United States
Department of
Agriculture

**Agricultural
Research
Service**

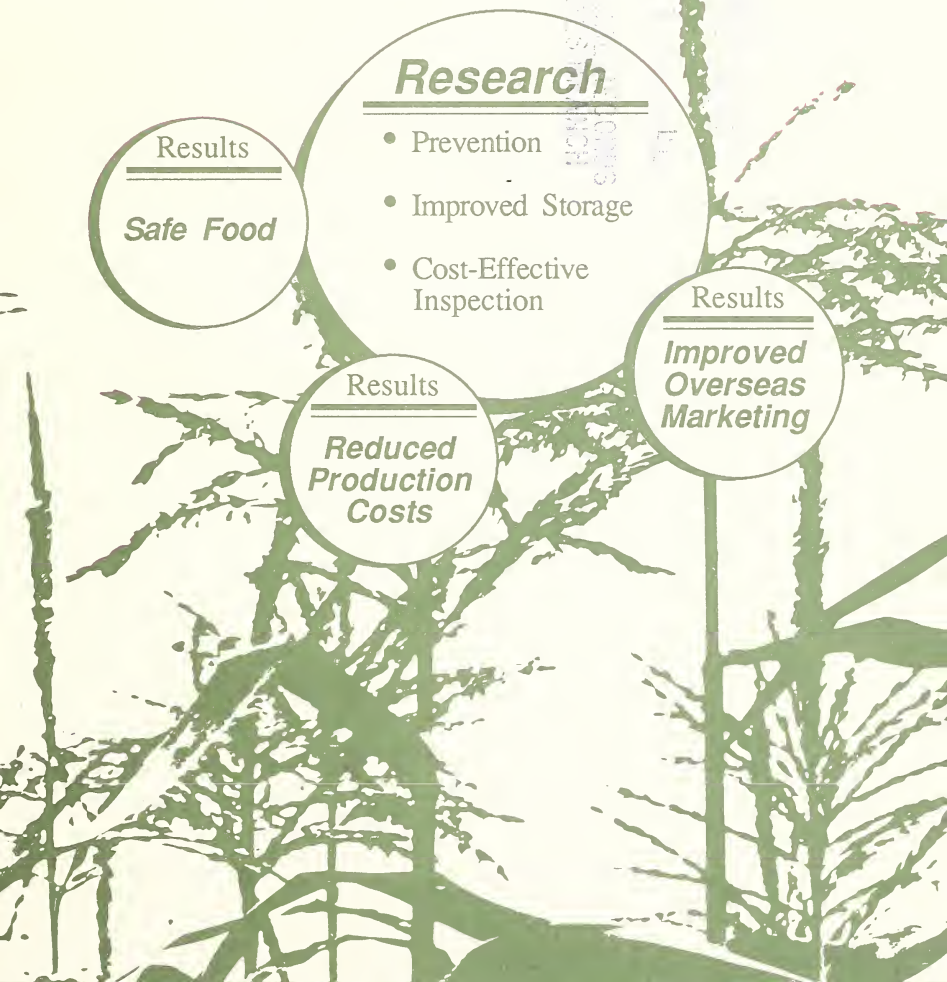
ARS - 83

June 1990

A Perspective on Aflatoxin in Field Crops and Animal Food Products in the United States

A Symposium

Washington, D.C.
January 23 - 24, 1990



Robens, Jane F. 1990. Chairperson. A Perspective on Aflatoxins in Field Crops and Animal Food Products in the United States: A Symposium. U.S. Department of Agriculture, Agricultural Research Service, ARS-83, pp. 157.

Keywords: aflatoxicoses, aflatoxin analyses, aflatoxin biocontrol, aflatoxin detoxification, corn, cottonseed, peanuts, residues.

While they last, single copies of this report are available upon request from the National Program Leader for Food Safety & Health; USDA-ARS; Bldg. 005, Rm. 210; Beltsville Agricultural Research Center-West; Beltsville, MD 20705.

Copies of this report may also be purchased from the National Technical Information Service; 5285 Port Royal Road; Springfield, VA 22161.

Acknowledgments

The Agricultural Research Service thanks the United States House Committee on Agriculture (Honorable E. (Kika) de la Garza, Chairman, and Honorable Edward R. Madigan, Ranking Minority Member) for their support and encouragement in the organizing of this symposium.

Symposium Organizing Committee

Jane F. Robens, William Huff, and John Richard.

Senior Contributing Authors

Kyd D. Brenner
Corn Refiners Association
Suite 1120
1100 Connecticut Avenue, NW.
Washington, DC 20036

T. E. Cleveland
USDA-ARS
Southern Regional Research Center
1100 Robert E. Lee Blvd.
P.O. Box 19687, Room 3006
New Orleans, LA 70179

Richard J. Cole
USDA-ARS
National Peanut Research Laboratory
1011 Forrester Drive, SE.
Dawson, GA 31742

Kimberly J. Cutchins
National Peanut Council
1500 King Street, Ste. 301
Alexandria, VA 22314

John A. Doerr
Department of Poultry Science
University of Maryland
College Park, MD 20742

Joe W. Dorner
USDA-ARS
National Peanut Research Laboratory
1011 Forrester Drive, SE.
Dawson, GA 31742

Winston M. Hagler, Jr.
Poultry Science Department
North Carolina State University
Raleigh, NC 27695-7636

Sara Hale Henry
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, SW.
Washington, DC 20204

John P. Honstead
USDA-Food and Safety and Inspection Service
Room 611, South Bldg.
Washington, DC 20250

William E. Huff
USDA-ARS
P.O. Drawer GE
College Station, TX 77841

Charles R. Hurburgh, Jr.
College of Agriculture
Iowa State University
Ames, IA 50011

George W. Latimer
Office of Texas State Chemist
Texas A&M University
College Station, TX 77843

William P. Norred
USDA-ARS
Russell Agricultural Research Center
P.O. Box 5677
Athens, GA 30613

Douglas L. Park
Department of Nutrition and Food Science
309 Chantz Blvd.
University of Arizona
Tucson, AZ 85721

Timothy D. Phillips
College of Veterinary Medicine
Texas A&M University
College Station, TX 77843

Craig A. Reed
USDA-Agricultural Marketing Service
Room 3064, South Bldg.
Washington, DC 20250

Robert D. Stubblefield
USDA-ARS
Northern Regional Research Center
1815 N. University Street
Peoria, IL 61604

Steven N. Tanner
USDA-FGIS
P.O. Box 96454
Washington, DC 20090-6454

D. Jesse Wagstaff
Center for Food and Safety and Applied Nutrition
Food and Drug Administration
200 C Street, SW.
Washington, DC 20204

Thomas B. Whitaker
USDA-ARS
126 Weaver Laboratory
North Carolina State University
Raleigh, NC 27695-7695

D.T. Wicklow
USDA-ARS
Northern Regional Research Center
1815 N. University Street
Peoria, IL 61604

R.D. Wyatt
Poultry Science Department
University of Georgia
Athens, GA 30602

Summary

Aflatoxin and its Control in U.S. Farm Commodities

Prepared by ARS for the United States House Committee on Agriculture

Aflatoxin is the most toxic of many naturally occurring toxins produced by fungi. It is a product of *Aspergillus flavus* and *Aspergillus parasiticus*, which coexist with and grow on almost any crop or food. However, aflatoxin is a problem because of its extensive preharvest contamination of corn, cotton, peanuts and tree nuts, and because residues from contaminated feed may appear in milk. Because of its extreme toxicity, the presence of even very low amounts of aflatoxin is believed to pose a risk to human health. Thus its presence in the major plant commodities, as well as in milk even in very minute amounts, is a perceived consumer food-safety issue and has caused severe economic losses to producers and to food handlers and processors.

The symposium, "A Perspective on Aflatoxins in Field Crops and Animal Food Products in the United States," was held to review (1) the identification of the aflatoxin problem through analysis and incidence of aflatoxin field crops, (2) aflatoxin presence in field crops and prevention strategies, (3) marketing strategies to reduce or eliminate aflatoxin in field crops, (4) monitoring aflatoxins in animal tissues and fluids as a method of assessing contamination of feeds and exposure of animals, (5) prevention strategies for aflatoxicosis in farm animals, and (6) guidelines for assessing risks associated with aflatoxins in field crops. The speakers at this symposium made several points that are very important to designing programs to reduce aflatoxin in animal feed and human food, which in turn will reduce economic losses and maintain the confidence of the American consumers in the safety of the food supply.

Agribusiness, agricultural research, and the regulatory agencies all have roles and responsibilities in making the changes necessary to greatly reduce the potential for the problem of aflatoxin. Some particular areas are as follows:

1. Aflatoxin contamination in field crops has always been associated with drought stress. Water is expensive and often unavailable at the critical time in crop production. We need increased research to develop drought resistant varieties of crops resistant to aflatoxin. Biocontrol of the fungal infection and/or aflatoxin production is another promising control technology that needs research.

2. Producers must take the responsibility to plant crops which are adapted to the environment and to manage them in the best possible manner to resist drought stress. Weather is never average, and we must plan for even the Midwest corn belt to have weather conducive to aflatoxin formation.

3. Contaminated product must be identified either on the farm or at the earliest possible handling and buying point before mixing and dilution. Options provided by agribusiness and the regulatory agencies for handling and for disposing of the contaminated product must be conducive to its detection, the economic viability of farmers, and the public health.

4. The regulatory agencies must be fully committed to reducing aflatoxin, understand the economic system in which commodities are produced, and provide viable and creative regulatory options for contaminated product. These decisions should be based on providing the safest possible total food supply, and not be constrained by former, narrowly drawn decisions.

Highlights of the presentations at the symposium follow.

Topic I
Identification of the Aflatoxin Problem through Analysis and Incidence of Aflatoxins in Field Crops

- The true aflatoxin concentration in a lot (a truckload, railway carful or other amount in commerce) cannot be determined with 100% certainty by measuring the concentration in appropriate samples. The toxin is not evenly distributed in commodities of plant origin, and the testing procedure needs to utilize statistical probabilities (Whitaker).

- The aflatoxin must be extracted from the matrix, that is, from corn, peanuts, meat, milk, etc., by a solvent or combination of solvents (Dorner), and then detected by some form of chromatography to separate interfering compounds and the aflatoxins from each other. Recently, immunoassays for aflatoxin have been shown to be highly specific, sensitive, and relatively simple to perform (Dorner).

- The U.S. Department of Agriculture's (USDA's) Federal Grain Inspection Service (FGIS) performed a comparison study in corn of several commercially available aflatoxin test methods utilizing immunochemistry. On the basis of their comparison study results, the FGIS approved all six of the methods tested for use in screening corn for aflatoxin at the 20-ppb level in their official inspection system (Tanner).

- Aflatoxin has been a periodic problem in midwestern corn for decades. There is no reason why each new outbreak should be treated as a new and surprising event, raising public fears over food safety. While aflatoxin occurs with greater regularity in the Southeastern States, the effect on the corn market is much more pronounced when midwestern corn is affected, because of the volume of the grains handled and their importance in the export market. Aflatoxin monitoring and test procedures should become part of the normal course of the grain business. The public would be better protected by a more open, flexible system that provides options for disposition at the raw commodity level, rather than by outright rejections. The grain industry must have sufficient, realistic disposal options to create an incentive for self-regulation at the local elevator level, and for FGIS monitoring at the export and processor levels (Hurburgh).

- Aflatoxin contamination of corn is decidedly more severe in the South because of the likelihood of drought/heat stress at silking. Unfortunately, irrigation which could almost eliminate contamination in the Southeast is not economically feasible for corn. The situation in the peanut industry is somewhat better than for southeastern corn or for southwestern cottonseed, since the peanut certification program buffers the industry economically and limits aflatoxin in food peanuts (Hagler).

- In Texas prior to 1988, there was only one serious incident of aflatoxin contamination in corn. Severe problems were encountered during both 1988 and 1989 and necessitated a change in survey and regulatory emphasis for commodities used as animal feed by the Office of the Texas State Chemist. (This office does not have responsibility for human food, and Dr. Latimer's presentation was limited to animal feed.) The 1988-1989 sampling was (1) very area specific as to where the evidence suggested there were problems and (2) based on a final-use monitoring strategy which started at the feed mills and worked

forward to the consumer or backwards to the grain supplier if contamination was found. However, final-use monitoring may not necessarily be the best strategy for the feed marketplace. The feed industry in Texas is made up of a spectrum of various sized firms which do not necessarily have better sampling and testing capabilities than their suppliers. Growers must now accept the fact that toxin-producing fungi represent a permanent and serious threat to their crops, and they should manage their crops to minimize such infestations. Likewise, producers of feed must now accept the fact that aflatoxins or other fungal-produced toxins may be present in products they buy and in products they produce. The agricultural community must work out an acceptable protocol for testing these products so that the interests of all, including that of the ultimate consumer, are protected (Latimer).

Topic II
Aflatoxin Production in Field Crops and Prevention Strategies

- Climate, sources of fungal inoculum, potential insect vectors, and the plant response interact to produce an aflatoxin outbreak. The screening of corn hybrids for resistance to aflatoxin contamination has been disappointing because of the variable amounts of aflatoxin at different geographical locations and from year to year. It is difficult to select for drought-stress resistance, since conditions necessary for its expression cannot be controlled (Wicklow).

- Biological control agents provide an attractive alternative to pesticides for many crop pests, and they show promise to prevent preharvest aflatoxin contamination of peanuts. A highly competitive, non-aflatoxin-producing strain of *Aspergillus parasiticus* has been shown to replace wild toxic strains in peanut test plots without a dramatic increase in the amount of fungus present in the soil. Nontoxigenic strains of the same organisms were chosen because they occupy the same ecological niche as the toxic strains. Bacteria appear to be an attractive strategy; however, they may not grow during the very critical hot dry periods (Cole).

- With the potential for a continued lowering of permissible levels of aflatoxin in crops by regulatory agencies, it seems unlikely that conventional control methods will be able to achieve the extremely low

levels of aflatoxin that are required in commercial food and feed. A complete understanding of the factors controlling aflatoxin biosynthesis in host plant tissues will lead the way to the development of novel biocontrol strategies and/or, in longer term research, development of elite crop lines "immune" to aflatoxin-producing fungi. Once identified, components involved in resistance could be used in traditional plant breeding to optimize selection for resistance against *Aspergillus flavus*, or as traits which could be introduced into any desirable germplasm through new genetic engineering techniques (Cleveland).

Topic III
Marketing Strategies To Reduce or Eliminate Aflatoxins in Field Crops

- All raw shelled peanuts in this country are marketed under a USDA/ industry agreement that requires analysis and certification by USDA of each lot for aflatoxin content. The agreement plays a very important role in the industry's quality-control efforts. Each lot of shelled peanuts for edible use must be officially sampled and chemically tested for aflatoxin by USDA's Agricultural Marketing Service (AMS) or in laboratories approved by the Peanut Administrative Committee. Provision is made for indemnification of sheller losses if the peanut administrative committee or the Food and Drug Administration deems the peanuts unsuitable for consumption because of aflatoxin (Reed).

- The Nation's grain processors and handlers have a quality-control system to detect and remove aflatoxin-contaminated grain from the food supply. Data from the Food and Drug Administration (FDA) surveillance of food products (1988-1989 crop year) indicated that this system was successful (Brenner).

Topic IV
Monitoring Aflatoxins in Animal Tissues and Fluids as a Method of Assessing Contamination of Feeds and Exposure of Animals

- One of the deficiencies in state-of-the art methodology for detecting aflatoxin in animal tissues and fluids has been the lack of a screening method. Recent methodology utilizing immunochemical techniques,

kits, and columns presents excellent possibilities for screening methods because the antibodies are very specific for aflatoxin. If urine is available for testing, little extraction is necessary except to detect very low ppb concentrations (Stubblefield).

- Many of the new immunochemical assays for aflatoxin are based on monoclonal antibodies which are genetically identical cells which are selected to be extremely specific to recognize the desired end point. These aflatoxin-specific antibodies form the basis for rapid and sensitive methods which are often available in inexpensive kits. Private and public sector scientists, commercial quality-control personnel, and others all can monitor aflatoxin in the food chain. When used correctly, these tests can assure buyers and consumers that grain purchased, feed manufactured, and poultry meat and eggs processed contain a minimum of mycotoxins (Doerr).

- Because of the large amount of aflatoxin in corn during the drought year of 1988, the USDA's Food Safety and Inspection Service formulated a worst-case sampling plan to detect any possible aflatoxin in the tissues of swine presented for slaughter. The analyses indicate that, even in a year of severe drought conditions with high aflatoxin contamination, a significant frequency or magnitude of aflatoxin residues do not occur in swine under routine slaughter conditions even in worst-case-type biased sampling. Swine appear to be the most sensitive species for the formation of tissue residues; that is, dietary aflatoxin is most likely to be deposited in their edible tissues. Thus, residues in other food-producing species should be significantly lower (Honstead).

Topic V
Prevention Strategies for Aflatoxicosis in Farm Animals

- Inorganic adsorbent materials in the diet act like "chemical sponges." These chemisorbents have been shown to sequester and immobilize aflatoxin in the gastrointestinal tract of livestock and poultry, thus preventing its normal uptake by the blood and distribution to target organs, such as the liver. Research is in progress to develop these materials for the practical control of mycotoxins (Phillips).

- Japanese quail that have extremely short generation times were genetically selected for resistance to acute aflatoxicosis. An 11-fold increase in resistance to acute aflatoxicosis was accomplished after only five generations of selection. Rapid and substantial genetic progress in selection for resistance to acute and chronic aflatoxicosis was also accomplished in chickens. These susceptible and resistant lines can serve as a basis for comparative studies of aflatoxin toxicity and metabolism in chickens (Wyatt).

- Treatment of aflatoxin-contaminated commodities with ammonia, in either gaseous or liquid form, is effective both in terms of aflatoxin destruction and in ease of use and cost effectiveness. In addition, the safety of the by-products of the process was determined through appropriately designed toxicological investigations at USDA's Agricultural Research Service. When the results of these and other toxicological studies on a variety of ammoniation methods in the past 20 years are viewed as a whole, the safety and efficacy of ammonia for reducing aflatoxin contamination are amply supported (Norred).

- In 1979, this safety and efficacy information developed by ARS was supplied to regulatory agencies for consideration in granting official approval of ammoniation as a practical detoxification procedure. Approval has still not been granted even though since that time, a number of research studies have been conducted to provide needed additional information. Regulation of the process rather than approval of the product created would answer needs of both regulatory agencies and feed and food processors (Park).

Topic VI
Guidelines for Assessing Risks Associated with Aflatoxins in Field Crops

- The toxicity of aflatoxin can vary greatly depending on additional factors such as the nutritional status of the animal. For instance, as the protein level of feed decreases, the toxicity of aflatoxin increases. Other variables include genetic variability in the animal's response to aflatoxin, a difference in susceptibility of males and females, environmental stressors such as extremes of hot and cold temperatures, and

the age of the animal that is exposed to aflatoxin with great susceptibility in younger animals. In addition, aflatoxin can disrupt the immune system and reduce the ability of animals to effectively fight off disease (Huff).

- Aflatoxin is a very potent cause of liver cancer in some, but not all, test-animal species. Testing the theory that aflatoxin causes cancer in humans is considerably more difficult, with the most common approach being to compare liver cancer rates of people in different geographic areas. To be accurate, all cases of liver cancer which occur in each study area must be found and compared with the base population taken from official census. Finally, accurate sampling and analyses of food for aflatoxin is necessary. The first major geographic human study started in Kenya in 1967, followed by others in Africa in Mozambique and Swaziland, and in Asia in Thailand and China. All of these studies provide some evidence of aflatoxin association with liver toxicity, but also they were done under less than ideal conditions; and there are reasons to doubt whether the correlations reported from Africa and Asia apply to other areas of the world. Correlations of liver cancer and aflatoxin have not been reported from the United States. Aflatoxin concentrations are higher in foods of the Southeastern States, but that region does not have higher liver cancer rates than other regions of the United States (Wagstaff).

- Up to the 1950's the public expected the Food and Drug Administration to make a decision and not to establish a scientific truth about the safety of components of or additives to food. But residual uncertainties were not usually considered in the public mind, and the illusion arose that absolute safety of these products was being promised. Gradually the public began to realize that these chemicals were not entirely free from risk, and risk communication became another problem area for FDA. The public demands an ever higher standard for risk, and the public also perceives involuntary vs. "chosen" risks differently and emotionally. In the 1970's quantitative risk assessment was developed utilizing four steps: hazard identification, dose-response, exposure assessment, and risk characterization. The basic assumptions upon which quantitative risk assessment (QRA) depends are the principle of dose-dependency and the assumption that laboratory animals are good models for humans. Animal assays bring with them several problems which affect QRA. The first is the insensitivity of whole animal bioassays. For instance, if the incidence of an

adverse effect is one in a thousand, it is very unlikely to be observed in a study with 100 animals. Thus, the only practical basis for estimating the safe dose was by extrapolation downward from results obtained in animal experiments at elevated doses far above the use level. Unfortunately, there are inherent large uncertainties in this extrapolation process. The FDA has considered possible alternatives to QRA. However, the first approach, that of Delaney no-risk, would in the case of aflatoxin, require complete prohibition of all the crops capable of being contaminated with aflatoxin - that is, corn, peanuts, sorghum, rice, wheat, soybeans, walnuts, almonds, pecans, pistachios, Brazil nuts, figs, and edible tissue (meat, milk, and eggs) from food-producing animals fed aflatoxin-containing animal feed. This option is clearly not feasible. The second option, the safety factor approach, is not scientifically defensible with a carcinogen such as aflatoxin which is so powerful in animal-susceptible species. Finally the third option, combining QRA with other scientific knowledge, is the one currently followed by the FDA. FDA believes it gives the best approach to their mission to protect the public health from food-borne hazards (Henry).

Contents

Topic I

Problems Associated With Testing Agricultural Commodities for Aflatoxin: Errors in Sampling, Sample Preparation, and Analysis

Thomas B. Whitaker

Introduction

It is difficult to accurately determine the aflatoxin concentration in a large quantity of material (lot) because of the large variability associated with the testing procedure (Whitaker et al., 1974; 1976; 1979). The testing procedure is a complicated process and generally consists of 3 steps: (a) a sample is taken from the lot and (b) in the case of a granular product the sample is comminuted to reduce particle size and a subsample is removed from the comminuted sample for analysis, and (c) the aflatoxin is extracted from the subsample and quantified. There are errors (the term error will be used to denote variability) associated with each of the above steps of the testing procedure. Because of these errors, the true aflatoxin concentration in the lot cannot be determined with 100 percent certainty by measuring the concentration in the sample taken from the lot.

Variation Among Test Results

Ten aflatoxin test results from each of 12 contaminated lots of shelled peanuts are shown in Table 1. Each test was made by comminuting a 5.45 kg sample in a subsampling mill developed by Dickens (Dickens et al., 1969; 1979), extracting aflatoxin from a 280 g subsample with the AOAC Method II and quantifying the aflatoxin densitometrically using TLC (Association of Official Analytical Chemists, 1980). The 10 test results from each lot are ranked from low to high to demonstrate several important characteristics about replicated aflatoxin test results taken from a contaminated lot.

First, the wide range among replicated test results from the same lot demonstrates the large variability among test result and is reflected in the large variance values shown in Table 1. Secondly, one can also see that the variance appears to be a function of the lot concentration. As the lot concentration increases, the variance among test results increases. The variability shown in Table 1 is equal to the sum of the sampling variance, subsampling variance, and analytical variance (Figure 1). Thirdly, the

distribution of the test results for each lot in Table 1 are not symmetrical about their mean (Whitaker et al. 1972). The distributions are positively skewed, meaning that more than half of the test results are below the mean. If a single sample is tested from a contaminated lot, there is more than 50% probability that the sample result will be lower than the true lot concentration. The skewness is greater for small sample sizes and the distribution becomes more symmetrical as sample size increases (Remington and Schrok, 1970). Also it can be seen in Table 1 that for a given sample size, the distribution of test results becomes more symmetrical as the lot concentration increases.

Table 1. Replicated test results for ten 5.45 kg samples from each of twelve lots of shelled peanuts.

Lot #	Observed aflatoxin test results ppb										Mean ppb	Variance ppb
1	0	0	0	0	0	0	0	6	10	14	3.0	26.9
2	0	0	0	0	2	4	8	14	28	43	9.9	214.8
3	0	0	0	0	0	0	0	16	40	69	12.5	561.6
4	0	0	0	0	0	3	8	26	52	70	15.9	647.2
5	0	0	0	0	3	13	19	41	43	69	18.8	588.4
6	0	0	3	12	12	12	12	26	63	103	24.2	1093.7
7	0	0	3	4	4	5	15	60	106	165	36.2	3249.7
8	0	0	32	32	34	37	55	67	77	134	46.8	1563.3
9	0	3	5	19	32	49	87	91	127	168	58.1	3353.4
10	4	7	40	41	55	60	75	95	99	230	70.6	4177.2
11	0	4	6	17	36	80	133	148	192	216	83.2	6871.7
12	18	50	53	72	82	108	112	127	182	191	99.5	3168.8

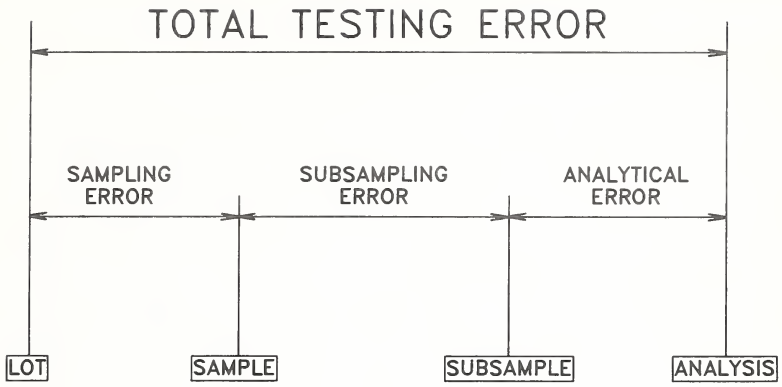


Figure 1.
Sources of sampling error in aflatoxin analyses.

Sampling Variability

Studies by Whitaker et al. (1974; 1976; 1979) on 3 granular products, peanuts, cottonseed, and shelled corn, indicate that with the exception of shelled corn, sampling variability, especially for small sample sizes, is the largest source of the three errors. Sampling error is large because aflatoxin is found only in a small percentage of the kernels in the lot (less than 0.1%) (Whitaker and Wiser, 1969), but the concentration in a single kernel may be extremely high. Cucullu et al. (1966; 1977) reported aflatoxin concentrations in excess of 1,000,000 ng/g (parts per billion, ppb) for individual peanut kernels and 5,000,000 ng/g for cottonseed. Shotwell et al. (1974) reported finding over 400,000 ng/g of aflatoxin in a corn kernel. Because of this extreme range in aflatoxin concentrations among individual kernels in a contaminated lot, variation among replicated samples tends to be large. The sampling variance associated with raw peanut kernels, cottonseed, and shelled corn, for a given sample size was estimated empirically by Whitaker et al. (1974; 1976; 1979).

Subsampling Variability

Once the sample has been taken from the lot, the sample must be prepared for aflatoxin extraction. It is essential that the entire sample be comminuted in a suitable mill before the subsample is removed from the sample (Dickens and Whitaker, 1982). Removing a subsample from the sample before the comminution process would eliminate the benefits associated with the larger size sample of granular product. It is assumed that the distribution of contaminated particles in the comminuted sample is similar to the distribution of contaminated kernels found in the lot. As a result, there is also variability among replicated subsamples taken from the same sample. However, the subsampling variance is not as large as the sampling variance due to the large number of comminuted particles in the subsample. The subsampling variance for peanuts, cottonseed, and shelled corn, for a given degree of comminution was estimated empirically by Whitaker et al. (1974; 1976; 1979). The subsampling variances measured by Whitaker et al. are specific for the particular mill used in the study to comminute the samples. Increasing the degree of comminution (more particles per unit mass) will decrease the subsampling variance.

Analytical Variability

Once the subsample is removed from the comminuted sample, the aflatoxin is extracted by official methods (Association of Official Analytical Chemists, 1980; Nesheim, 1979; Schuller et al., 1976). These methods are fairly complicated involving several steps such as solvent extraction, centrifugations, drying, dilutions, and quantification. As a result, there is considerable variation among replicated analyses on the same subsample extract. Whitaker et al. (1974; 1976; 1979) determined the analytical variance, associated with the Best Foods (BF) method used to extract aflatoxin from peanuts, the analytical variance associated with the Contaminants Branch of FDA (CB) method used to extract aflatoxin from corn, and the analytical variance associated with the Velasco method used to extract aflatoxin from cottonseed. Studies by Whitaker et al. (1981) on the BF method indicate that the thin layer chromatography quantification step is the major source of variability in the analytical process associated with testing peanuts for aflatoxin. While studies have not been conducted, the above results with peanuts probably apply to corn and cottonseed also.

Reducing Variability of Test Results

The only way to achieve a more precise estimate of the true lot concentration is to reduce the total variance associated with test results. The sampling variance can be reduced by increasing the size of the sample, and the subsampling variance can be reduced either by increasing the size of the subsample or by increasing the degree of comminution (increasing the number of particles per unit mass in the subsample). The analytical variance can be reduced by increasing the number of analyses. For example, if the sample size is doubled, then the sampling variance is cut in half (Walpole, 1974). Different costs are associated with each step of the aflatoxin testing procedure, and they vary among different commodities, for example a given volume of almonds is more costly than the same volume of corn. Careful study is needed to determine the optimum balance in the size of the sample, the degree of sample comminution, the size of the subsample and number of analyses needed to achieve a given precision.

Designing Aflatoxin Testing Programs

In a regulatory environment, lots are tested for aflatoxin and if the estimated lot concentration exceeds a defined guideline, the lot is removed from the food chain. With a perfect testing program, all good lots (a good lot has a concentration less than or equal to the guideline) would be accepted and all bad lots (a bad lot has a concentration in excess of the guideline) would be rejected by the testing plan. The acceptance curve associated with a perfect testing plan is shown graphically in Figure 2 where the probability of accepting a good lot is 100 percent and the probability of accepting a bad lot is zero percent. Because of the large variability among aflatoxin test results, two types of mistakes are associated with any aflatoxin testing program. First, good lots will test bad and be rejected by the testing program. This type of mistake is often called the processor's risk since these lots will be rejected at an unnecessary cost to the processor. Secondly, bad lots will test good and be accepted by the testing program. This type of mistake is called the consumer's risk since contaminated lots may get into the food chain posing a possible health hazard to the consumers. The acceptance probability associated with a less than perfect testing plan is shown in Figure 3 where less than 100 percent of the good lots will be accepted and more than zero percent of

the bad lots will be accepted by an aflatoxin testing plan. In Figure 3, the area above the acceptance curve for good lots represents good lots rejected (processor's risk) and the area below the acceptance curve for bad lots represents bad lots accepted (consumer's risk) by the testing plan. The magnitude of the processor's and consumer's risk (thus the shape of the acceptance curve as shown in Figure 3) is uniquely defined for a particular testing plan with designated values of sample size, subsample size, degree of comminution, type analytical method, number of analyses and the accept level. The accept level of a testing plan is defined as the highest test result allowed for a lot to be accepted.

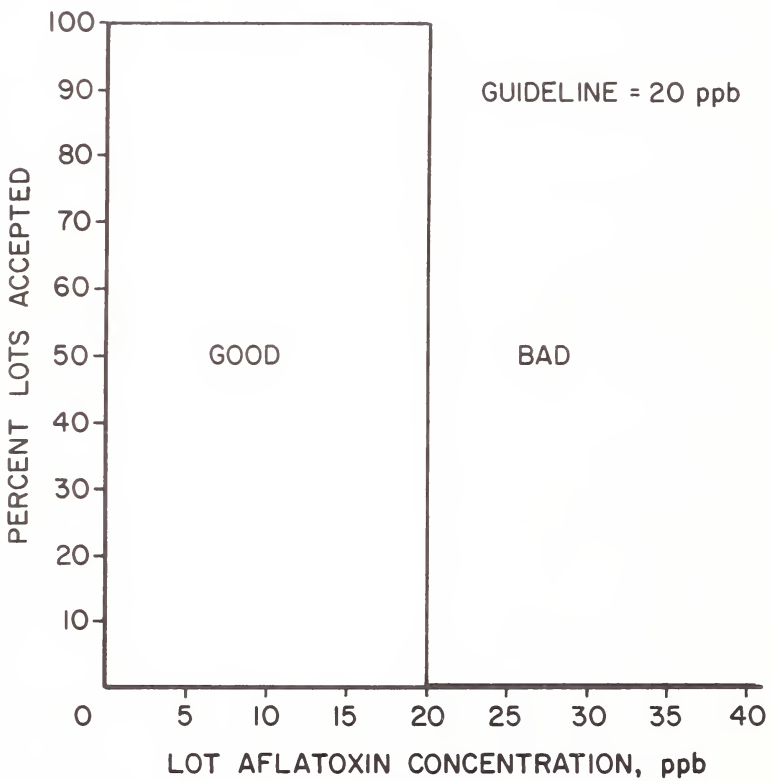


Figure 2.
Percent lots accepted by a perfect testing plan as a function of lot concentration.

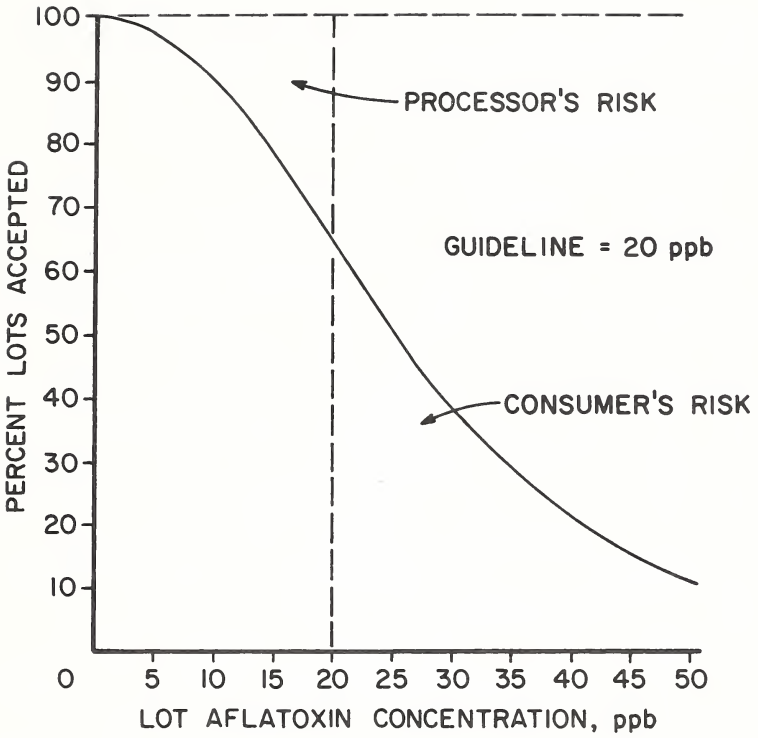


Figure 3.
Typical shape of an acceptance curve showing the processor's and consumer's risks associated with aflatoxin testing plans.

In order to maintain an effective control program, the above risks associated with a testing program must be evaluated and minimized. Based upon these evaluations, the costs and benefits (benefits refers to removal of aflatoxin contaminated lots) associated with a testing program can be evaluated. Different techniques can be used to design aflatoxin testing plans that will minimize the consumer's and processor's risk associated with a testing plan. Reducing the total variability associated with the testing plan will reduce both the consumer's and processor's risk. For example, increasing the sample size decreases both the processor's and consumer's risk. The effect of increasing sample size on the acceptance curve can be seen in Figure 4. The acceptance curve for the 45 lb (20.4 kg) sample is steeper than the acceptance curve for the 5 lb (2.3 kg) sample. As a result, the areas that reflect the processor's and consumer's

risks are smaller for the 45 lb (20.4 kg) sample than the 5 lb (2.3 kg) sample. The same effect can be obtained by increasing either the degree of sample comminution, subsample size or number of analyses.

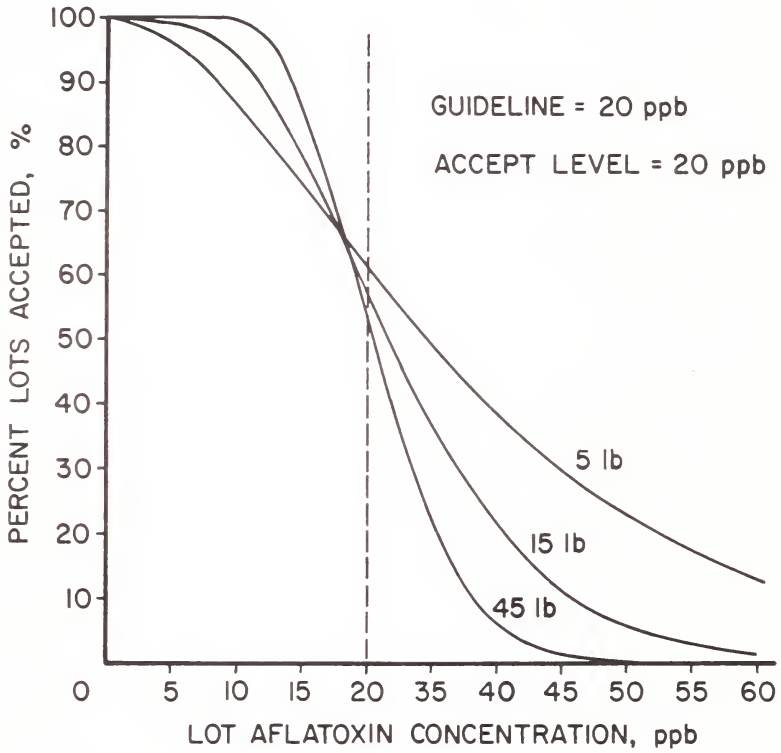


Figure 4.
The effect of sampling size on the processor's and consumer's risks associated with testing cottonseed for aflatoxin.

Changing the accept level of the testing plan relative to the guideline, also affects the magnitude of the processor's and consumer's risks. Figure 5 shows acceptance curves for three testing plans, each with a different accept level. The guideline is assumed to be 20 parts per billion. As shown in Figure 5, reducing the accept level to a value below the guideline, decreases the consumer's risk but increases the processor's risk. Conversely, increasing the accept level to a value above the guideline increases the consumer's risk but decreases the processor's risk. Only one of the two risks can be reduced by changing the accept level relative to the guideline value.

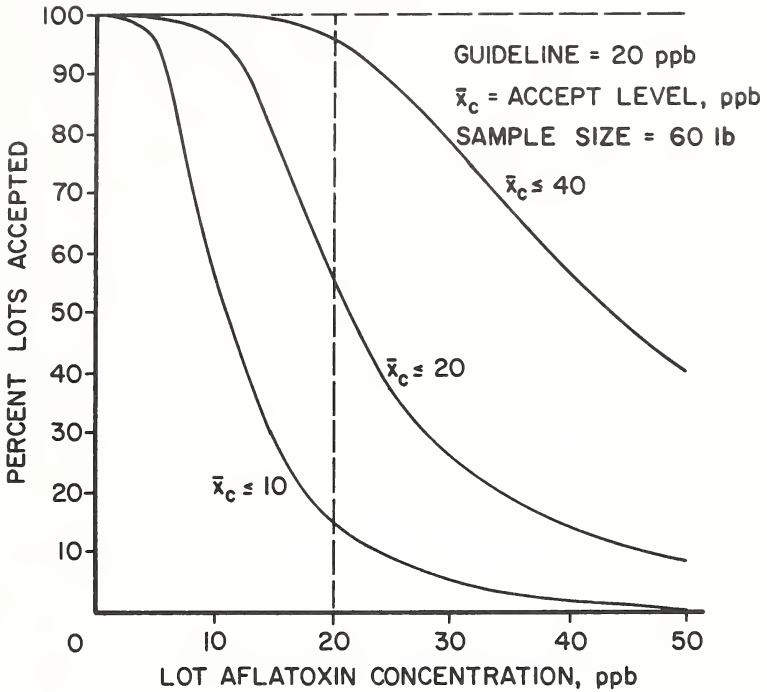


Figure 5.
The effect of acceptance level on the processor's and consumer's risks associated with testing peanuts for aflatoxin.

Whitaker et al. (1979) developed methods to predict the processor's and consumer's risk, the total number of lots accepted and rejected, the amount of aflatoxin in the accepted and rejected lots, and the costs associated with an aflatoxin testing plan for shelled peanuts, cottonseed, and shelled corn. These methods have been used by the peanut industry to design aflatoxin testing programs for shelled peanuts.

Selecting Samples

All of the above discussions assumed that there are no selection biases associated with drawing the sample from the lot. If the lot has been blended thoroughly from the various material handling operations, then the contaminated particles are probably distributed uniformly throughout

the lot. However, since we never can be sure that this is the case, and contaminated particles may not in fact be distributed uniformly throughout the lot, a sample should be an accumulation of small portions taken from many different locations throughout the lot (Bauwin and Ryan, 1982; Hurburgh and Bern, 1983).

References

Association of Official Analytical Chemists. 1980. Officials Methods of Analysis, 13th Ed. Assoc. Off. Anal. Chem., Washington, D.C. 229.

Bauwin, G.R. and H.L. Ryan. 1982. Sampling inspection and grading of grain. In Storage of Cereal Grains and Their Products, Vol. 5, 3rd ed., C.M. Christensen, Ed. Am. Assoc. Cereal Chem., St. Paul, Minnesota, 115.

Cucullu, A.F., L.S. Lee, and W.A. Pons. 1977. Relationship of physical appearance of individual mold damaged cottonseed to aflatoxin content. J. Am. Oil Chem. Soc. 54:235A.

Cucullu, A.F., L.S. Lee, R.Y. Mayne, and L.A. Goldblatt. 1966. Determination of aflatoxin in individual peanuts and peanut sections. J. Am. Oil Chem. Soc. 43:89.

Dickens, J.W. and J.B. Satterwhite. 1969. Subsampling mill for peanut kernels. Food Technol. 23:90.

Dickens, J.W. and T.B. Whitaker. 1982. Sampling and sampling preparation, in Environmental Carcinogens-Selected Methods of Analysis: Some Mycotoxins. Vol. 5, H. Egan, L. Stoloff, P. Scott, M. Costegnaro, I.K. O'Neill, and H. Bartsch, eds. ARC, Lyon, 17 pp.

Dickens, J.W., T.B. Whitaker, R.J. Monroe, and J.N. Weaver. 1979. Accuracy of subsampling mill for granular material. J. Am. Oil Chem. 56:842.

Hurburgh, C.R. and C.J. Bern. 1983. Sampling corn and soybeans. 1 Probing method. Trans. Am. Soc. Agric. Engineers 26:930.

Nesheim, S. 1979. Methods of Aflatoxin Analysis. NBS Spec. Publ. (US) No. 519, 355.

Remington, R.D. and M.A. Schrok. 1970. Statistics and Applications to the Biological and Health Sciences, Prentice-Hall, Englewood Cliffs, New Jersey, 105 pp.

Schuller, P.O., W. Horwitz, and L. Stoloff. 1976. A review of sampling and collaboratively studied methods of analysis for aflatoxin. J. Assoc. Off. Anal. Chem. 59:1315.

Shotwell, O.L., M.L. Goulden, and C.W. Hessletine. 1974. Aflatoxin: Distribution in contaminated corn. Cereal Chem. 51:492.

Walpole, R.E. 1974. Introduction to Statistics, MacMillan, New York, 156.

Whitaker, T.B. and E.H. Wiser. 1969. Theoretical investigation into the accuracy of sampling shelled peanuts for aflatoxin. J. Am. Oil Chem. Soc. 46:377.

Whitaker, T.B. and J.W. Dickens. 1979. Evaluation of the peanut administrative committee testing program for aflatoxins in shelled peanut. In Peanut Science, Vol. 6, pp. 7-9.

Whitaker, T.B. and J.W. Dickens. 1981. Errors in aflatoxin analyses of raw peanuts by thin layer chromatography. Peanut Sci. 8:92.

Whitaker, T.B., J.W. Dickens, and R.J. Monroe. 1972. Comparison of the observed distribution of aflatoxin in shelled peanuts to the negative binomial distribution. J. Am. Oil Chem. Soc. 49:590.

Whitaker, T.B., J.W. Dickens, and R.J. Monroe. 1974. Variability of aflatoxin test results. J. Am. Oil Chem. Soc. 49:590.

Whitaker, T.B., J.W. Dickens, and R.J. Monroe. 1979. Variability associated with testing corn for aflatoxin. J. Am. Oil Chem. Soc. 56:789.

Whitaker, T.B., M.E. Whitten, and R.J. Monroe. 1976. Variability associated with testing cottonseed for aflatoxin. J. Am. Oil Chem. Soc. 53:502.

Methods of Analysis and Confirmation of Aflatoxins in Commodities

Joe W. Dorner

Aflatoxins are toxic, secondary metabolites produced by the fungi, *Aspergillus flavus* and *A. parasiticus*. There are four naturally produced toxins designated B_1, B_2, G_1, and G_2. *A. flavus* is used in reference to both *A. flavus* and *A. parasiticus*.

Aflatoxin contamination of commodities such as peanuts, corn, and cottonseed occurs under specific environmental conditions that favor *A. flavus*. Many methods exist for determining whether or not commodities are contaminated with aflatoxin. It is often necessary to determine the quantity of aflatoxin in a commodity to satisfy FDA guidelines. The levels of aflatoxin that are important are in the parts per billion (ppb) range, i.e., the units of aflatoxin in a billion units of commodity. In this report a variety of methods that are used to analyze commodities for aflatoxin will be described.

I. Indirect Methods

Until recent years, chemical methods that were used to analyze samples for aflatoxin were generally time-consuming, costly or unavailable. Therefore, indirect methods to determine the probable presence of aflatoxin contamination were developed. These methods do not rely on a direct analysis of the commodity for aflatoxin, but rather make deductions about aflatoxin based on other criteria.

A. Visible A. flavus *Method*

As part of the grading procedure for farmers stock peanuts, the visible *A. flavus* method (VAF) is used to segregate aflatoxin-suspect loads from non-suspect loads at the farm level. A sample of peanuts from each load is visually inspected for visible *A. flavus* growth. If a kernel is found with visible *A. flavus* growth, the load is presumed to be at high risk for aflatoxin contamination and is diverted from the edible trade. No chemical analysis for aflatoxin is performed at this point; the indirect method of looking for the fungus is used to identify suspected aflatoxin-contaminated loads.

B. *BGYF Method*

An indirect method used as a presumptive test for aflatoxin in corn is known as the bright greenish-yellow fluorescent (BGYF) or black light test. Corn kernels, which have been infected by *A. flavus*, typically exhibit a bright fluorescence when placed under long-wave ultraviolet light. Unfortunately, many corn samples may exhibit the characteristic fluorescence without being contaminated with aflatoxin, thus creating false positive results. For this reason it is recommended that the BGYF test be used as a screening tool to identify lots or samples that are suspected of being contaminated with aflatoxin. When such samples are encountered, a confirmatory chemical analysis for aflatoxin should be performed. However, many grain elevators rely on the BGYF or black light test as the only tool for determining aflatoxin contamination in corn.

II. Direct Methods

Direct methods are those that chemically analyze directly for aflatoxin rather than relying on an indicator of aflatoxin contamination. Some of these methods are rapid, qualitative methods that determine the presence or absence of aflatoxin in the sample, but they do not indicate the concentration. Others are more rigorous, quantitative methods that provide for the accurate measurement of the amount of aflatoxin in the sample. All of these methods require the extraction of the aflatoxin from the sampled commodity with a solvent or combination of solvents. The extract is then processed in one of many different ways to establish the presence and/or amount of aflatoxin. Some of the methods estimate only the total concentration of the four aflatoxins present in a sample, whereas other methods determine the concentration of each individual toxin (B_1, B_2, G_1, G_2). Many of the methods rely on some form of chromatography to separate interfering compounds and the aflatoxins from each other. Most methods base the detection of aflatoxins on their fluorescent properties.

A. *TLC*

Thin-layer chromatography (TLC) was the first method developed to quantitate aflatoxins in extracts, and it remains the most widely used method. There are actually many TLC methods for aflatoxin quantitation, but while the basic methodology is the same, the differences among the methods are associated with extract purification and plate development.

Essentially, TLC methods are carried out by extracting the commodity sample with a solvent, usually composed of water in combination with an organic solvent such as methanol, acetone, chloroform, etc. The extraction is performed by either homogenizing the sample-solvent mixture in a blender for 1-2 minutes or by shaking a ground sample-solvent mixture for several minutes. That extract is then filtered and one of various purification procedures is performed to separate the aflatoxins from the many compounds in the extract that would interfere with the detection and quantitation of the aflatoxins. This partial purification procedure is the most time-consuming part of TLC methods. After the purification steps are completed, aliquots of the purified extract are placed on the TLC plate along with known amounts of pure aflatoxin standards. The TLC plate consists of a thin plate of glass that is coated on one side with a very thin layer of adsorbent material, typically silica gel. The aliquots of extract and aflatoxin standard are "spotted" onto the silica gel layer near one edge of the TLC plate. This edge of the plate is then placed into a shallow reservoir of a developing solvent. As the solvent migrates up the plate, the aflatoxins and other compounds still in the extract also move with the solvent through the silica gel layer. However, the aflatoxins and other compounds move at different rates; therefore, after development, they are at different locations on the plate. The aflatoxins are then detected by placing the plate in a darkened chamber exposed to long-wave ultraviolet light. Aflatoxins B_1 and B_2 emit a blue fluorescence and G_1 and G_2 emit a green fluorescence. A comparison of the intensities of the fluorescence of the aflatoxins in the extract spot with the aflatoxin standard spot is made either visually or with an instrument called a densitometer to determine the quantity of aflatoxin in the extract. The limit of detection of aflatoxin with TLC is about 2-3 ppb.

The simplest way to confirm that the fluorescent spots in an extract are in fact aflatoxin is to spray the plate with a sulfuric acid solution, which changes the fluorescence from blue or green to yellow. Other ways to confirm the presence of aflatoxin include spotting multiple TLC plates and developing them in different solvent mixtures or running two-dimensional TLC in which the plate is developed in one direction in solvent A and then developed in a perpendicular direction in solvent B. The latter method is quite useful when the extract contains interfering fluorescent compounds that are difficult to separate from aflatoxin. More specific and accurate confirmation is achieved by chemical derivatization. The most common method involves a reaction of aflatoxins B_1 and G_1 with water in the presence of trifluoroacetic acid (TFA) to form aflatoxins B_{2a} and G_{2a}. The derivatization can be carried out on a portion of the

extract prior to TLC, or it can be performed after TLC directly on the B_1 and G_1 spots followed by further TLC. The most accurate and non-ambiguous method of confirmation, however, is to recover the aflatoxin from the TLC plate after development and perform mass spectral analysis. Comparison of this mass spectrum with that of authentic aflatoxin serves as the best method of confirmation of the identity of the compound believed to be aflatoxin.

B. Minicolumn

Another semi-quantitative form of chromatography that has received widespread use as a screening tool to detect aflatoxin in commodities is minicolumn chromatography. This method also involves extraction of aflatoxin from the commodity with a solvent and a partial purification of the extract. The final determinative step requires that the extract be passed through a small glass tube (column) that contains at least two adsorbent materials, which are layered one on top of the other. The first, or upper, layer (typically alumina) serves to bind many of the interfering substances in the extract while allowing the aflatoxin to pass through to the second layer. The second, or lower layer (typically florisil) then binds the aflatoxin. When the minicolumn is then viewed under long-wave ultraviolet light, a blue fluorescent band is seen at the interface of the two adsorbent layers if aflatoxin is present. The intensity of the fluorescence can be used to roughly estimate the concentration of aflatoxin in the sample. The method is used primarily as a screening tool to identify samples that most likely contain aflatoxin and does not itself provide for confirmation. If confirmation is necessary, another method of analysis, such as TLC, would have to be carried out.

C. HPLC

High performance liquid chromatography (HPLC) is the most sensitive and technologically advanced method available for aflatoxin analysis. As the name indicates, aflatoxins are separated from each other and from interfering compounds chromatographically. In this case the separation takes place as a liquid flows through a column that is packed with a material similar to that which is applied as a thin layer to a TLC plate. In the case of HPLC, the particle size of the column packing material is extremely small and it is packed very tightly into, typically, a stainless steel column. The diameter of the column is usually a few millimeters and the length a few centimeters. The liquid is pumped

through the column under high pressure. A portion of the partially purified extract is introduced (injected) into the liquid flow prior to the column. As it flows through the column, the aflatoxins are separated. The aflatoxins eventually elute from the chromatography column and pass into a very sensitive ultraviolet or fluorescence detector. The detector converts that fluorescence into a r. As a fluorescent compound such as aflatoxin passes through the detector, a peak is produced on the recorder tracing. The time taken for the aflatoxins to pass through the column and into the detector is the primary basis for concluding that the chemical is aflatoxin. The area under the peak is compared to a standard curve of peak areas generated from injections of known quantities of pure aflatoxin, thus, producing accurate quantitation.

Confirmation of the presence of aflatoxin by HPLC is similar to TLC. Prior to injection of the sample, TFA derivatization can be carried out on a portion of the extract and both the derivatized and non-derivatized portions can be analyzed. The presence of aflatoxins B_1 and G_1 peaks in the non-derivatized sample along with presence of B_{2a} and G_{2a} peaks in the derivatized sample serves as confirmation. One HPLC method for aflatoxin employs continuous, on-line derivatization with a flow of aqueous iodine introduced between the column and the detector. This derivatization produces an enhanced fluorescence of B_1 and G_1, thus allowing more sensitive measurement. For confirmation, the sample is reinjected into the system with the iodine flow interrupted. The unreacted B_1 and G_1 again produce peaks on the recording, but their size is greatly reduced compared to the analysis of B_1 and G_1 derivatized with iodine (Fig. 1). As with TLC, however, if unequivocal confirmation is necessary, mass spectral analysis must be carried out on the isolated compound believed to be aflatoxin. The detection limit for aflatoxins by HPLC is about 0.1 ppb.

D. Immunoassays

In recent years, many types of immunoassays have been developed for the analysis of the aflatoxins. These assays are highly specific, sensitive, and relatively simple to perform. Several companies have developed immunoassay test kits that are currently marketed for aflatoxin analysis. While there are differences in the various immunoassays, the basic principle is the same and that will be discussed here.

In the production of an immunoassay test, aflatoxin is conjugated to a protein. An animal is immunized with the protein-aflatoxin conjugate,

which stimulates production of antibodies against the conjugate. These aflatoxin-specific antibodies are then isolated and become the basis for the immunoassay. The antibodies are bound to the type of support being used for the specific assay. After extraction of a commodity with an appropriate solvent, the extract is typically filtered and a portion of it is placed in contact with the antibody-support. The antibodies bind the aflatoxin in the sample and subsequent steps, which vary depending on the specific test kit, reveal whether or not aflatoxin was, in fact, present in the sample (Fig. 2).

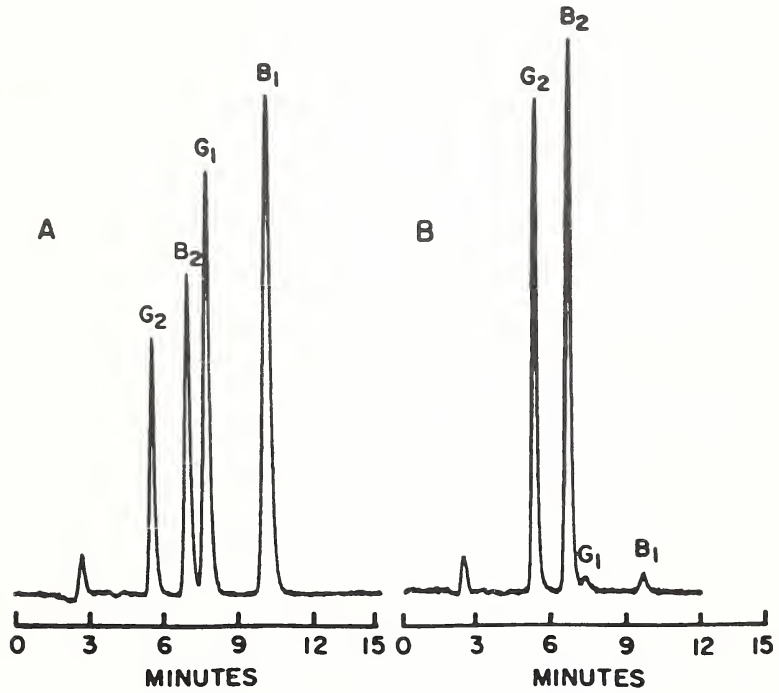


Figure 1.
HPLC separation of aflatoxins with fluorescence detection. A, chromatogram of aflatoxin standard solution with postcolumn iodine derivatization. B, chromatogram of aflatoxin standard solution with iodine flow interrupted.

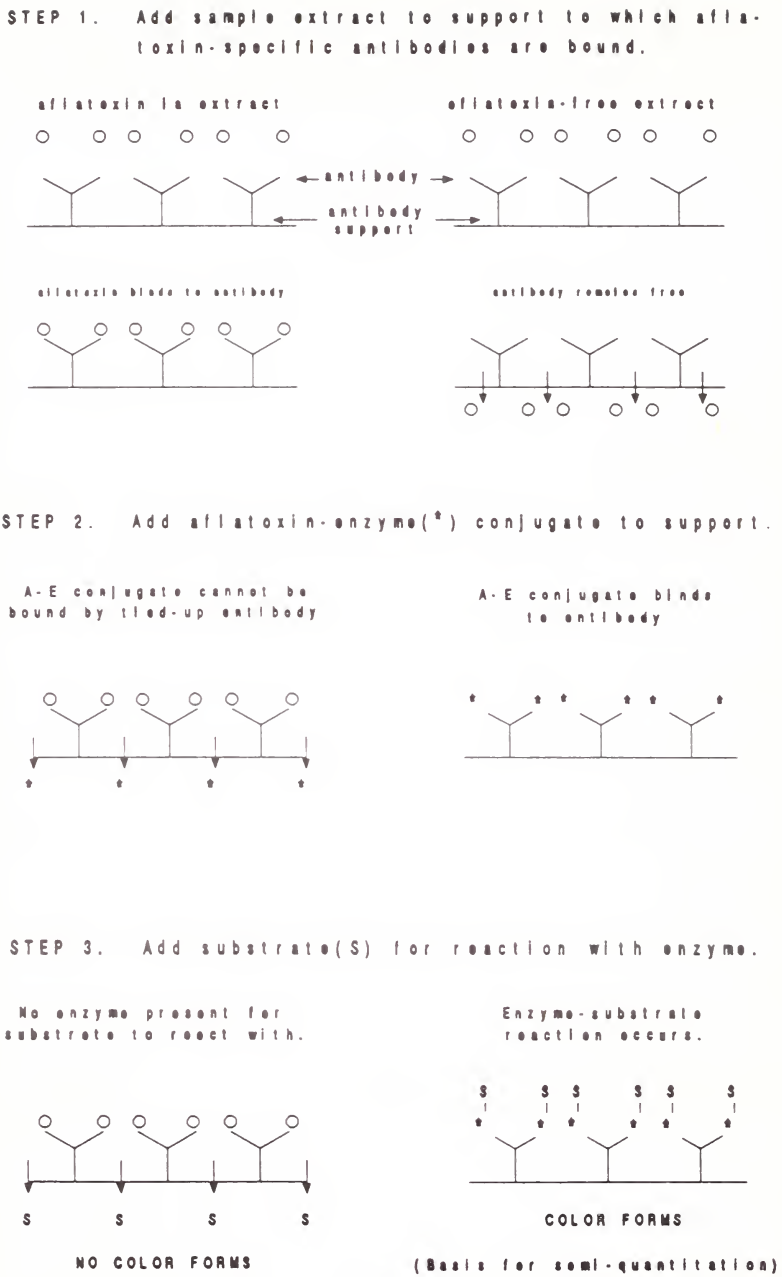


Figure 2.
Flowchart illustrating the principle of most aflatoxin immunoassay test kits.

One type of immunoassay test kit has the antibodies coated onto a microtiter plate containing many "wells." The sample extract is placed in the well allowing the antibodies to bind with any aflatoxin present in the sample. The sample is then rinsed out of the well and a solution of an aflatoxin-enzyme conjugate is added. The aflatoxin-enzyme conjugate binds to any free antibodies not bound by aflatoxin in the initial incubation of the sample. This material is then rinsed from the well and a substrate solution is added. The substrate reacts with any aflatoxin-enzyme conjugate bound during the second incubation to form a color change. The degree of color change is related to the amount of aflatoxin in the sample, and by comparison of sample well color with aflatoxin standard color, can be achieved either visually or spectrophotometrically.

Another type of immunoassay test has the antibodies bound to a matrix and packed as a layer in a short column. When the sample extract is passed through the column, any aflatoxin present is bound by the antibodies while other compounds pass on through. The aflatoxin is then eluted from the column with methanol. The methanol-aflatoxin solution can then be analyzed by HPLC to accurately quantitate the individual aflatoxins, or the solution can be placed in an instrument which measures the amount of fluorescence present to indicate the total aflatoxin concentration of the sample.

Other immunoassay kits are available that are designed for use in screening programs where it is only necessary to determine the presence or absence of aflatoxin above a specific value, such as 20 ppb. In these tests the antibody is bound to a membrane. The sample extract is applied to the membrane allowing the antibodies to bind the aflatoxin. This is followed by addition of an aflatoxin-enzyme conjugate and finally a substrate solution. The presence or absence of aflatoxin above the set value is determined by visual observation of a change in the color of the membrane from white to grey or blue. The development of color indicates that the sample contains no aflatoxin or an amount below that of the set value. When the membrane remains white, it indicates that the sample contained aflatoxin above the set value.

Because of the specific nature of the antibodies to aflatoxin, additional confirmation is not as important as with other methods. There may be isolated cases in which an extract might contain a substance that may interfere with the antibody. In cases where certainty of the presence of aflatoxin is necessary, the aflatoxin would be purified and isolated with TLC or HPLC and analyzed by mass spectrometry. However, these immunoassay tests are very specific for aflatoxin and usually do not require additional confirmatory steps.

Comparative Evaluation of Commercially Available Aflatoxin Test Methods

Steven N. Tanner and Donald E. Koeltzow

Introduction

In 1980, the Federal Grain Inspection Service adopted the Holaday-Velasco or HV minicolumn as the method for screening corn samples for total aflatoxin content at the 20 parts per billion (ppb) level in the official inspection system. In this method, aflatoxin is extracted from a ground corn sample with methyl alcohol and, after several steps, is finally concentrated on a small column containing the compound Florisil. The presence of aflatoxin is then detected by placing the column underneath an ultraviolet light.

One of the main problems with this test method is that it is often difficult to determine whether or not a sample is higher or lower than 20 ppb especially when aflatoxin concentrations are very close to the 20 ppb standard. A second concern is that the minicolumn is not specific for aflatoxin. While we don't know of any other compounds which might be detected by this method, it is still possible that there are compounds which will react like aflatoxin and give false readings in the test. Finally, the minicolumn test procedure is rather complex and requires the use of chemicals which are suspected of causing cancer.

Therefore, we decided to do a comparison study of commercially available aflatoxin test methods. The objective of this study was to compare the analytical performance of each of the methods evaluated with that obtained using the HV minicolumn.

A listing of the methods which were evaluated includes 1) the Afla-20-Cup analytical method developed by International Diagnostic Systems Corp., 2) the Aflatest developed by VICAM, 3) the Agri-Screen method developed by Neogen, 4) EZ-Screen developed by Environmental Diagnostics, Inc., 5) Oxoid developed by Oxoid, U.S.A. and 6) Sam-A developed by Rialdon Diagnostics.

Three sets of samples were used in the study. Sample Set #1 contained samples which were artificially contaminated with an aflatoxin mixture containing a B_1:B_2 ratio of 92:8. Aflatoxin B_1 is the more toxic of these two substances and this ratio of B_1 to B_2 is approximately what is found in naturally contaminated samples. Dosage levels used in this

sample set were 0, 10, 15, 20, 25, 30, and 40 ppb of total aflatoxin. Each laboratory received 3 samples at each aflatoxin level for a total of 21 samples in Sample Set #1.

Sample Set #2 was identical to Sample Set #1 except an aflatoxin standard containing only aflatoxin B_1 was used to spike each specimen. Again three samples were produced at each dose level for a total of 21 samples. This sample set was added to the study because the current inspection system was standardized using Aflatoxin B_1 only and we wanted to know if this made any difference.

Sample Set #3 contained samples of naturally contaminated corn which were obtained from one of our field offices. These samples were analyzed for their aflatoxin content by three different independent laboratories. Eighteen samples which contained approximately the same levels of aflatoxin used in the previous two sample sets were selected for inclusion in Sample Set #3. Again, 3 aflatoxin free samples were added bringing the total number of samples in Sample Set #3 to 21.

Therefore, each test site received 21 samples from each of 3 sample sets for a total of 63 samples. All samples were identified by random numbers and test participants did not know the sample set identification of any of the samples they received.

Six test sites were chosen to participate in the study. Four of them were FGIS laboratories and included the Technical Center at Kansas City, the Commodities Testing Laboratory at Beltsville and the field Offices at Lutcher and Belle Chasse, Louisiana. Two external labs - namely Dr. D. Park's Laboratory, University of Arizona, Tucson, AZ, and Dr. D. Wilson's Laboratory, University of Georgia, Tifton, GA - were also included.

All participants were given one week of training in the use of all tests at the Technical Center.

TLC analysis was carried out on a second set of 63 samples sent to each test site. The method used for analysis was the official method approved by the Association of Official Analytical Chemists. Samples used in Sample Set #3 were subportions of the same naturally contaminated samples extracted for analysis with the kits.

Results and Conclusions

To analyze the results of this study, the percentage of positive scores was plotted versus the dosage level for each of the methods tested. The current FDA action level is set at 20 ppb. As shown in Figure 1, in an

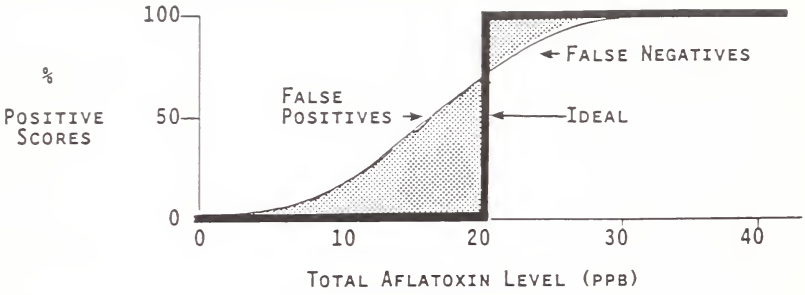


Figure 1.
Plot of % positive results versus aflatoxin content.

IDEAL test method, all test results would be negative at aflatoxin concentrations of 20 ppb or lower. However, at just a small amount over 20 ppb and higher, all results would be positive. In real life this rarely happens and the true results obtained with the HV minicolumn are demonstrated by the S-shaped curve. Those positive scores which are obtained at aflatoxin levels below 20 ppb are considered false positives because, according to the IDEAL case, they should all be negative, but they aren't. Those negative results from samples containing over 20 ppb aflatoxin are considered false negatives because, again according to the ideal case they should all be positive, but they aren't. Thus if the performance of a test method were to be better than the real life case illustrated here, it should move more toward the ideal curve. That is both the percentage of false positives and the percentage of false negatives should decrease.

The major goal of this study was to find a replacement for the HV minicolumn. Therefore, test accuracy was evaluated using the performance of the HV minicolumn as the standard. The percent positive responses from each test method were plotted versus the aflatoxin level. Next, the performance of each method was compared with that of the HV minicolumn to determine if it was different. This was done using a Chi-square goodness-of-fit test. If the performance of a particular method was found to be different from that of the HV minicolumn, the next question to be answered was, "Is the method better?" That is, are both the percentage of false negatives and the percentage of false positives lower when compared to the HV minicolumn?

In addition, Chi-square analysis was also used to compare the percentage of positive responses obtained from each method evaluated with TLC results. Again methods which gave results that were different from TLC analysis were further analyzed to determine if their performance was better than that of TLC.

For Sample Set #1 containing both aflatoxin B_1 and B_2, only two test methods - namely the Agri-Screen method and the TLC method - gave results which were statistically different from those obtained with the HV minicolumn. In neither case were these results classified as being better than those obtained with the HV minicolumn. When TLC was used as the standard, essentially the opposite picture was obtained. Only two methods namely the Afla-20-Cup and Agri-Screen gave results which were NOT statistically different from those obtained with TLC.

Essentially the same results were obtained with Sample Set #2. In this case three methods were statistically different from the HV minicolumn. The Agri-Screen and TLC methods gave the same results as they did in Sample Set #1. In addition, the Afla-20-Cup method was different as well; however, it also proved to be better than the minicolumn in that both the percentage of false positives and the percentage of false negatives were lower. With the exception of the Afla-20-Cup method, TLC results were identical to those obtained with Sample Set #1.

These data also suggest that whether or not a screening method is standardized using a standard containing a 92:8 mixture of B_1 and B_2 or just B_1 alone, really doesn't make any difference.

With few exceptions, the results from Sample Set #3 were the same as those obtained with Sample Sets #1 and #2. This time the TLC performance did not prove to be statistically different from that of the HV minicolumn. This is probably because TLC was one of the major methods used to determine the actual content of these naturally contaminated samples. When TLC was used as the standard, the Afla-20-Cup performance was not statistically different from TLC; however that of the Agri-Screen read by the microwell reader was different.

This separation of the test methods into two general populations - those which are statistically different from the HV minicolumn - namely the Agri-Screen method and TLC - and those which are similar to TLC can be partially explained by the fact that neither the Agri-Screen nor the TLC methods corrected for less than 100% recovery of the aflatoxin. When a corn sample is extracted with a solvent, not all of the aflatoxin is removed. In 1982 Stahr et al. reported that when corn was extracted with 80% methanol, only 60% of the aflatoxin was removed. The extraction procedure used for the TLC analysis removes only 67% of the aflatoxin present in the sample.

In August of 1989, the Agri-Screen method was retested using a modified procedure adjusted to 100% recovery of the aflatoxin. A sample set identical to Sample Set #1 was used for the test which was carried out at the Technical Center in Kansas City. Chi-square probability values for

the visual and microwell reader determinations showed that the performance of the modified Agri-Screen system was not statistically different from that of the HV minicolumn.

One of the most surprising results obtained in this study was the relatively poor performance of the TLC test. Individual values were omitted from calculations according to AOAC procedures for removing outliers. Statistical analyses of the data show a poor correlation between TLC and the actual aflatoxin levels in all three sample sets when results are taken individually. Correlation (r^2) values of 0.45, 0.41 and 0.57 were obtained for Sample Sets #1, #2 and #3 respectively. The higher correlation obtained with Sample Set #3 is probably due to the fact that the initial concentrations in the naturally contaminated samples were determined using TLC. This poor performance was not unique to any individual laboratory.

TLC results at each dosage level were averaged across all laboratories for each sample set. Statistical analyses of these results show that if you take the average of several TLC analyses on a particular sample, then the results are highly correlated with the actual aflatoxin content present in the samples.

Finally, it is important to note that all of the methods evaluated were also tested at 18 and 30°C. Such a test is necessary because most of these methods contain antibodies which are proteins and proteins are affected by different temperatures. Chi-square analysis of the results showed that test performance at these different temperatures was not statistically different from their performance at room temperature.

On the basis of these results, the Federal Grain Inspection Service has approved all six of the methods tested for use in screening corn for aflatoxin at the 20 ppb level in the official inspection system.

Aflatoxins in Midwestern Corn

Charles R. Hurburgh, Jr.

Aflatoxin has been a periodic problem in midwestern corn. Significant incidence occurred in 1983 and 1988. While aflatoxin is a chronic problem in Southeastern states, the effect on the corn market is much more pronounced when Midwestern corn is affected. In 1988, 9 corn belt states (SD, NE, MN, IA, MO, WI, IL, IN, OH) produced 3.8 billion bushels of corn, 78% of total U.S. production. Aflatoxin in these areas obviously creates major difficulties in finding clean stocks for export and sensitive domestic food uses.

Midwestern grain handlers receive corn very rapidly, especially at country elevators during harvest. Because aflatoxin test procedures are relatively slow and variable, identification of contaminated lots is nearly impossible on a large scale. Country elevators typically receive producer-deliveries at a rate about 1 load per minute.

The objectives of this discussion are:

1. To summarize available data on aflatoxin outbreaks in the midwest and

2. To relate reported aflatoxin levels to the needs and constraints of midwestern grain handlers.

Aflatoxin data for midwest corn

Incidence data is available for the two most recent aflatoxin outbreaks—1983 and 1988. However, any comparisons of data between states, or even within states, must be used with caution because sampling and analysis errors are large (coefficients of variation in the order of 25-50%).

Figure 1 shows the results of testing 99 samples of 1983 Iowa corn. Each sample was a composite of six 4-lb samples collected from randomly selected farm bins in the 99 Iowa counties. Composites were then subdivided into 10-lb analysis portions. July-August temperatures over the entire state were 2-3°C higher than normal, but the southern half of the state received about 6 inches less than normal rainfall as well. The aflatoxin was concentrated in areas with high temperature and low rainfall.

Figure 2 has the same type of data collected in Indiana. The samples were hand-harvested from fields just before harvest. Again, the concentration of aflatoxin in localized areas is evident.

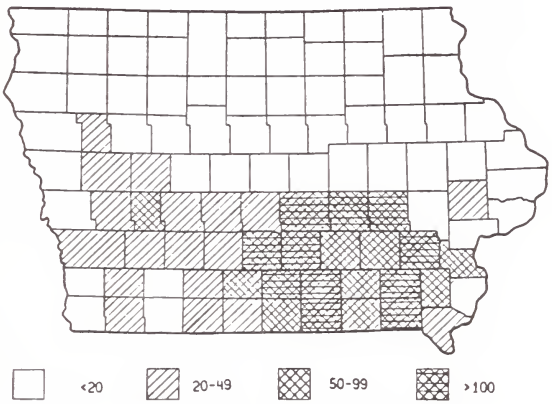


Figure 1. Aflatoxin level (ppb) in 1983 Iowa corn as estimated from TLC results on 10-11 lb county-composite samples.

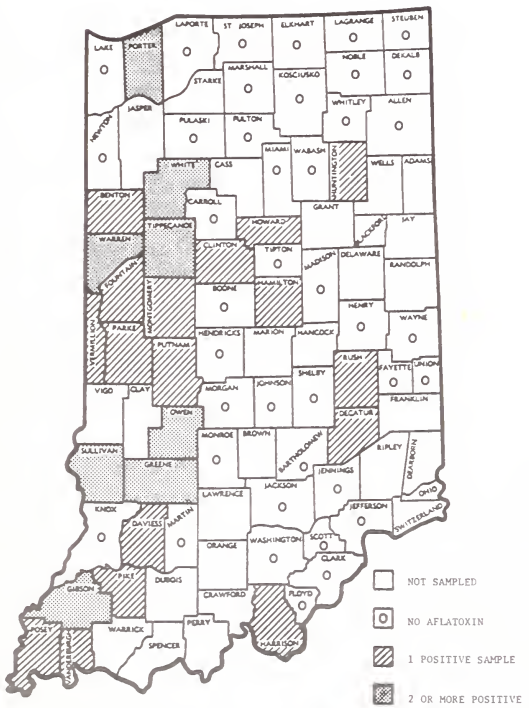


Figure 2. Distribution of positive aflatoxin (+20 ppb) samples in 1983 Indiana corn. 5 samples tested per county.

The usual method for presenting aflatoxin data is to tabulate the percentage of samples over certain levels (e.g. 20 ppb or 100 ppb). Iowa data from 1983 and 1988 is presented in this manner in Table 1.

This data illustrates the difficulty in generalizing about statewide or areawide averages. One might conclude that the average for all Iowa corn was 21 ppb, over the 20 ppb action level. Yet the map in Figure 1 clearly shows that large areas of Iowa were relatively free of aflatoxin. Aflatoxin results must be interpreted on a localized, case-by-case basis. Average aflatoxin figures are only meaningful to indicate the risk of creating indiscriminate mixes that will have aflatoxin levels in excess of action levels.

Table 1. Aflatoxin data for 1983 and 1988 Iowa corn[a]

	Year					
	1988			1983		
Aflatoxin range (ppb)	n	Percent of total	Average aflatoxin (ppb)	n	Percent of total	Average aflatoxin (ppb)
Above 100	7	7.2	64	11	11.1	73
20-99	22	22.9		26	26.3	
1-19	4	4.2		40	40.4	
None detected	63	65.7		22	22.2	
Totals	**96**	**100.0**	**21**	**99**	**100.0**	**21**

[a]Samples collected from farm bins in 1983, at country elevator dryers in 1988.

The local variability of aflatoxin is more apparent in Table 2. These are the individual subsample results from the ten counties that had a composite test in excess of 100 ppb (1983 crop). The composites weighed about 10-11 lbs, the individual subsamples 1.5-2.0 lbs. Even in these clearly high-risk areas, a significant portion of the samples tested below 20 ppb. It is inappropriate to apply generalizations about average levels to individual lots. The grain handler receiving many lots may experience the local average but individual grain sellers will be treated unfairly by averages. Unfortunately, summary reports to the public tend to accentuate such generalizations, to the detriment of the grower.

Table 2. Distribution of aflatoxin concentrations in county composite samples testing over 100 ppb. 1983 Iowa corn.

County number	Aflatoxin concentration in county composite ppb	Individual subsample results						
				Number of samples testing				
		Avg., ppb	C.V., %	0-20 ppb	21-100 ppb	101-200 ppb	201-500 ppb	501+ ppb
79	590	504	193	2	1	1	1	1
93	279	76	105	2	2	1	1	0
51	232	193	104	0	1	4	0	1
59	215	212	97	0	1	3	2	0
26	174	218	159	2	1	1	1	1
20	172	214	159	1	2	2	0	1
48	140	78	85	1	3	2	0	0
92	118	169	88	0	3	1	2	0
50	114	50	129	2	3	1	0	0
91	108	165	83	0	3	1	2	0
63	104	90	72	0	4	2	0	0
	207	181	105	10	24	19	9	4
				(15%)	(36%)	(29%)	(14%)	(6%)

Extensive publicity forced most midwestern states to conduct some sort of survey program in 1988. A summary of the results is given in Table 3. While Iowa and Illinois had higher incidence of aflatoxin, the diversity of survey methodologies and sampling frequencies greatly weakens any scientific conclusions that might be drawn. Given the variability within a county, even the state average percentages are uncertain. Unfortunately, the general public and media view such figures as indicative of the amount of contaminated grain in a state.

Other sources of public information are summaries of voluntarily submitted samples to either State laboratories or veterinary diagnostic laboratories. Both of these sources are completely biased because the samples were submitted because they were suspect, either from a black-light test, or from animal-health symptoms. In 1988 the Iowa Veterinary Diagnostic Laboratory found 75% of their submitted samples to be over 20 ppb, with 30% over 100 ppb. This was a totally unrealistic portrayal of incidence in Iowa, yet, absent more reliable data, this type of information has been used in the past to estimate levels.

The incidence problem is further compounded by the natural incentive for each state to downplay its situation. State government agencies are usually the agents for Food and Drug Administration in their respective states, and thus are called upon to do the testing. No state wants to portray itself as a hotbed of contamination, and thus risk market isolation. Clearly, if incidence surveys are to be done, they should be designed and managed by an agency with no local conflict of interest. Developments in testing technology may make marketplace screening so routine that there will be no need for government monitoring data.

Aflatoxin development is weather-related. Laboratory studies and field studies in the Southeast have provided information on critical weather parameters. High temperature (daytime high and nighttime low) is the necessary condition, with crop stress of various forms being magnifying factors. However, average weather conditions for a state or substate area have not been effective in predicting specific aflatoxin levels. Localized information is needed, as well as more knowledge of compounding agronomic factors. The extreme variation of aflatoxin in high-risk areas (Table 2) shows that factors in addition to average weather conditions have great influence over the presence or absence of aflatoxin.

However, weather data can play an important role in dealing with the sporadic incidence in midwestern corn. In years when the Iowa July-August mean temperature has exceeded 76-77° F, aflatoxin has been reported. These were also dry years because low rainfall gives little evaporative cooling, thus contributing to temperature rise. Macro-weather data may be very useful in estimating the risk of aflatoxin, even though it may

not predict specific levels or incidence percentages. The Iowa Aflatoxin Task Force, in its August 1989 report, suggested that the national weather database be used to forecast high-risk areas before harvest.

Table 3. A summary of aflatoxin survey results for the 1988 corn crop, midwestern states.

State	Percent of 1988 U.S. corn crop	No. of samples	Percentage with aflatoxin: 20-99 ppb	Above 100 ppb	Total	Pre-screening	Sample composition
IL	14.2	327	26.0%	9.8%	35.8%	None-all TLC	Farmer deliveries
IA	18.3	96	22.9%	7.2%	30.1%	Black light[a]	Dryer at country elevators
IN	8.4	373	6.2%	1.3%	7.5%	Black light[a] and Neogen column	Farmer deliveries, fields
MN	7.0	980	4.1%	1.9%	5.0%	Mini-column	Various
MO	3.1	———Monitoring program not statewide in 1988.———					
NE	16.6	141	5.0%	0.7%	5.7%	Black light[a], then mini-column	Farmer deliveries, fields

Table 3. A summary of aflatoxin survey results for the 1988 corn crop, midwestern states. (Cont.)

State	Percent of 1988 U.S. corn crop	No. of samples	Percentage with aflatoxin 20-99 ppb	Above 100 ppb	Total	Pre-screening	Sample composition
OH	5.2	241	5.4%	1.1%	6.5%	All TLC	Farmer deliveries
SD[b]	2.7	150	4.6%	6.0%	10.6%	Not available	Not available
WI	2.7	50	0	0	0	Neogen column	Various
Overall[b]	78.2		13.2	4.3	17.5		

[a]Percentage of false positives from black light: 1A-57%, IN-45%, NE-more than 50%

[b]Weighted by relative crop production, excluding Missouri.

Aflatoxin and grain handlers

The country grain handler faces a decision as to whether to accept or reject potentially aflatoxin-contaminated corn. At country elevators, that decision must be made in 1-2 minutes, the time normally available for grading. The more complete inspection done at river grain terminals, processing plants and export elevators allows more time for aflatoxin testing at those points.

Aflatoxin levels are also the most variable in farmer-deliveries, which often originate from an individual field. The variations shown in Table 2 are probably typical of what a country elevator in a high-risk area would receive. Lot-to-lot variations are compounded by the well-documented random error in sampling and testing, in the order of ± 50%.

The only truly rapid screening method available to country elevators is the black-light. The ELISA-based tests require 8-10 minutes per sample, which is too long for accept/reject decisions. Even if used properly, the black light gives 40-60% false positives (BGYF fluorescence without aflatoxin). Attempts to relate the number or weight of fluorescing particles (glowers) to aflatoxin concentration have been unsuccessful, with coefficients of variation over 100%. The black light test can only establish risk, not definitive presence or absence of aflatoxin.

Therefore, the country grain handler's problem is one of assessing risk and of estimating the average concentration of large lots consolidated from farmer deliveries. If more than 20-30% of individual lots are truly in excess of 20 ppb, then the risk of having a consolidated shipment test greater than 20 ppb is relatively high.

Judicious use of the black light can reduce this risk. For 1983 and 1988 Iowa corn, the average concentration of samples with fewer than 5 BGYF particles (unground sample) per kg was 10 ppb with 70% false positives, while samples with 5 or more BGYF particles averaged 65 ppb with only 20% false positives. Individual samples with low glowers per unit weight did have much higher levels (and vice-versa), but, overall, low glower counts had less aflatoxin. Using glower count to assess risk is not the same as using glower count to predict aflatoxin level.

The point is that country elevators in high-volume grain production areas need sampling and analysis techniques compatible with their handling constraints. Their decision process must necessarily be based on risk probabilities. Their legal options for handling grain must be flexible enough to accommodate the variability in sampling, testing and lot-to-lot aflatoxin levels. Perfect identification of <u>all</u> contaminated lots is not possible at this point.

The county grain elevator also acts as a warehouse for growers without sufficient on-farm storage to meet their marketing needs. Warehouses are licensed under either state or federal statutes, but in either case, warehouses must maintain corn of quality equal or better than that certified on warehouse receipts. Generally warehouse receipts are only issued for the standard Grades, which by implication means less than 20 ppb aflatoxin. Clearly, warehouses in high-volume areas must know the aflatoxin status of grain received. The cost of a warehouse examination showing a large storage contamination is very great. The sampling error is yet further magnified in a storage structure compared to a bulk carrier. In 1988, there were reports of uneven treatment of warehouses with suspect grain by the regulatory agencies, between various state jurisdictions and Federally licensed warehouses.

Analysis and Commentary

The incidence data demonstrate the difficulty of assessing aflatoxin levels in a growing area. So far, aflatoxin contamination in midwestern corn has been limited enough that the natural consolidation of grain lots into larger shipments eventually diluted aflatoxin concentrations below 20 ppb in most cases. This would not apply to users drawing from localized high-risk areas. A much better early-warning system is needed to identify potential high-risk areas.

The at-harvest surveys do respond to public pressure for information, but their methodology should be standardized. The estimated average aflatoxin levels, and distribution, are very uncertain, which means that data presentation formats should be carefully designed. Because each state has a vested interest in not finding aflatoxin, surveys should be administered at the Federal level.

The current FDA status of aflatoxin as an adulterant creates a strong incentive not to know aflatoxin levels. The U.S. high-volume grain industry is designed around the ability to adjust quality by mixing, blending, and dilution. Labelling of grain as unmarketable, particularly based on tests with great uncertainty, is a major problem for handlers. Therefore, it is sometimes perceived as advantageous not to know, or not to suspect. The public would be better protected by a more open, flexible system that provides disposition options at the raw commodity level, rather than outright rejections. One option offered by the Iowa Aflatoxin Task Force is to allow unrestricted blending of aflatoxin lots up to 50-75 ppb, so long as the blended mix met the applicable FDA limits for its intended use. High aflatoxin lots would be marketed separately as feed. The burden, in the present system, is placed mainly on the end-user preparing to convert corn into food, meat or dairy products. When aflatoxin is a problem in corn belt areas, FDA simply does not have the resources to police an adulterant-oriented approach to aflatoxin. The grain industry has to have enough realistic disposal options to create an incentive for self-regulation at the local elevator level, and for FGIS monitoring at the export and processor levels.

No handler will be able to make perfect identification of aflatoxin lots. Therefore, grain receival procedures should be designed to reduce risk, and remove with some certainty the lots with the highest aflatoxin levels. Successive application of such methods will relieve the burden on the final users and better protect the consuming public.

Aflatoxin should no longer be regarded as a rare event in the midwest. It has occurred 3 times in the past 12 years. Aflatoxin risk assessment, monitoring and test procedures should become part of the normal course of grain business. There is no reason why each new outbreak should be treated as a new and surprising event, raising public fears over food safety. The problems of aflatoxin in midwest corn are no different than are associated with aflatoxin in other areas, but the volume of grain handled in the midwest greatly accentuates both the public health and grain handling difficulties. Fortunately, technology exists, or could exist with appropriate research support, to deal with aflatoxin in a practical manner that will protect the public to a greater extent than is now done.

Aflatoxin in Field Crops in the Southeastern United States

Winston M. Hagler, Jr.

Introduction

Aflatoxins were discovered in the early 1960's during research on Turkey-X disease in England. This mycotoxicosis, which killed thousands of turkeys, was caused by moldy Brazilian peanut meal contaminated with aflatoxin. The four aflatoxins discovered in this work, aflatoxins B_1, B_2, G_1, and G_2, are most frequently encountered as contaminants of susceptible crops. Aflatoxin B_1 is the predominant aflatoxin in contaminated crops. Aflatoxins are produced by the closely related imperfect fungi. *Aspergillus flavus* Link ex. Fries produces aflatoxins B_1 and B_2, and *Aspergillus parasiticus* Speare produces all four toxins.

Since the beginning of aflatoxin research, the "aflatoxin problem" has been an economic burden for the corn, cotton, peanut, livestock, and poultry industries. Because aflatoxins are acutely toxic and carcinogenic in rats and other species, there has always been concern about contamination of food with aflatoxins. In the late 1960's, the U.S. Food and Drug Administration established action level (20 ppb) for aflatoxin in corn (*Zea mays* L.), peanuts (*Arachis hypogea* L.), cottonseed (*Gossypium hirsutum* L.), and other commodities. Because dairy cattle consuming contaminated diets convert aflatoxin B_1 into aflatoxin M_1 which is secreted into milk, a limit of 0.5 ppb M_1 was established for milk. Nichols (1983) reported a study of the economic effect of aflatoxin contamination of corn on the grain and livestock industries in the Southeast. Similar economic impact studies for aflatoxin in the peanut and cottonseed industries have apparently not been conducted.

Aflatoxin research was initially focused on post-harvest pathology, mycology, and animal toxicology. In the mid-1970's, aflatoxin was discovered before harvest in U.S. corn (Lillehoj et al., 1983). This discovery stimulated research in the plant sciences which has given a much clearer perspective and some techniques for management of the aflatoxin problem in corn. However, proper storage and handling of susceptible commodities remain crucial to maintaining them non-moldy and toxin free. Unfortunately, no useful genetic resistance to aflatoxin accumulation has been found for any of the susceptible crops.

Discussion

In corn, peanuts, and cotton the host/fungus/environment interaction is critical to predisposition for aflatoxin contamination. The dominant factor seems to be drought and/or temperature stress at critical times in the life cycles of these susceptible crops (Diener, 1989). Agronomic practices and insects also play major roles in exacerbation of aflatoxin contamination in the field (Stoloff, 1988; Diener, 1989).

Aflatoxin contamination of corn and cottonseed in the U.S. shows distinct regional differences (Lillehoj, 1983; Diener, 1989). Contamination of the corn crop with aflatoxin is decidedly more severe in the Southeast because of the likelihood of drought/heat stress at silking (Diener, 1989). Cotton is produced in the Southeast and Southwest, but aflatoxin contamination of cottonseed is most severe in the Southwest. Southeastern cottonseed seems to escape significant contamination because certain climatic conditions protect cotton from invasion by the fungus. There may be consistent intra-regional differences in peanut contamination in the Southeast due to climatic conditions, but these have not been reported.

Information about the extent of aflatoxin contamination from year to year in corn within the Southeast is not readily available. Unlike in peanuts, there has been no systematic record-keeping of aflatoxin contamination in Southeastern corn. Most corn buyers today monitor for aflatoxin contamination, but their records are not generally available. Random sample surveys were done for corn in a few states in the region from the late 1970's through the early 1980's, but these have been discontinued.

Peanuts

The history of aflatoxin contamination in Southeastern peanuts is available from the government/industry certification program. Reports derived from these data offer excellent perspectives on the problem of aflatoxin in peanuts in the U.S. (Stoloff, 1977, 1986; National Peanut Council, 1988; CAST, 1989). For example, Table 1 contains data on the extent of contamination of peanuts in Virginia for the crop years 1986-1988 (Mooney et al., 1990). Of 14,775 peanut lots tested over the three-year period, only 111 tested at above 25 ppb. Proper irrigation of peanuts during drought decreases the severity of aflatoxin contamination, but irrigation is an added production cost.

Table 1. Aflatoxin contamination in Virginia peanuts, 1986 - 1088[1].

Crop Year	Samples	Aflatoxin, ppb 0-15 %	16-25 %	26-100 %	>100 %
1986	6,225	96.7	2.1	1.1	0.1
1987	4,077	98.1	1.3	0.5	0.1
1988	4,473	99.0	0.7	0.2	0.1
Total	14,775	97.8	1.4	0.7	0.1

[1] From Mooney et al., 1990.

Corn

Information about the year-to-year aflatoxin contamination of corn in the Southeast is not easy to compile. A good impression of aflatoxin contamination in North Carolina corn from 1976 - 1985 can be obtained from the data in Tables 2 and 3 (Nichols, 1983, 1987). Since ca. 1985, this type of information has not been gathered, and there is little survey data available from the other states in the Southeast. Although the percentages of samples containing >20 ppb in all years are considerable, 1980, with nearly 66% of the lots tested exceeding 20 ppb, stands out as the worst year on record. According to Nichols (1983), Alabama, Virginia, and South Carolina had greater aflatoxin contamination in 1980 than in previous years. Unfortunately, irrigation, which can almost eliminate contamination, is not economically feasible for corn in the Southeast.

Table 2. Aflatoxin in North Carolina corn, 1976 - 1980. [1]

Crop	Aflatoxin (ppb) <20 %	20-100 %	>100 %
1976	64.2	27.7	8.0
1977	58.1	30.2	11.6
1978	87.0	12.0	1.0
1979	67.3	28.3	4.4
1980	34.3	48.1	17.6

[1] Source: N.C. State University/N.C. Department of Agriculture elevator surveys. (From Nichols, 1987)

Table 3. Aflatoxin in North Carolina corn, 1981 - 1985.[1]

Crop	Aflatoxin (ppb) <20 %	20 - 100 %	>100 %
1981	76.4	14.9	8.7
1982	91.8	5.8	2.4
1983	49.9	28.6	21.5
1984	79.6	11.6	8.8
1985	85.4	10.4	4.2

[1]Farmer samples submitted to N.C. Department of Agriculture. (From Nichols, 1987).

Small Grains

Domestic wheat, rye, barley, oats, and sorghum are probably not susceptible to extensive preharvest aflatoxin contamination. Stoloff (1977) reported that of 3,489 samples of these small grains analyzed by the USDA between 1968 and 1975, only 19 samples contained detectable aflatoxin; the average aflatoxin concentration in the positive samples was 5 ppb. Wheat, barley, and oats analyzed by the N.C. State University Mycotoxin Laboratory in last 5 years have shown some incidence of aflatoxin contamination, but the levels detected have been very low. These commodities have had higher incidence and concentrations of the mycotoxins deoxynivalenol and zearalenone (W.M. Hagler, Jr., unpublished).

There is more recent information on preharvest mycotoxin contamination in Southeastern grain sorghum (*Sorghum bicolor* L.) [McMillian et al., 1983; Babadoost et al., 1987; Hagler et al., 1987]. Aflatoxin does not seem to be a significant problem in Southeastern sorghum, but contamination with *Fusarium* toxins may be significant from year to year. Preharvest sorghum in 1980 and 1981 in Georgia's Coastal Plain and Mississippi was tested for aflatoxin and zearalenone by McMillian et al. (1983). Incidence of contamination in Georgia was rather high (56%), but concentrations were low, 0-99 ppb of aflatoxin B_1. The sorghum grown in Georgia exhibited significant contamination with zearalenone, 31% incidence ranging from 0-1,468 ppb. In 1980 and 1981 the Mississippi sorghum sampled had neither aflatoxin nor zearalenone contamination.

Hagler et al. (1987) examined North Carolina grain sorghum in crop years 1981-1985 for preharvest aflatoxin, zearalenone, and deoxynivalenol. Aflatoxin incidence was high, but levels in all years were very low. In 1981, aflatoxin incidence was 44%, but concentrations ranged from 0-13 ppb. Zearalenone incidence and concentrations in all years were high. Significant deoxynivalenol contamination was also found. Babadoost et al. (1987) sampled sorghum from 17 farmers' storage bins in 6 North Carolina counties for aflatoxin, zearalenone, and deoxynivalenol analysis. Aflatoxin was found in trace amounts (ca. 1 ppb) in 4 of 17 samples. Zearalenone incidence was 100% and concentrations ranged from 7-2,024 ppb; deoxynivalenol was found in 5 of the 17 samples ranging from 10-558 ppb. Rainfall at flowering was the predisposing factor for preharvest zearalenone and deoxynivalenol contamination (Hagler et al., 1987).

Soybeans

In the U.S., domestically produced soybeans are probably not subject to significant preharvest aflatoxin contamination. A USDA survey of 866 lots revealed only two aflatoxin-positive samples contaminated at 10 and 11 ppb (Stoloff, 1977). A number of soybean meal and moldy soybean samples from the 1985 crop in North Carolina were analyzed for aflatoxin (Hagler et al., 1989). Only two of these samples, one each of beans and soybean meal, were found to be contaminated and these showed <5 ppb of aflatoxin B_1.

Cottonseed

Diener (1989) outlined the probable reasons that preharvest aflatoxin contamination of cottonseed produced in the Southeast is not a serious problem when compared to that in cottonseed produced under irrigation in the arid Southwest. In North Carolina, analysis of 35 samples of suspect cottonseed, believed to be grown in the Southeast, collected 1987-1989 on dairy farms in Virginia, North Carolina, and Delaware for aflatoxin revealed 11 aflatoxin positive samples. Levels ranged from 0-480 ppb with an overall means only of 23 $\pm$ 85 ppb (W.M. Hagler, Jr., unpublished).

Conclusion

Mycotoxins, especially aflatoxins, are a serious agricultural and public health problem. The situation in the peanut industry is somewhat better than with Southeastern corn or Southwestern cottonseed. The peanut certification program buffers the industry economically and limits aflatoxin in food peanuts. However, the expense to the industry and taxpayers is great. Cottonseed and small grains in the Southeast are apparently not susceptible to serious preharvest aflatoxin contamination. The preharvest aflatoxin problem in Southeastern corn is acute. Research to-date has just begun to define the host/fungus/environment relationships involved in preharvest aflatoxin contamination. More research is needed so that solutions can be found which eliminate aflatoxin contamination from susceptible crops. Aflatoxin and other mycotoxins also arise in crops after harvest, during storage. Better handling and storage techniques are needed to protect the producer and consumer, and to improve our position in overseas markets.

Acknowledgements

The cooperation of Dr. Gerald C. Llewellyn, Bureau of Toxic Substances, Virginia Department of Health in providing some of the data herein is greatly appreciated.

Literature Cited

Babadoost, M., W.M. Hagler, Jr., D.T. Bowman, and P.E. Nelson. 1987. Field contamination of sorghum with zearalenone and deoxynivalenol in North Carolina: Density segregation to remove mycotoxins. pp. 99-109. In: "Biodeterioration Research 1", G.C. Llewellyn and C.E. O'Rear, eds., Plenum Press, New York. 388 pp.

Council for Agricultural Science and Technology (CAST). 1989. Mycotoxins: Economic and health risks. Task Force Report No. 116. CAST, Ames, Iowa. 91 pp.

Diener, U.L. 1989. Preharvest aflatoxin contamination of peanuts, corn, and cottonseed: A review. pp. 217-244. In: "Biodeterioration Research 2", G.C. Llewellyn and C.E. O'Rear, eds., Plenum Press, New York. 697 pp.

Hagler, W.M., Jr., M. Babadoost, S.P. Swanson, and D.T. Bowman. 1987. Aflatoxin, zearalenone, and deoxynivalenol in North Carolina grain sorghum, 1981-85. Crop Sci. 27:1273-1278.

Hagler, W.M., Jr., F.T. Jones, and D.T. Bowman. 1989. Mycotoxins in North Carolina 1985 crop soybeans. I. Zearalenone and deoxynivalenol in soybeans and soybean meal. pp. 337-349. In "Biodeterioration Research 2." G.C. Llewellyn and C.E. O'Rear, eds. Plenum Press, New York.
697 pp.

Lillehoj, E.B., M.S. Zuber, L.L. Darrah, W.F. Kwolek, W.R. Findley, E.S. Horner, G.E. Scott, A. Manwiller, D.B. Sauer, D. Thompson, H. Warren, D.R. West, and N.W. Widstrom. 1983. Aflatoxin occurrence and levels in preharvest corn kernels with varied endosperm characteristics grown at diverse locations. Crop Sci. 23:1181-1184.

McMillian, W.W., D.M. Wilson, C.J. Mirocha, and N.W. Widstrom. 1983. Mycotoxin contamination in grain sorghum in fields from Georgia and Mississippi. Cereal Chem. 60:226-227.

Mooney, R.L., G.C. Llewellyn, and C.E. O'Rear. 1990. Aflatoxin in Virginia peanuts for the crop years 1986-1988. In: "Biodeterioration Research 3", G.C. Llewellyn and C.E. O'Rear, eds. Plenum Press, New York. (in press).

National Peanut Council. Quality Task Force. 1988. U.S. peanut quality: An industry commitment: Consensus report of the peanut quality task force. National Peanut Council, Alexandria, Virginia. 48 pp.

Nichols, T.E., Jr. 1983. Economic effects of aflatoxin in corn. pp. 67-71. In: "Aflatoxin and *Aspergillus flavus* in Corn." Diener, U.L., R.L. Asquith, and J.W. Dickens, eds. Southern Cooperative Series Bulletin 279. Auburn Agricultural Experiment Station, Auburn Alabama. 112 pp.

Nichols, T.E., Jr. 1987. Aflatoxin in the southeastern USA. pp. 339-347. In: "Aflatoxin in Maize: A Proceedings of the Workshop." Zuber, M.S., E.B. Lillehoj, and B.L. Renfroe, eds. CYMMYT, Mexico, D.F. 389 pp.

Stoloff, L. 1977. Aflatoxins - an overview. pp. 8-28. In: "Mycotoxins in Human and Animal Health", J.V. Rodricks, C.W. Hesseltine, and M.A. Mehlman, eds. Pathotox Publishers, Inc., Park Forest South, Illinois. 807 pp.

Stoloff, L. 1986. A rationale for the control of aflatoxin in human foods. pp. 457-472. In: "Mycotoxins and Phycotoxins", P.S. Steyn and R. Vleggar, eds., Elsevier Scientific Publishing Co., Amsterdam, The Netherlands. 545 pp.

Incidence of Aflatoxin in the South Central United States

George W. Latimer

My name is Dr. George W. Latimer, Jr. I appreciate this opportunity to represent Dr. William Y. Cobb, the Texas State Chemist, and the Office of the Texas State Chemist in discussing the subject of the incidence of aflatoxin in the South Central United States. These remarks relate specifically to Texas. The feed law under which this Office operates restricts our regulatory efforts to products grown, manufactured or sold within the State so we have not conducted surveys outside of Texas. However, the State encompasses such a wide geographical and climatic range of conditions that we see crops and feeds grown under many different circumstances; thus, our observations can reasonably be generalized.

The technical aspects of this subject either have been or will be dealt with by other speakers; therefore, I should like to address these topics:

- First, the mycotoxin survey program that our Office has undertaken over the last five years.

- Second, our observations about the occurrence of aflatoxins in Texas feed crops over the last five years.

- Third, the regulatory program that we have in place for dealing with grains and animal feeds containing aflatoxin at various levels.

As introduction to our aflatoxin monitoring program, let me summarize some important aspects of Texas geography, climate and agricultural practice. The State itself stretches through 5 climate zones, i.e., from that characteristic of Southern Florida to that characteristic of Northern New Jersey, New York City and Connecticut. Soils vary from solid clay to sand. Rainfall patterns vary from a yearly average of 45 to 56 inches in East and Southeast Texas to 8 to 10 inches in West Texas. This creates a wide range of micro-environments and permits a variety of possible crop planting patterns if rainfall is normal; however, during the last several years Texas has experienced severe drought, with areas of the south and east several inches below normal. Further affecting agricultural practice is the equally important, but often ignored fact that economics as well as

federal agricultural policy often tempt the farmer or rancher to push nature to the limit in many areas by engaging in risky planting practices, e.g., planting corn in areas more suited to sorghum or other crops. When viewed from this perspective, and considering the ubiquitous nature of the *A. flavus* mold, it is perhaps surprising that Texas has not generally experienced the degree of aflatoxin crop contamination seen in the southeastern states.

Indeed, prior to 1988 there was only one serious incident of aflatoxin contamination - an episode requiring the Office of the State Chemist to seize and dispose of some 90,000 bushels of corn from a ranch in Southeast Texas. By serious incident, I mean that the grain growing, handling and using communities were severely affected. This instance was unique since it originated in bad management practice exacerbated by weather. Specifically, the owners, whose property was located in traditional rice country, chose, extremely late in the planting season, to plant corn instead of rice. They seeded and fertilized simultaneously thus obtaining quick growth, but not substantial rooting, just as the dry season arrived. Drought stress during the silking and dough stages was exacerbated by heavy rains at harvest, circumstances which induce *A. flavus* growth and aflatoxin production. While it is possible that neither improper management nor adverse weather by itself would alone have caused the problem, the combination certainly did. Therein, I believe, lies a cautionary tale worthy of attention.

In 1986 the Office of the Texas State Chemist initiated an on-going survey of grains, feed ingredients and finished feeds. This action was not so much a response to a specific incident as it was to (1) secure baseline information for mycotoxin regulatory program planning, and (2) to protect against the immediate presence of aflatoxin and other mycotoxins in both domestic and import products. While all grains were surveyed, emphasis was placed on collecting corn, cottonseed, oats, soybeans and sorghum in commerce. These are the chief grains used in Texas feeds, and corn and cottonseed are particularly sensitive to the *A. flavus* organism. We also sampled a spectrum of beef and dairy feeds; aflatoxin in dairy feeds is of great concern because metabolites of aflatoxin B_1 (and possibly others) find their way into the animal's milk.

The surveys were not designed to be statistically correct models due to resource limitations; however, when combined with other evidence, the collected data give a reasonable year-to-year picture of the problems experienced in the State.

The monitoring program was developed in a way which would best utilize the 12 inspectors at our disposal and our small laboratory, while

conforming to the limitations imposed by the Texas Feed Law. Thus, the Office of the Texas State Chemist made informed, rather than statistical decisions about sampling in the areas of interest. By informed, I mean we were guided by the experience of Dr. W. Y. Cobb, who had been deeply involved with the aflatoxin problems as North Carolina State Chemist in the late '70s and early '80s.

While suitable for a survey under conditions where no problems are suspected, the events of 1988 and 1989 necessitate a change in emphasis. In the first place, while in other years samples were taken at random, the 1988-1989 sampling was very area specific, i.e., we went where the evidence suggested there were problems. In the second place, sampling was based on a final use monitoring strategy, i.e., we sampled and tested at feed mills, working forward to the consumer or backward to the grain supplier, if contamination was found. This permitted us to help direct aflatoxin-containing materials to their ultimate end-use, i.e., cattle feedlot or burial. I emphasize again that our monitoring strategy was based on our work limited force and the situation; I do not recommend final-use monitoring as necessarily the best strategy for the feed market-place. While the recommendation to that effect made by the Iowa Aflatoxin Task Force may be best for the Midwest, the feed industry in Texas is made up of a spectrum of various sized firms and in our case it is not necessarily true that the small to medium-sized feed firms or even the mills of the corporate giants have better sampling and testing capabilities than their suppliers, if they have either at all.

One final caveat about discussions of the 1988 crop year needs to be entered into the record. Neither a historical nor a geographical summary really represents the full extent of the difficulties suffered nor the possible extent of the problems for two reasons.

1. In the 1988 corn crop year many grain elevators accepted corn without adequate testing. Thus, as this grain comes out of the silos the problems with 1988 corn may be worse than we now know.

2. Some grain crops, primarily corn, have been left in the field to attract and feed game, primarily migratory water fowl and deer. We have no idea, of the degree to which aflatoxins have infiltrated or affected Texas fauna.

Prior to conducting this survey, we found it both a scientific and an economic necessity to devise a rapid on-line high performance liquid chromatographic analytical procedure which would allow us to determine

six mycotoxins-aflatoxins B_1, B_2, G_1, G_2, ochratoxin, and zearalenone simultaneously. Using this technique, we surveyed 150 samples of whole grain in FY 86, i.e., the period September 85 to August 86. In FY 87, i.e., the period September '86 to the end of August '87, we surveyed some 200 grain samples taken statewide. In neither case were levels found above 20 ppb of any aflatoxin. Other toxins were likewise at insignificant levels.

By July 1988 we had acquired 200 samples and, although there were a few samples exceeding 20 ppb aflatoxin, these did not seem significant nor was there a pattern. Thus, to that point in time, four facts support the belief mentioned earlier, i.e., that there was a low level of incidence of contamination in the State of Texas:

1. The State Chemist surveys of FY 86 and 87.

2. The lack of claims filings with insurers under the Federal Crop Insurance Program.

3. The extremely low incidence of aflatoxin M_1 found in market milk by state health authorities.

4. A relative low (< 10% for all purposes) rejection of crops at elevators which handle corn for exports.

The 1988 new crop began coming to market in August and at that time I received a call from Mr. Ron George, a reporter for the Corpus Christi Call Times. Mr. George had heard reports that the Corpus Christi Grain Exchange was turning away "a lot" (ca. 50% of the loads) of Nueces County field corn based on positive aflatoxin tests, i.e., sample analyzing more than 20 ppb. Since we had been told anecdotally that in a "good" year only 10% or so of loads are so rejected, we immediately began a detailed sampling, first in the area around Corpus Christi and then throughout the State as the harvest moved north. It quickly became evident that corn throughout all of the drought-stricken areas in Texas was affected. Northeastern Texas, while not a huge corn producer, harvested corn which reflected extremely high levels of aflatoxin (some over 1,000 ppb) in virtually all lots tested. As a result of our surveys, we believe the 1988 aflatoxin problem was circumscribed in an area bounded by a line from Wichita Falls, Texas through Waco to the Rio Grande and across to either the Gulf Coast or the Louisiana line, and affected 90% of the feed corn crops (Fig. 1).

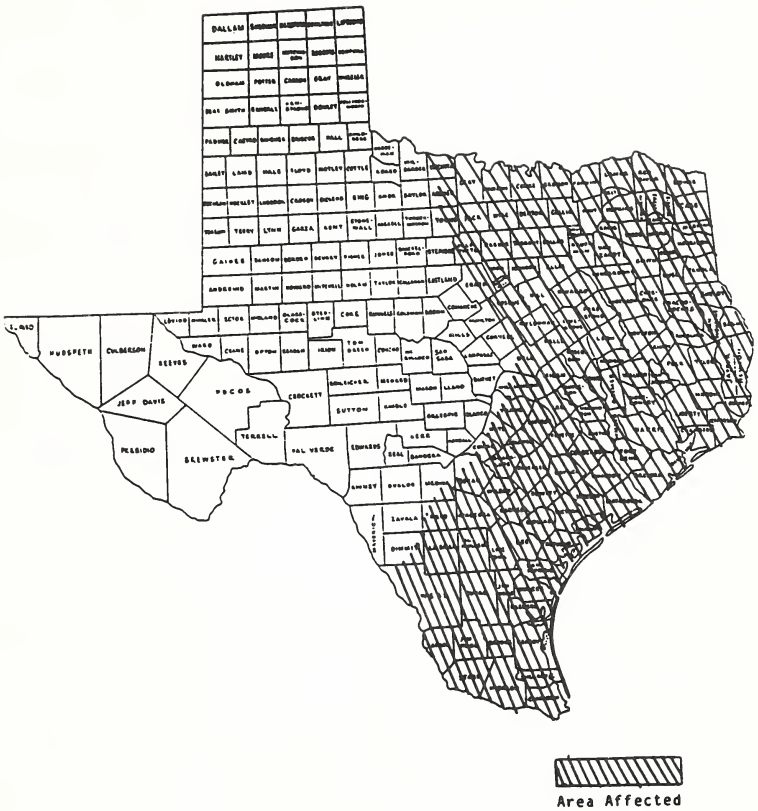


Figure 1.
Approximate extent of 1988 aflatoxin contamination.

Our 1989 crop year survey plus other sources of information indicate that it is the southern part of the State, roughly the area running on a line from Victoria through Uvalde to the Rio Grande River and then south to the border and the Gulf that has a severe aflatoxin problem. In the irrigated areas of the Rio Grande Valley, food corn has been very severely affected because the natural rainfall depended upon to supplement irrigation never came. Even here, we estimate that only 13-30% of the corn exceeded 300 ppb and there appears to be a considerable overall decrease of aflatoxin occurrence and levels from 1988 (Fig. 2).

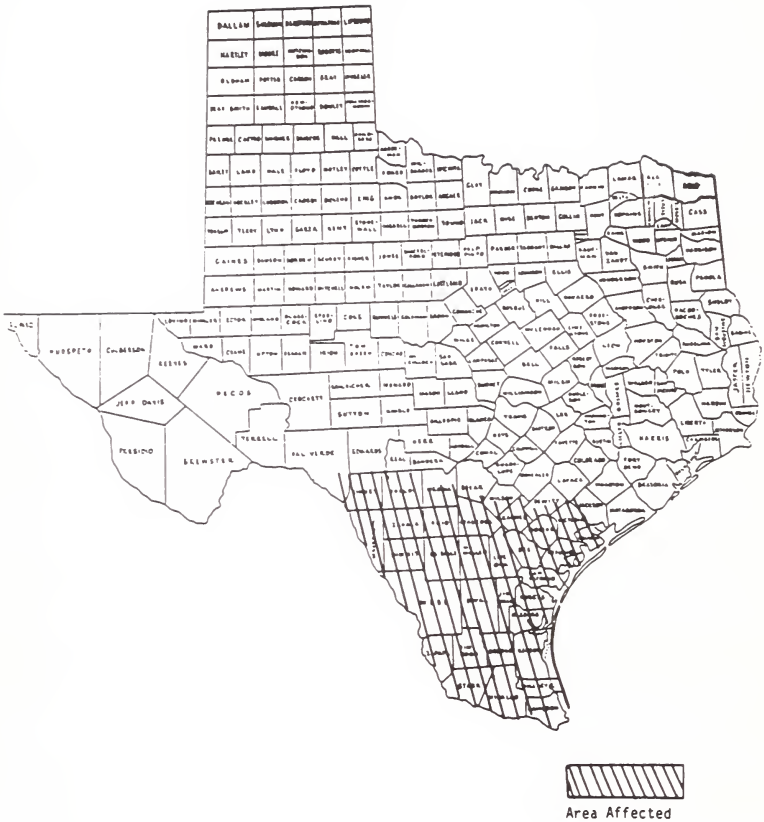


Figure 2.
Approximate extent of 1989 aflatoxin contamination.

These surveys (Tables 1 and 2) show that there is little aflatoxin infestation outside of corn. The aflatoxin present in dairy feeds comes primarily from corn, secondarily from cottonseed. Almost certainly soybean contamination arises from abuse of the beans after the harvest. There is also little evidence that ochratoxin and zearalenone are problems; however, in early November 1989 the Texas A&M Veterinary Diagnostic Laboratory identified Fusarium moniliforme and the mycotoxin Fumonisin B_1 as the causative agent in the death of horses in coastal counties between Houston and the Louisiana border. Fumonisin B_1 appears to induce lesions in the equine brain. So far, the outbreak appears to be

limited to a geographic area which received enormous late spring and early summer rains, subsequently topped by torrential rainfall from a tropical gulf storm. Since there are no readily available analytical methods for determining the level of Fumonisin in feeds, autopsies are needed to provide diagnosis and estimates of level.

Table 1. Mycotoxin Survey of Grains, FY 1985 Through FY 1989

Grains	Number of samples	Number Positive			Number of Regulatory Significance		
		# B1	# OTA	# ZER	# B1	# OTA	# ZER
Alfalfa	5	0	0	0	0	0	0
Barley	28	1	0	0	1	0	0
Chaff	1	0	0	0	0	0	0
Corn	447	215	14	3	168	5	0
Cottonseed	220	95	59	11	63	3	5
Oats	95	18	9	1	2	2	0
Peanuts	22	13	1	0	9	1	0
Rice	32	3	5	0	0	5	0
Sorghum	224	14	29	47	3	11	26
Soybean	40	3	0	2	0	0	1
Sunflower	1	1	0	0	1	0	0
Wheat	100	22	9	11	0	0	3

Table 2. Mycotoxin Survey of Feedstuffs, FY 1985 Through FY 1989

Feed-stuffs	Number of samples	Number Positive			Number of Regulatory Significance		
		# B1	# OTA	# ZER	# B1	# OTA	# ZER
Beef Feeds	15	5	0	1	4	0	0
Brewers Grains	2	0	0	0	0	0	0
Dairy Feed	112	35	0	0	16	0	0
Dog Food	9	3	0	0	2	0	0
Dried Milk	1	0	0	0	0	0	0
Horse Feed	3	0	0	0	0	0	0
Mix Feed	4	2	0	1	2	0	0
Poultry Feed	4	2	0	0	2	0	0
Sheep Feed	2	0	0	0	0	0	0
Swine Feed	8	3	0	0	1	0	0
Yeast	1	0	0	0	0	0	0

So much for history - what is our regulatory position?

First, we have made a concerted effort to educate the Texas grain and feed industry and the farmers-ranchers with our regulatory policies on aflatoxins, which parallel those of the FDA, as well as the circumstances favoring the production of aflatoxins. The Texas Agricultural Extension Service has been of great service to the State in updating an earlier brochure on the subject, in disseminating information to county agents, and in sponsoring workshops to discuss issues involving mycotoxins. The Office of the Texas State Chemist has made extensive efforts to notify appropriate industry representatives personally, to provide discussions and talks at various grain and feed conferences and meetings and to meet with groups of concerned farmer-ranchers where appropriate.

Second, we will continue extensive sampling and testing during each harvest year. Our 1990 action plan targets corn, whole cottonseed, cottonseed meal, milo, dairy and starter rations. Grain elevators and feed mills are encouraged to test incoming grain by lot before commingling with other grain. Much progress has been made. In 1987 few elevators or mills screened incoming grain for aflatoxin. Where screening occurred, most frequently only an ultraviolet light was used to search for glowing particles, a procedure which is quite unsatisfactory when used to detect aflatoxin contamination.

These steps, of course, are prospective in character. There is still a great deal of 1988 and 1989 corn in silos to be disposed of, much of which has not been tested. The intensified interest of feed mills and end users will ensure proper end use for a high percentage of it.

Where it is desirable to recondition corn containing high aflatoxin for use in less-sensitive species rations, e.g., mature beef cattle, the State Chemist has developed the following program.

1. If the corn was originally intended as food grade and has been placed under embargo by the Texas Department of Health, or is under seizure or withholding agreement by the Food and Drug Administration, the lot is released to the State Chemist for reconditioning following discussion with the cooperating regulatory agenciesand the owner or claimant of the grain.

2. A written reconditioning agreement is devised for each episode.

3. Actual conditions of reconditioning are predicated on original analyses of the material. If these analyses exceeded 200 ppb, constant on-site monitoring of testing and handling of data is undertaken by an agent of the State Chemist.

4. If original analyses are less than 200 ppb, approximately 20% of loads is to be sampled and analyzed. This extent of sampling should provide appropriate information about the aflatoxin levels in the grain being shipped.

5. Each load is to be labeled appropriately.

6. Individual loads exceeding 300 ppb must be immediately buried in a landfill or disced into farmland soil under supervision.

7. All shipments to feedlots are for finishing feeding of mature beef cattle, unless otherwise agreed.

8. There is no blending allowed for 1989 crop corn.

The agricultural community in Texas is still sorting out the events of the last two years to see what lessons can be learned. Several principles seem clear, however:

1. Growers must now accept the fact that toxin-producing molds and fungi represent a permanent and serious threat to their crops and manage their crops to minimize such infestations.

2. Producers of feed must now accept the fact that aflatoxins or other mold fungi-produced toxins may be present in products they buy and in products they produce.

3. The agricultural community must work out on acceptable protocol for testing these products so that their interests as well as the interests of the ultimate consumer of feeds may be protected. How this is to be managed I cannot yet visualize, but the outlines must start taking shape. The Office of the Texas State Chemist would be happy to work with any and all elements of the regulatory and agricultural communities to design and appropriate programs for this purpose.

Again, thank you for the opportunity to appear.

Topic II

Environmental Factors and Other Stresses Important in the Production of Aflatoxin in Corn

D.T. Wicklow

Aspergillus flavus produces yellow-green spores that function in dispersal and as infective inoculum, in addition to long-lived survival structures called sclerotia. Both types of propagules are associated with damaged corn kernels and are dispersed onto the ground during combine-harvesting (Wicklow and Horn, 1984; Wicklow et al., 1984). Sclerotia are able to survive overwintering in Georgia and Illinois (Wicklow, 1987). Upon germination, the sclerotia produce large numbers of yellow-green spores (Fig. 1; Wicklow and Donahue, 1984). Wicklow and Wilson (1986) found that sclerotium germination occurred in corn fields just prior to silking. Shade provided by the corn canopy helps to retain moisture at the soil surface, thus promoting germination. Stack and Pettit (1984) reported germination of buried sclerotia at soil moisture levels slightly below saturation with subsequent fungal colonization of dead roots. Therefore, simple burial of *A. flavus* sclerotia by plowing will not necessarily eliminate this important source of primary inoculum. Soil-inhabiting fungivorous mites disperse spores throughout the soil and carry them through cracks in the peanut fruit (Aucamp, 1969).

We recently contrasted the survival of both sclerotia and spores of *A. flavus* that were buried for 6 months to 3 years (Oct. 1986 - Oct. 1989) in sandy field soil in southern Georgia and central Illinois (D.T. Wicklow & D.M. Wilson, unpublished results). Nearly all of the *A. flavus* spores had disappeared within the first year, while many sclerotia remained viable and produced spores after 3 years. Another soil-inhabiting fungus, *Paecilomyces lilacinus* (Thom) Samson colonized and rotted many of the *A. flavus* sclerotia, thus pointing to a potential means of biological control (Wicklow and Wilson, 1990). Sclerotium survival is poor in heavier soils that become seasonally inundated (Wicklow, 1987). The opportunity to eliminate *A. flavus* inoculum from soil occurs in fields that are seasonally rotated from rice to peanuts as practiced in some areas of Thailand (Wicklow, 1989). Unfortunately, this approach is not feasible in sandy, well-drained soils typical of the Georgia Coastal Plain. An understanding of the dynamics of *A. flavus* populations in soils under cultivation (Angle et al., 1982; Griffin and Garren, 1976; Martynink and Wagner, 1978) is central to the design of agronomic/cultural practices with the objective of reducing levels of *A. flavus* inoculum in those soils.

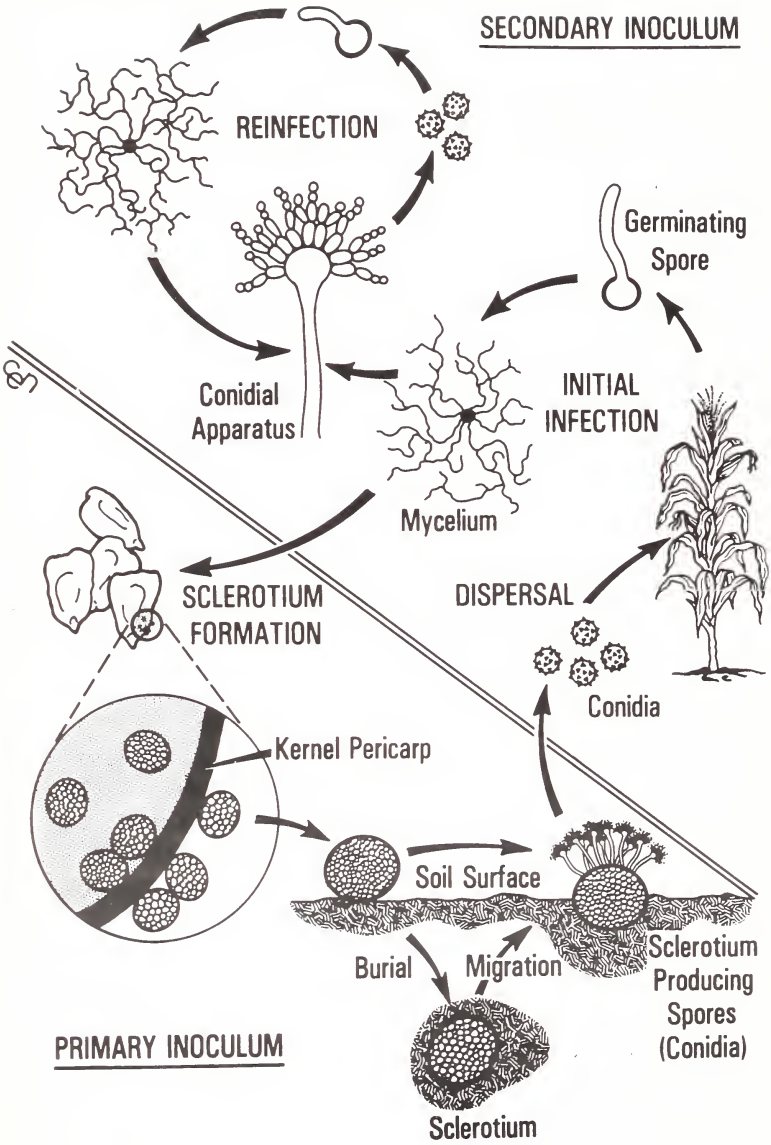


Figure 1.
Schematic diagram, not to scale, showing the relationship between primary and secondary inoculum in the life cycle of *A. flavus* (Wicklow and Donahue, 1984).

The most widely accepted model of *A. flavus* contamination of corn (Diener et al., 1987) involves three steps: (1) airborne or insect-transmitted spores contaminate corn silks, and the fungus then grows down through the silks into the developing ear; (2) portions of the ear having kernels damaged by insects or birds become rotted by *A. flavus* and contaminated with aflatoxin; (3) cultural factors that stress the corn plant, such as drought, increase susceptibility to fungal infection. Initial attempts to identify insects that might carry *A. flavus* to maize have proven inconclusive (Diener et al., 1987). While many kinds of insects collected from maize ears are reported to carry *A. flavus* (Fennell et al., 1977), collections have typically been made late in the season at a time when secondary inoculum of *A. flavus* is so abundant that insects are more likely to become contaminated. These studies did not identify the initial source(s) of *A. flavus* infective inoculum. A significant association between earworm damage, presence of 'picnic beetles' (Nitidulidae: Coleoptera), and incidence of aflatoxin contamination in corn has been reported in the southern United States (Lillehoj et al., 1975), but the authors did not consider the possibility that picnic beetles were potential vectors of *A. flavus*. Our research shows that these beetles overwinter in and feed on molded crop residues (e.g. lodged corn ears) and carry *A. flavus* spores to ripening corn ears (Fig. 2). The beetles gain entry to the ears through wounds caused by other insects, birds, etc. and are capable of entering some ears on their own (Connell, 1956; Tamaki et al., 1982; Wicklow, 1989). Increased losses in corn from insect damage is attributed to new early maturing hybrids with loose, open husks and quick dry-down attributes. These hybrids have recently been introduced in the southeast (Barry et al., 1986; McMillian, 1987), and offer ready access to picnic beetles (Barry, 1986).

When *A. flavus*-contaminated picnic beetles fly to damaged maize ears, they contaminate the wounds with *A. flavus* spores. The damaged kernels become contaminated with substantial quantities of aflatoxin (i.e. to @ 60,000 ppb). The fungus can then spread to infect the adjacent sound kernels (Wicklow et al., 1988). Aflatoxin also accumulates (to @ 4,000 ppb) in many of the 'sound kernels.' It takes only a few of these aflatoxin-contaminated kernels in a grain sample to register a bulk concentration > 20 ppb aflatoxin. Efforts to identify maize genotypes resistant to *A. flavus* kernel rot and aflatoxin contamination have been unsuccessful (Davis et al., 1986). No maize genotype has a complete defense against ear-feeding insects, and no half-eaten kernel is resistant to molding by *A. flavus*.

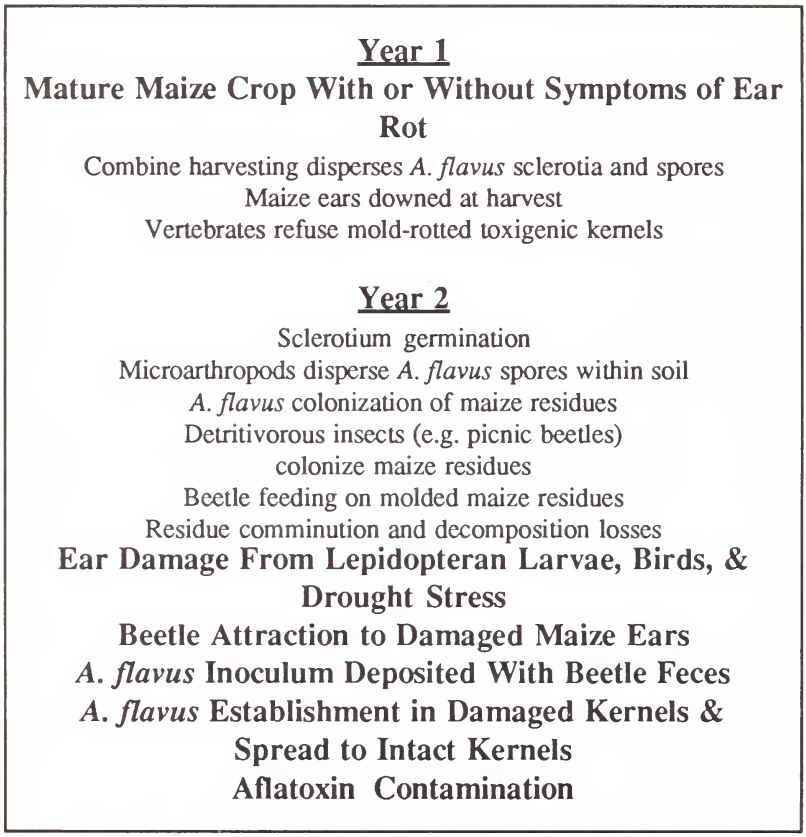


Figure 2.
A simplified conceptual model of how preharvest maize becomes infected with *A. flavus* and contaminated with aflatoxins. Events occurring in the standing maize crop depicted in UPPER CASE Letters, with action on ground indicated by lower case letters (Wicklow, 1989).

Picnic beetle aggregation patterns in corn fields could explain the irregular distribution of *A. flavus* and aflatoxin among damaged ears from the same field. One strategy that has proven effective in controlling picnic beetles, in fig orchards, is the use of bait trapping (Warner, 1960; Warner, 1961). Our discovery that picnic beetles produce aggregation pheromones that can be synthesized (Bartelt et al., 1990), and that plant and fungal volatiles, from decaying host tissues, synergize the attractancy of the beetle aggregation pheromone (Dowd and Bartelt, 1988), should enable us to develop superior traps.

Temperature stress (evening temperatures > 80 C), drought stress, nitrogen stress and crowding of corn plants are all associated with *A. flavus* contamination and aflatoxin formation (Anderson et al., 1975; Davis et al., 1986; Jones et al., 1981b; Smart et al., 1990; Thompson et al., 1980). The cause of this association is believed to be the greater susceptibility of corn plants to fungal invasion when they are stressed (Jones and Duncan, 1981a). Zuber et al. (1983) found that high temperatures, especially during grain-filling stages, were more important than moisture in enhancing the level of aflatoxin. Drought and high temperatures should also promote the proliferation of *A. flavus* populations in and around corn fields, although there is no experimental data showing that, with prolonged drought, corn insects become contaminated more likely with *A. flavus* before flying to the corn.

I have attempted to illustrate how variations in climate, sources of fungal inoculum, potential insect vectors and the response of corn plants to stress can interact in various ways to produce an aflatoxin outbreak. Our integrated disease management program seeks to reduce damage to corn ears from drought/temperature stress and from insects while attempting to eliminate natural reservoirs of *A. flavus* spores and sclerotia. The screening of corn hybrids for resistance to aflatoxin contamination has been disappointing because of the variable amounts of aflatoxin at different geographical locations and from year to year (Davis et al., 1986; Widstrom et al., 1984, 1987). It is difficult to select for drought stress resistance when conditions necessary for its expression cannot be controlled. Side-by-side dryland and irrigated trials (Jensen and Cavalieri, 1987) should be used in testing corn varieties for resistance to insect damage and *A. flavus* infection under drought and temperature stress at a location, where aflatoxin contamination of corn is a recurrent problem. In 1989 we began cooperative research with a major commercial corn seed company, to screen their corn hybrid genotypes for resistance to *A. flavus* infection and aflatoxin contamination of the kernels. We are encouraged by preliminary results showing that our screening procedure can pair rows of the same hybrid when planted as part of a blind screen.

References

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

Angle, J.S., K.A. Dunn, and G.H. Wagner. 1982. Effect of cultural practices on soil populations of *Aspergillus flavus* and *A. parasiticus*. J. Soil Sci. Soc. Amer. 46:301-304.

Aucamp, J.L. 1969. The role of mite vectors in the development of aflatoxin in groundnuts. J. Stored Product Research 5:245-249.

Barry, D., E.B. Lillehoj, N.W. Widstrom, W.W. McMillan, M.S. Zuber, W.F. Kwolek, and W.D. Guthrie. 1986. Effect on husk tightness and insect (Lepidoptera) infestation on aflatoxin contamination of preharvest maize. Environ. Entomol. 15:1116-1118.

Bartelt, R.J., P.F. Dowd, R.D. Plattner, and D. Weisleder. 1990. Aggregation pheromone of the driedfruit beetle, *Carpophilus hemipterus*: wind-tunnel bioassay and identification of two novel tetraene hydrocarbons. Chemical Ecology (in press).

Connell, W.A. 1956. Nitidulidae of Delaware. Univ. Delaware Agric. Exper. Sta. Bull. #318, 67 pp.

Davis, N.D., C.G. Currier, and U.L. Diener. 1986. Aflatoxin contamination of corn hybrids in Alabama. Cereal Chem. 63:467-470.

Diener, U.L., R.J. Cole, T.H. Sanders, G.A. Payne, L.S. Lee, and M.A. Klich. 1987. Epidemiology of aflatoxin formation by *Aspergillus flavus*. Ann. Rev. Phytopathol. 25:249-270.

Dowd, P.F. and R.J. Bartelt. 1988. Synergized attraction of *Carpophilus hemipterus* by combinations of aggregation pheromones and host volatiles. Entomol. Soc. Amer. Annual Meeting, Louisville, KY, Dec. 4-8, 1988. Abstract.

Fennell, D.I., W.F. Kwolek, E.B. Lillehoj, G.A. Adams, R.J. Bothast, M.S. Zuber, O.H. Calvert, W.D. Guthrie, A.J. Bockholt, A. Manwiller, and M.D. Jellum. 1977. *Aspergillus flavus* presence in silks and insects developing and mature corn ears. Cereal Chemistry 54:770-778.

Griffin, G.J. and K.H. Garren. 1976. Colonization of rye green manure and peanut fruit debris by *Aspergillus flavus* and *Aspergillus niger* group in field soils Appl. Environ. Microbiol. 32:28-32.

Jensen, S.D. and A.J. Cavalieri. 1987. Maize plant stress inheritance, pp. 178-186. In: Aflatoxin in maize: a proceedings of the workshop. M.S. Zuber, E.B. Lillehoj and B.L. Renfro, eds. CIMMYT, Mexico, D.F.

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

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

Lillehoj, E.B., W.F. Kwolek, E.E. Vandegraft, M.S. Zuber, O.H. Calvert, N.W. Widstrom, M.C. Futrell, and A.J. Bockholt. 1975. Aflatoxin production in *Aspergillus flavus* inoculated ears grown at diverse locations. Crop Science 15:267-270.

Martynink, S. and G.H. Wagner. 1978. Quantitative and qualitative examination of soil microflora associated with different management systems. Soil Sci. 125:343-350.

McMillian, W.W. 1987. Relation of insects to aflatoxin contamination in maize grown in the Southeastern USA, pp. 194-200. In: Aflatoxin in Maize: A proceedings of the workshop. M.S. Zuber, E.B. Lillehoj and B.L. Renfro (eds.), Mexico, D.F., The International Maize and Wheat Improvement Center (CIMMYT).

Smart, M.G., D.T. Wicklow, and R.W. Caldwell. 1990. Pathogenesis of *Aspergillus* ear rot of maize. 2. Light microscopy of fungal spread from wounds. Phytopathology (in press)

Stack, J. and R.E. Pettit. 1984a. Germination of *Aspergillus flavus* sclerotia in soil. Phytopathology 74:799. Abstract.

Stack, J.P. and R.E. Pettit. 1984b. Colonization of organic matter substrates in soil by *Aspergillus flavus*. Proc. Amer. Res. Ed. Soc. 16:45.

Tamaki, G., L. Fox and P. Featherston. 1982. Laboratory biology of the dusky sap beetle and field interaction with the corn earworm in ears of sweet corn. J. Entomol. Soc. Brit. Columbia 79:3-8.

Thompson, D.L., E.B. Lillehoj, K.J. Leonard, W.F. Kwolek, and M.S. Zuber. 1980. Aflatoxin concentration in corn as influenced by kernel development stage and post-inoculation temperature in controlled environments. Crop Science 20:609-612.
Warner, R.M. 1960. Area baiting to control *Drosophila* and Nitidulid beetles. Proc. Cal. Fig. Inst. 14:35-38.

Warner, R.M. 1961. Area baiting program - 1960 results.
Proc. Cal. Fig. Inst. 15:36-40.

Wicklow, D.T. 1987. Survival of *Aspergillus flavus* sclerotia in soil. Trans. Br. Mycol. Soc. 89:131-134.

Wicklow, D.T. 1989. Maize cultivation selects for mycotoxin-producing ability among kernel-rotting fungi, pp. 1-12. In: Phytochemical Ecology: Allelochemicals, Mycotoxins and Insect Pheromones and Allomones, C.H. Chou and G.H. Waller (eds.), Institute of Botany, Academia Sinica Monograph Series No. 9. Taipei, R.O.C.

Wicklow, D.T. and J.E. Donahue. 1984. Sporogenic germination of sclerotia in *Aspergillus flavus* and *A. parasiticus*. Trans. Br. Mycol. Soc. 82:621-624.

Wicklow, D.T. and B.W. Horn. 1984. *Aspergillus flavus* sclerotia form in wound-inoculated corn. Mycologia 76:503-505.

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

Wicklow, D.T., B.W. Horn, O.L. Shotwell, C.W. Hesseltine, and R.W. Caldwell. 1988. Fungal interference with *Aspergillus flavus* infection and aflatoxin contamination of maize grown in a controlled environment. Phytopathology 78:68-74.

Wicklow, D.T. and D.M. Wilson. 1986. Germination of *Aspergillus flavus* sclerotia in a Georgia maize field. Trans. Brit. Mycol. Soc. 87(4):651-653.

Wicklow, D.T. and D.M. Wilson. 1990. *Paecilomyces lilacinus* (Thom) Samson a colonist of *Aspergillus flavus* Link: Fr. sclerotia buried in Illinois and Georgia. Mycologia (in press).

Widstrom, N.W., E.B. Lillehoj, A.N. Sparks, and W.F. Kwolek. 1976. Corn earworm damage and aflatoxin B1 on corn ears protected with insecticide. J. Econ. Entomol. 69:677-679.

Widstrom, N.W., D.M. Wilson and W.W. McMillian. 1984. Ear resistance of maize inbreds to field aflatoxin contamination. Crop Sci. 24:1155-1157.

Widstrom, N.W., W.W. McMillian, and D.M. Wilson. 1987. Segregation for resistance to aflatoxin contamination among seeds on an ear of hybrid maize. Crop Sci. 27:961-962.

Zuber, M.S., L.L. Darrah, E.B. Lillehoj, L.M. Josephson, A. Manwiller, G.E. Scott, R.T. Gudauskas, E.S. Horner, N.W. Widstrom, D.L. Thompson, A.J. Bockholt and J.L. Brewbaker. 1983. Comparison of open-pollinated maize varieties and hybrids for preharvest aflatoxin contamination in the southern United States. Plant Disease 67: 185-187.

Biocontrol of Aflatoxin Production by Using Biocompetitive Agents

Richard J. Cole and Peter J. Cotty

Corn, peanuts, cottonseed and tree nuts are the important commodities in which aflatoxin contamination can occur in the United States (Diener et al., 1987). Advanced technology has been developed to detect and remove the aflatoxins from these commodities and this permits some segregation of contaminated components and serves as a guide for detoxification (Goto and Manabe, 1989). Storage conditions necessary to prevent postharvest contamination are known and detoxification procedures have been developed to treat contaminated commodities (Park et al., 1988). Decontamination procedures, however, are costly and can result in reduced product quality. Sound management of aflatoxin contamination should begin in the field prior to harvest. This is where the toxigenic fungi first become associated with the crop and where the contamination process begins. Contamination occurring prior to harvest is often the predominant problem in the United States. Techniques to manage aflatoxin contamination during crop production have been developed for peanuts and cottonseed in recent years, but these methods are not adequate to ensure aflatoxin free commodities (Cole et al., 1989; Cotty, 1989a).

Recent consumer concerns related to pesticide residues in the food supply require that alternative methods of pest control be developed. The use of biological control provides an attractive alternative to pesticides. Biological control can be of three basic types. (1) Use of an agent that destroys the pest such as a predator or parasite, (2) an agent that secretes a toxin(s) that destroys the pest and (3) the use of an agent that competes with the pest in its ecological niche. The topic of this discussion relates to the later; specifically, the use of biocompetitive agents (BA) as a biological control strategy for preharvest aflatoxin contamination of agricultural commodities. It has been demonstrated that *Aspergillus flavus* and *A. parasiticus* (the term aflatoxin-producing fungi) do not require the aflatoxins to invade plant tissues (Cotty, 1989b). This implies that non-toxigenic strains of *A. parasiticus* and *A. flavus* may be potentially useful as agents directed at competitively excluding toxigenic strains. Studies on prevention of aflatoxin contamination of peanuts with non-toxigenic strains of *A. parasiticus* have been conducted at the National Peanut

Research Laboratory (NPRL) and studies on the use of non-toxigenic strains of *A. flavus* to prevent contamination of cottonseed have been conducted at the Southern Regional Research Center (SRRC).

At NPRL studies have utilized highly competitive non-aflatoxin-producing strains of *A. parasiticus*, which replace the wild toxic strains of *A. parasiticus* and *A. flavus*. This is achieved without a dramatic increase in fungal propagules that would normally be present in the soil. The major advantage with this strategy is that the non-toxic strains of *A. parasiticus* or *A. flavus* occupy the same ecological niche as the toxic strains. The use of other biocompetitive agents such as bacteria would also appear to be an attractive strategy; however, these become inactive under the extremely hot and dry conditions associated with preharvest aflatoxin contamination and thus do not occupy the same or a similar ecological niche. Therefore, they are not ideal biocompetitive agents for this application. The only possible impact these agents could provide would be production of inhibitory chemicals secreted into the soil during times when soil conditions are favorable for bacterial growth (#2 above).

At NPRL we have a powerful experimental facility available that provides excellent control of soil moisture, soil temperature and soil microflora. The research facility (environmental control plots) was utilized initially to elucidate environmental parameters responsible for preharvest aflatoxin contamination of peanuts. These have been essentially elucidated and now the facility provides a valuable tool to develop and test preharvest prevention strategies, including the use of biocompetitive agents.

A brief summary of studies at NPRL using non-aflatoxigenic strains of *A. parasiticus* follows. In 1987 we added a BA to one of our environmental control plots to test the concept. The study was conducted over a three-year period. No additional BA was added to the soil the two subsequent growing seasons (1988-1989). Each of the three years the peanuts were subjected to ideal conditions for preharvest aflatoxin contamination, harvested and subsequently analyzed for aflatoxin. Populations of both BA and wild toxigenic strains were monitored. The results of the aflatoxin analyses demonstrated a dramatic reduction in the level of aflatoxin contamination all three years when compared to peanuts from non-BA treated soil. The three-year study provided evidence that the concept is effective and justifies continued research to develop and refine this prevention strategy. Additional BA have been identified and are currently being tested or will be tested over the next few years.

An important observation in addition to the effectiveness of BA in reducing aflatoxin was that the BA replaced the wild toxigenic species without dramatically increasing the total number of fungal propagules in the soil. This is an important environmental consideration.

At SRRC several non-toxigenic strains of *A. flavus* have been isolated from agricultural fields. These strains are highly pathogenic to cotton and yet do not produce detectable levels of aflatoxins in developing cottonseed (Cotty, 1989b). Aflatoxin contamination of cottonseed in agricultural fields is often associated with *A. flavus* infection of pink bollworm damaged bolls (Cotty and Lee, 1989; Ashworth et al., 1971) and a method of inoculating greenhouse grown bolls with *A. flavus* was developed which closely simulates this phenomenon (Cotty, 1989b). With this greenhouse inoculation technique, the ability of non-toxigenic strains of *A. flavus* to interfere with cottonseed infection and aflatoxin production by toxigenic strains was evaluated. Several non-toxigenic strains of *A. flavus* were effective at reducing aflatoxin contamination of developing cottonseed when inoculated into bolls simultaneously with virulent toxigenic strains (Cotty, 1989c). The most effective strain reduced toxin 10 to 100 fold in various experiments. When cotton bolls were inoculated with the non-toxigenic strain 24 hr prior to the toxigenic strain, the seed contained over 100 fold less toxin at maturity than when the cotton bolls were inoculated with the toxigenic strain alone. These greenhouse studies suggest that non-toxigenic strains of *A. flavus* endemic to agricultural fields may be useful as biocompetitive agents directed at preventing aflatoxin contamination of cottonseed.

In conclusion, results of a three-year field plot study at NPRL and of greenhouse studies at SRRC have shown that this strategy can significantly reduce preharvest aflatoxin contamination in peanuts and cottonseed. Additional studies to refine the use of non-toxigenic *A. flavus* and *A. parasiticus* strains, including addressing human health and environmental impact concerns, are therefore justified.

There are a number of advantages that the *A. parasiticus* and *A. flavus* biocompetitive approaches have over other biological control approaches and other conventional control approaches. Both toxigenic and non-toxigenic strains are adapted to exploiting the same environmental conditions; so conditions that favor increases in toxigenic strains will also favor increases in the non-toxigenic strains. If a drought occurs and we previously applied our *A. parasiticus* or *A. flavus* BA out in the field, the BA will increase in proportion with the toxigenic strains and continue to be effective. This probably would not be the case with other biocontrol agents. Therefore, the BA will be active under the same

conditions as toxigenic strains and thus will be most active when the BA is needed most. Also, the BA should protect both damaged and undamaged seed. We know that the contamination on crops is frequently associated with insect and other damage. Certain plant defense mechanisms are not effective in dead or damaged seed or in crops under severe and prolonged drought stress, whereas the BA would be effective. In addition, the BA strategy should protect the seed both prior to harvest and after harvest. This is because, even when there are postharvest problems, the toxigenic fungus usually became associated with the crop in the field prior to harvest. The BA that becomes associated with the crop prior to harvest should continue the association with the crop through all stages of potential vulnerability.

References

Ashworth, L.J., Jr., R.F. Rice, J.L. McMeans, and C.M. Brown. 1971. The relationship of insects to infection of cotton bolls. Phytopathology 61:488-493.

Cole, R.J., T.H., J.W. Dorner, and P.D. Blankenship. 1989. Environmental conditions required to induce preharvest aflatoxin contamination of groundnuts: summary of six years' research. In: Aflatoxin contamination of groundnuts. pp. 279-287. ICRISAT, Patancheru, India.

Cotty, P.J. 1989a. Aflatoxins in cottonseed—what can be done? Proc. 38th Oilseed Processing Clinic. pp. 30-38. Southern Regional Research Center, New Orleans, LA.

Cotty, P.J. 1989b. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79:808-814.

Cotty, P.J. 1989c. Prevention of aflatoxin contamination with strains of *Aspergillus flavus*. Phytopathology 79:1153.

Cotty, P.J. and L.S. Lee. 1989. Aflatoxin contamination of cottonseed: comparison of pink bollworm damaged and undamaged bolls. Trop. Sci. 29:273-277.

Diener, U.L., R.J. Cole, T.H. Sanders, G.A. Payne, L.S. Lee, and M.A. Klich. 1987. Epidemiology of aflatoxin formation by *Aspergillus flavus*. Ann. Rev. Phytopathol. 25:249-270.

Goto, T. and M. Manabe. 1989. Methods for analysis of aflatoxins in groundnut and other agricultural commodities. In: Aflatoxin contamination of groundnuts. pp. 173-182. ICRISAT, Patancheru, India.

Park, D.L., L.S. Lee, R.L. Price, and A.E. Pohland. 1988. Review of the decontamination of aflatoxins by ammoniation: current status and regulation. J. Assoc. Off. Anal. Chem. 71:685-703.

Control of Biosynthesis of Aflatoxin in Strains of *Aspergillus Flavus*

T. E. Cleveland, D. Bhatnagar and P. J. Cotty

Historical Background

Aflatoxins are toxic, carcinogenic chemicals produced by the fungal species, *Aspergillus flavus* and *A. parasiticus*. These fungi can infect crops before and after harvest and produce aflatoxins, thereby contaminating foods and feeds and threatening both human and animal health (Goldblatt, 1970; Jelinek et al., 1989). The first report of the toxicity of aflatoxins appeared nearly thirty years ago in Great Britain (Blount, 1961) when the cause of the extensively publicized turkey "X" disease was traced to contaminated peanut meal from Brazil. The disease caused the deaths of over 120,000 turkeys and other poultry and led to a huge research effort to investigate aflatoxin contamination of foods and feeds in the USA and other countries.

Aflatoxin was first detected in cottonseed and cottonseed meal about 25 years ago and, since then, Agricultural Research Service scientists at the Southern Regional Research Center have investigated methods to control aflatoxin contamination of food and feed commodities. Initial research by scientists at the Center centered on the development of analytical techniques to quantify aflatoxins and on the development of methods to detoxify contaminated products (Goldblatt, 1970; Park et al., 1988; Pons and Goldblatt, 1965). In the 1980's, scientists in our research Unit began research on Arizona-grown cottonseed to develop an understanding of the environmental and ecological factors that control the synthesis of aflatoxin in agricultural commodities. Research efforts centered on prevention of aflatoxin contamination before harvest to prevent the initial association of aflatoxin with crop tissues; this strategy was designed to alleviate the need to either discard contaminated crops or subject the crops to costly detoxification procedures. The research was carried out in Arizona cotton fields having some of the highest endemic levels of aflatoxin known. The knowledge generated by this research eventually led to the formulation by project scientists of certain recommended cultural practices, which if used by growers would reduce preharvest aflatoxin contamination in Arizona cottonseed (Cotty, 1989a). Briefly, these recommendations included: 1) prevention of insect damage of cotton bolls during certain defined periods in the growing season (boll

damage caused by certain specific insects is known to predispose cottonseed to aflatoxin contamination); and 2) early harvest to prevent amplification of aflatoxin in cottonseed in open bolls before harvest, and also to prevent late season increases of overwintering insects which may damage and predispose the crop to aflatoxin contamination early in the following year.

Unfortunately, traditional control methods (such as the use of certain cultural practices, pesticides and resistant varieties), which effectively reduce populations of many plant pests in the field, are not entirely effective in controlling aflatoxin producing fungi. With the potential for a continued lowering of permissible levels of aflatoxin in crops by regulatory agencies, it seems unlikely that conventional methods will achieve the extremely low levels of aflatoxin that are required in commercial food and feed. Therefore, our scientists have embarked on a three pronged research effort to understand how and why the fungus makes aflatoxin. Since the mid 1980's, our scientists have conducted research on three major projects to acquire knowledge of: 1) the molecular and biochemical mechanisms controlling aflatoxin formation within the fungus, 2) environmental factors and biocompetitive microbes controlling growth of *A. flavus* and aflatoxin biosynthesis in crops, and 3) enhancement of host plant resistance against aflatoxin through understanding the biochemistry host plant resistance responses. It is our objective to obtain a complete understanding of the factors controlling aflatoxin biosynthesis in host plant tissues. This understanding will lead to the development of novel biocontrol strategies and/or, in longer term research, development of elite crop lines "immune" to aflatoxin producing fungi.

Regulation and Control of Aflatoxin Biosynthesis

Many of the critical biochemical factors controlling aflatoxin formation by the fungus, *A. parasiticus*, have been elucidated by Food and Feed Safety researchers (Bhatnagar et al., 1989). Several of the chemical intermediates in the aflatoxin pathway have been identified by project scientists (Bennett and Lee, 1977). Identification of aflatoxin pathway intermediates allowed these compounds to be used as enzyme substrates to detect aflatoxin pathway enzymes. Several critical enzymes, including a methyltransferase (Bhatnagar et al., 1989), a reductase (Bhatnagar and Cleveland, 1990) and an oxidoreductase (Bhatnagar et al., 1989), catalyzing key steps in the aflatoxin pathway, have been identified, purified and characterized. An antiserum probe was made against the methyltrans-

ferase protein to study the molecular regulation of this catalyst (Cleveland and Bhatnagar, 1989) and to identify the gene coding for this aflatoxin pathway enzyme. Cloning of aflatoxin pathway genes will provide molecular probes for future applications in biotechnology involving: 1) investigation of the molecular trigger controlling the onset of aflatoxin biosynthesis, and 2) precision inactivation of a specific steps/genes in aflatoxin biosynthesis to produce aflatoxin non-producing fungi.

Biocompetitive Agents

The use of aflatoxin non-producing strains of A. flavus to exclude aflatoxin producing strains from field environments

The studies described above will assist us in developing stable, aflatoxin non-producing strains of *A. flavus* which could be used as biocompetitive agents. The cloning of aflatoxin pathway genes will, in the near future, allow precise and stable elimination of aflatoxin steps to produce domesticated fungal strains for use in biocontrol applications.

Previous research on native, non-toxigenic strains of *A. flavus* (their stability is still unknown) in this laboratory (Cotty, 1989c) has shown that aflatoxin production is apparently not required for infection of plant tissues and is apparently "disposable" (perhaps by genetic engineering) in the development of non-toxigenic, biocompetitive agents. The use of aflatoxin non-producing strains of *A. flavus* in a biocontrol approach is based on the premise that other conventional control measures or even certain novel methods (such as use of biocontrol strains of bacteria) will very likely yield only partial control of *A. flavus* and aflatoxin contamination under the environmental conditions favoring this fungus. Fungi in the *A. flavus* group are ubiquitous, will grow on practically any organic substrate (both living and dead plant tissues) and are superbly adapted to Arizona desert environments or areas subjected to periodic droughts. The adaptation of *A. flavus* for growth at high temperatures and low water potentials enable this fungus to outcompete other commonly occurring microflora (especially bacteria) under drought conditions. Preliminary results from this laboratory (Cotty, 1989c) have shown that non-toxigenic strains of *A. flavus*, adapted to the same ecological niche as toxigenic strains, can effectively exclude toxigenic strains of this same fungus from cotton boll tissues. The use of non-toxigenic strains of *A. flavus* as biocompetitive agents has been proposed as a generic approach to control aflatoxin in cotton, corn, peanut, and tree nuts; these strains are being

extensively tested on cotton as the model crop system in this laboratory and initial results are promising, but trials still need to be initiated to test fungal biocontrol strains on other commodities.

Application of soil amendments and/or soil bacteria to reduce A. flavus populations in field environments

Although bacteria may be poor biocontrol agents during drought conditions conducive to growth of aflatoxin producing fungi on commodities, certain of these microbes may be very effective in suppressing *A. flavus* in soils. Bacterial suppression of soil populations of toxigenic fungi could occur in soils not subjected to drought conditions or in soils of crops receiving periodic irrigation. A new project has been initiated in our laboratory to test certain soil amendments such as chitosan (derived from seafood waste such as crab and shrimp shells) to encourage the growth of certain beneficial soil bacteria (e.g. the actinomycetes). Chitosan amendments are known to induce soil microbes that are effective in suppressing populations of certain filamentous fungi (such as *A. flavus*) existing in agricultural soils (Sneh et al., 1971). Endogenous actinomycetes or other beneficial microbes existing in particular agricultural fields could, in theory, be isolated, identified and used to "seed" chitosan amendments during field application to promote optimum antifungal activity. If soil provides the primary *A. flavus* inoculum (conidia or sclerotia) that infects susceptible cottonseed (and other crops), then chitosan amendments may have a suppressive effect on the primary inoculum in the soil and, thus, reduce initial preharvest infection of cottonseed and other crops by *A. flavus*.

Another interesting biological effect of chitosan observed in the laboratory, is its direct inhibition of growth of *A. flavus* in culture (Cuero et al., 1988). Chitosan also induces plant resistance responses (phytoalexins) which may ward off aflatoxin producing fungi (Hadwiger et al., 1984). All of these biological effects of chitosan could be related to the observed protection of crops against fungal pests in soils amended with this substance (Sneh et al., 1971) and these effects are being further investigated.

Enhancement of Host Plant Resistance Against Aflatoxin Contamination

Traditional plant breeding

Researchers have identified varieties of corn (Scott and Zummo, 1988; Widstrom, 1987) and cotton (Cotty, 1989b) that demonstrate measurable levels of resistance which, in theory, could provide germplasm for enhancement of host plant resistance to useful levels against aflatoxin producing fungi. Efforts to breed for resistance against these toxigenic fungi have been hampered by high variability from sample to sample within a given genotype (Scott and Zummo, 1988) and the resistance that was identified is apparently multigenic and complex.

New molecular techniques for enhancing host plant resistance

Identification of biochemical "markers" for resistance against *A. flavus*, would greatly facilitate selection of resistant progeny derived by classical plant breeding or through new genetic engineering technology. It is possible that chemical barriers in crops, that impede fungal development and aflatoxin formation, may account for the partial level of resistance identified in corn (Scott and Zummo, 1988; Widstrom, 1987) or in cotton (Cotty, 1989b). Therefore, scientists in our laboratory are attempting to characterize fungal and/or aflatoxin inhibitors in corn and cotton tissues; certain hydrolytic enzymes (chitinases and glucanases) have been detected in corn kernels (Neucere and Cleveland, 1989) that have been suggested to be antifungal in other crop systems. Certain other proteins (presently unidentified) from these corn kernels have demonstrated fungal growth inhibitor properties. Volatile and non-volatile compounds have been identified in cotton leaves (Zeringue and McCormick, 1989) that inhibit both fungal growth and aflatoxin production. These fungal and aflatoxin inhibitor compounds could be used as markers to enhance resistance against aflatoxin producing fungi through classical plant breeding and/or new molecular techniques. Our scientists have begun a biochemical screening of corn germplasm demonstrating measurable resistance to aflatoxin producing fungi (Scott and Zummo, 1988; Widstrom, 1987); kernels are also being examined anatomically to identify structural barriers that may impede fungal development. Once identified, components involved in resistance could be used as selectable markers in traditional plant breeding to optimize selection for resistance against A. flavus or as traits which could be introduced into any desirable germplasm through new genetic engineering techniques.

References

Bennett, J.W. and L.S. Lee. 1977. Mycotoxins—their biosynthesis in fungi: aflatoxins and other bisfuranoids. J. Food Protec. 42:805.

Bhatnagar, D., T.E. Cleveland, and E.B. Lillehoj. 1989. Enzymes in late stages of aflatoxin B_1 biosynthesis: strategies for identifying pertinent genes. Mycopathologia. 107:75-83.

Bhatnagar, D. and T.E. Cleveland. 1990. Purification and characterization of a reductase from *Aspergillus flavus* SRRC 2043 involved in aflatoxin biosynthesis. Fed. Proc. (In press).

Blount, W.P. 1961. Turkey "X" disease. Turkeys. 9:52-57.

Cleveland, T.E. and D. Bhatnagar. 1989. Evidence for *de novo* synthesis of an aflatoxin pathway methyltransferase near the cessation of active growth and the onset of aflatoxin biosynthesis of *Aspergillus parasiticus* mycelia. Can. J. Microbiol. (In press).

Cotty, P.J. 1989a. Aflatoxins in cottonseed products—What can be done? Proceedings of the 38th Oilseed Processing Clinic. Southern Regional Research Center, New Orleans, LA.

Cotty, P.J. 1989b. Effects of cultivar and boll age on aflatoxin in cottonseed after inoculation with *Aspergillus flavus* at simulated exit holes of the pink bollworm. Plant Disease 73:489-492.

Cotty, P.J. 1989c. Prevention of aflatoxin contamination with strains of *Aspergillus flavus*. Phytopathology 79:1153.

Cuero, R.G., E.B. Lillehoj, T.E. Cleveland and A.H. Reine. 1988. Chitosan as a control agent of toxigenic fungal growth and aflatoxin production. In: Mycotoxins and Phycotoxins, Proceedings of the Japanese Association of Mycotoxicology. pp. 194-198. Published by the Japanese Association of Mycotoxicology.

Goldblatt, L.A. 1970. Chemistry and control of aflatoxin. Pure and Appl. Chem. 21:331-353.

Hadwiger, L.A., B. Fristensky, and R.C. Rigglemen. 1984. Chitosan, a natural regulator of plant-fungal pathogen interactions, increases crop yields. In: Chitin, Chitosan, and Related Enzymes. (J.P. Zikakis, ed.) pp. 291-302, Academic Press, Inc., New York.

Jelinek, C.F., A.E. Pohland, and G.E. Wood. 1989. Review of Mycotoxin contamination. Worldwide occurrence of mycotoxins in foods and feeds—an update. J. Assoc. Off. Anal. Chem. 72:223-230.

Neucere, J.N. and T.E. Cleveland. 1989. Existence of chitinase activity in mature corn kernels (*Zea mays*). Phytopathology 79:1192.

Park, D.L., L.S. Lee, R.L. Price, and A.E. Pohland. 1988. Review of the decontamination of aflatoxins by ammoniation: current status and regulation. J. Assoc. Off. Anal. Chem. 71:685-703.

Pons, W.A. Jr. and L.A. Goldblatt. 1965. The determination of aflatoxins in cottonseed products. J. Am. Oil Chem. Soc. 42:471-475.

Scott, G.E. and N. Zummo. 1988. Sources of resistance in maize to kernel infection by *Aspergillus flavus* in the field. Crop Science 28:504-507.

Sneh, B., J. Katan, and Y. Henis. 1971. Mode of inhibition of *Rhizoctonia solani* in chitin amended soil. Phytopathology 61:1113-1117.

Widstrom, N.W. 1987. Breeding strategies to control aflatoxin contamination of maize through host plant resistance. pp. 212-220. In: M.S. Zuber et al. (ed.). Aflatoxin in maize: A proceedings of the workshop. El Batan, Mexico. 7-11 Apr. 1986. CIMMYT, Mexico City, Mexico.

Zeringue, H.J., Jr. and S.P. McCormick. 1989. Relationships between cotton leaf-derived volatiles and growth of *Aspergillus flavus*. J. Am. Oil Chem. Soc. 66:581-585.

Topic III

AMS' Monitoring Program for the Post-Harvest Management of Peanuts To Reduce or Eliminate Aflatoxins

Craig A. Reed

Good afternoon, it's a pleasure to be with you today to discuss the AMS monitoring program for aflatoxin in a variety of peanut products.

Symposia such as this one greatly enhance our efforts to improve cooperation between government, industry and academia in meeting a common goal. That goal is to assure the consumer that our food supply is safe and wholesome. We can only do that through research and science applied in a logical manner.

We are all well aware of the recent food scares that have hit the press: Alar in apples, ethylenethiourea (ETU) a metabolite of ethylenebisdithiocarbamate (EBDC) used as a fungicide on fruits and vegetables, cyanide in grapes, and aflatoxins which contaminated large areas of the drought damage midwestern corn belt to name the most notorious. This past year has seen the most focused attention in recent memory on the matters of chemical contaminants and carcinogens in the food supply.

Today, we can detect chemical contaminants at levels which are multiples of fractions lower than what we could detect 10 years ago. Attempting to determine a substance's safe level is guaranteed to open a controversy when the scientific community cannot agree on data interpretation for low-level or long-term risks. In addition, the risk assessment process is often confused by the consumer with actual hazards, since they are unaware that risk calculations are based on maximal tolerance concentration, not on actual exposure. Thus, the process of communication is crucial to our attempt to overcome consumer concerns and instill public confidence. Consumers want scientific assurance that their food is safe and nutritious, and it is our goal to work toward providing that assurance.

It might be useful at this point to briefly describe our program for those of you who may not be familiar with the U.S. Department of Agriculture's (USDA'S) Agricultural Marketing Service (AMS).

How Does AMS Fit into this Perplexing Situation?

AMS administers more than 30 statutes which establish a wide array of programs to facilitate the marketing of agricultural products. These include the development of commercial grade standards and specifications

for foods, and the furnishing of inspection and grading services, including the issuance of certificates of quality and or/condition to producers, processors, shippers, buyers, or other interested parties. On a fee basis, our 13 laboratories perform numerous microbiological and chemical tests of processed dairy products, egg products, meat, poultry and poultry products, processed fruits and vegetables, and other food products. These tests support certification, acceptance, and regulatory programs, such as the military, the school lunch program and foreign government food commodity contract purchases. These purchasing programs contain microbiological and chemical food safety specifications which include detailed minimum requirements for acceptance of a food product. AMS also conducts laboratory quality assurance programs with commodity industries, as well as testing imported flue-cured and burley tobacco for pesticide residues.

Aflatoxin Testing

The aflatoxin control program for peanuts produced in the United States is administered by the Peanut Administrative Committee (PAC) under provisions of a USDA marketing agreement for peanuts that requires the analysis and certification of each lot of shelled nuts for aflatoxin content. The objective of the agreement is to ensure that only wholesome peanuts enter edible market channels. Since aflatoxins were found in peanuts in the mid-1960's, the domestic peanut industry has sought to minimize aflatoxin contamination in peanuts and peanut products.

The agreement plays a very important role in the industry's quality control efforts. It has been in place since 1965 with over 90 percent of U.S. shellers participating. The participating shellers handle about 95 percent of the crop. This agreement requires that farmer stock peanuts with the visible *Aspergillus flavus* or *Aspergillus parasiticus* mold be diverted to nonedible uses at the initial point of sale. In addition, at the shelling plant each lot of peanuts for edible use must be officially sampled and chemically tested for aflatoxin by AMS or in laboratories approved by the Peanut Administrative Committee. The sampling and chemical analysis inspection programs are administered by AMS. Shellers who comply with these requirements are indemnified for losses if the Peanut Administrative Committee or the Food and Drug Administration deems the peanuts unsuitable for consumption because of aflatoxin. This agreement is the basis for a memorandum of understanding between

USDA and FDA (FDA Compliance Policy Guides 7155a.11, 7155A.13, and 7155a.14). Thus, each lot of shelled peanuts must be sampled and the sample chemically analyzed for aflatoxin. If the chemical assay shows the lot to be positive for aflatoxin, the lot is not allowed to go to edible channels. These contaminated peanuts are then crushed for oil and meal. The end result is that only good quality peanuts end up in the human supply.

Since 1967, FDA and AMS have cooperated in a voluntary program with United States importers for aflatoxin testing of tree nuts. Under this voluntary program, importers of in-shell Brazil and pistachio (raw and unprocessed) nuts are subject to USDA inspection prior to product introduction into United States commerce. USDA/AMS is responsible for sampling and testing each lot for aflatoxin in accordance with procedures prescribed by FDA. USDA then issues an analysis certificate for each lot (which FDA accepts), allowing entry of that lot into United States commerce provided the aflatoxin level does not exceed the administrative guidelines prescribed by FDA. This guideline for aflatoxins in raw peanuts, peanut products, pistachio nuts and Brazil nuts is 20 ppb.

FDA announced in the Federal Register (53:5043, 1988) that current administrative guidelines are not binding on the courts and the public, including food producers. The guidelines do, however, represent the best guidance available on aflatoxin contamination levels that FDA considers to be of regulatory interest.

Analytical Methods

Suitable analytical methods for the detection and quantitation of the aflatoxins are necessary for an adequate monitoring program. These methods could be used under a variety of applications, that is, screening, surveying, and regulatory control. Various methods based on either biological responses or on the chemical characteristics of the toxins have been developed and validated by interlaboratory collaborative studies.

Our laboratories currently use two analytical procedures (AMS, 1988). The method used to analyze raw shelled peanuts, peanut meal, and roasted peanuts for aflatoxin is the Best Foods (BF) TLC method (Waltking et al., 1968). The method has been modified to incorporate the water slurry method of subsampling. An alternative Association of Official Analytical Chemists (AOAC) procedure used in our laboratories for products such as peanut butter and corn, is the Contaminants Branch (CB) method of the Food and Drug Administration (Eppley et al., 1968).

The procedure is used when doubt exists as to the effectiveness of the BF method for extracting aflatoxin from the sample or when background interferences exist that might mask thin-layer chromatography quantitation of aflatoxin.

We will soon have the ability to use reverse-phase chromatography with fluorescence detection after derivatization. This procedure is faster that TLC and has AOAC approval for the determination of aflatoxin B_1, B_2, G_1, and G_2.

During the summer of 1989, the Agency also conducted a pilot study to determine the feasibility of using aflatoxin rapid test kits for routine monitoring for peanut products in AMS laboratories conducting aflatoxin tests on peanuts, peanut products, and other nuts and miscellaneous products. Two quantitative rapid aflatoxin analysis test kits: Aflatest, manufactured by Vicam; and Agri-screen, manufactured by Neogen Corporation, were evaluated at the laboratory in Albany, Georgia. The study was not designed to test repeatability or reproducibility of the kits, since this had already been accomplished through interlaboratory collaborative studies conducted for the Association of Official Analytical Chemists. In our study, three analysts tested 200 samples in parallel using both rapid test kits and the official AOAC thin-layer chromatography procedures. Rapid test kit procedures were found to be feasible for routine laboratory monitoring of aflatoxin in peanut products. A final report is being prepared and copies will be available upon request. Potential users of rapid tests must decide, based on their own needs and resources, whether or not test kit methodologies are practical in their programs. Our use of the kits is currently under discussion within the Agency.

Improved methodologies for aflatoxin detection, from nonspecific biological responses to specific chemical and biological procedures, must be part of an overall program that focuses on research and prevention, genetic manipulation, use of agrichemicals, and the effects of various food processing techniques on contaminant levels. In a marketing environment, we believe the most effective control is accomplished by preventing problems, not by simply catching products of a failed process.

Aflatoxin Monitoring Data

The presence of aflatoxin on raw peanuts has four general characteristics (Hesseltine, 1967; Campbell et al., 1986):

1. Fungal growth and formation of aflatoxin occur both in the field and during storage;

2. In a contaminated lot of peanuts, few kernels (perhaps 1 in a thousand) contain aflatoxin, and success in detecting the toxin depends on collecting a relatively large sample, such as 22 kilograms for the assay; and

3. The most important factors affecting aflatoxin formation are moisture, temperature, and insect damage.

In fiscal year 1989 (Figure 1), AMS analyzed over 118,000 samples of raw peanuts, peanut butter, and miscellaneous products such as tree nuts, cottonseed meal, and canned corn products for total concentration of aflatoxins (the sum of aflatoxins B_1, B_2, G_1 and G_2).

Measurable levels of aflatoxin were found in shelled raw peanuts throughout the fiscal year 1989 (Figure 2). However, the majority of the samples tested fell below the FDA administrative guideline of 20 ppb. The percentage of samples above the FDA permissible aflatoxin contamination guideline was relatively small—3 percent. In peanut butter, 18 samples were above the FDA administrative guidelines. This was out of a total of 2,935 samples tested throughout FY 1989 (Figure 3).

Some samples of processed peanuts (roasted and blanched) and peanut meal had measurable levels of aflatoxin above the guidelines (Figure 4). The highest level of aflatoxin contamination was attributable to peanut meal. In fiscal year 1989 our laboratories tested 103 lots of in-shell Brazil nuts and 29 lots of in-shell pistachio nuts with a minimum sample size of 11 kilograms. All fell below the FDA guidelines and none were denied entry into the country.

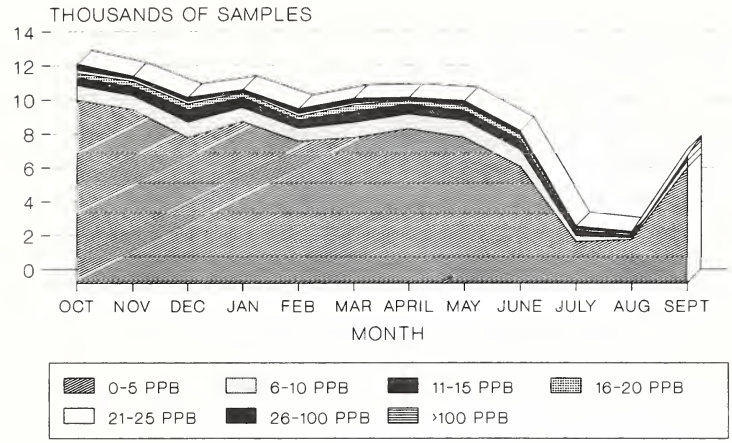
Figure 1.--AFLATOXIN TESTING

Commodities Scientific Support Division (CSSD)

Product	Total Tests
Peanuts (Raw & Processed)	113,729
Peanut Butter	2,935
Miscellaneous	1,839
Total	118,503

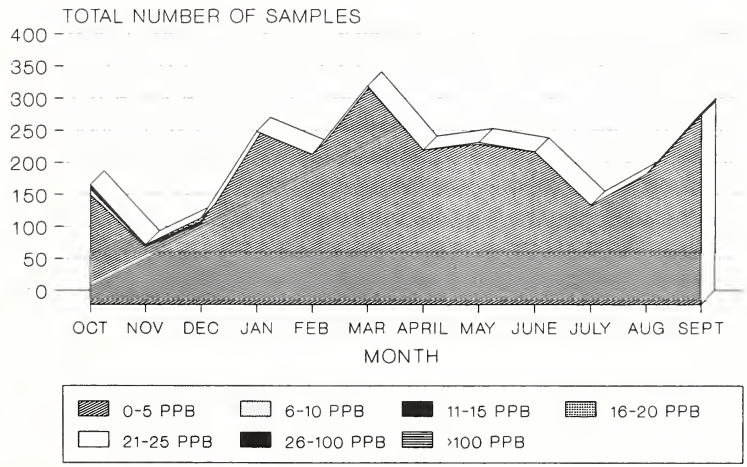
Fiscal Year 1989
(October 1988-September 1989)

Figure 2.--AFLATOXIN IN RAW PEANUTS FY 1989



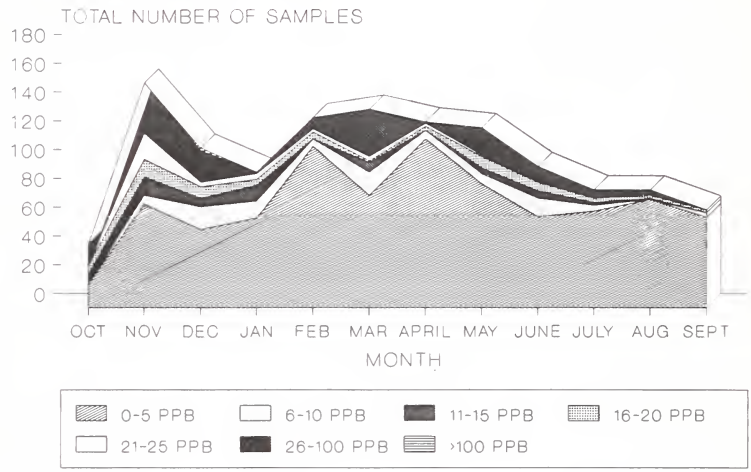
Data set 1

Figure 3.--AFLATOXIN IN PEANUT BUTTER FY 1989



Data set 3

Figure 4.-- AFLATOXIN IN PROCESSED PEANUTS AND PEANUT MEAL - FY1989



Data set 4

Conclusion

AMS will be reaching out more than we ever have before—to consumer groups, the producers who we service, and the professional community. We will communicate our findings about the safety of the domestic food supply we oversee so United States agriculture can compete effectively in global world economy.

References

AMS. 1988. Inspectors instructions for aflatoxin, Fruit and Vegetable Division, USDA, Washington, D.C. File Code 157-0-29.

Campbell, A.D., T.B. Whitaker, A.E. Pohland, J.W. Dickens, and D.L. Park. 1986. Sampling, sample preparation and sampling plans for foodstuffs for mycotoxins. Pure Appl. Chem. 58:305.

Eppley, R.M., L. Stoloff, and A.D. Campbell. 1968. Collaborative study of a versatile procedure for assay of aflatoxins in peanut products including preparatory separation and confirmation of identity. J. Assoc. Off. Anal. Chem. 51:67.

Hesseltine, C.W. 1967. Aflatoxins and other mycotoxins. Hlth. Lab. Sci. 4:222.

Rodrick, J.V., C.W. Hesseltine, and M.A. Mehlman (Eds). 1977. Mycotoxins in Human and Animal Health, Pathotox Publishers, Inc., Park Forest South, IL.

Waltking, A.E., G. Bleffert, and M. Kiernan. 1968. An improved rapid physicochemical assay method for aflatoxin in peanuts and peanut products. J. Amer. Oil Chemists Soc. 45:880.

Whitaker, T.B. and J.W. Dickens. 1989. Simulation of aflatoxin testing plans for shelled peanuts in the United States and in the export market. J. Assoc. Off. Anal. Chem. 72(4):644.

Wood, G.W. 1989. Aflatoxin in domestic and imported foods and feeds. J. Assoc. Off. Anal. Chem. 72(4):543.

Acknowledgment

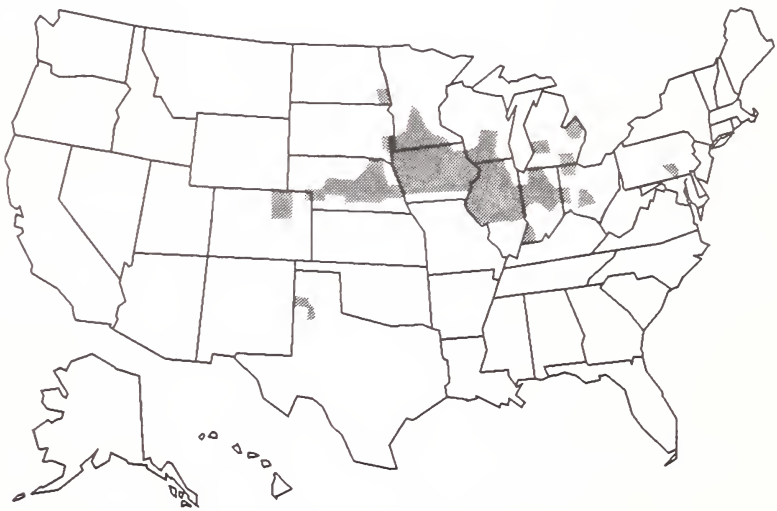
The author would like to acknowledge the assistance of Jeannette Coleman, Larz Kremer, Alan Post, and Martha Worley in the preparation of the manuscript.

Grain Handling and Processing Procedures To Reduce or Eliminate Aflatoxins

Kyd D. Brenner

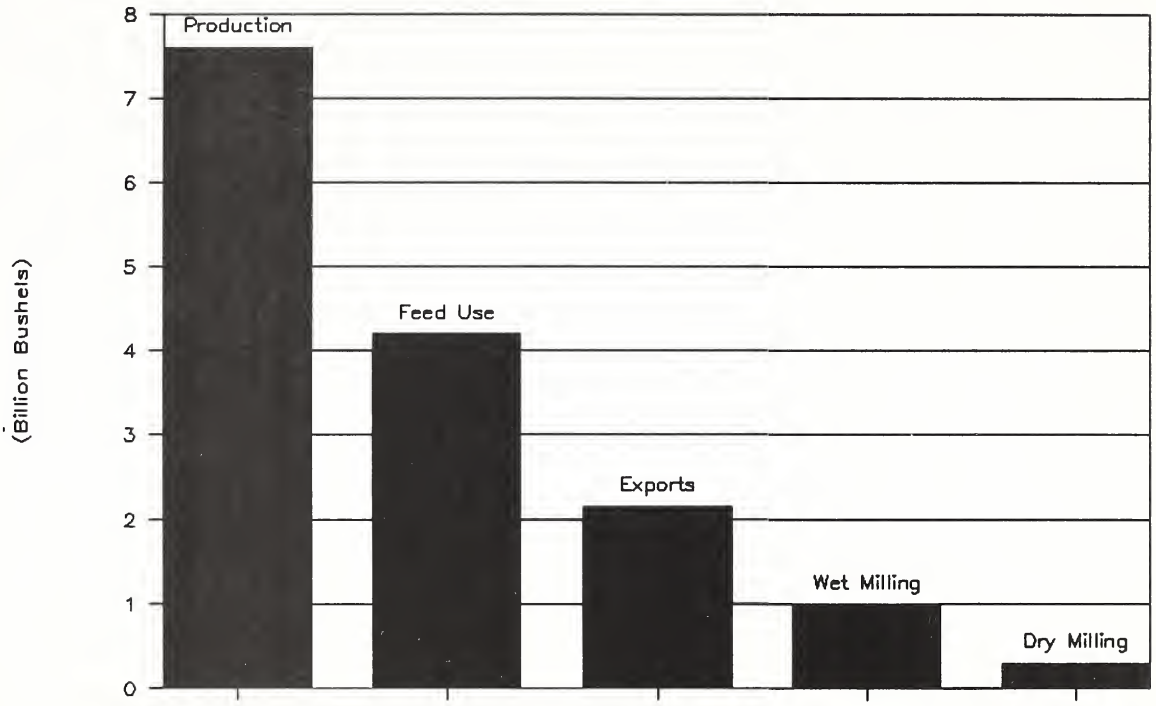
It is a pleasure to appear before you today. I would like to offer our appreciation to Chairman de la Garza, Mr. Madigan and the staffs of the House Agriculture Committee and the Agricultural Research Service who have made this program possible. In light of the tremendous public interest in food safety issues, we are very pleased at this tangible demonstration of the Committee's concern for a safe and wholesome food supply.

As the sole private sector speaker on the program, I am here not so much to discuss what we know about aflatoxin as how we deal with the knowledge we already have.



MAJOR CORN PRODUCING AREAS

CORN PRODUCTION AND UTILIZATION 1989/90



Before discussing grain industry procedures to manage aflatoxin, let me give you some background on the corn industry. The U.S. corn industry is composed of three major segments. First, of course, is the producer. There are over one million corn producers in the United States, with the major production centered in the central and upper Midwest. Next is the marketing and storage portion of the industry. Grain elevators range in size from small, local elevators which serve as gathering centers for grain from a limited number of farms to large facilities located at major ports which serve as gathering and transhipping facilities for corn and feed exports.

The final link in the chain is the processing industry, which is made up of three different parts. First is the feed compounding industry, which grinds corn for incorporation into finished feeds for livestock and poultry. Second is the corn dry milling industry, which grinds corn for corn meal, corn flour, grits, alcohol, corn oil and feed ingredients. Finally, there is the corn wet milling industry which processes corn for products such as corn sweeteners and oil for the food industry, ethanol for motor fuel, starch for use in food, textile, paper and plastic products and animal feed ingredients.

Most corn is used as livestock feed, either fed by the producer to his own livestock, or in the form of compound feeds. Domestic feed usually accounts for about 4.2 billion bushels out of a total corn crop of seven to eight billion bushels. Next, exports take about 2 billion bushels of corn, although this amount varies widely depending on market conditions. Third is the wet milling industry, which this year will use 960 million bushels. Finally, dry millers will use about 296 million bushels of corn this year.

In recent years the concept of total quality control as articulated by such industrial analysts as Arthur Deming has been widely adopted by the food industries. In addition to routine quality defects, these systems are also used to manage hazardous substances such as mycotoxins and microbial contaminants. In the case of food contaminants, the most widely used system is the Hazard Analyses-Critical Control Point system.

The HACCP system has seven major components. The first step in this system is the assessment of hazards and risks. In the case of aflatoxin, the hazard identification step is basically complete. The assessment of risk, however, is less advanced.

Aflatoxin has been well known to the grain industry since the 1960s. The identification of aflatoxin as the cause of "Turkey X disease" was enough for the food and feed industry to define aflatoxin as a hazard in need of monitoring and control. In 1969 this knowledge was incorporated

into our food laws when FDA established action levels for aflatoxin in corn, placing aflatoxin in the same category as other food adulterants. FDA's power to regulate adulterants under the Food, Drug and Cosmetic Act is quite broad and carries sanctions of criminal penalties, injunctions and product seizure. In addition to governmental sanctions, the potential product liability exposure for contamination of the food supply with aflatoxin is immense.

Much is known about the conditions under which aflatoxin occurs in corn. ARS researchers have identified specific conditions of air temperature and humidity, soil temperature, and insect damage which are necessary for formation of aflatoxin.

Tomorrow's speakers will discuss in detail the difficulties in assessment of human risk posed by aflatoxin. Despite these uncertainties, the grain industry is guided by the regulations of the FDA and the advisory of the International Agency for Research on Cancer that aflatoxin is a suspected human carcinogen.

Having identified the hazards of aflatoxin, the next step in the control system is to determine the critical control points.

The first of these is in the field, during development of the new crop. Because of our knowledge of the conditions under which aflatoxin is formed, the grain industry begins its control processes before harvest. For the country elevator, drought, sustained high temperatures and late-season rain in the receiving area are early signals that higher-than-normal levels of crop testing are warranted. Depending on the results of testing in the first few months after harvest, this program can be maintained, intensified or diminished. For the processor, these conditions will trigger intensive testing programs and can be used as a guide to areas to avoid in grain purchases. For example, because of the repeated occurrence of aflatoxin in corn grown in the southeast, corn refiners tend not to use corn from the region. During 1988-89, due to the occurrence of these conditions in many corn belt areas, corn processors tested every incoming shipment of corn, regardless of origin.

The development of rapid immunoassay-based test kits has brought the possibility of on-farm testing for aflatoxin much closer to reality. On-farm testing can serve as the first point of segregation for any grain found to be contaminated. Because these test kits are very new, they have not found a great deal of use on the farm to date, but this use could increase in coming years.

The next critical point is the location of the first sale of grain, the country elevator. Through their industry associations, extension agents and state regulatory agencies, country elevator operators are well trained

in procedures for sampling and screening incoming grain for aflatoxin. Although the black light has been traditionally used for this initial screening, new test kits have greatly increased the effectiveness of elevator testing. Last year, the National Grain and Feed Association found that over 400,000 immunoassay kits had been sold to elevator companies.

At the sub-terminal and terminal elevator levels the same sampling and testing systems are used, backed up in some facilities by the availability of automatic grain samplers and more sophisticated testing apparatus. Similar facilities for inbound corn testing are available at processing plants.

Because of the problems associated with sampling for minute quantities of a contaminant in bulk grain, processing facilities also test both in-process and finished products when there is reason to believe aflatoxin may be present in the grain supply. In the case of corn refiners, the wet milling process has been demonstrated to isolate aflatoxin from food products such as starch, sweeteners and oil. However, in-process and outbound feed products must be monitored. Because the dry milling process does not isolate aflatoxin in this manner, finished food products are intensely scrutinized as well. The location of critical control points within processing facilities will differ depending on the type and design of the process. In corn refining critical locations for sampling and testing include inbound unloading, steepwater and feed product streams. Since corn cleanings may contain aflatoxin levels above that of the original grain, they must be carefully scrutinized prior to reintroduction to any feed product stream.

The next step is to define the conditions which must be met at each control point. These conditions will be dictated by a variety of factors, including regulatory policies, requirements of end-users and the individual policies of buyers and sellers of grain and grain products.

First among these conditions is the regulatory system of the FDA. Although FDA's powers to regulate sales of grain are limited to products moving in interstate commerce, the fungible nature of grain creates an assumption that any sale is intended for interstate commerce, unless specific evidence exists to the contrary. Thus, FDA's guidelines are national in scope. Limited exemptions have been granted to these regulations by FDA for sale of grain and cottonseed products within some states.

FDA's basic action levels on aflatoxin have been designed to prohibit shipment of grain containing over 20 parts per billion aflatoxin. Beginning with the 1988 crop, FDA has allowed shipment of corn with up to

300 ppb aflatoxin, depending on its end use. For grain for food use and for use in feeding dairy and immature animals, the 20 ppb cutoff remains. For other species and uses different levels are permitted.

The introduction of a hierarchy of permitted aflatoxin levels has added knowledge of the end-use of grain to the determination of conditions to be met at testing facilities. When the end-use of the grain is not known, the 20 ppb cutoff remains in effect. If the end-use is one of the various categories of feed use for which higher limits are permitted, this becomes the controlling factor.

Other cut-off points may be established by policies of individual purchasers or by agreements between buyers and sellers. Many corn millers have established in-house guidelines for acceptance of corn which are below 20 ppb. Export grain contracts may require certification of aflatoxin free grain, or of levels dictated by regulatory requirements of the importing country. Grain which does not meet FDA requirements may be exported, if it meets the regulatory limits of the importing country and any purchase specifications. Because of importers' policies, in practice this results in export grain specifications of no more than 20 ppb.

Drs. Dorner and Koeltzow have already discussed the variety of analytical methods which are available for detection of aflatoxin. For the grain industry the major choice is which method to use at which stage in the control process and how to sample for aflatoxins.

Due to the large volume of material which moves through the grain handling system, it is imperative that a sampling system be used even with screening methods. While advances have been made in optical damage detection systems which are promising for use with some commodities, their use is impossible in a system which must handle, store and market nearly 200 million tons of corn per year.

Although there are a number of different sampling strategies available for aflatoxin, recent discussions in the Codex Alimentarius Commission indicate that no international consensus exists on the best plan available. Most governments, including the U.S., rely on a plan based on the principle of average-of-the-lot by a single, randomly selected composite. This system is used by the FDA and is the most commonly used sampling system in the grain industry.

At the country elevator level the black light screening method has traditionally been used, although it is rapidly being supplanted and supplemented by the rapid methods recently approved by the FGIS. However, the adoption of an end-use classification system makes it important to be able to quantify the level of aflatoxin in a contaminated load as soon

as possible in order to direct the load to an appropriate use. Only two of these rapid methods have this capability, and they are the most expensive of these systems.

At the processor and exporter level both black light and quick test methods are used as screening devices, although this sector is also moving rapidly to adopt the newer methods. Processors in particular are using the affinity column system which can be used for quantification either with the fluorometer supplied, or with in-house HPLC capability.

While TLC analysis remains the official method of the AOAC it is used only in the case of disputed samples or for internal cross-checks of rapid methods. The minicolumn method is also falling by the wayside as a result of the approval of equivalent rapid methods by FGIS.

Within processing plants many of the methods common for use with corn are inappropriate due to interfering substances which may be found in process streams. For internal process and finished product analysis, HPLC is the most commonly used procedure, with different cleanup and extraction systems used depending on the product being analyzed.

The next step in the process is the $64,000 question. What should be done when aflatoxin contamination over 20 ppb is detected? Until 1988, except in states where FDA had issued temporary exemptions, the choice was clear. The grain must be rejected.

Once grain has been rejected, the owner has several choices. Individual farmers may use the grain for livestock feed within the relevant FDA guidelines. Grain elevators were, until 1988, limited in their options. Today, however, they have the option of segregating the grain and providing it to either feedlots or feed manufacturers for use in mature animal feeding. In this case, it is the responsibility of the elevator to provide information on the aflatoxin content of the load to the end-user. If the load is contaminated above the highest action level of 300 ppb, destruction is the only alternative.

Food processors have only one response available, which is to reject the load. Although it is permitted to use contaminated grain for production of ethanol or other industrial products, most major processors use the same facilities for these products as are used for food production and cannot commingle contaminated grain in the process.

For the 1988 crop, FDA permitted blending of contaminated corn to reduced aflatoxin levels for feed use under stringent FDA control. This one-time exemption was not continued for the 1989 crop.

While the determination of what to do with contaminated grain depends on an admittedly imperfect sampling system, actual field results from 1988 indicate that it was effective in preventing contaminated corn

from reaching the food supply. FDA sampling data indicated that nationally only 2 percent of corn tested contained above 10 ppb aflatoxin. Only one in a hundred ready-to-eat corn products tested positively, and that sample was below 20 ppb.

The final two steps in the control system are record-keeping and audit functions. These measures serve a number of purposes. First, an accurate documentation system is critical to determine the severity of any quality problem and is used to allocate resources properly within a facility to the most critical quality and safety concerns. For example, an increasing level of rejection of corn for aflatoxin may be concentrated among grain from a particular region, although that may not be immediately apparent to the personnel at the receiving area. Proper record-keeping and statistics can often pinpoint these concerns quickly and enable the firm to adjust buying patterns or increase sampling of particular suppliers. A well-documented control plan also serves as an audit tool for company management, insurance carriers and regulatory authorities. Periodic unannounced inspections, both by internal staff and regulatory authorities serve to verify the compliance of operating personnel with the system.

Taken together, these programs operated through the grain industry have served to prevent contamination of the food supply with aflatoxin. Everyone in this chain, however, knows that improvements can and should be made. Improvements in aflatoxin management can come in four major areas.

First, the grain industry supports the actions of the Codex Alimentarius Commission to design a more effective sampling program for aflatoxins, either in a generic form, or commodity-by-commodity. Today's sampling system exposes producers, processors and end-users to risks which could be avoided. The difficult task is to design a program which takes into account the sporadic and scattered nature of aflatoxin and does so without disrupting the flow of grain from farm to market.

Second, although great strides have been made in rapid aflatoxin methods, these newer methods need to undergo the type of collaborative evaluation which qualifies them for acceptance by the AOAC as official methods. This evaluation should focus on method performance, the consistency of proprietary kits from test to test and any specialized sample preparation which may be necessary for use with rapid methods.

Third, a USDA-operated system which provides early warning of potential aflatoxin contamination would be very helpful in allowing sampling and analyses to focus more accurately on the areas with a high probability of contamination.

Finally, the grain industry supports the efforts of the Agricultural Research Service to develop crop varieties which are resistant to mold invasion and development of bio-competitive organisms which could result in non-aflatoxin forming varieties of the *Aspergillus flavus* mold. Detoxification systems under investigation may, pending FDA approval, be useful in managing already-contaminated crops. However, in the grain industry as elsewhere, there is no substitute for the rule of "Make it right, the first time."

Topic IV

Physical-Chemical Methods for the Applied Analysis of Aflatoxins in Animal Tissues and Fluids

Robert D. Stubblefield

Introduction

Aflatoxins are produced by a group of molds belonging to either *Aspergillus flavus* or *Aspergillus parasiticus*. In laboratory animals they are extremely potent carcinogens, and consequently, we must strive to keep them from entering our food supplies. Difficulties are encountered in trying to market crops that either are or are suspected to be contaminated with aflatoxin, e.g., the 1988 corn crop. Obviously, one solution is for individual farmers to feed the contaminated crop to animals, but this solution may result in losses due to mycotoxins in the fed animals, reduced resistance to other disease agents, and possibly contaminated edible meats, milk, and dairy products.

The first comprehensive study on the effects of feeding aflatoxin to livestock and poultry was published in 1971 by Keyl et al., of the Western Regional Research Center of the Agricultural Research Service (ARS) in cooperation with scientists at both the Northern and Southern Centers of ARS. Keyl's group established the levels of aflatoxin required to produce recognizable growth effects in swine, beef and dairy cattle, and broilers and laying hens. Unfortunately, methodology available then could not detect low part per billion levels; therefore, the data on the transmission of aflatoxin residues in the meat were not as definitive.

More recent studies establish that residues of aflatoxin B_1 and M_1 can be found in animal tissues (Furtado et al., 1982; Trucksess et al., 1982; Richard et al., 1983; Trucksess et al., 1983; Richard et al., 1986; Chen et al., 1984). These can be briefly summarized as follows: 1) low levels of aflatoxin-contaminated feed, i.e., 400 ppb for cattle and swine and 150 ppb for turkeys, will result in detectable aflatoxin residues in liver, kidney, and muscle, 2) aflatoxin is eliminated from the animal tissue in a relatively short time, i.e., 4 days for swine, 14 days for turkeys, and 21 days for cattle (residues found in urine and rumen contents), and 3) chickens can handle large doses (2000 ppb) with little effects, and after 2 days, no aflatoxin is detected in the tissue.

Tissue Methods

Analytical methods for determining aflatoxins in tissue have improved in sensitivity and accuracy since 1976. The official first action method of the Association of Official Analytical Chemists was developed at the Northern Regional Research Center (Stubblefield and Shotwell, 1981; Association of Analytical Chemists, 1984a). In contrast to grain, proper sampling is not a critical problem with animal tissues. The aflatoxin contamination is not located in "hot" spots, but it is distributed evenly throughout tissue. Tissue is either blended or ground to prepare it for extraction. The mixture is weighed into a 500 ml flask, denatured, and extracted with an organic solvent. The extract contains many compounds besides aflatoxins; therefore, it must undergo a clean-up step that will remove many of these compounds. The extract solution is added to a glass column that contains a small amount of silica gel (a sand-like material). The aflatoxins and other compounds in the solution are adsorbed by the silica gel. Three different organic solvent mixtures are percolated through the column to remove interfering pigments and other compounds, and aflatoxins are eluted with another organic solvent. The solvent is evaporated to prepare the extract for quantitation.

Amounts of aflatoxins are determined by a process known as Thin-Layer Chromatography (TLC). With TLC, small volumes of the silica column solution containing the aflatoxins and a standard aflatoxin solution are spotted on a silica gel-coated glass plate, and an organic solvent is wicked up the plate. Normally, TLC will separate aflatoxins from other impurities, and they are quantitated with either visual or machine comparisons of the fluorescent zones. Regular TLC will not give satisfactory quantitative data because fluorescent impurities remain after the silica gel column clean-up step. However, 2-dimensional TLC (2-D TLC) does give satisfactory resolution of aflatoxins and impurities. 2-D TLC is similar to regular TLC except a second organic solvent is allowed to wick up the plate at 90° to the first direction. After excess solvent is evaporated, the plate is examined under longwave ultraviolet light (366 nm). The fluorescent zones of the sample are matched against those of standard aflatoxins, either visually or densitometrically, and quantities of aflatoxin are calculated. This method is efficient, accurate, and sensitive (0.1 ppb). It is not a quick method because one sample requires approximately 4-6 hr to assay.

In a cooperative study with the Food Safety Inspection Service (FSIS), the author modified this method to permit final quantitation of pig liver extracts by an instrumental method known as reverse phase high performance liquid chromatography (RP-HPLC) (Stubblefield et al., in prep.

b). With HPLC, a specific solvent system is pumped through a closed system which includes a pump, an injector, a specially coated silica gel column, and a fluorescence detector. A sample (or standard aflatoxin solution) is introduced into the system via the injector and a syringe. Aflatoxins and other compounds pass through the column at different rates depending on their solubility in the solvent system and their ability to adsorb to the silica gel column. After the aflatoxins pass through the column, they flow through the fluorescence detector that measures their concentration. With the modified method, no changes in the extraction step were necessary, but instead of using the entire meat extract, only a small portion (10%) was put on the silica gel column for clean-up. This was permissible because smaller concentrations of aflatoxins can be measured by this method. Recovery studies gave data very similar to the values obtained in the official method described above. A significant increase in sensitivity was achieved. Minimum detection levels (MDL) of 0.010-0.030 ppb for aflatoxin B_1 and M_1, respectively, are attained by this method. There is no time savings because the extraction and clean-up steps are not changed; however, HPLC can be automatically operated overnight which permits many more samples to be assayed in a 24 hr time period.

Whenever a positive sample is determined, it has to be confirmed. This is done by 2D-TLC. The difference between the confirmation and the quantitation procedures occurs after the plate is developed the first time. A special chemical is sprayed on the area that contains the aflatoxins, and a new fluorescent compound is formed. The plate is rotated 90 degrees and developed in a second direction. The plate is examined under UV light for fluorescent zones, which must match those of the standards. This procedure is unique in that aflatoxins were developed in direction 1 while aflatoxin derivatives were developed in direction 2. This confirms the positive identity of aflatoxins in samples.

Improved Rapid Quantitative/Screening Tissue Method

Neither the official nor modified tissue methods described above can be readily converted to a screening method. As mentioned, neither are rapid to perform. Because a tissue screening method is an important necessity for regulatory agencies such as FSIS and the Food and Drug Administration, the author has developed an improved, rapid quantitative method that appears to be adaptable to a screening method (Stubblefield and Greer, in prep.). The extraction step is identical to the official method because it is efficient. After the filtration step, the solution is passed

through a small silica column. After this treatment, the residue is redissolved and added to an immunoaffinity column (VICAM, Cambridge, MA) to further clean-up the meat extract. This column contains the specific antibodies for aflatoxin B_1; therefore, only the aflatoxin B_1 is adsorbed by the column. After the aflatoxins are eluted from the column with an organic solvent, they are quantitated by HPLC. Recoveries of aflatoxin B_1 and M_1 have been very good (>85%). The other important achievement of this method is its apparent adaptability to a screening method. New methodology is available that utilizes immunochemical techniques, kits, and columns. These present excellent possibilities for screening methods because the antibodies used in the kits, etc. are specific for the aflatoxin(s) only. Examples of these kits are the Afla-20 Cup (International Diagnostics Inc., Michigan City, MI), the EZ Screen Card Test (Environmental Diagnostic Inc., Burlington, NC), and the CITE Probe Aflatoxin B_1 Test Kit (IDEX, Inc., Portland, MN). These kits will test for aflatoxin B_1 at levels of 5-20 ppb and above. We are working on testing the extract residue from the small silica column with one of the immunochemical kits.

Animal and Human Urine Methods

The author, in cooperation with FSIS, has developed a screening method for aflatoxin B_1 in human and animal urine (Stubblefield et al., in prep. a). Animals (including humans) that consume aflatoxin-contaminated feed will pass 1-2% of the aflatoxins in their urine (Zhu et al., 1987). An animal's urine could be tested for aflatoxin without killing the animal, and if positive, a high correlation of certainty would exist for contaminated tissues. The urine screening method offers the user the choice of three detection limits; 1.5, 0.2, and 0.02 ppb.

Urine samples are mixed with a filter aid and filtered. The samples may be tested at this stage with any of the rapid immunochemical test kits mentioned earlier, and aflatoxin B_1 can be detected at a 1.5 ppb level. Sensitivity can be lowered to 0.2 ppb by passing the urine through a coated-silica gel column. The column is washed with an organic solvent-water mixture and water, then aflatoxin is eluted with another solvent-water mixture. The column extract is tested with an immunochemical test kit for B_1. If the initial volume of urine is limited or if it is necessary to reduce the sensitivity to 0.02 ppb, an additional purification step can be incorporated with the use of an immunoaffinity column. After aflatoxins are eluted, they are tested with one of the kits. This extract can also be assayed by HPLC as described above if quantitative data is desired.

Milk Methods

There are currently 3 official methods for determining aflatoxin M_1 in milk (the Pons method, Association of Official Analytical Chemists, 1984b; the Stubblefield method, Association of Official Analytical Chemists, 1984c; the Foos-Warren method, Journal of Official Analytical Chemists, 1986); however, the Pons method is lengthy, is not used often, and will not be discussed. The extraction step for the Stubblefield method utilizes a vigorous shaking of either liquid or reconstituted milk with an immiscible organic solvent. For the Foos-Warren method, a coated-silica gel column/cartridge is used to adsorb the M_1 from the milk. The Stubblefield method is efficient, but it is subject to occasional emulsions. The Foos-Warren method is also efficient, but sometimes the cartridge will clog, and milk will not pass through it, in particular reconstituted milk which first needs to be centrifuged. Both methods use a silica gel column to clean-up the milk extract. The Stubblefield method makes identical use of the tissue-method column and is, therefore, more elaborate. The Foos-Warren method uses a finer grade silica gel and is very fast. Either method can use RP-HPLC to quantitate the aflatoxins. Originally, the Stubblefield method used TLC for the final assay. The minimum detection limit for method 1 by TLC is 0.1 ppb and for the Foos-Warren method by HPLC is 0.07 ppb. Actually, both methods are capable of detecting 10 ppt by HPLC with some adjustments of the methods. Recoveries are nearly identical, 80+/-15%. The Foos-Warren method is a faster method overall.

When the commercial immunoaffinity columns became available, Mortimer et al. (1987) published a method for the determination of M_1 in liquid and powdered milk. Others, including the author, had developed similar methods. All of these methods offer analysts very rapid assays and very clean extracts for quantitation by HPLC. The initial centrifugation of the milk to remove the fat is the only time-consuming step. The skim milk is passed through an immunoaffinity column which is then washed with water to eliminate excess milk. Aflatoxin M_1 (B_1, if present) is eluted with an organic solvent. The residue is analyzed by HPLC. Sensitivities down to 10 ppt are possible depending upon the initial volume of milk. Recoveries are >85% and exceptionally clean extracts are obtained for analysis. Mortimer's method is currently in the initial stages of an international collaborative study.

Future Research Needs

Faster and better methods for determining aflatoxins are always a necessity. In particular screening methods, especially those that can be conducted at the farm, mill, or dairy, need further development. The newer immunological kits/columns will be useful here. A tissue survey for beef cattle similar to the joint FSIS pig survey (Stubblefield et al., in prep. b) might give another view concerning the use of stored contaminated 1988 corn by farmers. Aflatoxin can be found in the rumen contents and urine of beef cattle after nearly 3 weeks (Richard et al., 1983) raising the possibility of tissue residues for 4 times longer than for swine. Supercritical extraction and chromatography of meat tissues, milk, and other dairy products should be investigated because this technique may provide cleaner extracts and improved recoveries.

Reports (Coulter et al., 1986; Lamplugh et al., 1988) have been published indicating aflatoxins have been found in liver biopsies of children and neonatal cord blood and serum from women in countries that annually have aflatoxin contaminated grain. Also, mother's milk (Lamplugh et al., 1988; Coulter et al., 1984) has been found to contain aflatoxin M_1 in these countries.

We recognize that these studies involve women from Africa and that local foods from that continent are more likely to contain aflatoxin. However, a similar study of women from specific areas of the U.S. might provide useful epidemiological information, if sufficient subjects could be found that consume a regionally produced diet, and if socio-economic relationships could be adequately handled. With the cooperation of medical facilities located in an area of interest, such a study could be accomplished in the author's laboratory using the newer more rapid assay methods.

References

Changes in Methods. 1986. J. Offic. Anal. Chem. 69:361-362, Secs. 26.B02-B06.

Chen, C., A.M. Pearson, T.H. Coleman, J.C. Gray, J.J. Pestka, and S.D. Aust. 1984. Tissue Deposition and Clearance of Aflatoxins from Broiler Chickens Fed a Contaminated Diet. Food Chem. Toxicol. 22:447- .

Coulter, J.B.S., S.M. Lamplugh, G.I. Suliman, M.I.A. Omer, and R.G. Hendrickse. 1984. Aflatoxins in Human Breast Milk. Ann. Trop. Paediatr. 4:61-66.

Coulter, J.B.S., G.I. Suliman, S.M. Lamplugh, B.I. Mukhtar, and R.G. Hendrickse. 1986. Aflatoxins in Liver Biopsies from Sudanese Children. Am. J. Trop. Med. Hyg. 69:360-365.

Furtado, R.M., A.M. Pearson, M.G. Hogberg, E.R.Miller, J.I. Gray, and S.D. Aust. 1982. Withdrawal Times Required for Clearance of Aflatoxins from Pig Tissues. J. Agr. Food Chem. 30:101-106.

Keyl, A.C. and A.N. Booth. 1971. Aflatoxin Effects in Livestock. J. Amer. Oil Chem. Soc. 48:599-604.

Lamplugh, S.M., R.G. Hendrickse, F. Apeagyei, and D.D. Mwanmut. 1988. Aflatoxins in Breast Milk, Neonatal Cord Blood, and Serum of Pregnant Women. Brit. Med. J. 296:968.

Mortimer, D.N., J. Gilbert, and M.J. Shepherd. 1987. Rapid and Highly Sensitive Analysis of Aflatoxin M_1 in Liquid and Powdered Milks Using an Affinity Column Cleanup. J. Chromatog. 407:393-398.

Official Methods of Analysis, Association of Official Analytical Chemists, 1984a. Arlington, Va., Chapter 26, Sec. 101-106.

Official Methods of Analysis, Association of Official Analytical Chemists, 1984b. Arlington, Va., 26.090-26.094.

Official Methods of Analysis, Association of Official Analytical Chemists, 1984c. Arlington, Va., 26.095-26.099.

Richard, J.L., A.C. Pier, R.D. Stubblefield, O.L. Shotwell, R.L. Lyon, and R.C. Cutlip. 1983. Effect of Feeding Corn Naturally Contaminated with Aflatoxin on Feed Efficiency, on Physiologic, Immunologic, and Pathologic Changes, and on Tissue Residues in Steers. Am. J. Vet. Res. 44:1294-1299.

Richard, J.L., R.D. Stubblefield, R.L. Lyon, W.M. Peden, J.R. Thurston, and R.B. Rimler. 1986. Distribution and Clearance of Aflatoxins B_1 and M_1 in Turkeys Fed Diets Containing 50 and 150 ppb Aflatoxin from Naturally Contaminated Corn. J. Avian Diseases. 30:788-793.

Stubblefield, R.D. and J.I. Greer. An Improved Rapid Determination of Aflatoxins B_1 and M_1 in Animal Tissue. In Preparation.

Stubblefield, R.D. and O.L. Shotwell. 1981. The Determination of Aflatoxins in Animal Tissue. J. Assoc. Offic. Anal. Chem. 64:464-468.

Stubblefield, R.D., J.I. Greer, O.L. Shotwell, and A.M. Aikens. A Rapid Immunochemical Screening Method For Aflatoxin B_1 in Human and Animal Urine. In Preparation a.

Stubblefield, R.D., J.P. Honstead, and O.L. Shotwell. Aflatoxins in Swine Tissues During Drought Conditions. In Preparation b.

Trucksess, M.W., L. Stoloff, W.C. Brumley, D.M. Wilson, O.M. Hale, L.T. Sangster, and D.M. Miller. 1982. Aflatoxicol and Aflatoxins B_1 and M_1 in the Tissues of Pigs Receiving Aflatoxin. J. Assoc. Offic. Anal. Chem. 65:884-887.

Trucksess, M.W., J.L. Richard, L. Stoloff, S. McDonald, and W.C. Brumley. 1983. Absorption and Distribution Patterns of Aflatoxicol and Aflatoxins B_1 and M_1 in Blood and Milk of Cows Following Oral Administration of Aflatoxin B_1. Am. J. Vet. Res. 44:1753.

Zhu, J.Q., X.H. Zhang, Y. Xiao, J.S. Chen, Y.C. Xu, J. Fremy, and F.S. Chu. 1987. Correlation of Dietary Aflatoxin B_1 Levels with Excretion of Aflatoxin M_1 in Human Urine. Cancer Res. 47:1848-1852.

Use of Monoclonal Antibodies in Assessing Aflatoxin Exposure in Poultry

John A. Doerr

I. Introduction

Aflatoxin and Antibodies

Cereal grains and other crops destined to provide food and fiber for farm animals or humans are often colonized by a variety of micro-organisms including certain kinds of fungi called molds. While some molds are directly parasitic to the host plant, others present a different kind of problem in that they leave behind residues of their metabolism, residues which may be toxic to the humans or animals which consume the contaminated material. Such metabolites are called mycotoxins, and AFLATOXIN, which derives its name from the name of the mold which produces it, *Aspergillus flavus*, is, perhaps, the best known of the economically significant mycotoxins. This mycotoxin can reduce farm animal productivity, compromise safety of meat and grain for human consumption, and adversely affect perception, and thus demand, for U.S. agricultural products in foreign markets.

Since analytical capability for qualitative and quantitative analysis of aflatoxin became available in the early 1960's, scientists, feed manufacturers, grain storage operators, and others have used direct testing or examination of grains and feeds as the approach of choice in determining aflatoxin contamination; and, thus, estimating risk or ascribing cause for problems seen in poultry and livestock.

In the 1970's reports were published describing production of polyclonal antibodies against aflatoxin (Chu and Ueno, 1977). This result was of particular importance, first, since there are difficulties attendant to generating immune globulins against so small a molecule[1], and second, since the implications of antibody use as reagent, prophylactic, or even therapeutic could herald new ways of dealing with these natural products. Subsequently, as the technology became more available, monoclonal antibodies were produced, and various testing applications using such antibodies were introduced. To date, most such tests have been offered by commercial sources and have been directed at quantitative or semi-quantitative examination of feeds and grains, although there is use in human diagnostics through analysis of blood or urinary metabolites (Gan et al., 1988).

[1] Aflatoxin B_1 has a molecular weight of 312.

An antibody is a protein produced by white blood cells. It possesses active sites which, by their structure and conformation, can "discriminate" amongst thousands of antigens to select and bind to that single antigen possessing the right "match". Antigens are foreign materials, often proteins, which stimulate the immune system to respond. An antigen, such as viral coat protein, possesses many topographical features sufficiently distinct to be a unique determinant for one antibody. When such an antigen is processed by the host, many white cells are stimulated to manufacture antibodies, each cell responding to just one of these many determinants. The final result is a large and varied mixture of antibodies, each recognizing some distinct piece of the total antigen. The production process is amplified as these stimulated cells divide; and, since many original cells are involved, the reactive antibody to a particular antigen is called polyclonal antibody—antibodies arising from many populations of white cells. The antibodies are in the blood of the immunized animal, and to use them, blood serum must be obtained.

It is possible, however, to select just one of these antibody-producing cells, to isolate it, and to cause it to reproduce or clone itself. Now all the cells are genetically identical and they all produce the same antibody, one reacting with but a single determinant of the original antigen. As long as the genetic purity of the producing cell is maintained, the uniqueness of the antibody will also be retained. The product is now called a monoclonal antibody. In order to accomplish this result, antibody-producing cells are removed from an animal and are joined with similar cells which have become cancerous. The cancerous cells do not produce antibody, but they can live forever in cell culture. The normal cells do produce antibody, but they cannot live in cell culture. Joining or fusing the two types yields a new cell, a hybrid, which carries the antibody-producing ability of the normal cell and the immortality feature of the cancerous cell. This "hybridoma" becomes the source of the desired, unique antibody, which can now be obtained from tissue culture media rather than from animal blood.

Test Formats

Two major ways of employing antibodies as a test reagent involve using the antibody to identify the desired item through procedures known as radio-immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). The former uses the antibody to deliver and attach a radio-isotopic marker to the chemical to be assayed, followed by counting of the radioactive emissions from the tagged chemical as a means of determining its concentration. The latter uses the antibody to identify and mark the

chemical to be tested, but with an enzyme rather than an isotope. The enzyme subsequently catalyzes a reaction with a chromogenic reagent yielding some change in color detectable by spectroscopic instruments. While much more complex than the foregoing would suggest, these tests are used routinely in detecting and quantitating disease agents in many animals, in finding minute amounts of hormones, and in other ways.

II. Direct Testing of Animals for Exposure to Aflatoxin

Serum Aflatoxin ELISA for Poultry

Given the availability of both antibody development techniques and the ELISA format for testing, a monoclonal antibody (mAby) was obtained to aflatoxin B_1, which had been derivatized, bound to bovine serum albumin (BSA), and injected into female mice (BALB/c). The antibody possessed high affinity for aflatoxin B_1 and a hydroxylated metabolite[2] of B_1 which is a major product of mixed function oxidase action on aflatoxin B_1 in chicken liver. The affinity of the antibody for various forms of aflatoxin was B_1 = DIOL > B_2 > G_1 > G_2. This means that the antibody would recognize B_1 and DIOL equally well but would have increasing degrees of difficulties reacting with the other forms.

The antibody was incorporated into a double antibody, competitive ELISA in which aflatoxin B_1 bound to BSA is the solid phase, and the mAby and quantified free aflatoxin are in the incubation mixture. Anti Mouse-IgG, the second antibody, labelled with horseradish peroxidase (HRP) was used to mark solid phase antigen which had reacted with mAby. Subsequent reaction of HRP with substrate yielded a green color, the intensity of which is related to the concentration of aflatoxin in a serum sample. Serum, from 21 day old chickens, was de-protenated by centrifugation through special membranes[3], and tested by the ELISA.

---

[2] 8,9-dihydroxy-8,9-dihydro-aflatoxin B_1 ("DIOL") and aflatoxicol are major oxidative and reductive metabolites, respectively, produced in the liver of chickens.

[3] Anisotropic, hydrophilic membranes. "Centrifree" Micropartitioners (Amicon Div., W.R. Grace & Co., Danvers, MA)

Both aflatoxin-spiked sera and sera from chickens previously fed diets containing aflatoxin were tested. With this ELISA, as little as 40 picograms of aflatoxin per cubic centimeter of blood could be quantified, and comparisons showed the ELISA to be as sensitive as an available RIA or a test involving chemical extraction and liquid chromatography (HPLC). In a practical test, blood was received from a layer operation in the mid-West. Some of the samples tested positive for aflatoxin at a level consistent with a 21 day feeding of 600 parts per billion (ppb). Feed and corn samples received at the same time tested positive at 45 and 26 ppb, respectively.

Elimination studies in chickens revealed that in birds with little or no prior intoxication with aflatoxin, a single dose is cleared rapidly from the blood and within 48 hours is no longer detectable by the serum ELISA. However, birds previously fed high levels of the toxin (e.g., > 2.5 parts per million) exhibited relatively high blood concentrations which declined only slowly over time. Partitioning of the blood showed that a large majority of aflatoxin in the blood is associated with protein (albumin and pre-albumin fractions) and is eliminated during preparation of the serum for ELISA. The test, therefore, monitors only "free" aflatoxin, which is dependent upon dose and on degree of compromise of the bird's health status. That is, sicker birds had higher levels of free toxin in the blood. (Study unpublished.)

Interpretation of Results

At this time, the ELISA permits qualitative assessment of exposure by birds to dietary aflatoxin. The concentration of free toxin in blood is dependent upon dose, health of the bird, and time after last dose, among various physical factors which affect the result. However, the distribution of the toxin in its original form among several metabolic forms raises additional questions. For example, the current test has not yet been validated for some other metabolites, such as aflatoxicol (also significant in poultry), M_1, B_{2a}, etc. Nor is it yet established in poultry whether the profile of aflatoxin metabolites changes with time; this may be likely. Since differences in affinity for the mAby and differences in ratio of metabolites with time may both have substantial effect on apparent concentration, the relationship of blood concentration to dietary exposure level remains unknown.

To date, tests have been conducted in broiler chickens and laying hens. The ELISA must be validated in other poultry; and, furthermore, other livestock should be included to determine whether the test in its present form is of use in large animal diagnostics.

III. Alternative Uses of Monoclonal Antibodies

Additional work is required to pursue other uses of anti-aflatoxin monoclonal antibodies. For example, special labelling of the antibody for use in "staining" tissue sections could have application in diagnostics, while more rapid methods are needed for manual and in-line detection of residues in food processing situations. Although testing of individual large animals for diagnostic purposes is generally cost effective, flock sizes generally restrict such testing of poultry. However, in modern poultry health care, vaccination program effectiveness against a variety of disease agents is monitored by a system called "flock profiling" in which a few birds in a flock are periodically sampled and blood submitted to a battery of ELISA tests. An equivalent battery test for aflatoxin and other important mycotoxins would be of great benefit in preventing substantial loss to the farmer and reducing the risk of exposed birds reaching the processing plant. It is also important to extend this kind of technology to other mycotoxins as well as aflatoxin. Trichothecenes, fusarins, and fumonisins are among the important *Fusarium* mycotoxins today, and, with ochratoxin, cyclopiazonic acid, zearalenone, etc., are of concern to poultry and livestock producers. Monoclonal antibody technology can be useful adjunct to diagnosis, prevention, and residue avoidance concerning mycotoxins in poultry and animal agriculture.

IV. Conclusions

The poultry industry in the United States is perhaps the most progressive and competitive of the meat producing industries. It consumes a significant portion of the Nation's feed grains and returns a significant portion of the American consumer's diet in the form of ready-to-cook, further processed, and value-added poultry products. It is an industry sensitive to the safety concerns of the American public, and it is an industry desirous of maintaining a profitable and competitive posture, not only domestically, but in foreign markets.

In part, the achievement of these goals requires rapid, sensitive, and inexpensive methods to insure that the mycotoxicologic quality of grain purchased, of feed manufactured, and of poultry meat and eggs processed is of the highest standard. Monoclonal antibodies offer one approach to providing such an edge by allowing private and public sector scientists, commercial quality control personnel, and others to monitor aflatoxin in the food chain. Recent attention to pre-harvest aflatoxin in corn and

indications of inclusion of mycotoxins in National Residue Avoidance programs suggest the degree of awareness of our citizens and public officials concerning this toxin. Efforts to develop and apply monoclonal antibody and other biotechnologies to these problems should be accelerated.

References

Chu, F.S. and I. Ueno, 1977. Production of antibody against aflatoxin B_1. Appl. Environ. Microbiol. 33:1125-1128.

Gan, L.S. et al. 1988. Serum albumin adducts in the molecular epidemiology of aflatoxin carcinogenesis: correlation with aflatoxin B_1 intake and urinary excretion of aflatoxin M_1. Carcinogenesis. 9:1323-1325.

Aflatoxins in Animal Tissues During Drought Conditions

John P. Honstead

The purpose of this joint Agricultural Research Service (ARS)/Food Safety and Inspection Service (FSIS) project was to determine the presence of aflatoxins in the United States meat supply in a drought year. The year in question was 1988, during which certain regions of the Midwestern and Eastern United States experienced a severe drought. A worst case sampling plan for aflatoxin from swine slaughtered in FSIS plants was conducted from samples associated with the 1988 severe drought. Six states were identified as having significant aflatoxins in their corn crop: Virginia, North Carolina, South Carolina, Texas, Iowa and Illinois.

Swine were sampled because they have the lowest feed-to-tissue ratio for aflatoxin among the food-producing animals. Liver was analyzed because it contains the highest amount of aflatoxin of the edible tissues. The tissues were first screened by high pressure liquid chromatography and confirmed by two-dimensional thin layer chromatography. The analysis was accomplished at the ARS Northern Regional Research Center in Peoria, Illinois.

The results of the analysis indicate that these animals effectively metabolize aflatoxins present in feed under routine slaughter conditions. Eight of 160 (5%) liver samples had confirmed aflatoxin, and only four (4) of these eight (8) exceeded 0.1 ppb. Only one (1) of 160 liver samples had total aflatoxin, B_1 and M_1, in excess of the milk enforcement level of 0.5 ppb for M_1 alone. In severe drought conditions, aflatoxins do not result in a significant frequency in or magnitude of tissue residues in swine even in worst-case type biased sampling. Since swine appears to be the most sensitive species insofar as tissue concentration of aflatoxins, it would be logical to conclude that residues in other food-producing species would be significantly lower.

Topic V

Use of Dietary Chemisorbents To Prevent Aflatoxicosis in Farm Animals

Timothy D. Phillips, Beverly A. Clement,
Leon F. Kubena, and Roger B. Harvey

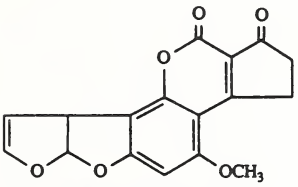
Abstract

Aflatoxins comprise a structurally similar group of naturally occurring fungal-elaborated poisons that have been strongly implicated in disease and death in man and animals and have resulted in substantial economic losses to agriculture. Consequently, there is a growing awareness of the dangers of these substances as frequent contaminants of animal feed and human food. Our recent research has led to the discovery of an exciting new technology for the control of aflatoxins (i.e., the use of inorganic adsorbent materials in the diet that act like "chemical sponges" to sequester and immobilize aflatoxin in the gastrointestinal tract of livestock and poultry. Our results have indicated that these materials possess the ability to chemisorb (tightly bind) aflatoxin, thus preventing its normal uptake by the blood and distribution to target organs (such as the liver). Research is in progress to develop and test novel chemisorbents for the practical control of mycotoxins.

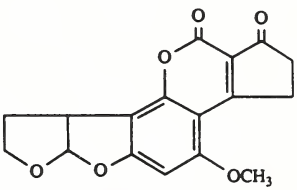
Introduction

The most thoroughly studied and best understood of the mycotoxins are the aflatoxins. The aflatoxins, a closely related group of polysubstituted bisfuranocoumarins (Cole and Cox, 1981), have invoked much concern due to their potent carcinogenicity. Aflatoxin B_1 and three naturally occurring homologues (aflatoxins B_2, G_1 and G_2) are frequently detected as contaminants of grain (Figure 1). The aflatoxins represent only one group of many mycotoxins which are currently known to exist. Because these poisons can significantly affect animal and human health, food and feed protection from mycotoxins is a critical need. Thus, practical methods to detoxify mycotoxin contaminated crops are in great demand. Although rigorous guidelines have been established for the preventive management of mycotoxins in crops (i.e., recommended practices for growing, harvesting, handling, and storage), significant mycotoxin contamination still occurs (Anderson, 1983). It is important to

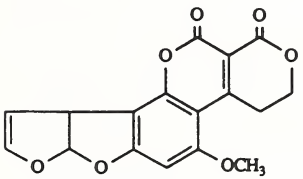
understand that good crop management practices and methods of mycotoxin detection do not provide a firm solution to the problem, since contamination can be unavoidable and sampling error may bias analysis. We must also be able to prevent the toxicity of these hazardous substances through the development and utilization of safe and effective procedures for the detoxification of mycotoxin-contaminated food and feed.



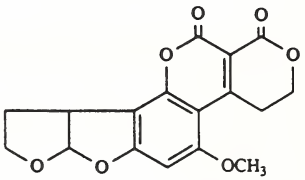
Aflatoxin B1



Aflatoxin B2



Aflatoxin G1



Aflatoxin G2

Figure 1.
Chemical structures of naturally occurring aflatoxins.

Detoxification Strategies

Numerous strategies for the detoxification/inactivation of mycotoxin contaminated food and feed have been proposed (e.g., physical separation, thermal inactivation, irradiation, microbial degradation and treatment with a variety of chemicals). The reader is referred to several excellent reviews on the subject (Anderson, 1983; Goldblatt, 1971; Goldblatt and Dollear, 1979). Many of the reported techniques are impractical and/or ineffective and/or potentially unsafe. The detoxification strategy which has received the most attention is the treatment of aflatoxin-contaminated feed with ammonia (i.e., ammoniation). The ammoniation procedure is currently being utilized in Arizona and California to reduce the parent aflatoxin levels in cottonseed products and in France, Senegal and Brazil

for the treatment of aflatoxin-contaminated peanut meal (Park et al., 1988). For a detailed review of the current status and regulation of ammoniation see Park et al., 1988.

A new approach to the detoxification of aflatoxin is the use of inorganic adsorptive compounds in the diet of farm animals. Many of these compounds, such as the aluminosilicates, are chemically complex materials, exhibiting a variety of functional properties. Aluminosilicates possess sizable surface areas, high porosity, and variable cation exchange activities along with active sites which can interact with and immobilize certain molecules via weak electrostatic forces or through the formation of strong bonds. We have recently evaluated a variety of adsorbent compounds (including aluminosilicates) and have ranked them according to their ability to bind aflatoxin in aqueous solution (Figure 2). We have also described the significant protective action of an aluminosilicate anticaking agent (HSCAS) in chickens (Phillips et al., 1987; Davidson et al., 1987; Phillips et al., 1988a; Phillips et al., 1988b; Phillips et al., 1989; Kubena et al., 1987; Kubena et al., 1988) and swine (Harvey et al., 1988a; Harvey et al., 1989).

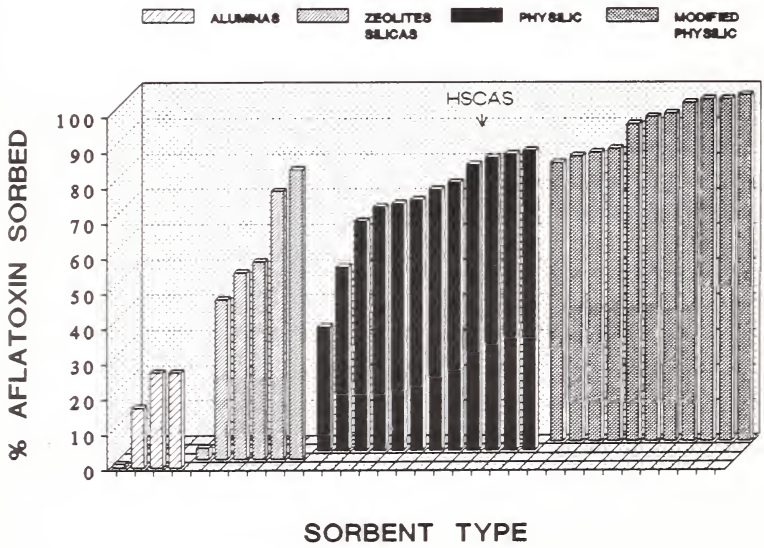


Figure 2.
Sorption of ^{3}H-aflatoxin B_1 in aqueous solution by aluminas, zeolites, silicas, phyllosilicates and chemically modified phyllosilicates (Phillips et al., 1987, 1988a, 1988b, 1989)

Studies in Vitro

Our results demonstrated that aluminas, zeolites, silicas, phyllosilicates and chemically modified phyllosilicates were capable of sorbing radiolabeled aflatoxin B_1 in aqueous solution (Figure 2). However, the binding capacities for aflatoxin and the stabilities of the sorption complex were markedly different for many of these compounds (Phillips et al., 1987; Phillips et al., 1988a). HSCAS (hydrated sodium calcium aluminosilicate), or NovaSil™ (Engelhard Chemicals, Cleveland, Ohio) possessed a propensity for aflatoxin and rapidly formed a stable sorption complex. Aflatoxin binding to HSCAS occurred on contact with negligible dissociation of the complex at equilibrium (which was complete after 30 min reaction time under the experimental conditions employed in our studies). The stability of the HSCAS/aflatoxin sorption complex was evaluated at different pHs and temperatures and in the presence of an eluotropic series of solvents. The complex was stable in water at pH 2, 7 and 10 and temperatures of 25°C and 37°C. Moreover, less that 10% of aflatoxin which was bound to HSCAS could be extracted by various organic solvents.

Studies in Vivo

Results from studies in Leghorn and broiler chicks demonstrated that HSCAS (when incorporated into the diet at a level of 0.5%) significantly prevented the toxicity of purified aflatoxin B_1 and a crude mixture of aflatoxins B_1, B_2, G_1 and G_2 (Phillips et al., 1988a; Kubena et al., 1987). The dietary addition of activated charcoal at the same level (0.5%) did not appear to have any protective action against the effects of aflatoxins (Kubena et al., 1988). In order to elucidate the protective mechanism of HSCAS, studies were designed to determine the effects of HSCAS on the bioavailability of ^{14}C-aflatoxin in the chicken (Davidson et al., 1987). Arbor Acres x Peterson chicks were dosed by gelatin capsules containing radiocarbon-labeled aflatoxin at a level calculated to deliver 20 and 80 ppb total aflatoxin. Samples of liver and blood were taken from each treatment group at 0.5, 1.0, 2.0, 4.0 and 6.0 hours and counted for radioactivity. Results were expressed as radioactivity relative to peak concentrations of the control (100%) and as absolute and relative corrected AUC (area-under-the-curve) values. AUC was utilized as a measure of the bioavailability of aflatoxin to the liver and blood. HSCAS in the diet at a level of 0.5% significantly reduced the amount of radioactivity available

to the blood (Figure 3) and to the liver (data not shown) of chickens throughout the test period. Radioactivity (associated with 80 ppb aflatoxin) in the blood and liver of chickens was greatly decreased in the presence of 0.1% and 0.5% HSCAS in the diet (Figure 4). Comparable reductions were observed in the 20 ppb treatment group. Results suggest that HSCAS acts to rapidly bind aflatoxin in the gastrointestinal tract, thus altering its uptake by the blood and distribution to the liver. Similar aluminosilicates and activated charcoal do not bind aflatoxin (*in vivo*) as effectively as HSCAS. These findings support the conclusion that the protective action of HSCAS in animals may be related to the stability of the HSCAS/aflatoxin sorption complex. Recent research has also indicated a protective action of HSCAS in swine (Harvey et al., 1988a; Harvey et al., 1989) and the ability of this aluminosilicate to greatly decrease the level of aflatoxin M_1 residues in the milk of lactating dairy cattle (Harvey et al., 1988b).

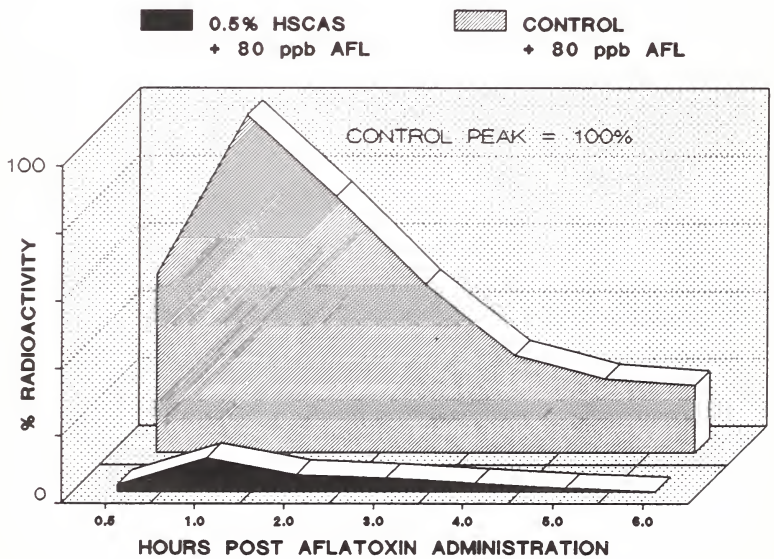


Figure 3.
^{14}C-aflatoxin B_1 in the blood of broiler chicks fed the equivalent of 80 ppb aflatoxin (with and without 0.5% HSCAS in their diet) (Davidson et al., 1987).

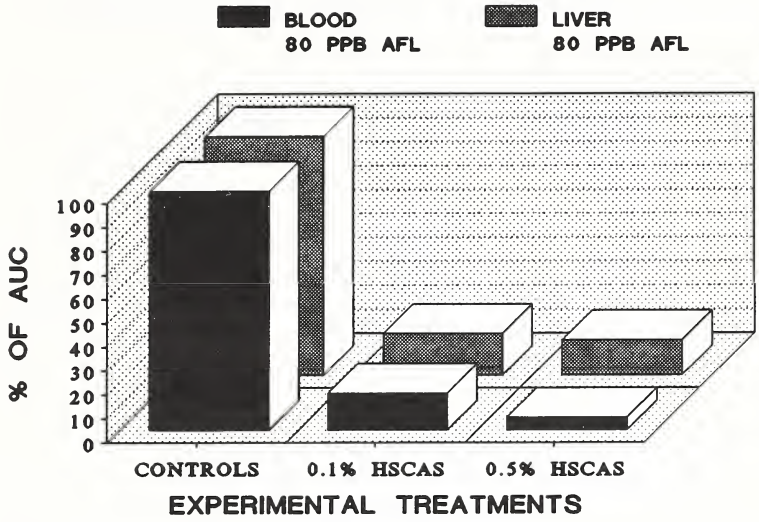


Figure 4.
Relative bioavailability of ^{14}C-aflatoxin B_1 to blood and liver of chicks fed the equivalent of 80 ppb total aflatoxin (with and without 0.1% and 0.5% HSCAS in their diet) (Davidson et al., 1987).

Summary and Conclusions

Our findings clearly demonstrate that HSCAS can prevent the adverse effects of aflatoxin in chickens. The mechanism for this action appears to involve sequestration of aflatoxin in the gastrointestinal tract of animals and chemisorption (i.e., tight binding) to HSCAS which results in a reduction in aflatoxin bioavailability.

In conclusion, mycotoxins have been implicated as significant health hazards on a worldwide scale. Consequently, there is a critical need for practical and effective solutions to this problem. A comprehensive effort on the part of academia, government, and industry to identify and implement state-of-the-art control and management strategies to solve the mycotoxin problem is highly desirable. The development and utilization of practical technologies for the prevention, detection and detoxification of these poisons will foster an integrated approach to the control of mycotoxins, with global implications to the health of animals and humans (Figure 5).

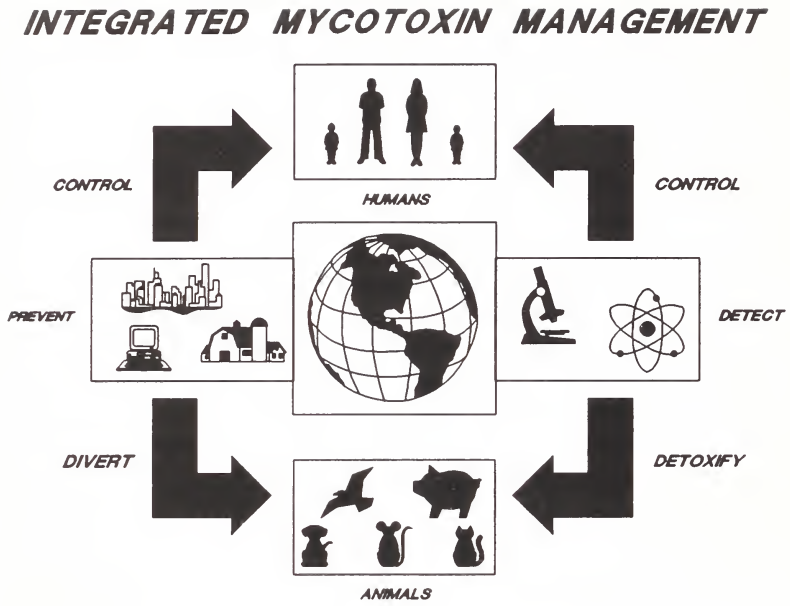


Figure 5.
Chart illustrating the essential components of an integrated approach to mycotoxin management.

Acknowledgements

The authors wish to express thanks to E.C. Shepherd, D.R. Taylor, W.E. Huff, N.D. Heidelbaugh and S.E. Hewins for their collaboration. Support of this research was provided in part by grants from the Engelhard Corporation (Project 6207), the Texas Agricultural Experiment Station (Project H6215) and the United States Agency for International Development (USAID) Peanut CRSP (Project 02-50305-2).

References

Anderson, R.A. 1983. Detoxification of aflatoxin-contaminated corn. pp. 87-90. In: Aflatoxin and *Aspergillus flavus* in Corn. U. Diener, R. Asquith and J. Dickens, eds. Southern Cooperative Series Bulletin 279, Auburn University, Auburn, Alabama.

Cole, R.J. and R.H. Cox. 1981. The aflatoxins. pp. 1-66. In: Handbook of Toxic Fungal Metabolites. Academic Press, New York.

Davidson, J.N., J.G. Babish, K.A. Delaney, D.R. Taylor, and T.D. Phillips. 1987. Hydrated sodium calcium aluminosilicate decreases the bioavailability of aflatoxin in the chicken. Poult. Sci. 66(1):89 (Abst).

Goldblatt, L.A. 1971. Control and removal of aflatoxin. J. Am. Oil Chem. Soc. 48:605-610.

Goldblatt, L.A. and F.G. Dollear. 1979. Modifying mycotoxin contamination in feeds: Use of mold inhibitors, ammoniation, roasting. pp. 167-184. In: Interactions of Mycotoxins in Animal Production. Natl. Acad. Sci., Washington, D.C.

Harvey, R.B., L.F. Kubena, T.D. Phillips, W.E. Huff, and D.E. Corrier. 1988a. Approaches to the prevention of aflatoxicosis. pp. 102-107. Proceedings of the Maryland Nutrition Conference.

Harvey, R.B., T.D. Phillips, L.F. Kubena, and W.E. Huff. 1988b. Dietary hydrated sodium calcium aluminosilicate and its impact on aflatoxin toxicity in pigs and milk residue in dairy cows. Dairy Agent Update, Department of Animal Science, Cornell University, Ithaca. 7(7):3-4.

Harvey, R.B., L.F. Kubena, T.D. Phillips, W.E. Huff, and D.E. Corrier. 1989. Prevention of aflatoxicosis by addition of hydrated sodium calcium aluminosilicate to the diets of growing barrows. Am. J. Vet. Res. 50:416-420.

Kubena, L.F., R.B. Harvey, T.D. Phillips, and N.D. Heidelbaugh. 1987. Novel approach to the preventive management of aflatoxicosis in poultry. pp. 302-304. Proceedings of the U.S. Animal Health Association.

Kubena, L.F., R.B. Harvey, T.D. Phillips, and W.E. Huff. 1988. Modulation of aflatoxins in growing chickens by dietary addition of a hydrated sodium calcium aluminosilicate. Poult. Sci. 67(1):106 (Abst.).

Park, D.L., L.S. Lee, R.L. Price, and A.E. Pohland. 1988. Review of the decontamination of aflatoxins by ammoniation: Current status and regulation. J. Assoc. Off. Anal. Chem. 71(4):685-703.

Phillips, T.D., L.F. Kubena, R.B. Harvey, D.R. Taylor, and N.D. Heidelbaugh. 1987. Mycotoxin hazards in agriculture: New approach to control. J. Am. Vet. Med. Assoc. 190(12):1617 (Abst.).

Phillips, T.D., L.F. Kubena, R.B. Harvey, D.R. Taylor, and N.D. Heidelbaugh. 1988a. Hydrated sodium calcium aluminosilicate: A high affinity sorbent for aflatoxin. Poult. Sci. 67:243-247.

Phillips, T.D., B.A. Clement, L.F. Kubena, and R.B. Harvey. 1988b. Mycotoxins: Detection and detoxification. pp. 94-106. Proceedings of the Delmarva Poultry Health and Condemnations Meeting.

Phillips, T.D., B.A. Clement, L.F. Kubena, and R.B. Harvey. Prevention of aflatoxicosis in animals and aflatoxin residues in food of animal origin with hydrated sodium calcium aluminosilicate. Proceedings of the World Assoc. Vet. Food Hygienists, Stockholm, Sweden, July 2-7, 1989.

Genetic Resistance in Chickens to Aflatoxin

R.D. Wyatt, R.O. Manning, R.A. Pegram and H.L. Marks

Introduction

The detrimental effects of aflatoxicosis in poultry are well documented and include growth depression (Smith and Hamilton, 1970), anemia (Tung et al., 1975a), increased susceptibility to bruising (Tung et al., 1971) and inhibition of protein synthesis (Tung et al., 1975b). Inhibition of protein synthesis is thought to be responsible, in part, for other effects of aflatoxicosis, such as immunosuppression (Thaxton et al., 1974), poor digestion (Osborne et al., 1982), and coagulopathies (Doerr et al., 1976; Doerr and Hamilton, 1981). The liver is the target organ in chickens, characterized by severe enlargement and fatty infiltration (Smith and Hamilton, 1970). Renal pathology and changes in function are also characteristic of aflatoxicosis in chickens; however, these changes are less severe than those noted in the liver (Tung et al., 1973).

It is well documented that aflatoxin is widely distributed in nature, is carcinogenic in laboratory animals, is highly toxic, affects numerous organ systems in chickens, and results in high economic losses within the poultry industry. Unfortunately, the poultry industry cannot prevent aflatoxin contamination in commodities used in the formulation of poultry feeds. In spite of rigorous quality control measures, the purchase of aflatoxin-contaminated feedstuffs can and does occur. Aflatoxin formation subsequent to feed manufacture is also well documented. Thus, several approaches aimed at ameliorating aflatoxicosis in chickens have been investigated. These include dietary modifications, environmental modifications, and genetic selection.

Dietary Modifications

Changes in the composition of poultry diets alleviate some of the adverse effects attributed to the consumption of aflatoxin. Dietary fortification with certain vitamins (Hamilton et al., 1974), protein (Smith et al., 1971), fat (Hamilton et al., 1972), and fatty acids (Lanza et al., 1981) have shown promise to lessen the effect of aflatoxin on the performance of poultry, as have modifications of a non-nutritive nature. One of the latter, dietary hydrated sodium calcium aluminosilicate, was highly effective in

alleviating aflatoxicosis in broiler chickens (Phillips et al., 1988). Its effect appears to result from non-reversible binding of the dietary additive to aflatoxin, thereby minimizing absorption of the aflatoxin from the intestinal tract.

Environmental Modifications

Acclimation of chickens to a low environmental temperature minimizes many of the acute (oral dosing) effects of aflatoxicosis (Wyatt et al., 1977; Manning and Wyatt, 1990). Acclimation-induced resistance was related to the acclimation temperature (i.e., the lower the acclimation temperature, the more resistance) and the length of acclimation (chicks acclimated for 20 days were more resistant than chicks acclimated for 10 days). The cold-induced resistance was temporarily lost when acclimated chicks were housed in a warmer environment) and could be overcome by administration of relatively high levels of aflatoxin. Decreased pentobarbital sleeping times and increased hepatic cytochrome P-450 in cold acclimated (resistant) chicks suggested that changes in the hepatic metabolism of aflatoxin B_1 were responsible.

Genetic Selection

Following the observation of differing mortality patterns from acute aflatoxicosis in various growth-selected lines of Japanese quail (Marks and Wyatt, 1979a), a specific effort was made to genetically select Japanese quail for resistance to acute aflatoxicosis (Marks and Wyatt, 1979b). After only 5 generations of selection, an 11-fold increase in resistance to acute aflatoxicosis was accomplished. Lanza et al. (1983) noted genetic variation in a nonselected population of chickens in regard to aflatoxin resistance. In view of the rapid and substantial progress accomplished in Japanese quail for resistance to aflatoxin and the genetic variability of aflatoxin resistance in chickens, we investigated whether chickens could be selected for resistance to aflatoxin and the basis for this resistance.

The technique of selection for aflatoxin resistance in chickens was similar to that used by Marks and Wyatt (1979b) in Japanese quail, that is, a nonselected (NS) line of chickens was maintained at the same time as the survivors from a similar population of chickens that had been admini-

stered an LD_{50} dose of aflatoxin. Survivors were utilized as the breeding stock for each subsequent generation. The resulting line of chickens is designated as the aflatoxin resistant (AR) line.

Two original populations of chickens were used: one, a commercial broiler stock and the other nonselected random-bred population (Athens-Canadian) of chickens maintained for experimental purposes (Wyatt et al., 1987). After each generation of selection, progress in selection for aflatoxin resistance was assessed by administering both the NS (control) and AR lines identical oral doses of aflatoxin capable of causing high mortality.

After 4 generations of selection in the Athens-Canadian population, administration of an oral dose of aflatoxin (20 mg/kg) resulted in 76% mortality in the NS line but only 18% mortality in the AR line. In the two lines derived from the commercial broiler stock, differences were present, but the magnitude was less. In both cases, sensitive indicators of aflatoxicosis, including plasma total protein, albumin, cholesterol concentrations, and gamma glutamyl transferase, were significantly altered in the NS chicks but not in the AR chicks fed aflatoxin. This selection for resistance to acute aflatoxicosis in chickens was rapid and substantial; also the resistance was maintained during more chronic exposure of chicks to aflatoxin.

In vitro microsomal metabolism of aflatoxin B_1 was investigated in the NS and AR lines derived from the Athens-Canadian population of chickens. The rate of aflatoxin B_1 metabolism was greater with microsomes from AR chicks than with microsomes from NS chicks. Additionally, in vivo pretreatment with sodium phenobarbital increased aflatoxin B_1 metabolism with NS microsomes but decreased aflatoxin B_1 metabolism with AR microsomes. In vivo pretreatment with beta-napthoflavone (a microsomal enzyme inducer) enhanced the metabolism of aflatoxin B_1 by AR microsomes compared to NS microsomes. Aflatoxin B_1-dihydrodiol was the major metabolite produced by both lines, and aflatoxin M_1 and Q_1 were produced in small quantities from beta-naphthoflavone pretreated AR microsomal incubations only. The data indicated that increased in vivo resistance of the AR line to acute aflatoxicosis may be related to increased hepatic aflatoxin B_1 metabolism. Thus, it can be concluded that genetic selection alters in vitro metabolism (both quantitative and qualitative) of aflatoxin B_1.

The significance of this project lies in the potential use of these lines (NS and AR) for comparative studies of aflatoxin toxicity and metabolism in the broiler chicken. Additional research is needed to investigate the metabolism of aflatoxin, including the metabolic profiles and clearance

rates in poultry before genetic selection can be a viable means to assist in successful management of aflatoxicosis in chickens. Nevertheless, the present knowledge of genetic selection for aflatoxin resistance, coupled with dietary and environmental modifications may help the commercial poultry industry successfully cope with aflatoxicosis.

References

Doerr, J.A. and P.B. Hamilton. 1981. Aflatoxicosis and intrinsic coagulation in broiler chickens. Poultry Sci. 60:1406-1411.

Doerr, J.A., R.D. Wyatt, and P.B. Hamilton. 1976. Impairment of coagulation function during aflatoxicosis in young chickens. Toxicol. Appl. Pharmacol. 35:437-446.

Hamilton, P.B., H.T. Tung, J.R. Harris, J.H. Gaines, and W.E. Donaldson. 1972. The effect of dietary fat on aflatoxicosis in turkeys. Poultry Sci. 51:165-170.

Lanza, G.M., K.W. Washburn, and R.D. Wyatt. 1981. The effect of linoleic acid on broiler response to graded levels of aflatoxin. Arch. Geflugelk 45:206-211.

Lanza, G.M., K.W. Washburn, R.D. Wyatt, and H.L. Marks. 1982. Genetic variation of physiological response to aflatoxin in *Gallus domesticus*. Theo. Appl. Genet. 63:207-212.

Manning, R.O. and R.D. Wyatt. Effect of cold acclimation on the broiler chicks' resistance to acute aflatoxicosis. Poultry Sci. 69: (in press).

Manning, R.O., R.D. Wyatt, and O.J. Fletcher. 1990. Effect of cold acclimation on the broiler chicks' resistance to dietary aflatoxin. Poultry Sci. 69: (in press).

Marks, H.L. and R.D. Wyatt. 1979a. Aflatoxin susceptibility of selected and control Japanese quail lines. British Poultry Sci. 21:29-36.

Marks, H.L. and R.D. Wyatt. 1979b. Genetic resistance to aflatoxin in Japanese quail. Science 206:1329-1330.

Osborne, D.J., W.E. Huff, P.B. Hamilton, and H.R. Burmeister. 1982. Comparison of ochratoxin, aflatoxin and T-2 toxin for their effects on selected parameters related to digestion and evidence for specific metabolism of carotenoids in chickens. Poultry Sci. 61:1646-1652.

Pegram, R.A. and R.D. Wyatt. 1985. Comparative responses of genetically resistant and nonselected Japanese quail to dietary aflatoxin. Poultry Sci. 64:266-272.

Phillips, T.D., L.F. Kubena, R.B. Harvey, D.R. Taylor and N.D. Heidelbaugh. 1988. Hydrated sodium calcium aluminosilicate: a high affinity sorbent for aflatoxin. Poultry Sci. 67:243-247.

Smith, J.W. and P.B. Hamilton. 1970. Aflatoxicosis in the broiler chicken. Poultry Sci. 49:207-215.

Smith, J.W., C.W. Hill, and P.B. Hamilton. 1971. The effects of dietary modifications on aflatoxicosis in the broiler chicken. Poultry Sci. 50:768-774.

Thaxton, P., H.T. Tung, and P.B. Hamilton. 1974. Immunosuppression in chickens by aflatoxicosis. Poultry Sci. 53:721-725.

Tung, H.T., J.W. Smith, and P.B. Hamilton. 1971. Aflatoxicosis and bruising in the chicken. Poultry Sci. 50:795-800.

Tung, H.T., R.D. Wyatt, P. Thaxton, and P.B. Hamilton. 1973. Impairment of kidney function during aflatoxicosis. Poultry Sci. 52:873-878.

Tung, H.T., F.W. Cook, R.D. Wyatt, and P.B. Hamilton. 1975a. The anemia caused by aflatoxin. Poultry Sci. 54:1962-1969.

Tung, H.T., R.D. Wyatt, P. Thaxton, and P.B. Hamilton. 1975b. Concentrations of serum proteins during aflatoxicosis. Toxicol. Appl. Pharmacol. 34:320-326.

Wyatt, R.D., W.C. Lockhart, and T.M. Huston. 1977. Increased resistance of chickens to acute aflatoxicosis by acclimation to low environmental temperatures. Poultry Sci. 56:1648-1651.

Wyatt, R.D., H.L. Marks, and R.O. Manning. 1987. Selection for resistance to aflatoxin in chickens. Poultry Sci. 66:1901-1904.

Animal Testing Procedures in Assessing the Efficacy of Ammoniation of Commodities

William P. Norred

Introduction

Aflatoxin contamination of corn, peanuts, cottonseed and other agricultural commodities is a problem of worldwide importance. The severe toxicity of this group of fungal toxins, including their proven ability to cause cancer in experimental animals, has caused regulatory agencies in many countries to set limits on the amount of aflatoxin allowable in commodities. Rejection of crops at grain elevators or at ports of entry results in severe economic losses, particularly in years when weather conditions such as drought are favorable to fungal growth and toxin production. Compounding the problem is the difficulty encountered, at least so far, in producing cultivars resistant to the fungi that produce aflatoxin. Although sound agricultural practices, such as proper drying and storing of grain, are highly effective in reducing levels of contamination, the fact is that toxin production can begin in the field before harvest begins. In summary, while current research on methods to reduce fungal growth and toxin production through genetic manipulation or biocontrol holds promise for the future, the aflatoxin problem is likely to remain with us for some time.

Because of the seemingly unavoidable contamination of some commodities with aflatoxin, a number of procedures have been proposed for decontamination of the product so that it may be utilized, at least for animal feeding purposes if not for human use. Of these, treatment with ammonia, in either gaseous or liquid form, appears to be the most effective both in terms of aflatoxin destruction and in ease of use and cost effectiveness. Ammoniation can be done at either atmospheric pressure and ambient temperatures or under elevated pressure and high temperature. A number of investigations have left little doubt as to the effectiveness of both procedures in chemically destroying the aflatoxin molecule. However, as with any decontamination process, the safety of the by-products of the process must be determined through appropriately designed toxicological investigations. In this brief review, the animal testing procedures that have been conducted at the Russell Research Center in an effort to establish whether ammoniation is safe and effective

will be described. Other laboratories have also investigated the efficacy of the ammoniation process through experiments with animals, and the reader is referred to the excellent reviews by Park et al. (1988) and Coker et al. (1985).

Acute Toxicity Studies

Aflatoxin can produce toxic symptoms within a short time (hours to days) of its consumption. The extent of the toxicity observed will depend, of course, on the amount consumed, but also will vary depending on the species, age, sex, and health of the animal. For example, ducklings and young pigs are very susceptible to acute poisoning with aflatoxin, but chickens and sheep are relatively resistant. The signs of toxicity produced by aflatoxin on the various organs of the exposed animal are varied. The effects are most pronounced in the liver, but also occur in the kidney. Clinical signs produced by acute aflatoxicosis include weight loss, decreased feed efficiency, jaundice, lethargy and death. When liver cells are killed by toxins, enzymes are released into the blood. The elevation in serum enzymes, such as alkaline phosphatase, can be measured and used as an indication of aflatoxicosis.

A study in 1979 (Norred, 1979a) was designed to determine whether the acute toxic effects to rats of aflatoxin were ameliorated by ammonia treatment. Finely ground corn was mixed with aflatoxin, and given to rats by stomach tube so that the rats received highly toxic doses (10 or 20 mg aflatoxin/kg body weight). Signs typical of aflatoxicosis were observed, including weight loss, elevated serum alkaline phosphatase, and death within 72 hours of 4 of 6 rats given the high dose. In addition, liver function was adversely affected, as determined by the prolonged duration of anaesthesia produced by a barbituate given to the rats 72 hours after they were dosed with the toxic corn. Flasks containing the aflatoxin-spiked corn were treated with ammonium hydroxide, and the excess ammonia was driven off by gently warming the flasks and blowing a stream of nitrogen over the corn. When this ammoniated, aflatoxin-contaminated corn was administered to rats, no toxic signs were observed. The rats appeared as healthy as rats that were given ground corn that was free of aflatoxin, or aflatoxin-free corn that was treated with ammonia.

Distribution Studies

The manner in which a toxin distributes in the various "compartments" of the body determines to a large degree the extent of the toxic injury that may occur. So, too, chemical properties of the toxin - those that determine its ability to cross membranes and enter cells, to react with receptor sites or other cell components such as DNA, to be stored in body fat or other tissues, to undergo metabolism by the biochemical factory of the invaded cell, and to be excreted from the body - are important determinants of toxicity. The fate of aflatoxin in experimental animals and in different tissues has been studied by many investigators, and as a result we know much about the mechanism by which aflatoxin can produce its toxicity at such low doses. Aflatoxin undergoes a metabolic process in which one of the products is a highly reactive, very unstable compound - an epoxide. The aflatoxin-epoxide can undergo very rapid reaction with macromolecules within cells, forming covalent bonds. These reactions that occur with cell constituents have an adverse effect, causing injury or death of the cell. When such reactions occur with DNA, the structure of the DNA is altered, the genetic code is misread and if the misinformation is not corrected by repair processes within the cell, cancer can be initiated.

In order for ammoniation to be effective, the ability of aflatoxin-ammonia reaction products to interact with cell components must be greatly reduced or eliminated compared to that of aflatoxin. In 1981, studies were conducted to determine the effect of ammonia treatment on distribution and excretion patterns of aflatoxin in rats (Norred, 1979b; Norred, 1982). These studies were done by means of a radioactive tracer (carbon-14) incorporated as part of the aflatoxin molecule. The radioactivity of various tissues or excreta at various times after administration of ammoniated aflatoxin could then be compared to that of rats given non-ammoniated aflatoxin. Flasks of ground corn containing equal amounts of radiolabelled aflatoxin were prepared, and one was treated with ammonium hydroxide. Doses of the corn were given to rats by stomach tube, and urine and feces were collected over a 72 hour period. Some of the rats were killed at 1, 3, 6, 12, 24, or 48 hours after they were dosed, and the amount of radioactivity in blood and tissues was determined.

The excretion of ammoniated aflatoxin in both urine and feces was more rapid than with non-ammoniated aflatoxin. More importantly, the absorption of ammoniated by-products from the gastrointestinal tract occurred to a much lesser degree than that of aflatoxin. After 12 hours, about 90% of the dose of ammoniated-aflatoxin products remained in the gastrointestinal tract, compared to only 50% of the aflatoxin dose. In

addition the levels of radioactivity found in the blood were very low (near 0% of the dose) when ammoniated-aflatoxin was administered, but reached as high as 6% of the dose 6 hours after dosing rats with the non-ammoniated aflatoxin. After 72 hours, blood, liver and other tissues contained measurable amounts of radioactivity if the non-ammoniated aflatoxin was given (as high as 5% of the dose in liver), but radioactivity could not be detected in any tissues after ammoniation. In summary, the distribution studies using radiolabelled aflatoxin indicated that treatment with ammonia resulted in reaction products that, compared to aflatoxin, were poorly absorbed, more rapidly excreted, and had considerably less affinity for tissue constituents.

Chronic Toxicity

Aflatoxin is well known for its ability to produce liver cancer in many different animals, and is strongly suspected of causing cancer in humans, particularly if there are predisposing factors, such as hepatitis, present. One of the species most sensitive to aflatoxin-induced liver cancer is the rainbow trout. Brekke et al. (1977) showed that ammoniation of corn contaminated with aflatoxin prevented the appearance of liver tumors in trout after 12 months feeding. An animal model more commonly used as an indicator of potential toxicity to humans is the rat, and in 1983, we published results of long-term feeding studies of ammoniated, aflatoxin-contaminated corn to Fischer 344 rats (Norred and Morrissey, 1983).

The Fischer rat has been shown to be extremely sensitive to the carcinogenic action of aflatoxin, with as little as 1 part per billion (ppb) of aflatoxin in the diet capable of causing liver tumors (Wogan et al., 1974). We selected this strain because of its susceptibility, and fed both male and female rats diets containing 20% corn that was naturally contaminated with aflatoxin. The amount of aflatoxin in the final ration was 176 ppb. Other rats were fed an identical ration, except that the corn was ammoniated prior to being mixed with the other dietary ingredients. Control groups of rats received equivalent amounts of corn free of aflatoxin, aflatoxin-free corn that had been ammoniated, or a standard laboratory rat chow. The rats were fed for 91 weeks, then killed and examined for any internal or microscopic abnormalities. All of the rats (24 of 24) that were fed the diet containing aflatoxin-contaminated corn had liver cancer. Liver tumors did not occur in any of the other treatment groups, including the rats fed ammoniated, aflatoxin-contaminated corn.

Relay Feeding Studies

Ammoniation is intended to render aflatoxin-contaminated commodities usable for animal feed. It is not intended to be used for food products destined for human consumption. An early concern for the efficacy of ammoniation was that the by-products of aflatoxin formed during ammoniation, if not toxic of and by themselves, might undergo either reformation to toxic compounds, or be metabolically converted to toxic agents by the farm animals which were fed the products, and appear in edible tissues of the animals. Therefore studies were designed in the early 1970's by FDA and USDA to determine the safety of ammoniation of corn in "relay" feeding studies, in which ammoniated, aflatoxin-contaminated corn was fed to farm animals (Phase I), and the meat or eggs from the animals was subsequently fed to rats (Phase II) (Norred, 1982).

The Phase I studies were intended to simulate "on-farm" conditions as much as possible. Naturally contaminated corn, containing about 1000 ppb of aflatoxin was purchased, thoroughly blended, and half of it ammoniated by scientists at the ARS/USDA laboratory in Peoria, IL. Control corn, free of aflatoxin was also ammoniated by the same process. The corn was then trucked to our laboratory in Athens where arrangements with the University of Georgia were made to feed pigs, and with Clemson University to feed broiler chickens and beef cattle. The amount of corn in the rations and the duration of feeding prior to slaughter followed standard procedures. Eggs collected during the experiment and the pork and chicken meat obtained after slaughter were cooked, freeze-dried, and mixed into rat rations. The beef was not cooked, but was freeze-dried. Fischer rats were fed a ration containing 20% of the meat or egg product. The rat feeding trials extended over a 5-year period, and included chronic (2 year) and multi-generation reproduction studies. The data collected, including daily observations, body weight, feed efficiency, serum enzymes, pathological observations, and the data from the reproduction studies, including fetal absorptions and skeletal or visceral abnormalities, were analyzed by scientists at the FDA National Center for Toxicological Research. The animals used in these studies included 120 pigs, 4,000 broiler chickens, 60 beef cattle, and over 5,000 rats. There was no indication of adverse effects in any of these animals due to ammoniation of either aflatoxin contaminated corn, or of corn free of aflatoxin.

Conclusion

This report summarizes the studies conducted at the Russell Research Center to evaluate the safety of the atmospheric ammoniation process developed by ARS/USDA. There are other toxicological studies that have been conducted at other locations on a variety of ammoniation methods, but space does not permit the detailing of these. As perhaps best stated by Park et al. (1988): "When the data reported in research papers published on detoxification and biological testing in the past 20 years are viewed as a whole, the safety and efficacy of ammonia for reducing aflatoxin contamination are amply supported."

References

Brekke, O.L., R.O. Sinnhuber, A.J. Peplinski, H.H. Wales, G.B. Putnam, D.J. Lee, and A. Ciegler. 1977. Aflatoxin in corn: Ammonia inactivation and bioassay with rainbow trout.
Appl. Environ. Microbiol. 34:34-37.

Coker, R.D., K. Jewers, and B.D. Jones. 1985. The destruction of aflatoxin by ammonia: practical possibilities. Trop. Sci. 25:139-154.

Norred, W.P. 1979a. Effect of ammoniation on the toxicity of corn artificially contaminated with aflatoxin B_1. Toxicol. Appl. Pharmacol. 51:411-416.

Norred, W.P. 1979b. Effect of ammoniation of aflatoxin B_1-spiked corn on acute aflatoxicosis. Fed. Proc. 38:534.

Norred, W.P. 1982. Ammonia treatment to destroy aflatoxins in corn. J. Food Protect. 45:972-976.

Norred, W.P. and R.E. Morrissey. 1983. Effects of long-term feeding of ammoniated, aflatoxin-contaminated corn to Fischer 344 rats.
Toxicol. Appl. Pharmacol. 70:96-104.

Park, D.L., L.S. Lee, R.L. Price, and A.E. Pohland. 1988. Review of decontamination of aflatoxins by ammoniation: current status and regulation. J. Assoc. Off. Anal. Chem. 71:685-703.

Wogan, G.N., S. Paglialunga, and P.W. Newberne. 1974. Carcinogenic effects of low dietary levels of aflatoxin B1 in rats. Fd. Cosmet. Toxicol. 12:681-685.

New Perspectives on the Ammonia Treatment for Decontamination of Aflatoxins

Douglas L. Park and Louise S. Lee

Abstract

Ammoniation of meals made from oilseeds contaminated with aflatoxin has received intense research attention over the last 20 years. In 1979, information was supplied to regulatory agencies for consideration in granting official approval of ammoniation as a practical detoxification procedure. Approval was not granted. Since that time a number of research studies have been conducted to provide needed additional information. This report summarizes that new information and provides new perspectives for approval of the process on meals that flow through interstate commerce. Regulation of the process rather than approval of the product created would answer needs of both regulatory agencies and feed and food processors.

Aflatoxins are potent hepato carcinogens and toxins produced as molds grow on seeds that are constituents of food and feed (Goldblatt, 1971). Primary agricultural commodities affected are corn, peanuts, cottonseed, and tree nuts. Fungal contamination by *Aspergillus flavus/ parasiticus* can occur prior to harvest and under adverse storage conditions (Diener et al., 1987). Four primary toxins are of health significance, aflatoxin B_1, B_2, G_1, and G_2. Human exposure to aflatoxins can result from direct consumption of the contaminated product, or indirectly by consumption of foods from animals previously exposed to aflatoxin in feeds, such as milk products.

Prevention is the best solution. However, if contamination occurs, other measures must be established and used in order to assure safe use of agricultural products. Regulatory agencies have established various prevention and control programs. Programs include monitoring the contamination in the various products and if necessary, redirecting the highly contaminated product into non-risk uses; the establishment of regulatory guidelines; and the use of decontamination procedures where appropriate (Fremy and Quillardet, 1985). Ammonia decontamination is the most promising approach (Park et al., 1988).

In order to evaluate the safety and efficacy of any aflatoxin decontamination process, research must focus on criteria which will determine whether the procedure will be acceptable, such as: (a) the capability to

destroy, inactivate or remove the toxin, (b) identification of aflatoxin/ decontamination reaction products, (c) determination of the effect of the feed matrix on aflatoxin; and (d) evaluation of any toxic residues in the ammoniated products or foods derived from animals fed the decontaminated products. Industry and trade associations have stressed strongly the desire and the need to have a decontamination process available should aflatoxin levels in the susceptible products reach unacceptable levels (Harper, 1972). In 1979, the National Cottonseed Products Association (NCPA) filed a food additive petition with the Food and Drug Administration (FDA) requesting approval of the ammoniation procedure to reduce aflatoxin levels in cottonseed products (Jones, 1979). The petition was denied.

FDA posed several questions. What are the details of the procedure, and is it effective in reducing aflatoxin levels? Two procedures are currently in use: (a) a high pressure, high temperature procedure and, (b) an atmospheric pressure, ambient temperature procedure. The details of these procedures are listed in Table 1. The next logical question would be: are these procedures effective, and do they reduce aflatoxin levels in the commodities treated? Studies have confirmed that the ammoniation procedure reduces aflatoxin levels in contaminated products by 99%. Factors affecting the efficacy of the ammoniation procedure have been identified as: (a) the level of the ammonia applied, (b) the moisture level in the treated products, (c) the holding temperature of the process, (d) the duration of exposure to ammonia, and, (e) the level of the original aflatoxin contamination.

FDA also asked: What about aflatoxin/ammonia reaction products? What compounds are formed, in what amounts, and what are the relative toxic potentials of these compounds with relation to the parent aflatoxin B_1 compound? Several studies have been carried out addressing the ammoniation chemistry of aflatoxin. These studies evaluated the formation of aflatoxin related compounds, the interaction of aflatoxin ammonia compounds with food matrices, and the distribution of the aflatoxin ammonia compounds in feed matrices (Park et al., 1988). Studies have shown that the aflatoxin structure is altered due to the exposure to ammonia, and that this reaction is irreversible if the exposure time is sufficient. Figure 1 presents the scheme of the ammonia/aflatoxin reaction. Aflatoxin D_1 is one of the reaction products identified (Lee et al., 1974). Loss of carbon dioxide insures no reversion to the original toxin. However, if reaction with ammonia is not sufficient, exposure of the product to an acidic condition may cause the aflatoxin molecule to

return to the original state. Studies also addressed the level of conversion of aflatoxin B_1 to aflatoxin D_1, in meals where components would influence the reaction (Park et al., 1984; Lee and Cucullu, 1978). Results are presented in Table 2. Percent conversion of B_1 to D_1 is minimal. Because of the lack of knowledge of reaction products of B_1 in ammoniated meal, the study using radiolabelled B_1 and cottonseed meal (Park et al., 1984) was undertaken. That study identified products other than D_1 formed by ammonia/heat treatment in a meal matrix.

Table 1. Parameters and application of ammonia/ aflatoxin decontamination procedures.

	Process	
	High Temperature/ High Pressure	**Ambient Temperature/ Atmospheric Pressure**
Ammonia Level (%)	1-4	1.5-2
Pressure (PSI)	45 (3 bars)	Atmospheric
Temperature (C)	80-120	Ambient
Duration	0.5-3.0 hrs	14-21 days
Moisture (%)	14-20	17
Commodities	Whole cottonseed Cottonseed meal Peanut meal Peanut cakes	Whole cottonseed Corn
Application	Feed mill	Farm

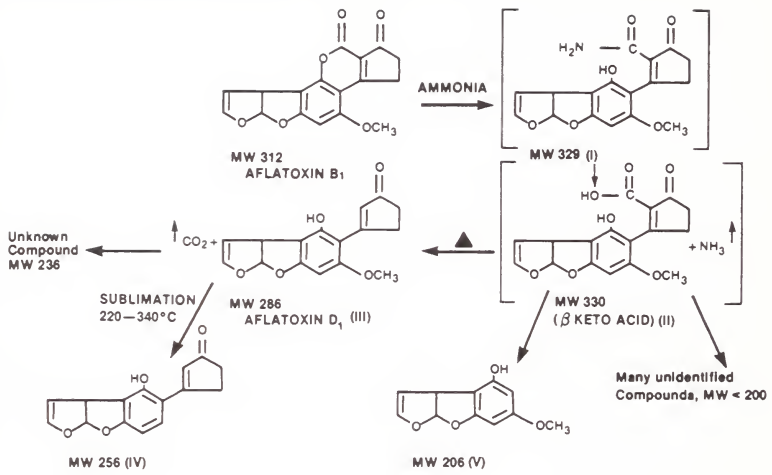


Figure 1.
Proposed formation of aflatoxin-related reaction products following exposure to ammonia. The major products: The MW 286 compound and the MW 206 compound have been isolated and biologically tested. From Park et al., J. Assoc. Off. Anal. Chem., 4:(4) 685-703 (1988).

Table 2. Relative levels of conversion of aflatoxin B_1 to aflatoxin D_1

Product/Substrate	Aflatoxin B_1 to D_1 (%)
Pure aflatoxin B_1	30[a]
Cultured aflatoxin-containing peanut meals	0.31[b]
Cultured/natural aflatoxin-containing cottonseed meals	0.1[b]
Aflatoxin-spiked peanut and cottonseed meals	0.9[b]

[a] Lee, L.S., et al. J. Assoc. Off. Anal. Chem. 52:626 (1974).
[b] Lee, L.S., and A.F. Cucullu, J. Agric. Food Chem. 26:881 (1978).

Extensive studies evaluated the effect of feeding ammonia-treated, aflatoxin-contaminated feeds to domestic and laboratory animals. These studies have evaluated the effect in dairy cows on production parameters, aflatoxin feed to tissue transfer, and the presence of aflatoxin/ammonia reaction products in milk (Price et al., 1982; Lough, 1979). Feeding studies with dairy cattle focused on production parameters (Hoversland et al., 1973), and poultry feeding studies evaluated the effect of the treated ration on egg production and quality (Waldroup et al., 1976). The nutritional quality of the feeds after treatment has shown decreased levels of lysine with no significant changes in other parameters (Mann et al., 1971). Toxicological properties of the isolated aflatoxin-related, decontaminated reaction products focused on comparing relative toxicities using the Salmonella/microsome mutagenic, the covalent binding index, and the developing chicken embryo assays (Lee et al., 1981). Comparative results of these studies show that, although a toxic potential still exists, that potential is several orders of magnitude lower than the potential for the parent aflatoxin B_1 compound (Table 3).

Approval of ammonia decontamination for aflatoxin has been received in several locations throughout the world. France, South Africa, Senegal, and Sudan use ammoniation for the treatment of peanut meal/cake. Brazil authorizes use of the procedure for incoming aflatoxin-contaminated feeds. Many European Economic Community member countries import ammonia-treated meals for animal feeds. The states of Arizona and California permit the use of ammoniation for cottonseed. North Carolina, Georgia, and Alabama permit ammoniation of corn. From 1968-1973 limited interstate approval was authorized by the Federal Drug Administration (FDA) for non-lactating ruminants and for laying hens. That approval, however, was for that five year period only. An estimate of the annual application of the ammoniation process is provided in Table 4.

FDA's next question: Which issues remain unresolved? FDA stated a lasting concern about the toxic and potential carcinogenicity of aflatoxin/ammonia reaction products in human foods, and which would be the appropriate legal mechanism to control the process? Studies evaluating the transfer of aflatoxin B_1 to foods from animals exposed to this contaminant have identified milk as the target showing the highest transfer rate (Table 5). In response to this concern, studies evaluating the safety of this process with respect to potential toxic residues in animal-derived foods have focused on the transfer rate (Jorgensen et al., in press; Frayssinet, 1989). Early studies examined the potential of transfer of toxic residues in tissues from chickens, eggs from chickens, swine, and beef by incorporation of these tissues in diets fed to rats. Studies were conducted under a

Table 3. Relative toxic potential of ammonia/ aflatoxin reaction products and animal metabolites

Compound	Salmonella Microsome Mutagenic Potential, ug/plate	Covalent Bindiing Index[a]	Chick Embryo,[b] ug/egg
Aflatoxin B_1	0.005[c]	22000	0.125[d]
Aflatoxin M_1	0.16[e]	2100	ND[f]
Aflatoxin D_1	2.25[d,g]	<70	2.5[d]
206 MW compound	3.3[h]	ND	ND
Isolate following Pronase digestion	180[c]	ND	ND

[a] Schroeder et al., J. Agric. Food Chem.33:311 (1985).

[b] Caused 40% embryo mortality.

[c] Lawlor et al., J. Am. Oil Chem. Soc.62:1136 (1985).

[d] Lee et al., Experientia, 37:16 (1981).

[e] Jorgensen, K.V. and R.L. Price, J. Agric. Food Chem. 29:555 (1981).

[f] Not determined

[g] Calculations based on 450-fold decrease in mutagenic potential.

[h] Haworth et al., J. Am. Oil Chem. Soc. 66:102 (1989).

Table taken from Park et al., J. Assoc. Off. Anal. Chem. 71(4) 685-703 (1988).

Table 4. Use of the ammoniation procedure for aflatoxin-contaminated commodities.

Location	Commodity	Annual Application (tons)	Daily Capacity (tons)
California	Cottonseed	200,000	—[a]
Arizona	Cottonseed	5,800[b]	500
France	Peanut meal/ cake	45,000	500
South Africa	Peanut meal	1,650	—[a]
Senegal	Peanut meal/ cake	—[a]	600
North Carolina	Corn	—[a]	—[a]
Georgia	Corn	—[a]	—[a]
Alabama	Corn	—[a]	—[a]

[a] Information not available

[b] October 1987-September 1988

Table 5. Relation of aflatoxin level in feed to aflatoxin residue levels in edible tissues[a].

Animal	Tissue	Aflatoxin	Feed/Tissue Ratio[b]
Beef Cattle	Liver	B_1	14000
Dairy Cattle	Milk	M_1	75
		Aflatoxicol	195000
Swine	Liver	B_1	800
Layers	Eggs	B_1	2000
Broilers	Liver	B_1	1200[c]

[a] Park, D.L. and A.E. Pohland in Mycotoxins and Phycotoxin, P. Steyn and R. Vleggaar, Ed. pp. 473-482 (1986).

[b] Level of aflatoxin B_1 in the feed divided by the level of the specified aflatoxin in the specified tissue.

[c] Study by Chen et al., Proc. V. Int. IUPAC Symp. Mycotoxins and Phycotoxins, Vienna, Austria p. 134 (1982) showed feed/tissue ratio in broiler livers to be 1-1200.

joint FDA/USDA research effort (Norrel, 1982). No toxic or carcinogenic effect was observed. Milks from lactating dairy animals fed ammonia-treated and untreated aflatoxin-contaminated cottonseed were tested for mutagenicity using the Salmonella/mammalian microsome mutagenicity assay. These studies, carried out at the University of Arizona, showed that the mutagenic potential in milk from cows fed the aflatoxin-contaminated feed was eliminated or significantly reduced following the ammoniation procedure (Jorgensen et al., in press; Lough et al., 1983). In studies currently underway evaluating trout carcinogenicity, where the diet of the trout contained milks from the previous study, preliminary results show that the ammoniation process shows no toxic or carcinogenic potential (R. L. Price, Personal Communication).

In summary, relative toxic potentials of ammonia-aflatoxin reaction products are several orders of magnitude less than aflatoxin B_1; aflatoxin D_1 is 450 times less mutagenic than aflatoxin B_1 (Lee et al., 1981). A larger portion of the reaction products bind to the feed components and are not biologically available. The safety of the aflatoxin/ammoniation reaction products in tissues and milk from animals fed ammonia-treated, aflatoxin contaminated feed have been evaluated in relay toxicity, Salmonella mutagenicity and trout carcinogenicity studies. No toxic affect has been attributed to the ammoniation procedure. Generally, production parameters, such as milk and egg quality, are better from animals fed the ammonia-treated product, due probably to the added nutritive value from the residual ammonia. Data demonstrate that the ammonia treated product poses less risk to animal health and reduced aflatoxin residues in human foods than non-treated products. When all parameters tested are evaluated in model systems, spike systems, ammoniation of aflatoxin-contaminated substances, and extensive feeding studies, results support the safety and efficacy of the ammoniation procedure.

The question now is: What must be done? Have enough research studies been made? Or is the issue one of regulatory options? Gaining approval of a food additive, as petitioned by NCPA in 1979 (Jones, 1979), requires that the safety of the product be established by evaluation of "probable exposure" and "appropriate toxicological tests." Over twenty years of research effort has not provided the basis for FDA approval of ammonia detoxification of aflatoxin from the food additive perspective. The time has now come to examine new perspectives. One could regulate the ammoniation procedure as a process rather than as a food additive. FDA could use the relative toxicity of the untreated product versus the relative non-toxicity of the ammonia-treated product as the basis for approval and set up a control program based either on (a) control of the

process, i.e., time, temperature, ammonia level, product moisture, etc., or (b) monitor aflatoxin levels before and after the ammoniation treatment. This regulatory mechanism is enforceable. Ammoniation provides a safer product to the consumer, and a product that does not have adverse affects on animal health. Primary goals of the FDA would be met and food processors would profit from the added value of an improved product.

References

Diener, U.L., R.J. Cole, T.H. Sanders, G.A. Payne, L.S. Lee, and M.A. Klich. 1987. Ann. Rev. Phytopathol. 25:249.

Frayssinet, C. and C. Lafarge-Frayssinet. 1989. Elev. Med. Vet. Pays Trop. 42:614.

Fremy, J.M. and P. Quillardet. 1985. Food Addit. Contam. 2:201.

Goldblatt, L.A. 1971. J. Am. Oil Chem. Soc. 48:605.

Harper, G.A. 1972. Summary of Proceedings of the Western Cotton Production Conference, Bakersfield, CA..

Hoversland, A.S., S. Bingana, G.C. Cavanagh, R.J. Selkirk, J. McKinney, J. Pearson, and D.M. Nelson. 1973. J. Anim. Sci. 36:1204.

Jones, L.A. 1979. Petition to Federal Drug Administration for Approval of Ammoniation as a Decontamination Procedure for Aflatoxin. National Cottonseed Products Assoc. Memphis, TN..

Jorgensen, K.V., D.L. Park, S.M. Rua, Jr., and R.L. Price. Journal Food Protection (in press).

Lee, L.S. and A.F. Cucullu. 1978. J. Agric. Food Chem. 26:881.

Lee, L.S., J.J. Dunn, A.J. DeLucca, Jr., and A. Ciegler. 1981. Experientia 37:16.

Lee, L.S., J.B. Stanley, A.F. Cucullu, W.A. Pons, Jr., and L.A. Goldblatt. 1974. J. Assoc. Off. Anal. Chem. 57:626.

Lough, O.G. 1979. Hoard's Dairyman 124:586.

Lough, O.G., C. Gingg, and M. Billotti. 1983. J. Dairy Sci. 62:171.

Mann, G.E., H.K. Gardner, Jr., A.N. Booth, and M.R. Gumbmann. 1971. J. Agric. Food Chem. 19:1155.

Norrel, W.P. 1982. J. Food Prot. 45:972.

Park, D.L., L.S. Lee, and S.P. Koltun. 1984. J. Am. Oil Chem. Soc. 61:1071.

Park, D.L., L.S. Lee, R.L. Price, and A.E. Pohland. 1988. J. Assoc. Off. Anal. Chem. 71:685.

Price, R.L., O.G. Lough, and W.H. Brown. 1982. J. Food Prot. 45:341.

Waldroup, P.W., K.P. Hazen, R.J. Mitchell, J.R. Payne, and J. Johnson. 1976. Poultry Sci. 55:1011.

Topic VI

Interactions as Complicating Factors in Assessing Risks of Aflatoxins

William E. Huff

One of the most frequently asked questions of animal producers concerned with aflatoxin is what is a safe level of aflatoxin. The answer to this question is that there is no "safe" level. Philosophically, safety is a concept that is based on the frame of mind of a given individual. For example, an individual may not feel "safe" on an I-beam during the construction of a building. However, some people work very comfortably on the same I-beam. It is more appropriate to talk about risk which can be quantitated in some manner. Aflatoxin is an extremely toxic compound. Therefore, anytime aflatoxin is fed to animals a measurable and calculated risk is taken that the level of aflatoxin ingested will not deleteriously affect the animals' health or production.

Unfortunately, the risk to animal producers due to aflatoxin is more complicated than simply evaluating the risk of this single compound. There are over 300 different mycotoxins and some of them can interact synergistically to increase the toxicity of aflatoxin (Huff et al., 1988a; Huff et al., 1988b). A good example of this type of interaction is that between the mycotoxins aflatoxin and ochratoxin A (Huff and Doerr, 1981). Furthermore, if a commodity is found to contain a given mycotoxin, for example aflatoxin, it indicates that at some time during the production, storage, and transportation of that commodity conditions were such that fungal growth occurred and aflatoxin was produced. Since the conditions that support fungal growth are not generally restrictive to a single fungus, we can suspect that finding a given mycotoxin as a contaminant of a commodity increases the probability that other mycotoxins are also present. As analytical laboratories have obtained procedures to evaluate samples for multiple mycotoxins, they have found that a number of samples do indeed contain more than just a single mycotoxin (McMillian et al., 1983; Abramson et al., 1983). Therefore, determining the risk associated with feeding a commodity on animal production based only on an aflatoxin analysis may significantly underestimate the true risk.

The answer to the question of what is a "safe" level of aflatoxin is even more complex than just a consideration of the numbers and quantities of mycotoxins present in feed. The toxicity of aflatoxin can vary greatly dependent on a number of additional factors. Aflatoxin can interact with disease agents by reducing the ability of the animal to

effectively fight-off diseases through disrupting the animals' immune system (Thaxton et al., 1974; Boonchuvit and Hamilton, 1975). The toxicity of aflatoxin can vary with the nutritional status of the animal, for example, as the protein level of feed decreases the toxicity of aflatoxin increases (Smith et al., 1971; Hamilton, 1977). Furthermore, the animals' response to aflatoxin is genetically determined both between and within species (Smith and Hamilton, 1970) and there is a difference in susceptibility of males and females (Wyatt et al., 1973). Environmental stressors such as extremes of hot and cold temperatures alter the toxicity of aflatoxin (Hamilton and Harris, 1971; Wyatt et al., 1975); and finally one of the most significant factors that affects the toxicity of aflatoxin is the age of the animal (Smith and Hamilton, 1970). In general, juvenile animals are much more susceptible to aflatoxin than mature animals.

Thus it is very difficult to answer the question of what is a "safe" level of aflatoxin that will not deleteriously affect animal production and health. Since so many factors affect the toxicity of aflatoxin, the amount of risk an individual takes by feeding aflatoxin depends on those factors unique to the given animal production system. Also the total mycotoxin exposure of the animal is very important and it may be very nearsighted to make recommendations based on a single mycotoxin analysis. The species and genetic makeup of the animals, the sex, age, nutritional status, disease challenges and the specific environmental stressors all dictate the animals response to aflatoxin. Thus the answer to a seemingly very simple question is very complex. We have made considerable progress in understanding these relationships and how they alter the toxicity of aflatoxin, however, there is a real need for further research to better understand the toxicity of aflatoxin as well as other mycotoxins.

References

Abramson, D., J.T. Mills, and B.R. Boycott. 1983. Mycotoxins and Mycoflora in animal feedstuffs in western Canada. Can. J. Comp. Med. 47:23-26.

Boonchuvit, Boonbungearn, and P.B. Hamilton. 1975. Interaction of aflatoxin and paratyphoid infections in broiler chickens. Poultry Sci. 54:1567-1573.

Hamilton, P.B. 1977. Interrelationships of mycotoxins with nutrition. Fed. Proc. 36:1899:1902.

Hamilton, P.B. and J.R. Harris. 1971. Interaction of aflatoxicosis with *Candida albicans* infections and other stresses in chickens. Poultry Sci. 50:906-912.

Huff, W.E. and J.A. Doerr. 1981. Synergism between aflatoxin and ochratoxin A in broiler chickens. Poultry Sci. 60:550-555.

Huff, W.E., R.B. Harvey, L.F. Kubena, and G.E. Rottinghaus. 1988a. Toxic synergism between aflatoxin and T-2 toxin in broiler chickens. Poultry Sci. 67:1418-1423.

Huff, W.E., L.F. Kubena, R.B. Harvey, and J.A. Doerr. 1988b. Mycotoxin interactions in poultry and swine. J. Animal Sci. 66:2351-2355.

McMillian, W.W., D.M. Wilson, C.J. Mirocha, and N.W. Widstrom. 1983. Mycotoxin contamination in grain sorghum from fields in Georgia and Mississippi. Cereal Chem. 60:226-227.

Smith, J.W. and P.B. Hamilton. 1970. Aflatoxicosis in the broiler chicken. Poultry Sci. 49:207-215.

Smith, J.W., C.H. Hill, and P.B. Hamilton. 1971. The effect of dietary modifications on aflatoxicosis in the broiler chicken. Poultry Sci. 50:768-774.

Thaxton, J.P., H.T. Tung, and P.B. Hamilton. 1974. Immunosuppression in chickens by aflatoxin. Poultry Sci. 53:721-725.

Wyatt, R.D., D.M. Briggs, and P.B. Hamilton. 1973. The effect of dietary aflatoxin on mature broiler breeder males. Poultry Sci. 52:1119-1123.

Wyatt, R.D., J.P. Thaxton, and P.B. Hamilton. 1975. Interaction of aflatoxicosis with heat stress. Poultry Sci. 54:1065-1070.

Epidemiologic Studies of the Association of Aflatoxin Exposure and Human Liver Cancer

D. Jesse Wagstaff

In Camelot we are told that King Arthur was so powerful that by his decree even the weather was controlled. Rain could not fall until after sundown and moonlight had to appear by 9 PM. But the powerful wise Arthur had humility as a watchword; he did not have all power or all knowledge. He sought out the counsel of Merlin the enchanter through clouds, through mist, and through the cobwebs of his own mind.

The king knew he must face the black knight who had vanquished so many good knights of the realm and displayed their shields upon an apple tree. On his way to the contest Arthur saved Merlin from attackers but Merlin told him that he was the one in the greatest danger. The humble king then applied two of his other watchwords, prudence and caution, and invited Merlin along. They passed the apple tree that rustled with the battered shields of the fallen knights and issued the challenge to battle. The two knights met with the violence of a thunderbolt, their great ashwood lances snapped like toothpicks. King Arthur was amazed that he had not overthrown his opponent, for he thought he was the very best knight in all the land. Although he unmasked the black knight, he was gravely wounded and was saved to fight another day only through Merlin's intervention.

Camelot is no longer with us but the spirit lives on. We in our own time in this country challenged the dragon of polio and marched our dimes into the battle from which we emerged victorious, later we conquered space and put a man on the moon. We declared war on the black knight of cancer but are amazed that we have not yet won.

Liver cancer has hung the shield of many on that symbolic apple tree. In this country the number of deaths each year is relatively small, being a little over 2000. But, in the world as a whole the toll is in the millions. The largest number of victims are men at the prime of life in their early adult years (Wagstaff, 1985). For example, among South African gold miners who had been carefully selected to perform heavy manual work, liver cancer killed over 90% of those who died of cancer (Berman, 1959). These men went from the peak of health to death in a few short months. Even last year in 1989 it was reported from a rural area of China that among young and middle aged men, over half of all deaths were due to liver cancer (Yeh et al., 1989).

We can't research this disease by putting people in cages like rats. Rather we are limited to observing people, their circumstances, their illnesses, and their deaths. From these observations we can draw conclusions and take steps to control the disease. Lest you be tempted to disdain observation as an impotent weapon, let me remind you that this was the basic method used to eradicate the only disease ever totally conquered by human effort; I speak of smallpox. The first step was the observation by a modern descendent of the Round Table, William Jenner, that cowpox immunized people against smallpox. Application of his vaccination technique led to the final victory in India many years after his death. Other simple observations of patterns and associations were made by William Withering, the discoverer of the heart drug digitalis. Even the observation of something that seemed out of place can be valuable, as exemplified by the discovery of penicillin by Sir Alexander Fleming. He is even titled a knight.

The father of epidemiology, which is the science of epidemics, is John Snow, a physician who helped control a 19th century cholera epidemic in London by counting cases of disease, calculating disease rates, and relating those rates to the consumption of sewage-contaminated water. King Arthur had his wonderful sword Excaliber but the modern epidemiologist has a simpler but more powerful weapon. It was best explained by Dr. Alexander Langmuir, founder of the Epidemic Intelligence Service, at the Centers for Disease Control. He said that the basic thing that an epidemiologist does is to count, count the number of cases of a disease, count the number of people in the base population in which the disease occurs, draw appropriate conclusions and take corrective action. A count is a powerful thing if done accurately and applied well.

In human research of liver cancer, cases are counted and the number of people in the population from which they come is also counted. Then a disease rate is calculated, i.e., the number of cases per unit of population such as 10 cases of liver cancer per 100,000 population. Evidence for disease causation comes from comparing rates for different groups of people. For example, lung cancer rates are greater for smokers than for nonsmokers and the rates increase with the amount of smoking.

A number of different approaches are used to investigate disease in humans. The first and easiest way is to observe a single case, one person with the disease. This is done by recording the signs, symptoms, lesions, living and working conditions, and laboratory analyses of blood and other samples. The definitive criterion for diagnosing liver cancer is microscopic examination of cancer tissue. The cancer tissue can be collected either before death by surgical means, a biopsy, or after death at an

autopsy. Other diagnostic procedures can suggest a diagnosis of liver cancer but only microscopic examination of cancer tissue can confirm it. Liver cancer was first recognized in the mid-1800's but only gradually since then have we learned its characteristics and still little is known about its causes. The black knight is not yet unmasked.

Survey of a group of cases, or a case series as these groups are called, provides further clues. Several decades ago evaluations of this type led to the broad conclusions that the number of cases of liver cancer was greater in males than females and that there were longterm epidemics of the disease in Africa and Asia. Explorers such as David Livingston found no cancer among native peoples. But we now know that liver cancer has been common for a long time. One of the best indications of this prevalence come from the meticulous records of Albert Cooke, a medical missionary in early Uganda (Davies et al., 1964). Similar reports have come from Asia.

The discovery of aflatoxin in the early 1960's coincided with an awareness from case series studies that liver cancer rates were high in some hot, humid climates where people ate mold-contaminated food. Thus the theory originated that aflatoxin causes human liver cancer. Animal studies showed aflatoxin to be a very potent cause of liver cancer in some but not all species. The rat is the most sensitive mammalian species.

Testing the theory that aflatoxin causes cancer in humans required more sophisticated types of research. The most common approach is the geographic or ecologic study. Basically the strategy is to compare liver cancer rates in people in different geographic areas. The goal is to find all cases of liver cancer which occur in each study area. Then the base population is taken from official census. And finally food is sampled and aflatoxin analyses are performed. This sounds simple enough and it might be if people would stand still to be counted. But many conditions interfere with doing high quality research including racial distrust, tribal jealousy, political unrest, and migration. Both the strengths and weakness of each study are based on the accuracy of the counts and the comparability of groups. Comparison of unlike groups of people can only lead to confusion, the technical term is confounding. Groups to be compared should be identical except for the element of interest, here that is the level of aflatoxin exposure. If the groups are dissimilar, if they are of different ages or have different exposures to alcohol, hepatitis B, herbal products or other things which can cause chronic liver injury, then it would be difficult if not impossible to sort out the different factors and come to any valid conclusions.

The first major geographic human study started in Kenya in 1967 (Peers and Linsell, 1973). Other studies have since been done in Africa in Mozambique and Swaziland and in Asia in Thailand and China. All of these studies provide some evidence but also all of them were done under less than ideal conditions. Therefore the data produced are less than perfect. Let us examine in more detail that first study in Kenya which is rather typical of the group of geographic human studies. The rural district of Murang'a which lies north of Nairobi on the eastern slope of the Aberdare Mountains was divided into 3 study areas by altitude. The low area was from 3500 to 5250 feet, the middle zone was 5250 to 6500 feet, and the high area was from 6500 to 12,000 feet. Liver cancer cases were registered over a 4 year period mainly at the hospital located in the low area. The only cases registered were those people who voluntarily presented themselves at a hospital or clinic for treatment of an illness. From other studies (Van Renzburg et al., 1985) there are indications that under reporting could have been as much as 80%, i.e., only 1 case was registered for every 5 which occurred. Even that 1 case could be a false positive, an erroneous diagnosis, if no microscopic examination were performed.

It was the original intent to microscopically confirm all cases but so few cases were found in the first year that the diagnostic criteria were relaxed and nearly half of the cases in the final report were not confirmed. One third of them were based solely on clinical appearance. Aflatoxin exposure was estimated from samples of food that were collected from randomly selected sites in the 3 study areas over a 21 month span. The samples were frozen and later taken to Nairobi for chemical determination of their aflatoxin content. Base population counts were taken from official national census. Liver cancer rates were then calculated for the 3 areas. The rates were found to be correlated with consumption of aflatoxin in the food. The low altitude area had the highest aflatoxin exposure and the highest liver cancer rate. The high altitude area had the lowest aflatoxin exposure corresponding to the lowest liver cancer rate. If the results are taken at face value the situation is comparable to smoking and lung cancer. We might conclude that aflatoxin causes liver cancer. But before we start cheering the unseating of the black knight let us look more closely. When examined carefully the first Kenyan study, and all of the other studies, are found to contain limitations which cause uncertainty regarding the comparability of the study groups and the accuracy of the counts. Humility and caution are in order. In the Kenya study the 3 study groups were not comparable in several ways. They had differences in altitude, proximity to the hospital, economic conditions, agricultural

conditions, the crops grown, and the diets. Also large numbers of the most susceptible class of people, adult males, were missing from the study areas presumably because they had migrated from their rural district to work in the urban area of Nairobi. Later research projects further south in Africa had to contend with the problem of migration to work in the South African gold mines. Any one or combination of these factors might be more highly associated with liver cancer than aflatoxin. I have already mentioned problems in finding cases and thus getting an accurate count of cases. The accuracy of the census are suspicious because in some areas the number of adult males recorded was only about one half that of adult females. Aflatoxin exposure estimates were compromised by failure to find one third of the selected households and by the assumption that aflatoxin content of food at each locality was the same at the time of sampling as it had been many years before that when the cancer process first started in the livers of the victims.

Many of the reports since the first one in Kenya have indicated that aflatoxin in the diet is related to liver cancer. But in other studies no such relationship has been found. No single study or combination of studies removes all doubt, there are methodologic problems in all the reports. Some problems such as case finding are common to all the projects but there were other differences. In the most recent report (Yeh et al., 1989) from a rural area of the Peoples Republic of China, the study populations were rather stable, there was little migration, and the census and vital statistics reporting systems seemed to be adequate. However, the published report is unclear regarding the comparability of groups, most of the liver cancer cases were not microscopically confirmed, and the details of sampling food for aflatoxin are not available.

There are reasons to doubt whether the correlations reported from Africa and Asia apply to other areas of the world. These correlations have not been reported from the United States. Aflatoxin concentrations are higher in foods of the southeastern states but that region does not have higher liver cancer rates than other regions of the United States. Some regions of the world such as Central America have high aflatoxin exposure but not corresponding high liver cancer rates. Another type of human study called case control has been widely used to estimate risk. The results for case control studies of aflatoxin and liver cancer have been widely divided. Some researchers found an association of aflatoxin with liver cancer but others reported no increased risk of liver cancer. For example, the risk of liver cancer in Taiwan was the same for people who ate peanuts compared to those who did not eat peanuts (Lu et al., 1988).

The actual or true rate of liver cancer associated with aflatoxin is not known. Probably it lies somewhere between the highest rate reported (Peers and Linsell, 1976) from human studies and the lowest rate (Stoloff, 1989). If the true rate were the highest reported then approximately 5 tablespoons of food containing 1 PPB of aflatoxin would be associated with 4 cases of liver cancer per million males in the population. On the other hand if the true rate were the same as the lowest estimate reported then there would be no cases of liver cancer. If Merlin were alive today perhaps he could tell where in this range from highest to lowest the truth really lies. But since he is not here we must continue on our own to search after truth.

After all of this it may seem that there is so much dust on the battlefield that it is difficult to tell whether the black knight or our hero is winning. Even though the black knight has not been unmasked or defeated, there are some things that we know with some degree of assurance. There is hope. Humans probably are not as susceptible to aflatoxin as are rats. The aflatoxin threat to human health may not be as great as once was feared. Despite problems, the range in which actual human risk lies is gradually being defined. And, many actions are known which could markedly reduce liver cancer rates in the world including vaccination for hepatitis B; improvement of socioeconomic conditions such as sanitation, nutrition, and health care; improvement of blood transfusion procedures; and encouragement of moderate lifestyles such as avoidance of excessive alcohol consumption, promiscuous sex and intravenous drugs.

The black knight of cancer will ultimately be defeated but the fight will be long and hard. The evidence that aflatoxin may cause human liver cancer is too strong to ignore but too weak to provide final confirmation. We must continue to apply the watchwords of the ancient king - humility, prudence, caution.

References

Berman, C. 1959. Primary carcinoma of the liver. Bull. New York Acad. Med. 35:275-292.

Davies, J.N.P., S. Elmes, M.S.R. Hutt, L.A.R. Mtimavalye, R. Owor, and L. Shaper. 1964. Cancer in an African community 1897-1956. Part 1. Br. Med. J. 1:259-264.

Lu, S.N., T.M. Lin, C.J. Chen, J.S. Chen, Y.F. Liaw, W. Chang, and S.T. Hsu. 1988. A case-control study of primary hepatocellular carcinoma in Taiwan. Cancer 62(9):2051-2055.

Peers, F.G., G.A. Gilman, and C.A. Linsell. 1976. Dietary aflatoxins and human liver cancer: A study in Swaziland. Intnl. J. Cancer. 17:167-176.

Peers, F.G., and C.A. Linsell. 1973. Dietary aflatoxins and liver cancer: A population based study in Kenya. Br. J. Cancer 27:473-484.

Van Rensburg, S.J., P. Cook-Mozaffari, D.J. Van Schalkwyk, J.J. Van der Watt, T.J. Vincent, and I.F. Purchase. 1985. Hepatocellular carcinoma and dietary aflatoxin in Mozambique and Transkei. Br. J. Cancer 51:713-726.

Wagstaff, D.J. 1985. The use of epidemiology, scientific data, and regulatory authority to determine risk factors in cancers of some organs of the digestive tract. 3. Liver cancer. Regulat. Toxicol. Pharmacol. 5:384-404.

Yeh, F.S., M.C. Yu, C.C. Mo, S. Luo, M.J. Tong, and B.E. Henderson. 1989. Hepatitis B virus, aflatoxins, and hepatocellular carcinoma in Southern Guangxi, China. Cancer Res. 49:2506-2509.

Risk Assessment Analysis: Requirements and Limitations

Sara Hale Henry

In March 1983, the National Academy of Sciences (NAS) published its report *Risk Assessment in the Federal Government: Managing the Process* (NAS, 1983). The report was commissioned by Congress in response to widespread criticism over the scientific objectivity and credibility of various safety decisions made by federal health regulatory agencies.

Why the interest in risk assessment in the 1980's when the Food and Drug Administration has been regulating the safety of food and drugs since 1906 and the safety of cosmetics and devices since 1938? Several changes have occurred in recent years to change our attitude toward risk and how risk is controlled (Scheuplein, 1987).

1. Absolute safety is not attainable; some risk is unavoidable.
2. Quantification of risk is useful in regulation of carcinogenic chemicals.
3. Scientists' ability to detect risks has outstripped their ability to evaluate these risks.

Drs. Fitzhugh and Lehman in the 1940's expressed FDA's traditional approach for setting safe levels of non-carcinogenic substances. An effect level was demonstrated in an animal study, a threshold or "no-effect" level was found at a lower dose, and then a still lower dose by a factor of 100 was set as the "safe dose" for humans. The safety factor of 100 was not unreasonable and was consistent with the limited data available. It took into account the fact that humans are generally more sensitive to chemical toxicity than laboratory animals given the same unit dose and also that humans are more diverse than animals. As long as the effects observed in animals showed a threshold and were reasonably akin to those seen in humans, the safety factor approach could be used to meet the statutory requirement of "a reasonable certainty of no harm" (Lehman and Fitzhugh, 1954).

The public expected the agency to make a decision and not to establish a scientific truth. But residual uncertainties were not usually considered in the public mind, and the illusion arose that absolute safety was being promised.

After World War II more and more chemicals began to be introduced into food, and more and more pesticides began to be used on food crops. Congress passed the Federal Insecticide, Fungicide, and Rodenticide Act in 1947 (Pub. L. 1947), the Pesticide Chemicals Act of 1954 (Pub. L., 1954), the Food Additives Amendment of 1958 (Pub. L., 1958), and the Color Additive Amendments of 1960 (Pub. L., 1960), including the Delaney Clause and preclearance regulations (21 U.S.C.). The public began to realize that environmental chemicals were not entirely free from risk.

The agency felt by the early 1970s that public and judicial acceptance of its regulatory decisions could best be secured by articulating their basis in greater detail, by proposing any requirements in written rules, and by assuring public participation in their development (Scheuplein, 1987). The first FDA document to make use of quantitative risk assessment for carcinogens was the Sensitivity of Method document of 1973 (38 Fed. Reg., 1973). This document prescribed the manner of determining the acceptable sensitivity of an analytical method to permit trace levels of carcinogens in commercial beef, pork, and poultry. "(A)bsolute safety can never be conclusively demonstrated experimentally. The level defined by the...procedure is an arbitrary, but conservative, level of maximum exposure resulting in a minimal probability of risk to an individual (e.g., 1/1,000,000)...."

"Here excess risk is rendered explicitly visible as a specified increase in the probability of harm. It does not necessarily come across to all members of the public that the harm that seems to be becoming more certain is also becoming smaller and often insignificant by any reasonable measure. Agency risk managers, in their turn, can also be misled.... By making risk numerically explicit, there is a tendency to exclude qualitative factors that do not readily fit the quantitative models and to also forget that estimates of risk are themselves uncertain. The residual uncertainties remain, and it is very unlikely that foreseeable improvements in the data base will ever completely resolve them." (Scheuplein, 1987).

Why then does the agency use quantitative risk assessment (QRA)? Despite its limitations, QRA can (Flamm, 1988; Scheuplein, 1987):

a. Provide consistency.
b. Help in setting priorities.
c. Permit the agency to perform risk/benefit balancing where allowed under the law.
d. Help in settling disputes.

e. Take into account that risk is related to dose or exposure.
f. Fit the nature of toxicological studies which in themselves nearly compel use of QRA.

What is QRA? As defined by the NAS book, QRA has four steps (NAS, 1983).

Hazard identification. The determination of whether a particular chemical is or is not causally linked to particular health effects.

Dose-response assessment. The determination of the relationship between the magnitude of exposure and the probability of occurrence of the health effects in question.

Exposure assessment. The determination of the extent of human exposure before or after application of regulatory controls.

Risk characterization. The description of the nature, and often the magnitude, of human risk, including attendant uncertainty.

How does the Center for Food Safety and Applied Nutrition/FDA perform QRA? The flow chart depicted in Figure 1 shows the pathway a contaminant would follow resulting in a possible QRA if the contaminant is deemed to be a carcinogen. Performing a QRA is an interdisciplinary process with input from the Divisions of Pathology, Mathematics, Nutrition, Toxicology, and Chemistry and scientific experts outside the Center if necessary (Kokoski et al., 1989).

It was stated earlier that the nature of toxicological studies nearly compels the use of QRA. To understand this, it is necessary to look at the basic assumptions upon which QRA depends:

- The principle of dose-dependency.
- The assumption that laboratory animals are good models for humans.

The commonly used experimental animals (rats, mice and hamsters), like humans, have viable mammalian cell and enzyme systems. These animals respond to toxic insults in ways that are comparable to humans, with the degree of the insult or injury generally increasing with the dose administered. Also, the overall patterns of chemical metabolism are generally similar in humans and experimental animals, although the rates of metabolism may differ. In the case of cancer, all 26 substances known to be

human carcinogens, as based on epidemiological studies, also are carcinogenic in experimental animals; furthermore, the six known human carcinogens were found to be animal carcinogens before they were discovered to be human carcinogens (Kokoski et al., 1989).

However, animal assays bring with them several problems which affect QRA. The first is the insensitivity of whole animal bioassays. If the incidence of an adverse effect is one in a thousand, it is very unlikely to be observed in a study with 100 animals. The experiment with 100 animals is statistically too insensitive to detect, at the chosen level of confidence, an incidence smaller than 3% (Scheuplein, 1987).

Why not use the threshold safety factor approach used for non-carcinogens? Cancer investigators have showed that exposure to small persistent doses of carcinogens could be more effective than single large doses in producing tumors. A single exposure to a carcinogenic initiator could sometimes produce a latent effect that was essentially permanent and that could be manifested later even if treatment with the promoter were delayed for up to half the lifetime of the animal. These experiments suggested that for carcinogens low doses are very important and the experimentally observed no-effect level might only reflect the statistical insensitivity at that dose. If thresholds do exist for carcinogens, they cannot be confidently determined in studies with a manageable number of animals (Scheuplein, 1987).

Thus the only practical basis for estimating the safe dose was by extrapolation downward from results obtained in animal experiments at elevated doses far above the use level. Unfortunately, there are inherent large uncertainties in the extrapolation process, such as:

- A 10^3 - 10^4-fold variation in low dose/risk estimates in current dose-response model.
- Neglect of the pharmacokinetic behavior of the toxic agent.
- Neglect of thresholds and/or repair processes.
- An uncertain animal to human scale-up method.
- Different animal vs. human exposure patterns.
- Different promotion environments for humans vs. animals.
- Uncertain human exposure level estimates.

Question of Potential Carcinogenicity of Contaminant Raised

↓

Toxicology evaluation

Input from internal experts

- Toxicologists
- Pathologists
- Chemists
- Statisticians
- Others

Input from external experts (where need is indicated)

CAC evaluation

CAC reviews input from internal and external experts.

Is the contaminant a likely carcinogen?

If Yes: CAC recommends the studies, tissue sites, species, and sex suitable for quantitative risk evaluation.

If No: No further consideration by CAC or QRAC needed.

↓

QRAC evaluation

QRAC reviews data and exposure potential.

QRAC chooses risk assessment model and procedure.

QRAC estimates magnitude of potential human risk.

1. Calculates the 10^{-6} dose.
2. Calculates the upper bound risk.

↓

Director of Office of Toxicological Sciences

Reviews QRAC evaluation.

Makes recommendation for risk management.

↓

Director of CFSAN, FDA

Makes risk management and policy decisions

Figure 1.
Typical steps in risk assessment at FDA. These are interactive steps, and at any point in the process issues may be referred back to previous step(s) for more information or clarification.

CFSAN/FDA's method of extrapolation is shown in Figure 2. The animal response in units of risk is plotted vs. dose. All experimental knowledge is compressed within a very narrow dose range. An extrapolation of possibly five orders of magnitude may be necessary to reach the level of use of the chemical in food (Lorentzen, 1984).

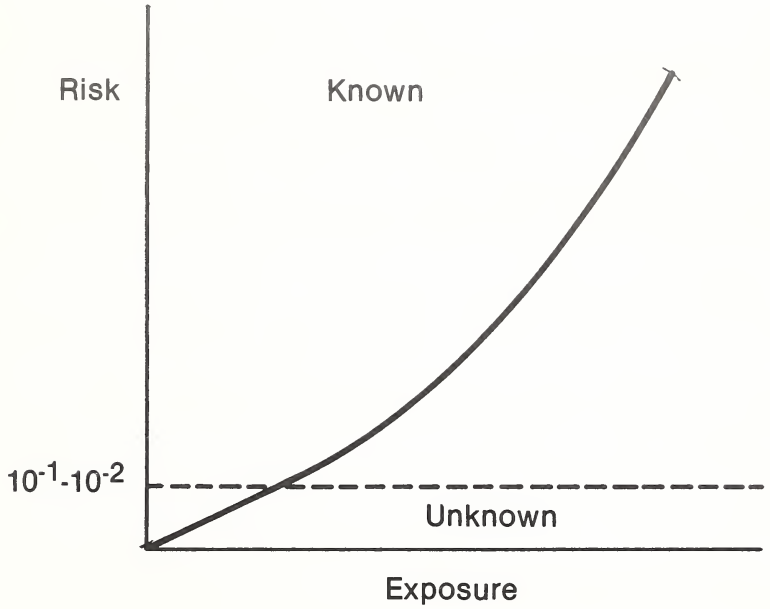


Figure 2.
Hypothetical dose-response curve for estimating the upper limit of risk using the linear-at-low-dose approach.

More problems with the QRA process as practiced by the agency may be summarized as follows (Scheuplein, 1987):

- Advances in analytical chemistry and hence our ability to detect possible carcinogens have outstripped the advance of toxicology and our ability to evaluate the significance of these chemicals.
- Science is advancing and more knowledge is often destabilizing.
- The law has lagged behind progress in science.

Risk communication is another area which gives FDA problems. Public demands create an ever-increasing standard for risk. The public also perceives involuntary vs. "chosen" risks differently, and this is a very emotional issue. Interactions of QRA with politics introduces another factor. Finally, the press often reports risk figures to the largely non-scientifically trained public without an adequate explanation of all the uncertainties accompanying those risk figures.

Does FDA have an alternative to QRA (Flamm, 1988)?

- Delaney-type no-risk approach.
- Safety factor approach.
- Combining QRA with other scientific knowledge.

The first approach would, in the case of aflatoxin, require complete prohibition of all the crops capable of being contaminated with aflatoxin - that is, corn, peanuts, sorghum, rice, wheat, soybeans, walnuts, almonds, pecans, pistachios, Brazil nuts, figs and edible tissue (meat, milk and eggs) from food-producing animals fed aflatoxin-containing animal feed. This option is clearly not feasible.

The second option is not scientifically defensible with a carcinogen such as aflatoxin which is so powerful in animal susceptible species.

The third option is the one currently followed by the agency which, we believe, gives the best approach to our mission to protect the public health from food-borne hazards.

References

Flamm, W.G. "Pros and cons of quantitative risk analysis". Presented at the IFT Basic Symposium, Institute of Food Technologists, New Orleans, LA, June 17-18, 1988. In press.

Kokoski, C.J., S.H. Henry, C.S. Lin, and K.A. Ekelman, (1989). p. 579 ff. Methods Used in Safety Evaluation. In: Food Additives. Branen, A.L., Davidson, P.M. and Salminen, S. (Eds.) Marcel Dekker, New York.

Lehman, A.J. and O.G. Fitzhugh, (1954). 100-fold margin of safety. Quar. Bull. Assoc. Food and Drug Off. of U.S. Jan. 33-35.

Lorentzen, R. J. FDA procedures for carcinogenic risk assessment. Food Tech. 38(10):108-111.

National Academy of Sciences, Risk Assessment in the Federal Government: Managing the Process (1983).

Scheuplein, R. J. (1987). Risk assessment and food safety: a scientist's and regulator's view. Fd. Drug Cosm. Law J. 42, 237-250.

Pub. L. No. 80-104, 61 Stat. 163 (1947).

Pub. L. No. 83-518, 68 Stat. 511 (1954).

Pub. L. No. 85-929, 72 Stat. 1784 (1958).

Publ L. No. 86-618, 74 Stat. 397 (1960).

21 U.S.C. @348. Food Additives Amendment.
21 U.S.C. @376 Color Additive Amendments.

38 Fed. Reg. 19, 226 (1973). Compounds used in food-producing animals.

Multicrop Aflatoxin Working Group Summary

Kimberly J. Cutchins

I think the understatement of the symposium would be that aflatoxin is a complex problem. This symposium has been extremely informative and the research presented very encouraging, and I would like to thank Chairman Kika de la Garza for calling together this symposium.

It was at just such a meeting, a little over a year ago in New Orleans, where the idea emerged of a Multi-crop Aflatoxin Working Group which would discuss the problems of aflatoxin within all commodities and seek ways to solve these problems. As a result of these discussions the National Peanut Council, the National Cotton Council, the National Corn Growers Association, the Corn Refiners Association, and the tree nut industry came together to form the Multi-crop Aflatoxin Working Group. Our primary goal is the elimination of aflatoxin, not just the reduction.

An ambitious research strategy plan for the elimination of preharvest aflatoxin was developed and endorsed by the Group. This plan focuses on two major approaches which Ed Cleveland mentioned yesterday; the enhancement of aflatoxin resistance in crops through classical plant breeding and genetic engineering and, secondly, the use of biocompetitive agents in the field. From the excellent research presented here, I'm sure the Working Group will consider expanding its strategy plan.

The Working Group quickly realized that its combined research resources would not begin to accomplish this goal of eliminating aflatoxin. So, the associations involved, with the assistance of many others, jointly approached Congress seeking funding for the necessary research. Our request was for a budget line item of $2.5 million. We are pleased to report that Congress responded to our request with a $750,000 line item. This is a terrific start, but not enough to accomplish our goal. Therefore, the Working Group will be seeking an increase to this line item for the next fiscal year.

The Congressional funds will be administered by ARS under the direction of Dr. Jane Robens (Chairman of the Committee responsible for the program for this symposium). They will be used for both in-house research and to support cooperative agreements with outside laboratories. Proposals from interested laboratories will be reviewed by a Technical Committee of commodity scientists and recommended for approval by the

Working Group. The ARS is currently seeking proposals from both the public and private sectors to control preharvest aflatoxin, that is by developing aflatoxin resistance in crops and/or developing biocompetitive agents.

I'd like to note that this money is (1) in addition to the ARS budget and does not take away from any current programs, and (2) is in addition to research supported by the individual commodities. Again, I want to reiterate that I am encouraged by what I have heard here; however, we have a long road to haul to eliminate aflatoxin as a source of concern for the food industry and I hope Congress will be listening when we request additional funding for this important research. Additionally, I would like to ask the attendees from the private sector to continue their support of the research being done on aflatoxin.