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Aflatoxin risk management in Texas: test kit approval for maize

Susie Y. Dai [a, b,*], Kyung-Min Lee [a], Wei Li [a], Jim Balthrop [a], and Timothy J. Herrman [a, c,*]

[a] *Office of the Texas State Chemist, Texas A&M AgriLife Research, Texas A&M University System, College Station, TX 77841, USA*

[b] *Texas A&M University, Department of Veterinary Pathobiology, College Station, TX 77843, USA*

c Texas A&M University, Department of Soil and Crop Sciences, College Station, TX 77843, USA

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ABSTRACT

Five commercially available quantitative test kits were evaluated for their capability to measure naturally contaminated maize samples at 59 µg/kg, 306 µg/kg, and 901 µg/kg aflatoxin levels. The aflatoxin contaminated maize samples, collected during the 2010 Texas corn harvest, were measured using high performance liquid chromatography (HPLC) following Association of Analytical Communities (AOAC) official method 2005.08. Five aflatoxin quantitative test kits approved for measuring aflatoxin contaminated maize containing ≤100 µg/kg by the United States Department of Agriculture (USDA) Grain Inspection Packers and Stockyard Administration (GIPSA) were evaluated by three analysts who analyzed seven 50 gram samples for each of the three sources of aflatoxin contaminated grain. The relative difference between the HPLC measurements and test kit results were analyzed using an analysis of variance. Significant two-way interactions were observed among the three explanatory variables including analysts, test kit, and aflatoxin level. No significant main effect was observed for analyst. All quantitative test kits performed within GIPSA prescribed performance criteria for the low and medium (59 µg/kg and 306 µg/kg) aflatoxin levels and were approved by the Texas State Chemist to measure aflatoxin contaminated maize as part of the Office of the Texas State Chemist's (OTSC) aflatoxin risk management program. One test kit failed to perform within extrapolated GIPSA criteria at the 901 µg/kg.

1. Introduction

Mycotoxins are produced by fungi as secondary metabolites. Aflatoxins belong to a common group of mycotoxins and are carcinogens (Krska et al, 2008). The United States (US) Food and Drug Administration (FDA) established a 20 µg/kg action level for many major human foods and animal feeds. Aflatoxin contamination of cereals and oilseeds pose a risk to the quality and safety of human food and animal feed, which in turn can lead to economic losses to growers, animal protein producers, grain handlers, and food and feed processors. It is important to monitor the aflatoxin levels in susceptible raw commodities

* Corresponding authors. Office of the Texas State Chemist, Texas A&M AgriLife Research, Texas A&M University System, College Station, TX 77841, USA. Tel: (979) 845-1121; fax: (979) 845-1389; E-mail: susie@otsc.tamu.edu (S. Dai); tjh@otsc.tamu.edu (T. Herrman).

in the market place to ensure fair competition and keep consumer confidence. Many methods developed to quantitatively measure aflatoxin in commodities (AOAC International 2006, Krska et al 2008, Papp et al 2002, Trucksess et al 1991) include thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and mass spectrometry (MS) methods (Li et al, 2011; Papp et al, 2002; Sforza et al 2006, Songsermsakul and Razzazi-Fazeli 2008). The aforementioned methods are difficult to perform at the first collection point of contaminated grain. Fast and inexpensive methods favored by the food and feed industry were approved for use by the GIPSA for measuring aflatoxin and are widely adopted (Zheng, 2006).

Different mechanisms have been utilized in various test kits, which include enzyme-linked immunosorbent assay (ELISA) based detection, immunoaffinity column based purification and fluorescence detection and such (Lupo et al, 2010; Zheng et al, 2005). USDA published guidelines for the performance requirements for aflatoxin test kit to achieve greater uniformity in mycotoxin test kit performance. The published requirements set the precision and accuracy standards and verification procedures (USDA, 2011; USDA, 2012a). Instructions for using USDA approved test kits have also been published in the aflatoxin handbook (USDA, 2012b).

The manufacturers of the test kit market their products according to the capability of the product to measure a specified aflatoxin concentration range. The claims of different kit products for aflatoxin concentration range may vary. For example, the USDA aflatoxin handbook indicates the Neogen Veratox® aflatoxin test kit is able to provide aflatoxin measurement between 5–50 µg/kg based on the test kit claim and for samples containing more than 50 µg/kg aflatoxin, a supplemental analysis to further dilute the sample extract before the test kit measurement is required to guarantee accurate results (USDA, 2012b). Particularly important, interpretation of the test kit results might be difficult when the test sample contains aflatoxin close to or exceed the cut-off level based on the test kit performance claim (Zheng et al, 2006). No prior knowledge of the aflatoxin concentration is available to the personnel who are doing the measurement in the field and they are frequently unaware of the test kits concentration range.

In an attempt to improve the performance of aflatoxin measurement accuracy within the Texas grain and feed industry, a "One Sample Strategy" for aflatoxin was launched by the state government agency responsible for regulating the distribution of feed, which includes raw material containing more than 20 µg/kg aflatoxin in collaboration with industry partners. Among the needs identified in this program involved the verification of quantitative test kits approved by FGIS for measuring heavily aflatoxin contaminated corns using GIPSA criteria for levels of aflatoxin (up to 900 µg/kg) commonly encountered in Texas. All five test kits approved by FGIS for quantitative measurement of aflatoxin were evaluated.

2. Materials and methods

Five quantitative aflatoxin test kits manufactured by four companies were evaluated using the FGIS specifications found in their document titled "Design Criteria and Test Performance Specifications for Quantitative Aflatoxin Test Kits." Those five kits are: VICAM AflaTest®, Romer FluoroQuant® aflatoxin test method from Romer® Labs, Inc., ROSA® aflatoxin (Quantitative) test kit from Charm Sciences, Inc., Neogen Veratox® aflatoxin test kit and Neogen Veratox AST® aflatoxin test kit from Neogen Corporation (Table 1).

Naturally occurring aflatoxin contaminated ground corns were collected by Texas Feed and Fertilizer Control Service regulatory officials. The corn was purchased from the local feed store at the 50 pound per bag. The whole bag of corn was processed with a RAS® mill from Romer® Labs, Inc., and then grounded through a commercial grinder (Retsch® Ultra Centrifugal

Table 1
Test kits receiving certificate of conformance by FGIS-GIPSA for quantitative measurement of aflatoxin in maize.

Test kit	ROSA®	Veratox®	Veratox AST®	FluoroQuant® Afla	AflaTest®
Manufacturer	Charm Sciences, Inc. ®	Neogen®	Neogen®	Romer® Labs, Inc.	VICAM®
Mechanism	Antigen/Antibody recognition and color reading of strip	Antigen/Antibody recognition and color reading of solution	Antigen/Antibody recognition and color reading of solution	Solid phase extraction and fluorescence detection	Immunoaffinity column purification and fluorescene detection
Extraction solvent MeOH/water	100 ml 70/30 (v/v)	250 ml 70/30 (v/v)	250 ml 70/30 (v/v)	100 ml 80/20 (v/v)	100 ml 80/20 (v/v)

Mill SR300) using a 0.75 mm screen. The ground sample was then mixed with a commercial Kobalt® mixer #0241568 for 2 hours. Certified AFB_1, AFB_2, AFG_1, and AFG_2 were purchased from Romer Labs, Inc.-Biopure (Tulln, Austria). All solutions are made with HPLC grade solvents and reagent grade materials unless otherwise noted. The concentration of AFB_1 and AFG_1 standard is 2 µg/mL in 5 mL acetonitrile. The concentration of AFB_2 and AFG_2 standard is 0.5 µg/mL in 5 mL acetonitrile.

2.1. Romer® Labs, Inc. FluoroQuant® (FQ Afla) test method

Fifty grams of each corn sample was extracted with 100 mL of 80% methanol/water and shaken for 1 hr at 200 rpm. Sample extracts were then filtered. One mL of the filtrate was passed onto the column and mixed with 1 mL of Romer® Labs, Inc., dilution buffer. The mixed solution was pushed through the column with a syringe plunger. The developer solution was prepared by mixing the developer solution provided in the kit with 25 mL of DI water. Five hundred µL of the column eluted solution was mixed with 1 mL developer solution and put into the Romer® Labs, Inc., reader for immediate reading.

2.2. Vicam AflaTest® test method

Fifty grams of each corn sample was mixed with 5 g 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 filtered. 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 twice with 5 mL of DI water. 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 by the kit) with 45 mL DI water. One mL of prepared developer solution was added to the methanol eluate and the mixed solution was vortexed and placed into the reader for immediate reading.

2.3. Neogen Veratox® aflatoxin test method

Fifty grams of each corn sample was extracted with 250 mL of 70% methanol/water and shaken for 1 hour 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 by the kit) was pipetted into a red-colored well provided in the kit.100 µL of the calibration

standard solution and the sample 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. The solution was incubated in the antibody coated well for two minutes and then rinsed out with DI water. One hundred liters of the substrate solution was added into the antibody coated well after all the rinsing water was removed from the well. The solution was incubated for 3 minutes before the 100 µL of the stop solution (provided by the kit) was added into the well. The mixed solution was then put into the reader for immediate reading.

The Veratox AST® kit was used with the similar procedure except the incubation time for the conjugate with the sample extract was five minutes and the incubation time for the substrate with the antibody coated well was 5 minutes as well.

2.4. Charm Sciences, Inc. ROSA® aflatoxin (quantitative) test method

Fifty grams of each corn sample was extracted with 100 mL of 70% methanol/water and shaken by hand for three minutes. The extract was then filtered. One hundred µL of the filtrate was transferred into an Eppendorf tube and 1 mL of the AFQ solution (provided by the kit) was mixed with the filtrate. Three hundred µL of the mixed solution was transferred onto the strip (supplied by the kit) and the strip was incubated at 40 degrees on the incubator provided with the kit for 10 minutes. After the incubation, the strip was inserted into the reader for reading.

2.5. HPLC analysis

Fifty grams of each ground corn sample was extracted with 250 mL of methanol/water (70:30, v/v) by mechanical shaker for 1 hour at 200 rpm. A 15 mL extract was filtered through a folded filter paper (Whatman #1), and 5 mL of the filtered extract was diluted with water with a dilution factor of 5 and 1 gram of sodium chloride was added into the diluted solution. After filtration, 2 mL of the solution was loaded onto an immunoaffinity column (AflaTest® affinity column, Vicam #12020). The solution was pushed through the column. After washing the column with 5 mL of water twice, 1 mL of methanol was used to elute the aflatoxins out from the column. Eluate was diluted with 1 mL of HPLC water. The mixture was then filtered through a 0.2 µm syringe filter prior to HPLC-fluorescence analysis. HPLC analysis of aflatoxins was carried out using a Waters® 2695 system (Milford, MA). The system also includes a fluorescence detector (parameters: 360 nm excitation, 420 nm emission) and a PHRED™ (Photochemical Reactor for Enhanced Detection) with 254 nm low pressure mercury bulb and 25 m x 0.25 mm ID knitted reaction coil. The chromatographic separation was achieved on a Waters Spherisorb® C18 column (5 µm, 4.6 x 150 mm) with a Waters® Spherisorb guard column (10x4.6 mm). Mobile phase is 3:1:1 water:acetonitrile:methanol. Isocratic flow rate is 1.0 ml/min. The separation of aflatoxin B_1, B_2, G_1 and G_2 was achieved with good resolution. The results were calculated using the HPLC software (Waters Empower™).

2.6. Statistical analysis

Three chemists analyzed seven samples per aflatoxin test kit for each of the three reference samples. The reference samples were also subject to HPLC analysis methodology (21 times per reference sample) yielding 63 independent HPLC analyses yielding official results of 59 µg/kg, 306 µg/kg, and 901 µg/kg. The results of these tests are summarized in Table 2 including the mean, relative standard deviation, and accuracy of the kit compared to HPLC results. A completely randomized design was employed with seven replications of three treatments (analyst, test kit, and aflatoxin level). The percent relative difference for each aflatoxin measurement was calculated by

Table 2
Statistical results of validating test kits by comparing with reference HPLC results.

Aflatoxin levels (μg/kg)[a]	Statistical parameters	Kit 1	Kit 2	Kit 3	Kit 4	Kit 5	HPLC
59	Mean (μg/kg)	48	64	56	52	65	59
	RSD[b]	0.16	0.13	0.15	0.13	0.15	0.13
	Accuracy (%)[c]	-17.8	7.9	-4.6	-12.1	10.9	
306	Mean (μg/kg)	266	312	293	284	301	306
	RSD	0.08	0.06	0.12	0.15	0.14	0.04
	Accuracy (%)	-12.9	1.9	-4.1	-7.3	-1.8	
901	Mean (μg/kg)	817	946	1199	799	829	901
	RSD	0.09	0.10	0.14	0.06	0.16	0.06
	Accuracy (%)	-9.3	5.0	33.0	-11.3	-7.9	

[a] Aflatoxin concentrations determined by HPLC
[b] RSD: relative standard deviation
[c] Accuracy (%) = [(estimated−HPLC value)/HPLC value]x100

subtracting the HPLC value from the test kit value, dividing the difference by the average HPLC value (e.g. 59, 306, and 901 μg/kg for the low, medium and high reference sample) and multiplying by 100. Relative differences for chemist, test kit, aflatoxin level were evaluated with a general linear model analysis using the SAS® Proc GLM procedure. The means of three treatments were compared using least significant difference (LSD) and least-squares means (LS Means) with the PDIFF and SLICE options. All statistical analyses were conducted using SAS® software (SAS®, 2009).

3. Results and discussion

In this study, the HPLC analysis reports the aflatoxin concentration as the sum of aflatoxin B_1, B_2, G_1 and G_2. Comparable to the HPLC measurements, all test kits under the current study are measuring total aflatoxin (B_1, B_2, G_1 and G_2) as well. GIPSA publishes dilution instructions for operating test kits were to evaluate aflatoxin over 100 μg/kg, however, their published verification reports for test kit approval do not exceed 100 μg/kg. The relative standard deviation (RSD) for the official HPLC method was 0.13, 0.04, and 0.06 for the three sample levels of 59 μg/kg, 306 μg/kg, and 901 μg/kg, respectively (Table 2). All test kit results displayed greater variability than the official method at the 306 μg/kg and 901 μg/kg aflatoxin concentration. Johansson et al (Johansson et al, 2000) estimated the variation attributed to sampling, sample preparation, and sample analysis of ground maize at 10 levels of aflatoxin concentration. They found a 26.5% RSD attributed to sample preparation and a 10.7% RSD for analytical variance in a 1.18 kg sample of maize. The USDA Aflatoxin Handbook permits 16% variability between aflatoxin test kits performance at the 100 μg/kg concentration. Study results conform to these results, verifying the analytical control of this experiment.

The significance of main effects and their interactions for the relative difference in aflatoxin measurement are presented in Table 3.

Table 3
Analysis of variance (ANOVA) for mycotoxin concentration measured by different mycotoxin test kits.

Source[a]	DF	F-value	P-value
Replication	6	0.8	0.5940
Analyst	2	1.4	0.2469
Kit	4	49.8	<.0001
Analyst x Kit	8	6.0	<.0001
Level	2	11.5	<.0001
Analyst x Level	4	14.9	<.0001
Level x Kit	8	25.1	<.0001
Analyst x Level x Kit	16	2.8	<.0004

[a] Analyst, three analysts performing sample analysis; Kit, five mycotoxin test kits used for the study; Level, different mycotoxin concentrations of samples (59, 306, and 901 µg/kg).

Significant two-way interactions were observed for test kit by analyst, test kit by aflatoxin level, and analyst by aflatoxin level. Main effects were observed for test kit and aflatoxin level, however, there was no significant main effect attributed to analyst.

The statistically significant (P<0.05) two-way interaction between test kit and analyst resulted from a difference in magnitude and direction in the results (Figure 1). For example, Kit 3 always displayed a positive difference compared to the official method and the deviation in the least square mean (LSM) values ranged between 5.2 and 12.9% among the three analysts. In comparison, Kit 1 yielded results consistently lower compared to the official method and the LSM values between analysts ranged between -8.6% and -16.5%. Kit 5 results were above and below the official method, depending upon analysts, and ranged between -5.5 and 9.2% LSM. Kit 2 displayed the most robust performance across analyst as evidenced by the absence of (no) significant difference (P>0.05) between the three analysts results. Kit 4 displayed significant difference between all three analysts. The consistence among test kits was also determined by the aflatoxin concentration among the samples (Figure 2).

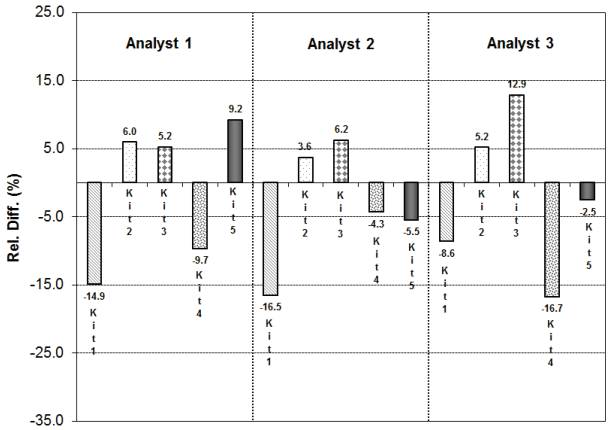

Fig. 1. Two-way interaction plot: difference in aflatoxin concentrations (µg/kg) among analysts with different test kits.

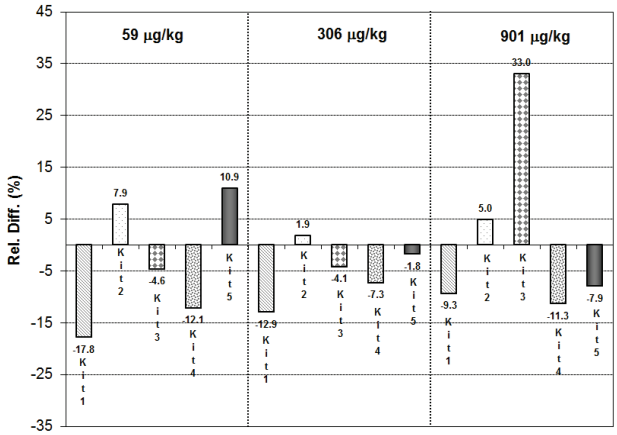

Fig. 2. Two-way interaction plot: difference in aflatoxin concentrations (µg/kg) among test kits at different aflatoxin levels determined by HPLC.

The statistically significant (P<0.05) test kit by aflatoxin concentration two-way interaction indicated the stability in relative difference between the test kit and official results for differing aflatoxin concentrations. Kit 3 displayed a negative relative difference compared to the official result for aflatoxin levels of 59 and 306 µg/kg and a positive relative difference at 901 µg/kg. The magnitude of difference between Kit 3 and the HPLC result varied from 4.1% to -33.0% at the low and high levels as measured by the LSM values. Kit 1 relative difference results were consistently

below the official method results and ranged in magnitude of relative difference between -17.8 and -9.3% from the lowest to highest aflatoxin sample concentration. Kit 2 and Kit 5 displayed no significant difference in percent relative difference at the three aflatoxin levels (59 µg/kg, 306 µg/kg, and 901 µg/kg).

The dilution schemes were performed according to the test kit performance claim following the methodology outlined in the USDA Aflatoxin Handbook. Comparing the relative difference between official and test kit results for the three aflatoxin concentrations across analyst also yielded a significant two-way interaction. For analyst 1, the relative difference in aflatoxin for the low concentration was significantly different from the two higher levels and the percent least square means ranged between 1.7% and -9.8%. For analyst 2, the relative difference in aflatoxin was not significantly different and the least square means ranged between -2.0 and -5.0%. These types of variations are commonly observed when more than one analyst performs a test, however, there was no significant main effect attributed to analyst. The LSM for the relative difference was -0.8%, -3.3% and -2.0% below the official test results among the three analysts.

A significant test kit main effect was observed with LSM values as follows: Kit 1, 13.3%; Kit 2, 4.9%; Kit 3, 8.0%; Kit 4, 10.2%; and Kit 5, 0.4%. Kit 1 and Kit 4 yielded consistently lower measures and Kit 3 gave consistently higher results than the official method.

According to the USDA-GIPSA requirements, the acceptable limits for the relative standard deviation (RSD) to measure samples that contain 20 and 100 µg/kg aflatoxin is 20% and 16%, respectively. USDA-GIPSA has not published acceptable limits for measuring aflatoxin at higher levels.

In our evaluation, all five kits yielded a RSD value smaller or equal to 16% at all measured concentration levels. Meanwhile, USDA-GIPSA also requires that at least 95% of the individual values at each concentration level must be within the applicable range specified for each concentration range. The range is calculated as the mean plus or minus twice the standard deviation. Thus for a sample that contains 100 ppb aflatoxin, at least 95% of the test kit measurement results should be in the range of 68-132 µg/kg. By using the same maximum 16% RSD at 100 µg/kg, a range of 612-1188 µg/kg is calculated for samples containing 901 µg/kg aflatoxin, however, USDA-GIPSA did not publish the acceptable limits for the 901 ppb concentration level. According to this requirement, one test kit would not meet the GPSIA requirements as 10 out of 21 measurement results were higher than 1188 µg/kg.

The Office of the Texas State Chemist is the agency tasked with protecting animal and human health involving regulatory oversight of commercial grain handlers and feed manufacturers for aflatoxin risk management in Texas. A key element in the Texas aflatoxin risk management program involves accurate quantification of aflatoxin by establishments licensed to distribute maize and maize products containing >20 µg/kg aflatoxin. As an outcome of this study, the state of Texas approved all test kits for measuring aflatoxin to 300 µg/kg and four of the kits for measuring aflatoxin at the 1000 µg/kg level. While the highest action level permitted for commercial distribution of aflatoxin contaminated maize by the FDA is 300 µg/kg, the Office of the Texas State Chemist has reported between 1.4% to 2.8% of the Texas maize crop exceeded 500 µg/kg from 2008-2010.

Prior to this study, no regulatory body validated test kits for measuring aflatoxin >100 µg/kg using the USDA-GIPSA dilution scheme and validation procedures. Since this study, GIPSA has validated the Romer® Labs, Inc., platform and approved its use by their agency and designees for measuring aflatoxin levels ≤1000 µg/kg without dilution.

Accurately measuring aflatoxin concentrations at the first commercial collection point is an important step toward protecting public health and managing risk associated with this naturally occurring and highly prevalent

carcinogen. In 2012, a severe drought in the Midwestern US resulted in aflatoxin concentrations more commonly observed in Texas. The adoption of validated dilution schemes and aflatoxin test kits presented in this study throughout the US and abroad will assist regulatory risk management programs and help maintain a safe food supply.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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