ANALYSIS OF AFLATOXINS IN GRAIN AND PEANUT PRODUCTS IN THE STATE OF GEORGIA, USA

by

AMANDA GAY SEAWRIGHT

(Under the Direction of Jia-Sheng Wang)

ABSTRACT

Aflatoxins (AFs) are a class of secondary metabolites produced by toxicogenic *Aspergillus* which are known to contaminate a broad range of crops and food products worldwide. Analyses of thirteen peanut and corn products collected from supermarkets in the State of Georgia, USA and 280 peanut and grain samples grown in the State of Georgia were conducted to determine the current AF contamination status in the State. The detection rate of AFB_1 in supermarket samples was 100 percent using HPLC and ELISA analysis methods, all below the FDA's 20 ppb regulatory limit. The detection rate of AFs in Georgia-grown samples using UHPLC and mReader analysis methods was 100%. Results for total AF levels (µg/kg) in oats, sorghum, wheat, corn, and peanuts were 23.48±36.79, 172.34±59.14, 51.81±86.45, 8.44±12.46, and 68.22±95.58, respectively. These results prove the need of closer monitoring of AF contamination in crops grown in the State of Georgia.

INDEX WORDS: Aflatoxin B_1, aflatoxin, food safety, Georgia-grown food crops

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DEDICATION

This work is dedicated to my husband, Chad Seawright, for his love, support, prayers, and guidance. To my parents, Lynn and Dennis Gay, who have helped me become who I am today and encourage me to reach for the stars and do what I love. I would also like to dedicate this to my grandparents, Ella and Wayne Howell, Billy Gay, and my late grandmother, Marilyn Gay. Finally, I dedicate this to family and friends who have shown their unending support every step of the way. May all glory be given to my Lord and Savior, Jesus Christ.

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CHAPTER 1

INTRODUCTION

Aflatoxins (AFs) are the most common naturally occurring mycotoxins produced mainly by *Aspergillus flavus* and *A. parasiticus.* AFs were discovered in England in the 1960s after an unknown disease outbreak in turkeys (turkey-X disease). The disease was later linked to peanut meal contaminated with *Aspergillus flavus,* which produced the toxins (Bennett and Klich 2003). The six major types of AFs are B_1, B_2, G_1, G_2, M_1, and M_2. AFB_1 is the most toxic and is carcinogenic, immunosuppressive, potentiates other diseases, mutagenic, and is harmful to both humans and animals that frequently consume AFB_1-contaminated foods (Richard 2007). AFs are common in corn, peanuts, wheat, cottonseed, and tree nuts. The formation of AFs begins while crops are growing in the fields and is prevalent throughout growth, harvest, and storage of the crops (Klich et al. 2000; Richard 2007). The State of Georgia's hot and humid climate during grain and peanut growing and harvest seasons provides ideal conditions for the growth of *A. flavus* and *A. parasiticus*.

This thesis focuses on the comparison of different methods and instruments used for AF determination in food samples and the current status of AF contamination in the State of Georgia, USA because of the lack of data in the last two decades. The literature review (Chapter 2) provides a detailed look at AFs, development of each of the methods and protocols used in these studies, and detailed information about AFs and their impact on crop, animal, and human health and how it specifically affects the State of Georgia. Chapter 3 describes the preliminary study used to develop the subsequent studies described in Chapter 4. The study in Chapter 3

focuses on the detection of AFB_1 in peanut and corn food products purchased from supermarkets in Athens, GA comparing enzyme-linked immunosorbent assay (ELISA) and high pressure liquid chromatography (HPLC) methods. Chapter 4 is a study that first compared different ultra-high performance liquid chromatography (UHPLC) methods and Mobile Assay's mobile diagnostic rapid test reader (mReader) methods for the analysis of AFs, B_1, B_2, G_1, and G_2, in peanut samples. Two of the methods were then selected to determine the levels of AFs in grain and peanuts grown in Georgia, U.S.A. Chapter 5 summarizes the findings and conclusions of these studies of AFs and the future plans for research.

CHAPTER 2

LITERATURE REVIEW

Introduction

Mycotoxins are secondary metabolites naturally produced by toxicogenic fungi (Bennett and Klich 2003). Aflatoxins (AFs) are a class of mycotoxins that were discovered after a deadly disease outbreak in turkey poults (turkey-X diseases) in England in the 1960s, which was linked to peanut meal contaminated with mold. It was determined that the meal was contaminated with AFs, which are secondary metabolites of *Aspergillus flavus* (Bradburn et al. 1994). This discovery led to the realization and increased research of possible hazards to humans and animals that ingest foodstuffs contaminated with mycotoxins (Wogan 1966). The six major types of AFs are B_1, B_2, G_1, G_2, M_1, and M_2 and are produced by *Aspergillus flavus* and *A. parasiticus* (Bennett and Klich 2003). AF contamination of peanuts, corn, and crops not properly dried post-harvest are the most significant among contamination problems in the United States (Diener et al. 1987). Crops can become contaminated with AFs in the field and also after harvest under conditions that favor growth of *A. flavus* and *A. parasiticus*. Over 100 countries have regulations for mycotoxins, including AFs, in food which are in place for human and animal safety (van Egmond et al. 2007). There are many methods that have been used throughout the years to determine the levels of AFs in food products and commodities. High pressure liquid chromatography (HPLC) is considered the gold standard for analysis of AFs (Wacoo et al. 2014). However, ELISA and chromatography-based methods such as those that use lateral flow devices

are also used because of the rapid detection, cost-effectiveness, and portability in the field (Wacoo et al. 2014).

Chemistry and Biology of Aflatoxins

AFs are produced by the strains of fungi *Aspergillus flavus* and *A. parasiticus* and are difuranocoumarin derivatives (Bennett and Klich 2003). Over 20 AF derivatives have been identified, but the most common AFs are B_1, B_2, G_1, G_2, M_1, and M_2 (Hussein and Brasel 2001). AFs, especially AFB_1, are immunosuppressive, toxic, mutagenic, carcinogenic, potentiate other disease, and increase mortality in humans and animals (Bennett and Klich 2003; Diener et al. 1987). AFB_1 is the most potent natural carcinogen and the most toxic AF (Marin et al. 2013). AFM_1 and AFM_2 are metabolites of AFB_1 and AFB_2 produced in milk (Goldblatt 1970). The Büchi group determined the five-ring structures of AFs B_1, B_2, G_1, and G_2 in 1963, which are shown in Figure 2.1 (Brase 2013; Wogan 1966).

AF B and G are both fluorescent under ultraviolent light and are named based on the color of light they fluoresce, blue and green, respectively (Wang 1998). Thin-layer chromatography was used to determine there are two components of each that differed in R_f, and are distinguished by the numerical superscripts, 1 and 2 (Goldblatt 1970). Spectral data for the four major AFs is shown in Table 2.1 and includes the ultraviolent absorption, infrared absorption, and fluorescence emission (Wogan 1966). This information is necessary for conduction analyses for AFs. Molecular data for the four major AFs is shown in Table 2.2.

AFs are metabolized mostly by the liver, which is also the main target organ (Bennett and Klich 2003; Marin et al. 2013). AFs are products of the polyketide pathway and the expression of 23 genes in a 75-kb gene cluster (Ehrlich et al. 2002). Versiconal hemiacetal acetate is the

precursor of each of the AFs, shown in the pathways of formation of the six major types of AFs are shown in Figure 2.2 (Brase 2013).

AFs are known to contaminate a variety of crops with corn, groundnuts, tree nuts, and oilseeds being the most common (Khlangwiset et al. 2011). Production and growth of the toxin is affected by rainfall, humidity, temperature, presence of other fungi, crop damage (Marin et al. 2013). Hot and humid conditions found in tropical and sub-tropical climates provide the most favorable conditions for AF growth, however temperate climates may provide risk of growth as well (Marin et al. 2013; Peraica et al. 1999). *A. flavus* and *A. parasiticus* produce AFs in a range of 15-37ºC, but 20-30 ºC has been found to be the temperature range with the highest levels of production (Fokunang et al. 2006; Pitt and Miscamble 1995). The stability of AFs makes decontamination of crops and food products difficult because they are often able to withstand processing and cooking conditions (Marin et al. 2013).

Contamination of Food Commodities with Aflatoxins

Due to mold growth, AFs contaminate commodities throughout growing, harvesting, and processing which then causes feed for animal and human consumption to be contaminated (Marin et al. 2013). AFs may contaminate crops and foodstuffs when the conditions are favorable to fungal growth (Monbaliu et al. 2010). *Aspergillus flavus* mainly grows in the leaves and flowers of plants, but is overall ubiquitous in nature (Marin et al. 2013). *Aspergillus parasiticus* has a smaller range of contamination sites, the main being the soil (Marin et al. 2013). Locations with hot and humid climates promote the growth of molds that produce AFs (Peraica et al. 1999). Drought and immoderate rainfall also increase possible contamination of AFs during crop seasons (IARC 1993). The fungi that produce AFs are known to contaminate

crops during growing and postharvest conditions, such as storage and processing (Khlangwiset et al. 2011). Storage conditions with proper moisture levels and temperatures are critical for prevention of AF contamination (Fokunang et al. 2006). AFs are known to contaminate peanuts, corn, grains and cereal derivatives, tree nuts, cottonseed, and dried fruits (Marin et al. 2013; Zain 2011).

Animal feed contaminated with AFs is the first risk of AF exposure to the food chain and can potentially cause contamination to biological fluids and animal tissues (Arroyo-Manzanares et al. 2015). Regulations of AFs in food and feed products are in place in over 90 countries (Adams and Whitaker 2004). The United States Food and Drug Administration limit is 20 ng/g as compared to the Canadian and European Union limits of 15 ng/g (Adams and Whitaker 2004). Lots of commodities can be misclassified by exporters and importers because of the nonhomogeneous contamination of AFs and the variance of testing procedures, from sampling to analysis (Adams and Whitaker 2004). Shipments of commodities that do not meet the regulatory limit will be rejected (Adams and Whitaker 2004). *A. flavus and A. parasiticus* can grow on both live and dead plants and animals worldwide (Diener and Davis 1977). *A. flavus* has also been reported to cause deterioration of wheat, corn, rice, barley, bran, flour, soybeans, and other seeds in storage conditions (Christensen 1957; Diener and Davis 1977). Table 2.3 shows the number of isolates of *A. flavus* that contaminated peanuts in eight different states in the U.S. in a range of 0 to 17,000 ppb AFB_1 (Diener and Davis 1977). Table 2.4 shows the 11 isolates of *A. flavus* that contaminate six different crops in four states in the U.S. with concentrations of AFB_1 ranging from 10- 16,560 ppb (Diener and Davis 1977). Commodities contaminated with AFB_1 in this study included but was not limited to wheat, corn and soybeans. AF production on different substrates varies based on the fungus strain, temperature and moisture conditions of the substrate

and surrounding area, incubation, and the analysis (Diener and Davis 1977). Diener *et al.* concluded that using 20-60 percent CO_2 in commercial storage areas with temperature below 20 ºC and humidity below 90 percent is a possible method to control aflatoxigenic fungi production during storage (Diener and Davis 1977). The fact that *Aspergillus spp.* are tolerant to a wide range of temperatures proves it to be extremely difficult to eliminate the molds that produce AFs, as well as eliminating the AFs (Guo et al. 2005). AFs are resistant to heat and are soluble in intermediate polar solvents, making them difficult to remove from commodities and food products (Hwang and Lee 2006).

Peanut Contamination

The fungi that produce AFs are prevalent in the soil and can contaminate the peanut pods at any time during growth (Bowen and Hagan 2015). The combination of drought and high temperatures in known to increase AF contamination in peanuts (Bowen and Hagan 2015). Wilson *et al.* determined in a study that increased moisture between the final 40 to 75 days before harvest showed higher risk of AF contamination than drought conditions early in the growing season (Wilson 1983). Hill *et al.* determined that AF contamination occurred during drought conditions when correlated with high soil temperatures (Hill 1983). Soil temperature, moisture, plant cover, soil texture and color, and air temperatures affect aflatoxigenic fungal growth (Bowen and Hagan 2015). Soon after the discovery of AFs, different studies determined there is a greater risk of *A. flavus* contamination in peanuts with shell damage from insects, mechanical damage, and cracking (Holbrook et al. 2008).

The Coastal Plain Experiment Station along with the University of Georgia studied the correlation of plant-parasitic nematodes in the soil with AF contamination to the peanut pods

pre-harvest (Holbrook et al. 2008). The conclusion of the studies was that there was an increase in AF contamination in kernels when there was a combined presence of *A. flavus* and nematodes, but the specific contribution of the nematode was not determined (Holbrook et al. 2008). These groups also studied how drought stress in combination with *A. flavus* and insect damage increased AF contamination in kernels, and concluded that damaged pods from these factors had increased AF concentrations (Holbrook et al. 2008). Overall, these studies concluded that combinations of drought, increased soil temperatures, and damage to the peanut pod can increase *A. flavus* growth and AF concentrations.

Corn Contamination

Corn is just one of the many agricultural commodities that can be contaminated by AF (Robens 2005). *A. flavus*, which is the producer of the carcinogen AFB_1, is the major source of AF contamination in corn crops in the United States (Wrather 2010). Damage to corn ears by insects and machinery, high temperatures and moisture levels, and drought are all factors that may increase the risk of aflatoxigenic mold growth in ears (Lillehoj et al. 1978). AF contamination in corn is most severe in the southern and midsouth areas of the United States (Wrather 2010). Contrary to original beliefs, it is now known through studies that *A. flavus* can grow on corn both in the field and in storage (Wrather 2010). This contamination is ubiquitous throughout a field and lots of corn, which proves testing for AF levels to be difficult (Hurbraugh 2005). *A. flavus* can sometimes be identified as a green, yellow, or brown powdery mold on the kernels (Wrather 2010). A species of corn that is genetically modified to be more resistant to conditions in the southern regions of the United States is one suggestion by researchers to help reduce aflatoxigenic mold growth, and therefore AF contamination in corn crops (Guo et al.

2005). Because inconsistent rainfall can contribute to AF contamination in corn, producers who are able to irrigate their corn crops can almost eliminate this as a factor of contamination (Guo et al. 2005).

Aflatoxin Contamination in Wheat, Oats, Sorghum and Other Grains

Wheat, oats, and sorghum are among the other cereal grains that can risk contamination by AFs (Hwang and Lee 2006). Although these grains are more likely to be contaminated by other mycotoxins, AF contamination is possible has been researched (Abbas 2005). Shortly after the 1960s AF outbreak in turkey poults, Stubblefield *et al.* determined that wheat and oats were possible substrates of *Aspergillus spp.* growth (Stubblefield R. D. et al. 1967). Wheat was known to be a good substrate for AFG_1 prior to the study but oats had not yet been studied. Samples were inoculated using a fermentation method and AFs were extracted using chloroform and analyzed using a TLC Densitometer. The maximum AF concentrations produced on wheat in flasks were 1,950 μg/g of substrate and AFG_1 was of highest concentrations produced on substrates among the four major AFs. AFB_1 and AFG_1 were produced in much higher quantities on both wheat and oats than AFB_2 and AFG_2. Although done in a laboratory setting, this study proves that contamination of oats and wheat with AFs is possible especially with higher moisture levels present. Hwang *et al*. successfully used varieties of US and Korean wheat as a substrate for AFB_1 in a study regarding cooking treatments to reduce AF contamination in 2005 (Hwang and Lee 2006).

Effects of Aflatoxins in Animal and Human Populations

Effects of Animal Exposure to Aflatoxins

Negative health effects of AF dietary exposure were first discovered in turkey poults in the 1960s. Since then, their carcinogenic effects have been seen in other animal species including fish, non-human primates, and rodents (Jackson and Groopman 1999). AF contaminated feed has caused deaths and negative health effects in farm animals (Zain 2011). These effects include a decrease in feed intake and refusal, weight gain, reproduction, and immune response to diseases (Binder et al. 2007). Animal studies have also shown that AF exposure may lead to growth impairments, decreased weight gain, and decreased appetites (Khlangwiset et al. 2011). Hussein *et al.* showed a dose-dependent response in a study in dairy cattle whose feed was contaminated at levels between 10-108.5 μg/kg each day (Hussein and Brasel 2001). Animal studies have also shown that baby animals may be exposed to AFM_1 contamination by ingestion of milk from maternal animals that were exposed to AFs through their feed (Khlangwiset et al. 2011). Table 2.5 shows results from three studies conducted in baby mice, chicks, and ducklings where there were decreases in weight gain in several of the groups fed AF contaminated feed or milk. AF exposure in animals including ducklings, Japanese quail, chickens, pigs, channel catfish, turkeys, and Nile tilapia have shown a decrease in weight gain and feed consumption after exposure to AFs in their diet (Khlangwiset et al. 2011). Khlangwiset *et al*. summarizes the results from forty-one different studies in different animal species that were exposed to AFs via their feed. The results conclude that decrease in weight gain and body weight is a result of animals being exposed to AFs in their diet (Khlangwiset et al. 2011). This will then result in an economic loss for farmers. Dairy cattle exhibit an added risk from AF consumption because AFB_1 and AFB_2 are metabolized into AFM_1 and AFM_2 in the milk (Boudra et al. 2007). This not only causes

exposure risk to humans that consume the contaminated milk, but also to the calves (Vanegmond 1989).

Effects of Aflatoxins on Human

Animal studies have been used to learn more about possible effects of human exposure to AFs, and have provided foundations for epidemiological studies in human populations. Humans who are exposed to AFs through ingestion or via their surroundings are at risk for increased health effects including growth impairments, aflatoxicosis, cancers including human hepatocellular carcinoma (HCC), and even death (Jackson and Groopman 1999; Khlangwiset et al. 2011). Age, nutrition, exposure, and overall health of each person are factors for the extent of AF damage (Peraica et al. 1999). Chronic dietary exposure is of the most concern and danger to humans (Hussein and Brasel 2001). The in utero effects of AFs have been examined in studies in the breast milk of women in Africa, where blood cord and breast milk samples revealed AF contamination (Peraica et al. 1999). A study comparing the effects of AF exposure in four different countries through three different exposure routes is shown in Table 2.7 (Peraica et al. 1999). Many children throughout the world encounter AF exposure from breastfeeding from a woman who has been exposed to AFs and her body metabolizes them to AFM_1 in her milk (Khlangwiset et al. 2011). Consumption of foodstuffs contaminated with AFB_1 has been linked to cases of hepatic cancer in Africa and the Far East (Miller and Miller 1986). The United States and other countries have regulations in place to monitor AF contamination in human and animal feeds because of the epidemiological studies and the confirmed carcinogenicity of AFB_1 (Miller and Miller 1986). It is currently impossible nor feasible to rid all foodstuffs and crops of AFs and other mycotoxins and the fungi that produce them, therefore it is important to have strong

regulations and monitoring programs in place to of the contamination of crops and food worldwide (Miller and Miller 1986). AF consumption is a risk factor for HCC, especially in countries where AF contamination is not monitored or regulated as strongly as in the US and other countries (Sudakin 2003). AFB_1 is often the highest of the AFs in food samples (Marin et al. 2013). AFs are known to potentiate other diseases and several studies have revealed children with Kwashiorkor and Reye's syndrome whom also had AF exposure and damage (Hussein and Brasel 2001). There are also many recent studies that show compounded exposure of hepatitis B virus and AF dietary exposure may increase incidence of HCC (Hussein and Brasel 2001). Hussein *et al* determined that a quarter of a million deaths in China and Africa from HCC are also correlated with an average dietary exposure of 1.4 g of AF daily (Hussein and Brasel 2001). Epidemiological studies in Africa and Asia in the 1960s and 1970s analyzed foodstuffs and results from dietary questionnaires to determine possible health effects of AF consumption (Jackson and Groopman 1999). It was determined there was an increase of liver cancer incidence from 2.0 to 35.0 cases per 100,000 population per year from an increase of AF consumption from 3 to 222 ng/kg body weight per day (Jackson and Groopman 1999).

History of Aflatoxin Contamination in Georgia

The climate in the State of Georgia presents ideal conditions for growth of aflatoxigenic fungi, especially in southern areas that are more common for cultivation of peanuts, corn, wheat, and other crops commonly associated with AF contamination. The Coastal Plain Experiment Station in Tifton, Georgia, has conducted research on the risks for AF contamination in crops and ways to address the problem over the past few decades, mainly focusing on peanuts (Holbrook et al. 2008).

In 1977 and 1978, McMillian *et al.* conducted a field survey of corn grown in the State of Georgia's coastal plain region (McMillian et al. 1980). In 1977, the range of AF contamination was from 0 to 4,708 μg/kg and averaged 622 μg/kg. In 1978, the AF contamination in corn sampled at the beginning of the growing season in July and just before harvest in September averaged 62 μg/kg and 51 μg/kg, respectively, and the range was 0 to 620 μg/kg. There was a significant decrease in AF contamination between these two years. Sampling procedures were the same for both years. Ears selected were between 20-30 percent moisture and in the first field within 6.4 km of each county line. Five ears were selected from different areas in the field, observed for *Aspergillus* mold growth, and then the two most representative ears were placed in a paper bag and shelled. After drying kernels below seven percent moisture content, an AOAC method was used for analysis of AF levels. There was more rainfall in 1978 between April and July and more in 1977 between August and October. The insect infestation was less in 1978 than 1977. Overall, there was a 94 percent detection rate of AFs in July 1978 and 76 percent in September 1978. There was positive correlation between AF contamination and visible presence of *A. flavus* in corn ears.

Horn *et al.* conducted a study on peanuts and corn planted in three fields in southwestern Georgia (Horn et al. 1995). Peanut pods and corn kernels were collected and prepared for analysis of AF levels. The results were that *A. flavus* and *A. parasiticus* were in the soil in close to equal amounts in the first half of pod development. The levels of each fungi species in the soil was consistent in two fields throughout the year study, but the *A. flavus* population increased significantly before harvest in the third field. In corn, it was determined that drought is favorable for AF contamination in corn during growth and development. Also, when corn contaminated with *A.* flavus is harvested with a combine, the fungi then contaminates the surface of the soil

and is airborne. In these studies, there was not significant effect on the AF contamination due to combine harvesting.

Methods of Determination of Aflatoxins

Early research of AFs found that methanol was a successful solvent used to extract the AFs, and that they had strong fluorescent properties under ultraviolent light (Wogan 1966). Commodities may be tested for possible AF contamination in the field using test strips or black lights, but analytical tests should be conducted in order to yield quantitative and accurate results (Hurbraugh 2005). HPLC, UHPLC, and ELISA are among several of the most prevalent methods used for the detection of AFs in foodstuffs. Mobile Assay's mReader is a new instrument and method that is useful for rapid and easy detection of AFs, especially in field applications. Solid-liquid extraction of foodstuffs is a common step in methods that analyze AF contamination (Arroyo-Manzanares et al. 2015). Immunoaffinity columns are often used for cleanup because of their ability to target specific analytes with antibodies (Vaclavikova et al. 2013). However, because many IACs are not capable of multi-mycotoxin analysis, other cleanup columns such as Sep-pak cartridges and MycoSep columns may be used.

HPLC Analysis of AFs

HPLC is the "gold standard" for analysis of AFs (Wacoo et al. 2014). Many different extraction and cleanup methods prior to analysis with HPLC have been researched and can be found in the literature. Using HPLC with fluorescence detection (FLD) is very common for AF analysis (Arroyo-Manzanares et al. 2015).

Arroyo-Manzanares *et al.* verified a method using solid-liquid extraction with acetonitrile followed by HPLC-FLD detection in five different animal feeds. The contents of the feed included corn, wheat, barley, and other grains. The sample was extracted by shaking 2-g sample and 10-mL acetonitrile for 3 minutes, removing and drying the upper layer under N_2, and then reconstituting it with methanol:water (50:50). A tertiary mobile phase of water, methanol, and acetonitrile was used for HPLC conditions with FLD. Samples were filtered with a 0.22μm syringe filter before injection into HPLC. With recoveries above 81 percent, this method was shown to have high accuracy even with its simple extraction.

UHPLC Analysis of AFs

Vaclavikova *et al.* implemented IACs capable of multi-mycotoxin selection and used UHPLC with mass spectrometry (Vaclavikova et al. 2013). Sample portions were extracted with a solution of acetic acid, acetonitrile, and water and mixed thoroughly. The crude extract was diluted with PBS buffer, passed through an IAC with multi-functional purposes, and then extracted using methanol. The extract was dried and reconstituted with a methanol:water (50:50) solution, filtered, and then analyzed with UHPLC-MS/MS. UHPLC was used for separation and detection of a variety of mycotoxins was done using mass spectrometry. This method is impressive because of the large differences of chemical properties of the mycotoxins identified. Recovery percentages ranged from 63 to 112 percent. This method proves the use of immunoaffinity columns for multi-mycotoxin analysis with adequate detection, in this case UHPLC-MS/MS.

In a study by Zachariášová *et al*. the levels of AFs were detected in beer samples using UHPLC detection (Zachariášová 2010). A portion of beer sample was extracted with acetonitrile

and mixed thoroughly. This mixture was then dried and reconstituted with methanol:water (50:50). Before injection into UHPLC, the extract was filtered with a 0.2 μm filter. This method was successful in the separation and detection of 32 mycotoxins. The UHPLC conditions and gradient method used in this study was used for the UHPLC analysis in this thesis, with some modifications.

Mobile Diagnostic Rapid Test Reader (mReader) by Mobile Assay

Mobile Assay Incorporated has developed software to make tablets and smart phones a mobile instrument with powerful analysis capabilities ([Anonymous] 2014). Any lateral flow test strip can be used with the mReader. Neogen Reveal Q+ for Aflatoxin lateral flow strips were used in this thesis study because they were recommended and provided by Mobile Assay. This test allows for rapid and inexpensive testing both in lab and field settings, especially where resources are limited.

Tables

Table 2.1 Spectral data summary for AFs, including the ultraviolent absorptions and fluorescence emissions.

Aflatoxin	**Ultraviolent absorption** *265 m_μ*	*363 m_μ*	**Fluorescence emission ©**
B_1	13,400	21,800	425
B_2	9,200	14,700	425
G_1	10,000	16,100	450
G_2	11,200	19,300	450

Table 2.2 Molecular data summary for AFs including molecular weight and formula, and melting point for AFs.

Aflatoxin	**Molecular formula**	**Molecular weight**	**Melting point (ºC)**
B_1	$C_{17}H_{12}O_6$	312	268-269
B_2	$C_{17}H_{14}O_6$	314	286-289
G_1	$C_{17}H_{12}O_7$	328	244-246
G_2	$C_{17}H_{14}O_7$	330	237-240

Table 2.3 Summary of study by Diener *et al.* showing the number of isolates of *A. flavus* that contaminated peanuts in eight different states in the U.S.

State or Country	No. of Isolates	AFB_1 in peanuts (ppb)
Alabama	7	2,870-17,000
Florida	1	6,900
Georgia	2	70-2,450
New Mexico	1	0
North Carolina	1	2,760
Texas	2	280-610
Uganda	1	2,400
Virginia	11	180-9,200

Table 2.4 Summary of study by Diener *et al.* of the AFB_1 contamination in six different crops in four states in the US.

Isolate source	State	No. of Isolates	AFB_1 in peanuts (ppb)
Chestnut	Alabama	1	2,300
Corn	Georgia	1	30
Poultry litter	Alabama	1	16,560
Rice	Texas	1	2,300
Soybean	Minnesota	3	10-70
Wheat	Minnesota	3	30-120

Table 2.5 Summary of study by Khlangwiset et al**.** showing effects of AF exposure to baby mice, chicks, and ducklings via food intake.

Animal	**N**	**AF dose**	**Experiment Duration**	**Results**	**Study**
6 wk old male Swiss albino mice	70	(A) 0μg AFB_1 + 5% protein diet (B) 0μg AFB_1 + 20% protein diet (C) 0.5μg AFB_1 + 5% protein diet (D) 0.5μg AFB_1 + 20% protein diet	7 weeks	% weight gain increase per dose: (A) 4%, (B) 18.2%, (C) 7.2%, (D) 12.2%. Decrease in % weight gain ($p<0.001$) in group D compared to group B	Kocabas et al. (2003)
1-d old broiler chicks	48	(A) 0 mg/kg AFB_1 (B) 5 mg/kg AFB_1 in feed	3 weeks	Decrease in weight gain: (A) 866±12.7g (B) 699±38.5g [$p<0.05$]; Decrease in feed intake: (A) 1369±45.7g (B) 957±183.5g [$p<0.05$]	Pimpukdee et al. (2004)
Cherry Valley commercial ducks	90	(A) 0 μg AFB_1 (B) 20 μg AFB_1 in contaminated rice (C) 40 μg AFB_1 in contaminated rice	6 weeks	Decrease in ADG: (A) 48.21±2.5g (B) 42.52±2.5g (C) 37.44±2.7g; Dec in feed intake in high-dose group: (C) 130.28±3.5g; Increase in FCR in groups B & C	Han et al. (2008)

Figures

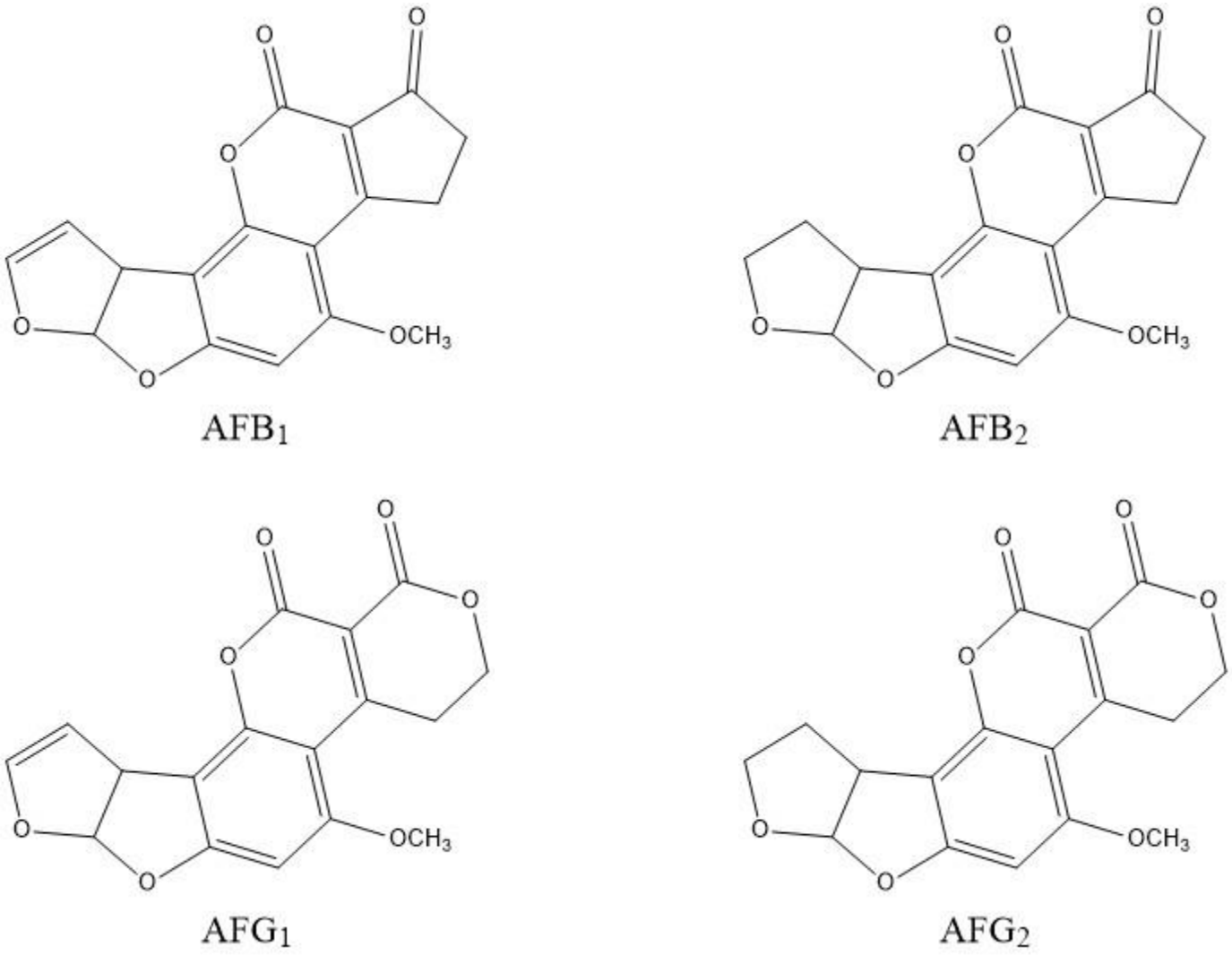

Figure 2.1 Molecular structures of AFB_1, AFB_2, AFG_1, and AFG_2.

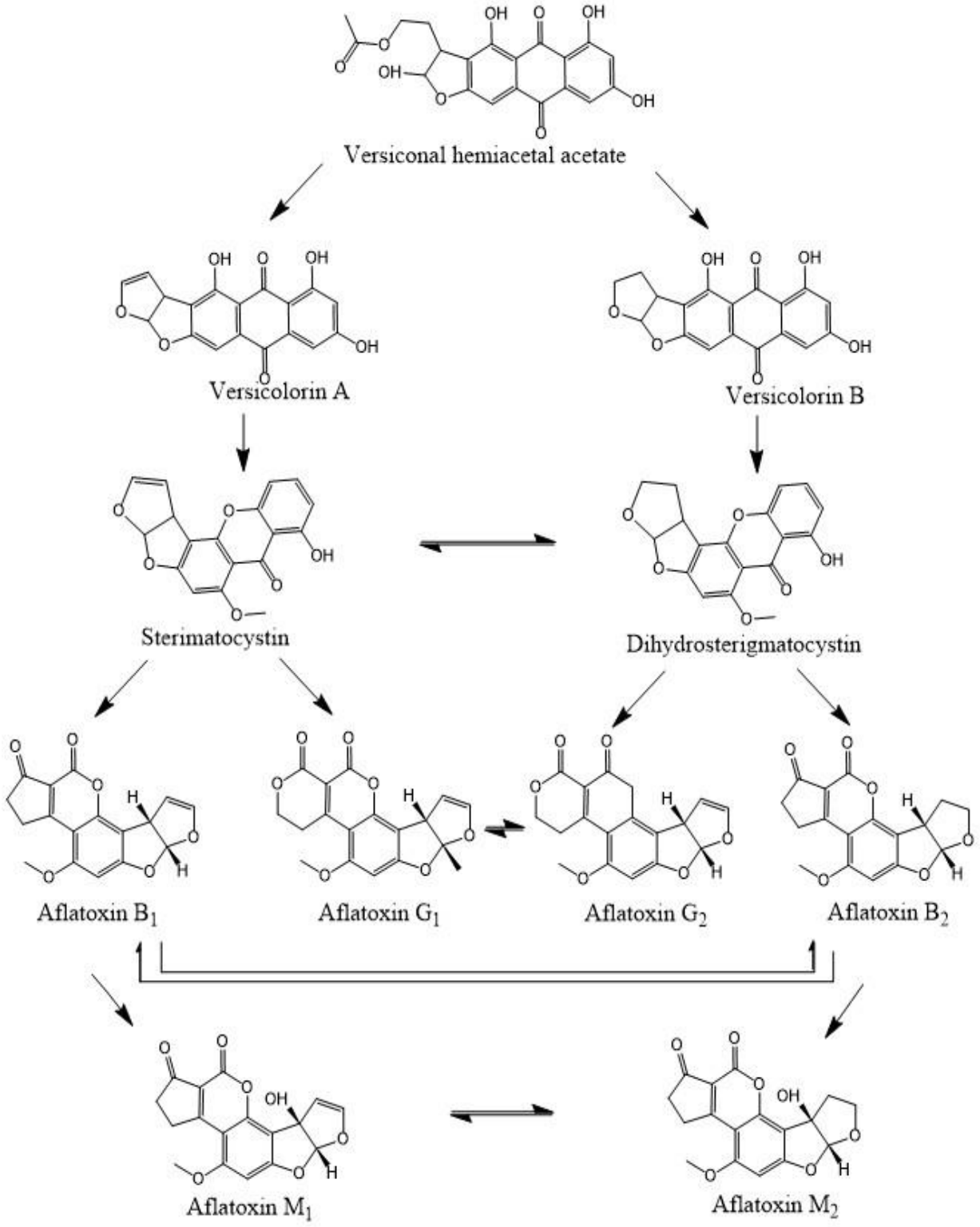

Figure 2.2 Pathway of formation for the six major types of AFs.

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CHAPTER 3

ANALYSIS OF AFB_1 LEVELS IN FOOD PRODUCTS COLLECTED FROM SUPERMARKETS IN ATHENS, GA

Abstract

Corn and peanuts represent a large contribution to the world's food supply and are two of the top ten commodities produced in the State of Georgia, USA (Georgia Farm Bureau 2013). The purpose of this study is to analyze AFB_1 contamination in peanut and corn food products purchased from supermarkets in Athens, Georgia using high pressure liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) methods. This study serves as a preliminary study used to develop and compare sampling methods as well as analysis and instrument methods used for AF determination in peanuts and grains. There was a 100% detection rate of AFB_1 in the thirteen samples collected, all with levels below the 20 ppb regulatory limit set by the USFDA. Corn products had a higher average of contamination than peanut products. HPLC is the "gold standard" for AF analysis and yielded lower results than ELISA methods. Both methods were successful in detecting AFs, however the HPLC method was preferred for its specificity of analyzing AFB_1 individually.

Introduction

Since their discovery in the 1960s, AFs have been studied, monitored, and regulated in the United States and worldwide. *Aspergillus flavus* and *A. parasiticus* are the two main strains

of fungi that produce AFs. Contamination may occur during growing, harvesting, storage or even processing. AFs are very heat stable and are not destroyed in many cooking and processing methods. Food contaminated with AFs may cause harm to humans and animals, especially if consumed regularly. Exposure to AFs has been shown to be carcinogenic in studies of different animal species and can lead to human hepatocellular carcinoma (HCC) (Jackson and Groopman 1999). AFs also have mutagenic, toxic, and immunosuppressive effects and potentiate other diseases (Brase 2013).

AFB_1 and total AFs in foods are strictly regulated in the United States by the FDA. The regulatory limit for AFs in milk is 0.5 ppb and 20 ppb in foods for human consumption, and for immature animals and dairy cattle, and feedstuffs other than corn. Other regulatory limits of 100 ppb, 200 ppb, and 300 ppb are designated for corn for mature or finishing animals or cottonseed meal as a feed supplement. The European Union regulatory limits for AFB_1 in foods for human consumption are 2-12 ppb and 4-15 ppb for total AFs in foods.

This study focuses on the comparison of different methods and protocol for sampling, extraction, and the analysis of AFs in peanut and corn food products. This serves as a preliminary study for research on the status of AF contamination in peanuts and grains grown in the State of Georgia, USA.

Materials and Methods

Study Design

This study was designed as a preliminary study to determine food product contamination of AFB_1 and to compare different methods for the analysis of AFB_1 in peanut and corn products. Different extraction and cleanup methods for HPLC and ELISA analysis methods were

compared as well. Samples were collected in October and analyzed through April of the following year. The specific location or State where commodities were grown is not stated on most products, so the packaging and distribution information was used to select the thirteen samples instead. The states of distribution locations included: Alabama, Florida, Georgia, Illinois, Iowa, North Carolina, Tennessee, and Texas. Food products using samples only grown in the State of Georgia were wanted for this study, however these samples still provided information about AFB_1 contamination in products sold in the State of Georgia and achieved the goal of comparing different extraction and analysis methods for AFB_1 determination.

Chemicals and Reagents

AFB_1 (>98% purity) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). HPLC grade water was purchased from Avantor Performance Materials (Center Valley, PA) and methanol was obtained from Honeywell (Morristown, NJ). Phosphate buffered saline (PBS) was purchased from American Type Culture Collection (Manassas, VA). 5mM triethylammonium formate (TEAF) buffer solution was prepared using 1M TEAF buffer and formic acid, both purchased from FLUKA Analytical. (St. Louis, MO). Veratox ELISA Test Kits for Aflatoxin were purchased from Neogen Corporation (Lansing, MI). VICAM AflaB immunoaffinity columns were purchased from Waters Corporation (Milford, MA). An extraction solution was prepared using a ratio of 70:30 methanol:HPLC grade water.

Sample Collection and Processing

Peanut and corn products were collected from three supermarkets in Athens, Georgia, USA: Publix, Walmart, and Kroger. Six corn products and seven peanut products were chosen based on the distribution and possible origination information stated on each food product package. Peanut products selected for analysis included roasted peanuts in shell, unsalted dry roasted peanuts, powdered peanut butter, natural creamy peanut butter, natural and raw redskin peanuts, jumbo raw natural peanuts, and natural and raw blanched peanuts. Corn products selected for analysis included stone ground medium enriched white corn meal, white self-rising corn meal, plain white enriched corn meal, yellow un-popped popcorn, white un-popped popcorn, and plain enriched yellow corn meal. At least 450 g, about 16 ounces, of each sample was purchased.

Each of the products were collected and stored in a dry, dark, cabinet as this was determined to prevent loss or increase of AF contamination and also not cause moisture build-up in products which could possibly increase the levels of AFs. Each total sample was ground into a fine, almost powder-like consistency using a Ninja Professional 1100 Watt Blender (Euro-Pro Operating LLC, Newton, MA). Samples were labeled so that the product brand, type, or origination information was not known during the analysis process. The blender and all parts were cleaned thoroughly between each sample with methanol and ultrapure water. All samples were refrigerated after being ground, but were kept in sealable plastic storage bags to prevent an increase of moisture within the sample. Sealed bags were placed inside a black plastic bag inside the refrigerator in order to protect the samples from light and maintain the levels of AFs present. Glassware was used when possible instead of plastic materials because AF can bind to plastic and detectable levels may be affected.

Sample Extraction, Filtration, and Analysis using ELISA Method

Five grams of each ground sample was weighed into a 50-mL disposable plastic centrifuge tube. Twenty-five mL of methanol:water (70:30) was added to each sample and vortexed for one minute. The sample was filtered into a clean centrifuge tube using Whatman No. 1 fluted filter paper and filtrate was allowed to drain thoroughly. The caps were replaced on the tubes containing the filtrate and aluminum foil was tightly wrapped around each tube.

A Veratox ELISA kit was used in order to determine the levels of AFB_1 in each sample extract. The lower limit of detection for this method is 5 ppb and has a range of quantitation between 5-50 ppb. The procedure was adapted from the Veratox ELISA manual and is shown in Figure 3.1. One hundred µL of conjugate and 100 µL of each sample and control was add to the red mixing wells and mixed thoroughly. One hundred µL of this solution was then transferred to the antibody wells included in the kit and incubated for two minutes. The liquid was emptied from the antibody wells, washed with DI water five times, and then emptied. After 100 µL of substrate was added to the antibody wells and allowed to incubate for two minutes, 100 µL of Red Stop was added to the wells. The wells were then placed in a Bio-tek EL_x808 IU Ultra Microplate Reader was used to determine results of ELISA analysis. Absorbance results were collected with a 650 nm filter. Beer's law was then used to determine the concentration of AFB_1 present in each sample. A blue color is produced when the substrate is added to the bound conjugate, which indicates the amount of AF present. A darker shade of blue indicates a higher concentration of AFB_1 present, whereas a lighter shade of blue correlates with a lower concentration of AFB_1 present in the sample extract.

Sample Extraction, Filtration, and Analysis using HPLC Method

Sample extraction and elution were completed within hours of HPLC analysis in order to preserve the AF structure and concentration in each extraction that was inserted into HPLC. HPLC analysis was used to determine the concentration of AFB_1 in each peanut and corn sample and results were then compared with those of the ELISA analyses. The extraction and analysis method used, as shown in Figure 3.2 was adapted from various AOAC methods. A 25-g portion of each ground sample was weighed into a blender jar. Five grams of laboratory grade sodium chloride and 125-mL of methanol:water (70:30) was added to the portion of sample in the blender jar and blended on high for 2 minutes. The extraction slurry was filtered into a 250-mL glass bottle using Whatman No. 1 filter paper and the extraction was collected. Fifteen-mL of each extraction solution was diluted using HPLC grade water, and vortexed for 1 minute. This solution was filtered using VICAM 11 cm microfibre filter paper (Waters Corporation; Milford, MA) and collected in a clean glass bottle.

Column chromatography was used to prepare each eluate for HPLC analysis. AflaTest immunoaffinity columns (IAC) were used in combination with a Waters extraction manifold. Each IAC was placed in the top connectors of the manifold and 10-mL glass syringe barrels were placed in the top of each column. Ten milliliter of PBS was filtered through each column followed by 15-mL of solution extract. Two portions of 10-mL of HPLC grade water were filtered through each column. One milliliter of 100% methanol was filtered through each column and the eluate is collected in glass collection vials. The eluate was diluted with 1-mL of HPLC grade water and the solution was vortexed for one minute. A micropipette was used to add 125-µL of prepared solution to an HPLC vial and then placed into the sample tray. One hundred microliters of sample was injected into HPLC for analysis. An Agilent 1100 HPLC system

(Agilent Technologies; Santa Clara, CA) with fluorescence detector (FLD) and diode array detector (DAD) was used with a flow rate of 1.0 mL/min and emission and excitation wavelengths of 366 and 425 nm, respectively. A Luna 5 μm C18 HPLC 250x4.6mm column was used. An isocratic mobile phase of 70% A (TEAF) and 30% B (HPLC Grade Methanol) was determined to yield best peak separation and results.

Recovery Test for HPLC Method

Four peanut and three corn samples were spiked using AFB_1 standard solution. A 50-gram portion of each sample was weighed into a glass beaker and 500 μL of 2.6 ng/μL AFB_1 standard in methanol was added to the sample using a micropipette and then thoroughly mixed. The spiked sample was allowed to dry for 30 minutes in the fume hood and then a 25-g portion was used for the HPLC procedure.

HPLC Peak Integration

AFB_1 standards were prepared to create a standard curve using the HPLC method described earlier. The standard curve, shown in Figure 3.3, was created using standard solutions of the following concentrations of AFB_1: 2.5, 5, 10, 20, and 40 μg/kg. The formula of y=0.2776x-0.375 was determined from the standard curve, where the "y" variable represents the peak area and the "x" variable represents the sample concentration of AFB_1 (μg/kg). The retention time determined was near 17.4 for AFB_1. After the analysis of each peanut and corn sample using HPLC, the peaks were then integrated by measuring the area of each peak at the determined retention time. The same formula was used to determine the AFB_1 concentration in each sample.

Results

ELISA Method

The overall average of AFB_1 contamination in all peanut and corn products collected and analyzed from the supermarkets using the ELISA method was 8.175 ppb, which is below the regulatory limit. Mean and standard deviation results, shown in Table 3.1, reveal that the AFB_1 contamination in corn was slightly higher than that of peanuts. The results of the AFB_1 concentrations in each sample as determined by the ELISA method are shown in Figure 3.4. AFB_1 was detected in 12 of the 13 samples, however all results were below the regulatory limit of 20 ppb for human consumption.

HPLC Method

The overall average of AFB_1 contamination in all peanut and corn products collected and analyzed from supermarkets using the HPLC method was 0.5714 ppb, which is significantly below the regulatory limit. Mean and standard deviation results, shown in Table 3.1 reveal that AFB_1 contamination in corn samples was over two-fold greater than that of peanut samples. The results of contamination in each sample are shown in Figure 3.5. The detection rate of AFB_1 in the thirteen samples using HPLC methods was 100 percent, however all results were below the regulatory limit of 20 ppb for human consumption.

Comparison of ELISA and HPLC Method

Levels of AFB_1 below the 20 ppb regulatory limit were detected in all 13 corn and peanut products collected from supermarkets in the State of Georgia using ELISA and HPLC analysis methods. The results of AFB_1 contamination in samples were higher using ELISA versus HPLC

methods. The averaged results of duplicate testing for each sample using the HPLC and ELISA methods are shown in Table 3.2. A graph comparing the results of the ELISA and HPLC methods is shown in Figure 3.6. ELISA is an immunoassay based on the binding of antibodies to a specific molecular structure (Cox 2012). Because of the nature of this test, it may yield higher results of AFB_1 than are actually present in a sample. The ELISA method is less reliable and less accurate because of the cross-reactivity of the antibodies. ELISA may detect other strains of AFs which contributes to the higher values detected. HPLC specifically allows for detection of AFB_1. These features of each test explain the lack of correlation between the two shown in Figure 3.6. HPLC is the gold standard for detection of AFs, which is why it was used as a comparison analysis in this study (Wacoo et al. 2014).

Discussion

Based on CPG Section 683.100 by the United States FDA, the action level for AF in peanut and corn food products for human consumption is 20 ppb. This regulatory limit was the basis for this study since all samples were collected in the United States. Samples from different countries may be subject to follow different regulatory limits. The strict regulation of levels of AFs in food products by the United States FDA is a critical factor in reducing the exposure of people who frequently consume these products to AFs, especially AFB_1. The regulatory limit for AFs in food products for human consumption set by the FDA is 20 ppb. In this study, all corn and peanut products selected from local supermarkets in the State of Georgia and analyzed using ELISA and HPLC methods had levels below the 20 ppb regulatory limit. Corn products had higher levels of AFB_1 detected using both methods. This result correlates with studies and

information about the nature of AFB_1 contamination that show it is usually more prevalent in corn than other crops.

The results using both methods were averaged for each sample in order to compare between samples. In both popcorn and corn meal, the "white" varieties had lower AFB_1 levels than the "yellow" varieties. Among peanut samples, products labeled as "natural" had the lowest levels of contamination. Powdered peanut butter had higher levels of contamination than creamy peanut butter. Packaging materials did not seem to be a factor of the contamination levels however more porous packaging may allow for an increase in moisture in the product that could affect AFB_1 levels. The age or storage period of commodities used for products and of the food product in a supermarket can vary significantly. Because AFB_1 can still contaminate products during storage, this may be a possible factor when analyzing food products from food markets.

It is important for consumers to be informed about the realities of AF contamination, especially those who regularly consume foodstuffs that are more susceptible to contamination. Although the toxin is closely regulated in the US and other countries, it is still present in foodstuffs and not completely eliminated. More studies should be conducted in order to further examine the effects of human consumption of foodstuffs contaminated with AFs below the regulatory limit set by the FDA on a regular basis. Also, it is important that research continues on possible ways to eliminate AFs from contaminated commodities and foodstuffs.

Since AFs are regulated in the US, food products from supermarkets should ideally measure below the regulatory limits. AF contamination is ubiquitous and a continuous risk throughout the food chain. Adequate sampling and testing in large lots of commodities and products can prove difficult because AF contamination is not consistent throughout a field or lot. Even with rigorous regulation and testing, it is still common to find detectable levels of AFs in

foodstuffs because of the difficulties with completing eliminating this toxin. Each of these factors of AF contamination shows the difficulty and seriousness of analysis of AFs in commodities and food products. HPLC methods are the gold standard for AF analysis. TLC and antibody-based analyses are also popular because of lower costs and more rapid testing, however the degree of accuracy may be compromised. Comparing different extraction and analysis techniques is important in order to ensure accuracy of results. HPLC analysis also allows for simultaneous testing of multiple AFs in commodities which is useful.

Conclusion

Results for the ELISA analysis of each sample were higher than the results for the HPLC analyses because ELISA is an antibody-based immunoassay that has lower accuracy and reliability. HPLC is the gold standard for AF detection was determined to be the most preferred of the methods used in this study. All peanut and corn samples collected and analyzed had levels of AFB_1 below the regulatory limit of 20 ppb set by the USFDA. The overall average of AFB_1 levels in corn was higher than that of peanuts, which historical patterns of greater prevalence of AFB_1 in corn among other crops. Corn products are also normally in packaging that is risks moisture permeating through, whereas peanut products are usually stored in sealed plastic. Overall, this study was successful in developing a method for AFB_1 detection in corn and peanut products, as well as examining contamination of samples sold in the State of Georgia, USA.

Tables

Table 3.1 Summary of comparison of ELISA and HPLC methods used to determine levels of AFB1 in corn and peanut products purchased from local supermarkets.

Corn Products

	Mean	SD	Range
ELISA	8.439	6.342	0.160,4.611
HPLC	0.745	0.954	0.000, 16.832

N=6

Peanut Products

	Mean	SD	Range
ELISA	7.911	5.557	0.106, 1.995
HPLC	0.368	0.339	0.971, 14.373

N=7

Table 3.2 Summary of averaged results using ELISA and HPLC methods to determine levels of AFB1 in 6 corn and 7 peanut products purchased from local supermarkets. There were two duplicate subsamples analyzed for each sample.

Sample ID	*HPLC Results (ppb)*	*ELISA Results (ppb)*	*Average (µg/kg)*
A	0.294	8.581	5.860
B	0.113	5.186	3.587
C	1.057	9.884	6.241
D	0.160	16.000	11.201
E	0.368	11.443	7.831
F	0.466	5.643	3.661
G	0.160	15.602	10.920
H	0.106	17.939	12.610
I	0.651	0.000	0.460
J	0.106	2.263	1.526
K	0.361	3.020	1.880
L	2.648	5.824	2.246
M	0.556	4.626	2.878

Figures

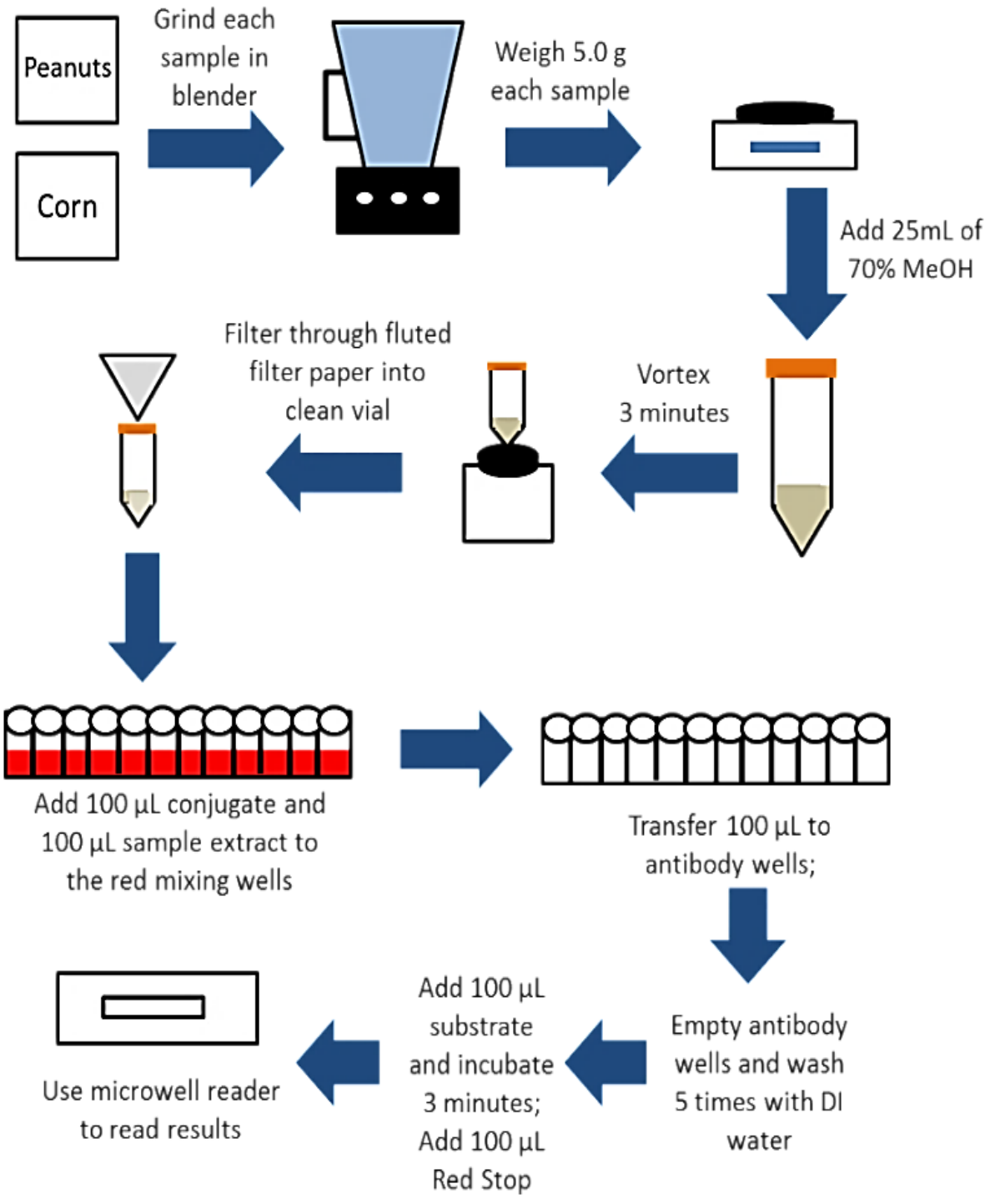

Figure 3.1 Flowchart of basic procedure of sample preparation and ELISA method based on the Veratox Procedure for Aflatoxin.

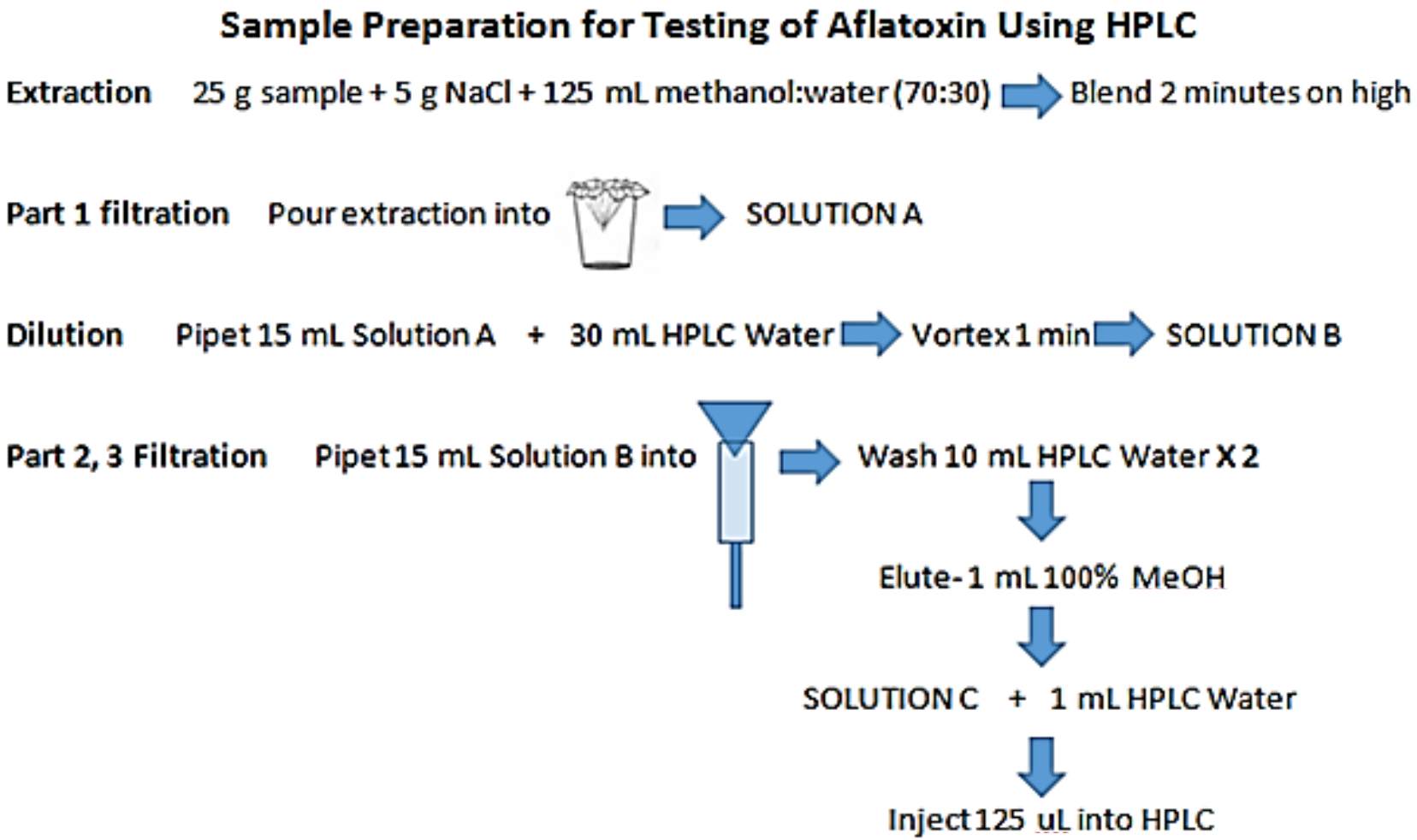

Figure 3.2 Flowchart of basic procedure for sample preparation and HPLC method based on the VICAM AflaTest HPLC Instruction Manual.

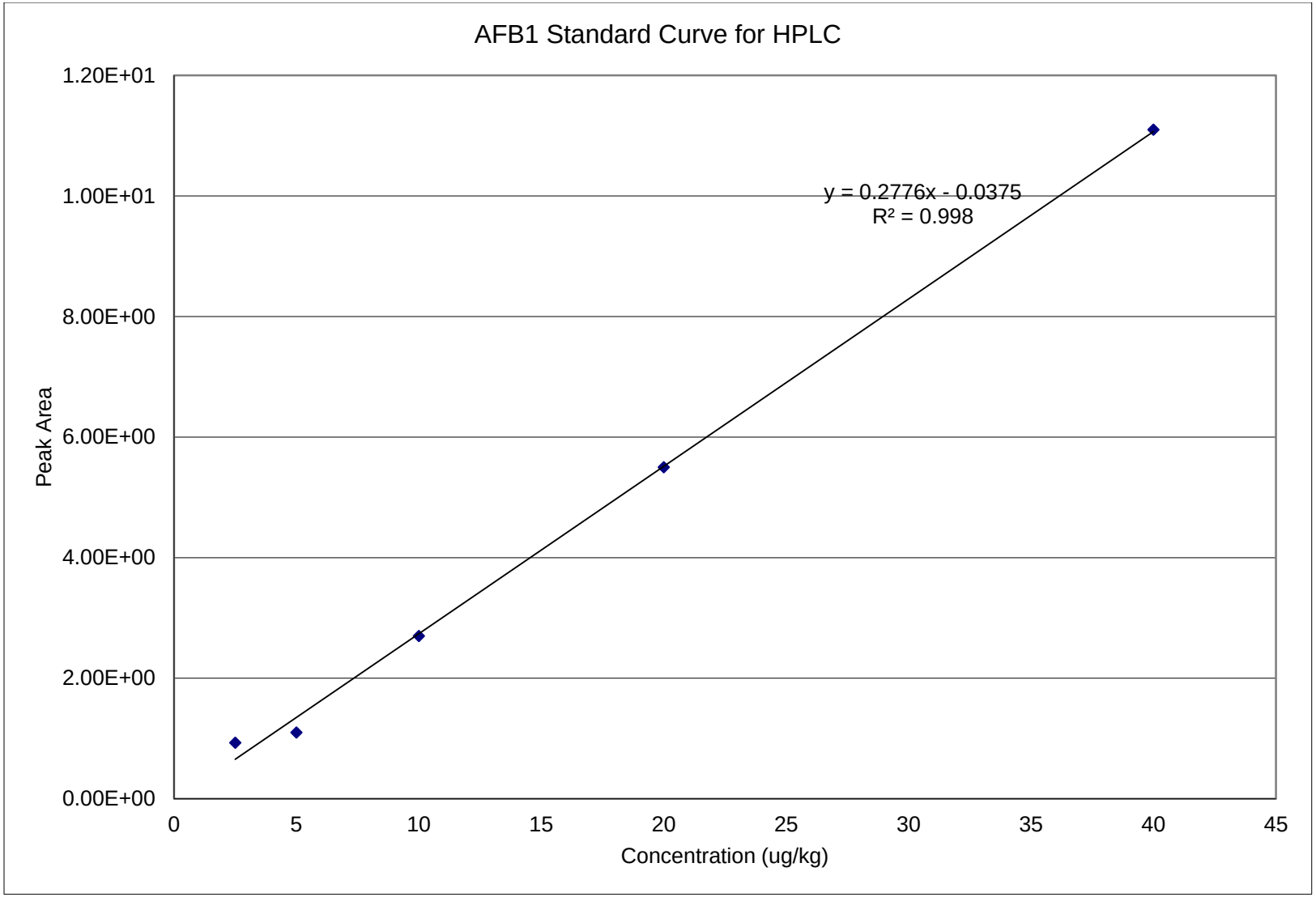

Figure 3.3 AFB_1 standard curve use for the determination of AFB_1 concentrations in each sample based on the peak area from the HPLC chromatogram.

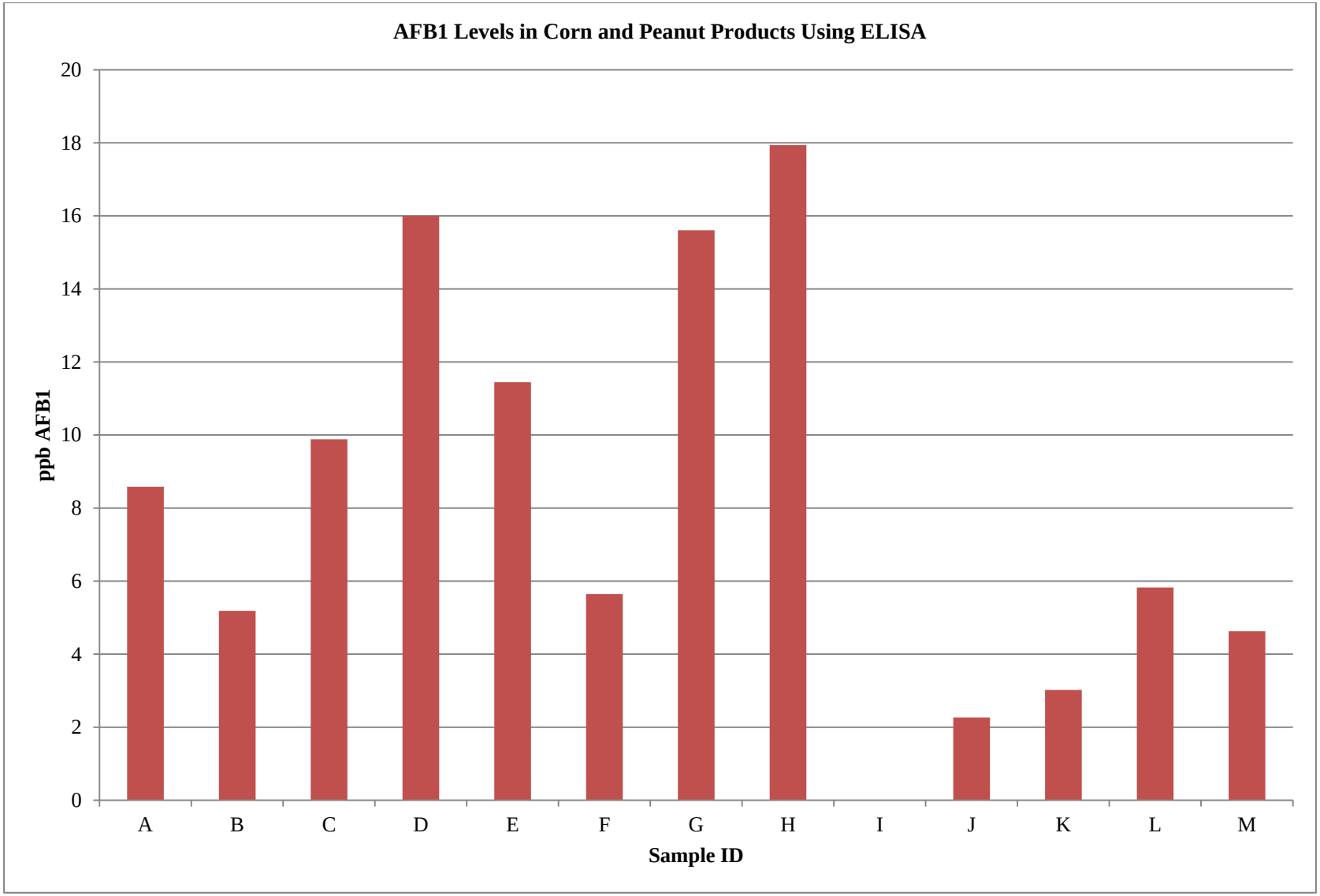

Figure 3.4 Levels of AFB1 (ppb) detected in corn and peanut products using ELISA method.

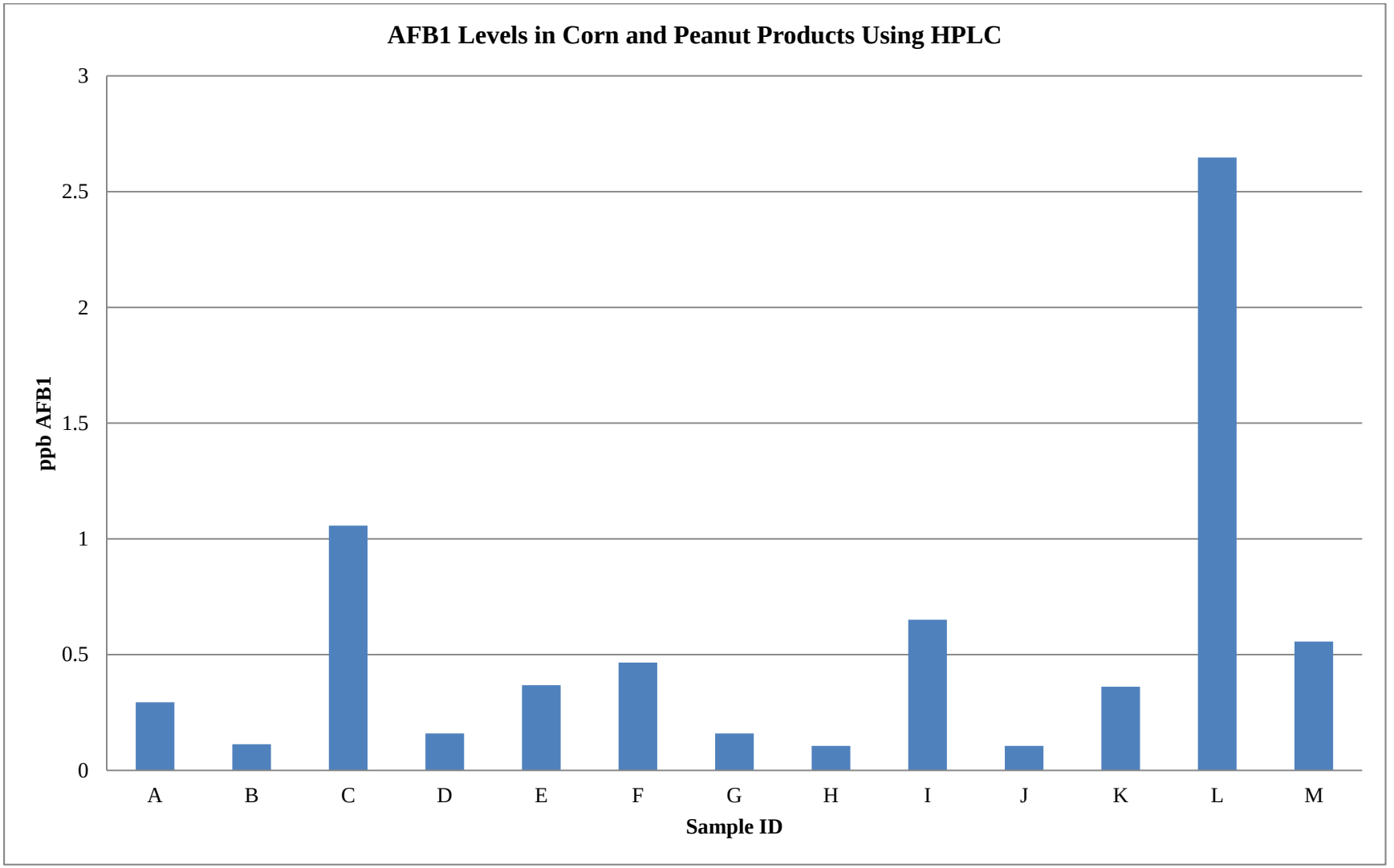

Figure 3.5 Levels of AFB1 (ppb) detected in corn and peanut products using HPLC method.

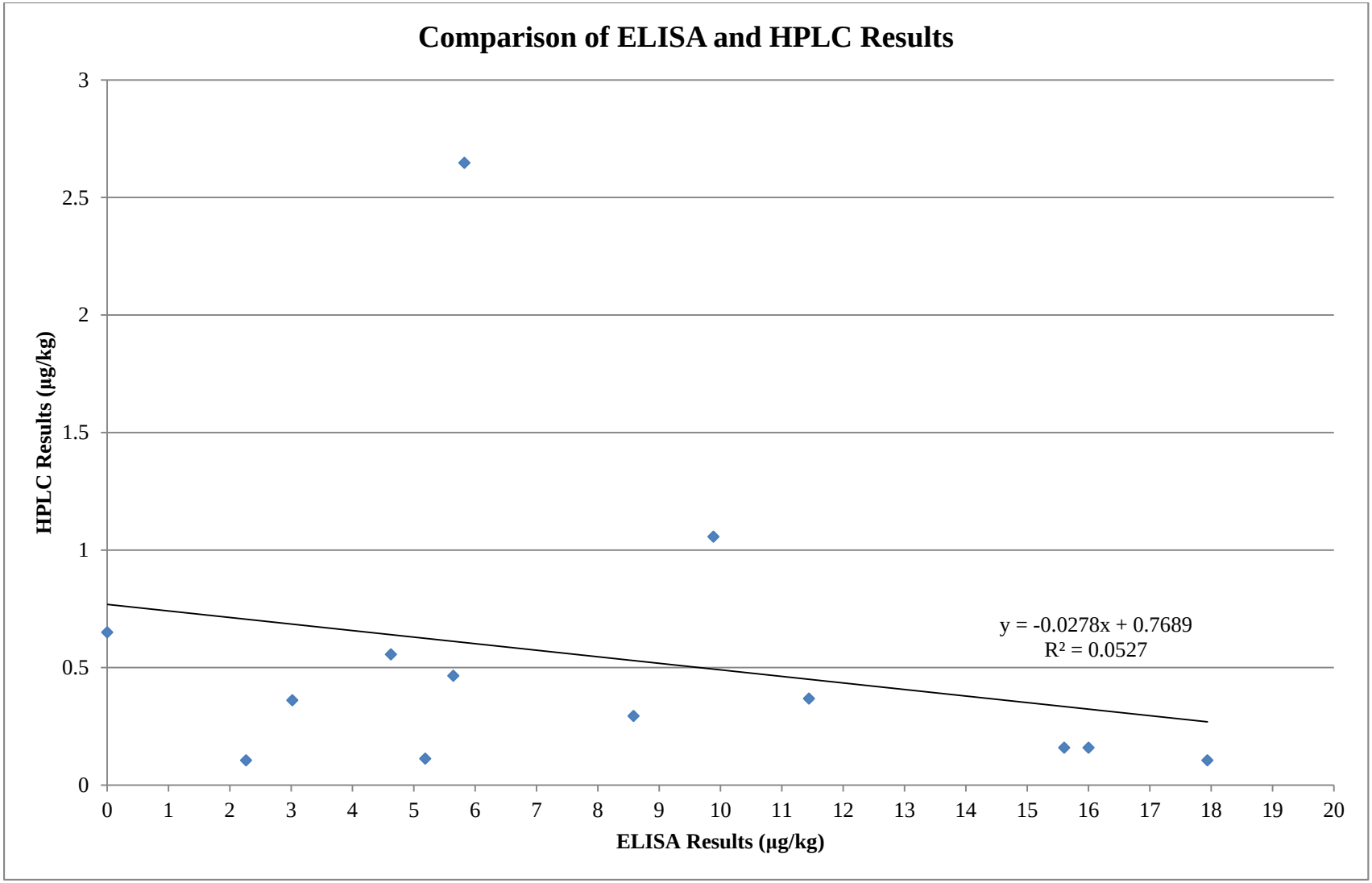

Figure 3.6 Comparison of HPLC and ELISA method results (ppb) of detection of AFB1 in corn and peanut food products purchased at local supermarkets.

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CHAPTER 4

CURRENT STATUS OF CONTAMINATION OF AFLATOXINS IN GRAIN AND PEANUTS GROWN IN GEORGIA, U.S.A

Abstract

Aflatoxins (AFs) are the most common naturally occurring mycotoxins produced mainly by *Aspergillus flavus* and *A. parasiticus.* AFs are carcinogenic, immunosuppressive, mutagenic, potentiates other diseases, and may be harmful to both animals and humans that frequently consume contaminated foods. The State of Georgia's climate during grain and peanut growing and harvesting seasons provides ideal conditions for growth of the aflatoxigenic fungi that produce AFs. The purpose of this study is to investigate the current state of AF contamination in Georgia-grown grains and peanuts because no such information is available over two decades. Analytical protocols were developed and validated via using spiked samples and selection of ratios of solvent extraction for different matrixes. Approximately 280 peanut and grain samples were collected and analyzed using ultra-high-performance liquid chromatography (UHPLC) and Mobile Assay's Mobile Diagnostic Rapid Test Reader (mReader) analysis methods. There was a 100% detection rate of AFs in Georgia-grown samples. Results for total AF levels (µg/kg) in oats, sorghum, wheat, corn, and peanuts were 23.48±36.79, 172.34±59.14, 51.81±86.45, 8.44±12.46, and 68.22±95.58, respectively. The results of this study conclude the need for closer monitoring of commodities produced the State of Georgia and other areas with higher risks for AF contamination.

Introduction

AFs are a class of mycotoxins that are naturally occurring secondary metabolites, mainly produced by *Aspergillus flavus* and *A. parasiticus* (Wogan 1966). AFs are the most potent natural toxins and are carcinogenic, immunosuppressive, mutagenic, and potentiate other diseases (Diener et al. 1987). The four most common strains of AFs are AFB_1, AFB_2, AFG_1, and AFG_2 (Hussein and Brasel 2001). They were originally named based on the blue or green colors they fluoresce (Wang 1998). These fungi are known to grow in soil, on plants, and also on commodities in storage containers (Khlangwiset et al. 2011). Different fungicides can be applied to crops to prevent aflatoxigenic fungi from growing on crops, however this does not eliminate the risk of AF contamination in later stages. Because of these properties, AFs can be produced and then contaminate commodities throughout the growing, harvesting, and storage processes. Therefore, the most effective way to control AFs currently is to monitor and control storage conditions of commodities in order to prevent production of AFs by aflatoxigenic fungi.

Corn, peanuts, groundnuts, and cereal grains are among the most common commodities contaminated with AFs (Marin et al. 2013; Zain 2011). Aflatoxigenic fungi have been known to grow on a wide variety of substrates, however. The optimum conditions for aflatoxigenic fungal growth are 86ºF, 85 percent humidity, and kernel moisture of 18% (Villers 2014). High humidity combined with drought conditions and warm temperatures encourage AF contamination. The humid subtropical climate in the State of Georgia provides hot summers, mild winters, and high average humidity. These conditions prove it hard to prevent AF contamination in the State of Georgia, especially in the southern regions where many of the commodities are grown.

There are approximately 48,000 farms in Georgia, totaling ten million acres (Georgia Farm Bureau 2013). Agriculture contributes $71 billion to Georgia's $763 billion economy each

year. Georgia ranks first in the production of peanuts, which are normally affected by AFs. Because of the large impact agriculture has on the economy of Georgia, the United States, and the world, determining and monitoring the levels of AFs in Georgia commodities and food products is beneficial and critical to understanding the current status of contamination and what steps should be taken to decrease levels of AFs in commodities and food products.

Mycotoxins in food for human and animal consumption are currently regulated or planned to be regulated in over sixty countries (Vanegmond 1989). Commodity lots in the State of Georgia and the United States are tested at buying points or during the buying and selling process, and must meet the USFDA's regulatory levels for AFs. Foods for human consumption in the United States must not exceed 20 ppb of AFs and 0.5 ppb for milk (FDA 1979). Even though the levels of AFs are regulated in the US, it is still important for current research to be conducted and for field and packaged samples to be tested with multiple methods in order to determine if there are problems or better methods to use. It has been over twenty years since the levels of AFs in Georgia commodities has been formally researched and reported. Therefore, this research study was designed to survey a variety of commodities throughout Georgia because of the large number of years no data was collected.

The objective of this research is to determine the current status of AF contamination of crops grown in the State of Georgia, U.S.A. Ultra-high performance liquid chromatography (UHPLC) and the Mobile Diagnostic Raid Test Reader (mReader) by Mobile Assay were the two analytical instruments compared. UHPLC is the "gold standard" with AF analysis and was chosen after the results of the preliminary study comparing HPLC and ELISA analysis in corn and peanut samples. Different extraction and cleanup methods were adopted and compared using each instrument for their capability in detecting AFs in grains and peanuts.

Materials and Methods

Study Design

The overall purpose of this study was to determine the current status of AF contamination in peanuts and grain grown in the State of Georgia, USA. This study also compared four different methods for the determination of AFs in peanuts and grains using peanut samples spiked with a mixture of AFB_1, AFB_2, AFG_1, and AFG_2. The methods compared were immunoaffinity column cleanup using UHPLC analysis, MycoSep cleanup using UHPLC analysis, Sep-pak cleanup using UHPLC analysis, and Neogen's Reveal Q+ method using mReader analysis. Based on the results, two methods were chosen to use for the analysis of the 280 samples grown in Georgia: Sep-pak cleanup using UHPLC analysis and Neogen's Reveal Q+ method using mReader analysis. After analysis of all samples, results were collected and compared.

Chemicals and Reagents

AFB_1, AFB_2, AFG_1, and AFG_2 standards were obtained and dissolved in dimethyl sulfoxide (DMSO), all purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). A Supleco AF mix analytical standard and ethanol (pure, HPLC grade) was also purchased from Sigma Aldrich Chemical Co. HPLC grade water was purchased from Avantor Performance Materials (Center Valley, PA) and methanol was purchased from Honeywell (Morristown, NJ).

VICAM AflaB immunoaffinity columns and Sep-pak Classic C18 Cartridges were purchased from Waters Corporation (Milford, MA). MycoSep columns were supplied by the Mallikarjunan lab group in the Department of Biological Systems Engineering at Virginia Tech (Blacksburg, VA). The mReader tablet and supplies and the Neogen Reveal Q+ for Aflatoxin kits were kindly provided by Dr. Don Cooper of Mobile Assay Inc. (Boulder, CO).

An extraction solution was prepared using a ratio of 65:35 ethanol:HPLC grade water. A second extraction solution was prepared using a ratio of 70:30 methanol:HPLC grade water.

Sample Collection and Processing

Peanut, corn, sorghum, wheat, and oat samples were collected from various sources in Georgia including farmers, USDA stocks, and local supermarkets. A total of 280 samples were collected, weighing a minimum of 500 g each. Each sample was then ground into a fine, almost powder-like texture using a Ninja Professional 1100 Watt Blender (Euro-Pro Operating LLC, Newton, MA). A 500 g subsample was weighed into sealable plastic storage bags, labeled numerically and with commodity identification, and stored in a dry, dark location.

Peanut samples spiked with an AF mixture were obtained from Virginia Tech. The types of samples included peanut oil, peanut paste, peanut flour, and RUTF (ready-to-use therapeutic food). Our lab group was blind to the actual concentrations of AFs in each sample. Each sample was stored in a plastic air-tight sealed bag, and some samples were in a centrifuge tube within the bag. Samples were stored at 4^{o}C throughout sampling and analysis.

Standard Solutions

The Supleco AF mix standard was originally used to develop the mReader method. Individual AF standards B_1, B_2, G_1, and G_2 were used to create a standard curve for each of these four strains of AF using the UHPLC method. Concentrations of each standard were also combined to create an AF mixture, which was also used to create a standard curve using the UHPLC method. The individual AFB_1 standard curve, as shown in Figure 4.1, was used for

recovery tests with Georgia peanut and grain samples. The standard curve for AFB_2 is shown in Figure 4.2; the AFG_1 is shown in Figure 4.3; and the AFG_2 is shown in Figure 4.4

UHPLC Conditions

The Thermo Scientific Dionex UltiMate 3000RS UHPLC system was used for separation of target analytes. Detection was completed using the Thermo Scientific Dionex UltiMate 3000 RS Diode Array detector and Fluorescence detector. The UHPLC and detector conditions are shown in Table 4.1. Excitation and emission wavelengths for fluorescence detection were 360 nm and 440 nm, respectively, and 362 nm and 440 nm for UV detection.

Sample Extraction, Filtration, and Analysis using Immunoaffinity Column Cleanup Method with UPHLC Analysis

Five gram portions of each sample were weighed into centrifuge tubes. The sample was extracted using methanol:water (70:30) and sodium chloride, and then vortexed. This mixture was then filtered using Whatman filter paper (No. 1). Three milliliters of filtrate was collected in a centrifuge tube and diluted using HPLC water. This solution was filtered using a microfibre filter and then five milliliters of the filtrate was passed through an immunoaffinity column. The sample was eluted with methanol. The eluate was dried under a gentle stream of ultra-pure nitrogen gas and reconstituted using 25% methanol. Ten microliters of reconstituted samples was analyzed using UHPLC. This method, as shown in Figure 4.5, was repeated with a duplicate of each sample

Sample Extraction, Filtration, and Analysis using MycoSep Cleanup Method with UHPLC Analysis

Five gram portions of each sample were weighed into centrifuge tubes. The sample was extracted with methanol:water (70:30) and vortexed. The supernatant was filtered using Whatman filter paper (No.1) and the filtrate was collected in a clean vial. Eight milliliters of filtrate was transferred to a glass tube and then pushed through the MycoSep column. A 1-mL portion was then evaporated to dryness under a gentle stream of ultra-pure nitrogen gas and reconstituted using 25% methanol. Ten microliters of reconstituted samples was analyzed using UHPLC. This method is summarized in Figure 4.6.

Sample Extraction, Filtration, and Analysis using Sep-pak Cleanup Method with UHPLC Analysis

Five gram portions of each sample were weighed into centrifuge tubes. The sample was extracted using methanol:water (70:30), and then vortexed. This mixture was then filtered using Whatman filter paper (No. 1). Two milliliters of filtrate was collected in a centrifuge tube and diluted using HPLC water. Two milliliters of diluted filtrate was then passed through a sep-pak cartridge and eluted with methanol. The eluate was dried under a gentle stream of ultra-pure nitrogen gas and reconstituted using 25% methanol. Ten microliters of reconstituted samples was analyzed using UHPLC. This method, as shown in Figure 4.7, was repeated with a duplicate of each sample.

Sample Extraction, Filtration, and Analysis using mReader Method and Analysis

Five gram portions of each sample were weighed into centrifuge tubes. The sample was extracted using ethanol:water (65:35) and then shaken vigorously for three minutes. This mixture was then filtered using Whatman filter paper (No. 1). Diluent was added to a portion of the sample and mixed. A portion of this mixture was transferred to a clean vial. A Reveal Q+ Aflatoxin strip was placed into the sample solution where it developed for five minutes. After developing, the strip was removed promptly and results were interpreted using the mReader device. This method, as shown in Figure 4.8, was repeated with a duplicate of each sample.

Before analysis the peanut or Georgia samples, the method described was used to analyze spiked peanut samples in order to determine the method and instrument accuracy. A sample of blanched, raw peanuts was ground according the procedure and five subsamples of 50 g were weighed. Three of the subsamples were spiked using the Supleco AF mix standard to concentrations of 5, 20, and 40 μg/kg. One subsample remained a control. Duplicates of each sample were analyzed based on the procedure and the results are reported in Figure 4.9.

Standard Curve Determination for the mReader Method and Analysis

Supleco Aflatoxin Mix standard was used to prepare standards for analysis using the mReader device. Neogen Reveal Q+ strips were used and then results were read by the mReader. The concentration of the mixed AF standard used was determined to 2.59 μg/mL. Standards were prepared using the Supleco AF mixed standard and 100% ethanol to the concentrations of 6, 12, 24, 48, and 96 μg/kg. The procedure for Reveal Q+ kit was followed and the results of the standard curve are reported in Figure 4.10. A standard curve with all values except for the 96 μg/kg was created and used to determine sample results, as shown in Figure 4.11. This

concentration was not included in the standard curve because this value is near the maximum capabilities of the test strip and tablet.

Statistical Analysis

STATA 12 and Microsoft Excel were both used to determine mean and standard deviation of the levels of AFB_1, AFB_2, AFG_1, and AFG_2 in the peanut and grain samples. Statistical summaries were created for each commodity as well.

Results

Method Comparison and Determination

There were two parts of the research study. The first part involved the comparison of different extraction and analysis methods in order to determine the levels of AFs in peanut samples. Twelve different spiked peanut samples were used. Sep-pak, immunoaffinity columns, and MycoSep columns were the different extraction and cleanup methods used to prepare for UHPLC analysis. A fourth method using the Reveal Q+ extraction method and mReader analysis was also used. Duplicates of each sample were analyzed using each of the four methods described. The results are shown in Table 4.2. Overall, results using the Sep-pak cleanup method with UHPLC analysis and Reveal Q+ method with mReader analysis were most closely related. The immunoaffinity column cleanup with UHPLC analysis yielded similar results. However, the expense of IACs makes this method less desirable when analyzing a large number of samples. The MycoSep extraction method proved to have poor extraction and cleanup results. These results also show the accuracy of results using the Reveal Q+ method with mReader analysis in peanut samples. Using spiked peanut samples for this portion of the study was helpful in order to

determine accuracy and reliability of different analysis and extraction methods, and compare results easily. Based on these results, the Sep-pak cleanup method with UHPLC analysis was chosen to analyze the peanut and grain samples grown in the State of Georgia.

Georgia-grown Peanut and Grain Samples

Total, there were 280 peanut and grain samples analyzed with the UHPLC method using Sep-pak cleanup. The summary of results for these samples is shown in Table 4.3. Overall, there was a 100% detection rate for total AFs in each commodity category. The detection rate varied among each category with each type of AF. Corn was the only commodity where the total average of AFs was below the regulatory limit of 20 ppb set by the United States FDA. The total average of AFs in oats was 23.484±36.791 ppb. Table 4.4 shows the individual results of each of the four types of AFs analyzed for each of the18 oat samples, along with the total AFs (µg/kg) detected. Table 4.5 shows the individual results of each of the four types of AFs analyzed for each of the 12 sorghum samples, along with the total AFs (µg/kg) detected. Table 4.6 shows the individual results of each of the four types of AFs analyzed for each of the 68 wheat samples, along with the total AFs (µg/kg) detected. Table 4.7 shows the individual results of each of the four types of AFs analyzed for each of the 32 corn samples, along with the total AFs (µg/kg) detected. Table 4.8 shows the individual results of each of the four types of AFs analyzed for each of the 150 peanut samples, along with the total AFs (µg/kg) detected. Repeatability tests were conducted for 19 of the peanut and grain samples and are reported in Table 4.9.

The mReader method was used to analyze 19 of these samples as part of a field analysis. Five peanut, corn, and wheat samples were analyzed and two oat and sorghum samples were analyzed. The results from these analyses are shown in Table 4.10. Based on these results, the

extraction and analysis protocol are effective for determining the total AFs in peanut samples. The strong correlation of mReader and UHPLC results for the peanut samples is shown in Figure 4.12. However, the methods need to be studied further in order to use this protocol and instrument for the detection of AFs in grains such as wheat, oats, sorghum, and corn. Figure 4.13 shows the lack of correlation between mReader and UHPLC results for the grain samples. Standard curves and the formulas used by the software to determine AF levels should be determined for each of these commodities.

Discussion

The State of Georgia's climate provides ideal conditions for growth of aflatoxigenic fungi and AF contamination in crops, both in the field and throughout storage and processing. While commodities and food products are strictly regulated by the United States FDA and private companies for the levels of AF present, this information is not reported to the public. AF contamination varies throughout a field or lot and is difficult to predict because of varying weather patterns and conditions from year to year. Adequate sampling of large fields and lots proves difficult because of these factors. Because AFs are not affected much by heating, cooking, or processing, it is important to prevent contamination by implementing better practices in the field and in storage that reduce occurrence of aflatoxigenic fungi. Adequate sampling and analysis methods are also important for accurate AF determination throughout the food chain.

The results for the analysis of AFs in peanut, corn, oat, sorghum, and wheat samples grown in the State of Georgia were surprising for several of the crops because of higher than expected levels of AFs detected. The exact county or even region of the state each sample originated from is not known because to the sample collection sources. Drought conditions

during the growing or harvest months for these crops may have contributed to the higher levels of AFs. Also, high humidity in storage containers may have played a role in contaminated sample results. Corn and wheat samples had information sheets from the USDA that allowed for a few possible conclusions to be drawn. The summary for characteristics of all 32 corn sample shown in Table 4.11 reveals an average percent moisture of 14.087±0.596 and an average of 3.797±3.840 damage kernels percent. The summary for characteristics of 55 wheat sample shown in Table 4.12 reveals an average percent moisture of 12.635±2.102, 2.116±1.933 average percent of damage, and 4.351±3.022 average percent of defects. Although the moisture level in wheat samples was lower on average than in corn, the range was much greater than in corn and four samples had moisture percentages above 18 percent. The ideal kernel moisture for AF production is 18 percent, however lower moisture levels can still encourage AF production. Damage and defects caused by weather, insects, animals, and processing increases risk for AF contamination in commodities. Because information was not known for all samples, it is not possible to determine definite conclusions based on this information.

These samples had been in storage so it is possible that AF contamination may have occurred from the containers samples were stored in. Previous storage conditions may also be an explanation for higher AF levels. Future studies need to be conducted in the State of Georgia that analyze samples at multiple time points between growing and processing. This would help determine if AF contamination was more prominent in field or storage conditions. Also, more wheat samples in the State of Georgia should be analyzed further since AF contamination is not as common in this commodity, especially at levels detected. The Georgia samples collected would have been from the 2014 growing season most likely. Drought conditions, high humidity,

and damaging winds during that year are potential conditions that could have also caused higher than normal levels of AFs.

A study on the status of AF contamination in commodities in the State of Georgia has not been conducted or reported in over two decades. Therefore, the status of contamination for these crops studied during the past few years is not known. More studies need to be completed on these crops in future years in order to make comparisons of AF contamination related to weather conditions and location in the State of Georgia. Because of reasons previously stated, it is important to consistently monitor crops for AF contamination especially in a location like Georgia that has the prime conditions for aflatoxigenic fungi and AF production. These monitoring practices will help to determine if more steps for prevention should be taken, better farming and storage practices implemented, or different sampling and analysis methods should be used. The results from this study show that more studies should be conducted throughout the State of Georgia to determine if the high levels of AF contamination determined form this study were from storage contamination, weather abnormalities during the growing year, or other reasons.

The mReader software and tablet analysis method uses Reveal Q+ test strips by Neogen. This newer method provides rapid and inexpensive results and would be useful in field analysis where resources are limited. The results closely correlated with the UHPLC method with Sep-pak cleanup for peanut samples and method used. Future studies should be conducted to modify the extraction method for different grains including sorghum, oats, and wheat. Different test strips for AFs are available from Neogen, and there may be one that provides better results with these grains. The extraction method recommended by Neogen for peanuts was used for the peanut samples, and the recommended corn method was used for grain samples. The only

difference between the two methods is the ratio of solvent to weight of sample used during extraction. Changes may need to be made to the calibration curves in the mReader software to correlate with each commodity. This instrument will help increase monitoring of crops for AF contamination both in the field, in storage, and throughout processing because of its rapid results that can be done in field conditions.

Conclusion

In conclusion, this study updated AF levels in Georgia-grown peanut, corn, wheat, oat, and sorghum samples via UHPLC analysis with Sep-pak cleanup. The mReader analysis method to determine levels of AFs was successful in peanut samples, but requires more research and method development for grain analysis. The levels of AFs in the commodities studied require future research to determine if contamination resulted from weather conditions, storage conditions, or other reasons. Corn was the only commodity that had a total average of AFs below the 20 ppb limit set by the United States FDA. Results from this study prove the need for continued yearly research on the levels of AFs in Georgia-grown peanuts and grain in order to determine reasons for increased contamination and protocols to decrease contamination both in the field and in storage.

Tables

Table 4.1 UHPLC conditions, gradient program, and column information.

Column	Acclaim RSLC 120C18 2.1x150 mm, 2.2 µm 120Å (Thermo Scientific, Waltham, MA)		
Mobile phase A	Methanol:HPLC grade water (10:90)		
Mobile phase B	Methanol		
Flow Rate	0.4 mL/min		
Column Temperature	50 °C		
Injection Volume	10 µL		
Gradient	Time (min)	A%	B%
	0.00	95	5
	6.00	50	50
	10.00	5	95
	15.00	5	95
	15.10	95	5
	18.00	95	5

Table 4.2 Summary of results of the comparison of different methods used to determine the total AF concentrations in peanut samples. Two replicates of each spike sample were used for analysis, and are described as "Sample Set 1" and "Sample Set 2".

Sample Set 1	RUTF	RUTF	RUTF	Peanut Flour	Peanut Flour	Peanut Oil	Peanut Oil	Peanut Oil	Peanut Oil
IAC Method	4.874; 4.916	15.512; 15.509	171.631; 171.073	309.971; 313.137	1628.401; 1630.068	45.540; 49.602	89.685; 93.710	14.236; 13.695	1.000; 1.033
Mycosep Method	3.192; 2.761	0.504; 0.681	5.395; 5.270	27.055; 28.118	148.703; 146.349	11.168; 11.194	24.100; 24.610	2.078; 1.912	0.096; 0.091
Mobile Assay mReader	4.7	31.000; 30.800	31.000; 30.800	414.700; 423.300	2215.900; 2210.500	56.600; 58.300	82.500; 84.900	11.800; 11.400	8.800; 2.700
Sample Set 2									
Sep-Pak Method	13.569; 8.30	53.529; 34.665	332.017; 223.819	423.499; 356.783	2203.453; 1764.917	44.718; 47.630	96.034; 85.617	17.262; 15.292	3.131; 3.124
Mobile Assay mReader	4.560; 4.200	37.100; 32.100	324.600; 329.700	358.500; 359.000	2463.000; 2504.00	44.300; 60.500	90.900; 83.800	16.950; 27.200	4.530; 5.800

Table 4.3 Summary of comparison of the results using mReader method and UHPLC method for the analysis of AFB_1, AFB_2, AFG_1, and AFG_2 in five different commodities that were grown in the State of Georgia, USA.

	Oats	Sorghum	Wheat	Corn	Peanuts
N	18	12	68	32	150
AFB1					
Detection Rate	0	0	68	32	150
Average	0.000±0.000	0.000±0.000	41.460±50.861	3.256±2.144	22.433±71.270
Range	0.000, 0.000	0.000, 0.000	11.451, 398.289	1.375, 11.912	1.080, 682.446
AFB2					
Detection Rate	15	12	33	32	141
Average	0.956±1.768	4.107±4.370	0.048±0.072	0.585±0.966	1.933±8.185
Range	0.000, 7.587	0.028, 0.550	0.000, 0.253	0.025, 5.729	0.000, 67.362
AFG1					
Detection Rate	13	12	14	12	149
Average	21.394±35.298	165.871±59.411	10.221±40.100	3.028±12.357	40.250±41.031
Range	0.000, 133.006	85.488, 263.360	0.000, 298.803	0.000, 70.230	0.000, 260.607
AFG2					
Detection Rate	15	12	10	31	91
Average	1.137±1.742	1.873±1.980	0.083±0.265	1.572±1.437	3.603±4.366
Range	0.000, 5.779	0.645, 7.813	0.000, 1.367	0.000, 5.616	0.000, 28.543
Total AFs					
Detection Rate	18	12	68	32	150
Average	23.484±36.791	172.344±59.138	51.811±86.446	8.440±12.455	68.219±95.579
Range	0.321, 140.560	88.666, 268.942	11.451, 697.816	2.272, 74.219	12.673, 828.249

Table 4.4 Summary of levels of AFs detected in oat samples collected in State of Georgia, USA using UHPLC method. Total average and range included.

Sample ID	AFB1 (ug/kg)	AFB2 (ug/kg)	AFG1 (ug/kg)	AFG2 (ug/kg)	Total AFs (ug/kg)
O1	0.000	0.652	1.129	1.464	3.245
O2	0.000	0.472	24.739	0.150	25.361
O3	0.000	1.424	13.674	0.598	15.697
O4	0.000	0.000	0.718	0.206	0.870
O5	0.000	7.587	0.000	0.000	7.587
O6	0.000	0.123	0.000	3.623	3.746
O7	0.000	1.965	49.517	0.067	51.548
O8	0.000	0.671	59.366	4.520	64.557
O9	0.000	0.620	67.553	2.112	70.285
O10	0.000	1.775	133.006	5.779	140.560
O11	0.000	0.000	0.000	0.654	0.654
O12	0.000	0.045	3.628	0.527	4.201
O13	0.000	1.153	13.063	0.274	14.490
O14	0.000	0.070	13.483	0.233	13.787
O15	0.000	0.000	0.430	0.000	0.430
O16	0.000	0.082	0.000	0.238	0.321
O17	0.000	0.225	4.783	0.000	5.007
O18	0.000	0.345	0.000	0.017	0.362
AVG±SD	0.000±0.000	0.956±1.768	21.394±35.298	1.137±1.742	23.484±36.791

Table 4.5 Summary of levels of AFs detected in sorghum samples collected in State of Georgia, USA using UHPLC method.

Sample ID	AFB1 (ug/kg)	AFB2 (ug/kg)	AFG1 (ug/kg)	AFG2 (ug/kg)	Total AFs (ug/kg)
S1	0.000	0.834	115.158	0.908	116.999
S2	0.000	0.028	85.488	3.146	88.666
S3	0.000	0.550	201.704	1.288	203.608
S4	0.000	2.654	190.445	1.355	194.773
S5	0.000	3.339	217.789	1.743	223.271
S6	0.000	2.968	143.257	1.422	148.003
S7	0.000	16.887	88.753	0.645	108.311
S8	0.000	4.065	263.360	1.030	268.942
S9	0.000	3.657	151.118	1.242	156.455
S10	0.000	5.559	136.892	1.174	144.291
S11	0.000	4.015	253.446	7.813	265.756
S12	0.000	4.729	143.045	0.707	149.049
AVG±SD	0.000±0.000	4.107±4.370	165.871±59.411	1.873±1.980	172.344±59.138

Table 4.6 Summary of levels of AFs detected in wheat samples collected in State of Georgia, USA using UHPLC method.

Sample ID	AFB1 (ug/kg)	AFB2 (ug/kg)	AFG1 (ug/kg)	AFG2 (ug/kg)	Total AFs (ug/kg)
W1	18.203	0.102	0.000	1.367	19.672
W2	15.089	0.083	34.075	0.000	49.248
W3	18.050	0.000	0.000	0.000	18.050
W4	40.752	0.000	107.512	0.000	148.264
W5	36.067	0.187	0.000	0.000	36.254
W6	35.052	0.161	0.000	0.000	35.212
W7	20.994	0.000	0.000	0.000	20.994
W8	28.966	0.000	0.000	0.000	28.966
W9	36.929	0.186	0.000	0.000	37.115
W10	61.976	0.141	0.000	0.000	62.117
W11	39.795	0.015	0.000	0.000	39.810
W12	32.166	0.030	0.000	0.000	32.196
W13	63.110	0.146	0.000	0.000	63.257
W14	104.384	0.000	40.668	0.031	145.083
W15	16.178	0.000	0.000	1.113	17.291
W16	28.917	0.000	0.000	0.000	28.917
W17	11.451	0.000	0.000	0.000	11.451
W18	16.988	0.038	0.000	0.000	17.025
W19	30.182	0.158	0.000	0.000	30.340
W20	27.154	0.120	0.000	0.000	27.274
W21	22.037	0.064	0.000	0.000	22.100
W22	102.270	0.000	24.322	0.000	126.592
W23	18.814	0.000	0.000	0.000	18.814
W24	26.360	0.015	0.000	0.000	26.375
W25	33.769	0.137	0.000	0.000	33.906
W26	34.879	0.002	0.000	0.000	34.881
W27	33.244	0.132	0.000	0.000	33.377
W28	26.874	0.008	0.000	0.000	26.882
W29	26.875	0.049	0.000	0.000	26.924
W30	32.692	0.017	0.000	0.000	32.710
W31	39.048	0.231	83.531	0.000	122.811
W32	21.557	0.000	0.000	0.000	21.557
W33	47.990	0.000	0.000	0.000	47.990
W34	36.529	0.002	0.784	0.000	37.291
W35	22.798	0.000	0.000	0.000	22.798
W36	32.919	0.144	55.129	0.000	88.192
W37	19.076	0.030	10.318	0.000	29.424
W38	15.974	0.000	0.000	0.000	15.974

W39	29.103	0.160	0.000	0.000	29.263
W40	14.564	0.000	0.000	0.000	14.564
W41	24.052	0.138	0.000	0.000	24.189
W42	61.466	0.017	0.000	0.000	61.483
W43	44.734	0.000	0.000	0.000	44.734
W44	24.553	0.000	0.000	0.000	24.553
W45	34.071	0.000	0.000	0.000	34.071
W46	17.526	0.000	0.000	0.000	17.526
W47	66.629	0.000	16.672	0.000	83.302
W48	21.001	0.000	0.000	0.000	21.001
W49	21.561	0.000	0.000	0.000	21.561
W50	43.846	0.000	0.000	0.000	43.846
W51	27.777	0.000	0.000	0.000	27.777
W52	27.008	0.013	0.000	0.000	27.021
W53	30.862	0.089	0.000	0.000	30.951
W54	27.870	0.000	0.000	0.000	27.870
W55	21.793	0.000	0.000	0.000	21.793
W56	22.540	0.000	0.000	0.000	22.540
W57	18.759	0.000	0.000	0.000	18.759
W58	25.096	0.131	0.000	0.000	25.227
W59	398.289	0.000	298.803	0.723	697.816
W60	33.408	0.227	11.653	0.765	46.052
W61	160.762	0.000	0.000	0.000	160.762
W62	43.939	0.000	1.827	0.000	45.767
W63	35.364	0.000	0.000	0.000	35.364
W64	24.824	0.000	0.000	0.767	25.591
W65	126.207	0.000	3.768	0.553	130.528
W66	51.146	0.000	0.000	0.022	51.168
W67	42.352	0.253	5.963	0.255	48.824
W68	22.066	0.022	0.000	0.023	22.111
AVG±SD	41.460±50.861	0.048±0.072	10.221±40.100	0.083±0.265	51.811±86.446

Table 4.7 Summary of levels of AFs detected in corn samples collected in State of Georgia, USA using UHPLC method.

Sample ID	AFB1 (ug/kg)	AFB2 (ug/kg)	AFG1 (ug/kg)	AFG2 (ug/kg)	Total AFs (ug/kg)
C1	3.356	0.194	0.000	4.742	8.292
C2	4.259	0.424	5.295	0.820	10.797
C3	11.912	5.729	0.000	2.311	19.952
C4	1.617	0.792	1.910	1.040	5.359
C5	2.217	0.090	0.000	0.000	2.272
C6	2.214	0.460	0.272	1.192	4.138
C7	3.243	0.333	0.000	0.956	4.533
C8	6.088	0.350	0.000	0.201	6.638
C9	3.927	0.025	0.000	0.374	4.325
C10	2.147	0.420	2.574	5.249	10.390
C11	4.109	0.130	0.000	0.417	4.657
C12	6.283	0.143	0.000	0.367	6.793
C13	2.585	0.521	4.769	1.094	8.969
C14	1.869	0.388	0.000	0.938	3.195
C15	6.257	0.248	1.017	0.550	8.072
C16	2.129	0.645	0.000	4.510	7.284
C17	2.223	0.631	0.000	1.630	4.484
C18	4.813	0.313	0.000	0.644	5.771
C19	4.997	0.145	0.000	0.668	5.810
C20	4.081	0.164	0.000	0.487	4.732
C21	1.622	0.697	0.000	1.588	3.908
C22	1.831	0.453	0.378	5.616	8.278
C23	2.116	0.794	1.270	1.271	5.451
C24	2.744	0.930	3.321	1.426	8.421
C25	3.045	0.709	4.021	1.208	8.983
C26	1.478	0.469	0.000	1.496	3.443
C27	2.132	0.308	0.000	1.393	3.833
C28	1.375	0.483	0.000	1.700	3.559
C29	2.455	0.193	1.846	1.431	5.925
C30	1.768	0.577	0.000	1.503	3.848
C31	1.709	0.408	70.230	1.871	74.219
C32	1.594	0.550	0.000	1.599	3.742
AVG±SD	3.256±2.144	0.585±0.966	3.028±12.357	1.572±1.437	8.440±12.455

Table 4.8 Summary of levels of AFs detected in peanut samples collected in State of Georgia, USA using UHPLC method.

Sample ID	AFB1 (ug/kg)	AFB2 (ug/kg)	AFG1 (ug/kg)	AFG2 (ug/kg)	Total AFs (ug/kg)
N1	4.573	0.037	9.259	1.649	15.517
N2	5.420	0.073	29.800	4.091	39.384
N3	4.069	0.055	35.051	6.613	45.788
N4	5.248	0.078	21.031	5.558	31.916
N5	4.473	0.025	16.722	2.660	23.880
N6	3.937	0.149	23.150	7.537	34.773
N7	4.219	0.071	19.768	12.854	36.912
N8	4.105	0.031	13.105	5.591	22.832
N9	4.825	0.038	10.712	3.732	19.307
N10	5.749	0.113	66.736	6.096	78.694
N11	16.772	0.123	17.252	0.000	34.147
N12	8.291	0.068	22.437	0.000	30.796
N13	5.620	0.093	10.307	0.000	16.021
N14	7.977	0.124	20.345	0.000	28.446
N15	4.046	1.435	31.868	0.000	37.350
N16	3.847	0.590	34.328	0.000	38.765
N17	3.667	0.084	17.435	0.000	21.186
N18	9.109	0.071	36.237	0.000	45.417
N19	4.210	0.177	31.729	0.000	36.116
N20	5.385	0.258	44.843	0.021	50.507
N21	36.053	2.943	74.890	0.000	113.885
N22	218.085	24.032	13.058	0.000	255.174
N23	6.027	0.019	34.250	0.000	40.296
N24	9.182	0.073	14.158	0.000	23.413
N25	7.264	0.294	16.391	0.000	23.949
N26	6.700	0.503	20.953	0.000	28.156
N27	4.165	0.063	60.039	0.000	64.266
N28	7.118	0.055	23.115	0.000	30.288
N29	8.180	0.321	17.015	0.000	25.516
N30	8.440	0.043	12.875	0.000	21.359
N31	3.776	0.065	16.152	0.000	19.993
N32	3.274	0.123	27.283	0.000	30.680
N33	5.886	0.095	10.512	0.000	16.493
N34	345.961	54.240	0.000	1.171	401.372
N35	5.782	0.232	10.506	0.000	16.519
N36	6.667	0.141	13.960	0.000	20.767
N37	3.352	0.181	13.780	0.000	17.313
N38	4.554	0.153	9.092	0.000	13.799

N39	5.894	0.366	9.090	0.000	15.350
N40	21.940	2.074	115.928	0.000	139.941
N41	10.151	0.276	21.181	0.000	31.609
N42	3.762	0.118	42.232	0.000	46.112
N43	4.763	0.139	11.218	0.000	16.120
N44	174.878	15.370	13.508	0.000	203.755
N45	6.233	0.313	13.763	0.000	20.309
N46	1.971	0.043	12.234	0.000	14.248
N47	6.134	0.106	32.501	5.584	44.326
N48	5.649	0.190	25.163	0.000	31.002
N49	4.182	0.124	19.692	0.000	23.998
N50	8.404	0.080	18.150	0.000	26.634
N51	102.297	10.435	14.018	0.000	126.749
N52	9.442	0.202	16.139	0.000	25.783
N53	10.708	0.024	19.387	0.000	30.119
N54	11.859	0.104	13.895	0.000	25.857
N55	13.564	0.098	22.513	18.383	54.557
N56	12.560	0.099	20.372	0.000	33.031
N57	9.654	0.114	21.341	10.439	41.548
N58	10.231	0.114	260.607	0.000	270.952
N59	10.156	0.142	26.841	2.766	39.905
N60	13.410	0.578	22.322	0.000	36.309
N61	9.865	0.147	59.367	4.161	73.539
N62	9.071	0.123	13.720	3.058	25.971
N63	8.089	0.137	15.675	0.000	23.901
N64	9.735	0.235	10.074	0.000	20.045
N65	24.479	1.622	17.410	0.000	43.511
N66	11.781	0.116	27.398	4.916	44.212
N67	7.087	0.408	25.555	0.000	33.050
N68	5.752	0.219	41.171	3.702	50.843
N69	6.317	0.205	62.212	7.848	76.582
N70	14.999	0.781	41.835	4.458	62.072
N71	11.999	0.288	48.881	3.482	64.649
N72	36.142	2.460	72.248	5.354	116.204
N73	4.507	0.081	60.282	2.906	67.776
N74	4.647	0.270	19.375	5.811	30.103
N75	6.778	0.312	76.914	5.581	89.585
N76	17.465	2.430	88.781	3.309	111.985
N77	5.674	0.036	49.726	2.281	57.718
N78	5.001	0.072	30.735	6.683	42.490
N79	8.684	0.154	60.760	2.810	72.409
N80	13.567	0.120	94.910	4.097	112.694

N81	7.220	0.201	129.222	8.836	145.479
N82	6.896	0.323	61.168	8.682	77.070
N83	15.790	0.386	164.916	3.215	184.307
N84	6.143	0.071	34.149	5.547	45.910
N85	6.872	0.132	26.755	9.984	43.743
N86	27.099	2.037	75.046	7.218	111.400
N87	8.639	0.074	52.361	10.706	71.780
N88	12.395	2.324	61.239	2.801	78.760
N89	6.645	0.095	50.123	6.608	63.470
N90	3.860	0.199	60.647	6.679	71.385
N91	2.348	0.077	192.633	4.454	199.511
N92	2.700	0.088	99.797	2.822	105.407
N93	7.499	0.770	61.350	2.380	72.000
N94	3.603	0.000	75.789	2.730	82.122
N95	2.833	0.005	224.409	4.076	231.323
N96	6.256	0.028	45.047	4.456	55.787
N97	4.602	0.054	54.982	5.960	65.598
N98	4.784	0.061	83.995	6.959	95.799
N99	6.597	0.484	74.920	7.662	89.662
N100	6.819	0.194	55.568	6.281	68.862
N101	3.049	0.058	48.011	3.342	54.460
N102	17.881	0.116	21.095	4.455	43.547
N103	6.376	0.061	14.718	2.827	23.982
N104	9.194	0.107	24.794	0.135	34.228
N105	9.652	0.102	62.371	0.057	72.183
N106	10.237	0.170	14.959	0.055	25.420
N107	8.656	0.121	10.494	0.000	19.271
N108	12.313	0.483	8.224	0.000	21.021
N109	11.315	0.388	29.958	0.000	41.662
N110	12.665	0.285	13.094	0.000	26.043
N111	52.393	4.100	21.312	0.000	77.806
N112	93.305	12.699	27.445	0.000	133.449
N113	25.976	2.406	26.324	0.000	54.706
N114	26.597	1.685	27.433	0.000	55.715
N115	10.179	0.196	26.400	0.000	36.775
N116	11.465	0.406	28.862	0.000	40.733
N117	321.130	40.492	256.768	28.543	646.934
N118	16.650	1.555	32.718	4.581	55.504
N119	9.252	0.000	43.449	0.574	53.275
N120	7.354	0.114	53.963	0.000	61.432
N121	5.189	0.265	51.911	8.573	65.938
N122	4.865	0.000	35.900	8.226	48.991

N123	10.979	0.303	41.214	11.133	63.629
N124	16.042	0.240	34.900	16.385	67.567
N125	682.446	67.362	70.780	7.662	828.249
N126	5.606	0.000	33.586	5.774	44.966
N127	9.585	0.131	19.819	12.688	42.222
N128	7.299	0.000	25.320	5.995	38.614
N129	5.360	0.436	38.588	8.091	52.475
N130	10.428	0.088	59.353	5.122	74.991
N131	13.138	0.101	30.879	10.089	54.208
N132	12.711	0.096	43.930	7.871	64.607
N133	6.497	0.024	28.464	6.953	41.938
N134	13.706	1.416	44.853	8.504	68.480
N135	7.048	0.235	43.642	10.373	61.297
N136	5.413	0.058	32.847	5.798	44.117
N137	23.058	0.145	17.647	5.304	46.155
N138	8.973	0.660	18.168	8.529	36.329
N139	6.248	0.000	25.016	8.834	40.098
N140	6.939	0.012	53.640	11.362	71.953
N141	168.183	16.275	25.347	3.016	212.820
N142	2.379	0.000	21.955	2.110	26.444
N143	1.207	0.000	14.509	5.654	21.370
N144	4.419	0.176	22.784	2.121	29.500
N145	1.638	0.059	9.540	1.437	12.673
N146	1.080	0.063	19.868	5.022	26.034
N147	2.804	0.000	13.672	3.310	19.787
N148	3.771	0.081	38.422	9.626	51.900
N149	3.721	0.006	32.911	4.985	41.623
N150	3.456	0.012	22.212	2.076	27.756
AVG±SD	22.433±71.270	1.933±8.185	40.250±41.031	3.603±4.366	68.219±95.579

Table 4.9 Repeatability test for UHPLC method in peanut and grain samples.

Sample ID	UHPLC Test 1 (ug/kg)	UHPLC Test 2 (ug/kg)
O5	7.59	2.90
O16	0.32	28.07
S3	1400.12	848.51
S8	1284.95	952.42
C1	8.29	6.34
C8	6.64	9.20
C12	6.79	7.69
C20	4.73	6.20
C29	5.93	37.78
W1	19.67	34.63
W7	20.99	7.85
W27	33.38	15.01
W29	26.92	8.52
W37	29.42	17.74
N1	15.52	18.03
N55	54.56	53.11
N65	43.51	43.16
N102	43.55	48.95
N148	51.90	54.04

Table 4.10 Summary of comparison results using mReader method and UHPLC method.

Sample ID	Sep-pak and UHPLC Method (ppb)	Mobile Assay mReader Method (ppb)	Std. Deviation
O5	7.59	1.61	0.09
O6	3.75	1.83	0.80
O7	51.55	3.64	0.49
O10	140.56	3.18	0.19
O11	0.65	3.31	1.49
O12	4.20	2.93	0.11
O15	0.43	2.86	0.42
O16	0.32	2.64	0.21
O18	0.36	3.41	0.49
O12	4.20	2.93	0.11
O15	0.43	2.86	0.42
O16	0.32	2.64	0.21
O18	0.36	3.41	0.49
S1	1180.28	3.17	0.13
S2	762.73	3.37	1.09
S3	1400.12	3.38	0.67
S4	1679.02	2.9	0.00
S5	661.39	2.50	0.86
S6	1336.22	2.08	0.54
S7	940.00	1.70	0.29
S8	1284.95	1.41	0.01
S10	390.68	2.54	0.64
S12	1029.34	3.48	1.00
W1	19.67	1.44	0.62
W7	20.99	2.36	0.08
W27	33.38	1.60	0.85
W29	26.92	1.85	0.62
W30	32.71	1.10	0.07
W31	122.81	2.01	0.53
W37	29.42	2.69	0.45
W48	21.00	1.51	0.21
W55	21.79	1.17	0.24
W61	160.76	1.90	0.14
C1	8.29	1.01	0.01
C2	10.80	1.13	0.18
C5	2.27	1.56	0.50
C8	6.63	5.60	0.52
C10	10.39	1.30	0.18
C12	6.79	5.45	6.16
C20	4.73	1.29	0.11
C23	5.45	0.98	0.03
C29	5.93	1.39	0.55
C30	3.85	1.11	0.16

Table 4.11 Summary of information given with each corn sample from USDA. Not all information categories were reported for each sample.

	Moisture (%)	Test Weight Per Bushel	Damaged Kernels Total (%)	Aflatoxin
N	32	32	32	12
AVG	14.081	60.591	3.797	8-DNE 5; 1-16ppb; 2-exceed 20
STD	0.596	1.409	3.840	NA
Range	(12.4, 14.8)	(57.8, 62)	(0.8, 17.6)	NA

Table 4.12 Summary of information given with each wheat sample from USDA. Not all information categories were reported for each sample.

	Moisture (%)	Test Weight Per Bushel	Damaged Kernels Total (%)	Insect Damage/100 g	Defects (%)
N	55	68	55	55	55
AVG	12.635	53.374	2.116	0.291	4.351
STD	2.103	6.376	1.933	0.567	3.022
Range	(10.7, 20)	(41, 68)	(0.5, 9.6)	(0,2)	(0.7, 12.4)

Figures

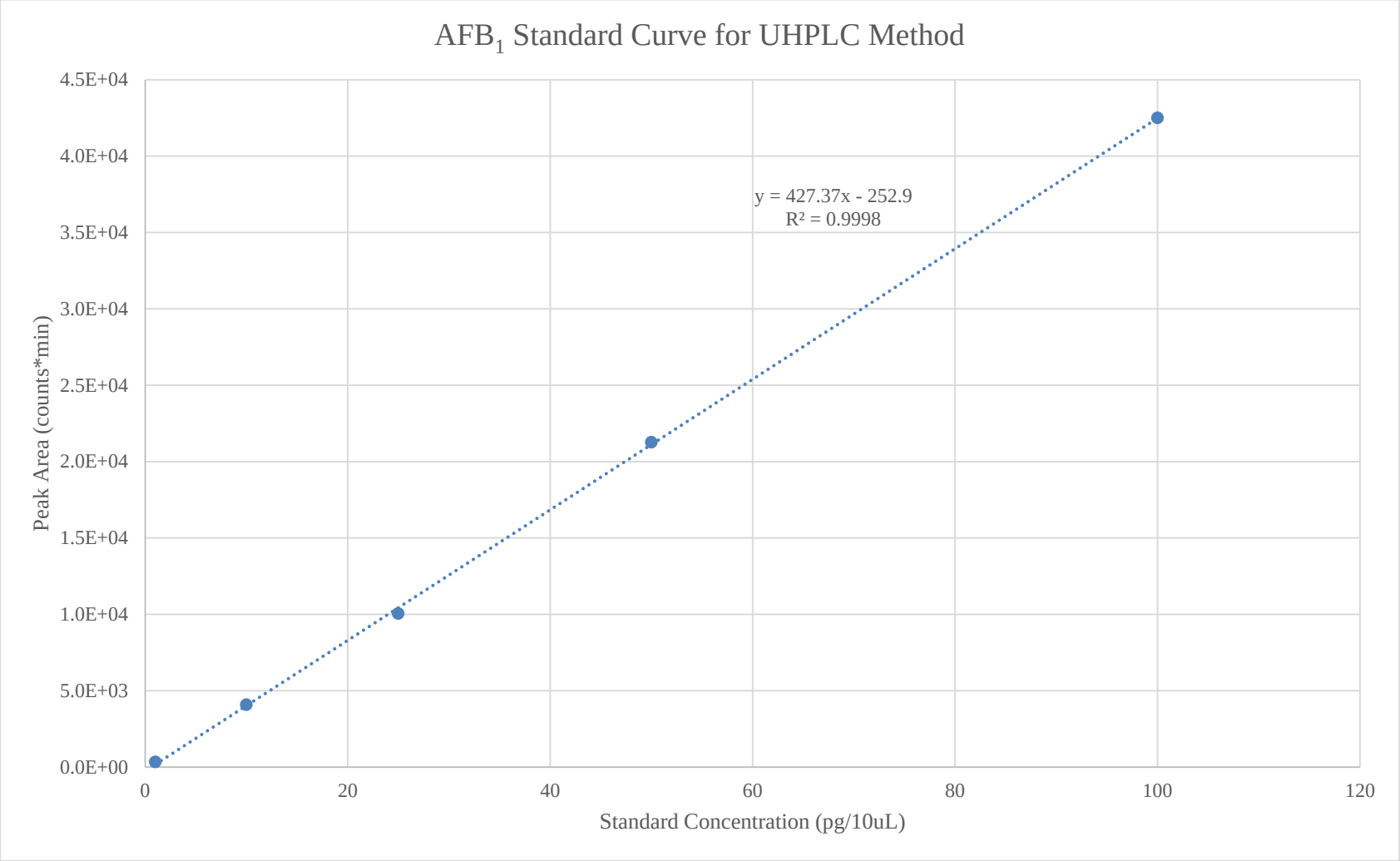

Figure 4.1 Standard curve for AFB_1 for UHPLC using conditions shown in Table 4.1.

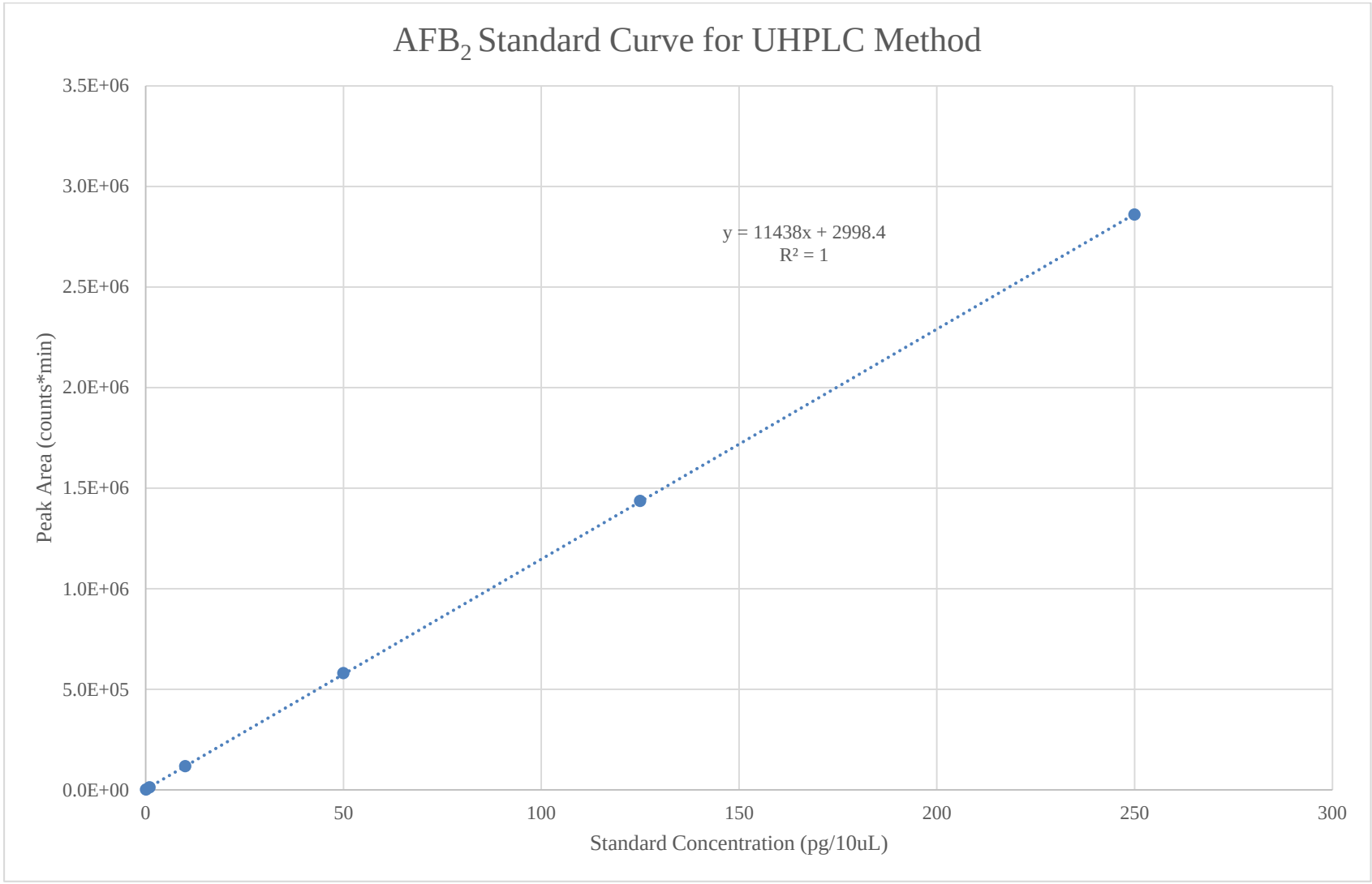

Figure 4.2 Standard curve for AFB_2 for UHPLC using conditions shown in Table 1.

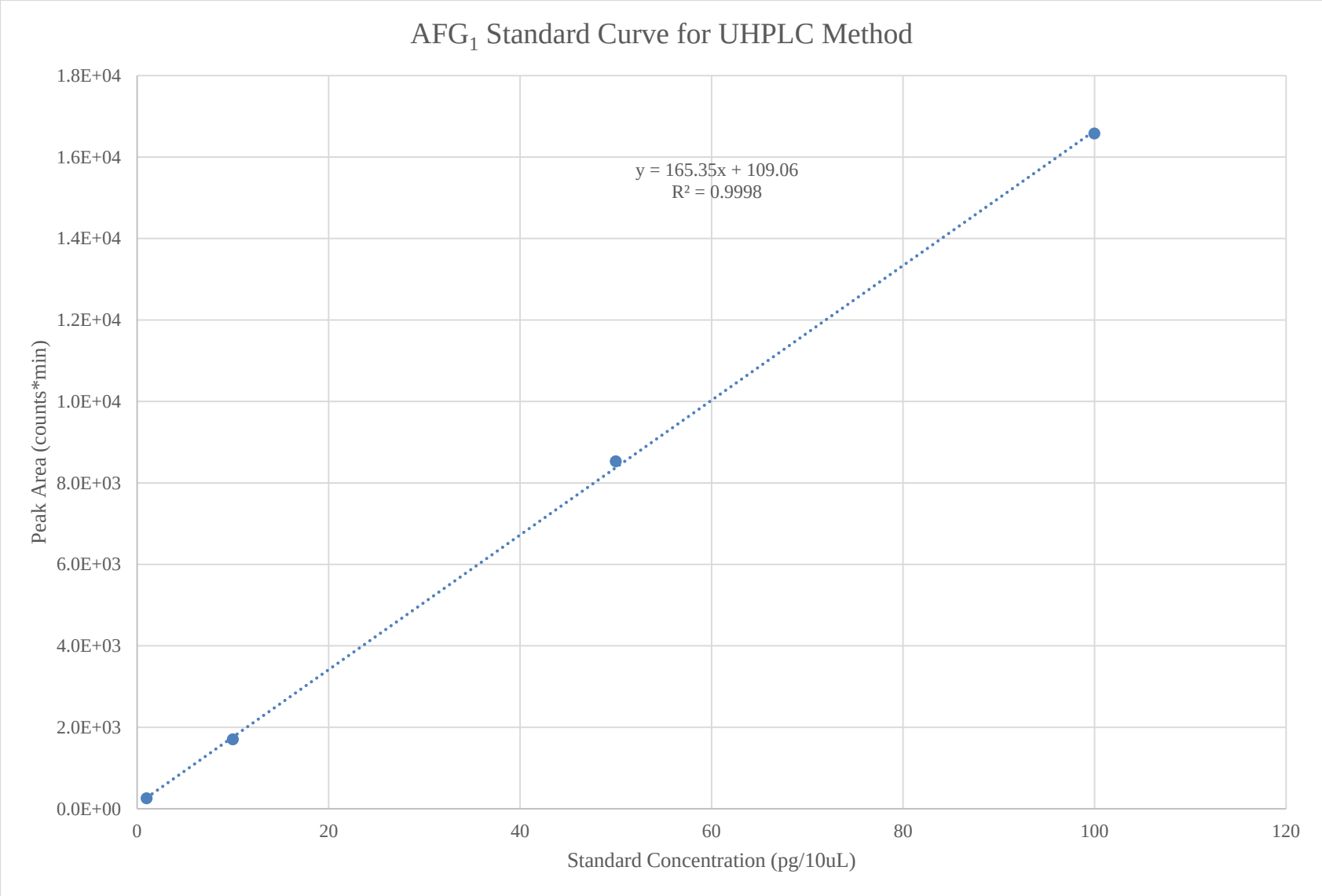

Figure 4.3 Standard curve for AFG_1 for UHPLC using conditions shown in Table 1.

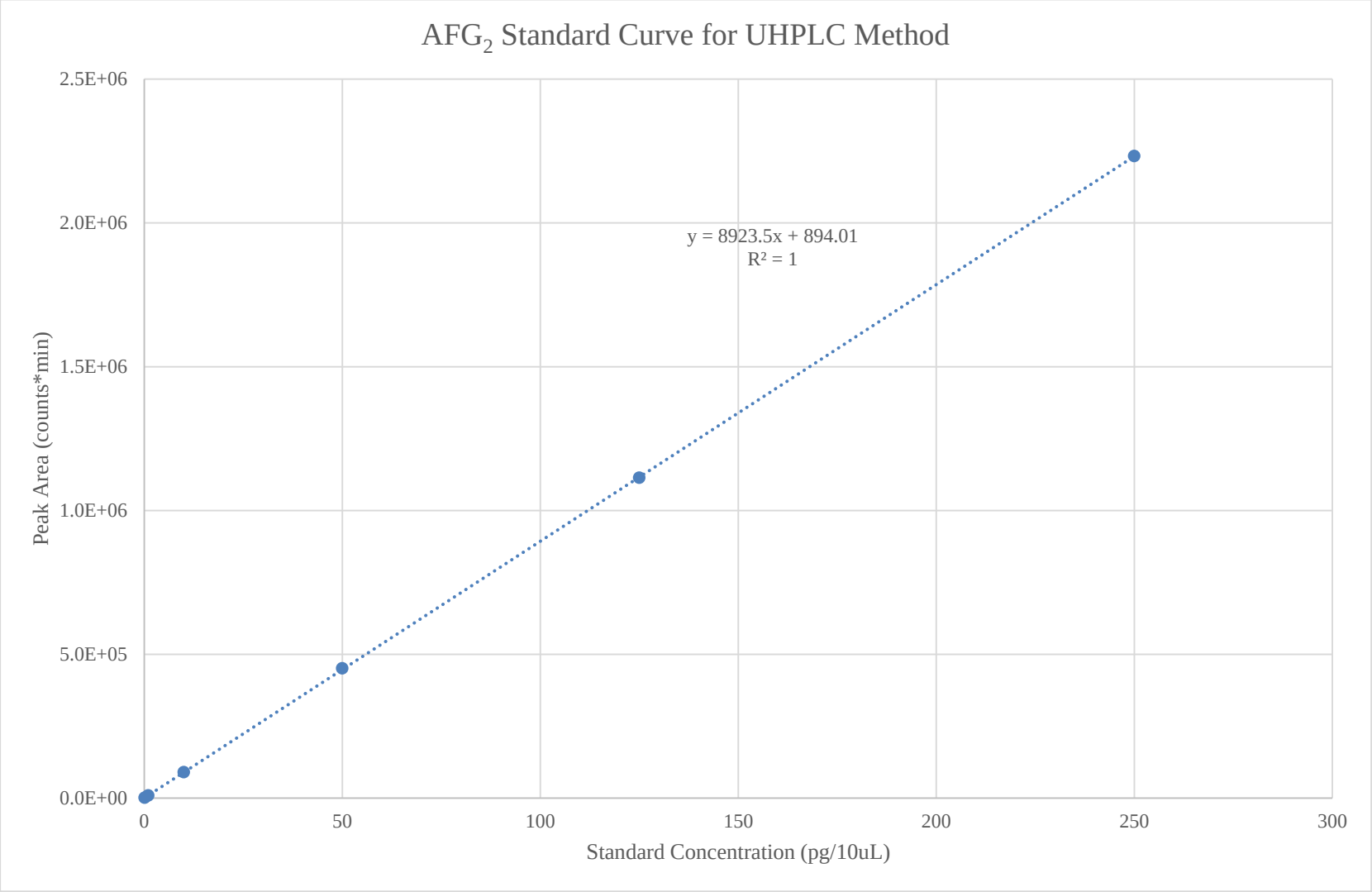

Figure 4.4 Standard curve for AFG_2 for UHPLC using conditions shown in Table 1.

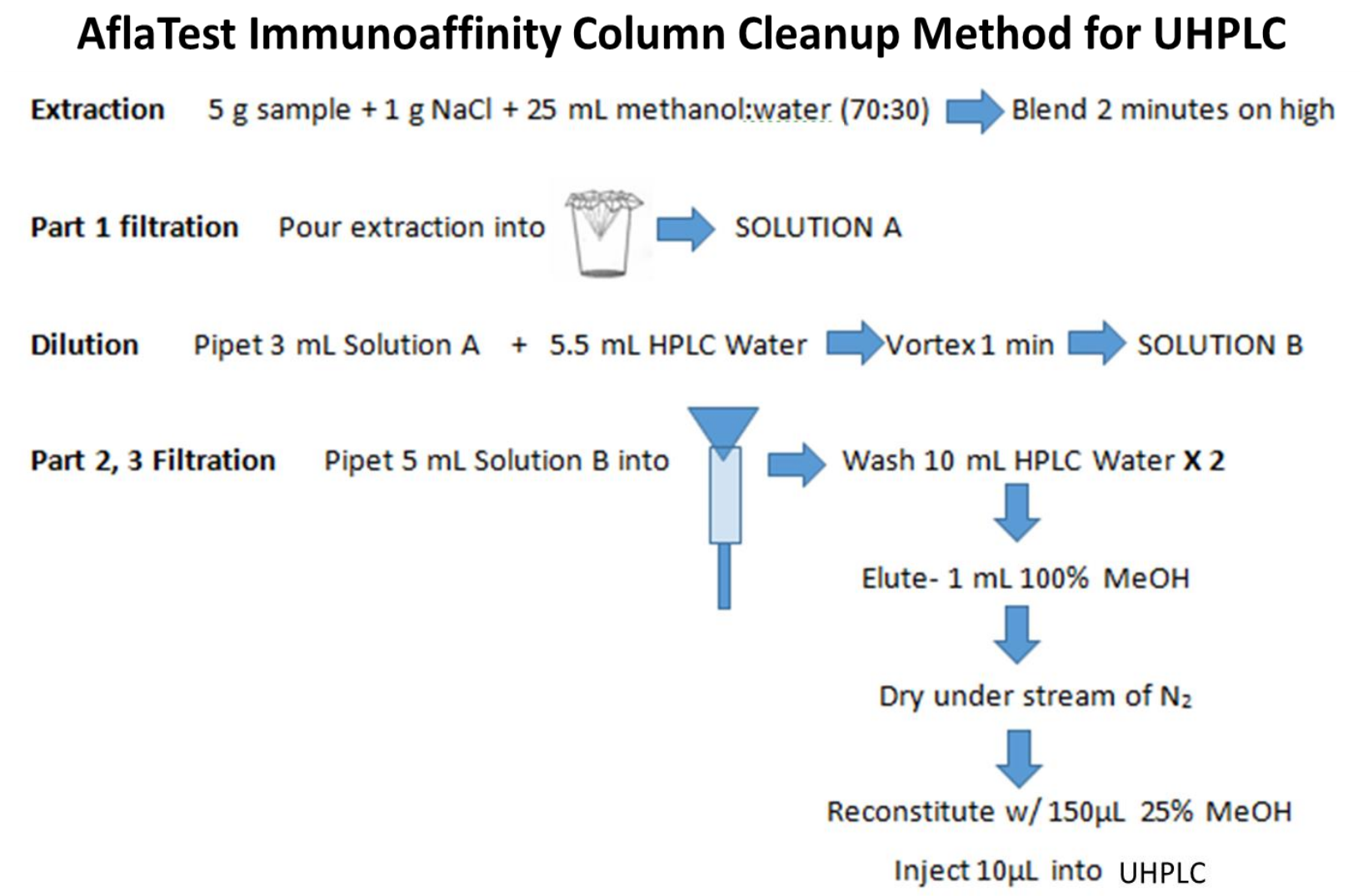

Figure 4.5 Flow chart of AflaTest Immunoaffinity column cleanup method used to determine the levels of aflatoxins B_1, B_2, G_1, and G_2 in peanut and grain samples using UHPLC.

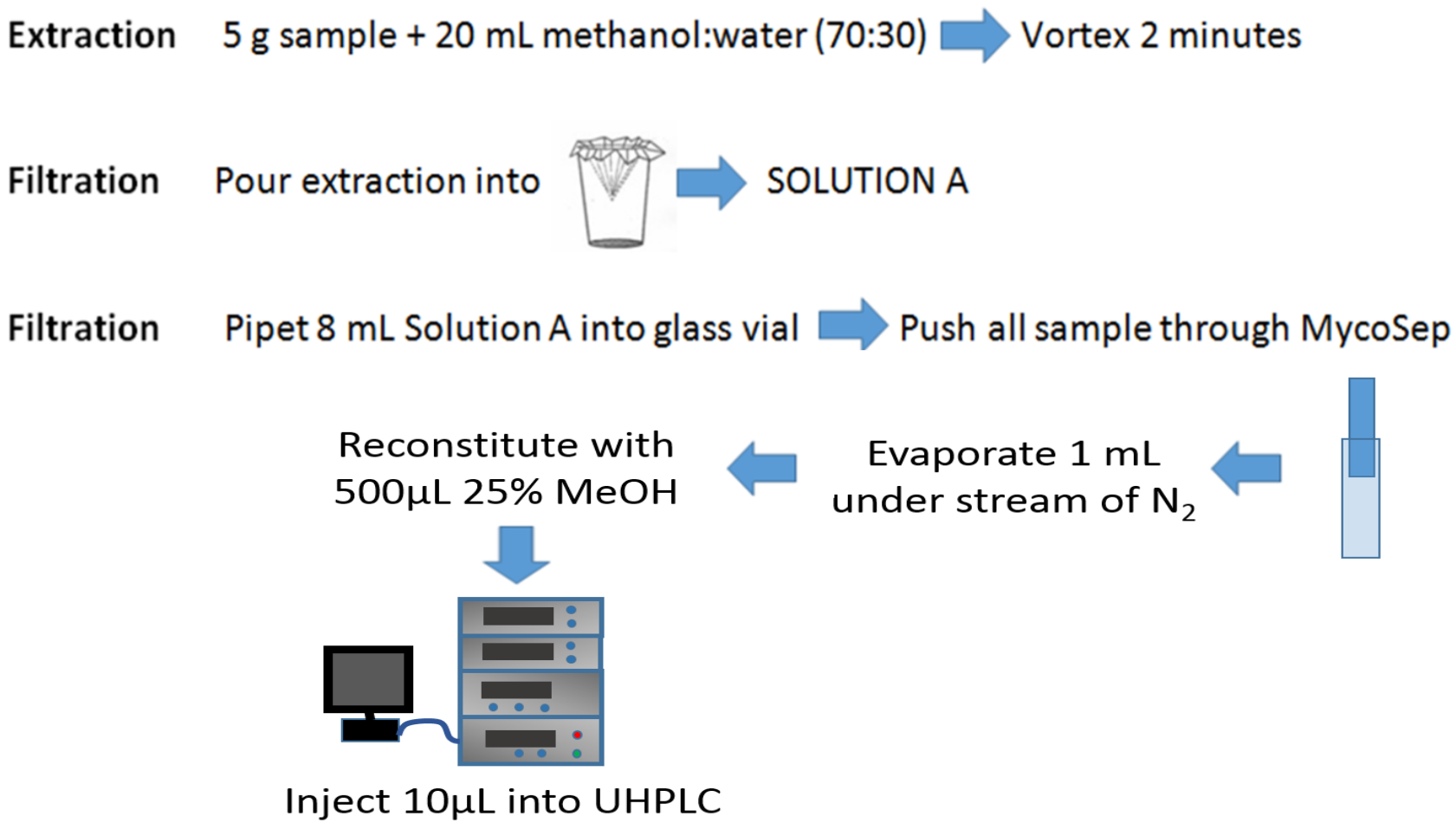

Figure 4.6 Flow chart of MycoSep cleanup method used to determine the levels of AFs B_1, B_2, G_1, and G_2 in peanut and grain samples using UHPLC.

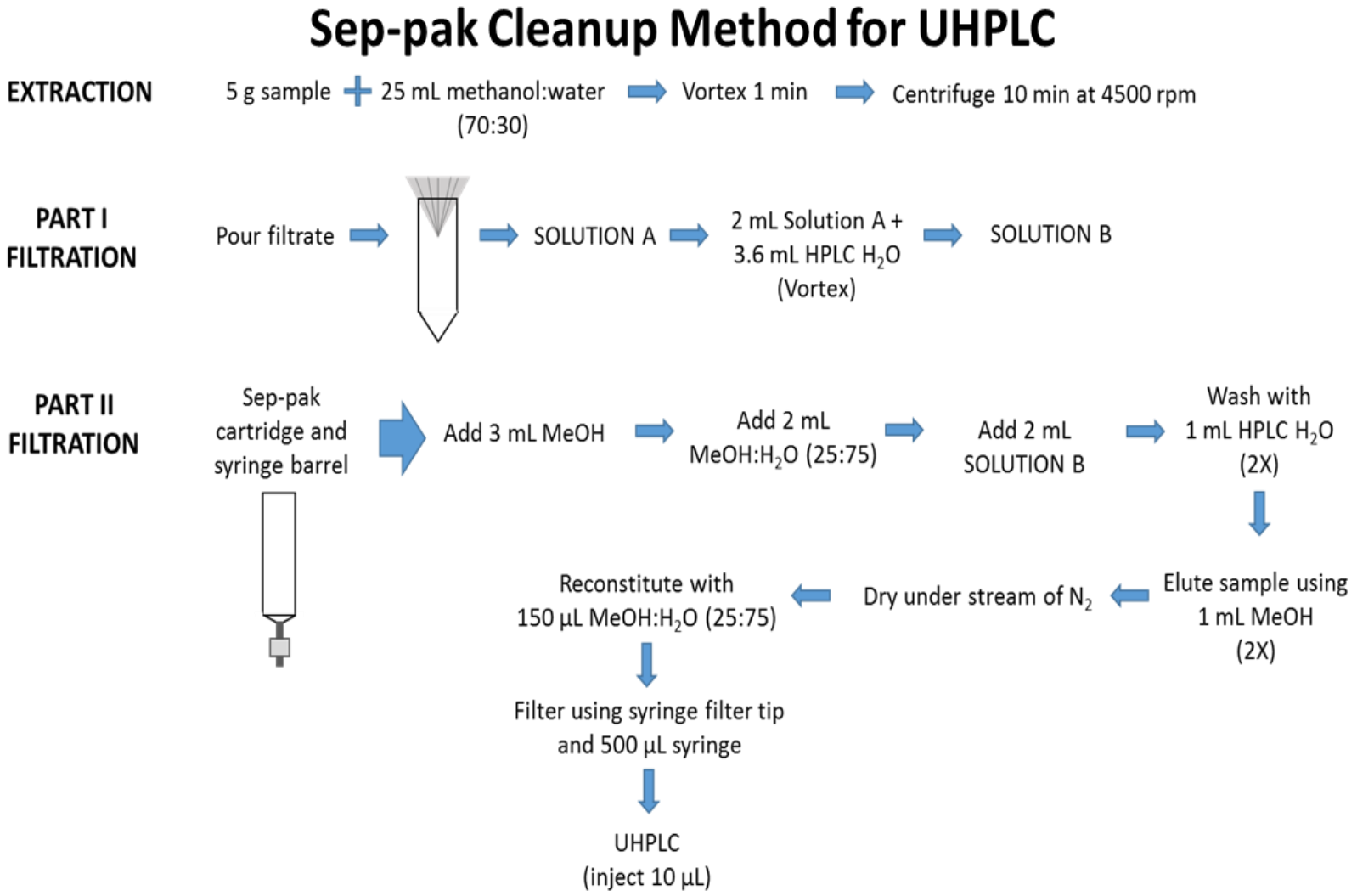

Figure 4.7 Flow chart of Sep-pak cleanup method used to determine the levels of aflatoxins B_1, B_2, G_1, and G_2 in peanut and grain samples using UHPLC.

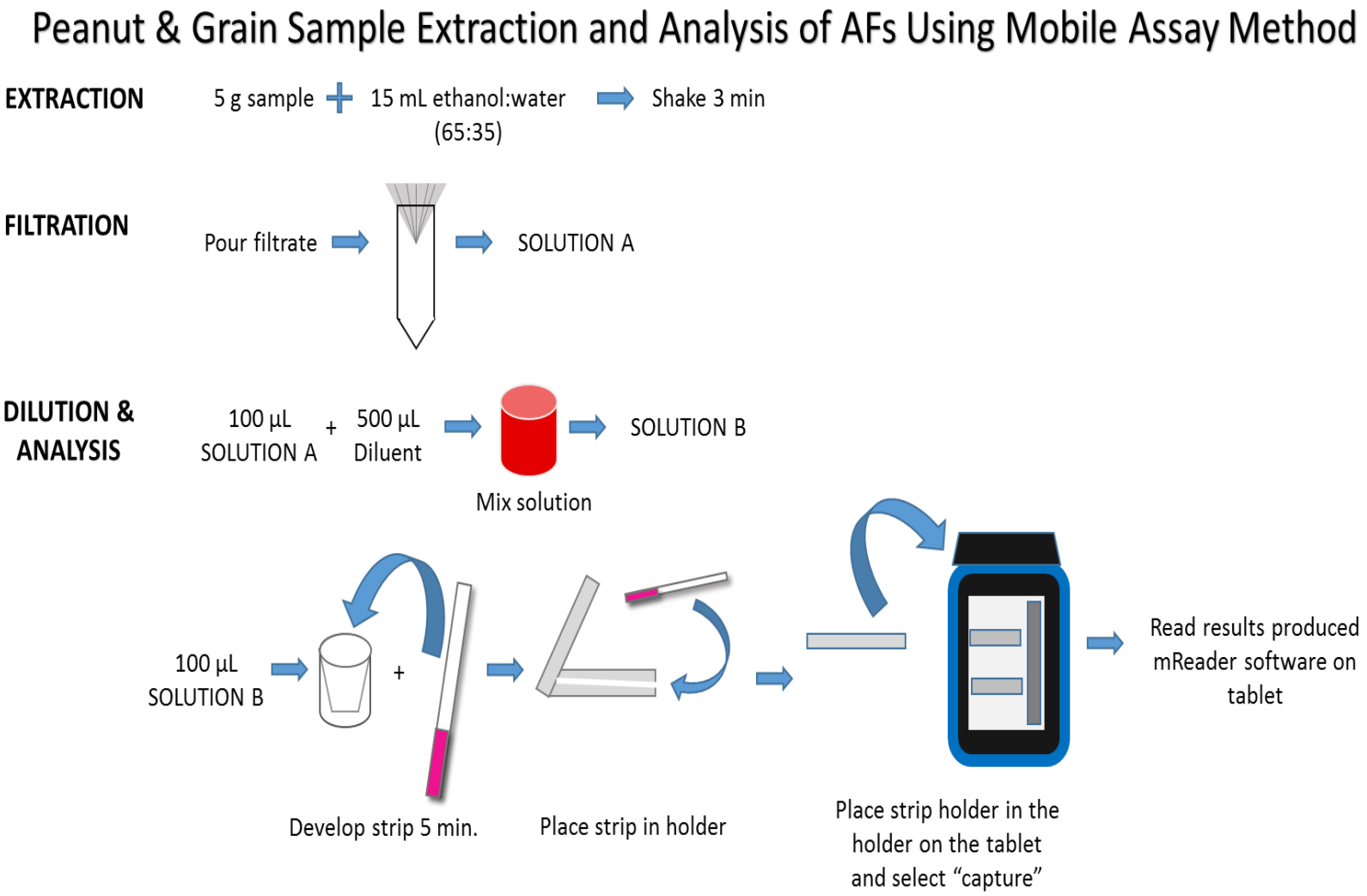

Figure 4.8 Flow chart of mReader method for analysis of levels of AFs in peanuts and grains.

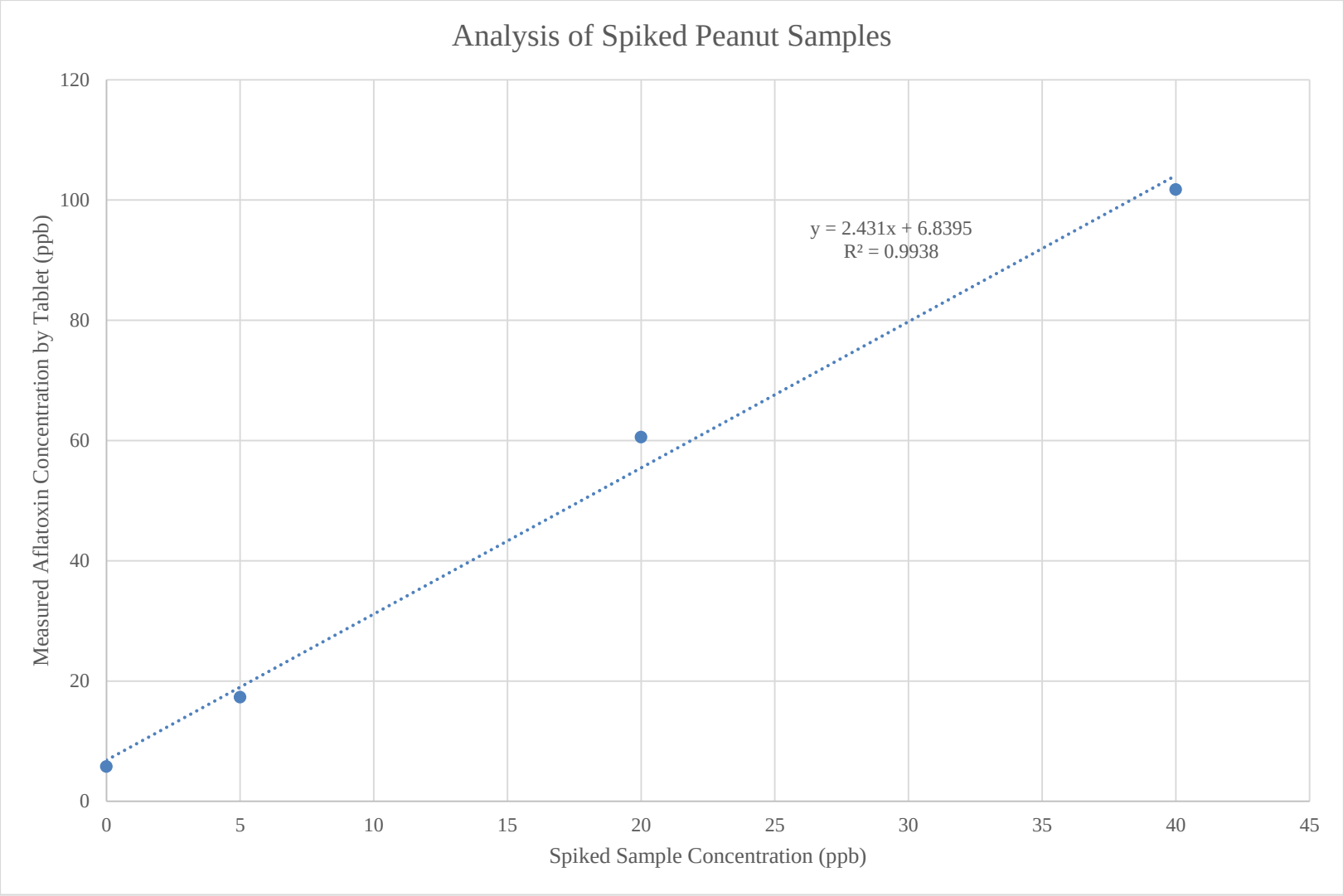

Figure 4.9 Results of analysis of spiked peanut samples using Supleco Aflatoxin Mix standard and determined by mReader device.

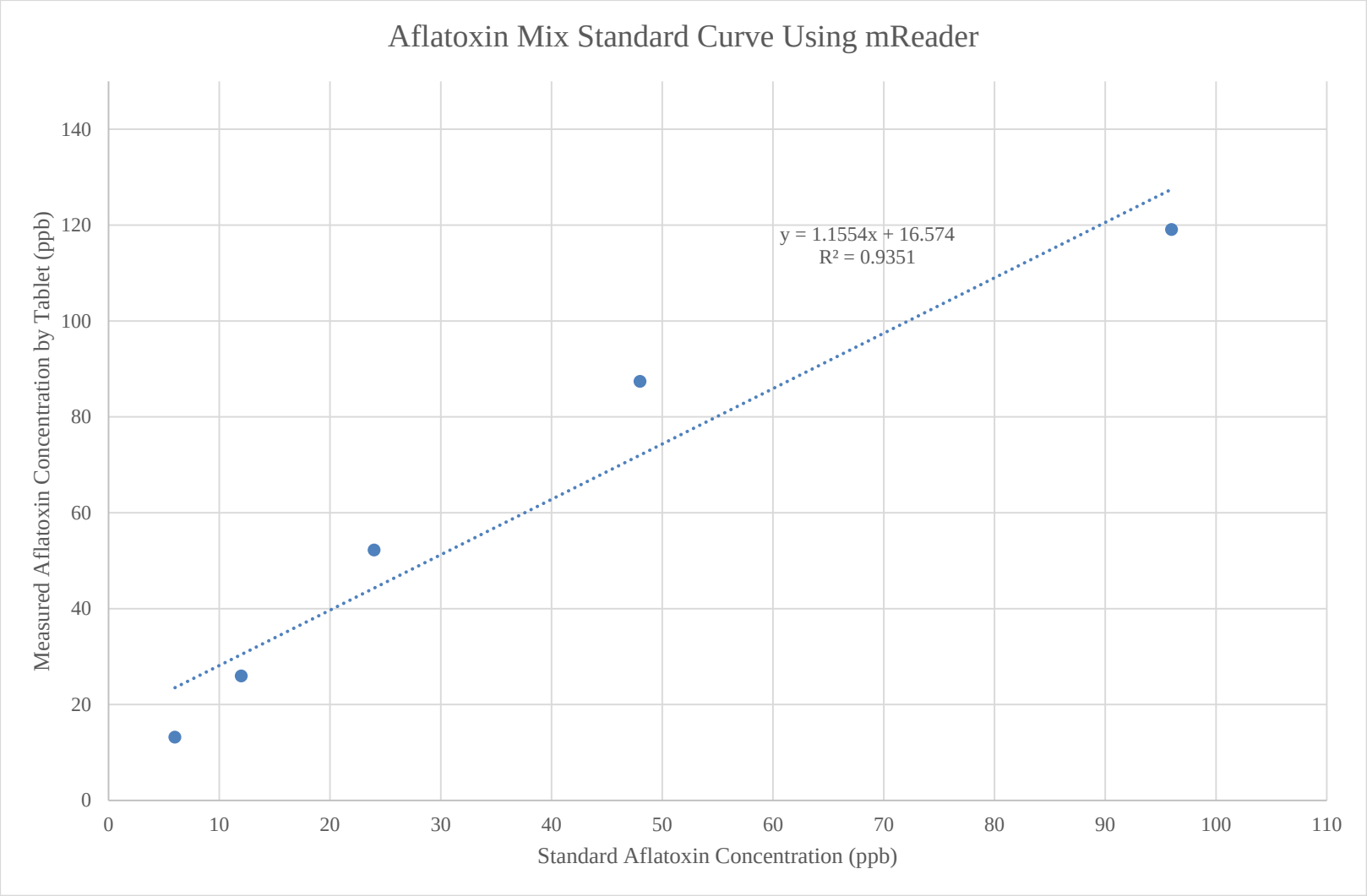

Figure 4.10 Results of standard curve using Supleco Aflatoxin Mix standard and determined by mReader device.

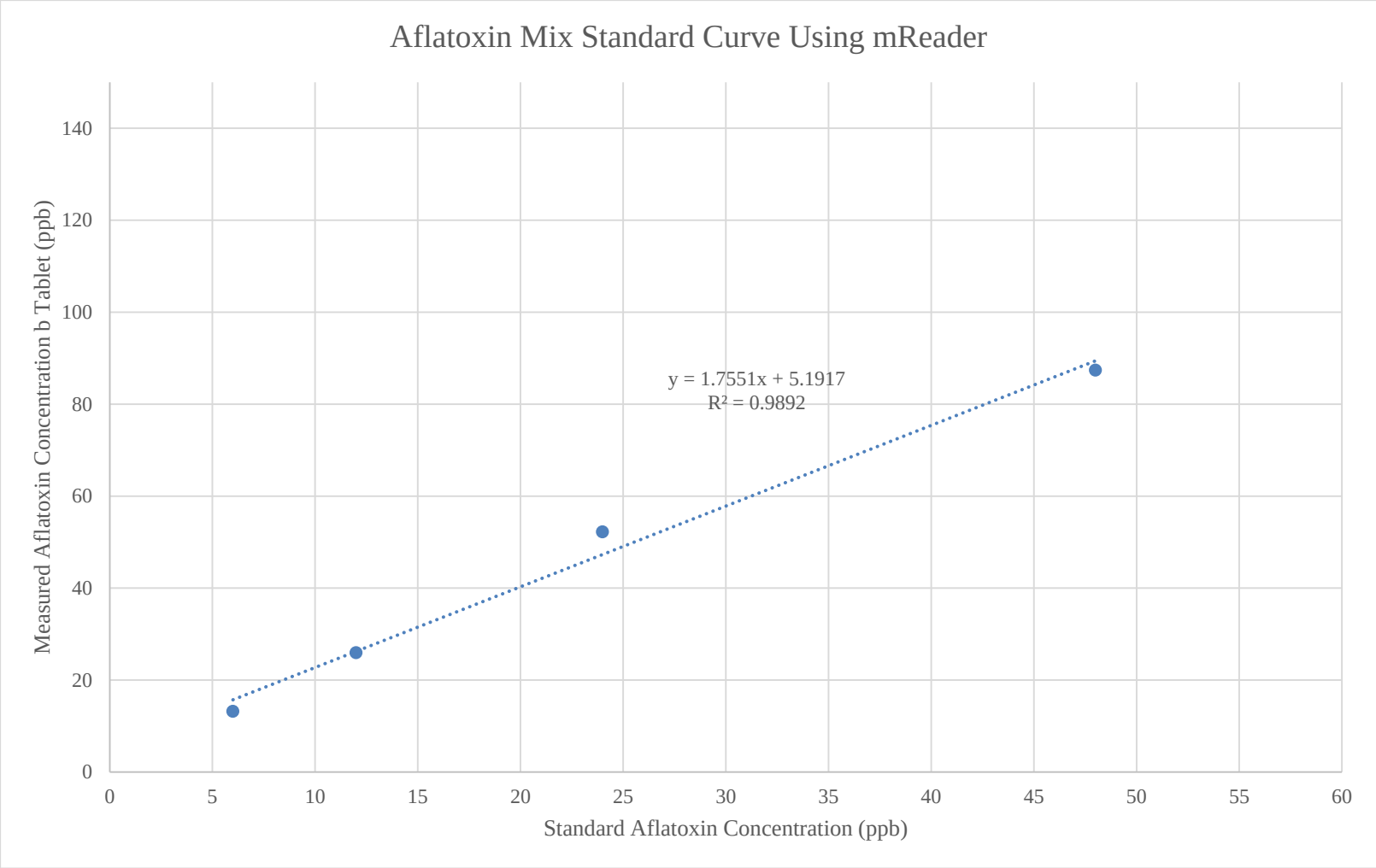

Figure 4.11 Results of standard curve using and Supleco Aflatoxin Mix standard. Results of 96 ppb solution not included and determined by mReader.

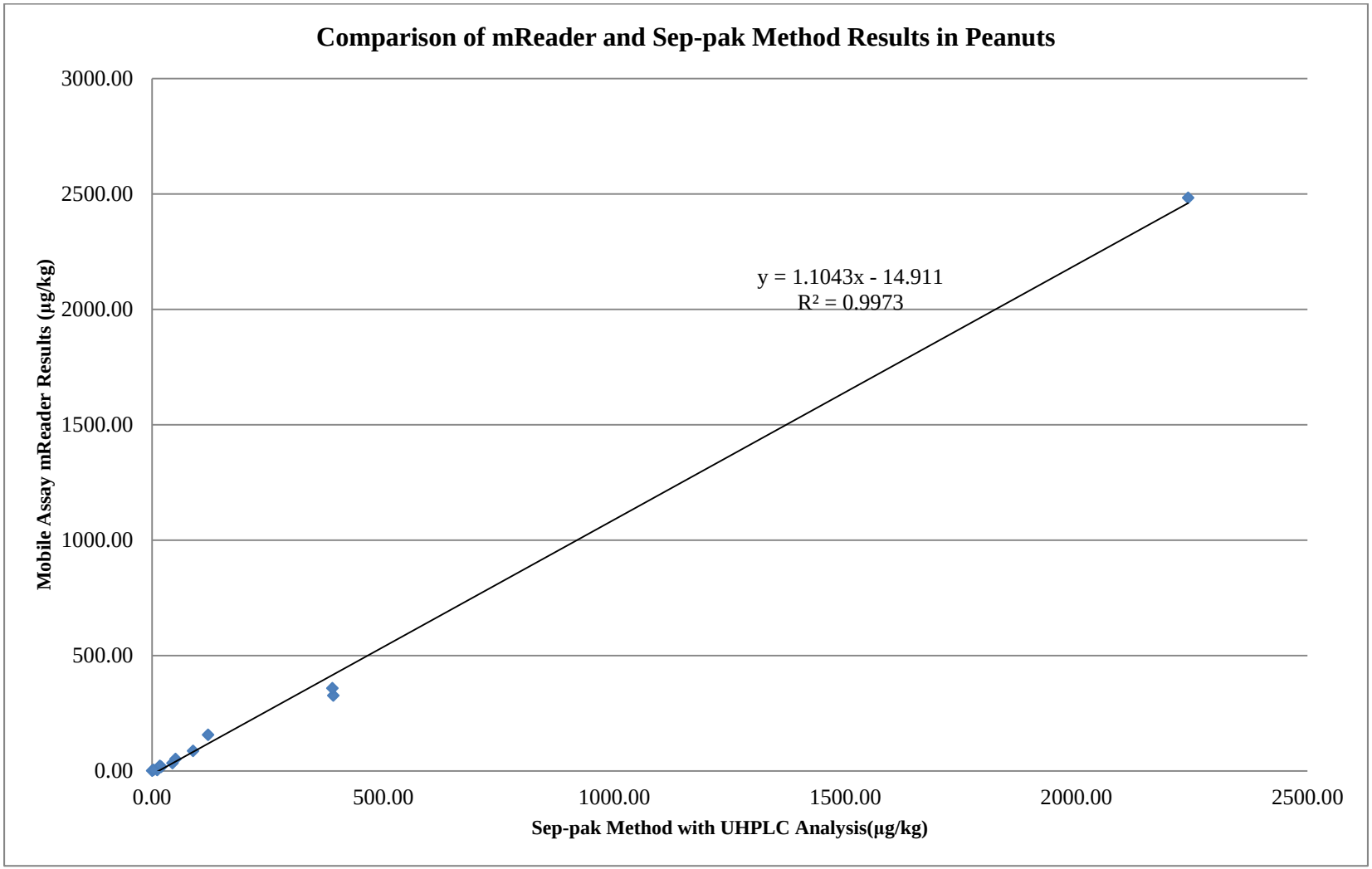

Figure 4.12 Comparison of results using mReader and UHPLC methods for the analysis of AFs in peanut samples shows the strong correlation of results.

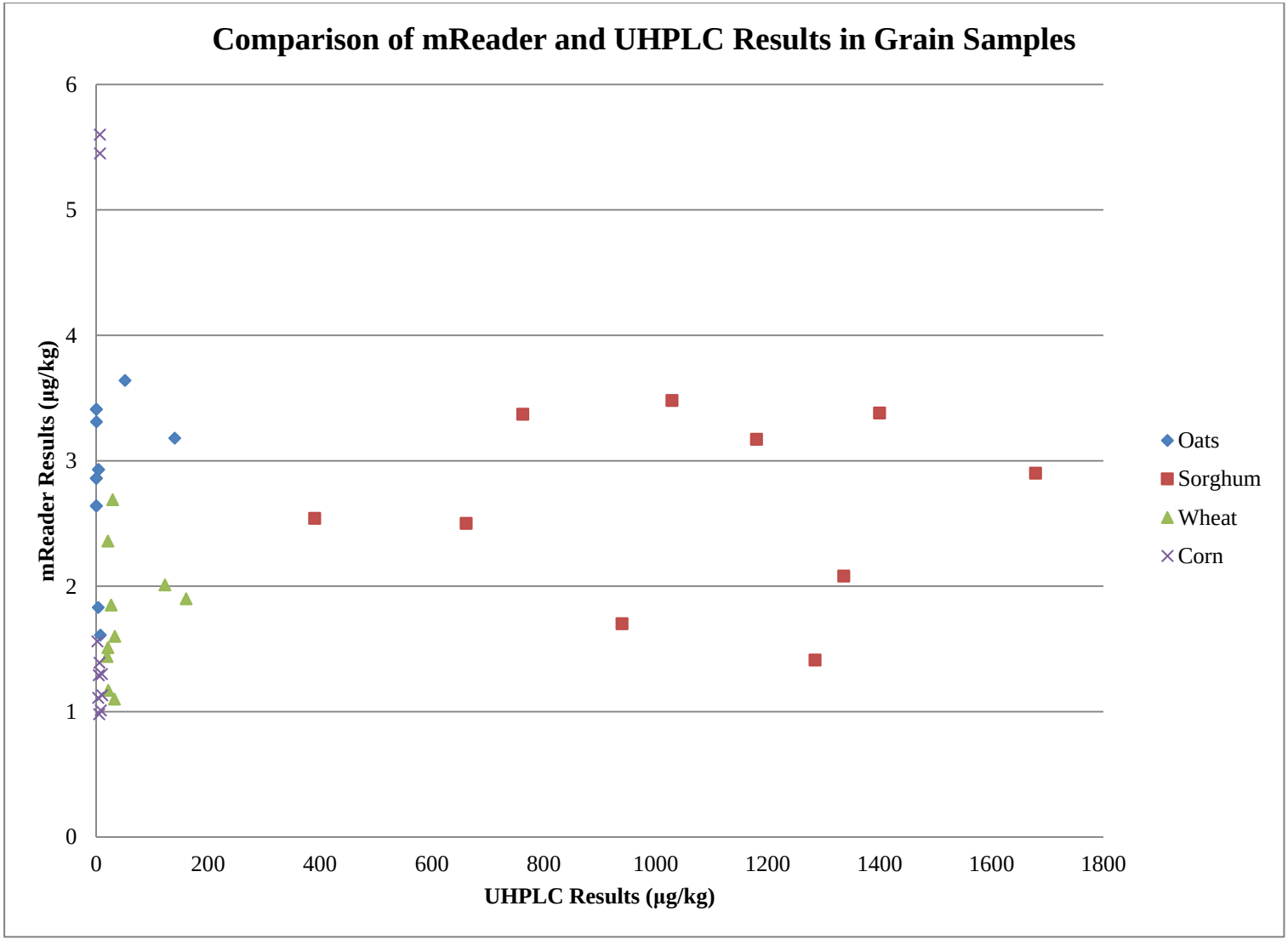

Figure 4.13 Comparison of results for mReader and UHPLC methods for analysis of AFs in oats, sorghum, wheat, and corn showing need for further development of mReader methods for grains.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTION

Multiple methods for analysis of AFs in peanut and grain samples, including HPLC, UHPLC, ELISA, and mReader detections, were compared in this research. Different extraction methods, including the use of MycoSep columns, immunoaffinity columns, and Sep-pak columns were also compared for UHPLC analysis. Overall, UHPLC analysis with Sep-pak cleanup was chosen to be used to analyze the contamination of AFs in over 280 peanut and grain samples grown in the State of Georgia, USA. Mobile Assay's mReader analysis with Reveal Q+ by Neogen methods were used to analyze the Georgia-grown samples as well in order to develop and confirm this new method. Because of its rapid detection and ability to easily analyze samples in field or remote conditions, the mReader device and method can make analysis of AFs in commodity much easier and possible throughout growing and processing. The implementation of instruments and devices such as this will help to determine the stages where crops are most at risk for AF contamination and also reduce the time from sample collection to analysis.

Study samples collected in supermarkets in the State of Georgia shows that AF levels in food products are below the regulatory limit set by the United States FDA. This study was also effective in comparing different extraction and analysis methods for AF determination in peanuts and grains. The research of AF contamination in crops grown in the State of Georgia shows the need for more research to help determine the pattern of AF contamination yearly, throughout different stages of growth, harvesting, and storage, and also in different commodities. Because of the lack of data of AF contamination in the State of Georgia throughout last two decades, this

study is a starting point to help determine crops that are at higher risk for contamination and weather patterns that increase risk for contamination in different areas of the State. This study was effective in comparing different methods of extraction and analysis, as well as exploring sample collection procedures and challenges. Because of the lack of data of AF contamination in the State of Georgia throughout the last two decades, this study is a starting point to help determine crops that are at higher risk for contamination and weather patterns that increase risk for contamination in different areas of the State. Therefore, there is a need for future monitoring of peanuts and grains grown in the State of Georgia and examination of current protocols being used to help decrease AF contamination in these commodities. Such studies should be continued with a larger sample size, and identification of where each sample was grown in order to further correlate weather data with contamination levels. Wheat, specifically, should be studied further for its potential for AF contamination in certain conditions.