SPECIAL GUEST EDITOR SECTION

Testing Shelled Corn for Aflatoxin, Part I: Estimation of Variance Components

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The variability associated with testing lots of shelled corn for aflatoxin was investigated. Eighteen lots of shelled corn were tested for aflatoxin contamination. The total variance associated with testing shelled corn was estimated and partitioned into sampling, sample preparation, and analytical variances. All variances increased as aflatoxin concentration increased. With the use of regression analysis, mathematical expressions were developed to model the relationship between aflatoxin concentration and the total, sampling, sample preparation, and analytical variances. The expressions for these relationships were used to estimate the variance for any sample size, subsample size, and number of analyses for a specific aflatoxin concentration. Test results on a lot with 20 parts per billion aflatoxin using a 1.13 kg sample, a Romer mill, 50 g subsamples, and liquid chromatographic analysis showed that the total, sampling, sample preparation, and analytical variances were 274.9 (CV = 82.9%), 214.0 (CV = 73.1%), 56.3 (CV = 37.5%), and 4.6 (CV = 10.7%), respectively. The percentage of the total variance for sampling, sample preparation, and analytical was 77.8, 20.5, and 1.7, respectively.

Aflatoxin is a naturally occurring mycotoxin that has been proven toxic and carcinogenic (1). This toxin was first discovered in the 1960s when the deaths of thousands of turkey poults were traced to aflatoxin-contaminated feed (1).

Aflatoxin is mainly produced by 2 fungi, *Aspergillus flavus* and *Aspergillus parasiticus* (2), which can easily invade agricultural commodities under favorable environmental conditions, such as high temperature and moisture. The occurence of aflatoxin in corn and its products is a potential threat to animal and human health. The U.S. Food and Drug Administration (FDA) has currently set an aflatoxin guideline of 20 parts per billion (ppb) in products for all commodities destined for human consumption (3).

An aflatoxin testing procedure consists of 3 steps: sampling, sample preparation, and analysis. Aflatoxin inspection and sampling programs have been developed for commodity industries to help meet FDA legal limits. Processing plants voluntarily test domestic lots of shelled corn, and some states offer voluntary aflatoxin testing programs. In addition, the Grain Inspection, Packers and Stockyards Administration's (GIPSA) Federal Grain Inspection Service (FGIS) tests all lots of shelled corn that are destined for export for the presence of aflatoxin. The FGIS currently uses a 908 g (2 lb) representative test sample for trucks, 1362 g (3 lb) representative test sample for railcars, and 4540 g (10 lb) representative test sample for barges. The test samples are comminuted in a Romer mill and a 50 g subsample is riffled out for analysis. The aflatoxin in the 50 g subsample is extracted with solvents such as methanol–water. Aflatoxin in the solvent is quantitated by various methods such as liquid chromatography (LC) and immunoassay (4).

Combining an aflatoxin threshold or a sample acceptance level with a testing procedure defines a sampling plan. Some sampling plans use a sample acceptance level below the FDA legal limit of 20 ppb to ensure that a finished product will meet FDA requirements.

Estimating the true amount of aflatoxin in a lot of shelled corn is difficult because of the distribution of contaminated

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kernels in a lot. Cucullu et al. (5) showed that a small percentage of peanuts in a lot were contaminated, and some contaminated peanuts had extremely high concentrations of aflatoxin. It is assumed that aflatoxin in shelled corn would behave in a similar manner. The variability associated with testing samples of shelled corn for aflatoxin was studied to provide a base for statistically measuring the effectiveness of sampling plans. Each component of the total variance (sampling, sample preparation, and analysis) was investigated to show how much each step of the testing procedure contributes to the total testing variability (6, 7).

The objectives of this study were to determine the total variance associated with testing commercial shelled corn for aflatoxin; partition total variance into sampling, sample preparation, and analytical variability components; and determine functional relationships between the variance components and aflatoxin concentration.

Experimental

Eighteen lots of shelled corn, suspected of being contaminated with aflatoxin, were collected from 8 counties in North Carolina.

Theoretical Considerations

We assumed that each lot consists of N individual corn kernels, each corn kernel has the same mass and physical characteristics, and that variation of aflatoxin concentration occurs between kernels. With shelled corn, it is common practice to estimate the aflatoxin concentration of a sample of *n* kernels, represented by $\hat{C}$, instead of analyzing aflatoxin on individual kernels $\hat{C}_i$. Cucullu (5) showed that most individual peanuts have an aflatoxin concentration of zero, but occasionally a peanut may have an extremely high aflatoxin concentration. It is assumed that aflatoxin in shelled corn behaves in the same manner.

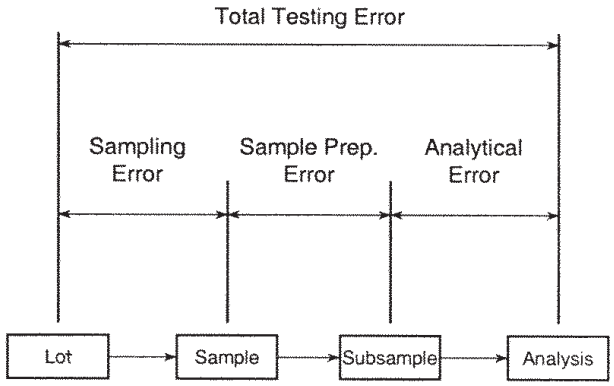

Figure 1. Total variance partitioned into sample, sample preparation, and analytical components.

Figure 1 shows the relationships between the 3 major components of the total variation associated with testing shelled corn for aflatoxin: sampling, sample preparation, and analysis.

A lot of corn with an aflatoxin concentration C is estimated by using a sample of individual corn kernels denoted as $\hat{C}$. A statistical model for the variability among aflatoxin test results $\hat{C}$ taken from the same lot can be represented by:

$$\hat{C} = \mu + S + SS + A \quad (1)$$

where μ = the true aflatoxin concentration in the lot being tested, S = random deviations of sample concentrations about the true lot concentration with expected value equal to zero and variance $\sigma^2_{\hat{C}(s)}$, SS = random deviations of subsample concentrations about the comminuted sample concentration with expected value equal to zero and variance $\sigma^2_{\hat{C}(ss)}$, and A = random deviations of analytical assay results about subsample concentration with the expected value zero and variance $\sigma^2_{\hat{C}(a)}$. If independence among the random deviations in Equation 1 is assumed, the model for variance can be obtained:

$$\sigma^2_{\hat{C}(t)} = \sigma^2_{\hat{C}(s)} + \sigma^2_{\hat{C}(ss)} + \sigma^2_{\hat{C}(a)} \quad (2)$$

where $\sigma^2_{\hat{C}(t)}$ is the total variance associated with the measured aflatoxin concentration $\hat{C}$.

Total variance $\sigma^2_{\hat{C}(t)}$ is the sum of sampling, sample preparation, and analytical variance and depends on sample size, mill type, subsample size, number of aliquots, and analytical procedure.

Because analytical procedures are required to measure aflatoxin in a sample or subsample, variance components, $\sigma^2_{\hat{C}(s)}$ and $\sigma^2_{\hat{C}(ss)}$ cannot be measured directly because of the nested design. However, $\sigma^2_{\hat{C}(t)}$, $\sigma^2_{\hat{C}(ssa)}$, and $\sigma^2_{\hat{C}(a)}$ can be measured directly where $\sigma^2_{\hat{C}(ssa)}$ is the combination of sample preparation and analytical variances as shown in Equation 3.

$$\sigma^2_{\hat{C}(ssa)} = \sigma^2_{\hat{C}(ss)} + \sigma^2_{\hat{C}(a)} \quad (3)$$

Then sampling and sample preparation variances can be calculated by subtraction.

$$\sigma^2_{\hat{C}(s)} = \sigma^2_{\hat{C}(t)} - \sigma^2_{\hat{C}(ssa)} \quad (4)$$

$$\sigma^2_{\hat{C}(ss)} = \sigma^2_{\hat{C}(ssa)} - \sigma^2_{\hat{C}(a)} \quad (5)$$

The sampling variance, $\sigma^2_{\hat{C}(s)}$, represents the variability among replicate test samples taken from the same lot of shelled corn. Sample preparation variance $\sigma^2_{\hat{C}(ss)}$ represents the variability among replicate subsamples taken from the same sample comminuted in a suitable mill. The analytical variance, $\sigma^2_{\hat{C}(a)}$, represents the variability among replicate aliquots of extracts of a single subsample.

Experimental Design

Two experiments were designed to estimate the variance components in Equation 2. The first experimental design was an unbalanced nested procedure designed to produce estimates of $\sigma^2_{\hat{C}(t)}$, $\sigma^2_{\hat{C}(ssa)}$, and $\sigma^2_{\hat{C}(s)}$. The notation $s^2_{\hat{C}}$ denotes an estimate of $\sigma^2_{\hat{C}}$. A bulk sample weighing ca 45.4 kg (100 lb) was

removed from each lot and divided into 32 test samples of 1.13 kg (2.5 lb). Each sample was comminuted in a Romer mill. Two 50 g subsamples were removed from 16 of the 32 samples and one 50 g subsample was removed from the remaining 16 samples. Aflatoxin was extracted from each 50 g sample with methanol–water (75 + 25, v/v) in a 2:1 ratio. All subsamples were analyzed with a single aliquot per subsample. To purify the extract (0.5 mL), it was passed through a Mycosep No. 224 column (8). The aflatoxins were derivatized by a bromine postcolumn derivatization process and quantitated by LC (9). The unbalanced design was used to keep costs minimal while still providing enough degrees of freedom for adequate variance estimation.

A second experiment was designed to obtain estimates of $\sigma^2_{\hat{C}(a)}$. Ten subsamples were chosen from the selected samples in the first experimental design to produce a wide range of aflatoxin concentrations. Analytical variance $\sigma^2_{\hat{C}(a)}$ is the estimate of the variance among 15 replicate aliquots of extract taken from the blender after the extraction process from a single subsample. All aliquot testing was conducted in the same laboratory to produce an analytical variance that reflects within-laboratory variance. The results are recorded in total parts per billion (ppb) and contain the sum of aflatoxins B_1, B_2, G_1, and G_2.

From the unbalanced nested design, $s^2_{\hat{C}(t)}$, $s^2_{\hat{C}(s)}$, $s^2_{\hat{C}(ssa)}$, and the lot aflatoxin concentration $\hat{C}$ were determined for each of the 18 lots using the NESTED Procedure in SAS (10). From the second experimental design, $s^2_{\hat{C}(a)}$ and the lot aflatoxin concentration $\hat{C}$ were determined for the 15 aliquots from each of the 10 subsamples using the NESTED Procedure in SAS (10).

Results and Discussion

Aflatoxin test results for all 18 lots and 32 samples are available upon request. Table 1 reports aflatoxin concentration, total variance $s^2_{\hat{C}(t)}$, sampling variance $s^2_{\hat{C}(s)}$, and combined sample preparation and analytical variance $s^2_{\hat{C}(ssa)}$ values for each of the 18 lots of shelled corn. The 18 lots, ranked by aflatoxin concentration in Table 1, range from about 6 to 677 ppb. In general, as the aflatoxin concentration increases, each variance estimate increases. This reflects the results of similar variance relationship studies conducted on other commodities (6, 11–15).

Sampling Variance

The sampling variance estimates from Table 1 show a linear relationship with the mean aflatoxin concentration in a full log plot (Figure 2). Therefore, sampling variance was modeled by the following mathematical expression

$$s^2_{\hat{C}(s)} = a\hat{C}^b \qquad (6)$$

where a and b are constants determined by regression analysis, and $\hat{C}$ is the estimate of aflatoxin concentration measured

Table 1. Average aflatoxin concentration, sample variance, and combined sample preparation and analytical variance components for all 18 lots of shelled corn[a]

Lot No.	Aflatoxin concentration, ppb	Total variance	Sample variance	Sample prep. + analytical variance
1	5.8	77.4	28.2	49.2
2	6.4	121.0	114.7	6.3
3	6.7	150.9	131.8	19.1
4	8.6	149.7	109.4	40.3
5	11.8	203.0	193.0	10.0
6	15.9	353.0	108.4	244.6
7	18.2	194.1	103.9	90.2
8	25.6	413.8	371.9	42.0
9	27.3	590.4	508.2	82.2
10	32.9	557.0	469.5	87.5
11	56.7	370.7	258.9	111.8
12	57.1	887.9	474.8	413.1
13	94.7	1277.4	1106.8	170.5
14	95.6	515.9	444.5	71.5
15	113.8	1452.8	1173.6	279.2
16	276.9	5393.1	2933.3	2459.8
17	298.9	7160.9	4012.7	3148.1
18	676.6	31308.1	9096.1	22212.0

[a] Testing plan = 1.13 kg sample, Romer Mill, 50 g subsample, and one aliquot quantitated by LC.

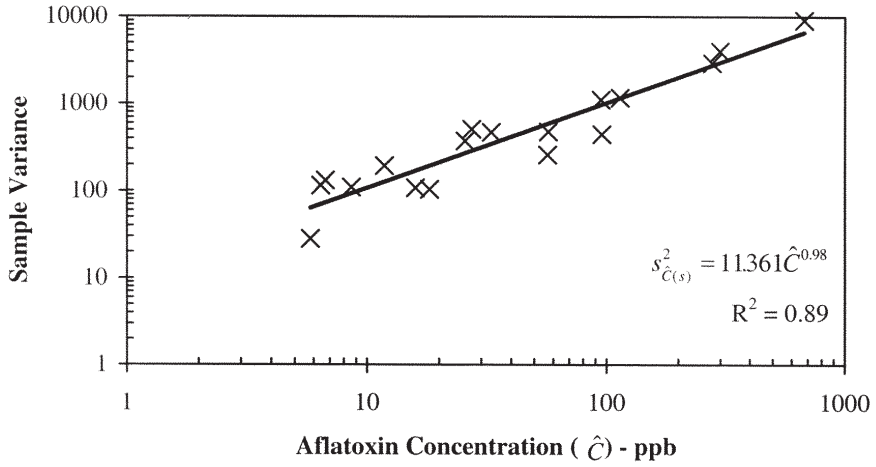

Figure 2. Sampling variance versus aflatoxin concentration for 1.13 kg test samples of shelled corn.

in ppb. Using regression analysis, the relationship between sampling variance and aflatoxin concentration is

$$s^2_{\hat{C}(s)} = 11.361\hat{C}^{0.98} \quad (7)$$

with a coefficient of determination of 0.89 in the full log scale (Figure 2).

Combined Sample Preparation and Analytical Variance

Table 1 reports the combined sample preparation and analytical variance estimates $s^2_{\hat{C}(ssa)}$. Figure 3 shows the combined sample preparation and analytical variance estimates versus aflatoxin concentration in a full log plot. Generally, combined sample preparation and analytical variance estimates increased with an increase in aflatoxin concentration. Using regression analysis, the following relationship was developed between the combined sample preparation and analytical variance and aflatoxin concentration with a coefficient of determination of 0.78 in the full log scale (Figure 3).

$$s^2_{\hat{C}(ssa)} = 1.383\hat{C}^{1.27} \quad (8)$$

Analytical Variance

Table 2 shows analytical variance estimates $s^2_{\hat{C}(a)}$ among the 15 replicated test results for each of the 10 subsamples analyzed. Generally, as the aflatoxin concentration increased, analytical variance also increased. Figure 4 shows the linear relationship between the analytical variance and the aflatoxin concentration in a full log plot. With the use of regression analysis, the following mathematical expression provided a suitable relationship between the analytical variance and aflatoxin concentration with a coefficient of determination of 0.92 in the full log scale.

$$s^2_{\hat{C}(a)} = 0.143\hat{C}^{1.16} \quad (9)$$

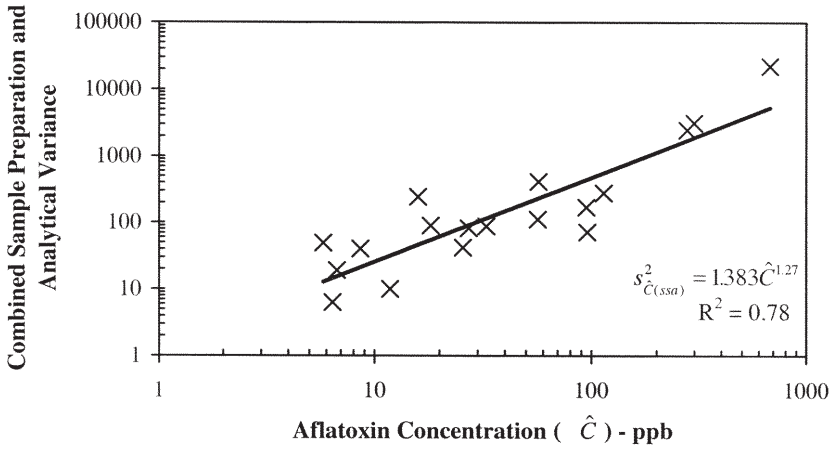

Figure 3. Combined sample preparation and analytical variance versus aflatoxin concentration for test subsamples of shelled corn using 50 g subsamples comminuted in the Romer mill, one aliquot per subsample, and LC.

Table 2. Average aflatoxin concentration, analytical variance, and coefficient of variation among replicate aflatoxin test results on 15 aliquots quantitated by LC[a]

Subsample No.	Aflatoxin concentration	Analytical variance	CV, %
1	28.3	9.1	10.6
2	58.1	20.7	7.8
3	58.7	24.5	8.4
4	68.4	14.5	5.6
5	103.7	22.7	4.6
6	117.3	15.8	3.4
7	189.0	63.0	4.2
8	433.2	230.9	3.5
9	876.7	266.6	1.9
10	937.8	608.4	2.6

[a] Analytical procedure = 15 aliquots taken from blender after extraction and quantitated by LC.

Sample Preparation Variance

Once relationships are developed for $s^2_{\hat{C}(ssa)}$ and $s^2_{\hat{C}(a)}$, Equation 5 can be used to determine sample preparation variance $s^2_{\hat{C}(ss)}$. An equation to estimate sample preparation variance can be calculated by subtraction of Equation 9 from Equation 8.

$$s^2_{\hat{C}(ss)} = 1.383\hat{C}^{1.27} - 0.143\hat{C}^{1.16} \quad (10)$$

Equation 10 can be simplified by regressing the difference, $s^2_{\hat{C}(ss)}$, on aflatoxin concentration $\hat{C}$. A suitable expression is shown in Equation 11.

$$s^2_{\hat{C}(ss)} = 1.254\hat{C}^{1.27} \quad (11)$$

Application of Results

Equations 7 through 11 estimate variances associated with testing a lot of shelled corn for aflatoxin using a 1.13 kg sample, Romer Mill, 50 g subsample, and LC. Reducing one or more of the 3 variance components, sampling, sample preparation, or analytical, reduces the total variance associated with a testing procedure. Statistical theory indicates that an increase in quantity of material tested decreases variance associated with that step of the testing procedure. This helps to estimate better the true aflatoxin concentration of the lot of corn. For example, increasing sample size or number of sample units reduces sampling variance; increasing subsample size or number of subsample units reduces sample preparation variance; and increasing the size of the aliquot or number of aliquots taken from the blender after the extraction process to be quantitated by LC reduces analytical variance.

Equation 7 can be modified to predict the sampling variance for a given sample size.

$$s^2_{\hat{C}(s)} = \left(\frac{1.13}{ns}\right) \cdot 11.361\hat{C}^{0.98} \quad (12)$$

where *ns* is the sample size in kg.

The sample preparation variance in Equation 11 can be modified to predict the effect of any size subsample comminuted in the Romer mill.

$$s^2_{\hat{C}(ss)} = \left(\frac{50}{nss}\right) \cdot 1.254\hat{C}^{1.27} \quad (13)$$

where *nss* is the subsample size in g.

A similar expression can be derived for the analytical variance described in Equation 9. Modification of Equation 9 shows the effect of any number of aliquots quantitated by LC.

$$s^2_{\hat{C}(a)} = \left(\frac{1}{na}\right) \cdot 0.143\hat{C}^{1.16} \quad (14)$$

where *na* is the number of aliquots.

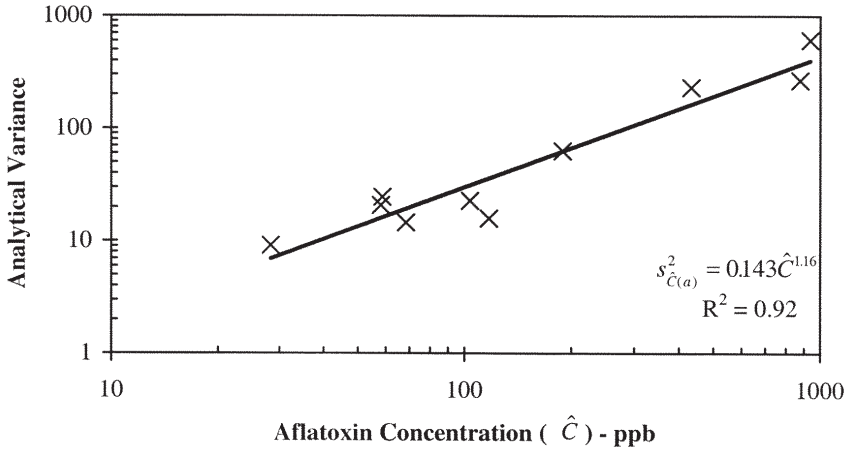

Figure 4. Analytical variance versus aflatoxin concentration for test subsamples of shelled corn using 15 aliquots per subsample and LC.

Total variance can be estimated for any sample size, subsample size comminuted in a Romer mill, and number of aliquots quantitated by LC by summing Equations 12 through 14.

$$s^2_{\hat{C}(t)} = \left[\left(\frac{12.95}{ns}\right)\hat{C}^{0.98}\right] + \left[\left(\frac{62.70}{nss}\right)\hat{C}^{1.27}\right] + \left[\left(\frac{0.143}{na}\right)\hat{C}^{1.16}\right] \quad (15)$$

With Equation 15, the total variance associated with testing a contaminated lot of shelled corn for aflatoxin at 20 ppb using a 1.13 kg sample, Romer Mill, 50 g subsamples, and quantitating 1 aliquot per subsample by LC is 274.9 (CV = 82.9%). Sampling, sample preparation, and analytical variances are 214.0 (CV = 73.1%), 56.3 (CV = 37.5%), and 4.6 (CV = 10.7%), respectively and account for 77.8, 20.5, and 1.7% of the total variation. Sampling variance accounts for the majority of the total variance, with sample preparation accounting for the next largest amount, and analysis with the least. This follows the same pattern observed with other commodities (6, 11–15).

The effect of increasing sample size on reducing testing variability can be demonstrated with Equation 15. Testing a contaminated lot of shelled corn for aflatoxin at 20 ppb using a 5 kg sample, Romer Mill, 100 g subsamples, and quantitating 1 aliquot per subsample by LC produces a total variance value of 81.6 (CV = 45.2%). Sampling, sample preparation, and analytical variances are 48.8 (CV = 34.9%), 28.2 (CV = 26.5%), and 4.6 (CV = 10.7%), respectively, and account for 59.8, 34.5, and 5.7% of the total variation.

Assuming that aflatoxin test results from shelled corn follow the theory of normally distributed variables, a lot with an aflatoxin concentration of 20 ppb and a total variance of 81.6 implies that aflatoxin test results will fall in the range of 20 ± 18 ppb, or 2–38 ppb, 95% of the time. Research has demonstrated with peanuts and cottonseed that the distribution among aflatoxin test results is highly skewed. Studies need to be developed to determine which theoretical distribution would best describe the distribution of aflatoxin test results for shelled corn.

Summary

Estimates of the total variability associated with testing 18 lots of shelled corn for aflatoxin increased as aflatoxin concentration increased. This also held true for each step of the test procedure: sampling, sample preparation, and analytical variability. With the use of regression analysis, mathematical expressions were developed to model all 3 variance components. The expressions were used to estimate the variance for any sample size, subsample size, and number of analyses for a specific aflatoxin concentration. For example, testing a lot with 20 ppb aflatoxin using a 1.13 kg sample, Romer mill, 50 g subsamples, and LC analysis, the total, sampling, sample preparation, and analytical variances were 274.9 (CV = 82.9%), 214.0 (CV = 73.1%), 56.3 (CV = 37.5%), and 4.6 (CV = 10.7%), respectively. The percentage of the total variance for sampling, sample preparation, and analytical was 77.8, 20.5, and 1.7, respectively. As with testing of aflatoxins in other commodities, sampling contributes the most variability followed by sample preparation and then analysis.

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