Research Proposal: Mycotoxin Detection Options

Description

Aflatoxin in Peanut and Peanut Products: Comparative Study on Analytical Methods for Detection of Aflatoxin

Principal Investigator

Kumar Mallikarjunan
Virginia Polytechnic Institute and State University
Biological Systems Engineering Department
Seitz Hall
Room 308
155 Ag Quad Lane
Blacksburg, VA USA 24061
Phone: +1 (540) 231-7937
Email: kumar@vt.edu

Testing Laboratories

ELISA

(ICRISAT-Malawi; Virginia Tech; Bunda College, Malawi)

HPLC

(KNUST, Ghana; UGA)

TLC

(UniLurio, Mozambique; University of Ghana, Ghana)

AflaTest

(Makerere University, Uganda; UniLurio)

FTIR

(Virginia Tech)

Geographical Locations

Ghana, Malawi, Mozambique, Uganda, USA

Project Duration

07/01/2014 – 02/28/2015

Executive Summary

The PMIL program over the years has supported many projects and each of those projects has used variety of methods to evaluate aflatoxin contamination level in peanuts and peanut products and always receive requests from USAID missions, NGOs and other organizations in the region on recommendations for Aflatoxin evaluation. However, we find it difficult to provide a sound science based recommendation as several research publications provide only one method of evaluation and could be difficult to compare among the studies due to variations among analytical methods. In addition, some methods considered to be approved standards are often time consuming, labor intensive and expensive. With developmental projects requiring a large number of samples, many projects allocate major portion of their funding for aflatoxin evaluation. There is also lack of standards for sample preparation and data interpretation. This warrants a need for a comparative study among various projects and come up with recommended procedures for aflatoxin analysis and reporting to the PMIL program.

The proposed study will compare existing detection methods and will prepare an information booklet and associated web-based decision tree for anyone (NARS, NGOs, USAID missions, Buyers, Processors, Traders, etc.) requesting to establish an aflatoxin detection facility in their organization/facility or simply interested in how best to determine aflatoxin levels in their materials/products. The proposed study will also compare existing and emerging analytical methods with the same samples shared among various project groups and will establish both SOPs and SSOPs to be adopted across all the PMIL projects. This will provide the necessary data to determine what facilities require further support to establish appropriate procedures and quality assurance testing. Such testing facilities will receive a 'PMIL stamp of approval' for quality testing. It is envisioned that such assessments will be continued in all future years of PMIL to assure proper quality control of PMIL research results.

Project Description

Goal

The goal of this project is to develop a decision support tool to aid in determining which detection system is optimal for mycotoxin determinations in samples collected by the Peanut and Mycotoxin Innovation Lab and others.

Relevance and Justification

Together with the economical inadequacy, lack of proper sampling and analytical procedures, inadequate enforcements of laws and poor awareness of farmers, traders, processors and consumers to seriousness of aflatoxicosis [1] aggravates the difficulties to control and manage the aflatoxin incidence

in peanuts, in Africa. Kaaya and Warren (2005) have pointed out the necessity for proper management of aflatoxins in food products like peanuts and adequate measurement techniques on the quality of peanuts. It was also reported that, there is a serious problem in aflatoxin analysis by individual farmers, traders or organizations handling aflatoxicosis-prone crops because of the cost of the analysis and unavailability of laboratories specifically constructed to handle aflatoxin analysis [2].

Besides the insufficient aflatoxin management at field and market level, detection, tracing and elimination of the aflatoxin presence in peanut products were being held by aflatoxin testing facilities or laboratories lacking proper equipment and supply to report reliable results in Africa. In her technical report, Wiana Louw (2011) has revealed the appalling scene of current situation in some of the aflatoxin testing laboratories located in Malawi and Zambia (She summarized that beside the financial problems to meet the expenses to operate the aflatoxin testing equipment, cleaning and maintenance of the equipment, lack in service engineers and inefficient laboratory management skills are the other concerns related with the assessment of aflatoxin testing facilities [3]. It is clear that in most part of the Africa, accessibility to accredited aflatoxin testing services is an urgent need to comply with regional and international market standards; and to work off the constraints standing against the trade in both region and international. Considering these necessities, a manual describing aflatoxin measurement techniques would be beneficial, in which laboratories may choose the most cost effective, rapid and efficient method that can be applied according to the supplies present in their laboratory. It is apparent that, together with a booklet describing the existing and emerging aflatoxin testing methodologies, standard operating procedures needs to be addressed to improve the facilities for more reliable testing results in Africa.

Methods for assessing the toxicity of fungal-contaminated crops are numerous and vary as the compounds produced. Difficulties encountered in sample preparation including the isolation of mycotoxin of interest from biological matrices make most of the current analytical methods laborious, slow, complex and expensive [4].

Presumptive aflatoxin detection can be performed with thin-layer chromatography (TLC) as this method is a simple, robust technique, which is relatively inexpensive compared to high-performance liquid chromatography (HPLC) methods [5]. That's why TLC is a preferential choice of method in many parts of the world, especially in developing countries; yet this method requires a reference method for confirmation and has been only tested at high aflatoxin levels [5]. Analytical methods present for mycotoxins usually need to extract the toxin of interest from the sample matrix with an

adequate extraction solvent followed by a clean-up step to remove the impurities to achieve high sensitivity and reproducibility [6]. The most important drawback in TLC is the incapability of the discrimination of mycotoxins of interest because of the interferences present in the medium after extraction of toxins from the product [5]. The separating power of chromatography can be improved by application of modern clean-up techniques, such as immunoaffinity columns. The AflaTest fluorometric method developed by VICOM uses monoclonal antibody affinity chromatography to separate the aflatoxins in extraction solution to provide a quantitative measurement of aflatoxins in parts per billion (ppb). AflaTest columns offer an adequate clean-up for samples prepared by mixing with an extraction solution, blending and filtering. Aflatoxins bind to the antibodies on the column and impurities are washed away by water. Later, elute is collected by passing the methanol solution through the column, which can directly be analyzed by TLC, fluorometer or HPLC for quantification (www.vicom.com).

Over the last two decades, HPLC coupled with ultraviolet (UV), diode array (DAD), fluorescence (FD) or mass spectrometry (MS) detectors have been popular for quantitative determination of mycotoxins in cereals, and some have been adopted as official or standard methods by the AOAC International or the European Standardization Committee (CEN); as chromatographic methods offer a great advantage of analyzing with good accuracy and precision [6, 7].

In some cases, fast and accurate screening methods based on enzyme-linked immunosorbent assay (ELISA) were applied instead of the more labor intensive and time-consuming chromatography methods. ELISA test has an advantage of not requiring any clean-up step and offering easier operation, and also it is portable for use in the field for the detection of mycotoxins in foods and feeds applications. Additionally, compared to labor intensive HPLC and MS methods, ELISA techniques do not require skilled technical staff [8, 9]. As a disadvantage, it is dependent on the individual matrices of interest [8]. Besides, quantitatively misleading results were reported. Owing to the posible interaction of antibodies to chemically similar substances in food matrix to aflatoxin, false posive results may be observed [10].

Optical methods for detecting and separating seeds severely contaminated by fungi have been widely studied, mainly focusing on changes (differences

or ratios) in spectral characteristics of fluorescence [11-13], transmittance and reflectance in the visible and near infrared regions [14-16].

NIR spectroscopy has been widely used for non-destructive quality classification of broad range of foods and has many applications in industry such as malting and brewing industry for process control purposes [17]. Near-infrared region is for overtones, in other words, to see relatively weak and broad overtone or combination bands of fundamental stretching bands which occur in the range of 14000-4000cm^{-1}, thence, well-resolved bands are very rarely obtained and cannot normally be assigned to a specific chemical entity. On the other hand, in mid-infrared region (4000-400cm^{-1}), absorption frequencies can be assigned to a particular deformation of the molecule, consequently, can be used as useful tool for analysis of functional groups and their contribution to the total structural elucidation of the molecules studied [17].

The combination of the reflectance methods with FTIR has been proposed for the *Fusarium* and/or *Aspergillus* contamination detection in corn kernels in several researches using accessories of photoacoustic (PAS), diffuse reflectance infrared (DRS) [18, 19] and transient infrared spectroscopy (TIRS) [20]. In more recent studies of Kos et. al. (2002, 2003), application of mid-infrared spectroscopy with ATR for determination of fungal infection with *Fusarium graminearum* on corn has been illustrated [21, 22]. There is a study using a FTIR-ATR based method for aflatoxin contamination in groundnuts has been reported [23]. In this study, aflatoxin spiked (in the range of 0-1200ppb) peanut paste samples were analyzed for four major aflatoxins, AFB_1, AFB_2, AFG_1, and AFG_2; and calibration models were developed using the subtracted spectra from solvent spectra. Another research group, led by the Principal Investigator, has shown that the sample preparation step can be minimized to grinding, and peanut paste can directly be loaded to FTIR-ATR system developed for aflatoxin detection. Same group has worked on the use of FTIR-ATR technology for the detection of mold type and amount of AF and found acceptable prediction models that will separate the peanut and peanut samples as clean, moldy (moderate or highly infested) and toxic samples [24].

Many of the listed analysis methods have advantages and some has drawbacks, yet little has been adopted as standard methods by AOAC/ or

CEN. The advantages of spectroscopic methods compared to other methods are the ease of operations, rapidity of analysis and non-destruction of samples [6]. However, there is a need to validate the calibration model; otherwise poor sensitivity of FTIR system will be a problem. With varying requirements for sample preparation and analysis, it is critical that these methods be compared among themselves and evaluated for their ease and cost of operation, sensitivity, repeatability and come up with recommendations for adoption for a specific need.

Research Plan

The overall objective of the study is to compare existing analytical methods (Thin Layer Chromatography, Enzyme-Linked Immunosorbent Assay (ELISA), Fluorometric method (AflaTest), and High Performance Liquid Chromatography) on the basis of ease of use (time required for sample prep, training, and data interpretation), cost per sample, sensitivity, and repeatability and develop a recommended procedures for adoption by interested parties for a specific application need. The methods will also be compared to develop SOP and SSOP to be adopted by PMIL projects across the program. We also will include emerging methods like Fourier Transform Infrared Spectroscopy (FTIR) equipped with Attenuated Total Reflection (FTIR-ATR) as rapid and non-destructive assessment. The methods will be tested on different peanut and peanut products spiked with aflatoxin.

Objective(s)

1. Information Booklet/Web-based recommendation Application Development
    a. Performance comparison of existing and emerging analytical methods (TLC, ELISA, AflaTest, HPLC and FTIR) with respect to aflatoxin detection in different peanut products (peanut paste, peanut butter, peanut cake and Ready to Eat Therapeutic Food (RETF))
    b. Preparation of an information booklet for answering questions related to specific application need (prepare a web-based recommendation application to include information on detection methods, local testing labs and local regulatory compliances)

2. Standardization across PMIL Programs
    a. Investigation of the effect of interferences/impurities in food matrix at different stages of sample preparation (extraction,

recovery and purification) and on the prediction capability of methods under consideration.

b. Performance comparison of existing and emerging analytical methods with respect to aflatoxin detection in aged (rancid) and fresh peanuts of different peanut varieties (e.g. high oleic).
c. Performance comparison of current detection procedures used across PMIL programs with respect to aflatoxin detection in different peanut products (peanut paste, peanut butter, peanut cake and Ready to Eat Therapeutic Food (RETF)) for standardization.

Methodology

Sample Preparation

Peanut Paste

Smooth, blanched, fresh and raw peanuts will be obtained from local retailers and stored at 4^{o}C in re-closable plastic bags until analysis. Paste will be made by grinding raw peanuts with a food processor (Butterfly Emerald Mixer, Gandhimathi Appliances Ltd., Tamil Nadu, India) equipped with metal cutting blade and stainless steel container. Aflatoxin free peanut samples will be spiked with defined amounts of aflatoxins (BioPure Aflatoxin Mix Standard (2 µg/mL AFB_1 & AFG_1, 0.5 µg/mL AFB_2 & AFG_2 in acetonitrile), Tulln, Austria) covering the range of 0-500 ppb (n=50). n=40 of the samples will be used for calibration of the FTIR system, while n=10 of the samples will be used for prediction and analysis with other proposed methodologies. Aflatoxin standards will be diluted to desired concentration with water and 10 ml of diluted aflatoxin will added into peanut paste. Moistened peanut paste sample will be incubated and exposed to air at room temperature until initial moisture level is attained. Same procedure will be applied to Spanish peanuts to prepare prediction set of samples (n=10) at varying levels of aflatoxin.

Other peanut products

Ready-to-Use Therapeutic Foods (RUTF) (e.g. Plumpy'nut) are consumed for treatment of severe acute malnutrition. In order to see the effect of other ingredients on aflatoxin determination performance of proposed analytic methods, commercially available peanut butter and RUTF samples will be homogenized and spiked with AF as described for peanut paste samples. n=10 representative samples from each product will further be analyzed with TLC, AflaTest Fluorometry, HPLC, ELISA and FTIR-ATR.

Rancid Peanut Paste

Both clean and contaminated (AF spiked), fresh ground peanut samples (50 mg each) will be placed in incubator for three weeks at 63^{o}C and data is collected at 0, 8, 14, 20, 28, 36, 42, 50, 60 days for short term rancidity analysis. Under accelerated conditions, it is known that one day of incubation at 63^{o}C is equal to one week of storage at room temperature. Thus 60 days incubation represents a year of storage at room temperature. n=10 of representative samples at different level of rancidity and AF contamination will be analyzed by TLC, AflaTest Fluorometry, HPLC, ELISA and FTIR-ATR.

Analysis of rancidity of peanut paste samples

Aged peanut paste samples contaminated at varying levels of aflatoxin (0-500ppb) will be exracted using hexane. The pellet will be removed by centrifuging and the upper layer containing peanut oil (and small amount of aflatoxin) will be used for Conjugated Diene Hydroperoxides (CDHP) and Free Fatty Acids (FFA) analyses. CDHP will be determined by measuring the absorbance of hexane extract using UV-Vis spectrophotometer. FFA will be determined by titrating the hexane extract with 0.01 N NaOH.

For the thiobarbituric acid (TBA) test, 50µL of peanut oil (after hexane is evaporated) will be mixed with TBA working solution (10ml), heated for 45 min in a boiling water bath, be mixed with trichloroacetic acid (TCA) solution (5ml), centrifuged and absorbance of upper layer will measured using UV-Vis spectrophotometer.

Peroxide value experiments will be performed by mixing 0.5ml extracted peanut oil with 3ml acetic acid:chloroform (3:2) solution, 3ml dH2O and mixture will be titrated with 0.01N sodium thiosulfate.

Sample Extraction, Filter and Cleanup

AF spiked peanut paste samples will be solvent extract with aqueous methanol solution after grinding up the peanuts. A 25 g of sample will be placed into food processor and 5 g iodine free salt together with 125ml methanol: water (70:30) solution will be added. Mixture will be blended for 2 minutes at high speed. Extract will be poured into fluted filter paper and filtrate will be collected in clean vessel. Some of the filtered extract will be used for aflatoxin analysis by ELISA test.

Filtered extract will be diluted with water and filtered through 1.5µm glass microfiber filter. 15 ml of filtered and diluted extract will be passed through the AflaTest column for cleanup procedure. After passing the all of the extract completely, column will be washed with water twice and eluate will be collected in a clean glass vessel by passing 1 ml HPLC grade methanol through the column. The 1 ml methanol eluate from AflaTest Fluorometer procedure will be split to be analyzed by TLC, Fluorometer and HPLC.

AFLATEST FLUOROMETER

Elute collected from AflaTest immunoafinity column will be mixed with AflaTest developer solution in the cuvette and aflatoxin concentration will be determined by placing the cuvette in a calibrated fluorometer.

THIN LAYER CHROMATOGRAPHY (TLC)

100 µL aflatoxin extract and aflatoxin standards will be spotted on silica gel G coated activated TLC plates and plated will be developed in toluene/isoamyl alcohol/methanol (90:32:2; v/v). Later, plates will be air dried and examined under long-wavelength UV light (360nm). The aflatoxins were chemically confirmed by spraying trifluoroacetic acid. For quantitative analysis, each spot will be eluted by methanol and quantified using UV–Vis spectrophotometer at 360.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Extracted and cleaned-up (with AflaTest columns, VICAM) eluates (AF in methanol solution) were quantified for aflatoxins by HPLC, following the detection method proposed by Trucksess and colleagues (1994) after derivatization with trifluoracetic acid [25, 26].

ELISA TEST

Aflatoxin content characterization was performed by ELISA test (AgraQuant Total Aflatoxin Test Kit, Romer Labs). Briefly, 20g ground portion of each sample was mixed with 100 ml 70% methanol extraction solvent for final extraction solvent ratio of 1:5 (w/v) in sealed vials. After shaking 2 min, mixture was filtered and the filtrate was directly tested with ELISA kit as manufacturer describes.

FTIR- ATR MEASUREMENTS

Spectra of peanut paste samples will be collected using Thermo Nicolet Nexus 470 FT-IR spectrometer (Thermo Electron North America, LLC)

equipped with attenuated total reflectance (ATR) accessorize. FTIR data will be obtained using the Omnic Spectra (Thermo Scientific) software**.** For the calibration, spectra will be collected as absorbance from 64 scans through the frequency region of 4000-625cm^{-1} at a resolution of 4cm^{-1} and a gain of 2.0. Background measurements will be made against air and collected before scanning each sample. After each measurement, crystal sample compartment will be cleaned with 70% methanol and dried.

Standardization across PMIL Programs

The samples (Table 1) will be prepared with known amount of aflatoxin and will be shipped to participating labs for analysis. In addition to the analysis results, a survey will be included to obtain information about the standard operating procedures (SOPs) and standard safe operating procedures (SSOPs) in order to assess the laboratory environmental quality. The SOPs will focus on the use of appropriate documentation to ensure the standards and chemicals used are within the date of expiry and properly stored until use and correct reporting procedures of the analyzed data. The SSOPs will focus on the operator safety, lab safety and safe disposal procedures employed to discard aflatoxin contaminated and/or *Aspergillus* infested products and protocols in place to provide corrective measures when violations have been observed. The labs will be evaluated based on the results from aflatoxin evaluation of products to be analyzed, the SOPs and SSOPs and in the case of inaccurate results, a corrective measure could be suggested based on the analysis of the methods and protocols being practiced.

Table 2. Types of products to be tested by participating labs

Type of product	AF Levels
Peanut flour	3
Peanut paste	3
RUTF	3
Peanut butter	3
Peanuts (raw)	3
Rancid nuts	2
Rancid paste	3
Total number of products	20

PROJECT TIME LINE

Calendar starting in:	Jul	Aug	Sep	Oct	No	Dec	Jan	Feb
Information Booklet/Web-based recommendation Application Development								
Sample collection and preparation								
Sample extraction, filtration and clean-up								
Analysis with HPLC, TLC, ELISA, AflaTest and FTIR-ATR (Cost, Ease of use, Sensitivity, etc.)								
Information booklet/web application								
Standardization across PMIL Programs								
Sample collection and preparation								
Sample extraction, filtration and clean-up								
Aging AF- free and AF spiked peanut paste samples								
Analysis with HPLC, TLC, ELISA, AflaTest and FTIR-ATR (AF detection across PMIL programs)								
Spectral data collection of oxidized samples								
Rancidity analysis by TBARs, CDHP, FFA, PV								
Data analysis of AF-spiked peanut samples								
Data analysis of lipid oxidized peanut samples								
Statistical evaluation of performances of analytic methodologies to measure the AF								
Report and Journal writing								

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