Aflatoxin Detection in Corn: A Simple Screening Test[1]

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ABSTRACT

A simple screening test for aflatoxin in corn is described. The test can be completed in about 15 min. and will detect aflatoxin at levels as low as 5 p.p.b. The method involves extraction of the grain with methanol in a blender, removal of fat and pigments by partitioning into hexane (or Freon-113) from an aqueous ammonium sulfate layer, and transfer of the aflatoxin from the aqueous layer into methylene chloride. Final detection is accomplished by a TLC procedure requiring only 3 min. developing time. The presence of aflatoxin is indicated by a blue fluorescent spot at a specified position on the plate. Aflatoxin concentration can be estimated by comparison of the sample spot with standards.

We describe a simple screening test which will detect aflatoxin at the 5 p.p.b. level. We believe the test offers greater overall flexibility and simplicity than minicolumn procedures (1-6) and is quicker and generally more desirable than screening test procedures proposed by Dantzman and Stoloff (7) and by Knake et al. (8).

Although the procedure was specifically designed for use with corn, several collaborating laboratories have applied it to other grains, feedstuffs, and peanuts (personal communications). We have applied it to milo, although in this case we recommend more complete development of the TLC plate to separate the aflatoxins from possible interfering substances. Detailed information concerning detection of aflatoxin in milo will be the subject of a forthcoming publication.

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[1]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

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MATERIALS AND METHODS

Although the solvents employed are no more flammable or toxic than those used in other available procedures (5–8), we recommend that the assay be performed in a well-ventilated area. The use of the rather toxic solvents chloroform and benzene has been minimized. Flammability can be reduced by the use of Freon-113 in place of hexane as discussed below.

Screening Test Procedure

1. *Whole Corn.* Place 50 g. grain in a Waring Blendor with sharp blades and grind without extraction solvent at high speed for 15. sec. Then add 150 ml. methanol and blend at high speed for 2 min.

Ground Corn. Place 50 g. ground corn and 150 ml. methanol in a Waring Blendor and blend at high speed for 2 min.

2. Gravity filter the extract through qualitative filter paper in a 100-mm. or similar filter funnel. We have found 18.5 cm. Fisher No. 28320-201 filter paper to work well. Alternatively, allow the solids to settle and draw the clear extract from the top with a pipet or syringe.

3. Into a 250-ml. separatory funnel, place 30 ml. methanol extract, 60 ml. of 20% ammonium sulfate solution (preferably warmed to 40 to 50°C.), and 30 ml. hexane. Shake the separatory funnel for about 20 sec. with venting and allow 1 min. for layers to separate.

4. Drain the lower layer into a beaker. Discard the upper phase (hexane) and then return the lower layer to the separatory funnel.

5. Add 5 ml. methylene chloride and shake the funnel with venting for 20 to 30 sec. Let the funnel stand about 1 min. or until it appears that most of the methylene chloride has collected in the lower tip of the funnel. While the layers are separating, use a Q-tip or pipecleaner to absorb any water which may be in the drain tube of the funnel. Carefully drain off the methylene chloride extract into a 10-ml. glass-stoppered Erlenmeyer flask.

6. Place flask on a steam bath, or other heating device set for about 100°C., in a fume hood and flush the flask with a slow stream of nitrogen gas. Remove the flask just when the methylene chloride has completely evaporated (usually 2 min. or less). Add 0.5 ml. benzene:acetonitrile (98:2, v./v.) and swirl the flask to allow the benzene to completely wet the sides of the flask. A 4-dram vial could be substituted for the flask if desired.

7. Spot 25 μl. benzene solution onto a Brinkman SILG-HR-25 precoated plate for aflatoxin and drug screening. Separately, spot the following volumes of 1 ng. per μl. B_1 standard: 3, 6, 9, and 15 μl. Although not essential, use a flow of warm air over the plate to evaporate the benzene and keep the spot small. Activation of the TLC plate before spotting is not necessary. It is important to use the plate specified above. The plate can be cut to a size just large enough to accommodate the number of samples to be spotted.

8. Place plate in developing solvent (acetone:chloroform, 12:88, v./v.), for 3 min. The solvent will usually rise at least 3 cm. above the spotting origin, which is enough to separate the aflatoxin from interfering materials.

9. Examine the plate under a long-wave UV light. A *blue* fluorescent spot at about 0.66, and/or at the same height as the B_1 standard spot, indicates the presence of aflatoxin B_1. The sample contains 10, 20, 30, or 50 p.p.b. B_1 when a

TABLE I. A COMPARISON OF METHODS FOR DETECTION OF AFLATOXIN[a]

Sample	Seitz-Mohr Method	AOAC Screening[b] Method II	CB Method
A	ND[c]	...	ND
B	ND	...	ND
C	8	...	6
D	16	...	12
E	5	...	12
F	15	...	16
G	20	...	21
H	28	...	23
I	35	...	31
J	55	...	48
K	47	...	61
L	ND	ND	ND
M	8	7	5
N	8	12	5
O	12	8	11
P	8	20	11
Q	17	20	25

[a] Values represent p.p.b. aflatoxin B_1.
[b] Reference 6.
[c] Not detected.

sample B_1 spot is equal in intensity to the first, second, third, or fourth standards, respectively, in step 7. Aflatoxins B_2,G_1, or G_2, if present, will be visible below the B_1 spot in this order. Aflatoxin G_1 will be completely separated from B_1, whereas B_2 will overlap B_1 and G_2 will overlap G_1. The aflatoxin spots are to be expected just above a nonfluorescent yellow (that is, yellow in ordinary visible light) spot usually present in analysis of yellow corn.

To analyze a large number of samples, final extracts from step 6 and the standards (step 7) can be spotted separately on one 20 X 20 cm. TLC plate (or 10 X 20 cm. plates spotted along the length of the plates). The plates can be developed for a longer period of time (about 10 min.) to achieve better resolution with very little increase in time spent per sample.

Freon-113 as a Substitute for Hexane

Freon-113 (1,1,2-trichlorotrifluoroethane) is a nonflammable, nontoxic solvent[3] that can be used as a substitute for hexane. Freon-113 has a specific gravity of 1.51. It is the bottom layer in the clean-up extraction (step 3) and is easily drained off. It is easy to wash the aqueous layer a second time with Freon-113 and we recommend that this be done. Use about 15 ml. for the first wash and about 5 ml. for the second wash.

Freon-113 is sufficiently expensive to merit recycling, especially when large numbers of samples are run routinely. This can easily be accomplished by using a simple distillation apparatus to distill the used Freon-113. Its boiling point is only 47.6°C. and the distillation presents no fire hazard because Freon-113 is nonflammable.

[3] According to the Occupational Safety and Health Administration, Freon-113 is one of the least toxic solvents available: it is slightly less toxic than hexane.

RESULTS AND DISCUSSION

We have found that recovery of B_1 and G_1 from spiked, yellow corn samples ranges from 68 to 81% and 59 to 81%, respectively. Recovery can be increased 7 to 9 percentage points by washing the aqueous-methanol layer (step 5) a second time. With natural mold-damaged samples, better agreement with the CB Method (9, 10) is attained if our B_1 p.p.b. values, as based on 100% recovery, are adjusted upward by a factor of 1.7. This adjustment factor has been taken into account in the instructions to spot the quantities of standard specified above. Therefore, the p.p.b. value arrived at by visual comparison of the B_1 spot with the standards will be close to the p.p.b. value obtained by the CB method.

Typical results from our procedure, adjusted as recommended above, are shown in Table I along with the CB method results. Samples A through K were analyzed in our laboratory. To obtain an additional evaluation, samples of yellow corn were sent to O. L. Shotwell at the USDA, ARS, Northern Regional Research laboratory at Peoria, Ill., for analyses by our procedure, the AOAC Screening Method II (6), and the CB method. Samples L through Q represent Dr. Shotwell's results; the results from our procedure have been adjusted as described above. It is noteworthy that our procedure was effective in distinguishing one sample which did not contain aflatoxin from five samples containing 5 to 25 p.p.b. aflatoxin.

The screening test described here can be completed in approximately the same time as required to complete the AOAC Screening Method I (5), and takes less time to complete than the AOAC Screening Method II (6). These comparisons apply to samples analyzed routinely. The detection limits of AOAC Methods I and II are 10 and 5 p.p.b., respectively. Therefore, our procedure is timewise similar to AOAC Method I, but has a detection limit approaching that of the longer AOAC Method II.

Besides routine analyses one should also consider the setup required to run a single sample or a small group of samples in a well-equipped laboratory. To properly use the minicolumn methods (AOAC Methods I and II), one should have on hand a "blank" minicolumn and "standard" minicolumns containing aflatoxin equivalent to specific p.p.b. levels. If not already available, one has to complete the procedure several times to obtain these minicolumns. Our procedure eliminates the need to have available minicolumns, and silica gel, Florisil, or alumina at specific activities. Activation of the adsorbents requires a minimum of 2 hr. and as long as overnight to adjust to the required activity. Our procedure employs unactivated precoated TLC plates. Therefore, we feel many laboratories will find our procedure convenient for either routine or intermittent use.

In addition, our procedure offers a greater degree of selectivity at the final detection step. One can distinguish aflatoxin from other blue fluorescent compounds which might be present in the sample and cause either a false positive minicolumn result or indicate a higher aflatoxin level than actually present. The 3-min. TLC development we use provides complete separation of B_1 from G_1 and partial separation of B_2 from B_1, and G_2 from G_1. Thus, one can obtain an estimate of G_1 concentration as well as B_1 concentration. The minicolumn methods do not separate the aflatoxins. Most laboratories have available standards which contain either B_1 and G_1 or all four of the aflatoxins, B_1, B_2, G_1, and G_2. When these standards are used to prepare "standard" minicolumns required by the AOAC

Screening Methods to estimate aflatoxin levels, an error is built in because the sample may only contain aflatoxin B_1.

We believe our procedure is of adequate simplicity to be useful in field applications. It is at least as simple as the AOAC Screening Method I recently adopted by AOAC for field use, while at the same time it offers improved sensitivity and differentiation between aflatoxins. We believe it would be as easy to train personnel to perform the procedure described here as either of the AOAC methods.

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