Comparing the USDA/AMS Subsampling Mill to a Vertical Cutter Mixer Type Mill Used to Comminute Shelled Peanut Samples for Aflatoxin Analysis[1]

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ABSTRACT

The US peanut industry can use up to three 21.8 kg samples per lot to determine if shelled peanut lots are acceptable or unacceptable due to aflatoxin content. If a lot is accepted by the first 21.8 kg sample (1AB≤8 ng/g) prepared with the USDA/AMS Subsampling mill (DM), then some peanut buyers request that the sheller prepare the second sample (2AB) and in some cases the 3AB sample (called special samples in the trade) with a vertical cutter mixer (VCM) type mill. These requests to specifically use the VCM instead of the DM to prepare official (1AB) and special (2AB or 3AB) samples is based in part on a perception that analytical results associated with a test portion taken from the 21.8 kg sample comminuted with a DM does not detect the full magnitude of aflatoxin in the 21.8 kg sample and that negative aflatoxin certificates (lot acceptance) are more likely to occur when samples are prepared with a DM than a VCM. Analysis of aflatoxin test results from two shellers along with Monte Carlo simulation indicate that differences between the 1AB and special sample test results are due to the use of a cut-off limit (≤ 8 ng/g associated with the 1AB) requested by the buyer as part of the acceptance criteria and not due to any bias associated with the DM. Operating characteristic curves were used to demonstrate that the performance of the USDA/AMS aflatoxin sampling plan is about the same regardless of the use of a DM or a VCM for sample preparation. The performances are similar because the DM, with an 1100 g test portion, account for only 8% of the total variability of the aflatoxin test procedure (sampling and analysis account for about 92%). A sampling plan that requires two 21.8 kg samples to test less than a limit, regardless of mill used to prepare the two samples, has a very low risk of accepting bad lots above the FDA limit of 20 ng/g, but has a very high risk of rejecting good lots, which makes for an extremely high economic burden on the sheller.

Key Words: aflatoxin, peanuts, sample preparation, test portion, mills, USDA/AMS Subsampling mill, and vertical cutter mixer.

Aflatoxin is a toxic and carcinogenic compound produced by fungi such as *A. flavus*. About 100 countries have established regulatory limits or maximum levels for aflatoxin and other mycotoxins in food and feed products (Food and Agriculture Organization, 2003). Because of these regulatory limits, bulk products are often tested for aflatoxin in the market chain between the buyer and seller. The aflatoxin concentration in a bulk lot is estimated by measuring aflatoxin in a sample taken from the bulk lot (Whitaker, 2006). Then the measured aflatoxin concentration in the sample is used to estimate aflatoxin concentration in the bulk lot or is compared to a maximum level established by a regulatory agency or members of the market system such as the buyer to determine if the lot is acceptable for further processing.

The Agricultural Marketing Service of the US Department of Agriculture (AMS/USDA) and the peanut industry estimate aflatoxin in all domestic raw shelled peanut lots before being shipped by the seller to a buyer. In the peanut industry the seller is typically a sheller, but the buyer can vary from exporters and importers to food manufacturers. The accepted aflatoxin test procedure used by USDA/AMS to estimate the aflatoxin concentration in a bulk lot of raw shelled peanuts consist of sampling, sample preparation, and analytical steps. It is important to understand the role and importance that each step has in the overall aflatoxin test procedure. These three steps are briefly described below.

Sampling. When possible, automatic sampling equipment is used to select samples from moving streams of shelled peanuts. A diverter cup cuts through a moving a stream at specified intervals removing approximately 50 to 100 small incremental samples from the beginning to the end of the lot. For a 20 metric ton lot (about 44,053 lbs), the diverter cup takes an incremental sample about every 400 kg (50 cuts) to 200 kg (100 cuts). The incremental samples are accumulated to form an aggregate sample of approximately 70 to 75 kg. Using an USDA/AMS approved divider; three 21.8-kg (48-lb) laboratory samples are taken from

[1]The use of trade names in this publication does not imply endorsement by the N.C. Agricultural Research Service of the products named nor criticism of similar ones not mentioned.

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the aggregate sample for aflatoxin analysis (the remainder of the aggregate sample is used as a grade sample).

Sample Preparation. Each 21.8-kg laboratory sample is comminuted in a USDA/AMS approved mill and an 1100 g test portion is removed for aflatoxin analysis. Typically, two types of mills are approved by AMS to comminute the laboratory sample. A USDA/AMS Subsampling mill (often called the Dickens Mill or DM for short), developed by the Agricultural Research Service (ARS) of USDA for the USDA/AMS, comminutes and automatically subsamples the 21.8-kg laboratory sample and provides an 1100 g comminuted test portion for aflatoxin analysis (Dickens and Satterwhite, 1969; Dickens *et al.*, 1979). The DM comminutes the laboratory sample into a meal or powder and not a paste and is constructed with vanes to automatically provide an 1100 g test portion. The second type mill is a vertical cutter mixer (VCM), which is commercially available. The VCM comminutes the laboratory sample into a paste because the comminution process creates a much smaller particle size than the DM. The test portion must be randomly removed from the comminuted laboratory sample by manual methods.

Analysis. Because the 1100-g test portion is considered to be a large test portion size, a water slurry method was developed by USDA/ARS for AMS that provides additional particle size reduction and saves on organic solvents (Whitaker *et al.*, 1980; United States Department of Agriculture, 1991). The 1100-g test portion is blended with 1600 ml of water for three minutes. A 196 g portion of the water blend (containing 80 g of peanuts) is removed from the slurry blend. Then the appropriate amount of an organic solvent is added to the 196 g portion of water and peanuts and blended for an additional 30 seconds. Two aliquots (called A and B) are removed from the blend and each aliquot is quantified for aflatoxin and the results are averaged to obtain a sample test result. The aflatoxin is extracted from the peanut solvent blend and quantified using an approved USDA/AMS analytical methods such as thin layer chromatography (TLC), immunoassay, or high performance liquid chromatography (HPLC).

USDA/AMS Accept/Reject Rule. The USDA/AMS uses a sequential sampling plan where three 21.8-kg laboratory samples are removed form each lot and tested for aflatoxin (called 1AB, 2AB, and 3AB). If the 1AB laboratory sample test result is less than or equal to 8 ng/g total aflatoxins, the lot is accepted into the domestic market with no further tests. If the 1AB laboratory sample test result is greater than 45 ng/g, the lot is rejected with no further tests. If the 1AB sample test result is between 8 and 45 ng/g, the 2AB is analyzed for aflatoxin and the two sample test results are averaged (Avg 1AB/2AB = (1AB+2AB)/2). If the average of the two sample test results is less than or equal to 12 ng/g, the lot is accepted with no further tests. If the average of the two sample test result is greater than 22 ng/g, the lot is rejected with no further tests. If the average of the two sample test result is between 12 and 22 ng/g, the 3AB is analyzed for aflatoxin and the three sample test results are averaged (Avg 1AB/2AB/3AB = (1AB+2AB+3AB)/3). If the average of the three sample test results is less than or equal to 15 ng/g, the lot is accepted, else the lot is rejected. The sequential test was designed to save on the amount of sample required to accept or reject a lot. Lots with very low or very high levels of aflatoxin are accepted or rejected on the first 1AB sample. Lots with aflatoxin concentration near the USDA/AMS limit of 15 ng/g may require two or all three samples to be tested to make a decision to accept or reject the lot.

Some buyers of raw shelled peanuts will request that all official 21.8-kg laboratory samples used to accept or reject a lot be comminuted with a VCM. In that case, USDA/AMS still requires that an 1100 g test portion be removed from the 21.8 kg laboratory sample comminuted with a VCM for aflatoxin analysis. Some buyers also request that lots accepted by the first 21.8 kg laboratory sample (1AB≤8 ng/g) where the DM was used to prepare the official sample undergo additional testing before the buyer considers the lot acceptable for purchase. If the first 21.8-kg sample tests 8 ng/g or less when the sample is prepared by the DM, then the seller prepares the 2AB and/or 3AB samples (called Specials or special samples in the trade) with a VCM and the sample test result must be less than an accept/reject limit defined by the buyer. Accept/reject limits defined by the buyer may vary, but are generally in the 5 to 10 ng/g range. Once a lot has been accepted by the official USDA/AMS test (1AB≤8ng/g), any additional testing is strictly between the seller and the buyer. The buyer may also request that a 50 g test portion (not the USDA/AMS required 1100 g) be taken from the special sample prepared with a VCM for aflatoxin analysis.

Sellers and buyers have observed that on some occasions when the official 1AB prepared with the DM tested 8 ng/g or less, the corresponding Specials prepared with the VCM tested greater than 8 ng/g or some limit defined by the buyer. They also noticed that over many lots, the average aflatoxin among all 1AB samples prepared with the DM that tested below the USDA/AMS limit of

8 ng/g was lower than the average aflatoxin among all corresponding Special samples prepared with the VCM. These observations led to several concerns on part of both sellers and buyers about sample test results when the sample was prepared with the DM versus the VCM. There are concerns that (a) analytical results associated with 21.8-kg samples prepared with the DM may be lower than analytical results from subsequent tests of the 21.8 kg special samples prepared with a VCM and (b) that lots may be more likely to receive negative certificates (more likely to accept the lot) when a 21.8-kg sample is prepared by the DM than when prepared by the VCM. These observations have led some sellers and buyers to believe that analysis of 21.8-kg samples prepared by the DM produces results that are biased to the low side of the true 21.8-kg sample concentration. The DM can't be biased to the low side of the true sample concentration unless aflatoxin is destroyed during the comminution process and/or the extraction efficiency associated with the analytical method that is used to quantify aflatoxin in the test portion is less for the DM after the water slurry process than for the VCM.

The objective of this study was to use actual sample test results supplied by two commercial shellers that were prepared by the DM and VCM along with sampling theory to determine why buyers and sellers are observing that (a) a greater percentage of 1AB samples prepared by the DM test 8 ng/g or less than Special samples prepared by the VCM and (b) why the average aflatoxin among official sample test results (1AB) prepared with the DM are lower than the average aflatoxin among Special samples prepared with the VCM.

Materials and Methods

Theoretical Considerations

As described above, the USDA/AMS aflatoxin test procedure consists of a sampling, sample preparation, and analytical steps. Even when using accepted sampling, sample preparation, and analytical procedures that minimize bias, there is variability associated with each of the steps of the aflatoxin test procedure. Because of the variability associated with each step of the USDA/AMS aflatoxin test procedure for peanuts, the true aflatoxin concentration in a bulk lot can't be determined with 100% certainty by measuring aflatoxin in laboratory samples taken from the lot. As shown in Fig. 1, the sampling, sample preparation, and analytical steps of the aflatoxin test procedure each contribute to the total variability among aflatoxin test results. The total variability is the sum of the sampling variability, sample preparation variability, and analytical variability.

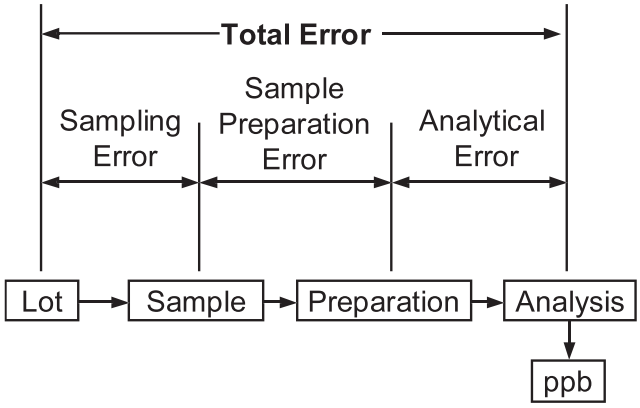

Fig. 1. Total error of the mycotoxin-test procedure is the sum of sampling, sample preparation, and analytical errors. The term error denotes variability as measured by the variance.

Among the various statistical measures of variability, only the variance is additive. Therefore, the total variance (s^2_t) associated with a specific aflatoxin test procedure for peanut is the sum of the sampling, sample preparation, and analytical variances (equation 1).

$$s_t^2 = s_s^2 + s_{sp}^2 + s_a^2 \quad (1)$$

To understand how much each step of the aflatoxin test procedure contributes to the total variability and to be able to predict confidence limits associated with estimating the true lot aflatoxin concentration, Whitaker *et al.*, 1974 measured the sampling (s^2_s), sample preparation (s^2_{sp}), and analytical (s^2_a) variances using USDA/AMS approved aflatoxin test procedures. The magnitude of the variability of each step and its contribution to the total variability and reasons why each step contributes to the overall variability is discussed below.

Sampling Variance (s^2_s). Studies (Whitaker *et al.*, 1974) indicate that, especially for small laboratory sample sizes, the sampling step is usually the largest source of variability associated with the aflatoxin test procedure. Even when using accepted sample selection equipment and random sample selection procedures, sampling variability is large because of the aflatoxin distribution among contaminated kernels within a lot. Studies indicate that a very small percentage (0.1%) of the kernels in the lot is contaminated and the concentration on a single kernel may be extremely high. (Cucullu *et al.*, 1986) reported an aflatoxin concentration in excess of 1,000,000 ng/g (parts per billion, ppb) for an individual peanut kernel.

Because of this extreme range in aflatoxin concentrations among a few contaminated kernels in a lot, variation among replicated laboratory sample test results tends to be large. The sampling variance, s^2_s, associated with testing shelled peanuts is shown in equation 2.

$$s_s^2 = (10{,}638/ns)9.19C^{1.336} \quad (2)$$

where ns is the laboratory sample size in number of shelled kernels and C is the aflatoxin concentration in ng/g total aflatoxins. If sample size is expressed in units of mass (kg), the mass can be converted to number of kernels knowing the count per unit mass.

Sample Preparation Variance (s^2_{sp}). Once the laboratory sample has been taken from the lot, the sample must be prepared for aflatoxin quantification. Since it is not practical to extract the aflatoxin from a large laboratory sample of kernels, the sample is comminuted in a suitable mill and the aflatoxin is extracted from a small test portion (sometimes called a subsample) taken from the comminuted laboratory sample. If the commodity is a granular product such as shelled peanuts, it is essential that the entire laboratory sample be comminuted in a suitable mill before a test portion is removed from the laboratory sample. Removing a test portion of whole seed from the laboratory sample before the comminuting process is a sample size reduction process and eliminates the benefits associated with the larger size laboratory sample of kernels.

Due to the aflatoxin distribution among contaminated particles in the comminuted laboratory sample, there is also variability among replicated test portions taken from the same comminuted laboratory sample. The sample preparation variance is not as large as the sampling variance due to the large number of comminuted particles in the test portion. An example of sample preparation variance for aflatoxin and shelled peanuts is shown below in equation 3.

$$s_{sp}^2|_d = (275/nss)\ 0.294C^{1.729} \quad (3)$$

where nss is the comminuted test portion size in g. The sample preparation variance in equation 3 is specific to the USDA/AMS Subsampling mill or DM (identified by the "d" subscript) and its corresponding particle size distribution.

Dorner and Cole (1993) and Whitaker *et al.* (1994) measured the sample preparation variance associated with various VCM type mills. The sample preparation variance associated with a Robot Coup VCM is shown in equation 4.

$$s_{sp}^2|_v = (200/nss)\ 0.0216C^{1.940} \quad (4)$$

Analytical Variance (s^2_a). Once the test portion is removed from the comminuted laboratory sample, the aflatoxin is extracted from the test portion by blending the test portion with a suitable solvent. Analytical methods usually involve several steps such as solvent extraction, clean-up, dilution, and quantification. As a result, there can be variation among replicated analyses on the same solvent/test portion blend. The analytical variance (s^2_a) associated with various analytical methods are shown below for TLC, immunoassay, and HPLC, respectively.

$$s_a^2|_t = (1/na)\ 0.685C^{1.641} \quad (5)$$

where na is the number of aliquots taken from the peanut solvent blend and quantified for aflatoxin by TLC (Whitaker *et al.*, 1974).

$$s_a^2|_i = (1/na)\ 0.294C^{1.729} \quad (6)$$

where na is the number of aliquots taken from the peanut solvent blend and quantified for aflatoxin by immunoassay (Whitaker *et al.*, 1996).

$$s_a^2|_h = (1/na)\ 0.083C^{1.654} \quad (7)$$

where na is the number of aliquots taken from the peanut solvent blend and quantified for aflatoxin by HPLC (Whitaker *et al.*, 1996).

Comparison of Aflatoxin Test Results When Samples Are Prepared by DM and VCM

Sample Test Results from Two Shellers. Two shellers in the southeastern United States (called sheller #1 and #2) provided one or more aflatoxin test results (1AB, and Specials) from each of 1677 and 2289 lots, respectively, of runner peanuts harvested in 2010. Along with the aflatoxin test result for each sample, the type mill used to comminute each of the 21.8-kg laboratory samples was also identified in the database. For each sheller, the database was sorted into two groups where the lots in group 1 had the 1AB and Special samples prepared by the DM and VCM, respectively, and group 2 had lots where both 1AB and Specials were prepared by the VCM. In both groups, the 1AB samples tested 8 ng/g or less. The percent of lots in each group where Specials tested 8 ng/g or less was determined (remember all 1AB samples tested 8 ng/g or less). The average aflatoxin among all 1AB and Special samples was determined for each group. Comparing DM to VCM results from group 1 illustrates what sellers and buyers are observing when the DM

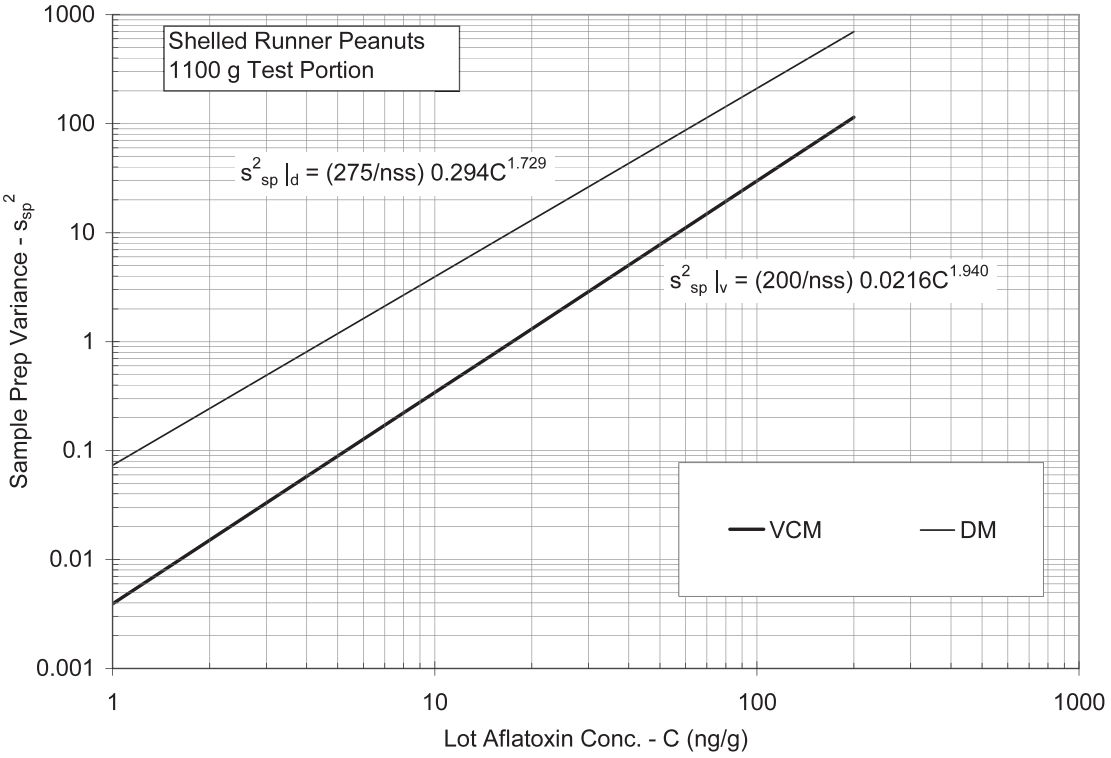

Fig. 2. Sample preparation variance associated with the USDA/AMS Subsampling mill (DM) and vertical cutter mill (VCM) and an 1100 g test portion.

and the VCM are used to prepare 1AB and Special samples from the same lot. Comparing the results from group 1 to group 2 would give an indication if the DM behaved differently from the VCM when the VCM was used to prepare samples 1AB and Specials.

Monte Carlo Simulation. One possible problem with using actual sample test results in the above analysis is that lot concentrations vary over a wide range in each group and the aflatoxin distribution among lot concentrations may be different between group 1 and group 2 for each sheller. It would be desirable to compare the aflatoxin test results for 1AB and Specials for a large number of lots at the same aflatoxin concentration. Therefore, Monte Carlo simulation methods (Whitaker *et al.*, 1976) were used to theoretically sample a lot at 10 ng/g. Forty replicates of three 21.8-kg samples (1AB, 2AB, and 3AB) were taken from a lot at 10 ng/g using Monte Carlo methods. Sample 1AB was designated as the official sample and samples 2AB and 3AB were designated as the two special samples. All three samples were prepared with a VCM and aflatoxin was quantified in two aliquots by HPLC and averaged. If all three samples were prepared by the VCM in the simulation (no DM involved) behaved in a manner similar that shown with actual results where 1AB and 2AB were prepared by the DM and VCM, respectively, then no further simulations are required.

The forty lots were sorted in a similar manner to that described for actual data. The percentage of official and special samples testing 8 ng/g or less and the average aflatoxin among the official and special samples was computed as above for actual sample test results. One advantage of the Monte Carlo simulation is that 2AB and 3AB sample test results are shown when 1AB test greater than 8 ng/g unlike the database of actual sample test results (2AB and 3 AB would not be tested if the 1AB prepared by the DM tested greater than 8 ng/g).

Results and Discussion

Mill Performance

A full-log plot of the sample preparation variance for the DM and VCM are shown in Fig. 2 for an 1100 g test portion. These two variance curves were computed from equations 3 and 4 for an 1100 g test portion. It can be seen in Fig. 2 that the sample preparation variance for the VCM is smaller than that for the DM at all aflatoxin concentration levels when using an 1100 g test portion. For an aflatoxin concentration of 15 ng/g (C=15 ng/g) and an 1100 g test portion, the sample preparation variance for the VCM is 0.8 and for the DM is 7.9. The sample preparation variance is smaller for the VCM because the grinding process creates smaller particles or more

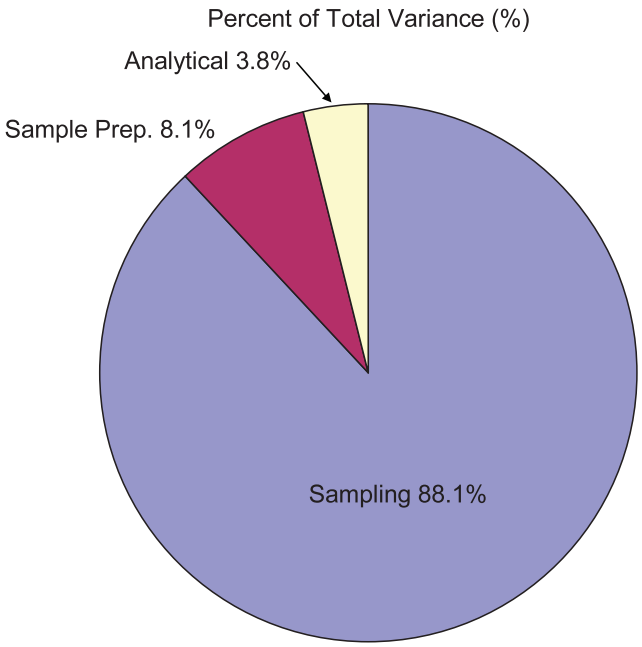

Fig. 3. Contribution of the sampling, sample preparation, and analytical steps to the total variability associated with USDA/AMS aflatoxin test procedures when using 21.8 kg sample, USDA/AMS Subsampling mill (DM), 1100 g test portion, and analyzing aflatoxin in two aliquots using HPLC.

particles per unit mass than the DM. USDA/AMS and the peanut industry reduced the sample preparation variance associated with the DM over a period of many years by increasing the test portion size from 275 g to the current 1100 g. Increasing the size of the test portion from 275 to 1100 g reduced the sample preparation variance for the DM by a factor of 4, which is the ratio of the two test portion sizes (1100/275).

Since sample preparation is just one of the three steps associated with the aflatoxin test procedure for peanuts, it is important to understand what the contribution of the sample preparation step is to the total variability of the aflatoxin test procedure. The total variance (s^2_t) associated with the USDA/AMS aflatoxin sampling plan to detect aflatoxin in a peanut lot at 15 ng/g (C=15 ng/g) when using the DM to prepare the sample is the sum of equations 2, 3, and 7 (ns=42,554 kernels or 21.8 kg for runner peanuts, nss=1100 g, na=2, and HPLC).

$$97.2 = 85.6 + 7.9 + 3.7 \quad (8)$$

When using the DM to prepare the 21.8 kg sample (Equation 8), the sampling, sample preparation, and analytical steps account for 88.1, 8.1, and 3.8 percent of the total variance, respectively (Fig 3). Assuming the distribution among sample test results is Normal, the range among replicated sample test results associated with the aflatoxin test procedure described above where the lot concentration is 15 ng/g is ± 19.5 ng/g (19.5 = $1.98\sqrt{97.2}$ for 95% confidence limits). The sampling step is the largest source of variability and the analytical step is the smallest source of variability. The sample preparation variance for the DM and an 1100 g test portion constitutes only about 8% of the total variability.

When the seller uses a VCM instead of the DM to comminute the 21.8 kg laboratory sample at 15 ng/g (C=15 ng/g), the sample preparation variance for an 1100 g test portion (nss=1100 g) is reduced from 7.9 (DM, equation 3) to 0.8 (VCM, equation 4). The total variance of the aflatoxin test procedure (equation 9) is reduced from 97.2 to 90.1.

$$90.1 = 85.6 + 0.8 + 3.7 \quad (9)$$

Assuming the distribution among sample test results is Normal, the range among replicated sample test results associated with the aflatoxin test procedure described above when a VCM is used to prepare the sample and lot concentration is 15 ng/g is ± 18.8 ng/g (18.8 = $1.98\sqrt{90.1}$ for 95% confidence limits). Use of a VCM instead of the DM reduces the range of sample test results about the true lot concentration of 15 ng/g from ±19.5 to ±18.8 ng/g, respectively.

Using a 50 g test portion when an additional 21.8 kg special sample is comminuted outside the USDA/AMS testing program risks increasing the sample preparation variance associated with the VCM to a value greater than that associated with the DM and an 1100 g test portion. If a 50 g test portion is taken from a special 21.8 kg sample comminuted with a VCM, the sample preparation variance increases from 0.8 (for an 1100g test portion) to 16.9 (for C=15 ng/g in equation 4). The sample preparation variance for the VCM and a 50 g test portion is greater than the sample preparation variance for the DM and an 1100 g test portion (16.9 vs. 7.9, respectively). The total variance of the aflatoxin test procedure increases from 97.2 to 105.2 due to the increase in the sample preparation variance with a 50 g test portion.

The 1100 g test portion defined by USDA/AMS was originally specified to lower the sample preparation variance associated with the DM. It would be interesting to determine the test portion size associated with a VCM such that the VCM sample preparation variance is the same as the sample preparation variance for the DM with 1100 g test portion. This can be calculated by setting equation 3 equal to equation 4. When C=15 ng/g, the sample preparation variance for the VCM and 104.1 g test portion is equal to the sample preparation variance for the DM and an 1100 g test portion, which is 7.9. To avoid

Table 1. Average aflatoxin and percent lots testing 8 ng/g or less by the official 1AB and special samples (2AB) by type mill for sheller #1.

Mill used 1AB, 2AB, 3AB	Number of lots	1AB Average aflatoxin	1AB Aflatoxin range	1AB Lots < 9 ng/g	Special 1 (VCM) Average aflatoxin	Special 1 (VCM) Aflatoxin range	Special 1 (VCM) Lots < 9 ng/g
		(ng/g)		(%)	(ng/g)		(%)
DM	115	1.3	0–8	100.0	12.2	0–99	64.3
VCM	416	1.0	0–8	100.0	5.1	0–74	82.5
All mills	531	1.1	0–8	100.0	6.7	0–99	78.3

DM=USDA/AMS Subsampling mill.
VCM=USDA/AMS approved vertical cutter mixer type mill.

increasing the sample preparation or total variance associated with the DM and an 1100 g test portion, then the test portion size associated with the VCM should be greater than 104 g.

Comparison of Aflatoxin Test Results When Samples Are Prepared by DM and VCM

Sheller Results. From the database provided by sheller#1, 531 lots were accepted by the USDA/AMS plan on the first sample (1AB≤8 ng/g). Of these 531 official 1AB samples that tested 8 ng/g or less, 115 and 416 were comminuted with a DM and VCM, respectively. Buyers requested that the 2AB sample from each of these 531 lots be tested for aflatoxin using the VCM. Depending on the buyer's specified limit (typically in the range of 5 to 10 ng/g total aflatoxin), the lot was accepted by the buyer if the special sample (2AB) tested less than or equal to the buyer limit. As a result, 115 lots had the 1AB and 2AB prepared by the DM and VCM, respectively, and the remaining 416 lots had both the 1AB and 2AB prepared by the VCM. These results allowed for sample test results to be compared for 115 lots where two different mills were used on the 1AB and 2AB (DM vs. VCM) and 416 lots where the same mill (VCM) was used on the 1AB and 2AB (VCM vs. VCM).

Table 1 show that for group 1 all 115 (100%) 1AB sample test results were 8 ng/g or less (1AB ≤8 ng/g), but when the 115 special samples (2AB) were tested at the request of the buyer, only 64.3% or 74 of the 115 special samples tested 8 ng/g or less. Table 1 also show that the average aflatoxin among the 115 1AB and special samples (2AB) was 1.3 and 12.2 ng/g, respectively (DM vs. VCM). The results shown in Table 1 reflect the observations made by sellers and buyers that (a) 100% of the 1AB prepared by the DM were accepted (115/115) while only 64.3% (74/115) of the special samples (2AB) prepared by the VCM were accepted and (b) the average aflatoxin level among the 115 1AB was 1.3 ng/g compared to 12.2 ng/g for the special samples.

Table 1 show that for group 2 all 416 (100%) 1AB samples tested 8 ng/g or less (1AB ≤8 ng/g), but when the additional special samples (2AB) were tested at the request of the food manufacturer, only 82.5% or 343 of the 416 special samples tested 8 ng/g or less. Table 1 also show that the 416 lots where the VCM was used to prepare both the 1AB and 2AB (VCM vs. VCM) that the average aflatoxin among the 416 1AB and special samples (2AB) was 1.0 and 5.1 ng/g, respectively.

Comparing group 2 to group 1 results in Table 1 indicate that the 416 lots where the VCM was used to prepare both the 1AB and 2AB behave in a similar way as the 115 lots where the DM was used on 1AB and the VCM on 2AB. Since the same mill (VCM) was used to prepare both the 416 1AB and 2AB samples, the results can't reflect a bias associated with the DM specifically or an effect due to the type mill used to prepare the 1AB sample in general.

The average aflatoxin among the 2AB samples was lower for the 416 lots (VCM vs. VCM) than the 2AB for the 115 lots (DM vs. VCM). The special samples prepared by the VCM in Table 1 (either group) were not restricted by a cut-off level and are therefore an unbiased estimate of the average aflatoxin among lots selected by the sheller in the two groups in Table 1 (DM vs VCM and VCM vs VCM). The lower average aflatoxin among the 2AB samples from the 416 lots (group 2 or VCM vs VCM) reflect a lot selection bias where samples were taken from lots suspected of having less aflatoxin than lots in group 1 (*confirmed by personal communications with sheller#1*). Sampling theory also predicts that the percentage of samples testing 8 ng/g or less will be higher among lots with the lowest average aflatoxin concentration. As shown in Table 1, the average aflatoxin among lots in group 1 was higher than among lots in group 2, (12.2 vs 5.1 ng/g) but had the lowest percentage of special testing 8 ng/g or less (64.2 vs 82.5%).

Table 2. Average aflatoxin and percent lots accepted by the official 1AB and special samples for sheller #2.

Mill used 1AB, 2AB, 3AB	Number of lots	1AB Average aflatoxin	1AB Aflatoxin range	1AB Lots < 9 ng/g	Special 1 (VCM) Average aflatoxin	Special 1 (VCM) Aflatoxin range	Special 1 (VCM) Lots < 9 ng/g
		(ng/g)		(%)	(ng/g)		(%)
DM	2081	1.1	0–8	100.0	7.5	0–440	81.0
VCM	208	1.2	0–8	100.0	3.5	0–72	87.5
All mills	2289	1.2	0–8	100.0	7.1	0–440	81.6

DM = USDA/AMS Subsampling mill.
VCM = USDA/AMS approved vertical cutter mixer type mill.

A second sheller (Sheller #2) provided sample test results in a similar data format as sheller #1 and the results are shown in Table 2. Results from Sheller #2 (Table 2) are similar to results from Sheller #1 (Table 1). Analysis of data from Sheller #2 also supports the conclusion that it makes no difference which mill (either DM or VCM) is used to prepare sample 1AB for aflatoxin analysis. Results for DM vs. VCM lots are similar to results for VCM vs. VCM lots.

The fact that the VCM vs. VCM lots behave in a similar manner to the DM vs. VCM lots (both Tables 1 and 2) supports the assumption that neither mill is biased and specifically that the DM doesn't under estimate the aflatoxin in the sample or provides more negative certificates than the VCM. The reason for the results observed in Tables 1 and 2 is probably due to the use of a sample cut-off limit of 8 ng/g used by the USDA/AMS on the first official sample (1AB≤8 ng/g) to accept, reject, or continue sampling a lot. Using data provided by Sheller #1, many more than 115 lots in Table 1 (682 lots not shown) had the 1AB prepared with a DM and testing greater than 8 ng/g, but only a subset of those 682 lots (115) tested 8 ng/g or less and qualified to have a special sample (2AB) tested for aflatoxin. As a result, the 1AB samples from the 115 lots were all 8 ng/g or less and would have to average less than 8 ng/g (1.3 ng/g). The 115 special samples (2AB) were not constrained by a limit and randomly varied from 0 to 99 ng/g and averaged 12.2 ng/g. Since 2AB aflatoxin test results in group 1 varied from 0 to 99 ng/g, only 64.3% of the 115 special samples (2AB) tested 8 ng/g or less. The same explanation can be used to explain results for the 416 VCM vs. VCM lots (group 2) in Table 1.

From the database provided by Sheller #1, 93 lots had two special samples (Special 1 = 2AB and Special 2 = 3AB) tested for aflatoxin in addition to having the official 1AB tested for aflatoxin. For each lot, Special 1 (2AB) and Special 2 (3AB) samples were prepared by with a VCM. Without considering the 1AB, these two special samples could also be used to demonstrate the effects of a sample cut-off limit on these 93 lots and show that the results are similar to Tables 1 and 2 when the VCM is used to prepare both samples (VCM vs. VCM). There are no constraints on the sample test results among the 93 2AB and 3AB sample test results. Sample test results for the 93 2AB varied from 0 to 80 ng/g and from 0 to 150 ng/g for the 93 3AB samples. The aflatoxin among the 93 2AB and 3AB samples averaged 10.2 and 12.9 ng/g, respectively (Table 3).

A sample cut-off limit of 8 ng/g or less was chosen to screen the 93 2AB samples (Special 1) since it is the first USDA/AMS accept limit. A total of 63 of the 93 lots had 2AB≤8 ng/g (not shown). Thirty lots had 2AB>8 ng/g. The 2AB and 3AB results among these 63 lots are summarized in Table 4. Results among the 63 VCM vs. VCM sample test results in Table 4 are similar to results among the VCM vs. VCM results in Tables 1 and 2. The average aflatoxin among the 63 Special 1 (2AB) and 63 Special 2 (3AB) samples was 2.1 and 8.6 ng/g, respectively. While 100% of the 63 2AB samples were 8 ng/g or less, only 45 of the 63 3AB samples (71.4%) were 8 ng/g or less. The range among sample test results for the 2AB samples varied from 0 to 8 ng/g and from 0 to 56 ng/g for 3AB samples. These 93 lots also demonstrate that results observed in Tables 1 and 2 can be reproduced when two random samples are both prepared with the same mill (VCM).

Monte Carlo Simulation of Aflatoxin Test Results for DM and VCM. To expand on information in Table 1, 2, and 4, Monte Carlo simulation methods (Whitaker *et al.*, 1976) were used to illustrate in more detail with individual sample test results how a cut-off limit (use of 8 ng/g in this simulation) affects the distribution among sample test results for official samples (1AB) and special samples (2AB) when both are prepared by the VCM.

Forty replicates of three 21.8 kg samples (1AB, 2AB, and 3AB) are taken from a lot at 10 ng/g using Monte Carlo simulation methods. By taking samples from a lot at a constant concentration

Table 3. Summary of aflatoxin test results for 93 lots with two special samples before sorting into categories using a cut-off limit of 8 ng/g.

	Special 1	Special 2
Number of lots	93	93
Average aflatoxin (ng/g)	10.2	12.9
Minimum aflatoxin (ng/g)	0.0	0.0
Maximum aflatoxin (ng/g)	80.0	150.0
Lots < 9 ng/g (%)	67.7	64.5

(10 ng/g), we can remove the effect that sampling many lots with varying aflatoxin concentrations have on the comparison of the two mill (groups 1 and 2 in Tables 1 and 2). The 1AB sample is the official sample and the 2AB and 3AB are the two special samples. All three samples were prepared with a VCM and aflatoxin is quantified in two aliquots by HPLC and averaged. All sample test results, sorted by the 1AB aflatoxin value, are shown in Table 5.

Replicates where 1AB sample test results were 8 ng/g or less are shown in bold/italics along with the respective specials (2AB and 3AB). The average aflatoxin among the 40 1AB, 2AB, and 3AB along with the number of times that 1AB and 2AB and 3AB each tested 8 ng/g or less is shown at the bottom of the table. Since all samples are randomly chosen from the lot, the average among each of the 40 samples should be reasonably close to the true lot concentration of 10 ng/g and the number of samples testing 8 ng/g or less should also be about the same for 1AB, 2AB, and 3AB. The average among all 40 1AB, 2AB, and 3AB samples are 10.7, 10.3, and 10.3 ng/g, respectively. The more samples chosen from the lot, the closer the sample average should be to the lot average of 10 ng/g. The number of times that the 40 1AB, 2AB, and 3AB samples test 8 ng/g or less is 18 (45.0%), 22 (55.0%), and 23 (57.5%), respectively.

The domain of all sample test results (40 sets of 3 sample test results) in Table 5 is divided into two subsections where 1AB≤8 ng/g (subsection 1) and 1AB>8 ng/g (subsection 2). The summary statistics for each subsection are also shown at the bottom of Table 5. Eighteen of the 40 1AB samples tested 8 ng/g or less and 22 of the 40 1AB samples tested greater than 8 ng/g. For subsection 1 (bold/italics), the average aflatoxin among the 18 1AB, 2AB and 3AB samples are 3.2 ng/g, 9.4 ng/g, and 9.7 ng/g, respectively. Within subsection 1 (1AB≤8 ng/g), the number of 1AB, 2AB, and 3AB samples testing 8 ng/g or less is 18 (18/18=100%), 12 (12/18=66.7%), and 10 (10/18=55.6%), respectively. This example shows the 18 1AB samples testing 8 ng/g or less, which are a subset of the total

Table 4. Summary of aflatoxin sample test results when a cut-off limit of 8 ng/g is used on special sample 1.

	Special 1	Special 2
Number of lots	63	63
Average aflatoxin (ng/g)	2.1	8.6
Minimum aflatoxin (ng/g)	0.0	0.0
Maximum aflatoxin (ng/g)	8.5	56.0
Lots < 9 ng/g (%)	100.0	71.4

domain (40), are restricted to samples values below 8 ng/g while the 18 2AB and 18 3AB sample test results are not restricted and vary in a random manner. The 1AB samples that are greater than 8 ng/g are not included in the summary statistics for subsection 1 (1AB≤8 ng/g). The 2AB and 3AB samples in Table 5 for subsection 2 (1AB>8 ng/g) would not have been quantified in the real world since 1AB>8 ng/g. But we can see that the average among the 2AB and 3AB samples in subsections 1 and 2 still average close to the true lot concentration of 10 ng/g. The summary statistics from the Monte Carlo simulation in Table 5 are very similar to the results shown in Tables 1, 2, and 4, but Table 5 show all the sample test results for 1AB, 2AB, and 3AB that make up the summary statistics.

Sampling Plan Performance When Samples Are Prepared by A VCM and DM

Because of the total variability (s^2_t) associated with the aflatoxin test procedure, some lots will be misclassified by the sampling plan. Some good lots (true lot concentration below the USDA/AMS limit of 15 ng/g) will be rejected by the sampling plan (samples will test greater than the USDA/AMS limit). This is often called the seller's risk. Also, some bad lots (true lot concentration above the USDA/AMS limit of 15 ng/g) will be accepted by the sampling plan (samples will test less than the USDA/AMS limit). This is often called the buyer's risk. The magnitude of these two risks are a function of the aflatoxin sampling plan design (variability of the aflatoxin test procedure and the accept/reject limit) and can be estimated from operating characteristic (OC) curves that are calculated knowing the variability and distribution among sample test results (Whitaker and Dickens, 1989; Whitaker *et al.*, 2006).

USDA/AMS Sampling Plan. Two OC curves are plotted in Fig. 4 and show the performance of the current USDA/AMS aflatoxin sampling plan when all 21.8 kg laboratory samples are prepared by a DM and by a VCM mill with an 1100 g test portion. Since the sample preparation variance for the DM and an 1100 g test portion accounts for only 8.1% of the total variability, the VCM and an

Table 5. Monte Carlo simulation used to sample a lot at 10 ng/g using the USDA/AMS aflatoxin test procedure (21.8 kg sample, VCM to prepare samples, 1100 g test portion, and HPLC to quantify aflatoxin in two aliquots.

Rep		VCM mill 1AB	VCM mill 2AB	VCM mill 3AB	Average of 1AB, 2AB, 3AB
1		***0***	***4***	***6***	***3.3***
2		***0***	***33***	***2***	***11.7***
3		***1***	***6***	***13***	***6.7***
4		***1***	***19***	***12***	***10.7***
5		***1***	***8***	***2***	***3.7***
6		***2***	***0***	***9***	***3.7***
7		***2***	***1***	***27***	***10.0***
8		***2***	***5***	***2***	***3.0***
9		***3***	***14***	***1***	***6.0***
10		***3***	***1***	***12***	***5.3***
11		***4***	***1***	***7***	***4.0***
12		***4***	***8***	***1***	***4.3***
13		***4***	***2***	***36***	***14.0***
14		***4***	***20***	***2***	***8.7***
15		***5***	***2***	***12***	***6.3***
16		***6***	***2***	***7***	***5.0***
17		***7***	***31***	***2***	***13.3***
18		***8***	***13***	***22***	***14.3***
19		9	4	10	7.7
20		9	4	13	8.7
21		10	15	3	9.3
22		11	18	6	11.7
23		12	18	4	11.3
24		12	7	12	10.3
25		13	3	8	8.0
26		13	23	42	26.0
27		13	29	6	16.0
28		14	12	23	16.3
29		15	13	24	17.3
30		17	5	10	10.7
31		17	9	6	10.7
32		18	0	8	8.7
33		19	8	5	10.7
34		20	3	7	10.0
35		20	13	2	11.7
36		21	9	16	15.3
37		25	10	3	12.7
38		25	4	20	16.3
39		26	4	2	10.7
40		31	29	7	22.3
All reps	Avg ppb	10.7	10.3	10.3	10.4
	Count $\leq$ 8	18	22	23	
	Count $>$ 8	22	18	17	
1AB $\leq$ 8	Avg ppb	3.2	9.4	9.7	
	Count $\leq$ 8	18	12	10	
	Count $>$ 8	0	6	8	
1AB $>$ 8	Avg ppb	16.8	10.9	10.8	
	Count $\leq$ 8	0	10	13	
	Count $>$ 8	22	12	9	

1100 g test portion reduce only a small part of the total variability and as a result the two OC curves are very similar.

Theoretically, the smaller the total variance of the aflatoxin test procedure, the steeper the OC curve about the final accept/reject limit of 15 ng/g (Whitaker *et al.*, 2006: Vargas, *et al.*, 2006). For the same test portion size (ie. 1100 g), the sample preparation variance is smaller for the VCM than the DM. Therefore, the total variance is smaller when a VCM is used to prepare the sample (compared to the DM) and as a result the OC curve for the VCM should be steeper than the OC curve where the DM is used to prepare the 21.8 kg sample. The consequences of a steeper OC curve are that fewer misclassifications will occur with the use of the VCM when compared to the DM for the same test portion size. Fewer misclassification implies that fewer good lots will be rejected (lower sheller's risk) and fewer bad lots will be accepted (lower buyer's risk). It is difficult to observe in Fig. 4 that the OC curve where the sample is prepared with a VCM is steeper than the OC curve where the sample is prepared with a DM since the sample preparation variance for the DM and an 1100 g test portion constitutes only about 8% of the total variability associated with the USDA/AMS aflatoxin test procedure.

Performance of Sampling Plan That Use Special Samples. When a lot is accepted on the first official USDA/AMS sample (1AB$\leq$8 ng/g) and the buyer request that a second special sample (2AB) must also test less than a defined limit for acceptance of the lot, the performance of that sampling plan is very different from the USDA/AMS sequential sampling plan shown in Fig. 4. The sampling plan where a buyer requests a special sample must test less than or equal to 8 ng/g is now defined as taking two 21.8 kg samples from the lot and both samples must test 8 ng/g or less to accept the lot (2$\times$21.8kg$\leq$8ng/g). The operating characteristic curves for two sampling plan where one 21.8 kg sample must test 8 ng/g or less (1$\times$21.8kg$\leq$8ng/g) and where two 21.8 kg samples must both test 8 ng/g or less (2$\times$21.8kg$\leq$8 ng/g) are shown in Fig. 5 when the samples are prepared by the DM and the VCM. A vertical line is plotted on the graph in Fig. 5 to show the FDA limit of 20 ng/g that the buyer's products must meet even though the accept/reject limit agreed upon by the seller and buyer in this example is 8 ng/g.

From Fig. 5 it can be seen that increasing the number of 21.8 kg samples that must test 8 ng/g or less from one to two shifts the OC curve to the left. and as a consequence, the seller's risk (good lots rejected) increases, but the buyer's risk (bad lots

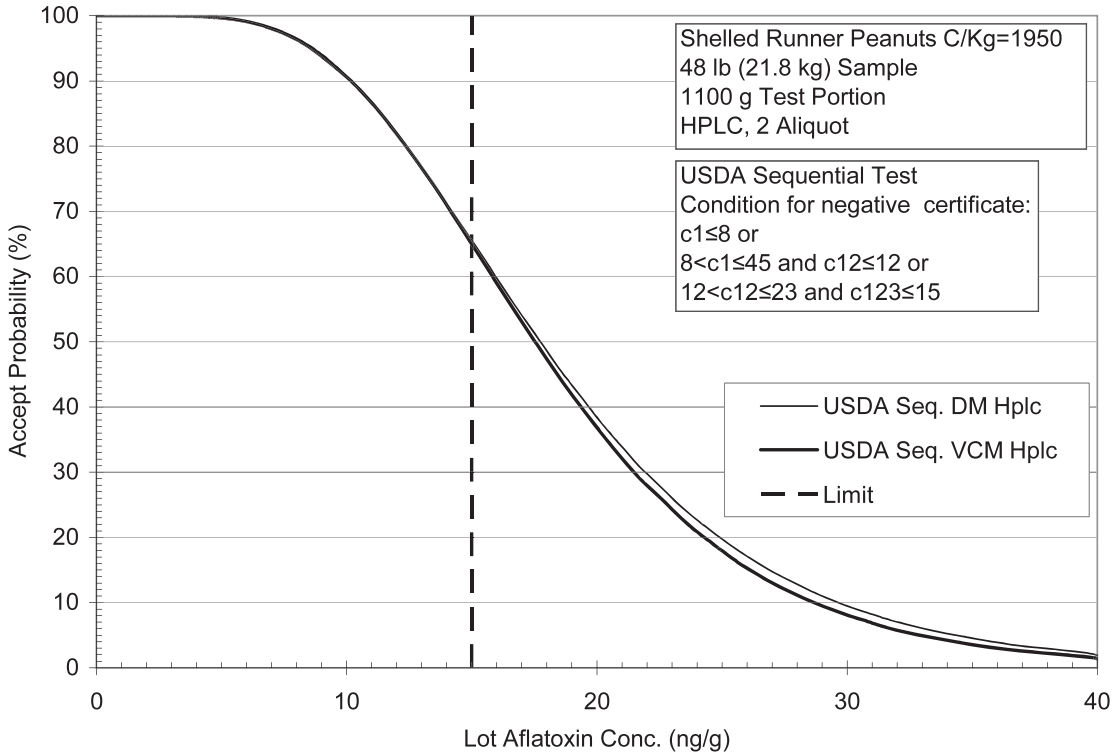

Fig. 4. Operating characteristic curves show the chances of accepting peanut lots by the USDA/AMS aflatoxin sampling plan when samples are prepared by the USDA/AMS Subsampling mill (DM) and a vertical cutter mixer (VCM) type mills.

accepted) decreases. When a special sample is added to the first USDA/AMS sample test (2×21.8kg ≤8 ng/g), the chances that the buyer will accept a lot above the FDA limit of 20 ng/g is less than 2 to 3% (Fig. 5). The seller, however, has a very large risk of rejecting good lots (lot concentrations below 20 ng/g). For example in Fig. 5, the chance of rejecting a lot at 10 ng/g is about 75% (100%-25%).

With the USDA/AMS sequential sampling plan (Fig. 4), both the seller and the buyer share in the two risks associated with the sampling plan. However, the sampling plan that requires an additional 21.8 kg special sample to accept the lot, the seller bares most of the risk associated with misclassifying a lot while the buyer has very little risk of accepting a bad lot or lots > 20 ng/g. In crop years where aflatoxin levels are elevated, the large seller's risk can impose a large economic burden on the seller. The performance of a sampling plan that requires two 21.8 kg samples to both test 8 ng/g or less is approximately the same regardless of the type mill used to prepare the samples (Fig 5).

There are almost no benefits (lower variability) associated with using a VCM to prepare samples when both 21.8 kg samples must test 8 ng/g or less to accept or reject a lot (Fig. 5). For example, the probability of accepting a lot at 20 ng/g with one 21.8 kg sample using the DM and VCM to prepare the sample is 0.17 and 0.15, respectively, or a difference of 0.02. When two 21.8 kg samples must both test 8 ng/g or less, the difference in the accept probability becomes $(0.02)^2$ or 0.0004. As seen in Fig. 5, the difference between the two OC curves is almost nil for the VCM and DM at 20 ng/g for the sampling plan that requires two samples to both test 8 ng/g or less.

Summary and Conclusions

When the Official USDA/AMS 1AB sample is prepared for analysis by either the DM or the VCM, the average aflatoxin among official samples testing ≤ 8 ng/g (or any other limit) will be less than the average aflatoxin among special samples. The difference in the average aflatoxin between official and special sample test results is not due to the type of mill used to prepare samples, but due to the use of a cut-off limit that restricts official sample test results to values below the cut-off limit while not restricting sample test results of the special samples. Even when the same mill is used to prepare both the official and special samples, the average aflatoxin among official samples is less than that of the special samples. The cut-off limit restricts high values for the official sample (1AB) from being included while there is no restriction on the special sample values.

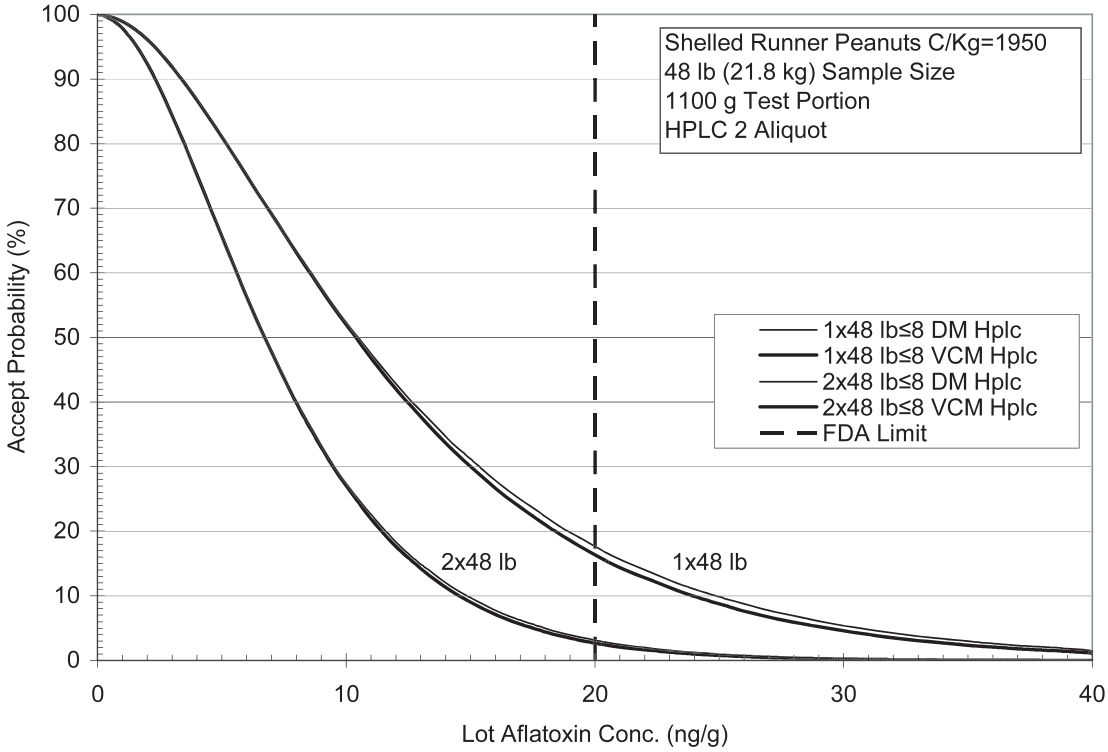

Fig. 5. Operating characteristic curves show the chances of accepting peanut lots by sampling plans that require either one or two 21.8 kg samples to test 8 ng/g or less when samples are prepared by the USDA/AMS Subsampling mill and the vertical cutter mixer type mills.

The only way that a test portion prepared by the VCM will measure more aflatoxin than a test portion prepared by the DM is for (a) the VCM creates aflatoxin, (b) the DM destroy aflatoxin, or (c) the analytical method has a higher extraction efficiency associated with the test portions prepared with a VCM than with a DM after the water-slurry process. The last option is the most plausible, but not likely since the 1100 g test portion from the DM is further comminuted in the blender during the water slurry preparation and has decreased particle size even further.

An unintended consequence of a sampling plan that uses the VCM and test portions less than 104 g to prepare 21.8 kg samples is the total variance of the aflatoxin test procedure is larger than a similar sampling plan that uses a DM and an 1100 g test portion. If the test portion associated with the VCM is greater than 104 g, there will be slightly fewer lots misclassified by the sampling plan than one that uses a DM with 1100 g test portion. However, since the sample preparation variance of the DM and 1100 g test portion accounts for only about 8% of the total variability of the USDA/AMS aflatoxin test procedure, the decrease in the number of misclassification is minimal for one 21.8 kg sample and non existent when two 21.8 kg samples are used to accept or reject a lot.

Another consequence of requiring two 21.8 kg samples (the special and official samples) to both test less than a limit (ie., the USDA/AMS limit of 8 ng/g) is to have a sampling plan design that has a very low buyer's risk (bad lots accepted), but a very high seller's risk (good lots rejected). In years where aflatoxin levels are elevated, the large seller's risk can impose a large economic burden on the seller. The performance of a sampling plan that requires two 21.8 kg samples to both test 8 ng/g or less is approximately the same regardless of the type mill used to prepare the samples.

Each mill has it strengths and weaknesses, which provide a basis for choosing a specific mill to prepare samples. Since the contribution of the sample preparation step to the total variability of the aflatoxin test procedure is small, either mill can be chosen without significantly affecting the total variability of the overall aflatoxin test procedure. A more effective method of reducing the total variability of the aflatoxin test procedure is to select more samples from the lot since the sampling step contributes about 88% of the total variability.

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