Testing Green Coffee for Ochratoxin A, Part I: Estimation of Variance Components

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The variability associated with testing lots of green coffee beans for ochratoxin A (OTA) was investigated. Twenty-five lots of green coffee were tested for OTA contamination. The total variance associated with testing green coffee was estimated and partitioned into sampling, sample preparation, and analytical variances. All variances increased with an increase in OTA concentration. Using regression analysis, mathematical expressions were developed to model the relationship between OTA 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 OTA concentration. Testing a lot with 5 µg/kg OTA using a 1 kg sample, Romer RAS mill, 25 g subsamples, and liquid chromatography analysis, the total, sampling, sample preparation, and analytical variances were 10.75 (coefficient of variation [CV] = 65.6%), 7.80 (CV = 55.8%), 2.84 (CV = 33.7%), and 0.11 (CV = 6.6%), respectively. The total variance for sampling, sample preparation, and analytical were 73, 26, and 1%, respectively.

Ochratoxin A (OTA) is a naturally occurring mycotoxin that has been shown to be nephrotoxic in mammalian species, exerting cytotoxic and carcinogenic effects and classified as a substance of the group 2B. OTA has been extensively studied in coffee since the first report of Levi et al. in 1974 (1). Cereals and their derivatives have been reported as the major contributors for the ingestion of OTA. Other sources of OTA in the diet are beer, wine, grape juices, tea, cocoa, pork, poultry, dried fruits, pulses, and roasted coffee (2). Aspergillus ochraceus (Aspergillus subgenus Circundati section Circundati) is the main producer of OTA contamination in green coffee (1–3) especially during drying and storage under favorable growth conditions.

The Joint Expert Committee on Food Additives (JECFA) has established the Provisional Tolerable Weekly Intake (PTWI) of 100 ng/kg body weight based on the lower amount of toxin that causes deterioration of renal functional of pig kidney (2). Regulation and guidelines on OTA in food have been established in many countries, with levels ranging from 2 to 50 ng/g, and a few countries such as Greece and Uruguay have specific regulation for coffee (4). It is envisaged that the European Community Commission will, in the future, establish regulations for the presence of OTA in green and roasted coffee, along with sampling plans that include analytical method performance criteria, as a modification of EC Directives Nos. 2002/472/CE (5) and 2002/26/CE (6). The establishment of OTA in coffee by importers requires that coffee-producing countries have a scientific-based sampling plan.
Although numerous sampling plans for aflatoxins (7–15), deoxynivalenol (16), fumonisins (17), and OTA have been published (5, 6), none of them was designed for coffee. Usually, 100% of the coffee bags (ca 60 kg) in the lot marketed are sampled by taking small incremental portions (ca 10 g). The increments are combined into a composite sample that is homogenized and usually divided into 3 test samples on which different types of analysis, such as physical quality (grading and defects), and sensorial (test cup), are conducted.

An OTA sampling plan is defined by an accept/reject limit and an OTA test procedure. The accept/reject limit is a threshold concentration that separates good lots from bad lots and is usually equal to a legal or maximum limit established by a regulatory agency. The test procedure consists of 3 steps: sampling, sample preparation, and analysis. Because of the uncertainty associated with each step of the OTA test procedure, the true OTA concentration in a bulk lot cannot be determined with 100% certainty. As a result, some good lots will be classified as bad by the sampling plan (exporter’s risk or false positives) and some bad lots will be classified as good by the sampling plan (importer’s risk or false negatives). These 2 risks can be reduced if the uncertainty or variability of the OTA test procedure can be reduced. Precise estimates of the true OTA concentration in a lot are difficult because of the usually skewed distribution among contaminated coffee beans in a lot (18). Sample and subsample size (19, 20), particle size, and type of mill also influence the uncertainty associated with measuring the true level of OTA contamination in a green coffee (21, 22). Sampling plan designs are usually a compromise of these aspects (23).

This study aimed to determine the total variability associated with testing samples of green coffee for OTA contamination; partition the total variability into sampling, sample preparation, and analytical variance components; investigate how much each step of the testing procedure contributes for the total testing variability; and use cost/benefits to make effective recommendations to best reduce the variability and achieve more precise estimates of the true OTA concentration in a lot.

![Figure 1](image-url)

**Figure 1.** Total variance partitioned into sample, sample preparation, and analytical components.

### Experimental

Twenty-five lots of green coffee contaminated with OTA were procured and identified for the study in Brazil. These 25 lots of *Arabica* coffee, “Bica corrida,” usually type 7 (ca 160 defects), were chosen because they had a wide range of OTA contamination and represent a regular type coffee.

### Theoretical Considerations

It was assumed that each lot consists of individual coffee beans, each coffee bean has the same mass and physical characteristics, and variation of OTA concentration occurs between beans. With coffee beans, the OTA concentration of a sample of $n$ beans, represented by $C$, was measured, not the OTA on individual beans, $C_i$. In peanuts, Cucullu et al. (24) showed that most individual peanuts have an aflatoxin concentration of 0, but occasionally a peanut may have an extremely high aflatoxin concentration. It was assumed that OTA in coffee beans behaves in the same manner.

Figure 1 shows the relationships between the 3 major components of the total variation associated with testing coffee beans for OTA: sampling, sample preparation, and analysis.

A lot of coffee beans with an OTA concentration $C$ is estimated by using a sample of individual coffee beans denoted as $C$. A statistical model for the variability among OTA test results $C$ taken from the same lot can be represented by Equation 1:

$$\hat{C} = \mu + S + SS + A \tag{1}$$

where $\mu$ = the true OTA concentration in the lot being tested, $S$ = random deviations of sample concentrations about the true lot concentration with expected value equal to 0 and variance $\sigma_{C_{(i)}}^2$, $SS = \text{random deviations of subsample concentrations about the comminuted sample concentration with expected value equal to 0 and variance } \sigma_{C_{(m)}}^2$, and $A = \text{random deviations of analytical assay results about subsample concentration with the expected value 0 and variance } \sigma_{C_{(a)}}^2$. If independence among the random deviations in Equation 1 is assumed, the model for variance can be obtained by Equation 2:

$$\sigma_{C_{(o)}}^2 = \sigma_{C_{(i)}}^2 + \sigma_{C_{(m)}}^2 + \sigma_{C_{(a)}}^2 \tag{2}$$

where $\sigma_{C_{(o)}}^2$ is the total variance associated with the measured OTA concentration $\hat{C}$.

Total variance $\sigma_{C_{(o)}}^2$ is the sum of sampling, sample preparation, and analytical variance and depends on sample size, mill type, subsample size, number of aliquots, and analytical procedure.

The sampling variance, $\sigma_{C_{(i)}}^2$, represents the variability among replicate test samples taken from the same lot of coffee beans. Sample preparation variance $\sigma_{C_{(m)}}^2$ represents the variability among replicate subsamples taken from the same sample comminuted in a suitable mill. The analytical variance, $\sigma_{C_{(a)}}^2$, represents the variability among replicate aliquots of extract taken from a single subsample. The variance
components were estimated experimentally and are represented by \( S_{42}^2 \), \( S_{43}^2 \), and \( S_{45}^2 \).

**Design**

An unbalanced experimental design was used to keep experimental cost at a minimum while providing the necessary degree of freedom for an adequate variance estimation (Figure 2). The lots were sampled by using probing devices and collecting about 200 g per increment. The increments were collected, preferably from every 4th bag, and pooled together as a composite sample (Table 1).

The composite sample was thoroughly blended and divided in a sample divider type purchased from the U.S. Department of Agriculture to obtain 16 test samples, 1 kg each. The schematic division of a given composite sample and the subsampling were performed according to the flow chart in Figure 2. Composite samples >16 kg were homogenized and duly fractionated down to 16 kg. Each 1 kg test sample of coffee beans was packed, put into a plastic bag, sealed, and kept under −15°C until grinding.

Systematically, the 16 test samples, coded as shown in the flow chart in Figure 2, were analyzed for determination of OTA, with the process performed until the 25 lots were completed. In each lot, the odd test samples 1, 3, 5, 7, 9, 11, 13, and 15 had 2 subsamples (25 g each), which were extracted, and a total of 3 aliquots was taken (2 + 1). In each lot, the even test samples 2, 4, 6, 8, 10, 12, 14, and 16 each had 1 subsample (25 g) and 1 aliquot. A total of 32 aliquots or 32 OTA analyses was performed per each lot. For all the 25 lots, a total of 25 × 32, or 800 analyses were performed.

Each 1 kg test sample was comminuted through a Romer RAS type Mill (Marconi 920/CF) and a subsample of 25 g (<20 mesh) was taken from every test sample. OTA was extracted from each 25 g subsample with 200 mL methanol and 3% aqueous sodium hydrogen carbonate solution (50 + 50, v/v). A 4 mL aliquot of filtered extract was transferred to a volumetric flask and diluted to 100 mL with phosphate-buffered saline solution, and homogenized. The diluted extract was transferred and passed through an immunoaffinity column (OchraTest™ columns, Vicam, Watertown, MA), at a flow rate of 2–3 mL/min. The column was washed with 10 mL deionized water, and dried; OTA was eluted with 4 mL methanol at flow rate of 2–3 mL/min and quantified by liquid chromatography (LC) with fluorescence detector (25, 26).

Blank (<0.12 μg/kg), spiked materials (5 μg/kg), and naturally contaminated materials (13.46 ± 1.18 μg/kg; \( n = 30 \)) were used as controls during the analysis.

From the unbalanced design (Figure 2), the OTA lot concentration \( C \) was determined for each of the 25 lots using the Nested procedure in Statistical Analysis System Institute (27) and Excel software.

**Figure 2.** Flow chart of the experimental design.
Table 1. Collecting plan to assess OTA contamination in green coffee according to lot size, with incremental sample size of 200 g

<table>
<thead>
<tr>
<th>Size of lot</th>
<th>Minimum number of samples collected from each 4th bag with an increment sample of 200 g</th>
<th>Composite sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;320 bags</td>
<td>Over 80</td>
<td>If the composite sample is larger than 16 kg, homogenize the composite sample and fractionate down to obtain 16 kg</td>
</tr>
<tr>
<td>=320 bags</td>
<td>80</td>
<td>16 kg</td>
</tr>
<tr>
<td>&lt;320 bags</td>
<td>As many samples as necessary to complete 16 kg</td>
<td>16 kg</td>
</tr>
</tbody>
</table>

Table 2. Average OTA, sample, sample preparation, analytical, and total variance component for each of the 25 lots sorted by OTA concentration*

<table>
<thead>
<tr>
<th>Lot</th>
<th>Lot OTA concentration, µg/kg</th>
<th>Sample</th>
<th>Sample preparation</th>
<th>Analytical</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.636</td>
<td>1.605</td>
<td>0.164</td>
<td>0.006</td>
<td>1.775</td>
</tr>
<tr>
<td>1</td>
<td>1.748</td>
<td>10.534</td>
<td>1.878</td>
<td>0.013</td>
<td>12.424</td>
</tr>
<tr>
<td>4</td>
<td>2.625</td>
<td>2.078</td>
<td>0.548</td>
<td>0.151</td>
<td>2.776</td>
</tr>
<tr>
<td>3</td>
<td>4.077</td>
<td>6.716</td>
<td>1.342</td>
<td>0.038</td>
<td>8.096</td>
</tr>
<tr>
<td>9</td>
<td>5.460</td>
<td>b</td>
<td>3.806</td>
<td>0.054</td>
<td>3.859</td>
</tr>
<tr>
<td>11</td>
<td>5.721</td>
<td>17.323</td>
<td>7.364</td>
<td>0.265</td>
<td>24.953</td>
</tr>
<tr>
<td>8</td>
<td>6.209</td>
<td>2.274</td>
<td>6.880</td>
<td>0.383</td>
<td>9.537</td>
</tr>
<tr>
<td>5</td>
<td>6.229</td>
<td>2.308</td>
<td>6.263</td>
<td>0.093</td>
<td>8.664</td>
</tr>
<tr>
<td>10</td>
<td>6.707</td>
<td>6.703</td>
<td>2.141</td>
<td>0.009</td>
<td>8.854</td>
</tr>
<tr>
<td>19</td>
<td>7.887</td>
<td>15.845</td>
<td>2.113</td>
<td>0.404</td>
<td>18.362</td>
</tr>
<tr>
<td>14</td>
<td>9.171</td>
<td>4.137</td>
<td>5.749</td>
<td>0.477</td>
<td>10.363</td>
</tr>
<tr>
<td>7</td>
<td>9.848</td>
<td>9.094</td>
<td>4.566</td>
<td>0.300</td>
<td>13.960</td>
</tr>
<tr>
<td>12</td>
<td>12.324</td>
<td>27.714</td>
<td>7.989</td>
<td>0.285</td>
<td>35.998</td>
</tr>
<tr>
<td>6</td>
<td>13.626</td>
<td>15.293</td>
<td>11.172</td>
<td>0.311</td>
<td>26.777</td>
</tr>
<tr>
<td>18</td>
<td>13.805</td>
<td>57.401</td>
<td>8.720</td>
<td>1.469</td>
<td>67.589</td>
</tr>
<tr>
<td>20</td>
<td>17.368</td>
<td>28.727</td>
<td>14.786</td>
<td>1.381</td>
<td>44.895</td>
</tr>
<tr>
<td>13</td>
<td>22.970</td>
<td>b</td>
<td>81.483</td>
<td>0.599</td>
<td>82.081</td>
</tr>
<tr>
<td>23</td>
<td>23.682</td>
<td>111.570</td>
<td>9.696</td>
<td>2.205</td>
<td>123.471</td>
</tr>
<tr>
<td>15</td>
<td>23.974</td>
<td>27.219</td>
<td>13.253</td>
<td>3.537</td>
<td>44.009</td>
</tr>
<tr>
<td>17</td>
<td>24.110</td>
<td>103.170</td>
<td>30.806</td>
<td>1.894</td>
<td>135.670</td>
</tr>
<tr>
<td>16</td>
<td>24.112</td>
<td>b</td>
<td>24.613</td>
<td>3.200</td>
<td>27.813</td>
</tr>
<tr>
<td>25</td>
<td>29.939</td>
<td>179.800</td>
<td>63.134</td>
<td>3.287</td>
<td>246.221</td>
</tr>
<tr>
<td>21</td>
<td>31.453</td>
<td>27.526</td>
<td>47.347</td>
<td>1.751</td>
<td>76.624</td>
</tr>
<tr>
<td>22</td>
<td>38.700</td>
<td>78.344</td>
<td>61.271</td>
<td>0.785</td>
<td>140.400</td>
</tr>
<tr>
<td>24</td>
<td>40.131</td>
<td>99.833</td>
<td>190.650</td>
<td>3.399</td>
<td>293.882</td>
</tr>
</tbody>
</table>

* Testing plan: 1 kg sample, Romer RAS type mill, 25 g subsample, and LC analytical method.

b Sampling variance could not be computed because of random experimental error.
Results and Discussion

The sampling, sample preparation, analytical, and total variances are reported in Table 2 for each of the 25 lots. The 25 lots, sorted by OTA concentration (Table 2), range from approximately 0.6 to 40.1 µg/kg. Each variance estimate increases with OTA concentration. This response is similar to what was observed with other mycotoxins such as aflatoxins in corn (7) and peanuts (28, 29), deoxynivalenol in wheat (16), and fumonisins in corn (17).

A full-log plot of all variance data in Table 2 is shown in Figure 3. Because the plots are somewhat linear, a regression equation is represented by a power function

\[ S^2 = aC^b \]  

(3)

where \( a \) and \( b \) are constants determined by regression analysis, and \( \hat{C} \) is the estimate of OTA concentration measured in µg/kg.

**Sampling Variance**

The sampling variance estimates, \( S^2_{(s)} \), from Table 2 show a linear relationship with the OTA concentration in a full-log plot (Figure 4). The relationship between sampling variance and OTA concentration is

\[ S^2_{(s)} = 1.350\hat{C}^{1.090} \]  

(4)

with a coefficient of determination (R²) of 0.650. The sampling variance in Equation 4 is unique for 1 kg samples.

**Sample Preparation Variance**

Sample preparation variance, \( S^2_{(sp)} \) from Table 2 shows a linear relationship with the OTA concentration in a full log-plot (Figure 5). Sample preparation variance increases with OTA concentration, as demonstrated by Equation 5.

\[ S^2_{(sp)} = 0.272\hat{C}^{1.457} \]  

(5)

with a coefficient of determination (R²) of 0.846. The sample preparation variance in Equation 5 is unique for a 25 g subsample taken from a sample comminuted in a Romer RAS type mill.

**Analytical Variance**

The analytical variance estimates, \( S^2_{(a)} \), reported in Table 2 for the 25 lots, are shown in a full-log plot in Figure 6. The relationship between the analytical variances and OTA concentration is

\[ S^2_{(a)} = 0.008\hat{C}^{1.05} \]  

(6)

with a coefficient of determination (R²) of 0.758. The analytical variance in Equation 6 is unique for the quantification of OTA in 1 aliquot by LC.

**Total Variance**

A full-log plot scale (Figure 7) of the total variance estimates versus OTA concentration (Table 2) showed a linear relationship. Using a regression analysis, it was possible to model the following mathematical expression giving a suitable relationship between total variance and OTA concentration with a coefficient of determination (R²) of 0.777.

\[ S^2 = 1.630\hat{C}^{1.18} \]  

(7)

The total variance in Equation 7 is unique for a 1 kg sample, Romer RAS type mill, 25 g subsample, and OTA quantified in 1 aliquot by LC.
Sample Preparation Variance vs OTA Concentration

![Sample Preparation Variance vs OTA Concentration](image)

Figure 5. Observed sample preparation variance vs mean OTA concentration for 1 kg sample, Romer RAS type mill, 25 g subsample, and LC analytical method, and predicted sample preparation variance by log/log power regression.

Application of Results

Mathematical Equations 4–6 estimate variances associated with testing a lot of green coffee for OTA using a 1 kg sample, Romer RAS type mill, 25 g subsample, and LC analytical method. For example, the estimated variance at 5 μg/kg using 1 kg sample, Romer RAS type mill, 25 g subsample, and quantifying 1 aliquot per subsample by LC was 10.75. Sampling, sample preparation, and analytical variances were 7.80, 2.84, and 0.11, respectively, and account for about 73, 26, and only 1% of the total variability, respectively. Sampling variance accounts for the majority of the total variance, followed by sample preparation and analytical variance. This is consistent with what has been observed with other mycotoxins and other commodities (7–15, 28, 29). For small sample sizes, sampling is usually the largest source of error.

By reducing one or more of the variance components (sample, sample preparation, and analytical variances) total variability of the test procedure can be reduced. Variance Equations 4–6 can be modified to predict the variances for different sample sizes, different subsample sizes, and different numbers of aliquots than those used in the experiment with statistical theory. For example, increasing the sample size can reduce the sampling variance; sample preparation variance can be reduced by increasing subsample size; and analytical variance can be reduced by increasing the number of aliquots quantified by LC from the same extract. Therefore, Equation 4 can be modified to predict the sampling variance for a given sample size

\[ S^2_{(s)} = (1/\text{ns})^{1.350C^{1.900}} \]  

where \( \text{ns} = \) sample size (kg).

The sample preparation variance in Equation 5 can be modified to predict the effect of any subsample size in the sample preparation variance comminuted in the Romer RAS type mill.

\[ S^2_{(sp)} = (25/\text{ns})^{0.272C^{1.457}} \]  

where \( \text{ns} = \) subsample size (g).

In the same way, the analytical variance can be modified in Equation 6 to predict any effect of any change of the number of aliquots in the quantification of OTA by the present LC method.

\[ S^2_{(a)} = (1/\text{na})^{0.008C^{1.605}} \]  

where \( \text{na} = \) number of aliquots quantified by LC.

As shown in Equation 7, the total variance can be estimated by summing the sampling, sample preparation, and analytical variances (Equations 8–10).

\[ S^2_{(t)} = (1/\text{ns})^{1.350C^{1.090}} + (25/\text{ns})^{0.272C^{1.457}} + (1/\text{na})^{0.008C^{1.605}} \]  

Unlike Equation 7, which is the total variance for the specific test procedure used in the study, Equation 11 predicts the total variance associated with testing a contaminated lot of green coffee for OTA for any size sample, size subsample, and number of aliquots quantified by LC. For example, the total, sampling, sample preparation, and analytical variances associated with testing of a lot of green coffee at 5 μg/kg using 5 kg sample, Romer RAS type mill, 50 g subsample, and quantifying 1 aliquot per sample by LC are 3.09, 1.56, 1.42, and 0.11, respectively, and account for about 51, 46, and only 3.4% of the total variability, respectively.

Analytical Variance vs OTA Concentration

![Analytical Variance vs OTA Concentration](image)

Figure 6. Observed analytical variance vs mean OTA for 1 kg sample, Romer RAS type mill, 25 g subsample, and LC analytical method, and predicted analytical variance by log/log power regression.
Figure 7. Observed total variance vs OTA concentration for 1 kg sample, Romer RAS type mill, 25 g subsample, and LC analytical method, and predicted total variance by log/log power regression.

Figure 8 shows the variances for each step of the test procedure plotted together on one graph. The variance was converted to coefficient of variation (CV) as another way to present variability in Figure 9.

Assuming that OTA test results from a green coffee lot follow the theory of normally distributed variables, a lot with an OTA concentration of 5 μg/kg using 5 kg sample, Romer RAS type mill, 25 g subsample, and quantifying 1 aliquot per sample by LC and a total variance of 3.09, implies that OTA test results will fall in the range of 5 ± 3.45 or 1.55–8.45 μg/kg 95% of the time. Further studies need to be implemented in order to determine the distribution among of OTA test results for green coffee.

Figure 9. Coefficient of variation associated with each step of the OTA test procedure vs OTA concentration.

Conclusions

Estimates of the total variance associated with testing 25 lots of green coffee for OTA were shown to increase as OTA concentration increased. This also held true for the uncertainty associated with each step of the test procedure: sampling, sample preparation, and analytical variances. Using regression analysis, mathematical expressions were developed to model all 3 variance components as a function of OTA concentration. The expressions were used to estimate the variances for any sample size, subsample size, number of analyses, and specific OTA concentration. For example, testing a lot with 5 ng/g using a 1 kg sample, Romer mill, 25 g subsamples, and LC analysis, the total, sampling, sample preparation, and analytical variances are 10.75 (CV = 65.6%), 7.80 (CV = 55.8%), 2.84 (CV = 33.7%), and 0.11 (CV = 6.6%), respectively. The sampling, sample preparation, and analytical steps of the OTA test procedure accounted for 73, 26, and 1%, of the total variability, respectively. As with the testing of other commodities for other mycotoxins, sampling variance contributes the most variability followed by sample preparation and then analytical variability. The best use of resources to reduce the total variability of the OTA test procedure would be to increase the size of the test sample.

Acknowledgments

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