Food Safety—Risk Assessment Strategies for Mycotoxin Analysis

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Mycotoxins are toxic secondary metabolites produced by molds that have detrimental effects on human and animal health (1–3). Mycotoxin-producing fungi colonize on stressed or improperly stored crops and can generate high levels of mycotoxins. Aflatoxins are produced by *Aspergillus* species of fungi and are carcinogenic, with aflatoxin B_1 being significantly more carcinogenic than aflatoxins B_2, G_1, and G_2 (19). Ochratoxins, which are produced by *Aspergillus* and *Penicillium* species of fungi, are neurotoxins and immunosuppressants. Fusarium toxins are produced by *Fusarium* species of fungi and include mycotoxins such as fumonisin, which affects the nervous system of horses; trichothecenes such as deoxynivalenol (vomitoxin or DON), which have chronic and fatal toxic effects such as vomiting, diarrhea, and feed refusal; and zearalenone, an estrogenic toxin that affects reproduction. Alone, each mycotoxin adversely affects human and animal health; however, the synergistic effects of mycotoxins can increase their toxicity (9). Mycotoxin-producing molds are ever-present, and mold growth can occur both pre- and postharvest in localized regions or "hot spots" within a crop. Prior to harvest, mold growth and subsequent mycotoxin production can affect a single ear or kernel of corn while not affecting others. During storage, improper grain drying can cause wet spots that promote mold growth, resulting in increased mycotoxin concentrations. Mycotoxin contamination from mold growth also can result from improper harvesting techniques, such as kernel damage or soil contamination from harvesting equipment. Localized contamination can result in significant variability in mycotoxin concentrations throughout a grain lot.

To perform a mycotoxin analysis, a commodity is sampled with the expectation that the test sample is representative of the bulk grain lot. However, inhomogeneous distribution of mycotoxins throughout a bulk lot is the largest source of error in the mycotoxin analysis (18). Biased sampling can lead to both false negative and false positive determinations. A false negative occurs when a positive sample yields a test result below the determined threshold and the contaminated sample is accepted. Conversely, a false positive occurs when the test result for a negative sample is greater than the threshold and the sample is rejected. The U.S. Department of Agriculture, Grain Inspection, Packers and Stockyards Administration (USDA-GIPSA) has established sampling method specifications to reduce sampling bias (11).

Sampling Methods

Mycotoxin determination necessitates that surveyed samples be ground and subsampled and that mycotoxins be extracted using suitable solvents. The solvent used affects mycotoxin extraction efficiency, while particle and test sample size affect sampling variability. USDA-GIPSA has established specifications for sample size, sample grinding, and subsampling for aflatoxin (13), DON (12), fumonisin (15), ochratoxin (14), and zearalenone (16). In each instance, the specifications require that samples be ground such that 60–75% of the particles pass through a No. 20 sieve and that a 50 g test sample be used for extraction of the mycotoxin.

Measurement variability associated with sampling corn naturally contaminated with aflatoxin is shown in Table I to demonstrate how particle size, test sample size, and multiple analyses affect the mycotoxin determination. Aflatoxin quantitation was performed using a modified HPLC reference method (10). A 2 kg corn sample was ground such that 60% of the particles passed through a No. 20 sieve (coarse grind) and then were mixed and sampled to obtain 10 and 50 g samples for analysis. The remaining sample was ground further such that 99% of the particles passed through a No. 20 sieve (fine grind) and then were similarly mixed and sampled. Finally, the remaining sample was passed

Table I. Variability in aflatoxin determination associated with particle and sample sizes

	60% 20 mesh Sample		99% 20 mesh Sample		Hammer Mill Ground Sample
Sample Number	10 g	50 g	10 g	50 g	10 g
1	91	151	140	149	160
2	222	125	154	148	154
3	167	136	136	135	151
4	217	110	138	144	155
5	135	123	155	151	156
6	95	139	141	140	153
7	86	111	147	148	162
8	108	115	167	149	171
Average (ppb)	140	126	147	145	158
SD (ppb)	56	15	11	5	7
RSD (%)	40	12	7	4	4

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through a hammer mill (laboratory mill 3100, Perten), and the particles were mixed and sampled (10 g samples only). For official USDA-GIPSA testing, the required sample size is 50 g. However, users sometimes decrease this sample size (e.g., 10 g) to save on solvent costs and disposal fees and should be cognizant of the potential for a higher bias with a reduced sample size.

For the 50 g samples, the coarse ground material had a 12% relative standard deviation (RSD) versus a 4% RSD for the fine ground material, demonstrating the importance of grinding the sample to a fine particle size before testing. The 10 g sample demonstrated this more emphatically, as the RSD shifted from 40% for the coarse ground material to 7% for the fine ground material. Grinding the sample to a fine powder using a hammer mill reduced the RSD to 4% when using 10 g samples for analysis and was equivalent to that of the 50 g fine ground sample. The measurement error of the test method was <1%, showing that test variability was associated with the sampling method and not the test method. These results show that grinding and subsampling as specified by USDA-GIPSA for good laboratory practices can minimize errors in mycotoxin determinations. A more detailed discussion on sampling and associated variability is provided by Whitaker et al. (18).

Analytical Methods

Reference Methods. HPLC reference methods exist for a broad range of mycotoxins and commodities that are not suitable for field or nonlaboratory settings due to the time required for sample analysis and the need for hazardous chemicals, expensive equipment, assay expense, and operator expertise. These methods typically require hazardous solvents (e.g., acetonitrile) for extraction and as mobile phase constituents. Reference methods also require expensive instrumentation, including a high-performance liquid chromatograph and detectors, disposable equipment such as solid-phase extraction columns for sample cleanup, and trained operators. Finally, reference methods are time-consuming due to the time required to perform chromatographic runs on calibration standards, matrix recovery sample, and test sample(s), as well as the time required for sample cleanup and data processing.

Rapid Methods. Rapid methods of analysis enable testing for mycotoxin contamination in the field and nonlaboratory settings. Such methods focus on customer needs, including time and space requirements, cost, and ease of use. Rapid test methods, including ELISA, immunoaffinity column, and lateral flow methods, have been developed to meet these demands. These types of methods provide cost-effective testing platforms that utilize solvents that are more user-friendly and require fewer analysis steps that provide results in minutes.

As part of its Rapid Test Kit Evaluation program, USDA-GIPSA has developed design criteria and test performance specifications for quantitative rapid test kits for mycotoxins, including aflatoxin, DON, fumonisin, ochratoxin, and zearalenone (17). These specifications require that a quantitative test result be obtained in under 30 min using a preground sample, that 95% of the test results for 21 naturally contaminated samples are within the defined acceptance limits, and that users are not subjected to toxic or hazardous substances at levels higher than the standards specified by OSHA. Design criteria and test performance specifications must be consistent with the customer's need to make decisions in a timely manner and validated using naturally contaminated grains that contain mycotoxin concentrations certified by HPLC reference methods.

Reference Materials. Validation of rapid test methods using naturally contaminated reference materials emphasizes the importance of preparing reference materials for validation and calibration of test methods. Reference materials are also crucial for training and proficiency testing and for inter- and intralaboratory studies performed to compare test methods. USDA-GIPSA has published a synopsis/solicitation (Solicitation Number AG-6395-5-10-0145; www.fbo.gov/index?s=opportunity&mode=form&id=a4fc16dea86fef48db28b593c579a7b7&tab=core&_cview=1) to procure naturally contaminated mycotoxin reference materials. The solicitation requires that 95% of the particles pass through a No. 20 sieve, that testing be conducted on 21 samples over a 3 day period, and that the mycotoxin be homogeneously dispersed.

Mycotoxin reference materials (provided by Charm Sciences) for 20 ppb aflatoxin in corn are shown in Table II. These reference materials meet the USDA-GIPSA specifications for particle size and homogeneity (e.g., RSD < 15% at 20 ppb aflatoxin). Mixing was conducted over several days to homogenize the materials, and the test results demonstrate the expected variability observed for aflatoxin reference materials in 10–15 kg lots. The higher variability of these test results relative to similar 50 g subsample data in Table I was due to the larger lot size of the reference materials and testing over a 3 day period. For sample 2, one subsample showed an aflatoxin content of 13.8 ppb, which was 3 standard deviations (SD) from the mean result of 21.2 ppb. The USDA-GIPSA allowable test range for 20 ppb aflatoxin reference materials is based on a maximum RSD of 20% from the mean concentration (17). Rapid test kits using this 21.2 ppb aflatoxin reference material must test between 12.7 and 29.7 ppb (2 SD from the mean concentration). The RSD for the 21 analyses performed for sample 1 was less than half that for sample 2. However, further testing of an additional 33 samples from sample 1 was conducted and found 2 results that were >2 SD from the mean response and still within the defined specifications. The observed variability was consistent with normal distribution. The sampling variability and strict acceptable limits set by USDA-GIPSA provide confidence in the reliability of the rapid tests approved by USDA-GIPSA.

USDA-GIPSA Approved Test Kits. Charm Sciences developed a Rapid One

Table II. HPLC test results for aflatoxin reference materials when using a nominal concentration of 20 ppb aflatoxin

	Sample 1 (ppb)			Sample 2 (ppb)		
Sample Number	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
1	20.3	19.4	20.9	20.0	20.8	20.0
2	19.1	17.3	19.7	24.3	24.1	19.9
3	20.1	19.1	20.4	18.5	22.4	22.7
4	21.8	17.8	19.6	13.8	19.0	21.2
5	20.6	16.9	20.3	22.4	23.8	22.0
6	20.1	18.5	20.2	24.7	19.0	22.9
7	20.5	18.1	19.7	21.3	21.0	21.9
Average (ppb)		19.5			21.2	
SD (ppb)		1.2			2.5	
RSD (%)		6.3			11.7	

Step Assay (ROSA) lateral flow device for rapid testing of antibiotics in milk (6,7). The test platform includes a lateral flow strip, incubator, and reader. The lateral flow strip is self-contained in a protective plastic case that consists of a sample pad, conjugate pad, test zone, and control zone. Sample is added to the sample pad, which expands and pushes sample to the conjugate pad. The conjugate pad contains receptor-labeled gold beads that enable the receptor to bind to the target analyte. The receptor-labeled beads are reconstituted by the sample and react with the available analyte before passing to the membrane layer, which has a test zone and a control zone. The test zone captures receptor-labeled beads that are not bound by the target analyte, and the control zone then captures both bound and unbound receptor-labeled beads. The control zone also acts as a sample flow monitor. In the absence of target analyte, the beads bind strongly to the test zone and deplete the amount of beads available for control zone binding. For positive samples, the target analyte binds to the receptor-labeled beads, resulting in inhibited test zone binding and strong binding to the control zone due to the higher availability of bound and unbound receptor-labeled beads. The color intensity of the gold beads bound to the test and control zones depends on the target analyte concentration. Finally, the reader interprets the color intensity and includes a calibration curve to calculate the concentration of analyte in the assayed strip.

The Charm ROSA Aflatoxin Quantitative Test was the first quantitative lateral flow test approved by USDA-GIPSA in 2005 (Certificate FGIS 2005-101). The test method is simple to perform and requires four steps: 1) sample extraction; 2) extract dilution of 100 µL of extract with 1.0 mL of dilution buffer; 3) assay of 300 µL of diluted extract with 10 min of incubation at 45°C; and 4) measurement of the developed assay strip in the reader (8). This test methodology was followed by USDA-GIPSA approval of quantitative assays for DON, fumonisin, ochratoxin, and zearalenone and an unofficial quantitative test for T-2 and HT-2 toxins.

Table III. Rapid quantitative test (DONQ-FAST5) results compared with HPLC results for deoxynivalenol in wheat and corn[a]

Grain	HPLC (ppm)	DONQ-FAST5 (ppm)
Wheat	0.52 ± 0.02	0.48 ± 0.06
Wheat	1.02 ± 0.07	0.96 ± 0.13
Wheat	2.00 ± 0.05	1.95 ± 0.24
Wheat	4.80 ± 0.18	4.51 ± 0.48
Corn	0.55 ± 0.04	0.55 ± 0.09
Corn	1.04 ± 0.11	1.06 ± 0.22
Corn	2.04 ± 0.09	2.17 ± 0.24
Corn	4.38 ± 0.22	4.63 ± 0.51

[a] Test results are the average ± standard deviation of 21 independent analyses using each method.

The Charm ROSA DONQ-FAST5 Quantitative Test received USDA-GIPSA approval (Certificate FGIS 2012-008) following the new test kit specifications (17). The test method is similar to the previously approved DON quantitative lateral flow test, with the incubation time reduced from 10 to 5 min for the quantitative detection of 0.5–5 ppm DON in 11 commodities. The test results for 21 independent analyses using the HPLC reference method (4) and the rapid quantitative test for DON are presented in Table III. Both methods accurately determined the DON concentration in wheat and corn samples; however, the rapid quantitative test was more user-friendly, and test results were obtained in less than 7 min after extraction.

Sources of Error

Both buyers and sellers are affected by the outcome of mycotoxin analyses, because the results determine the quality of the product and, subsequently, the value of the commodity. Differences in assessed value between buyer and seller can be due to the use of different test methodologies, failure to perform the test method accurately, and/or sampling and subsampling errors. In these instances, reference laboratories are used to provide a validated mycotoxin analysis, including rapid test and HPLC reference methods. This validated result effectively acts as the final mediator between the parties.

The sampling and subsampling process can be a source of error in mycotoxin determinations. In one study, as much as 75% of the analytical error was associated with the sampling of aflatoxin (18). Performing sampling and subsampling properly is critical to the outcome of mycotoxin analyses because prior assessments of mycotoxin contamination may not predict the outcome of future test results. Mycotoxin analysis requires that a sample be ground prior to extraction of mycotoxin(s). As is shown in Table I, variability in results can be reduced by more finely grinding the sample. A finer particle size produces a more homogenized sample that more accurately defines the sample and reduces possible biases between subsamples. Furthermore, the retained sample is more homogeneous and reduces bias in any future testing, including reference validated testing. The test sample size should be representative of the surveyed sample to reduce any bias (Table I).

Reference and rapid test method errors also can bias the determined mycotoxin concentration. Proficiency testing using mycotoxin reference materials is integral to an effective quality control program to monitor performance and minimize method and user errors. A collaborative study conducted on aflatoxin M_1 in milk that compared the quantitative lateral flow test and HPLC reference methods showed that the repeatability and reproducibility of the rapid method were lower than those of the HPLC method for interlaboratory testing (5). Data from one reference laboratory were biased due to incorrect concentration assignment to the aflatoxin M_1 standard, which was identified only after sending each reference laboratory all the data pertaining to the testing. The increased number of steps required by reference methods is another source of error. Rapid test methods can minimize some systematic errors by limiting the number of steps involved in the assay; however, instrumental and method errors can occur due to the shortened assay times and detection methods used to achieve rapid test results. As these examples show, performance monitoring using mycotoxin reference materials is fundamental to verifying the accuracy of both reference and rapid test methods used to determine mycotoxin contamination levels on any given day.

Conclusions

Mycotoxin risk assessment is challenging. Proper sampling and subsampling procedures must be followed to obtain a representative sample for testing. Testing in the field or nonlaboratory settings requires the use of rapid methods to screen samples for multiple mycotoxins. These methods should be validated by USDA-GIPSA, or other governing bodies, to provide confidence in the test results. The testing program also should be monitored using proficiency samples to validate the testing system as part of the quality control system. A successful testing program that follows these guidelines promotes the overall safety of food and feed products.

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