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Variance Structure of Aflatoxin Contaminated Maize In Incoming Trucks at Commercial Mills in Kenya

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Abstract

Aflatoxin contamination in maize represents a significant food safety hazard in Kenya. To manage this risk, millers need to properly sample incoming bags of maize. In this study, three firms allowed researchers access to sample three incoming trucks each to quantify sources of variability using a hierarchical sampling design. A total of 180 samples consisting of two samples per bag, 10 bags per truck, three trucks per mill, and three mills were sampled in April of 2014 and analyzed for aflatoxins using the Romer-FluorQuant test method. Samples from three trucks were analyzed twice for aflatoxin to define intra-bag variability, and extracts were analyzed in duplicate to quantify analytical variability. The variance was partitioned as follows: mill (1.9%), truck (4.1%), bag (60.8%), in-bag (26.6%), analytical error (6.3%) and residual error (0.3%). Using these data, a power study was conducted to optimize the number of bags per truck that should be sampled. The power test, depicted using a power curve, indicated that 20 bags provided a statistical probability of 0.83. This study characterizes the sources of variability in sampling, and the inclusion of a power study highlights the tradeoffs in the number of bags sampled and the ability to detect small effects in estimating the level of aflatoxin contamination in maize.

Keywords: Kenya, aflatoxin, maize, variance component analysis, mill, risk management, regulatory, agency

1. Introduction

Aflatoxins are a group I carcinogen, as defined by the International Agency for Research on Cancer [25], and have been documented to occur at fatal levels in humans consuming maize in Kenya [24]. The most prominent aflatoxin-producing fungal species are *Aspergillus flavus* and *A. parasiticus*, which can attack many different types of crops and tree nuts [16]. High aflatoxin levels in maize have caused widespread health and economic problems in Kenya [10], and a regulatory limit of 10 μg/kg (total aflatoxin) has been established in Kenya for food and feed products [1]. A science-based sampling system using data collected from incoming bags of maize in trucks delivering to commercial mills was used to identify the sources of variability and to perform a power study, to help manage tradeoffs in sampling cost versus the ability to detect small effects in aflatoxin levels between truckloads of maize.

At present, no sampling scheme for aflatoxin analysis has been developed by the Codex Alimentarius; however, a sampling standard does exist for fumonisins [5]. Prescribed sampling schemes for aflatoxin contamination exist for maize in the United States [21]. Specifically, the sampling scheme for a truckload containing up to 30 tons of maize would be probed nine times and a minimum of 0.9 kg of maize would be collected and ground for aflatoxin analysis. The East African Community (EAC) adopted a standard for sampling in 2008 following the ISO 13680:1999 standard [7], that applies to bag and bulk containers. The latter standard prescribes a sample number for trucks containing 200 bags at 14 bags and a minimum mass of 1 kg. An updated version of the EAC standard for Cereal and Pulses Sampling was adopted by Kenya in 2017 [8]. This standard prescribes a minimum mass of the laboratory sample of 10 kg, and the number of bags sampled for a consignment containing 101 to 1000 bags would require that no less than 50 bags be sampled [8].

Prior studies using a hierarchical study design identified sources of variability in a controlled experiment [15] and in a commercial setting involving bulk loads of grain containing approximately 30 tons [11]. The present study evaluated the variance structure in commercial truckloads of bagged maize delivered to commercial mills in Nairobi, Kenya. The data also lent itself to performing a power analysis to identify the number of bags to sample at a specified probability level. (Power studies are used to determine a sample size that would give the

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analysis enough statistical power.) The objective was to support marketing research exploring consumer demand for third-party verified maize flour that was tested for aflatoxin [14]. Toward that end, the present study included two goals: 1) identify the main contributors to sampling variability, and 2) identify an economically-feasible number of samples that would provide sufficient power to the sampling plan to assess aflatoxin contamination in a truckload of maize.

2. Materials and Methods

2.1. Sampling

Three mills in Kenya permitted a technician employed by the International Food Policy Research Institute to sample three incoming trucks containing approximately 200 bags of maize weighing 90 kg each. For each one of the three mills, three trucks were randomly selected and 10 bags were sampled twice. Samples were collected using a Seedburo (Des Plaines, IL) handgrip bag trier (17 1/2 x 1 1/8 in.). Samples weighed approximately 500 g and were taken at two locations within each bag, resulting in a total of 180 samples.

2.2. Sample Preparation and Analysis

Aflatoxin-contaminated maize samples were ground in a Romer mill (Model 2A, Romer Labs, Inc., Washington, MO). The aflatoxin testing procedure was performed using a Romer FlouroQuant system at the Texas A&M AgriLife ISO 17025-accredited laboratory, at the Biosciences eastern and central Africa (BecA) International Livestock Research Center (ILRI) hub, in Nairobi, Kenya. Prior to the aflatoxin testing in Kenya, the Romer-FluorQuant test method had already been validated based on the United States Deaprtment of Agriculture-Federal Grain Inspection Service (USDA-FGIS) criteria [23] using in-house validation data for maize samples at three aflatoxin levels (59, 306, and 901 μg/kg). The performance of the test method was evaluated by comparing it with the high-performance liquid chromatography (HPLC) method (an ISO 17025 accredited method), as described in an earlier study [6]. The validation study showed that accuracy, prediction, and robustness of the test method were comparable to those of the HPLC method, with no statistical significant difference in the results ($p > 0.05$) between the two methods. The ruggedness test also revealed that the recovery of aflatoxins ranged from 80 to 120 percent. The limits of detection (LOD) and quantitation (LOQ) of the Romer-FluorQuant test method for aflatoxins in maize were 0.7% and 2.3 μg/kg, respectively.

Two 50-g portions of ground maize were analyzed for each sample, resulting in four analyses per bag and a total of 360 aflatoxin analyses. In addition, samples were run in duplicate for one truck at each mill, resulting in eight analyses per bag for three trucks and a total of 480 aflatoxin analyses. This analysis scheme was performed to enable researchers to partition variance down to analytical error. At the beginning and at the end of each day, a 50-g portion of reference material was analyzed, and the results were plotted on a control chart. (The control chart plotted over time provides historical and cumulative data and information that are useful for determining if a measurement is in statistical control and whether there is a need for improvement in the analytical procedure.)

The reference material used for this study was developed by the Office of the Texas State Chemist (OTSC) at its headquarters in College Station, Texas, USA. Reference material was prepared by analyzing 12 samples per 20-kg batch for aflatoxin by the HPLC-fluorescence detection method of the Association of Official Analytical Chemists [2]. In the HPLC method, a 50-g sample of ground maize was extracted with 250 mL of 70% methanol (v/v) and shaken for 1 hr at 200 rpm. A 10-mL aliquot of extract was mixed with about 1 g of NaCl and 20 mL of deionized water for dilution. After filtration of the diluted extract, 2 mL were loaded onto an Aflatest™ immunoaffinity column (Vicam, Somerville, MA) and eluted by washing twice with water and then with methanol. The eluent was placed in an autosampler vial and injected into a Waters 2695 HPLC system (Waters, Milford, MA). The HPLC instrumentation consisted of a Waters model 746 data module integrator, tube-and-shell membrane reactor module, a Waters model 2695 autosampler, a Waters model 2695 LC pump, and a Water 2475 Multi -fluorescence detector set at 360 nm for excitation and 420 nm for emission. Sample data acquisition and analysis were carried out using the Empower software (Milford, MA). Other detailed analytical conditions and procedures are given in earlier studies [6, 17]. Certified aflatoxin standards (B_1, B_2, G_1, and G_2) used in the study were purchased from Romer Labs-Biopure (Tulln, Austria). The concentration of aflatoxin standards B_1 and G_1 was 2 μg/mL acetonitrile, while aflatoxin standards B_2 and G_2 had a concentration of 0.5 μg/mL acetonitrile. All chemicals, solvents, and reagents were of analytical grade and used as supplied.

2.3. Statistical Analyses

Descriptive statistics were calculated using Excel® software. The variance structure analysis was performed using OTSC individual probe and composite probe data, which were subsequently analyzed using mixed models in the NESTED and GLM procedures of SAS® software, respectively (ver. 9.2, SAS Institute, Cary, NC). The p-values corresponding to F-statistics were calculated for each variable (*i.e.*, mill, truck, bag, in-bag, and analysis) using the GLM procedure.

A power study was used to determine the optimal number of bags required to estimate the aflatoxin level for each truck. Effect size was determined based on the pilot study data. The procedure was performed using the pwr package in R software [4]. The power study used was for two proportions.

3. Results and Discussion

3.1. Descriptive Statistics

The descriptive statistics for each of the nine trucks in the study are recorded in Table 1. Aflatoxin levels ranged from 0 to 1,141 μg/kg. Aflatoxin was detected in 60 (66.7%) of the 90

Aflatoxin Descriptive Statistics	**Mill 1**			**Mill 2**			**Mill 3**		
Truck	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
Samples number	45	45	70	45	70	45	70	45	45
Mean (μg/kg)	1.8	0.6	1.5	0.4	111.4	0.9	0.9	0.4	0.1
Percent of samples above 10 μg/kg	2.2	0.0	2.9	0.0	20.0	2.2	2.9	0.0	0.0
Standard deviation (μg/kg)	2.3	1.5	6.8	1.4	268.2	2.0	2.7	1.5	0.3
Standard error (μg/kg)	0.3	0.2	0.8	0.2	32.1	0.3	0.3	0.2	0.1
Median (μg/kg)	1.3	0	0	0	0	0	0	0	0
Kurtosis	14.2	6.8	47.9	25.7	6.4	9.6	9.3	12.0	18.2
Skewness	3.3	2.7	6.7	4.9	2.6	2.9	3.2	3.5	4.4
Minimum (μg/kg)	0	0	0	0	0	0	0	0	0
Maximum (μg/kg)	13.9	6.8	54.0	8.6	1,140.7	10.2	13.7	7.5	1.7

Table 1: Descriptive statistics for each of the sampled trucks, by mill.

sampled bags. Five of the nine incoming trucks sampled contained at least one sampled bag that had an aflatoxin level over 10 μg/kg, the upper regulatory limit for aflatoxin contamination in Kenya. There was one truck in which two of the 10 bags had aflatoxin levels above the 10 μ/kg regulatory limit. One of the bags had a mean aflatoxin level of 433 μg/kg (standard deviation of ±163 μg/kg), whereas the other bag had a mean of 856 μg/kg (standard deviation of ±512 μg/kg).

In all trucks, the minimum aflatoxin level was zero and the maximum aflatoxin concentration by truck ranged from 1.7 to 1,141 μg/kg. The coefficient of kurtosis was greater than three for all trucks, indicating a lighter-tailed distribution, and the positive coefficient of skewness indicated fewer samples per truck with high aflatoxin concentration. These results differ from an examination of bulk truckloads of maize highly contaminated with aflatoxin reclaimed from commercial grain elevators in Texas, which displayed a heavy concentration around the mean aflatoxin value (heavy tail) and a coefficient of skewness of less than one [11]. A partial explanation of the difference between studies includes: lower aflatoxin concentration in the Kenya study; maize sampled from bags versus bulk; mixing in a grain bin in Texas; and possibly a wider range of farms and growing conditions for the maize in the Kenya study.

3.2. Variance Components of Aflatoxin Distribution

The variance component analysis for an unbalanced nested design partitioned aflatoxin variances into mill (1.9%), truck (4.1%), bag (60.8%), in-bag (26.6%), analytical error (6.3%), and residual error (0.3%). Table 2 has the percent of total variance and F-values for each variance component, which explains the amount of variability contributed by each variable (e.g. mill, truck, bag) and whether the variable significantly contributed to the total amount of variability. The total variance of aflatoxin concentration mainly consists of bag and in-bag variance. The unbalanced nested experimental design occurred due to duplicate analyses performed on sample extracts for three trucks, one from each mill.

The sampling error characterizes the intra-truck variation captured through the individual probes of maize. The coefficient of variation among individual probes within a bag ranged between 1.24 and 4.74%, with an average CV of 3.02% across nine trucks.

3.3. Design Structure

A power study was used to evaluate the tradeoff between the number of bags that need to be sampled to detect aflatoxin contaminated maize at a 0.8 probability. To calculate sample size, the dichotomous variable for aflatoxin levels above 10 μg/kg was used. A two-proportion power test was used with the greatest truck proportion and the smallest truck proportion of aflatoxin contaminated maize. Four of the nine trucks had proportions of zero, while the greatest proportion was 20 percent. An alpha level of 0.05 and a power of at least 0.8 were used. With a sample size of 20 bags, the statistical power was 0.83. Figure 1 shows the power curve with different sample size values at different probability levels. Twenty bags per truck was found to be the minimum number of bags necessary to obtain an 0.8 probability and represents the tradeoff between sampling/detection and what is feasible for commercial millers to detect aflatoxin.

3.4. Limitations

One limitation in the study relates to the hierarchical design of the research. Specifically, the study was designed to answer two questions: first, to characterize the variance structure of aflatoxin contamination, and second, to identify a sample number that provides sufficient power in estimating the aflatoxin

Variance Source [a]	F-value	p-value	Variance Component	Percent (%) of Total Variance
Mill	0.91	0.45	229.3	1.9
Truck	2.16	0.06	498.8	4.1
Bag	3.75	<0.0001	7,360.5	60.8
In-bag	10.23	<0.0001	3,221.3	26.6
Analysis	27.07	<0.0001	762.5	6.3
Error			34.7	0.3
Total			12,107.1	100.0

Table 2: Variance of aflatoxin distribution by variance source.

[a] Values presented in this table are obtained from both NESTED and GLM procedures of SAS.

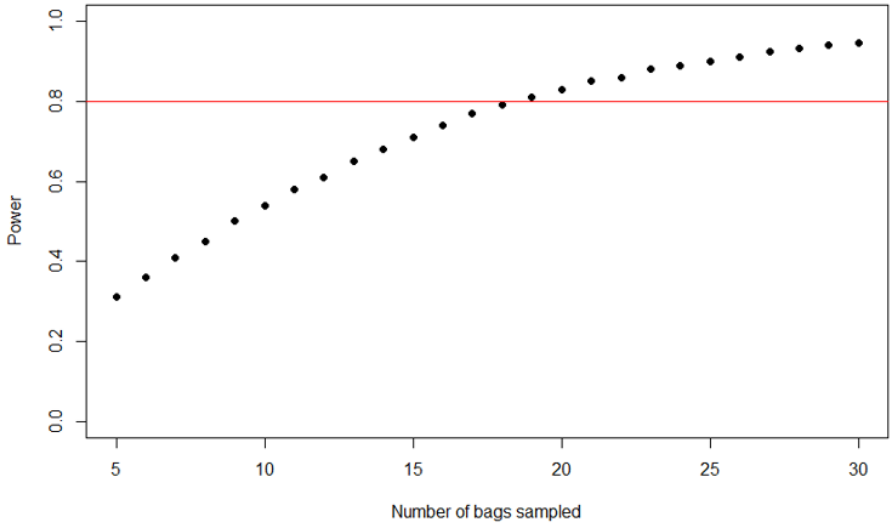

Figure 1: Statistical power curve for the number of bags sampled per truck.

contamination. Due to the high number of samples analyzed per truck, the study was limited to a total of nine trucks. Aflatoxin levels vary by year and season, and in this particular study the levels of aflatoxin were relatively low. Another limitation was that the data were not normally distributed and was zero inflated. A third limitation was that the nature of aflatoxin detection is highly variable. For example, it would not be unusual to analyze one sample from a bag of maize and obtain an aflatoxin level of 50 μg/kg and then analyze another sample from the same bag and not detect aflatoxin. These results do not, however, deviate from prior studies, and as such, are inherent to the nature of aflatoxin distribution [15, 18]. Multiple sampling was performed to address such a problem, but the data were still quite variable.

3.5. Risk Management Implication

This study documents the aflatoxin variance structure of bagged maize delivered to commercial mills. While limited in scope, the results provide an insight into how to develop a sampling program to manage risk based on samples collected in commerce. The application of a power study method seeks to find a tradeoff between the cost of sampling and the feasibility of implementing an aflatoxin risk management program in the Kenya market. The power study based on data from this study led researchers to conclude that a feasible number of samples to detect aflatoxin is 20 samples. This number is greater than the 2008 sampling recommendation of 14 bags and 1 kg [7], and less than the newly adopted method of 50+ bags and 10 kg [8]. Sampling bags is more cumbersome than bulk; nevertheless, the deviation between samples size required by USDA versus Kenya is approximately 10-fold. While the consumption of maize by humans in Kenya and by animals in the US can partially explain the need for additional efforts to control aflatoxin contamination in Kenya, the level and frequency of aflatoxin contamination in maize between the two countries are not that great, particularly when examining the level of afla-

toxin contamination in Texas [12]. While it may be feasible to implement the new Kenya standard for aflatoxin sampling at commercial mills, the study helps define the uncertainty associated with sampling to manage aflatoxin risk.

This study, similar to others, documents that analytical variability is low (6.3%) in a controlled experiment. However, it would be incorrect to interpret these results to suggest that the aflatoxin analysis is a minor contributor to the overall variability in aflatoxin testing in the market place. Rather, it reaffirms that it is possible to control testing variability through the use of reference material and control charts. Inter-lab variability remains a major concern in managing aflatoxin risk, as documented in Texas [12, 20] and, through proficiency testing in Kenya [13].

An amount of uncertainty with any design setup is expected. In this study, trucks may contain bags of harvested maize from multiple farms from different locations, with variable results attributed to weather conditions and drying practices, both of which impact aflatoxin levels. Despite this uncertainty, this study provided insight into how to manage aflatoxin risk at a commercial mill through sampling, and indicated that the Kenya sampling standard [7] used at the time of this study prescribed too few bags be sampled.

4. Declaration of Conflicting Interest

The authors declare no conflicts of interest.

5. Acknowledgement

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6. Article Information

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