DEVELOPMENT OF A SCREENING METHOD FOR DETERMINATION OF

AFLATOXINS

by

JOAO AUGUSTO

(Under Direction of David M. Wilson)

ABSTRACT

Toxigenic fungal invasion and subsequent production of aflatoxins in peanut and corn can be a serious problem before and after harvest. The objectives of this study were to: (i) enhance the fluorescence of aflatoxin B_1 with cyclodextrins for detection using a fluorometer, and (ii) develop an easy and inexpensive method to screen for aflatoxins in peanut and corn. Improvement of fluorescence was accomplished by using β-cyclodextrin, succinyl-(2-hydroxy)propyl-β-cyclodextrin and dimethyl-β-cyclodextrin using different concentrations of aflatoxin B_1 standard. Fluorescence of aflatoxin was greater and more stable over time with β-cyclodextrin and its derivatives than a bromine (control). The method involved a clean-up procedure with neutral alumina and quantification with a fluorometer. Statistics showed a high correlation between the developed method and the "Aflatest" in peanut and corn samples with R^2 of 0.977 and 0.997, respectively. The new method could be extremely important when monitoring high levels of aflatoxins, reducing the risk to human health.

INDEX WORDS: Screening Method, Cyclodextrins, Fluorescence, Aflatoxins, Peanut, Corn.

DEVELOPMENT OF A SCREENING METHOD FOR DETERMINATION OF

AFLATOXINS

by

JOAO AUGUSTO

B.S., Universidade Eduardo Mondlane, Mozambique, 1996

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment

of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2004

DEVELOPMENT OF A SCREENING METHOD FOR DETERMINATION OF

AFLATOXINS

by

JOAO AUGUSTO

Major Professor: David M. Wilson

Committee: Scott E. Gold
                     Albert K. Culbreath

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2004

DEDICATION

Maria Fernando…………

I owe you everything I earn in my life. Thank you mom!

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Aflatoxin contamination of food and feeds remains a worldwide problem. The United

Nation's Food and Agriculture Organization (FAO) has estimated that as much as 25 per cent of

the world's food is significantly contaminated with mycotoxins (CAST, 1989), much of this

contamination being by aflatoxins. The aflatoxins were first identified after cases of livestock

poisoning in 1960 in the United Kingdom. More than 100,000 turkeys died from acute necrosis

of the liver and hyperplasia of the bile duct (D'Mello and Macdonald, 1997) termed "Turkey X

Disease" that was finally traced to the peanut component of their feed. It turned out that the

consumed peanuts were contaminated with *Aspergillus flavus* group fungi (i.e. *A. flavus*, or *A.

parasiticus*, or both) whose metabolites, later called aflatoxins, were responsible for the

occurrence of the disease. It is now known that these secondary metabolites are produced by

fungi belonging to *Aspergillus* Section *Flavi*, more commonly known as the *Aspergillus flavus*

group fungi (Klich and Pitt, 1988; Varga *et al*., 2003).

Chemically, aflatoxins are difurocoumarolactone compounds because they possess furan,

coumarin and lactone rings (Brown *et al*., 2001). In the word *aflatoxin*, the first syllable 'a' was

derived from the genus Aspergillus, the 'fla', from the species *flavus* and the term 'toxin' came

from the adjective 'poison' (Papp *et al*., 2002). The major aflatoxins of concern and routinely

monitored in foods and feeds in commerce are designated B_1, B_2, G_1, G_2, and M_1 (Figure 1)

(Holcomb *et al*., 1992). The letters B and G indicate the color of the fluorescent emissions from

1

the two categories of aflatoxins (i.e., blue or green) (Phillips, 1999), and M indicates that the metabolites of the B and G aflatoxins are found in milk.

It has been suggested that almost all toxigenic isolates of *A. parasiticus* produce the B and G aflatoxins, whereas the toxigenic *A. flavus* isolates generally produce only the B aflatoxins (Wilson and Payne, 1994). However, there are exceptions to this tendency depending on strain and geographic location. The most common aflatoxin-producing species, *A. flavus*, can be divided into two strains. The S strain produces numerous small sclerotia (average diameter, < 400 μm) and high levels of aflatoxins. The L strain produces fewer, larger sclerotia and, on average, less aflatoxin. Within the S strain, some isolates, termed S_B, produce only B aflatoxins, while others, termed S_{BG}, produce both B and G aflatoxins (Cotty and Cardwell, 1999; Varga *et al.*, 2003). Molecular phylogenetics suggests that S_B isolates are closely related to the *A. flavus* type culture and other L strain isolates. Cotty and Cardwell (1999) compared S strain communities in West Africa with those previously characterized in North America. They found that West African *A. flavus* S isolates differed from North American isolates. Both produced aflatoxin B_1. However, 40% and 100% of West African isolates also produced aflatoxin G_1 in NH_4 medium and urea medium, respectively. No North American S strain isolates produced aflatoxin G_1.

Aspergillus flavus and *A. parasiticus* form the toxins at temperatures ranging from 12 to 42°C and relative humidity greater than 85%. Chemically, aflatoxins are crystalline substances, freely soluble in moderately polar organic solvents such as acetone, acetonitrile, isopropanol and methanol, dissolve in water to the extent of 10-20 mg/liter, and are intensely fluorescent in ultraviolet light and do not get destroyed even at very high temperatures (260° C) (Leatherhead Food Research Association, 2004; Bilgrami and Choudhary, 1998).

Aflatoxins are found in a number of foods and feeds, particularly in peanuts, cereals, cottonseed, tree nuts, meat, milk products, eggs and different oilseeds (Brown *et al*., 2001; Papp *et al*., 2002; Palmgren and Hayes, 1987). Humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth. Such exposure is difficult to avoid since fungal growth in foods is not easy to prevent (CAST, 1989; Viquez *et al*., 1994).

The consumption of an aflatoxin-contaminated diet may induce acute and long-term chronic effects in humans and animals resulting in a teratogenic, carcinogenic, or immunosuppressive impact (Huwig *et al*., 2001). Evidence of acute aflatoxicosis in humans has been reported including vomiting, abdominal pain, pulmonary edema, convulsions, coma, and death with cerebral edema and fatty infiltration and necrosis of the liver, kidney, and heart (Cullen and Newberne, 1994). Aflatoxins have also been implicated in sub-acute and chronic effects in humans. These effects include primary liver cancer, chronic hepatitis, jaundice, hepatomegaly and cirrhosis. Conditions increasing the likelihood of acute aflatoxicosis in humans include limited availability of food, environmental conditions that favor fungal growth, and the lack of regulatory control systems (CAST, 1989). Moreover, aflatoxin-related diseases may be influenced by age, sex, nutritional status, and exposure to the causative agents such as viral hepatitis or parasite infestation (CAST, 1989).

Aflatoxins B_1, B_2, G_1, and G_2 are listed as Group I carcinogens. Aflatoxin M_1, just as toxic as aflatoxin B_1 (Papp *et al*., 2002), is listed as a Group 2B carcinogen by the International Agency for Research on Cancer. These toxins are implicated in human primary hepatocellular carcinoma (Ioannou-Kakouri *et al*., 1999; Li *et al*., 2001). Aflatoxin B_1, the most potent and commonly occurring of the aflatoxins, has also been recognized as a teratogen, mutagen, hepatocarcinogen, immunosupressant and potent inhibitor of protein synthesis (Methenitov *et al*.,

2001). Aflatoxin M_1 is a hydroxylated metabolite of aflatoxin B_1 and is produced when cows or

other mammals ingest feed contaminated with aflatoxin B_1. It is then excreted in the milk and

may subsequently contaminate other dairy products such as cheese and yogurt. Because of

potential health hazards for humans, threshold levels of aflatoxins in commodities have been

established worldwide. The European Commission has set maximum levels of 2 µg/kg for

aflatoxin B_1 and 4 µg/kg for total aflatoxins (B_1, B_2, G_1, and G_2) in peanuts, nuts, dried fruits and

cereals (Stroka and Anklam, 2002; Sobolev and Dorner, 2002; Gilbert, 2002). These levels are

about five times lower than those established in the USA (Gilbert, 2002). The maximum level set

by FDA for aflatoxin M_1 in milk is 0.05 µg/kg (Stroka and Anklam, 2002).
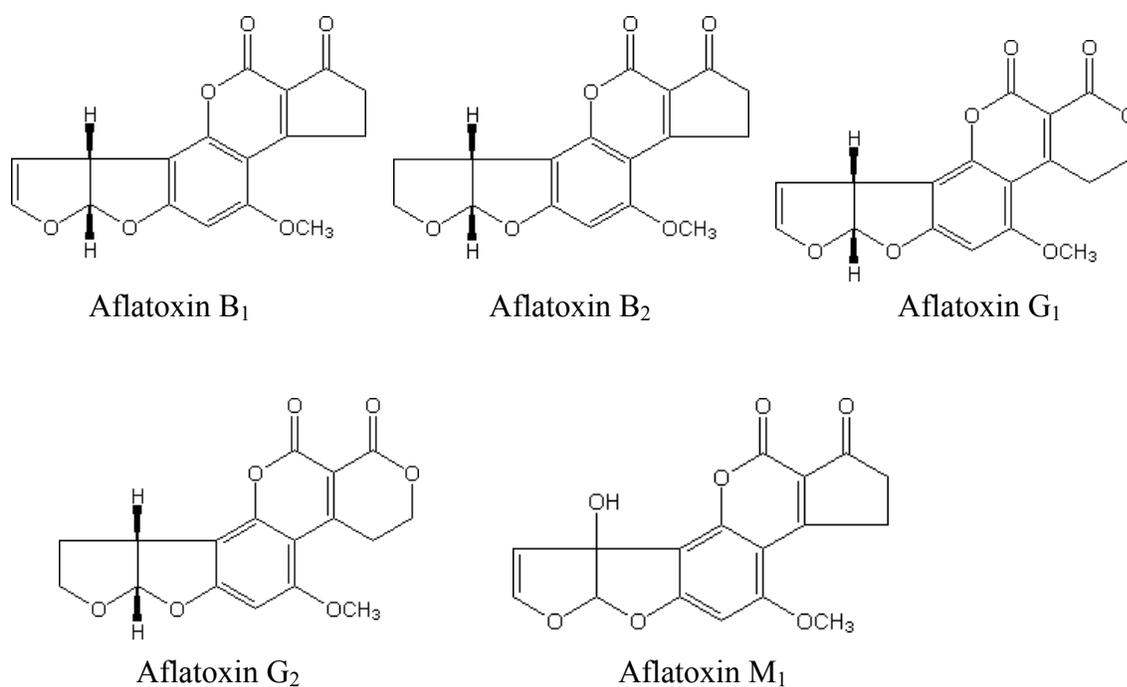
Figure 1. Structures of aflatoxins B_1, G_1, B_2, G_2, and M_1.

TOXIGENIC FUNGAL INVASION AND AFLATOXIN CONTAMINATION OF CROPS

Historically, fungi have been divided into two distinct groups with regard to the quality of grains as they come from the field at harvest and its potential for worsening in storage. The first comprises those that invade before harvest, which are frequently called "field fungi". The other group becomes a problem after harvest and these fungi are known as "storage fungi".

Christensen and Kaufmann (1969) defined "field fungi" as the fungi that invade the kernels or seeds while the plants are still growing in the field, or after the grain is cut and swathed but before it is threshed. On the other hand, with a few exceptions, storage fungi do not infect seeds of cereals or other crops to any significant degree or extent before harvest. About the only important exception to this is the infection of the developing ears of corn or pods of peanuts by *A. flavus* and *A. parasiticus* (Sauer *et al*., 1992). Pre- and postharvest invasion of toxigenic fungi and aflatoxin contamination of corn and peanut are discussed next.

(i) Preharvest Aflatoxin Contamination of Crops

Preharvest Aflatoxin Contamination of Peanut (Arachis hypogea L.).

Even though aflatoxin contamination of peanuts can occur before and after harvesting, the contamination may occur prior to harvest and increase in storage (Parmar *et al*., 1997; Dorner *et al*., 1992; Cole, 1989). Preharvest aflatoxin contamination of peanut is believed to be positively associated to a large extent with drought stress and elevated soil temperatures during the latter part of the growing season (Umeh *et al*., 2000; Parmar *et al*., 1997). A possible explanation for drought stress induced preharvest aflatoxin contamination is the elimination of microbial competitors of *A. flavus* and *A. parasiticus* and the elevation of the soil temperature in the peanut geocarposphere (Cole *et al*., 1985). The mechanism for this is probably that toxigenic fungi gain entrance into the peanut kernel at an early developmental stage (probably after the

pegs enter the soil), and then remain inactive or dormant, presumably as a result of the phytoalexin-based defense system. Drought stress results in a loss of moisture from the peanut kernels that cannot be maintained by the stressed plant, and as a result, the capability of the kernels to produce phytoalexins is reduced, allowing toxin-producing fungi to grow until the low moisture content becomes limiting for fungal growth (Cole, 1989). Alternatively, Holbrook *et al.* (2000) suggested that less aflatoxin contamination might be associated with drought tolerance. Thus, the correlation between drought tolerance and aflatoxin contamination remains unclear.

Studies on factors related to aflatoxin contamination during the late-season reveal that: (a) the mean threshold geocarposphere temperature required for significant aflatoxin development is between 25.7 and 27° C; (b) kernel damage is not a prerequisite for aflatoxin contamination; and (c) sound kernels from irrigated peanuts are not likely to be contaminated (Pohland and Wood, 1987). In previous studies by Blankenship *et al.* (1984), the incidence of *A. flavus* group fungi in drought-stressed, sound mature kernels at harvest was relative to the 5 cm deep, mean geocarposphere temperatures. Cole *et al.* (1985) reported that undamaged peanuts grown under drought stress and subjected to mean geocarposphere temperatures below 25.7° C were not contaminated with aflatoxin, but those grown at geocarposphere temperatures at or above 27° C were likely to become contaminated in the absence of visible damage. *Aspergillus flavus* or *A. parasiticus* invasion of drought-stressed, visibly undamaged peanut kernels takes place mostly in the soil rather than from the flowers and aerial pegs (Cole *et al.*, 1986).

Another important means of peanut contamination by aflatoxin is the positive association between peanut pod damage caused by termites and other insects and *Aspergillus* infection and aflatoxin contamination. Seeds in broken and insect-damaged pods are directly exposed to invasion by *A. flavus* group fungi. Pod-scarifying termites such as *Armitermes* spp., *Microtermes*

spp. and *Odontotermes* spp. also weaken the pods and render them vulnerable to invasion by *A. flavus*, or *A. parasiticus*, or both (Manzo and Misari, 1987). Drought and high soil temperatures, characteristics of the latter part of the cropping season in many peanut production areas, play important roles in increasing termite attack in the semi-arid tropics (Umeh *et al*., 2000).

Lesser cornstalk borer (*Elasmopalpus lignosellus*) is also responsible for peanut kernel damage in the soil during drought periods before harvest, and this insect is likely to occur with dry soil and elevated soil temperatures (Blankenship *et al*., 1984). Lynch and Wilson (1991) examined the role of the lesser cornstalk borer as a vector of an *A. parasiticus* color mutant (ATCC 24690), the relationship between the extent of pod injury by the lesser cornstalk borer in the field and contamination of pods and seeds with the *A. flavus* group fungi, and the subsequent contamination of seeds with aflatoxin. In the laboratory, lesser cornstalk borer larvae were excellent vectors of the *A. parasiticus* color mutant to all developmental stages of peanut pods. Contamination of seeds with ATCC 24690 was directly related to the extent of pod injury by larvae of the lesser cornstalk borer. In field studies, increased pod injury by the lesser cornstalk borer significantly increased the percentage of seeds infected with species of the *A. flavus* group fungi. Seeds in pods with only external scarification from larval feeding had a significantly higher percentage of *A. flavus* group fungi infection than seeds from uninjured pods.

Different strategies of reducing peanut preharvest contamination have been suggested. The possible use of genetic resistance to kernel invasion by *A. flavus* group fungi has been investigated, and the significant postharvest varietal resistance to *A. flavus* group fungi invasion of rehydrated mature peanut kernels has been interpreted to indicate a possible resistance, or tolerance, to preharvest invasion under field conditions (Blankenship *et al*., 1985).

Non-aflatoxin-producing strains of *A. parasiticus* may reduce aflatoxin in edible-grade peanuts. Here the biocompetitive agent might dominate the soil microflora and prevent the buildup of native, aflatoxin-producing strains of *A. flavus* and *A. parasiticus* that normally occurs during late-season drought (Dorner *et al*., 1992). Analysis of soil in a two-year study to evaluate the efficacy of three formulations of non-toxigenic strains of *A. flavus* and *A. parasiticus* to reduce preharvest aflatoxin contamination in peanuts showed that a large soil population of the non-toxigenic strains resulted from all formulations, namely solid state fermented rice, fungal conidia encapsulated in "pesta", and conidia encapsulated in pregelatinized corn flour granules. Aflatoxin concentrations in peanuts were significantly reduced in year two by all formulation treatments with an average reduction of 92% (Dorner *et al*., 2003). However, although significant aflatoxin reductions were observed with all formulations, the mean total aflatoxin with rice, "pesta", and corn flour formulations were 43.9, 20.4, and 29.9 ppb, respectively. This is still likely to adversely affect the marketability of the peanut since these levels of aflatoxin contamination are still above the threshold of 20 ppb enforced by FDA.

The use of irrigation in peanut fields during the latter part of growing season can be an effective measure to reduce drought stress and decrease preharvest aflatoxin contamination of peanuts (Brenneman *et al*., 1993). Wilson and Stansell (1983) investigated the effect of irrigation regimes on aflatoxin contamination of peanut pods in Georgia for 4 years. Florunner and Florigiant peanuts were grown and foliar inoculated with an aflatoxin producing isolate of *A. parasiticus* (NRRL 2999) 30 days after planting. Peanuts were grown in plots under rainfall-controlled shelters with six irrigation treatments. In all treatments where irrigation was applied during the last 40 days of the season, no significant aflatoxin contamination was detected in any cultivar any year of the test. Wilson *et al*. (1989), studying the effect of irrigation on percent

fungi recovered from NC-7 peanut kernels and hulls, found that irrigation significantly reduced the numbers of kernels and hulls from which members of the *A. flavus* group fungi were recovered from 19.5 to 7.8%.

Mineral nutrition also seems to affect aflatoxin contamination of peanuts. Studies on effect of calcium on colonization of Florunner peanut seed by *A. flavus* group fungi showed that application of calcium as gypsum at early bloom reduced percent colonization of seed (kernels) from 7.38% to 4.06% with 112 and 336 kg ha^{-1} of Ca (Wilson *et al.*, 1989).

In addition, good management practices, such as using sound, fungus-free seeds for planting, controlling insects and plant diseases, harvesting plants at maturity, and proper adjustment and operation of harvesting equipment to avoid damage to peanut pods also prevent contamination (Ellis *et al.*, 1991).

Preharvest Aflatoxin Contamination of Corn (Zea mays L.).

In grains, aflatoxins are primarily a problem in corn. The colonization of corn in the field by toxigenic fungi depends on environmental conditions (Miller, 1995). Increased levels of airborne conidia have been linked to increased kernel infection (Jones *et al.*, 1981). Payne *et al.* (1986) suggested that because *A. flavus* is predominately soilborne, plant debris in the soil might be the primary source of airborne inoculum. *Aspergillus flavus* can also produce sporogenic sclerotia, which have been found in infected corn seeds (Wicklow *et al.*, 1984).

Even though the main route of infection of corn by *A. flavus* and *A. parasiticus* seems to vary in different geographical areas of the world, the aflatoxin-producing fungi invade plants in the field with the help of wind and insects (Machinski *et al.*, 2001). The mode of entry of *A. flavus* group fungi into kernels of corn is distinct from that into agricultural crops such as peanut. Exposed silk tissue is susceptible to colonization by the fungus and provides a suitable infection

court for entry of airborne spores into intact seeds if environmental conditions favor *A. flavus* group fungi (Jones *et al.*, 1980).

In field and greenhouse studies used to determine the nature of preharvest infection of corn by *A. flavus*, inoculation of external silks that were yellow-brown resulted in more extensive colonization of the silks and a greater number of infected kernels than inoculation of brown silks. In silk-inoculated and uninoculated ears, tissue colonization proceeded from the ear tip towards the base, colonizing the silks first, then the glumes and the kernels' surfaces but rarely penetrating the cob pith (Marsh and Payne, 1984).

Payne *et al.* (1986) using either silk-inoculated, wound-inoculated, or naturally infected corn ears found that kernel infection and aflatoxin contamination were always greater in silk-inoculated plots than in naturally infected plots, and wound inoculation resulted in the greatest amount of aflatoxin.

Smart *et al.* (1990) developed a model of pathogenesis for *A. flavus* in corn by following the growth of *A. flavus* hyphae from a wound-inoculation site to the adjacent, unwounded spikelets. They focused on how *A. flavus* arrived in the rachilla and on subsequent entry into the seed proper. The fungus spread from the wound sometime after 14 days postinoculation, and at 28 days postinoculation it could be found in small amounts throughout all rachis tissues except the pith and lignified fibers. *Aspergillus flavus* entered the rachillae of adjacent spikelets from the rachis and also from the bracts at their insertion point. Then, it grew through the aerenchyma (outer layer of the rachilla) to the floral axis and innermost layers of the pericarp, but the hyphae did not penetrate to the endocarp from the exterior of the pericarp.

Several factors have been associated with high levels of aflatoxin in preharvest corn, including high temperatures, insect damage, and plant stress (McMillian *et al.*, 1985). Lower

aflatoxin contamination has been associated with the expression of secondary traits such as good husk coverage and tightness, insect resistance, kernel integrity under environmental stress, and drought tolerance (Betran and Isakeit, 2004).

Payne *et al*. (1988), studying the effect of temperature on the preharvest infection of corn kernels by *A. flavus*, suggested that the parasitic ability of *A. flavus* is enhanced at high temperatures. The percentage of infected kernels increased greatly between 28-32 days after inoculation when kernel moisture was less than 32%. Thompson *et al*. (1980) found that the highest aflatoxin levels were in kernels inoculated at the latest kernel-development stage and grown at the highest postinoculation temperatures. Aflatoxin levels were low in kernels inoculated at earlier development stages and grown at lower temperatures. Rambo *et al*. (1974) had previously found similar results in yellow and white dent corn where ears inoculated at later stages of maturity, late milk and early dough, were more susceptible to infection than ears inoculated at the silking or early milk stages.

Lillehoj *et al*. (1987) linked the variations in moisture in preharvest corn kernels with the ability of *A. parasiticus* to infect the seed and produce aflatoxin. During the period 21 to 49 days after flowering corn kernel moisture content decreased from 58 to 28%, and the osmotic pressure of seed sap increased from 450 kPa at 21 days after flowering to 683 kPa at 35 days after flowering. During the same period, the maximum level of aflatoxin (141 µg/kg) in the field-inoculated corn kernels was detected in those inoculated at 28 days postflowering (52% MC) and no aflatoxin was detected in those inoculated at 49 days postflowering. Widstrom *et al*. (1981) had previously found the same trend of results in which delaying inoculation until 40 days after silking significantly reduced the aflatoxin contamination level of samples harvested at maturity.

In addition, less than one-half the ears inoculated at 40 days after silking (35.3%) exhibited visible signs of infection compared with ears inoculated 20 days after silking (82.9%).

Another important factor influencing corn invasion by *A. flavus* and *A. parasiticus* is insect damage. Damage to corn kernels by European corn borer (*Ostrinia nubilalis* Hubner), fall armyworm (*Spodoptera frugiperda* J.E. Smith), and corn earworm (*Helicoverpa zea* Boddie), has been associated with high aflatoxin levels (Gorman and Kang, 1991). Jones *et al.* (1980) pointed out that insects might play a role in increasing infection by spreading the toxigenic fungi from kernel to kernel within the ears or by providing sites for extensive growth and sporulation on the surface of kernels injured by insect feeding. They also suggested that the rapid dehydration and consequent lowered moisture content of injured kernels might result in higher concentrations of aflatoxins in damaged kernels than in their undamaged counterparts. Lillehoj *et al.* (1980) also found a trend of increased aflatoxin levels associated with greater insect damage. In earlier study by Anderson *et al.* (1975) on aflatoxin contamination of corn in the field, insect damage was observed in 90% of the samples that showed bright greenish-yellow fluorescence normally associated with the presence of aflatoxin. McMillian *et al.* (1980) determined the degree of enhancement of fungal infection on corn ears visited by corn weevils contaminated with *A. flavus*. Ears of corn hybrids infested with corn weevils, *Sitophilus zeamais* Motschulsky, previously exposed to spores of *A. flavus*, had almost twice as much fungal infection as ears infested with unexposed weevils. They concluded that the corn weevil could contribute significantly to increased *A. flavus* infection on corn ears by transporting spores and damaging corn kernels.

Information on association between plant stress and aflatoxin accumulation has been extensively reviewed. Stress can be induced by water deprivation, inadequate fertility, or weed

competition. Plant stress seems to enhance the susceptibility of developing kernels to infection by *A. flavus* and *A. parasiticus* and subsequent aflatoxin biosyntheses. In fertilization studies (Jones and Duncan, 1981), aflatoxin B_1 concentrations were consistently higher in corn grown in plots low in nitrogen. Wilson *et al*. (1989) investigated the effects of initial fertility level (NPK) and supplemental fertilizer on aflatoxin in corn. They found that late applications of large amounts of N or excessive fertilization with N, P and K resulted in elevated aflatoxin concentrations. Application of S as ammonium-thiosulfate at the 12-leaf stage also increased aflatoxin contamination. No clear explanation was given for these phenomena.

Water deprivation is another key factor in preharvest fungi invasion and aflatoxin contamination. Payne *et al*. (1986) studying the reduction of aflatoxin contamination in corn by irrigation and tillage concluded that although several factors might contribute to high amounts of aflatoxin in the field, water stress appeared to be a major factor affecting aflatoxin contamination, because subsoiling as well as irrigation reduced aflatoxin contamination.

The most promising method for control of aflatoxin contamination in preharvest corn is currently the development of resistant hybrids. Diallel analysis showed that general combining ability effects (a measure of additive effects) were more important than specific combining ability effects (a measure of dominance effects), therefore suggesting that the genetic control of Aspergillus ear rot and aflatoxin accumulation was of an additive nature (Naidoo *et al*., 2002; Darrah *et al*., 1987). Sprague and Tatum (1942) described the term "general combining ability" as the average performance of a line in hybrid combinations and, "specific combining ability" as those cases in which certain combinations do relatively better or worse than would be expected on the basis of the average performance of the lines involved.

Windham and Williams (2002) evaluated eighteen corn inbred lines and advanced breeding lines for resistance to aflatoxin contamination when artificially inoculated with *A. flavus* in 1998, 1999, and 2000 at Mississippi State University. The top ear of each plant was inoculated with the *A. flavus* isolate NRRL 3357 seven days after midsilk (50% of the plants in a plot had silks emerged) using the side-needle technique. They found that four lines (Mp81:112, Mp92:673, Mp92:679, and Mp494) supported the lowest levels of aflatoxin contamination, and suggested that these lines might provide potential sources of resistance that could be used to move aflatoxin resistance into commercial corn hybrids.

Two germplasm lines had been previously released as sources of resistance to aflatoxin accumulation. GT-MAS:gk (reg. no. GP-241, PI561859) was released cooperatively by USDA-ARS and the Georgia Agricultural Experiment Station in 1992. GT-MAS:gk was described as having had consistently lesser amounts of kernel aflatoxin contamination, whether obtained from field- or laboratory-inoculated samples, when compared with the sister population (Widstrom *et al.*, 1987). Testcrosses of GT-MAS:gk with southern adapted lines had equal or less contamination than the testcrosses with its sister counterpart (McMillian *et al.*, 1991). Germplasm line Mp715 (Reg. no. GP-362, PI 614819) was released by USDA-ARS and the Mississippi Agricultural and Forestry Experiment Station in March 1999. Mp715 was developed from Tuxpan by selfing for eight generations and selecting for reduced aflatoxin accumulation following inoculation of developing ears with an *A. flavus* spore suspension (Zummo and Scott, 1989).

Even though several researchers have identified corn genotypes with varying degrees of resistance, no commercial hybrids with resistance are currently available (Campbell and White,

1994). This is due to the lack of uniform environmental conditions from year to year, making field selection of resistance difficult (Woloshuk *et al.*, 1997).

(ii) Postharvest Aflatoxin Contamination of Crops

Postharvest Aflatoxin Contamination of Peanut.

Peanuts are susceptible to invasion by molds during production, between harvest and drying, in storage, and after processing and manufacturing (Kubena *et al.*, 1998; CAST, 1989). If mold growth occurs, then there is also the possibility of aflatoxin production. Nevertheless, the presence of toxigenic molds in a food product does not automatically mean the presence of aflatoxins, especially if growth has not occurred. Conversely, the absence of toxinogenic molds does not guarantee that the product is free of aflatoxins, since the toxins may persist long after the molds have disappeared (Bullerman, 1986; Ellis *et al.*, 1991).

The natural contamination of peanut is likely to persist whenever warm and moist weather conditions, faulty or inadequate storage facilities, and human error combine to produce circumstances favorable for fungal growth and toxin production. Under favorable conditions for mold growth, aflatoxin production is also possible. The most important factors influencing growth of, and aflatoxin production by, *A. flavus* and *A. parasiticus* are a relative humidity between 88 and 95% and a storage temperature of 25 to 30˚C (Ellis *et al.*, 1991).

Mold growth on peanut can be minimized by good sanitation and handling throughout the entire production chain, from producer to consumer (Bullerman, 1986). Storage conditions play an important role in the physicochemical and microbiological quality of peanut. The specific fungal species that develop in a given environment depend on moisture, temperature, presence of competing microorganisms, and the nature and physiological state of the produce. Moisture levels and temperature are the most important factors in the protection of stored grains against

mold growth and aflatoxin production. *Aspergillus flavus* is unlikely to invade grain and oilseeds when the moisture content is in equilibrium with a relative humidity of 70% or less (Ellis *et al.*, 1991). At 70% relative humidity, the moisture content of commodities rich in oil, such as peanuts, is 7 to 8% (Table 1).

Table 1. Equilibrium moisture content of peanut with temperature and relative humidity.

	Equilibrium Moisture Content of Stored Peanut[a]							
	Temp. (°C)	Relative Humidity (%)						
		20	30	40	50	60	70	80	90
Peanuts, kernels	10	4.0	4.9	5.7	6.4	7.2	8.1	9.1	10.5
	21	3.4	4.3	5.1	5.9	6.7	7.7	8.9	10.6
	32	3.0	3.9	4.7	5.6	6.5	7.5	8.8	10.6
Peanuts, hull	10	7.3	9.1	10.7	12.3	13.9	15.8	18.0	21.2
	21	6.2	7.9	9.4	11.0	12.6	14.5	16.8	20.1
	32	5.6	7.1	8.6	10.2	11.8	13.6	15.9	19.3

[a] Data from ASAE Standards (1986)

Other important measures to control mold growth and aflatoxin production in stored commodities susceptible to fungal and insect attack are to maintain temperatures at 0°C (although not economically feasible for large scale storage) and increase the level of carbon dioxide and nitrogen in the atmosphere. This modification of the gaseous atmosphere decreases the oxygen content of the air causing the environment in the storage facility to be less suitable for aerobic pathogens and pests (Ellis *et al.*, 1991).

Aflatoxins may also be produced in the field before harvest and stay in the peanuts for years (Bullerman, 1986). At lifting, pod and kernel moisture can range from as high as 48% to

below 15% depending on when or if soil drying occurs prior to harvest. When peanuts with high moisture contents are lifted and placed in stacks in the field, rapid invasion of the kernels by *A. flavus* group fungi and subsequent aflatoxin contamination can occur. In addition, rapid fluctuations in seed moisture content during drying in stacks can result in aflatoxin contamination, especially when rain falls on partially dried peanuts. Rewetting peanuts after drying greatly increases the susceptibility of seeds to *A. flavus* group fungi infection and aflatoxin contamination (Manzo and Misari, 1987).

Peanuts entering storage may contain foreign material such as sticks, soil, rocks and weeds collected during the harvesting process. Although the current United States peanut agreement requires that kernel moisture be less than 10.5% (wet basis) at marketing, no regulations have been established for foreign material. These foreign materials may rewet surrounding kernels and provide moisture for growth of *A. flavus* (Dowell and Smith, 1995).

Therefore, postharvest drying should be rapid but not so fast as to lead to seed damage, and storage should be under clean, dry, pest-free conditions (Swindale, 1987). The death of the testa during curing and drying may contribute to the loss of resistance to seed infection (Manzo and Misari, 1987). Produce should be monitored for aflatoxin contamination by screening or quantitative methods as it leaves the farm, on arrival at buying stations or processing plants, and lots with aflatoxin levels above those permissible should be diverted to nonfood use, or be subject to some detoxification process before use as food or livestock feed (Swindale, 1987).

Postharvest Aflatoxin Contamination of Corn.

Despite devoted research efforts worldwide, aflatoxin contamination of corn after harvest continues to be a major problem (Miller, 1995). However, the extent of the problem seems to vary by location and country. In the USA, for instance, storage conditions are typically adequate

and the issue is the management of preharvest contamination of corn (Payne, 1992), in tropical

countries however, storage of corn is an additional and substantial problem (Siriacha *et al*.,

1991). Most attention has been paid to the management of aflatoxin in corn by the development

of systems to detect and segregate contaminated kernels or better storage systems (Miller, 1995).

Colonization of stored products by the Aspergilli is primarily a function of water activity,

temperature, length of time the grain is stored, seed damage, and foreign material present. Also,

the degree to which the grain is invaded by the *A. flavus* group fungi in storage is influenced by

aeration, fungal inoculation, microbial interactions, and moisture content, exacerbated by insect

and rodent activity (CAST, 2003; Wilson *et al*., 2002; Christensen and Kaufmann, 1969). All of

these factors interact with one another to some extent, but the major risk determinants in storage

are moisture content, temperature, and time.

The combinations of moisture content, temperature, and time that make for low risk of

damage to stored corn by fungi are known (Table 2).

Table 2. Corn storability as a function of moisture content, temperature, and time.

	Allowable Storage Time for Corn[a]						
	Corn Moisture Content (%)						
Temperature	14	15	16	17	20	25	30
(° C)	Months				Days		
5	58	28	14	9.0	90	33	19
10	34	16	9	5.3	48	17	10
15	21	10	5	3.2	29	11	6
20	13	6	3	2.0	18	7	4
25	8	4	2	1.2	10	3	2.5

[a] Data from Saul and Steele (1966)

In general, if the corn is sound when it is put into storage, it can be kept for a year at moisture contents of 15.0-15.5% at a temperature of 15° C. During this time, however, it will have been invaded to some extent by storage fungi and therefore is a poorer risk for continued storage than when it was previously stored. If the product is too dry to allow fungal growth and it is kept dry, no further deterioration will occur. However, if there is insect or rodent activity, moisture migration, condensation, or water leaks, fungal growth and possible aflatoxin contamination will occur. Germination and growth of *A. flavus* require water activity greater than 0.85 and temperatures greater than 10°C (CAST, 2003). Sauer (1987) suggested that *A. flavus* does not grow at relative humidities below 85% or moisture contents below 16%. Nevertheless, as moisture increases even slightly above these levels, aflatoxin risks increases greatly. Besides, if moisture is adequate, aflatoxin can be produced at temperatures ranging from 11° to 40°C, with 25° to 35°C the optimum range. Studies by Lopez and Christensen (1967) on the effects of moisture and temperature on invasion of stored corn by *A. flavus* in the Midwest US found that *A. flavus* did not invade any samples of stored corn with moisture contents below 17.5%, wet weight basis, but did invade those stored with moisture contents of 18.5% and above. Furthermore, a storage temperature of 35°C did not reduce the moisture content limit for invasion of the corn by *A. flavus*.

Beti *et al*. (1995), investigating the role of corn weevils, *Sitophilus zeamais* Motschulsky, in enhancing aflatoxin B_1 content in stored corn, found that corn kernels infested with *A. flavus*-contaminated weevils had significantly higher levels of aflatoxin B_1 than *A. flavus*-inoculated corn without weevils. The presence of corn weevils resulted in increased kernel moisture content during incubation, and grain moisture was positively correlated with aflatoxin content across treatments receiving spores. They further concluded that corn weevils facilitated the growth of *A.*

flavus and aflatoxin production in corn by increasing surface area susceptible to fungal infection and increasing moisture content as a result of weevil metabolic activity. Therefore, insect and rodent control are important because their activity in stored corn creates favorable microclimates for fungal growth (CAST, 2003). Insect and rodent control in stored grain requires an integrated approach, including sanitation, good control of grain moisture and temperature, frequent monitoring, and chemical treatments (Munkvold, 2003).

It has been recognized that while plant stress, temperature, and insect damage play an important role on preharvest invasion and subsequent production of aflatoxins in either corn or peanut, moisture content and temperature are the most important factors in postharvest contamination. Therefore, controlling these factors accompanied by the development of simple and inexpensive methods to screen for aflatoxins may substantially reduce aflatoxin exposure to humans.

LITERATURE CITED

Anderson, H.W.; Nehring, E.W.; Wichser, W.R. Aflatoxin contamination of corn in the field. *J. Agric. Food Chem*. **1975;** 23:775-782.

ASAE Standards. Moisture relationships of grains. Hahn, R.H.; Rosentreter, E.E., Ed(s). St. Joseph, MI: The American Society of Agricultural Engineers, 1986. p. 63-67.

Beti, J.A.; Phillips, T.W.; Smalley, E.B. Effects of maize weevils (Coleoptora: Curculionidae) on production of aflatoxin B_1 by *Aspergillus flavus* in stored corn. *J. Econ. Entomol*. **1995**; 88: 1776-1782.

Betran, F.J.; Isakeit, T. Aflatoxin accumulation in maize hybrids of different maturities. *Agron. J.* **2004**; 96:565-570.

Bilgrami, K.S.; Choudhary, A.K. Mycotoxins in preharvest contamination of agricultural crops. In *Mycotoxins in Agriculture and Food Safety*. Sinha, K.K.; Bhatnagar, D., Ed(s). New York: Marcel Dekker, Inc., 1998. p. 1-43.

Blankenship, P.D.; Cole, R.J.; Sanders, T.H. Comparative susceptibility of four experimental peanut lines and the cultivar Florunner to preharvest aflatoxin contamination. *Peanut Sci.* **1985**; 12:70-72.

Blankenship, P.D.; Cole, R.J.; Sanders, T.H. Effect of geocarposphere temperature on preharvest colonization of drought stressed peanut by *Aspergillus flavus* and subsequent aflatoxin contamination. *Mycopathologia* **1984**; 85:69-74.

Brenneman, T.B.; Wilson, D.M.; Beaver, R.W. Effects of diniconazole on *Aspergillus* populations and aflatoxin formation in peanut under irrigated and nonirrigated conditions. *Plant Dis.* **1993**; 77:608-612.

Brown, R.L., Chen, Z.Y., Menkir, A., Cleveland, T.E., Cardwell, K., Kling, J., White, D.G. Resistance to aflatoxin accumulation in kernels of maize inbreds selected for ear rot resistance in West and Central Africa. *J. Food Prot.* **2001**; 64:396-400.

Bullerman, L.B. Mycotoxins and food safety. *Food Technol.* **1986**; 40:59-66.

Campbell, K.W.; White, D.G. An inoculation device to evaluate maize for resistance to ear rot and aflatoxin production by *Aspergillus flavus*. *Plant Dis*. **1994**; 78:778-781.

CAST. *Mycotoxins: Risks in Plant, Animal, and Human Systems*. Task force report (Council for Agricultural Science and Technology); no. 139. Ames, IA: Council for Agricultural Science and Technology, 2002. 199 p.

CAST. *Mycotoxins: Economic and Health Risks*. Task force report; no. 116. Ames, IA: Council for Agricultural Science and Technology, 1989. 91 p.

Christensen, C.M.; Kaufmann, H.H. *Grain storage: the role of fungi in quality loss*. Minneapolis: University of Minnesota Press, 1969. 153 p.

Cole, R.J. Preharvest aflatoxin in peanuts. *Int. Biodeterior.* **1989**; 25:253-257.

Cole, R.J.; Hill, R.A.; Blankenship, P.D.; Sanders, T.H. Color mutants of *Aspergillus flavus* and *Aspergillus parasiticus* in a study of preharvest invasion of peanuts. *Appl. Environ. Microbiol*. **1986**; 52:1128-1131.

Cole, R.J.; Sanders, T.H; Hill, R.A.; Blankenship, P.D. Mean geocarposphere temperatures that induce preharvest aflatoxin contamination of peanuts under drought stress. *Mycopathologia* **1985**; 91:41-46.

Cotty, P.J.; Cardwell, K.F. Divergence of West African and North American communities of *Aspergillus* Section *Flavi*. *Appl. Environ. Microbiol*. **1999**; 65:2264-2266.

Cullen, J.M.; Newberne, P.M. Acute hepatotoxicity of aflatoxins. In *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance*; Eaton, D.L.; Groopman, J.D., Ed(s). San Diego: Academic Press, Inc., 1994. p. 3-26.

Darrah, L.L.; Lillehoj, E.B.; Zuber, M.S.; Scott, G.E.; Thompson, D.; West, D.R.; Widstrom, N.W.; Fortnum, B.A. Inheritance of aflatoxin B_1 levels in maize kernels under modified natural inoculation with *Aspergillus flavus*. *Crop Sci*. **1987**; 27:869-872.

D'Mello, J.P.F.; MacDonald, A.M.C. Mycotoxins. *Anim. Feed Sci. Technol*. **1997**; 69:155-166.

Dorner, J.W.; Cole, R.J.; Connick, W.J.; Daigle, D.J.; McGuire, M.R.; Shasha, B.S. Evaluation of biological control formulations to reduce aflatoxin contamination in peanuts. *Biol. Control*. **2003**; 26:318-324.

Dorner, J.W.; Cole, R.J.; Blankenship, P.D. Use of a biocompetitive agent to control preharvest aflatoxin in drought stressed peanuts. *J. Food Prot.* **1992**; 55:888-892.

Dowell, F.; Smith, J., Jr. A note on high moisture content foreign material effects on aflatoxin in peanuts during storage. *Peanut Sci*. **1995**; 22:166-168.

Ellis, W.O.; Smith, J.P.; Simpson, B.K.; Oldham, J.H. Aflatoxins in food: occurrence, biosynthesis, effects on organisms, detection, and methods of control. *Crit. Rev. Food Sci. Nutri*. **1991**; 30:403-439.

Gilbert, J.; Anklam, E. Validation of analytical methods for determining mycotoxins in foodstuffs. *Trends in Analytical Chemistry*. **2002**; 21:468-486.

Gorman, D.P.; Kang, M.S. Preharvest aflatoxin contamination in maize: resistance and genetics. *Plant Breed*. **1991**; 107:1-10.

Holbrook, C.C.; Kvien, C.K.; Rucker, K.S.; Wilson, D.M.; Hook, J.E.; Matheron, M.E. Preharvest aflatoxin contamination in drought-tolerant and drought-intolerant peanut genotypes. *Peanut Sci*. **2000**; 27:45-48.

Holcomb, M.; Wilson, D.M.; Trucksess, M.W.; Thompson Jr., H.C. Determination of aflatoxins in food products by chromatography. *J. Chromatogr*. **1992**; 624:341-352.

Huwig, A.; Freimund, S.; Kappeli, O.; Dutler, H. Mycotoxin detoxification of animal feed by different adsorbents. *Toxicol. Lett*. **2001**; 122:179-188.

Ioannou-Kakouri, E.; Aletrari, M.; Christou, E.; Hadjioannou-Ralli, A.; Koliou, A.; Akkelidou, D. Surveillance and control of aflatoxins B1, B2, G1, G2, and M1 in foodstuffs in the Republic of Cyprus: 1992-1996. *J. AOAC Int.* **1999**; 82:883-892.

Jones, R.K.; Duncan, H.E. Effect of nitrogen fertilizer, planting date, and harvest date on aflatoxin production in corn inoculated with *Aspergillus flavus*. *Plant Dis*. **1981**, 65, 741-744.

Jones, R.K.; Duncan, H.E.; Hamilton, P.B. Planting date, harvest date, and irrigation effects on infection and aflatoxin production by *Aspergillus flavus* in field corn. *Phytopathology*. **1981**; 71:810-816.

Jones, R.K.; Duncan, H.E.; Payne, G.A.; Leonard, K.J. Factors influencing infection by *Aspergillus flavus* in silk-inoculated corn. *Plant Dis*. **1980**; 64:859-863.

Klich, M.A.; Pitt, J.I. Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. *Trans. Br. Mycol. Soc*. **1988**; 91:99-108.

Kubena, L.F.; Harvey, R.B.; Bailey, R.H.; Buckley, S.A.; Rottinghaus, G.E. Effects of a hydrated sodium calcium aluminosilicate (T-Bind™) on mycotoxicosis in young broiler chickens. *Poultry Sci*. **1998**; 77:1502-1509.

Leatherhead Food Research Association (2004, April 15). *The Aflatoxins*. Retrieved June 17,

2004 from "Micotoxinas" online on the World Wide Web:

http://www.micotoxinas.com.br/aflafacts.pdf.

Li, F.Q.; Yoshizawa, T.; Kawamura, O.; Luo, X.Y.; Li, Y.W. Aflatoxins and fumonisins in corn

from the high-incidence area for human hepatocellular carcinoma in Guangxi, China. *J. Agric.

Food Chem*. **2001**; 49:4122-4126.

Lillehoj, E.B.; Wall, J.H.; Bowers, E.J. Preharvest aflatoxin contamination: effect of moisture

and substrate variation in developing cottonseed and corn kernels. *Appl. Environ. Microbiol*.

1987; 53:584-586.

Lillehoj, E.B.; Kwolek, W.F.; Horner, E.S.; Widstrom, N.W.; Josephson, L.M.; Franz, A.O.;

Catalano, E.A. Aflatoxin contamination of preharvest corn: role of *Aspergillus flavus* inoculum

and insect damage. *Cereal Chem*. **1980**; 57:255-257.

Lopez, L.C.; Christensen, C.M. Effect of moisture content and temperature on invasion of stored

corn by *Aspergillus flavus*. *Phytopathology*. **1967**; 57:588-590.

Lynch, R.E.; Wilson, D.M. Enhanced infection of peanut, *Arachis hypogaea* L., seeds with

Aspergillus flavus group fungi due to external scarification of peanut pods by the lesser cornstalk

borer, *Elasmopalpus lignosellus* (Zeller). *Peanut Sci*. **1991**; 18:110-116.

Machinski Jr., M.; Soares, L.M.V.; Sawazaki, E.; Bolonhezi, D.; Castro, J.L.; Bortolleto, N. Aflatoxins, ochratoxin A and zearalenone in Brazilian corn cultivars. *J. Sci. Food Agric*. **2001**; 81:1001-1007.

Manzo, S.K.; Misari, S.M. Status and management of aflatoxins in groundnuts in Nigeria. In *Aflatoxin contamination of groundnuts: proceedings of the international workshop, 6-9 Oct. 1987, ICRISAT Center, India*. McDonald, D.; Mehan, V.K., Ed(s). Patancheru, A.P., India: ICRISAT, 1989. p. 77-90.

Marsh, S.F.; Payne, G.A. Preharvest infection of corn silks and kernels by *Aspergillus flavus*. *Phytopathology*. **1984**; 74:1284-1289.

McMillian, W.W.; Widstrom, N.W.; Beaver, R.W.; Wilson, D.M. Aflatoxin in Georgia: factors associated with its formation in corn. In *Research bulletin 599*, Iowa Agriculture and Home Economics Experiment Station. Ames, Iowa: The Station, 1991. p. 329-334.

McMillian, W.W.; Wilson, D.M.; Widstrom, N.W. Aflatoxin contamination of preharvest corn in Georgia: a six-year study of insect damage on visible *Aspergillus flavus*. *J. Environ. Qual*. **1985**; 14:200-202.

McMillian, W.W.; Widstrom, N.W.; Wilson, D.M.; Hill, R.A. Transmission by maize weevils of *Aspergillus flavus* and its survival on selected corn hybrids. *J. Econ. Entomol*. **1980**; 73:793-794.

Methenitou, G.; Maravelias, C.; Athanaselis, S.; Dona, A.; Koutselinis, A. Immunomodulative effects of aflatoxins and selenium on human natural killer cells. *Vet. Hum. Toxicol.* **2001**; 43:232-234.

Miller, J.D. Fungi and mycotoxins in grain: implications for stored product research. *J. Stored Prod. Res*. **1995**; 31:1-16.

Munkvold, G.P. Cultural and genetic approaches to managing mycotoxins in maize. *Annu. Rev. Phytopathol*. **2003**; 41:99-116.

Naidoo, G.; Forbes, A.M.; Paul, C.; White, D.G.; Rocheford, T.R. Resistance to Aspergillus ear rot and aflatoxin accumulation in maize F_1 hybrids. *Crop Sci*. **2002**; 42:360-364.

Palmgren, M.S.; Hayes, A.W. Aflatoxins in food. In *Mycotoxins in food*; Krogh, P., Ed. London; San Diego: Academic Press, 1987. p. 65-71.

Papp, E.; H-Otta, K.; Zaray, G.; Mincsovics, E. Liquid chromatographic determination of aflatoxins. *Microchem. J*. **2002**; 73:39-46.

Parmar, R.S.; McClendon, R.W.; Hoogenboom, G.; Blankenship, P.D.; Cole, R.J.; Dorner, J.W. Estimation of aflatoxin contamination in preharvest peanuts using neural networks. *Trans. ASAE*. **1997**; 40:809-811.

Payne, G.A. Aflatoxin in maize. *Crit. Rev. Plant Sci*. **1992**; 10:423-440

Payne, G.A.; Thompson, D.L.; Lillehoj, E.B.; Zuber, M.S.; Adkins, C.R. Effect of temperature on the preharvest infection of maize kernels by *Aspergillus flavus*. *Phytopathology*. **1988**; 78:1376-1380.

Payne, G.A.; Cassel, D.K.; Adkins, C.R. Reduction of aflatoxin contamination in corn by irrigation and tillage. *Phytopathology*. **1986**; 76:679-684.

Phillips, T.D. Dietary clay in the chemoprevention of aflatoxin-induced disease. *Toxicol. Sci*. **1999**; 52:118-126.

Pohland, A.E.; Wood, G.E. Occurrence of mycotoxins in food. In *Mycotoxins in food*; Krogh, P., Ed. London; San Diego: Academic Press, 1987. p. 37-40.

Rambo, G.W.; Tuite, J.; Crane, P. Preharvest inoculation and infection of dent corn ears with *Aspergillus flavus* and *A. parasiticus*. *Phytopathology*. **1974**; 64:797-800.

Sauer, D.B.; Meronuck, R.A.; Christensen, C.M. Microflora. In *Storage of cereal grains and their products*, 4th ed. Sauer, D.B., Ed. St. Paul, Minn.: American Association of Cereal Chemists, 1992. p. 313-340.

Sauer, D.B. Conditions that affect growth of *Aspergillus flavus* and production of aflatoxin in stored maize. In *Aflatoxin in maize*: a proceedings of the workshop, El Batan, Mexico, April 7-11, 1986 / sponsored by CIMMYT, UNDP, and USAID. Mexico, D.F., Mexico: International Maize and Wheat Improvement Center, 1987. p. 41-50.

Saul, R.A.; Steele, J.L. Why damaged shelled corn costs more to dry. *Agric. Eng*. **1966**; 47:326-329.

Siriacha, P.; Tanboon-Ek, P.; Buangsuwon, D. Aflatoxin in maize in Thailand. *ACIAR Proc*. **1991**; 36:187-193.

Smart, M.G.; Wicklow, D.T.; Caldwell, R.W. Pathogenesis in Aspergillus ear rot of maize: light microscopy of fungal spread from wounds. *Phytopathology*. **1990**; 80:1287-1294.

Sobolev, V.S.; Dorner, J.W. Cleanup procedure for determination of aflatoxins in major agricultural commodities by liquid chromatography. *J. AOAC Int*. **2002**; 85:642-645.

Spraque, G.F.; Tatum, L.A. General vs. specific combining ability in single crosses of corn. *J. Am. Soc. Agron*. **1942**; 34:923-932.

Stroka, J.; Anklam, E. New strategies for the screening and determination of aflatoxins and the detection of aflatoxin-producing moulds in food and feed. *Trends in analytical chemistry*. **2002**; 21:90-95.

Swindale, L.D. A general overview of the problem of aflatoxin contamination of groundnut. In *Summary and recommendations of the international Workshop on Aflatoxin Contamination of Groundnut, 6-9 Oct 1987, ICRISAT Center, India*; McDonald, D.; Mehan, V.K.; Hall, S.D., Ed(s). Patancheru, A.P., India: International Crops Research Institute for the Semi-Arid Tropics, 1987. p. 1-4.

Thompson, D.L.; Lillehoj, E.B.; Leonard, K.J.; Kwolek, W.F.; Zuber, M.S. Aflatoxin concentration in corn as influenced by kernel development stage and postinoculation temperature in controlled environments. *Crop Sci*. **1980**; 20:609-612.

Umeh, V.C.; Waliyar, F.; Traore, A. *Aspergillus* species colonization of termite-damaged peanuts in parts of West Africa and its control prospects. *Peanut Sci.* **2000**; 27:1-6.

Varga, J.; Rigo, K.; Toth, B.; Teren, J.; Kozakiewicz, Z. Evolutionary relationships among *Aspergillus* species producing economically important mycotoxins. *Food Technol. Biotechnol*. **2003**; 41:29-36.

Viquez, O.M.; Castell-Perez, M.E.; Shelby, R.A.; Brown, G. Aflatoxin contamination in corn samples due to environmental conditions, aflatoxin-producing strains, and nutrients in grain grown in Costa Rica. *J. Agric. Food Chem*. **1994**; 42:2551-2555.

Wicklow, D.T.; Horn, B.W.; Burg, W.R.; Cole, R.J. Sclerotium dispersal of *Aspergillus flavus* and *Eupenicillium ochrosalmoneum* from maize during harvest. *Trans. Br. Mycol. Soc*. **1984**; 83:299-303.

Widstrom, N.W.; McMillian, W.W.; Wilson, D.M. Segregation for resistance to aflatoxin contamination among seeds on an ear of hybrid maize. *Crop Sci*. **1987**; 27:961-963.

Widstrom, N.W.; Wilson, D.M.; McMillian, W.W. Aflatoxin contamination of preharvest corn as influenced by timing and method of inoculation. *Appl. Environ. Microbiol*. **1981**; 42:249-251.

Wilson, D.M.; Mubatanhema, W.; Jurjevic, Z. Biology and ecology of mycotoxigenic *Aspergillus* species as related to economic and health concerns. In *Mycotoxins and food safety*; DeVries, J.W.; Trucksess, M.W.; Jackson, L.S., Ed(s). (Advances in experimental medicine and biology; v. 504). New York: Kluwer Academic / Plenum Publishers, 2002. p. 3-17.

Wilson, D.M.; Payne, G.A. Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of crops. In *The toxicology of aflatoxins: human health, veterinary, and agricultural significance*; Eaton, D.L.; Groopman, J.D., Ed(s). San Diego: Academic Press, 1994. p. 309-325.

Wilson, D.M.; Walker, M.E.; Gascho, G.J. Some effects of mineral nutrition on aflatoxin contamination of corn and peanuts. In *Soilborne plant pathogens: management of diseases with macro- and microelements*; Engelhard, A.W., Ed. St. Paul, Minn.: APS Press, 1989. p. 137-151.

Wilson, D.M.; Stansell, J.R. Effect of irrigation regimes on aflatoxin contamination of peanut pods. *Peanut Sci*. **1983**; 10:54-56.

Windham, G.L.; Williams, W.P. Evaluation of corn inbreds and advanced breeding lines for resistance to aflatoxin contamination in the field. *Plant Dis*. **2002**; 86:232-234.

Woloshuk, C.P.; Cavaletto, J.R.; Cleveland, T.E. Inducers of aflatoxin biosynthesis from colonized maize kernels are generated by an amylase activity from *Aspergillus flavus*. *Phytopathology*. **1997**; 87:164-169.

Zummo, N.; Scott, G.E. Evaluation of field inoculation techniques for screening maize genotypes against kernel infection by Aspergillus flavus in Mississippi. *Plant Dis*. **1989**; 73:313-316.

CHAPTER 2

FLUORESCENCE ENHANCEMENT OF AFLATOXIN B_1 WITH CYCLODEXTRINS

USING A FLUOROMETER AS THE DETECTOR

AFLATOXIN DETECTION APPROACHES

There are usually three approaches for the detection of the aflatoxins namely by UV

absorption at about 365 nm, fluorescence detection in non-polar solvents, and fluorescence

detection in polar solvents. Seitz (1975) investigated the sensitivity of different UV absorptions

and fluorescence detection of aflatoxins in yellow corn. He found that detection was more

effective at 350 nm (or 365 nm used by other available detectors) than at 254 nm because it

provided greater selectivity for aflatoxins, was easier to establish and maintain a steady baseline,

and tolerated a variety of injection solvents. The fluorescence detector exhibited much greater

sensitivity for aflatoxins G_1 and G_2 than for B_1 and B_2. Emission from G_1 and G_2 (about 450 nm)

was nearer the wavelength at which the sensitivity of the detector was optimum. Emission from

aflatoxins B_1 and B_2, on the other hand, was closer to the lower wavelength limit (approximately

400 nm) of the detector.

Whereas UV detection at 365 nm used in normal phase high-performance liquid

chromatography (HPLC) affords peaks for all four aflatoxins, fluorescence detection (365 nm

excitation, > 415 nm emission) is more selective and has greater sensitivity for the B_2 and G_2

than for B_1 and G_1 (Knutti *et al.*, 1979; Joshua, 1993). Panalaks and Scott (1977) suggested the

use in series of UV detector for higher amounts of aflatoxins and a fluorimetric method for

quantification of aflatoxins at lower levels. However, the problem with fluorescence detection is

that the sensitivities for the four major aflatoxins (B_1, B_2, G_1, and G_2) in solution strongly depend on the composition of the solvent. For instance, in the chloroform or dichloromethane containing mobile phases originally used for normal-phase chromatography, the aflatoxins B_1 and B_2 show little fluorescence (Kok, 1994).

Dirr (1987) investigated the solvent-dependent changes in the spectral properties of aflatoxin B_1 in order to evaluate the effects of solvents of different polarities on the ground and excited singlet state energy levels of aflatoxin B_1. The absorption and emission maxima extended from 359 nm (in chloroform) to 365 nm (in water) and 407 nm (chloroform) to 440 nm (water), respectively, indicating that the excited state of aflatoxin B_1 is more sensitive to solvent effects than the ground state. In a given polar solvent, the native fluorescence capacity of the saturated aflatoxins B_2 and G_2 is much larger (ca. ten times) than that of the unsaturated aflatoxins B_1 and G_1 (Vazquez *et al.*, 1992). It is suggested that increasing solvent polarity may lead to the quenching of fluorescence intensity of aflatoxins B_1 and G_1 (Chang-Yen *et al.*, 1984).

Any variation in the purity of solvents that change their polarity significantly may affect both the fluorescence wavelength maxima and intensities of aflatoxins. Besides, maximum fluorescence intensity is observed for B_1, B_2, G_1 and G_2 in different solvent mixtures. Therefore, sensitivity of analysis may be improved by use of different systems giving the best sensitivity for a specific aflatoxin. For example, according to Chang-Yen *et al.* (1984), the fluorescence intensity of B_1 in a 50% methanol in chloroform solution is more than 12 times that in pure chloroform and more than 6 times that in pure methanol. B_2 is over 6 times more fluorescent in pure methanol than in chloroform, and G_1 fluorescence intensity in chloroform is over 27 times that in methanol. Manabe *et al.* (1978) observed that HPLC detection of aflatoxins in nanogram levels by fluorescence emission had been possible only with G_1 and G_2 if chloroform or

dichloromethane was used for the mobile phase. Aflatoxins B_1 and B_2, however, under these conditions are barely detected because their fluorescence is markedly quenched in the mobile phase, while the fluorescence of G_1 and G_2 is not (Panalaks and Scott, 1977).

On the other hand, in reversed-phase HPLC analysis of aflatoxins using fluorescence detection, aflatoxins B_2 and G_2 fluoresce naturally in aqueous solution while aflatoxins B_1 and G_1 do not (Holcomb *et al.*, 1991). The natural fluorescence of aflatoxins arises from the oxygenated pentaheterocyclic structure of these coumarin derivatives, which involves different luminescence characteristics in solution creating a clear distinction between furano-saturated derivatives (B_2 and G_2) and furano-unsaturated derivatives (B_1 and G_1) (Vazquez *et al.*, 1992).

Vazquez *et al.* (1991) had previously noted that the saturation of the 8,9- or 9,10-double bond in the furan ring (aflatoxins B_2 and G_2, respectively) is of major importance with respect to the luminescence properties, with these compounds exhibiting relative quantum fluorescence values 10-40 times higher, depending on the solvent, than the corresponding furan-unsaturated aflatoxins B_1 and G_1. Unfortunately, the latter are more carcinogenic toxins, especially B_1. Hence, many attempts have been made to improve the detection limits of the aflatoxins in liquid chromatography. Improved fluorescence detection of aflatoxins B_1 and G_1 by reversed-phase liquid chromatographic analysis is accomplished by conversion to the corresponding hemiacetal aflatoxins B_{2a} and G_{2a}, respectively (Orti *et al*, 1989). When using aqueous solvents aflatoxins B_1 and G_1 can be derivatized to increase their fluorescence intensity by iodine, trifluoracetic acid, or bromine reaction with the double bond (Cepeda *et al.*, 1996; Jansen *et al.*, 1987). The resulting derivatives have fluorescence intensities similar to aflatoxins B_2 and G_2 (Davis and Diener, 1980).

Thorpe *et al*. (1982) showed that the fluorescence of aflatoxins B_1 and G_1 could be enhanced after reversed-phase separations by post-column derivatization with aqueous iodine. They demonstrated that a 50-fold increase in fluorescence of aflatoxins B_1 and G_1 could be obtained without affecting the response of aflatoxins B_2 and G_2. Tuinstra and Haasnoot (1983) also suggested a 50-fold increase in fluorescence of aflatoxin B_1 treated with a saturated aqueous solution of iodine, compared to the original aflatoxin B_1 fluorescence in reversed-phase HPLC systems. Thiel *et al*. (1986) determined aflatoxin concentrations in corn, peanut butter, sorghum malt and duckling mash extracts by reverse phase HPLC and fluorescence detection incorporating post-column derivatization with iodine. The procedure was found to be sensitive and reproducible with a dramatic increase in fluorescence of aflatoxins B_1 and G_1 of the same order (50 times) as reported by other authors. Jansen *et al*. (1987) found a 20-fold increase in the fluorescence intensity of the aflatoxins B_1 and G_1 in the post-column addition of iodine to a HPLC mobile phase by the use of a solid-phase iodine column mounted in parallel with the analytical column. Beaver *et al*. (1990) used post-column iodine derivatization to quantify aflatoxins by liquid chromatography in naturally contaminated corn samples. Shepherd and Gilbert (1984) investigated the conditions favoring the enhancement of aflatoxin B_1 fluorescence by HPLC post-column iodination.

Bromine (Kok *et al*., 1986) and trifluoracetic acid (Tarter *et al*., 1984) have also been used to enhance the fluorescence signal of aflatoxins by chemical derivatization in order to improve the limit of detection of the native fluorescence of aflatoxins B_1 and G_1. This approach utilizes the reaction of the 8,9- or 9,10-furan double bond of unsaturated aflatoxins (B_1 or G_1, respectively) with bromine or trifluoracetic acid (Vazquez *et al*., 1991, Dunne *et al*., 1993) to convert these compounds to aflatoxin B_{2a} or G_{2a}, respectively, which are known to be highly

fluorescent in hydrogen-bonded solvents (Diebold and Zare, 1977) and much less toxic than B_1 or G_1 (Pons *et al*., 1972). In a collaborative study involving 16 European countries, aflatoxins were quantified in peanut butter, pistachio paste, fig paste, and paprika powder by reversed-phase liquid chromatography with post-column derivatization involving bromination. The method showed acceptable within-laboratory and between-laboratory precision for all four matrixes (Stroka *et al*., 2000).

Although good results are reported with iodine, trifluoracetic acid, and bromine, there is still a need to search for an alternative derivatization. Iodine, trifluoracetic acid and bromine solutions are not stable and have to be prepared freshly every day (Jansen *et al*., 1987; Traag *et al*., 1987; Kok *et al*., 1986). The derivatives are also unstable and rapidly degrade losing the fluorescence properties. These disadvantages may be overcome by the possibility of using cyclodextrins as more convenient fluorescence enhancers with the aim of including the aflatoxins in the hydrophobic internal cavity of cyclodextrins (Vazquez *et al*., 1992).

CYCLODEXTRINS

Cyclodextrins have been known for more than a century. In 1881, Villiers first produced cyclodextrins by digesting starch with *Bacillus amylobacter* and in 1903, Schardinger demonstrated the cyclic structure of these compounds (Singh *et al*., 2002).

Enzymatic degradation of starch generally results in the production of a long series of linear or branched chain malto-oligomers such as glucose, maltose, maltotriose, etc., known as dextrins. This type of starch degradation is a true hydrolytic process, as the primary product from the splitting of the glycosidic linkage reacts with one molecule of water. If however, the starch is degraded by the glucosyltransferase enzyme, the primary product of the chain splitting undergoes an intramolecular reaction without the participation of a water molecule and α-1,4-

linked cyclic products are formed. These products are known as cyclodextrins (Szejtli, 1988). The cyclodextrin glucosyltransferase enzyme is produced by various microorganisms such as *Bacillus macerans*, or *B. circulans* (Francis *et al*., 1988, Singh *et al*., 2002).

The cyclodextrins are macrocyclic, non-reducing malto-oligosaccharides, toroidal-shaped molecules composed of glucose units. The cyclodextrins are naturally occurring water-soluble glucans. The readily available cyclodextrins contain six, seven, or eight glucopyranose units in an α(1-4) configuration, according to which they are named α-, β-, or γ-cyclodextrin (Figure 2) (Saenger and Steiner, 1998; Armspach *et al*., 1994; Szejtli, 1982). The origin of the cyclodextrin glycosyltransferase enzyme will determine the ratio of the different cyclodextrins (Horikoshi and Akiba, 1982; Szejtli, 1988). The α: β: γ cyclodextrin ratio of cyclodextrin production is as follows: cyclodextrin glucosyltransferase enzyme from *B. macerans* (2.7 : 1.0 : 1.0), from *B. megaterium* (1.0 : 2.4 : 1.0), and from Bacillus Sp. No. 38-2 (1.0 : 11.0 : 1.5), respectively.

However, Bender (1983) noted that these ratios should not be considered as fixed values. Most cyclodextrin glycosyltransferase enzymes will initially form α-cyclodextrin, the rate of the formation of the higher cyclodextrins being much slower. The β-cyclodextrin scarcely participates in the reverse reactions, and therefore accumulates at the cost of α-cyclodextrin in the course of secondary transfer reactions. Accordingly, only the incubation time determines which cyclodextrin is obtained as the main product.

Sundararajan and Rao (1970) demonstrated by conformation-energy map calculations that cyclodextrins with less than six ring members (glucopyranose units) cannot be formed for steric reasons. The higher cyclodextrin homologues, i.e. δ-, ε-, ς-, and η-cyclodextrins were also reported to exist by Pulley and French (1961). The δ-cyclodextrin is a ring that consists of nine

glucose units. However, this and cyclodextrins with more than nine units have not yet been

characterized satisfactorily (Szejtli, 1988).
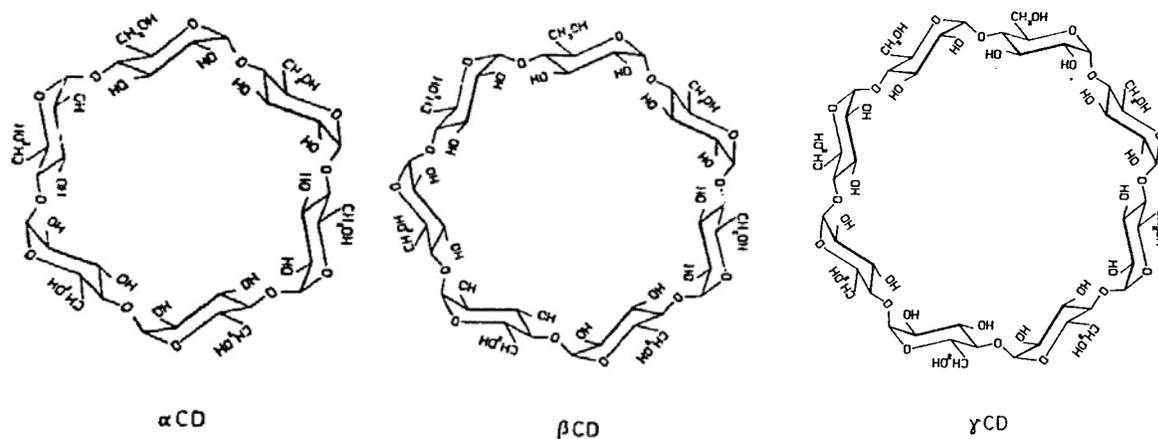
Figure 2. Structures of α-, β-, and γ-cyclodextrins: naturally tailored host molecules (source:

Szejtli, 1988).

Cyclodextrins are inexpensive and are produced in large quantities (Armstrong, 1988; Szejtli,

1985). Their exterior is relatively hydrophilic with a polar character due to the hydroxyl groups

present (Cepeda-Saez *et al*., 1988), thus the cyclodextrins are fairly soluble in water. In contrast, their

cavity is relatively hydrophobic, allowing them to include nonpolar molecules of appropriate

dimensions and bind them through hydrophobic interactions (Buchanan *et al*., 2002; Szejtli *et al*.,

1987; Harada *et al.*, 1977). No covalent bonds are broken or formed during formation of the inclusion

complex (Schneiderman and Stalcup, 2000). The main driving force for complex formation is the

release of enthalpy-rich water molecules from the cavity. These water molecules are displaced by

more hydrophobic guest molecules present in the solution to attain a hydrophobic association and

decrease of cyclodextrin ring strain resulting in a more stable lower energy state (Szetjli, 1998; Singh *et al*., 2002).

Cyclodextrins are currently used in a large number of applications where they can change, enhance, and improve a variety of analytical techniques (Armstrong, 1988; Hoshino *et al*., 1981). In HPLC, the cyclodextrins or highly soluble methylated cyclodextrins in the mobile phase, as well as the silica-bonded cyclodextrins as stationary phase, have been successfully used (Armstrong, 1988; Szejtli *et al*., 1987). In affinity chromatography, they are either immobilized or are dissolved in the eluent (Szejtli *et al*., 1987). The α-cyclodextrin and the soluble β-cyclodextrin polymers have been studied as components of the mobile phase in TLC (Armstrong, 1988; Szejtli *et al*., 1987). Besides cyclodextrin or cyclodextrin derivatives, acetylated or methylated cyclodextrins may function as highly effective and specific stationary phases (Szejtli *et al*., 1987). Cyclodextrins are stable within a large pH range, resist photolysis, and do not absorb in the UV ranges commonly used in chromatographic detection (Sybilska, 1987).

As fluorescence enhancers, cyclodextrins are mainly post-column additives to improve aflatoxin detection (Chiavaro *et al*., 2001). The use of cyclodextrins in a post-column reaction system has been developed to improve the detection limit of aflatoxin B_1, owing to the high fluorescence enhancement obtained with β-cyclodextrin derivatives (Franco *et al*., 1998). However, β-cyclodextrin is currently the most widely available and of the lowest cost (Singh *et al*., 2002; Buchanan *et al*., 2002), and its methylated derivatives provide an excellent cavity for aflatoxins to form aflatoxin-β-cyclodextrin inclusion complexes (Fente *et al*., 2001). In addition, the transparency of β-cyclodextrin allows the partially encased aflatoxin to be excited by UV irradiation and enhance the weak fluorescence exhibited by aflatoxins B_1 and G_1 in aqueous systems.

A substantial enhancement of the fluorescence emission has been reported for aflatoxins with an unsaturated furan ring, while the emission properties of aflatoxins with a saturated furan ring have been shown to remain practically unchanged. Francis *et al.* (1988) developed a unique reverse-phase liquid chromatographic method for quantification of aflatoxins B_1, B_2, G_1, and G_2 in corn without preparing derivatives of B_1 and G_1. Using a mobile phase of methanol-β-cyclodextrin (1+1), the aflatoxins were resolved on a C_{18} column. Fluorescence responses for B_1 and G_1 standards were linear over the concentration range 0.5-10 ng, yielding correlation coefficients of 0.9989 and 1.000, respectively.

In a study on fluorimetric detection of aflatoxins B_1, Q_1 and P_1 using heptakis-di-*O*-methyl-β-cyclodextrin as a post-column HPLC reagent, the fluorescence enhancement achieved was 37- and 27-fold for aflatoxins Q_1 and B_1, respectively, whereas the aflatoxin P_1 signal was increased about 2-fold (Vazquez *et al.*, 1999). Seidel *et al.* (1993) developed a reversed-phase high-performance liquid chromatography method for simultaneous quantification of ochratoxin A and zearalenone in corn with fluorescence detection and beta-cyclodextrin as a mobile phase additive.

Current studies with cyclodextrins are often focused on analytical methods to quantify aflatoxins with liquid chromatographic methods. Due to the high toxicity of aflatoxin B_1 at low concentrations, a method useful for trace analysis is needed. In this study, beta-cyclodextrin, dimethyl-beta-cyclodextrin, and succinyl-(2-hydroxy)propyl-beta-cyclodextrin were investigated to determine if they would enhance fluorescence of aflatoxin B_1 using a fluorometer as the detection mechanism. The present study is of importance because the use of relatively inexpensive and stable beta-cyclodextrins as reagents to enhance the fluorescence of aflatoxins B_1 and G_1, may make it possible to devise a less expensive method to screen aflatoxins using a fluorometer as a detector.

MATERIALS AND METHODS

Apparatus

Vicam Series 4 Fluorometer equipped with a printer, a silicon detector, a broad

wavelength pulsed xenon lamp, selected source filters (365-380 nm excitation, 450-550 nm

emission) and RS-232 output for a printer was obtained from Source Scientific Systems Inc.,

Garden Grove, CA. MLA^{TM} pipettes (500 μL) were obtained from Medical Laboratory

Automation, Inc., 270 Marble Avenue, Pleasantville, NY. Disposable polypropylene (MLA^{TM})

pipette tips (200 – 1000 μL) were purchased from VistaLab^{TM} Technologies, Inc., 27 Radio

Circle Drive, Mt. Kisco, NY. Disposable culture tubes- Durex^{TM} Borosilicate Glass, size: 12 x

75mm were purchased from VWR Scientific Products, West Chester, PA. Fisher vortex mixer

(Model G-560) was purchased from Scientific Industries, Inc., Bohemia NY.

Chemicals

All reagents were of analytical reagent grade. Beta-cyclodextrin hydrate and dimethyl-

beta-cyclodextrin were purchased from ACROS Organics, NJ, and succinyl-(2-hydroxy)propyl-

beta-cyclodextrin from Sigma-Aldrich, St. Louis, MO. Aflatoxin B$_1$ from *Aspergillus flavus* was

purchased from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO. Afla Test$^®$ Developer

(50mL concentrated solution) containing 0.03% bromine and mycotoxin standards for the

fluorometer calibration with red calibration standard, green calibration blank and yellow

calibration standard were purchased from VICAM, 313 Pleasant Street, Watertown, MA 02172

USA. HPLC grade methanol and water were obtained from Fisher Scientific, Fair Lawn, NJ.

Precautions

"Aflatoxins should be handled as very toxic substances. Perform manipulations under

hood whenever possible, and take particular precautions, such as use of glove box, when toxins

are in dry form because of electrostatic nature and resulting tendency to disperse in working

areas. Swab accidental spills of toxin with 1% NaOCl bleach, leave 10 min, and then add 5%

aqueous acetone. Rinse all glassware exposed to aflatoxins with methanol, add 1% NaOCl

solution, and after 2 h add acetone to 5% of total volume. Let react 30 min and then wash

thoroughly" (AOAC, 1995).

Methods

The solubility of β-cyclodextrin is 1.85 g/100mL at ambient temperature, whereas the α-

and γ-cyclodextrins are considerably higher: 14.5 and 23.2 g/100mL, respectively (Wiedenhof

and Lammers, 1967; Wiedenhof and Lammers, 1968; French *et al*., 1949). The lower solubility

of β-cyclodextrin (Figure 3) is a consequence of a less favorable (more positive) enthalpy of

solution and a less favorable (more negative) entropy of solution. In addition, the solubility of

the anhydrous forms of β-cyclodextrin is greater than that of the hydrates and is positively

associated with the temperature (Jozwiakowski and Connors, 1985). Therefore, after mixing with

water, beta-cyclodextrin hydrate was heated to increase the solubility. There was no need,

however, to heat dimethyl-beta-cyclodextrin and succinyl-(2-hydroxy)propyl-beta-cyclodextrin

solutions due to their high solubility in water (Chiavaro *et al*., 2001). The working solutions were

prepared by dissolving 600mg of the selected cyclodextrin in 100mL water as the recommended

dilution ratio of the cyclodextrin reagents.

Solubility of cyclodextrins at various temperatures[a]
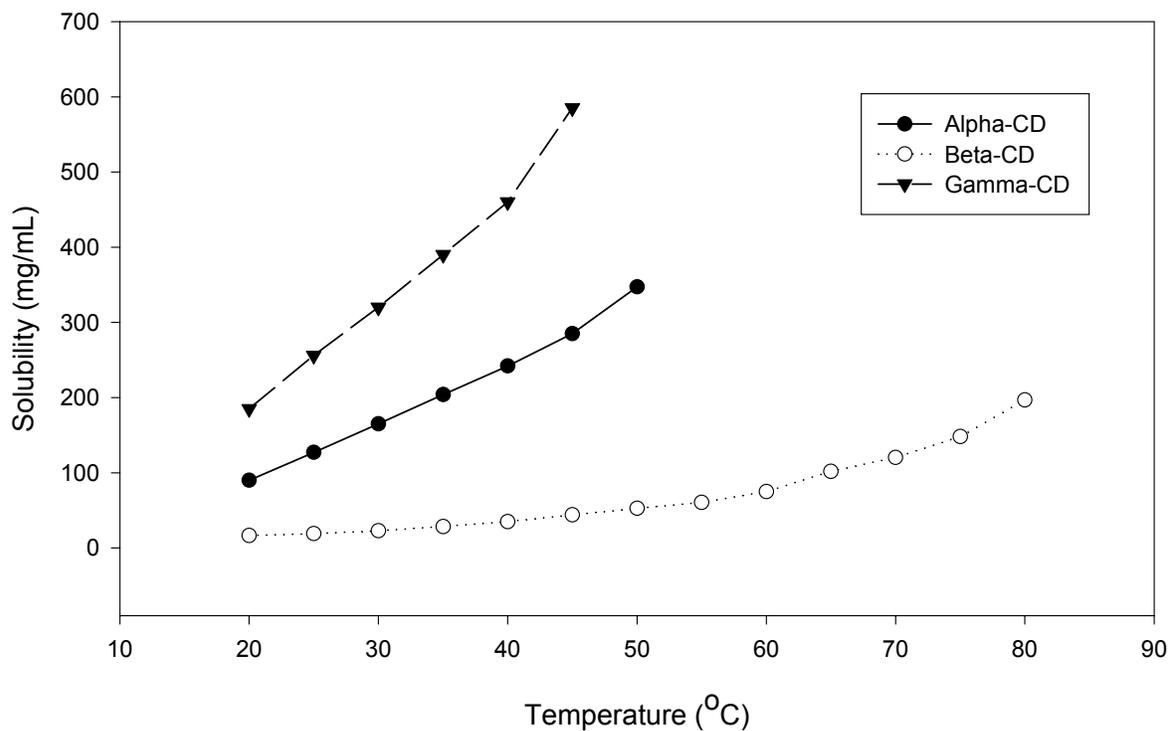
[a] Data from Szejtli (1988)

Figure 3. Solubility of α-, β-, and γ-cyclodextrins in water at various temperatures.

To make working solution of bromine (0.003%), 5mL of concentrated developer (50mL concentrated solution, containing 0.03% bromine) were mixed with 45mL-distilled water.

The aflatoxin B_1 standard was serially diluted with methanol to give concentrations of 10, 5, 2.5, 0.5 and 0.05 μg/mL. The stock solutions were protected from light by aluminum foil and stored at 4° C for further analyses when necessary. Then, 1mL of working solution developer

(cyclodextrins or bromine) was added to 1mL of stock standard solution of aflatoxin B_1 in a glass vial and mixed with a vortex mixer before measurement with the calibrated fluorometer.

Fluorescence

All the measurements of fluorescence of the aflatoxin B_1 standard plus cyclodextrins or bromine were made with a calibrated Vicam Series 4 Fluorometer. The fluorometer was calibrated prior to use with red calibration standard and green calibration blank. A control calibrator (yellow calibration standard) was used to ensure accuracy of the fluorometer and 240 parts per billion value was recorded as indicated in the instructions for calibration (Appendix 1). Fluorometer calibration standards are made to use with Afla Test-P[TM], Afla B[TM], DON Test[TM], Ochra Test[TM] and Zearala Test[TM] Columns. After a sample was inserted into the fluorometer, a 60-second delay occurred before the measurement was made and the result printed.

RESULTS

Fluorometer Response to Cyclodextrin and Bromine Reagents

The response of the calibrated fluorometer to working solutions of bromine, β-cyclodextrin, dimethyl-beta-cyclodextrin and succinyl-(2-hydroxy)propyl-beta-cyclodextrin with different concentrations of aflatoxin B_1 standard was investigated. Bromine working solution is the common developer used for the derivatization of aflatoxin B_1 and G_1 for the detection of aflatoxins with a fluorometer using immunoaffinity methods. Therefore, bromine developer was used as a control. The results showed an increase of the fluorescence response with increased concentrations of aflatoxin B_1 for all four developers (Figure 4). β-Cyclodextrin and succinyl-(2-hydroxy)propyl-beta-cyclodextrin working solutions showed higher fluorescence enhancement of aflatoxin B_1 compared to the bromine. Moreover, dimethyl-β-cyclodextrin working solution showed even better fluorescence enhancement than β-cyclodextrin and succinyl-(2-

hydroxy)propyl-beta-cyclodextrin. However, no practical differences were noted on fluorescence

enhancement of aflatoxin B_1 between β-cyclodextrin and succinyl-(2-hydroxy)propyl-beta-

cyclodextrin developers for concentrations less than 2.5 µg/mL of aflatoxin B_1 (Figure 4).

Fluorometer response to different reagents
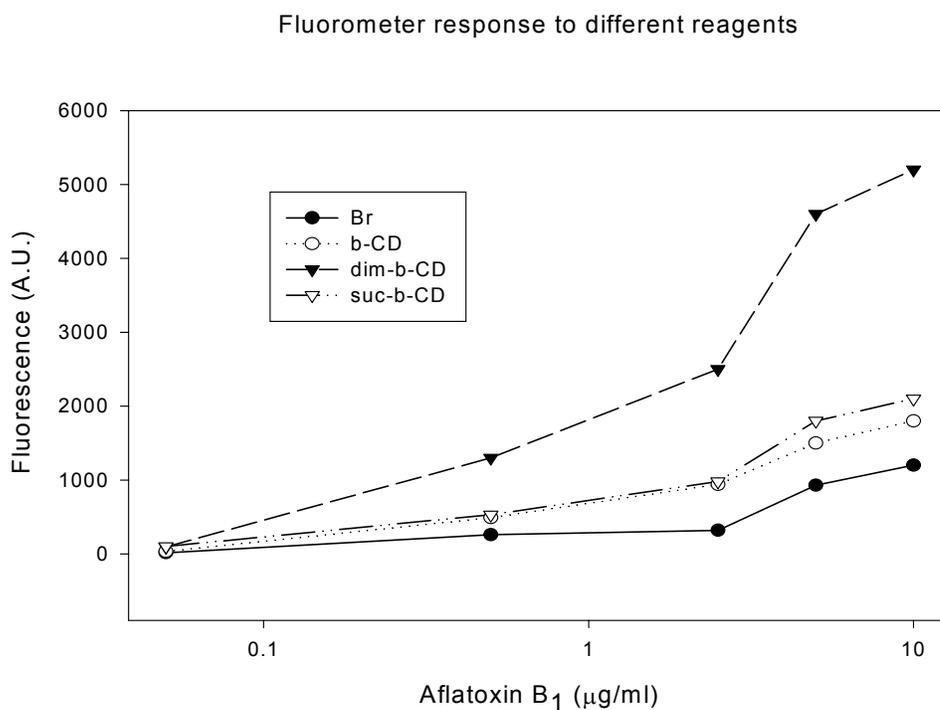
Figure 4. Effect of different concentrations of aflatoxin B_1 standard on fluorescence response

with bromine (Br), beta-cyclodextrin (b-CD), dimethyl-beta-cyclodextrin (dim-b-CD) and

succinyl-(2-hydroxy)propyl-beta-cyclodextrin (suc-b-CD).

Fluorometer Response to Bromine Over Time

Bromine developer has the disadvantage of being unstable in aqueous solutions and has to be prepared freshly every day (Jansen *et al.*, 1987; Kok *et al.*, 1986). In this study, stability of the bromine derivative over time was investigated and compared to the performance of cyclodextrins. The result showed a smooth decline in fluorescence response during the first 2 minutes (Figure 5a), and a more pronounced decline in fluorescence response during the subsequent period of evaluation (Figure 5b).

(a)   (b)
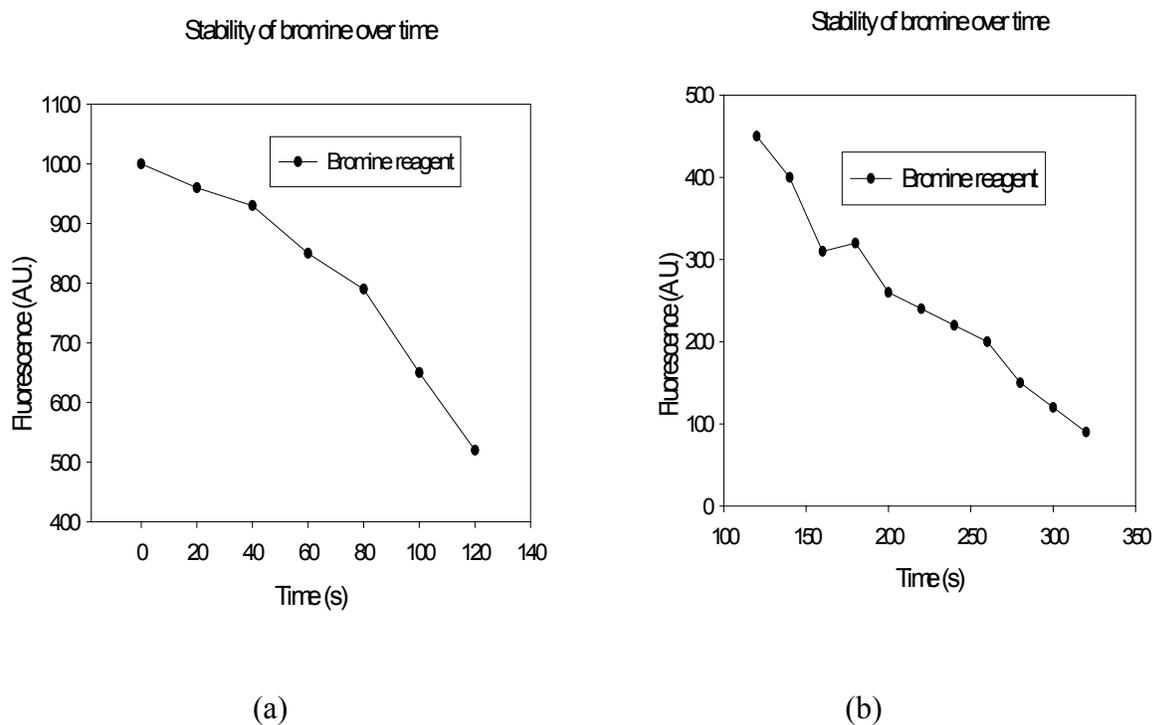
Figure 5. Effect of bromine on initial (a) and prolonged (b) fluorescence response.

Fluorometer Response to Cyclodextrins and Bromine Over Time

The results from stability of fluorescence enhancers of aflatoxin B_1 over time showed a lower fluorescence enhancement with the bromine reagent than cyclodextrins. Moreover, the fluorescence response to bromine working solution decreased over time to an almost insignificant response after three hours. Beta-cyclodextrin hydrate, dimethyl-beta-cyclodextrin and succinyl-(2-hydroxy)propyl-beta-cyclodextrin working solutions not only showed higher fluorescence response, but also a more stable response over time than bromine. In addition, dimethyl-β-cyclodextrin reagent showed a much higher fluorescence response compared to other developers (Figure 6).
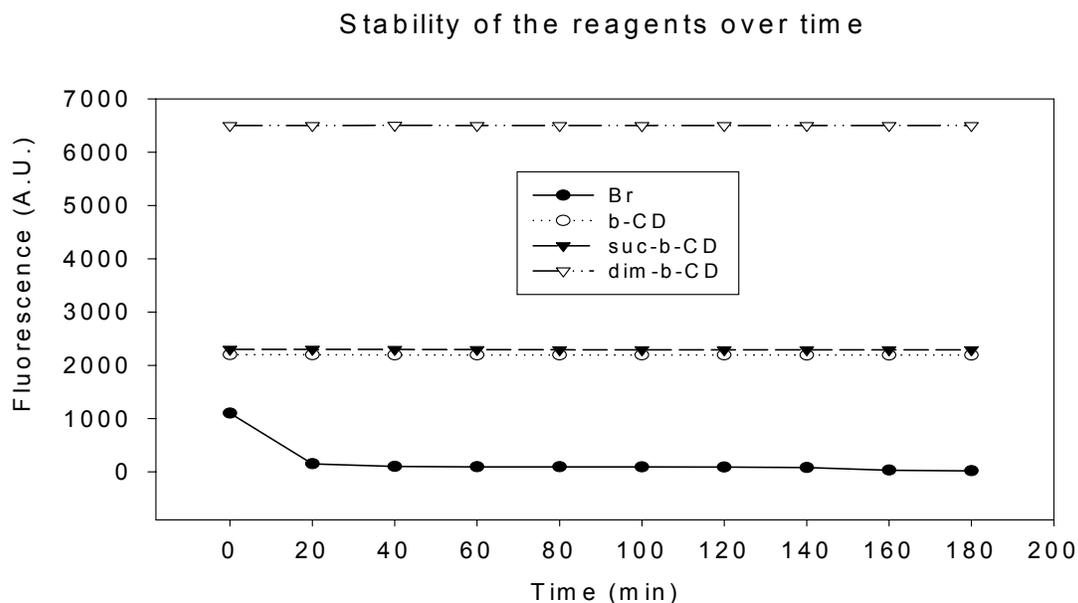
Figure 6. Effect of time on fluorescence stability of aflatoxin B_1 with bromine (Br), beta-cyclodextrin (b-CD), dimethyl-beta-cyclodextrin (dim-b-CD) and succinyl-(2-hydroxy)propyl-beta-cyclodextrin (suc-b-CD).

DISCUSSION

Methods to detect aflatoxins in food and feedstuffs rely on fluorescence of the aflatoxins. However, aflatoxins B_1 and G_1 have poor natural fluorescence in aqueous solutions. Therefore, their fluorescence enhancement is important for detection. The bromine enhancer, although widely used in immunoaffinity and liquid chromatographic methods, has the disadvantage of the reaction product being unstable in aqueous systems. In this study beta-cyclodextrin, dimethyl-beta-cyclodextrin and succinyl-(2-hydroxy)propyl-beta-cyclodextrin all showed higher enhancement of fluorescence of aflatoxin B_1 than bromine with concentrations of aflatoxin B_1 ranging from 0 to 10 μg/mL (Figure 3). Francis *et al*. (1988) investigating β-cyclodextrin post-column fluorescence enhancement of aflatoxins in corn, also found that the complexes formed with aflatoxins B_1 and G_1 showed a strong increase in the intensity of fluorescence.

Studies by Chiavaro *et al*. (2001) on effect of succinyl-β-cyclodextrin, dimethyl-β-cyclodextrin and β-cyclodextrin on the fluorescence of aflatoxins B_1, B_2, G_1, G_2 and M_1 showed enhanced fluorescence of aflatoxins B_1 and M_1 with succinyl-β-cyclodextrin while dimethyl-β-cyclodextrin showed better results for aflatoxin G_1. Based on the results, a new reversed-phase HPLC method for detecting aflatoxins B_1, B_2, G_1, G_2 and M_1 was developed using cyclodextrins directly dissolved in the liquid chromatography eluent. Chromatographic responses of aflatoxins B_1 and G_1 achieved using β-cyclodextrin dissolved in the mobile phase were enhanced 8- and 12-fold, respectively, and 10- and 15-fold with succinyl-β-cyclodextrin. In the present study, dimethyl-β-cyclodextrin showed higher fluorescence enhancement of aflatoxin B_1 than did β-cyclodextrin or succinyl-(2-hydroxy)propyl-beta-cyclodextrin. It is important to note however, that results from Chiavaro *et al*. (2001) were based on chromatographic method, while in this study a fluorometer was used to measure the fluorescence response.

Results from this study (Figure 4) show that succinyl-(2-hydroxy)propyl-beta-cyclodextrin and dimethyl-β-cyclodextrin reagents allowed more fluorescence enhancement of aflatoxin B_1 than did β-cyclodextrin. Harada *et al*. (1977) noted that derivatives of β-cyclodextrin result in an even higher fluorescence-enhancing effect than β-cyclodextrin. They suggested that an explanation for this phenomenon might be that, as a consequence of complex formation, the rotation of the guest molecule is hindered, and the relaxation of the solvent molecules is considerably decreased.

The decreased fluorescence response of aflatoxin B_1 over time with bromine reagent corroborates previous findings that bromine stock solutions and reaction products are unstable in aqueous systems (Jansen *et al*., 1987; Kok *et al*., 1986). On the other hand, β-cyclodextrin, succinyl-(2-hydroxy)propyl-beta-cyclodextrin and dimethyl-β-cyclodextrin stock solutions showed stability in fluorescence enhancement over time (Figure 6). Tests were performed with β-cyclodextrin and its derivative working solutions stored for a week at 4° C and no significant reduction in fluorescence enhancement was observed (results not shown). Therefore, the much higher effect and increased stability of β-cyclodextrin, succinyl-(2-hydroxy)propyl-beta-cyclodextrin and dimethyl-β-cyclodextrin stock solutions on fluorescence enhancement of aflatoxin B_1 using a fluorometer should be an important consideration in developing an inexpensive and less complex method to screen for aflatoxins in food and feedstuffs.

LITERATURE CITED

AOAC. Official methods of analysis of AOAC International. 16th Edition, Volume II. Food composition, additives, natural contaminants. Cunniff, P., Ed. Arlington, Virginia: AOAC International, 1995. Chapter 49.

Armspach, D.; Ashton, P.R.; Spencer, N.; Stoddard, J.F.; Williams, D.J. Cyclodextrins: 'Linking lampshades'. *Pestic. Sci*. **1994**; 41:232-235.

Armstrong, D.W. Cyclodextrins in catalysis and analytical chemistry. In *Proceedings of the Fourth International Symposium on Cyclodextrins*; Huber, O.; Szejtli, J., Ed(s). Dordrecht, The Netherlands: Kluwer Academic Publishers, 1988. p. 437-449.

Beaver, R.W.; Wilson, D.M.; Trucksess, M.W. Comparison of postcolumn derivatization-liquid chromatography with thin-layer chromatography for determination of aflatoxins in naturally contaminated corn. *J. Assoc. Off. Anal. Chem*. **1990**; 73:579-581.

Bender, H. Cyclodextrin glycosyltransferases from *Klebsiella pneumoniae* M 5 al and *Bacillus macerans*: quantitative analysis by high-performance liquid chromatography of the $(1 \rightarrow 4)$-α-D-glucopyranosyl transfer-products from some linear and cyclic substrates. *Carbohydr. Res*. **1983**; 117:1-11.

Buchanan, C.M.; Alderson, S.R.; Cleven, C.D.; Dixon, D.W.; Ivanyi, R.; Lambert, J.L.; Lowman, D.W.; Offerman, R.J.; Szejtli, J.; Szente, L. Synthesis and characterization of water-soluble hydroxybutenyl cyclomaltooligosaccharides (cyclodextrins). *Carbohydr. Res*. **2002**; 337:493-507.

Cepeda, A.; Franco, C.M.; Fente, C.A.; Vazquez, B.I.; Rodriguez, J.L.; Prognon, P.; Mahuzier, G. Postcolumn excitation of aflatoxins using cyclodextrins in liquid chromatography for food analysis. *J. Chromatogr. A* **1996**; 721:69-74.

Cepeda-Saez, A.; Prognon, P.; Mahuzier, G.; Blais, J. Improvement in the fluorimetric detection of 5-methoxypsoralen by using β-cyclodextrin in the mobile phase and a cross-linked β-cyclodextrin column. *Anal. Chim. Acta*. **1988**; 211:333-337.

Chang-Yen, I.; Stoute, V.A.; Felmine, J.B. Effect of solvent composition on aflatoxin fluorescence. *J. Assoc. Off. Anal. Chem*. **1984**; 67:306-308.

Chiavaro, E.; Dall' Asta, C.; Galaverna, G.; Biancardi, A.; Gambarelli, E.; Dossena, A.; Marchelli, R. New reversed-phase liquid chromatographic method to detect aflatoxins in food and feed with cyclodextrins as fluorescence enhancers added to the eluent. *J. Chromatogr. A* **2001**; 937:31-40.

Davis, N.D.; Diener, U.L. Confirmatory test for the high-pressure liquid chromatographic determination of aflatoxin B_1. *J. Assoc. Off. Anal. Chem*. **1980**; 63:107-109.

Diebold, G.J.; Zare, R.N. Laser fluorimetry: Subpicogram detection of aflatoxins using high-pressure liquid chromatography. *Science*. **1977**; 196:1439-1441.

Dirr, H.W. Solvent effects on the spectroscopic properties of aflatoxin B_1. *Int. J. Biochem.* **1987**; 19:1137-1140.

Dunne, C.; Meaney, M.; Smyth, M.; Tuinstra, L.G.M.Th. Multimycotoxin detection and clean-up method for aflatoxins, ochratoxin and zearalenone in animal feed ingredients using high-performance liquid chromatography and gel permeation chromatography. *J. Chromatogr.* **1993**; 629:229-235.

Fente, C.A.; Ordaz, J.J.; Vazquez, B.I.; Franco, C.M.; Cepeda, A. New additive for culture media for rapid identification of aflatoxin-producing *Aspergillus* strains. *Appl. Environ. Microbiol.* **2001**; 67:4858-4862.

Francis, O.J.; Kirschenheuter, G.P.; Ware, G.M.; Carman, A.S.; Kuan, S.S. β-cyclodextrin post-column fluorescence enhancement of aflatoxins for reverse-phase liquid chromatographic determination in corn. *J. Assoc. Off. Anal. Chem.* **1988**; 71:725-728.

Franco, C.M.; Fente, C.A.; Vazquez, B.I.; Cepeda, A.; Mahuzier, G.; Prognon, P. Interaction between cyclodextrins and aflatoxins Q_1, M_1 and P_1: fluorescence and chromatographic studies. *J. Chromatogr. A* **1998**; 815:21-29.

French, D.; Levine, M.L.; Pazur, J.H.; Norberg, E. Studies on the Schardinger dextrins. The preparation and solubility characteristics of alpha, beta and gamma dextrins. *J. Amer. Chem. Soc.* **1949**; 71:353-356.

Harada, A.; Furue, M.; Nozakura, S. Interaction of cyclodextrin-containing polymers with fluorescence compounds. *Macromolecules*. **1977**; 10:676-681.

Holcomb, M.; Korfmacher, W.A.; Thompson Jr., H.C. Characterization of iodine derivatives of aflatoxin B_1 and G_1 by thermospray mass spectrometry. *J. Anal. Toxicol*. **1991**; 15:289-292.

Horikoshi, K.; Akiba, T. Alkalophilic microorganisms. A new microbial world. Tokyo: SpringerVerlag; Berlin, Heidelberg, New York: Japan Sci. Soc. Press, 1982. 215 p.

Hoshino, M.; Imamura, M.; Ikehara, K.; Hama, Y. Fluorescence enhancement of benzene derivatives by forming inclusion complexes with beta-cyclodextrin in aqueous solutions. *J. Phys. Chem*. **1981**; 85:1820-1823.

Jansen, H.; Jansen, R.; Brinkman, U.A.Th.; Frei, R.W. Fluorescence enhancement for aflatoxins in HPLC by post-column split-flow iIodine addition from a solid-phase iodine reservoir. *Chromatographia*. **1987**; 24:555-559.

Joshua, H. Determination of aflatoxins by reversed-phase high-performance liquid chromatography with post-column in-line photochemical derivatization and fluorescence detection. *J. Chromatogr. A* **1993**; 654:247-254.

Jozwiakowski, M.J.; Connors, K.A. Aqueous solubility behavior of three cyclodextrins. *Carbohydr. Res*. **1985**; 143:51-60.

Knutti, R.; Balsiger, Ch.; Sutter, K. Routine application of HPLC for quantification of aflatoxins in whole peanut kernels. *Chromatographia*. **1979**; 12:349-353.

Kok, W.Th. Derivatization reactions for the determination of aflatoxins by liquid chromatography with fluorescence detection. *J. Chromatogr. B* **1994**; 659:127-137.

Kok, W.Th.; Van Neer, Th.C.H.; Traag, W.A.; Tuinstra, L.G.M.Th. Determination of aflatoxins in cattle feed by liquid chromatography and post-column derivatization with electrochemically generated bromine. *J. Chromatogr*. **1986**; 367:231-236.

Manabe, M.; Goto, T.; Matsuura, S. High-performance liquid chromatography of aflatoxins with fluorescence detection. *Agric. Biol. Chem*. **1978**; 42:2003-2007.

Orti, D.L.; Grainger, J.; Ashley, D.L.; Hill, R.H. Chromatographic and spectroscopic properties of hemiacetals of aflatoxin and sterigmatocystin metabolites. *J. Chromatogr*. **1989**; 462:269-279.

Panalaks, T.; Scott, P.M. Sensitive silica gel-packed flowcell for fluorometric detection of aflatoxins by high-pressure liquid chromatography. *J. Assoc. Off. Anal. Chem.* **1977**; 60:583-589.

Pons, W.A., Jr.; Cucullu, A.F.; Lee, L.S.; Janssen, H.J. Kinetic study of acid-catalyzed conversion of aflatoxins B_1 and G_1 to B_{2a} and G_{2a}. *J. Am. Oil Chem. Soc*. **1972**; 49:124-128.

Pulley, A.O.; French, D. Studies of the Schardinger dextrins XI. The isolation of new Schardinger dextrins. *Bichem. Biophys. Res. Commun.* **1961**; 5:11-15.

Saenger, W.; Steiner, T. Cyclodextrin inclusion complexes: host-guest interactions and hydrogen-bonding networks. *Acta Crystallogr. A* **1998**; 54:798-805.

Schneiderman, E.; Stalcup, A.M. Cyclodextrins: a versatile tool in separation science. *J. Chromatogr. B*. **2000**; 745:83-102.

Seidel, V.; Poglits, E.; Schiller, K.; Lindner, W. Simultaneous determination of ochratoxin A and zearalenone in maize by reversed-phase high-performance liquid chromatography with fluorescence detection and beta-cyclodextrin as mobile phase additive. *J. Chromatogr*. **1993**; 635:227-235.

Seitz, L.M. Comparison of methods for aflatoxin analysis by high-pressure liquid chromatography. *J. Chromatogr*. **1975**; 104:81-89.

Shepherd, M.J.; Gilbert, J. An investigation of HPLC post-column iodination conditions for enhancement of aflatoxin B_1 fluorescence. *Food Addit. Contam.* **1984**; 1:325-335.

Singh, M.; Sharma, R.; Banerjee, U.C. Biotechnological applications of cyclodextrins: research review paper. *Biotech. Adv*. **2002**; 20:341-359.

Stroka, J.; Anklam, E.; Jorissen, U.; Gilbert, J. Immunoaffinity column cleanup with liquid chromatography using post-column bromination for determination of aflatoxins in peanut butter, pistachio paste, fig paste, and paprika powder: collaborative study. *J. AOAC Int*. **2000**; 83:320-340.

Sundararajan, P.R.; Rao, V.S.R. Conformational studies on cycloamyloses. *Carbohydr. Res*. **1970**; 13:351-358.

Sybilska, D. Cyclodextrins as mobile-phase components for separation of isomers by reversed-phase high-performance liquid chromatography. In *Ordered media in chemical separations*; Hinze, W.L.; Armstrong, D.W., Ed(s). Washington, D.C: American Chemical Society, 1987. p. 218-234.

Szejtli, J. Introduction and general overview of cyclodextrin chemistry. *Chem. Rev*. **1998**; 98:1743-53.

Szejtli, J. Cyclodextrins. In *Cyclodextrin technology*; Szejtli, J., Ed. Dordrecht, The Netherlands: Kluwer Academic Publishers, 1988. p. 1-78.

Szejtli, J. Cyclodextrins: a new group of industrial basic materials. *Die Nahrung*. **1985**; 29:911-924.

Szejtli, J. Introduction: fundamentals. In *Cyclodextrins and their inclusion complexes*; Szejtli, J., Ed. Budapest, Hungary: Akademiai Kiado, 1982. p. 13-16.

Szejtli, J.; Zsadon, B.; Cserhati, T. Cyclodextrins use in separations. In *Ordered media in chemical separations*; Hinze, W.L.; Armstrong, D.W., Ed(s). Washington, D.C: American Chemical Society, 1987. p. 200-216.

Tarter, E.J.; Hanchay, J.P.; Scott, P.M. Improved liquid chromatographic method for determination of aflatoxins in peanut butter and other commodities. *J. Assoc. Off. Anal. Chem.* **1984**; 67:597-600.

Thiel, P.G.; Stockenstrom, S.; Gathercole, P.S. Aflatoxin analysis by reverse phase HPLC using post-column derivatization for enhancement of fluorescence. *J. Liq. Chromatogr*. **1986**; 9:103-112.

Thorpe, C.W.; Ware, G.M.; Pohland, A.E. Determination of aflatoxins by HPLC with a fluorescence detector and using post-column derivatization. In *V International IUPAC Symposium on Mycotoxins and Phycotoxins*; Pfannhauser, W.; Czedic-Eysenberg, P.B., Ed(s). Vienna: Technical University, 1982. p. 52-55.

Traag, W.A.; Van Trijp, J.M.P.; Tuinstra, L.G.M.Th.; Kok, W.Th. Sample clean-up and post-column derivatization for the determination of aflatoxin B_1 in feedstuffs by liquid chromatography. *J. Chromatogr*. **1987**; 396:389-394.

Tuinstra, L.G.M.Th.; Haasnoot, W. Rapid determination of aflatoxin B_1 in Dutch feeding stuffs by high-performance liquid chromatography and post-column derivatization. *J. Chromatogr*. **1983**; 282:457-462.

Vazquez, B.I.; Fente, C.A.; Franco, C.M.; Cepeda, A.; Mahuzier, G.; Prognon, P. Preliminary study on fluorimetric detection of aflatoxins Q_1, P_1 and B_1 using heptakis-di-*O*-methyl-β-cyclodextrin as post-column HPLC reagent. *Anal. Commun*. **1999**; 36:5-7.

Vazquez, M.L.; Franco, C.M.; Cepeda, A.; Prognon, P.; Mahuzier, G. Liquid chromatographic study of the interaction between aflatoxins and β-cyclodextrin. *Anal. Chim. Acta*. **1992**; 269:239-247.

Vazquez, M.L.; Cepeda, A.; Prognon, P.; Mahuzier, G.; Blais, J. Cyclodextrins as modifiers of the luminescence characteristics of aflatoxins. *Anal. Chim. Acta*. **1991**; 255:343-350.

Wiedenhof, N.; Lammers, J.N.J.J. Properties of Cyclodextrins: Part I. Solubility measurements. *Carbohydr. Res*. **1967**; 4:318-325.

Wiedenhof, N.; Lammers, J.N.J.J. Properties of cyclodextrins: part II. Preparation of a stable β-cyclodextrin hydrate and determination of its water content and enthalpy of solution in water from 15-30°. *Carbohydr. Res*. **1968**; 7:1-6.

CHAPTER 3

A SCREENING METHOD FOR DETERMINATION OF AFLATOXINS IN PEANUT AND

CORN

Although different analytical (i.e., thin layer, gas and liquid chromatography) and immunological (affinity column, enzyme-linked immunosorbent assay and radioimmunoassay) methods have been recommended to quantify aflatoxins in food and feeds (Leszczynska *et al*., 2000; Ellis *et al*., 1991), most of these methods are costly and time-consuming. Each method for aflatoxin analysis proposed to maintain effective control of aflatoxins in peanut and corn has its advantages and disadvantages. Some detection methods require only presumptive or screening tests, whereas others require the quantification of just aflatoxin B_1 or several of the aflatoxins (Wilson, 1989). However, quantitative approaches to measure aflatoxins in farmers' stock peanuts at the buying points have not been adopted yet since it is expensive and time-consuming to extract and quantify the aflatoxins (Whitaker and Dickens, 1986). Such measures would be of importance by helping divert highly contaminated lots to non-consumption purposes and possibly reducing risks to human health by employing detoxification processes.

IMMUNOLOGICAL AND ANALYTICAL APPROACHES

Immunological Techniques

Three specific types of immunochemical methods are presently used for quantification of aflatoxins in food and feed namely, enzyme-linked immunosorbent assay (ELISA), immunoaffinity columns (IC) and radioimmunoassay (RIA) methods (Trucksess *et al*., 1991).

Enzyme-Linked Immunosorbent Assay. Presently ELISA procedures for aflatoxin analysis are qualitative or semi-quantitative and are temperature sensitive. This method is mostly used to screen for aflatoxin B_1 below a predetermined concentration, and the color developed by the enzyme-mediated reaction gives an indication of the amount of B_1 present (Wilson, 1989). In ELISA either the antibody or the (conjugated) antigen is immobilized on a solid support. Often transparent microtitre plates or microtitre strips are used as solid support (FAO, 1990). There are primarily two types of ELISA used for aflatoxin detection, homogeneous ELISA and heterogeneous ELISA. In homogeneous ELISA, enzyme activity is altered after binding to specific antibodies, and it is not necessary to separate free and bound forms of the enzyme-ligand conjugate in the assay. In the heterogeneous ELISA, enzyme activity remains unchanged and separation of the free and bound enzyme-ligand is necessary. The heterogeneous ELISA is most frequently used for analysis of aflatoxins (Ellis *et al*., 1991).

ELISA methods typically employ an initial aqueous methanol extraction followed by a simple dilution of the extract in buffer, with aliquots of this solution being pipetted into the wells of a microtitre plate. This is a standard format, transparent, plastic unit with an 8 X 12 array of wells moulded into it. In the commercial double-antibody ELISA kits, the plate is supplied with a dried layer of aflatoxin-protein conjugate adsorbed onto the surface of the wells. The sample is added to each well of the plate, followed by a limited amount of anti-aflatoxin antibody (first antibody). The plate is then incubated to allow antibody-antigen binding to occur. Aflatoxin-antibody complexes in solution are discarded, and the plate is thoroughly washed. A second antibody, containing an enzyme covalently attached to it, is then added, which recognizes and binds to the anti-aflatoxin antibody now attached to the plate wall. Finally, an enzyme substrate is added to the well. After incubation, the optical density of the colored product of the enzymatic

reaction is measured using an ELISA-reader. In a single antibody ELISA, a microtitre plate is coated with a known amount of antibody against the aflatoxin looked for (antigen). After being washed, the test solution containing an unknown quantity of the aflatoxin is added together with a known amount of enzyme-labelled aflatoxin. Labelled and non-labelled aflatoxins compete for the active sites of the found antibody. After incubation, the plate is washed again and the captured enzyme is determined by adding chromogenic substrate. The intensity of the resulting color can be measured either photometrically using an ELISA-reader, or visually (FAO, 1990). In this type of ELISA, the color-producing enzyme is conjugated directly to the first anti-aflatoxin antibody (Shepherd *et al*., 1987).

Ammida *et al*. (2004) developed a disposable electrochemical immunosensor based on the indirect competitive ELISA for simple and fast measurement of aflatoxin B_1 in barley using differential pulse voltammetry. The immunosensor strip was assembled immobilizing the aflatoxin B_1 conjugated to bovine serum albumin and incubation of the sample with the monoclonal antibody anti-aflatoxin B_1. The sensitivity was 2.5 ng/g, and the recovery ranged from 93 to 117% with an average value of 105 ± 8 %.

ELISA methods are faster, usually requiring around 20 to 30 minutes, more sensitive, less expensive, and there are no radioactive hazards compared to RIA methods (Malone *et al*., 2000; Ellis *et al*., 1991), but they are sometimes less quantitative than high-performance liquid chromatographic methods (Malone *et al*., 2000).

Immunoaffinity Columns. Affinity column methods are fast, easy to handle and good for routine analyses. Immunoaffinity procedures are used for clean-up of an extract prior to quantification of aflatoxins. They involve reversible binding between antigens (aflatoxins) and selective antibodies, leading to a specific antigen-antibody complex. The anti-aflatoxin

antibodies are bound to a gel material contained in a small plastic cartridge. An extract of the produce is forced through the column and the aflatoxins (antigens) are left bound to the recognition site of the immunoglobulin. Non-aflatoxin material is washed off the column with water or aqueous buffer, and the aflatoxins are recovered in purified form by applying a strong elution organic polar solvent such as methanol or acetonitrile (Shepherd *et al*., 1987; Sharman and Gilbert, 1991). Aflatoxins are then quantified with a fluorometer, Florisil tip fluorescence or liquid chromatograph following a pre- or post-column derivatization (Scott and Trucksess, 1997; Kussak *et al*., 1993).

Immunoaffinity columns based on monoclonal antibodies have been developed commercially in the United States and in the United Kingdom (Scott and Trucksess, 1997) and have greatly simplified the sample clean-up procedures (Leszczynska *et al*., 2000). An immunoaffinity column called Aflaprep, developed by Vicam (Watertown, MA) and marketed by Rhône-Poulenc Diagnostics (Glasgow, Scotland) has been used in Europe to clean-up extracts of various foods and feeds. Vicam currently sells two immunoaffinity columns: Aflatest-10, used for aflatoxins B_1 and B_2 only, and Aflatest P, used for aflatoxins B_1, B_2, G_1, G_2, and M_1 (Scott and Trucksess, 1997). Another widely used immunoaffinity column is the Easi-Extract column, developed by Biocode Ltd. (York, United Kingdom), formerly Microtest Research. Easi-Extract gives concentrations of aflatoxins in nuts and nut products, grains, edible oils, dried fruits and other foods (Scott and Trucksess, 1997). Patey *et al*. (1991) evaluated the Biocode "Total Aflatoxin Easi-Extract" columns for clean-up of peanut butter for aflatoxin determination. Recovery levels were between 51 and 67%.

Radioimmunoassay. In this method, antigen and specific antibody form a reversible soluble antigen-antibody complex. The RIA method involves simultaneous incubation of the

unknown sample or known standard dissolved in phosphate buffer with a constant amount of

labeled toxin and specified antibody. Free aflatoxins and bound aflatoxins are then separated,

and, using scintillation counters, radioactivity levels determined. RIA is a very sensitive method

but has several disadvantages. The radioisotopes, which can be a health hazard, present disposal

difficulties and may have short shelf lives. Hence, nonisotopic labels, e.g., enzymes, have been

used in place of radioisotopes (Trucksess *et al*., 1991).

Analytical Methods

Thin Layer Chromatography (TLC). Thin layer chromatography, also known as *flat bed*

chromatography or *planar chromatography*, is one of the most widely used separation

techniques in aflatoxin analysis. It is based on coating a glass plate with silica gel and applying a

concentrated sample of aflatoxins on a baseline. As the solvent migrates, compounds in solution

separate. After drying, characterization of the resultant spots takes place. Detection methods

based on the fluorescent properties of aflatoxin are used in developing the spots. Quantification

can be achieved by several methods, with one of the most common methods being the visual

estimation technique (Takahashi, D.M., 1977; Ellis *et al*., 1991). Such techniques are only semi-

quantitative, with considerable variation in results from laboratory to laboratory (Garner, 1975).

Whatever TLC method is used, it is necessary to confirm the identity of aflatoxins. The

Association of Official Analytical Chemists (AOAC) aflatoxin confirmation method is based on

the trifluoroacetic acid reaction with aflatoxins B_1, G_1, or M_1 (Wilson, 1989). New methods

involve the use of the fluorodensitometer, in which the TLC plates are examined under UV light

and scanned with a photometer that determines the exact location of the position of fluorescent

spots, as well as a precise measurement of the intensity of their fluorescence (Ellis *et al*., 1991).

The most significant advantage of TLC is that it can be a very inexpensive technique, although in

its more sophisticated forms it requires a substantial capital investment in items such as spotters

and densitometers. If one-dimensional TLC gives adequate resolution, a considerable number of

samples may be analyzed in parallel on one plate. Where two-dimensional TLC is found

necessary, several plates can be developed simultaneously (Shepherd *et al.*, 1987). However,

even though a powerful method, TLC lacks the sensitivity and quantification of the other

methods (Malone *et al.*, 2000).

     High-Performance Liquid Chromatography (HPLC). High-performance liquid

chromatography is now increasingly used for analysis of aflatoxins because of its increased

sensitivity, improved accuracy, automation, and precision compared with the TLC method (Ellis

et al., 1991; Wilson, 1989). Early confirmation methods involved the use of high-performance

liquid chromatography for separation and the collection of eluents representing the peaks for

analysis by mass spectrometry (Rao and Anders, 1973). Another confirmation method in which

fluorescent bands observed on TLC plates coated with silica gel were scraped off and examined

further by HPLC using a fluorescence detector, was described by Hunt *et al.* (1978). HPLC

methods have been developed using both normal and reverse phase systems in conjunction with

UV adsorption and fluorescence detection techniques. Pons and Franz (1977) devised the first

HPLC method for analysis of aflatoxins in cottonseed products. The method incorporated

aqueous acetone extraction, lead acetate treatment, partition of aflatoxins into methylene

chloride, and sample purification in silica gel. Aflatoxins B_1 and B_2 were then resolved on silica

gel column using a water-saturated chloroform-cyclohexane-acetonitrile solvent with detection

by UV absorbance at 365 nm. Recovery of added aflatoxins B_1 and B_2 was 90 - 95% at levels of

$5 - 100$ µg/kg. Then, Pons and Franz (1978) and Pons (1979) used HPLC methods for

determination of aflatoxins in peanut products and corn, respectively. Beebe (1978) used an

HPLC method with fluorescence detection after CB (Contaminants Branch) extraction and clean-up for determination of aflatoxins in green coffee, peanut butter, tree nuts, seeds, grains, chocolate-covered peanut butter candy, and roasted, salted-in-shell peanuts.

Currently, reverse-phase HPLC separations of aflatoxins are more widely used than normal phase separations due to easier manipulation as well as the reduced toxicity of aqueous mobile phases. Since the fluorescence of aflatoxins B_1 and G_1 diminishes in these types of solutions, different derivatization procedures have been tested (Jaimez *et al*., 2000). The early stages of this new era involved pre-column derivatization with trifluoroacetic acid and then post-column derivatization with iodine to produce more intensely fluorescing derivatives (Davis and Diener, 1980). Later on, increase in sensitivity for B_1 and G_1 in aqueous solutions was brought about by much stronger oxidizing reagents than iodine, such as bromine (Kok, 1994).

Hurst *et al*. (1982) described an HPLC method, which combined CB (Contaminants Branch) extraction method and a modified Pons column chromatographic clean-up step with chromatography on a reverse phase column and UV detection at 365 nm for aflatoxins B_1 and G_1 and fluorescence detection for aflatoxins B_2 and G_2 (365 nm excitation and 455 nm emission) for quantification of aflatoxins in artificially contaminated cocoa beans. Recoveries varied from 77 to 107 %. Francis *et al*. (1982) developed an HPLC method for quantification of aflatoxins in peanut butter using a silica gel-packed flowcell for fluorescence detection. The method combined the extraction and clean-up of Pons and Franz (1978) with the packed flowcell fluorescence detection system developed by Panalaks and Scott (1977) and later used by Pons (1979). The combined procedure eliminated the need for two detectors for determining the four aflatoxins by using UV absorbance and fluorescence detector.

Tarter *et al*. (1984) combined the extraction and clean-up procedures of Pons and Franz (1978) with the liquid chromatographic derivatization technique of Beebe (1978) to quantify aflatoxins in peanut butter, shelled peanuts, peanut meal, shelled Brazil nuts, pistachios, cashews, walnuts, yellow corn, and filberts. Awe and Schranz (1981) devised an HPLC method with fluorescence detector containing a silica gel-packed flowcell for determination of aflatoxins in spices.

Generally, HPLC techniques involve the separation of sample constituents, followed by their detection and quantification. Separation is achieved by a competitive distribution of the sample between a mobile liquid-phase and a stationary liquid- or solid-phase that is supported in a column. The most commonly used extraction technique is the solid-phase, which replaced the traditional use of column chromatography and liquid-liquid partition for clean-up. The most popular stationary phases of the solid phase extraction columns used are silica gel, C_{18} bonded-phase and magnesium silicate commercialized as Florisil (Papp *et al*., 2002). The mobile phase moves under pressure by use of a pump and passes through a column that contains the extract and then flows to a UV absorption fluorescence detector (Ellis *et al*., 1991). A variety of detectors are available for HPLC that can be both selective and sensitive to the aflatoxins (Beaver, 1989). During the mobile phase movement, a change in electrical output is produced, which is recorded on a moving chart to give a chromatogram. A comparison of the retention times with those of the standards enables results to be compared on a quantitative basis as the area under each peak on the chromatogram is proportional to the concentration of the particular type of aflatoxin (Ellis *et al*., 1991). Otta *et al*. (2000) and Papp *et al*. (2002) described overpressured-layer chromatography (OPLC) methods, which combine the advantages of HPLC and high-performance thin layer chromatography (HPTLC). Liquid chromatography analyses

have high precision, good recovery and high sensitivity. However, these techniques are lengthy, and the cost of equipment is high (Mirghani *et al*., 2001; Malone *et al*., 2000; Ellis *et al*., 1991).

HPLC methods can be used to quantify all the aflatoxins in a wide range of products. Beebe and Takahashi (1980) determined aflatoxin M_1 in fluid milk, sour cream, cottage cheese, and buttermilk by HPLC using fluorescence detection. Roch *et al*. (1992) proposed a non-polar bonded phase clean-up procedure of an acetone-water (85:15) extract followed by HPLC quantification for the determination of aflatoxins in peanut meal. Although the proposed method was described as having higher recoveries of aflatoxin B_1 than those recorded using the validated AOAC (CB) method, large quantities of the solvents (500mL) were used for extraction of aflatoxins from just 50g of sample.

Capillary Gas Chromatography (GC). This method has been considered unsuitable for the analysis of aflatoxins because of high polarity, molecular weight, low volatility and thermal instability of the aflatoxin molecule (Ellis *et al*., 19991). Nevertheless, the advent of fused silica capillary columns and the use of a mass spectrometer as a detector resulted in use of on-column injection to chromatograph aflatoxin B_1 standard.

In the past, confirmation of the identity of aflatoxins by means of mass spectrometric analysis required additional clean-up such as TLC isolation or solid-phase extraction, as the presence of impurities in the sample extracts caused problems. Then, new approaches involved interfacing gas chromatography with mass spectrometry (GC-MS), which uses GC to separate the impurities in the extracts and MS to confirm the identities of the aflatoxins (Holcomb *et al*., 1992). Ellis *et al*. (1991) successfully used a flame ionization detection method for analysis of aflatoxins. Gas chromatography methods are rapid and automated, but they require expensive equipment and are not suitable for field-testing (Malone *et al*., 2000; Ellis *et al*., 1991).

With the goal of assessing and keeping highly contaminated foods and feeds away from the marketplace, the search for new methods to screen aflatoxins is still a priority. Therefore, the objective of this study was to develop a rapid and inexpensive method to screen aflatoxins in peanut and corn.

The method proposed in this study is based on the Immunoaffinity Column (Aflatest) Method (49.2.18) for aflatoxins in corn, raw peanuts, and peanut butter (AOAC, 1995). The approach was to substitute the relatively expensive anti-aflatoxin antibody columns bound to a gel matrix contained in small plastic cartridges (Patey *et al*., 1991) with a less expensive aflatoxin binding material composed of activated alumina packed in polyethylene filtration tubes. The method utilizes 80% aqueous acetone and 10% NaCl to extract aflatoxins from peanut and corn, anhydrous Na_2SO_4 to separate lipids and pigments from aqueous acetone layer, and neutral activated alumina to adsorb aflatoxins. Then aflatoxins are eluted with HPLC grade methanol, derivatized with bromine and measured in a calibrated fluorometer. However, measurements must be performed rapidly after derivatization with bromine since this reagent is unstable over time. The significance of this more affordable and easy to handle method is that it can be used to screen and monitor high levels of aflatoxins in peanuts and corn, thereby helping reduce the risk to human health.

MATERIALS AND METHODS

Apparatus

Equipment specified is not restrictive. Other suitable equipment can be substituted.

Vicam Series 4 Fluorometer equipped with a printer, a silicon detector, a broad wavelength pulsed Xenon lamp, selected source filters (365-380nm excitation, 450-550nm emission) and RS-232 output for a printer was obtained from Source Scientific Systems Inc.,

Garden Grove, CA. Mycotoxin standards with red calibration standard, green calibration blank and yellow calibration standard were purchased from 313 Pleasant Street, Watertown, MA. Fisher vortex mixer, model G-560, was obtained from Scientific Industries, Inc., Bohemia, NY. Waring Blender was obtained from Dynamic Corporation of America. Balance Model METTLER MTS was purchased from US Mettler Instrument Corporation, Hightstown, NJ. Crepe fluted filter papers, 315–folded (size: 24cm) and glass microfiber filter (size: 11cm) were purchased from VWR Scientific Products, West Chester, PA. Plastic filtration tubes (6mL) without frits and polyethylene frits were purchased from Supelco Inc., 595 North Harrison Road, Bellefonte, PA. AflaTest P clean-up columns were purchased from VICAM Science Technology, 313 Pleasant Street, Watertown, MA.

Reagents

Solvents – Acetone for aflatoxin extraction, reagent grade and HPLC grade methanol were purchased from Mallinkrodt Chemical Words, St Louis, MO. Afla Test® developer (50mL concentrated solution) containing 0.03% bromine was obtained from VICAM, 313 Pleasant Street, Watertown, MA. Anhydrous sodium sulfate, Baker Analyzed Reagent, was purchased from Baker Chemical Co., Phillipsburg, N.J. Sodium chloride (Crystals, Reagent, ACS) was purchased from VWR International, West Chester, PA. Neutral alumina (Brockman Activity I, Fisher ChemAlert® Guide) was purchased from Fisher Scientific, Fair Lawn, NJ.

Precautions

Daylight should be excluded as much as possible during all procedures, since aflatoxins gradually breakdown under the influence of ultraviolet light. In addition, as the distribution of aflatoxin is extremely non-homogeneous, samples must be prepared and homogenized with extreme care. "Aflatoxins should be handled as very toxic substances. Perform manipulations

under hood whenever possible, and take particular precautions, such as use of glove box, when toxins are in dry form because of electrostatic nature and resulting tendency to disperse in working areas. Swab accidental spills of toxin with 1% NaOCl bleach, leave 10 min, and then add 5% aqueous acetone. Rinse all glassware exposed to aflatoxins with methanol, add 1% NaOCl solution, and after 2 h add acetone to 5% of total volume. Let react 30 min and then wash thoroughly" (AOAC, 1995).

Proposed Method vs. "Aflatest"

In order to compare the methods, 10 separate representative samples of peanut and corn were analyzed for aflatoxins using both techniques. The results were analyzed to find the correlations using parametric methods.

Data Analysis

The data obtained with the proposed method for quantification of aflatoxins in peanuts and corn were processed by the SAS program (Spector, 2001). The results of the methods comparison were determined by SPSS™ version 11.0/11.5 (Babbie *et al*., 2003).

Principle

Aflatoxins are extracted from peanut and corn samples by a weak organic polar solvent (acetone), and the extract is then filtered by glass microfiber filter. Anhydrous sodium sulfate separates lipids and pigments from filtrate. Then, neutral activated alumina in the columns separates the aflatoxins (compounds of interest) from impurities by binding the aflatoxins through dipole-dipole interactions and leaving sample impurities when the sample passes through the packed alumina in the polyethylene filtration tube. The remaining unwanted materials in the filtration tube are washed off with HPLC grade water. Then, aflatoxins are eluted from packed alumina using a strong organic polar solvent in this case, HPLC grade methanol.

Finally, after derivatization with bromine working solution, aflatoxins are quantified using a calibrated fluorometer (Appendix A).

Sample Preparation and Extraction

Peanut and corn samples (500-2000 grams) were obtained already ground. A 100g subsample of each peanut or corn sample, with 10 g of sodium chloride, was placed in a blender jar along with 200mL acetone-water (80:20 v/v). The extraction/blending was performed for 1 min at high speed in the blender. The extraction solvent was decanted into a glass container through 24 cm diameter filter paper.

Clean-up

Five milliliters of extract filtrate from the glass container was mixed thoroughly with 20mL diluent (deionized water) by hand shaking for 5 seconds and filtered through an 11 cm diameter glass microfiber filter. Then, four grams of anhydrous sodium sulfate were added into the 20mL filtrate and hand shaken for 30 seconds. Most of the anhydrous sodium sulfate remained undissolved but this did not impair the salt from performing the necessary effect. Lipid, pigments and other unwanted materials were lifted up to the top of filtrate, and the layer of interest, i.e., aqueous acetone containing aflatoxins, remained at the bottom of the filtrate. After inserting a polyethylene frit into the lower end of the filtration tube, 800mg of neutral alumina, activated at 120° C for 24 hours, were carefully poured into the tube and, another polyethylene frit was inserted tightly on top of the alumina. Subsequently, 2mL from aqueous acetone layer was passed through the filtration tube packed with alumina. The tube was then washed with 10mL of HPLC grade water to remove interferences. Finally, aflatoxins were eluted with 2mL of HPLC grade methanol, and the eluent was collected in a small disposable tube.

Derivatization

A 1mL portion of cleaned-up extract was placed in a clean 12 x 75mm disposable tube, and 1mL of bromine developer working reagent was added. The tube was then mixed thoroughly with a vortex mixer for 5 seconds. After the tube was wiped with lint-free paper, it was placed immediately into a calibrated fluorometer.

Fluorometry

A Vicam Series 4 Fluorometer was calibrated prior use with red calibration standard and green calibration blank. A control calibrator (yellow calibration standard) was used to ensure accuracy of the fluorometer and 240 parts per billion value was recorded as indicated in the instructions for calibration (Appendix B). After a sample was inserted into the calibrated fluorometer, a 60-second delay (fluorometer-reader) occurred before the result appeared on the screen and was printed. Comparisons with "Aflatest" method (Appendix C) were made on naturally contaminated peanut and corn samples.

The Role of Acetone as an Extraction Solvent for Aflatoxins

Aflatoxins are crystalline substances, freely soluble in moderately polar solvents such as acetone, chloroform, acetonitrile and methanol, and dissolve in water to the extent of 10-20 mg/liter. However, due to the international agreement to move away from using chlorinated solvents, and other toxic solvents, acetone was the extraction solvent of choice. Acetone, also called 2-propanone or dimethyl ketone, is a colorless, flammable liquid that is miscible with water and most organic solvents.

Acetone occurs naturally in the environment and is biodegradable. It has comparatively low acute and chronic toxicity as compared to other organic solvents. High vapor concentrations

produce anesthesia and may irritate the eyes, nose, and throat. Generally, there are no injurious effects other than skin irritation or headaches from prolonged exposure.

The Role of Sodium Chloride (NaCl) on Extraction of Aflatoxins

Though aflatoxins are soluble in acetone and most organic solvents, they are poorly soluble in water. Sodium chloride when added into the sample has the ability to of break down hydrogen bonds of aflatoxins thus improving the distribution coefficient between acetone and water for extraction. Although other inorganic salts have the same effect as sodium chloride, the latter is the least expensive salt available (Fessenden & Fessenden, 1993).

The Role of Neutral Activated Alumina as an Adsorbent of Aflatoxins

Activated alumina, a polar adsorbent, has found wide and varied uses in chemical analysis and chromatography. It retains hydrophilic organic material much more strongly than do compounds that are more hydrophobic. Alumina (Al_2O_3) is activated by heating to a high temperature to increase its adsorptive properties and render it insoluble in water.

The most important feature of the adsorption is the physical structure of the adsorbent, i.e. the total charge and charge distribution, the size of the pores and the accessible surface area (Huwig *et al*., 2001). The thermal activation of a polar adsorbent (to remove adsorbed water) also results in an enhanced selectivity. Conversely, the addition of water to polar adsorbents leads to a selective covering or blocking of the most active parts of the adsorbent surface. This causes a reduction in volume of adsorbed phase per unit weight of adsorbent, because less surface is exposed for adsorption, and there is also a decrease in the ability of a unit of adsorbent surface to bind adsorbed molecules, because the remaining unblocked surface interacts less strongly with adsorbing molecules. Other than by an ion exchange, alumina clay retains analytes (e.g. aflatoxins and other mycotoxins) by dipole-dipole interaction (Fritz, 1999). Mycotoxins are

dipolar molecules; therefore only dipolar clays can adsorb them. Aflatoxin is the only mycotoxin that has a strong positive charge; hence polar clays can adsorb it.

The Role of Anhydrous Sodium Sulfate (Na_2SO_4)

The anhydrous sodium sulfate, Na_2SO_4, chemically binds any water found in the sample to form the decahydrate of sodium sulfate $Na_2SO_4 \bullet 10H_2O$. The longer the sample remains in contact with the adsorbent bed, the more water will be removed. It also separates unwanted compounds such as lipids and pigments from layer of interest, in this case, the aqueous acetone layer.

RESULTS

Ten separate representative naturally contaminated peanut and corn samples, after being cleaned-up with alumina packed in polyethylene cartridges, were analyzed using a calibrated fluorometer. Results (Table 3 and 4) show the means of total aflatoxins recorded at each level of contamination in both peanut and corn samples, together with the standard deviation and coefficient of variation. The level of aflatoxin contamination in peanut samples detected by the proposed method ranged from 6 to 741 ppb, corresponding to 2 to 580 ppb with the AOAC approved "Aflatest" method, respectively (Table 3). Likewise, in corn samples, the range was from 17 to 231 ppb (proposed method), corresponding to 11 to 240 ppb (Aflatest), respectively (Table 4).

Larger variations of the recorded aflatoxins by the proposed method are found in peanut than corn samples (Table 3 and 4). This is probably due to the higher content of lipids and pigments in peanut samples requiring much effort on the clean-up procedure to remove the impurities of the extract before the detection of the aflatoxins is made. This fact is likely to reduce the repeatability of the method for determination of aflatoxins in peanut samples. Within

the peanut and corn samples, results show larger coefficients of variation in less contaminated

samples, whereas highly contaminated samples show smaller coefficients of variation (Table 3

and 4). The smallest coefficient of variation (3.3 %) occurred at 50 parts per billion and the

largest (22.2 %) was at 8 parts per billion for peanut samples (Table 3), whereas for corn samples

the smallest (2.7 %) occurred at 88 ppb and the largest (20.7 %) was at 17 ppb (Table 4).

Table 3. Aflatoxin content (ppb) in naturally contaminated peanut samples cleaned-up with

neutral alumina (Brockman Activity I) and quantified with a fluorometer.

	Peanut samples									
Rep.	A	B	C	D	E	F	G	H	I	J
1	10	170	52	5	98	124	10	20	62	750
2	7	160	50	6	108	120	7	18	50	710
3	8	150	50	6	110	122	8	16	54	790
4	7	160	48	7	114	116	6	16	60	714
Mean	8	160	50	6	108	121	8	18	57	741
Std dev.	1.41	8.16	1.63	0.82	6.81	3.41	1.71	1.91	5.51	37.29
CV (%)	17.6	5.1	3.3	13.7	6.3	2.8	22.2	10.9	9.7	5.0
Aflatest (control)	6	190	48	2	120	130	5	10	92	580

Std dev. = standard deviation; CV = coefficient of variation; "Aflatest" = an official

immunoaffinity column method.

Table 4. Aflatoxin content (ppb) in naturally contaminated corn samples cleaned-up with neutral alumina (Brockman Activity I) and quantified with a fluorometer.

					Corn samples					
Rep.	A	B	C	D	E	F	G	H	I	J
1	44	18	98	20	50	220	22	52	20	85
2	43	20	90	19	55	230	14	60	12	86
3	45	17	89	18	57	235	15	62	17	90
4	46	19	88	21	56	237	18	61	18	89
Mean	45	19	91	20	55	231	17	59	17	88
Std dev.	1.29	1.29	4.57	1.29	3.11	7.59	3.59	4.57	3.40	2.38
CV (%)	2.90	6.97	5.00	6.61	5.71	3.29	20.75	7.77	20.24	2.72
Aflatest (control)	41	11	86	17	59	240	12	59	11	89

Std dev. = standard deviation; CV = coefficient of variation; "Aflatest" = an official immunoaffinity column method.

Results also show good agreement between the proposed procedure and "Aflatest" estimates in both corn (Figure 7b) and peanut (Figure 8b) samples. The correlation coefficients for the 10 samples, packed alumina vs. "Aflatest", were 0.997 and 0.977 for corn and peanut samples, respectively (Figure 7a, 8a).

Corn Samples

Packed alumina vs Aflatest
Y = -5.576 + 1.065X ; R^2 = 0.997

Methods to screen aflatoxins

Packed alumina   Aflatest

(a)   (b)

Figure 7. Linear regression analysis of "Aflatest" as function of packed alumina procedure (a)

and agreement between the two methods in paired results of each corn sample (b).

Peanut Samples

Packed alumina vs Aflatest
Y = 19.113 + 0.778X ;
R^2 = 0.977

Methods to screen aflatoxins

Packed alumina   Aflatest

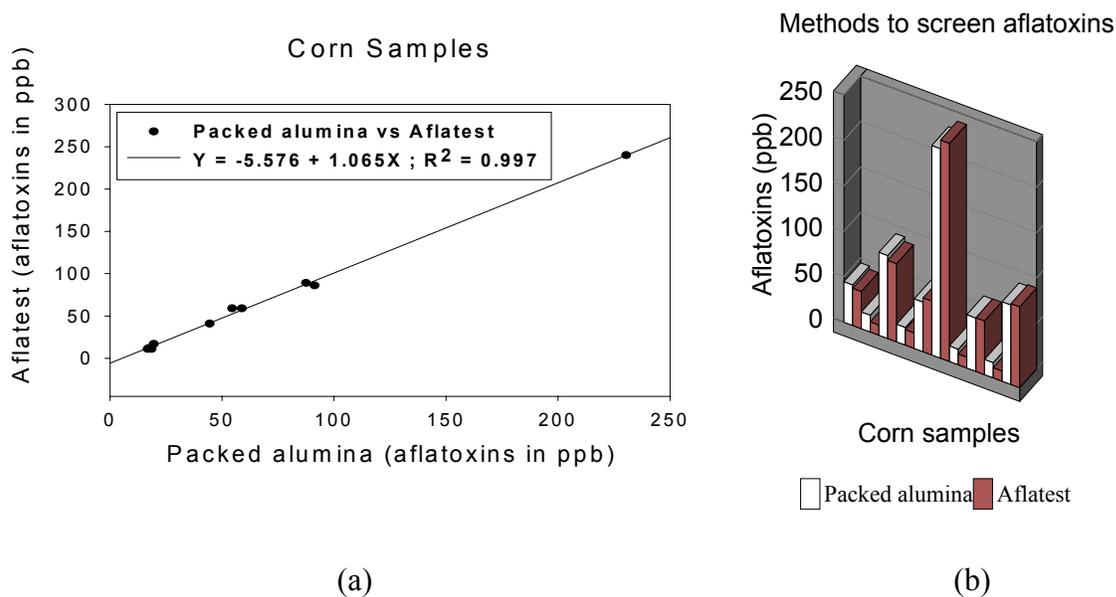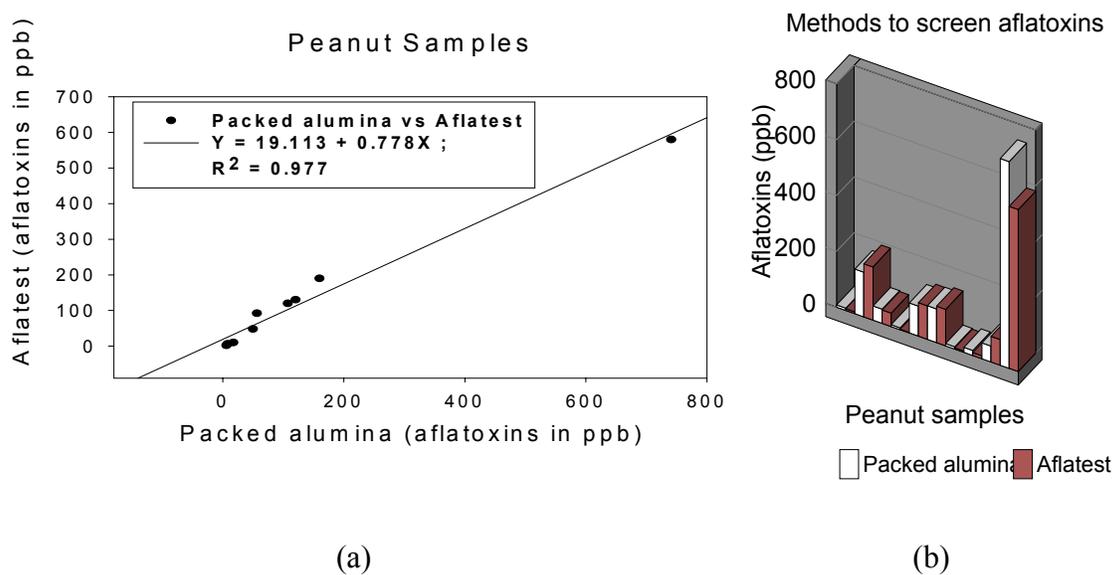(a)   (b)

Figure 8. Linear regression analysis of "Aflatest" as function of packed alumina procedure (a)

and agreement between the two methods in paired results of each peanut sample (b).

DISCUSSION

Data developed in this study indicate the expected degree of precision in the determination of aflatoxins with coefficients of variation ranging from 2.8 – 22.2% and 2.7 – 20.7% in peanut and corn samples, respectively (Table 3 and 4). The American Society for Testing and Materials (ASTM) has evaluated the quantitative precision of other analytical methods and found that coefficients of variation of 3 – 8 per cent for HPLC, 10 and 25 per cent for TLC with densitometric and visual quantification respectively may be expected, depending upon the complexity of the mixture to be separated (Shepherd *et al*., 1987).

Shepherd *et al*. (1987) observed that whatever method is used for the final measurement, the purity of the residue obtained from the sample during the clean-up step will have a major influence upon both the detection limit achievable and the degree of confidence that may be placed on the result. It is possible that different solvents used in the extraction step could account for the variations in the levels of aflatoxins recorded.

Other extraction solvents, such as methanol and acetonitrile, and aqueous acetone ratios were tested but failed to yield optimum results. Alumina, basic and alumina, acid were also tested as adsorbant materials for the clean-up procedure however; they did not provide the expected results. In such cases, some sample cloudiness and concomitant inaccuracy was evident, possibly due to the concentration of one or more impurities and/or to the low solubility of aflatoxins in water.

Haghighi *et al*. (1981) devised a HPLC method for aflatoxin quantification in pistachio nuts where the sample extract from Official Methods (BF method) was further purified on a small clean-up column to remove the non-aflatoxin impurities and aflatoxins were quantified by HPLC on a C_{18} reverse-phase column. The limit detection found was 0.5 ppb. Sobolev and

Dorner (2002) also developed a clean-up procedure with a minicolumn packed with basic aluminum oxide for quantification of aflatoxins in peanut, corn, cottonseed, almonds, Brazil nuts, pistachios and walnuts using liquid chromatography. The quantification limit of aflatoxin B_1 was 1 μg/kg. It is important to note that in this research a fluorometer was used as a detector. Unfortunately, it was not possible to determine the limit of quantification with the proposed procedure since all the samples were naturally contaminated. Nevertheless, good agreement was found in both peanut and corn samples between the packed alumina procedure and "Aflatest" method (an official method with a limit of quantification at less than 1 ppb), suggesting, therefore, high sensitivity of the proposed method.

While the "Aflatest" method has been subjected to interlaboratory testing and evaluation, this is not the case with the proposed procedure. This study was carried out in one laboratory only, therefore the reproducibility of the method cannot be concluded. However, the proposed method was far less expensive on the clean-up step. For example, clean-up affinity columns used in the "Aflatest" procedure are currently about $10 and cannot be reused. On the other hand, polyethylene cartridges used for clean-up in this study packed with neutral activated alumina are about $1. In addition, alumina can be washed off with water after use and cartridges can be recycled by washing in 50% aqueous methanol followed by several rinses in water.

The easy-to-handle and inexpensive proposed method could be an extremely effective technique when monitoring high levels of aflatoxins in peanut and corn samples to reduce the risk of these toxins to human health. In addition, this approach can easily be used for research studies and can help in the regulatory arena.

The method proposed in this study used bromine as the developer to convert the less fluorescent aflatoxins B_1 and G_1 (in aqueous systems) to their more fluorescent derivative forms

B_{2a} and G_{2a}, respectively. However, since these derivatives are not stable in these systems, the next challenge for further research is to use the more stable cyclodextrins as the developer, and calibrate the fluorometer to cyclodextrins.

LITERATURE CITED

Ammida, N.H.S.; Micheli, L.; Palleschi, G. Electrochemical immunosensor for determination of aflatoxin B_1 in barley. *Anal. Chim. Acta*. **2004**; 520:159-164.

AOAC. Official methods of analysis of AOAC International. 16th Edition, Volume II. Food composition, additives, natural contaminants. Cunniff, P., Ed. Arlington, Virginia: AOAC International, 1995. Chapter 49.

Awe, M.J.; Schranz, J.L. High pressure liquid chromatographic determination of aflatoxins in spices. *J. Assoc. Off. Anal. Chem*. **1981**; 64:1377-1382.

Babbie, E.; Halley, F.; Zaino, J. Advances in social research: data analysis using SPSS[TM] 11.0/11.5 for windows[®]. 5th edition, Thousand Oaks (California), London, New Delhi: Sage Publicans, Inc., 2003. 514p.

Beaver, R.W. Determination of aflatoxins in corn and peanuts using high performance liquid chromatography. *Arch. Environ. Contam. Toxicol.* **1989**; 18:315-318.

Beebe, R.M.; Takahashi, D.M. Determination of aflatoxin M_1 by high-pressure liquid chromatography using fluorescence detection. *J. Agric. Food. Chem*. **1980**; 28:481-482.

Beebe, R.M. Reverse phase high-pressure liquid chromatographic determination of aflatoxins in foods. *J. Assoc. Off. Anal. Chem*. **1978**; 61:1347-1352.

Davis, N.D.; Diener, U.L. Confirmatory test for the high-pressure liquid chromatographic determination of aflatoxin B_1. *J. Assoc. Off. Anal. Chem*. **1980**; 63:107-109.

Ellis, W.O.; Smith, J.P.; Simpson, B.K.; Oldham, J.H. Aflatoxins in food: occurrence, biosynthesis, effects on organisms, detection, and methods of control. *Crit. Rev. Food Sci. Nutri*. **1991**; 30:403-439.

FAO. Manuals of food quality control 10. Training in mycotoxins analysis. Rome: Food and Agriculture Organization of the United Nations, 1990. 109p.

Fessenden, R.J.; Fessenden, J.S. Organic laboratory techniques. 2nd edition, Pacific Grove, California: Brooks/Cole Publishing Company, 1993. 253p.

Francis, O.J., Jr.; Lipinski, L.J.; Gaul, J.A.; Campbell, A.D. High pressure liquid chromatographic determination of aflatoxins in peanut butter using a silica gel-packed flowcell for fluorescence detection. *J. Assoc. Off. Anal. Chem*. **1982**; 65:672-676.

Garner, R.C. Aflatoxin separation by high-pressure liquid chromatography. *J. Chromatogr*. **1975**; 103:186-188.

Haghighi, B.; Thorpe, C.; Pohland, A.E.; Barnett, R. Development of a sensitive high-performance liquid chromatographic method for detection of aflatoxins in pistachio nuts. *J. Chromatogr*. **1981**; 206:101-108.

Hunt, D.C.; Bourdon, A.T.; Wild, P.J.; Crosby, N.T. Use of high performance liquid chromatography combined with fluorescence detection for the identification and estimation of aflatoxins and ochratoxin in food. *J. Sci. Food Agric*. **1978**; 29:234-238.

Hurst, W.J.; Lenovich, L.M.; Martin, R.A., Jr. Liquid chromatographic determination of aflatoxins in artificially contaminated cocoa beans. *J. Assoc. Off. Anal. Chem*. **1982**; 65:888-891.

Huwig, A.; Freimund, S.; Kappeli, O.; Dutler, H. Mycotoxin detoxification of animal feed by different adsorbents. *Toxicol. Lett*. **2001**; 122:179-188.

Jaimez, J.; Fente, C.A.; Vazquez, B.I.; Franco, C.M.; Cepeda, A.; Mahuzier, G.; Prognon, P. Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis. *J. Chromatogr. A* **2000**; 882:1-10.

Kok, W.Th. Derivatization reactions for the determination of aflatoxins by liquid chromatography with fluorescence detection. *J. Chromatogr. B* **1994**; 659:127-137.

Kussak, A.; Andersson, B.; Andersson, K. Automated sample clean-up with solid-phase extraction for the determination of aflatoxins in urine by liquid chromatography. *J. Chromatogr*. **1993**; 616:235-241.

Leszczynska, J.; Kucharska, U.; Zegota, H. Aflatoxins in nuts assayed by immunological methods. *Eur. Food Res. Technol*. **2000**; 210:213-215.

Malone, B.R.; Humphrey, C.W.; Romer, T.R.; Richard, J.L. Determination of aflatoxins in grains and raw peanuts by a rapid procedure with fluorometric analysis. *J. AOAC Int*. **2000**; 83:95-98.

Mirghani, M.E.S.; Man, Y.B.C.; Jinap, S.; Baharin, B.S.; Bakar, J. A new method for determining aflatoxins in groundnut and groundnut cake using fourier transform infrared spectroscopy with attenuated total reflectance. *J. Am. Oil Chem. Soc*. **2001**; 78:985-992.

Otta, K.H.; Papp, E.; Bagocsi, B. Determination of aflatoxins in food by overpressured-layer chromatography. *J. Chromatogr. A* **2000**; 882:11-16.

Panalaks, T.; Scott, P.M. Sensitive silica gel-packed flowcell for fluorometric detection of aflatoxins by high-pressure liquid chromatography. *J. Assoc. Off. Anal. Chem.* **1977**; 60:583-589.

Papp, E.; H-Otta, K.; Zaray, G.; Mincsovics, E. Liquid chromatographic determination of aflatoxins. *Microchem. J*. **2002**; 73:39-46.

Patey, A.L.; Sharman, M.; Gilbert, J. Liquid chromatographic determination of aflatoxin levels in peanut butters using an immunoaffinity column cleanup method: international collaborative trial. *J. Assoc. Off. Anal. Chem.* **1991**; 74:76-81.

Pons, W.A., Jr. High-pressure liquid chromatographic determination of aflatoxins in corn. *J. Assoc. Off. Anal. Chem.* **1979**; 62:586-594.

Pons, W.A., Jr.; Franz, A.O., Jr. High-pressure liquid chromatographic determination of aflatoxins in peanut products. *J. Assoc. Off. Anal. Chem.* **1978**; 61:793-800.

Pons, W.A., Jr.; Franz, A.O., Jr. High performance liquid chromatography of aflatoxins in cottonseed products. *J. Assoc. Off. Anal. Chem.* **1977**; 60:89-95.

Rao, G.H.R.; Anders, M.W. Aflatoxin detection by high-speed liquid chromatography and mass spectrometry. *J. Chromatogr.* **1973**; 84:402-406.

Roch, O.G.; Blunden, G.; Coker, R.D.; Nawaz, S. The development and validation of a solid phase extraction/HPLC method for the determination of aflatoxins in groundnut meal. *Chromatographia*. **1992**; 33:208-212.

Scott, P.M.; Trucksess, M.W. Application of immunoaffinity columns to mycotoxin analysis. *J. AOAC Int.* **1997**; 80:941-949.

Sharman, M.; Gilbert, J. Automated aflatoxin analysis of foods and animal feeds using immunoaffinity column clean-up and high-performance liquid chromatographic determination. *J. Chromatogr*. **1991**; 543:220-225.

Shepherd, M.J.; Mortimer, D.N.; Gilbert, J. A review of approaches to the rapid analysis of aflatoxins in foods. *J. Assoc. Publ. Anal*. **1987**; 25:129-142.

Sobolev, V.S.; Dorner, J.W. Cleanup procedure for determination of aflatoxins in major agricultural commodities by liquid chromatography. *J. AOAC Int*. **2002**; 85:642-645.

Spector, P.E. SAS® programming for researchers and social scientists. 2nd edition, Thousand Oaks (California), London, New Delhi: Sage Publications, 2001. 231p.

Takahashi, D.M. Reversed-phase high-performance liquid chromatographic analytical system for aflatoxins in wines with fluorescence detection. *J. Chromatogr*. **1977**; 131:147-156.

Tarter, E.J.; Hanchay, J.-P.; Scott, P. Improved liquid chromatographic method for determination of Aflatoxins in peanut butter and other commodities. *J. Assoc. Off. Anal. Chem*. **1984**; 67:597-600.

Trucksess, M.W.; Stack, M.E.; Nesheim, S.; Page, S.W.; Albert, R.H.; Hansen, T.J.; Donahue, K.F. Immunoaffinity column coupled with solution fluorometry or liquid chromatography

postcolumn derivatization for determination of aflatoxins in corn, peanuts, and peanut butter: collaborative study. *J. Assoc. Off. Anal. Chem.* **1991**; 74:81-88.

Whitaker, T.B.; Dickens, J.W. Efficacy of the visual, minicolumn, and thin layer chromatography methods to test farmers' stock peanuts for aflatoxin. *Peanut Sci.* **1986**; 13:74-77.

Wilson, D.M. Analytical methods for aflatoxins in corn and peanuts. *Arch. Environ. Contam. Toxicol.* **1989**; 18:308-314.

APPENDICES

Appendix A. Procedure of the Proposed Method for Aflatoxin Analysis in Peanut and Corn.

Ground (corn, peanut) sample (100g)

Sodium chloride (10g)
Aqueous acetone
(160mL HPLC acetone + 40mL D.I. water)
Blend for 1 min
Filter

Filtrate (5mL)

HPLC water (20mL), mix well
Filter

Filtrate (20mL)

Anhydrous sodium sulfate (4g)
Shake 30 seconds

Lipids plus pigments upper layer (discard)   Aqueous acetone lower layer

Polyethylene filtration tube w/o frit

Insert polyethylene frit into the lower end
Activated neutral alumina (800mg)
Insert polyethylene frit into the top
Pipette aqueous acetone layer (2mL)
Wash off column with 10mL water HPLC grade
Elute with 2mL methanol HPLC grade, and collect

Filtrate (1mL)

1mL of developer working reagent: (0.003% bromine)
(0.003% bromine: 5mL of 0.03% bromine plus 45mL distilled water)
Shake 5 seconds

Test solution ———— Run using a calibrated Fluorometer

Appendix B. Calibration of the Fluorometer with Mycotoxin Standards Prior to Quantification of Aflatoxins.

1. Turn on Fluorometer: "**Vicam V1.3 Ready**"

2. Press **OPTION**: "**Calibrate Test**"

3. Press **ENTER**: "**AFLATEST**"

4. Press **ENTER**:

   "**Open the lid**"

   "**Insert RED vial**"

5. Insert the RED vial: "**High CAL 240 ppb**"

   If no type: 240

6. Press **ENTER**:

   "**Reading RED vial**"

   "**Remove RED vial**"

   "**Insert GREEN vial**"

7. Insert GREEN vial: "**Low CAL −1**"

   "**Vicam V1.3 Ready**"

8. Press **SELECT TEST**

   "**Aflatest**"

9. Press **ENTER**

   "**Insert vial**"

10. Insert Yellow vial:

    Read 120 or 110 ppb.

Reference: VICAM. Aflatest Instruction Manual, 1999.

Appendix C. Procedure of the "Aflatest" Method for Aflatoxin Analysis in Peanut and Corn.

Sample Extraction

1. Weigh out 100 g of sample and place in blender.

   If less than 100 g, use 1g / 2mL solvent; if less than 10 g, use 20mL solvent and correct.

2. Add 10g of NaCl.

3. Add 200mL of 80% Methanol (ASC Methanol + DI water).

4. Cover blender and blend at high speed for 1 minute.

5. Pour about 50mL extract into fluted filter paper; collect filtrate in a clean container.

   Extraction Dilution

6. Pipet 5mL filtered extract into test tube.

7. Dilute extract with 20mL HPLC water, mix well.

8. Filter dilute extract through glass microfibre filter.

   Calibration of Fluorometer (Appendix B)

9. Insert red vial and set at 240 ppb, press enter.

10. Insert green vial and press enter.

11. Insert yellow vial. The reading should be 120 ppb.

    Aflatest Affinity Chromatography Procedure

12. Rinse syringe with HPLC water.

13. Place column on syringe and add filtered extract.

10mL for 0 – 40 ppb	0 – 40	divide by 10
1mL for 0 – 400 ppb	0 – 400	results are correct
100µL for 0 – 4000 ppb	0 – 4000	multiply by 10
10µL for 0 – 40000 ppb	0 – 40000	multiply by 100

14. Pass extract halfway through column.

15. Wash column with 10mL HPLC water stopping at the top of the packing.

16. Repeat the column wash with an equal volume of HPLC water. Pass all water completely through the column.

17. Collect the aflatoxin in a test tube by passing 1mL of HPLC grade Methanol through the column.

18. Add 1mL fresh Aflatest Developer (made with HPLC water), mix well, and place test tube in calibrated Fluorometer (Appendix B).

Reference: Wilson, D.M. Personal Communication.