1997

Reduction and Management of Risks Associated With Aflatoxin and Fumonisin Contamination in Corn.

Socrates Trujillo
Louisiana State University and Agricultural & Mechanical College

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

REDUCTION AND MANAGEMENT OF RISKS ASSOCIATED WITH AFLATOXIN AND FUMONISIN CONTAMINATION IN CORN

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Food Science

by

Socrates Trujillo
B.S. Universidad Autonoma de Sinaloa (Mexico, 1992)
May, 1997

DEDICATION

For the three most important women in my life; My Mother, my Grandmother, and my Aunt. Your love and support have been imnumerable. I am what I am because of you.

ACKNOWLEDGMENT

I would like to express my greatest gratitude to Dr. Douglas L.Park. His advise, knowledge, and support through the completion of this study is invaluable. Special thanks to the members of my committe: Dr. Robert Grodner, Dr. Wanda Lyon, Dr. Leslie Plhak, Dr. Ralph Portier, and Dr. Vince Wilson. I would not have been able to complete the study without their participation. The guidance they gave me is unaccountable. Thank you to Mr. Hershel Morris and Dr. Janet Simmonson for allowing me the use of the Microbiology Laboratory facilities at the Agriculture Experimental Station. Also, I would like give thanks to Mr. Scott Floyd (Poultry Science), Dr. Steven Nicholson (Extension Service), and Dr. Barbara Shane (Institute for Environmental Studies), for their support and help. This study would not be possible without the help of Dr. Ralph Price, who gave me the first opportunity in Tucson, Arizona. Fr. Miguel de las Casas, O.P., Miss Griselda Romero, Miss Rebeca Lopez-Garcia, Dr. Linda Andrews, Mrs. Denise Craig, Mrs. Lara da Silva Carrilho, and Armando Burgos-Hernandez who were an important part in all the spiritual, moral, and technical support I had. I would like to give special thanks to all my friends of the University of Arizona Newman Center, Christ the King LSU Catholic Student Center, and LSU Food Science Department. If I write all their names, I would need one hundred more pages. All their support was very important.

To my family and other friends, for their support and understanding. To Jude Bourque, who came to my life almost at the end of this work, but stayed through the most difficult times. He has an important place in my life now. Thank you. I cannot finish my list without giving thanks to God, who taught me how to love, and continue walking in spite of the stormy weather.

The author was supported by the Consejo Nacional de Ciencia y Tecnologia-Mexico (CONACyT) Universidad Autonoma de Sinaloa (UAS), and Louisiana State University (LSU).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Aflatoxins and fumonisins, secondary mold metabolites, occur naturally as co-contaminants in corn. Health and economic risks associated with this contamination of food and feed have been the focus of much research worldwide. Research programs resulting from the discovery of aflatoxins in the 1960's has been used to guide study programs for fumonisins. The establishment of food safety programs, including mycotoxin decontamination procedures, must be evaluated for further application. The evaluation of selected procedures which reduce the risks associated with the contamination of these mycotoxins in food utilized for human and animal consumption was the focus of this study. This study investigated the possible risk reduction of naturally contaminated and spiked corn with aflatoxin B_1 and fumonisin B_1, utilizing conventional and modified industrial processes. Several ammonium based FDA-approved food additives (ammonium chloride, ammonium persulfate, and ammonium hydroxide) and their combination with hydrogen peroxide were utilized during normal fermentation and nixtamalization processes. HPLC C_{18} reverse phase procedures were utilized for the detection and quantification of both toxins. Reduction of aflatoxin B_1 levels was observed with all chemicals evaluated in this study. Fumonisin B_1 was not detected in any of the final products. The combination of ammonium persulfate with hydrogen peroxide, and ammonium hydroxide alone were the most effective additives. Tortillas prepared with modified nixtamalization (ammonium hydroxide, 1% v/w) was selected for evaluation, i.e., determination of teratogenicity and

mutagenicity of reaction products, and sensory evaluation of prepared tortillas. Teratogenicity and acute toxicity potentials of naturally contaminated samples were determined using a chicken embryo assay. Mutagenicity of the samples was measured with the *Salmonella*/microsomal mutagenicity assay utilizing test Strains TA 98, TA 100, and TA 102. The sensory evaluation study used semi-trained panelists for using the triangle test protocol. Results of the study showed no teratogenic potential in normal nixtamalized versus the modified nixtamalized processes for aflatoxin contaminated corn. Mutagenicity assay results were inconclusive and will require further study. Sensory evaluation demonstrated high acceptable levels of the product. These results support the need for more research in the area of decontamination procedures utilizing conventional processes and food additives.

1. INTRODUCTION

Naturally occurring toxicants produced by organisms (algae, bacteria, and fungi) can contaminate the food chain. One school of thought treats naturally occurring toxicants as a separate group of substances that established methods of studying chemical contamination of food cannot be applied. Since there is a systematic approach to studying other environmental contaminants, i.e., dioxins or agrochemical residues (pesticides, fertilizers, fungicides, and herbicides), the use of this knowledge can provide a firm basis for studying naturally occurring toxicants in food. A systematic approach is needed to study these diverse natural toxins. Several investigators believe that natural toxins pose a greater health risk to consumers than manmade chemical contaminants in food. Naturally occurring toxicants are difficult to control and monitor. Because of this, they can only be controlled by intervention methods.

Naturally occurring toxicants are chemical contaminants. Chemical contaminants (Table 1.1) are substances not intentionally added to food, but can be present as a result of production (including operations carried out in crop production, animal husbandry and veterinary medicine), manufacturing, processing, preparation, treatment, packing, packaging, transporting or holding of foods, or as a result of environmental contamination by living organisms.

Table 1.1. A classification of chemical contaminants in food. Modified from Watson, 1993.

Activity	Related chemical contaminants that can occur in food.
Crop production	Pesticides, nitrate, metals, naturally occurring toxicants.
Animal production	Pesticides, veterinary drugs, metals.
Food manufacture	Pesticides, metals, nitrate, nitrite, nitrosamines.
Packaging of food	Chemicals migrating from packaging.
Food storage	Pesticides, metals, naturally occurring toxicants.
Industrial	Environmental organic chemical contaminants, metals, pesticides.

Natural toxicants in food are usually classified by the types of organisms that produce them. The main groups are: higher plant toxicants, algal toxicants, bacterial toxins, and fungal toxins (mycotoxins).

This study will be focus on the fungal toxins, or mycotoxins, principally aflatoxins and fumonisins. Table 1.2 lists the fungi known to produce mycotoxins that are toxic to mammals. Acutely toxic metabolites from higher fungi (notably toadstools) are not included.

It is necessary to clarify that the term aflatoxins refers to a family of compounds with similar structures. The most toxic and prominent of these is alfatoxin B_1 (AFB_1). The terms aflatoxin and AFB_1 will be used interchangeably unless otherwise specified. With respect to fumonisins, fumonisin B_1 is the most prominent and toxic, and this term also will be used freely. This review is formatted by mycotoxin by addressing important issues, i.e., toxicity, biosynthesis, health hazard, and identification. Decontamination procedures will also play an important role in the development of this study. Although decontamination procedures are mentioned at the end of each topic, the development of this study was based on data collected for this literature review.

1.1. AFLATOXINS AND FUMONISINS

1.1.1. Genus *Aspergillus* and Genus *Fusarium*

The binomial classification of *Aspergillus flavus* and *Fusarium moniliforme* are presented (Table 1. 3).

Table 1.2. Sources of mycotoxins that are known or suspected toxins for mammals. Metabolites in bold have been found in food. Modified from Watson, 1993.

Fungi	Toxic metabolites
Alternaria	**alternariol and related compounds, tenuazonic acid.**
Aspergillus	**aflatoxins**, aflatrem, ascladiol, asp-haemolysin, aversin, chrysophanl (=chrysophanate), cytochalasins, **cyclopiazonic acid**, emodin, fumitremorgens, gliotoxin, malformin C, **naphto-γ-pyrones**, β-nitropropanoic acid, **oxalic acid**, paspaline, physcion and related metabolites, secalonic acid D, **sterigmatocystin and related metabolites**, terreic acid, territrems, TR-1 and TR-2 toxins, tryptoquivaline, tryptoquivalone, viomellein, xanthoascin, xanthocillins, xanthomegnin.
Cephalosporium	ophiobolins
Cercospora	unidentified toxins
Chaetomium	chetomin
Claviceps	**ergot alkaloids**, paspaline
Cochliobolus	ophibolins
Curvularia	cytochalasins
Diplodia	diplodiol
Dreschlera	chrysophanol, ophiobolins
Engleromyces	cytocholasins
Fusarium	fusarenon-X, **fumonisins**, **moniliformin**, sporofusarines, **tricothecens**, **zearalenone.**
Helminthosporium	cytochalasins, ophiobolins
Hormiscium	cytochalasins
Metarrhizium	cytochalasins
Micronectriella	**trichothecens**
Microsporium	viomellein, xanthomegnin
Nigrosabalum	cytochalasins
Penicillium	brefeldin, chrysophanol, citreoviridin, **citrinin**, **cyclopiazonic acid**, cytochalasins, funiculosin (=islandicin), hadacidin, janthitrems, **ochratoxins**, **patulin**, paxilline, **penicillic acid**, **penitrems** (=tremortins), PR toxin, **roquefortine**, rubratoxin B, secalonic acid D, simatoxin, viridicatum toxin, viomellein, xanthocillins, xanthomegning
Phoma	brefeldin, cytochalasins
Phomopsis	cytochalasins
Pithomyces	sporidesmins
Rhizoctonia	slaframine
Rhizopus	unidentified toxins
Rosellinia	cytochalasins
Scopulariopsis	unidentified toxins
Stachybotrys	satratoxins, verrucarin A
Trichophyton	viomellein, xanthomegnin
Trichothecium	**trichothecens**
Verticimonisporium	satratoxins, verrucarin A
Zygosporium	cytochalasins

Table 1.3. Binomial classification of *Aspergillus flavus* and *Fusarium moniliforme* (Alexopoulus and Mims, 1979).

Classification	*Aspergillus flavus*	*Fusarium moniliforme*
Superkingdom	Eukaryonta	Eukaryonta
Kingdom	Myceteae	Myceteae
Division	Amastigomycota	Amastigomycota
Subdivision	Deuteromycotina	Deuteromycotina
Class	Ascomycetes	Deuteromycetes
Subclass	Plectomycetidae	Hyphomycetidae
Order	Eurotiales	Moniliales
Family	Eurotiaceae	Tuberculariaceae
Genus	*Aspergillus*	*Fusarium*
Species	*flavus*	*moniliforme*

The genus *Aspergillus* is widely distributed from the arctic region to the tropics. The conidia of these organisms are present everywhere in the atmosphere. Soil also contains the spores of aspergilli, but whether these organisms play an important role in soil economy has not been determined with certainty yet. Aspergilli are capable of utilizing an enormous variety of substances for nutrients because of the large number of enzymes they produce. *Aspergillus flavus* is adapted to use a broad assortment of organic resources (Bhatnagar *et al.*, 1994). The aspergilli affect the world welfare in a variety of ways. Some species produce mycotoxins, of which the most significant are aflatoxins. *Aspergillus* and *Penicillium* are utilized in the Orient to prepare various fermented foods. Consequently, the danger of food poisoning from moldy rice, for example, as well as from other products, is quite real. *Aspergillus fumigatus*, *A. flavus*, *A. niger*, and other species are animal and human pathogens cause a group of diseases collectively known as aspergilloses (sing. aspergillosis).

About eight species have been identified as causes of this complex disease known as aspergillosis, of which *Aspergillus fumigatus* accounts for the majority of all infections. Other species involved in various types of aspergillosis are *Aspergillus terreus*, *A. flavus*, and *A. niger*. Aspergillosis of the lungs is probably the most serious of these diseases and is quite prevalent in birds and various mammals including humans (Alexopoulus and Mims, 1979).

The division Amastigomycota consists of a tremendous assemblage of common and familiar fungi. It includeds yeasts, molds, mildews, cup fungi, rusts, smuts, bracket or shelf fungi, puffballs, and mushrooms. The genus *Fusarium* classified by form is the largest in the Tuberculariaceae family and, taxonomically, one of the most difficult of all fungal groups. Few mycologists attempt to identify species of *Fusarium* because of the great variability in the forms within each species. This variability makes identification uncertain for all but the few specialists.

A number of Fusaria are parasitic, generally causing wilt of the host plant. Members of the form-genus *Fusarium* typically produce two types of conidia that are termed macroconidia and microconidia because of their respective sizes. When the fungus invades vascular tissue, the mycelium and conidia physically block xylem vessels and prevents the translocation of water. If enough vessels are clogged, wilting results. In addition, *Fusarium* is also known to produce toxins thought to contribute to wilting by affecting the permeability of cell membranes and disrupting cell metabolism (Alexopoulus and Mims, 1979).

Fusaria have a versatile biosynthetic apparatus capable of producing secondary metabolites such as isoprenoids, trichodienoids and polyketioles (Apsimon, 1994). They produce several toxins, *e.g.*, fusarins, fusaric acid, chlamydosporol, acuminatopyrone, fumonisins, moniliformin, zearalenone and zearalenols, deoxynivalenol and nivalenol, diacetoxyscirpenol, butenolide,

trichothecenes, and enniantins. These fungi are ubiquitous in wheat, sorghum, corn, and barley crops (Miller, 1994).

Miller (1994) studied infestation of corn by *Fusarium* species. The toxins produced by different species and their pathogenicities are shown in Table 1.4. The study mentions *F. moniliforme* and *F. proliferatum* were found to be moderately pathogenic in corn. These two strains have been found to produce fumonisins in different conditions (Miller, 1994).

1.1.2. Background Information.

Aspergillus flavus is a saprobe, opportunistic pathogen of plants, insects, and vertebrates including humans and domestic animals. Together with *Aspergillus parasiticus*, they produce aflatoxins, contaminants of food and feed worldwide. Aflatoxins (which comes from A= *Aspergillus*, fla= *flavus*, and toxins) were discovered in 1960 by English researchers, when turkeys and ducklings were fed animal feed containing peanut meal contaminated with these compounds. High levels of aflatoxins were found in corn crops in the United States during the years of 1977 and 1983, resulting in high economic losses. Although some species of *Fusarium* produce fumonisins, *Fusarium moniliforme* section Liseola is the most common fungus producing them. *F. moniliforme* has been estimated to occur in 80 to 100 percent of all corn harvested in the United States (Haschek *et al.*, 1986). This specific fungus has six different mating populations which can present bisexualism. The sexual

Table 1.4. Pathogenicity of *Fusarium* spp. in corn. Adapted from Miller, 1994.

SPECIES	PATHOGENICITY	MYCOTOXINS
F. subglutinans	High	Moniliformin
F. miniliforme	Moderate	Fumonisins
F. graminearum	High	Deoxynivalenol or nivalenol, zearalenone
F. culmorum	High to moderate	Deoxylivalenol, zearalenone
F. sporotrichiodes	Low	T-2, HT-2
F. crookwellense	Moderate	Nivalenol, zearalenone
F. proliferatum	Moderate	Fumonisins, moniliformin
F. avenaceum	Low	Moniliformin
F. poae	Very low	Diacetoxyscirpenol

stage for this fungus is *Gibberella fujikuroi* (Riley *et al.*, 1993). The genus *Gibberella* belongs to the family Nectriaceae. *Gibberella fujikuroi* (also designated as *Fusarium moniliforme*), causes foolish seedling disease of oriental rice in the Orient. Gibberellic acid produced by *G. fujikuroi* is extensively used as a growth-promoting substance for flowering, growth, and seed germination. *Gibberella zeae* (also desiganted as *Fusarium graminearum*) causes red ear-rot in corn. Not all mating populations of *Gibberella fujikuroi* share the *Fusarium moniliforme* anamorph. Only A and F mating populations share the anamorph stage and these types produce higher amounts of fumonisin B_1 when compared with mating type E and D (Leslie *et al.*, 1992). Furthermore, A and F mating types are host specific. Mating type A is primarily found in corn and mating type F is found in sorghum (Riley *et al.*, 1993). Incidence of *Fusarium* kernel rot is higher in warmer climates under dry conditions and insects appear to promote its occurrence (Miller, 1994).

Fusarium moniliforme presents the following characteristics when grown in laboratory conditions (Nelson *et al.*, 1983):

1. Rapid rate of growth.
2. Light purple aerial mycelium.
3. Colony light to purple diffusing into the agar, with a tan to orange color of spore masses.
4. Thin, long macroconidia from sporodocia with thin wall shape.
5. Macroconidia from aerial mycelium is abundant in chains and flase. heads, oval to kidney-shaped (reniform) and clavate.
6. Presents long monophiliades producing microconidia conidiophore.
7. Does not present chlamydiospores.

The presence of fumonisins, a family of toxins produced by *Fusarium moniliforme,* in food and feeds has become a daily concern for toxicologists, government, and farmers. Since the first identification of these compounds in 1989, fumonisins have been found all over the world, mainly in corn (Snijders, 1994; Miller, 1994; Norred *et al.*, 1991; Thiel *et al.*, 1991; Sydenham, 1991). The development of illnesses such as equine leucoencephalomalacia (ELEM) and porcine pulmonary edema (PPE) has been successfully connected with the presence of fumonisins (Brown *et al.*, 1992; Ficham *et al.*, 1992; Harrison *et al.*, 1990; Hendrich *et al.*, 1993). Identification of fumonisins is not as well defined as it is for aflatoxins which makes the research associated with these compounds more difficult. Basic research is still being conducted for a more successful method of fumonisin detection and identification. The Food and Drug Administration (FDA) has yet to establish regulatory limits for these compounds resulting in the discarding of highly contaminated crops.

Recently, the Department of Health of the State of Virginia detected an outbreak of ELEM caused by the presence of fumonisins in corn used for horse feed. This department's recommendation was to stop feeding the animals only corn, and to combine the corn with other feeds, or buy the corn from fumonisin tested providers (Diener, 1995).

Fusarium spp. occur throughout the world, and reports of fumonisin naturally contaminated animal and human feeds come from South Africa (Norred *et al.*,1991), China (Thiel *et al.*,1991), Peru (Sydenham *et al.*,1991) and

the United States (Hendrich *et al.*, 1993). Commodities affected include corn, peanuts, sorghum, wheat and millet (Nelson et. al.,1992). A map of the United States depicting where fumonisin-contaminated corn and/or peanut and ELEM have been reportly found, is shown in Figure 1.1. A screening method for their presence in the food supply is necessary.

1.1.3. Structures and Toxicity.

Aflatoxins, secondary metabolites, are potent hepatocarcinogens and toxins produced as molds grow on food and feed. Aflatoxin contamination in agricultural commodities can result from fungal contamination prior to harvest or when the product is stored under improper conditions. There are four different classes of naturally occurring aflatoxins (Figure 1.2): aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2). Aflatoxin M_1 (AFM_1), and aflatoxin M_2 (AFM_2) are hydroxylated metabolites of AFB_1 and AFB_2, respectively. AFM_1 and AFM_2 are excreted in milk after consumption of aflatoxin-contaminated feed. The conversion into the more hydrophilic derivatives occurs in the presence of the hepatic mixed function oxidase systems (Lynch, 1979). It has been found that approximately 1.6 percent of all the AFB_1 that an animal consumes is excreted in the milk (Price *et al.*, 1985; Frobish *et al.*, 1986). The FDA regulates feed containing aflatoxin under Section 402 (a)(1) of the Federal Food Drug and Cosmetic Act (FDA, 1989), which is the adulteration section.

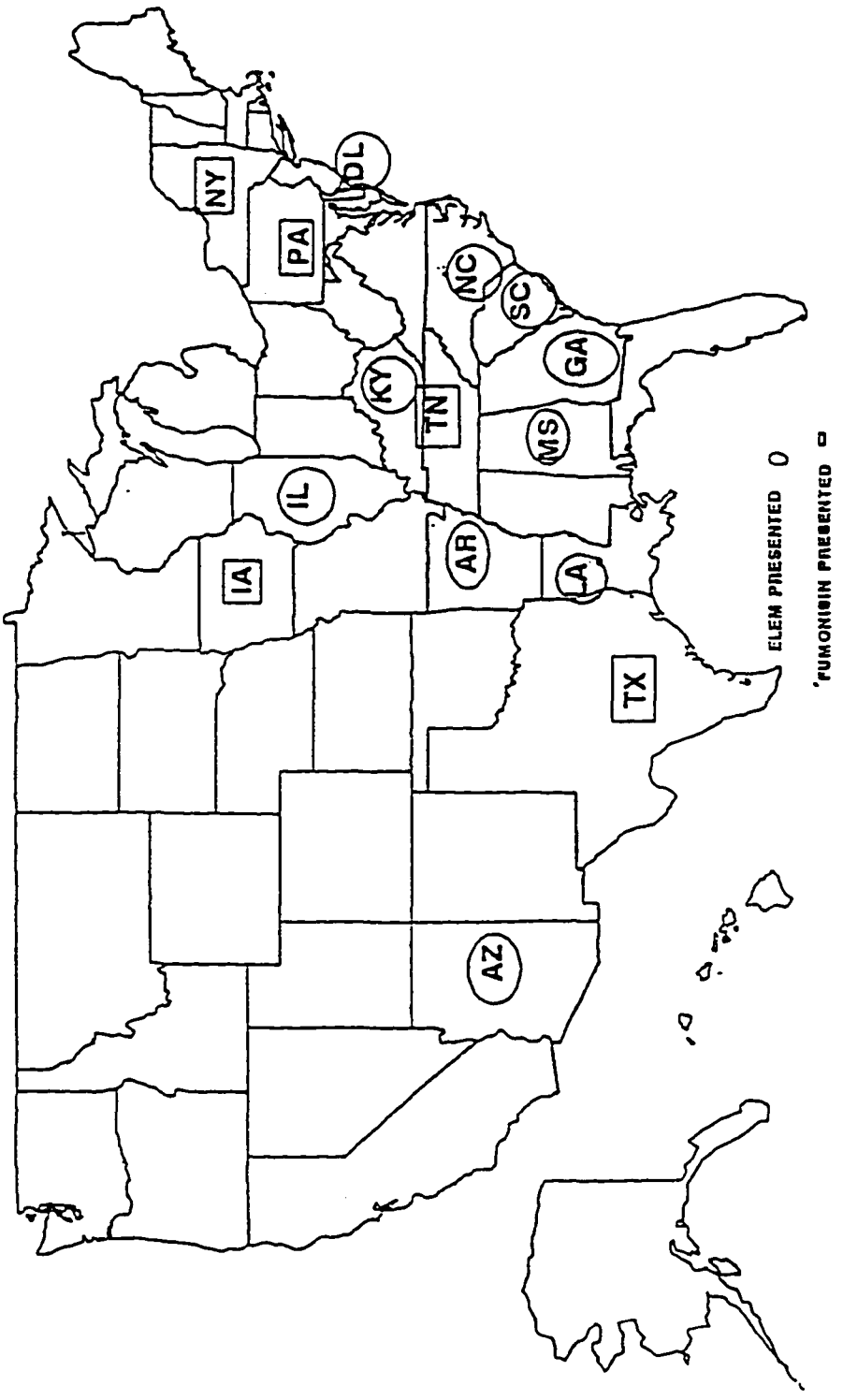

Figure 1.1. Fumonisin, ELEM and the U.S.A.

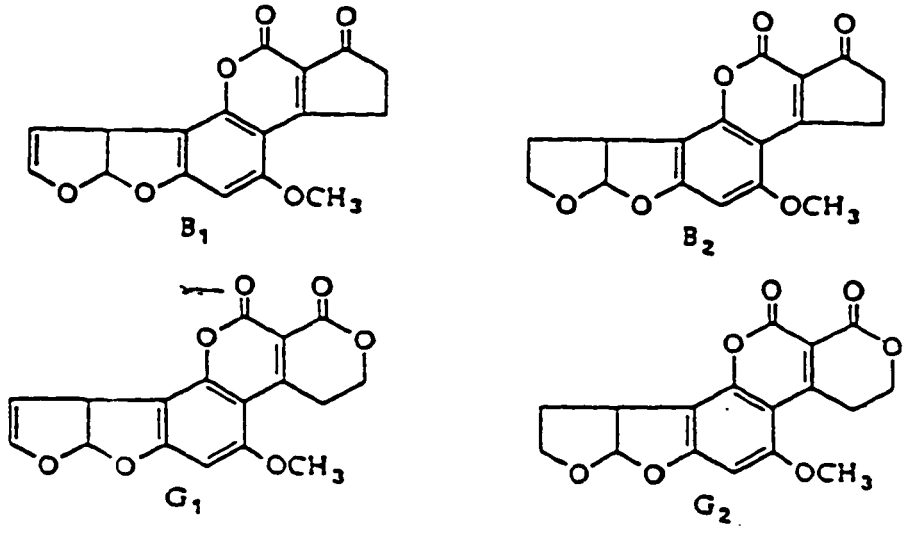

Figure 1.2. Naturally occurring aflatoxins.

Specifically, aflatoxin is regulated as an added (rather than not added) poisonous and deleterious substance for two reasons. First, the FDA believes that aflatoxin contamination can often be controlled by appropriate storage conditions. Second, as an added substance, the Agency only needs to prove that aflatoxins may render the food injurious to health (Price *et al.*, 1993).

Fumonisins (which name comes from Fu= *Fusarium*, moni= *moniliforme*, and sins= toxins) belong to a group of mycotoxins produced by certain strains of *Fusarium moniliforme*, section Liseola, *Fusarium nygamy* (Nelson *et al.*,1992) and *Alternaria alternata* (Chen *et al.*,1992). This relatively new group of toxins is responsible for equine leucoencephalomalacia (ELEM) and pulmonary edema in swine (Thiel *et al.*,1991).

Fumonisins (Figure 1.3) are hydrocarbons diesters containing various hydroxyl and carboxyl substitutions and a primary amine moiety (Shepard *et al.*, 1990). They are water soluble and heat stable (Ficham *et al.*, 1992; Dupuy *et al.*, 1993). It is believed that FB_1 undergoes typical ester reactions. If this is the case, it is expected that FB_1 will react with ammonia in the presence of ethanol to produce amides, which will break down the fumonisin molecule. Table 1.5 establishes the effects of heat treatment on the stability of FB_1 in a dried-corn culture of *F. moniliforme*. Data is expressed as percent recovery. This study illustrates that dry heating does not affect the structure of fumonisins (Dupuy *et al.*, 1993). Fumonisin structures are similar to the long chain base backbone of sphingolipids, and the inhibition of the conversion of sphinganine

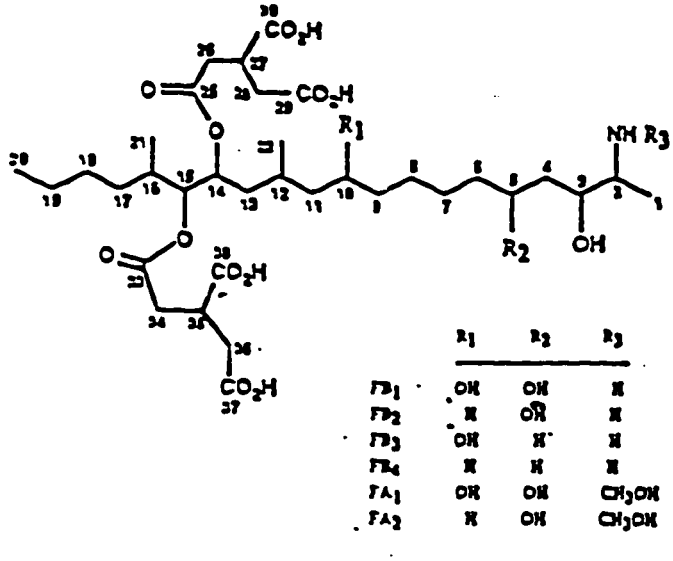

Figure 1.3. Chemical structures of the fumonisins.

Table 1.5. Effects of heat treatment on the stability of FB_1 in a dried-corn culture of *Fusarium moniliforme*. Data is expressed as percent recovery (Dupuy *et al.*, 1993).

t (min)	50°C	75°C	100°C	125°C	150°C
5		103±4	94±5	85±4	53±1
10				85±1	41±3.5
15		100±5	94±7		
20				56±4	36±1
40				55±4	13±2
45		104±1	83±6		
60	100±7				
80				25±2	
135		83±1	58±13		
240	90±3				
960	97±3				

to N-acyl-sphinganines may explain a possible toxicologic mechanism (Wang *et al.*, 1991).

1.1.3.1. Aflatoxin toxicity

Acute structural and functional damage to the liver, the principal target organ for aflatoxins as observed in field outbreaks, has been reproduced experimentally in most laboratory animals and several domestic animal species (Cullen and Newbern, 1994).

A review of the effects of acute exposure to aflatoxins reveals that a wide variety of vertebrates, invertebrates, plants, bacteria, and fungi are sensitive to these toxins and the degree of sensitivity is wide (Cullen and Newbern, 1994). Acute effects of aflatoxins on plants and microorganisms are important components of the total effect of these mycotoxins on the environment. Acute inhibitory effects of AFB_1 on microorganisms growth has been determined for several species of *Bacillus*, one strain of *Clostridium*, and one species of *Streptomyces* (Cullen and Newbern, 1994). The aflatoxins have been examined for toxicity in a range of primary cultures as well as in established cell lines (*e.g.* Ames test). Liver cell cultures from chick embryos demonstrated cytotoxicity of AFB_1 to both mesenchymal and parenchymal cells (Cullen and Newbern, 1994). Human embryo cells are also susceptible to AFB_1 toxicity. Electron microscopy reveals nucleolar capping of the chromatin, rounding of the cells, and degranulation of the endoplasmic reticulum (Cullen and Newbern, 1994). Although AFB_1 is a classic hepatotoxicant and hepatocarcinogen,

tissues other than the liver are affected at various degrees. Extrahepatic effects probably contribute to the sequelae of this natural toxicant. Factors such as route of administration, dose, frequency of dose, species, strain, age, and sex of the animal appear to affect the degree of extrahepatic involvement (Coulombe, 1994). Nonhepatic systems include the respiratory, renal, gastrointestinal, nervous, reproductive, and immune system (Coulombe, 1994).

The effects caused by the toxin, in most cases, are secondary for nonhepatic tissues. In any event, AFB_1 disperses into a variety of extrahepatic tissues (Coulombe, 1994). Subsequent effects, such as neoplasm, are common in several nonhepatic organs. Synergestic factors also appear to increase tissue involvement in response to AFB_1 exposure. In addition to the important route of exposure, dietary inhalation of AFB_1-contaminated grain dusts may result in hepatic and extrahepatic tumors (Coulombe, 1994). The immune system has been observed during aflatoxin intoxication. Repeated low-dose administration of AFB_1 is now known to modulate several parameters of cell- and antibody-mediated immune functions in avian and mammalian species. Importantly, immunotoxic effects often are seen in the absence of gross clinical pathology.

Aflatoxicosis alters the reproductive efficiency of both male and female domestic animals, particularly poultry, and several reports have shown that AFB_1 alters homeostasis of the central nervous system biogenic amines (Coulombe, 1994).

1.1.3.2. Fumonisin toxicity.

Fumonisins are known to produce illnesses in horses and swine. These naturally occurring compounds are produced by the genera *Fusarium*, a well known fungi related to the production of other mycotoxins (T-2 toxins, fusarin, moniliformin, zearalelone, etc.). Fumonisins possess the same basic arrangement; however, some have different functional groups. The chemical composition of fumonisins resembles the central structure of a sphingolipid, a long chain carbon molecule with a polar end. Sphingolipids constitute parts of the cell membrane and are important for its function. Diseases produced by fumonisins are based on cell disruption. Fumonisins are also associated with the presence of esophageal cancer in the region of Transkei (South Africa) and China (Thiel *et al.*, 1991).

1.1.3.2.1. Porcine Pulmonary Edema.

Fumonisin B1 intoxication in pigs results in porcine pulmonary edema (PPE). This illness is characterized by the formation of a massive hydrothorax, pulmonary edema, and a golden-yellow liquid presented in thoraxic cavities (Haliburton *et al.*, 1986). Death occurs as a result of this infection. Although PPE is also induced by alpha-naphthyl thiourea, a rodenticide, the confirmation in 1989 that this disease can also be caused by fumonisins led to the discovery of this group of toxins (Lenn *et al.*, 1990). Microscopic examination of sections of lung tissue revealed edema so severe that individual lobules and pleura were separated from the parenchyma. The alveolar septa were congested, but

without hyperplasia or fibroplasia (Haliburton *et al.*, 1986). The absence of epithelial hyperplasia or fibroplasia suggested that these conditions were perhaps due to an unusual toxin (Colvin *et al.*, 1992). Lesions induced by FB1 are remarkably distinctive and generally are confused with other conditions that induce pulmonary and/or thoraxic effusion Colvin *et al.*, 1992). Lower doses of FB1 caused hepatic disease in swine in longer periods of time. While at higher doses, acute pulmonary edema was superimposed on hepatic injury and caused death (Ledoux *et al.*, 1992).

Chemical and mycological investigations have revealed the presence of FB1 at concentrations of 20 to 360 parts per million (ppm) in suspected swine feeds (Marasas *et al.*, 1992). PPE has been induced by the injection of 0.4 mg of FB1 of percentage body weight daily for four days. Animals fed with corn screening naturally contaminated with 175 ppm fumonisins developed PPE with single dosis, but presented hepatotoxicity when fed doses higher than 23 ppm for 14 days (Ledoux *et al.*, 1992; Colvin *et al.*, 1992; Ross *et al.*, 1991). Purified FB1 administrated intravenously has been shown to produce PPE (Ledoux *et al.*, 1992; Colvin *et al.*, 1992; Ross *et al.*, 1991).

1.1.3.2.2. Equine Leucoencephalomalacia.

When horses are fed with infected grain, two manifestations of toxicosis can occur (Haschek *et al.*, 1986). One manifestation is called equine leucoencephalomalacia (ELEM). It is characterized by the destruction of the white and gray matter in the brain. The second manifestation is hepatotoxicity.

It causes icterus, hemorrhages, and edema. ELEM is a syndrome clinically characterized by an acute neurological disorder proceeded by lethargy and inappetence with presence of liquefactive necrolitic lesions in the white matter of the cerebrum. Necrosis of the gray matter may also be involved (Harrison *et al.*, 1990; Shier *et al.*, 1991). It appears that ELEM occurs only in equine. ELEM has been recognized since the 19th century as sporadic, seasonal, "epidemic like" condition (Ross *et al.*, 1992). Since its first report in the United States in 1891, ELEM has been recognized in South America, China, Greece, Egypt, South Africa, and Germany (Ross *et al.*, 1992). Historically, the diagnosis of ELEM has been based on tissue lesions in conjunction with clinical signs and the isolation of *F. moniliforme* from suspected feeds (Marasas *et al.*, 1992). Since the recognition of *F. moniliforme* as the causative fungus, ELEM has been reproduced several times under diverse experimental conditions. Marasas *et al.* (1992) produced ELEM in a horse by intravenous administration (7 daily doses of 0.125 mg FB1/g liver mass spread over 10 days) while trying to avoid hepatotoxicity as much as possible. Wilson *et al.* (1991) reported that for levels above 10-20 ppm range, there should be concern for toxicity in horses. However, ponies fed 8 ppm FB1 (Osweiler *et al.*, 1992) did show minor non-specific lesions in liver, kidney, and brain stem. Current data suggest that horses which consume feed containing levels as low as 8 ppm FB1 may be at risk for developing ELEM (Wilson *et al.*, 1991). Researchers have found that in equine, high dosage levels cause fatal hepatotoxicity and mild brain lesions

while low dosage levels produce mild hepatotoxicity and severe brain lesions (Shier *et al.*, 1991).

1.1.3.2.3. Esophageal Cancer.

In humans, development of esophageal cancer has been correlated with these toxins (Thiel *et al.*, 1992). *F. moniliforme* infection of corn has been correlated with human esophageal cancer (EC) risk in Transkei, South Africa, where corn is a dietary staple (Haschek *et al.*, 1992). EC has occurred in higher than normal concentrations in portions of China, Iran (Kmet *et al.*, 1972), and the Charleston, South Carolina, area of the United States (Fraumeni *et al.*, 1977). Transkei has a rate of 50-100 EC cases per 100,000 population while normal occurrence for EC is normally less than 5 cases per 100,000 population (Shier *et al.*, 1992). Contamination of corn with *F. moniliforme* and fumonisins in the areas of high EC incidence is statistically higher than that found in areas of the Trasnkei with lower cancer incidence (Hascheck *et al.*, 1992; Sydenham *et al.*, 1990). A daily consumption of 460 g corn/day by a 70 Kg person in Transkei, results in the consumption of ca 0.44 mg/g day FB1 (Hascheck *et al.*, 1992).

1.1.3.2.4. Other Diseases.

Non-primates develop thrombotic, hepatotoxic, carcinogenic, and cerebral problems (Ficham *et al.*, 1992). Poultry is affected by this toxin group with multifocal hepatic necrosis, biliary hyperplasia, muscle necrosis, intestinal globet-cell hyperplasia, and rickets (Brown *et al.*, 1992).

Riley *et al.* (1994) in the study "Mechanism of fumonisin toxicity and carcinogenesis", addresses the gathering of data for a possible mechanism for understanding the development of illnesses such as equine leucoencephalomalacia and porcine pulmonary edema produced by acute fumonisin toxicity. It was found that the inhibition of the enzyme known to work with sphingolipid biosynthesis is affected by these toxins.

Studies have shown that hydrolyzed fumonisin presents higher toxicity than fumonisin B1 (Hendrich *et al.*, 1993; Hopmans *et al.*, 1993). Hendrich *et al.* (1993) also mentioned the importance of further studies about the production of hydrolyzed fumonisin during food processing. Riley *et al.* (1994) presented data showing the severe consequences *in vitro* due to fumonisins contamination. These include the following:

1. Inhibition of *the novo* sphingosine biosynthesis.
2. Accumulation of the free sphinganine.
3. Depletion of complex sphingolipids.
4. Increase in degradation products from catabolism of free sphingoid bases.
5. Increase in lipid products derived from the increase in sphingoid base degradation products.
6. Increase of free sphingosine.

The data suggests that the enzyme ceramide synthase is being affected during fumonisin intoxication (Riley *et al.*, 1994). *In vitro* studies have shown that the accumulation of sphingosine is due to the blocking action of fumonisins on ceramide synthase which also degrades complex sphingolipids (Riley *et al.*, 1994). Figure 1.4 shows the disruption of normal sphingolipid metabolism as it occurs in mammalian cells exposed to fumonisins or the AAL- toxin (compound produced by *Alternaria alternata* in tomato plants).

Data obtained from *in vivo* studies suggests the disruption of sphingolipid biosynthesis. Riley *et al.* (1994) presented the following conclusions based on studies of swine exposure to FB_1:

1. FB_1 causes disruption of sphingolipid biosynthesis in liver, lung and kidney at low concentrations.
2. FB_1 causes elevation of free sphinganine in plasma before indication of tissue damage.
3. disruption of sphingolipid biosynthesis in liver, lung and kidney occurs to a much greater extent than in other tissues (brain, pancreas, lymph nodes, and skeletal muscle) when pure FB_1 or naturally contaminated corn diets were used.

1.1.3.3. Fumonisins and Sphingolipids

The structure of fumonisins are similar to the long chain base backbone of sphingolipid (an acylglycerol). The inhibition of the conversion of sphinganine to N-acyl-sphinganines may explain a possible toxicologic

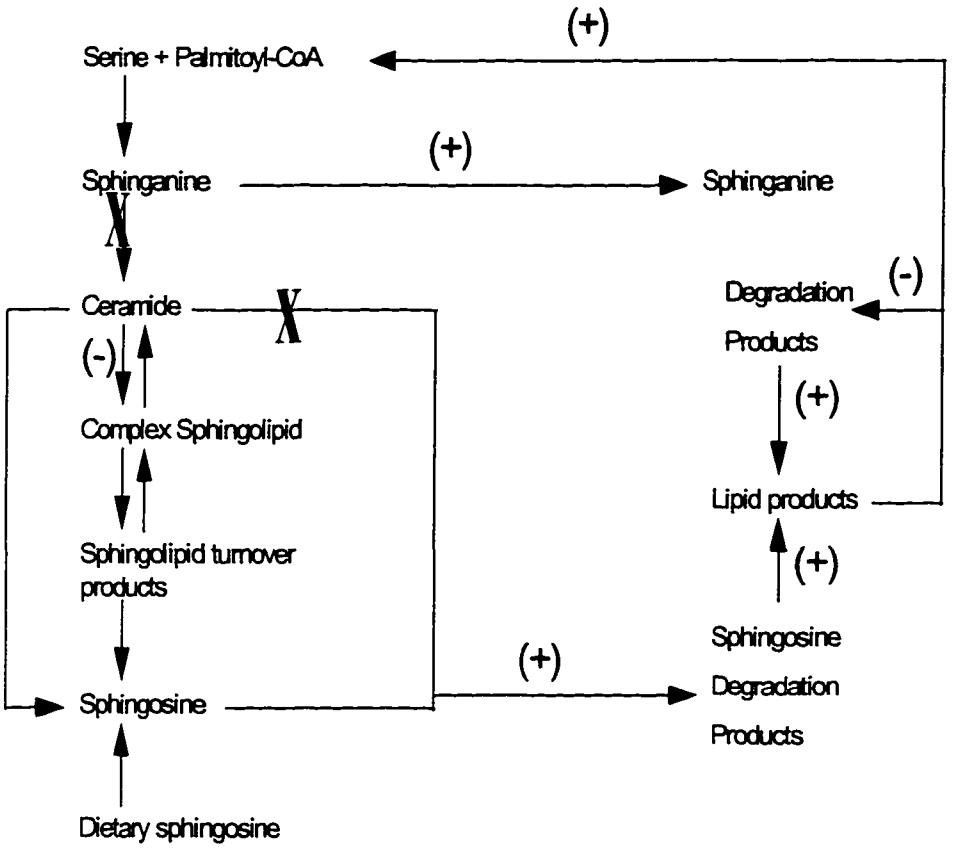

Figure 1.4. Disruption of normal sphingolipid metabolism in mammalian cells exposed to fumonisins or AAL-toxin. From Riley *et al.*, 1994.

mechanism (Wang *et al.*, 1991). Acylglycerols constitute the majority of lipids in the body and have a role in lipid transport and storage and in various diseases such as obesity, diabetes, and hyperlipoproteinemia (Mayes, 1993). Triacylglycerols are the major lipids in fat deposits and food. In addition, acylglycerols, particularly phospholipids, are major components of the plasma and other membranes. Phospholipids also take part in the metabolism of many lipids. Glycosphingolipids, which contain sphingosine and sugar residues as well as fatty acids, account for 5-10 percent of the lipids of the plasma membrane (Mayes, 1993). Phosphoglycerols, phosphosphingolipids, and glycosphingolipids are all amphipatic lipids. Consequently, they are ideally suited as the main lipid constituents on the plasma membrane. Some phospholipids have specialized functions; *e.g.* dipalmitoyl lecithin is a major component of lung surfactant, the lack of which in premature infants is responsible for respiratory distress syndrome of the newborn. Inositol phospholipids in the cell membrane act as precursors of hormone second messengers, and platelet-activating factor is an alkyl-phospholipid. Glycosphingolipids, found in the outer leaflet of the plasma membrane with their oligosaccharide chains facing outward, form part the glycocalyx of the cell surface and are considered to be important (1) in intracellular communication and contact; (2) as receptors for bacterial toxins (*e.g.*, the toxin that causes cholera); and (3) as ABO blood group substances. A dozen or so glycolipid storage diseases have been described (*e.g.*, Gaucher's disease, Tay-Sachs

disease), each due to a specific deficiency in a hydrolase enzyme in the pathway of glycolipid breakdown in lysosomes (Mayes, 1993).

Certain diseases are characterized by abnormal quantities of these lipids in the tissues, often in the nervous system (Mayes, 1993).

High amounts of free sphingolipid-based compounds can affect cell development; be toxic to cells; and be associated with genetic defects in sphingolipid biosynthesis (Mayes, 1993). In Swiss 3T3 cells, the addition of sphingosine or sphinganine to the cell culture medium stimulates DNA synthesis (Riley *et al.*, 1994).

1.1.4. Biosynthesis

Aflatoxins are secondary metabolites with no known physiological role in primary growth and metabolism of *Aspergillus* spp. yet. However, aflatoxins represent a possible defense mechanism for the fungi. Studies of the biosynthesis of aflatoxin B_1 have shown that the basic skeleton of the toxin molecule is derived entirely from acetate units via the polyketide pathway. Bhatnagar *et al.* (1994) present a complete study in the biosynthesis of aflatoxins. Figure 1.5 presents the generally accepted scheme of known precursors in the biosynthesis of aflatoxin B_1. Aflatoxin biosynthesis is not linked unequivocally to fungal infection of seed (Bhatnagar, 1994). Several observations indicate that the aflatoxin biosynthetic pathway is very sensitive to certain genetic, biochemical, or environmental influences (Bhatnagar, 1994). Pitt (1993) presented a descriptive model of mold growth and aflatoxin

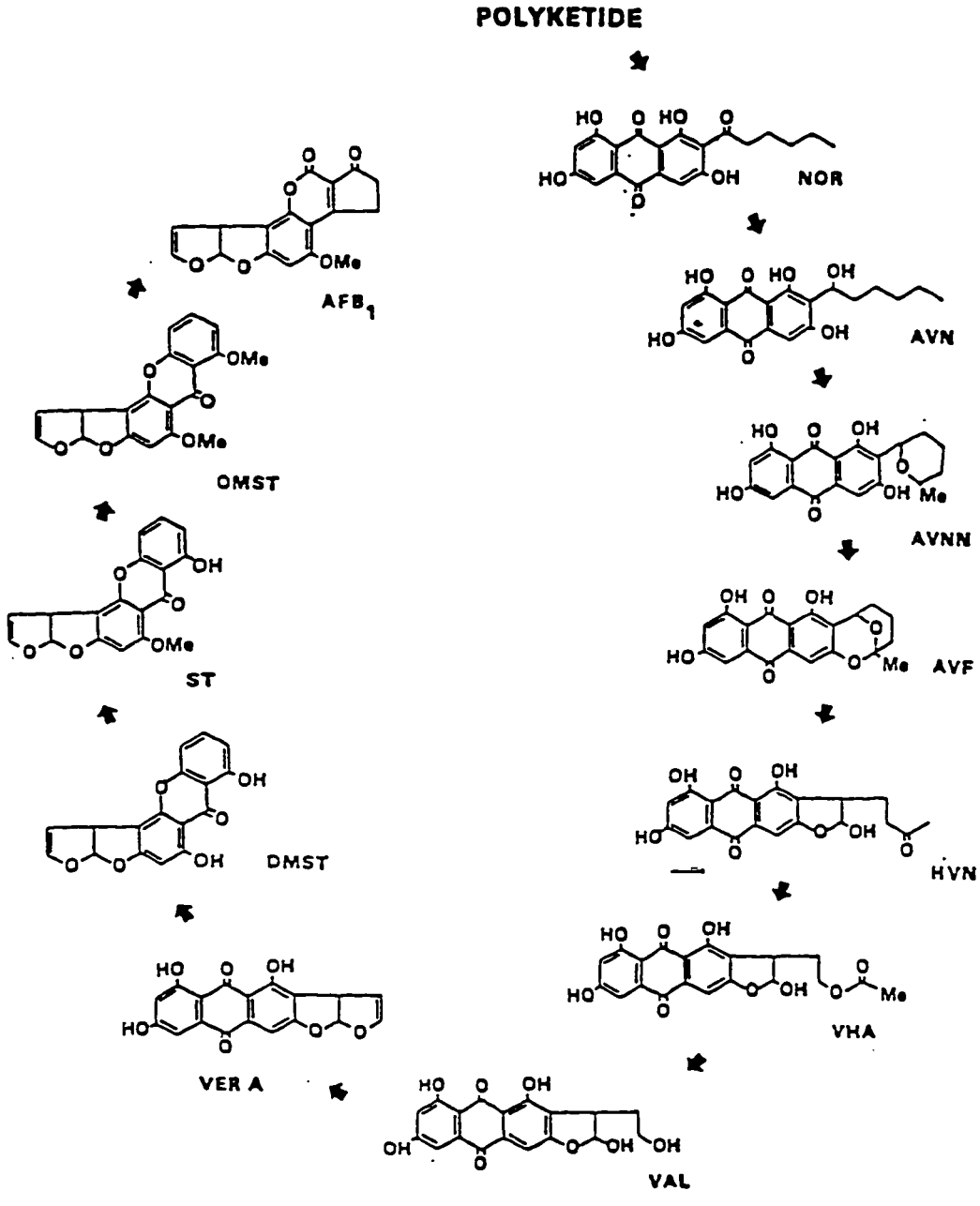

Figure 1.5. Biosynthesis of aflatoxin B_1 as described by Bhatnagar *et al.*, 1994.

formation as affected by environmental conditions. The rate of toxin formation is assumed to be proportional to the rate of production of new cell mass. Thus, the rate of toxin degradation is assumed to be proportional to the product of the concentrations of dead cell mass and aflatoxin. Temperature and water activity have an interactive effect on growth and toxigenesis in the model. The model provides a theoretical explanation for observed temporal shifts in the optimum temperature for toxigenesis, and for a hyperbolic relationship between heat units and time to toxicogenesis with and without temperature cycling.

Branham and Plattner (1993) studied the biosynthesis of fumonisin B_1 by *Fusarium moniliforme* in liquid culture. The study results suggest that alanine is directly incorporated into FB_1.

1.1.5. Risk Management

The primary agricultural commodities affected with aflatoxin contamination are corn, peanuts, cottonseed and tree nuts (i.e., pecans, walnuts, etc.). Human exposure to aflatoxins can be from direct consumption of contaminated commodities, or consumption of foods from animals previously exposed to aflatoxin through feeds (milk and egg products). In an effort to limit man's exposure to these toxins, prevention and control programs have been studied and established. These include mycotoxin monitoring programs, the establishment of regulatory guidelines and decontamination procedures (Park, 1993b).

The necessity to establish a regulatory program to manage the risk of exposure to mycotoxins is well known. The Aflatoxins Regulatory Program is a very good example to follow (Stoloff *et al.*, 1990). A program for fumonisins should include the establishment of regulatory limits according to end use. The LD_{50} for fumonisins has not yet been determined but some studies for acute toxicity give the following data:

Horses	8 ppm
Poultry	75 ppm
Swine	23 ppm

These concentrations are the minimum doses of FB_1 to produce or develop symptoms (Riley *et al.*, 1993).

The principal crop affected by fumonisin contamination is corn. Hopmans *et al.* (1993) conducted a study of food products (yellow and white cornmeal, canned corn, tortilla chips, masa, and dog and cat foods) and the levels of fumonisins in these products were determined. All of the products under study tested positive for at least one of the fumonisins.

Fumonisins and *Fusarium moniliforme* are a unique problem because of the following characteristics:

- *F. moniliforme* mating type A can exist as an endophyte. No symptoms are present in corn infected by this fungus and fumonisin production might be possible. This characteristic depends upon several factors such as the genetic nature of the fungus and environment (Riley *et al.*, 1993).

- *F. moniliforme* is seed and soil-borne and not an obligate parasite. It does not produce a sclerotium which survives in the soil, but it survives in corn fragments as thickened hyphae (Riley et al., 1993).
- A high variability in disease expression exists. This is caused by heterozygosity within a corn cultivar and genetic variation within the fungus (Riley *et al.*, 1993).

1.1.6. Methods of Analysis

Table 1.6 includes all the methods of analysis for aflatoxins approved by Association Of Analitical Chemists-International Union of Pure Analytical Chemists (AOAC-IUPAC). This is an important tool for accepted methods of analysis.

Several methods including HPLC, GC-MS, TLC and ELISA have been developed to detect fumonisins (Pestka *et al.*,1992, Shephard *et al.*,1990). All of them are useful and their results are acceptable. However, they continue under study.

1.1.7. Industrial Processes and Mycotoxins

Detoxification methods have not been as successful for fumonisins as hoped. Ammoniation (Park *et al.*,1992) and fermentation (Bothast *et al.*,1992) are not as effective for fumonisins as they are for aflatoxins. Detoxification procedures designed to reduce fumonisin B_1 (FB_1) levels are under evaluation to determine chemical modification and reduction in toxic/carcinogenic potentials. There are also methods to avoid post-harvest contamination by

Table 1.6. Approved AOAC-IUPAC methods of analysis for aflatoxins.

PRODUCT	METHOD	AOAC No.
Cottonseed	Thin layer and liquid chromatographic method	980.20 15th Ed. 1990
Peanuts and Peanut products	BF Method	970.45 15th Ed. 1990
Peanuts and Peanut products	CB Method	968.22 15th Ed. 1990
B_1 in Corn and Roasted Peanuts	Enzyme-linked immunosorbent (Agri-Screen) screening assay	990.32 1st supplement, 1990 15th Ed. 1990
Corn and Peanut butter	Liquid chromatographic method	990.33 1st supplement, 1990 15th Ed. 1990
B_1, B_2, and G_1 in Corn, cottonseed, peanuts, and peanut butter	Enzyme-linked immunosorbent (immunoDot Screen cup) Screening Assay	990.34 1st supplement, 1990 15th Ed. 1990
Corn, raw peanuts and peanut butter	Immunoaffinity column (Aflatest) Method	991.31 2nd supplement 1991 15th Ed. 1990
Peanut butter	Enzyme-linked immunosorbent Assay Method (Biokits)	991.45 3rd Supplement 1992 15th Ed, 1990
Corn	Enzyme-linked immunosorbent assay method (Afla-20 cup test).	993.16 5th supplement 1994 15th Ed. 1990
Corn and Peanuts	Thin layer chromatographic method	993.17 5th supplement 1994 15th Ed. 1990

fumonisin during storage. Modifying the atmosphere by reduction of oxygen content as well as reducing air moisture to less than 22 percent decreases the production of this toxin (Riley *et al.*, 1993). The fact that fumonisins are aflatoxin co-contaminants is a further consideration (Park *et al.*, 1995). Therefore, it would be of interest to develop a process effective for the detoxification of both mycotoxins.

1.1.7.1. Fermentation

The stability of aflatoxin B1 during industrial processes has been studied. Chu *et al.* (1975) examined the stability of aflatoxin B1 and ochratoxin A in brewing. The researchers utilized spiked samples during mashing in a conventional micro-brewing process. The results indicated that both toxins are heat stable and are insensitive to cooker mash treatment. However, it is necessary to specify that spiked samples do not perform as naturally contaminated ones. The matrix plays an important role in the development of the treatment. Nofsinger and Bothast (1981) studied ethanol production by *Zymomonas mobilis* and *Saccharomyces uvarum* on alfatoxin-contaminated and ammonia-detoxified corn. *Z. mobilis* demonstrated greater fermentative activity than *S. uvarum* during the first day in the fermentation of two lots of alfatoxin-contaminated corn and two lots of ammonia-detoxified corn. Values in the post-fermentation solids obtained from the ammonia-detoxified corn were low in comparison with the non-treated substrate. Along the same line of

research, Lagoda and Maisch (1979) utilized corn naturally contaminated with aflatoxin in the ethanol fermentation process. Distribution of toxin in several process and recovery fractions was identified. Although little degradation of the mycotoxin occurred during fermentation, no toxin appeared in the distilled alcohol. As accumulation of toxin in spent grains represents a potential problem in use of the material as animal feed, several decontamination procedures were tested. Sodium hydroxide, ammonium hydroxide, sodium hypochlorite, and hydrogen peroxide were identified as efficient agents for toxin degradation. In another study, Bothast *et al.* (1982) described a process for converting aflatoxin-contaminated corn to ethanol via combining ammonia inactivation with the liquefaction step of the ethanol fermentation process. Better ethanol yields were obtained when ammonia was added during liquefaction than when no ammonia was added. Aflatoxin B_1 levels were reduced 80 to 85 percent in the solid fraction by this process.

1.1.7.2. Nixtamalization

Nixtamalization (Nixtamalización in Spanish) is an ancient and traditional process used in Mexico to make corn cakes (tortillas), which constitute the basic Mexican food. Corn used to make tortillas is prepared using a lime/heat treatment. The physical and chemical effect of the lime over corn has been reported by different authors (Bresani, 1958; Paredes, 1982; Guzman-de-Pena *et al.*, 1995). Physically, it causes corrugations and partial dissolution of the

outermost layer while the aleurone layers keep enclosing the endosperm (Paredes *et al.*, 1982). Chemically, there is leaching of proteins. Most of the protein leached seems to consist of albumins and globulins of low molecular weight. This process also increases the rate of release of the most essential amino acids (Bresani, 1958). The loss of leucine during the process improves the biological value of tortilla protein by partially correcting the isoleucine to leucine disproportion (Bresani, 1958). Furthermore, cook books refer to this method as "... a fundamental process for improving the nutritional value of this cereal. The purpose of this process is to remove the cuticle of the grain, which not only is difficult to digest, but interferes with the assimilation of other foodstuffs taken at the same time." (van Rhijin, 1993).

Nixtamalization as it is traditionally performed in Mexico, uses the following steps:

1. Corn is washed 2 or 3 times to discard floating debris;
2. Corn is covered with water;
3. One percent (w/w) of lime is added;
4. Mixture is boiled at approximately 94°C until water gets turbid at approximately 50 min;
5. Mixture is left to soak over night (approximately 17 hr);
6. Corn is washed 2 or 3 times and mill-ground to obtain the dough.

As it can be observed, the process eliminates the possibility of highly contaminated kernels through physical selection . The broken kernels are eliminated due to difference in density, and thus contaminated kernels are eliminated. Broken kernels have been found to be highly contaminated when a crop tests positive for aflatoxin contamination. Removal of broken kernels is a possible method suggested for physical decontamination procedures (Park, 1992). Several reports about the effect of lime ($Ca(OH)_2$) over aflatoxin in corn have been published, and widely varying estimates of the effectiveness of nixtamalization in removing aflatoxin B_1 contamination from corn have been published. Ulloa-Sosa and Shroeder (1969), on one hand, reported that about 70 percent of the aflatoxin is removed during the process. On the other hand, Arriola *et al.* (1986), indicated that nixtamalization does not reduce aflatoxin levels substancially. Other authors, Rosiles (1979), Machorro and Valdivia (1987), and Price and Jorgensen (1985) report results somewhere in between. However, according to Guzman-de-Pena *et al.* (1995), these studies utilized artificially-contaminated corn, the normal nixtamalization process was not followed, and they failed to determine the fate of the toxin during the process. Guzman-de-Pena *et al.* (1995) presented a study using naturally contaminated corn, following the traditional process of nixtamalization employed in Mexico. The fate of aflatoxin during the process was followed employing radiolabelled aflatoxin B_1.

Guzman-de-Pena *et al.* (1995) demonstrated that only a small amount of aflatoxin (less that 17 percent) survive the nixtamalization process (Table 1.7). Most of the toxin was destroyed during the alkaline cooking, and is transformed into products which do not display the characteristic fluorescent properties of the original compound. Other studies mention the possible acidification of the molecule by the gastric acids. That will reconstitute the molecule of aflatoxin and its toxicological properties. Nixtamalization and modified nixtamalization (addition of FDA approved additives such as sodium bicarbonate and hydrogen peroxide) have been studied to determine toxicity modification of fumonisin contaminated corn (Park *et al.*, 1995). The traditional process provides a physical decontamination through the removing of broken contaminated kernels. However, fumonisin-producing fungi (*Fusarium* sp.) have the quality of being asymptomatic. Thus, it is possible to have physically acceptable kernels which may be contaminated by fumonisin. To assure that there is no contamination within remaining kernels is more difficult. The effectiveness of the treatment to chemically modify the fumonisin molecule and to reduce the toxic potential obtained varied according to the exposure and combination of chemicals. Reduction of the levels of fumonisin B_1 in the samples was determined by reversed phase high performance liquid chromatography (a separation technique based upon chemical characteristics of the compounds). The highest degree of modification (100 percent) was observed with the $Ca(OH)_2$.

Table 1.7. Effect of the Process of Nixtamalization over naturally aflatoxin-contaminated corn. From Guzman-de-Pena *et al.*, 1995.

Corn Sample	Level of aflatoxin B_1 Before "Nixtamalización"	After	Aflatoxin destruction by nixtamalization (%)
Less contaminated	37 ng/g	0 ng/g	100
Highly contaminated	251 ng/g	6±1.0 ng/g	97

The treatment of calcium hydroxide (23°C, 12 hours) combined with hydrogen peroxide and sodium bicarbonate (23°C, 1 hour) was the most successful. Toxic and mutagenic modifications of the samples were monitored using the exposure of brine shrimp to the toxin and contaminated corn (*Artemia* spp. bioassay) and the *Salmonella*/microsomal mutagenicity assay (Ames Test). The results showed 90 percent reduction in toxicity and no evidence of mutagenicity formation. The mutagenicity assay is a measurement of the carcinogenic or cytotoxic potential of fumonisin and treatment- related by-products. Decontamination procedures utilizing established techniques and/or modifications offer practical methods for assuring economic and health confidence. The use of decontaminated crops (*e.g.* corn) which result in reduced health risks, needs to be available in the near future.

1.1.8. Detoxification Methods

Since their discovery, aflatoxins have been subject of numerous research studies. Because of the serious economic problems they cause, their elimination by destruction or inactivation has been a principal area of research.

Detoxification processing methods based on physical, biological and/or chemical elimination have been developed. Ammoniation has been the most successful procedure (Park, 1992). This procedure is extensively used as a practical means for decontaminating oilseed meals and corn for use in animal feeds.

Following the Food and Agriculture Organization (FAO) set of criteria for determining the acceptability of a decontamination process (Park *et al.*, 1988; Park, 1993b), the elimination of aflatoxin in human food products is not possible except by direct destruction. The process to decontaminate commodities must:

1. destroy, inactivate, or remove the mycotoxin;
2. not produce or leave toxic or carcinogenic/mutagenic residues in the final products or in food products obtained from animals fed decontaminated feed;
3. retain the nutritive value and acceptability of the product;
4. not alter important technological properties; and ideally,
5. destroy fungal spores and mycelia which could, under favorable conditions, proliferate and form new toxins.

The public health is at risk when any product is contaminated by these compounds. Although the FDA, research, and industry are working together to aliviate this problem, the actual human ingestion of aflatoxin cannot be estimated. The adverse effects of aflatoxin call for constant monitoring of aflatoxin levels in human foods and animal feed stuffs, especially, in tropical environments, where conditions are optimal for the growth and elaboration of mycotoxins by many fungal species (Atawodi *et al.*, 1994). Following this line, Atawodi *et al.* (1994) designed a study to establish the status of the aflatoxin problem in Nigeria, thus, providing an empirical basis for the formulation of appropriate regulations and/or the modification of those already existing, for the effective control of aflatoxin contamination. Groundnut and groundnut-containing materials were the most heavily contaminated with the

highest value (1862 ppb) being found in a groundnut cake sample. Aflatoxin was also detected occasionally, but to a lesser extent, in some grains and cereals that are of nutritional importance in human foods and the livestock industry in Nigeria (Atawodi *et al.*, 1994).

Aflatoxins cannot be completely prevented or eliminated from food or feeds by current good manufacturing practices. Therefore, from a regulatory point of view, they are considered to be added poisonous and unavoidable contaminants (Wood, 1989). Experiments have demonstrated that AFB_1 above 20 ppb in feed of livestock other than dairy animals does not result in significant aflatoxin residues in human food nor effect animal health. Therefore, FDA establishes the regulatory limit for aflatoxin in cottonseed meal to 300 ppb (Park *et al.*, 1988). Furthermore, states are being urged to monitor corn and corn products intended for human consumption to comply with a maximum of 20 ppb. The compliance limit for aflatoxin in milk was set to 0.5 ppb due to the higher susceptibility of infants to aflatoxins and other toxins (FDA, 1983). Levels of aflatoxin contamination vary from year to year, depending on the commodity, weather conditions, and other factors (Wood, 1989). Current technology still cannot prevent aflatoxin contamination of field crops before harvest. Research is aimed at controlling preharvest contamination of corn and peanuts through genetic manipulation and the use of chemicals (Wood, 1989). It should not be expected that all foods are free from aflatoxins; however, as an

alternative, FDA's surveillance and regulatory programs help to keep aflatoxin food contamination at the lowest practical levels consistent with preserving an adequate food supply at a reasonable cost.

Decontamination procedures have focused on physical, chemical, or biological removal, or physical or chemical inactivation of mycotoxins (Park, 1993a). With respect to physical methods of separation, electronic sorting of contaminated peanuts has been used extensively by the peanut industry to reduce aflatoxin levels in peanut products destined for human consumption (Dickens and Whitaker, 1975). However, there is usually a low level of contamination remaining in the final product. Since contaminated kernels usually have a different density than non-contaminated products, density separation has been used successfully (Huff, 1980; Cole *et al.*, 1989). Various food processing procedures such as dry and wet milling of corn can result in lower aflatoxin levels in the final product (Yahl *et al.*, 1971; Brekke *et al.*, 1975).

Aflatoxins are heat-stable, therefore, thermal inactivation procedures only result in modest reduction in aflatoxin levels following boiling water and autoclaving (Christensen *et al.*, 1977).

Microbial inactivation and fermentation have been studied for degradation and removal of aflatoxin (Ciegler *et al.*, 1966; Ciegler 1978; Marth and Doyle, 1979; Hao *et al.*, 1987).

The use of dietary supplements and chemisorbents has been evaluated as potential chemical methods of decontamination (Monroe and Eaton, 1987; Newberne, 1987; Phillips *et al.*, 1987, 1988a,b 1989). Although these chemicals show promise, data supporting efficacy and safety of their use are lacking. Other than ammoniation, most of the techniques studied are impractical, ineffective, or unproven with respect to safety for long-term use (Park, 1993b).

1.1.8.1. Ammoniation.

The ammoniation process using either ammonium hydroxide or gaseous ammonia has been shown to reduce aflatoxin levels in corn, peanut meal-cakes, whole cottonseed, and cottonseed products by greater than 99 percent (Park, 1993b). If the reaction is allowed to proceed sufficiently, the process is irreversible. Primarily, two procedures are used; a high pressure and temperature process (HP/HT) used at feed mills or an atmospheric pressure and ambient temperature procedure (AP/AT) that can be used on the farm (Table 1.8). Tentative definitions for the HP/HT procedure for whole cottonseed and cotton seed meal and the AP/AT process for whole cottonseed have been approved by the Association of American Feed Control Officials (Park, 1993b).

Studies on the chemistry of the aflatoxin B_1 ammonia reaction have identified the formation of several decomposition compounds (Figure 1.6).

Table 1.8. Parameters and application of ammonia/aflatoxin decontamination procedures (From Park, 1993b).

	Process	
	High pressure/high temperature	Ambient pressure/ atmospheric temperature
Ammonia level (%)	0.2-2	1-5
Pressure (psi)	35-50	Atmospheric
Temperature (°C)	80-120	Ambient
Duration	20-60 min	14-21days
Moisture (%)	12-16	12-16
Commodities	Whole cottonseed, cottonseed meal, corn, peanut meal	Whole cottonseed,corn
Application	Feed mill	Farm

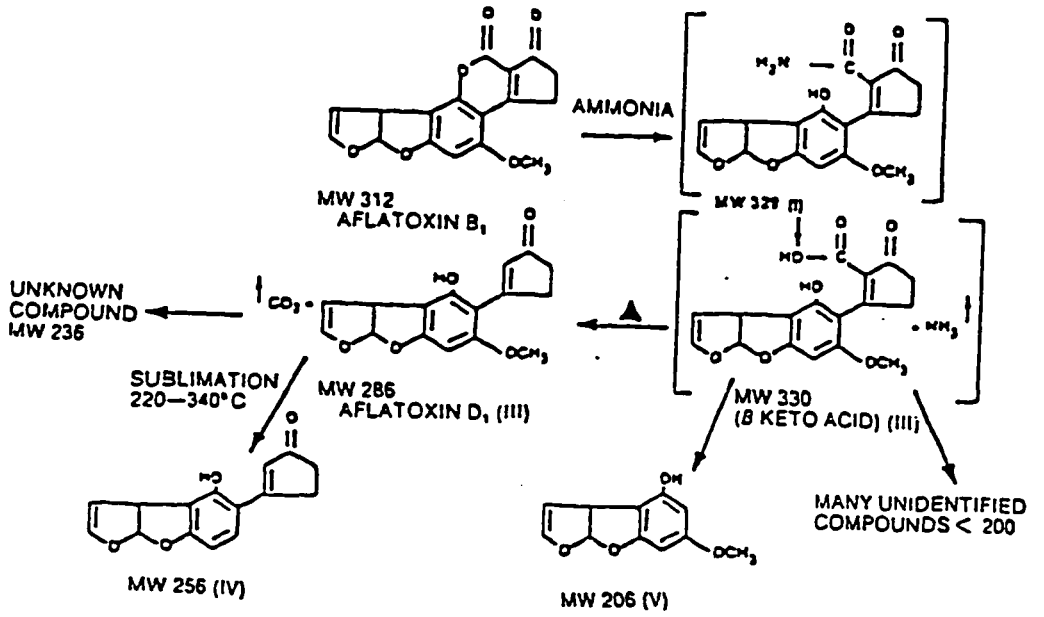

Figure 1.6. Proposed formation of aflatoxin-related reaction products following exposure to ammonia. The major products: the MW 286 compound and the MW 206 compound have been isolated and biologically tested. From Park, 1993a.

Hydrolysis of the AFB_1 lactone, the first step in the reaction (compound I), is reversible if the ammonia process is carried out under mild conditions. When the reaction is allowed to proceed further, the compounds formed do not revert back to AFB_1. Reaction products are not formed in the same relative amounts and are dependent on the temperature and pressure conditions used and whether ammonium hydroxide or ammonia gas is the ammonia source (Park, 1993b). The ammoniation process is usually advantageous, resulting in increased levels of total and non-protein nitrogen, protein, ash, and soluble solids, and reduced level of sulphur-containing aminoacids, available lysine, and non-reducing sugars (Park, 1993b). Chronic, sub-chronic, relay, and multigeneration feeding studies showed toxic effects or lesions related to the ammoniation procedure (Park, 1993b). Fremy *et al.* (1988) studied the effect of the ammonia treatment on the feed/tissue carryover for milk. AFB_1 residues in the treated diet were below 10 ug/Kg and no AFM_1 residues were detected in the milk collected throughout the experiment.

Selected isolated decontamination reaction products demonstrated some degree of toxicity or mutagenicity potential (chicken embryo, or Salmonella/microsomal mutagenicity assays). However, a large portion of the reaction products is bound to feed components such as protein and is potentially not biologically available to animals. Researchers at the University of Arizona compared mutagenic and tumorgenic potentials in milk from cows

fed rations containing aflatoxin-contaminated and ammonia-treated aflatoxin-contaminated cottonseed (Price, 1989; Jorgensen *et al.*, 1990). A low level of mutagenic potential was evident in whole milk from the ammonia-treated group using the AMES-TA100 tester strain. Lawlor *et al.* (1985) performed a study in the risks associated with mutagenic potential of ammonia-related aflatoxin reaction products in cottonseed meal. In the study, ammonia treatment of aflatoxin-contaminated cottonseed meal significantly decreased aflatoxin levels, and the aflatoxin decontamination products formed by the treatment had little or not mutagenic potential.

Weng *et al.* (1994) performed a study on the efficacy and permanency of ammonia treatment in reducing aflatoxin levels in corn. In the study, treatment with ammonium hydroxide alone at elevated temperatures resulted in a reduction of the AFB_1 content by greater than 99 percent. The results showed no significant reversion of aflatoxin. These findings suggest that at high temperature aqueous ammonium hydroxide or gaseous ammonia can be used effectively to reduce AFB_1 in corn (the temperatures were up to 121 °C). The study revealed that the moisture level of the product and holding temperature were the crucial factors that influenced the efficacy of aflatoxin decontamination by ammoniation (moisture content of the corn was adjusted from 8 to 16 percent).

The ammoniation procedure has been used successfully for many years in the U.S., France, and Africa (Park, 1996. Personal comunication). This practical application as well as research results strongly support the use of the ammonia treatment to reduce risks associated with aflatoxin contamination.

A detoxification study performed by Mercado *et al.* (1991) utilizing copra as the matrix with ammonium hydroxide showed promising results. Copra is the raw material for the production of coconut oil and copra meal. The oil is used as cooking oil as well as raw material in the manufacture of food products. Aflatoxin-containing copra at moisture contents of 24% and 7% was effectively detoxified by ammonium hydroxide (> 97% and 89% reduction, respectively). Detoxification was accomplished in 5 days using 1.5% ammonium hydroxide (ammonia/copra); in 10 days using 1.0%and in 15 days using 0.5%. The initial aflatoxin B_1 concentration of 500 ppb (9% moisture) was reduced to $\leq$ 20 ppb. Reversibility test (acidification of the treated samples) observed no change in aflatoxin content of copra before and after acidification.

1.1.8.2. Food Additives.

Tabata *et al.* (1994) presented a study where aflatoxin was degraded by using food additives. Pure aflatoxins were degraded by treatment with solutions of various acidic, alkaline, and neutral food additives. The aflatoxins were treated with food additives under several conditions, and the effects of treatment temperature, time, and concentration of food additives on aflatoxin

degradation were studied. Potassium bromate, potassium nitrate, and sodium nitrite had no effect on aflatoxins. All the aflatoxins were degraded by sodium hypochlorite (0.25% pH 4, 48 h) and ammonium persulfate (0.25%, 49 h) at 60 °C. These findings suggested that aflatoxins can be degraded or removed by treatment with food additives during food processing.

1.1.8.3. Other Methods of Detoxification.

Decontamination of aflatoxin-contaminated peanut meal using monomethylamine:$Ca(OH)_2$ was studied (Park *et al.*, 1981). The decontamination process resulted in a 94-100% reduction in aflatoxin levels, depending on the level of contamination and the chemical structure of the aflatoxin. Comparative toxicity tests showed that, although some decontamination by-products exhibited elevated responses to specific toxicity tests, the relative toxicity was inferior to AFB_1. There was a significant change in the mold flora following the decontamination process, and the product was susceptible to recontamination.

Other methods of risk reduction include risk management as a solution. Avoiding the usage of foods that are highly contaminated with aflatoxins may reduce the danger posed to human health and animal production, reducing the economic losses as a result. In addition, it may be advisable to use alternative protein supplements such as soy beans, in regions where contaminated crops are the basic source of food. Furthermore, it is possible to reduce the overall

contamination level of human foods and animal feeds through adequate education of farmers and traders involved in the handling of agricultural commodities, with regard to rapid post-harvest-drying procedures; use of appropriate storage conditions; and feed management practices. These measurements have been shown to be effective in the control of aflatoxin contamination (Busby and Wogan, 1984). Other studies have used modified atmosphere during storage on non-contaminated grains for the reduction on mycotoxin production. 2-Chloroethylphosphonic acid (CEPA, ethrel, ethephon), an ethylene generating compound, was found to inhibit aflatoxin biosynthesis in the cultures (Sharma *et al.*, 1985). Sharma *et al.* (1987) assessed the efficacy of CEPA for aflatoxin-free storage of peanuts and corn. Treatment of the samples with an aqueous solution of 2-chloroethylphosphonic acid (40%) prevented aflatoxin formation in both commodities, whereas, the untreated lots supported aflatoxin formation. No toxicological data were presented in this study.

1.1.9. Further Applications in other Mycotoxins

Other mycotoxins, in addition to aflatoxins, may play a role in human and animal diseases. These include ergot toxins, T-2 toxin and related trichothecens, citreoviridin, ochratoxins, and the fumonisins. Using the knowledge gained with aflatoxin, regulatory, monitoring, and decontamination programs, similar food and feed safety programs can be established for these

toxicants which will reduce risks/hazards associated with the contaminant to practical levels and help preserve an adequate food supply (Park, 1993a).

Park and Stoloff (1989) provide an example of the varying forces that affect risk assessment and management by a regulatory Agency. The authors presented work which described how the FDA responded to the initial discovery of a potential carcinogenic hazard to humans in a domestic commodity, to the developing information concerning the nature of the hazard, to the economic and political pressures that are created by the impact of natural forces on regulatory controls, and to the restraints of laws within which the Agency must work. This has been the foundation for the development of other monitoring programs for risks associated with the great variety of mycotoxins present. Thus, directing researchers to develop quick steps to identifying possible contaminants and setting up corrective actions plan to provide a safe and adequate food supply.

2. INDUSTRIAL PROCESSES, TRADITIONAL METHODS

Traditional methods for food preparation are part of the roots of a culture. It has been said that you are what you eat. From another point of view, culture is how it is prepared what it is eaten. Ancient cultures have developed different processes such as smoking , alcohol production, yeast utilization (fermentation), pickling, and spicing. These processes traditionally have been used to extend the shelf life of foods and modify their natural nutritional value. Although we do not know nowadays if our ancestors knew the reason for food modification, it is obvious that some knowledge was achieved. However, the changes of life style do not seem to find a place for the traditional methods. Alternatives that fit the life style followed by modern life do not have to be far from traditional methods. Modifications of the traditional processes are frequently done for reduction of time. A time-consuming procedure is the reason why traditional processes have been left aside. The wisdom of our ancestors is not understood completely today. It is surprising how the traditional methods have their scientific explanation for improving the quality of a product. Nixtamalization is one of them. On the other hand, the production of alcoholic beverages has followed the development of entire civilizations. No or little modification has occurred in the preparation of alcoholic beverages. The variation observed is usually the type of grain utilized, the form of the grain (dried, wet, or germinated), or the presentation of the beverage at the end.

2.1. Fermentation, Preparation of Beverages.

Corn is fundamental in the preparation of traditional beverages throughout the world, as mentioned above. Mexico, as well as several Afican countries, has the preparation of several alcoholic beverages as traditional drinks that are based on corn. However, fermentation of contaminated corn for the production of beverages can have fatal consequences. The problem arises when contaminated grain is utilized for preparing these traditional alcoholic beverages. Latin America, Africa, and Asia utilize corn to produce these beverages. Here, the kernel is placed in a open surface on a fiber sack and water is added to wet the grain for germination purposes. The humidity is controlled covering the grains with another sack and adding water as needed. The conditions generated for germination also allow the molds present in the commodity to grow, and the production of toxins can increase 5 fold in the 10-day period required for germination (Njapau, 1996, Personal communication). Modifications in the process of fermentation to improve the quality of the product, once contaminated corn has been utilized would be the goal to achieve. That would not be a problem for industry. The focus is to obtain a simple and affordable modification that can be applied in home-scale production. Although it has been proven that some mycotoxins stay in the distilled drain solid grains (DDSG), when utilizing contaminated grain, the use for ethanol production has been suggested. In the United States, fermentation

of contaminated kernels results in a problem for production of feed. DDSG are used as base for cattle and other farm animals feed. Furthermore, alcohol production could be affected by the presence of mycotoxins. Yeast can be affected by cytotoxic compounds such as fumonisins, and alcohol yield can decrease dramatically. The elimination or reduction of these compounds is not only beneficial due to feed production, but for improving the economic losses produced by the presence of these compounds during ethanol production.

2.2. Nixtamalization

Although traditional nixtamalization may take place in many places of the central and south part of Mexico, the small factories at Mexico City are trying to reduce the time of process and sacrifice the goodness of the traditional process. The alternative could be the addition of an additive that may help in the reduction or elimination of risks associated with the contamination of commodities. Moreover, the utilization of broken kernels without problems of producing a contaminated product may be possible. It must be mentioned that industry does not follow the traditional method. The method followed in a medium-size tortilla plant has been closely observed. The process does not involve the washing step discarding the floating debris (usually highly contaminated by aflatoxins), and the washing step after nixtamalization is done only once. Furthermore, big plants only eliminate the water of the process without having the second washing step (the first washing step is not done).

Thus, there is a tendency to modify the traditional method in order to decrease time and cost.

At the same time, nixtamalization is the base of other dishes prepared in Mexico. Pozol (Nahuatl pozolli meaning frothy) is a beverage prepared in the southeastern states of Mexico. The corn previously washed is cooked with lime, as nixtamalization (following all the traditional steps), but instead of cooking for the production of tortillas, the cooked corn is ground and mixed with cocoa nuts. The dough is blended with water, and the result is a beverage of a light consistency. It can be noted that corn is basic in the nutrition of some cultures.

2.3. Food Additives

Continued consumer demand for modified or entirely new types of foods having improved flavor, color, convenience, stability, or nutritional qualities has resulted in the introduction and utilization of both natural and synthetic compounds, in the form of food additives, during the last 50 years (Maga, 1993). As a result, today there are 2922 total food additives of which 1755 are regulated food additives, including direct, secondary direct, color and Generally Recognized As Safe (GRAS) additives (USDA, 1993). They are approved for food use in the United States that can be legally incorporated into over 20,000 different food items.

It is generally accepted that a food additive can be defined as a single compound or mixture of substances, other than a basic foodstuff, which finds its way into a food during any aspect of its production, processing, storage, packaging, or preparation for consumption. By applying this definition to our food supply, it is quite obvious that food additives have been used for centuries to enhance and preserve the quality of numerous foods. Some individuals have preconceived notion that food additives are restricted to various forms of synthetic and perhaps toxic compounds that are indiscriminately added to every commercial food. However, it should be remembered that compounds such as smoke, alcohol, vinegar, and spices, have traditionally been used to extend the shelf life of foods and, as such, are also considered to be food additives. Most would agree that numerous potential benefits can be derived from the use of certain food additives. High on the list of benefits is increased food safety through the utilization of antimicrobial agents that minimize the risk of certain types of food poisoning. It can also be argued that the use of food additives has resulted in overall lower food prices since spoilage during processing, distribution and storage is reduced and lower-cost packaging can be utilized. That is the reason why the addition of approved food additives into traditional processes may provide a solution to reduce or eliminate risks associated with mycotoxin presence in commodities used for food or feed. The additives chosen for this study are ammonium hydroxide, ammonium chloride and ammonium persulfate. The reason is that ammonium compounds have been

proven to reduce toxicity of aflatoxin and fumonisin contaminated corn (Park, 1992). Hydrogen peroxide, on the other hand, is a potent oxidizer. Today, food grade hydrogen peroxide is GRAS with limitations as both a direct and indirect food ingredient. The modifications in the toxicity of aflatoxins and fumonisins that these food additives can produce are to be determined. The mentioned food additives currently are used in the United States. As an example, the treatments for bleaching starch are covered under section 21 of the Code of Federal Regulations -21CFR172.892(b). Normally they involve treating suspensions of ungelatinized starch granules with minimal amounts of oxidizing agents such as hydrogen peroxide or peracetic acid (active oxygen 0.45% max.); ammonium persulfate (0.075% max.) and 0.05% max. sulfur dioxide. According to the Food Chemicals Codex, the food additives utilized in this study have been approved for use in the United States (Table 2.1).

2.3.1. Ammonium Chloride

Ammonium chloride is used as yeast food and dough conditioner in the bakery industry. It is a colorless crystal, or a white, fine or coarse crystalline powder. It has a cool, saline taste and is somewhat hygroscopic. One g dissolves in 2.6 mL of water at 25 °C, in 1.4 mL of boiling water, in about 100 mL of alcohol, and in about 8 mL of glycerin. The pH of a 1 in 20 solution is between 4.5 and 6.0 and its molecular weight is 53.49. It is strongly

Table 2.1. Food additives utilized in this study, according to USDA, From: Everything added to food in the United States. U.S. Food and Drug Administration. C.K. Smoley, Boca Raton, Florida, 1993.

MAINTERM[a]	CAS[b]	CFR[c]
Ammonium Chloride	012125029	184.1138
Ammonium Hydroxide	001336216	184.1139
		163.110
Ammonium Persulfate	007727540	172.892
Hydrogen Peroxide	007722841	172.814
		175.105
		172.892
		160.145
		160.185
		133.113
		184.1366
		178.1010

[a]MAINTERM: Name of the chemical as recognized by the Center for Food Safety and Applied Nutrition (CFSAN) of the U.S. Food and Drug Administration.

[b]CAS: Chemical Abstract Service (CAS) registry code for the chemical or CAS-like code assignes by CFSAN to those substances that do not have a CAS number(8\9777nnnnnn-series).

[c]CFR: Regulation numbers in Title 21 of the U.S. Code of Federal Regulations where the chemical is listed.

endothermic. LD50 i.m. in rats: 30 mg/Kg. Therapeutic cat (vet): expectorant; diaphoratic; acidifying diuretic.

2.3.2. Ammonium Hydroxide

Ammonium hydroxide is also known as strong ammonia solution, or stronger ammonia water. Its molecular weight is 35.05. It is a clear, colorless solution of ammonia having an exceedingly pungent, characteristic odor. Upon exposure to air it loses ammonia rapidly. Its specific gravity is about 0.90. It is usually utilized as alkali for pH modifications.

2.3.3. Ammonium Persulfate

Ammonium persulfate (also known as peroxydisulfate) is found as odorless plate-like or prismatic (monoclinic) crystals, or white granular powder. This compound is stable for months when pure and dry. It decomposes in the presence of moisture, gradually evolving ozone containing oxygen. It also decomposes on heating, evolving oxygen and forming $(NH_4)_2S_2O_7$. Its density is 1.98. Ammonium persulfate is a strong oxidizing agent. It is freely soluble in water. Aqueous solution is acid and decomposes slowly at room temperature and rapidly at higher temperatures evolving oxygen and forming NH_4HSO_4. LD_{50} orally in rats: 820 mg/Kg, and none carcinogenicity has been related to it. The uses of ammonium persulfate in food are due to its characteristic oxidizing properties: Use as oxidizer and bleacher; decolorizing and deodorizing oils; washing infected yeast; and making soluble starch.

2.3.4. Hydrogen Peroxide

Its molecular weight is 34.01. Hydrogen peroxide is a clear, colorless liquid having a slightly pungent odor. It is miscible with water. The grades of hydrogen peroxide suitable for food use usually have a concentration between 30% and 50%. Approved uses of food grade hydrogen peroxide include bleaching of tripe, instant tea and cheese whey. Hydrogen peroxide is an effective antimicrobial agent in cheese making and in the manufacture of starch. It is also approved as a sterilizing agent for aseptic packaging. Hydrogen peroxide has been used to lighten foods at least since the turn of the century, when Swedes and Norwegians living in Minnesota bleached salted fish that had darkened during the Atlantic crossing (McNiellie and Wetmur, 1994). Concentrations in bleaching applications will range from 0.1% to 8%, depending on the time and temperature available to the process. For example, fish requires very low concentrations of peroxide. At the other extreme is nut processing, which allows only short time for peroxide contact. These applications generally require hydrogen peroxide concentrations between 5% and 10% (McNiellie and Wetmur, 1994). The toxicological information, according to MSDS is as follows:

Acute toxicity

1. Oral route, LD_{50}, rat, 841 mg/Kg. Test Substance: Hydrogen peroxide 60%.

2. Oral route, LD_{50}, rat 1232 mg/Kg.Test substance: Hydrogen peroxide 35%.

3. Dermal route, LD_{50}, rabbit, >2000 mg/Kg. Test substance: Hydrogen peroxide 35%.

4. Inhalation, LC_{50}, 4 hour(s), rat, 2000 mg/Kg.

Irritation

1. Rabbit, corrosive (eyes). Test substance: Hydrogen peroxide 70%.

2. Rabbit, irritant (skin). Test substance: Hydrogen peroxide 35%.

3. Rabbit, corrosive (skin). Test substance: Hydrogen peroxide 50%.

Chronic toxicity

1. In vitro, without metabolic activation, mutagenic effect.

2. In vivo, No genotoxic affect.

3. Oral route, prolonged administration (>6 months), mouse, Target organ: duodenum, carcinogenic effect.

4. Dermal route, prolonged administration (>6 months), mouse, no carcinogenic effect.

5. Oral route, prolonged administration (>6 months), rat, no carcinogenic effect.

6. Oral route, prolonged administration (>6 months), rat, gastro-intestinal effect. Test Substance: Hydrogen peroxide 70%.

7. Oral route, prolonged administration (>6 months), mouse, gastro-intestinal effect.

8. Oral route, effect on reproduction/insufficient data.

9. Inhalation, irritating effect. (LOAEL: 7ppm).

3. MATERIALS AND METHODS

3.1 Nixtamalization/Modified Nixtamalization

3.1.1 Principle

The purpose of nixtamalization is to eliminate the cuticle of the corn kernel, which interferes with the assimilation of the other nutrients consumed (Paredes *et al.*, 1982). The process causes corrugations and partial dissolution of the outermost layer while the aleurone layers keep enclosing the endosperm (Paredes *et al.*, 1982). There is a leaching of proteins (most of them seem to consist of albumins and globulins of low molecular weight). It is a fundamental process of improving the nutritional value of this cereal.

3.1.2 Apparatus

- Heat sealable pouch - Heavy duty 4 ½ mils thick heat sealable pouch (KAPAK/SCOTCHPAK stock No. 504) size 8" x 12", St. Paul, Michigan.
- Water bath - Precision water bath, Model 270. 890 L capacity. Max temp. 100°C. Ext. dimensions: 40.0x114x30 cm. Dallas, Texas.
- Hammer mill - Laboratory Mill, Thomas-Wiley, Model No. 4. Dallas, Texas.
- Hot plate - Corning Low Profile Hot Plate, Model PC-300, Temp. range 65 - 510°C. Dallas, Texas.
- Tortilla press. Azteca Brand. Mexico, D.F.

3.1.3 Reagents

- Distilled water - SIGMA Chemical Company, St. Louis, Missouri.
- $Ca(OH)_2$ - SIGMA Chemical Company, St. Louis, Missouri.

- NH_4OH 1% v/w - SIGMA Chemical Company, St. Louis, Missouri.
- NH_4Cl 1% w/w - SIGMA Chemical Company, St. Louis, Missouri.
- $(NH_4)S_2O_8$ 1% w/w - SIGMA Chemical Company, St. Louis, Missouri.
- H_2O_2 1% v/w - Solvay Interox, Houston, Texas.

3.1.4 Process

Approximately 150g of corn (aflatoxin naturally contaminated-fumonisin spiked) was placed in a heat resistant pouch. Distilled water (450 mL) and 0.75% (w/w) $Ca(OH)_2$ were added. One of the ammonia based chemicals was added. The pouch was sealed and placed into the hot water bath (100°C) for approximately 75 min. The corn was left soaking over night at room temperature (22°C). The "nixtamal" was ground using a hammer mill, and the dough ("masa") was made into individual tortillas (approximately 2 mm thick), using a tortilla press. The tortillas were cooked on a hot plate (300°C, 8 min), cooled down to room temperature, and frozen (-4°C) for aflatoxin-fumonisin determination, and performance of biotoxicological assays.

3.2 Fermentation/Modified Fermentation

3.2.1 Principle

One hundred grams of ground corn (drymill were mixed with 400 mL of distilled water in 1000 mL Erlenmeyer flask and 214 µL of thermostable α-amylase (Spezyme GA 400, GENECOR International, Houston, Texas), 1% (w/w or v/w) of the ammonium chemicals (for modified fermentation only), and 0.03 g of calcium chloride ($CaCl_2$) were added. The pH was adjusted to 6.2 using 1N NaOH.

Samples were cooked in an autoclave at 105°C during five min. For liquefaction, samples were held in agitation in a 90°C water bath during 2 h. The, 100 mL of distilled water were added and samples were cooled to approximately 65°C and 1.068 mL of glucoamylase (Spezyme AA-L GENECOR International, Houston, Texas) were added. The pH was adjusted to 4.3 using either NaOH or HCL, depending on the pH of the sample. Samples were placed on a 60°C water bath for two hours. For fermentation, dried brewing yeast was grown in a 10% saccharose solution at approximately 40°C during 15 min. Samples were cooled down to approximately 45°C and inoculated with 1% (v/v) yeast solution. Samples were placed for fermentation in a 30°C water bath for 72 hours.

3.3 Reversibility Test

3.3.1 Principle

The reversion of inactivated AFB_1 to the parent compound, and effect of acid on FB_1 were studied by exposing the ammonia treated corn (50 g) to 0.2N HCL (40 mL), mixed (pH = 2.0), and keeping in a 37°C water bath for 2 h. Mycotoxin levels in the HCl-treated sample were determined as described without the addtion of HCl.

3.4 High Performance Liquid Chromatography

3.4.1 Modified 991.31 Aflatoxins in Corn, Raw Peanuts, and Peanut Butter. Immunoaffinity Column (Aflatest) Method. First Action 1991. AOAC-IUPAC Method.

3.4.1.1 Principle

Test portion is extracted with 80% methanol-H_2O. The extracted sample was filtered, diluted with water, and applied to an affinity column containing monoclonal antibodies specific for aflatoxins B_1, B_2, G_1, and G_2. Aflatoxins are isolated, purified, and concentrated on the column and eluted using methanol. Total aflatoxins are quantitated by fluorescence detection and precolumn trifluoroacetic acid derivatization (THF method).

3.4.1.2 Apparatus

- Blender - UL High speed 500 mL blender jar, Houston, Texas.
- Filter paper 0 24 cm, prefolded Whatman Grade #4, Houston, Texas.
- Glass microfiber filter paper - 11 cm Whatman 934AH, Houston, Texas.
- Affinity column - Aflatest P column (Vicam, 29 Mystic Ave., Somerville, MA 02145).
- Syringe - 20 mL Luer tip for sample reservoir.
- LC pump - Waters 510, flow rate 2 mL/min, Chicago, Illinois.
- Injection system - Waters 717 Autosampler, Chicago, Illinois.
- LC column - Reverse phase Waters 150 mm C_{18}, Chicago, Illinois.
- Fluorescence detector - Waters 470. 360 nm excitation filter and 420 nm cutoff emission filter, Chicago, Illinois.

3.4.1.3 Reagents

- Solvents - Distilled-in-glass methanol, LC grade methanol. Acetonitrile, and water. SIGMA Chemical Company, St. Louis, Missouri.
- Extraction solvent - Methanol/water (8:2).
- LC mobile phase - Acetonitrile/water (2:8).
- Aflatoxin standard solutions for LC - Romer Laboratories Aflatoxins Standards. 50 ppb were diluted in acetonitrile and stored frozen.
- Derivatizing Reagent - 14 mL distilled deionized water, 4 mL Trifluoroacetic acid, 2 mL glacial acetic acid.

3.4.1.4 Preparation and Extraction of Samples

Ground treated sample (50 g) and 5 g NaCl were weighted and placed in the blender jar. Methanol (100 mL, 80%) was added to the jar. The blender jar was covered and blended at high speed for 1 min. The extract was poured into fluted filter paper and the filtrate was collected in a clean vessel. The filtrate was diluted with 40.0 mL distilled water, mixed well, and filtered through a glass microfibre filter (Vicam #31955) in a small funnel into clean collecting vessel.

3.4.1.5 Affinity Column Chromatography

The syringe reservoir was secured to a ring stand. The top cap was removed from the column, the tip was cut off and used as a connector between the column and reservoir. the filtered extract (2 mL, equivalent to 0.2 g sample equivalent) were added into the reservoir. The reservoir was connected to an air-filled hand pump and the end cap was removed from column. The extract was

pushed through column at flow rate of ca. 2 drops/s (6 mL/min). The hand pump was disconnected form reservoir and filled with air, then reconnected to the reservoir and 2-3 mL of air were passed through column. Hand pump was disconnected and 5 mL of water were added to reservoir. Hand pump was filled with air and reconnected. Water was pushed through column at flow rate of ca 6 mL/min. The last step was repeated with another 5 mL of water. The water washings were discarded. Hand pump was disconnected and filled with air. Once reconnected, 2-3 mL or air were passed through column. Hand pump was disconnected and 1.0 mL LC grade methanol was added to reservoir. The eluate was collected in appropriate container. Hand pump was filled with air. Once reconnected, methanol was passed through column. Additional 2-3 mL of air were passed through column. The eluate was divided into 4 vials with 250 µL of eluate each. Methanol was dried out from each vial with a water bath at 90°C. The vials were labeled and stored in -20°C for further analysis.

3.4.1.6 High Performance Liquid Chromatography Analysis (HPLC)

The eluate was reconstituted with 200 µL acetonitrile/water (9:1), and 700 µL derivatizing agent were added. The sample was heated at 65°C for 8.5 min. After heating, samples were cooled down in ice water. The samples were ready for analysis in the HPLC. Samples were placed into the autosampler. Run time, 20 min/sample.

3.4.2 999.15 Fumonisins B_1, B_2, and B_3 Corn, Liquid Chromatography Method, AOAC-IUPAC Method

3.4.2.1 Principle

Fumonisins were extracted from corn with a methanol-water solution. The filtered extract was purified on a strong-anion-exchange (SAX) solid-phase extraction (SPE) cartridge, and fumonisins were eluted with acetic acid-methanol solution, which was then removed by evaporation. The residue was dissolved in methanol, and *o*-phthaldialdehyde (OPA)/2-mercaptoethanol was added to form fluorescent fumonisin derivatives. The derivatives were analyzed by reversed-phase liquid chromatography (LC) with fluorescence detection.

3.4.2.2 Apparatus

- Liquid chromatograph - LC pump (Waters, Chicago, IL) capable of delivering 1 mL/min constant flow rate, equipped with injection system calibrated to deliver 10 µL.
- LC column - (1) 150 x 4.6 mm id, C_{18} reverse-phase, stainless steel, packed with 5 µm ODS material, and (2) suitable corresponding reversed-phase guard column. LC column was maintained at ambient temperature (23°C), Waters, Chicago, IL.
- Fluorescence detector - Variable wavelength; set at 335 nm (excitation) and 440 nm (emission), Waters, Chicago, IL.
- SPE cartridges - 10 mL capacity; containing 500 mg silica-based SAX sorbent (Varian Bond Elut).

- SPE manifold.
- Solvent evaporator - To hold 4 mL capacity glass vials; capable of operating at 60°C.
- Membrane filter - 0.45 µm porosity.

3.4.2.3 Reagents

- Methanol.
- *o*-Phosphoric acid - Concentration > 85%.
- 2-Mercaptoethanol.
- Acetonitrile-water solution (1+1, v/v).
- Acetic acid-methanol solution (1+99, v/v) - Glacial acetic acid was used when preparing solution.
- Sodium dihydrogen phosphate solution - 0.1M. 13.8 g $NaH_2PO_4 \cdot H_2O$ were dissolved in 1 L H_2O.
- Methanol-water solution (3+1, v/v).
- Sodium hydroxyde - 1 M.
- Disodium tetraborate solution - 0.1 M, 3.8 g $Na_2B_4O_7 \cdot 10H_2O$ were dissolved in 100 mL H_2O.
- LC mobile phase - Methanol - 0.1M sodium dihydrogen phosphate (77+23, v/v), adjusted to apparent pH 3.3 with *o*-phosphoric acid. Mobile phase was filtered through membrane filter and pumped at 1 mL/min flow rate.
- Phthalaldehyde (*o*-Phthaldeialdehyde, OPA) reagent - 40 mg OPA were dissolved in 1 mL methanol, and diluted with 5 mL 0.1M disodium

tetraborate solution. 50 µL 2-mercaptoethanol were added, and mixed. OPA reagent was stored at room temperature in a capped amber or aluminum foil-covered vial up to 1 week.

- Fumonisin standard solution - Stock solution of fumonisin B_1 at concentrations of 250 µg/mL was prepared in acetonitrile-water solution. 100 µL aliquot of stock solution was transferred to clean glass vial and 400 µL acetonitrile-water solution were added, to yield standard working solution containing concentration of 50 µg/mL. Fumonisin stock and working standard solutions are stable up to 6 months when stored at 4°C.

3.4.2.4 Extraction and Purification of Sample

Fifty g ground sample were weight and placed in the blender jar. 100 mL 75% Methanol were added to the jar. The blender jar was covered and blended at high speed for 5 min. Extract was centrifuged 10 min at 500 x g. Supernatant was filtered through fluted filter paper. Filtrate (apparent pH ca 5.8) was collected in a clean vessel. If necessary, pH was adjusted to 5.8-6.5 with 1M NaOH (only 2-3 drops were required). SPE cartridge was fitted to manifold. Cartridge was conditioned by washing successively with 5 mL methanol followed by 5 mL methanol-water solution (3+1). 10 mL filtered extract were applied to cartridge while maintaining flow rate ≤ 2 mL/min. Cartridge was washed with 5 mL methanol-water solution followed by 3 mL methanol. Cartridge was not allowed to dry. Fumonisins were eluted with 10 mL acetic acid-methanol solution at flow rate ≤ 1 mL/min. Eluate was collected in 20 mL glass collection vial.

Four mL aliquots were transferred to glass vial, while evaporating solvent to dryness under stream of nitrogen at ca 60°C. Collection vial was rinsed with 1 mL methanol and rinsing solvent was added to 4 mL vial, washing sides of vial to concentrate residue at the base. Additional methanol was evaporated to dryness to ensure that all acetic acid is removed. Dried sample residues could be stored up to 1 week at 4°C prior to LC analysis.

3.4.2.5 Derivatization and LC Analysis

- Preparation of standard derivative - 25 µL fumonisin standard working solution were transferred to base of small test tube. 225 µL OPA reagent were added. 10 µL solution were injected into LC system within 1 min after addition of OPA reagent.
- Corn extracts - Purified residue was redissolved in 200 µL methanol. 25 µL of the solution were transferred to base of small test tube and 225 µL OPA reagent were added. 10 µL solution were injected into LC system within 1 min of adding OPA reagent.

3.4.2.6 Calculations

Fumonisin B_1 in aliquot injected (F) into LC system was calculated by using peak areas for fumonisin B_1 as follows:

$$F, ng = (P_u/P_f) \times S$$

where P_u = individual fumonisin B_1 peak area of test sample; P_f = fumonisin B_1 peak area of standard solution; S = amount of fumonisin B_1 standard injected into LC system; 50 ng/fumonisin (based on concentration in fumonisin standard

working solution). Concentrations of fumonisin present in corn were calculated as follows:

$$C, ng/g = (F \times V_t \times D)/(V_i \times W)$$

where V_t = total volume of derivatized solution, 250 µL; D = any dilution factor that may have been used; V_i = injection volume, 10 µL; W = sample equivalent weight derivatized, 0.625 g.

3.5 Toxicological Bioassays

3.5.1 Chicken Embryo Bioassay

3.5.1.1 Principle

Chicken embryo assay is based on the sensitivity of the embryo to external compounds. It was first developed for aflatoxin identification. Fertile eggs are exposed to the chemical and development and mortality of the embryo is observed during the normal process of development. Growth retardation, hemorrhaging, short feet, edema, and mortality of the embryos are observed. Harchability power is accountable for the assay.

3.5.1.2 Apparatus

- Egg candling light. 100 watts UL, Houston, Texas.
- Drill. Black and Decker, 120 V, Houston, Texas.
- Forceps. Rochester-Ochsner Hemostatic 5½", Dallas, Texas.
- Syringe. 20 mL Plastipak, B-D. Dallas, Texas.
- Needles. Dissecting needle, plain wooden handle. Houston, Texas.
- Sealing was.

- Incubator. No data available.
- Hatcher. No data available.

3.5.1.3 Reagents

- Aflatoxin B_1 std. Soln. Romer Laboratories Aflatoxins Standards. %0 ppb diluted in DMSO.
- Fumonisin B_1 std. DMSO Soln. 98% purity, South Africa.
- Sterile distilled deionized water. Sigma Chemical Company, St. Louis, Missouri.
- Sterile corn oil. Commercial Brand.
- Treatments.

3.5.1.4 Egg Supply

Eggs, fertile, from inbred Single-Comb White Leghorn flock (ConAgra, Nachitoches, LA).

3.5.1.5 General Technique

3.5.1.5.1 Egg Selection

First, fertile eggs to be injected were candled and those that were defective discarded -- i.e. have cracked or improperly classified shells, tremulous or misplaced air cells, or blood clots. The exact location of the air cell was outlined with a pencil.

3.5.1.5.2 Injection Technique

Pure chemical to be injected, chemical solution, or suspension into the air cell to be a total volume of 50 µL. When necessary, the substance was diluted with corn oil, or sterile distilled deionized water.

Air Cell Injection. A sterile atmosphere was not necessary for injections into the air cell. A hole was drilled about 5 mm in diameter into the shell over the air cell. The air cell membrane was removed with the needle bent at a 90° angle, and the hole sealed with wax. The eggs were left undisturbed in a vertical position (air cell up) for about an hour to let the material disperse.

In each experiment, an adequate number of injected eggs for control were included. Also, a number of eggs injected only with the mycotoxin (aflatoxin, fumonisin) were included (positive controls). These eggs provided a basis of comparison with the eggs injected with the drug studied.

3.5.1.5.3 Incubation

Injected eggs were placed into incubator trays with the large end up, day 0. Temperature was maintained at 38°C and a relative humidity of 85% in the incubator. The eggs were candled on the fifth day of incubation and every day thereafter. Clear eggs and dead embryos were removed to be examined.

3.5.1.5.4 Hatching

On the 18th day, the eggs were placed into hatching baskets and put into the hatcher. They were separated by compound and dose level so that the

hatched chicks could be properly identified. Chicks and unhatched eggs were removed on the 22nd day and put in labelled boxes.

Upon hatching, the embryos from unhatched eggs and hatched chicks were examined for gross deformation, such as length of the legs, form of the beak and of the rump, and compared with the controls.

3.5.2 *Salmonella*/Microsome Mutagenicity Assay

3.5.2.1 Principle

The Ames *Salmonella*/microsomal mutagenicity assay is a short-term assay used to detect chemically induced mutagenesis caused by two types of DNA alters *Salmonella typhimurium*. The types of mutation detected are the base-pair substitution and the frame-shift. Both of these alterations cause changes is the reading frame of the DNA and result in three kinds of coding error: (1) Sense - this is where the codon had been altered but it still reads for the same protein; (2) Non-sense - the codon has been changed and makes no sense and reading stops; (3) Missense - the codon reads for a different protein. This short-term assay is classified as a reverse mutation assay because mutagenicity is determined by the reversion of the *Salmonella* from histidine-requiring to histidine independent (wild type).

Sample preparation for the *Salmonella*/microsome mutagenicity assay involved 50 g of each subsample being extracted with 100 mL methanol:water (8:2), for the inorganic and acid portion, and 100 mL chloroform (for the organic portion). These extract were filtered with Whatman #4 filter paper with the filtrate

collected for use in the mutagenicity assay. The inorganic extracts were freeze-dried to remove the water. The chloroform extracts were evaporated in a water bath at 60°C. The freeze-dried samples were resuspended in DMSO and serially diluted for incorporation into the mutagenicity assay. The fumonisin B_1 and aflatoxin B_1 standards were brought up (separately) in DMSO; aflatoxin B_1 was used as a positive control. Fumonisin B_1 standard was kindly donated by Dr Eric W. Sydenham, Medical Research Council, Tygerberg, Republic of South Africa. The mutagenicity assay was performed using tester strain TA 98, TA 100, and TA 102, plate incorporation and S9 activation according to Maron and Ames, 1983; pre-tests and controls were all acceptable.

3.6 Sensory Evaluation

Sensory evaluation was performed using Triangle Test protocol. The samples compared were the traditional nixtamalization and the modified nixtamalization (1% NH_4OH) processes. Semi-trained panelists (trained during the test) performed the sensory evaluation on color, odor, flavor, and texture of the samples.

3.7 Statistical Analysis

Statistical analysis were performed using the Minitab Statistical Software Release 8 (Minitab, Inc.), and Quattro Pro version 6.01 (Novell, Inc.).

3.8 Fumonisin Determination in Corn and Corn Products from the Tucson Area

3.8.1 Principle

It has been attempted to develop a procedure that allows the elimination fumonisins from crops without leaving toxic residues that may affect consumers, both humans and animals. Some studies show that the process of nixtamalization (tortilla production) hydrolyses fumonisins. Those studies suggest the possibility of creating more toxic compounds called hydrolyzed fumonisins or aminopentols (Hendrich *et al.*, 1993; Hopmans *et al.*, 1993). Samples from a Tucson-area corn products factory were tested for the presence of fumonisins. Fumonisin B_1 (the most toxic compound out of the group) was detected in the corn used for corn products production. Fumonisin B_1 was not detected in any of the treated products, but due to the lack of hydrolyzed fumonisin B_1 standard (HFB_1) we could not confirm the presence of it.

3.8.2 Apparatus

The HPLC system consisted of Beckman Liquid Chromatograph (Beckman Instruments, Inc., Berkeley, CA) 421A System Controller, a 110B Solvent Delivery Module, a 210A Sample Injection Valve with a 10 Ul loop, and a Varian Fluorichrom™ Fluorescence Detector (Varian Associates, Inc., Walnut Creek, CA). The reversed-phase column was a Whatman Partisil DXS 10/25 ODS.

3.8.3 Chemical Standards

- Fumonisin B_1 standard was donated by the FDA, Washington, DC. A stock solution of 1 mg/mL of FB_1 in methanol was prepared. From these, standard solutions of 100, 75, 50, and 25 µg/mL of fumonisin B_1 in methanol were prepared.
- Reagents. The HPLC mobile phase was 0.1 M monobasic sodium phosphate, HPLC grade methanol, and HPLC grade acetonitrile (6:3:3). The mobile phase was degassed using vacuum.

3.8.4 Derivatization

For the derivatization, 1 mL of 100% methanol, 0.5 mL of OPA regent, and 0.5 mL of 2-mercaptoethanol solution were added to the cleaned up sample extracts and to the standards. Each reaction mixture was allowed to stand for 10 min at room temperature.

3.8.5 Preparation of Standard Curve

Standard curves were constructed by injection of derivatized standard samples using 100, 75, 50, and 25 µg/mL of fumonisin B_1. The curve was constructed by plotting peak areas versus nanograms of fumonisin B_1.

3.8.6 Toxin Extraction of Corn and Corn Product Samples

Corn and corn product samples obtained in El Zarape Mexican Food Inc. (Tucson, AZ, Table 3.1). This small industry distributes the corn products consumed by the university community of Tucson, Arizona. The samples were extracted and purified using a modification of the method of Bennett (1994).

Kernels and corn products were freeze-dried to eliminate water, ground, and 25 g of ground material was extracted by wrist-action shaking for 60 min with 100 mL of acetonitrile/water (1+1) and 5 g NaCl. The slurry was passed through Whatman No. 2 filter paper, and a 2 mL portion in 4 mL methanol/water (20+80) was applied to a Bond-Elut C18 cartridge (Varian Bond-Elut) which had been conditioned with methanol (5 mL), followed by 8 mL of methanol/water (20+80). The cartridge was washed with 5 mL acetone/ethylacetate (1+1), and the toxin was eluted with 10 mL of chloroform/methanol/acetic acid (6+4+1). The eluate was evaporated to dryness under nitrogen in a water bath at < 60°C. Fumonisin concentrations of the corn and corn product samples were calculated from the chromatographic peak area as follows:

$$\mu g/kg = (B \times Y \times S \times V)/(Z \times X \times W)$$

where B = average area fumonisin B_1 peaks in sample aliquot; Y = concentration of fumonisin B_1 standard (µg/mL); S = µL fumonisin β standard injected onto HPLC; V = final dilution of sample extract (µL); Z = average area of fumonisin B_1 peaks in standard aliquot; X = µL sample extract injected onto HPLC; and W = g sample represented by final extract.

Table 3.1. Corn and corn products analyzed for Fumonisin B_1.

Sample	Description
Corn	natural yellow kernels
Boiled corn	corn + $CA(OH)_2$ boiled for 45 min
Soak corn	boiled corn, soaked for 18 h
White corn	rinsed, soaked corn dough
Yellow corn	soaked corn dough
MASECA	commercial corn meal dough
White corn tortilla	tortilla made of white corn
Yellow corn - Jumbo	thick tortilla made of yellow corn
Yellow corn tortilla	tortilla made of yellow corn
MASECA tortilla	tortilla made of MASECA
MASECA gordita	medium size tortilla made of MASECA
MASECA Jumbo	thick tortilla made of MASECA

3.8.7 Statistical Analysis

Quattro Pro version 5.0, Borland International, was used for descriptive statistics, and linear regression was calculated using Harvard Graphics version 3.0.

4. RESULTS AND DISCUSSION

All applied treatments studied for reducing the levels of naturally incurred aflatoxin in contaminated corn showed some degree of success. The concentration of the starting material (naturally contaminated corn) was 300 ng/g. When comparing aflatoxin levels in the initial material and the final products other factors, i.e., moisture content, can have an effect on the analytical result. However, the procedures evaluated significantly lowered the levels of aflatoxin and toxic potentials (see appendix A for statistical analysis).

The most successful treatment for aflatoxin reduction during fermentation was ammonium persulfate with hydrogen peroxide (Figure 4.1). That is in accordance with data obtained in the University of Arizona (Burgos-Hernandez *et al.*, 1996). The basic fermentation treatment (hear treatment) also presented the lowest level value for aflatoxin with the combination of these chemicals (Figure 4.2). Heat treatment is referred to the fermentation process minus the addition of yeast.

Almost all the ammonia based chemicals, with the exception of ammonium persulfate, decreased the levels of aflatoxin by HPLC analysis for modified nixtamalization treatments (Figure 4.3). Modification in the structure of aflatoxins avoiding the formation of the derivative could have occurred. The boiling treatment by itself produced more reduction on the aflatoxin levels than the heat treatment (Figure 4.4).

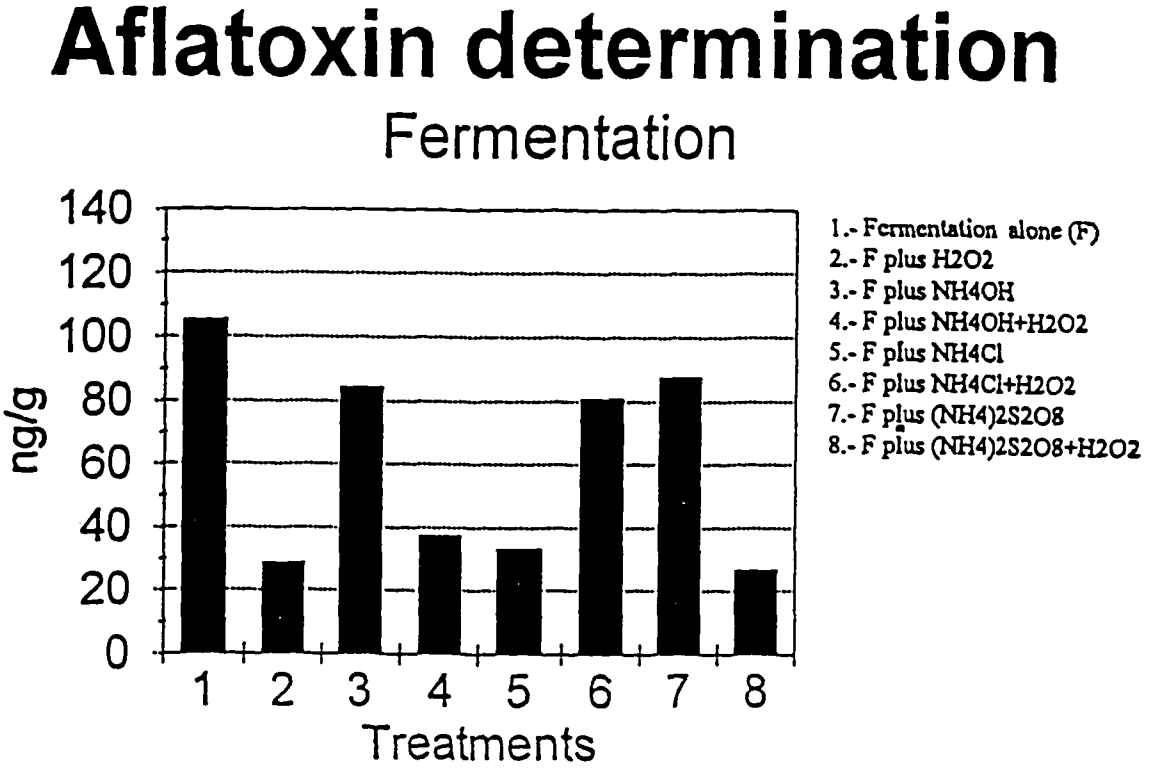

Figure 4.1 Effect of fermentation treatment with and without additional chemical mixtures in naturally contaminated corn. Original concentration aflatoxin B_1 level in corn was 300 ng/g.

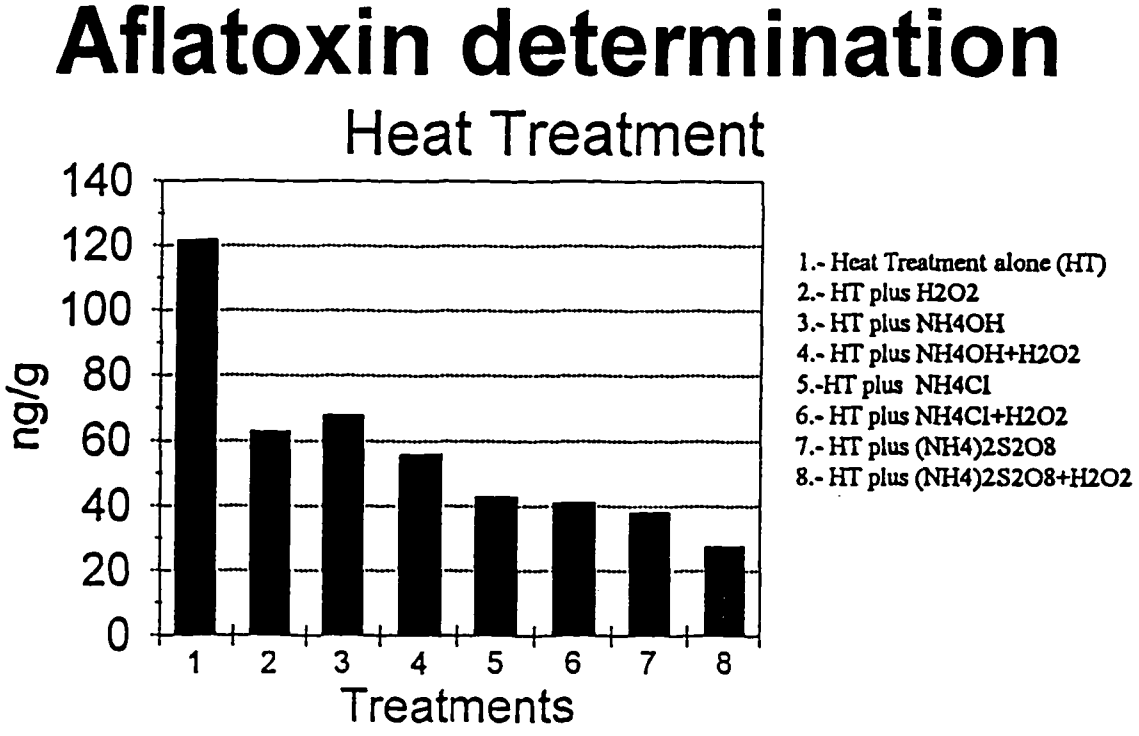

Figure 4.2 Effect of heat treatment (comparable to fermentation process minus yeast addition) with and without additional chemical mixtures on aflatoxin levels in naturally contaminated corn. Original concentration aflatoxin B_1 level in corn was 300 ng/g.

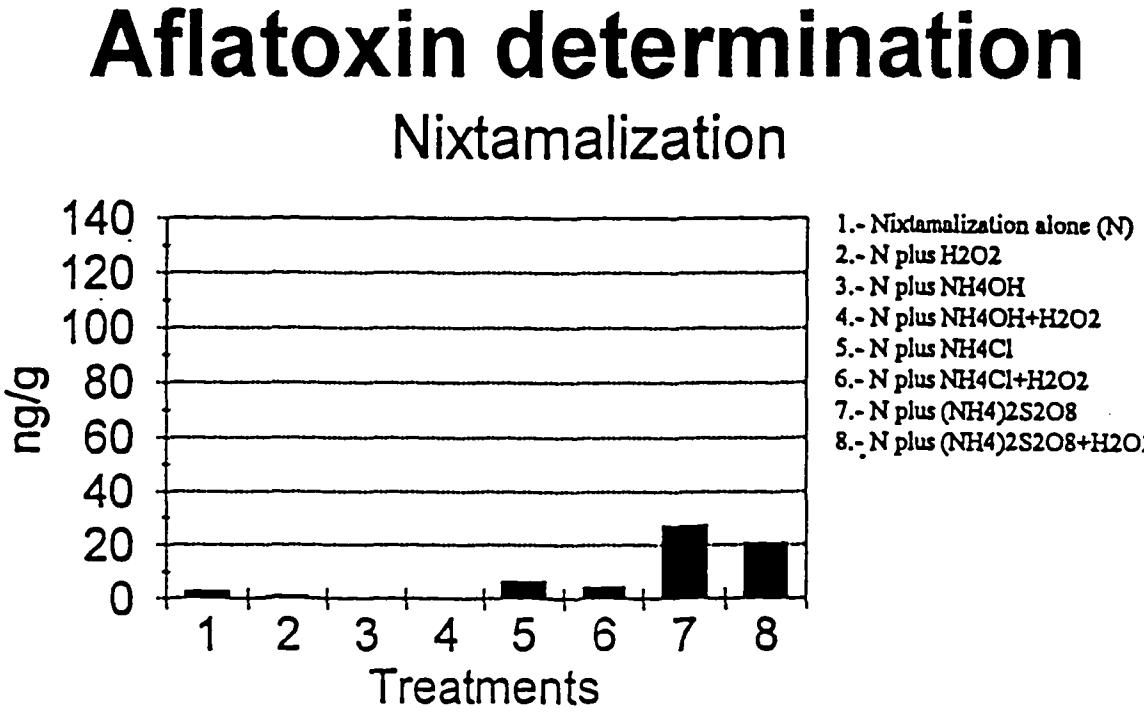

Figure 4.3 Effect of nixtamalization treatment with and without additional chemical mixtures in naturally contaminated corn. Original concentration aflatoxin B_1 level in corn was ng/g.

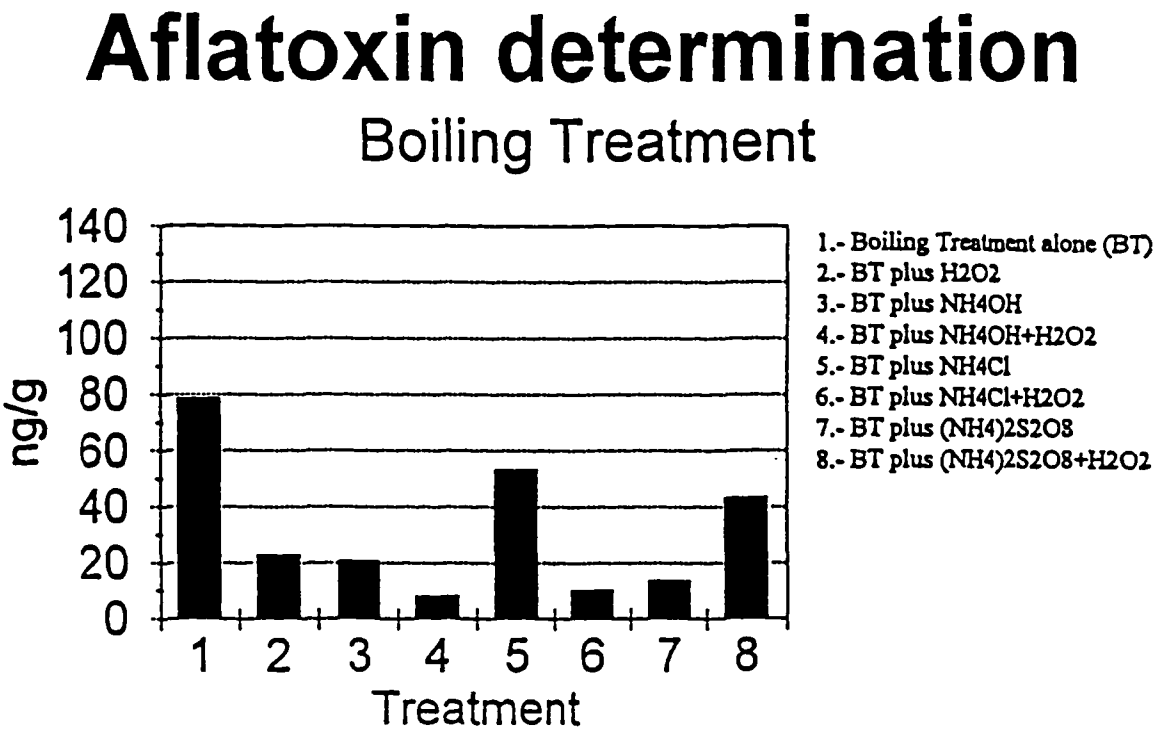

Figure 4.4 Effect of boiling treatment (100°C for 75 min; comparable to nixtamalization process minus $Ca(OH)_2$) with and without additional chemical mixtures on aflatoxin levels in naturally contaminated corn. Original concentration aflatoxin B_1 level in corn was 300 ng/g.

The importance of the presence of calcium hydroxide during the reduction of aflatoxin levels can be observed in Figure 4.1 and 4.2. The boiling treatment simulates the nixtamalization process minus the addition of calcium hydroxide. Aflatoxin levels decreased dramatically when the chemical was present in the matrix. At the same time, the presence of ammonium persulfate resulted in less reduction of aflatoxin in the naturally contaminated nixtamalized corn (Figure 4.1). This peculiar situation suggests that ammonium persulfate either stops the effect of calcium hydroxide, or frees the bound aflatoxin in the matrix. A similar effect is observed with the presence of ammonium chloride in the system when treated at 100°C for 75 minutes (Figure 4.4).

In the reversibility test, for all treatments which contained ammonium hydroxide, ammonium chloride, ammonium persulfate, calcium hydroxide, and hydrogen peroxide, the results showed greater increase of aflatoxin for persulfate when compared to calcium hydroxide. There was no evidence of reversibility of the decontamination processes. The reversibility test consisted of exposing the tortillas to 0.1N HCl for two hours at 37°C for almost all the treatments (Figure 4.5).

Fumonisin reduction was apparent for all procedures tested. The levels of reduction were 100% for all treatments. The samples used in this study were spiked with 100 ng/Kg. Naturally contaminated corn was not available for the analysis. The process of nixtamalization involves water soaks, therefore, it is possible that the toxins are extracted and discarded in the wash since fumonisins

Aflatoxin determination
Reversibility Study

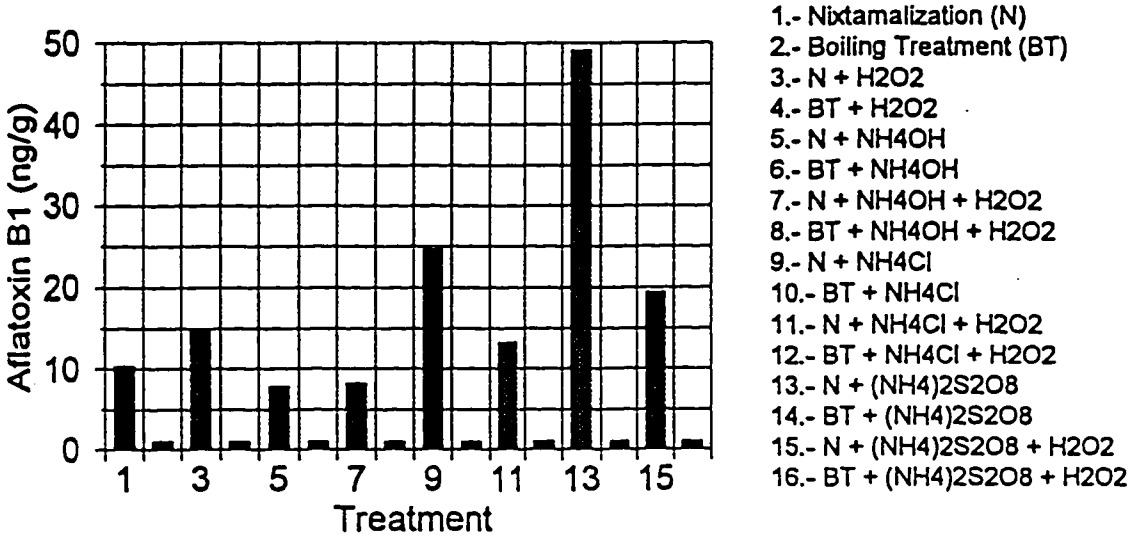

Figure 4.5 Effect of reversibility study (exposure of tortillas to 0.1 N HCl for two hours at 37°C) with and without additional chemical mixtures on aflatoxin levels in naturally contaminated corn. Original concentration aflatoxin B_1 level in corn was 300 ng/g.

are water soluble compounds. The spiked procedure was done by adding fumonisin culture material to the aflatoxin contaminated corn. After the process of modified nixtamalization, the water was discarded, and the formation of the dough involved a triple washing step. The analysis of the discarded water was not performed. Chemical modification or physical separation is unknown.

During the modified fermentation study, the preparation of sample for analysis required centrifugation and decantation of the liquid portion of the sample followed by a freeze-drying step. It is possible that fumonisin could have been eliminated. For this case, the apparent reduction in FB_1 levels may have been due to the analytical procedures rather than the fermentation procedures. Further studies are required. Determination of fumonisin in these steps was not detected.

Comparison of optimal chemical and thermal treatments on aflatoxin B_1 levels in naturally contaminated corn products is observed in Figure 4.6. Based on chemical and analytical results, the most effective procedure for reducing AFB_1 levels was selected for further toxicity/mutagenicity evaluation. Modified nixtamalization utilizing ammonium hydroxide alone was chosen.

Tortillas obtained from modified nixtamalization with ammonium hydroxide (1% v/w) were tested for teratogenicity, mutagenicity, and sensory evaluation. Three extracts were obtained for the biological assays (teratogenicity and mutagenicity). The first two extracts were from the methanol-water extraction, and reversibility test-methanol-water extraction. These extracts represented the

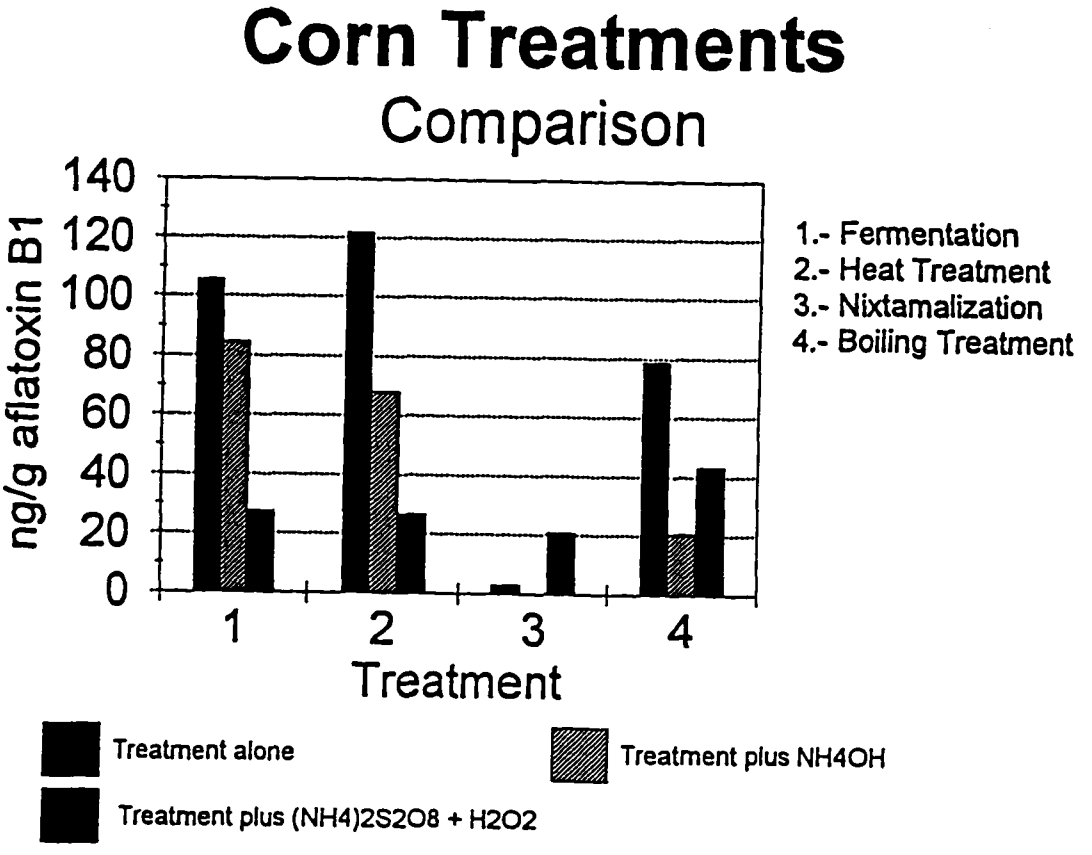

Figure 4.6 Comparison of optimal chemical and thermal treatment on aflatoxin B_1 levels in naturally contaminated corn. Original aflatoxin B contamination level in corn was 300 ng/g. Heat treatment is comparable to fermentation process minus addition of yeast. Boiling treatment if comparable to nixtamalization process minus addition of $Ca(OH)_2$.

inorganic portion of the sample. The third extract, chloroform extraction, represented the organic portion of the sample.

Results from the chicken embryo assay for toxicity and teratogenicity showed no significant difference between normal nixtamalization and modified nixtamalization modification treatment (Figures 4.7 - 4.9). There were differences in mortality of the developing embryo based on extraction solvent used. The methanol extraction showed no variation of the extreme concentrations between the treatments (Figure 4.10). Interestingly, an inverse response was observed, i.e., as the concentration increased, embryo mortality decreased. The reversibility study-methanol extract resulted in low embryo mortality when compared with the chloroform extract fractions (Figure 4.11).

The *Salmonella*/microsomal mutagenicity assay was performed on the modified tortilla (1% NH_4OH) and the normal nixtamalization process tortilla. The results are observed in Figures 4.12-4.21.

Mutagenicity is observed in the samples when the number of revertants double the natural revertants. Problems during the performance of the assay were observed. The number of natural revertants were extremely low compared to reported studies (Park *et al.*, 1992; Park *et al.*, 1996). Mutagenicity was observed then, in all the extracts tested with strain TA 98 without metabolic activation (Figure 4.12). No mutagenicity was observed when the extracts were tested with the same strain with metabolic activation (Figure 4.14). No mutagenicity was observed with TA 100 without metabolic activation (Figure 4.16).

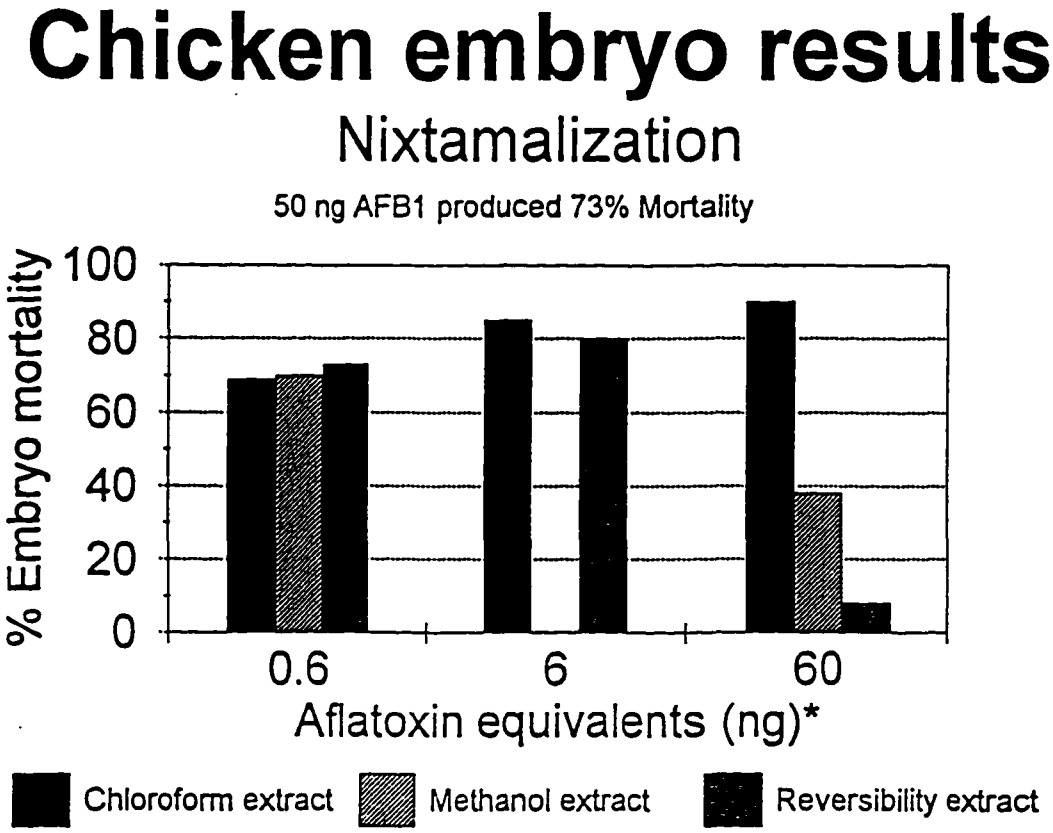

Figure 4.7 Effects of organic and inorganic extracts from tortilla samples on chicken embryo mortality. Nixtamalization process was used for sample preparation. Chloroform extract represents organic portion of the samples. Methanol extract represents inorganic portion of the sample. Reversibility extract was obtained from the reversibility study followed by methanol-water extraction. Control embryos had 0 percent mortality.

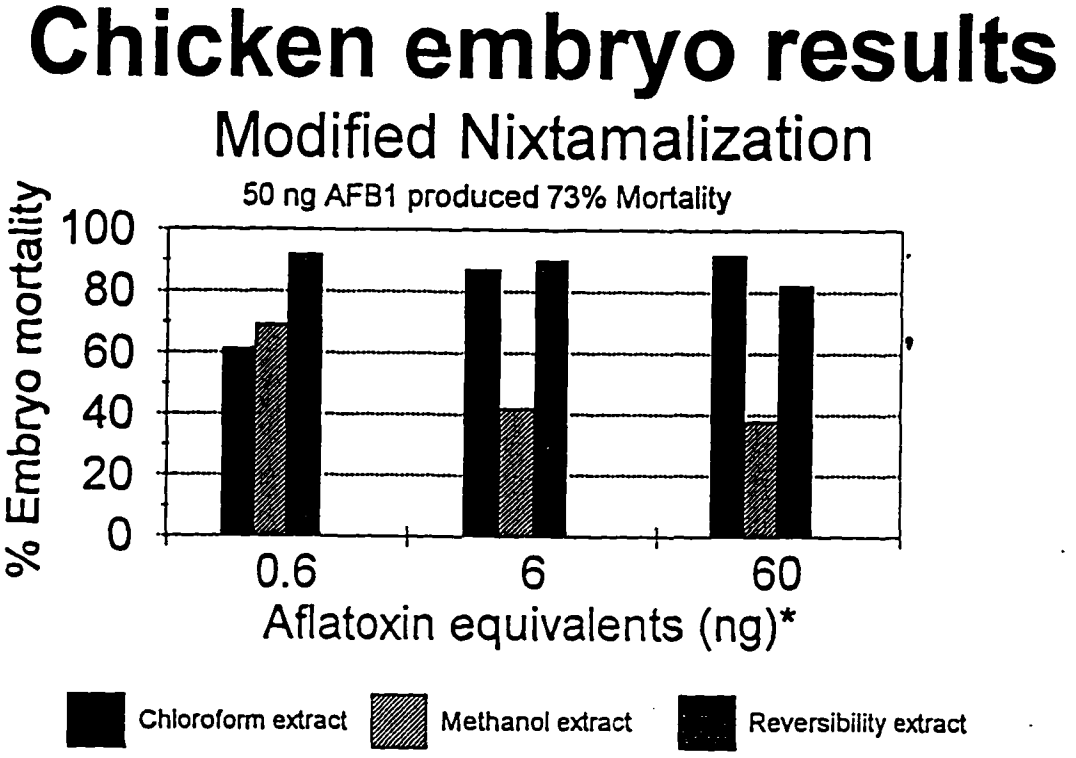

Figure 4.8 Effects of organic and inorganic extracts from tortilla samples on chicken embryo mortality. Modified nixtamalization process (addition of 1% NH_4OH) was used for sample preparation. Chloroform extract represents organic portion of the samples. Methanol extract represents inorganic portion of the sample. Reversibility extract was obtained from the reversibility study (0.1N HCl for two hours at 37°C) followed by methanol-water extraction. Control embryos had 0 percent mortality.

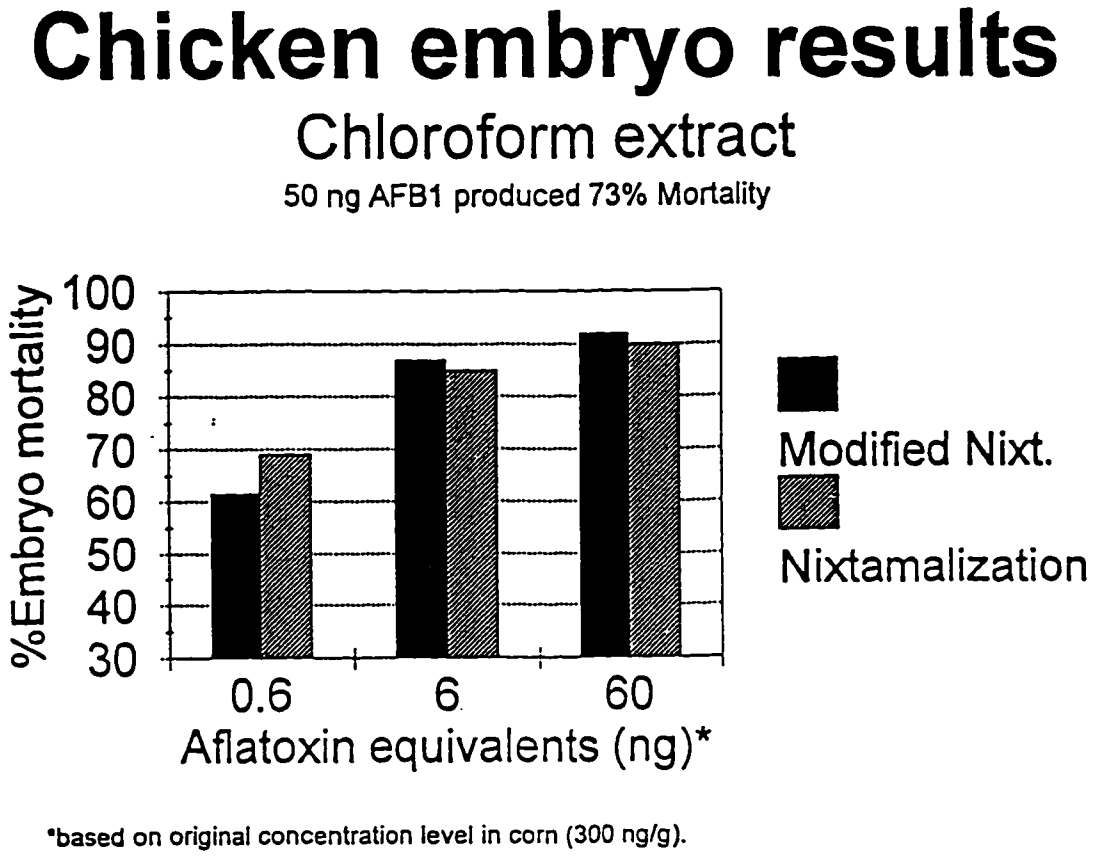

Figure 4.9 Comparison of the effects of chloroform extracts from tortilla samples prepared by normal nixtamalization and modified nixtamalization (addition of 1% NH_4OH) processes on chicken embryo hatchability. Control embryos had 0 percent mortality.

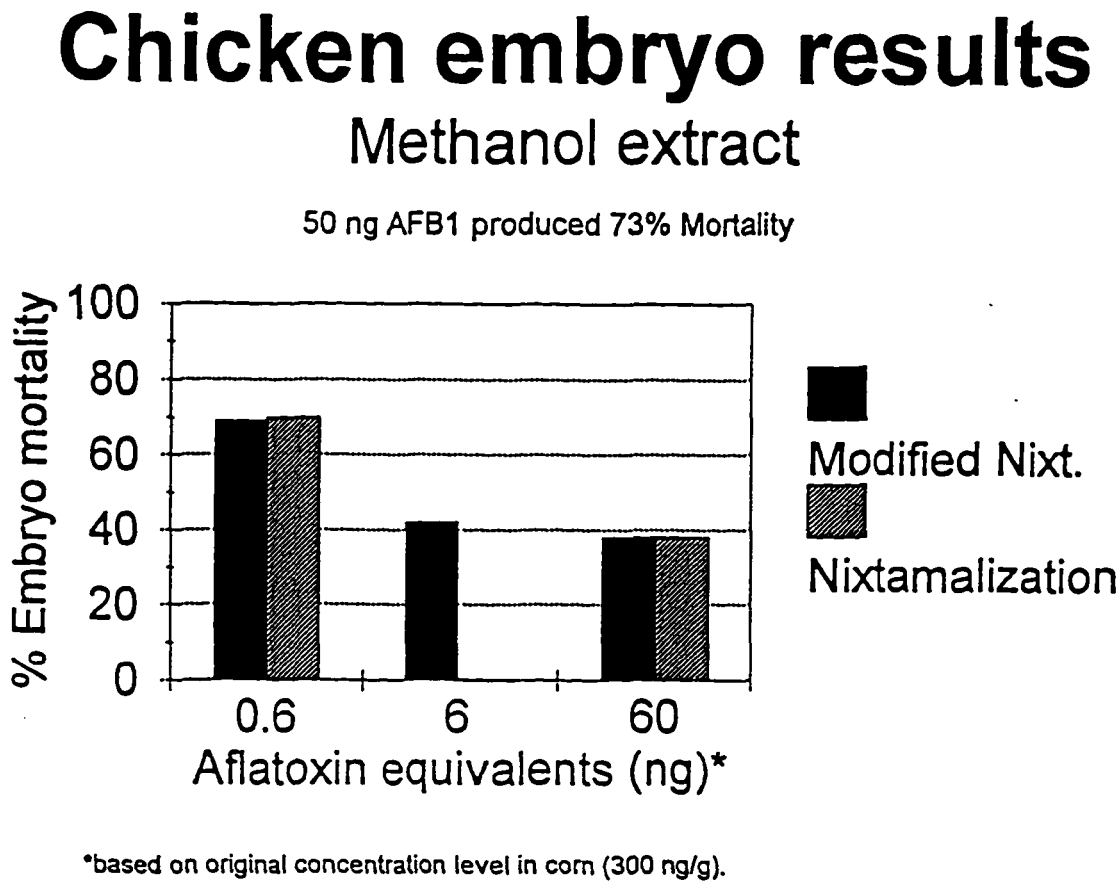

Figure 4.10 Comparison of the effects of methanol extracts from tortilla samples prepared by normal nixtamalization and modified nixtamalization (addition of 1% NH_4OH) processes on chicken embryo mortality. Control embryos had 0 percent mortality.

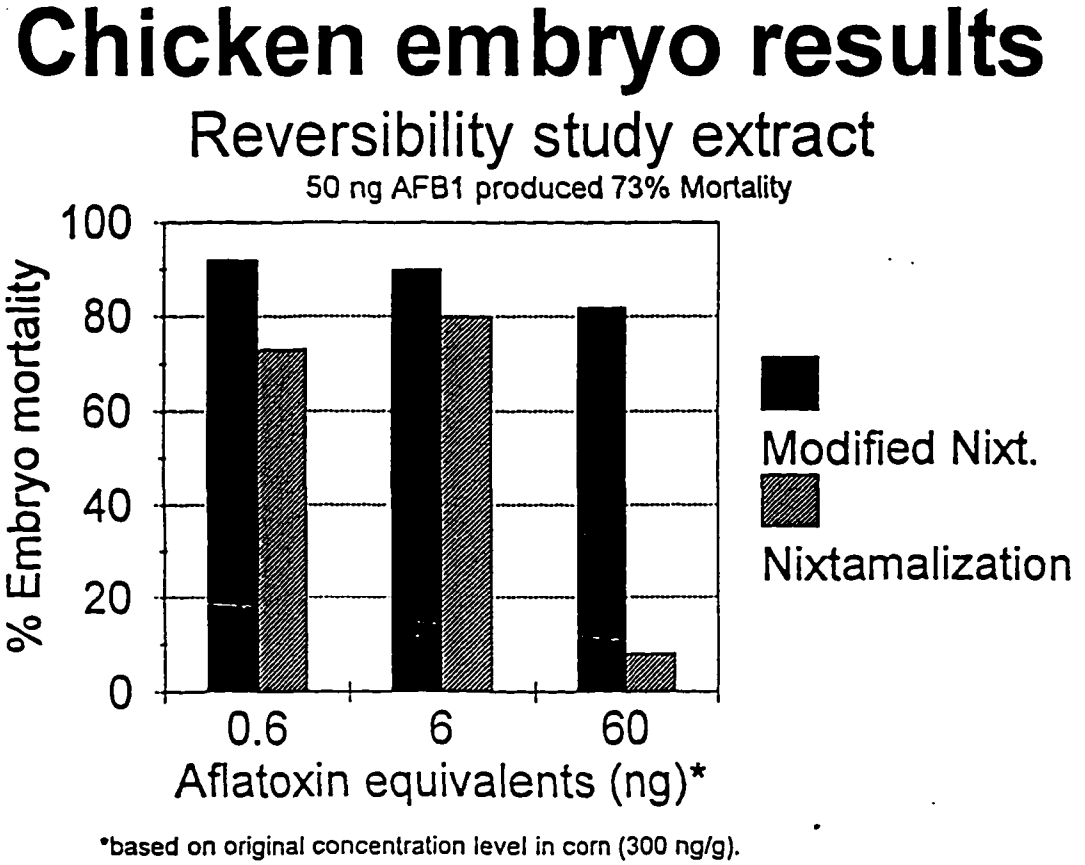

Figure 4.11 Comparison of the effects of reversibility study extracts from tortilla samples prepared by normal nixtamalization and modified nixtamalization (addition of 1% NH_4OH) processes on chicken embryo mortality. Reversibility study consisted in the exposure of tortilla to 0.1N HCl for two hours at 37°C. Control embryos had 0 percent mortality.

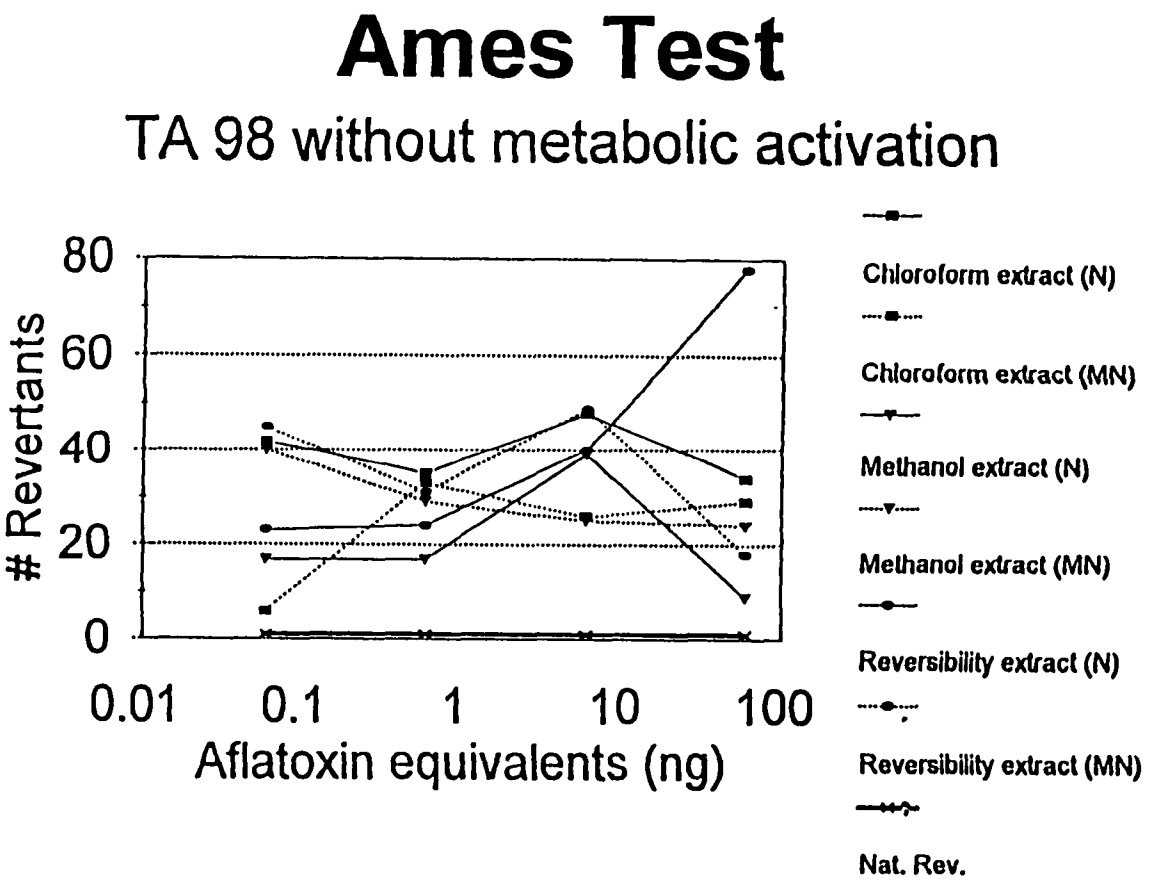

Figure 4.12 Effects of nixtamalized and modified nixtamalized tortillas on the *Salmonella*/microsomal mutagenicity assay (AMES), strain TA 98 without metabolic activation. Aflatoxin equivalents are based on original contamination level in corn 300 ng/g. N stands for nixtamalization process. MN stands for modified nixtamalization.

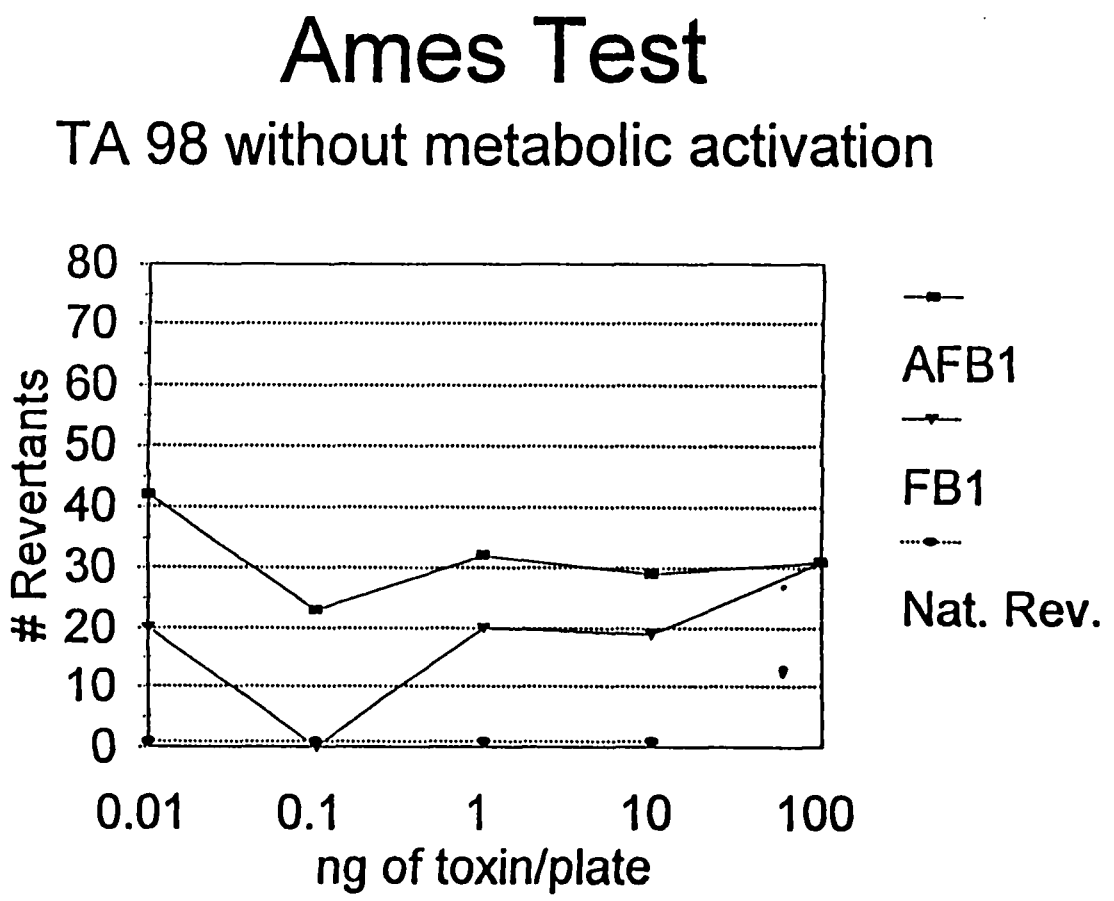

Figure 4.13 Effects of aflatoxin B and fumonisin B standards on the *Salmonella*/microsomal mutagenicity assay (AMES), strain TA 98 without metabolic activation. A volume on 100 µL of toxin dilution was applied to the plates.

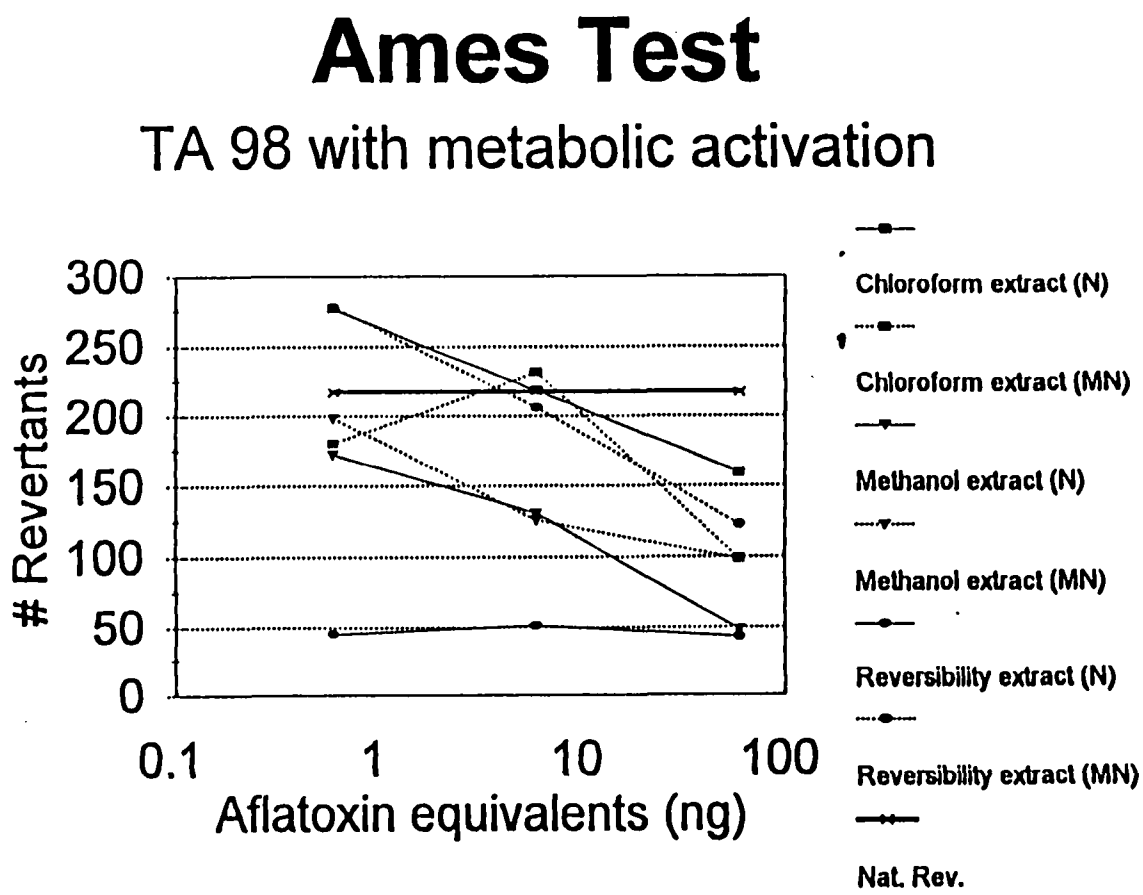

Figure 4.14 Effects of nixtamalized and modified nixtamalized tortillas on the *Salmonella*/microsomal mutagenicity assay (AMES), strain TA 98 with metabolic activation. Aflatoxin equivalents are based on original contamination level in corn 300 ng/g. N stands for nixtamalization process. MN stands for modified nixtamalization.

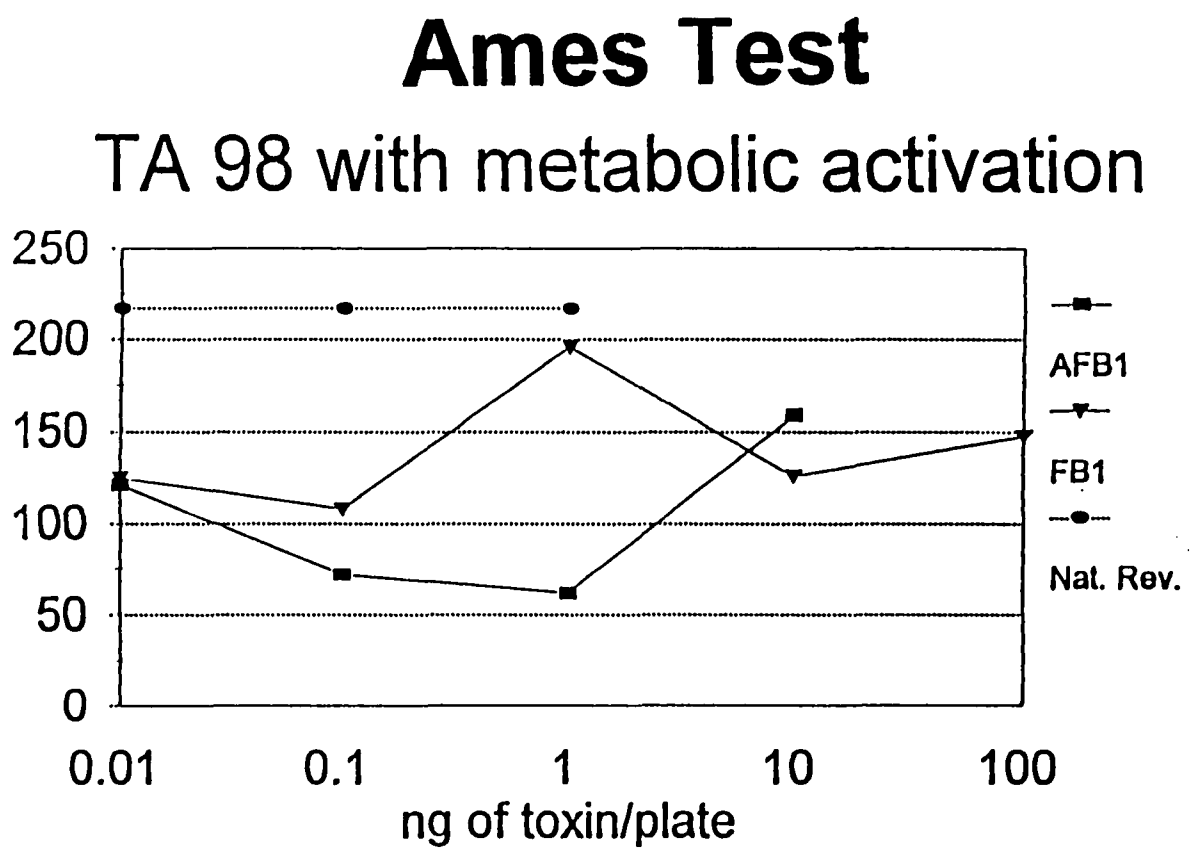

Figure 4.15 Effects of aflatoxin B and fumonisin B standards on the *Salmonella*/microsomal mutagenicity assay (AMES), strain TA 98 with metabolic activation. A volume on 100 µL of toxin dilution was applied to the plates.

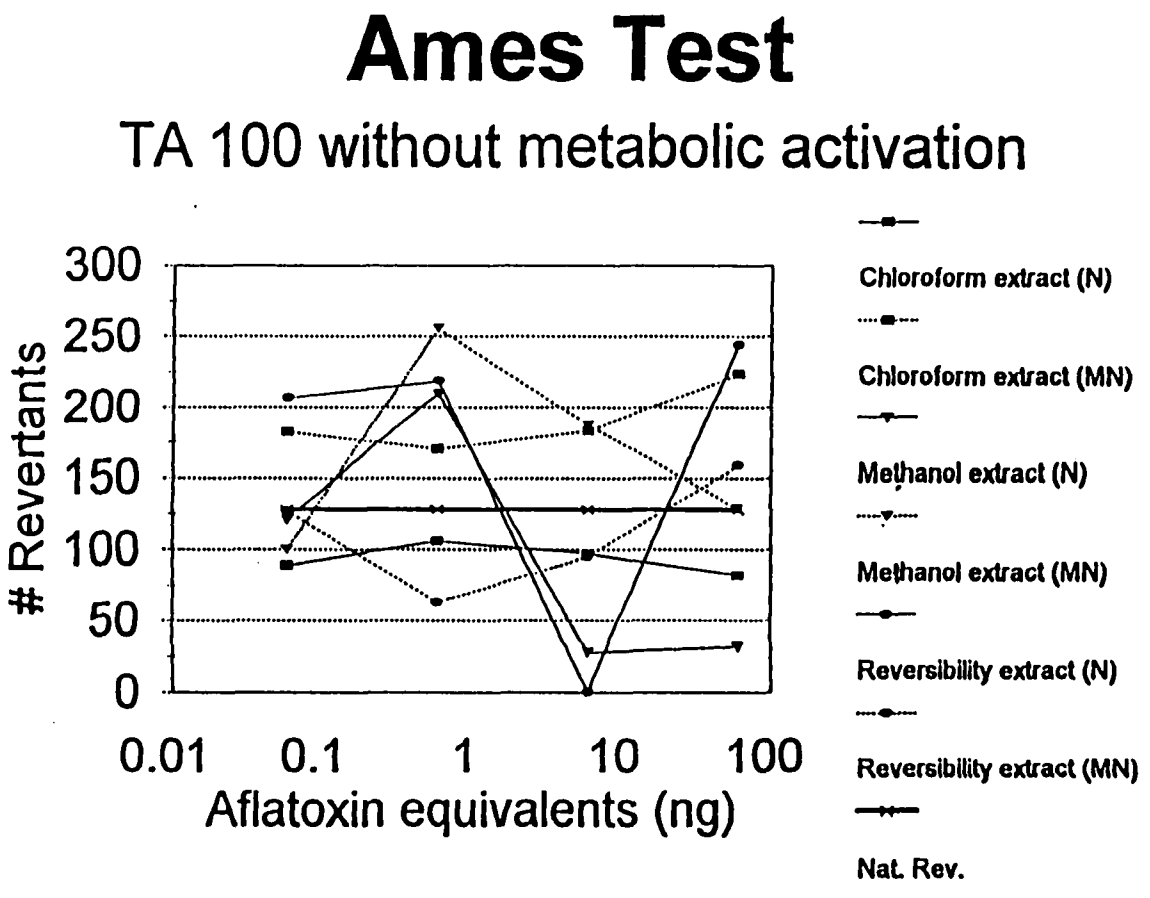

Figure 4.16 Effects of nixtamalized and modified nixtamalized tortillas on the *Salmonella*/microsomal mutagenicity assay (AMES), strain TA 100 without metabolic activation. Aflatoxin equivalents are based on original contamination level in corn 300 ng/g. N stands for nixtamalization process. MN stands for modified nixtamalization.

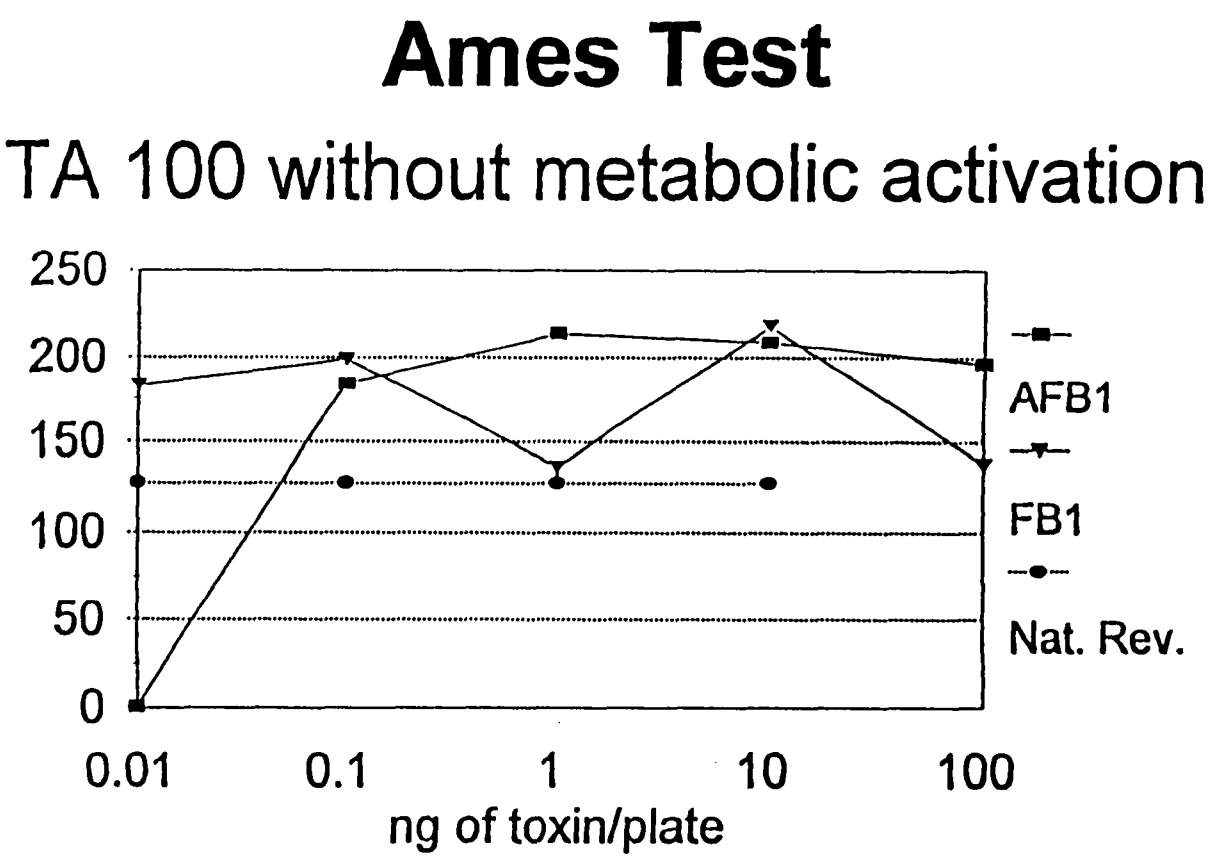

Figure 4.17 Effects of aflatoxin B and fumonisin B standards on the *Salmonella*/microsomal mutagenicity assay (AMES), strain TA 100 without metabolic activation. A volume on 100 µL of toxin dilution was applied to the plates.

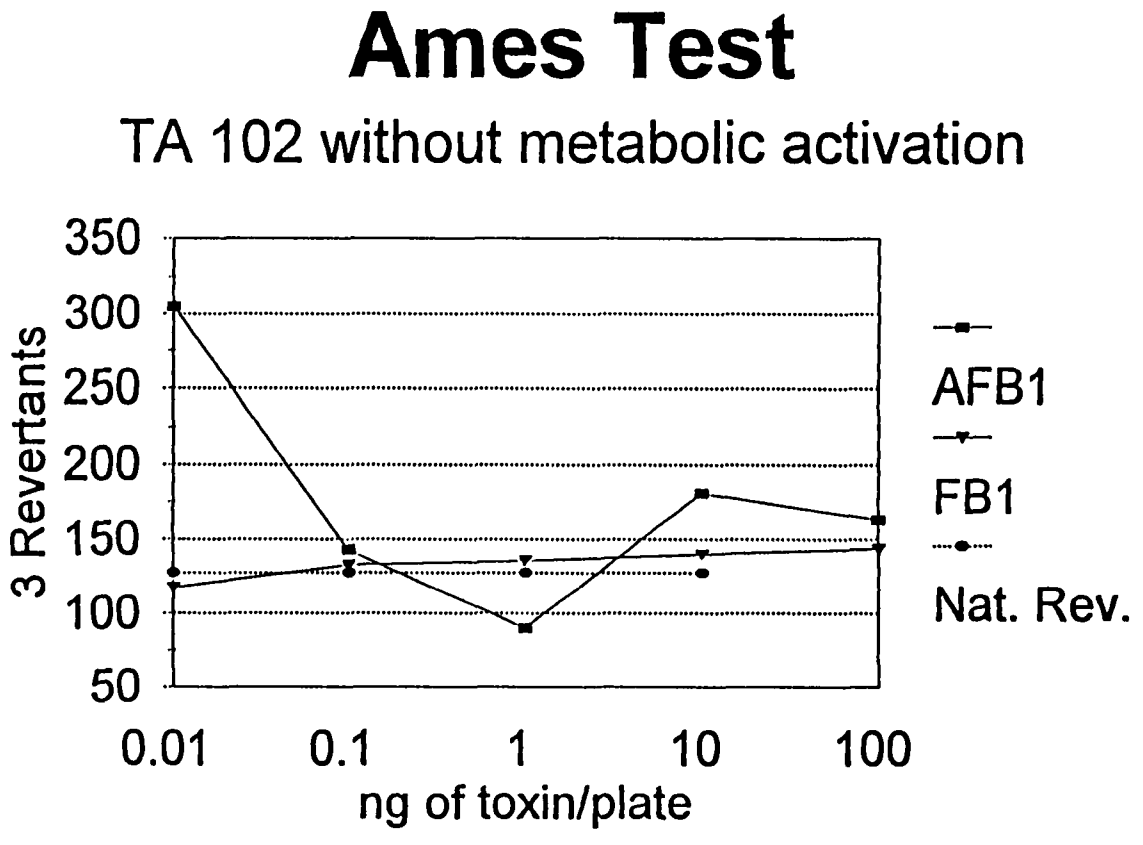

Figure 4.18 Effects of nixtamalized and modified nixtamalized tortillas on the *Salmonella*/microsomal mutagenicity assay (AMES), strain TA 102 without metabolic activation. Aflatoxin equivalents are based on original contamination level in corn 300 ng/g. N stands for nixtamalization process. MN stands for modified nixtamalization.

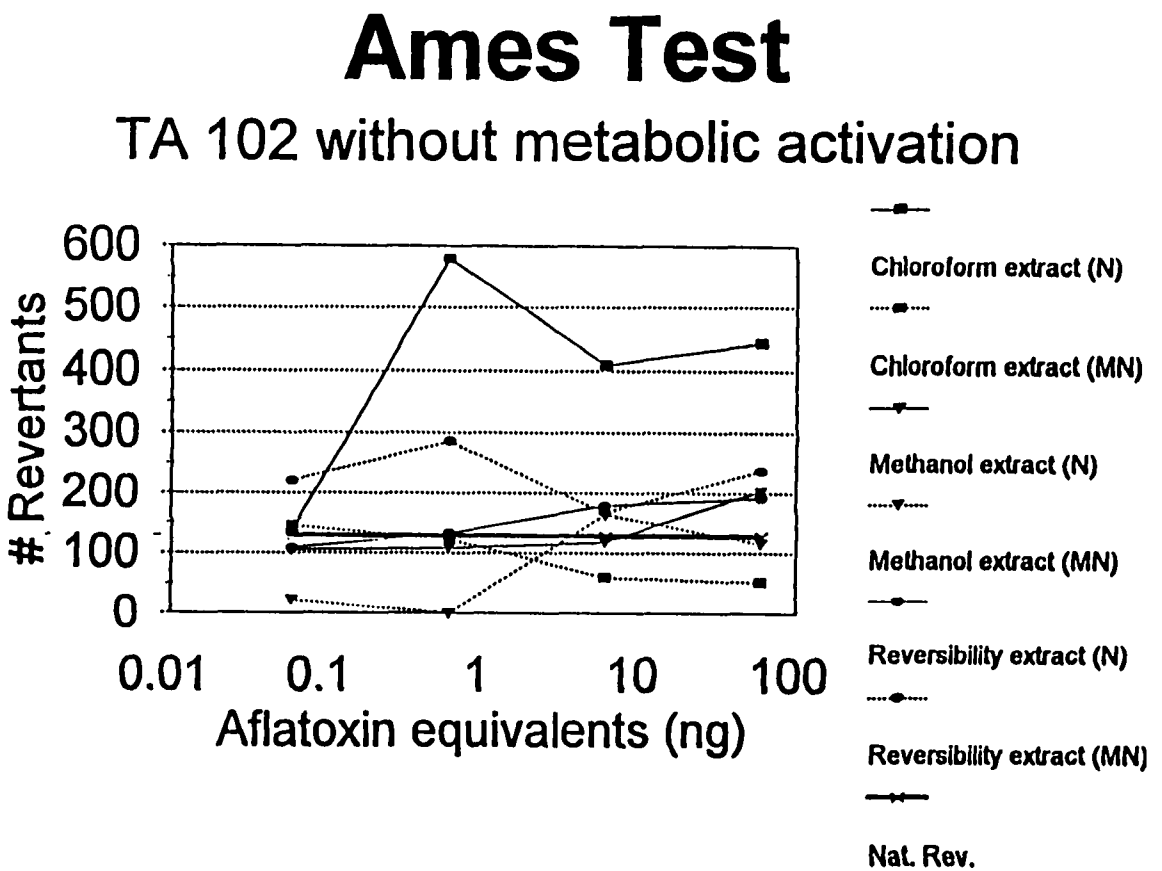

Figure 4.19 Effects of aflatoxin B and fumonisin B standards on the *Salmonella*/microsomal mutagenicity assay (AMES), strain TA 102 without metabolic activation. A volume on 100 µL of toxin dilution was applied to the plates.

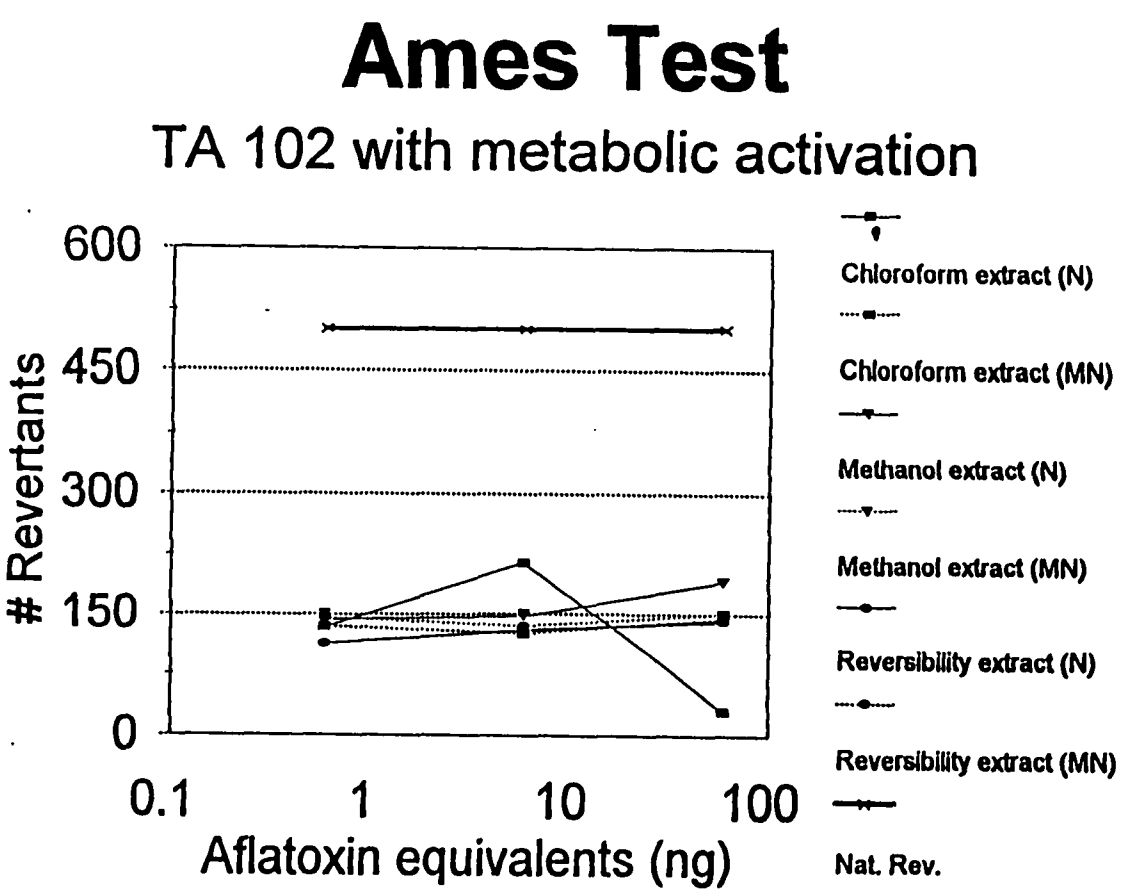

Figure 4.20 Effects of nixtamalized and modified nixtamalized tortillas on the *Salmonella*/microsomal mutagenicity assay (AMES), strain TA 102 without metabolic activation. Aflatoxin equivalents are based on original contamination level in corn 300 ng/g. N stands for nixtamalization process. MN stands for modified nixtamalization.

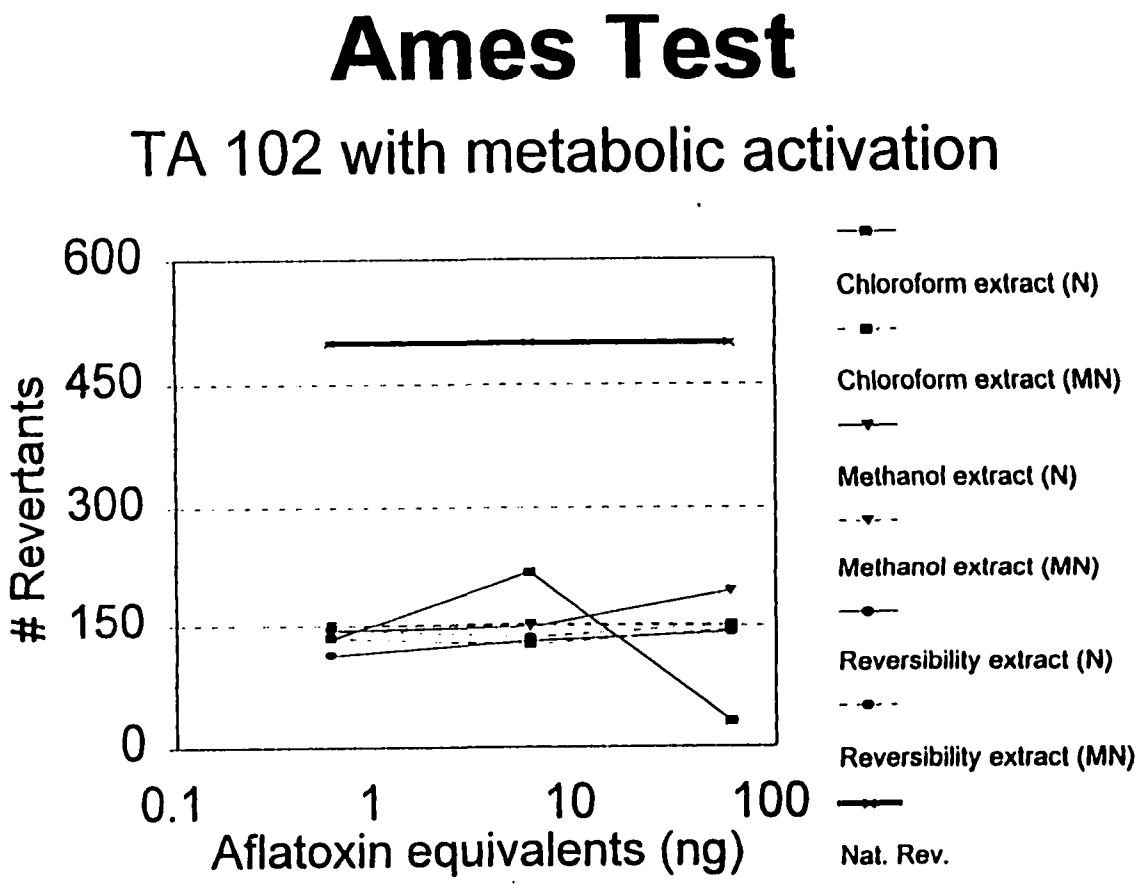

Figure 4.21 Effects of aflatoxin B and fumonisin B standards on the *Salmonella*/microsomal mutagenicity assay (AMES), strain TA 102 with metabolic activation. A volume on 100 µL of toxin dilution was applied to the plates.

No data was obtained from the same strain with metabolic activation. Figure 4.18 shows the extracts tested for mutagenicity with TA 102 without metabolic activation. Chloroform extract of the normal nixtamalization process resulted mutagenic at 0.6 ng aflatoxin equivalents (original sample concentration aflatoxin B_1 300 ng/g). Natural revertants data was obtained for the same strain with metabolic activation from Park *et al.*, 1996. The data showed no mutagenicity for either of the extracts (Figure 4.20).

Sensory evaluation showed no statistical difference in odor, flavor, or taste between the modified nixtamalization and the nixtamalization processes (Figure 4.22). A triangle test protocol was performed by the panelists to detect differences between the samples (see appendixes B and C). A total of 96 trials were conducted. According to Roessler *et al.* (1978), 44 correct responses were necessary to observe a significant difference between the compared samples ($p \leq 0.005$).

Data showed that the treated tortillas are more blue-green than the normal tortillas. Panelists comments, however, indicated that the darker color was not offensive. Texture, on the other hand, presented differences between the untreated and the treated tortillas easily to detect by the panelists. During the preparation of the treated tortillas, ammonia odor was detected. However, the odor decreased during the process of cooking. Ammonia odor was not detected by the panelists in any of the cooked samples. The sensory evaluation overall gave a good feedback for the modified nixtamalization.

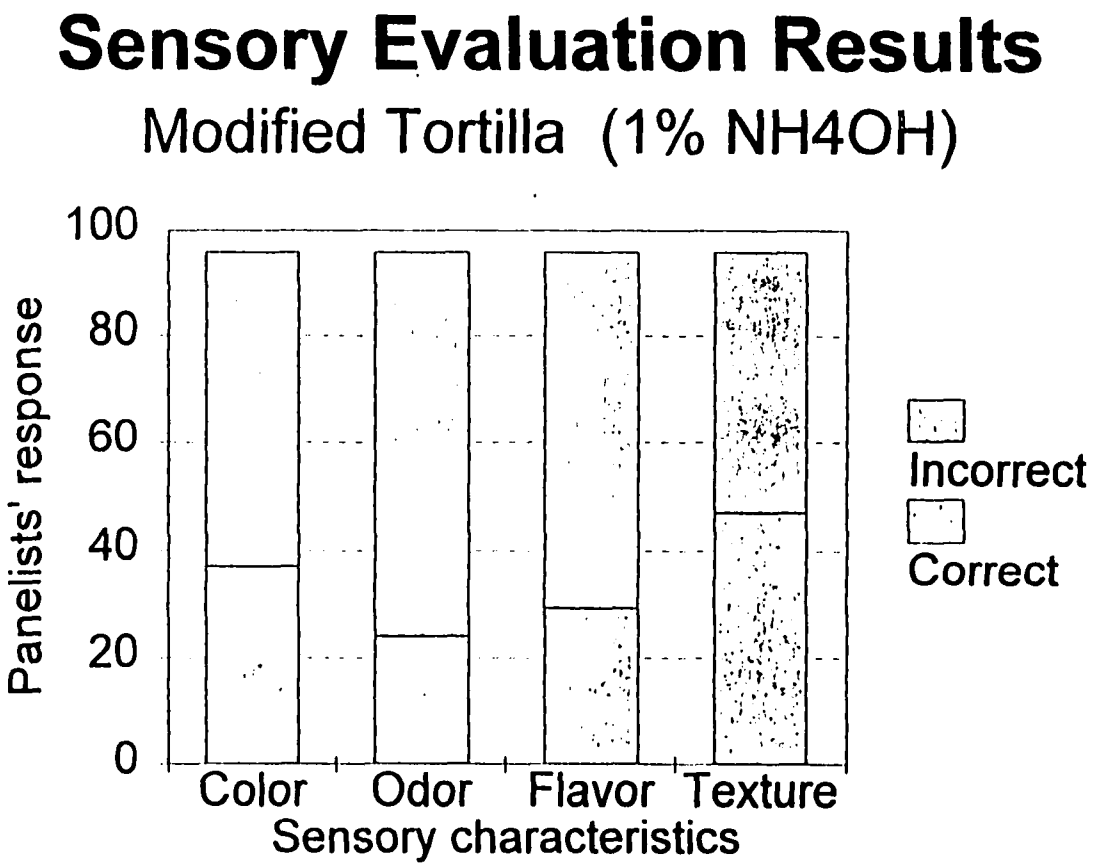

Figure 4.22 Sensory evaluation response on modified nixtamalization (1% NH OH) tortilla versus normal nixtamalization tortilla. The protocol performed is known as triangle test (two even samples, one odd sample). Significance difference was demonstrated when 44 or more positive responses were obtained ($p \leq 0.005$). A total of 96 trials were performed.

During the evaluation of corn products obtained from the Tucson area, fumonisin levels in the range of 2 ppm were detected in the starting material. Levels of FB_1 decreased to undetectable in the final products (Table 3.1). This data confirms that the process of nixtamalization decreases levels of fumonisin in naturally contaminated corn. Hopmas *et al.* (1993) suggested the possibility of producing a more toxic compound after hydrolysis of fumonisins through nixtamalization. However, embryo toxicity was not changed during this process. More research is required.

5. CONCLUSIONS

All ammonium-based chemicals resulted in a reduction in the levels of aflatoxin and fumonisin in both processes (nixtamalization and fermentation).

The best treatment chosen for the performance of safety evaluation tests was 1% ammonium hydroxide during nixtamalization.

No teratogenic or toxic potential modification were observed in the avian embryo assay when treated samples were utilized.

No mutagenic potential was observed during the performance of the *Salmonella*/microsomal mutagenicity assay while strain TA 102 was utilized. However, inconclusive results were obtained while strains TA 98 and TA 100 were used.

An acceptable product was the overall grade of the semi-trained panelists during sensory evaluation.

Although these studies need to be continued, results support the addition of 1% ammonium hydroxyde to the nixtamalization process for corn to reduce risks posed by aflatoxin and fumonisin contamination.

LITERATURE CITATIONS

Adegoke, G.O., Otumu, E.J., and Akanni, A.O. 1994. Influence of grain quality, heat, and processing time on the reduction of aflatoxin B1 levels in "tuwo" and "ogi": two cereal based products. *Plant Foods for Human Nutrition*. 45: 115-117.

Alexopoulus, C.J., and Mims, C.W. 1979. Introductory Mycology. Third Edition. John Wiley and Sons. New York, NY. 632.

Ames, N.B., and Maron, M.D. 1983. Revised methods for the Salmonella mutagenicity test. *Mutation Research*. 113: 173-215.

Apsimon, J.W. 1994. The biosynthetic diversity of secondary metabolites. In: *Mycotoxin in Grain: Compounds other than aflatoxins*. J.D. Miller and H.L. Trenholm, Eds. Eagan Press, St. Paul, Minnesota. 48.

Arriola, M.C., Porres, E., Cagrera, S., Zepeda, M., Rolz, C. 1988. Aflatoxin fate during alkaline cooking of corn for tortilla preparation. *J. Agric. Food Chem*. 36: 530-533.

Atawodi, S.E., Atiku, A.A., and Lamorde, A.G. 1994. Aflatoxin contamination of nigerian foods and feedingstuffs. *Food Chemical Toxicology*. 32 (1): 61-63.

Bacon, C.W., Porter, J.K., and Norred, W.P. 1995. Toxic interaction of fumonisin B1 and fusaric acid by injection into fertile chicken eggs. *Mycopathologia* 129: 29-35.

Bhatnagar, D., Cleveland, T.E., and Cotty,P.J. 1994. Mycological aspects of aflatoxin formation. In: *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance*. 327-346

Black, L.T. 1982. Integrated process for ammonia inactivation of aflatoxin-contaminated corn and ethanol fermentation. *Appl. Eviron. Microbiol.* 43 (4): 961-963.

Bothast, R.J., Nofsinger, G.W., Lagoda, A.A., and Black, L.T. 1982. Integrated process ofr ammonia inactivation of aflatoxin-contaminated corn and ethanol fermentation. *Appl. Environ. Microbiol.* 43(4): 961-963.

Bothast, R.J., Bennett, G.A., Vancauwenberge, J.E., and Richard, J.L. 1992. Fate of fumonisins B1 in naturally contaminated corn during ethanol fermentation. *Appl. Environ. Microbiol.* 58(1): 233-236.

Branham, B.E., and Plattner, R.D. 1993. Alanine is a precursor in the biosynthesis of fumonisin B1 by Fusarium moniliforme. *Mycopathologia* 124: 99-104.

Bresani, R., Schrimshaw, N.S. 1958. Effect of lime treatment on in vitro availability of essential aminoacids and solubility of protein fraction in corn. *J. Agric. Food Chem.* 6: 774-778.

Brekke, O.L., Peplinski, A.F., Nofsinger, G.W., Conway, H.F., Stringfellow, A.C., Montgomery, R.R., Silman, R.W., Sohns, V.E., and Bagley, E.G. 1975. Pilot-plant dry milling of corn containing aflatoxin. *Cereal Chemistry.* 52: 205-211.

Brown, T.P., Rottinghaus, G.E., Williams, M.E. 1992. Fumonisins mycotoxicosis in broilers:Performance and pathology. *Am. Assoc. Avian Pathol.* 36: 450-454.

Busby, W.F., and Wogan, G.N. 1984. Aflatoxins. In Chemical Carcinogenes. ACS Monograph 182 (2). C.E. Searle, Ed. ACS, Washington, DC. 945-1048.

Chang, P.K., Skory, C.D., and Linz, J.E. 1992. Cloning of a gene associated with aflatoxin B1 biosynthesis in Aspergillus parasiticus. *Current Genetics.* 21: 231-233.

Chen, J., Mirocha, C.J., Xie, W., Hogge, L., Olson, D. 1992. Production of the mycotoxin fumonisin B1 by Alternaria alternata f. sp. lycopersici. *Appl. Environ. Microbiol.* 58: 3928-3931.

Christensen, C.M., Mirocha, C.J., and Meronuck, R.A. 1977. Mold, mycotoxinsm and mycotoxicoses. *Agricultural Experiment Station Miscellaneous Report* 142. University of Minnesota, St. Paul, Minnesota.

Chu, F.S., Chang, C.C., Ashoor, S.H., and Prentice, N. 1975. Stability of aflatoxin B1and ochratoxin A in brewing. *Appl. Environ. Microbiol.* 29 (3): 313: 316.

Ciegler, A. 1978. Trichothecens: occurrence and toxicoses. *J. Food Prot.* 41: 399-403.

Ciegler, A., Lillehoj, E.B., Peterson, R.E., and Hall, H.H. 1966. Microbial detoxification of aflatoxin. *Applied Microbiology.* 14: 934-939.

Cole, R.J. 1989. Technology of aflatoxin decontamination. Mycotoxins and Phycotoxins '88. S. Natori, K. Hashimoto, and Y. Ueno, Eds., Amsterdan. 177-184.

Colvin, B.M., and Harrison, L.R. 1992. Fumonisin induced pulmonary edema and hydrothorax in swine. *Mycopathologia*. 117: 79-82.

Coulombe, R.A. 1994. Nonhepatic disposition and effects of aflatoxin B1. In *The Toxicology of Aflatoxins*. David L. Eaton and John, D. Grropman, Eds. Academic Press. San Diego, California. 89-101.

Cullen, J.M., and Newbern, P.M. 1994. Experimental hepatotoxicity of aflatoxins. In *The Toxicology of Aflatoxins*. David L. Eaton and John, D. Groopman, Eds. Academic Press. San Diego, California. 3-26.

Dashwood, R.H., Arbogast, D.N., Fong, A.T., Hendricks, J.D., and Bailey, G.S. 1988. Mechanisms of anti-carcinogenesis by indole-3-carbinole: detailed in vivo DNA binding dose-response studies after dietary administration with aflatoxin B1. *Carcinogenesis*. 9(3): 427-432.

Dickens, J.W. and Whitaker, T.B. 1975. Efficacy of electronic color sorting and hand picking to remove aflatoxin-contaminated kernels from contaminated lots of shelled peanuts. *Peanut Science*. 2: 45-50.

Diener, U.L. 1995. Mycotoxicology Newsletter. An International Forum for Mycotoxins. VI(1): 1.

Dupuy, J., Le bars, P., Boundra, H., Le bars, J. 1993. Thermostability of fumonisin B1, a mycotoxin from Fusarium moniliforme in corn. *Appl. Environ. Microbiol.* 59(9): 2864-2867.

Flannigan, B. 1989. Importance of mycotoxins in the brewing process. In *Biodeterioration Research* 2: 191-200. Plenum Press, New York.

Ficham, J.E., Marasas, W.F.O., Taljaard, J.J.F., Krierk, N.P.J., Badenhorst, C.J., Gelderblom, W.C.A., Seier, J.V., Smuts, C.M., Feber, M., and Weight, M.J. 1992. Atherogenic effects in non-human primate of Fusarium moniliforme cultures added to a carbohydrate diet. *Atherosclerosis*. 94: 13-25.

Food and Drug Admnistration. 1983. Aflatoxin contaminated corn: FDA's policy regarding interstate shipment of corn harvested in 1983. *Federal Register 48*. 53175-53176.

Food and Drug Administration. 1993. Everything added to food in the United States. C.K. Smoley, Ed. Boca Raton, Florida.

Fraumeni, J.F., and Blot, W.J. 1977. Geographic variation in esophageal cancer mortality in the Unites States. *J. Chron. Dis.* 30: 759-767.

Fremy, J.M., Gautier, J.P., Herry, M.P., Terrier, C., and Calet, C. 1988. Effects of ammoniation on the "carry over" of aflatoxins into bovine milk. *Food Additives and Contaminants.* 5: 39-44.

Frobish, R.A., Bradley, B.D., Wagner, D.D., Long-Bradley, P.E., and Hairston, H. 1986. *J. Food Prot.* 49: 781-785.

Fukayama, M.Y., and Hsieh, D.P.H. 1985. Effect of butylated hydroxytoluene pretreatment on the excretion, tissue distribution, and DNA binding of [14C] aflatoxin B1 in the rat. *Food Chem. Tox.* 23 (6): 567-573.

Glew, R.H. 1992. Textbook of biochemistry with clinical correlations. Thomas M. Devlin, Ed. Third Edition. Wiley-Liss. New York, NY. 449-461.

Guo, B.Z., Russin, J.S., Brown, R.L., Cleveland, T.E., and Widstrom, N.W. 1995. Resistance to aflatoxin contamination in corn as influenced by relative humidity and kernel germination. *J. Food Protect.* 59 (3): 276-281.

Guzman-de-Pena, D., Trudel, L., and Wogan, G.N. 1995. Corn "nixtamalization" and the fate of radiolabelled aflatoxin B1 in hte tortilla making process. *Bulletin of Environ. Cont. Toxicol.* 55: 858-864.

Haliburton, J.C., and Buck, W.B. 1986. Equine leucoencephalomalacia: An historical review. *Vet. Med. Anim. Sci.* 33: 75-79.

Harrison, L.R., Colvin, B.M., Greene, J.T., Newman, L.E., Cole, J.R. 1990. Pulmonary edema and hydrothorax in swine produced by fumonisin B1, a toxic metabolite of Fusarium moniliforme. *J. Vet. Diagn. Invest.* 2: 217-221.

Haschek, W.M., and Haliburton, J.C. 1986. Fusarium moniliforme and zearalenone toxicoses in domestic animals: A review. Diagnosis of *Mycotoxicoses.* 83: 213-235.

Hao, D.Y.Y., Brackett, R.E., and Nakayama, T.O.M. 1987. Removal of aflatoxin B1 from peanut milk by Flavobacterium aurantiacum. Summary and recommendations of the International Workshop on Aflatoxin Contamination of Groundnut. (India: ICRISAT Center).

Hendrich, S., Miller, K.A., Wilson, T.M., Murphy, P.A. 1993. Toxicity of Fusarium proliferatum-fermented nixtamalized corn-based diets fed to rats: Effects of nutritional status. *J. Agric. Food Chem.* 41: 1649.

Hopmans, E.C., Murphy, P.A. 1993. Detection of fumonisins B1, B2 and B3 and hydrolyzed fumonisin B1 in corn-containing foods. J. *Agric. Food Chem.* 41: 1655.

Huff, W.E., 1980. A physical method for the segregation of aflatoxin contaminated corn. *Cereal Chemistry.* 57: 236-238.

Javed, T., Richard, J.L., Bennett, G.A., Dombrink-Kurtzman, M.A., Bunte, R.M.,Koelkebeck, K.W., Cote, L.M., Leeper, R.W., and Buck, W.B. 1993. Embryopathic and embryocidal effects of purified fumonisin B1 or Fusarium proliferatum culture material extract on chicken embryos. *Mycopathologia* 123: 185-193.

Jorgensen, K.V., Park, D.L., Rua, S.M. Jr, and Price, R.L. 1990. Reduction of mutagenic potentials in milk: Effects of ammonia treatment on aflatoxin-contaminated cottonseed. *J. Food Prot.* 53: 777-779.

Jorgensen, K.V. and Price, L.R. 1981. Atmospheric pressure-ambient temperature reduction of aflatoxin B1 in ammoniated cottonseed. *J. Ag. Fd. Chem.* 29: 555-558.

Kmet, J., and Mahboudi, E. 1972. Esophaegeal cancer in the caspian litoral of Iran: Initial studies. *Science.* 175: 846-853.

Lawlord, T.E., Haworth, S.R., Zeiger, E., Park, D.L. amd Lee, L.S. 1985. Mutagenic potential of ammonia-related aflatoxin reaction products in cottonseed meal. *J. AOCS.* 62(7): 1136-1138.

Ledoux, D.R., Brown, T.P., Weibking., T.S. 1992. Fumonisin toxicity in broiler chicks. *J. Vet. Diagn. Invest.* 4: 330-333.

Leslie, J.F., Plattner, R.D., Desjardins, A.E., and Klittich, C.J.R. 1992. Fumonisin B1 production by strains from different mating populations of Gibberella fujikuroi (Fusarium section liseola). *Mycotoxicology.* 82(3): 341-345.

Leslie, J.F., Marasas, W.F., Shephard, G.S., Sydenham, E.W., Stockenstrom, S., and Thiel, P.G. 1996. Duckling toxicity and the production of fumonisin and moniliformin by isolates in the A and F mating populations of Gibberella fujikuroi (Fusarium moniliforme). *Appl. Environ. Microbiol.* 62 (4): 1182-1187.

Lynch, G.P. 1979. Biological effects of mycotoxin on ruminants. In *Proc. Symp. Interaction of Mycotoxin in Anim. Production.* Am. Dairy Sci. Assoc. and Am. Soc. Anim.Sci. Michigan State University, Natl. Acad. Sci. Washington, DC.

Machorro, V.L., and Valdivia, L.A. 1987. Cambios cuantitativos en las aflatoxinas durante el proces de la nixtamalizacion y elaboracion de tortillas. *Tecnol. Alimnent.* 19: 10-12.

Maga, J.A. 1993. Types of food additives. In *Food Additive Toxicology.* Joseph A. Maga and Anthony T. Tu, Eds. New York, NY. 1-9.

Marasas, W.F.O., Kellerman, T.S., Gelderblom, W.C.A., Coetzer, J.A., Thiel, P.G. 1992. Leucoencephalomalacia in a horse induced by fumonisin B1 isolated from Fusarium moniliforme. Ondersteport. *J. Vet. Res.* 55: 197-203.

Maron, D.M., and Ames, B.N. 1983. Revised methods for *Salmonella* mutagenicity test. *Mutat. Res.* 113: 173-215.

Marth, E.H., and Doyle, M.P. 1979. Updated on molds: Degradation aflatoxin. *Food Technology.* 33: 81-87.

Mayes, P.A. 1993. Harper's Biochemistry. 23rd. Edition. Murray, R.K., Granner, D.K., Mayes, P.A., and Rodwell, V.W., Eds. Appleton abd Lange. Norwalk, Connecticut. 241-249.

Mercado, C.J., Real, M.P.N., Del Rosario, R.R. 1991. Chemical detoxification of aflatoxin-containing copra. *J. Food Science.* 53(3): 733-735.

Miller, J.D. 1994. Epidemiology of Fusarium ear disease of cereals. In *Mycotoxin in Grain Compounds other than aflatoxins.* J.D. Miller and H.L. Trenholm, Eds. Eagan Press, St. Paul, Minnesota. 19-36.

Monroe, D.H. and Eaton, D.L. 1987. Comparative effects of butylated hydroxyanisole on hepatic in vivo DNA and in vitro biotransformation of aflatoxin B1 in the rat and mouse. *Tox. Appl. Pharm.* 90: 401-409.

Nelson, P.E.., Toussoun, T.A., Marasas, W.F.O. 1983. Fusarium species. An illustrated manual for identification. The Pennsylvania State University Press. 48.

Nelson, P.E., Plattner, R.D., Shackelford, D.D., and Desjardins, A.E. 1992. Production of fumonisins by Fusarium species other than F. Moniliforme in section Liseola and by some related species. *Appl. Environ. Microbiol.* 58: 948-989.

Newberne, P.M. 1987. Interaction of nutrients and other factors with mycotoxins. In *Mycotoxins in food.* P. Krogh, Ed. New York, NY. 177-216.

Nofsinger, G.W. and Bothast, R.J. 1981. Ethanol production by Zymomonas mobilis and Saccharomyces uvarum on aflatoxin-contaminated and ammonia-detoxified corn. *Canadian Journal of Microbiology.* 27: 162-167.

Norred, W.P., Voss, K.A., Bacon, C.W., and Riley, R.T. 1991. Effectiveness of ammonia treatment in detoxification of fumonisin-contaminated corn. *Fd. Chem. Toxic.* 29: 815.

Osweiler, G.D., Ross, P.F., Wilson, T.M., Nelson, P.E., Witte, S.T. 1992. Characterization of an epizootic of pulmonary edema in swine associated with fumonisin in corn screening. *J. Vet. Diag. Invest.* 4: 53-59.

Paredes-Lopez, O., and Saharopulos, M.E. 1982. A review of tortilla production technology. *Bakers Digest.* Sept: 13-25.

Park, D.L., LaFarge-Frayssinet, C., Yvon, M. 1981. Decontamination of aflatoxin-contaminated peanut meal using monimethylamine:Ca(OH)2. *J. AOCS.* December, 995-1002.

Park, D.L., Lee, L.S., Price, R.L., and Pohland, A.E. 1988. Review of the decontamination of aflatoxins by ammoniaton: Current status and regulation. *J. AOAC.* 71:685-703.

Park, D.L., and Stoloff, L. 1989. Aflatoxin control-how a regulatory agency manage risks from an unavoidable natural toxicant in food and feed. *Reg. Tox. Pharm.* 9: 109-130.

Park, D.L. 1992. Perspective on mycotoxin decontamination procesures. *Microb. Hyg. Ali.* 4: 21-27.

Park, D.L., Rua, S.M., Mirocha, C.J., Abd-Alla, E.A.M., and Weng, C.Y. 1992. Mutagenic potentials of fumonsin contaminated corn following ammonia decontamination procedure. *Mycopathologia*. 117: 105-108.

Park, D.L. 1993a. Controlling aflatoxin in food and feed. *Food Technology*. October, 92-96.

Park, 1993b. Perspectives on mycotoxin decontamination procedures. *Food Add. Cont.* 10(1): 49-60.

Park, D.L. 1995. Surveillance programmes for managing risks from naturally occurring toxicants. *Food Add. Cont.* 12(3): 361-371.

Pestka, J.J., Azcona-Olivera, J.I., Plattner, R.D., Minervini, F., Doko, M.B., Visconti, A. 1992. Comparative assessment of fumonisin in grain-based foods by ELISA, GC/MS and HPLC. *Research note*. Michigan State University.

Pitt, R.E. 1993. A dercriptive model of mold growth and alfatoxin formation as affected by environmental conditions. *J. Food Prot.* 56(2): 139-146.

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

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

Phillips, T.D., Clement, B.A., Kubena, L.F., and Harvey, R.B. 1988b. Mycotoxins: Detection and detoxification. Proceedings of the 23rd National Meeting. *Poultry Health and Condemnations*, October 20 and 21, Ocean City, Maryland.

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

Price, D.W., Lovell, R.A., and McChesney, D.G. 1993. Naturally occurring toxins in feedstuffs: center for veterinary medicine perspective. J. Animal Science. 71: 2556-2562.

Price, R,L. 1989. Improve food safety through discovery and control of natural and induced toxicants. Progress Report, USDA/CSRS/ARZT, October 17.

Price, R.L., and Jorgensen, K.V. 1985. Effects of processing on aflatoxin levels and on mutagenic potential of tortilla made from naturally contaminated corn. *J. Food Sci.* 50: 347-349.

Price, R.L., Paulson, J.H., Lough, O.G., Gingg, C. and Kurtz, A.G. 1985. Aflatoxin conversion by diary cattle consuming naturally-contaminated whole cottonseed. *J. Food Prot.* 48(1): 11-15.

Riley, R.T., Norred, W.P., Bacon, C.W. 1993. Fungal toxins in foods: Recent concerns. *Annu. Rev. Nutr.* 13: 167-189.

Riley, R.T., Voss, K.A., Yoo, H-S., Gelderbloom, W.C.A., Merril, A.H. Jr. 1994. Mechanism of fumonisin toxicity and carcinogenesis. *J. Food Prot.* 57: 683-645.

Rosiles, M.R. 1979. Las aflatoxinas en las tortillas. *Veterinaria Med.* 10: 37-44.

Ross, P.F., Rice, L.G., Plattner, R.D., Wilson, T.M. 1991. Concentrations of fumonisin B1 in feeds associated with animal health problems. *Mycopathologia.* 114: 129-135.

Ross, F.P., Rice, L.G., Oswieler, G.D., Nelson, P.E., Richard, J.L., Wilson, T.M. 1992. A review and update of animal toxicoses associated with fumonisin-contaminated feeds and production of fumonisins by Fusarium isolates. *Mycopathologia.* 117: 109-114.

Sharma, A., Padwal-Desai, S.R., and Nadkarni, G.B. 1985. Possible implications of reciprocity between ethulene and aflatoxin biogenesis in Aspergillus flavus and Aspergillus parasiticus. *Appl. Environ. Microbiol.* 49: 79.

Sharma, A., Padwal-Desai, S.R., and Madkarni, G.B. 1987. A new method for aflatoxin-free storage of agricultural commodities. *J. Food Sc.* 52(2): 497-499.

Shephard, G.S., Sydenham, E.W., Thiel, P.G., Gelderblom, C.A. 1990. Quantitative determination of fumonisin B1 by high-performance liquid chromatography with fluorescence detection. *J. Liquid Chromat.* 13: 2077-2087.

Shephard, G.S., Thiel, P.G., Stockenstrom, S., and Sydenham, E.W. 1996. Worldwide survey of fumonisin contamination of corn and corn-based products. *J. AOAC* Int. 79 (3): 671-687.

Shier, W.T., Abbas, H.K., Mirocha, C.J. 1991. Toxicity of mycotoxins fumonisin B1 and B2 and Alternaria alternata f. sp. lycopersici toxin (AAL) in cultured mammalian cells. *Mycopathologia*. 116: 97-104.

Snijders, C.H.A. 1994. Breeding for resistance to Fusarium in wheat and maize. In *Mycotoxins in Grain: Compounds other than aflatoxin*. J.D. Miller and H.L. Trenholm, Eds. Eagan Press, St. Paul, Minnesota. 37-.

Stoloff, L., van Egmod, H.P., Park, D.L. 1990. Rationales for the establishment of limits and regulations for mycotoxins. *Food Add. Cont.* 8: 213-222.

Sydenham, E.W., Shephard, G.S., Thiel, P.G., Marasas, W.F.O., and Stockenstrom, S. 1991. Fumonisin contamination of commercial corn-based human foodstuffs. *J. Agric. Food. Chem.* 39: 2014.

Sydenham, E.W., Shephard, G.S., Thiel, P.G., Bird, C., and Miller, B.M. 1996a. Determination of fumonisin in corn: evaluation of competitive immunoassay and HPLC techniques. *J. Agric. Food Chem.* 44(1): 159-164.

Sydenham, E.W., Shephard, G.S., Thiel, P.G., Stockenstrom, S., and Snijman, P.W. 1996b. Liquid chromatographic determination of fumonisins B1, B2, and B3 in corn: AOAC-IUPAC collaborative study. *J. AOAC* Int. 79 (3): 688-696.

Tabata, S., Kamimura, H., Ibe, A., Hashimoto, H., and Tamura, Y. 1994. Degradation of aflatoxins bt food additives. *J. Food Prot.* 57(1):42-47.

Thiel, P.G., Marasas, W.F.O., Sydenham, E.W., Shephard, G.S., Gelderblom, W.C.A., Nieuwenhuis, J.J., 1991. Survey of fumonisin production by Fusarium species. *Appl. Environ. Microbiol.* 57: 1089.

Ulloa-Sosa, M., and Shroeder, H.W. 1969. Note on aflatoxin decomposition in the process of making tortilla from corn. *Cereal Chem.* 46: 397.

Van Rhijin,P. 1993. Maize Cookery. Corn-Based Recipes from Mexico. La Galera. Mexico. 191.

Watson, D. 1993. Chemical contaminants. In *Safety of Chemicals in Food*. Ellis Horwood, Ed. Great Britain.

Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T., Merril, A.H. Jr. 1991. Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with Fusarium moniliforme. *J. Biol. Chem.* 266: 14486-14490.

Wilson, T.M., Ross, P.F., Oswelter, G.D., Nelson, H. 1991. ELEM and its relationship to fumonisins in feed. Symposium. Raleigh, N.C. April 24-25.

Weng, C.Y., Martinez, A.J., and Park, D.L. 1994. Efficacy and permanency of ammonia treatment in reducing aflatoxin levels in corn. *Food Add. Cont.* 11(6): 649-658.

Wood, E. Garnett. 1989. Aflatoxin in domestic and imported food and feeds. *J. AOAC.* 72(4): 543-548.

Yahl, K.R., Watson, S.A., Smith, R.J., and Barabolak, R. 1971. Laboratory wet-milling of corn containing high levels of aflatoxin and a survey of commercial wet-milling products. *Cereal Chemistry.* 48: 385-391.

Yeagle, P.L. 1993. The membranes of cells. Phillip L. Yeagle, Ed. Second Edition. Academic Press, INC. San Diego, California. 33-37, 315-319.

APPENDIX A

Statistical Analysis

```
Worksheet retrieved from file: socrates.MTW
MTB > Set 'Chemmtr'
DATA>   1( 1 : 2 / 1 )32
DATA>   End.
MTB > Set 'ChemTr'
DATA>   2( 1 : 8 / 1 )4
DATA>   End.
MTB > Twoway 'Afla' 'Phytreat' 'ChemTr';
SUBC>   Means 'Phytreat' 'ChemTr'.

ANALYSIS OF VARIANCE  Afla

SOURCE        DF        SS        MS
Phytreat       1     10450     10450
ChemTr         7     13490      1927
INTERACTION    7     13941      1992
ERROR         48     23080       481
TOTAL         63     60960

                      Individual 95% CI
Phytreat       Mean   -+---------+---------+---------+---------+
      1         7.6    (-------*------)
      2        33.1                                (-------*-------)
               -+---------+---------+---------+---------+---------+
              0.0       10.0      20.0      30.0      40.0

                      Individual 95% CI
  ChemTr       Mean   ------+---------+---------+---------+---------
       1       48.7                                (------*-------)
       2       12.0        (-------*-------)
       3        9.4       (-------*-------)
       4        4.1   (-------*-------)
       5       29.9                 (-------*-------)
       6        6.2    (-------*-------)
       7       20.5           (-------*-------)
       8       32.1                 (-------*-------)
               ------+---------+---------+---------+---------
                   0.0       20.0      40.0      60.0

MTB > Unstack ('Afla') (c10) (c11);
SUBC>   Subscripts 'Phytreat'.
MTB > Set 'CheTreat'
DATA>   1( 1 : 8 / 1 )4
DATA>   End.
MTB > Describe 'Nixtam'-'Boiling';
SUBC>   By 'CheTreat'.

          CheTreat     N      MEAN    MEDIAN    TRMEAN     STDEV    SEMEAN
Nixtam           1     4     3.022     2.951     3.022     0.834     0.417
                 2     4     1.054     1.006     1.054     1.219     0.610
                 3     4   0.00000   0.00000   0.00000   0.00000   0.00000
                 4     4   0.00000   0.00000   0.00000   0.00000   0.00000
                 5     4     6.357     7.064     6.357     1.648     0.824
                 6     4      2.22      0.00      2.22      4.45      2.22
                 7     4    27.139    27.077    27.139     0.778     0.389
                 8     4     20.89     21.00     20.89     11.75      5.87
Boiling          1     4      94.4     104.3      94.4      66.7      33.3
                 2     4     22.85     22.85     22.85     15.80      7.90
                 3     4     18.85     18.81     18.85      2.37      1.19
                 4     4     8.160     8.190     8.160     1.370     0.685
```

	5	4	53.4	52.6	53.4	32.9	16.4
	6	4	10.26	10.07	10.26	2.51	1.25
	7	4	13.90	13.94	13.90	12.27	6.13
	8	4	43.4	42.5	43.4	39.9	19.9

	CheTreat	MIN	MAX	Q1	Q3
Nixtam	1	2.293	3.892	2.300	3.813
	2	0.000	2.203	0.000	2.155
	3	0.00000	0.00000	0.00000	0.00000
	4	0.00000	0.00000	0.00000	0.00000
	5	3.896	7.402	4.680	7.326
	6	0.00	8.89	0.00	6.67
	7	26.404	28.000	26.444	27.897
	8	10.47	31.08	10.59	31.07
Boiling	1	17.3	151.4	28.0	150.8
	2	8.81	36.90	8.99	36.71
	3	16.60	21.17	16.70	21.03
	4	6.715	9.547	6.859	9.433
	5	24.8	83.5	24.8	82.7
	6	7.89	13.04	8.02	12.71
	7	3.16	24.57	3.22	24.55
	8	8.8	79.7	8.8	78.8

```
MTB > Oneway 'Nixtam' 'CheTreat';
SUBC>   Tukey 5.

ANALYSIS OF VARIANCE ON Nixtam
SOURCE      DF        SS         MS         F        p
CheTreat     7    3072.9      439.0     21.51    0.000
ERROR       24     489.7       20.4
TOTAL       31    3562.6

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL       N      MEAN      STDEV  ------+---------+---------+---------
     1       4     3.022      0.834     (----*----)
     2       4     1.054      1.219   (----*----)
     3       4     0.000      0.000  (----*----)
     4       4     0.000      0.000  (----*----)
     5       4     6.357      1.648         (---*----)
     6       4     2.223      4.445     (---*----)
     7       4    27.139      0.778                          (----*----)
     8       4    20.890     11.746                    (----*----)
                                      ------+---------+---------+---------
POOLED STDEV =     4.517                 0        10        20        30

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00294

Critical value = 4.68

Intervals for (column level mean) - (row level mean)

              1         2         3         4         5         6         7

     2    -8.602
          12.538

     3    -7.549    -9.516
```

```
          13.592    11.624

     4    -7.549    -9.516   -10.570
          13.592    11.624    10.570

     5   -13.905   -15.873   -16.927   -16.927
           7.235     5.267     4.214     4.214

     6    -9.771   -11.739   -12.793   -12.793    -6.436
          11.369     9.401     8.348     8.348    14.704

     7   -34.688   -36.656   -37.710   -37.710   -31.353   -35.487
         -13.548   -15.516   -16.569   -16.569   -10.213   -14.347

     8   -28.439   -30.406   -31.460   -31.460   -25.104   -29.238    -4.321
          -7.298    -9.266   -10.320   -10.320    -3.963    -8.097    16.820
```

```
MTB > Oneway 'Boiling' 'CheTreat';
SUBC>   Tukey 5.

ANALYSIS OF VARIANCE ON Boiling
SOURCE      DF         SS         MS         F         p
CheTreat     7      24358       3480      3.70     0.008
ERROR       24      22590        941
TOTAL       31      46948

                                       INDIVIDUAL 95 PCT CI'S FOR MEAN
                                       BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV  ----+---------+---------+---------+-----
     1       4      94.36      66.68                          (-----*-----)
     2       4      22.85      15.80             (------*-----)
     3       4      18.85       2.37            (------*-----)
     4       4       8.16       1.37  (------*-----)
     5       4      53.36      32.88                    (------*-----)
     6       4      10.26       2.51           (-----*-----)
     7       4      13.90      12.27           (------*-----)
     8       4      43.39      39.87                  (------*-----)
                                       ----+---------+---------+---------+-----
POOLED STDEV =      30.68                0        50       100       150

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00294

Critical value = 4.68

Intervals for (column level mean) - (row level mean)

               1         2         3         4         5         6
     2      -0.3
           143.3

     3       3.7     -67.8
           147.3      75.8

     4      14.4     -57.1     -61.1
           158.0      86.5      82.5
```

```
5     -30.8    -102.3    -106.3    -117.0
      112.8     41.3      37.3      26.6

6      12.3     -59.2     -63.2     -73.9     -28.7
      155.9      84.4      80.4      69.7     114.9

7       8.7     -62.8     -66.8     -77.5     -32.3     -75.4
      152.2      80.7      76.7      66.0     111.3      68.2

8     -20.8     -92.3     -96.3    -107.0     -61.8    -104.9    -101.3
      122.8      51.3      47.3      36.6      81.8      38.7      42.3

MTB >
```

```
Worksheet size: 38000 cells
MTB > RETRIEVE 'socrates.MTW'
 WORKSHEET SAVED  1/ 1/1904

Worksheet retrieved from file: socrates.MTW
MTB > Set 'Treat'
DATA>   1( 1 : 2 / 1 )16
DATA>   End.
MTB > Set 'Chem'
DATA>   2( 1 : 8 / 1 )2
DATA>   End.
MTB > Twoway 'AFB1' 'Chem' 'Treat';
SUBC>   Means 'Chem' 'Treat'.

ANALYSIS OF VARIANCE  AFB1 .

SOURCE        DF        SS        MS
Chem           7     19576      2797
Treat          1       393       393
INTERACTION    7      4590       656
ERROR         16     13322       833
TOTAL         31     37882

                      Individual 95% CI
    Chem      Mean    -+---------+---------+---------+---------
       1       114                                (------*-------)
       2        46          (------*-------)
       3        55            (-------*------)
       4        47          (-------*------)
       5        38        (-------*------)
       6        41         (------*-------)
       7        63                 (-------*------)
       8        27     (-------*------)
                       -+---------+---------+---------+---------
                        0        40        80       120       160

                      Individual 95% CI
   Treat      Mean    -----+---------+---------+---------+------
       1      57.3                (--------------*--------------)
       2      50.3     (--------------*--------------)
                       -----+---------+---------+---------+------
                          40.0      50.0      60.0      70.0

MTB > Unstack ('AFB1') (c5) (c6);
SUBC>   Subscripts 'Treat'.
MTB > Set c4
DATA>   1( 1 : 8 / 1 )2
DATA>   End.
MTB > Describe 'Heat'-'Fermen';
SUBC>   By 'ChemTrea'.

          ChemTrea     N     MEAN   MEDIAN   TRMEAN    STDEV   SEMEAN
Heat             1     2    121.6    121.6    121.6     22.3     15.7
                 2     2     62.9     62.9     62.9     38.2     27.0
                 3     2    68.07    68.07    68.07    11.95     8.45
                 4     2     56.0     56.0     56.0     30.9     21.8
                 5     2     42.9     42.9     42.9     16.6     11.7
                 6     2    41.18    41.18    41.18    12.46     8.81
                 7     2    38.22    38.22    38.22     7.65     5.41
                 8     2    27.64    27.64    27.64    10.18     7.20
```

Fermen	1	2	105.5	105.5	105.5	67.8	47.9
	2	2	28.8	28.8	28.8	26.4	18.7
	3	2	42.2	42.2	42.2	25.2	17.8
	4	2	37.5	37.5	37.5	14.8	10.4
	5	2	33.42	33.42	33.42	2.25	1.59
	6	2	40.5	40.5	40.5	57.3	40.5
	7	2	87.8	87.8	87.8	15.3	10.8
	8	2	26.71	26.71	26.71	5.08	3.60

	ChemTrea	MIN	MAX	Q1	Q3
Heat	1	105.9	137.4	*	*
	2	35.9	89.9	*	*
	3	59.61	76.52	*	*
	4	34.2	77.9	*	*
	5	31.2	54.6	*	*
	6	32.37	49.99	*	*
	7	32.82	43.63	*	*
	8	20.44	34.84	*	*
Fermen	1	57.6	153.4	*	*
	2	10.1	47.5	*	*
	3	24.5	60.0	*	*
	4	27.1	48.0	*	*
	5	31.83	35.01	*	*
	6	0.0	81.0	*	*
	7	77.0	98.6	*	*
	8	23.11	30.30	*	*

```
MTB > Oneway 'Heat' 'ChemTrea';
SUBC>   Tukey 5.

ANALYSIS OF VARIANCE ON Heat
SOURCE      DF         SS         MS         F         p
ChemTrea     7      11994       1713      3.77     0.041
ERROR        8       3640        455
TOTAL       15      15635

                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL       N       MEAN      STDEV  ----+---------+---------+---------+-----
     1       2     121.62      22.27                          (------*------)
     2       2      62.92      38.19               (------*------)
     3       2      68.07      11.95                (------*------)
     4       2      56.05      30.85             (------*------)
     5       2      42.91      16.55          (------*------)
     6       2      41.18      12.46         (------*------)
     7       2      38.22       7.65         (------*------)
     8       2      27.64      10.18      (------*------)
                                        ----+---------+---------+---------+-----
POOLED STDEV =      21.33                  0        50       100       150

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00425

Critical value = 5.60

Intervals for (column level mean) - (row level mean)

               1         2         3         4         5         6         7
```

```
      2     -25.8
            143.2

      3     -30.9      -89.6
            138.0       79.3

      4     -18.9      -77.6      -72.5
            150.0       91.3       96.5

      5      -5.8      -64.5      -59.3      -71.3
            163.2      104.5      109.6       97.6

      6      -4.0      -62.7      -57.6      -69.6      -82.7
            164.9      106.2      111.4       99.3       86.2

      7      -1.1      -59.8      -54.6      -66.6      -79.8      -81.5
            167.9      109.2      114.3      102.3       89.2       87.4

      8       9.5      -49.2      -44.0      -56.1      -69.2      -70.9      -73.9
            178.5      119.8      124.9      112.9       99.7       98.0       95.1

MTB > Oneway 'Fermen' 'ChemTrea';
SUBC>   Tukey 5.

ANALYSIS OF VARIANCE ON Fermen
SOURCE     DF        SS        MS        F        p
ChemTrea    7     12172      1739     1.44    0.310
ERROR       8      9682      1210
TOTAL      15     21854

                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+
     1      2    105.53     67.75                 (---------*---------)
     2      2     28.81     26.41   (---------*---------)
     3      2     42.24     25.16      (---------*---------)
     4      2     37.51     14.77     (---------*---------)
     5      2     33.42      2.25    (---------*---------)
     6      2     40.49     57.26      (---------*---------)
     7      2     87.81     15.27                (---------*---------)
     8      2     26.71      5.08   (---------*---------)
                                     ------+---------+---------+---------+
POOLED STDEV =    34.79              0        60       120       180

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.00425

Critical value = 5.60

Intervals for (column level mean) - (row level mean)

              1         2         3         4         5         6         7

      2     -61.0
            214.5

      3     -74.5    -151.2
            201.0     124.3
```

```
4     -69.7    -146.5    -133.0
      205.8     129.1     142.5

5     -65.6    -142.4    -128.9    -133.7
      209.9     133.1     146.6     141.8

6     -72.7    -149.4    -136.0    -140.7    -144.8
      202.8     126.1     139.5     134.8     130.7

7    -120.0    -196.8    -183.3    -188.1    -192.1    -185.1
      155.5      78.8      92.2      87.4      83.4      90.4

8     -58.9    -135.6    -122.2    -127.0    -131.0    -124.0     -76.6
      216.6     139.9     153.3     148.6     144.5     151.5     198.9

MTB >
```

APPENDIX B

Sensory Evaluation Response Sheet

Tortilla Sensory Evaluation Trial

Date: _____ SSN#: _____

Follow the instructions.

Compare: Indicate if same (No Difference -ND) or one is different (ODD).

Sample #		Color		Odor		Flavor	
		ND	ODD	ND	ODD	ND	ODD
123	133						
334	555						
222	111						
669	233						

123	133	122
334	555	999
222	111	556
669	233	888

Comments:

APPENDIX C

Triangle Test Results

Triangle Test Results

Date	Color		Odor		Flavor		Texture	
Panelist	+	-	+	-	+	-	+	-
09/30/96								
9287	1	1	1	1	1	1	0	2
7685	0	2	0	2	0	2	0	2
9238	0	2	0	2	0	2	1	1
6734	1	1	0	2	0	2	2	0
0485	0	2	0	2	0	2	0	2
7191	2	0	0	2	0	2	2	0
8274	2	0	1	1	0	2	1	1
9371	0	2	0	2	0	2	0	2
1478	2	0	1	1	1	1	2	2
2370	2	0	2	0	1	1	2	0
7522	0	2	0	2	0	2	0	2
4760	0	2	0	2	0	2	0	2
Total	10/24	14/24	5/24	19/24	3/24	21/24	10/24	14/24
10/02/96								
9287	0	2	2	0	0	2	0	2
9371	0	2	0	2	1	1	0	2
7191	2	0	0	2	0	2	1	1
7685	0	2	0	2	0	2	0	2
4760	0	2	0	2	1	1	0	2
0485	1	1	0	2	0	2	0	2
1478	0	2	0	2	1	1	0	2
8274	2	0	0	2	1	1	0	2
6734	0	2	0	2	0	2	0	2
7522	0	2	1	1	1	1	1	1
9238	0	2	0	2	0	2	0	2
2370	1	1	0	2	0	2	0	2
Total	6/24	18/24	3/24	21/24	5/24	19/24	2/24	22/24

10/04/96								
9371	0	2	0	2	0	2	2	0
6734	0	2	0	2	0	2	2	0
4760	0	2	0	2	2	0	2	0
7191	2	0	2	0	1	1	1	1
9238	0	2	0	2	0	2	2	0
0485	1	1	0	2	1	1	1	1
1478	2	0	0	2	2	0	1	1
7685	1	1	0	2	0	2	2	0
8274	0	2	0	2	1	1	0	2
9287	1	1	2	0	0	2	1	1
7522	0	2	0	2	1	1	2	0
2370	1	1	1	1	1	1	2	0
Total	8/24	16/24	5/24	19/24	9/24	15/24	18/24	6/24
10/07/96								
9371	1	1	1	1	0	2	2	0
8274	0	2	2	0	2	0	1	1
1478	0	2	1	1	0	2	0	2
6734	0	2	0	2	0	2	1	1
9287	2	0	2	0	1	1	1	1
7191	2	0	2	0	1	1	2	0
4760	2	0	0	2	2	0	2	0
9238	0	2	0	2	1	1	1	1
7522	1	1	1	1	1	1	1	1
0485	2	0	1	1	2	0	2	0
2370	2	0	1	1	2	0	2	0
7685	1	1	0	2	0	2	2	0
Total	13/24	11/24	11/24	13/24	12/24	12/24	17/24	7/24
TOTAL	37/96	59/96	24/96	72/96	29/96	67/96	47/96	49/96

From Roessler et al., 1978, The minimum numbers of correct judgments to establish significance at various probability levels for the triangle test (one-tailed, p=1/3) may be derived from:

$$x=0.4714z\sqrt{n}+[(2n + 3)/6]$$

where n = 96
z = From Roessler et al., 1978.

For a α= 0.05, z=1.64, From Roessler et al. (1978).

$x=(0.471)(1.64)(\sqrt{96})+[(2)(96)+3]/6$

X= 7.56 + 32.5

x= 40.06

For a α= 0.01, z=2.33, From Roessler et al. (1978).

X= 10.76 + 32.5

x= 43.26

For a α= 0.005, z=2.58, From Roessler et al. (1978).

x= 11.9 + 32.5

x= 44.4

For n = 24, x =15, for α= 0.005

n= 24, x= 13, for α= 0.05

From Roessler et al., 1978.

VITA

Socrates Trujillo-Preciado was born in Culiacan, Sinaloa, Mexico, in 1969. He has two brothers and one sister. He studied at the University of Sinaloa and traveled to the United States to continue with his master's program. He changed programs at the University of Arizona and continued with his doctoral work at Louisiana State University. He has worked with safety programs including mycotoxins and seafood toxins. He wants to continue this area of research mostly in underdeveloped countries.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Socrates Trujillo

Major Field: Food Science

Title of Dissertation: Reduction and Management of Risks Associated with Aflatoxin and Fumonisin Contamination in Corn

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Robert M. Grodner

Date of Examination:

November 13, 1996