Development of Statistical Models to Simulate the Testing of Farmers Stock Peanuts for Aflatoxin Using Visual, Thin Layer Chromatography, and Minicolumn Methods[1]

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ABSTRACT

The negative binomial probability function was used to model the distribution of sample aflatoxin test results when replicated grade samples from farmers stock peanuts are analyzed by thin layer chromatography and minicolumn methods. The Poisson probability funtion was used to model the distribution of the number of kernels with visible *Aspergillus flavus* growth found in replicated grade samples of farmers stock peanuts when the visible *A. flavus* method is used. The probabilities of accepting a lot of farmers stock peanuts with given aflatoxin concentrations when using a 465-g grade sample and 2 different accept/reject levels were predicted with the models and compared to observed acceptance probabilities computed from previously published data for each of the 3 methods. The comparisons showed good agreement between the predicted acceptance probabilities and the observed acceptance probabilities.

Key Words: Aflatoxin, sampling, farmers stock peanuts, methods, minicolumn, visible *A. flavus*, *A. flavus*.

A provision of the U. S. Department of Agriculture Peanut Marketing Agreement requires that all of the kernels from each grade sample of farmers stock peanuts be examined for visible growth of the aflatoxin-producing mold *Aspergillus flavus* (6). Lots found to contain kernels with *A. flavus* growth (VAF kernels) are classified Segreation-3. Lots with none of these kernels but with more than 2% damaged kernels or more than 1% concealed damage caused by rancidity, mold, or decay are designated Segregation-2 peanuts. All other peanuts are designated Segregation-1. The Segreation-3 peanuts are crushed for oil, which is aflatoxin free after refining, and the meal is used for non-food purposes. Segregation-2 peanuts are crushed for oil and the meal is used in animal feed if a chemical assay does not indicate aflatoxin. While most Segregation-1 peanuts are marketed as shelled raw peanuts some may be cleaned and marketed in the shell. The cleaned inshell peanuts are not analyzed for aflatoxin unless 1.0% or more of the seed are damaged by mold. All raw shelled peanuts are analyzed by chemical assay methods to determine if the aflatoxin concentration is acceptable ($\leq$ 25 ppb).

A recent study by Davidson *et al.* (5) compared the performance of the current visual *A. flavus* examination method (VAF method) with two other methods that were used to estimate the amount of aflatoxin that was solvent-extracted from the samples. One method employed thin-layer chromatography (TLC method) (8), and the other method used a minicolumn (MCL method) (7) to quantify the aflatoxin in the solvent extract. For each of the 3 methods, the study estimated the relationship between the average and the variability of 16 replicated test results for each of 20 lots of farmers stock peanuts. The variance S_T^2 and mean X_T of sample aflatoxin concentrations for the TLC method, the variance S_M^2 and mean X_M of sample aflatoxin concentrations for the MCL method, the variance S_V^2 and mean X_V of the number of VAF kernels per sample for the VAF method, and the lot aflatoxin concentration M for 20 lots are given in Table 1. The variance of aflatoxin test results shown in the table reflects both sampling variance associated with a 465-g grade sample of shelled kernels and the analytical variance associated with each method. As discussed by Davidson, *et al.* (5), the average of the aflatoxin concentrations for the 16 replicated grade samples from each lot are higher than the lot concentration, because the 465-g samples have a higher percentage of loose shelled kernels (LSK) than the lot. The LSK generally contain higher concentrations of aflatoxin than other kernels in the lot.

Because of the varability among test results, two kinds of errors are associated with an aflatoxin testing program for farmers stock peanuts: a Type I error where samples from a good lot will test bad (farmer's risk) and a Type II error where samples from a bad lot will test good (sheller's risk). For a given lot, the farmer's and sheller's risks are functions of the sample size N and the definition of good and bad sample quality. A sample is termed "bad" when the sample concentration X is above some predefined critical level X_c and "good" when $X \leq X_c$. Lots with an aflatoxin concentration M will be accepted with a probability $P(M) = \text{prob}(X \leq X_c | M)$. A plot of P(M) versus M is called an Operating Characteristics (OC) curve. Figure 1 depicts the general shape of an OC curve. As M approaches zero P(M) approaches 1, and as M becomes large P(M) approaches zero. The shape of the OC curve is uniquely defined for a particular testing program with designated values of N and X_c and a given probability distribution.

The objective of this study is to use the experimental data reported by Davidson *et al.* (5) to develop statistical models that accurately describe the varability associated with estimating aflatoxin concentrations in lots of farmers stock peanuts by VAF, TLC, and MCL methods and to develop OC curves for the three methods.

Materials and Methods

Theoretical Models

The negative binomial probability (NBP) function was used to simulate the distribution of sample aflatoxin concentrations for the TLC and for the MCL method. This function has been used to simulate the distribution of sample aflatoxin concentrations from contaminated lots of shelled peanuts (18,15,13,16). The NBP function is suitable for a

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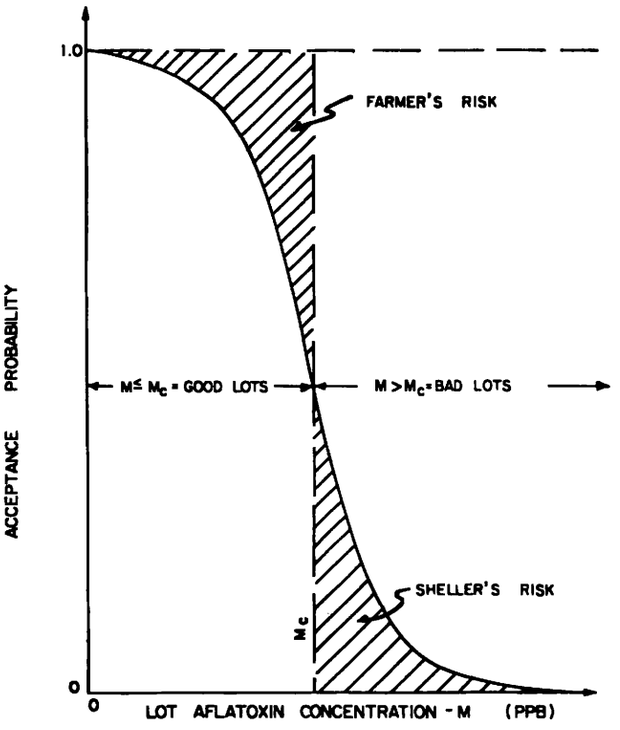

Fig. 1. Typical operating characteristic (OC) curve for evalutating sampling plans.

distribution where there is a high incidence of non-contaminated kernels and a low incidence of kernels with very high aflatoxin concentrations. This type of distribution among kernels in an aflatoxin-contaminated lot of shelled peanuts has been reported by Cucullu *et al.* (4). Equation 1 is the NBP function.

$$\text{Prob}\,(NX \leq r) = F(r) = \sum_{t=0}^{r} (\Gamma(t+NK)/(t!\Gamma(NK)))p^{NK}q^{t} \qquad (1)$$

where Γ is gamma function, K is a shape parameter, M is the lot aflatoxin concentration, N is the sample size in number of kernels, X is the sample aflatoxin concentration, $p=(K/M+K)$, and $q=(1-p) = (M/(M+K)$. The cumulative distribution of sample concentrations F(X) can be determined from a scale transformation of Equation 1.

If a lot of peanuts contains a specific proportion, P, of VAF kernels, the probability of obtaining at least z VAF kernels in a random sample of size N is described by the binomial probability function. However, for large N and small P, the binomial equation is approximated by the Poisson equation (11). Because the data in Table 1 reflects an N of 1129 and a P ranging from 0.0002 to 0.0076, the Poisson probability function (Equation 2) was used to simulate the distribution of the number of VAF kernels in replicated samples taken from farmers stock peanut lots.

$$\text{Prob}\,(X \leq z) = F(z) = \sum_{t=0}^{z} (e^{-NP}(NP)^{t})/t! \qquad (2)$$

where X is a positive integer less than or equal to N.

Model Parameters

The negative binomial equation is completely defined by the two parameters M and K. By assigning values to these parameters, the distribution of sample concentrations X for replicated samples of N kernels from a lot with a specified concentration M can be determined from Equation 1. The accuracy of predicting the distribution of X for a given value of M and N is dependent upon a correct choice of K. A method described as the "method of moments" (1) may be used to estimate K.

$$K = M^2/(NS^2 - M) \qquad (3)$$

where S^2 is the variance among replicated grade sample concentrations. The Statistical Analysis System (12) was used with data from Table 1 to compute regresseion equations which describe the relationship between the variance S^2 and mean M for the TLC and MCL methods. The regression equations for TLC and MCL variances were substituted into Equation 3 to determine a shape parameter K for the TLC and MCL methods. Equation 1 was then used to compute the distribution of sample test results about their mean when using the TLC and MCL methods and 465-g samples each of which contained about 1129 kernels.

As previously mentioned, S^2_M, and S^2_T reflects both analytical and sampling variance. Since analytical variance probably is not related to a negative binomial distribution, the distribution of sample test results about their means which were computed with Equation 1 is not precisely correct. However studies by Whitaker *et al.* (13) indicated that for aflatoxin tests on shelled peanuts the analytical variance is less than 1% of the total variance among tests with the TLC method when 465-g samples of kernels are analyzed from a lot with 50 ppb aflatoxin. Because studies by Holiday and Lansden (7) indicate good agreement between analysis with MCL and TLC, analytical variance by MCL is probably about 1% of the total variance among tests with the MCL method. Even after differences in the capabilities of analytical laboratories are considered, it is unlikely that the analytical variance for either the TLC or the MCL method would exceed 5% of the total variance when 465-g samples are analysed. Since the analytical variance is a small proportion of the total variance, the modeling error introduced by this procedure is negligible and would not justify the more complicated Monte Carlo solution technique which would be required if the analytical variance were handled separately (16).

The Poisson probability function (Equation 2) is completely described by the single parameter NP which is equal to the mean number of VAF kernels in the lot. By specifying the mean (NP) Equation 2 can be used to compute the distribution of test results about their mean when using the VAF method for replicated samples of 465-g (1129 kernels) each.

Comparing Observed and Theoretical Distributions

The Kolmogorov-Smirnov (K-S) test was used to determine the probability that an observed cumulative distribution of test results C(r) came from a population having a true but unknown distribution function F*(r) that can be specified by Equation 1 for the TLC and MCL methods and Equation 2 for the VAF method. The test is based upon the greatest absolute difference D_{max} between the observed distribution and the theoretical distribution. If D_{max} is greater than some critical value D_{nn}, then the null hypothesis H_o that F*(r) is equal to the theoretical distribution is rejected with significance α. Values of D_{nn} for various significance levels α and number of samples nn are presented in several texts (10,3).

The K-S test is exact when the hypothesized theoretical distribution function is continuous; otherwise the test is conservative (3). Also the K-S test is valid only when the parameters of F*(r) are evaluated independent of the observed data (9,2). However Kendall and Stuart (9) indicated that when the parameters are determined from the observed data the Kolmogorov two-sided test statistic D_{nn} may be used to form a confidence band for the true unknown distribution function F*(r) for any significance level 1-α. The confidence band is a band of width $\pm D_{nn}$ around the observed cumulative distribution function C(r), and the probalility that the true unknown distribution function F*(r) lies entirely in the band is 1-α. Therefore, if the theoretical distribution lies completely within the band $C(r) \pm D_{nn}$, then the null hypothesis H_o that F*(r) is equal to the theoretical distribution cannot be rejected with significance α.

Plots of Theoretical OC Curves and Experimental Data

The NBP distribution (Equation 1) was used to generate OC curves for the TLC and MCL methods with critical levels X_c of 25 and 50 ppb aflatoxin. The Poisson distribution (Equation 2) was used to generate OC curves for the VAF method with critical levels of 0 and 1 VAF kernels. The proportion of the 16 test results that were equal to or less than the designated critical levels X_c of 25 and 50 ppb aflatoxin for the TLC and MCL method and 0 and 1 VAF kernels for the VAF method were determined from the data on 20 lots reported by Davidson et al. (5). The observed data were plotted on corresponding OC curves as another check of the agreement between the experimental data and the theoretical OC curves computed from the models simulating each method.

As mentioned in the introduction, the mean sample aflatoxin concentration are generally higher than the lot aflatoxin concentrations (Table

1) because the samples contain a higher percentage of LSK. In order to plot OC curves for lot aflatoxin concentrations it was necessary to transform mean sample aflatoxin concentrations to corresponding lot concentrations for the VAF method. The Statistical Analysis System (12) was used in conjunction with data from Table 1 to make these transformations. The OC curves generated by Equations 1 and 2 were plotted versus the corresponding lot aflatoxin concentrations.

Table 1. Mean and variance among 16 grade samples for each lot as estimated by the TLC, MCL, and VAF methods.

LOT NUMBER	LOT CONCEN. (PPB)	TLC MEAN (PPB)	TLC VARIANCE	MCL MEAN (PPB)	MCL VARIANCE	VAF MEAN (# OF VAF KERNELS)	VAF VARIANCE
1	8	16	1374	96	97213	0.2	0.6
2	10	21	3947	22	2766	0.5	0.82
3	14	33	2135	29	970	1.2	2.56
4	21	22	977	67	23703	2.9	5.59
5	25	40	4420	22	1245	0.3	0.73
6	27	20	3981	19	2445	0.2	0.57
7	34	92	16572	66	3404	1.4	1.30
8	42	113	31344	134	41688	2.0	3.82
9	42	119	92420	32	9340	0.7	1.14
10	59	97	13163	116	8981	2.0	2.51
11	64	154	64599	130	47309	2.2	8.45
12	111	119	24675	113	9104	2.4	4.98
13	128	182	44591	190	100352	2.8	6.18
14	157	173	23437	217	39300	4.4	3.95
15	166	365	148473	355	155852	8.6	9.78
16	166	172	29549	356	125764	3.4	5.08
17	179	383	90870	451	148665	7.8	13.85
18	198	170	20804	287	45290	5.6	5.82
19	242	175	32613	335	72484	3.1	2.29
20	255	245	67170	294	91916	5.5	3.05

Source: Davidson, et al., Peanut Sci., 11:77-82, 1984.

Results and Discussion

Equations 4,5, and 6 are regression equations computed with the Statistical Analysis System (12) from the data in Table 1. The mean and variance data for lots 9 and 1 were deleted from the regression analysis for equations 4 and 5, respectively, since they appeared to be outliers (difference between the observed and predicted variances was greater than 2 standard deviations) when compared to the mean and variance data associated with the other 19 lots. These equations describe the relationship between the variance and mean for the TLC, MCL, and VAF methods, respectively.

$$S_T^2 = 142.4630\, X_T + 0.4583\, X_T^2 \qquad (4)$$

$$S_M^2 = 203.5240\, X_M + 0.3131\, X_M^2 \text{ , and} \qquad (5)$$

$$S_V^2 = 0.8097 + 1.1954\, X_V \qquad (6)$$

with coefficients of determination of 0.913, 0.904, and 0.642, respectively.

As discussed previously, the total variance indicated by both Equations 4 and 5 are predominantly sampling variance, and they should be almost equal since both MCL and TLC tests were made on the same samples. The differences between Equation 4 and 5 may be due to the fact that the TLC test indicated a more precise aflatoxin concentration for each grade sample, while the MCL test indicated that the concentration was greater than or less than the following 11 concentrations: 0, 15, 25, 50, 75, 100, 150, 250, 500, 750, and 1000 parts per billion (ppb). Davidson *et al.* (5) assigned midpoint values for all MCL tests that fell within the indicated ranges. For example, if a test showed the aflatoxin concentration was between 250 and 500 ppb, the test was designated as 375 ppb. The MCL variances listed in Table 1 were computed from these midpoint values and may not be as correct as the variance estimates for TLC which were computed from more exact test results. In view of the possibility that errors were introduced through the use of midpoint values to compute the MCL variances, Equation 4 was used in this study to estimate variances for both the TLC and the MCL methods. Equation 4 shows that the total variance increases as the mean increases which is consistent with previous studies (13,14,17). The variance is greater than the average aflatoxin concentration which is a necessary condition for the negative binomial distribution (1).

From the regression analysis for Equation 6, the standard errors associated with the intercept and linear coefficient did not show that the intercept value was significantly (95% C.L.) different from zero or that the linear coefficient was significantly (95% C.L.) different from 1.0. Therefore, it appears that the variance and mean number of VAF kernels are approximately equal, which is a necessary condition for the Poisson distribution (11).

The individual test results used to compute the data shown in Table 1 have been tabulated by Davidson *et al.* (5), and they were used to construct observed cumulative distributions for the TLC, MCL, and VAF methods for each of the 20 lots. The Kolmogorov-Smirnov two-sided test statistic Dnn of $\pm$ 0.327 for nn = 16 replicated test results and 5% significance level were used to determine an upper and lower bound for each of the the 60 (20 lots by 3 test methods) observed cumulative distributions C(r). For each lot and test method, the theoretical distributions were generated for the mean and variance values listed in Table 1 and compared to C(r). Equation 1 was used to generate theoretical distributions for the TLC and MCL methods, and Equation 2 was used for the VAF method. Due to the detection limits associated with the TLC and MCL assay procedures used in this study, samples that had 2 ppb or less total aflatoxin tested O ppb aflatoxin, Therefore all observed zero values for the TLC and MCL methods were treated as 2 ppb aflatoxin or less when calculating the cumulative probability distributions of observed values for the TLC and MCL methods.

An example of the Kilmogorov-Smirnov two-sided test comparing the NBP function with the observed distribution of sample aflatoxin concentrations determined by TLC for lot 10 in Table 1 are shown in Fig. 2. The theoretical NBP function falls within the upper and lower bounds computed for the observed cumulative distribution of the 16 sample aflatoxin concentrations. For the TLC and the MCL methods the theoretical NBP distribution fell within the upper and lower bounds of all 20 observed distributions for each method. For the VAF method, the theoretical Poisson distribution fell within the upper and lower bounds for all 20 observed distributions. Consequently, neither the NBP function or the Poisson probability function can be rejected at the 95% condidence level as being the distribution for the TLC and MCL methods or for the VAF method, respectively.

Equation 7 is a regression equation computed from the data in Table 1 to transform the means of sample TLC test results, X_T, to lot concentrations, M_T.

$$M_T = 0.6753\, X_T. \qquad (7)$$

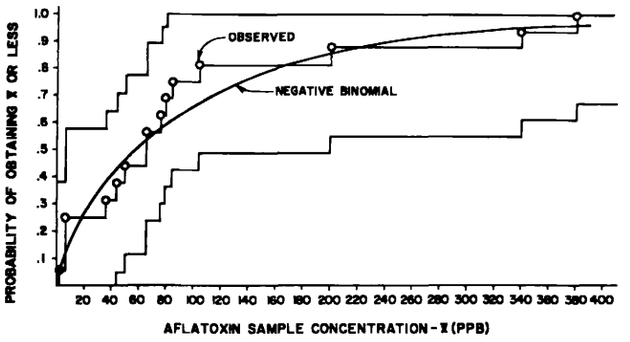

Fig. 2. Comparison of the cumulative distribution of aflatoxin concentrations in grade samples of farmers stock peanuts as predicted by the negative bionomial probability function and the observed cumulative distribution of aflatoxin test results on 16 grade samples from lot number 10. Upper and lower bounds around the observed distribution are shown for the 5% significance level.

The coefficient of determination for equation 7 was 0.831. Equation 8 is a regression equation computed from the data in Table 1 to transform the means of MCL test results, X_M, to lot concentrations, M_M.

$$M_M = 0.5647\, X_M. \qquad (8)$$

The coefficient of determination for Equation 8 was 0.910.

Equation 9 is a regression equation computed from data in Table 1 to transform the lot aflatoxin concentration M_V to the mean number of VAF kernels in the 16 replicated samples, X_V.

$$X_V = 0.02662\, M_V \qquad (9)$$

The coefficient of determination for equation 9 was 0.817.

Figures 3 through 6 show OC curves computed with the NBP function (Equation 1) for the TLC and MCL methods, respectively, when critical level X_c of 25 and 50 ppb aflatoxin are used. Figures 7 and 8 shows OC curves computed with the Poisson function (Equation 2) when critical levels X_c of 0 and 1 VAF kernel are used. The observed acceptance probabilities which correspond to the theoretical OC curves are plotted on the figures. As previously mentioned, the OC curves are plotted versus the lot concentrations computed with Equations 7,8, and 9.

For all three methods there is considerable scatter among the 20 observed acceptance probabilities about the OC curve. The scatter is probably due to the high variance among the test results and the fact than only 16 test results were used to compute the observed acceptance probabilities and the corresponding lot aflatoxin concentrations. Even with this degree of scatter the overall fit between the OC curves computed with the statistical models and the observed acceptance probabilities appear to be as good as could be expected for OC curves which would generally have the shape of an exponential decay curve or a sigmoidal decay curve and which would have a y intercept of 1.0 probability of acceptance. The models developed in this study make it possible to compare the efficacy of the TLC, MCL, and VAF methods to separate lots of farmers stock peanuts according to their aflatoxin concentration. A study to make this comparison is underway.

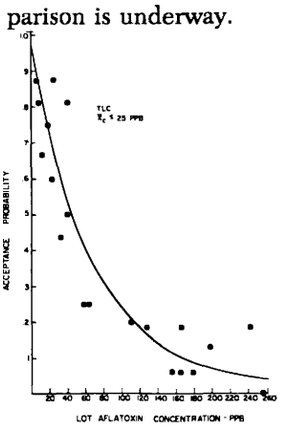

Fig. 3. (left) Observed and predicted acceptance probabilities associated with the TLC method, 465-g sample, and 25 ppb critical level.

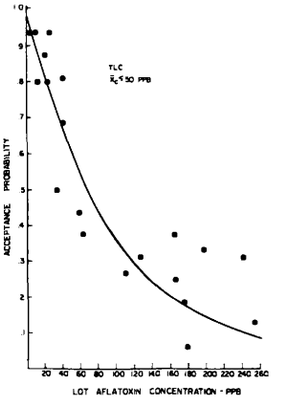

Fig. 4. (right) Observed and predicted acceptance probabilities associated with the TLC method, 465-g sample, and 50 ppb critical level.

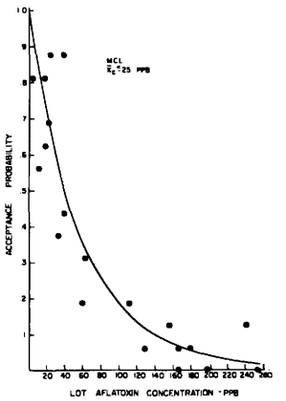

Fig. 5. (left) Observed and predicted acceptance probabities associated with the minicolum method, 465-g sample, and 25 ppb critical level.

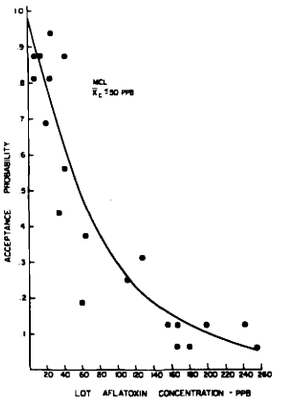

Fig. 6. (right) Observed and predicted acceptance probabilities associated with the minicolumn method, 465-g sample, and 50 ppb critical level.

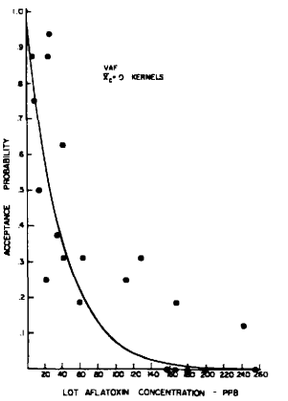

Fig. 7. (left) Observed and predicted acceptance probabilities associated with the visible *A. flavus* method (VAF), 465-g sample, and a critical level of O VAF kernels.

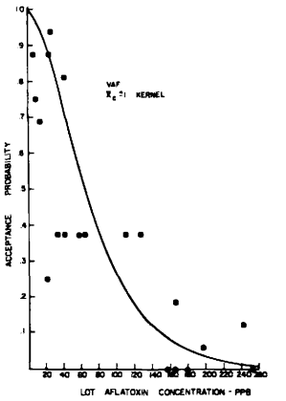

Fig. 8. (right) Observed and predicted acceptance probabilities associated with the visible *A. flavus* method (VAF), 465-g sample, and a critical level of 1 VAF kernel.

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