NEW METHODS FOR RAPID DETECTION OF AFLATOXIN

C. W. HESSELTINE and O. SHOTWELL

Northern Regional Research Laboratory†, Peoria, Illinois, 61604, USA

ABSTRACT

The rapid detection of mycotoxin contamination in agricultural commodities is an area of research that requires more attention. The many companies buying hundreds of bushels of cereal grains daily, need to inspect their purchases immediately and to make a decision in minutes. Arising from this need to detect aflatoxin, we have developed two methods for rapid identification of aflatoxin in corn. The first is based upon a glowing greenish-gold fluorescence produced under ultraviolet light (365 nm) by corn kernels that contain aflatoxin. The fluorescent material is not aflatoxin but a compound associated with it. Fluorescence depends upon the interaction between enzymes in corn and a compound produced by members of the *Aspergillus flavus* series. Heat-sterilized corn inoculated with *A. parasiticus* does not fluoresce, although aflatoxin is formed. Fluorescence is seen in broken kernels but it is not visible in intact corn kernels until they are broken. Laboratory studies show that this method of detection is applicable for other cereals, including wheat, rice, oats and barley, but is apparently not effective for soybeans and peanuts. The second is a modification of the chromatographic mini-column method devised for corn.

The rapid detection of mycotoxin in contamination of agricultural commodities and products is an area of research that requires much more attention. Elevator operators, grain buyers and processing companies buy thousands of bushels of cereal grains daily, and in buying, they need to inspect their purchases immediately and to make a decision in minutes. To give you an idea of the magnitude of this problem, remember that this year the corn crop in the United States is estimated to be more than 5 billion bushels. In response to the need to detect aflatoxin rapidly in corn (maize), we have developed two methods within the past year.

Until 1971, levels and incidence of aflatoxin detected in corn from commercial markets caused no alarm. The levels of contamination were less than 50 ppb. However during 1971, it became apparent that a problem did exist, at least for that crop year, in certain areas where white corn is grown.

During our earlier corn surveys, we had hoped to make a visual observation of grains that would enable us to detect toxin-contaminated grains. We

† Agricultural Research Service, US Department of Agriculture. Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

thought perhaps the blue fluorescence of aflatoxin B_1 under ultraviolet (u.v.) light would be a helpful property. However, very few aflatoxin-contaminated corns were available and nothing in their appearance appeared to be unusual. It was only when more corn samples containing aflatoxin became available that Mrs Marion Goulden, one of our technicians, noticed that they contained kernels which gave a bright greenish, yellow colour under u.v. light. The kernels appear to glow and the fluorescence has been described as 'firefly-like'.

We then started studies on the possibility of detecting kernels with aflatoxin in them by a rapid scanning under u.v. light at 365 nm. The corn sample is spread out in a flat container and inspected for pieces or kernels of corn having the characteristic glowing fluorescence. The flat container must be shaken in order to turn each piece over to expose all sides to the light. We also found that coarse grinding of the sample was desirable before inspection, as is discussed later.

Johnson and co-workers[1] claimed that aflatoxin might be concentrated in the 'dockage'—broken kernels and foreign material—and could be easily removed by physical means. In their study they had added 12-year-old aflatoxin-contaminated corn to uncontaminated corn samples. When the dockage was removed from the blended mixture, the aflatoxin was in the dockage. We now know that this is not typical of a naturally contaminated corn.

In our initial studies, we established a correlation between a characteristic greenish-yellow glowing fluorescence in damaged corn and the presence of aflatoxin. Our findings on aflatoxin in broken corn-foreign material (BCFM) and other fractions of corn moving in commercial channels have only just been published (Shotwell *et al.*[2]).

In this study, we examined 34 corn samples, both aflatoxin positive and negative, which came from commercial markets and had been collected in previous surveys. As samples were received, each was divided into two 1-kg portions, one of which was ground for the original assay of the total sample. Unground portions were shaken on a grain-grading 12/64 sieve. The material that passed through the sieve (BCFM) was collected and weighed. BCFM is defined as pieces of corn and small particles which pass readily through a 12/64 sieve as well as large particles obviously not corn. Particles that had a greenish-yellow glowing fluorescence, as detected with a Blak-Ray B-100 high-intensity light or a UVL-22 hand lamp (each 365 nm wavelength), and that were large enough to pick out with tweezers were removed, weighed and extracted. Particles and pieces of corn with other kinds of fluorescence were also separated for study. From all samples, bigger pieces of corn and kernels not passing through the 12/64 sieve, but having the same kind of greenish-yellow fluorescence, were also picked out for extraction. The following fractions were hand-selected from portions of seven samples that did not pass through the sieve: large broken pieces, whole kernels with mould damage or cracks and whole undamaged kernels. The scheme used to separate corn into the various fractions (*Scheme 1.*) is taken from our earlier article (Shotwell *et al.*[2]).

How the kernels of corn appear under u.v. is shown in *Figure 1* in black and white. Unfortunately even with the best photography, the glowing

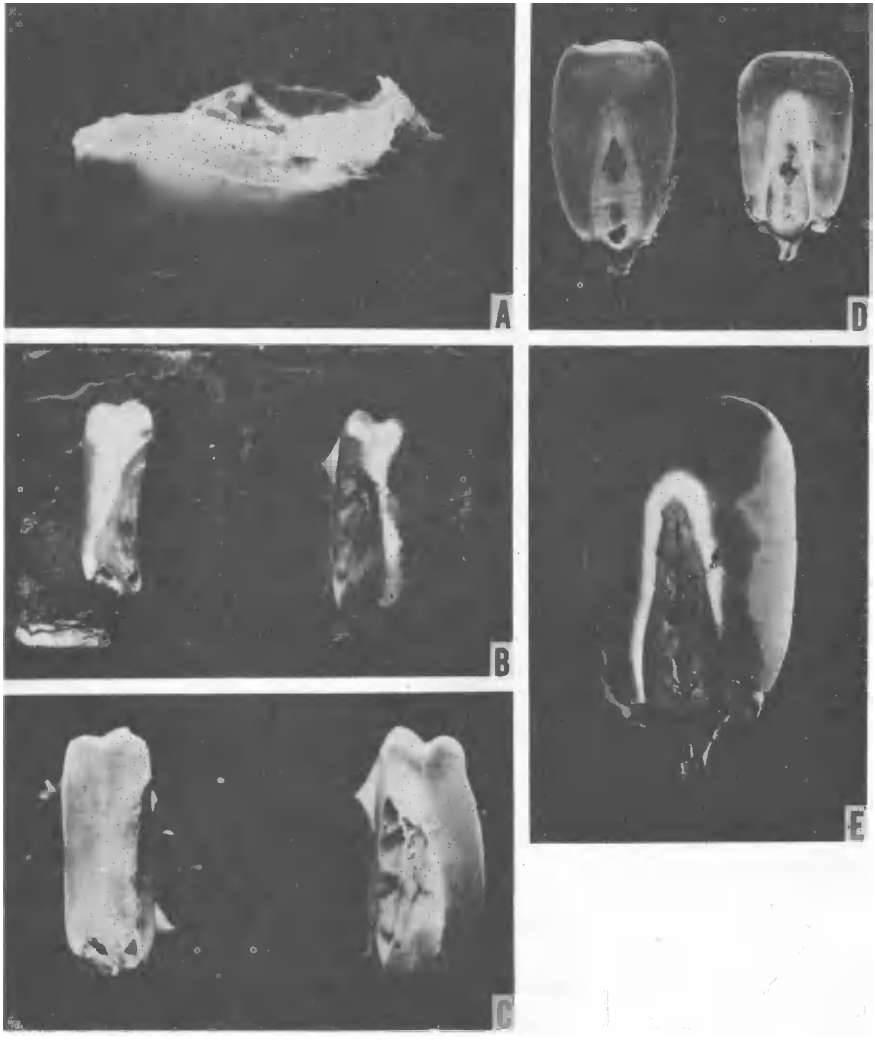
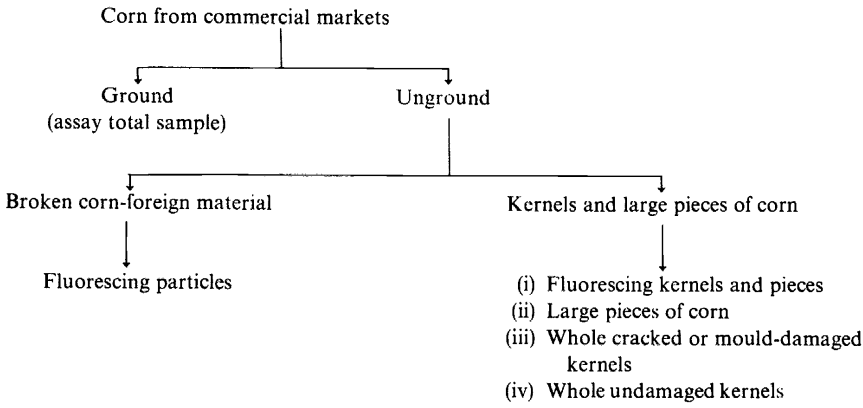


Figure 1. A, naturally broken corn kernel with fluorescence; B, two corn kernels photographed in daylight; C, the same kernels as in B photographed under u.v. light, the one on the left showing fluorescence; D, two sectioned corn kernels, one without fluorescence (right) and one with fluorescence outlining the germ; E, sectioned corn kernel showing area of intense fluorescence about the germ. All pictures were photographed under ultraviolet (u.v.) light except B



Scheme 1.

particles cannot be adequately reproduced. A shows a naturally broken corn kernel under u.v. light. We soon recognized that not all the aflatoxin occurred in dockage and in broken kernels, and that often the fluorescence was hidden until the kernel was broken open. B shows two corn kernels split open and photographed in daylight. One notes that the germs are broken and dead in both pictures. The same kernels are photographed under u.v. light (C); the light parts had the characteristic bright greenish-yellow fluorescence. D shows two kernels under u.v. light, one without fluorescence and the other with a definite fluorescence outlining the germ area. E contains a kernel cut in the same plane to reveal the area of intense fluorescence.

Here it is necessary to point out that much corn will fluoresce many different colours, including blue, orange, yellow and bright red. These colours are perhaps from other mould metabolites or they arise from corn varietal differences or even extraneous chemicals or a combination of these factors.

The difficulty we have encountered with people using this detection method is the incorrect interpretation of different kinds of fluorescence as being the glowing bright greenish-yellow fluorescence. We have conducted several training courses to teach technical and non-technical people what are positive kernels. Due to the efforts of Mr Jack Swarthout (Chairman, Research Committee, American Corn Millers Federation) a chemical used as an optical brightener was found which under u.v. light appears almost identical to the glowing fluorescent corn kernels. We have now prepared small samples sealed in glass vials, which are available from the Northern Laboratory, as visual standards. The optical brightener has to be protected from high humidity because it tends to adsorb water and the hydrated compound fluoresces pale blue. This material is sold by Ciba-Geigy as Tinopal BV. The compound responsible for the greenish-yellow fluorescence in corn is not stable to u.v. light; contaminated corn kernels lose their fluorescence rapidly when exposed to u.v. light and daylight. The optical brightener is more stable.

Besides being able to identify the colour of the characteristic fluorescence, one should be aware of the fact that highly contaminated kernels may be relatively undamaged and have 'hidden' fluorescence—that is the kernels must be broken for the fluorescence to become visible. We recommend

cracking the corn before inspection under u.v. light. By shaking the cracked corn kernels two or three times to bring up the various sides of the cracked corn to show fluorescence, any fluorescing kernels can be detected in a minute or two.

Should the corn be 'fluorescent' positive, one of the official methods for analysis of aflatoxin needs to be run. The analyst may use either the rapid mini-column method we shall describe or the CB method. These methods will confirm whether aflatoxin is present and will give the actual amount. The bright greenish-yellow fluorescence method is not quantitative, nor is it a direct detection of aflatoxin itself. The bright fluorescence is caused by one or more compounds formed when the mould grows on the corn.

We next had to prove that the firefly-like particles and kernels contained aflatoxin.

Particles from BCFM and damaged corn kernels were placed in small vials and steeped for 48 h in chloroform (2–3 ml) and a drop of water. The chloroform layer was removed, and solids were washed with small portions of chloroform until the chloroform wash did not fluoresce the typical bluish colour associated with aflatoxin B_1. The combined extract and washes were evaporated on a steam bath under nitrogen for thin-layer chromatography (t.l.c.). If t.l.c. of extracts indicated aflatoxin could be present, identity of the mycotoxin was confirmed by water and acetate adjuncts.

Extraction of particles and damaged kernels possessing the bright greenish-yellow fluorescence revealed that they contained aflatoxin. All of the available unground samples of corn in which aflatoxin had been detected in previous surveys were inspected for particles and damaged kernels that fluoresced bright greenish-yellow. Eighteen particles and damaged kernels with greenish-yellow fluorescence were selected by hand from the unground corn. Seventeen contained high levels of aflatoxin B_1 after being extracted as described. The fragment that contained no aflatoxin was so small (0.002 g) that B_1 would have had to be present at levels of 3000 ppb to be detected. The greenish-yellow fluorescence disappears when aflatoxin is removed by chloroform extraction. Some of the other damaged kernels fluoresced white or blue, and a number of these were hand-selected and extracted. Although a white or blue fluorescence is typical of aflatoxin B_1 itself, extracts of samples fluorescing white or blue contain no B_1.

Material responsible for the glowing greenish-yellow fluorescence that we observed in corn may be identical to that observed in cotton fibres by Marsh *et al.*[3,4] Evidence indicates that the fluorescing substance in cotton fibres is formed by a heat-labile enzyme in the living plant that oxidizes kojic acid produced concurrently with aflatoxin by *Aspergillus flavus* and *A. parasiticus*. Participation by enzymes in the corn kernel or germ was indicated by our failure to obtain the glowing greenish-yellow fluorescence when rice and wheat that we inoculated and incubated had been autoclaved, and thus sterilized. The enzyme undoubtedly had been destroyed by the autoclaving. However, good production of aflatoxin occurred, showing that the fluorescing material is not a direct product of metabolism of the micro-organism during aflatoxin production. We are now attempting to establish a relationship between the greenish-yellow fluorescence we observe in corn and that found in cotton fibres and other living plant tissues.

The greenish-yellow fluorescing material in damaged kernels appeared to be located next to the germ in many instances. Kernel parts that fluoresced were structurally weakened and tended to crumble easily. Levels at 284–101 000 ppb B_1 were detected in separated damaged kernels (*Table 1*).

Table 1. Aflatoxin B_1 (ppb) in damaged corn kernels and large pieces with bright greenish-yellow fluorescence

Original corn sample		B_1 in kernels
Grade US No.	B_1 contamination	and pieces
1	0.7	88 500
2	47	5320
3	25, 18, 15	2080, 1060, 8090
4	16, <6	284, 285
Sample grade	8	101 000

The entire ground sample of US Grade No. 1 contained 0.7 ppb B_1 (average of four values ranging from 0 to 1.5 ppb). From the unground portion, a kernel with 88 500 ppb B_1 was removed. If this kernel had been in the sample originally ground and had been the only aflatoxin-containing kernel in the sample, the resulting meal would have analysed 9 ppb B_1. The problems involved in obtaining a representative sample of corn as discussed by Johnson *et al.*[5] are understandable.

The BCFM from available unground portions of 13 aflatoxin-positive samples from previous studies was assayed for the presence of aflatoxin. Seven samples had higher levels of B_1 in the BCFM than was determined in the total sample; two about the same levels of toxin in both; and four, less in the BCFM than in the total sample. Assuming that the level of B_1 in the separated BCFM analysed was the same as the BCFM of the original total sample, we calculated the percentage of total toxin accounted for in the BCFM. In two samples, almost all the aflatoxin B_1 was in the BCFM. However, B_1 was not always concentrated in this part of a corn sample. As seen before, damaged kernels and large pieces of corn that do not pass through a 12/64 sieve can be highly contaminated with B_1.

Several possibilities exist in which the fluorescence method may not be applicable. If corn were overheated to the point of destroying the enzyme during drying followed by wetting due to moisture condensation, roof leaking, or moistened in some other fashion, and if the corn is reinoculated with *A. flavus*, or *A. flavus* spores survive the heating, then aflatoxin may be formed without the corn showing fluorescence. At present, we know an enzyme(s) in the corn must be present to give the glowing greenish-yellow fluorescence. As yet we do not know how much heat is required to inactivate the corn enzyme(s). We also know that the fluorescing kernels, when exposed to light, gradually lose their fluorescence although stability seems to be good in the dark. Even after 12 years in storage, corn containing aflatoxin still fluoresces.

The following conclusions can be drawn from our study of this fluorescence

method. (*a*) All fragments or kernels of corn that have the characteristic fluorescence contain aflatoxin. The corn sample in which they occur usually will contain appreciable amounts of toxin, but in some samples levels may not be appreciable or even detectable. (*b*) The obviously glowing fragments or kernels generally do not contain all of the aflatoxin. Much of the toxin occurs in relatively undamaged kernels that must be cracked to observe the fluorescence. (*c*) The glowing material is not aflatoxin, but a compound normally associated with it. Fluorescence depends upon the interaction between an enzyme in corn and a compound, possibly kojic acid, produced by members of the *A. flavus* series. (*d*) Heat-sterilized corn inoculated with *A. parasiticus* does not fluoresce bright greenish-yellow but aflatoxin is produced. (*e*) Laboratory work shows the method is applicable for predicting the presence of aflatoxin for other cereals including wheat, sorghum, rice, oats and barley, but not such oilseeds as peanuts or soybeans. (*f*) All particles of glowing material from naturally contaminated corn when surface sterilized, placed on nutrient agar, and incubated at 28°C show the presence of members of the *A. flavus* series. (*g*) Although fluorescence is often associated with the germ area, some kernels may be found in which the other parts of the kernel fluoresces. (*h*) The time required to make this u.v. test is only a few minutes. Not more than 5 min are required to check a 5–10 lb sample. (*i*) The method is so simple that unskilled persons, if they have fluorescent standards, can be trained in an hour to do the test reliably. (*j*) Mechanical separation of dockage will not remove all contaminated corn. (*k*) This method of aflatoxin detection has already been widely accepted in the United States by corn-milling companies with follow-up by analysis of suspect samples to establish whether aflatoxin is present. (*l*) This detection method is used also to monitor mill streams as a further check on possible contamination of corn products.

The second rapid method is a direct detection of aflatoxin and makes use of the mini-column originally devised for use in detection of aflatoxin in cottonseed meal by USDA's Southern Laboratory. Currently, we are conducting a collaborative study for the Association of Official Analytical Chemists on three methods involving the mini-column method—the Southern Regional Research Laboratory, the Northern Regional Research Laboratory and the Velasco methods. The mini-column method involves the use of small glass mini-columns made from Pyrex tubing cut into 20 cm lengths and requires about 20 min to detect aflatoxin levels down to 10 ppb. The Northern Regional Research Laboratory's method (Shannon *et al.*[6]) involves the use of ammonium sulphate to precipitate impurities instead of lead acetate, and use of acetone–water extractant instead of acetonitrile–water in order to avoid disadvantages relative to personnel safety and water pollution presented by the use of acetonitrile and lead acetate. Plans are to publish both the Southern (Pons *et al.*[7]) and Northern methods in the *Journal of Association of Official Analytical Chemists*. We know that some of the US corn-processing companies with laboratory facilities are using our method for detecting aflatoxin.

We suggest that the fluorescence method be used as a preliminary check on all samples of corn. If no glowing fluorescent particles are seen in a properly collected and coarsely ground sample, no further tests need to be made. If

glowing particles are found, then the mini-column method should be used to see if detectable aflatoxin is present. This second examination will eliminate the possibility of rejecting aflatoxin-free lots of corn.

REFERENCES

[1] R. M. Johnson, W. T. Greenaway and W. P. Dolan, *J. Assoc. Offic. Anal. Chemists*, **52**, 1304 (1969).

[2] O. L. Shotwell, M. L. Goulden and C. W. Hesseltine, *Cereal Chem.*, **49**, 458 (1972).

[3] P. B. Marsh, M. E. Simpson, R. J. Ferretti, T. C. Campbell and J. Donoso, *J. Agr. Food Chem.*, **17**, 462 (1969).

[4] P. B. Marsh, M. E. Simpson, R. J. Ferretti, G. V. Merola, J. Donoso, G. O. Craig, M. W. Trucksess and P. S. Work, *J. Agr. Food Chem.*, **17**, 468 (1969).

[5] R. M. Johnson, W. T. Greenaway and C. Golumbic, *Cereal Sci. Today*, **14**, 25 (1969).

[6] G. M. Shannon, R. D. Stubblefield and O. Shotwell, *J. Assoc. Offic. Anal. Chemists.* in the press.

[7] W. A. Pons, Jr., A. F. Cucullu, A. O. Franz, Jr., L. S. Lee and L. A. Goldblatt, *J. Assoc. Offic. Anal. Chemists*, in the press.