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Rapid Screening Methods for Aflatoxin in Corn

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Aflatoxin B₁, a liver toxin and carcinogen, may be produced by the fungi Aspergillus flavus and A. parasiticus as they grow on many agricultural products at certain conditions and temperatures. The present FDA administrative action guideline is 20 parts per billion (ppb) total aflatoxins for all products intended for feed or food. However, this guideline may be changed in the future depending upon whether the product is intended for human or animal use, and, possibly, categorized according to the age and species of animal to be fed. Young animals are more susceptible than older ones, and species of animals vary markedly in their susceptibility. Generally, mature cattle and sheep are the least affected. As aflatoxin is excreted in milk, a stringent consideration will be given for dairy cattle rations.

Methods of Detection

The blacklight technique (BLT) does not determine the presence of aflatoxin. However, it is widely used to indicate the growth of the fungi that may have resulted in the production of aflatoxin. Thus, the BLT is strictly a presumptive but useful test. Samples which give a positive blacklight reaction should be checked by a chemical assay procedure to determine the presence of aflatoxin.

Three chemical assay methods are used to determine the presence of aflatoxin in most grains and feeds: the minicolumn (MC), thin layer chromatography (TLC), and high pressure liquid chromatography (HPLC). These tests, except for HPLC, are described briefly in Table 1 along with their recommended use.

The blacklight and the minicolumn techniques are discussed in detail because of their adaptability to field use. The thin layer chromatography procedures are detailed in the Official Methods of Analysis of the Association of the Official Analytical Chemists (Chapter 26).

A partial list of firms that perform aflatoxin analyses by MC or TLC is available. See your local county Extension agent or write the Dept. of Botany and Plant Path-

*Grateful thanks are given to Dr. Odette Shotwell, Dr. Leonard Stoloff, and Dr. Charles Holaday for assistance in preparation of this manuscript.

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ology, Lilly Hall of Life Sciences, Purdue University, for the current list.

Proper Sampling and Sample Preparation

Proper sampling and sample preparation is extremely important! A representative sample must be obtained regardless of which chemical assay method is used. Sampling is important because a relatively few kernels in a lot may be the source of significant contamination. Occasionally, a biased sample may be more revealing than a representative one; for example, corn selected from an area of a bin where leaks, caked feed, or heating have occurred may be more appropriate than a composite bin sample. When it is necessary to detect aflatoxin levels of fewer than 50 to 100 ppb, a 10-pound properly probed or grain stream sample should be obtained. Statistical studies indicate there is a decreasing probability of detecting 20 ppb in corn with decreasing sample size. The total sample should be coarse ground. If positive with the blacklight technique, the sample should be mixed and 2 pounds finely ground for the analytical sample.

Blacklight Technique

The blacklight technique is the quickest method of detecting grain that could contain aflatoxin, but remember, this technique does not detect aflatoxin. This presumptive test detects parts of kernels that glow with a bright green-yellow fluorescence (BGF). The BGF is usually produced by *A. flavus* or *A. parasiticus* when growing on living corn, cottonseed, sorghum, wheat or barley kernels. Seed that has been killed by high temperature drying usually will not produce BGF. No BGF is produced in soybeans although healthy soybean seeds without the seed coat fluoresce a dull yellow green in UV light. BGF is usually confined to the starchy part of the kernel and the peripheral parts of the germ. BGF is apparently produced by a reaction of fungal-produced kojic acid and a seed enzyme, peroxidase. The latter is associated with living seed. Thus, BGF is not from aflatoxin, but is a unique fluorescence associated with the growth of *A. flavus* or *A. parasiticus* in living seed. BGF may be associated with the presence of aflatoxin, but there are individual strains of the fungus that produce BGF but produce little aflatoxin. Most, if not all strains of the fungus that produce aflatoxin, produce BGF. Aflatoxin may be produced without BGF in seed that has been previously killed. However, there often appears to be an approximate positive relationship with the number of BGF particles and the amount of aflatoxin. Some operators concern themselves only with samples that have at least 3 to 5 positive BGF particles.

Errors (false positives) may result from a faulty interpretation of fluorescent materials in a grain sample, or, particularly in a feed mixture. Certain foreign materials and some weed seeds (jimson weed at the hilum end or without a seed coat) may fluoresce bright green yellow similar to that of BGF. Corn kernels and cob tips, especially when exposed to weather, may fluoresce yellow. Tips of corn kernels and glumes (bees wings) may fluoresce a light yellow. Soybeans without seed coats fluoresce a dull green yellow. In general, false positives do not fluoresce with the color of BGF, or they do not fluoresce as brightly (glowers) as BGF. False positive errors may be reduced by the following procedures: (1) Refer to an authentic BGF particle that has not been overly exposed to light or UV light. (2) Refer to a color standard*. (3) Check the solubility of the fluorescent material. It should be water soluble. Note that false BGF from cobs and glumes is not water soluble. The fluorescence from jimson weed seed is water soluble, but usually the seed can be recognized. (4) Confirm that the fluorescing particle is from the starchy endosperm or germ fragments of a corn kernel.

*Color standard available from USDA. Regional Laboratories, Peoria, Illinois 61604.
It is recommended that grain be cracked before inspection with the blacklight. Some positive samples may be missed by examination of whole kernels and screenings only, but the latter is helpful as it allows the operator to identify glumes, pieces of cobs or weed seeds that may be fluorescing. Ground samples permit detection of more positive samples, but more false positives may be detected if care is not taken. A vibrating table, enclosed in a box, with a viewer has been designed by CPC International and is used to inspect intact samples for BGYF without any preparation.

Samples can be cracked or coarsely ground with a Straub disk mill, coffee grinder or a Tag-Heppenstahl moisture meter equipped with a large soybean shim. A grain mill attachment on a Hobart-Plantary mixer is also useful. Food blenders, such as a Waring blender, may be used, but they grind the sample too fine, and their capacity is limited. Fine grinding is usually accomplished with a Hammer mill or Wiley mill. The corn should pass a no. 20 sieve to be a proper analytical sample.

Blacklight examination should be made in the dark or in a darkened chamber with a long wave ultraviolet (about 365nm) lamp. Do not use short wave ultraviolet or germicidal lamps. A high intensity lamp is recommended but a suitable, inexpensive substitute is a fluorescent fixture fitted with a BLB lamp shielded to keep the light out of the operator’s eyes. Goggles that screen out UV light increase contrast and lessen eye strain and possible eye damage with continued exposure.

When a positive BGYF sample is encountered, a chemical assay is required to determine if aflatoxin is actually present. It is important not to make a final judgment of whether the grain contains aflatoxin without actually testing for aflatoxin by chemical means. The minicolumn does this quickly but with some sacrifice in precision as compared to TLC.

Minicolumn Technique

The Holaday and Lansden minicolumn technique described below detects aflatoxin in amounts perhaps as low as 2 ppb in a variety of agricultural products. However, it does not work with some feeds. Another minicolumn technique (Roemer’s) is designed and used extensively for feeds. It is more involved and requires about 30 minutes, while the Holaday and Lansden method requires about 10 minutes. For details of Roemer’s technique refer to the Journal of AOAC 58:500-506, 1975.

Our experience with the Holaday and Lansden technique indicates it is valuable for corn because it requires inexpensive equipment, is simple to perform and requires a minimum of time. This technique does not distinguish the different aflatoxins although B1 is almost always predominant, and aflatoxin is not unequivocably demonstrated. Occasionally false positives have been encountered with this technique.

Procedure for Holaday and Lansden Technique**

Rinse all reusable glass containers with bleach between samples, then wash thoroughly. Disposable plastic ware may be substituted to avoid washing and possible contamination. Handle samples with care. If extracts contact skin, immediately wash thoroughly with soap and cold water. The use of disposable, lightweight, plastic polyvinyl gloves is recommended. Analysis should be performed in a well-ventilated room and solvents added under a ventilated chemical hood, or efficient

*According to a recent study by M. Goulden and O. L. Shotwell (personal communication) this method can be relied on by any laboratory for 10 ppb and 5 ppb for some laboratories.

exhaust system. Benzene originally recommended may be leukemogenic, but toluene is a good substitute for benzene. Toluene should be handled with care. Dispose used chemicals in such a manner that vaporization does not occur. See Figure 1 and List of Chemicals and Equipment at end of this section.

1. Grind sample finely enough to pass through a no. 20 mesh screen.

2. Blend 100 grams of finely ground sample with 200 milliliters of methanol-water solution for 1 minute at high speed in a blender (any size sample may be used as long as the ratio of sample weight to solvent volume remains the same; 50g is often used).

3. Fold fast filtering paper into a funnel and filter 10 ml of sample into a culture tube fitted with a plastic-lined screw cap or rubber stopper. Tubes can be calibrated for 10 ml and marked with tape.

4. Add 10 ml of salt solution to test tube and shake vigorously for 5 to 10 minutes.

5. Filter 15 ml of contents through a glass filter fiber into a second culture tube.

6. Add 3 ml of toluene to solution, close tube and shake vigorously for 10 seconds. If problem with emulsion, shake less vigorously.

7. Let layers separate and pipet (use bulb or mechanical device on pipet, do not aspirate by mouth) 1 ml of upper layer toluene into the top of a minicolumn. Be sure not to pipet material from lower level.

8. After the toluene has been pulled through the minicolumn by vacuum, add 5 ml of the hexane-acetone washing solution to the top of the minicolumn and pull through. Evacuate column for an additional 2 minutes or until all of the washing solution has evaporated from the minicolumn. Do not use too high a vacuum, as part of the column packing may be disrupted. Also, too high a vacuum may diffuse the fluorescent zone and increase difficulty in reading the minicolumn. If no vacuum is available, the column may be drained by gravity which will increase the time by 20 to 30 minutes.

9. Observe minicolumn under long wave ultraviolet light. Use a high intensity light, such as a B-100 A BLAK-RAY lamp. A high intensity lamp is very important in the detection of borderline samples.

10. A blue fluorescent band appearing in the center of the column, below the interface of the florisil and alumina, indicates at least 4 ppb of aflatoxin.

The amount of liquid from the toluene layer determines the detection limit of the method and can be used to design a "go" or "no go" system at various approximate levels as indicated as follows: 2 ml, 2 ppb; 1 ml, 4 ppb; 0.5 ml, 8 ppb; 0.2 ml, 20 ppb; and 0.1, 40 ppb. Keep in mind these are approximate levels. Instead of adding 0.1 or 0.2 ml directly to column it may be advisable to dilute to 1 ml with toluene and then add 1 ml of dilution. This is more accurate and will give a more uniform zone. Alternatively, add 1 ml of hexane-acetone washing solution before the 0.1 or 0.2 ml of the toluene extract is added to the column.

Chemicals and Equipment Required (all reagent grade)

Glassware
(May be of disposable plastic to eliminate washing, but a rapid rinse in bleach and water wash works well.)

Toluene or benzene
Methanol-water solution (80:20, v/v)
Salt solution
(600g NaCl, 600g zinc acetate and 15 ml of glacial acetic acid dissolved in 4 liters distilled H₂O).
Hexane-Acetone solution (80:20 v/v)
Waring blender
(an explosion-proof model preferred)

Vacuum source
(aspirator on water line or vacuum pump)

Minicolumn
Prepared, ready to use minicolumns (be sure to specify which kind, Holaday, CPC, Roemer) may be purchased from:

- Ag-Science
  P.O. Box 253
  Shellman, GA 31786

- Schuman Chemical Laboratories
  P.O. Box 196
  Battle Ground, IN 47920

- The Myco-Lab Co.
  P.O. Box 321
  Chesterfield, MO 63017

- Tudor Scientific Glass Co.
  555 Edgefield Road
  Belvedere, SC 29841

Keep minicolumns dry and out of light.

Holaday minicolumns may be made with glass tubing 5.5 mm id and 160 mm long with 15 mm florisil (100-200 mesh) on bottom and 15 mm of neutral alumina (100-200 mesh activity IV or E. Merck Activity I, dry at 100°C for 2 hrs. then add 15% distilled water by weight. Mix well, let stand at least 2 hrs.) immediately above, held in place with 4 to 6 mm of paper pulp packing at bottom of florisil and at top of alumina.

Another minicolumn that may be used and considered by some to give a more distinct band and more sensitivity is the CPC column. It is more expensive than the Holaday column, see JAOAC 57:764 1974.

Test tubes with screw or watertight plastic caps

Graduated cylinders, 25 ml or 10 ml and a 100 ml

Pipettes
(1 ml and mechanical device to draw up fluid).
Do not pipette solvents by mouth. Short pipettes are easier to handle than long. Automatic pipette devices will speed up dispensing of salt solution and toluene.

Glass fiber filter, 12 cm
Filter paper
(such as Whatman 2V or 114 pleated, 18.5 cm, but size is not critical).

Funnels to fit filters listed above.

Blacklight

May be purchased from:

- LaPine Scientific Co.
  6001 South Knox Avenue
  Chicago, IL 60624

- Seedboro Equipment Co.
  1022 West Jackson Blvd.
  Chicago, IL 60607

- Scientific Products
  1210 Leon Place
  Evanston, IL 60201

- Ultra-Violet Products, Inc.
  5100 Walnut Grove Avenue
  San Gabriel, CA 91778
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<tr>
<td>Blacklight</td>
<td>A presumptive test only. Cracked corn or screenings are viewed in dark with long wave ultraviolet light (about 365 nm). A bright green yellow fluorescence (BGYF) &quot;glow&quot; in floury endosperm indicates the growth of Aspergillus flavus and possible aflatoxin contamination. A color standard or an authentic sample of BGYF should be used for purpose of comparison; the latter fades. Authentic BGYF is water soluble and can be eluted from fluorescing particle.</td>
<td>A quick presumptive test--does not tell if aflatoxin is actually present</td>
<td>Quick, but only indicative of Aspergillus flavus. Aflatoxin must be confirmed by one of the following methods. Positive samples may not have aflatoxin or be below the guideline. Fluorescent substances such as soybean fragments, weed seed, foreign material may be confusing, particularly in feed.</td>
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<td>Holaday Minicolum</td>
<td>Ground corn is extracted with methanol and filtered, extraneous materials are precipitated with salts and remaining solution re-extracted with another solvent and a small amount added to a minicolum containing two absorbent layers. The extract is washed down the column with acetone-hexane. Aflatoxins migrate to juncture of absorbents and are detected by a blue fluorescent band under (long wave) ultraviolet light.</td>
<td>Semi-quantitative procedure that reveals aflatoxin levels as low as 2ppb.</td>
<td>Relatively fast--takes about 9-11 minutes. Does not measure precise amounts of aflatoxin but can be modified to give a &quot;go&quot; or &quot;no go&quot; at various levels from 4-6 ppb. This shortened minicolum technique is probably not suitable for mixed feeds.</td>
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<tr>
<td>Thin Layer Chromatography (TLC)</td>
<td>Corn is extracted with chloroform, filtered and evaporated almost to dryness. The corn oil is spotted on a glass plate, thinly coated with an absorbent. An aflatoxin standard is also spotted. The bottom of the plate is placed in a solvent usually for 10 to 15 minutes. Initial development in ethyl ether solvent before the regular solvents helps to eliminate interfering substances. This last procedure eliminates the use of a clean-up column that is recommended in the official AOAC procedure. The plate is allowed to dry and examined under UV light. The spot from the unknown is compared with standard aflatoxin for color and migration distance. A more elaborate extraction and purification procedure is required for mixed feeds.</td>
<td>A quantitative method and the different common aflatoxins -- B₁, B₂, G₁, G₂, M₁ can be identified and if necessary, readily confirmed by additional chemical tests on the TLC plate.</td>
<td>Slow and somewhat expensive. However, precise and reasonably accurate, particularly when used with a densitometer.</td>
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**Figure 1.**

**RAPID SCREENING METHODS FOR AFLATOXIN IN CORN**

1. Blend a 100 gram sample with 200 milliliters of methanol-water solution for 1 minute at high speed in a blender (any size sample may be used as long as the ratio of sample weight to solvent volume remains the same).

2. Fold fast filtering paper into a funnel and filter 10 ml of sample into a culture tube fitted with a plastic lined screw cap or rubber stopper can be used.

3. Add 10 ml of the salt solution to test tube and shake vigorously for 5 to 10 sec.

4. Filter 15 ml of contents through a glass filter fiber into a second culture tube.

5. Add 3 ml toluene to solution, close tube and shake vigorously for 10 seconds.

6. Let layers separate and pipet (use bulb or mechanical device on pipet) 1 ml of upper layer benzene into the top of a minicolumn.

7. After the toluene has been pulled through by vacuum add 5 ml of the hexane-acetone washing solution to the top of the minicolumn and pull through. Use vacuum for an additional 2 minutes or until all of the washing solution has evaporated from the minicolumn. Do not use too high a vacuum.

8. Observe minicolumn under long wave ultraviolet light.

9. A blue fluorescent band in the center of the column, at the interface of the florisoril and alumina, indicates at least 4 ppb of aflatoxin.
The information given herein is supplied with the understanding that no discrimination is intended and no endorsement by the Indiana Cooperative Extension Service is implied.