Variability Among Aflatoxin Test Results on Runner Peanuts Harvested From Small Field Plots[1]

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ABSTRACT

An experiment was conducted to determine the variability associated with aflatoxin contamination of peanuts from plants grown in specified row lengths. Runner peanuts (cv. Georgia Green) were planted in 10, 76.2 m rows (20 seed/m) and grown using standard production practices. Plants were exposed to natural late-season drought conditions making the peanuts susceptible to preharvest aflatoxin contamination. Plants were mechanically dug, inverted, and separated into 500 plots of 1.5 m single rows. Peanuts from each numerically identified plot were harvested with a mechanical picker, dried to 8% kernel moisture (wet basis), shelled, and analyzed for aflatoxin by high performance liquid chromatography (HPLC). The average kernel mass and weighted average aflatoxin concentration for all plots was 131 g and 2278 ng/g, respectively. The kernel mass varied among the 500 plots from a low of 4 g to a maximum of 283 g. The aflatoxin concentration among the 500 plots varied from a low of 0 ng/g to a maximum of 32,142 ng/g. The standard deviation among the 500 plot aflatoxin values was 4061. The standard deviation among sample concentrations for this field study was very similar to previous studies that measured the standard deviation among sample concentrations taken from bulk farmers' stock lots. Increasing plot length decreased the standard deviation among plot aflatoxin values as predicted by statistical theory. For example, increasing plot row length by a factor of four, or from 1.5 to 6 m, decreased the standard deviation by a factor of two, or from 4061 to 2031. A regression equation was developed to predict the effect of plot row length on the variability among aflatoxin plot values. This information is useful for designing field plot experiments to test various strategies for reducing or preventing preharvest aflatoxin contamination.

Key Words: Sample variability, field variability, uncertainty, treatment effects, *Aspergillus* spp.

The U.S. peanut industry has an aggressive and multifaceted aflatoxin control program that attempts to prevent aflatoxin formation, detect contaminated lots, and reduce aflatoxin contamination through processing (Dickens, 1977a,b). This multifaceted control program attacks the aflatoxin problem from production fields to the manufacture of consumer goods. Prevention methods are the most effective approach, and if successful they can eliminate the need for detection and decontamination methods associated with the overall control program. However, it is extremely difficult to completely prevent aflatoxin contamination. Some preventative methods, such as irrigation, inverted windrows, timely drying to safe moisture levels, and preventing moisture accumulation during storage, reduce (but do not eliminate) the growth of aflatoxin-producing fungi and aflatoxin contamination. Scientists continue to develop methods that can be incorporated into field production practices that will reduce or prevent the growth of aflatoxin-producing fungi and aflatoxin contamination in peanuts. For example, researchers are investigating the use of competitive fungi that do not produce aflatoxin (atoxigenic fungi) as a method of reducing aflatoxin contamination in peanuts (Dorner *et al.*, 1992; Dorner *et al.* 1998). Also, peanut breeders are investigating different peanut genotypes for resistance to aflatoxin contamination (Anderson *et al.*, 1995; Holbrook *et al.*, 2000). Scientists study these control methods using full-scale field trials.

Accurate and precise measurements of aflatoxin contamination among treated peanuts in a production or field setting are required to determine if agronomic practices or use of more resistance genotypes can reduce aflatoxin contamination. For large field experiments, not all peanuts grown in a treated field can be tested for aflatoxin. Therefore, aflatoxin is usually measured among selected samples of peanuts taken from plots in a treated field. The sample concentrations are used to estimate field contamination and the effect of a specific treatment on aflatoxin reduction. Because of the variability among replicated sample concentrations taken from a population, the true aflatoxin concentration of a population cannot be determined with 100% certainty. Studies to measure the variability among sample concentrations have been limited to testing bulk farmers' stock (Whitaker *et al.*, 1994a,b,c; Whitaker *et al.*, 1999) and shelled peanut lots (Whitaker *et al.*, 1974). For example, the coefficient of variation among replicated sample concentrations taken from a contaminated bulk farmers' stock lot at 20 ppb has been measured at 244% for 2.27 kg samples (Whitaker *et al.*, 1994a). For small sample sizes, the sampling step accounts for most of the variability associated with the aflatoxin test procedure used to estimate aflatoxin in a

[1]The use of trade names in this publication does not imply endorsement by the USDA or the N.C. Agric. Res. Serv. of the products named nor criticism of similar ones not mentioned.

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lot. The sampling step can account for 90% of the total variability while sample preparation and analysis can account for the remaining 10% of the variability associated with the test procedure (Whitaker *et al.*, 1994a). With such high variability, it is difficult to determine the effect of different agronomic treatments on aflatoxin reduction in a field setting.

The objective of this study was to determine the effect of plot size on the variability associated with measuring aflatoxin in peanuts sampled from a production field. Knowing the aflatoxin variability among peanut samples taken from production fields will help scientists design better experiments to test and determine various strategies for reducing or preventing preharvest aflatoxin contamination in peanuts.

Materials and Methods

Runner peanuts (cv. Georgia Green) were planted in ten 76.2 m rows at a seed density of 20 seed/m on Americus Sand soil near the National Peanut Research Laboratory in southwest Georgia. Plants were grown using standard production practices (Beasley *et al.*, 1997). Plants were not irrigated and by chance were exposed to natural late-season drought conditions making the peanuts susceptible to preharvest aflatoxin contamination (Diener *et al.*, 1982). Peanuts were mechanically dug with a two-row digger shaker inverter (Kelly Manufacturing Co. Tifton, GA), inverted (10 single rows were maintained), and partially dried in the windrow. Fifty consecutive 1.5 m sections were marked or identified in each 76.2 m windrow of inverted plants for a total of 500 individual 1.5 m single-row sections in the field (Fig. 1). Peanut vines from a single 1.5 m section were hand fed into a peanut thresher (Kincaid Equipment Manufacturing, Haven, KS) and threshed peanut pods were placed into a mesh bag. Each of the 500 mesh bags was numerically identified with a plot identification code that represented the row number (i = 1 to 10) and within row section number (j = 1 to 50) that defined the location of each plot within the field. The layout of the 500 plots is shown in Figure 1.

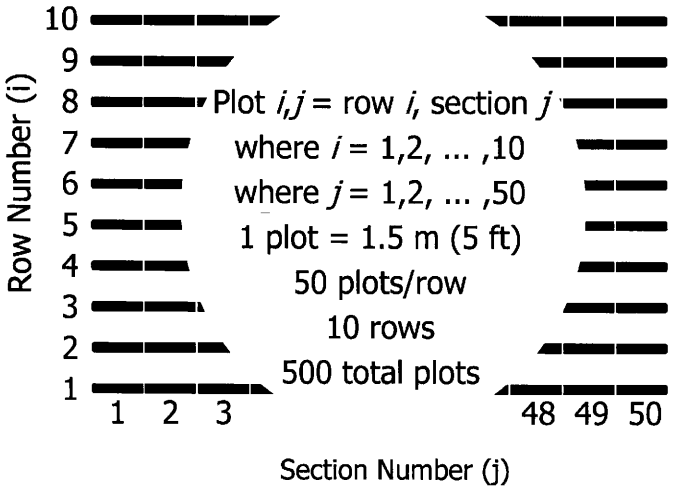

Fig. 1. Plot identification among the 500 field plots.

Peanuts were artificially dried in the meshed bags to about 8% kernel moisture wet basis. Once the peanuts were dried, peanuts in each meshed bag were shelled and the kernel mass and aflatoxin concentration was determined. The sample was considered to be all peanut pods taken from a 1.5 m plot. As a result, 500 plot samples were used to determine the aflatoxin contamination of the field. All shelled kernels from each plot were homogenized in a blender with methanol-water (80 + 20, v/v; 2 mL/g) so that no subsampling error was introduced. Aflatoxins were quantified by the high performance liquid chromatographic (HPLC) method of Dorner and Cole (1988) with slight modifications. The HPLC system consisted of a Waters 3.9 × 150 mm Nova-PAK C_{18} column (Waters Inc, Milford, MA) with a mobile phase of water-methanol-butanol (700 + 355 + 12; v/v/v). Instead of using postcolumn iodination to enhance fluorescence of aflatoxins B_1 and G_1, postcolumn derivatization was achieved with a photochemical reactor (Joshua, 1993) placed between the column and a Shimadzu Model RF551 fluorescence detector (Shimadzu, Kyoto, Japan) with excitation and emission wavelengths of 365 and 440 nm, respectively. Injection solvent consisted of methanol-water (62 + 38, v/v) with 0.1% acetic acid. Aflatoxin standards were prepared from crystals according to AOAC method 970.44 (AOAC, 1995), and aflatoxin determinations were not corrected for recovery.

Aflatoxin was recorded as a concentration, ng of aflatoxin per g of peanuts (ng/g) or parts per billion (ppb). The kernel mass, aflatoxin concentration, and plot identification code were recorded in a spreadsheet for statistical analysis. The standard deviation among the 500 plot concentrations was used as the measure of variability associated with measuring aflatoxin in peanuts from 1.5 m plots in a field.

Results and Discussion

Simple statistics describing the variability in kernel mass and aflatoxin concentration from plot to plot is shown in Table 1. Peanut kernel mass varied widely among the 500 samples (plots) from a low of 4 g to a high of 283 g and averaged 131 g. The standard deviation and coefficient of variation among the 500 plot kernel masses were 53 and 40%, respectively. The aflatoxin concentration among the 500 plots varied widely from a low of 0 ppb to a high of 32,142 ppb and averaged 2657 ppb (weighted average was 2278 ppb). The standard deviation and coefficient of variation among the 500 plot aflatoxin concentrations were 4061 and 153%, respectively. Because the mass of kernels varied from plot to plot, the weighted average aflatoxin concentration of 2278

Table 1. Peanut sample statistics for weight and aflatoxin among 500 1.5 m plots.

	Number of plots	Average	Std. dev.	C.V. (%)	Min.	Max.
Kernel weight (g)	500	131.4	52.9	40.3	3.7	283.4
Aflatoxin concentration (ppb)	500	2277.6[a]	4061.1	178.3	0.0	32141.8

[a]Weighted average.

ppb was computed and used in all calculations. In an ideal sampling experiment, it would be desirable for all replicated samples taken from a population have the same kernel mass. But when using a uniform plot length of 1.5 m, biological variation from plant to plant makes it impossible to maintain a constant kernel mass per plot. So the variability or standard deviation among plot aflatoxin concentrations reflects an average sample size or kernel mass of 131 g (1.5 m plot length) and an aflatoxin concentration of 2278 ppb.

Statistical theory states that if the standard deviation (S_n) is known for a specific sample size n, the standard deviation (S_N) at any other sample size N, can be predicted from Equation 1 (Huntsberger and Billingsley, 1987).

$$S_N = (n/N)^{0.5} S_n \quad \text{[Eq. 1]}$$

For this study, sample size (n and N) units can be expressed either as (a) number of 1.5 m plots, (b) number of 131 g samples, (c) plot length (m), and/or (d) total kernel sample mass (g). The values of n and N will depend on the units chosen. Several examples are shown below for different units of n and N.

If N is the number of 1.5 m plots or the number of 131 g samples, then Equation 1 can be written as

$$S_N = (1/N)^{0.5} 4061 \quad \text{[Eq. 2]}$$

where n = 1 and S_n = 4061. Or

$$S_N = 4061\ N^{-0.5} \quad \text{[Eq. 3]}$$

For example, the standard deviation among aflatoxin values from the combination of two 1.5 m plots (N = 2) computed from Equation 3 is 2872.

It may be more convenient for scientists to think in terms of plots length. Then N is total plot length in meters and n = 1.5 m in Equation 2.

$$S_N = (1.5/N)^{0.5} 4061 \quad \text{[Eq. 4]}$$

$$S_N = 4974\ N^{-0.5} \quad \text{[Eq. 5]}$$

For example, the standard deviation among aflatoxin values from 3 m plots (N = 3), computed from Equation 5 is 2872.

Finally, if N is expressed in kg of peanuts, then Equation 1 becomes

$$S_N = (0.131/N)^{0.5} 4061 \quad \text{[Eq. 6]}$$

$$S_N = 1472\ (N)^{-0.5} \quad \text{[Eq. 7]}$$

For example, the standard deviation among aflatoxin values from samples of 2.27 kg of peanuts, computed from Equation 7 for N = 2.27 is 977.

To test the theory that governs Equation 1 (increasing sample size decreases standard deviation), the standard deviation among 3 m plots was determined by randomly selecting two 1.5 m plots from the 500, 1.5 m plots and calculating the weighted aflatoxin concentration among the two plots to represent a 3 m plot. This process was repeated 250 times, without replacement, to obtain 250-aflatoxin values representing 250, 3 m plots. The weighted average aflatoxin concentration and standard deviation among the 250-aflatoxin concentrations from the 250 plots that are 3 m long were computed and the results are shown in Table 2. The process was repeated for several plot lengths ranging from 1.5 to 15 meters. The number of plots at a given length is dependent on plot length. As plot length increases, the number of plots that can be created from the 500, 1.5 m plots decreases. Only 50 plots of 15 m length can be created from the pool of 500, 1.5 m plots. The weighted average and standard deviation among aflatoxin values from plots of various lengths are shown in Table 2. As expected, the average aflatoxin concentration appears to be constant or independent of plot length. However, as Equation 1 predicts, the standard deviation does decrease as plot length increases. A linear regression in the log scale was run on the 10 data points in Table 2 and the regression curve is shown in Figure 2 with the observed values. Results of the regression analysis are shown in Equation 8.

$$S_N = 4653\ N^{-0.53} \quad \text{[Eq. 8]}$$

The coefficient of determination is 0.97. The regression coefficients in Equation 8 are very close to what theory would predict when sample size N is expressed as plot length (Equation 5). The coefficients -0.53 and 4653 in Equation 8 should be -0.50 and 4974, respectively. Since the coefficients of Equations 8 and 5 are so close, the variation among aflatoxin values among the 500, 1.5 m plots appears to behave in a random manner.

The effect of increasing plot length by combining together randomly selected 1.5 m plots to form plots of various lengths demonstrated that Equation 5 holds in a field environment. However, scientists conducting field trials traditionally lay out field plots in multiple rows of a certain length. It was decided to repeat the above study where plots of various lengths would be constructed by sequentially combining 1.5 m plots from each row. For example, a single 76.2 m row would yield 50, 1.5 m plots. If two sequential or consecutive 1.5 m plots are combined, then a 76.2 m row will yield 25, 3 m plots. A 76 m row

will yield other combinations such as 16 plots of 4.5 m, 12 plots of 6 m, and eventually one 76 m plot. The weighted average and standard deviation among aflatoxin values from plots of various lengths are shown in Table 3 for all 10 rows. The average aflatoxin concentration appears to be constant or independent of plot length and the standard deviation decrease as plot length increases. A linear regression in the log scale was run on the 10 data points in Table 3 and the regression curve is shown in Figure 3 with the observed values. Results of the regression analysis are shown in Equation 9.

$$S_N = 5009\ N^{-0.49} \quad \text{[Eq. 9]}$$

The coefficient of determination is 0.97. The regression coefficients in Equation 9 are very close to what theory would predict when sample size N is expressed as plot length (Equation 5). The coefficients in Equation 9 (-0.49 and 5009) should be -0.50 and 4974, respectively. Since the coefficients of Equations 9 and 5 are similar, the reduction in the variation among aflatoxin values among plots of increasing length behave as predicted by statistical theory even though plots were combined in a non-random or systematic manner.

Previous studies that measured the variability associated with sampling bulk lots of farmers' stock peanuts for aflatoxin indicated that the standard deviation was a function of aflatoxin concentration (Whitaker, 1994a). The standard deviation and average aflatoxin among the 50 samples weighing 2.27 kg were measured for each of the 40 farmers' stock lots. The functional relationship between standard deviation, S_N,and aflatoxin concentration, C, was

$$S_N = (66.04*2.27/N)^{0.5}\ C^{0.599} \quad \text{[Eq. 10]}$$

where N was peanut pod mass in kg. A plot of standard deviation versus aflatoxin concentration (Equation 10) is shown in Figure 4 for N = 2.27 kg pod sample and a range of C values from 0 to 5000 ppb. The standard deviation from the current field study, adjusted to reflect a 2.27 kg sample of pods (Equation 7), is also plotted as a single point in Figure 4. In Equation 7, N is set equal to 1.7 kg kernels to represent 2.27 kg pods. It is assumed that 2.27 kg of pods yields about 1.7 kg kernels because hulls account for 25% of the pod mass. As Figure 4 shows, S_N at C = 2278 ppb and N = 2.27 kg pods for the bulk study (Equation 10) and the field study (Equation 7) are 834 and 1132, respectively.

In the current field study, since only one field or one population was sampled, there is no way to determine if a functional relationship exists between standard deviation and aflatoxin concentration as with the bulk lot study. Since all studies to determine the variability associated with aflatoxin measurements in peanuts (as well as for other mycotoxins and other commodities) have shown that the standard deviation among sample concentrations is a function of aflatoxin concentration, one would expect the standard deviation among sample concentrations taken from contaminated fields to also increase with aflatoxin concentration. It may be reasonable to assume that the distribution of aflatoxin from kernel to kernel in the field study is similar to the distribution in the bulk lot study and that Equation 10, developed for bulk lots, may also describe the relationship between standard deviation and aflatoxin concentration among sample concentrations taken from a field setting. Also, it appears that estimates of the standard deviation are not biased when rows within a plot are physically located adjacent to each other and not randomly located throughout the field. Until such time that resources can be found to sample additional fields at different aflatoxin levels, Equation 10 may give scientists an approximate estimate of the relationship between sample size (plot length), aflatoxin concentration, and standard deviation associated with estimating treatment effects under field settings.

Table 2. Standard deviation among plot aflatoxin concentration vs. plot length when longer plots are generated by combining 1.5 m plots randomly without replacement.

No. 1.5 m sections per plot	Plot length	Total number of plots	Weighted average	Standard dev.
	m		ppb	ppb
1	1.5	500	2277.6	4061.1
2	3.0	250	2277.6	2314.2
3	4.6	160	2277.9	2118.3
4	6.1	120	2277.6	1818.6
5	7.6	100	2277.6	1481.4
6	9.1	80	2269.1	1357.4
7	10.7	70	2267.5	1307.8
8	12.2	60	2270.8	1321.7
9	13.7	50	2291.3	1159.1
10	15.2	50	2277.6	1164.7

Table 3. Standard deviation among plot aflatoxin concentration vs. plot length when longer plots are generated by combining 1.5 m plots sequentially.

No. 1.5 m sections per plot	Plot length	Total number of plots	Weighted average	Standard dev.
	m		ppb	ppb
1	1.5	500	2277.6	4061.1
2	3.0	250	2277.6	3018.2
3	4.6	160	2301.2	2545.3
4	6.1	120	2301.2	1886.5
5	7.6	100	2277.6	1761.0
6	9.1	80	2301.2	1591.8
7	10.7	70	2289.9	1462.6
8	12.2	60	2301.2	1390.1
9	13.7	50	2255.8	1520.0
10	15.2	50	2277.6	1402.3

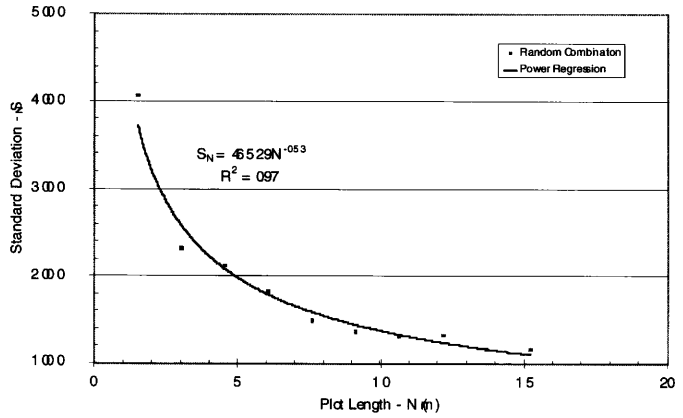

Fig. 2. Effects of increasing plot length on reducing standard deviation among plot aflatoxin values when longer plots are generated by combining 1.5 m plots randomly without replacement.

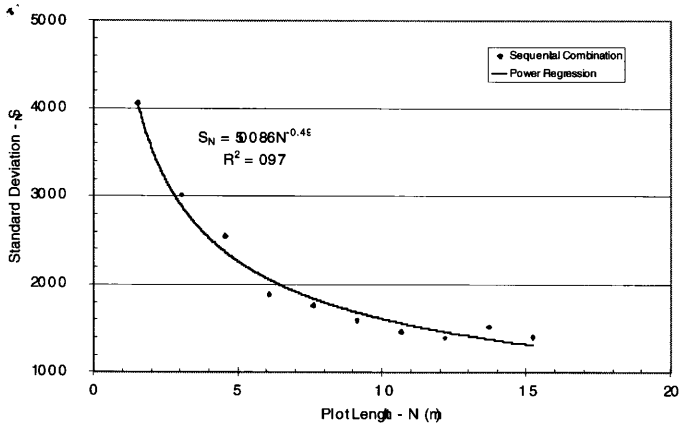

Fig. 3. Effects of increasing plot length on reducing standard deviation among plot aflatoxin values when longer plots are generated by combining 1.5 m plots sequentially.

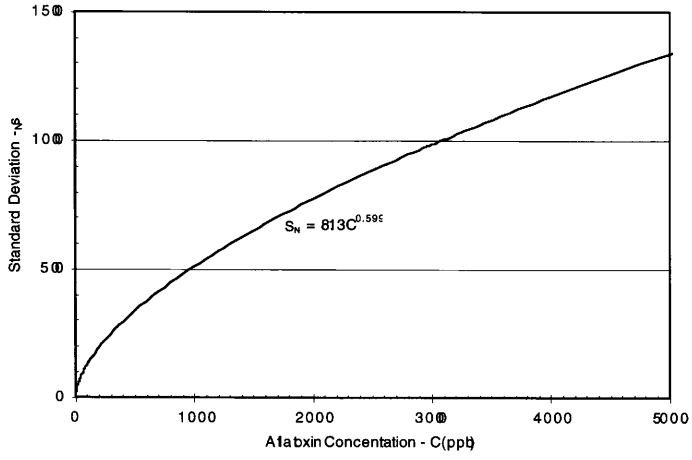

Fig. 4. Effect of aflatoxin concentration on the standard deviation among peanut pod samples of 2.27 kg taken from bulk lots. The single point represents the standard deviation among 2.27 kg pods taken from a field setting.

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