



Standardisation of mycotoxin sampling procedures: an urgent necessity

Thomas B. Whitaker *

USDA, ARS, Box 7625, North Carolina State University, Raleigh, NC 27695-7625, USA

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Abstract

A mycotoxin sampling plan is defined by the mycotoxin test procedure (sample size, sample preparation method, and analytical method) and the accept/reject limit. Because of the variability associated with each step of the mycotoxin test procedure, the true mycotoxin concentration of a bulk lot cannot be determined with 100% certainty. As a result, some lots will be misclassified by the sampling program. Some good lots will be rejected by the sampling plan (seller's risk or false positives) and some bad lots will be accepted by the sampling plan (buyer's risk or false negatives). The magnitude of these risks is directly related to the magnitude of the variability associated with the mycotoxin test procedure. It is difficult for an exporter to have an effective control program when regulatory limits and sample designs differ greatly among trading countries. In order to facilitate trade and provide protection for the consumer, it would be desirable for all trading countries to have the same mycotoxin limits and sample plan. While standardization of sampling plans among trading nations is important, any standardised sampling plan must be designed to minimize both the seller's and buyer's risks to the lowest possible levels that resources will allow. Reducing the variability of the mycotoxin test procedure will reduce both the buyer's and seller's risks. It is important to understand the sources of error in the mycotoxin test procedure so the errors can be effectively reduced. The sampling step usually is the largest source of error due to the extreme mycotoxin distribution among kernels in the lot. As an example, sampling (5 kg), sample preparation (USDA subsampling mill and 250 g subsample), and analysis (TLC) accounted for 83%, 9%, and 8% of the total aflatoxin testing error, respectively, when testing raw shelled peanuts for aflatoxin. Examples are given to show how increasing sample size reduces sampling error; increasing the fineness of grind and using larger subsamples reduces sample preparation error, and increasing the number of aliquots analyzed and using improved technology (HPLC versus TLC) decreases analytical error. International organizations such as FAO/WHO have used scientific techniques to evaluate and design aflatoxin sampling plans for raw shelled peanuts traded in the export market. Published by Elsevier Science Ltd.

1. Introduction

In a 1995, a global survey was conducted that showed at least 77 nations had mycotoxin regulations, mainly for the control of aflatoxin in both food and feed (Food & Agriculture Organization, 1997). These regulations, published in the FAO Food and Nutrition Paper 64, varied widely among the 77 countries. Mycotoxin regulations are generally implemented using a defined maximum limit and a sampling plan to detect and divert mycotoxin-contaminated products from food and feed markets. Variations among maximum limits and sampling plans make it difficult for exporters and importers to market commodities in the world market. The world

community, working through FAO/WHO, has readily acknowledged the need to harmonize or standardize mycotoxin sampling plans and maximum limits in order to facilitate international trade and improve consumer protection. Standardization of mycotoxin sampling plans implies: (a) developing a uniform sampling plan to be used by all buyers and sellers of agricultural commodities and (b) developing a uniform mycotoxin sampling plan that has a higher performance than most sampling plans currently being used in the marketplace. Performance of a sampling plan generally refers to several factors such as the frequency that false positives and false negatives occur, accuracy of detecting contaminated lots, and amount of contamination removed from the marketplace. It is important to know how to design standardized sampling plans in order to maximize performance. The objectives of this paper are: (a) identify the design elements of a mycotoxin sampling

* Tel.: +1-919-515-6731; fax: +1-919-515-7760.

URL: <http://www5.bae.ncsu.edu/usda/www/whitaker1.htm>.

plan that have to be considered in any standardisation process and (b) show how to make choices for these elements to maximize performance.

2. Definition

A mycotoxin sampling plan can be defined by a test procedure and a maximum limit. The test procedure is associated with steps used to quantify the mycotoxin in a sample. The maximum limit is a predefined threshold that separates acceptable lots from unacceptable lots. The mycotoxin level in a bulk lot is usually estimated by measuring the mycotoxin in a small portion of the lot called a sample. It is assumed that the lot concentration is the same as the sample concentration and decisions about the bulk lot are made based upon the sample value. Generally the decisions concerning a bulk lot are to: (a) estimate the true lot concentration or (b) determine if the lot is above or below some maximum limit. Often the exact lot concentration is needed in research situations, such as feeding studies. However, sampling plans used in regulatory environments are primarily concerned with knowing if the lot concentration is above or below a maximum limit and not knowing the exact lot concentration.

3. Test procedure

A mycotoxin test procedure generally consists of three steps: (a) sampling step, (b) sample preparation step, and (c) analytical step. The sampling step consists of selecting a sample of a given size from a bulk lot. The sample preparation step is a two-step process that includes: (a) grinding the sample in a mill and (b) taking a subsample of ground kernels from the comminuted sample. The analytical step consists of several steps where the mycotoxin is solvent extracted from the subsample, the solvent is purified, and the mycotoxin in the solvent is quantified. The mycotoxin value, measured in the analytical step, is then used to estimate the lot concentration or is compared to a maximum limit in order to classify the lot as acceptable or unacceptable.

The test procedure is an amazing process from two aspects. A very small quantity of the lot is finally used in the quantification step to estimate the mycotoxin concentration in the lot. The mass (weight) of product used at each step of the test procedure is continually reduced to smaller amounts. As an example, if a 25-kg sample is taken from a 25,000-kg lot, we have reduced the quantity inspected by a factor of 1000 at the sampling step. Then the 25-kg sample is comminuted in a mill and a 250-g subsample is removed for quantification of the mycotoxin. The quantity of product inspected at the sample preparation step has been reduced by a factor of

100,000. Finally, about 1 g of product is represented in the solvent mixture from which the mycotoxin is quantified. In this example, only 1 g out of the original 25,000,000 g is used to estimate the mycotoxin concentration in the lot. Second, a mycotoxin such as aflatoxin is measured in ng (10^{-9} g) of aflatoxin per g of product or parts per billion (ppb). For example, 1 ppb is 1 ng of aflatoxin per g of product. This is equivalent to measuring 40 cm in the distance from the earth to the moon or 1 s in 32 years. The fate of a 25-ton lot is decided on 1 g of product measured in the units of ppb.

4. Standardisation process

Based upon the definition of a mycotoxin sampling plan, the following elements of a sampling plan must be considered in any standardization process:

- (a) method of selecting the sample
- (b) sample size
- (c) degree of sample grind (particle size reduction)
- (d) subsample size
- (e) type analytical method
- (f) number of analytical measurements
- (g) maximum limit

There are many choices that can be made for each of the above elements. However, some choices are better than others. In any standardization process, choices should be made that improve the performance of a standardized mycotoxin sampling plan. Sampling plans with poor performances can easily occur because you can never determine the true concentration of a lot with 100% certainty by measuring the concentration in samples taken from the lot. The problem with estimating the true lot concentration is illustrated below. This example illustrates taking ten 5-kg samples from the same lot of raw shelled peanuts and measuring aflatoxin in each sample (Whitaker, Dickens, Monroe, & Wiser, 1972).

0 0 0 0 3 13 19 41 43 69

The best estimate of the true aflatoxin concentration in the bulk lot of shelled peanuts is the average of the 10 sample test results or 19 ppb. You can see how difficult it would be to take a single 5-kg sample from this peanut lot and try to make an intelligent decision about either the exact concentration in this lot or if the lot concentration is above or below a maximum limit such as the EU 15 total ppb for shelled peanuts destined for further processing. Assuming the true aflatoxin concentration in this lot is 19 ppb, the lot should be classified as unacceptable. However, 6 of the 10 sample test results are below the maximum limit of 15 ppb. As a result, the lot would be incorrectly classified as being acceptable 60%

of the time and correctly classified as unacceptable 40% of the time.

Because of the variability among sample test results, two types of mistakes will be made when classifying lots as acceptable or unacceptable based on a defined maximum limit. Some unacceptable lots will be classified as acceptable (seller's risk or false positive) by the sampling plan and some unacceptable lots will be classified as acceptable (buyer's risk or false negative) by the sampling plan. The frequency with which the buyer's and seller's risks occur is dependent on the design of the sampling plan (choice of test procedure and maximum limit). If all mycotoxin sampling plans were standardized to use the same test procedure and maximum limit, then all sampling plans should have the about the same level of buyer's and seller's risks. In addition, design elements could then be chosen to maximize performance by reducing the variability of the test procedure, which will reduce the buyer's and seller's risk associated with a standardized sampling plan.

To reduce variability associated with a test procedure, the sources of variability must first be understood, then elements of the sampling plan can be chosen to reduce the variability. There is random variation associated with each step of the test procedure. Replicated sample concentrations will vary about the true lot concentration; replicated subsample concentrations will vary about the true sample concentration; and replicated analytical measurements will vary about the true subsample concentration. Each step of the test procedure contributes to the overall variability of the test procedure. The total variability, as measured by the variance, is equal to the sum of sampling, sample preparation, and analytical variances.

In any standardisation process, the design elements should be chosen to reduce variability of the test procedure. Since there is a different cost associated with reducing the variability associated with each step, it is important to know how to reduce the variation and how much each step contributes to the total variability associated with a test procedure. Error reduction costs money, so the best use of resources should be based on providing the greatest reduction in error. An example of the variability associated with measuring aflatoxin in shelled peanuts is shown in Table 1. The test procedure consisted of using a 5-kg sample, United States Department of Agriculture, Agricultural Marketing Service (USDA/AMS) approved subsampling mill, 250-g subsample, TLC analysis, and one measurement. Studies (Whitaker, Dickens, & Monroe, 1974) show that sampling variance is 521.4, sample preparation variance is 59.2; and analytical variance is 50.1. The total variance associated with the test procedure is the sum of the sampling, sample preparation, and analytical variances or 630.7. We can see that for a 5-kg sample, USDA/AMS subsampling mill, 250-g subsample, TLC analysis,

Table 1
Variability associated with a test procedure that uses a 5-kg sample

Test procedure	Variance	Ratio (%)
5 kg	521.4	82.7
AMS, 250 g	59.2	9.4
TLC 1	50.1	7.9
Total	630.7	100.0

Agricultural Marketing Service (AMS) of the US Department of Agriculture subsampling mill, 250-g subsample, and thin layer chromatography (TLC) to estimate aflatoxin in shelled peanut lots at 20 ng/g.

and one measurement that sampling, sample preparation, and analysis accounts for 83%, 9%, and 8% of the total variation, respectively.

5. Error reduction of test procedure

5.1. Sampling

Sampling is usually the largest source of error. This is because a small percentage of the kernels are contaminated and a contaminated kernel can have extremely high levels of the mycotoxin (Whitaker & Wiser, 1969). With small samples, it is difficult to get a contaminated kernel in the sample. Studies have shown that less than 1 kernel per 1000 (0.1%) is contaminated in a lot of raw-shelled peanuts (Whitaker et al., 1974). The sample has to be selected in such a way that every kernel in the lot has an equal chance of being chosen. The sample should be the accumulation of many small incremental portions taken at many different locations throughout the lot (Whitaker, 2000). It is easier to select a representative (lack of any bias) sample from a moving stream of product than from a static lot such as trucks or rail cars. Even if the sample is representative, sample concentrations will vary about the lot concentration. Increasing sample size can reduce sampling error. For example, if the sample is increased from 5 to 20 kg, sampling error is reduced from 521.4 to 130.4 (Table 2). Increasing sample size by a factor of 4 reduces sampling variance to 1/4 of the original amount or 130.4. In any standardization process, one should always consider using large sample sizes.

5.2. Sample preparation

Next, the sample is ground in a mill. The kernels are broken into many small pieces. Then a small subsample of ground product is removed from the comminuted sample. The mycotoxin concentration in replicated subsamples will also vary about the sample concentration. Sample preparation error is reduced by: (a) increasing subsample size and (b) grinding the sample into finer particles. Increasing subsample from 250 to 1000 g

Table 2
Comparison of variability associated with two different test procedures used to estimate aflatoxin in shelled peanuts

Test procedure	Variance
5 kg	521.4
AMS, 250 g	59.2
TLC 1	50.1
Total	630.7
20 kg	130.4
VCM, 100 g	25.5
HPLC 1	20.1
Total	176.0

Sample size is increased from 5 to 20 kg; subsampling mill used by the Agricultural Marketing Service (AMS) is replaced by a vertical cutter mixer (VCM); subsample size is decreased from 250 to 100 g; and one aliquot analyzed by thin layer chromatography (TLC) is replaced by one aliquot analyzed by high performance liquid chromatography (HPLC).

for the USDA/AMS mill reduces sample preparation error from 59.2 to 14.8. Using a vertical cutter mixer (VCM) with a 100-g subsample instead of the USDA/AMS mill with 250 g, reduces the error from 59.2 to 25.5 (Table 2). In the standardization process, smaller subsample sizes can be used with mills or grinders that give a very fine particle size. Larger subsamples sizes are needed when using a mill that gives larger particles sizes.

5.3. Analysis

Finally the mycotoxin in the subsample is quantified using an analytical procedure. Most analytical procedures are complex procedures involving several steps where errors can occur. Analytical error is reduced by: (a) increasing the number of measurements made on the subsample extract and (b) using analytical methods with superior technology (i.e., HPLC vs. TLC). Increasing the number of measurements from one to two using TLC analytical methods reduces the analytical error from 50.1 to 25.0. Changing from TLC to HPLC reduces the error from 50.1 to 20.1 (Table 2).

5.4. Test procedure

The first test procedure (5-kg sample, USDA/AMS mill, 250-g subsample, TLC analytical method, and one measurement) used to estimate aflatoxin in a shelled peanut lot at 20 ppb has a variance of 630.7. Assuming normality, a variance of 630.7 indicates that repeated sample test results will vary 95% of the time from 20 ± 50 ppb or from 0 to 70 ppb. Once elements of the first test procedure are changed in a way described above (20-kg sample, VCM mill, 100-g subsample, HPLC analytical method, and one measurement), the variance associated with the second test procedure is

reduced to 176.0. A variance of 176.0 indicates that repeated sample test results from a shell peanut lot at 20 ppb will vary 95% of the time from 20 ± 26 ppb or 0 to 46 ppb. As a result of reducing the variability, both buyer's and seller's risks will be reduced with improved precision among sample test results.

6. Maximum limit

Maximum limits are established to reduce the amount of contaminated food and feed products that reach consumers or animals. The dilemma in any standardization process is how low to set the maximum limit. Lowering the maximum limit will (a) reduce contamination in the food and feed market and (b) increase the amount of product rejected in the testing program. Any standardized maximum limit should be low enough to protect the health of the consumer, but not so low as to limit the supply of food. The FAO and WHO sponsor Joint Expert Committees on Food Additives (JECFA) to conduct risk assessments of various mycotoxins to help establish maximum limits for mycotoxins.

7. Standardized sampling plan

To show that standardisation can work, FAO/WHO working through the CODEX committee structure, has developed an aflatoxin-sampling plan for raw shelled peanuts traded in the export market and destined for further processing. The sampling plan calls for a 20-kg sample and a maximum limit of 15 ng/g total aflatoxins. Currently, the CODEX Committees on Methods of Analysis and Sampling and Committee on Food Additives and Contaminants are developing a document for member nations that demonstrates how to implement the FAO/WHO aflatoxin sampling plan for raw-shelled peanuts. The document explains how to select a representative sample from a bulk lot, prepare the sample, and sets minimum performance standards for analytical methods.

Standardizing mycotoxin-sampling plans will uniformly establish the levels of buyer's risk (false negatives) and seller's risk (false positives) associated with sampling plans used by all traders of agricultural commodities in the export market. In addition, increasing sample size, subsample size, and number of analytical measurements will reduce variability (increase precision) associated with a mycotoxin test result, reduce the buyer's and seller's risks, and increase costs of the mycotoxin test procedure. Reducing the maximum limit will reduce contamination in accepted lots, increase lots rejected in the marketplace, and increase costs to reclaim rejected lots. Methods to evaluate the performance of aflatoxin-sampling plans have been developed for shelled peanuts

(Food & Agriculture Organization, 1993). The evaluation method was used to design aflatoxin-sampling plans with improved performance for FAO/WHO.

References

- Food and Agriculture Organization (1993). Sampling plans for aflatoxin analysis in peanuts and corn. FAO Food and Nutrition Paper 55 (75pp), Rome, Italy.
- Food and Agriculture Organization (1997). Worldwide Regulations for Mycotoxins 1995, A compendium. FAO Food and Nutrition Paper 64 (43pp), Rome, Italy.
- Whitaker, T. B. (2000). Sampling techniques. In M. W. Trucksess, & A. E. Pohland (Eds.), *Methods in molecular biology mycotoxin protocols: vol. 157* (pp. 11–24). Totowa, NJ: Humana Press.
- Whitaker, T. B., Dickens, J. W., & Monroe, R. J. (1974). Variability of aflatoxin test results. *Journal of American Oil Chemists' Society*, 51(5), 214–218.
- Whitaker, T. B., Dickens, J. W., Monroe, R. J., & Wiser, E. H. (1972). Comparison of the observed distribution of aflatoxin in shelled peanuts to the negative binomial distribution. *Journal of American Oil Chemists' Society*, 49(10), 590–593.
- Whitaker, T. B., & Wiser, E. H. (1969). Theoretical investigations into the accuracy of sampling shelled peanuts for aflatoxin. *Journal of American Oil Chemists' Society*, 46(7), 377–379.