Variability of Aflatoxin Test Results[1]

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ABSTRACT

Using 12 lb samples, 280 g subsamples, the Waltking method of analysis, and densitometric procedures, the sampling, subsampling, and analytical variances associated with aflatoxin test procedures were estimated. Regression analysis indicated that each of the above variance components is a function of the concentration of aflatoxin in the population being tested. Results, for the test procedures given above, showed that sampling constitutes the greatest single source of error, followed by subsampling and analysis. Functional relationships are presented to determine the sampling, subsampling, and analytical variance for any size sample, subsample, and number of analyses.

INTRODUCTION

All commercial lots of shelled peanuts in the U.S. are tested for aflatoxin prior to processing for food use (1). The concentration of aflatoxin in a lot of shelled peanuts is estimated by measuring the aflatoxin concentration in a random sample of kernels drawn from the lot. The sample is comminuted in a subsampling mill (2), and a subsample of ca. 280 g is analyzed by the Waltking procedure (3). Accurate estimates are difficult to achieve since replicated aflatoxin determinations for a given lot are highly variable (4). The above test procedures, illustrated in Figure 1, indicated that the total variance of aflatoxin test results can be composed of at least three variance components: sampling, subsampling, and analysis. An observed aflatoxin test result $\bar{x}$, may be represented as follows:

$$\bar{x} = \mu + SP + SS + A \qquad [1]$$

where μ = the true aflatoxin concentration of the population tested; SP = random error due to sampling with expected value zero and variance $\delta^2_{\bar{x}(s)}$; SS = random error due to subsampling with expected value zero and variance $\delta^2_{\bar{x}(ss)}$; and A = random error due to analysis with expected value zero and variance $\delta^2_{\bar{x}(a)}$.

The notation $\delta^2_{\bar{x}}$ (neglecting the subscripts s, ss, and a for component identification) indicates the variance of a population of $\bar{x}$ values obtained by sampling a parent population of individual items where the variance among the individual items is δ^2. The variance δ^2, by definition, can be related to $\delta^2_{\bar{x}}$ by the following equation:

$$\delta^2_{\bar{x}} = \delta^2 / n, \qquad [2]$$

where n is the number of individual items drawn from the population from which $\bar{x}$ is evaluated. By assuming both stochastic and functional independence among the random errors in equation [1], the following variance relationship is obtained:

$$\delta^2_{\bar{x}(t)} = \delta^2_{\bar{x}(s)} + \delta^2_{\bar{x}(ss)} + \delta^2_{\bar{x}(a)}, \qquad [3]$$

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

The objective of this study was to quantify empirically the sampling, subsampling, and analytical variance associated with aflatoxin test results.

EXPERIMENTAL PROCEDURES

Method of Analysis

The total variance $\delta^2_{\bar{x}(t)}$, combined subsampling, and analytical variance $\delta^2_{\bar{x}(ssa)}$ where:

$$\delta^2_{\bar{x}(ssa)} = \delta^2_{\bar{x}(ss)} + \delta^2_{\bar{x}(a)} \qquad [4]$$

and analytical variance $\delta^2_{\bar{x}(a)}$ were estimated by direct measurements. Once $\delta^2_{\bar{x}(t)}$, $\delta^2_{\bar{x}(ssa)}$, and $\delta^2_{\bar{x}(a)}$ were estimated, the remaining variance terms for sampling $\delta^2_{\bar{x}(s)}$ and subsampling $\delta^2_{\bar{x}(ss)}$ were determined indirectly using the summation property shown in equations [3] and [4]. Estimates of $\delta^2_{\bar{x}}$ and μ by experimental values are denoted by $s^2_{\bar{x}}$ and $\bar{\bar{x}}$ where $\bar{\bar{x}}$ is the average of observed $\bar{x}$ values.

Peanut samples were comminuted with a subsampling mill similar to that used in most aflatoxin laboratories for peanuts (2). The Waltking method (3) was used for analysis, and spot intensities were quantified by densitometric procedures using a Photovolt detector and amplifier. All analyses were made in the same laboratory. All variance estimates are based upon one analysis/aliquot, 280 g subsamples, and 12 lb samples. Kernel counts indicated an average of 10,634 kernels/12 lb sample or ca. 886 kernels/lb. The sample size in variance relationships were based upon the number of kernels/sample.

Total Variance

The total variance $\delta^2_{\bar{x}(t)}$ is defined as variance among aflatoxin determinations on replicated samples from the same lot of shelled peanuts. The estimated total variance $s^2_{\bar{x}(t)}$ was computed from data obtained in a previous study (4). For that study, 29 minilots weighing ca. 120 lb each were drawn from 29 commercial lots of shelled peanuts which were contaminated with aflatoxin. Using a riffle divider with 1 in. wide slots, each minilot was divided into 10 samples of ca. 12 lb with an average of 10,634 kernels/sample. Ten 280 g subsamples, each representing a

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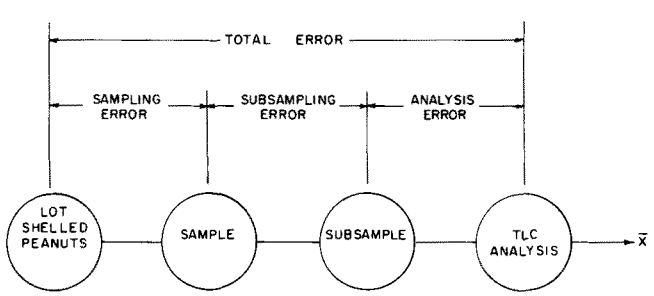

FIG. 1. Typical steps employed to estimate the aflatoxin concentration $\bar{x}$ and the associated variance components. TLC = thin layer chromatography.

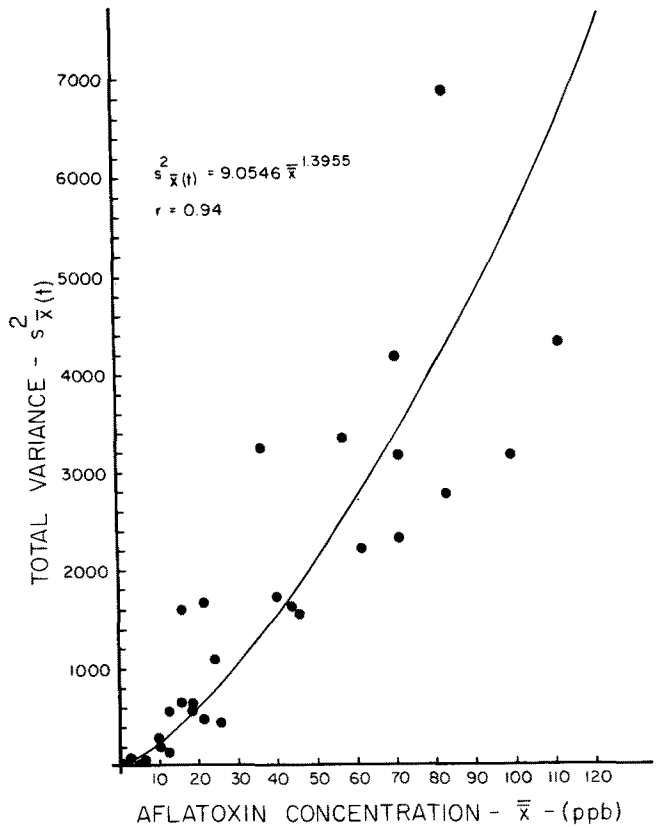

FIG. 2. Relationship between the total variance $s^2_{\bar{x}(t)}$ and the aflatoxin concentration $\bar{\bar{x}}$ in ppb. Correlation coefficient r = 0.94.

12 lb sample of kernels comminuted in a subsampling mill (2), were analyzed for aflatoxin from each minilot. The total variance $s^2_{\bar{x}(t)}$ and the average aflatoxin concentration $\bar{\bar{x}}$ of the 10 test results were calculated for each of the 29 minilots.

Combined Subsampling and Analytical Variance

The combined subsampling and analytical variance $\delta^2_{\bar{x}(ssa)}$ is defined as the variance among aflatoxin determinations on replicated subsamples taken from a sample of aflatoxin contaminated peanuts. A 5 lb sample was taken from each of 32 commercial lots of shelled peanuts contaminated with aflatoxin. The 32 samples were combined into a 160 lb minilot considered to be representative of typical commercial lots of aflatoxin contaminated shelled peanuts. A riffle divider with 1 in. slots was used to subdivide the minilot into 13 samples (12 lb). The 12 lb comminuted material from each sample were blended and subdivided into 20 subsamples of ca. 280 g each. The comminuted material was subdivided over a small riffle divider with 0.25 in. slots. The variance $s^2_{\bar{x}(ssa)}$ and the average aflatoxin concentration $\bar{\bar{x}}$ of the 20 test results were calculated for each of the 13 samples.

Analytical Variance

Analytical variance $\delta^2_{\bar{x}(a)}$ is defined as the variance among aflatoxin determinations on equal aliquots of extract taken from the blender after the extraction step specified in the Waltking method. Subsamples of comminuted peanuts weighing 560 g were blended with 2,800 ml methanol-water-hexane solution for 2 min. The blended material was divided equally among 16 centrifuge bottles. The content of each centrifuge bottle (ca. 250 ml meal and solvent) was analyzed as specified by the Waltking method. Replicated aflatoxin determinations (16 determinations)

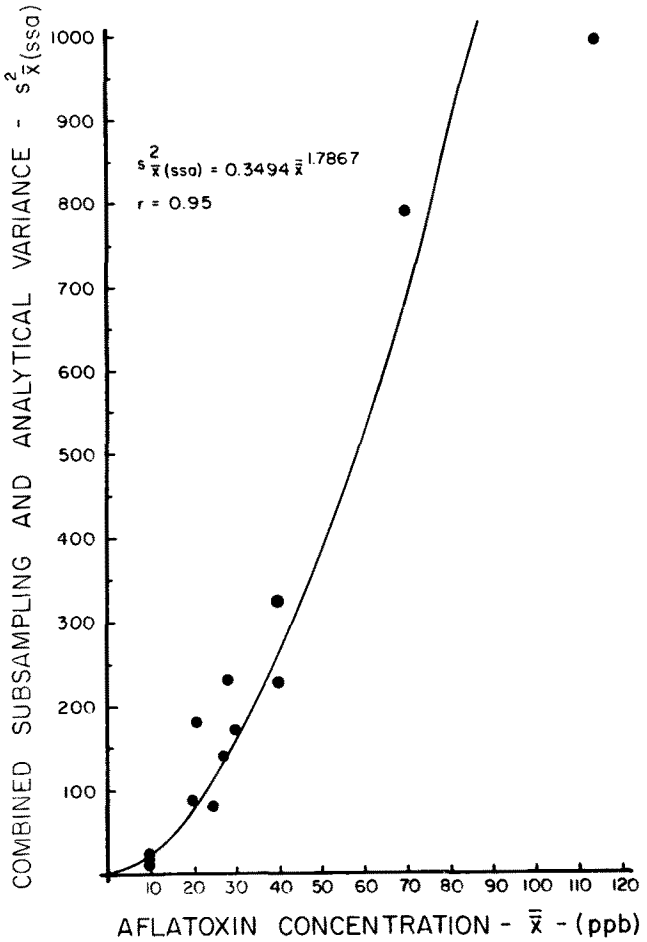

FIG. 3. Relationship between the combined subsampling and analytical variance $s^2_{\bar{x}(ssa)}$ and the aflatoxin concentration $\bar{\bar{x}}$ in ppb. Correlation coefficient r = 0.95.

were made on each of 11 subsamples (560 g). The variance $s^2_{\bar{x}(a)}$ and the average aflatoxin concentration $\bar{\bar{x}}$ indicated by the 16 test results were calculated for each of the 11 subsamples.

DISCUSSION OF RESULTS

Total Variability

The average $\bar{\bar{x}}$ and the total variance $s^2_{\bar{x}(t)}$ for each of the 29 minilots are plotted in Figure 2. For 26 of the 29 minilots, $\bar{\bar{x}}$ and $s^2_{\bar{x}(t)}$ were computed from 10 replicated test results. In the remaining 3 cases, $\bar{\bar{x}}$ and $s^2_{\bar{x}(t)}$ were computed from 9 replicated test results. One test result $\bar{x}$ was neglected in each of the three minilots by applying outlier theory (5). The results indicated that the total variance may be a function of the aflatoxin concentration, since $s^2_{\bar{x}(t)}$ appears to increase as $\bar{\bar{x}}$ increases. It was assumed that a power function of the general form:

$$s^2_{\bar{x}} = C_1 \bar{\bar{x}}^{C_2}, \qquad [5]$$

where C_1 and C_2 are constants independent of $\bar{\bar{x}}$, could describe the empirical relationship between the variance and aflatoxin concentration. Equation [5] was chosen because it fits the requirement that $s^2_{\bar{x}(t)} = 0$ when $\bar{\bar{x}} = 0$, and the literature (6,7) suggests that a power function is appropriate.

Using the Statistical Analysis System, the constants C_1

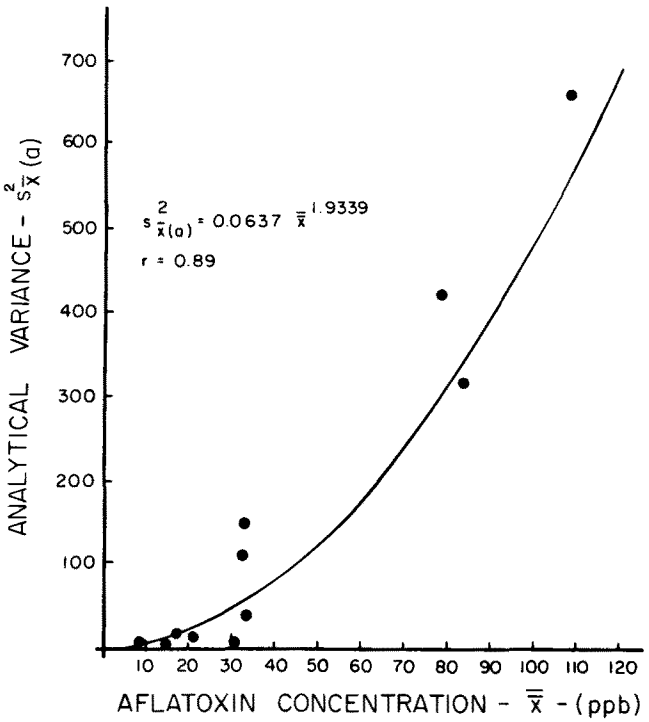

FIG. 4. Relationship between the analytical variance $s^2_{\bar{x}(a)}$ and the aflatoxin concentration $\bar{\bar{x}}$ in ppb. Correlation coefficient r = 0.89.

and C_2 were determined by a least squares fit of $\log_e (s^2_{\bar{x}(t)})$ to $\log_e (\bar{\bar{x}})$ which gives equal wt to the residuals from the line in the logarithmic scale, thus minimizing the effect of an occasional large deviation in the scale for $s^2_{\bar{x}(t)}$. The regression analysis gave the expression:

$$s^2_{\bar{x}(t)} = 9.0546\,\bar{\bar{x}}^{1.3955}, \qquad [6]$$

with a correlation coefficient of 0.94 in the log scale. A plot of regression equation [6] also is shown in Figure 2.

Combined Subsampling and Analytical Variance

The average $\bar{\bar{x}}$ and the combined subsampling and analytical variance $s^2_{\bar{x}(ssa)}$ of the 20 replicated test results for each of the 13 samples are plotted in Figure 3. The increase of $s^2_{\bar{x}(ssa)}$ with $\bar{\bar{x}}$ indicates that $s^2_{\bar{x}(ssa)}$ may be a function of $\bar{\bar{x}}$ as was $s^2_{\bar{x}(t)}$. Regression equation [5] was fitted to the data using log values. From the regression analysis, the expression:

$$s^2_{\bar{x}(ssa)} = 0.3494\,\bar{\bar{x}}^{1.7867}, \qquad [7]$$

with a correlation coefficient of 0.95 in the log scale was obtained. A plot of regression equation [7] also is shown in Figure 3.

Analytical Variance

The average $\bar{\bar{x}}$ and the analytical variance $s^2_{\bar{x}(a)}$ of the 16 replicated test results for each of the 11 subsamples are plotted in Figure 4. As in the 2 previous cases, regression equation [5] was fitted to the data giving:

$$s^2_{\bar{x}(a)} = 0.0637\,\bar{\bar{x}}^{1.9339}, \qquad [8]$$

with a correlation coefficient of 0.89 in the log scale. A plot of equation [8] is shown in Figure 4.

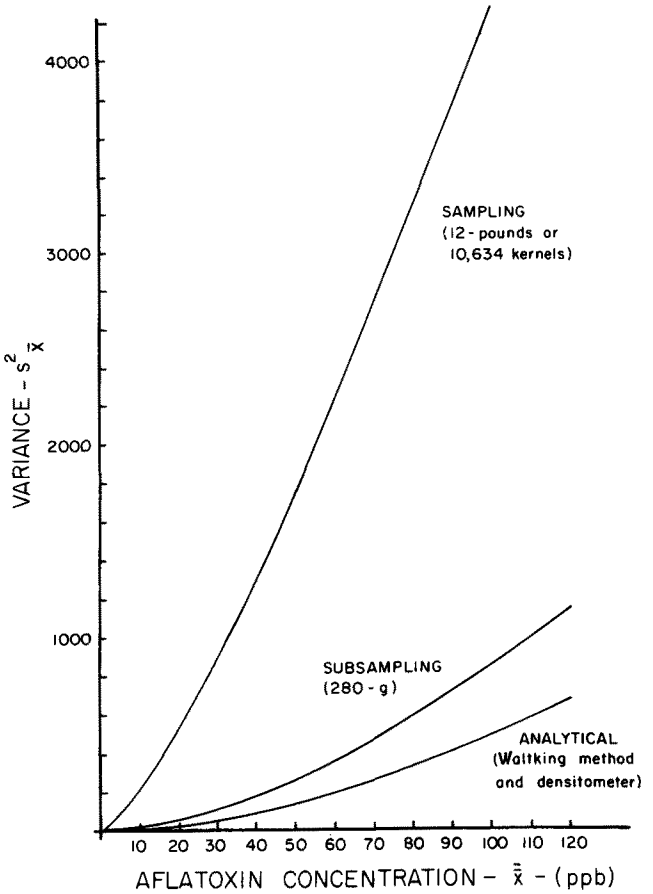

FIG. 5. Expected value of the sampling, subsampling, and analytical variance shown as a function of aflatoxin concentration $\bar{\bar{x}}$ in ppb.

The sampling and subsampling variance components were evaluated using the summation property given in equations [3] and [4]. The sampling variance is estimated by subtracting equation [7] from equation [6]:

$$s^2_{\bar{x}(s)} = 9.0546\,\bar{\bar{x}}^{1.3955} - 0.3494\,\bar{\bar{x}}^{1.7867}. \qquad [9]$$

The subsampling variance was estimated in a similar manner by subtracting equation [8] from equation [7]:

$$s^2_{\bar{x}(ss)} = 0.3494\,\bar{\bar{x}}^{1.7867} - 0.0637\,\bar{\bar{x}}^{1.9339}. \qquad [10]$$

The sampling, subsampling, and analytical variance are shown together in Figure 5.

The variances can be used to determine the coefficient of variation (CV) associated with each step of the aflatoxin test procedure. The CV expressed as a percent is defined as:

$$CV = 100\,\delta_{\bar{x}}/\mu \qquad [11]$$

where $\delta_{\bar{x}}$ is the standard deviation or the square root of the variance $\delta^2_{\bar{x}}$. Substituting the square root of the appropriate variance in equation [8], [9], or [10] into equation [11] provides the CV associated with each step of the aflatoxin test procedure. The coefficient of variation representing the sampling, subsampling, and analytical procedures are shown graphically in Figure 6.

For the condition stated in the procedure (12 lb sample, 280 g subsample, and use of densitometer), Figures 5 and 6 indicate that the total variance of aflatoxin test results primarily reflects sampling variability. At $\bar{\bar{x}}$ = 20 ppb, the

sampling variance is ca. 88% total variance. The ratio, s_s^2 / s_t^2, decreases slightly as $\bar{\bar{x}}$ increases. For example, at $\bar{\bar{x}}$ = 60 ppb, sampling variance makes up ca. 81% total. The CV associated with sampling is large especially at low $\bar{\bar{x}}$ values. For $\bar{\bar{x}}$ = 20 ppb, $CV_s \simeq 114\%$. Figures 5 and 6 also indicate that subsampling constitutes the next largest variance component, with the analytical procedure being the smallest component. CV_{ss} and CV_a do not decrease as rapidly with an increase in $\bar{\bar{x}}$ as does the sampling coefficient of variation. The CV_a at ca. 22% is almost constant with $\bar{\bar{x}}$.

The total variance associated with aflatoxin test results can be reduced by reducing 1 or more of the variance components in equation [3]. One way the variance components can be reduced is to increase the quantity of material inspected. The effect of sample size upon the variance of the mean of n items $\delta^2_{\bar{x}}$ can be illustrated using equation [2]. Since the variance among the individual items of a population δ^2 is a fixed parameter, the variance of the mean of n items $\delta^2_{\bar{x}}$ varies inversely with the number of items drawn from the population. By evaluating δ^2 where:

$$\delta^2 = n\,\delta^2_{\bar{x}}, \qquad [12]$$

the variance of the mean of any quantity of material N can be estimated using the following expression:

$$\delta^2_{\bar{x}}\Big|_N = n\,\delta^2_{\bar{x}}\Big|_n / N. \qquad [13]$$

Using equation [13], the sampling variance for any given sample size becomes:

$$\delta^2_{\bar{x}(s)}\Big|_{ns} = 10{,}634\,\delta^2_{\bar{x}(s)}\Big|_{10{,}634} / ns, \qquad [14]$$

where $\delta^2_{\bar{x}(s)}\Big|_{ns}$ is the sampling variance for a sample of size ns; $\delta^2_{\bar{x}(s)}\Big|_{10{,}634}$ is the sampling variance for a 12 lb sample of 10,634 kernels given by equation [9]; and ns is the sample size in number of kernels. Therefore:

$$\delta^2_{\bar{x}(s)}\Big|_{ns} = (10{,}634/ns)\,(9.0546\,\mu^{1.3955} - 0.3494\,\mu^{1.7867}). \qquad [15]$$

A similar expression exists for the subsampling variance $\delta^2_{\bar{x}(ss)}$:

$$\delta^2_{\bar{x}(ss)}\Big|_{nss} = 280\,\delta^2_{\bar{x}(ss)}\Big|_{280} / nss, \qquad [16]$$

where $\delta^2_{\bar{x}(ss)}\Big|_{nss}$ is the subsampling variance for a given subsample size nss; $\delta^2_{\bar{x}(ss)}\Big|_{280}$ is the subsampling variance associated with a 280 g subsample given by equation [10], and nss is the subsample wt in g. Therefore:

$$\delta^2_{\bar{x}(ss)}\Big|_{nss} = (280/nss)\,(0.3494\,\mu^{1.7867} - 0.0637\,\mu^{1.9339}. \qquad [17]$$

Since the subsample size may approach the size of the population (sample) from which it was taken, a finite population correction term is added to equation [17] giving:

$$\delta^2_{\bar{x}(ss)}\Big|_{nss} = (280/nss)\,(1 - (nss/xs))\,(0.3494\,\mu^{1.7867} - 0.0637\,\mu^{1.9339}), \qquad [18]$$

where xs is the sample wt in g.

The analytical variance given by equation [8] is for an analysis of a 250 ml aliquot carried through all of the steps in the Waltking procedure subsequent to the blending step. Doubling the number of analyses (carrying two aliquots through the Waltking procedure) would halve the analytical variance $\delta^2_{\bar{x}(a)}$. The effect of the number of analyses upon the analytical variance is shown by the following equation:

$$\delta^2_{\bar{x}(a)}\Big|_{na} = \delta^2_{\bar{x}(a)}\Big|_1 / na, \qquad [19]$$

where $\delta^2_{\bar{x}(a)}\Big|_{na}$ is the analytical variance for na analyses; $\delta^2_{\bar{x}(a)}\Big|_1$ is the analytical variance given by equation [8] associated with an analysis; and na is the number of analyses. Equation [19], becomes:

$$\delta^2_{\bar{x}(a)}\Big|_{na} = (1/na)\,(0.0637\,\mu^{1.9339}). \qquad [20]$$

By adding equations [15], [18], and [20] together, the total variance can be estimated for a given size sample, subsample, and number of analyses.

The variances estimated in this study reflect the following: (A) 12 lb samples averaging 10,634 kernels, (B) subsampling mill used to comminute the samples, (C) 280 g subsample, (D) Waltking method of analysis, (E) use of densitometric equipment to quantify spot intensities for the thin layer chromatographic analysis, and (F) use of a particular laboratory for analyses. Visual, rather than

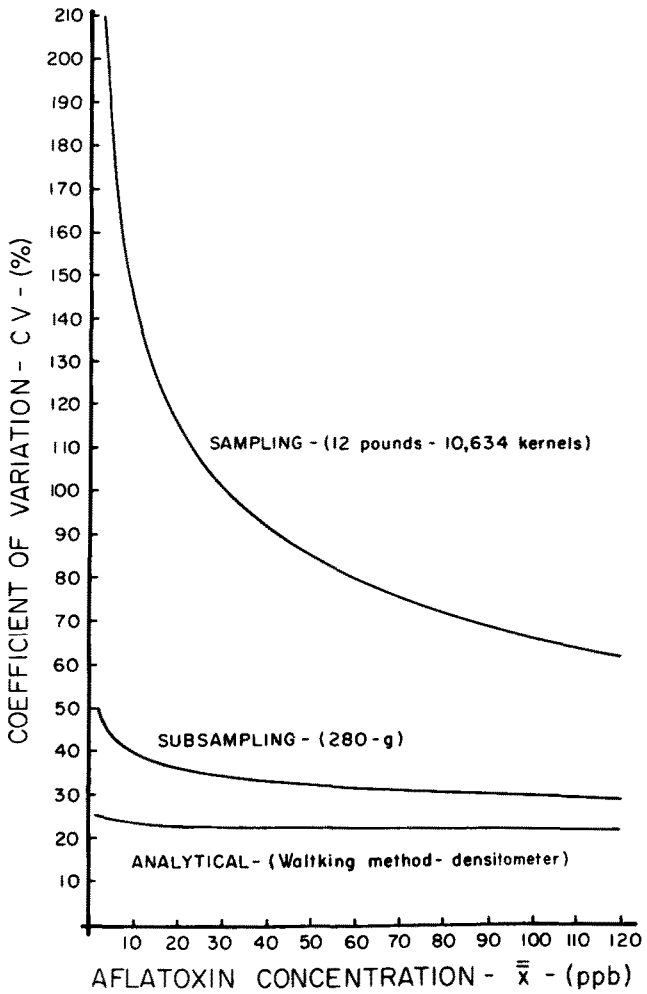

FIG. 6. The coefficient of variation characterizing sampling, subsampling, and analysis is shown as a function of aflatoxin concentration $\bar{\bar{x}}$ in ppb.

densitometric, procedures are used in most laboratories for routine analysis of samples from commercial lots. Use of the densitometer in this study probably reduced analytical variance.

Even with clearly defined aflatoxin test procedures, variability in test results among different laboratories can be high (8,9). Since all measurements made in this study were made in the same laboratory, the variances do not reflect differences in laboratories and may not be representative of all laboratories. However, the variance relationships presented in this article should indicate the major sources of error in aflatoxin tests and provide insight concerning ways to reduce the total variability associated with aflatoxin test results.

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