



Aflatoxin Sampling and Testing Proficiency in the Texas Grain Industry

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ARTICLE INFORMATION

Article History:

Received Aug 6 2014

Received in revised form

Sept 2 2014

Accepted Sept 16 2014

Keywords:

Aflatoxin

Risk management

Variability

Sampling

Analysis

ABSTRACT

Aflatoxin is a group I carcinogen and represents a significant public health and food safety risk throughout the world. Aflatoxin contaminated cereals and oilseeds that contain greater than 20 µg/kg of the toxin (B₁, B₂, G₁, G₂) are defined as adulterated by the Texas Commercial Feed Rules and are regulated by the Texas Feed and Fertilizer Control Service. This study was performed to assess sampling and testing of maize for aflatoxin contamination. During 2010, 87 Texas grain elevators' samples with respect to their sampling procedures were assessed and a second investigation documented aflatoxin analysis procedures at 41 grain elevator establishments. The average sample size was 1.8 kg (4 pounds) and fewer than 20% of the grain elevators collected samples using an official (prescribed) sampling pattern by the grain inspection, crop insurance or regulatory authorities within the United States. Proficiency materials were analyzed by 39 cooperating grain elevators and 7 firms accurately analyzed samples containing greater than 300 µg/kg aflatoxin. While sampling contributes to variability in measuring aflatoxin in grain, this study highlights that aflatoxin analysis using commercially available test kits is a major contributor to variation in aflatoxin test results among commercial grain handlers.

1. Introduction

Aflatoxin is a toxic fungal metabolite produced by *Aspergillus flavus*, and *A. parasiticus* that occurs in cereals and oilseeds during production and storage. Aflatoxin is a group I carcinogen as designed by the IARC (2002). Chronic symptoms include liver cancer while acute aflatoxicosis leads to liver cirrhosis and death in animals and humans (CAST, 2003). The transfer of aflatoxin in animal feed to milk has been well documented, with a rate of transfer of aflatoxin to milk between 1 to 3% (Jouany and Diaz, 2005).

Multiple agencies within the United States (US) sample and test for the presence of aflatoxin in the food system including the Food and Drug Administration (FDA), the United States Department of Agriculture (USDA) Grain Inspection, Packers, and Stockyard Administration (GIPSA), the USDA Risk Management Agency (RMA), as well as state government agencies. The FDA published non-regulatory action levels for aflatoxin in a compliance guidance format that are voluntarily observed by the United States (US) food and feed industry (FDA, 1994). Raw grain is sampled and tested for aflatoxin by GIPSA or their

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designated private agency upon request to facilitate commerce. The grain sampling and testing protocols developed by GIPSA (2002) are included in the RMA Loss Adjustment Manual (LAM) and represent the protocol that is to be followed by crop insurers for quality adjustments involving aflatoxin (RMA, 2011). Procedures within the LAM permit grain elevator personnel to collect samples for the purpose of crop insurance; however, testing for the presence of aflatoxin is to be performed by a disinterested party (RMA, 2011).

To manage aflatoxin risk in the state of Texas, the Office of the Texas State Chemist (OTSC) Feed and Fertilizer Control Service (FFCS) published regulatory limits for aflatoxin contamination and performs an active surveillance of the state's maize crop during harvest. Cereal and oilseeds containing greater than 20 µg/kg aflatoxin are defined as commercial feed and considered adulterated by Texas Administrative Code (TAC, 2011). All commercial firms that distribute aflatoxin contaminated cereal and oilseeds in Texas must be licensed with FFCS.

RMA assists farmers in managing financial risk including weather related crop loss or quality deterioration through the crop insurance industry. RMA's mission is to "promote, support, and regulate sound risk management solutions to preserve and strengthen the economic stability of America's agricultural producers" (RMA, 2011). RMA operates and manages the Federal Crop Insurance Corporation (RMA, 2013). OTSC is a regulatory risk manager with the mission of "Protecting consumers and enhancing agribusiness through its feed and fertilizer regulatory compliance program" and through its surveillance and monitoring of animal and human health hazards (<http://otscweb.tamu.edu/>).

OTSC explored the compatibility of these two agencies approach involving aflatoxin risk management in 2010. Both RMA and OTSC have mechanisms in place to sample and test maize for aflatoxin to fulfill their mission. Consequently, a truckload of maize delivered to a commercial grain elevator could be sampled and tested for aflatoxin three times for grain purchasing, crop insurance, and regulatory oversight. Multiple tests yield different aflatoxin results and create uncertainty in the market. Aflatoxin is not uniformly distributed in maize (Johansson et al, 2000; Herrman et al, 2013), accuracy among field tests approved by GIPSA may be variable (Dai et al, 2013) and grain elevator

employee capability to run these aflatoxin test kits at commercial grain elevators has, heretofore, not been documented.

The GIPSA aflatoxin handbook prescribes a 908g grain sample for official testing procedure, collected from trucks using a seven probe pattern and ground using a Romer mill (GIPSA, 2002). Aflatoxin field tests are approved by GIPSA to measure aflatoxin levels < 100 µg/kg. The RMA (LAM) references the GIPSA procedures for the collection of samples by grain elevator operators. However, an assessment of adherence to the LAM sample collection procedures has, heretofore, not been previously performed. Nor has an evaluation been performed to evaluate the accuracy of aflatoxin testing by the grain industry that adopted GIPSA approved aflatoxin tests.

The following study was performed in 2010 to assess the capability of the Texas commercial grain industry to sample and measure aflatoxin contaminated maize. The intent of this study was to benchmark industry performance as a first step toward aligning sampling and testing procedures in Texas to provide greater uniformity between crop insurance, regulatory, and grain elevator aflatoxin test results.

2. Materials and methods

2.1. Field investigation and sample preparation

OTSC field investigators monitored grain elevator sampling procedures at 87 grain elevators to assess conformance to the LAM during the 2010 harvest and recorded: 1) grain elevator knowledge of the LAM; 2) sampling pattern, sample size and sample mass reduction; 3) employee training involving official sampling procedures; 4) sample identification procedure; 5) sample delivered to approved testing laboratory; 6) receipt of test results; 7) identity of the outside laboratory performing the testing; and 8) percent of farmers using crop insurance.

A second field investigation was performed to ascertain aflatoxin testing accuracy at 41 commercial grain elevators. Field investigators were equipped with 50g individually packaged aflatoxin control materials at three levels (52 µg/kg, 378 µg/kg and 580 µg/kg) for grain elevator laboratory personnel to analyze. Field investigators recorded: 1) test kit name and manufacturer; 2) expiration date on the test kit; 3) storage conditions for the test kit; 4) operators training and experience; 5) sample preparation; 6) grinder cleanout; 7) maximum

aflatoxin measuring limit in the test kit instructions; 8) procedure used when the maximum concentration of the test kit is exceeded; 9) condition of the analytical equipment; and 10) results of three control materials run by the firm.

The proficiency materials were comprised of naturally occurring aflatoxin contaminated maize ground using a Romer mill (Union, MO) and reground through a Retch mill (Haan, Germany) through a 1 mm screen opening. The proficiency materials were subdivided into 50g portions. Thirty portions for each aflatoxin concentration were selected at random and analyzed using high-performance liquid chromatography-fluorescence detection (HPLC-FLD), Vicam AflaTest® (Watertown, MA), and Neogen Veratox® (Lansing, MI). The HPLC-FLD test was run in duplicate. Certified AFB1, AFB2, AFG1, and AFG2 were purchased from Romer Lab Inc-Biopure (Tulln, Austria) were used to prepare standard solutions. All solutions are made with HPLC grade solvents and reagent grade materials. The concentration of AFB1 and AFG1 standard is 2 µg/mL in 5 mL acetonitrile. The concentration of AFB2 and AFG2 standard is 0.5 µg/mL in 5 mL acetonitrile.

2.2. HPLC-FLD analysis

A detailed procedure of HPLC-FLD analysis for aflatoxin in maize was described in our previous study (Dai et al, 2013). Briefly, a 50g test portion of the ground maize samples was extracted with 250 mL of methanol/water (70:30, v/v) by mechanical shaker for 1 hr at 200 rpm. A 15 mL aliquot of the extract was filtered through a folded filter paper (Whatman #1), and a 5 mL aliquot of the filtrate was diluted with water to 25 mL (dilution factor of 5) and 1g of sodium chloride was added. After filtration, 2 mL was loaded onto an immunoaffinity column (AflaTest® affinity column, Vicam #12020) and passed through the column. After washing the column with 5 mL of water twice, 1 mL of methanol was used to elute the aflatoxins from the column. Eluent was diluted with 1 mL of HPLC water and filtered through a 0.2 µm syringe filter prior to HPLC-FLD analysis.

2.3. Vicam test method

A 50g test portion of maize samples was mixed with 5g of analytical grade sodium chloride and the mixture was extracted with 250 mL of 80%

methanol/ water with shaking for 1 hr at 200 rpm. The extract was filtrated. Ten mL filtrate was mixed with 40 mL of DI water and the mixture was filtered again. Two mL of the filtrate was loaded onto the affinity column and passed through the column. The column was then washed with 5 mL of DI water twice. One mL of methanol was used to elute the aflatoxin from the column. The developer solution was prepared by mixing 5 mL developer solution (supplied with the kit) with 44 mL DI water. One mL of prepared developer solution was added into the methanol elute and the mixed solution was vortexed and placed into the reader for immediate reading.

2.4. Veratox® aflatoxin test kit

A 50g portion of maize samples was extracted with 250 mL of 70% methanol/ water and shaken for 1 hr on a shaker at 200 rpm. The extract was filtered and the pH of the solution was adjusted to fall into the range of 6-8. One hundred µL of conjugate (supplied with the kit) was pipetted into a red-colored well provided with the kit. One hundred µL of the calibration standard solution and the extract was transferred into the red-colored well and mixed with the conjugate. After mixing, 100 µL of the mixed solution was transferred into the antibody coated well and incubated for two minutes and then rinsed out with DI water. One hundred µL of the substrate solution was added into the antibody coated well after all the rinsing water had been moved out of the well. The solution was incubated for 3 minutes before the 100 µL of the stop solution (provided with the kit) was added into the well. The mixed solution was then put into the reader for immediate reading.

2.5. Statistical analysis

Proficiency material preparation and grain elevator proficiency test results were analyzed using descriptive statistics in Microsoft® Excel. The different treatment effects for aflatoxin level and testing procedures were analyzed using the GLM procedures in SAS® v 9.1.3 (SAS® Institute, Cary, NC) and least squared means to identify significance. A paired t-test was performed to evaluate significance between percent relative standard deviation between aflatoxin testing platforms and aflatoxin level for the proficiency samples.

Table 1. Aflatoxin average concentration in µg/kg, standard deviation, and relative standard deviation for samples measured by the proficiency test provider using Veratox[®], Aflatest[®], and HPLC platforms.

Testing method	Descriptive statistics	Control		
		A	B	C
Neogen Veratox [®]	Average (µg/kg)	47	329	577
	Standard deviation (µg/kg)	2.80	30.87	44.60
	RSD (%) ^a	6	9	8
Vicom AflaTest [®]	Average (µg/kg)	47	375	585
	Standard deviation (µg/kg)	3.22	16.57	48.45
	RSD (%)	7	4	8
HPLC	Average (µg/kg)	52	378	580
	Standard deviation (µg/kg)	3.4	17.6	33.7
	RSD (%)	6	5	6

^a RSD (%): relative standard deviation.

3. Results and discussion

3.1. Analysis of aflatoxin proficiency samples

Personnel at 9 of the 87 grain elevators were familiar with the RMA LAM. Of the 87 firms, 8 collected samples without a sampling probe, 8 collected a one-probe sample, 29 collected a two-probe sample, 5 collected a three-probe sample, 18 collected a four-probe sample and 16 collect five or more probes. The average sample weight was 1.8 kg (4 pounds) and was correlated with the number of probes ($r = 0.74$) with an average of 0.59 kg (1.3 pounds) per probe. The GIPSA aflatoxin handbook (2002) prescribes a 7 or 10 probe pattern (dictated by grain depth) for trucks and a minimum of a 0.908g (2 pounds) sample from trucks to test for aflatoxin. The LAM (2011) adheres to the 7 or 10 probe pattern prescribed by GIPSA and states on

page 305 that the “Sample size to be submitted for testing will be dictated by the approved testing facility. (For aflatoxin, most facilities will likely require at least a ten-pound sample).”

The use of a common sample, split for use by the grain elevator and crop insurance, occurred at 36% of the establishments. Only 5% of the individuals performing the truck sampling were trained in the GIPSA/LAM sampling procedure. The collection and sampling of maize for crop insurance is performed for the growers as a service. Forty percent of the grain elevators received aflatoxin analysis results from the approved testing laboratories and fewer than 50% of those could reference these test results back to grain placement in the elevators. Grain elevator operators estimated that approximately 50% of their producers did not use crop insurance for managing aflatoxin risk.

Table 2. Statistical significance for the effects of testing method and aflatoxin level on aflatoxin measured in samples prepared by the proficiency test provider.

Source	F-value	P-value
Testing method (M)	5.5	0.0059
Aflatoxin level (L)	3355.9	< 0.001
M x L	3.3	0.016

A total of 41 grain elevators were revisited to conduct a second evaluation focusing on aflatoxin testing methodology and to request firms analyze three proficiency materials. In preparation for the second evaluation, OTSC prepared proficiency materials with levels of naturally occurring aflatoxin in ground maize reported in Table 1. The analysis of variance (ANOVA) results showed the significance of testing method x aflatoxin level ($P < 0.05$) (Table 2). The OTSC Veratox[®] aflatoxin measurement for proficiency material B (329 µg/kg) was significantly ($P < 0.01$) lower than the HPLC (378 µg/kg) and Vicam (374 µg/kg) results. The variability within and between tests, measured using the RSD%, was not significantly different ($P > 0.05$). Whitaker et al (1996)

reported variability associated with analytical methods used to measure aflatoxin in various commodities among and within laboratories for enzyme-linked immunosorbent assay (ELISA), thin layer chromatography and liquid chromatography (LC). The reported RSD% within laboratories for ELISA and LC were approximately 27% and 13%, respectively, for an aflatoxin concentration approaching 50 µg/kg. The 6% RSD for ELISA (Veratox[®]) and HPLC testing platforms reported by OTSC for the 50 µg/kg aflatoxin concentration sample, while substantially lower than that reported by the Whitaker group, provide an accurate baseline for proficiency test result variability and the capability of different testing platforms to achieve a reproducible result.

Table 3. Descriptive statistics of proficiency sample test results from Texas grain elevators.

Aflatoxin control (µg/kg)	Number of measures (n)	Descriptive statistics			
		Average (µg/kg)	Low (µg/kg)	High (µg/kg)	RSD (%) ^a
52	38	50	2	102	45
378	35	282	4	660	44
580	28	488	64	1056	45

^a RSD (%): relative standard deviation.

Field investigators recorded testing methodology at the 41 grain elevators. A total of 38, 35, and 28 proficiency materials were analyzed by collaborating grain elevators for samples A, B, and C, respectively (Table 3). Aflatoxin testing platforms employed by the Texas grain elevator industry included the Romer FluoroQuant[®] (10), Vicam AflaTest[®] (2), Vicam Series 4EX Fluorometer (13), Vicam FX-100 (4), Vicam Sequioia (1), Charm ROSA (6), Neogen Veratox[®] 8030 (2), and Neogen Veratox[®] AST (3). The average results for the grain elevator proficiency test displayed a low bias for all samples with relative differences of -4%, -25%, and -16% for the 52 µg/kg, 378 µg/kg, and 588 µg/kg samples, respectively. Two of the 41 grain elevators did not participate in the study by

analyzing proficiency materials and two performed qualitative tests that are not included in the data set. Seven of the grain elevators that did possess the test kits capable of measuring >300 µg/kg aflatoxin yielded results within +20% the 378 µg/kg and 580 µg/kg aflatoxin proficiency material. The LAM (2011) requires that “The quantitative test kits used to perform the test must be verified by FGIS and must have a test-kit range of 5-300 µg/kg.” At the time of the manual’s publication, GIPSA had only approved quantitative test kits for measurement up to 100 µg/kg. All of the aflatoxin test kits observed at commercial grain elevators were approved for measuring aflatoxin at or below 100 µg/kg.

The operating characteristics observed and recorded by field investigators were as follows: one firm was using an expired test kit, one firm's scale was not calibrated, the average years of experience performing aflatoxin analysis was 8.2 years, and 6 grain elevators did not clean their grinder between samples.

3.2. Regulatory Consideration

This investigation revealed that fewer than 20% of the grain elevators collect a representative sample as defined by LAM procedures even though the grain elevator personnel were aware that samples were collected for use for crop insurance. The initial intent of the investigation was to assess the feasibility of using a single sample collected for crop insurance to serve a dual purpose of monitoring aflatoxin for regulatory purposes. The inconsistent implementation of LAM sampling procedures precluded adoption of this strategy.

The investigation revealed that aflatoxin analysis contributed significantly to the variability in aflatoxin test results, a common complaint by maize growers and elevator operators. The investigation did not examine sampling error, and it did not enable a partition of variance components between sampling, sample preparation and analysis variation. The variation in proficiency test results were higher than is commonly reported in the literature and indicates the need to focus attention on testing accuracy in addition to sampling and sample preparation protocol.

Several outcomes occurred in response to this investigation. First, the Office of the Texas State Chemist validated aflatoxin test kits used by the Texas grain industry to assess their capability to measure up to 1000 µg/µl in 2010 (Dai et al, 2013). During 2010, 15.4% of the maize samples collected and tested by OTSC contained greater than 100 µg/kg aflatoxin, the threshold of GIPSA aflatoxin test kit approval. In 2012, GIPSA approved the Romer FluoroQuant® Afla for measuring 1000 µg/kg aflatoxin and in 2013, GIPSA approved the Vicam AflaTest® for measuring 1000 µg/kg aflatoxin.

Second, OTSC prepared and distributed control samples to grain elevator operators in Texas so that they could verify their accuracy in testing for aflatoxin. The absence of working control samples is nearly universal in the grain industry.

A third outcome of this investigation involved the director of OTSC preparing a white paper outlining a plan to align industry, state and federal agency sampling and measuring efforts to manage aflatoxin risk (Herrman, 2010). Through a collaborative effort between the RMA, OTSC and the Texas grain industry, a program was launched to reduce risk through the use of a single sample for purchasing, crop insurance, and regulatory risk management referred to as the "One Sample Strategy." To maximize adoption, the One Sample Strategy utilizes protocol within the GIPSA Aflatoxin Handbook (2002). In 2013, 30 Texas firms were enrolled in the program, they measured over 13,000 samples by analysts approved by OTSC, and 600 of these were collected and analyzed by OTSC to verify testing performance. This program represents the first implementation of a co-regulation governance option by OTSC. The participating grain elevators receive working control materials with defined duplication limits that are run daily by the analyst. The results from these elevators are analyzed using statistical process control. These control charts are shared with the participating firms and analysts trained in their use.

This study offers a different perspective on factors contributing to variability in aflatoxin measurement. While sampling is a major contributor to aflatoxin variability, this study supports the premise that aflatoxin analysis (test kit operation) is also a primary contributor to aflatoxin variability and needs greater focus by test kit manufacturers, regulators, and industry.

Acknowledgments

The authors thank Linda Casey, James Embry, Erin Walker, Roy Hendley, Brent Sexton, Scott Hendley, Scott Sanders, Gregory McKinney, Ronald Rinn, Curtin Hinton, Mark Withers, and William Umphres of the Office of the Texas State Chemist.

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