Detecting Aflatoxin in Agricultural Commodities

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Dr. Dowell is a Research Agricultural Engineer with the USDA ARS. He has about 30 years of experience developing technology to measure grain quality and improving food security in developing countries. He has over 20 years of experience in international agriculture, and is President of Planting Hope International, a non-profit charity that provides agricultural, medical, and educational support to people in developing countries. He has seen issues with aflatoxin in countries such as Kenya and Laos, where the lack of aflatoxin testing programs has affected human and animal health. Much of his experience is in measuring and maintaining grain quality. His interest in learning more about production agriculture in the tropical climates of developing countries led him to recently take the ECHO TAD course.

Introduction

Aflatoxin, a mycotoxin produced by the fungi *Aspergillus flavus* and *A. parasiticus*, can negatively affect animal and human health. Its presence in many agricultural crops is a concern, and levels are often regulated in domestic and international trade. Aflatoxin is typically not a problem in healthy crops that are handled and stored properly. However, when crops become stressed (such as when damaged by insects or drought, or when stored improperly at high moisture content), the fungi that produce aflatoxin can infect the seeds in the field or in storage (Figure 1). The conditions favorable for aflatoxin contamination and the resulting health concerns are reviewed in EDN 87 (https://c.ymcdn.com/sites/echocommunity.site-ym.com/resource/collection/9EE3A8EE-FF5C-45A6-9BA9-0AB3A3E7652E/edn87.pdf). While the occurrence of aflatoxin is relatively rare and levels in food and feed are usually very low, it can be a concern in cereals, oilseeds, tree nuts, fruits, and spices.

Aflatoxin testing programs are a routine part of many import and export trade policies, and an established component of the farmer-marketing process for commodities like peanuts in some countries. However, testing is less common in countries where there is little or no financial incentive to reward the seller for delivering a high-quality crop. As economies and crop yields in developing countries improve, opportunities arise for sellers to be rewarded for delivering high-quality commodities. This has been realized in countries like Laos, Kyrgyzstan, and Kenya, where private and government grain buyers and feed producers are seeing the financial advantages of guaranteeing a safe and wholesome product. As in the U.S., an individual farmer may not be likely to implement an aflatoxin testing

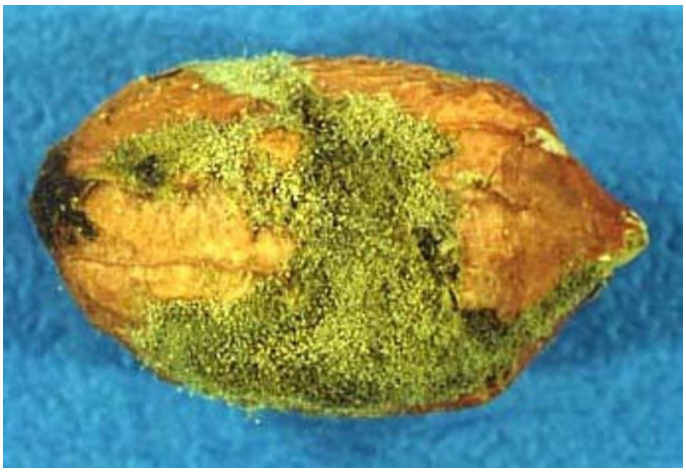

Figure 1. Moldy peanut kernel that likely has high levels of aflatoxin.
Photo: Floyd Dowell

program. Those that buy grain and sell it as food or feed are perhaps best positioned to implement a sampling and testing program that can reward sellers for delivering a safe wholesome product; these grain buyers can then in turn sell the grain as higher-quality feed or food.

Approved aflatoxin sampling and testing programs for official inspection and international trade are published by Codex (http://www.fao.org/fileadmin/user_upload/agns/pdf/CXS_193e.pdf (http://www.fao.org/fileadmin/user_upload/agns/pdf/CXS_193e.pdf)) and Grain Inspectors Packers and Stockyards Administration (GIPSA) (https://www.gipsa.usda.gov/fgis/public_handbooks.aspx (https://www.gipsa.usda.gov/fgis/public_handbooks.aspx)). However, those wishing to implement local testing programs, or to test in countries where aflatoxin is not regulated, may want to investigate testing procedures for their unique situations. Thus, this article will provide new users with basic information on sampling and testing for aflatoxin, including the benefits and limitations of various strategies.

Sampling for Aflatoxin

Often when discussing the subject of aflatoxin testing, the accuracies of various testing methods are debated. However, most error in measuring aflatoxin is due to sampling variability, rather than the accuracy of the testing method (Whitaker et al., 1994). This is because aflatoxin is typically concentrated in a small percentage of the kernels. For example, if a portion of a field is stressed from drought or disease, seeds from those plants are more likely to become infected with *A. flavus* and aflatoxin. Likewise, seeds that are damaged by insects in the field are more likely to contain the invading fungus. In storage, pockets of high moisture (attractive to *A. flavus*) can occur where a roof leaks, seeds are stored at high moisture content, or insect populations create conditions favorable for fungal growth. The rest of the seeds can be free from aflatoxin, but the very high levels in the few moldy kernels can cause high average levels of aflatoxin in the entire lot. If sampling does not include infected kernels, false negatives will give buyers an impression that the lot contains no aflatoxin. Conversely, if the sample contains mostly moldy kernels whereas the rest of the lot is relatively free from aflatoxin, the very high aflatoxin levels in the sample will lead to rejection of the whole lot, even though the rest of the lot may consist of very good, high-quality seeds.

Traditional sampling plans

If the objective of an aflatoxin testing plan is to have a level of confidence that average aflatoxin levels are below some defined threshold, the sampling plan should follow one of the standard procedures published by organizations such as the United States Food and Drug Administration or the European Commission. These plans typically require that multiple samples of specific size be drawn from a moving stream of grain, or from probes inserted into the grain pile. These large samples are then mixed and subsampled until a size suitable for analyzing is obtained. These traditional sampling plans give buyers and sellers some level of confidence that resulting aflatoxin values represent the grain mass. Of course, the aflatoxin measured in the sample can be higher or lower than the level of the entire lot, but repeatedly obtaining samples during the harvesting and handling process can result in more confidence in the average aflatoxin level of the commodity being bought or sold.

Sampling high-risk seeds

If the objective of the aflatoxin testing plan is to have a level of confidence that the lot has no, or very low, levels of aflatoxin, then only those seeds that are most likely to contain aflatoxin should be tested (Whitaker *et al.* 1998). If it is present, aflatoxin is often concentrated in seeds that have been damaged by insects or disease. These seeds will tend to be smaller, so testing those smaller seeds (obtained by running the sample or lot over a screen so that the smaller seeds fall through) can indicate if there is a potential aflatoxin problem. If no aflatoxin is found in this fraction, the rest of the lot is unlikely to contain aflatoxin.

Cleaning and re-sampling

If an unacceptable level of aflatoxin is detected in a sample, the lot can be cleaned to remove the smaller and discolored seed. The remaining seed can then be tested for aflatoxin to see if levels are below acceptable thresholds. As previously mentioned, smaller seeds are more likely to contain aflatoxin; the same is true of discolored seeds. Thus, removing discolored kernels by hand picking or with an electronic sorter can reduce aflatoxin in the remaining portion. The correlation between small, damaged kernels and high aflatoxin levels has been confirmed in tree nuts, ground nuts, and cereal grains. Discolored or small kernels had aflatoxin levels 10 to 1000 times higher than larger, healthy seeds (Dowell et al. 1990; Johansson *et al.* 2006). Although not a recommended method, another option to reduce average aflatoxin levels is to blend contaminated kernels with higher-quality seeds to dilute the aflatoxin to safe levels.

Detecting Aflatoxin

Many direct and indirect, quantitative and qualitative methods can be used to evaluate samples for aflatoxin. All require grinding the sample and extracting the aflatoxin with a solvent or aqueous-based solution for subsequent analysis, with exception of the black light visual method. All methods listed here are either American Association of Cereal Chemists International (AACCI) approved methods (http://methods.aaccnet.org/toc.aspx (http://methods.aaccnet.org/toc.aspx)) or USDA (GIPSA) performance-verified tests (http://www.gipsa.usda.gov/fgis/rapidtestkit.aspx

(https://www.gipsa.usda.gov/fgis/rapidtestkit.aspx)). The methods vary in their accuracy, instrumentation cost, per-sample cost, and level of required technical expertise. Some were reviewed in EDN 87 (https://c.ymcdn.com/sites/echocommunity.site-ym.com/resource/collection/9EE3A8EE-FF5C-45A6-9BA9-0AB3A3E7652E/edn87.pdf). Below they are grouped into three categories: 1) The Visual Method, 2) Chromatographic Methods, and 3) Quick Tests.

Visual Method

The simplest and quickest method to determine if samples may contain aflatoxin is to visually examine kernels under an ultraviolet, or "black," light (365 nm). The method is based on the assumption that bright greenish yellow fluorescence (BGYF) is correlated to the presence of aflatoxin. However, other material can fluoresce, which can result in false positives. Also, contaminated kernels do not always fluoresce, which can give a false negative result. Due to the possibility of false negatives and false positives associated with this test, GIPSA states that this visual method should not be used for mycotoxin screening. Despite these limitations, counting the number of BGYF kernels has been used to accept or reject corn lots with some success. The visual test is the simplest one available for a resource-limited situation, requiring no sample preparation and no per-sample cost. The only instrumentation cost is an ultraviolet lamp (~$600). If this method is used, any positive results should be confirmed by a chemical test.

Chromatographic Methods

Methods that use chromatography are the most accurate, but also require considerable skill and time. The sample is ground, then aflatoxin is extracted from the ground sample using a solvent. The aflatoxin in the solvent is then moved through a chromatography column or placed on a chromatography plate that contains a substance that attracts the aflatoxin based on the latter's polarity. All compounds have a unique polarity, so the strength of the attraction of the compounds to the solvent or to the column or plate determines how quickly the aflatoxin flows with the solvent. Each compound, including aflatoxin, will be separated from other compounds as it moves through the column or across a plate. It can then be quantified as described below.

- **High Performance Liquid Chromatography (HPLC)**. This method gives accurate and quantitative results, and is often used as a method to which all other aflatoxin testing methods are compared. However, it requires a significant capital investment (>$100,000), considerable training in a chemical laboratory to carry out the procedures, and skilled technicians to maintain the instrument. The per-sample cost at a commercial lab is ~$85/sample, including labor. The technique requires several hours per sample, although some of the process can be automated. With this method, the aflatoxin is attracted either to the solvent moving through the instrument or to the HPLC column through which it moves. The amount of aflatoxin in the sample is measured as it moves past a sensor at the end of the HPLC column. The HPLC method has a very sensitive detection limit of less than 1 ppb, and is trusted by many buyers and sellers; it is a good option if very accurate results are needed (such as for establishing a reference lab).

- **Thin Layer Chromatography (TLC)**. This method was once a popular alternative to HPLC, since it is somewhat field-portable. However, it requires several days of training and practice, attention to detail, and lab equipment that includes spotters, beakers, and TLC plates. It works on the same principle as HPLC, but the solvent is allowed to move up a stationary plate coated with a specific material, rather than flow through a column as with HPLC. This method is no longer approved by GIPSA. It requires several hours to complete one test, and is neither as accurate as HPLC, nor as fast and accurate as the quick tests listed below.

Quick Tests

Quick tests are some of the most popular current methods for testing commodities for aflatoxin. They involve extracting the aflatoxin from the ground sample, then adding a substance that causes a color change correlated to the aflatoxin level. In some tests, the color change indicates if the aflatoxin is above a specified level (for example, 20 ppb), while in other tests the intensity of the color can be used to quantify the aflatoxin level using a reader. These tests can be done in 5 to 20 minutes, require minimal training and equipment, and usually cost less than $10/test for consumable supplies. Necessary equipment can vary according to the test, but can include a small grinder (like a coffee grinder), balance, incubator, and basic glassware and pipettes. The one-time cost to begin testing generally ranges from $1000-$5000. The three basic types of tests are listed below.

- **Microwell tests**. The enzyme-linked immunosorbent assay (ELISA) microwell tests measure aflatoxin extracted from a ground sample with a solvent like methanol or (more recently) a more environmentally friendly aqueous-based solution. The solvent is then mixed with a known quantity of enzyme-labeled aflatoxin and the mixture is added to an antibody-coated microwell. The antibodies coated on the microwell will capture either the aflatoxin in the solvent or the enzyme-labeled aflatoxin. If a lot of aflatoxin is extracted from the sample, the antibodies will capture more of the sample aflatoxin than the enzyme-labeled aflatoxin. If no aflatoxin was extracted from the sample, then only the enzyme-labeled aflatoxin will be captured by the antibodies. A substrate is then added which causes a color change in only the enzyme-labeled aflatoxin that was captured by the antibodies, and the color is inversely correlated to the amount of aflatoxin in the extracted sample. A lighter color means more aflatoxin was extracted from the sample and thus captured by the antibodies. The process requires several steps and takes between 5 and 20 minutes. It can be used to screen samples to determine if they are below a specified level, or the color change can be quantified with a reader to indicate the actual aflatoxin level. The limit of detection is 2 to 5 ppb. Microwell tests cost about $10 each for consumable supplies. However, many microwell tests can be done at once, and some steps can be automated; this can lower the time and cost per test, and makes the technology preferred in some labs that regularly conduct many tests per day.
- **Lateral flow strips**. These “dip stick” type tests also require aflatoxin to be extracted from a ground sample with a solvent or aqueous-based solution and are gaining popularity. A lateral flow strip is simply placed into the solution (Figure 2), or the solution is applied to the strip. The solution then

flows by capillary action through a zone where an antibody, bound to colored particles, will bind to the aflatoxin. If no aflatoxin is present, the antibody with the colored particle will move into a zone where it can be captured, and a bright colored line will form. If sufficient aflatoxin is present to bind with all the antibodies, then no unbound antibodies remain to form the colored line. Thus, the brightness of the line is inversely related to the amount of aflatoxin in the sample. The brightness of the line can be measured with a reader in some versions of the tests so that the aflatoxin can be quantified. Lateral flow strip tests can be quicker (3.5 to 10 minutes) and simpler than the microwell test, since they require fewer steps. The procedures are simple enough that most users can quickly learn them. The limit of detection is similar to microwell tests, and the cost is similar to or less. These facts, combined with lateral flow strips' simplicity of use, make the strips preferable to ELISA microwell tests for occasional testing.

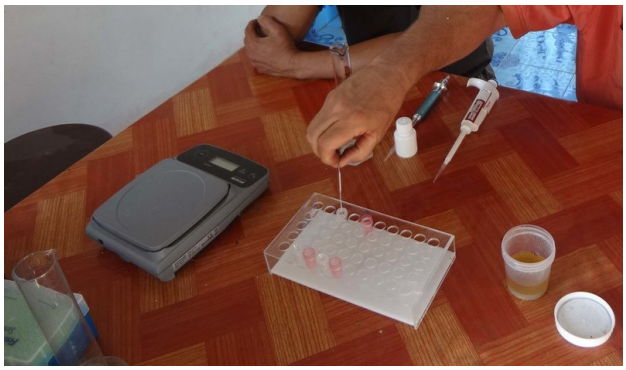

Figure 2. Placing a lateral flow test strip into an extract obtained from a ground sample. *Photo: Floyd Dowell*

- **Fluorometric tests**. These tests require extracting aflatoxin from ground-up samples as with the other quick tests, but the extract is then passed through a column that either binds impurities and passes only aflatoxin, or binds the aflatoxin and passes the impurities. In the latter case, the aflatoxin is then flushed from the column using a solvent. In both instances, a developer is added to the extracted aflatoxin which causes the aflatoxin to fluoresce. The amount of fluorescence is correlated to aflatoxin levels and can be read using a fluorometer. Several steps are required to filter and dilute the extract, but the test gives accurate, quantitative results. It can be completed in 5 to 15 minutes, and has a detection limit of <1 ppb. Fluorometric tests are more accurate over a wider range of aflatoxin levels than the other quick tests, but the costs (which include solvents) tend to be higher—over $10/test for consumable supplies.

Selection of an aflatoxin quick test will likely be influenced by the accuracy, cost, simplicity, and speed of the testing method. However, since sampling is by far the biggest source of error, and since all quick tests that are verified by GIPSA meet specific accuracy criteria, accuracy should perhaps not be a major factor in selecting a test. That leaves cost, simplicity, and speed. If many tests are routinely done per day, and if an experienced person can be dedicated to running the tests, the ELISA microwells may offer speed and cost advantages. If few tests are done, whether regularly or sporadically, lateral flow tests are easiest to learn and low cost. These lateral flow tests are steadily becoming cheaper and simpler, and may offer the best choice for the occasional user in the foreseeable future. Fluorometric tests are accurate, but can be tedious and require more solvent than other tests.

Summary

When testing for aflatoxin, sampling variability is the largest source of error. To determine if the lot meets an average aflatoxin threshold, take care to analyze a representative sample obtained using an approved sampling plan. To determine if the lot has any risk of aflatoxin at all, sample and measure only the portion that is most likely to have aflatoxin—the damaged or discolored kernels; if this sample has no or very low aflatoxin, the user can have good confidence that the entire lot has little or no aflatoxin. If a sample contains aflatoxin above a specified level, the lot can be cleaned to remove suspect kernels and then retested, or blended with good product. The aflatoxin testing method that is chosen will depend on the cost, accuracy, speed, and simplicity requirements of the user. For the occasional user, lateral flow tests generally offer advantages over other tests in simplicity, speed, cost, and accuracy.

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