Determining the Variability Associated with Testing Shelled Corn for Aflatoxin Using Different Analytical Procedures in Louisiana in 1998

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The number of elevator facilities with laboratories to test shelled corn for aflatoxin on site is increasing. The inherent difficulty in accurately determining the true aflatoxin concentration of a lot of corn may have serious implications. Deviations from the true value are of even greater significance at busy locations where a high throughput is desired. This study was instituted to measure (1) the differences in aflatoxin test results between elevator laboratories and the Louisiana Agricultural Chemistry (LAC) laboratory and (2) the variability in aflatoxin test results associated with sampling, sample preparation, and analysis of shelled corn at such locations. One hundred lots of shelled corn from 10 elevators in Louisiana were analyzed for aflatoxin using the Aflatest method (at elevators and at the LAC laboratory) and high-performance column liquid chromatography (HPLC; LAC laboratory only). Mean aflatoxin levels determined at elevator laboratories were significantly ($P < 0.05$) lower from those obtained in the LAC laboratory using the Aflatest method. Overall, Aflatest method results were lower than those obtained by HPLC. This difference may be attributed to analyst technical dexterity, difficulty in providing careful attention to detail in a high throughput environment; and/or substandard facilities found at elevators. The total variance was partitioned into the combined sampling plus subsampling variance and analytical variance. The sampling and sample preparation steps accounted for about 91.5% of the total variability. When using the HPLC analytical method, the analytical step contributed only 8.5% to the total variance.

Aflatoxins are toxic, mutagenic, and carcinogenic compounds produced by *Aspergillus flavus* and *A. parasiticus* that can contaminate selected agricultural commodities, such as corn, cottonseed, and peanuts, as they grow in the field or if the commodities are stored under improper conditions (1). Animals and humans can be at risk through exposure to these toxins and/or their metabolites. The principal toxins encountered include aflatoxins B$_1$ and G$_1$ for agricultural commodities and aflatoxin M$_1$ in dairy products (2, 3).

Because the U.S. Food and Drug Administration has established action limits for aflatoxin in shelled corn, elevator operators are adding an aflatoxin laboratory to test incoming lots on site for aflatoxin. Estimating the true aflatoxin concentration in bulk shipments of shelled corn is difficult in a high throughput environment, where rapid turnaround is required. Also, the true aflatoxin concentration of a producer's lot cannot be determined with 100% confidence because of the variability associated with the test procedure used to measure aflatoxin in a lot (4). As a result, lots will be misclassified when placed into categories based upon the lot's aflatoxin concentration. Some good lots are rejected (seller's risk or false positives) and some bad lots are accepted (buyer's risk or false negatives) by the aflatoxin sampling plan, causing economic losses for both the seller and the buyer.

The total variability associated with the test procedure is the sum of the variability associated with sampling, sample preparation, and analytical steps. Sampling variability is usually the largest source of variation, due to the distribution of contamination among individual kernels in a lot. Generally
a small percentage of the kernels are contaminated, and contamination levels can be extremely high (5). To reduce the variability associated with an aflatoxin test procedure, the variability associated with each step of the test procedure must be first determined. The objectives of this study were to (1) measure the differences in aflatoxin test results between elevator laboratories and the Louisiana Agricultural Chemistry (LAC) laboratory, (2) measure the total variability associated with testing shelled corn for aflatoxin, and (3) partition the total variance into combined sampling and subsampling variance and analytical variance.

**Experimental**

Two 4.5 kg composite samples (accumulation of corn from 10 probes, each 450 g) were collected from each of 10 lots (trucks) of shelled corn being processed at 10 different elevators in Louisiana. The cooperating elevator ground 1 sample, while the Agricultural Chemistry Laboratory of the Louisiana Department of Agriculture ground the other. The samples were ground in a Romer mill (Romer Labs, Inc., Union, MT) or equivalent. Three 50 g subsamples were then taken from each comminuted 4.5 kg sample at each location. The cooperating elevator quantified aflatoxin using the Aflatest immunoaffinity column method (Vicam Corp., Somerville, MA; 6) in two 50 g subsamples (one from the sample ground at the elevator and the second from the sample ground at the Agricultural Chemistry facility). The Agricultural Chemistry Laboratory assayed 4 subsamples (2 from the sample ground at an elevator and 2 from the sample ground at the laboratory) for aflatoxin using column high-performance liquid chromatography (HPLC; 7) and the same immunoaffinity test procedure as the elevators. Each elevator quantified aflatoxin in only 1 aliquot taken from the subsample extract, while the Agricultural Chemistry Laboratory quantified aflatoxin in 2 aliquots taken from the subsample extract. A diagrammatic illustration of the sample preparation and distribution is shown in Figure 1.

The variability associated with the aflatoxin test procedure was measured using the variance statistic. The total variance associated with the aflatoxin test procedure is the sum of the sampling (s), sample preparation (sp), and analytical (a) variances (Equation 1):

$$S_t^2 = S_s^2 + S_{sp}^2 + S_a^2 \tag{1}$$

The experimental design in this study provided estimates of the analytical variance and total variance associated with testing corn for aflatoxin. Subtracting analytical variance from total variance in Equation 1 gave an estimate of the combined sampling and sample preparation variance:

$$S_{sp}^2 = S_s^2 + S_{sp}^2 \tag{2}$$

Differences among aflatoxin values of the 2 aliquots from the same subsample gave an estimate of the analytical variability. The analytical variability associated with both the Aflatest and HPLC methods was estimated from the Agricultural Chemistry Laboratory results. Furthermore, differences among aflatoxin values for each lot gave an estimate of the total variance associated with the aflatoxin test

**Table 1. Average aflatoxin concentration (ng/g) among corn samples for all trucks and all elevators**

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Analytical method</th>
<th>Aliquot 1</th>
<th>Aliquot 2</th>
<th>Aliquot 1</th>
<th>Aliquot 2</th>
<th>Avg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agricultural Chemistry Laboratory</td>
<td>Aflatest</td>
<td>143.4</td>
<td>138.0</td>
<td>119.8</td>
<td>—</td>
<td>133.7</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>170.0</td>
<td>173.0</td>
<td>144.6</td>
<td>—</td>
<td>162.6</td>
</tr>
<tr>
<td>Elevators (all)</td>
<td>Aflatest</td>
<td>79.3</td>
<td>—</td>
<td>64.4</td>
<td>—</td>
<td>71.9</td>
</tr>
</tbody>
</table>

* — Not determined.
procedure used by the Agricultural Chemistry Laboratory and the elevators.

The Agricultural Chemistry Laboratory used both HPLC and Aflatest analytical methods to estimate aflatoxin in Samples 1 and 2 from material ground in the laboratory and at elevators. As a result, the null hypothesis that there was no difference between aflatoxin measured by the 2 methods was tested. The traditional analysis of variance (ANOVA) procedure was not considered acceptable since the aflatoxin variation from sample to sample was probably larger than the aflatoxin variation between the 2 analytical methods used within each sample. So a paired data analysis was used to test if the difference in aflatoxin measured by the 2 analytical methods was significantly different from 0. A total of 200 paired differences (100 for each sample) were tested.

Since both the Agricultural Chemistry Laboratory and the elevators used the Aflatest analytical method, the differences between aflatoxin values obtained by the Agricultural Chemistry Laboratory and each elevator were tested using paired data analysis. The Aflatest results paired differences test was performed for 9 of the 10 elevators. Data from Elevator B could not be used because it was reported in a noncompatible form (less or more than different levels).

Results and Discussion

Analytical Methods

The average aflatoxin content among all 100 lots sorted by laboratory, analytical method, and aliquot are shown in Table 1. There was good agreement between aflatoxin levels quantified by the Agricultural Chemistry Laboratory in Aliquots 1 and 2 when using either the HPLC or Aflatest analytical method. However, mean aflatoxin values measured by HPLC were about 18% (162.6-133.7/162.6) higher than those measured with the Aflatest method. A paired data ANOVA indicated that the null hypothesis of no differences between Agricultural Chemistry Laboratory HPLC and Aflatest was rejected at the 95% confidence limit (Table 2).

Table 2. Paired data analysis of variance to determine difference between values obtained by Aflatest and HPLC for the Agricultural Chemistry Laboratory only

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>No of paired data</th>
<th>Avg. difference</th>
<th>Standard error</th>
<th>T*</th>
<th>Probability &gt;T</th>
<th>Significant difference at 5% CL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agricultural Chemistry Laboratory</td>
<td>200</td>
<td>-27.8</td>
<td>5.35</td>
<td>-5.205</td>
<td>0.0001</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* T = T statistic.

* CL = Confidence limit.

Table 3. Paired data analysis of variance to determine difference between Aflatest results obtained by grain elevators and the Agricultural Chemistry Laboratory

<table>
<thead>
<tr>
<th>Elevator</th>
<th>No of paired data</th>
<th>Avg. difference</th>
<th>Standard error</th>
<th>T*</th>
<th>Probability &gt;T</th>
<th>Significant difference at 5% CL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20</td>
<td>29.2</td>
<td>21.9</td>
<td>1.330</td>
<td>0.199</td>
<td>No</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>-73.4</td>
<td>26.3</td>
<td>-2.785</td>
<td>0.012</td>
<td>Yes</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>-15.0</td>
<td>8.4</td>
<td>-1.781</td>
<td>0.091</td>
<td>No</td>
</tr>
<tr>
<td>E</td>
<td>20</td>
<td>19.8</td>
<td>10.4</td>
<td>1.908</td>
<td>0.072</td>
<td>No</td>
</tr>
<tr>
<td>F</td>
<td>20</td>
<td>13.6</td>
<td>18.5</td>
<td>0.734</td>
<td>0.472</td>
<td>No</td>
</tr>
<tr>
<td>G</td>
<td>20</td>
<td>16.2</td>
<td>12.4</td>
<td>1.304</td>
<td>0.208</td>
<td>No</td>
</tr>
<tr>
<td>H</td>
<td>20</td>
<td>146.3</td>
<td>30.8</td>
<td>4.747</td>
<td>0.000</td>
<td>Yes</td>
</tr>
<tr>
<td>I</td>
<td>20</td>
<td>29.6</td>
<td>11.7</td>
<td>2.534</td>
<td>0.020</td>
<td>Yes</td>
</tr>
<tr>
<td>Y</td>
<td>20</td>
<td>72.2</td>
<td>22.9</td>
<td>3.154</td>
<td>0.005</td>
<td>Yes</td>
</tr>
</tbody>
</table>

a Difference between first aliquot Aflatest results from Agricultural Chemistry Laboratory and elevator (i.e., Agricultural Chemistry Laboratory – elevator). Negative value indicates Agricultural Chemistry Laboratory ppb < elevator ppb.

b T = T statistic.

* CL = Confidence limit.

d — Data not used because of incompatible qualitative format (i.e., expressed as < or >).
The average among the 200 difference values (Aflatest-HPLC) was -27.8 ng/g.

The differences in laboratory and elevator Aflatest results were varied. The aflatoxin values measured by the Agricultural Chemistry Laboratory were 46.2% (133.7-71.9/133.7) higher than mean aflatoxin values measured by elevators using the Aflatest method, with the exception of Elevators C and D (Table 3). A paired data ANOVA indicated that the null hypothesis of no difference between elevators and the Agricultural Chemistry Laboratory Aflatest values was rejected at the 95% confidence limit for 4 elevators (C, H, I, and Y). No significant difference was detected for 5 elevators (A and D–G).

Variability

Analytical variability.—The analytical variances associated with the Aflatest and HPLC analytical methods used by the Agricultural Chemistry Laboratory were plotted in full log plots versus aflatoxin concentration (C), in Figures 2 and 3, respectively. The analytical variance appeared to be a function of aflatoxin concentration. From regression analysis, Equations 3 and 4 describe the analytical variances for the Aflatest ($S^2_{a1a}$) and HPLC ($S^2_{alh}$) methods, respectively:

$$S^2_{a1a} = 0.111 \times C^{1.535}$$  (3)

$$S^2_{alh} = 0.248 \times C^{1.363}$$  (4)

where C is the aflatoxin concentration in ng of aflatoxin/g of corn, or parts per billion (ppb). The correlation coefficient associated with regression Equations 3 and 4 are 0.62 and 0.79, respectively. Equations 3 and 4 indicate that the variability associated with HPLC and Aflatest were about the same magnitude. For an aflatoxin concentration of 20 ng/g, the HPLC and Aflatest variances were 14.7 and 11.0, respectively. The coefficient of variation (CV) values were 19.2 and 16.6% for the HPLC and Aflatest methods, respectively.

Using the CV, the analytical variability associated with testing corn for aflatoxin are shown in Figure 4 for Aflatest and HPLC at the LAC Laboratory.

Total variability.—Two estimates of the total variability associated with testing corn for aflatoxins were obtained. Both estimates reflected a 4.5 kg sample, use of the Romer mill, and a 50 g subsample. One test procedure reflected the Aflatest analytical method and the other test procedure reflected the HPLC analytical method. The total variances associated with the HPLC and Aflatest analytical methods were plotted in full log plots versus aflatoxin concentration (C) in Figures 5 and 6, respectively. The total variances also appeared to be a function of aflatoxin concentration. From regression analysis, Equations 5 and 6 describe the total variances for the Aflatest ($S^2_{tla}$) and HPLC ($S^2_{lth}$) methods, respectively:

$$S^2_{tla} = 2.08 \times C^{1.282}$$  (5)

$$S^2_{lth} = 0.248 \times C^{1.363}$$  (6)
The correlation coefficient associated with regression Equations 5 and 6 were 0.72 and 0.74, respectively. Equations 5 and 6 indicate that the total variability associated with the HPLC and Aflatest analytical methods were about the same magnitude. For an aflatoxin concentration of 20 ng/g, the total variance associated with the HPLC and Aflatest analytical methods (both used a 4.5 kg sample, Romer mill, and 50 g subsample) were 173.2 and 130.7, respectively. The CV values were about 65.8 and 57.2% for test procedures that used HPLC and Aflatest analytical methods, respectively.

Sample plus subsample variability.—Two estimates of the variability associated with the combined sampling plus sample preparation variance ($S^2_{sp}$) were obtained by first subtracting Equation 3 from Equation 5, and then subtracting Equation 4 from Equation 6. Both estimates reflect a 4.5 kg sample, Romer mill, and a 50 g subsample:

$$S^2_{sp} = (4.714 \times C^{1.203}) - (0.248 \times C^{1.363})$$  
$$S^2_{sp} = (2.808 \times C^{1.282}) - (0.111 \times C^{1.535})$$

Equations 7 and 8 provide 2 estimates of the combined sampling plus sample preparation variability associated with a 4.5 kg sample, Romer mill, and 50 g subsample. For an aflatoxin concentration of 20 ng/g, the 2 variance estimates were 158.5 and 119.7, respectively. The CV values were 62.9 and 54.7%. While the variability associated with the sample preparation step could not be determined in this study, other studies have shown sample preparation variability to be relatively small compared to the sampling variance (8).

At an aflatoxin concentration of 20 ng/g, the analytical variance associated with the Aflatest and HPLC methods contributed only 8.4 (11.0/130.7) and 8.5% (14.7/173.2) of the total variability, respectively. The combined sampling and subsampling variance contributed 91.6 and 91.5% of the total variability, respectively, depending on which analytical methods were used in the test procedure. While the combined sampling plus subsampling variance could not be partitioned into 2 variance components in this study, sampling is probably the largest source of variability associated with the aflatoxin test procedure.

**Conclusions**

Numerous elevators with aflatoxin testing facilities have been established in Louisiana. These facilities are supposed to rapidly provide aflatoxin results upon which tested lots may either be accepted or rejected for a specific use. Rejection of a lot may have serious economic implications because of the lower monetary value assigned to such commodities. Rapid turnaround of aflatoxin test results is important in a high throughput environment while maintaining accuracy and precision. If accuracy and precision are sacrificed for rapid turnaround, more lots will be misclassified, and both the producer and elevator operator will suffer an economic penalty.

On the average, aflatoxin test results from elevators were significantly ($P < 0.05$) lower than aflatoxin test results from the LAC Laboratory. This difference may be attributed to analyst technical dexterity, difficulty in providing careful attention to detail in a high throughput environment, and/or substandard facilities found at elevators.

Because of the nature of aflatoxin distribution, it is difficult to accurately estimate the true aflatoxin concentration in a bulk lot. As a result, estimates of aflatoxin content in a lot may vary from one testing facility to the other. Previous investigators (8) have shown that variability in aflatoxin test results is highly influenced by the sampling and sample preparation steps.

Results from this study have confirmed the major source of uncertainty associated with the aflatoxin test procedure is
associated with the sampling and sample preparation steps of an aflatoxin test procedure. The sampling and sample preparation steps accounted for about 91.5% of the total variability. When using the HPLC method, the analytical step contributed only 8.5% to the total variance.

For lots where the aflatoxin concentration is close to the permissible limit, consumers may be placed at risk of exposure to hazardous aflatoxin levels and processors placed at the risk of falsely rejecting an otherwise acceptable lot.

Acknowledgments

We thank the following contributing participants:
Philip Bible, Wisner Elevator, Wisner, LA
Brent Bordelon, Bunge Elevator, Lettsworth, LA
Rod Daggett, Bunge Elevator, Jonesville, LA
Kimble Hayes, Bunge Elevator, St. Joseph, LA
Ronnie Mulberry, Morehouse Grain Elevator, Mer Rouge, LA
John O'Neal, Central Louisiana Grain Elevator, Boyce, LA
Don Raley, Monticello Elevator, Epps, LA
Gordon Raley, Monticello Elevator, Epps, LA

Ronnie Swazye, Delta Gin Co. Elevator, Newelton, LA
Brad Terral, Terral Framers Elevator, Delhi, LA

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