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Errors in Aflatoxin Analyses of Raw Peanuts By Thin Layer Chromatography[1]

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ABSTRACT

Estimates were made of the errors associated with the extraction, cleanup, drying, and quantification steps of the analytical procedure used by the Food Safety and Quality Service to test peanuts for aflatoxin. An analysis of variance indicated that the error associated with the extraction, cleanup, and drying steps were each negligible and that the quantification step was the major source of the analytical error. The error associated with the quantification step (coefficient of variation average 18.6%) probably comes from three sources((1) differences in fluorescence among replicated spots on a TLC plate (spot to spot error), (2) differences in fluorescence among spots on different TLC plates (spot to spot plus plate to plate error), and (3) errors in measuring the fluorescence of the spots (reading error). The fluorescence spots on the TLC plates were read densitometrically.

Key Words: Peanuts, Aflatoxin, Variability, FSQS, Aflatoxin Analyses, Extraction, Thin Layer Chromatography

The Food Safety and Quality Service (FSQS) of the United States Department of Agriculture has defined the procedure used for official aflatoxin analyses on raw peanuts (1). A 21.8 kg sample of peanuts is comminuted in a subsampling mill (2), and a 1100-g subsample is extracted in 3000 ml methanol-water (55:45) and 1000 ml hexane or technical-grade petroleum ether. Duplicate 50-ml portions of the methanol-water extract are analysed for aflatoxin by Method II of AOAC (3). The FSQS quantifies the fluorescence of spots on the thin layer chromatography (TLC) plate by visual methods. Previous studies have shown that the coefficient of variation among replicated anlayses of the methanol-water extract is 22.8% for subsamples with an aflatoxin concentration of 20 parts per billion (ppb) total aflatoxin when the fluorescent intensities were read densitometrically (4, 5). The large samples, large subsamples and duplicate analyses employed by the FSQS method contribute to the accuracy of the method. Further improvement may be possible by reducing the error associated with the aflatoxin assay of the subsample.

Analysis of the subsample by the FSQS method consists of an extraction step, a clean-up step, a drying step and a quantification step by thin layer chromatography (TLC). An observed aflatoxin assay x may be represented by definition as follows:

$$x = \mu + E + C + D + Q \qquad (1)$$

where μ is the true aflatoxin concentration in the subsample being tested, E is the random error due to extraction with expected value zero and variance σ^2_E, C is the random error due to clean-up with expected value zero and variance σ^2_C, D is the random error due to drying with expected value zero and variance σ^2_D, and Q is the random error due to quantification with expected value zero and variance σ^2_Q. By assuming independence among the random errors in equation 1, the total variance σ^2_T associated with the FSQS method becomes

$$\sigma^2_T = \sigma^2_E + \sigma^2_C + \sigma^2_D + \sigma^2_Q \qquad (2)$$

The objective of this study is to empirically estimate the variance associated with each of the above steps in the analytical procedure.

Experimental Procedure

Method of Analysis - The total variance σ^2_T, the combined clean-up, drying and quantification variance σ^2_{CDQ}, the combined drying and quantification variance σ^2_{DQ}, and the quantification variance σ^2_Q were estimated by direct measurements. The variance terms σ^2_{CDQ} and σ^2_{DQ} can be expressed as

$$\sigma^2_{CDQ} = \sigma^2_C + \sigma^2_D + \sigma^2_Q, \qquad (3)$$

and

$$\sigma^2_{DQ} = \sigma^2_D + \sigma^2_Q. \qquad (4)$$

After σ^2_T, σ^2_{CDQ}, σ^2_{DQ}, and σ^2_Q were estimated, the remaining variance terms σ^2_C, σ^2_D and σ^2_E were estimated using summation properties.

$$\sigma^2_D = \sigma^2_{DQ} - \sigma^2_Q, \qquad (5)$$

$$\sigma^2_C = \sigma^2_{CDQ} - \sigma^2_{DQ}, \qquad (6)$$

and

$$\sigma^2_E = \sigma^2_T - \sigma^2_{CDQ}. \qquad (7)$$

Estimates of the statistical parameters σ^2 and μ by experimental values are denoted by s^2 and $\bar{x}$, where $\bar{x}$ is the average of replicated assay values x.

Nine tests were made in the study. In each test, peanut kernels that were naturally contaminated with aflatoxin were comminuted with a subsampling mill according to the FSQS method. Four 1100-g portions of the comminuted meal were each blended in a 3.78l Waring Blendor[1] with 1000 ml of hexane, 1650 ml of methanol, 1350 ml of water, and 22 g of sodium chloride at medium speed for 3 min. The four blends were poured into a suitable container and stirred until thoroughly mixed. Seventy-five portions of the blend were centrifuged in 250-ml centrifuge bottles. Using a volumetric pipette, at least 50 ml of the methanol-water-aflatoxin solution was transferred from each centrifuge bottle to a separate sample bottle. The 75 bottles were stored no longer than 8 days in a refrigerator before analysis. (Subsequent analyses of the extract did not demonstrate a reduction in aflatoxin with storage time.)

Three groups of 20 sample bottles each were selected at random. One group of 20 bottles was marked "T" and held for use to estimate σ^2_T. A second group of 20 bottles was marked "CDQ" and held for use to estimate σ^2_{CDQ}. A third group of bottles was marked "DQ" and held for use

[1]Paper Number 6884 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27650. The use of trade names in this publication does not imply endorsement by the United States Department of Agricultural or by the N.C. Research Service of the produce named, nor criticism of similar ones not mentioned.

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to estimate σ^2_{DQ}. The remaining 15 bottles were marked "Q" and held for use to estimate σ^2_Q.

Total Assay Variance - The total variance σ^2_T is defined as the variance among aflatoxin determinations on equal volumes of material taken from the blender. A 50-ml portion of the methanol-water-aflatoxin solution from each of the 20 "T" bottles was carried through the clean-up step of the FSQS method (Figure 1). Each of the 50-ml portions of chloroform-aflatoxin solution was sealed in a separate bottle marked "T", and stored in a refrigerator.

Combined Clean-up, Drying, and Quantification Variance - The combined clean-up drying, and quantification variance σ^2_{CDQ} is defined as the variance among aflatoxin determinations on equal volumes of the aqueous methanol solution. The methanol-water-aflatoxin solution from the 20 "CDQ" bottles was combined in a beaker and stirred thoroughly. This step provided over 1000 ml of an aqueous methanol solution which was divided into twenty 50-ml portions. Each 50-ml portion was carried through the clean-up step of the FSQS method (Figure 1). Each of the 50-ml portions of chloroform-aflatoxin solution was sealed in a separate bottle marked "CDQ" and stored in a refrigerator.

Combined Drying and Quantification Variance - The combined drying and quantification variance σ^2_{DQ} is defined as the variance among aflatoxin determinations on equal volumes of the aflatoxin-chloroform solution. A 50-ml portion of the methanol-water-aflatoxin solution from each of the 20 "DQ" bottles was carried through the FSQS clean-up step and all twenty 50-ml portions of the chloroform-aflatoxin solution were combined in a beaker and stirred thoroughly. The 1000 ml of chloroform-aflatoxin solution was divided into twenty 50-ml portions which were sealed in bottles marked "DQ" and stored in a refrigerator (Figure 1).

Quantification Variance - The quantification variance σ^2_Q is defined as the variance among aflatoxin determinations when equal quantities of the same benzene-acetonitrile-

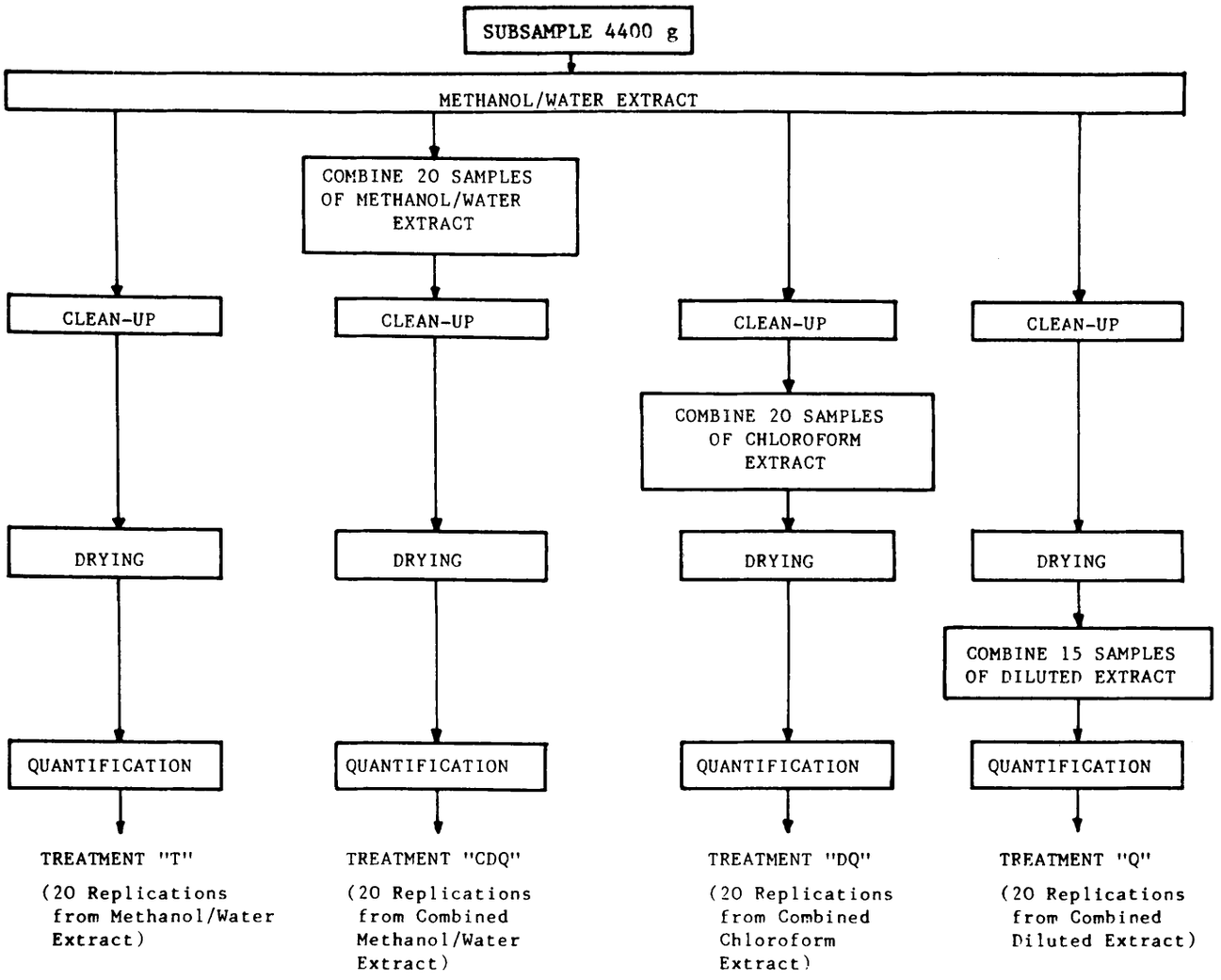

Fig. 1. Procedure by which the 75 samples of aqueous methanol extract was used to estimate the mean and variance of the four parameters T, CDQ, DQ and Q.

aflatoxin solution are placed on TLC plates. A 50-ml portion of the methanol-water-aflatoxin solution from each of the 15 "Q" bottles was carried through the FSQS clean-up step and all fifteen 50-ml portions of the chloroform-aflatoxin solution were combined and carried through the FSQS drying step where the aflatoxin was taken to dryness in a 10 ml vial (Figure 1). The aflatoxin in the 10-ml vial was dissolved with 4.5 ml of benzene-actonitrile. The vial was marked "Q", sealed and placed in a refrigerator.

TLC Quantification - The 50 ml of chloroform-aflatoxin solution from 1 bottle of each of the 3 treatments (T, CDQ, and DQ) were simultaneously carried through the FSQS drying step and the aflatoxin in each vial was dissolved with 0.3 ml of benzene-acetonitrile. A portion of the contents of each of the 3 vials and the contents of the vial marked "Q" were spotted on the same TLC plate. The spot intensities were quantified by densitometric procedures using a densitometer developed in this laboratory. This instrument has been described and compared to a commercially available instrument (5). This drying, spotting and quantification procedure was repeated until all 20 bottles from each treatment were used. The 20 replicated aflatoxin determinations for each of the four treatments were used to obtain 1 estimate of variance for each treatment (s^2_T, s^2_{CDQ}, s^2_{DQ} and s^2_Q). The entire test was repeated 9 times to give 9 estimates of variance for each treatment.

Results and Discussion

The variances s^2_T, s^2_{CDQ}, s^2_{DQ} and s^2_Q, the associated average aflatoxin concentrations $\bar{x}$, and the associated coefficients of variation (CV) for each of the 9 tests are given in Table 1 for B1 and total (B1 + B2 + G1 + G2) aflatoxin. The average total aflatoxin concentration varied from about 35 ppb in test 1 to 180 ppb in test 9. It can be seen that the variances, regardless of treatment, increase with $\bar{x}$. Previous studies with peanuts and corn (4,6) demonstrated that the CV for the analytical procedure is constant over the range of $\bar{x}$ values measured in this study.

$$s^2 = a_1\bar{x}^2, \quad (8)$$

$$CV = s/\bar{x}, \quad (9)$$

$$CV = (\sqrt{a_1})\,\bar{x}/\bar{x} = \sqrt{a_1}, \quad (10)$$

where a_1 is a constant.

Inspection of Table 1 shows that for each type of aflatoxin the CV's appear to be independent of $\bar{x}$ for each treatment. To verify this observation, a linear regression was run between CV and $\bar{x}$ for each treatment and type of aflatoxin.

Table 1. Mean aflatoxin concentrations and related statistical data for four experimental treatments (T, CDQ, DQ, and Q).

	T			CDQ			DQ			Q		
Test Number	$\bar{x}$ (ppb)	s^2_{TA}	CV_T (%)	$\bar{x}$ (ppb)	s^2_{CDQ}	CV_{CDQ} (%)	$\bar{x}$ (ppb)	s^2_{DQ}	CV_{DQ} (%)	$\bar{x}$ (ppb)	s^2_Q	CV_Q (%)
					B1 Aflatoxin							
1	19.2	14.8	20.0	20.5	39.4	30.6	19.5	13.3	18.7	17.7	6.8	14.7
2	27.0	22.1	17.4	26.4	14.5	14.4	24.6	26.7	21.0	25.1	44.2	26.5
3	37.5	64.7	21.4	38.4	63.3	20.7	37.9	78.6	23.4	35.3	19.4	12.5
4	22.2	10.2	14.4	23.5	16.8	17.4	23.2	16.1	17.3	22.8	11.1	14.6
5	66.5	185.1	20.5	71.3	159.3	17.7	73.4	85.3	12.6	55.6	84.7	16.6
6	54.8	156.6	22.8	51.2	84.9	18.0	53.2	85.1	17.3	44.3	75.3	19.6
7	31.5	34.7	18.7	32.3	35.4	18.5	32.2	51.0	22.2	33.6	44.3	19.8
8	64.9	303.8	26.9	73.3	307.9	23.9	73.1	216.6	20.1	52.1	338.5	35.3
9	86.0	124.4	13.0	76.4	258.2	21.0	90.8	374.3	21.3	80.8	156.8	15.5
Avg.			19.5			20.3			19.3			19.5
Slope			.0158			-.0044			-.0247			.0320
± S.E.			.0683			.0788			.0464			.1373
Intercept			18.7			20.5			20.5			18.1
R^2			.007			.000			.039			.008
					Total Aflatoxin							
1	34.3	32.3	16.6	37.2	136.6	31.4	35.8	50.9	19.9	32.3	23.0	14.8
2	44.0	58.5	17.4	42.4	31.8	13.3	39.5	42.8	16.6	40.7	127.5	27.7
3	71.9	137.8	16.3	72.1	138.4	16.3	70.0	185.2	19.4	67.3	53.2	10.8
4	46.6	40.1	13.6	47.9	64.0	16.7	46.9	54.6	15.7	46.5	36.7	13.0
5	117.9	504.4	19.0	124.5	416.4	16.4	127.3	291.2	13.4	101.8	192.9	13.6
6	75.2	264.5	21.6	70.3	129.0	16.2	73.0	147.7	16.6	60.8	114.4	17.6
7	40.3	42.9	16.3	41.8	54.3	17.6	41.3	68.3	20.0	44.2	84.1	20.8
8	112.4	869.7	26.2	126.2	842.3	23.0	127.1	614.9	19.5	91.6	975.9	34.1
9	189.4	489.9	11.7	166.2	1359.4	22.2	191.4	1889.0	22.7	176.3	650.9	14.5
Avg.			17.6			19.2			18.2			18.6
Slope			-.0039			.0115			.0145			-.0172
± S.E.			.0320			.0444			.0191			.0644
Intercept			17.9			18.3			17.0			19.8
R^2			.002			.009			.076			.010

$$CV = a_2 + a_3\bar{x}, \quad (11)$$

where a_2 and a_3 are the intercept and slope respectively. If the CV is constant over the range of $\bar{x}$ values in this study, the slope a_3 in equation 11 is zero. The intercept, slope, standard error associated with the slope, and coefficient of determination (R^2) for each treatment and type of aflatoxin are shown in Table 1. It can be seen that all values of the slope are approximately zero and less than the standard error. Therefore, it can be concluded that the CV is independent of $\bar{x}$ and can be averaged over the 9 tests.

An analysis of variance did not show a significant difference among the CV values for the 4 treatments in Table 1 for either aflatoxin B_1 or for total aflatoxin at the 5% significance level. Also, a significant difference was not shown between the CV values for aflatoxin B_1 or the CV values for total aflatoxin at the 5% significance level. Because of the functional relationship between CV and s^2 one may therefore assume that there is no difference in the variance for the 4 treatments at a given aflatoxin concentration.

$$s^2_T = s^2_{CDQ} = s^2_{DQ} = s^2_Q \quad (12)$$

Then it follows from equations 2, 3, and 4 that the errors associated with extraction, clean-up, and drying are approximately zero.

$$s^2_E = s^2_C = s^2_D = 0 \quad (13)$$

Therefore, the major source of error in the FSQS procedure is associated with the quantification step.

The average CV for all 4 treatments and the 2 types of aflatoxin (19.0%) along with equations 8, 9, and 10 can be used to develop an expression relating s^2_T or s^2_Q to $\bar{x}$.

$$s^2_T = s^2_Q = 0.0339\bar{x}^2. \quad (14)$$

The error associated with the quantification step (CV = 18.6% for total aflatoxin) probably comes from three sources: (1) differences in fluorescence among replicated spots on a single TLC plate (spot to spot error), (2) Differences in fluorescence among replicated spots on different TLC plates (spot to spot plus plate to plate error), and (3) errors in measuring the fluorescence of the spots (reading error). Future studies are required to partition these types of quantification error and to determine the magnitude of each type. The results of this study suggest that the best way to reduce the error associated with the FSQS procedure is to replicate the quantification step on different TLC plates and to average the results.

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