FOOD CHEMICAL CONTAMINANTS

Sampling Hazelnuts for Aflatoxin: Uncertainty Associated with Sampling, Sample Preparation, and Analysis

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The variability associated with the aflatoxin test procedure used to estimate aflatoxin levels in bulk shipments of hazelnuts was investigated. Sixteen 10 kg samples of shelled hazelnuts were taken from each of 20 lots that were suspected of aflatoxin contamination. The total variance associated with testing shelled hazelnuts was estimated and partitioned into sampling, sample preparation, and analytical variance components. Each variance component increased as aflatoxin concentration (either B1 or total) increased. With the use of regression analysis, mathematical expressions were developed to model the relationship between aflatoxin concentration and the total, sampling, sample preparation, and analytical variances. The expressions for these relationships were used to estimate the variance for any sample size, subsample size, and number of analyses for a specific aflatoxin concentration. The sampling, sample preparation, and analytical variances associated with estimating aflatoxin in a hazelnut lot at a total aflatoxin level of 10 ng/g and using a 10 kg sample, a 50 g subsample, dry comminution with a Robot Coupe mill, and a high-performance liquid chromatographic analytical method are 174.40, 0.74, and 0.27, respectively. The sampling, sample preparation, and analytical steps of the aflatoxin test procedure accounted for 99.4, 0.4, and 0.2% of the total variability, respectively.

The global production of hazelnuts is increasing by >10% annually. Globally, hazelnut producers are Turkey (73% market share), followed by Italy (14%), Spain (3%), the United States (4%), and all others (6%; percentages are averages of world hazelnut production for the last 7 years; 1). A major problem associated with hazelnut production is the growth of aflatoxin-producing molds. Aflatoxin is toxic and carcinogenic, and has subacute and chronic effects such as primary liver cancer, chronic hepatitis, jaundice, hepatomegaly, and cirrhosis (2, 3). Because aflatoxin is a serious global problem, studies on prevention, detection, and improvement of analytical test methods continue.

Regulatory limits for aflatoxin have been established in about 100 countries (4). In the United States, a maximum guidance limit for total aflatoxins of 20 ng/g for foods intended for human consumption has been set by the U.S. Food and Drug Administration (4), whereas the limits set by the European Commission for consumer-ready treenuts are 4 ng/g for total aflatoxins and 2 ng/g for aflatoxin B1 (5). Sampling and analysis procedures for detection of aflatoxin are therefore very important in relation to rejection of shipments (6). Because variable levels of aflatoxin may be present on a very small portion of nuts in a lot, sampling presents a serious problem (7–9).

An aflatoxin-sampling plan consists of an aflatoxin test procedure and an accept/reject limit. The aflatoxin test procedure generally consists of 3 steps: sampling, sample preparation, and analytical quantification. The accept/reject limit is a threshold value, which may or may not equal the regulatory limit, which is used to classify lots into acceptable and unacceptable categories. Aflatoxin sampling plan designs can vary tremendously, depending on the objectives of an industry or a regulatory agency. Sample size is usually the key issue when an aflatoxin-sampling plan is designed. Because of the random errors (variability) associated with each step of the aflatoxin test procedure, the true aflatoxin concentration of a bulk lot cannot be determined with 100% confidence by measuring aflatoxin in a sample taken from the lot (Figure 1). Because of the variability associated with the aflatoxin test procedure, some lots will be misclassified by the sampling plan. Some good lots will test as bad and be rejected by the sampling plan. The chance of rejecting a good lot is

Received January 3, 2006. Accepted by AP February 6, 2006.

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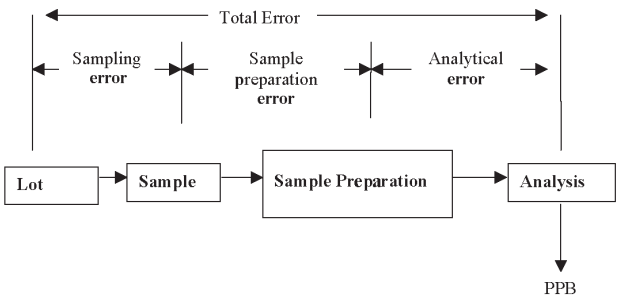

Figure 1. Errors associated with each step of the aflatoxin test procedure to estimate aflatoxin in a hazelnut lot.

sometimes called a false-positive or seller's risk. Also, some bad lots will test as good and be accepted by the sampling plan. The chance of accepting a bad lot is sometimes called a false-negative or buyer's risk (10).

Studies by researchers on a wide variety of agricultural products (peanut, cottonseed, shelled corn, wheat, barley, and pistachio nuts) indicate that the sampling error is generally the largest source of error (variability). The aflatoxin distribution among individual kernels is extremely skewed. A very small percentage of the kernels in the lot are contaminated, and the concentration on a single kernel may be extremely high (11, 12). Because of this extreme mycotoxin distribution among individual kernels in a contaminated lot, it is easy to miss the contaminated kernel with a small sample and underestimate the true concentration in the lot. On the other hand, if the test sample contains one or more highly contaminated kernels, then the test sample will overestimate the true mycotoxin contamination in the lot. Even with the use of proper sample selection techniques, the variation among test sample concentrations is largely due to the mycotoxin distribution among individual kernels (13).

It is assumed that the variability associated with measuring aflatoxin in hazelnuts is similar to that observed in other commodities (Figure 1). It is important to know the magnitude of the variabilty associated with each step of the aflatoxin test procedure so that resources can be used efficiently to reduce variability and reduce the number of lots misclassified by the sampling plan. The objectives of the study were to measure the total variability associated with testing samples of shelled hazelnuts for aflatoxin, which will provide a base for statistically measuring the effectiveness of sampling plans; partition the total variance associated with the aflatoxin test procedure into sampling, sample preparation, and analytical variance components; and determine and compare the variability associated with dry-grind and water-slurry sample preparation procedures.

Experimental

Theoretical Considerations

An aflatoxin test procedure used to estimate the true aflatoxin concentration of a bulk lot of in-shell hazelnuts consists of sampling, sample preparation, and analytical steps (Figure 1). The sampling step consists of selecting ≥1 sample of a given size from the bulk lot. The sample preparation step is a 2-step process in which the sample is ground in a suitable mill to reduce the particle size and a subsample is taken from the comminuted sample. The analytical step quantifies the aflatoxin in the comminuted subsample. It is assumed that the total uncertainty associated with the aflatoxin test procedure is the sum of the uncertainty associated with sampling, sample preparation, and analytical steps.

It is further assumed that (*1*) each in-shell hazelnut lot consists of a shell and an individual hazelnut kernel, (*2*) no aflatoxin contamination is associated with the shell, (3*)* all shelled kernels have about the same mass and physical characteristics, and (*4*) aflatoxin concentration varies from kernel to kernel. For shelled hazelnut kernels, the aflatoxin concentration of a sample of *n* kernels, represented by $\hat{C}$, was measured. No measurements were made of the aflatoxin concentration on individual kernels, $\hat{C}_i$. The true aflatoxin concentration in a hazelnut lot, μ, is estimated by measuring the aflatoxin concentration in a sample of individual hazelnuts denoted as $\hat{C}$. A statistical model describing the variability among aflatoxin test results $\hat{C}$ taken from the same lot can be represented by Equation 1:

$$\hat{C} = \mu + s + sp + a \qquad (1)$$

where μ = the true aflatoxin concentration in the lot being tested, *s* = random deviations of sample concentrations about the true lot concentration with the expected value equal to zero and variance $\sigma^2_{\hat{C}(s)}$, *sp* = random deviations of subsample concentrations about the comminuted sample concentration with the expected value equal to zero and variance $\sigma^2_{\hat{C}(sp)}$, and *a* = random deviations of analytical assay results about the subsample concentration with the expected value zero and variance $\sigma^2_{\hat{C}(a)}$. If independence among the random deviations in Equation 1 is assumed, the model for variance can be obtained by Equation 2:

$$\sigma^2_{\hat{C}(t)} = \sigma^2_{\hat{C}(s)} + \sigma^2_{\hat{C}(sp)} + \sigma^2_{\hat{C}(a)} \qquad (2)$$

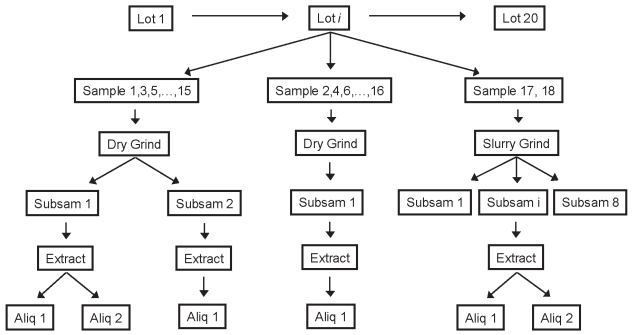

Figure 2. Unbalanced nested experimental design to measure sampling, sample preparation, and analytical variances associated with testing hazelnuts for aflatoxin.

Table 1. Sample, sample preparation, and analytical variances associated with measuring total aflatoxins in a 10 kg shelled hazelnut sample by using dry-grind sample preparation, a 50 g subsample, and HPLC

Lot[a]	Total aflatoxins, ng/g	Total variance	Sample variance	Sample prep. variance	Analytical variance
6	0.10	0.1047	0.1046	—[b]	—
4	0.14	0.2282	0.2281	—	—
7	0.25	0.3575	0.3573	0.0002	—
5	0.33	0.5646	0.5583	0.0050	0.0012
15	0.36	0.5034	0.4786	0.0246	0.0002
3	0.40	6.1732	6.1731	—	0.0001
10	0.48	2.1194	1.6522	0.4650	0.0022
2	0.48	0.7461	0.7357	0.0084	0.0019
16	1.10	2.0369	2.0350	0.0015	0.0004
14	1.72	6.5313	6.5286	—	0.0027
9	1.73	41.3789	41.2874	0.0837	0.0078
1	2.50	2.1301	1.9970	0.1275	0.0057
17	4.08	81.6585	81.1506	0.3847	0.1232
11	6.36	145.7921	144.7255	0.8864	0.1802
8	14.28	1581.3246	1579.7583	0.0830	1.4833
12	15.15	267.2035	266.1596	0.8200	0.2239
21	182.00	2950.1898	2468.3137	—	481.8761

[a] Only lots with measurable levels of aflatoxin are shown.
[b] — = Variance estimate was not measurable.

where $\sigma^2_{\hat{C}(t)}$ is the total variance associated with the measured aflatoxin concentration $\hat{C}$. Total variance $\sigma^2_{\hat{C}(t)}$ is the sum of sampling, sample preparation, and analytical variances and depends on sample size, type of mill used to grind the sample (affects particle size), subsample size, number of aliquots, and type of analytical procedure.

The sampling variance, $\sigma^2_{\hat{C}(s)}$, represents the variability among replicate test sample concentrations taken from the same lot of hazelnuts. Sample preparation variance, $\sigma^2_{\hat{C}(sp)}$, represents the variability among replicate subsample concentrations taken from the same sample comminuted in a suitable mill. The analytical variance, $\sigma^2_{\hat{C}(a)}$, represents the variability among replicate aliquot concentrations when the extract is taken from a single subsample. The variance components were estimated experimentally for both B1 and total aflatoxins and are represented by s^2_s, s^2_{sp}, and s^2_a.

Experimental Design

Based on the theoretical considerations described above, a nested experimental design was developed in which multiple samples would be taken from each lot, multiple subsamples would be taken from each comminuted sample, and aflatoxin would be measured in multiple aliquots taken from each subsample/solvent blend. The nested design for each lot provided estimates of the sampling, sample preparation, and analytical variances for both B1 and total aflatoxins for each lot. A modification of an unbalanced experimental design used by Vargas et al. (14) to measure uncertainty associated with detecting ochratoxin A in green coffee was used in this study to reduce analytical costs while providing the necessary degree of freedom for an adequate estimate of variance components (Figure 2).

Sample Selection

Twenty hazelnut lots, suspected of being contaminated with aflatoxin, were collected from Turkey (17 lots), Italy (2 lots), and Oregon (1 lot). Incremental portions were removed from each lot as the lot was moved from one holding bin to another. The increments were collected and pooled together as a composite or bulk sample of about 180 kg after shelling. The composite sample was thoroughly blended and divided into eighteen 10 kg test samples. Sixteen of the 10 kg samples were used to measure the sampling, dry-grind sample preparation, and analytical variances for each lot. The remaining two 10 kg samples per lot were used to measure the water-slurry sample preparation variance and analytical variance for each sample. All test samples were kept in refrigerated storage until time for the sample preparation procedure.

Table 2. Sample, sample preparation, and analytical variances associated with measuring aflatoxin B1 in a 10 kg shelled hazelnut sample by using dry-grind sample preparation, a 50 g subsample, and HPLC

Lot[a]	Aflatoxin B1, ng/g	Total variance	Sample variance	Sample prep. variance	Analytical variance
4	0.02	0.0062	0.0062	—[b]	—
6	0.08	0.0806	0.0806	—	—
3	0.09	0.1990	0.1990	—	—
7	0.17	0.2032	0.2029	0.0002	—
10	0.20	0.6693	0.6479	0.0213	0.0002
2	0.20	0.3110	0.3063	0.0047	—
5	0.23	0.3853	0.3130	—	—
15	0.24	0.1757	0.1757	—	—
1	0.74	0.1875	0.1779	0.0082	0.0013
16	0.82	1.3966	1.3951	0.0013	0.0002
14	0.97	3.1705	3.1686	—	0.0019
9	1.06	17.3775	17.3292	0.0455	0.0027
17	3.24	64.2105	63.8058	0.3048	0.0999
11	4.34	106.1891	105.4326	0.6080	0.1485
8	9.01	1265.8758	1265.6971	0.0066	0.1720
12	11.46	163.0188	162.4702	0.4495	0.0992
21	82.15	1478.7802	1400.9631	0.0000	77.8171

[a] Only lots with measurable levels of aflatoxin are shown.
[b] — = Variance estimate was not measurable.

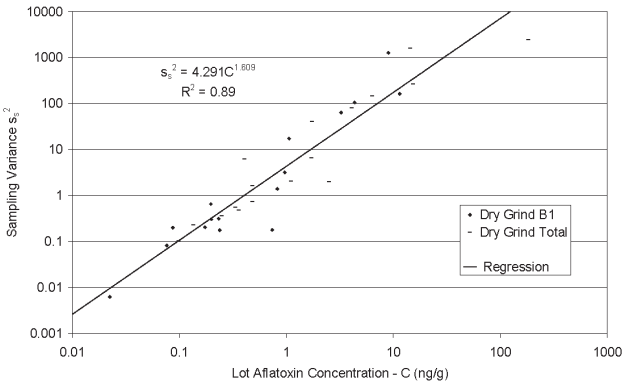

Figure 3. Full-log plot of sampling variance versus aflatoxin concentration. Sampling variance reflects a 10 kg sample size or 10 000 shelled kernels.

Sample Preparation, Dry Grind

Each of the sixteen 10 kg test samples was comminuted with a Robot Coupe vertical cutter (R-60) grinder that produced a paste. Either 1 or 2 comminuted subsamples of 50 g were taken from each 10 kg test sample. For each lot, two 50 g comminuted subsamples were removed from the odd-numbered test samples (1, 3, 5, 7, 9, 11, 13, and 15). Each subsample was blended with a solvent, 2 aliquots were taken from 1 subsample blend, and 1 aliquot was taken from the second subsample blend. A total of 3 aliquots were taken (2 + 1) from each odd-numbered sample (Figure 2). For each lot, one 50 g comminuted subsample was removed from the even-numbered test samples (2, 4, 6, 8, 10, 12, 14, and 16). One aliquot was removed and quantified from the single subsample taken from the even-numbered samples. A total of 32 aliquots or 32 analyses were performed per each lot (compared with a total of 64 analyses for a balanced nested design). For all 20 lots, a total of 20 × 32 or 640 aliquots were analyzed for B1 and total aflatoxins. From the unbalanced nested design, a single estimate of sampling variance (s^2_s), dry-grind sample preparation variance (s^2_{spd}), and analytical variance (s^2_{ad}) was measured for both B1 and total aflatoxins for each lot.

Sample Preparation, Water Slurry

The remaining two 10 kg samples (samples 17 and 18) from each lot were used for the water-slurry sample preparation study. Each 10 kg sample was blended with water on a 1:1 basis. Eight 100 g water-slurry subsamples (50 mL water:50 g hazelnuts) were removed from each water-sample blend. Duplicate aliquots were removed from each 100 g slurry subsample/solvent blend and quantified for aflatoxin (Figure 2). A total of 16 analyses were performed on each water-slurry sample. The water-slurry sample preparation variance (s^2_{spw}) and analytical variance (s^2_{aw}) were determined for B1 and total aflatoxins for each of the 40 samples (20 lots × 2 samples per lot).

Analytical Method

For all of the 20 lots, a total of 1280 analyses (20 lots × 32 analyses/lot × 2 sample preparation methods) were performed. Aflatoxin was extracted from each 50 g dry-grind subsample and each 100 g water-slurry subsample by using 200 mL methanol–water (2 + 1; the methanol–water solvent ratio was adjusted for the water in the water-slurry subsample). To purify the extract (2 mL), it was passed through a Rhone Diagnostics Aflatoxin column. The aflatoxins were derivatized by a postcolumn derivatization process and quantified by high-performance liquid chromatography (HPLC) with fluorescence detection. Aflatoxin B1 and total aflatoxin concentrations were recorded. The method described above, AOAC Method **999.07**, was validated in TUBITAK Marmara Research Centre Food Institute Mycotoxin Laboratory and was accredited by Deutscher Akkreditierungs Rat (German Accreditation Body or DAR). For internal quality control, 1 quality control sample in each 20 analyses of 1280 samples was also analyzed and recorded in the quality control chart.

From the unbalanced nested experimental design (Figure 2), the sampling variance, dry-grind sample preparation variance, water-slurry sample preparation variance, analytical variance, and lot aflatoxin concentration $\hat{C}$ were determined for B1 and total aflatoxins for each of the 20 lots by using the NESTED procedure of the Statistical Analysis System Institute, Inc. (15).

Results and Discussion

Dry-Grind Test Procedure

The sampling, dry-grind sample preparation, and analytical variances and aflatoxin concentrations are shown in Tables 1 and 2 for total aflatoxins and aflatoxin B1, respectively, for each lot. Several of the lots had such low levels of aflatoxin that variance estimates were not measurable and are not shown in Tables 1 and 2. The sampling, dry-grind sample preparation, and analytical variances in Tables 1 and 2 are plotted versus aflatoxin

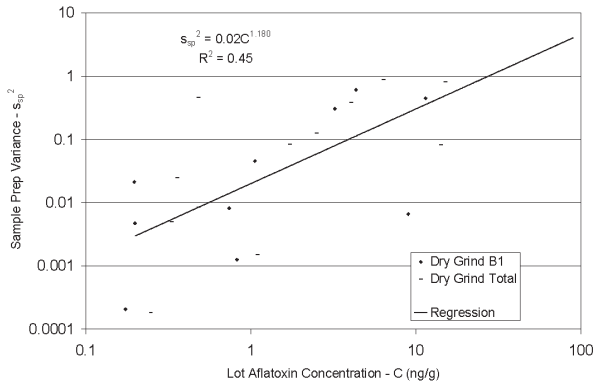

Figure 4. Full-log plot of sample preparation variance versus aflatoxin concentration. Sample preparation reflects a dry-grinding process and 50 g comminuted subsample for extraction.

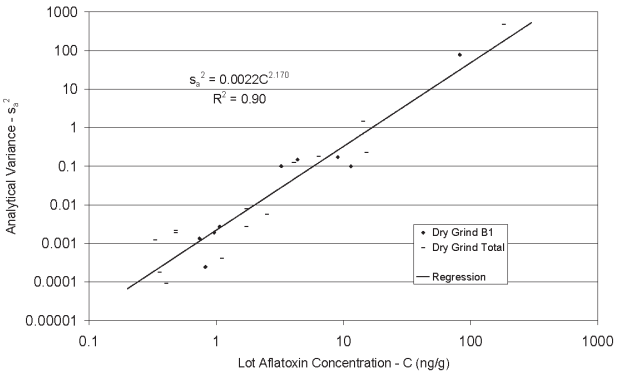

Figure 5. Full-log plot of analytical variance versus aflatoxin concentration. Analytical variance reflects HPLC analytical methods.

concentration in Figures 3–5, respectively. The points in the figures are identified by type of aflatoxin, either total or B1. There appears to be no difference in variance estimates for B1 or total aflatoxins. Each variance component increases with an increase in aflatoxin concentration. Because the plots for each variance are approximately linear in a full-log plot (or log-log plot), the relationship between variance and aflatoxin concentration is described by a power function in Equation 3:

$$s^2 = a\hat{C}^b \quad (3)$$

where a and b are constants determined by the regression analysis. From a linear regression performed on the log values (both B1 and total aflatoxins), regression equations describing the sampling variance (s^2_s), dry-grind sample preparation variance (s^2_{spd}), and analytical variance (s^2_{ad}) are given below in Equations 4–6, respectively. Each regression equation is shown on each plot along with the observed variances in Figures 3–5:

$$s^2_s = 4.291\hat{C}^{1.609} \quad (4)$$

$$s^2_{spd} = 0.020\hat{C}^{1.180} \quad (5)$$

$$s^2_{ad} = 0.0022\hat{C}^{2.170} \quad (6)$$

where $\hat{C}$ is aflatoxin concentration (either B1 or total) in ng/g or parts per billion (ppb). As shown in Equation 2, the total variance (s^2_t) is the sum of Equations 4–6:

$$s^2_t = 4.291\hat{C}^{1.609} + 0.020\hat{C}^{1.180} + 0.0022\hat{C}^{2.170} \quad (7)$$

Equation 7 is unique for the aflatoxin test procedure used in the experiment (10 kg sample of shelled hazelnuts, dry grind with a Robot Coupe R-60 mill, 50 g comminuted subsample, and aflatoxin quantified in 1 aliquot by HPLC analytical methods).

Water-Slurry Test Procedure

The water-slurry sample preparation variance (s^2_{spw}), analytical variance (s^2_{aw}), and aflatoxin concentration ($\hat{C}$) are shown for each sample in Tables 3 and 4 for total aflatoxins and aflatoxin B1, respectively, for each sample. Several of the samples had such low levels of aflatoxin that variance estimates were not measurable and are not shown in Tables 3 and 4. The water-slurry sample preparation variance (s^2_{spw}) and analytical variance (s^2_{aw}) in Tables 3 and 4 are plotted versus aflatoxin concentration in Figures 6 and 7, respectively. The points in the figures are identified by type of aflatoxin, either B1 or total aflatoxins. There appears to be no difference in variance estimates for B1 or total aflatoxins. Each variance increases with an increase in aflatoxin concentration. Because the plots are approximately linear in a full log plot (or log-log plot), a linear regression was run on the log values (both total aflatoxins and aflatoxin B1) by using Equation 3. The regression equations are shown in each plot along with the measured variances:

$$s^2_{spw} = 0.0202\hat{C}^{1.671} \quad (8)$$

Table 3. Sample preparation and analytical variances associated with measuring total aflatoxins in a 10 kg sample of shelled hazelnuts by using a water-slurry grinding process and HPLC

Lot[a]	Total aflatoxin, ng/g	Sample prep. variance	Analytical variance	Total variance
9	0.06	0.0099	—[b]	0.0099
14	0.23	0.0002	0.0002	0.0005
17	0.35	—	0.0015	0.0015
11	0.44	0.0007	0.0012	0.0019
2	0.50	0.0295	0.0007	0.0302
14	0.54	0.0007	0.0006	0.0013
17	0.94	0.0009	0.0017	0.0026
4	1.04	0.1159	0.0247	0.1406
7	2.06	0.0152	0.0012	0.0164
6	4.15	0.5748	0.0192	0.5940
3	4.22	0.9234	0.8894	1.8128
16	7.16	3.6191	0.0813	3.7004
8	13.31	0.4209	0.0750	0.4959
8	14.44	3.3019	0.1656	3.4675
11	16.79	6.5833	0.3149	6.8982
5	27.38	4.0781	11.8838	15.9620
7	30.93	4.3458	1.4353	5.7811
21	129.33	60.7856	161.0003	221.7859
21	146.37	96.2084	179.9911	276.1994

[a] Two samples were analyzed for aflatoxin per lot. Samples from lots with no measurable level of aflatoxin are shown.

[b] — = Variance estimate was not measurable.

Table 4. Sample preparation and analytical variances associated with measuring aflatoxin B1 in a 10 kg sample of shelled hazelnuts by using a water-slurry grinding process and HPLC

Lot[a]	Aflatoxin B1, ng/g	Sample prep. variance	Analytical variance	Total variance
4	0.11	0.0020	0.0002	0.0022
17	0.24	0.0007	0.0003	0.0010
11	0.29	0.0003	0.0004	0.0007
17	0.31	—[b]	0.0002	0.0002
14	0.43	0.0052	0.0005	0.0058
2	0.48	0.0260	0.0006	0.0265
3	1.60	0.1489	0.1234	0.2722
7	1.63	0.0091	0.0009	0.0101
6	3.48	0.4306	0.0133	0.4438
8	3.96	0.0386	0.0092	0.0479
8	4.09	0.3375	0.0156	0.3531
5	6.45	0.0919	0.7963	0.8882
16	6.62	3.1656	0.0682	3.2338
11	6.73	0.9309	0.0573	0.9882
7	24.22	2.6608	0.9054	3.5662
21	52.16	7.0431	12.2655	19.3086
21	61.42	35.0883	18.6572	53.7455

[a] Two samples were analyzed for aflatoxin per lot. Samples from lots with no measurable level of aflatoxin are shown.

[b] — = Variance estimate was not measurable.

$$s^2_{aw} = 0.0032\hat{C}^{1.915} \quad (9)$$

The mechanical grinding process for the water-slurry sample preparation procedure and the dry-grind sample preparation procedure are very different. However, when the sample preparation variances for both the dry-grind and water-slurry procedures are identified and plotted together in Figure 8, it appears that there is no significant difference in the variances associated with the 2 sample preparation methods. Sample preparation variance is affected by particle size and subsample size. The similarity among variance measurements implies that the sizes of the comminuted sample particles from the 2 grinding methods are similar because the subsample mass of hazelnuts (50 g) was the same for the dry-grind and the slurry preparations. As a result, a regression analysis was run on the combined dry-grind and water-slurry sample preparation variances. The resulting equation is:

$$s^2_{sp} = 0.021\hat{C}^{1.545} \quad (10)$$

Equation 10 is plotted in Figure 8 along with the measured variances.

Because the same analytical procedure was used to quantify aflatoxin in both the dry-grind and water-slurry subsamples, analytical variances from both studies should be about the same. The analytical variances from the dry-grind and water-slurry sample preparation measurements are identified and plotted together in Figure 9. As one would expect, there appears to be no differences in the analytical variances computed from the 2 experiments. As a result, regression analysis was run on the combined analytical variances from the dry-grind and water-slurry sample preparation studies. The resulting equation is:

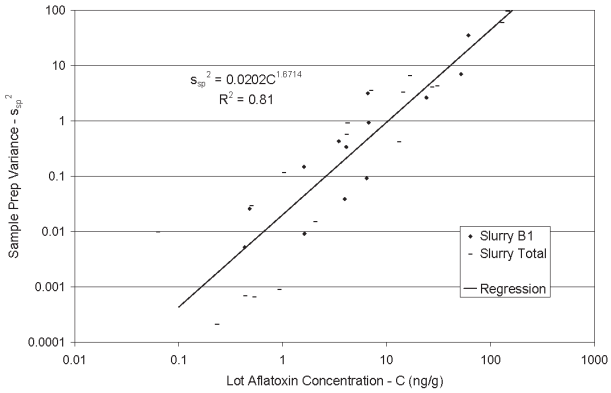

Figure 6. Full-log plot of water-slurry sample preparation variance versus aflatoxin concentration. Slurry subsample consisted of 50 mL water and 50 g comminuted hazelnuts.

$$s^2_a = 0.0028\hat{C}^{1.990} \quad (11)$$

Equation 11 is plotted in Figure 9 along with the observed analytical variances.

Equation 10 gives a better estimate of sample preparation variance than either Equation 5 or 8, and Equation 11 gives a better estimate of analytical variance than either Equation 6 or 9. Equation 7 can be improved upon by using Equations 9 and 11 in place of Equations 5 and 6:

$$s^2_t = 4.291\hat{C}^{1.609} + 0.021\hat{C}^{1.545} + 0.0028\hat{C}^{1.990} \quad (12)$$

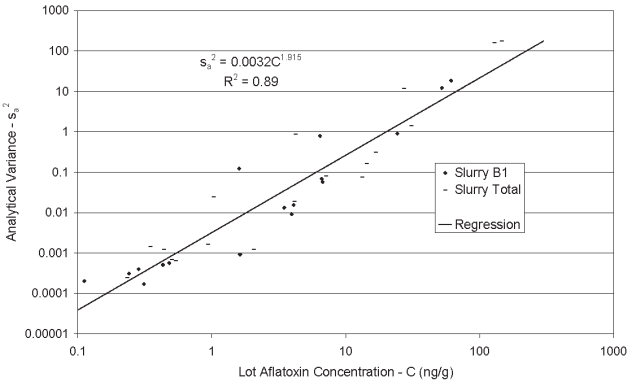

Figure 7. Full-log plot of analytical variance versus aflatoxin concentration. Analytical variance reflects HPLC analytical methods used to quantify aflatoxin in a water-slurry subsample.

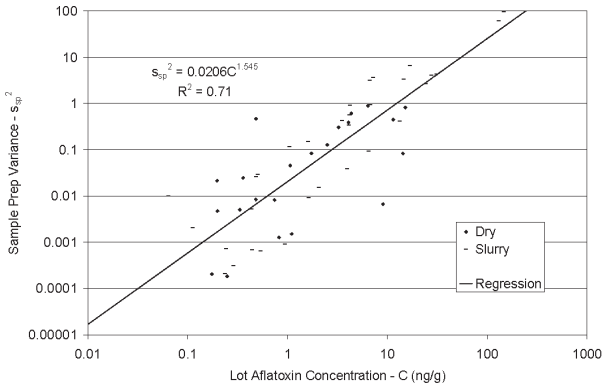

Figure 8. Full-log plot of sample preparation variance versus aflatoxin concentration for both dry and slurry sample preparation methods and 50 g hazelnuts. Regression analysis was run on a combined database.

The variances associated with the test procedure used in this study to detect aflatoxin in a lot with concentration, $\hat{C}$ (either total or B1), can be calculated from Equation 12. The sampling, sample preparation, and analytical variances are shown in Table 5 for a total aflatoxins concentration of 10 ng/g. Variances associated with other aflatoxin concentrations can be determined by using Equation 12. Table 5 shows how much each step of the aflatoxin test procedure used in this study contributes to the total variability. As Table 5 shows, sampling contributes >99% of the total error. The sample preparation method (either dry or slurry) and analytical methods combined contribute <1% of the total error.

Equation 12 provides an estimate of the variability of the aflatoxin test procedure that is specific to a 10 kg sample; either dry-grind with a Robot Coupe or use a water-slurry blend, take a 50 g subsample, and measure aflatoxin in 1 aliquot by HPLC. Statistical theory states that variance is inversely proportional to sample size. For example, if sample size is doubled from 10 to 20 kg, the sampling variance is cut in half (10/20 = 0.5). Equation 4 can be modified to calculate the variance associated with any size sample when the variance associated with a 10 kg sample is known:

$$s^2_s = (10/ns)\ 4.291\hat{C}^{1.609} \qquad (13)$$

where ns is sample size in kg. As Equation 13 indicates, if sample size is doubled, ns, from 10 to 20 kg, the sampling variance is cut in half.

The sample-size rule also applies to the sample preparation and analytical variances. For a given grinding process, the sample preparation variance decreases as the subsample size, nss, increases. Equation 10 can be modified to reflect the variance associated with subsample sizes other than 50 g:

$$s^2_{sp} = (50/nss)\ 0.021\hat{C}^{1.545} \qquad (14)$$

For a given analytical process (HPLC in this study), the analytical variance decreases as the number of aliquots, na, quantified for aflatoxin increases. Equation 11 can be modified to reflect the variance associated with averaging aflatoxin results for >1 aliquot:

$$s^2_a = (1/na)\ 0.0028\hat{C}^{1.990} \qquad (15)$$

The total variance associated with an aflatoxin test procedure that uses a sample of size ns, a Robot Coupe R-60 grinder to dry-grind the sample, a subsample of size nss, HPLC, and any number of aliquots, na, is the sum of Equations 13–15:

$$s^2_t = (10/ns)\ 4.291\hat{C}^{1.609} + (50/nss)\ 0.021\hat{C}^{1.545} + (1/na)\ 0.0028\hat{C}^{1.990} \qquad (16)$$

Reducing Uncertainty

The total uncertainty associated with an aflatoxin test procedure can be reduced by reducing the variability associated with the sampling, sample preparation, and/or analytical steps of the test procedure. As shown in Equation 16, sampling variance can be reduced by increasing sample size, ns; sample preparation variance (for a given type of grinder) can be reduced by increasing subsample size, nss; and analytical variance (for a given type analytical method) can be reduced by increasing the number of aliquots quantified. The total variance for the test procedure used in this study (ns = 10 kg, nss = 50 g, and na = 1) is 175.42 (Table 5). Because the sampling step contributes >99% of the total error, the most efficient way (best use of resources) to reduce the variability of the test procedure used in this study would be to increase sample size. If the sample size, ns, in Equation 16 is increased to 30 kg, the sampling variance is reduced to 58.1, and the total variance in Equation 16 for ns = 30, nss = 50, and na = 1 is reduced to 59.1.

The variances in Table 5 can be used to predict the range among replicated sample test results one would expect when taking 10 kg samples from a lot with a true aflatoxin concentration of 10 ng/g and using the same sample

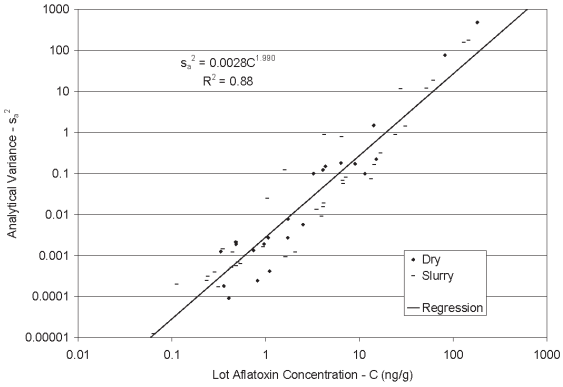

Figure 9. Full-log plots of analytical variance versus aflatoxin concentration for both dry and slurry preparation methods.

Table 5. Total, sampling, sample preparation, and analytical variances associated with estimation of aflatoxin in a hazelnut lot at a total aflatoxins concentration of 10 ng/g by using a 10 kg shelled sample, a 50 g subsample, and HPLC

Test procedure	Variance	Ratio, %
Sample, 10 kg	174.40	99.42
Sample prep., 50 g	0.74	0.42
Analysis, HPLC, 1 aliquot	0.27	0.16
Total	175.42	100.00

preparation and analytical methods of this study. Assuming a normal distribution among replicated sample test results, 95% of all sample test results would vary about the true lot concentration of 10 ng/g by 1.96 times the square root of the variance. Range is equal to $10 \pm (1.96 \times (175.4)^{0.5})$ or 10 ± 26.0 or from a low of 0.0 to 36.0 ng/g. If sample size is increased from 10 to 30 kg, the total variance is reduced from 175.4 to 59.1. The range among 30 kg samples taken from a lot with a true concentration of 10 ng/g is $10 \pm (1.96 \times (59.1)^{0.5})$ or 10 ± 15.1 or from a low of 0.0 to 25.1. Reducing the variability of the aflatoxin test procedure reduces the range of sample test results and will reduce the number of lots misclassified by an aflatoxin sampling plan to detect aflatoxin in hazelnuts.

The estimate of the range among sample test results from the variance is based upon the assumption that the aflatoxin distribution among sample test results is normal. However, past studies with aflatoxin and other mycotoxins have shown that the distribution is positively skewed. The next phase of the statistical analysis is to compare several theoretical distributions with the observed aflatoxin distribution among the 16 sample test results for each lot. The variability and distribution among sample test results will be used to calculate the performance (operating characteristic curves) associated with aflatoxin sampling plan designs for hazelnuts.

Summary

Estimates of the total variance associated with testing 20 hazelnut lots for aflatoxin were shown to increase as aflatoxin concentration increased. This also held true for the uncertainty associated with each step of the test procedure: sampling, sample preparation, and analytical variances. By using regression analysis, mathematical expressions were developed to model all 3 variance components as a function of aflatoxin concentration. The mathematical relationships between variance and aflatoxin concentration were the same for B1 and total aflatoxins. The expressions were used to estimate the variances for any sample size, subsample size, number of analyses, and specific aflatoxin concentration. Total, sampling, sample preparation, and analytical variances associated with estimating aflatoxin in a hazelnut lot at a total aflatoxin concentration of 10 ng/g and using a 10 kg sample, 50 g subsample, and an HPLC analytical method are 175.41, 174.40, 0.74, and 0.27, respectively. The sampling, sample preparation, and analytical steps of the aflatoxin test procedure accounted for 99.4, 0.4, and 0.2% of the total variability, respectively. Because sampling contributes >99% of the total variability, the best use of resources to reduce variability would be to increase sample size. The dry-grind and water-slurry sample preparation variances were similar, indicating that the dry-grinding method produces a particle size similar to that produced by the water-slurry procedure.

Acknowledgments

We thank technicians of TUBITAK Marmara Research Center for their help in the tests, and the International Treenut Council and Hazelnut Promotion Group for its financial support for implementation of the present study.

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