample have been discussed in AOAC Method 1, Section 26.003 (1).

All reagents were analytical reagent grade (American Chemical Society-certified) or better. Aflatoxin standard was supplied by USDA-ARS Southern Regional Research Center, New Orleans, La.

Procedure

Extraction and Cleanup. Blend 50 g ground corn and 100 ml methanol-water (75:25, v/v) at high speed for 2 min. Pour blended mixture into a 250-ml centrifuge bottle and centrifuge at about 1500 rpm; usually only 1–3 min at this

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speed is necessary. Pour off and save supernatant. Add another 100 ml of methanol-water (75:25, v/v), and thoroughly resuspend the residue by vigorously shaking the bottle for about 1 min. Centrifuge again and combine the supernatant with that of the previous centrifugation. Into a 250-ml separatory funnel, place 40 ml of the methanol-water extract, 80 ml of 20% ammonium sulfate solution, and 40 ml of hexane. Shake the funnel about 30 sec (vent several times) and allow layers to separate. Draw off the upper layer (hexane) with a Pasteur pipet connected to a vacuum via a trap. Also, a small lump of gummy, proteinaceous material at the interface of the two phases can be removed easily with the aid of the vacuum pipet. Then add 6 ml of methylene chloride to the aqueous phase and shake thoroughly (vent frequently). Allow layers to separate and drain the lower layer (methylene chloride) into a 2-dram vial. Extract again with 4 ml of methylene chloride. Evaporate the combined methylene chloride extracts to dryness over a steam bath with a stream of nitrogen from a Pasteur pipet directed into the vial. Then add 0.5 ml of benzene-acetonitrile (98:2, v/v) to the vial and shake to dissolve residue. This extract is ready for tlc.

Thin-Layer Chromatography. Detection of aflatoxins by tlc has been described in AOAC Method 1, Sections 26.037 and 26.039 (1). We used Brinkman SIL G-HR-25 (Cat. No. 6614600-6) precoated plates activated by heating to 110° C for about 1 hr, and usually developed the plates in chloroform-acetone (88:12, v/v). For samples of unknown aflatoxin content, we spot 10 and 25 μl extract, and also 4, 8, and 12 μl of standard (0.5 ng/μl each B_1, G_1, and 0.15 ng/μl each B_2, G_2). Developed plates are viewed in a cabinet (Brinkman C-5, or equivalent) equipped with short- (254 nm) and longwave (366 nm) lights. Blue fluorescent spots at the same height as aflatoxin B_1 standard are compared to the standard in terms of brightness and color under each light. If aflatoxin B_1 is not present, aflatoxins B_2, G_1, or G_2 are not expected. Then, if necessary, samples which appear to contain aflatoxin are respotted with adjusted amounts of final extract and standard for better quantitation and/or confirmation by either cochromatography, described in AOAC method 1, sections 26.037 and 26.039 (1) or derivative formation (3,4).

Calculation of Results. Concentration of aflatoxin in ppb (ng aflatoxin/g of grain) is calculated from the following formula:

$$\text{ppb} = \frac{A \cdot B \cdot C}{D \cdot E}$$

where

A = ng of aflatoxin in the standard spot to which the sample spot is compared
B = intensity of sample spot/intensity of standard spot
C = total μl of final extract (benzene-acetonitrile)
D = μl of final extract spotted
E = 10 g of grain, based on 50 g (40 ml extract/200 ml methanol-water added)

Test Samples

Preparation. For preparation of corn naturally contaminated with aflatoxin, we used an *Aspergillus flavus* isolate which produced aflatoxins B_1 and B_2, although the latter was produced at <0.1 the amount of B_1. Very clean yellow dent corn was spread on trays (10 trays, 80 g each) and placed in an

environmental chamber for 3 days to equilibrate at 90–92% humidity and 30° C. Then the corn was inoculated with the *A. flavus* isolate, mixed to assure equal dispersion of inoculum, and returned to the environmental chamber. By removing trays from the chamber at half-day intervals on days 5 and 6 of *A. flavus* growth, we obtained corn with aflatoxin B_1 levels of about 50, 100, 500, and 1000 ppb. The 50-ppb corn (ground to 20-mesh) was blended with aflatoxin-free corn (ground to 20-mesh) to make lots A and B. Similarly, corn containing 100, 500, and 1000 ppb was used to prepare lots C, D, and E–G, respectively. Each lot was mixed in a mechanical tumbler for at least 45 min. We prepared the test samples as described here to minimize variations from sampling. In particular, we avoided blending corn with a very high concentration (*i.e.*, >1000 ppb) of aflatoxin with aflatoxin-free corn to prepare samples with a low aflatoxin level (*i.e.*, <50 ppb).

Analyses. Each lot, A–G, was sampled 16 times, 50 g each. Eight samples were analyzed (for aflatoxin B_1 only) by the CB method, and eight by our method. For each lot, final extracts from both methods were spotted on one tlc plate and aflatoxin B_1 spots were compared to a set of standards on that plate. An Aminco fluorodensitometer was used to measure intensity of fluorescence of aflatoxin B_1 spots. To assure uniform quality and dryness, diethyl ether for the CB method was taken from a freshly opened can and stirred with anhydrous calcium chloride immediately before use.

TABLE I
Aflatoxin B_1 Concentrations (ppb) in Seven Lots of Naturally Contaminated Yellow Corn Determined by Seitz-Mohr (SM) and CB Methods

Lot	Method	Mean, ppb B_1[a]	Std. Dev.	Coef. of Var. %
A	SM	13.3	4.8	36.1
A	CB	15.4	7.5	48.7
B	SM	37.7	3.7	9.8
B	CB	36.6	16.6	45.4
C	SM	69.2	15.3	22.1
C	CB	68.3	13.8	20.2
D	SM	91.6[b]	16.8	18.3
D	CB	117.9	13.8	11.7
E	SM	74.6	15.4	20.6
E	CB	64.6	10.1	15.6
F	SM	165.7	12.6	7.6
F	CB	146.5	26.2	17.9
G	SM	208.6[b]	10.6	5.1
G	CB	161.8	16.5	10.2

[a]Each lot was analyzed eight times by each method.
[b]SM and CB results statistically different at the 0.05 level of significance.

RESULTS AND DISCUSSION

At the 0.05 level of significance as determined by "t" tests, results from the two methods did not differ significantly for 5 of the 7 lots (Table I). For lot D, results were higher from the CB method and for lot G, results were higher from our method. The two methods were in close agreement at levels of 70 ppb and lower. Standard deviations and coefficients of variation were lower with our method in 4 of the 7 lots. Considering "cleanliness" or absence of fluorescent spots other than aflatoxin in the test samples, our method was as good as (or better than) the CB method.

From our overall experiences with both methods, we concluded they are similar in terms of sensitivity, analysis for B and G aflatoxins, and interferences. Substances that interfered with aflatoxin identification with our method usually interfered with the CB method too. However, in terms of analysis time (30–45 min/sample), expense, efficient use of solvents, and avoidance of toxic and/or flammable solvents, our method has significant advantages over the CB method. Our method is shorter by about 30 min/sample, more convenient, and requires fewer and smaller amounts of chemicals per sample than the CB method. For chemicals used in initial extraction and cleanup, our method costs about one-seventh as much as the CB method, *i.e.*, \$0.33 compared to \$2.30/sample. Most of the reduction was accounted for by elimination of chloroform and diethyl ether. These latter solvents are also undesirable, because chloroform is fairly toxic (5) and diethyl ether is very volatile, flammable, and susceptible to peroxide formation. Costs are reduced and laboratory manipulations made easier by elimination of the chromatography column required by the CB method.

Several factors were considered in the choice of methanol-water (74:25, v/v) for initial extraction. Water was included to minimize possible effects from variation in sample moisture contents, to reduce volatility, and to lower costs by reducing the amount of methanol used. Fifty per cent water in methanol was not used because it extracted too much protein and starch, which slowed separation of phases in cleanup. If the residue in the centrifuge bottle were not washed with the second 100-ml portion of methanol-water (75:25, v/v), results would be 10 to 15% lower.

Ammonium sulfate was particularly suitable for enhancing polarity difference between aqueous-methanol and organic phases, because it was inexpensive and very soluble in water. Petroleum ether (30–60 bpt) could be substituted for hexane, but more polar solvents or aromatic solvents such as benzene would prematurely extract aflatoxins. Since hexane was the top layer, we used the aspiration method as described to discard the hexane without draining the separatory funnel. This saved time, avoided additional glassware, and decreased chance of spillage. As a dense, nonflammable solvent to extract aflatoxins into a bottom layer for easy removal from the separatory funnel, either methylene chloride or chloroform could have been used. We selected methylene chloride because it was cheaper, less toxic (5), and more volatile. The latter property was important because final extracts were evaporated to dryness. We collected methylene chloride extracts in a 2-dram vial and evaporated to dryness in that vial. This avoided transfer of final extract from a large flask to a vial required by the CB method.

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