# 24 Monitoring for Spoilage and Mycotoxin

*Ernesto Moreno Martinez*
*Charles Woloshuk*

Ideally, grain placed in storage should be high quality, without disease or structural damage. Grain entering the storage facility usually has some damage due to preharvest diseases. Damage and breakage also occur during harvest, drying, and grain transfer. Keeping grain moisture content below the level required by fungi (mold) to grow (Chapter 6) will minimize spoilage. Poor quality grain is more likely to spoil, especially during the warmer months. Managers should monitor grain diligently. When mycotoxins become an issue, managers should have a plan for testing grain to assure mycotoxins are below levels acceptable for marketing or safely feeding livestock.

## Grain Odor

All grain has odor. Distinguishing good quality grain from grain spoiled because of fungal growth is easy. Grain odor comes from volatile metabolites (chemicals) naturally produced by the grain. They are a mixture of numerous classes of small molecules including short chain hydrocarbons, terpenes, aldehydes, and alcohols (Buśko et al. 2010). Grain spoiled by fungal growth has an off-odor that can be described as musty, sour, earthy, or even putrid. Each of these odors is caused by one or more volatile fungal metabolites. The molecules often responsible for grain with musty and earthy odor have been identified as geosmin (a terpene) and 2-methylisoborneol (structure similar to camphor) (Jelenä et al., 2003). The human olfactory threshold for these metabolites is low, and the average person can detect concentrations that are in the part-per-trillion range (Polak and Provasi, 1992). Facility managers should monitor the headspace of grain storage units for these easy to smell off-odors

Research on electronic nose technologies suggests that equipment that can provide early warning of grain spoilage and mycotoxin production will be available in the future (Campagnoli et al. 2009, 2011). Some sensors include small semiconductors coated with materials that interact with volatile compounds. Computer programs are needed to recognize the signature of the targeted odor molecules. Research shows that fungal metabolites can be detected, but it is not possible to establish the relationship between a measured amount of metabolite and the level of fungal growth or spoilage. Producing equipment to withstand the rough conditions of the grain facility is also a challenge.

Off-odor is pervasive. When off-odor grain is mixed with good quality grain, the good grain will not mask the off-odor. Eliminating odor can be difficult. Extensive aeration can reduce the off-odor if damage is not severe (see Chapter 11). Treating grain with ozone ($O_3$) can remove off-odor. Ozone is a strong oxidizing gas that can be produced by electrical corona discharge in air (Hosselet, 1973). Ozone can destroy off-odor volatiles, but the treatment also will reduce or change the other molecules that give grain its particular smell. When the treatment is done correctly, the off-odor will be removed and the grain will smell like good quality grain. The treatment will not affect the quality of the grain for its end-product usage (Mendez et al. 2003).

## Other Methods of Monitoring Fungi

Chapter 23 describes the production of carbon dioxide ($CO_2$) and heat by fungi and insects during the formation of hot spots of spoilage in a grain mass. Temperature monitoring by a sensor within the grain is unlikely to detect a hot spot early unless it is located within inches. $CO_2$ detection appears to have great potential as a means of monitoring for spoilage (Ileleji et al., 2006). Sensors placed in the headspace of a grain storage unit or at fan exhausts can monitor normal $CO_2$ levels and detect significant increases due to fungal or insect activity.

Routine monitoring of the average moisture and temperature during grain storage can alert managers to changes that may lead to spoilage. Chapter 6, Table 1 provides equilibrium moisture content for corn and wheat. Maintaining low relative humidity (65%) between grain kernels will assure that spoilage by fungi will not be an issue. See Chapter 11 for a discussion about using aeration to maintain proper temperature and moisture conditions.

## Mycotoxin Contamination

Numerous mycotoxins have been identified in grain, including citrinin, cyclopyazonic acid, moniliformin, patulin, sterigmatocystin, trichothecenes, zearalenone, fumonisins, ochratoxins, and aflatoxins. Only a few of these occur often enough to warrant routine screening. Chapter 6 describes the major mycotoxins — aflatoxin, fumonisin, zearalenone, and deoxnivalenol — produced by fungi that cause preharvest diseases of corn and wheat.

Monitoring grain in disease affected areas at harvest is necessary to prevent contaminated grain from entering the food and feed supply chains. Aflatoxin and ochratoxin are also found in hot-spot formations within stored grain. It is impossible to determine if grain is contaminated with mycotoxins by looking at it with the naked eye. Aflatoxin-producing fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) can be detected on corn by examining kernels with a black light (long-wave ultraviolet) to indicate potential contamination. If kernals show a bright green-yellow fluorescence (BGYF) aflatoxin is indicated, and grain should be tested. The greater the percentage of kernels that show BGYF, the higher the probability that aflatoxin is at significant levels. This test is prone to false positives and negatives, and it does not indicate aflatoxin concentration. The only way to determine if grain is contaminated is to have it analyzed for mycotoxins.

### Sampling grain for mycotoxin analysis

Once the decision has been made to test grain for mycotoxins, the most important step is to obtain a sample that is representative of the entire grain mass. Mycotoxin-contaminated grain will not be uniformly distributed. Some areas will be highly contaminated, and other areas will have little or no contamination. Individual kernels will vary greatly in the amount of mycotoxin they contain. As a result, the probability for obtaining an accurate assessment from just a handful of grain is zero.

Most of the error in the mycotoxin analysis will be attributable to sampling error. An online reference, *Grain Inspection Handbook*, that describes various sampling methods for grain structures, trucks, and railcars is available from the USDA Grain Inspection, Packers, and Stockyards Administration (GIPSA). The handbook includes several chapters on grain sampling. It recommends taking multiple samples — usually 10 but no less than three — from different locations and depths in the grain mass and periodically collecting samples from the moving stream of grain during loading or unloading. Samples should be combined and mixed well to form a composite sample that is ground and tested for mycotoxin. GIPSA recommends a sample size of 10 pounds (4.5 kg) for corn. Taking fewer samples or collecting less grain will increase the probability for error in determining mycotoxin level.

### Mycotoxin analysis

Locating a laboratory that provides mycotoxin testing of grain samples can be difficult. Check with the local extension service or grain association for a list of laboratories that offer testing services. Most university veterinary schools have a toxicology laboratory that provides mycotoxin analysis. Also many of the grain certification offices perform mycotoxin testing. Drawbacks to sending grain out for testing are high cost and time required to receive the results, which can be several days. The alternative is to use one of the commercial rapid test kits. Some of the best known companies include Romer Labs,

Vicam, Charm, and Neogen. All the commercial test kits use some form of immunoassays. This technology takes advantage of antibodies that are specific to one mycotoxin. Antibodies can bind to a particular mycotoxin in a mixture of grain extract, making detection and quantification possible.

## Analysis of mycotoxins by chromatography

Traditional mycotoxins analysis methods are based on some form of chemical chromatography. These technologies, which include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC), separate mycotoxins by their chemical interaction with silica-based, solid-phase materials. Silica, which is primarily silicon dioxide, is what makes up sand at the beach.

**Thin-layer chromatography (TLC) –** TLC is the least sophisticated of the chromatography methods for mycotoxin analysis (Betina 1985). Extracts from grain samples are spotted onto a plate of glass (also metal or plastic) that is coated on one side with a thin layer of silica gel. Depending on the size of the plate, multiple samples can be placed in a line near the bottom of a plate. Once the samples are spotted, the plate is placed on edge into a tank containing a shallow level of organic solvent(s). The solvent moves up the plate by capillary action, carrying the mycotoxin.

Separation is achieved by the relative solubility of the mycotoxin in the solvent mixture and its interaction with the silica gel. The mycotoxin will migrate to a specific distance from its spotted origin. This is measured as the $R_f$, which is relative to the solvent front. Mycotoxins are visualized on the plate several ways, including examination under UV, as with aflatoxin, and by spraying the plate with a reagent followed by heating in an oven, as with fumonisins. Running mycotoxin standards of known quantity on the TLC plate allows for either quantitative or semi-quantitative measurements.

**High-performance liquid chromatography (HPLC) –** An HPLC is a fairly expensive machine, which in its basic form consists of one or two pumps, a separation column, and a detector. Higher end machines include add-on devices such as auto-sample-injectors. The silica material used in HPLC is packed into a small steel column that can withstand high pressure. Reverse-phase columns often are used for mycotoxin analysis. A molecule such as a C18 alkyl chain is attached to the silica, creating a hydrophobic layer. Extracts from grain samples are injected into the solvent stream that is flowing through the column. Movement of the mycotoxin molecules is slowed by their interaction with the column material, and by the time they reach the end of the column, separation from other metabolites in the sample has been achieved. Once the mycotoxin leaves the column, the solvent carries it to some type of detector. The most common detectors measure UV absorbance or fluorescence. Identification of the mycotoxin is based on the retention time on the column, which is compared to a standard. A mass spectrometer also can be used as detector. Regardless of which detector is used, mycotoxin quanity is determined by comparison with a concentration curve obtained from known mycotoxin standards.

**Gas chromatography (GC) –** A GC machine is commonly used for the analysis of the trichothecene mycotoxins such as deoxynivalenol and T-2 toxin (Kientz and Verwej 1986). This machine, which is also quite expensive, consists of a high temperature oven, a separation column, and a detector. GC columns are long coils of tubing that are often lined with fused silica coated with various silicon derivatives, which give columns specific separation properties. For GC analysis, the mycotoxin must be volatile because the carrier through the column is an inert gas such as helium. Mycotoxins are not volatile compounds, so extracts from grain must be treated with trimethylsilane (or other reagents) that bind to the mycotoxin making the mixture volatile when heated to the operating temperature of the GC (above 200˚C). A grain sample is injected into the gas flow that is running through the GC column. Separation is achieved by the interaction of the mycotoxin with the column material, which slows movement of the mycotoxin. From the column, the mycotoxin flows into a detector, which can be a mass spectrometer, a flame ionization detector (FID) or an infrared spectrometer (FTIR). As with HPLC, identification of the mycotoxin is based on the retention time on the column, and quantification is determined by comparison with a concentration curve made with mycotoxin standards.

## Analysis of mycotoxins by rapid-test kits

There are two main formats used in commercial rapid-test kits sold for mycotoxin analysis: enzyme linked immune-sorbent assay (ELISA) and lateral flow strips (Figure 1). Both are based on competition for binding to specific antibodies, which are fused to the bottom of the ELISA assay cup and at a location on the lateral flow strips. Mycotoxins in a grain sample compete with a known amount of standard mycotoxin that is mixed with the grain sample extract. Depending on the type of test, the standard mycotoxin is conjugated to a molecule which facilitates the optical reporting of the mycotoxin level (Zheng et al. 2006). Thus, more mycotoxin in the grain sample results in less binding of the standard mycotoxin conjugate to the antibodies in the assay.

In the ELISA assay, samples with more mycotoxin will have less color (Figure 1A). For the lateral flow assays, more mycotoxin results in a toxin line that is absent or one with much less intensity (Figure 1C). These tests kits are sold as a quick-screen, semi-quantitative or quantitative. The quick-screen kits test for a threshold level of mycotoxin. These tell the user whether a sample has more than the threshold level, such as 20 ppb aflatoxin. Kits are convenient for testing feed and are often used at grain elevators to test incoming grain. ELISA kits can be quantitative, and the lateral-flow assays can be semi-quantitative. Measurements will require the user to purchase a device from the kit provider that is specific to the kit manufacturer. Readers currently cost around two thousand dollars. Companies that sell these kits also provide or sell materials needed for sample extraction, clean up, and running the tests.

Immunoaffinity columns (IAC) are also commercially available and widely used for sample clean-up and mycotoxin analysis (Scott and Trucksess, 1997; Zheng et al. 2006). The IAC contains antibodies that are immobilized onto a solid support such as agarose gel in phosphate buffer contained in a small plastic cartridge. The sample extract is applied to an IAC containing specific antibodies to a certain mycotoxin. Then the mycotoxin binds to the antibody and water is passed through the column to remove any impurities. Finally, by passing a solvent through the column, the captured mycotoxin is removed from the antibody and eluted from the column. The mycotoxin is then further developed by addition of a chemical substance to either enhance fluorescence or render

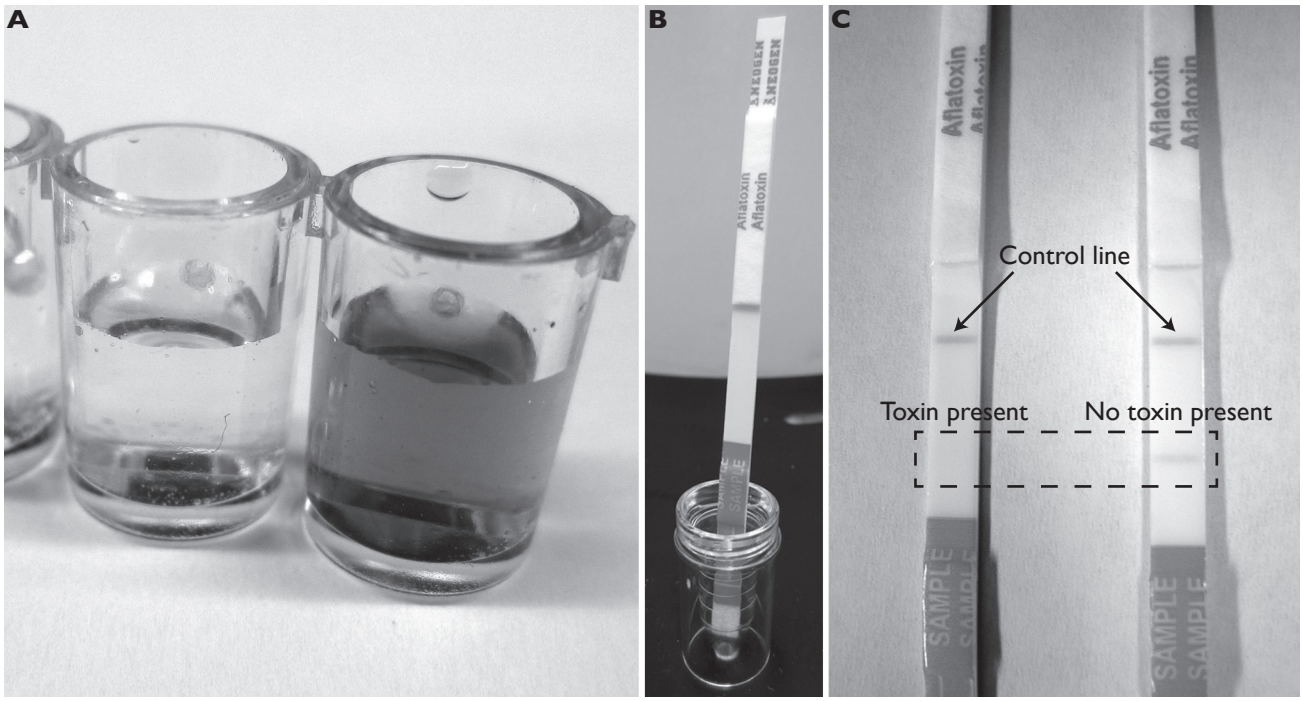


***Figure 1.*** *A) ELISA results showing the difference between a grain sample containing mycotoxin (left) and one without mycotoxin (right). B) Lateral flow strip placed into a cup containing extract from a grain sample. C) Lateral flow strip results showing the difference between a grain sample containing mycotoxin (left) and one without mycotoxin (right).*

the mycotoxin fluorescent before measuring in a fluorometer.

## Future assays

Research continues to apply new technologies to the task of measuring mycotoxins. One of these new technologies is called fluorescence polarization immunoassay (FPI) (Maragos and Plattner 2002; Zheng et al. 2006). As with technologies already discussed, mycotoxin-specific antibodies are used to bind mycotoxins in the grain sample extract. In the FPI system, this binding prevents the subsequent binding of a tracer molecule, which allows it to freely rotate, exhibiting non-polarized fluorescence. The more mycotoxin in the sample, the less polarized fluorescence is measured (Zheng et al. 2006).

Research is also being conducted on evanescent wave technologies such as surface plasmon resonance biosensors (Zheng et al., 2006). The principle of surface plasmon resonance is based on the detection of a change of the refractive index of the medium when an analyte binds to an immobilized partner molecule (antibody). The number of analyte molecules bonded by antibodies to a thin metal layer correlates with the changing of the resonance angle. This application has several advantages such as small sample volumes, reusable metal chips, and the potential for measuring several different mycotoxins simultaneously.

# References


Betina. V. 1985. Thin-layer chromatography of mycotoxins. J. Chromotogr. 334:211-276.

Buśko, M.; H. Jelen,; T. Goral, J. Chmielewski; K. Stuper, L. Szwajkowska-Michałek, B. Tyrakowska, and J. Perkowski. 2010. Volatile metabolites in various cereal grains. Food Additives and Contaminants 27:1574-1581.

Campagnoli, A., F. Cheli, G. Savoini, A. Crotti, A. G. M. Pastori, and V. Dell'Orto. 2009. Application of an electronic nose to detection of aflatoxins in corn. Vet Res Commun. 33:S273–S275.

Campagnoli, A., F. Cheli, C. Polidori, M. Zaninelli, O. Zecca, G. Savoini, L. Pinotti, and V. Dell'Orto. 2011. Use of the electronic nose as a screening tool for the recognition of Durum wheat naturally contaminated by deoxynivalenol: a preliminary approach. *Sensors 11*: 4899-4916.

Hosselet, L. M. L. 1973. Increased efficiency of ozone-production by electric discharges. Electrochimica Acta. 18:1033-1041.

Ileleji, K.E., D.E., Maier, C. Bhat, and C.P. Woloshuk. 2006. Detection of a developing hot spot in stored maize with a $CO_2$ sensor. Applied Engineering 22: 275-289.

Jelenä, H. H., M. Majcher, R. Zawirska-Wojtasia, M.W. Rowska, and E.W. Sowicz. 2003. Determination of geosmin, 2-methylisoborneol, and a musty-earthy odor in wheat grain by SPME-GC-MS, profiling volatiles, and sensory analysis J. Agric. Food Chem. 51: 7079-7085.

Kientz, C. E., and A. Verwej. 1986. Trimmethylsilyation and trifluoroacetylation of a number of trichothecenes followed by gas chromatographic analysis on fused-silica capillary columns. J. Chromatogr. 355:229-240.

Maragos, C. M., and R. D. Plattner. 2002. Rapid fluorescence polarization immunoassay for mycotoxin deoxynivalenol in wheat. J. Agric. Food Chem. 50:1827-1832.

Mendez, F., D.E. Maier, L.J. Mason, and C.P. Woloshuk. 2003. Penetration of ozone into columns of stored grains and effects on chemical composition and processing performance. J. Stored Prod. Res. 39:33-34.

Polak, E. H., and J. Provasi. 1992. Odor sensitivity to geosmin enantiomers. Chem. Senses 17:23-26.

Scott, P. M. and M.W. Trucksess. 1997. Application of immunoaffinity columns to mycotoxin analysis. J. AOAC Int, 80: 941-949.

Zheng, M. Z., J. L. Richard, and J. Binder. 2006. A review of rapid methods for the analysis of mycotoxins. Mycopathologia, 161: 261-273.



K-STATE Research and Extension

Publications from Kansas State University are available at: ***www.ksre.ksu.edu***



Publications are reviewed or revised annually by appropriate faculty to reflect current research and practice. Date shown is that of publication or last revision. Contents of this publication may be freely reproduced for educational purposes. All other rights reserved. In each case, credit the authors, Stored Product Protection, Kansas State University, March 2012.



**Kansas State University Agricultural Experiment Station and Cooperative Extension Service**

K-State Research and Extension is an equal opportunity provider and employer. Issued in furtherance of Cooperative Extension Work, Acts of May 8 and June 30, 1914, as amended. Kansas State University, County Extension Councils, Extension Districts, and United States Department of Agriculture Cooperating, Gary Pierzynski, Interim Director.

**S156 – 24 March 2012**