NON-DESTRUCTIVE DETECTION OF AFLATOXIN B1 IN BEANS

By

Omotola Folarin-ottun

Department of Bioresource Engineering

McGill University

Sainte-Anne-de-Bellevue, Québec, Canada

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ABSTRACT

The serious detrimental effects of aflatoxin contamination in the food chain demand an appropriate method of detection. There are various analytical methods of detection that have been established and are effective; however, those methods are destructive and time consuming. Thus, the need for a rapid and non-destructive method of detection. The aim of this study was to investigate the use of non-destructive technologies such as near infra-red (NIR) Hyperspectral imaging (900-1700 nm) and a luminescence bioassay sensor for detection of aflatoxin contamination in beans. Bean seeds were artificially inoculated with aflatoxin B1 by immersing them in different concentration (10, 20, 100 and 500 ppb) of stock solutions. They were dried naturally and then examined by near-infrared hyperspectral imaging, to determine its feasibility in detecting aflatoxin B1 on the bean seeds. The samples were imaged in reflection mode using a near-infrared hyperspectral imaging system operating between 900 and 1700 nm. Spectral images were processed to extract important features for further data processing. Average mean spectra showed differences in reflectance values of the different levels of toxin in an increasing order. The control group had the lowest reflectance value whereas the highest toxin level (500 ppb) had the highest reflectance value. Classification models were developed to discriminate between the uncontaminated and contaminated group. The classification accuracy was satisfactory with sensitivity and specificity values ranging from 78 - 100% and 94 - 100% respectively.

A luminescence assay was performed to detect different concentrations of aflatoxin based on their ability for prophage induction. An induction test was conducted using the indicator strain (*E. coli* BR513) which has the *lac*Z gene responsible for the production of the indicator enzyme β-galactosidase, and the control strain (*E. coli* K12) which lacks this gene. The presence of the

enzyme is indicative of prophage induction, which can be detected by measuring luminescence. A set of ten aflatoxin solutions (0.07, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20 and 200 ppb) were tested in the assay. The indicator and control strain were treated with the inducing agents (aflatoxin) and incubated for 6 hours at 37˚C. Aliquots of 100 µl treated cultures were measured into labelled wells in a 96-well plate reader, and 100 µl of the luminescence substrate which produces the light (Beta-Glo) was added to the same wells. The luminescence of the plates were measured using a plate reader (Synergy HTX). The relative light units (RLU) of the indicator and control strains were compared. Results showed that this luminescence bioassay can be used to detect aflatoxin B1 at concentrations as low as 0.21 ppb.

RÉSUMÉ

Les effets néfastes associés à la contamination aux aflatoxines dans la chaîne alimentaire exigent une méthode de détection appropriée. Il existe diverses méthodes analytiques de détection établies et efficaces; Cependant, ces méthodes sont destructrices et prennent beaucoup de temps. Ainsi, la nécessité d'une méthode de détection rapide et non- destructive. Le but de cette étude était d'étudier l'utilisation de technologies non destructives telles que l'imagerie hyperspectrale proche infrarouge (900-1700 nm) et un capteur de bioessais de luminescence pour la détection de la contamination par les aflatoxines dans les haricots. Les graines de haricot ont été artificiellement inoculées avec de l'aflatoxine B1 en les immergeant à différentes concentrations (10, 20, 100 et 500 ppb) de solutions mères. Ils ont été séchés naturellement et ensuite examinés par imagerie hyperspectrale proche infrarouge, afin de déterminer la faisabilité de détection de l'aflatoxine B1 sur les graines de haricot. Les échantillons ont été imagés en mode réflexion en utilisant un système d'imagerie hyperspectrale proche infrarouge fonctionnant entre 900 et 1700 nm. Les images spectrales ont été traitées pour extraire des caractéristiques importantes pour un traitement ultérieur des données. Les spectres moyens ont montré des différences dans les valeurs de réflectance des différents niveaux de toxine dans un ordre croissant. Le groupe témoin ayant la valeur de réflectance la plus faible et le groupe ayant un niveau de toxine le plus élevé (500 ppb) ayant la valeur de réflectance la plus élevée. Des modèles de classification ont été élaborés pour faire la distinction entre le groupe non contaminé et le groupe contaminé. La précision de la classification était satisfaisante avec des valeurs de sensibilité et de spécificité allant de 78% à 100% et de 94% à 100% respectivement.

Un test de luminescence a été effectué pour détecter différentes concentrations d'aflatoxine en fonction de leur capacité à induire le prophage. Un test d'induction a été réalisé en utilisant la

souche indicatrice (*E. coli* BR513) possédant le gène *lacZ* responsable de la production de l'enzyme indicatrice β-galactosidase, et la souche témoin (*E. coli* K12) qui n'a pas ce gène. La présence de l'enzyme est indicative de l'induction du prophage, qui peut être détectée en mesurant la luminescence. Une série de dix solutions d'aflatoxine (0.07, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20 et 200 ppb) ont été testées dans l'essai. L'indicateur et la souche témoin ont été traités avec les agents inducteurs (aflatoxine) et incubés pendant 6 heures à 37 ° C. Des aliquotes de 100 ul de cultures traitées ont été mesurées dans des puits marqués dans un lecteur de plaque à 96 puits, et 100 ul du substrat de luminescence qui produit la lumière (Bêta-Glo) ont été ajoutés aux mêmes puits. La luminescence des plaques a été mesurée à l'aide d'un lecteur de plaques (Synergy HTX). Les unités de lumière relatives (RLU) de l'indicateur et des souches témoins ont été comparées. Les résultats ont montré que ce bioessai de luminescence peut être utilisé pour détecter l'aflatoxine B1 à des concentrations aussi faibles que 0.21 ppb.

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CONTRIBUTION OF AUTHORS

This thesis was written according to the rules and regulations of the Faculty of Graduate Studies and Research of McGill university and consists of two manuscripts, both of which I am the primary author. The manuscripts were co-authored with Dr. Michael Ngadi, my thesis supervisor, and Dr. Goodridge, my thesis co-supervisor. The thesis was also reviewed by Dr. M. Ngadi and Dr. Li Liu.

Manuscripts of these chapters are under preparation and will be submitted for publication in a scientific journal in 2018.

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NOMENCLATURE

HSI	Hyperspectral Imaging System
SWIR	Short wave Infrared
NIR	Near Infrared
RLU	Relative Light Unit
ROI	Region of Interest
PCA	Principal Component Analysis
LDA	Linear Discriminant Analysis
PLSDA	Partial Least Squares Discriminant Analysis
SNV	Standard Normal Vitiate
MSC	Multiple Scatter Correction
PCs	Principal Components

I GENERAL INTRODUCTION

The importance of food quality and safety cannot be overemphasized. According to the World Health Organization, foodborne illnesses are "infectious or toxic diseases caused by agents that gain entry into the body via ingestion of food." The European Union legislation (Commission Regulation (EC) No. 2073/2005) also stated that "foodstuffs should not contain microorganisms or their toxins or metabolites in quantities that present an unacceptable risk for human health." Pathogenic fungal, algal, and bacterial cells and their associated toxins are ubiquitous in nature, reducing the quality of food and posing major risk to human and animal health (Byrne *et al*., 2015).

A wide variety of agricultural products including peanuts, maize, wheat, rice, cotton seed and chilli pepper are subjected to infection by aflatoxin producing toxigenic fungi (Abbas *et al*., 2006). Aflatoxins are highly toxic metabolites of fungi that belong to a family of mycotoxin (Piva *et al*., 1995). They are produced mainly by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Bryden, 2012). Aflatoxins are produced by Fungi on the field or during storage under favourable environmental conditions (Hesseltine, 1974). The existence of contaminated agricultural products has a negative economic impact in addition to the chronic damages that result upon consumption in human and animals (Filazi and Tansel, 2013). The most recent outbreak of acute human aflatoxicosis occurred in Kenya's eastern and central provinces where 125 deaths were recorded after the consumption of contaminated commercial maize products in April 2004 (Gieseker *et al*., 2005). The adverse effects associated with the occurrence of aflatoxin in agricultural commodities have made the research on aflatoxin reduction and elimination a global concern (Chu *et al*., 2017). Aflatoxin B1 is known to be the most common

and potent carcinogen (Berthiller *et al*., 2011), and as therefore been classified as a class 1 human carcinogen by the International Agency for Research Cancer (IARC 2002). Therefore, early and efficient detection of aflatoxins in commodities is required to prevent entry of contaminated products along the food supply chain (Delfiore *et al*., 2010).

Currently, the official standard methods that are available for quantification of aflatoxins include; High performance liquid chromatography (HPLC), Thin layer chromatography (TLC), Immunosorbent assays (ELISA) and plate counting methods (Kandpal *et al*., 2015). These analytical methods have high accuracies and sensitivities for aflatoxin detection in agricultural products. However, despite the advantages of these methods for detection, they require trained personnel, they are time consuming, expensive, destructive in nature, and can be harmful due to the use of unfriendly chemicals (Wang *et al*., 2015). These disadvantages have led to efforts being made to develop simple, rapid, accurate and non-destructive methods for aflatoxin detection.

Recently, there has been an advanced interest in the use of spectroscopy techniques for aflatoxin detection owing to its application in rapid and non-destructive analysis, requiring little or no sample preparation (Chu *et al*., 2017). The different spectroscopic methods include raman spectroscopy, fourier transform infrared (FTIR) spectroscopy and hyperspectral imaging (HSI) (Alexandrakis *et al*., 2012; Lu *et al*., 2011; Yoshimura *et al*., 2014). Unlike raman spectroscopy and fourier transform infrared spectroscopy, hyperspectral imaging can be used for the analysis of a large sample size because of its ability to integrate both imaging and spectral information for every pixel in an image (Barbin *et al*., 2012; Costa *et al*., 2011; Elmasy *et al*., 2012a; Elmasy *et al*., 2012b; Jackman *et al*., 2009; Kamruzzaman *et al*., 2012; Sun 2004; Wang and Sun, 2002; Wu, Sun and He, 2012; Wu and Sun, 2013). The use of HSI for aflatoxin detection on maize kernels

have been shown by some authors (Kimuli *et al*., 2018; Chu *et al*., 2017; Kandpal *et al*., 2015; Wang *et al*., 2014; Wang *et al*., 2015).

In addition, several authors have demonstrated the development and utilization of biosensors for aflatoxin detection. Hosseini *et al*. (2015), developed a novel colorimetric and chemiluminescence assay for the detection of aflatoxin B1 with a detection limit of 8 nm. The novel technique is similar to others used for aflatoxin detection (Shim *et al*., 2014; Li *et al*., 2016; Zangheri *et al*., 2015). This study focuses on the ability to directly detect aflatoxin using a non-destructive method.

1.1 Objectives

The aim of this research was to determine the use of a rapid and non-destructive method for aflatoxin detection. The specific objectives were to;

1. Assess the use of hyperspectral imaging to obtain characteristic wavelengths of AFB1 inoculated directly on beans.
2. Develop and compare accuracies of classification models for detection of AFB1 contaminated beans by investigating the optimal results produced by the correct discrimination rate of the models.
3. Develop a novel luminescence bioassay to determine whether aflatoxin is an inducing agent that can be detected by prophage induction.
4. Determine the detection limit of the luminescence bioassay.

II. GENERAL LITERATURE REVIEW

2.1 Introduction

Food security is effectively accomplished when the food pillars such as food availability, food access, food utilization and food stability are at levels that permit all individuals always to have physical and economic access to affordable, safe and nutritious food to meet the requirements for an active and healthy life (FAO, 1996). If by any chance one of these pillars weakens, then a society threatens its food security. Factors associated with food insecurity and malnutrition do affect not only human health but also influence social, economic, and political aspects of the society. Having outlined these factors, pre-and post-harvest loses due to mycotoxin contamination are recorded as one of the driving factors of food insecurity since these toxins occur along most food chains from farm to fork (Udomkun *et al*., 2017).

Various crops and food items are infected by micro organisms during the growth, harvesting, drying, processing and storage of the food items. Most important of these organisms infecting food are fungi producing mycotoxins (Dichter, 1984). Mycotoxins are secondary metabolites produced by fungi (Jan and Samson, 2007). Mycotoxins contaminate crops and foods and they show detrimental effects in animals and humans such as mutagenic, teratogenic, carcinogenic and immunosuppressive effects (Creppy, 2002). The fungi producing these toxins include Penicillium, Aspergillus, and Fusarium. They are commonly found growing on agricultural food products such as corn, wheat, rice, maize, spices, dried fruits and nuts (Laszczynska *et al*., 2001). In 1961, Aflatoxins were discovered following the severe outbreak in England that caused the deaths of over 100,000 turkeys and farm animals. The outbreak was attributed to a feed, and with the use of thin-layer chromatography technique which brought to

light a series of fluorescent compounds later called aflatoxins, they were discovered to be responsible for the disease outbreak. The disease resulted from a peanut meal in the diet, which was contaminated with a toxin produced by Aspergillus flavus, thus, the name aflatoxins (Rustom, 1997).

Aflatoxins are mutagenic natural compounds that are extremely dangerous and linked with different diseases in livestock-domestic animals and humans (Brown *et al*., 1999). Aflatoxins are toxic metabolites produced primarily by two species of Aspergillus, i.e., *Aspergillus Flavus* and *Aspergillus parasiticus*, which are mostly found in hot and humid areas (Filazi and Sireli, 2013). There are 18 different types of aflatoxins, and amongst these, only six are majorly found in food items; aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1) and M2 (AFM2) (Filazi and Sireli 2013). Of these six, four major naturally occurring aflatoxins are AFB1, AFB2, AFG1, and AFG2, and their chemical structures are shown in Fig 2.1. Aflatoxin B1 is predominant in cultures and food products. It is an odorless solid and pale-white to yellow crystalline in its pure form (Filazi and Sireli, 2013).

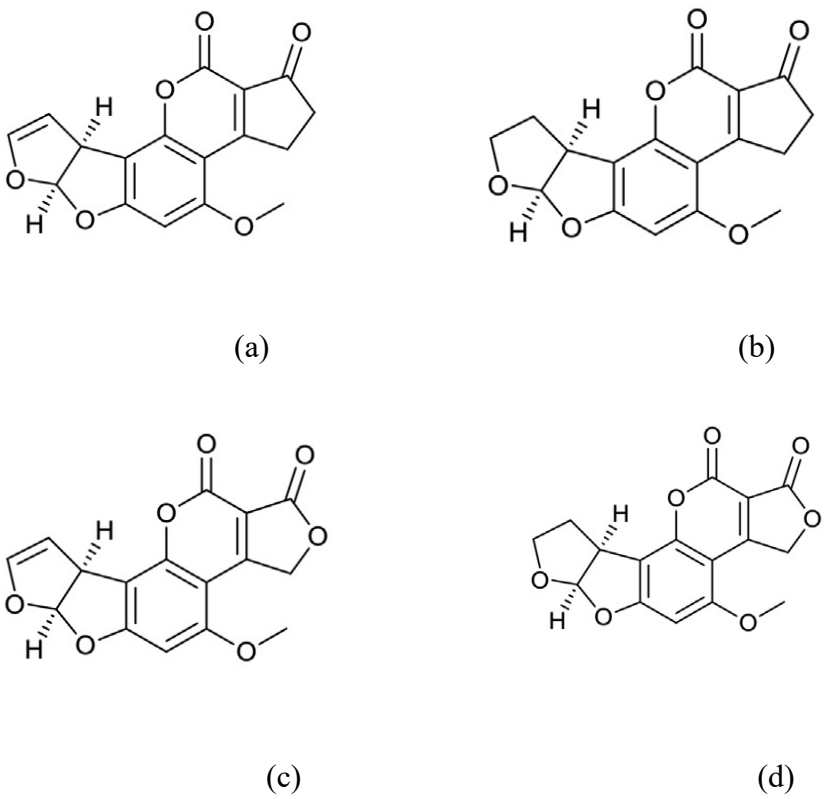

Figure 2.1 Chemical structure of the four major Aflatoxins, i.e. (a) AFB1, (b) AFB2, (c) AFG1 and (d) AFG2.

Having identified aflatoxins as the most common and universal source of chemical contamination, they have been found in raw and processed foods (EU Rapid Alert System for Food and Feed, 2013). The safety of the public health is of paramount importance, and to protect the public health, maximum limits (MLs) for aflatoxins in various food items have been established by government agencies. The maximum and permitted levels set by the European Commission are 2 µg/kg for AFB1 and 4 µg/kg for total aflatoxins (B1, B2, G1, and G2) in groundnuts, nuts, dried fruits, and cereal intended for direct consumption (European Commission, 2006). Likewise, the Codex Alimentarius Commission, which is a Joint FAO/WHO food Standards programme has set the legal limit of total aflatoxins in peanut at 15 µg/kg (Codex Alimentarius Commission, 2001).

Aflatoxin toxicity occurs at minute levels, and so it requires a sensitive and reliable method for its detection and measurement. Many efforts have been put into developing an effective and reliable assay for the identification and quantification of aflatoxins. Various analytical methods have been reported for the determination of aflatoxins, and these methods can be divided into 3 different categories;

1. Chromatographic methods: Chromatographic procedures rely on interactions between a mobile and stationary phase. Major chromatographic methods for aflatoxin analysis include; Thin layer chromatography (TLC) (Stroka *et al*., 2000), Gas chromatography (GC) (Goto *et al*., 1988), Liquid chromatography (LC) (Sobolev and Dorner, 2002), Liquid chromatography–mass spectrometry (Zhu *et al*., 2013), High performance liquid chromatography–fluorescence detection (Skrbic *et al*., 2014), and Ultra-high-pressure liquid chromatography (UPLC) with UV detection (Fu *et al*., 2008).
2. Immunochemical methods: Immunochemical techniques are based on the binding specificity between antibodies and antigens. Various immunochemical methods have been developed as a result of the high affinity and specificity of antibodies for antigens. The major immunochemical methods include; Radioimmunoassay (RIA), Electrochemical immune sensors (Tan *et al*., 2009), Enzyme-linked immunosorbent assay (ELISA), Immunoaffinity column assay (ICA), and Immunosensors (Iqbal *et al*., 2015; Prieto-Simón and Campàs, 2009; Jiang *et al*., 2013; Anfossi *et al*., 2008).
3. Spectroscopic methods: The spectroscopic techniques employed for aflatoxin analysis are; Fluorescence spectroscopy (Nasir and Jolley, 2002), and Infrared spectroscopy (Wacoo *et al*., 2014).

2.2 Aflatoxin detection

2.2.1 Aflatoxin extraction from food samples

Detection and measurement of aflatoxins in food require an effective extraction step. Aflatoxins are soluble in polar protic solvents such as methanol, acetone, chloroform, and acetonitrile, and they are required for its extraction (Bertuzzi *et al*., 2012; Taylor *et al*., 1993). Most detection methods depend on an efficient extraction and clean up method except ELISA which may not require clean up (Chu, 1992). The method of aflatoxin extraction from the biological matrix depends on the structure of the toxin, and the choice of extraction solvent is also dependent on the matrix from which the extraction is required because different chemical mixtures can affect the extraction (Wilkes *et al*., 1998). Aflatoxins are hydrophobic, and as such, they require organic solvents for their extraction (Holcomb *et al*., 1992; AQAC 1997). The use of chlorinated chemicals has been proven to be ecological hazardous and so therefore have been reduced (Montreal protocol 1998). The most crucial step in the extraction process is the clean up procedure, as the purity of the sample affects the sensitivity of results obtained. Trace aggregates of the target molecule may be concealed by interfering compounds that are found in the matrix, chemicals, materials and solvents used (AQAC 1997). Several methods of extraction have been recorded, and these are some of the most used methods;

1. Liquid-liquid extraction (LLE): In liquid-liquid extraction, the various solubility of the toxin in the aqueous phase and immiscible organic phase are exploited to extract the compound in one solvent while the rest of the matrix is left in the other solvent. As such, hexane and cyclo-hexane are common solvents used to remove non-polar contaminants such lipids and cholesterol. This extraction procedure is efficient for several toxins and

works effectively in small-scale preparations (Bauer and Gareis, 1987). However, this procedure is time-consuming and it depends on the type of matrix used and compounds being determined. A significant disadvantage is the possible loss of sample by adsorption onto the glassware.

2. Supercritical fluid extraction (SFE): Supercritical fluid extraction involves using a supercritical fluid such as CO_2 to extract the target compound from the matrix. It works effectively owing to the high solvating power and density of the solvating liquid. Supercritical fluid chromatography on fused silica capillary columns was applied by Young and Games (1992) to separate toxins, but it was reported as an unsuccessful technique due to problems associated with SFE. Also, SFE is not appropriate for routine analysis because it is expensive and requires a specialised equipment (Holcomb *et al*., 1996).
3. Solid phase extraction (SPE): The underlying principle of solid phase extraction is a variation of chromatographic techniques based around small disposable cartridges enveloped with silica gel or bonded phases which are located in the stationary phase. Under the condition of low pressure, the sample is loaded in one solvent, rinsed and most of the contaminants are removed before being eluted in another solvent (European Mycotoxin Awareness Network EMAN 2003). SPE has many advantages compared to LLE, they operate faster and require less solvent. Also, they can be used to pre-concentrate the sample to provide better detection results. It is widely used and has become an integral part of many extraction and detection procedures. A major disadvantage of SPE is the difficulty in finding a single universal type of cartridge useful for the extraction of all types of toxins. Silica gel is the most popular and frequently used material for SPE.

A clean up step follows the extraction step. The general clean up procedure used is immunoaffinity column chromatography IAC (Ma *et al*., 2013). IAC has been adjudged as the method of choice for purification and concentration of aflatoxins (Scott and Trucksess, 1997) before their determination. IAC uses the high specificity and reversibility of binding between an antibody and antigen to separate and purify target compounds from matrices (Shelver *et al*., 1998). In this process, the crude sample extracted is applied to the immunoaffinity column containing specific antibodies to the aflatoxin immobilized on a solid support like agarose or silica. While the cruse sample is moving down the column, the aflatoxin binds to the antibody and gets retained on the column. Impurities and unbound proteins are further removed by another washing procedure. This procedure employs the use of suitable buffers and ionic strengths, after which aflatoxin recovery is achieved by using solvents like acetonitrile which dissolves the bond between the antibody and the aflatoxin (Wacoo *et al*., 2014).

2.3 Traditional methods for aflatoxin detection

Since the discovery of aflatoxin in the 1960s, they have received substantial attention from analytical chemists, and there has been an advancement in a considerable number of analytical methods for the detection and quantification of aflatoxins. These methods range from those requiring technical skilled personnel to those that can be performed with very little training (Chris, 2002). The methodical and complete monitoring of aflatoxins is a major challenge most particularly for the future as food production is increasing (global peanut production has doubled within the last 20 years) (FAO 2000). There are established requirements for analytical methods at the national and international levels such as the European Committee for Standardisation, and the Association of Official Analytical Chemists International. For a proposed method to be adopted

as an official method, it should be corroborated in a combined trial study. The minimum method performance characteristics, the framework for the performance of combined trial studies, and the statistical evaluation of the results, are well defined in suitable protocols (Horwitz, 1995). The minimum requirements for the method performance to be validated are dependent on the level of contamination. Table 2.1 shows the typical validation parameters required for analytical methods.

Table 2.1. Validation parameters for analytical methods

Parameter	Description
Accuracy	Closeness of results to true value
Precision	Variation of results after repetition of measurements under similar conditions
Linearity	Ability to obtain results directly proportional to concentration
Specificity	Selectivity of the method for the target analyte
Sensitivity	A linear relationship; change in the analytical response per change in analyte concentration
Practicability	Ease of use and the number of covered analytes
Robustness	Indication for the reliability of the procedure against variations in method parameters
The limit of detections (LOD) and Limit of quantification (LOQ) (FAO 1998).	Smallest concentration of an analyte that can be reliably measured by an analytical procedure

Methods that have been established and tested as stated by these protocols can be recognized as an official method. The various methods of aflatoxin detection are broad and complex, and this complexity is due to the diverse chemical structures (Yong and Cousin, 2001)

The first method of detection was done by TLC but was replaced by HPLC, ELISA and fluorimetric techniques in the 1980s due to the technical progress of these methods which also

required more instruments. After reviewing TLC, HPLC and ELISA methods, it was concluded that the analytical challenge of determining less than 1 ng/g of aflatoxin B1 in support of the European legislation and other worldwide legislation could be done using these methods. Classical analytical methods for mycotoxin analysis include Thin layer chromatography (TLC) (Stroka and Anklam, 2000.), Enzyme linked immunosorbent assays (ELISA) (Patey *et al*., 1989; Trucksess *et al*., 1990), high performance liquid chromatography (HPLC) coupled to diode array (DAD), fluorescence (FLD), single mass spectrometry (MS) or tandem mass spectrometric (MS/MS) detection (Seitz, 1975; Sobolev and Dorner, 2002; Trucksess *et al*., 1994), gas chromatography (GC) coupled with single mass spectrometric detection (MS).

2.3.1 Thin layer chromatography (TLC)

Thin layer chromatography is traditionally the most used method for mycotoxin analysis due to its ability to screen enormous numbers of samples economically. TLC is a method used commonly in developing countries due to its simplicity and practicability. (Stroka *et al*., 2000). TLC technique avoids the use of chlorinated organic solvents, and it requires normal silica gel plates (Stroka, 2002). Its use is popular for both quantitative and qualitative application, and this is attributed to its low operating cost and ease of identifying the target compounds using UV-vis spectral analysis. TLC involves coating a glass plate with silica gel and the application of a concentrated aflatoxin sample on a baseline. Separation by solvent migration is followed by drying and categorization of the resultant spots. The plates are coated using silica gels, and high-purified grades are needed to obtain adequate resolution (Bullerman, 1987). A thin absorbent layer is spread onto a glass plate and then initiated by drying. The activation is time and temperature dependent. Prepared TLC plates which are normally purchased, are spotted with microliter quantities of the extract and

placed uprightly with the lower edge immersed in the solvent. The sorbent layer allows the solvent to migrate by capillary action which effects its separation into single spots perpendicular to the baseline. After migration, the plate is removed and dried, then detection methods are used in developing spots (Pomeranz and Meloan, 1987). The detection of the spots is based on the fluorescent properties of aflatoxin. Several methods can be used for quantification, of which visual estimation is the most common. Visual estimation involves comparison of aflatoxin standards with the color and intensity of fluorescence of the sample over a range of concentrations. TLC has been reported to be about 20% accurate (Moss and Smith, 1985). A major requirement with the application of TLC is the intrinsic need for sample preparation, a clean up protocol that is dependent on properties and type of toxin being detected (Holocomb *et al*., 1992; Lin *et al*., 1998). Several methods of cleaning mycotoxin samples have been recorded and the widely used methods include Liquid-liquid extraction (LLE), supercritical fluid extraction (SFE) and solid phase extraction (SPE). SPE is the most used method in regular analysis of mycotoxins, although it has some disadvantages. Currently, it is unlikely to find a universal cartridge for the extraction of all toxins, and its performance can be affected by factors such as pH, solvent and ion concentration (Turner *et al*., 2009).

TLC is not frequently used with examples in literature because HPLC has superseded it. TLC was first used by De Iongh (De Iongh *et al*., 1964). It has been used widely for the detection of aflatoxins in different food (Gulyas, 1985; Abdel-Gawad and Zohri, 1993; Younis and Malik, 2003), and concentrations as low as 1-20 ppb of aflatoxin have been recorded (Trucksess *et al*., 1984). A report by (Frisvad and Thrane, 1987) showed that the accuracy of TLC was greater when compared with more flexible HPLC for ochratoxin A detection. A very good example of the TLC

techniques is that of Dawlatana *et al*. (1996), where OTA was separated from rice following a series of solvent steps and quantified by fluorescence.

TLC methods involve tedious procedures, and an enormous amount of solvent and various types of chemicals are required for the method (Stroka *et al*., 2000). More issues with TLC technique are an inadequate limit of quantification and low chromatographic resolution (Gilbert, 1999). Also, it requires a skilled technician, presample treatment an expensive instrument (Stroka and Anklam, 2002; Papp *et al*., 2002). TLC lacks accuracy due to amassed errors during sample application, plate development, and plate interpretation. An advantage of TLC technique is the ability to screen an enormous number of samples with low operating cost, as well as the ability to identify target compounds using UV-vis spectral analysis (Krska and Josephs, 2001; Turner *et al*., 2009; Cigic and Prosen, 2009). TLC has excellent sensitivities; it can detect several types of mycotoxins in a single sample test (Trucksess *et al*., 1984; Balzer *et al*., 1978).

A study by Kamika and Takoy (2011) assessed the natural occurrence of AFB1 in raw peanuts. TLC method was used to determine aflatoxin B1 level in peanut samples that were collected from different locations during the rainy and dry season. The limit of detection was >1µg/kg, and the recovery values ranged from 71-87%, and these values were higher than those obtained by Kamika 2005 where he used TLC method for aflatoxin detection in roasted and raw peanuts (Kamika and Takoy, 2011).

2.3.2 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography is being used increasingly for aflatoxin analysis as well as other toxins, owing to its increased sensitivity and better accuracy compared with the TLC method. HPLC is an analytical technique that involves the separation, detection and quantification of sample constituents. Separation is attained by the competitive distribution of the sample between a mobile liquid phase and a stationary liquid or solid phase which is aided in a column. The efficiency of the separation is dependent on the optimized column parameters, most importantly the particle size. The mobile phase moves under pressure by the use of a pump and passes via a column containing the extract and then flows to a UV absorption fluorescence detector. A change in electrical output is produced and recorded on a moving chart to obtain a chromatogram. The retention time for aflatoxin is constant under fixed conditions, and the comparison of the retention times with those of the standards allows results to be differentiated on a quantitative basis as the area under each peak on the chromatogram is proportional to the concentration of the precise type of aflatoxin. Tremendously low levels of aflatoxin can be detected by connecting sensitive detection and sophisticated data retrieval equipment to the HPLC (Moss and Smith, 1985). HPLC has been used in assaying aflatoxins is various foods and agricultural crops such as cotton seeds (Association of Official Analytical Chemists (AOAC) 1984), peanut products, figs, corn (Pons and Franz, 1977), milk and milk products for AFM1 and in the blood of mammals (Thiel, 1986). The use of HPLC technique frequently using DAD or FLD is more widespread because fluorescence detection depends on the presence of a chromophore in the target toxin and only some toxins may be analysed directly for example zearalenone, ochratoxin A, and ergot alkaloids, while others such as fumonisins, aflatoxins, and trichothecenes are require pre- or post-column derivatization (Krska *et al*., 2008). Over the years, HPLC hyphenated to tandem MS

(HPLC-MS/MS) or MS detection has become the most chosen method for mycotoxin and food analysis because of its high sensitivity, set up accuracy and ease of handling (Sforza *et al*., 2006; Zolllner and Mayer-Helm, 2006; Berthiller *et al*., 2007; Songsermsakul and Razzazzi-Fazeli, 2008). Using the hyphenated method, it is possible to separate and detect all important mycotoxins in a single run without derivatization. The above-mentioned advantages are the reasons why HPLC-MS/MS is constantly gaining recognition in multi-mycotoxin analysis (Sagawa *et al*., 2008; Di Mavungu *et al*., 2009; Monbaliu *et al*., 2009).

In practice, HPLC-FLD is used for the detection of OTA in different foods such a red wine (Aresta *et al*., 2006), dried fruits (Zinedine *et al*., 2007), green or roasted coffee (La Pera *et al*., 2008), rice (Zinedine *et al*., 2007), and blue cheese (Dall'Asta *et al*., 2008). Likewise, aflatoxins can also be detected in food commodities using HPLC-FLD, although the native fluorescence emission of aflatoxins is significantly put out by aqueous mixtures used for reversed-phased chromatography (Koppen *et al*., 2010). Fluorescence can be enhanced by pre- or post-column addition of cyclodextrins to the HPLC eluent (Chiavaro *et al*., 2001; Maragos *et al*., 2008) or by pre-column derivatization of the hemiacetal using trifluoroacetic acid (D'Ovidio *et al*., 2006; Hernandez Hierro *et al*., 2008).

A specific disadvantage of the frequently used HPLC with fluorescence detection (FLD) methods is that pre- or post-column derivatization is necessary to enhance detection (Papp *et al*., 2002; Manetta *et al*., 2005). Immunoaffinity columns are regularly used in combination with HPLC because they have excellent selectivity and good robustness. Also, a higher level of pre-concentration can be attained with IAC, while significantly reducing the LOQ. This is of great concern, particularly in aflatoxin analysis due to legal limits as low as 25 ng/kg for aflatoxin M1 in infant food (EC 2006; Gilbert and Anklam, 2002; Shephard, 2009).

In a bid to measure the aflatoxin contamination level in raw peanuts, 1040 samples were collected and analyzed for aflatoxin B1 using HPLC and immunoaffinity columns. The HPLC analysis was performed with an HPLC system that was equipped with a fluorescence detector, and the recovery limits of detection and limits of quantification were determined to verify the validity of the HPLC procedure. It was revealed that AFB1 was detected in 25% of the samples (Ding *et al*., 2012). A similar determination of aflatoxins was carried out by Zahn *et al*. (2009), Herzallah (2009), and Khayoon *et al*. (2010). They used the traditional C18 reverse phase or multifunctional column HPLC coupled with either UV or fluorescence detection. With these methods, they could detect the target toxins within the legal limits of 0.5 ppb.

Aflatoxin M1 is a subsidiary AF which is not traditionally considered, however in specific matrices such as milk and other diary products it is a significant risk. An effective protocol was initiated by Wang for the detection of a specific toxin and chloramphenicol using HPLC-MS/MS technique (Wang *et al*., 2011), while another researcher used HPLC linked to a fluorescence detection method for AfM1 and ochratoxin A detection in dairy products (Iha *et al*., 2011) and human milk (Iha *et al*., 2014). HPLC produces fast and accurate aflatoxin detection results under a short time frame. It is highly sensitive, a sensitivity detection as low as 0.1 ng/kg using FLD was reported by (Herzallah, 2009). Some disadvantages of using HPLC for aflatoxin analysis are; rigorous sample purification using IAC, and tedious pre- and post-column derivatization process to enhance the limit of detection of AFB1 and G1 (Li *et al*., 2011). To control the challenges related to the derivatization process in aflatoxin analysis, HPLC was modified by connecting it to mass spectroscopy, and this method is currently used for aflatoxin detection (Takino and Tanaka, 2008). The mass spectrometer does not require the use of UV fluorescence nor the absorbance of an analyte, therefore the need for chemical derivatization is annihilated. The HPLC-MS/MS

effectively uses a small sample to provide structural information and exhibits low detection limits (Rahmani *et al*., 2009). Nevertheless, HPLC-MS/MS is bulky and requires a very expensive equipment which can only be operated by skilled personnel and is therefore limited to the laboratory and unsuitable for field applications.

2.3.3 Gas chromatography (GC)

In gas chromatography, the mobile phase is a carrier gas and the stationary phase is a liquid coated onto inert solid particles (Wacoo *et al*., 2014). Various mycotoxins such as trichothecenes, patulin, and zearalenone have been analyzed using as chromatography. Gas chromatography has been found inapplicable for the analysis of aflatoxins due to the high polarity, molecular weight, low volatility and thermal instability of the aflatoxin molecule (Beaver, 1986). Also, the existence of other cheaper chromatographic methods has made GC less common for aflatoxin analysis (Liang *et al*., 2005). The advent of fused silica capillary columns and the application of mass spectrometer as a detector resulted in capillary gas chromatography using on-column injection which is employed for chromatography of aflatoxin B1 standard (Friedll, 1981). Gas chromatography has been used for quantitative determination of mycotoxins, however there are some specific problems with the application of this method for mycotoxin analysis. Non-linearity of calibration curves, poor repeatability, matrix-induced over estimation, and memory effects from previous sample injections are some of the disadvantages of GC which have led to increased use of HPLC (Pettersson and Langseth, 2002). Usually, GC is suitable for thermally stable, non-and semi-polar, volatile and semi-volatile compounds such as oils and sterols. Most mycotoxins are however small non-volatile and polar molecules which have to be chemically derivatized before GC analysis. Gas chromatography also requires a preliminary clean up step before analysis, and it is therefore limited

to analysis of few mycotoxins such as A-trichothecenes and B-trichothecenes (Wacoo *et al*., 2014). More studies have been done using GC for the analysis of mycotoxins such as Trichothecenes and few for the analysis of zearalenone, ochratoxin A, patulin and citrinin. Silylating and acylating agents are preferably used to increase the volatility of mycotoxins by derivatization (Scott 1995; Langseth and Rundberget, 1998; Schollenberger *et al*., 1998; Cirillo *et al*., 2003. Trucksess *et al*., 1984, applied this method for the detection of aflatoxinB1 in corn and peanut butter using a methyl silicone fused silica column. In a study by Rosen *et al*. (1984), a bonded-phase fused silica capillary column was used to detect aflatoxin B1 and B2 in peanuts. Success has been reported with the use of a flame ionization detection method (FID) for aflatoxin analysis. An FID method with a capillary on-column injector and a fused silica capillary column was used by Goto and colleagues to separate aflatoxins B1 B2 G1 G2, although lower sensitivity was recorded for G1 and G2 compared to B1 and B2 when longer columns were used (Goto *et al*., 1988).

2.3.4 Enzyme-Linked Immunosorbent Assay (ELISA)

There are two types of Elisa used for aflatoxin detection; homogenous ELISA and heterogenous ELISA. In homogenous ELISA method, there is an alteration of enzyme activity after binding to specific antibodies, and it is not important to separate the free and bound form of the enzyme-ligand conjugate in the assay. Whereas in heterogenous ELISA method, enzyme activity stays unaltered and separation of the free and bound enzyme-ligand is necessary. The heterogenous method is most commonly used for aflatoxin analysis (Lawellin *et al*., 1977). Here, a coating of certain antibodies occurs on two solid phases with the use of either a microplate (Pestka *et al*., 1983) or a polystyrene tube method (Lawellin *et al*., 1977). The antibodies are coated on the solid phase using glutaraldehyde or bicarbonate (Pestka *et al*., 1983), afterward dried and then washed

with buffer before use. The sample solution or the aflatoxin standard solution is incubated concurrently with the enzyme conjugate or separately in two steps (Pestka *et al*., 1981). The plate is then washed, and the residual enzyme that is bound to the solid phase is determined by incubation using a substrate solution containing hydrogen peroxide and appropriate chromogens. The end color is measured spectrophotometrically or by visual comparison with aflatoxin standard. ELISA technique is currently used in the determination and measurement of aflatoxins in crops (Anjaiah *et al*., 1989; Devi *et al*., 1999; Thirumala-Devi *et al*., 2002; Ondiekil *et al*., 2014) and there are quite a number of commercially available ELISA kits based on competitive immunoassay format being employed for aflatoxin analysis (Ostadrahimi *et al*., 2014; Huybrechts, 2011; Stroka and Anklam, 2002). ELISA tests have also been used for the analysis of various mycotoxin in different food matrices such as ZON (Burmistrova *et al*., 2009; Thongrussamee *et al*., 2008), DON in barley (Hill *et al*., 2006), OTA in kidneys of swine (Matrella *et al*., 2006) and OTA in green coffee (Fukii *et al*., 2006). ELISA has some advantages over the Radioimmunoassay (RIA) method; it is more rapid, less expensive and no radioactive hazards attached to the method (Chu 1984). A large number of samples can be analysed using ELISA because it is a 96-well assay platform (Huybrechts, 2011), and the kits are cheap, easy to use and no sample clean up a step. ELISA has some disadvantages, it cannot certainly confirm the existence of toxins nor can it accurately quantify the toxins (Calleri *et al*., 2007), and it requires multiple washing steps which may be time-consuming (Wacoo *et al*., 2014).

Table 2.2. Analytical methods of aflatoxin detection in food

Method	Advantages	Limitations
Thin layer chromatography (TLC)	Simple, rapid, inexpensive, robust, widely used in various matrices	Requires clean-up, not precise, laborious
Gas chromatography(GC)	Selective, sensitive, accurate	Requires clean-up, time consuming, laborious, expensive
High-performance liquid chromatography (HPLC)	Selective, sensitive, accurate	Requires clean-up, time consuming, laborious, complex, expensive
Liquid chromatography-mass spectrometry (LC-MS)	Selective, sensitive, accurate	Time consuming, laborious, complex, expensive, requires skilled personnel
Enzyme-linked immunosorbent assay (ELISA)	Requires low sample volume, rapid, simple, specific, sensitive, cheap, and portable	Matrix dependent, laborious, limited application
Immunoaffinity column assay	Easy to use, rapid, simple, specific, selective, sensitive, and portable	Matrix dependent, laborious, limited application
Immunosensors	Rapid, sensitive, cost effective	Might not be applicable for detection of small sizes of mycotoxins
NIR Spectroscopy	Non-destructive, low cost, and rapid	Data analysis
Hyperspectral	Non-destructive, low cost and rapid	Data analysis

The conventional methods such as TLC, HPLC, GC and ELISA have been discussed earlier. These methods are expensive, difficult, tedious sample preparation and require skilled and trained personnel. They inevitably require the use of unfriendly chemicals and are not suitable for field applications. For accurate detection of aflatoxins at grain handling facilities, there is a need for a non-destructive, rapid, and objective method (Fermandez-ibanez *et al*., 2009). The growth of mold on grains results in odor or colour changes of kernels, various non-invasive methods such as ultrasound, machine version, and electronic nose were tested to replace the manual as an automatic fungus testing tool. Results showed that many of these techniques were not able to detect reduced levels of infection where obvious physiological changes were not detectable (Singh *et al*., 2012). Moreover, the detection of single kernel instead of bulk samples may be problematic, therefore it is imperative to develop a non-destructive and real-time method to screen crops, especially grains.

2.4. Non-destructive methods for aflatoxin detection

The non-destructive methods of detection include spectroscopy, hyperspectral imaging, thermal imaging, X-ray imaging, neutron tomography, biosensors and bioassays. Hyperspectral imaging and bioassays will be further discussed in a different section.

2.4.1 Spectroscopy

Over the years, evolving studies have been continually directed towards NIR spectroscopy for the detection of fungal contamination and toxins on cereals (Berardo *et al*., 2005; Dlwichie and Gaines, 2005; Dowell *et al*., 1999; Pearson *et al*., 2001; Peiris *et al*., 2009). NIR spectroscopy makes use of the electromagnetic spectrum in the range of 780-2500 nm (Cen and He, 2007). The principle of this technique is based on the measurement of bond vibrations between the atoms of

organic molecules involving mainly C-H, C-O, O-H, and N-H. NIR absorption takes place when the vibrations at a particular frequency coincides with those of a molecular bond in the sample under investigation (Manley, 2014). Pearson *et al*. (2001) showed that the differences in absorbance spectra for different levels of aflatoxin could be explained by the scattering and absorbance characteristics caused by the presence of fungus in the grain kernel. A contaminated kernel would scatter more light than an uncontaminated kernel because the invasion of fungi results in the kernel endosperm becoming powdery. In 2005, a study by Berardo and colleagues indicated the possibility of quantifying fungal infection and metabolites such as mycotoxins in maize grains using NIR spectroscopy. The potential of NIR methodology for the development of a screening test which is easier and faster compared with conventional methods for aflatoxin detection in raw materials was demonstrated by Fernandez-Ibanex *et al*. (2009). The regular NIR spectroscopic method can only generate one average spectrum without any distribution information of the chemical composition of the sample, that is, it cannot determine if the identified concentrations resulted from a bulk sample, a single kernel, a local region of one kernel, or from any other special distribution. Thus, to determine both the distribution and chemical composition of contaminated samples, a probable method to be used could be NIR hyperspectral imaging (HIS), which provides both localization information and a complete spectrum in each pixel in the NIR wavelength region (Manley *et al*., 2009).

2.4.2 Thermal imaging

The principle behind this technique is based on the fact that all materials are capable of emitting infrared irradiation, therefore, it uses the radiation to form a pseudo image of the thermal distribution of the body surface (Chen *et al*., 2013; Vadivambal and Jayas, 2011). An infrared

thermal imaging system is typically made up of the following: camera, an optical system which includes focussing lens, collimating lenses and filters, detector array, signal processing, and an image processing system (Chen *et al*., 2013). A conversion of infrared energy emitted from the object into an electrical signal through IR detectors in the camera produces a coloured or monochrome thermal image. The thermal images are processed to improve the contrast, thus highlighting regions of interest. Extracted statistical and textural features are therefore used in classification procedures. The different types of data mining methods commonly used in imaging techniques can also be applied in thermal image processing (Gowen *et al*., 2010).

Infrared thermal imaging was used to identify bulk wheat grain infected with *Aspergillus glaucus*, *Aspergillus niger*, and *Penicillium spp*, with a classification accuracy greater than 97% (Chelladurai *et al*., 2010). The differences in thermal properties between the healthy and infected samples were the basis for their discrimination. The disadvantages of thermal imaging method include: heating and cooling processes (this may affect heat sensitive commodities in a negative way), and variation in heat distribution may also result in the addition of unwanted variability to the thermogram (Orina *et al*., 2017).

2.4.3 Neutron tomography

This method is based on the principle of absorption and scattering of a neutron beam as it passes through a sample. The application of this technique helps to visualize the inner macroscopic structure and material composition of the sample (Vontobel *et al*., 2006). A tomographic system comprises of a neutron source, an object turntable, a scintillator, a mirror, a cooled CCD camera and computer support (Gibbons *et al*., 1996). The examined object is rotated in angular steps, either 180˚ or 360˚ in the illuminating neutron field, to produce a 2-dimensional image which is a

map of the neutrons attenuated within the sample (Perfect *et al*., 2014; Strobl *et al*., 2009). Cleveland and colleagues were able to distinguish between clean samples and samples that had been infected with *Aspergillus flavus*, using histograms of neutron attenuation coefficients (Cleveland *et al*., 2008). The contaminated kernels had lower neutron attenuation in their scutellum and embryo parts compared to the healthy kernels. Some of the limitations of using neutron imaging are: limited quantitative information obtainable from the images, lower spatial resolution of approximately 10 to 50 μm, and limited access to reactor companies that produce neutrons (Defraeye *et al*., 2013; Lehmann *et al*., 2004).

2.4.4 X-ray imaging and computed tomography

X-rays are electromagnetic radiations with wavelengths that range from 0.01-10 nm. X-rays can move through an object and produce an image that directly reflects internal defects, contamination, and internal structural changes (Chen *et al*., 2013). Soft X-rays (electromagnetic waves ranging from 0.1-10 nm with corresponding energies of 0.12-12KeV) are more suitable for examining agricultural products due to their low penetrating power and ability to display internal density changes (Kotwaliwale *et al*., 2014). X-ray imaging is a highly rapid technique that produces an X-ray radiograph in 3-5 seconds (Neehirajan et al., 2007). The radiographs produced display a 3-D object on a 2-D detector plane, which causes a loss of depth information (Cnudde and Boone, 2013). To prevent loss of information, computed tomography (CT) was developed in the x-ray imaging technique to enable 3-D images. When compared to the traditional x-ray imaging system, computed tomography produces images of superior quality, and allow in-depth analysis of the structure of an object. However, they are expensive and require a longer duration of scanning and data processing (Haff and Toyofuku, 2008). X-ray imaging can detect fungal infection due to the

changes that occur in grain density, because of the infection. The change in density can be detected by comparing the features obtained from the radiographs of healthy and contaminated kernels (Narvankar *et al*., 2009). A cabinet X-ray system was used for the detection of infected maize kernels, and the X-ray films showed that the mean X-ray intensity of the fungal infected kernels was significantly lower than that of the healthy kernels at 95% confidence level. The lower intensity indicated lower density due to the fungal infected kernels absorbing less X-ray energy. The classification was done by stepwise discriminant analysis using features such as the mean, standard deviation, and maximum pixel intensity. An accuracy of 82% was obtained (Pearson and Wicklow, 2006). This technique is rapid and efficient; however, it is expensive, and image analysis procedures are time-consuming (Schoeman *et al*., 2016).

2.4.5 Biosensors

Generically, a biosensor is a device that utilizes a biological sensing element in close contact with a transducer which can convert an alteration in the sensing element to a measurable physical response (Newman and Turner, 2005). Biosensors are sensitive, fast and portable improved methods of analysis that have been adopted for the detection of toxins (Malhotra *et al*., 2015). The different types of biosensors are classified based on their transduction technique. They may be: electrochemical, optical, piezoelectric, optical, calorimetric, impedimetric, amperometric, potentiometric, acoustic, and thermal biosensors (Pohanka *et al*., 2007; Christofi, 2005). Biosensors are composed of three main parts: sensor platform, transduction platform, and amplifier (Mokhtarzadeh *et al*., 2015). During the examination of samples, signals that result from sensor platform recognition, following the transduction operation, are eventually amplified (Geschwindner *et al*., 2012). Some authors have demonstrated the ability to detect aflatoxins in

agricultural products using biosensors (Maragos and Thompson, 1999; Carlson *et al*., 2000; Sapsford *et al*., 2006; Molina-Garcia *et al*., 2012). Maragos *et al*. (1999) used an Evanescent wave-based fiber-optic immunosensor for the detection of AF in maize. They employed a non-competitive assay using the natural fluorescence of AFB1. Aflatoxin B1 has a native fluorescence that is best possible for the construction of a non-competitive immunosensor. Because of its native florescence, the response of the sensor was directly proportional to the toxin concentration. The sensor had a detection limit as small as 2 ng/ml. This method showed potential for rapid screening of individual maize samples, however a previous clean-up step was required for it to be completely effective.

Another study described a chemiluminescence immunoassay for the detection of AFB1 in food and feed samples. A multiplex chemiluminescent biosensor was designed for AFB1 and type B-fumonisins in maize. An extraction of the analytes from the samples was required for this procedure. Their results showed that chemiluminescence detection was accurate and sensitive with a detection limit of 6 µg/kg and 1.5 µg/kg for fumonisins and AFB1, respectively (Zangheri *et al*., 2015). In comparison to conventional methods, some biosensors are able to detect large amounts of analytes with ease and at affordable costs. Numerous biosensors have been developed and are being utilized for aflatoxin detection.

2.5 Hyperspectral imaging

Spectroscopy obtains limited information from the areas on samples, which is a major set back because it offers no information about spatial dimension (William *et al*., 2012). Mycotoxins have been discovered to be unevenly distributed in grains, therefore removing a small percentage of contaminated grains is a better and more conservative method, other than discarding an entire

batch of kernels (Pearson *et al*., 2004). This is a crucial reason to be able to identify mycotoxins in individual kernels. Hyperspectral imaging is an emerging technique that combines the conventional imaging (spatial) and spectroscopy (spectral) method (Senthilkumar et al., 2015). HSI has three dimensional arrays (m × n × γ), where m and n are the spatial axes and γ is the spectral information (Williams *et al*., 2012). Spectral wavelength range can be divided into; visual near infrared (VIS-NIR) hyperspectral imaging (400-1000 nm) and short-wave infrared (SWIR) hyperspectral imaging (1000-2500 nm). HSI is a method that can be used to detect the distribution and composition of mycotoxins in contaminated foods, especially grains. HSI can produce both localized information and a complete NIR spectrum in each pixel (Manley *et al*., 2009). Hyperspectral images are large, but data reduction is an effective way of controlling the large amounts of data. Hyperspectral imaging is being widely applied in all aspects of agricultural and food sciences.

Yao *et al*. (2010) used HSI technique to determine aflatoxin contamination in maize kernels inoculated with *A. flavus* spores. Wang *et al*. (2014) and Wang *et al*. (2015) also exhibited the potential of HSI based in the Vis/NIR range for quantitative identification and distinction of AFs in inoculated maize kernels. They deposited different concentrations of AFB1 on the surface of maize kernels and acquired spectral images using SWIR and Vis-NIR. Regions of interest that had the AFB1 drips on the kernels were selected, and the mean spectra were calculated. They developed discriminant models to classify the samples and obtained classification accuracies of 88% and 98% respectively. It was concluded that it was possible to discriminate different concentrations of AFB1 applied on the samples. Similarly, Pearson *et al*., 2001, demonstrated that the spectral reflectance with a ratio between Vis and NIR can be analysed to detect highly contaminated AF corn kernels from those with a contamination level lower than 10 ppb. This

review agreed with other studies by Del Fiore *et al*. (2010) and Singh *et al*. (2012). They all reported that Aspergillus fungi in maize and wheat were observable by analyzing the HSI in 400-1000 nm or the combination of HSI and digital images. (Udomkun *et al*., 2017). In another study, Kandpal *et al*. (2015) used a SWIR hyperspectral imaging to detect AFB1 artificially inoculated on corn kernels. Images were acquired over the spectral range of 1100-1700 nm. A partial least squares discriminant model (PLSDA) model was developed for classification, and it yielded an accuracy of 96.9%. This predicted the system could detect toxic metabolites in grains. Some authors have also shown results which demonstrated that HSI with a wavelength range between 1000 and 2500 nm could be used for aflatoxin B1 detection at a very low concentration, as low as 10 ppb, if applied directly on maize kernel surfaces (Chu *et al*., 2017; Kimuli *et al*., 2018). Wavelengths such as 1729 nm and 2344 nm were identified to be employed for the characterization of the objective existence of AFB1 (Wang *et al*., 2014).

In order to detect insect infestation in mung beans, Kaliramesh *et al*. (2013) used NIR hyperspectral imaging over the range of 1000-1600 nm. They developed two classification models (LDA and QDA) to identify infested and uninfested bean kernels. The classification results were satisfactory with accuracies of 82 and 85%, respectively. In 2013, Huang *et al*. utilized hyperspectral imaging to detect insect damage in soybeans. The support vector data description (SVDD) approach was used to categorize the samples as damaged or healthy. Classification accuracies of 100% were reported. A vast number of researches have employed the use of hyperspectral imaging because of the advantages it confers. In addition to the rapid and non-destructive advantage of HSI and NIR spectroscopy, they are easy to use and require no sample preparation.

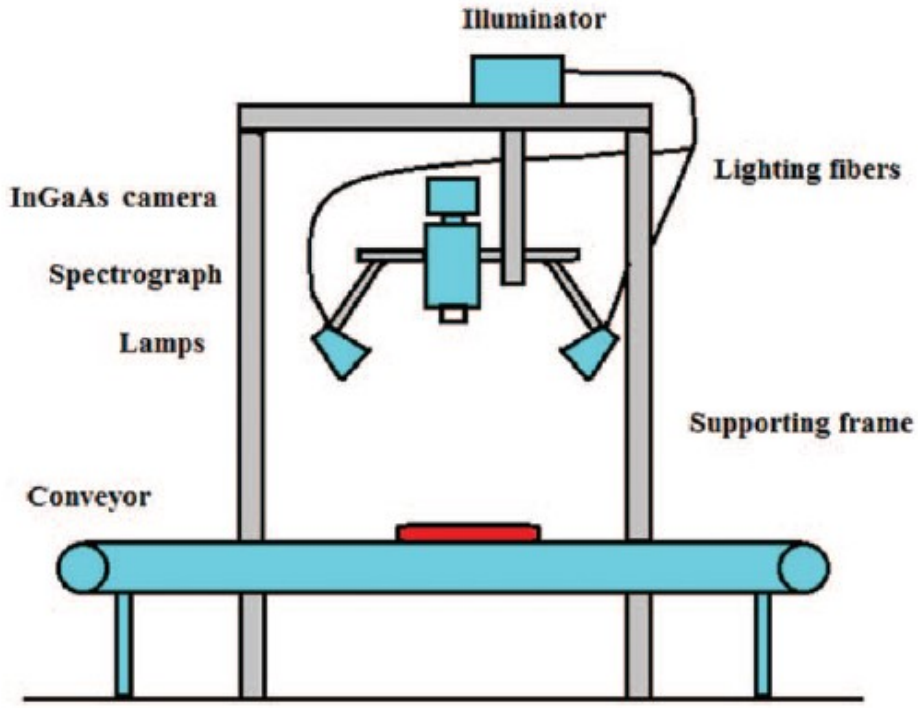

Figure 2.2 Hyperspectral imaging system (Liu *et al*., 2013).

2.6 Bioassays

Bioassays use living materials to detect and determine potential toxicity of substances. They are widely applied for the monitoring of potential hazardous substances in soils, potable and waste water, foods, and other sources (Christofi, 2005; Chayen and Bitensky, 1988). There are different measuring principles in bioassays, they include;

1. Bioassays using changes in growth rate, biomass and numbers: Assays that utilize changes in growth rate have been used to test the effect of chemicals on microorganisms. These tests require a long duration and are labour intensive, because they involve the use of

methods screening changes in microbial populations and biomass indicators that are difficult to automate. The methods of monitoring the growth rates and biomass include biochemical analyses, turbidometry, spectrophotometry, potentiometry, electron counting, flow cytometry, viable and total cell counting using microscopic techniques.

2. Respirometry: This involves the quantification and explanation of the biological oxygen consumption. Oxygen consumption is directly linked to microbial growth and substrate removal. Respirometers are used to measure the respiration rate. Respirometric methods have been widely used for the determination of biokinetic parameters. It has been developed for the respiration inhibition kinetics analysis to detect and measure the toxic effects of xenobiotic compounds on the biogenic-carbon removal in biological wastewater treatment systems. Many respirometers are available for bioassays. A common respirometer is the Arthur Respirometer which is used in toxicity testing.
3. Enzyme Bioassays: Enzymes play an important role in the metabolism of all microorganisms. They induce the chemical reactions that occur within anabolism, catabolism, or energy transfer. Enzyme tests are normally conducted in food analysis and clinical chemistry. Enzymatic activity can be measured in two ways; *in vitro* and *in vivo* tests. *In vitro* tests utilize commercially available isolated enzymes that are pure and characterized. In food analysis, carbohydrates, organic acids, and alcohols are measured using isolated enzymes. *In vivo* tests use a synthetic substrate which is labeled with a chromophore, and adds it to the sample. The enzyme present in the sample catalyses the cleavage of the substrate bond, and the chromophore is then detected using photometry or fluorometry. The application of *in vivo* enzymatic tests for detecting toxic substances has conducted and compared to other microbiological testing techniques.

4. Bioluminescence/Chemiluminescence: Chemiluminescence is the emission of light when chemically excited molecules are degraded after a chemical reaction. Bioluminescence on the other hand is the emission of light in response to reactions in living organisms. An example is the marine luminescent bacterium *Vibrio fischeri,* which produces light in a luciferin-luciferase system, associated with the energy transfer and respiration taking place in the cell. The light produced can be measured using a suitable luminometer. In the *V. fischeri* bioassay, the reduced light produced from a suspension of the bacteria on exposure to a toxic substance is measured. A control bacterium is used to monitor natural light decrease over time, and their results are compared. Bioluminescence genes are mostly used in biosensors for screening toxic substances and pollutants. Bioassays utilizing bioluminescence are available for a wide range of applications to detect and quantify toxic chemicals and pollutants.

A number of researches have been done applying bioluminescence assay to detect carcinogens, mutagens and other compounds. Elespuru and Yarmolinsky, (1979) developed a colorimetric assay of lysogenic induction for the screening of potential carcinogenic and carcinostatic agents. The principle of the assay involved galactosidase synthesis by an *E. coli* strain, which is in turn measured to determine if the target substance was detected. The assay was able to detect some harmful substances and their results indicated that aflatoxin B1 in addition to other chemicals are potential agents capable of DNA damage. A biochemical prophage induction assay which is adapted for detecting inducing chemicals has been used by a number of authors to detect antitumor agents, potential carcinogens, and other toxic chemicals. The galactosidase produced as a result of induction can be measure either by colorimetry or luminescence. (Moreau *et al*., 1976; Elespuru and White, 1983; McCann 1975; and Maya *et al*., 2005).

2.7 Conclusion

The effects of aflatoxins on food safety and food security are posing a great threat to the health of humans and animals. Due to aflatoxin's detrimental and frequent occurrence in agricultural products, the research on aflatoxin reduction and elimination has procured global attention. Thus, mycotoxin analysis continues to be critically important. A wide range of detection and determination methods used for practical analysis and detection of a broad spectrum of mycotoxin are available, many of which are highly sensitive. The various analytical methods for aflatoxin detection have been explored in this literature review. While some of the techniques are considered gold standard and widely used, they remain largely burdensome, destructive, requiring extensive sample preparation, very expensive equipment and require trained personnel. All these make their applications confined to the laboratory, and on this account, rapid and non-destructive methods such as NIR spectroscopy and hyperspectral imaging are necessary for aflatoxin detection.

CONNECTING TEXT

A comprehensive review of literature showed that hyperspectral imaging is a potential non-destructive method for rapid and accurate detection of aflatoxin. No report has shown the application of the technology on beans.

Chapter 3 addresses the first and second objective of the thesis, *i.e*., determining the feasibility of detecting aflatoxin B1 in bean seeds using hyperspectral imaging, and assessing different classification models. Part of this chapter has been submitted for a presentation at the ASABE conference in July 2018. A paper based on this chapter will be submitted for publication. The manuscript will be co-authored by my supervisor Dr. Michael Ngadi and his Research Associate Dr. Li Liu. The format of the original manuscript has been modified to remain consistent with the thesis format.

III. HYPERSPECTRAL DETECTION OF AFLATOXIN B1 CONTAMINATION ON BEAN SEEDS

3.1 Abstract

NIR hyperspectral imaging (900 – 1700 nm) combined with chemometrics and two classification techniques (PCA-LDA and PLSDA) were applied to develop a rapid, inexpensive, and non-invasive method for detection of aflatoxin (within the concentration range of 0 – 500 ppb) on the surface of bean seeds. Partial least square discriminant analysis (PLSDA), principal component analysis (PCA), and linear discriminant analysis (LDA) were used for classification of the contaminated bean seeds. Two models were developed to classify the uncontaminated and contaminated bean seeds. The best performing classification method was PCA-LDA, which yielded an accuracy ranging from 60 - 100% in terms of sensitivity and specificity in calibration and validation. However, PLS-DA also yielded a satisfactory classification accuracy greater than 71%, making the both of them reliable classification techniques. Overall, the results indicated that hyperspectral imaging is a feasible method for non-destructive detection on aflatoxins.

3.2 Introduction

Beans are grain legumes and currently estimated to be one of the most important legumes globally. (Messina 2014). As a whole food, they contain high levels of dietary fiber, starch, protein, vitamins, minerals and phenolic compounds. The consumption of beans confers numerous health benefits, making it an important source of nutrients for over 300 million people in Eastern Africa and Latin America (Harvest plus, 2009). Approximately 12 million metric tons of beans are produced yearly, with 5.5 and 2.5 million metric tons allocated to Latin America Caribbean (LAC) and Africa alone. Despite its benefits, bean production and consumption are often underestimated

because intercropping and consumption of beans occurs more frequently in remote rural areas that lack accurate dietary intake data (Jones, 1999). Losses occur after harvest and before consumption in the under developed areas, and these losses vary annually due to changes in environmental factors (Senthilkumar *et al*., 2016). The major cause of spoilage of stored products are moisture content and temperature at which they are stored. The favorable moisture content and temperature facilitates the growth and multiplication of mites and fungi. Fungi are potentially more harmful than insects and mites because they can contaminate the grains with their secondary metabolites known as mycotoxins. The major losses encountered due to mycotoxin contamination are: potential health problems, livestock poisoning, and economic loss. The disease outbreaks caused by mycotoxins are not frequently occurring in developing countries, however, the major concern is the detrimental effects of long term ingestion of low levels of mycotoxins. Aflatoxin is one of the major mycotoxins found in agricultural products. There are different of aflatoxins, and aflatoxin B1 is the most potent of all (Van *et al*., 2004). Aflatoxin B1 can cause severe damages to human health and a lethal dose of 10-20 mg of aflatoxin can lead to death (Etzel, 2002). Accordingly, it is highly crucial and necessary to detect it accurately.

Over the past several decades, accurate methods of detection based on enzyme linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC), and thin layer chromatography were broadly applied to guarantee the quality and safety of food (Maragos and Busman, 2010; Selvaraj *et al*., 2015). However, these conventional methods are laborious, time consuming, expensive and destructive in nature. In the last few years, various non-destructive methods have been developed to overcome these setbacks.

Spectroscopy is a non-destructive and potential technique that is commonly used for the quality assessment of food products (Dowell *et al*., 1999; Pearson *et al*., 2001; Dowell *et al*., 2002;

Wang *et al*., 2004; Berardo *et al*., 2005). The application of NIR spectroscopy for mycotoxin detection has been investigated since the late 1990s (Peiris *et al*., 2009). Fernandez *et al*. (2009) used NIR spectroscopy to detect aflatoxin B1 in maize and barley. The best predictive model they used to detect AFB1 in maize was developed using standard normal variate and detrending (SNVD), which gave satisfactory results (r^2 = 0.80 and 0.82; SECV = 0.211 and 0.200).

Despite the accuracy of spectroscopy, it provided only one spectrum of the target sample, without information about the spatial distribution of the chemical constituents of the sample. Unlike spectroscopy, hyperspectral imaging provides both spectral and spatial information of an object (Gowen *et al*., 2007; Chen *et al*., 2013; Manley 2014).

Some authors have researched on the potential of detecting aflatoxin on maize kernels with the use of hyperspectral imaging (Wang *et al*., 2014; Kandpal *et al*., 2015). The spectra data obtained can be improved using different preprocessing techniques to reduce or eliminate useless information and produce accurate results. For example, Wang *et al*. (2014) applied a short-wave infrared (SWIR) hyperspectral imaging system to evaluate the possibility of detecting AF B1 on the surface of contaminated maize kernels. They used standard normal variate (SNV) for the preprocessing of spectra. Factorial discriminant analysis (FDA) was applied on the principal component analysis (PCA) scores obtained in this study. Their results showed that it was possible to detect AFB1, and discriminate between the control and contaminated samples with a minimum classification accuracy of 88% using PCA-FDA method. In another NIR HSI study by Kimuli *et al*. (2018), chemometric techniques like PCA and FDA as well as preprocessing techniques such as SNV and Savitzky-Golay smoothing (SGS) were applied on spectra data to minimize or eliminate unwanted information to focus on aflatoxin information. They developed a PCA-FDA model which was able to predict AFB1 contamination with an accuracy greater than 96% in

validation. A similar study by the same author employed the use of SWIR HSI to identify AFB1 in contaminated maize kernels. Transformation methods such as SNV, first and second derivatives were performed. They applied PCA, partial least squares discriminant analysis (PLSDA), and FDA to the extracted spectra data. These methods were to develop and compare two classification models. Results indicated that a combination of SNV and first derivative produced a satisfactory result for PLSDA classification model with accuracies of 96 and 100% in validation and calibration respectively. However, the best AFB1 classification results was produced by the FDA model on raw spectra, with an accuracy of 100% in calibration and validation.

The objectives of this study were to: 1) Assess the feasibility of detecting aflatoxin contamination on bean seeds using the spectral characteristics obtained from NIR hyperspectral imaging. 2) Develop and compare the sensitivity of classification models for optimal classification of different levels of aflatoxin contaminations.

3.3 Materials and Method

3.3.1 Sample preparation

Maine bean seeds harvested in the 2016 season were imported from Lufita and Kameme communities of the Chitipa District in Northern Malawi. Aflatoxin B1 purchased from Sigma Aldrich was used to prepare stock solutions of aflatoxin at concentrations 10, 20, 100 and 500 ppb by diluting with 100% methanol. The preparatory procedure for the stock solutions was done according to a previous research by Wang *et al*. (2014). After the stock solution preparation, artificial contamination was carried out using a total of 180 clean and physically intact beans seeds which were divided into six groups of thirty seeds. Four of these groups were inoculated with

different concentrations of aflatoxin by immersing in the stock solutions for approximately 15 hours. The samples were divided into two control groups. The first group was treated with methanol whereas the second group was not treated. The samples were left to dry naturally for 2 days in a class II biosafety cabinet, after which they were imaged using a sample holder. The sample holder was made locally using a polystyrene material. To have a better understanding of the result, we had two control groups. One group was treated with methanol to ensure they were clean, and the last group was not subjected to any treatment. The samples were left to dry naturally for 2 days in a class II biosafety cabinet, after which they were imaged.

3.3.2 Hyperspectral imaging system

The NIR hyperspectral imaging system used was composed of an InGaAs camera, an imaging spectrograph (NIR hyperspec 900-1700 nm, Headwall photonics, Fitchburg, MA, USA,), a moving conveyor (MDIP22314, Intelligent motion system Inc., USA), two 50W tungsten-halogen lamps (JDR-C GU10, 120 V, 50 W), and a PC as shown in Fig 3.1. The hyperspectral imaging system consist of a software that scans the sample line by line, forming a hypercube with two spatial axes and one spectral axis. Within the obtained hypercube were individual images over the range of wavelengths 900 – 1700 nm with a spectra resolution of 4.8 nm. A couple of seconds were required to image the beans samples.

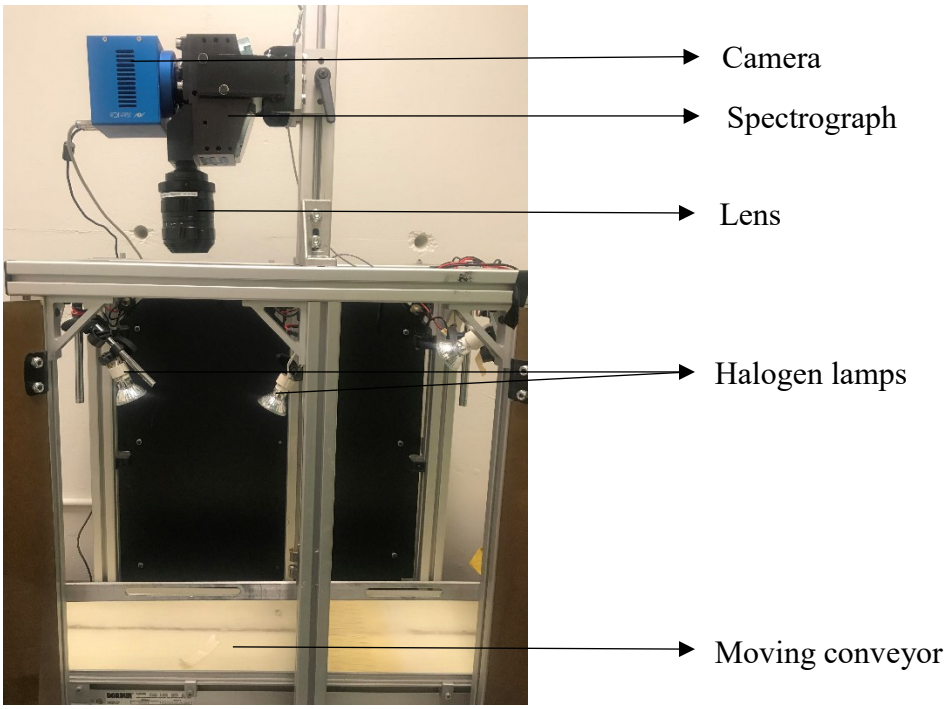

Figure 3.1 Hyperspectral imaging system

3.3.3 Image acquisition and calibration

The control and contaminated samples were aligned in the shallow wells of sample plate holder, and were set for image acquisition. The sample was placed on a computer controlled linear motion conveyor that moved the beans samples across the field of view (FOV) of the camera. The captured images of the beans samples were saved and transferred to the computer for pre-processing. To correct the spectral images, dark and white calibrations were done by covering the camera lens with a cap to obtain a dark image while a white image was obtained by scanning a standard ceramic reference (Spectralon, Labsphere North Sutton, NH).

3.3.4 Spectral pre-treatment

The acquired hyperspectral images consisted of the background of the sample plate holder. Therefore, to obtain the regions of interest, MATLAB 7.13.0.564 (The MathWorks, Inc., Mass., USA) was used for region of interest (ROI) selection. Different preprocessing techniques were employed to determine which of the method would perform better. Based on their performance, spectra were pretreated using two transformation techniques, namely; standard normal vitiate (SNV), and multiple scatter correction (MSC). These techniques were used to reduce the scattering noise. SNV is a mathematical spectra correction method that was used to eliminate the slope variation and to correct for scatter and variation effects. MSC was used to correct the level of spectra scattering by removing data spectral differences.

3.3.5 Multivariate analysis

In this study, chemometric techniques such as principal component analysis (PCA), linear discriminant analysis (LDA), and partial least squares discriminant analysis (PLS-DA) were used for data analysis. Principal component analysis (PCA) is a powerful and robust technique for reducing dimensions, selecting variables in spectral data and resolving multicollinearity drawback. On the other hand, Linear Discriminant Analysis (LDA) was used for classification of spectral data into mutually exclusive classes based on a set of measurable features.

3.3.6 Principal component analysis (PCA)

Principal component analysis (PCA) is the frequently used chemometric tool for data exploration (Alamprese *et al*., 2016). PCA was applied in this study as an unsupervised technique that converted the original variables in a data set into smaller number of new linear uncorrelated variables known as principal components (PCs). The main purpose of PCA was to reduce dimensionality of the HSI imagery which contains highly correlated information in neighboring bands (Williams and Norris, 1987, Alamprese *et al*., 2016). Data dimensionality reduction was necessary for the discriminant technique that followed thereafter (Castelbow *et al*., 2007; Karoui *et al*., 2011; Wang *et al*., 2014).

3.3.7 Partial Least Square Regression and Discriminant model (PLS-DA)

PLS-DA is one of the most important and used discriminant classification method to solve both regression and classification problems. It is a multivariate data analysis method which is principally suited to work with enormous spectral data (multicollinearity) and gives quantitative and qualitative information of the sample (Barker and Rayens, 2003). The PLS-DA model was constructed according to Kandpal *et al*. (2015). The model was based on 70:30 calibration to validation set using 180 samples. The calibration samples were used to construct the model and the validation set was used as a reference to test the model. The validation of the model was important to determine the potential of NIR HSI for future predictions. This technique was established for the grading of samples according to AFB1 contamination levels.

3.4 Results and Discussion

3.4.1 Spectral characteristics

The mean raw spectra of control and contaminated samples (i.e. the control group and seeds inoculated with 10, 20, 100 and 500 ppb aflatoxin B1) are presented in Fig 3.2. Spectra difference was fairly noticed between the control and contaminated group at the beginning of the illustrated spectra, but the separation was improved across 1000-17000 nm. Major differences in reflectance was observed between the control and contaminated groups, apparently due to the increase in AFB1 concentrations in the samples (Wang *et al*., 2014). The average reflectance intensity of all groups of seeds reduced as the wavelength increased, ranging from 1000 to 1700 nm as was also observed by Kimuli *et al*. (2018). It was observed that the within the spectral range of 1400 – 1700 nm, reflectance of the bean samples appeared in the order of increasing aflatoxin concentration (control group, 10, 20, 100 and 500 ppb). The control group had the lowest reflectance whereas the highest concentration (500 ppb) had the highest reflectance.

Similar spectra shapes, characteristic peaks, and valleys were observed throughout the spectra range, however, there were differences in their respective reflectance values. Some significant peaks were observed around 990, 1100, 1200, 1300, 1450, and 1650 nm. The differences in the positions of the peaks are likely correlated to differences between a profusion of chemical components in the control and aflatoxin contaminated seeds. The spectra around 990, 1110, and 1200 nm could be related to the second overtone C-H stretching (Kandpal *et al*., 2015), while 1300 and 1650 nm may correlate with combination C-H stretching (Kimuli *et al*., 2018). Previous studies by Chu *et al*. (2017) and Kimuli *et al*. (2018) revealed that 1459 nm was associated with AFB1 content in maize kernels. According to Stuart (2004) and Wang *et al*. (2015),

the first overtone of O-H stretching and first overtone of N-H stretching were also associated to 1459 nm, which may be linked to the spectra observed around 1450 nm in our results.

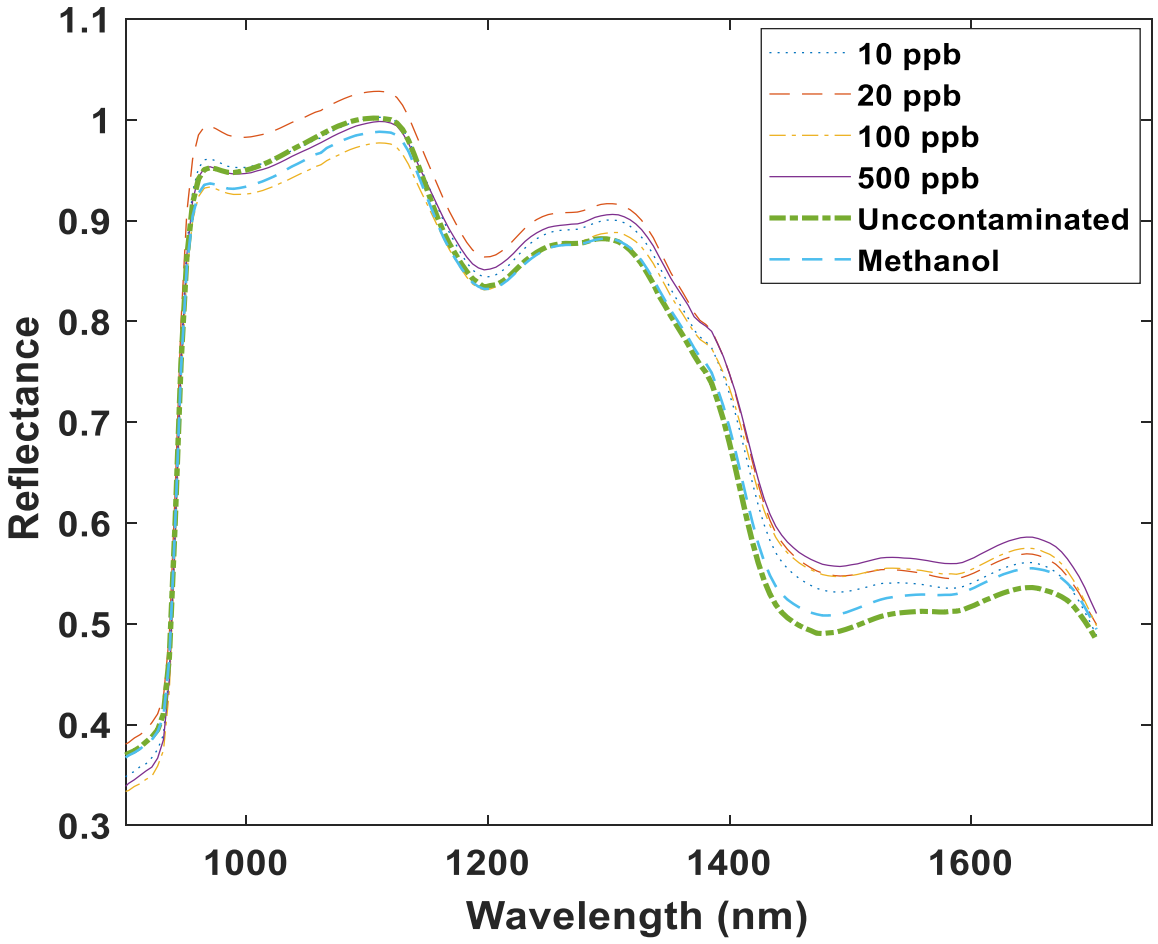

Figure 3.2 Mean spectra of beans with different aflatoxin concentrations

3.4.2 Classification analysis

3.4.2.1 Principal Component Analysis

PCA was done in order to explore the spectral differences. The spectra were pretreated with different processing method and were evaluated, and it was discovered that SNV had the best performance in terms of explained variance. The score plot of the raw spectra of bean seeds coloured on the basis of the different concentrations is shown in Figure 3.3. From the score plot obtained using SNV, there was a visible pattern of separation of the control group from the contaminated samples as shown in Figure 3.4. The score plot of raw spectra showed partial separation of the samples. The higher concentrations of AFB1 were more prominent above while the control and the lower concentrations (10 and 20 ppb) were below with some overlap. It was discovered that PC1 and PC2 accounted for more than 93.97% variability of the raw data, while PC1 and PC2 accounted for more than 96% variability in SNV transformed data. The remaining data were residual and unexplained. This result can be supported by a similar research which showed the separation of control samples from AFB1 contaminated seeds using PC1, PC2, and PC3, although they indicated that the maize kernels could not be separated according to their respective AFB1 contamination group 10, 20, 100, and 500 ppb (Wang *et al*., 2014). Contrary to this result, a more recent research by Kimuli *et al*. (2018) showed that the first 3 higher variance principal components (PC1, PC2 and PC3) did not provide a clear separation of the control from AFB1 contaminated maize kernels, so they analyzed the lower variance PCs (PC9, PC10, PC11, PC12, PC13, PC14 and PC16) to separte the control samples from aflatoxin contaminated maize kernels (Kimuli *et al*., 2018).

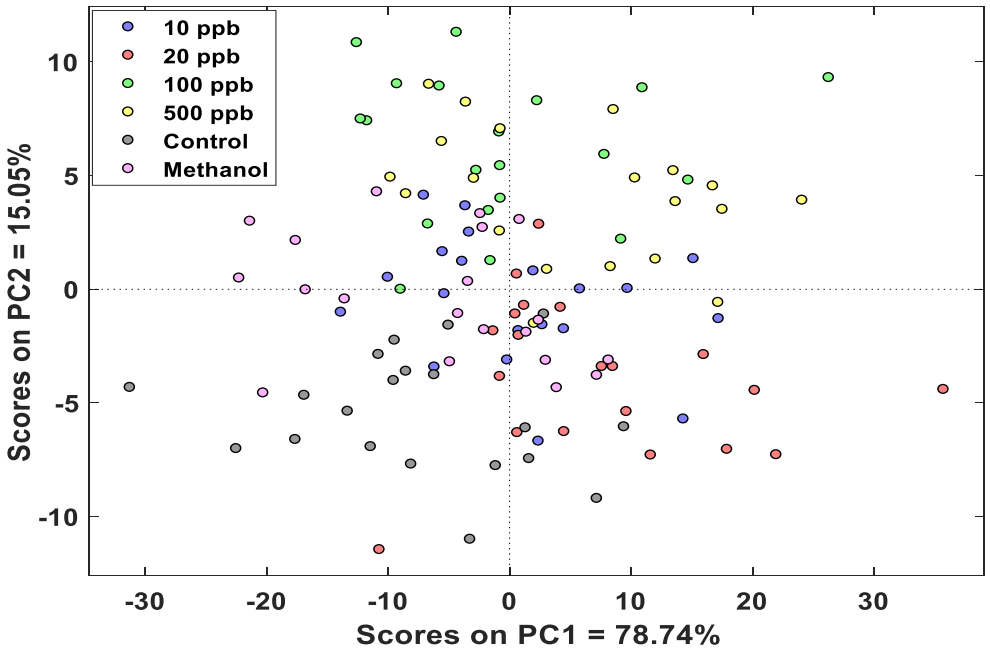

Figure 3.3. Raw spectra PCA score plot on PC1 and PC2 of contaminated and uncontaminated beans

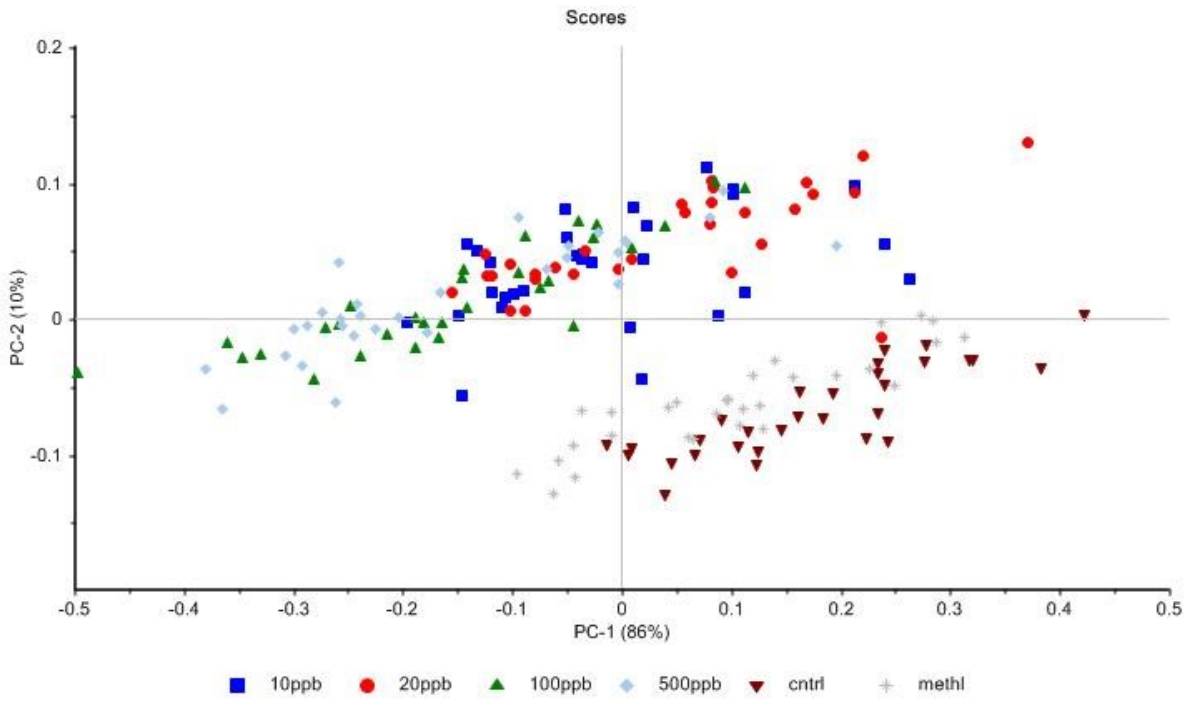

Figure 3.4. SNV pretreated PCA score plot on PC1 and PC2 of contaminated and uncontaminated beans

3.4.2.2 PLSDA and PCA-LDA

The possibility of identifying bean seeds that were contaminated with AFB1 was evaluated using the different classification approaches based on PLSDA and PCA-LDA. The spectra from the 180 samples were used for the development of the PCA-LDA and PLSDA models. Table 3.1 and Table 3.2 shows the classification results of the models in terms of sensitivity and specificity for calibration, cross-validation and prediction on raw spectra. Sensitivity is a measure of true positive rate while specificity constitutes true negative rates.

The models were calculated without considering pre-processing of the raw data because when pre-treatment using SNV and MSC were applied, accuracy deteriorated (data not shown). It was discovered that the raw data performed better, therefore, only results obtained from raw data were presented. The best discriminant model was selected based on the least number of errors in validation. The validation samples had identical aflatoxin concentrations as the calibration samples. The results showed that PCA-LDA proved to be the best discriminant model, showing higher accuracy and lower false errors in classifying the samples into their respective aflatoxin contamination groups. Accuracies ranged from 80 - 100% for calibration, 60 - 100% for prediction, and 75 - 100% for cross validation. When the calibration models were assessed using an independent sample set, there was a reduction in accuracy of prediction. The lowest error rates were found in the control groups, 10, and 20 ppb, showing almost 100% predictive accuracy. The correct classification rate for 100 and 500 ppb was slightly lower. The predictive ability of the model was demonstrated to be very high, yielding high values of sensitivity ranging from 75 - 100% and specificity values ranging from 92 - 100% which guaranteed a low number of misclassified samples.

The calculated classification results of PLS-DA models also showed an acceptable discriminant ability, with consistent sensitivity values higher than 86% in calibration, 67% in prediction and 71% in cross validation. The PLSDA model achieved 100% accuracies for the control groups and 20 ppb. The percentage of correctly classified samples was observed to decrease in the prediction set for 100 and 500 ppb. The PLSDA calibration models with the prediction set showed the classification results comparable to the LDA calibration models. The non-contaminated samples were correctly classified with 100% accuracy in both models, except for methanol group in the PLSDA model with 71% accuracy. A recent study investigated the use of SWIR to detect AFB1 on maize kernels by assessing two classification models (PLSDA and FDA). The models exhibited satisfactory results with accuracies higher than 96% in calibration and validation, however, the best discriminatory ability was observed in FDA model. From our models, the high sensitivity and specificity values can be explained to be a guarantee of a low number of misclassified samples. Thus, a reliable classification between the uncontaminated and contaminated groups. The differences in performance of the models may be attributed to differences in data manipulation methods in the algorithms of the techniques employed for classification.

The beta coefficient of the PLSDA model demonstrated significant wavelengths that are likely to be responsible for the separation of the control samples from the contaminated samples, and therefore depicts useful information about AFB1. Several distinct peaks around wavelengths such as 1100, 1200, 1300, 1450, and 1650 nm were also identified in Fig 3.5. The wavelengths around 1100 and 1200 nm are attributed to the C – H (carbohydrate) component (Agelet *et al*., 2012). The peaks ranging from 1400 – 1650 nm could be related to the O – H stretching of the first overtone and to the presence of starch and protein components (Sirisomboon *et al*., 2013). In

addition, peaks within the region 1400 – 1500 nm that contains 1459 nm may be related to phenolic compounds in beans (Chu *et al*., 2017), and the wavelength 1451 nm was related to the third overtone of C=O stretching (Kimuli *et al*., 2018). Lastly, Chu *et al*., (2017) revealed that 1459 nm was associated with AFB1 content in samples. These results indicate that the models developed were based on the chemical composition of bean seeds related to aflatoxin B1 content.

Table 3.1: PCA-LDA model for classification of aflatoxins in different concentrations

Aflatoxin	**Opt. latent**	**Calibration (%)**		**Cross-validation (%)**		**Prediction (%)**	
Concentration	**Variables**	**SENC**	**SPECC**	**SENC**	**SPECC**	**SENP**	**SPECP**
10 ppb	13	95	98	90	95	100	92
20 ppb	13	95	100	90	100	90	100
100 ppb	13	80	97	75	97	60	96
500 ppb	13	80	95	75	94	60	94
Control	13	100	100	100	100	100	98
Methanol	13	100	100	100	100	90	100

Table 3.2: PLS-DA model for classification of aflatoxins in different concentrations

Aflatoxin Concentration	Opt. latent Variables	Calibration (%)		Cross-validation (%)		Prediction (%)	
		SENC	SPECC	SENC	SPECC	SENP	SPECP
10 ppb	9	90	98	80	94	71	95
20 ppb	9	100	100	78	100	100	100
100 ppb	9	86	98	71	94	50	92
500 ppb	9	92	98	75	95	67	88
Control	9	100	100	100	98	100	96
Methanol	9	100	100	90	100	71	100

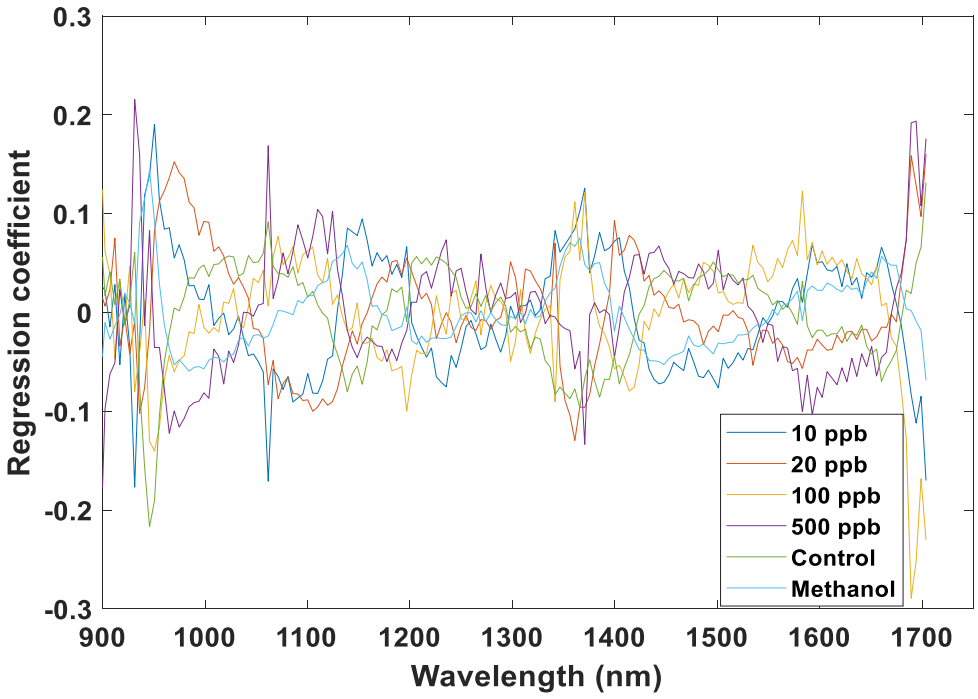

Figure 3.5 PLS regression vectors for prediction models for aflatoxin contaminated and uncontaminated beans

3.5 Conclusion

The present study using rapid and non-invasive NIR hyperspectral imaging with the adequate multivariate discriminant models and cross validation procedure, has demonstrated its potential of becoming a reliable tool for the rapid identification of aflatoxin, in beans. We examined low (10 ppb) and high (500 ppb) aflatoxin concentrations using NIR hyperspectral imaging. The selected models produced satisfactory results for the discrimination of contaminated and uncontaminated samples with high sensitivity and specificity values ranging from 75 - 100% in calibration and validation. Some misclassifications were observed, and these influenced the accuracies of the models, nevertheless their performances were ideal for a good model.

CONNECTING TEXT

Review of literature revealed that there have been advances in the application of luminescence bioassay to detect toxicants that include aflatoxin B1. In chapter 3, the feasibility of detecting aflatoxin B1 on bean seeds was determined. Chapter 4 deals with feasibility of developing a novel luminescence bioassay for detecting aflatoxin.

A paper based on this chapter will be submitted for publication. The manuscript is co-authored by my supervisors Dr. Michael Ngadi, Dr. Lawrence Goodridge, and a Research Associate, Dr Li Liu. The format of the original manuscript has been modified to remain consistent with the thesis format.

IV. LUMINESCENCE ASSAY FOR DETECTION OF AFLATOXIN B1

4.1 Abstract

Aflatoxin contamination is a serious global agricultural and health problem. Under suitable environmental conditions, agricultural products may be infected by different species of fungi. Accurate detection and control of aflatoxin is essential for ensuring the safety of food. The long-term objective of this study was to develop a new technology for monitoring aflatoxin B1 based on the ability of aflatoxin to induce prophage induction. The specific goals of the study were to determine the potential of a luminescence assay for the detection of aflatoxin, and to establish the detection limit. A high throughput luminescence assay of prophage induction was applied in the detection of aflatoxin B1. Two different strains of *E. coli* were used to detect the presence of aflatoxin B1. The indicator strain possesses the ability to produce beta galactosidase upon exposure to toxic chemicals, while the control strain lacks this ability. Duplicate cultures were inoculated with different concentrations of aflatoxin B1 (0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20 and 200 ppb) and incubated for 6 hours, followed by the addition of the luminescent reagent before measuring luminescence in a plate reader. The relative light units of the respective strains were compared, and a positive detection of aflatoxin was determined by a ratio value greater than 1 (>1). The aflatoxin concentrations of 0.312, 0.625, 1.25, 2.5, 5, 10, 20 and 200 ppb were positive for detection and thus classified as inducers. The results suggest that the luminescence prophage induction assay may be useful to detect aflatoxin B1. This inductest is suitable for the development of an advanced rapid and field based technique for the detection of aflatoxin.

4.2 Introduction

Aflatoxins are polyaromatic secondary metabolites of fungi, formed majorly by *Aspergillus flavus* and *Aspergillus parasiticus* (Rasooly *et al*., 2016). Aflatoxins are of global concern owing to their detrimental effects on health and substantial losses to food and agricultural commodities. Hamid et al (2013) reported the world's population exposed to aflatoxin from ingestion of contaminated plant and animal products to be 4.5 billion. The results of consuming fairly contaminated food products are unknown and are difficult to estimate because of the difficulty in recognizing the symptoms (Rasooly *et al*., 2016). Some authors (Josse *et al*., 2008; Pena and Duran, 1990) showed that aflatoxins are capable of bioaccumulation and bioconcentration, hence consumption of extremely low levels in food can have consequences over time. Aflatoxin B1 is the most common and widespread in the world, accounting for about 75% of all contaminations in food and feeds, and causing acute toxicity, teratogenicity, mutagenicity and carcinogenicity (Hussein and Brasel, 2001; Kok, 1994).

Traditional methods used for the detection of aflatoxins include: culture and colony techniques (Gourama and Bullerman, 1995), chemical analyses (Lin and Cousin, 1985), enzyme linked immunosorbent assay (ELISA) (Meirelles *et al*., 2006), and chromatographic techniques (Pereira *et al*., 2014). Although these methods are accurate, reliable, specific, and sensitive; they are expensive, time consuming, labour intensive, requiring tedious sample preparation which results in sample destruction. In the search for the development of a simple, rapid, and non-destructive method, the accurate detection of aflatoxin is crucial. The emergence of modern imaging methods and biosensors has provided numerous avenues for non-destructive screening of agricultural products. Common non-destructive methods of detection are; spectroscopy,

hyperspectral imaging, thermal imaging, colour imaging, X-ray imaging, biosensors and bioassays.

Bioassays are techniques that use living materials to detect substances or determine the probable toxicity of chemicals. They are mostly used to monitor potential hazardous chemicals in foods and other materials (Christofi, 2005). Toxicity bioassays are based on the application of bioluminescence which is the emission of light from living cells (vertebrates, invertebrates, and bacteria) when biological-mediated chemical reactions occur (Steinberg *et al*., 1995). Bacterial bioluminescence is based on the principle that some microorganism harbor genes that produce a 'reporter' signal in response to a specific recognition situation. This can be accomplished by the fusion of the promoter and reporter gene. The promoter gene is activated by the presence of a toxic substance, which in turn activates the reporter gene. The activated reporter gene codes for proteins such as alkaline phosphatase, bacterial luciferase, green fluorescent protein, and beta galactosidase. They are used to recognize transcriptional activities occurring within cells, and also in the production of signals to indicate the presence of the target analyte (toxicants and pollutants) (Christofi, 2005). Bioluminescence reactions have showed significance in its application to measure specific environmental compounds, pollutants and toxicity (Tescione and Belfort, 1993; Selifonova *et al*., 1993; and King *et al*., 1990).

In a study aimed at detecting DNA damaging agents, Singh *et al*. (2005) developed a chemiluminometric biochemical induction assay (CBIA) to detect production of β-galactosidase. The assay was run in a 96-well plate using *E. coli* BR513 as the test organism and a chemiluminescent substrate to detect beta galactosidase. The culture was added to the test samples in the plate and incubated for 3 hours at 37˚C. The substrate was added to the plate after incubation and measured for luminescence. Their results were interpreted based on the luminescence emitted

by the test samples. From the luminescence measurement obtained, they were able to determine toxic samples capable of DNA damage by calculating the beta galactosidase induction ratios.

The application of luminescence based assays exhibits a great potential for detecting contaminants. In the particular case of aflatoxin, the application of these assays might aid as an alternative, cost effective, and reliable tool for aflatoxin detection, compared to the sophisticated, expensive and destructive analytical methods. Nonetheless, there is limited literature reporting the application of this bioassay for determination of aflatoxin.

An assay by McCann *et al*. (1975) demonstrated the detection of aflatoxin B1 based on its prophage inducing ability in a simple Salmonella/microsome test. The test used bacteria as sensitive indicators for DNA damage and liver extracts of a rat for metabolic conversion of carcinogenic AFB1 to its active mutagenic state. An agar plate containing the indicator organism were spotted with the test samples and incubated for 3 hours. Afterwards, the plate was laid with a substrate and production of plaques around the spots indicated DNA-damaging activity. Similarly, a simple, inexpensive, and sensitive inductest was conducted by Moreau *et al*. (1976) to determine potential carcinogens and results showed that with metabolic activation aflatoxin B1 was detected based upon its capacityto induce prophage λ induction. The purpose of this study was to investigate the feasibility of applying a high throughput luminescent assay of prophage-induction to detect aflatoxin in solutions, and to assess the limit of the detection.

4.3 Materials and Methods

4.3.1 Bacterial strains

E. coli BR513 (ATCC 33312) and *E. coli* K12 strains were obtained from American Type Culture Collection. *E. coli* BR513 was used as the indicator/test organism while *E. coli* K12 which does not carry the *lacZ*-prophage lambda gene fusion was used as a control.

4.3.2 Media, reagents and supplies

The media used for bacterial growth were Trypticase Soy Agar (TSA) and Trypticase Soy Broth containing 0.2 M glucose (TSB 0.2 M glucose). To make 100 ml of TSB 0.2 M glucose, 18 ml of 20% glucose stock was aseptically added to 82 ml of TSB. The luminescence substrate Beta- Glo® was produced by Promega, Aflatoxin B1 was purchased from Sigma Aldrich, 70% ethanol and 10% hypochlorite were prepared in the laboratory for decontamination. Test tubes, white 96-well plates, a shaker incubator (Excella E25 Incubator shaker series, Eppendorf Canada), a spectrophotometer (Ultrospec 100 pro visible spectrophotometer, Thermo Fisher Scientific), and a plate reader (Synergy HTX Multi-Mode Microplate reader, Biotek Instruments) were utilized in the assay.

4.3.3 Luminescence bacteria induction assay

Two TSA plates were streaked with either *E. coli* BR513 or *E. coli* K12 and incubated overnight at 37˚C. Four test tubes were filled with 5 ml TSB 0.2 M glucose, and two separate colonies of each *E. coli* strain were added to two tubes. The tubes were then incubated at 37˚C overnight in a

shaker at 250 rpm. Thus, tubes 1 and 2 contained colonies 1 and 2 of *E. coli* BR513, respectively whereas tubes 3 and 4 contained colonies 1 and 2 of *E. coli* K12, respectively.

Following incubation, the overnight strains were diluted and duplicate cultures of each strain were prepared for each aflatoxin solution. The cultures were grown at 37˚C to exponential growth by measuring optical density of each culture in a spectrophotometer at 600 nm (OD_{600}= 0.5). At the point an OD of 0.5 was reached, 50 µl was measured and added to an equal volume of each inducing agent (aflatoxin concentrations 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20 and 200 ppb) to make 100 µl of induced cultures, except for those cultures designated as un-induced controls. The tubes were incubated for 6 hours at 37˚C. At the end of the incubation period, aliquots of 100µl induced and uninduced cultures were dispensed into labelled wells in a white 96-well plate, and an equal volume of Beta-Glo reagent was added to the wells. Luminescence of the plates were read using Synergy HTX plate reader. All raw data were exported into an Excel spread sheet and induction ratios for each aflatoxin concentration were calculated. The Beta-Glo Assay system contains a lysis buffer and a luminescent substrate for beta-galactosidase, which results in the production of light (Hannah *et al*., 2003).

Due to differences in growth kinetics of the two *E. coli* strains, a simple subtraction of beta-galactosidase produced by the control strain does not accurately depict the amount of the enzyme produced due to prophage induction in the BR513 strain. Instead, average relative light unit (RLU) of both induced strains are compared to average RLU of both un-induced strains. The average BR513 RLU is divided by the average K12 RLU for both induced and un-induced triplicate cultures (See Equation 4.1). The value determined from the un-induced cultures serves as a threshold, thus a value determined from induced cultures that is higher than the threshold is said to indicate that the compound added was an inducing agent, where a greater value corresponds to

a stronger inducer. In other words, when a ratio of induced over un-induced is greater than 1, the compound added is said to be an inducing agent. As for the application of the assay in aflatoxin detection, a positive result for an inducing agent may be interpreted as presence of the toxin.

$$IR = \frac{RLU_{513}}{RLU_{12}} \times \frac{RLU^{*}_{12}}{RLU^{*}_{513}} \quad (4.1)$$

Where IR is the induction ratio used to interpret whether prophage lambda induction has occurred in the high throughput luminescent assay, RLU_{513} is the average RLU of induced *E. coli* BR513, RLU_{12} is the average RLU of induced *E. coli* K12, RLU^{*}_{513} is the average RLU of uninduced *E. coli* BR513, and RLU^{*}_{12} is the average RLU of uninduced *E. coli* K12.

4.4 Results and Discussion

The assay used *E. coli* BR513 and *E. coli* K12 derivatives that produce beta-galactosidase upon induction of temperate bacteriophage lambda. *E. coli* BR513 carries a *lacZ*-prophage lambda gene fusion, where DNA damage triggers the cleavage of a prophage repressor, CI (Elespuru and Yarmolinsky, 1979). This in turn initiates transcription through the *lacZ* operon, causing subsequent production of beta galactosidase, which is detectable by luminescence. It is therefore possible to identify compounds that cause induction of prophage lambda in *E. coli* BR513 by measuring the relative quantity of the indicator enzyme, beta-galactosidase.

By comparing the relative light units (RLU) of the two strains against each other as well as against un-induced cultures, it was possible to interpret the occurrence of induction in this assay. The control strain will not produce beta-galactosidase when prophages are induced and thus its production of the enzyme represents relative background levels.

Fig. 4.1 and Fig. 4.2 show the average RLU values of the induced and uninduced strains, respectively for the different concentrations. The change in RLU was modeled using an n order power function namely:

$$RLU = \alpha C^{-\delta} \tag{4.2}$$

Where C is the concentration of aflatoxin, α is the change constant and δ is exponent . The results for induced and uninduced strains are as follows;

Induced *E. coli* BR513:

$RLU = 1E+06C^{-0.096}$ (4.3)

Uninduced *E. coli* BR513:

$RLU = 1E+06C^{-0.024}$ (4.4)

Uninduced *E. coli* K12:

$RLU = 234702C^{-0.006}$ (4.5)

Induced *E. coli* K12:

$RLU = 222302C^{-0.113}$ (4.6)

The respective values for each strain were inputted into the aforementioned equation (Equation 4.1) to determine their induction ratios. The β-galactosidase induction ratios for known aflatoxin concentrations indicative of prophage induction were calculated as shown in Fig 4.3, where 1 is the induction threshold. Ratios greater than one (> 1) signified induction and were classified as inducers, while ratios less than 1 (<1) meant there was no induction and were classified as non-inducers. It was observed that aflatoxin concentrations 0.31, 0.62, 1.25, 2.5, 5, 10, 20, and 200 ppb were positive for detection in the assay with ratios greater than 1. Concentrations 0.078 and 0.156 ppb had ratios lower than 1, and were categorized as undetected. The concentrations that were detected are said to be inducers, and thus detectable by this method, while undetected concentrations are not inducers. Highest induction ratios were observed at 10,

20, and 200 ppb in order of increasing aflatoxin concentration. To have an explicit observation of the detection limit, data of the lower concentrations were represented in a chart. As shown in Fig. 4.3, a reliable limit of detection using this assay is 0.21 ppb, which is the smallest concentration that can be reliably measured by this assay.

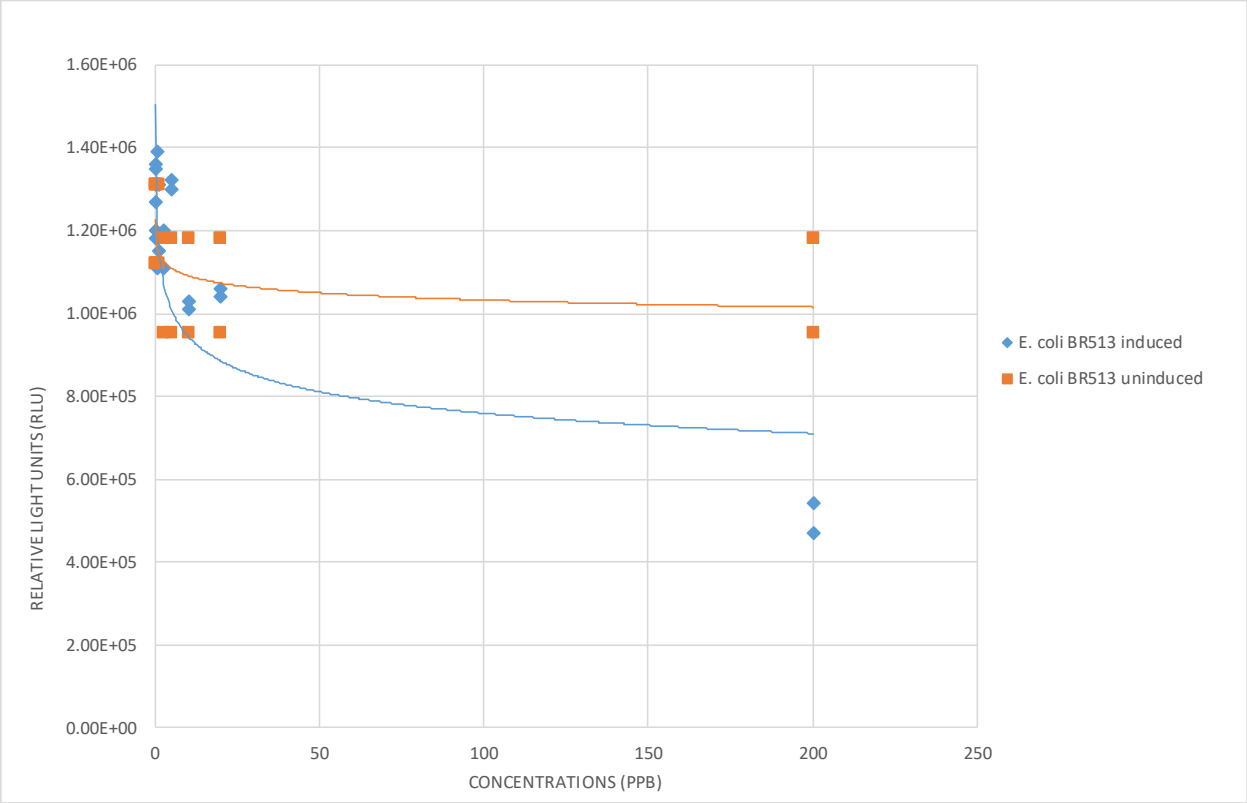

Figure 4.1 Relative light units (RLU) of induced and uninduced *E. coli* BR513

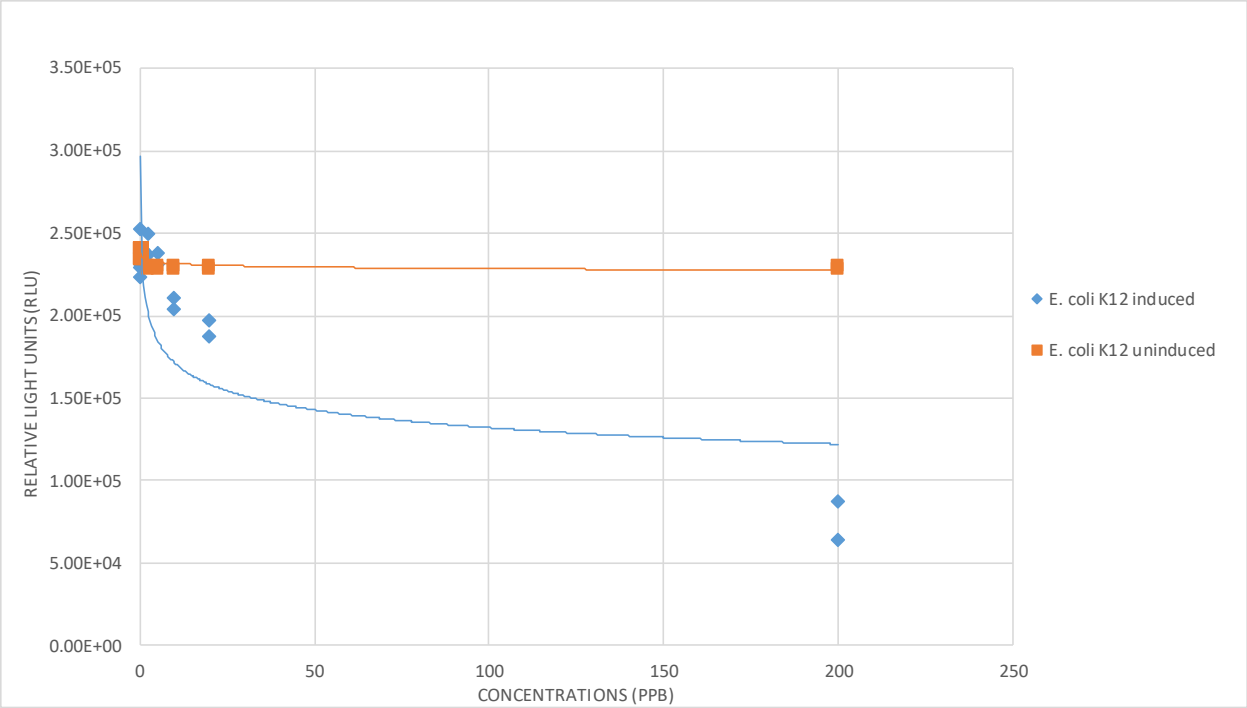

Figure 4.2 Relative light units (RLU) of induced and uninduced *E. coli* K12

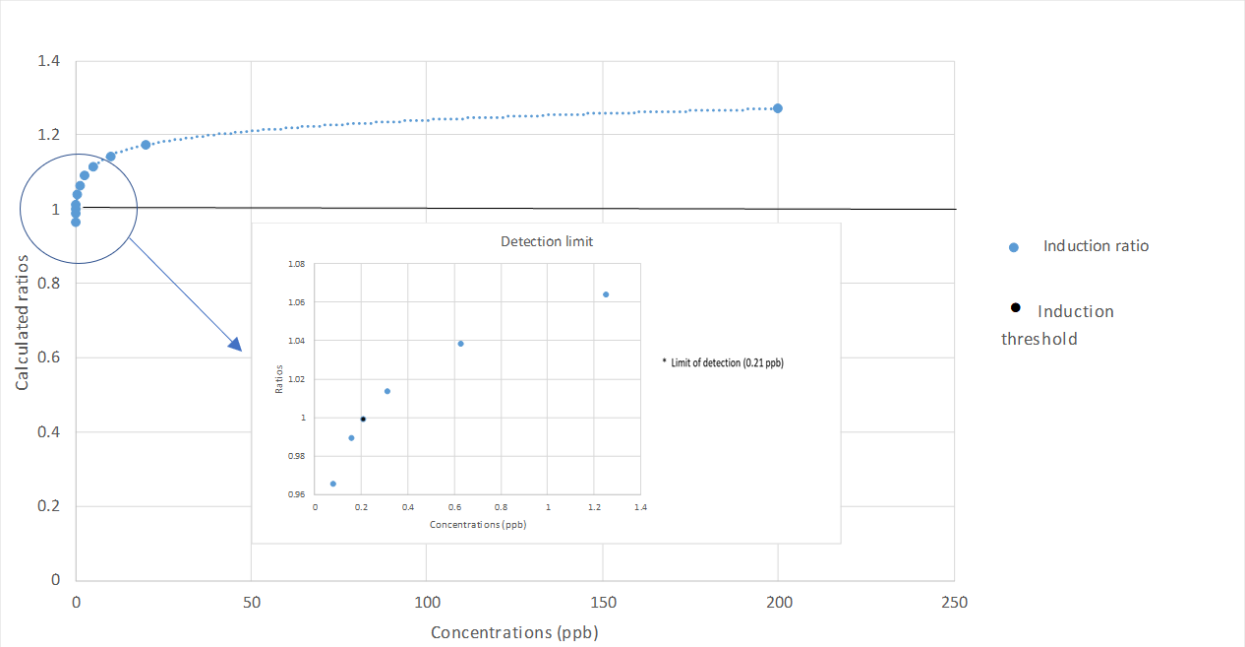

FIG 4.3 Induction threshold and limit of detection

These results are similar to a study conducted using antibiotics as the inducing agent. The experiments by Maya *et al*. (2005) evaluated the potential of antibiotics as inducers in an aerobic environment. In this study, the cells were grown anaerobically to prevent volatilization of aflatoxin, to avoid exposure and health risks to laboratory personnel. The cluster observed in the lower concentrations of aflatoxin can be attributed to the very close range of the toxins as it was observed that more prominent and reliable differences were seen in the higher concentrations.

The positive detection of aflatoxin from our result agrees with the result obtained by Elespuru and White (1983), where they employed a principle identical to the one applied in this study. They spot tested aflatoxin B1 directly on E. coli strain BR513 and after 3-5 hours of

incubation at 38˚C, the plates were covered with β-galactosidase. Their results showed that aflatoxin B1 exhibited chemical induction of the phage as monitored by the presence of β-galactosidase. The authors also reported that aflatoxin B1 is a stronger inducing agent in comparison with other agents such as ampicillin, chloramphenicol, actinomycin D, mitomycin C and many more. In a similar research Moreau *et al*. (1976) attempted to identify potential carcinogens by conducting a spot test for induction by aflatoxin B1 employing the same principle of prophage induction. They used GY 4015 as the indicator bacteria and the plates were incubated overnight at 37˚C after the addition of aflatoxin B1. It was reported that aflatoxin B1 is a potent carcinogen as well as a potent phage inducer. Other authors demonstrated the detection of aflatoxin B1 at a concentration of 0.1 µg/ml owing to its phage inducing action (Moreau *et al*., 1977; Magee, 1974; Miller and Miller, 1971). Metabolic activation using rat live homogenates to make the aflatoxin more active was a common method observed from the experiments carried out by these authors. However, this does not contribute significantly to the β-galactosidase activity (Elespuru and Yarmolinsky, 1979).

When carrying out an inductest, underestimation of induction in the treated bacteria strain is possible if its growth is inhibited, because induction is measured by comparing the β-galactosidase activity in the treated indicator strain with the enzyme activity in untreated bacteria at the end of an equivalent expression period (Elespuru and Yarmolinsky, 1979). Since growth plays a vital role in induction, hence carrying out this experiment under aerobic conditions suitable for bacteria growth could improve the results.

Another important factor to consider with the result is sensitivity. The question of sensitivity in aflatoxin detection is very important because of the low concentrations at which aflatoxins are likely to occur in food products (Maximum concentrations at 15 ppb for total

aflatoxins and 8 ppb for AFB1 has been imposed by the European commission, 2006). The length of the induction period may be used to vary the sensitivity of the induction assay. Although 3 hrs is a general optimum period for the detection of most inducers, reduction or extension of the induction periods may be used to monitor the inducing abilities of different substances (Elespuru and Yarmolinsky, 1979). To the best of our knowledge, our study is the first demonstration of a luminescent β-galactose assay that can be used to detect aflatoxins at different concentrations. This luminescence assay is a high throughput, low cost, easy to perform, and sensitive technique, therefore it is distinctly suited to detect the presence of aflatoxin.

4.5 Conclusion and Future development

The results presented here suggest that this bioassay shows potential as an effective tool for detecting aflatoxin. The study showed that concentrations in the range from 0.21-200 ppb could be detected. A threshold concentration of 0.21 ppb was determined, below which AFB1 could not be assessed based on the method adopted in this study. The result could be further developed satisfactorily for rapid and consistent assessment of aflatoxin in the field. Another propitious area of progress would be to extend this principle for the development of a rapid tool such as a hand held luminometer for detection of AFB1.

V. GENERAL CONCLUSION

This study demonstrated the potential of using near infrared hyperspectral to detect aflatoxin B1 in artificially contaminated bean seeds, and the development of a novel bioassay for aflatoxin detection. The first and second objectives were met by contaminating bean seeds and acquiring spectral images for data analysis. The classification models developed using the wavelength region from 900-1700 nm could accurately discriminate between contaminated and uncontaminated samples. PLSDA models showed good performance in calibration and prediction with sensitivity and specificity values greater than 75%. Likewise, PCA-LDA models manifested a similar and even better performance in classifying the samples with higher sensitivity and specificity values greater than 86%.

The third and fourth objectives were met by testing the prophage inducing ability of aflatoxins using two strains of *E. coli* in luminescence bioassay. The different concentrations of aflatoxin were detected by measuring their luminescence in a plate reader. Average RLUs were calculated and compared to determine the threshold of detection. A reliable limit of 0.21 ppb was determined.

Adequate sample preparation and chemometric techniques that will improve the accuracy of discrimination should be used in order to build a more robust classification model for future research in hyperspectral imaging. For the luminescence assay, a longer incubation time and an aerobic environment to support the growth of the cultures could increase the sensitivity of the assay. Generally, these results indicate that both techniques are promising methods for detection.

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