Variability Associated with Chemically Testing Screened Farmers Stock Peanuts For Aflatoxin[1]

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ABSTRACT

Forty farmers stock lots of runner peanuts suspected of containing aflatoxin were identified by the Federal State Inspection Service using the visual *Aspergillus flavus* method. A 227-kg portion was removed from each of the 40 lots. Each 227-kg portion was screened over a belt screening device with 0.953-cm (24/64 inch) spacing to remove loose shelled kernels, foreign material, and small pods. Each screened portion was divided into ten 9.5-kg samples. Each sample was shelled, all kernels in the sample were comminuted in the Federal State subsampling mill, and the aflatoxin in duplicate 356-g subsamples per sample was extracted and quantified using HPLC methods. The total variability among the 10 aflatoxin test results was determined for each lot. The total variability was partitioned into sampling, subsampling, and analytical variability components for each lot. All variance components were shown to be functions of the aflatoxin concentration. Using regression techniques the functional relationship for each variance components and aflatoxin concentration was developed. The total variance associated with a 9.5-kg sample, 356-g subsample, and HPLC quantification when testing a screened farmers stock lot at 20 ppb is 295.2 and the CV is 89.5%.

Key Words: Aflatoxin, peanuts, sampling, variability.

The peanut industry represented by the National Peanut Council has supported two major projects that should improve quality and reduce the aflatoxin content of farmers stock peanuts. The first study examined the feasibility of screening farmers stock peanuts over a device to remove foreign material, small pods, and loose shelled kernels (LSK) at the buying point. Studies by Dickens *et al.* (3, 4, 7) and Blankenship *et al.* (1) demonstrated that screening farmers stock peanuts would improve storage conditions and reduce lot aflatoxin concentrations. The second study examined the feasibility of replacing the present visual (VAF) method used to detect aflatoxin contaminated lots with a testing program where aflatoxin is chemically extracted from a sample drawn from a farmer's lot that has been screened at the buying point.

With the VAF method, Federal-State Inspectors look for the presence of *Aspergillus flavus* mold on all LSK from a 1800-g grade sample and on kernels shelled from a 500-g grade sample of pods (6, 10). If one or more kernels are found with the *A. flavus* mold, the lot is diverted from the edible market and classified as Segregation three. The VAF method is inexpensive, rapid, and easy to administer in the busy environment of the buying point. However, the VAF method does not measure aflatoxin directly and the variability associated with a 500-g sample is large (2).

In order to design an effective aflatoxin testing plan the cost/benefits associated with various testing designs (sample size and tolerance levels) needs to be determined. To determine the cost/benefits of a testing plan, the variability associated with each step of the testing procedure needs to be estimated. The objective of this study was to measure the variability associated with sampling, sample preparation, and analytical steps of an aflatoxin testing procedure for screened farmers stock peanuts. From the variability estimates, models can be subsequently developed the simulate aflatoxin testing plans which can be used to estimate the farmer's risk (percentage of good lots rejected), sheller's risk (percentage of bad lots accepted), amount of aflatoxin removed from the crop, and costs of a testing plan for various size samples and tolerance levels.

Materials and Methods

Sample Preparation - Forty farmers stock lots of runner-type peanuts suspected of containing aflatoxin were identified by the Federal State Inspection Service (FSIS) using the VAF method. A 227-kg portion of each lot was removed as the peanuts were being unloaded. Each 227-kg portion was screened over a belt-screening device with a 0.953-cm (24/64 inch) spacing to remove LSK, FM, and small pods from each lot (11). A riffle divider was used to divide the screened portion into twenty 9.5-kg samples. Ten samples were used for this study, while the remaining 10 samples were used in a separate study.

Each 9.5-kg sample was shelled and yielded on the average about 7.5 kg of kernels. These kernels were comminuted in the subsampling mill used by FSIS with two subsampling spouts (5, 8, 10). Each spout provided a 356-g subsample of comminuted peanuts. Each subsample was extracted in a 80/20 volume/volume solution of methanol/water with a 2/1 volume/mass solvent /peanut ratio. The aflatoxin in the solvent extract was quantified using high performance liquid chromatography (HPLC) methods (9).

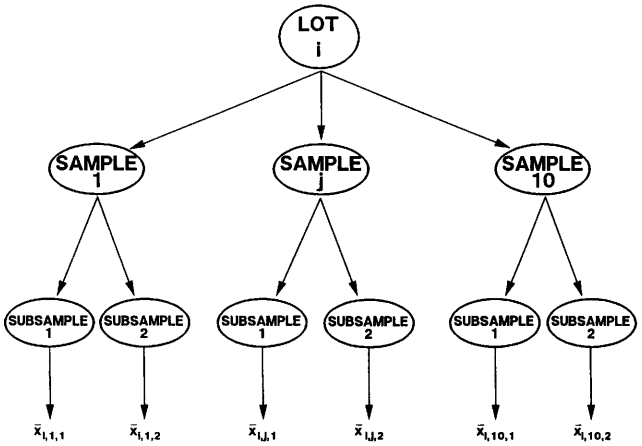

Fig. 1. Schematic diagram of experimental procedure showing how the test result $x_{i,j,k}$ was obtained. The identification for lot is i where i=1, 2,...,40, for sample is j where j-1,2,...,10 and for subsample is k where k=1 or 2.

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A schematic diagram of the experimental procedure described above is shown in Fig. 1. The $\bar{x}_{i,j,k}$ shown in the figure represents an aflatoxin test result in parts per billion (ppb) from the i^{th} lot, the j^{th} sample, and the k^{th} subsample. Each of the 800 test results were identified where i took on values from 1 to 40, j took on values from 1 to 10, and k took on values 1 or 2.

Variance Equations - The error structure or sources of the variability associated with the above aflatoxin test procedure is also illustrated in Fig. 1. As the figure indicates, the total variance among aflatoxin test results is composed of at least three variance components: sampling, subsampling, and analysis (12). An observed aflatoxin test result may be represented as follows:

$$\bar{x} = \mu + S + SS + A \quad (1)$$

where μ = the true aflatoxin concentration in the lot being tested; s = random deviation of sample concentrations about the lot concentration with expected value zero and variance $\sigma^2_{\bar{x}(s)}$; SS = random deviation of subsample concentrations about the sample concentration with expected value zero and variance $\sigma^2_{\bar{x}(ss)}$; and A = random deviation of analytical assay results about the subsample concentration with expected value zero and variance $\sigma^2_{\bar{x}(a)}$. By assuming independence among the random deviations in equation 1, the following variance relationship is obtained:

$$\sigma^2_{\bar{x}(t)} = \sigma^2_{\bar{x}(s)} + \sigma^2_{\bar{x}(ss)} + \sigma^2_{\bar{x}(a)}, \quad (2)$$

where $\sigma^2_{\bar{x}(t)}$ is the total variance associated with aflatoxin test results.

The sampling variance, $\sigma^2_{\bar{x}(s)}$, and the combined subsampling and analytical variance, $\sigma^2_{\bar{x}(ssa)}$, where

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

were estimated from the 800 $\bar{x}_{i,j,k}$ ppb values (Fig 1). Using a nested analysis of variance procedure (12) for each lot, 40 estimates of $\sigma^2_{\bar{x}(s)}$ and 40 estimates of $\sigma^2_{\bar{x}(ssa)}$ were obtained.

The analytical variance, $\sigma^2_{\bar{x}(a)}$, defined as the variance among aflatoxin determinations on equal aliquots of extract taken from the blender after the extraction step, was estimated from data obtained in a previous study (9). The extract was divided into 10 equal portions. Each portion was analyzed by HPLC procedures. Ten replicated aflatoxin determinations were made on each of the three subsamples.

The total variance, $\sigma^2_{\bar{x}(t)}$, and the subsampling variance, $\sigma^2_{\bar{x}(ss)}$ were estimated using the summation property in equations 2 and 3, respectively. Estimates of the true variance components $\sigma^2_{\bar{x}}$ and the true aflatoxin concentration μ by experimental values are denoted by $s^2_{\bar{x}}$ and $\bar{\bar{x}}$, respectively, where $\bar{\bar{x}}$ is the average of the observed $\bar{x}$ values.

Results

Table 1 show the sampling variance $s^2_{\bar{x}(s)}$ and the combined subsampling and analytical variance $s^2_{\bar{x}(ssa)}$ for each of the 40 lots. The results have been ordered by the average lot aflatoxin concentration. Three of the lots had less than 1 ppb aflatoxin while six lots had more than 1000 ppb aflatoxin. All 10 samples of lot 20 had no measurable aflatoxin and was deleted from the analysis.

Sampling variance - Figure 2 shows the average aflatoxin concentration $\bar{\bar{x}}$ and the estimated sampling variance $s^2_{\bar{x}(s)}$ for each of the 40 lots. The results indicate that the sampling variance is a function of aflatoxin concentration. Previous studies by Whitaker *et al.* concerning the variance associated with testing shelled peanuts (13), shelled corn (14), and cottonseeds (15) for aflatoxin suggested the relationship to be

$$\sigma^2_{\bar{x}} = A\mu^B, \quad (4)$$

where A and B are constants. Equation 4 would appear to be appropriate since the plot of $\log(s^2_{\bar{x}(s)})$ and $\log(\bar{\bar{x}})$ in Fig. 2 is approximately linear on a full log graph. Using the Statistical Analysis System (12), a model based upon equation 4 was fitted by regressing $\log(s^2_{\bar{x}(s)})$ on $\log(\bar{\bar{x}})$ data in Table 1. The following relationship between sampling variance and aflatoxin concentration was developed.

$$s^2_{\bar{x}(s)} = 3.5483\bar{\bar{x}}^{1.3981} \quad (5)$$

with a coefficient of determination of 0.927 in the log scale. The standard error of estimate on the (log A) and B regression coefficients are 0.3223 and 0.0644, respectively.

Combined subsampling and analytical variance - The average aflatoxin concentration $\bar{\bar{x}}$ and the estimated combined subsampling and analytical variance $s^2_{\bar{x}(ssa)}$ for each of the 40 lots are plotted in Fig. 3. The increase in

Table 1. Lot aflatoxin concentration, $\bar{\bar{x}}$, sampling variance, $s^2_{\bar{x}(s)}$, combined subsampling and analytical variance, $s^2_{\bar{x}(ssa)}$ for each of 40 lots of screened farmers stock peanuts[1].

LOT NUMBER	LOT CONCENTRATION (PPB)	SAMPLING VARIANCE ($s^2_{\bar{x}(s)}$)	COMBINED SUBSAMPLING AND ANALYTICAL VARIANCE ($s^2_{\bar{x}(ssa)}$)
20	0.00	0.00	0.00[2]
41	0.09	0.07	0.00
24	0.37	0.35	0.04
21	0.97	3.74	0.12
26	0.98	4.31	0.16
23	1.00	4.45	6.74
37	2.24	0.22	24.45
22	2.98	25.43	70.19
42	3.71	52.12	40.89
25	5.38	62.79	9.10
17	5.39	16.21	3.19
28	5.53	144.94	16.99
38	11.27	483.14	46.92
43	11.54	867.87	61.06
27	12.35	20.47	373.35
18	22.25	249.62	27.33
40	22.75	481.78	33.10
29	23.86	1668.34	171.69
35	47.19	772.64	247.06
36	56.34	2169.01	81.98
19	128.59	11461.84	901.68
34	208.48	8909.07	749.00
16	210.84	5350.22	498.40
4	237.53	4022.30	2217.83
33	240.32	7929.95	2025.04
32	258.68	20724.40	6241.18
15	301.45	8525.88	3326.46
10	466.30	22634.16	4669.75
14	483.61	27754.34	3958.01
7	591.99	6641.57	6931.78
1	598.32	8929.22	5233.90
5	598.76	17423.76	5536.18
11	851.65	11371.59	9026.47
6	854.39	27116.87	11504.61
9	1138.35	20711.22	20336.86
3	1453.00	161794.60	112509.00
2	3047.26	1464107.00	121532.10
8	4239.42	199253.40	121895.00
12	4609.75	200818.30	388864.90
13	6307.94	1689575.00	220168.90

1/ Variances reflect runner peanuts screened over a belt screening device with 0.953 cm (24/64 inch) spacing, 9.5-kg samples, 356-g subsamples comminuted in the FSIS subsampling mill, and HPLC quantification.
2/ Combined subsampling and analytical variance = 0.004.

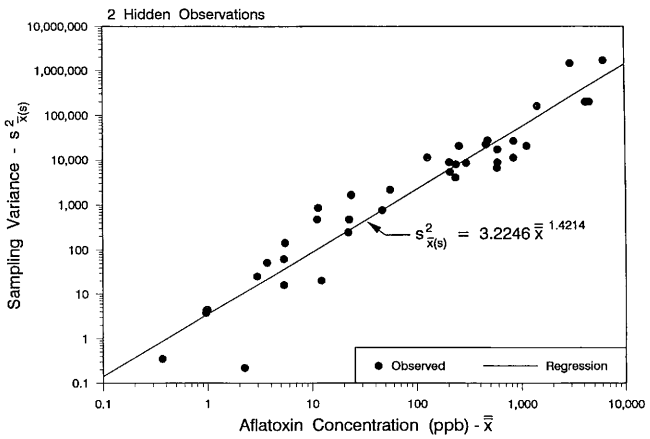

Fig. 2. Relationship between the sampling variance $s^2_{\bar{x}(s)}$ and aflatoxin concentration $\bar{\bar{x}}$ in parts per billion (ppb). Coefficient of determination = 0.928.

$s^2_{\bar{x}(ssa)}$ with $\bar{\bar{x}}$ indicates that $\sigma^2_{\bar{x}(ssa)}$ may be a function of aflatoxin concentration μ. A model based upon equation 4 was fitted by regressing log ($s^2_{\bar{x}(ssa)}$) on log ($\bar{\bar{x}}$) data in table 1. The following relationship between combined subsampling and analytical variance and aflatoxin concentration was developed.

$$s^2_{\bar{x}(ssa)} = 0.8653\ \bar{\bar{x}}^{1.4447} \qquad (6)$$

with a coefficient of determination of 0.927 in the log scale. The standard error of estimate for the (log A) and B regression coefficients are 0.3326 and 0.0648, respectively.

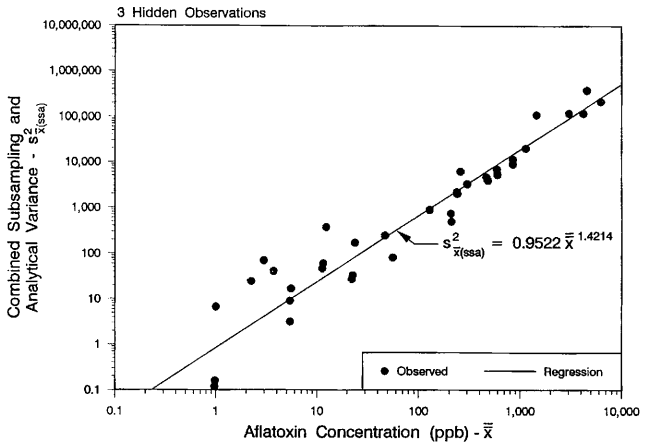

Fig. 3. Relationship between the combined subsampling and analytical variance $s^2_{\bar{x}(ssa)}$ and aflatoxin concentration $\bar{\bar{x}}$ in parts per billion (ppb). Coefficient of determination = 0.928.

The estimated B coefficients in equations 5 and 6 are similar in magnitude. From the standard error of estimates for the B coefficients, a "t" value of -0.5077 with 74 degrees of freedom suggest that the two B coefficients in equations 5 and 6 are not significantly different. Therefore, two models based upon equation 4 were fitted to the pooled variance observations in table 1 ($s^2_{\bar{x}(s)}$ and $s^2_{\bar{x}(ssa)}$) with the restriction of one common B coefficient and two separate A coefficients. The A coefficient was allowed to vary with the type variance component. The following relationships were developed.

$$s^2_{\bar{x}(s)} = 3.2246\ \bar{\bar{x}}^{1.4214} \qquad (7)$$

and

$$s^2_{\bar{x}(ssa)} = 0.9522\ \bar{\bar{x}}^{1.4214} \qquad (8)$$

with a coefficient of determination of 0.928 in the log scale. The standard error of estimate for (log A) in equations 7 and 8 is 0.2654 and for B is 0.0461. Regression equations 7 and 8 are plotted in Figs. 2 and 3, respectively.

Total Variance - As equation 2 and 3 indicate, the total variance, $s^2_{\bar{x}(t)}$, can be estimated by summing equations 7 and 8.

$$s^2_{\bar{x}(t)} = 4.1768\ \bar{\bar{x}}^{1.4214} \qquad (9)$$

The 9.5-kg sample accounts for 77.2% of the total variation while the combined sample preparation and analytical methods accounts for 22.8% of the total variation. These results are consistant the variability associated with testing shelled peanuts for aflatoxin where sampling, especially for small sample sizes, is the largest source of variation (13).

Analytical variance - The analytical variance associated with replicated HPLC analyses on the same extract, determined by Dorner and Cole (9), are shown in Table 2. The results indicate that the analytical variance increases with concentration. With only three data points, the variance information is shown in Table 2 instead of a log plot. Studies by Whitaker et al. (13, 14) indicate that the CV for analytical variation is approximately constant with aflatoxin concentration when using Association of Official Analytical Chemists (AOAC) Methods I and II extraction procedures and thin layer chromatography (TLC) quantification methods. The coefficient of variation (CV) associated with the HPLC analytical methods used in the above study was assumed to be constant with aflatoxin concentration and equal to the average of the three CV values in Table 2 or 3.5%. As a result, the estimated analytical variance $s^2_{\bar{x}(a)}$ can be derived as a function of aflatoxin concentration from the CV relationship to be

$$s^2_{\bar{x}(a)} = 0.0012\ \bar{\bar{x}}^2. \qquad (10)$$

Table 2. Variability associated with quantifying aflatoxin by HPLC methods[1].

AFLATOXIN CONCENTRATION (PPB)	VARIANCE	STANDARD DEVIATION (%)	COEFFICIENT OF VARIATION
11.2	0.1	0.3	2.7
78.6	8.4	2.9	3.7
489.1	.2	20.4	4.2

1/Dorner, J.W. and R.J. Cole. (9). Rapid determination of aflatoxins in raw peanuts by liquid chromatography with postcolumn iodination and modified minicolumn cleanup, J. Assoc. Off. Anal. Chem., 71:43-47.

Subsampling Variance - Once the analytical variance has been estimated, the remaining variance component or the subsampling variance can be estimated by subtracting the analytical variance, equation 10, from the combined subsampling and analytical variance, equation 8.

$$s^2_{\bar{x}(ss)} = 0.9522\ \bar{\bar{x}}^{1.4214} - 0.0012\ \bar{\bar{x}}^2. \qquad (11)$$

The total variance associated with aflatoxin test results can be reduced by decreasing one or more of the variance components in equation 2. The variance components in equation 2 can be reduced by increasing the quantity of material inspected. The sampling variance can be reduced by increasing sample size; the subsampling variance can be reduced by increasing subsample size; and the analytical variance can be reduced by increasing the number of aliquots quantified by HPLC. The sampling variance described by equation 7 can be modified to predict the effect of any size sample, n_s, in kg on the sampling variance.

$$s^2_{\bar{x}(s)} = (9.5/n_s)\ (3.2246\ \bar{\bar{x}}^{1.4214}). \qquad (12)$$

A similar expression exists for the subsampling variance for any size subsample, n_{ss}, in grams is

$$s^2_{\bar{x}(ss)} = (356/n_{ss})\ (0.9522\ \bar{\bar{x}}^{1.4214} - .0012\ \bar{\bar{x}}^2). \qquad (13)$$

The analytical variance, described by equation 10 for the analysis of a single aliquot quantified by HPLC, can be modified to predict the analytical variance for any number of aliquots, n_a, carried through the HPLC procedure.

$$s^2_{\bar{x}(a)} = (1/n_a)\ (0.0012\ \bar{\bar{x}}^2). \qquad (14)$$

From equation 12, 13, and 14, the total variance associated with testing screened farmers stock peanuts for any size sample, any size subsample, and any number of analyses can

be estimated.

$$s^2_{\bar{x}(t)} = (30.63/n_s+338.98/n_{ss})\bar{\bar{x}}^{1.4214}+(0.0012/n_a-0.4272/n_{ss})\bar{\bar{x}}^2 \quad (15)$$

Equation 9 estimates the total variance associated with the specific conditions of this study (n_s=9.5 kg, n_{ss}=356 g, and n_a=1) and does not have the flexibility of equation 15 to estimate the total variability associated with any size sample, any size subsample, and any number of aliquots. For example, the total variability associated with testing farmers stock lot of peanuts with 50 ppb aflatoxin using a 27-kg sample of pods, a 500-g subsample from the FSIS mill, and HPLC analytical methods is 437.1. A concentration of 50 ppb and a variance of 437.1 suggests that if sample assay values are normally distributed (which is true only for large sample sizes), aflatoxin test results will fall in the range of 50 ppb ± 41 ppb 95% of the time. The sample variance is 260.0 and accounts for 59.5% of the total variance. The subsampling variance is 174.1 and accounts for 39.8% of the total variance. The analytical variance is 3.0 and account for less than 1% of the total variance.

The variance estimated in this study reflect the following: (a) 9.5-kg samples of screened farmers stock runner peanuts, (b) FSIS subsampling mill used to comminute the kernels, (c) 356-g subsamples, (d) use of extraction procedures described by Dorner and Cole (9) with HPLC quantification, and (e) use of one particular laboratory for analysis.

With screened farmers stock peanuts, the majority of LSK and small kernels are removed. Since LSK and small kernels are a major source of aflatoxin, it is not clear whether the variance relationships developed in this study also reflect the variance relationships expected for unscreened farmers stock peanuts. Further study is required to determine the variance relationships for unscreened farmers stock peanuts.

Since the experimentally determined variance components appear to be functionally related to the aflatoxin concentration, the assumption made concerning the nature of the random errors S, SS, and A in equation 1 may be open to questions. Other statistical models, such as the multiplicative model, have been investigated but have not provided a workable alternative. However, the variance relationships presented in this paper indicate the major sources of error in testing screened farmers stock peanuts for aflatoxin and provide insights concerning ways to reduce the total variability.

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