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 OF AFLATOXINS
IN AFRICAN PEANUT AND PEANUT PRODUCTS

by

Salah Aldin Mahdi

A Dissertation Submitted to the Faculty of the
COMMITTEE ON NUTRITIONAL SCIENCES (GRADUATE)
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1995
THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have
read the dissertation prepared by SALAH AL DIN MA HDI
entitled REDUCTION OF A FLATOXINS IN AFRICAN PEANUT AND
PEANUT PRODUCTS

and recommend that it be accepted as fulfilling the dissertation
requirement for the Degree of DOCTOR OF PHILOSOPHY

Ralph L. Price

Date 4/7/95

Charles M. Weber

Date 4/7/95

Maria Luz Fernandez

Date 4/7/95

Ann M. Tinsley

Date 4/7/95

Charles P. Gerba

Date 3/8/95

Final approval and acceptance of this dissertation is contingent upon
the candidate's submission of the final copy of the dissertation to the
Graduate College.

I hereby certify that I have read this dissertation prepared under my
direction and recommend that it be accepted as fulfilling the dissertation
requirement

Dissertation Director

Date 10/24/95
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: [Signature]
ACKNOWLEDGMENTS

I wish to express my deepest and sincerest gratitude to my advisor and dissertation director, Dr. Ralph L. Price, for his invaluable guidance, patience, and friendship during this study.

Also I would like to express my deep appreciation to all my committee members, Dr. Bobby Reid, Dr. Charles Weber, Dr. Charles Gerba, Dr. Maria Luz Fernandez, and Dr. Ann Tinsley for their assistance and their participation on the examining committee.

Special thanks to Dr. Fernando Antonio Cardoso for his advise, tolerance and help. Thanks to Socrates Trujillo for his friendship and help, to Joan Weber for friendship and typing, and to Karen Kornman for help with the graphs.
DEDICATION

This work is dedicated to the memory of my Mother, Halima, and Grandfather, Khurshid.
With love.
TABLE OF CONTENTS

LIST OF FIGURES ........................................ 9

ABSTRACT .................................................. 10

CHAPTER 1 .................................................. 11

INTRODUCTION ............................................. 11

CHAPTER 2 .................................................. 14

LITERATURE REVIEW ..................................... 14
   Chemical Structure of the Aflatoxins .................. 14
   Description of Aspergillus ................................ 16
   History of the discovery of Mycotoxins ................. 17
   Metabolism and Carcinogenicity .......................... 18
   Fungal Contamination during Growth and Storage of Foodstuffs ................. 25
   Reduction of Aflatoxin Exposure ......................... 28
   Chemistry of Detoxification of Aflatoxins .............. 31
   Toxicity Estimation ..................................... 33
   Mutagenicity of Aflatoxins ................................ 34
   Biological Detoxification - Microbial Inactivation .... 35
   Water Treatment ......................................... 36
   Water Re-fluxing ....................................... 36
   Heat Treatment .......................................... 37
   Ultraviolet Light ......................................... 37
   Bright Sunlight .......................................... 38
   Gamma Rays .............................................. 38
   Adsorption ............................................... 38
   Food Processing ......................................... 39
   Extrusion ................................................. 40
   Acid Treatment .......................................... 41
   Treatment with Organic and Inorganic Bases ............. 41
   Calcium Hydroxide ...................................... 42
   Sodium Hydroxide ....................................... 42
   Ozonization of Aflatoxin ................................ 43
   Ammonia Detoxification ................................ 43
   Bisulfite Degradation ................................... 45
   Hydrogen Peroxide Detoxification ....................... 46
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen Peroxide and Calcium Hydroxide</td>
<td>47</td>
</tr>
<tr>
<td>Sodium Hypochlorite Detoxification</td>
<td>48</td>
</tr>
<tr>
<td>Sodium Hypochlorite and Calcium Hydroxide</td>
<td>49</td>
</tr>
<tr>
<td>Detoxification using Extrusion and Chemicals</td>
<td>49</td>
</tr>
<tr>
<td>CHAPTER 3</td>
<td>51</td>
</tr>
<tr>
<td>MATERIAL AND METHODS</td>
<td></td>
</tr>
<tr>
<td>Source of Chemicals and Solvents</td>
<td>51</td>
</tr>
<tr>
<td>Preparation of the Samples</td>
<td>52</td>
</tr>
<tr>
<td>Moisture Determination</td>
<td>52</td>
</tr>
<tr>
<td>Chemical Treatment of the Samples</td>
<td>52</td>
</tr>
<tr>
<td>TREATMENT 1: Hydrogen Peroxide</td>
<td>53</td>
</tr>
<tr>
<td>TREATMENT 2: Sodium Bicarbonate and Hydrogen Peroxide</td>
<td>53</td>
</tr>
<tr>
<td>TREATMENT 3: Ammonium Bicarbonate and Hydrogen Peroxide</td>
<td>53</td>
</tr>
<tr>
<td>TREATMENT 4: Sodium Hydroxide and Hydrogen Peroxide</td>
<td>55</td>
</tr>
<tr>
<td>TREATMENT 5: Ammonium Persulfate and Hydrogen Peroxide</td>
<td>55</td>
</tr>
<tr>
<td>TREATMENT 6: Ammonium Hydroxide and Hydrogen Peroxide</td>
<td>55</td>
</tr>
<tr>
<td>Aflatoxin Extraction</td>
<td>55</td>
</tr>
<tr>
<td>Aflatoxin Analysis</td>
<td>58</td>
</tr>
<tr>
<td>Thin-Layer Chromatography</td>
<td>58</td>
</tr>
<tr>
<td>Thin Layer Chromatography and HPLC</td>
<td>58</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>59</td>
</tr>
<tr>
<td>CHAPTER 4</td>
<td>60</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>61</td>
</tr>
<tr>
<td>Hydrogen Peroxide Treatment</td>
<td>63</td>
</tr>
<tr>
<td>Sodium Bicarbonate Treatment</td>
<td>66</td>
</tr>
<tr>
<td>Ammonium Bicarbonate Treatment</td>
<td>69</td>
</tr>
<tr>
<td>Sodium Hydroxide Treatment</td>
<td>72</td>
</tr>
<tr>
<td>Ammonium Persulfate Treatment</td>
<td>76</td>
</tr>
<tr>
<td>Ammonium Hydroxide Treatment</td>
<td>78</td>
</tr>
<tr>
<td>CHAPTER 5</td>
<td>83</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>83</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. Structure of Aflatoxins ........................................ 15
Figure 2. Metabolism of Aflatoxin B₁ (from Busby and Wogan, 1981) .... 20
Figure 3. Proposed Formation of Aflatoxin-Related Reaction Products Following Exposure to Ammonia .................... 32
Figure 4. Preparation of Peanut Sauce according to a Typical African Recipe with Addition of Chemical Reagents .................... 54
Figure 5. Diagram of the Extraction of Aflatoxin (CB Method) from Peanut Sauce .................................................. 56
Figure 6. Diagram of the Extraction of Aflatoxin (Methanol Method) from Peanut Sauce .................................................. 57
Figure 7. Aflatoxin Content in Peanut Meal. Control Uncooked and Control Cooked ........................................ 62
Figure 8. Aflatoxin Content in Peanut Meal. Treatment 1. Methanol Method. CB Method ........................................ 64
Figure 9. Aflatoxin Content in Peanut Meal. Treatment 1- (H₂O₂) Methanol Method (HPLC) (TLC) ............................... 65
Figure 10. Aflatoxin Content in Peanut Meal. Treatment 2. (NaHCO₃) - Methanol Method (HPLC) ............................... 67
Figure 11. Aflatoxin Content in Peanut Meal. Treatment 2. (NaHCO₃) - Methanol Method (TLC) ............................... 68
Figure 12. Aflatoxin Content in Peanut Meal. Treatment 3. (NH₄HCO₃) - Methanol Method CB Method - (TLC) .................. 70
Figure 13. Aflatoxin Content in Peanut Meal. Treatment 3. (NH₄HCO₃) - Methanol Method - CB Method - HPLC TLC ............. 71
Figure 14. Aflatoxin Content in Peanut Meal. Treatment 3. (NH₄HCO₃) - CB Method - (HPLC) ............................... 73
Figure 15. Aflatoxin Content in Peanut Meal. Treatment 3. (NH₄HCO₃) - Methanol Method - (TLC) ............................... 74
Figure 16. Aflatoxin Content in Peanut Meal. Treatment 3. (NH₄HCO₃) - CB Method - (TLC) ............................... 75
Figure 17. Aflatoxin Content in Peanut Meal. Treatment 4. (NaOH 2%) - Methanol Method, CB Method .............................. 77
Figure 18. Aflatoxin Content in Peanut Meal. Treatment 5. [(NH₄)₂S₂O₃] - CB Method - (HPLC) ............................... 79
Figure 19. Aflatoxin Content in Peanut Meal. Treatment 6. (NH₄OH 2%) Methanol Method HPLC - TLC .............................. 81
Figure 20. Aflatoxin Content in Peanut Meal. Treatment 6. (NH₄OH 2%) CB Method - (HPLC) ............................... 82
ABSTRACT

Naturally-contaminated roasted peanuts containing approximately 1500 ppb total aflatoxins were ground and cooked with water to simulate the preparation of a typical African sauce. The peanut sauce was prepared by mixing 200 g. ground peanuts with 700 ml of water, then bringing the mixture to a boil. Five minutes after the peanut sauce began to boil under slow heat, hydrogen peroxide at 0%, 1% or 5% was added in combination with other food additives and the mixture was cooked for 30 minutes. Additives used were: 2% sodium bicarbonate (NaHCO₃); 2% ammonium bicarbonate (NH₄CO₃); 2% sodium hydroxide (NaOH); ammonium persulfate [(NH₄)₂S₂O₈] and 2% ammonium hydroxide.

For analysis two methods of aflatoxin extraction were used. The AOAC-approved CB method produced a stable emulsion with sauces treated with sodium and ammonium hydroxide, so that extraction was not possible. The second, more successful method used methanol extraction. Both TLC and HPLC were used for aflatoxin analysis.

Cooking alone reduced aflatoxins B₁, B₂ and G₂ to half or more of the original values, but no significant reduction was observed for aflatoxin G₁. Although cooking with food additives further reduced aflatoxin, none of the chemicals alone or in combination with hydrogen peroxide were successful in eliminating aflatoxins from the peanut sauce. However, the peanut sauce treated with sodium bicarbonate and 1% hydrogen peroxide had the aflatoxins reduced to nearly the 20 ppb FDA action level.
CHAPTER 1

INTRODUCTION

Peanuts (*Arachis hypogaca*) are grown in tropical, subtropical and warm climates in more than 70 countries. In 1987-1988, an estimated 20.3 million metric tons were harvested from about 18.1 million hectares worldwide (USDA, 1988). Traditionally, peanuts are eaten raw, boiled, roasted, cooked as peanut sauce and in confectionery and snack foods. They are a good source of nutrients, containing about 26% protein, niacin, thiamin, vitamin E, magnesium, potassium and phosphorus (GACCP, undated).

Molds are beneficial to mankind in many ways where they play significant roles in the production of cheese, antibiotics, vitamins, enzymes and others. However, many of them produce mycotoxins, compounds which may be harmful to humans and animals. The family of aflatoxins is generally considered to be as one of the greatest concern. They are produced by *Aspergillus flavus* and *Aspergillus parasiticus*, molds that occur in many agricultural commodities, such as peanut, corn, tree nuts, cottonseed, pistachios, and spices, etc. Although those commodities are important substrates for the fungi, the optimum moisture content of the substrate and temperatures are the main factors that regulate the fungal growth and toxin production (Calvi, 1990).

Aflatoxin, although highly carcinogenic in all experimental animal species studied and tumorigenic in many organs, acts predominantly in the liver and biliary tract after oral, subcutaneous or intraperitoneal administration. The International Agency for
Research on Cancer (IARC) has classified aflatoxin as a probable human carcinogen (IARC, 1987).

The potential hazard of aflatoxins to animal and human health has led to worldwide monitoring programs of the toxin in various commodities as well as regulatory actions by many countries around the world. Levels from zero tolerance to 50 ppb have been reported for total aflatoxins. Most countries have a regulatory level around 20 ppb. Details regarding the regulatory levels for mycotoxins in foods and feeds in different countries have been documented by Van Egmond (1989).

The aflatoxins that produce the most severe pre- and postharvest contamination of foods and feeds are aflatoxins B₁, B₂, G₁ and G₂ (Figure 1). The aflatoxin B₁ is the most toxic and carcinogenic of the four.

Furthermore, a variety of chemical derivaties of the four basic aflatoxins (Detroy et al., 1971; Bhatnagar et al; 1991a) can be produced synthetically by physicochemical methods or after contact with enzymes in living system, such as the detoxifying enzymes (cytochrome P 450) present in human and animal liver tissue.

Aflatoxins are world-wide contaminants of food and feed (Jelinek et al., 1989) and are among the most carcinogenic of naturally occurring substances; various efforts are under way to remove or detoxify the toxins in animal and human food (Park et al., 1988; Hagler, 1991; Phillips et al., 1991). However, the prevention of aflatoxin contamination before harvest is the best long-term method and would insure consumer safety.
The success of a detoxification process is dependent first on the degree of destruction of the mycotoxin and the production of no toxic residues. Second, it must not drastically effect the nutritive value of the treated commodity. In this study the removal or inactivation of the aflatoxins in peanut sauce was the main area of focus. The objective was to develop a practical, inexpensive, and easy method to reduce the toxicity of aflatoxins in African diets by introducing various USFDA-approved chemical additives to the cooking process without taking into consideration the nutritive value of the product., since there, the most important factor to be considered would be the toxicity and the health hazards of aflatoxin ingestion, along with the dietary protein deficiency that leads to severe malnutrition in those countries. The objective of this work was to determine the effects of chemical additives upon aflatoxins present in naturally contaminated peanut products.

This project could have far reaching impact on the health of people, especially the poor, throughout portions of the world where the food supply is contaminated with aflatoxins. The addition of a simple powder or liquid to a food during its preparation which would not cause any alteration in its functional, nutritive, or sensory properties, but which would eliminate most of the toxin could lead to significant reductions in the incidence of African hepatocellular carcinomas, Reyes syndrome (Hendickese, 1983) and perhaps, kwashiorkor.
CHAPTER 2
LITERATURE REVIEW

A review of the literature pertinent to the detoxification of aflatoxins entails preliminary reviews of the following: a presentation of the chemical structure of the aflatoxins, aflatoxin toxicity, a review of the history of global and African human awareness of aflatoxins and their biological effects, fungal contamination of crops during growth and storage, the effects of poverty on the economies of sorting and destruction of aflatoxin contaminated foodstuffs in industrialized and African nations, the chemistry of detoxification, methods of detoxification, and a review of aflatoxin detection methods.

Chemical Structure of the Aflatoxins

Aflatoxins are a group of naturally occurring toxins produced by the widespread fungi *Aspergillus flavus* and *A. parasiticus*. Thirteen aflatoxin compounds are known to occur naturally. All of the various aflatoxins have a coumarin nucleus with a bifuran moiety and also contain either a pentenone ring or an additional six membered lactone (Nesbitt et al., 1962; Hartley et al., 1963; Holzapfel et al., 1966). When the mold grows on peanuts as a substrate four metabolites are produced; aflatoxins B₁, B₂, G₁ and G₂ (Fig. 1). The letters correspond to the color of florescence upon long-wave ultra-violet exposure of thin layer chromatoplates when these aflatoxins are present. The B is associated with a blue florescence and G for a yellow-green florescence (Natarajan, 1974).

Aflatoxin M₁ and M₂ are found in the milk of animals that are fed aflatoxin
Figure 1. Structure of aflatoxin (AF)
contaminated feed. They are the hydroxylated forms of aflatoxin B₁ and B₂. Aflatoxin B₉ and G₂ are formed by an hydration at the vinylene group of the terminal furan ring of aflatoxins B₂ and G₂. This is a different position than that of the milk toxins. Aflatoxin P₁ can be isolated from monkey urine. The methoxy group in the coumarin nucleus of aflatoxin B₁ is substituted by a hydroxyl group making P₁ the only aflatoxin that is a phenol. Aflatoxin D₁, D standing for detoxified, is a major product during the detoxification of aflatoxin with ammonia under pressure. "Aflatoxin Q₁ is the major product of in vitro conversion of aflatoxin B₁ by monkey liver tissue and accounts for up to 55% of the B₁ converted." (Goldblatt, 1977) Another major product of in vitro conversion using human and monkey liver tissues is aflatoxin H₁. Aflatoxicol is produced when the carbonyl group of the cyclopentenone group of aflatoxin B₁ is reduced to an alcohol. Similarly, aflatoxin H₁ is aflatoxin Q₁ with the carbonyl group reduced. (Goldblatt, 1977)

Description of Aspergillus

The aflatoxin fungi, *Aspergillus flavus* and *A. parasiticus*, which are widely distributed in the soil, are capable of invading important animal and human food crops, including corn, peanut, cottonseed, cassava, rice, sorghum and tree nuts (Moreau, 1979). The mold is capable of invading seed, kernal or nut, through the flower, or on the surface of the developed seed. Although generally considered a saprophyte, post-germination parasitization was observed in the Sudan (Ochomogo, 1978). Parasitized seed contains aflatoxin distributed throughout the seed mass and not just on the surface. *Aspergillus*
flavus and A. parasiticus aflatoxins production vary according to substrate and temperature (Park and Bullerman, 1983).

History of the discovery of Mycotoxins

Our current knowledge concerning mycotoxicology is of recent origin. Although disease caused by mycotoxins have been known since antiquity, (the ancient Chinese, Greeks, Romans, and Arabs knew of ergotism), comparatively little interest in mycotoxicology in modern scientific literature was apparent until the explosion of interest elicited by the discovery of aflatoxins in the early 1960's (Van Rensburg et al., 1985). The history of this discovery began when more than 100,000 turkeys died of "Turkey X disease" in Great Britain. At postmortem autopsies the livers of these animals were pale, fatty and had extensive necrosis and biliary proliferation. Outbreaks of disease showing similar symptomology involving pheasants, partridges, ducklings, calves and pigs occurred later that year. The initial search for the cause of the disease among known toxins was unsuccessful. The common factor was eventually found in the incorporation of batches of peanut meal in animal feed originally imported from Brazil in 1959. Extractions taken from the contaminated Brazilian peanuts led to the isolation and characterization of the compounds now called aflatoxins. In the United States an increasing incidence of hepatomas in hatchery-reared trout was linked with the same etiologic agent found in the Brazilian peanuts but this time in contaminated cottonseed meal (Butler, 1969). Most reports of aflatoxin contaminated foodstuffs and aflatoxicosis, since then, have originated
from tropical or semi-arid countries of Africa, Asia and South America where the
temperature and relative humidity are favorable for the growth of molds and production
of their toxic metabolites.

**Metabolism and Carcinogenicity**

Extensive studies have shown that aflatoxin is cytotoxic, carcinogenic and
mutagenic in a wide variety of living things. Aflatoxin is metabolized in the liver by the
cytochrome-P-450 isozymes (C-P-450) and the mixed function oxidase system. Aflatoxin
B<sub>1</sub> is activated and converted by the C-P-450 isozymes to the AFB-8,9-epoxide. This
epoxide metabolite is able to bind covalently to DNA, RNA and other macromolecules
causing mutagenicity/carcinogenicity. It can also react with glutathione aided by
 glutathione-S-transferase to form the AFB-GSH conjugate (8,9-dihydro-8-S-glutathionyl-9-
hydroxy-AFB<sub>1</sub>) which is then excreted or it can undergo reduction, hydroxylation,
hydration or o-dimethylation (Fukayma and Hsieh, 1985). It has been mentioned that the
high level of water soluble metabolites in the urine and feces of AFB<sub>1</sub> derivatives, at
earlier stages after AFB<sub>1</sub> treatment indicate increased levels of metabolism and
detoxification of AFB<sub>1</sub>. The compounds resulting from detoxification of the AFB<sub>1</sub>-epoxide
by binding with glutathione are found in the feces.

The AFB<sub>1</sub>-8,9-epoxide has been identified as the major biliary metabolite in the rat
(Degen and Neumann, 1978). Several studies have shown that the difference in resistance
to AF induced damage is mainly in the detoxification mechanism. For example, Monroe
and Eaton (1987) showed that control rats had higher levels of the enzyme glutathione-S-transferase than treated rats when higher levels of the conjugate were formed. There was also an increased metabolism to produce water soluble conjugates (Fig. 2). The syntheses of DNA, RNA, protein, and lipids are inhibited by aflatoxin. Alteration of steroid hormone mechanism and inhibition of membrane transport are also associated with aflatoxin (Heathcote and Hibbert, 1978; Busby and Wagon, 1981).

Chronic exposure to low levels of aflatoxins is likely to occur more commonly than actual toxic exposure, and the chronic exposure to aflatoxins more readily leads to cancer than does acute exposure; this represents a serious public health concern. Loss of weight or poor growth is one of the first and most consistent signs of intoxication (Edds, 1973). Although AFB1 is an example of a classic hepatotoxicant and hepatocarcinogen, it also affects other than the liver several organ systems such as pulmonary, gastrointestinal, renal, nervous, reproductive and immune systems (Coulombe, 1994).

Evidence linking inhaled aflatoxin B1 to human lung cancer is tenuous. Two epidemiological studies found that workers in a Dutch peanut oil processing plant exposed to aflatoxin B1-contaminated dusts experienced a significantly greater incidence of upper respiratory (trachea and bronchus) as well as liver tumors than unexposed workers (Van Nieuwenhuize et.al., 1973; Hayes et.al., 1984). Other case studies also have described the development of tumors in people working with aflatoxin B1-contaminated dusts (Deger, 1976; Dvorakova, 1976). Cusumano (1991) has found significantly higher amounts of
Figure 2. Metabolism of Aflatoxin B₁ (from Busby and Wogan, 1981)
aflatoxins in the sera of patients with lung cancer.

The effects of AFB₁ on the nervous system were investigated by finding this toxin in tissue samples from patients with Reye's syndrome, a pediatric disease characterized by cerebral edema and neuronal degeneration (Chaves-Carballo et al., 1979). Ryan, et al. (1979) reported finding AFB₁ in the blood of six of seven patients during the acute phase of this disease, as well as in the livers of those that subsequently expired. However, because of enormous outbreaks of acute hepatitis in Africa, Latin American and Asia, aflatoxin has been blamed for these outbreaks. The most extensively studied, in northwest India in 1974, has been the subject of two investigations and many reports (Krishnamachari et al., 1975; Tandon et al., 1978). Clearly the possibility existed for very high levels of exposure to aflatoxin in moldy maize. Aflatoxin was found in some of the individuals studied, particularly in their tissues. A subsequent outbreak of fatal hepatitis in Kenya, which also was attributed to aflatoxin-contaminated maize, lends support to this finding (Ngindu et al., 1982).

Several other case reports have been reviewed in the literature (Campbell and Stoloff, 1974). Establishing casuality in these situations is very difficult, but the circumstantial evidence, the measurably high levels of aflatoxins in food and human tissues, and the hostology are all persuasive (Hall and Wild, 1994).

Studies have shown that aflatoxin B₁ is the most common and most potent form of the aflatoxins. Using the chicken embryo test, lethal dosages have been determined to be
0.025, 0.125, 1.1, and 2.7 micrograms/egg respectively for aflatoxins B₁, B₂, G₁, and G₂ (Goldblatt and Dollear, 1977). Lethal doses vary from as little as 0.335 mg/kg in the duckling to as much as 17.9 mg/kg in female rats. Variation within a species and between species with respect to aflatoxin's carcinogenic potency may be large also. Although the rat is relatively resistant to acute levels, aflatoxin ..."is an extremely potent carcinogen for the rat" where "levels of 0.015 ppb may induce carcinoma ... with continuous ingestion" (Butler, 1969). Dietary levels of 0.1 ppm aflatoxin induced liver carcinoma in more than 50% of the rats studied, after 80 weeks (Ito, 1982). After extensive studies aflatoxins have been found to be cytotoxic, carcinogenic, and mutagenic. They inhibit DNA, RNA, protein, lipid and steroid syntheses. Chemically, aflatoxins covalently bind to nucleic acids, DNA, RNA and protein. (Ito, 1982).

Human tolerance may differ significantly. "It is not known if man is as acutely susceptible as the guinea pig, duckling or monkey, or relatively resistant as the mouse and rat" (Butler, 1969). Although the lethal dosage for man is not known, epidemiological studies which correlated average aflatoxin intake by humans with official liver cancer mortality statistics in Kenya (Peers and Linsell, 1973), Mozambique (Van Resensburg et al., 1974), China (Yeh et al., 1989), and Swaziland (Peers, 1976) have strongly linked the frequency of primary liver cancer to the consumption of AF-contaminated peanuts and peanut products. The incidence of primary liver cancer in Africa, Southeast Asia, and China is many times that of the United States or Western European countries (Rodricks et
al., 1977). Among the Bantu populations of Africa, for example, over 2000 cases of liver cancer per 100,000 inhabitants are reported annually, with less than one case per year being found among the same number of people of Holland, Norway or Canada (FDA, Jan. 1978, Moreau, 1979). Cases of liver cancer are 58 times more frequent in Mozambique than in the United States. Although chronic hepatitis B carriage is now recognized as contributing to the majority of liver cancer seen today, hepatic toxins (such as aflatoxin) which appear in many staple foods in areas of high frequency are also suspect, and it is likely that an interaction between hepatitis B virus and aflatoxin is responsible for the exceptionally high rates in parts of African and Asia (Van Rensburg et al., 1985). A recent finding is that aflatoxin can exacerbate the nutritional disease, kwashiorkor (Hendrickse et al., 1983). The etiology of protein-energy malnutrition and recommendation for its control have been illustrated by (Latham, 1990).

Kwashiorkor as a form of malnutrition, Hendrickse (1982, 1991), has pointed out the relationship between kwashiorkor and aflatoxin; and expressed that this disease is limited to the tropics and has an increased incidence in the wet season, both features of aflatoxin exposure (Hendrickse, 1991). Studies in Kenya (DeVries et al., 1987) and Sudan (Hendrickse et al., 1982) have demonstrated high levels of urinary excretion of aflatoxin in affected children compared with marasmic or normal children. Aflatoxin contamination has a crucial importance because of possible human and animal health problems caused by ingestion of contaminated food or feed. Children in Sudan suffering from this condition
had much higher levels of aflatoxin in their diet and in their urine than did normal children or those suffering from marasmus, another nutritional disease, caused by protein and calorie deprivation. Kwashiorkor is very common in the tropical areas of the world where aflatoxin is found in the food supply but is rare or absent in areas with aflatoxin-free food, even though the protein-calorie intake of individuals in the two areas is similar (Hendickese et al., 1983).

The hepatotoxicity of aflatoxin from foods is well documented. However, there is a possible relationship between dietary aflatoxins and mental retardation. Several areas of southern Georgia were studied with taking in consideration the associations between dietary intake of aflatoxin-containing foods by mothers, and performance of children with varying levels of achievement and IQ scores. Based on results of IQ tests, the researchers concluded that the diet pattern of the mother and the amount of aflatoxins in the food supply are two distinct factors involved in the diet correlation. These findings were similar to reports of mental retardation in a region of East Africa known to have high contents of aflatoxins in the food supply (Caster, 1986).

FDA reports and actions indicate uncertainty where to set tolerance levels for human foods, for example, ... "In 1971 the USFDA was ready to lower the action level to 5 ppb based upon new improved analytical methodology and announced that it was targeting for the 1 ppb level to reduce human health hazards", yet a study referred to in FDA litigation found that "at 20 ppb the human risk was 1.1. to 2.7 carcinomas per
100,000 people lifetimes and at 15 ppb it was reduced to 0.8 to 2.1. This reduction, it was said, is minuscule in comparison to cost for lowering it" (LaBuza, 1983; FDA, 1978). Although health risks are great, the economic cost of the destruction of contaminated food and animal feeds can be extensive, therefore, special exemptions have been implemented in the United States. Normally 20 ppb is also the limit for animal feeds; for cottonseed meal the official action level is 300 ppb. This level was rationalized on the basis of cottonseed meal only comprising about 11% of the feed formulation. So at a 300 ppb level of aflatoxins, an animal would be ingesting only about 30 ppb. This exemption, which was requested by the state of Arizona allowed 77,000 tons of cottonseed, worth nearly $10.7 million, or 70% of the contaminated meal in the state to be marketed (Labuza, 1983). The Protein Advisory Group, sponsored by the United Nation Children's Fund, the Food and Agriculture Organization and World Health Organization recommended in 1961 that 30 ppb total aflatoxins be the maximum level tolerated in peanut protein supplements (Gardner et al., 1970).

**Fungal Contamination during Growth and Storage of Foodstuffs**

As recently as the early 1970's, most people believed that aflatoxin was primarily a storage problem. "Aflatoxin can be frequently be found both in peanuts in the ground and in freshly dug peanuts. We know it can be found in corn growing in the field and at least in some tree nuts while the nuts are on the tree" (Goldblatt, 1977).

The fungi that produce the aflatoxins will grow and contaminate stored products
with aflatoxins if *Aspergillus flavus* is present in condition when the relative humidity of the stored product is above 85% for an extended period of time. Wilson and Abramson (1992) reviewed the factors leading to and the commodities susceptible to aflatoxin contamination in storage. Water activity (a_w) roughly corresponds to the equilibrium relative humidity in stored products. Fungi generally will not grow at water activities below 0.70. In addition, the contamination in storage may be affected by the commodity itself, the temperature, oxygen availability and the initial inoculum density. Therefore, the crops are susceptible to the aflatoxin contamination if the storage of the commodity at an a_w of 0.85 or above.

"The best approach to contain aflatoxin contamination is prevention" (Goldblatt and Dollear, 1977). Fungal contamination can be present in seed at the beginning of the agricultural cycle, hence it is up to the farmer to insure that seedstock is not infected with *Aspergillus* spp. A simple method to check for mold growth, although not necessarily *Aspergillus*, is to take and crack one kilo of suspected grain and then view it under a ultraviolet light. If mold growth has occurred in the grain, individual yellow-blue-green pieces will be seen fluorescing. Such 'sparklers' are not a positive indication of aflatoxin, but the more fluorescent pieces present, the more probable that the grain is contaminated. It should be used as a preliminary survey method only, but it is inexpensive and easy to use. In remote areas of the Orient or Africa, the farmer may not have the educational or material means to inspect his or her seedstock or simple poverty might require the use of
the only seeds available. In industrialized areas of the world commercial seed outlets are able to provide non-contaminated seed.

Contaminated plant debris in the soil surrounding the developing peanut embryo that has been colonized by *A. flavus* are considered to be the major source of potential aflatoxin contamination in peanuts. (Diener, 1989). *A. flavus* is able to invade peanut cotyledons at moisture levels ranging from 9 to 25%. Germinated peanut seeds are resistant to *A. flavus* invasion (Goldblatt and Dollear, 1977) Cleaning up contaminated plant materials and adequate watering after planting would therefore seem to be prime preventative agricultural measures. For rain dependent agriculture it may be impossible to supply adequate soil moisture levels after planting.

Aflatoxin may increase tremendously during storage. Cottonseed which averaged 30 ppb aflatoxins and ranged from 0 to 113 ppb when stored with a 21.8% moisture content and aerated had a mean aflatoxin content of 17,194 ppb after 30 days in one of the study lots. Decreasing moisture contents reduced the amount of aflatoxins produced. It is still important to emphasize that this is an exception, most aflatoxin is produced in the field when the plant is under some sort of stress such as dry weather or drought, insect or mechanical damage. Another factor which must be taken into consideration, especially when sampling for analysis, is that only one seed in several hundred may be contaminated (Whitten and Smith, 1972). One very highly contaminated seed may condemn an entire lot.
It is widely accepted that intact kernels are rarely invaded by *A. flavus* but when the kernel is damaged, either by insects or direct mechanical damage, it is more frequently contaminated by *A. flavus* and also frequently contains aflatoxin" (Butler, 1969). So it seems likely that the "sorting of nuts to remove damaged or discolored kernels is probably a more feasible and effective means than any detoxification process (Wang, 1982). both for the United States, where the majority of nuts pass through commercial channels and for traditional non-commercial channels, where foods are produced for local consumption. Yet there is no final safeguard to insure traditonal compliance with sound aflatoxin control measures, which government bodies such as the USFDA regulate by determining mycotoxin levels in typically large lots at a reasonably low cost in labor and materials. In rural African and similar social systems many more people per capita would be needed to gather samples, test for toxin levels and impound or destroy contaminated food or food products. To make things worse, most African countries have no agencies with enforcing powers similar to the USFDA (Van Resenburg et al., 1974.

**Reduction of Aflatoxin Exposure**

It is "possible to reduce the incidence of aflatoxin contamination at the lowest level by proper attention to agricultural, handling and storage practices." (Wang, 1982) Regulation of sound mold contamination control procedures in African commercial enterprises should not be more difficult than in the United States, however, much African agriculture is still pursued using traditional methods. Such traditional social and ethnic
groups often store and consume their own farming produce. If there is a surplus beyond family nutritional needs, this may be sold in the market place directly to the consumer. To alter or regulate such traditional patterns involves a major educational effort relative to commercial practice where legal regulation is practical. Butler, reports that other studies have shown that "from any batch of groundnuts only a relatively small number of the groundnuts were contaminated with aflatoxin. As a result it is widely accepted that intact kernels are rarely invaded by *A. flavus* but when the kernel is damaged, either by termites or direct mechanical damage, it is more frequently contaminated by *A. flavus* and also frequently contains aflatoxin". (Butler, 1969) Hence, the "sorting of nuts to remove damaged or discolored kernels is probably a more feasible and effective means than any detoxification process" (Wang, 1982).

For social groups threatened by the specter of starvation and hunger, mold damaged nuts will still be consumed, although with trepidation by those who understand the nature of aflatoxin toxicity. "For developing countries or where the nuts are raised for local consumption, chemical treatment with simple readily available reagents" (Wang, 1982) for the purpose of aflatoxin detoxification provides an alternative or complementary approach with the practice of sorting peanuts. In the bleakest of times it may provide the only known and practical means of reducing aflatoxin related health risks.

Ideally, in any social system, consumers and producers would be able to recognize the signs of fungal contamination of individual peanuts and other food staples and would
then discard them but the discovery of the amazingly toxic effects of aflatoxins is recent and largely confined to the scientific and food industry communities. The dissemination of current scientific knowledge concerning the grave health risks associated with mold contamination is still not extensive. For example, a storage method of great antiquity still used in areas of Swaziland in Africa, is to store grains in pits in the earth. The pits are only lined with a thin layer of cattle dung. Small quantities of grain in large pits often become caked with fungal mycelium. Such moldy grain is sometimes washed and eaten (Peers et al., 1976).

Even when people understand the grave danger of aflatoxin poisoning, hunger and starvation may force them to consume mold damaged nuts. "For developing countries or where the nuts are raised for local consumption, chemical treatment with simple readily available reagents" for the purpose of aflatoxin detoxification provides an alternative or complementary approach with the practice of sorting peanuts (Wang, 1982). In the bleakest of times it may provide the only known and practical means of reducing aflatoxin related health risks.

Intervention against aflatoxin can be articulated in several ways:

1. Preharvest, by controlling irrigation and pest infestation and by producing genetic crop variants resistant to fungal infection;
2. Postharvest, by improved storage and sorting techniques; and
3. Postingestion, by modifying the biological effects of aflatoxins.
Chemistry of Detoxification of Aflatoxins

Studies concerning decontamination of aflatoxin-contaminated cottonseed and peanut meals have been reviewed in several papers (CAST, 1979; Goldblatt, 1969; Goldblatt, 1977). A few products have been commercially acceptable, especially for the feeding of livestock. Of particular interest is an on-farm method of detoxification with ammonia developed by the University of Arizona and tested in this laboratory (Jorgensen and Price, 1981). This procedure can be used for the detoxification of feeds used by polygastric animals since the residual ammonia is harmless to these animals. Because of the inability of humans to tolerate ammonia, this method is not suitable for use on foods.

Decontamination procedures have focused on removal- physical, chemical or biological, or inactivation- physical or chemical (Park, 1993). In evaluating the acceptance of a given decontamination procedure specific criteria have been established. The process must: a) inactivate, destroy or remove the toxin; b) not produce or leave toxic residues in foods from animal fed the decontaminated product; c) retain nutritive value and feed acceptability of the product; d) not alter significantly the technological properties of the product; and if possible, e) destroy fungal spores (Park, 1993).

Reductions in toxicity and carcinogenicity should follow from hydrolysis and subsequent reactions such as decarboxylation or oxidation of aflatoxins (Dollear, 1969; Ochomogo, 1978). The lactone ring(s) of aflatoxin are susceptible to hydrolysis, as are esters, lactones being a cyclic ester (Fig. 3). The methoxy (methyl ether) and furan ether
Figure 3. Proposed formation of aflatoxin-related reaction products following exposure to ammonia. The major products: The MW 286 compound and the MW 206 compound have been isolated and biologically tested (from Park et al., 1988).
functional groups are not as susceptible to attack, so a strong acid such as hydroiodic acid is required for their degradation. Electrophilic agents will react with the double bonds of the vinylene group in the terminal furan ring in aflatoxins B₁ and G₁. And finally, the carbonyl group in the cyclopentenone rings of the aflatoxin B₁ and B₂ are capable of participating in classical carbonyl reactions (Natarajan, 1974 and Ochomogo, 1978).

Most chemical detoxification approaches have been empirical in nature and their reaction mechanisms are therefore not known although postulated (Ochomogo, 1978).

Aflatoxins in contaminated foods are of course not in the same chemical environment as a pure substance in solution. Treatments intended to detoxify should degrade the nutritional quality of foods as little as possible, use easily available substances and devices yet be simple to perform and of course not create new toxins as products of the decontamination procedure.

**Toxicity Estimation**

In order to establish whether a treatment or reaction has truly detoxified a contaminated food or food product has in the past been a fairly involved procedure. Chemical analysis for the presence of end-products known to be toxic does not necessarily insure that an unknown mutagen or carcinogen has not been produced. This is one reason for the popularity of bioassays in estimating the toxicity of treated food or feed. Feeding trials using all manner of animals to study weight gain, fertility, and liver lesion incidence and many more indices of toxicity have been used to gauge the efficacy of treatments. In
the late 60s and 70s, the presence of aflatoxins were usually verified by bio-assays concerning "the acute toxicity mainly to one-day old ducklings or chick embryos" (Natarajan, 1974).

Acute toxicity is not indicative of carcinogenicity. Feeding trials with animals can take a long time to complete. Consider the 80 week trial length in the study mentioned earlier concerning the carcinogenicity of 0.1 ppm aflatoxin in the rat diet (Ito, 1982). Due to this difficulty, alternative means for screening for carcinogenicity in complex mixtures have been developed.

A close correlation has been established by extensive studies between mutagenicity and carcinogenicity. Although this correlation is empirically established, the theoretical bridge is provided by the somatic mutation theory of carcinogenicity which holds that a cellular genetic change may allow tumor induction. Cellular genetic changes imply change in DNA sequencing. The studies of Ames and his colleagues "have indicated that almost all chemical carcinogens are also mutagens and have provided evidence for the somatic mutation theory of carcinogenicity" (Ames et al., 1975).

**Mutagenicity of Aflatoxins**

Mutation mechanisms may be either base substitutions, frameshifts, and larger lesions in DNA. In Ames' frameshift mutation theory for aromatic hydrocarbons, polycyclic aromatic compounds with a suitably planar ring structure are able to intercalate in DNA base-pair stacking and thereby allow, in repetitive sequences of bases, a stabilzed
shifted pairing. During the repair or replication of DNA this may allow the addition or
deletion of base pairs in DNA sequences. Aflatoxin is a frameshift mutagen (Ito, 1982;
Ames, Durston, Yamaski and Lee, 1973; and Isono and Tourne, 1974).

With the general acceptance of the correlation of mutagenicity and carcinogenicty
a simple test for mutagenicity suitable for testing complex mixtures has been developed
called the *Salmonella/mammalian-microsome* mutagenic test or Ames test. Mutant
*Salmonella typhimurium* which require histidine and which have been selected for both
sensitivity and specificity are used in the Ames test. Mutagenic activity is ascertained by
counting the number of colonies which revert back to wild strains (which require no
histidine) upon exposure to suspected mutagens. As many carcinogens/mutagens are not
specifically carcinogenic until being metabolically transformed, mammalian liver
homogenate which provides the microsomal mixed function oxidase system which can
convert a "proximate" carcinogen/mutagen into the "ultimate" toxic derivative is also used
(Ito, 1982).

In the following review of detoxificiation techniques and studies, references to
measured mutagenicity will refer to Ames' tests data.

**Biological Detoxification – Microbial Inactivation**

Of the approximately 1000 microorganisms tested for the destruction of aflatoxin,
only one, *Flavobacterium aurantiacum* was known to be capable of significant
detoxification in the late 70's (Goldblatt and Dollear, 1977). In several studies using *F.*
*aurantiacum* on contaminated peanut milk no toxic by-products were found in the treated peanut milk (Ciegler, 1978; Marth and Doyle, 1979; Ciegler et al., 1966; Hao et al., 1987).

Animals when fed aflatoxin contaminated feed are able to serve as a sort of filter for aflatoxins. Although metabolites of aflatoxin, notably aflatoxins M₁ and M₂ in cow milk, may be present in animal products they are usually a small percentage of the amount present in the feedstock (Goldblatt and Dollear, 1977). However, a promising new approach to the prevention (or elimination) of preharvest aflatoxin contamination is the molecular regulation of aflatoxin biosynthesis by toxigenic aspergillus fungi. Enzymes that govern in the biosynthetic pathway of aflatoxin have been purified (Bhatnagar et al., 1989; Bhatnagar and Cleveland, 1990), providing the key to characterizing aflatoxin pathway genes and their regulation at the molecular level (Cleveland and Bhatnagar, 1991).

**Water Treatment**

Using water as the only treatment, Ito (1982) found a significant decrease of aflatoxins after 24 hours treatment with varying moisture contents. "However, no significant difference in mutagenic activity was recognized between the two different temperatures nor within the two sets (20,30) (40,50) of moisture levels".

**Water Re-fluxing**

When aflatoxin B₁ was re-fluxed with water for 10 hours, "a compound whose ultraviolet spectrum was identical with that of O-coumaric acid was obtained."
Presumably, hydrolysis of the lactone ring, produced a substituted O-coumaric acid (Coomes et al., 1965; Feuell, 1966).

**Heat Treatment**

Pure aflatoxins are heat stable up to their melting point of approximately 250°C. Autoclaving a moist peanut meal at 120°C for four hours reduced aflatoxin content from 7000 to 350 ppb (Crosslin, 1980). Mann et al. (1967) found that "greater aflatoxin reduction took place with increasing temperature and increasing moisture content. ... definite reduction was obtained at 100°C. The effect was enhanced by increasing time of heating and by increasing moisture content from 6.6% to 15.0% and to 20%. Heating peanut meal containing 20% moisture for 2 hours at 100°C resulted in 80% reduction in aflatoxin while there was no loss if the water content was 6.6%" (Wang, 1982).

**Ultraviolet Light**

Exposure of a thin layer of contaminated peanut meal to ultraviolet light for a period of eight hours did not apparently alter either the fluorescence nor the toxicity of the meal (Wei and Chu, 1973). Numerous studies have shown that photo-modification can detoxify aflatoxins significantly (Crosslin, 1980, Andrellos et al, 1967). In particular, Shantha and Sreenivasa Murthy (1981), exposed contaminated peanut oil to short and longwave ultraviolet light and found significant reduction of aflatoxin, although not as much reduction as was found with sunlight.
Bright Sunlight

"Bright direct sunlight exposure on contaminated millet and maize for four hour periods decreased the aflatoxin contents from 16-30%" (Crosslin, 1980). Exposure of edible oil contaminated with aflatoxin to sunlight for 15 minutes completely destroyed all aflatoxin (Shantha and Murthy, 1977). Eighty three percent of aflatoxin B₁ added to casein was destroyed by sunlight. Half of the toxin added to groundnut meal was destroyed by sunlight (Shantha and Murthy, 1981).

Gamma Rays

Gamma rays at a 2.5 megarad dosage produced no significant destruction of aflatoxin B₁ (Crosslin, 1980).

Adsorption

Absorbent materials including activated carbon (Decker, 1980) and clay and zeolitic minerals (Masimanco et al., 1973) have been shown to bind and remove aflatoxins from aqueous solutions such as water, whole milk, sorghum beer and skimmed milk. The particular importance is the adsorption of aflatoxins by clay (which used to remove pigments from crude oils) is in the significant reduction of aflatoxins in refined peanut and corn oils.

Bentonite, a kind of clay was found to adsorb aflatoxin B₁ when it is added to a liquid. When the bentonite was removed, it was found that almost all of the aflatoxin B₁ had been removed from milk with it (Masmiango et al., 1978, Doyle et al., 1982).
Applebaum and Marth (unpublished data) treated milk that was contaminated with aflatoxin $M_1$ with bentonite. The bentonite was found to have adsorbed most of the aflatoxin from the milk. Increasing the amount of bentonite increased the proportion of aflatoxin removed (Doyle et al., 1982).

**Food Processing**

Aflatoxin reduction from peanut products, corn, milk and others by food processing have the highest attention concerning of aflatoxins. Aflatoxins are resistant to thermal inactivation and are not destroyed by boiling water, autoclaving or other processing procedures (Christensen et al., 1977). Aflatoxins may be destroyed partially by processing procedures such as oil- and dry-roasting of peanuts to be used as salted nuts, in confections, or in peanut butter (Lee et al., 1969). The reduction in aflatoxin content was dependent on roasting conditions and initial aflatoxin contamination in raw peanuts (King, 1971). In other studies, roasting conditions resulted in a significant decrease in the aflatoxin content of peanuts, oilseed meals (Marth and Doyle, 1979) and corn (Conway et al., 1978). The degradation of aflatoxins was a direct function of temperature, heating interval and moisture content (Mann et al., 1967).

A range of reduction (32-87%) in the level of aflatoxin $M_1$ in freeze-dried milk (resulting from pasteurization, sterilization, preparation of evaporated milk and spray drying) was reported by Purchase et al. (1972). In other studies, aflatoxin $M_1$ was apparently stable in raw milk and was resistant to pasteurization and processing (Stoloff
et al., 1975; Stoloff, 1980).

Traditional roasting reduces toxin levels in peanuts. Reductions from 45% to 83% during dry and oil roasting in toxin levels have been observed (Lee et al., 1969). Given the heat stability of pure aflatoxins it would seem likely that some residual moisture exists even in the dry roasting process, at least initially.

**Extrusion**

In a French study, extrusion was found to remove a maximum of 66% of the aflatoxin in peanut meal at 185°C. Aflatoxin destruction increased with increasing temperatures, but apparently was unaffected by varying the water content over a range of 11.5 to 38% (Grehaigne et al., 1983).

Giddey et al. (1977) used Ca(OH)$_2$ and monomethylamine to degrade AF in natural, contaminated peanut meal. They operated under atmospheric pressure at 80-100°C for 30-60 min in a mixer or an extruder. Initial AF levels of 600 to 2000 ppb were decreased by 95%.

Thiesen (1977) found that extrusion cooking of naturally contaminated peanut meal destroyed up to 12% of the AF present (2500 mg/kg), which was not a significant difference. Grehaigne et al. (1982) found that extrusion cooking of naturally AF-contaminated peanut meal with and without NH$_4$OH resulted in a decrease in the amount of AF. As the amount of NH$_4$OH increased, the amount of AF decreased. This was also affected by the operation temperature. Optimal moisture content was between 20 and
The AF levels decreased from approximately 290 to 50 ppb when using 1.5% NH$_4$OH. When no ammonia was added the residual AF was 120 to 212 ppb.

Price (1991) found that the extrusion process lowered aflatoxin levels in contaminated ground corn significantly, but the destruction varied from 33 to more than 80% depending upon extrusion conditions. Extrusion by itself did not lower the contamination level to the United States legal limit of 20 ppb (Price, 1991).

**Acid Treatment**

Dilute solutions of aflatoxins B$_1$ and G$_1$ when adjusted to a pH of 3 or less and heated over a range of 40 - 100°C react to produce aflatoxins B$_{2a}$ and G$_{2a}$. Apparently, as mentioned above, strong acid catalyzes an addition reaction at the terminal furan group double bond and so incorporates a hydroxyl group into the respective molecules (Ciegler, 1966). It is a first order rate reaction with respect to aflatoxin (Pons et al., 1972). Aflatoxins B$_{2a}$ and G$_{2a}$ are much less potent than B$_2$ and G$_2$ (Natarajan, 1974).

Acid treatment does not seem to be a practical treatment due to the likelihood of such strong acids causing degradation of the nutritional quality of foods. In addition, aflatoxins B$_2$ and G$_2$ are unaffected since they do not have a double bond at the terminal furan ring (Crosslin, 1980).

**Treatment with Organic and Inorganic Bases**

Preparation and storage of foods with alkali has been practiced traditionally for centuries and perhaps millenia. American Indian recipes for the preparation of breads,
tortillas and tamales incorporate basic aqueous solutions. Usually the alkali used is derived from ashes, the term alkali deriving from the Arabic term for ashes, al-kali. The Pueblo Indians add ashes to water and then strain the non-soluble solids off and dispose of them. The ash-water is then mixed with finely ground corn flour. The resulting batter is then spread thinly on a very hot (around 700 F) stone and cooked for a few seconds. A few layers of this are put together to make Piki bread, whose recipe has probably not varied for many centuries. Alkali treatment is used in the production many foods besides that of corn such as dates and olives. Contemporary preparation of some food products is accompanied by the destruction of aflatoxins.

**Calcium Hydroxide**

The boiling and soaking of corn in limewater during the manufacture of tortillas was thought to greatly reduce aflatoxin levels or to even eliminate them. Price and Jorgensen (1985), suggested that increasing the concentration of calcium hydroxide did not cause a complete reduction of aflatoxin in tortillas. The authors also suggested that this effect could be in part due to the solubility of calcium hydroxide being decreased at the boiling temperature of water.

**Sodium Hydroxide**

Camou-Arriola and Price (1989) found during the production of a corn snack, that treatment with lye reduced aflatoxin levels in corn products. Acidification of the corn snack prior to the final stage of autoclaving and frying caused "reformation of as much as
18% of the original aflatoxin, however, the acidified final product showed no reformation at all.

Another alkaline treatment for the destruction of aflatoxin is in the refining of crude edible oils. Crude oil is washed with a solution of 0.3 - 8.3 N NaOH "followed by a water wash and bleaching procedure at elevated temperatures ..." Aflatoxin content is reduced to below 1 ppb (Dollear, 1969; Crosslin, 1980).

**Ozonization of Aflatoxin**

Peanut and cottonseed meal was treated with ozone gas. Aflatoxins B₁ and G₁ were readily destroyed although aflatoxin B₂ was relatively resistant to this treatment. In cottonseed meal aflatoxin levels were reduced from 214 ppb to 20 ppb and in the peanut meal levels were reduced from 82 to 18 ppb in 1 hour. Aflatoxin B₁ was totally inactivated in both meals (Dwarakanath et al., 1968)

**Ammonia Detoxification**

Ammonia and related compounds appear to be the most practical reagents for the chemical inactivation for the decontamination of agricultural products. Within the United States, Arizona, California, Texas, North Carolina, Georgia and Alabama have approved ammoniation procedures for aflatoxin contaminated agricultural produce. Mexico also has approved ammoniation decontamination for corn. France, South Africa, and Senegal routinely use the ammoniation process and several members of the European Economic Community import ammonia treated peanut meal regularly (Park, 1992).
The ammoniation process, using ammonia hydroxide or gaseous ammonia, has been shown to reduce aflatoxin levels in corn, peanut-meal cake and whole cottonseed and cottonseed products by more than 99%. If the reaction is allowed to proceed sufficiently, the process is irreversible.

The safety of ammoniated corn has been tested in rainbow trout (Brekke et al., 1979), chickens (Hughes et al., 1979) and rats (Norred, 1979). In a long-term feeding study in rats, Norred and Morrissey (1983) reported that ammoniation of corn containing 750 ppb aflatoxins resulted in significant protection from toxicity and hepatic neoplasia in experimental animals.

Ammoniation procedures can be categorized as either high pressure and high temperature (HP/HT) or as atmospheric pressure and ambient temperature (AP/AT) techniques. HP/HT methodology is applicable for feed mills, whereas the AP/AT method is suitable as an on-farm decontamination procedure (Park, 1992). Price et al. (1982) tested an on-farm method of detoxification using ammonia at atmospheric pressure. This procedure can be used for the detoxification of feeds used by polygastric animal since the residual ammonia is harmless to these animals. Ammoniation at elevated pressures and temperatures using a pilot plant reduced aflatoxin levels from an average of 519 ppb to below 5 ppb and non-detectable levels (Gardner et al., 1971). Because of the inability of humans to tolerate ammonia, this method is not suitable for use on foods (Jorgensen and Price, 1985).
Bisulfite Degradation

Bisulfite is a common food additive, and is added to wines, fruit juices dried fruits, jams and several other food products. It is a highly reactive chemical and inhibits browning caused by enzymatic and non-enzymatic reactions. It also acts as a reducing agent and antioxidant while effectively controlling the growth of microorganisms. It reacts with aflatoxin B₁ and G₁ with the loss of fluorescence of the products of the reaction. Aflatoxin G₁ reacts more rapidly than does B₁ with bisulfite. Elevation of temperature and concentration increased the amounts of aflatoxin that were destroyed. Quantitative data suggest that at the concentrations of bisulfite used in wines aflatoxin destruction is negligible but with the higher concentrations and temperatures used in drying and preserving fruit, significant detoxification may occur (Doyle and Marth, 1978a). They also found that citric acid retarded the degradation of aflatoxin B₁ and G by bisulfite. Methanol also retarded the degradation by bisulfite. This suggested that bisulfite degradation of the susceptible aflatoxins is dependent upon bisulfite oxidation (Doyle and Marth, 1978b).

Under certain conditions, aflatoxin B₁ can be completely destroyed in whole-kernel corn by treatment 10% bisulfite solution for over 21 days. "Aflatoxin B₂, on the other hand, was resistant to sodium bisulfite and never over about 50% was destroyed. Moisture, sodium bisulfite level, time, as well as temperature had significant effects on aflatoxin degradation" (Hagler et al., 1982).
Experiments done on raw whole milk indicate that sulfite can inactivate aflatoxin M₁. Additional experiments need to be done before bisulfite or sulfite should be used in inactivating aflatoxin to demonstrate that such treated foods are still nutritional and biologically safe (Doyle et al., 1982).

Mutagenic activity was effectively decreased by sodium bisulfite treatment. Increasing temperatures and concentrations of bisulfite increased reductions of measured mutagenicity, although no treatments achieved a reduction equal to that of background (Ito, 1982).

Wang found that all four aflatoxins that occur in contaminated peanuts were degraded by treatment with bisulfite. Increasing concentrations increased observed degradation. Increased temperature also increased degradation efficacy (Wang, 1982).

**Hydrogen Peroxide Detoxification**

Hydrogen peroxide at 6% concentration was found to reduce aflatoxin M₁ levels in milk by up to 71%. Treatment of contaminated milk with hydrogen peroxide combined with riboflavin and hydrogen peroxide with lactoperoxidase resulted in deactivation of 100% of the aflatoxin M₁ (Applebaum and Marth, 1982).

In another study, hydrogen peroxide was used to detoxify aflatoxin in peanut meal. Total aflatoxin levels were reduced from 90 ppm to 3ppm. Nutritional quality was evidently not impaired and the treated meal was not toxic to ducklings (Crisan and Grefig, 1967, Wang, 1982).
Ito (1982) found that hydrogen peroxide treatment of peanut meal contaminated with aflatoxin reduced mutagenicity. Using TLC methods she found a close association with the reduction of revertant colonies and the amount of aflatoxin residues present after treatment. Increased hydrogen peroxide concentrations from 0.5% to 2% had no significant effect, although the least mutagenic activity was found at 30% moisture, 2% hydrogen peroxide and 62°C.

In Wang's experiments with the inactivation of aflatoxin using hydrogen peroxide no effective action was found (Wang, 1982). At concentrations of up to 2% hydrogen peroxide at pH 8 and 60°C. It was thought that "the failure to obtain aflatoxin inactivation must be due to the reaction parameters such as pH and temperature. Since the addition of hydrogen peroxide to the alkaline suspension at the extraction step produced large volumes of foam, further investigation of hydrogen peroxide treatment did not seem feasible" (Wang, 1982).

**Hydrogen Peroxide and Calcium Hydroxide**

Although treatment with the combination of 1% calcium hydroxide and 2% hydrogen peroxide resulted in a significant reduction of mutagenesis and decrease of aflatoxin using the TLC method, the addition of the 1% calcium hydroxide only produced a slight decrease in mutagenicity from treatment using only 2% hydrogen peroxide (Ito, 1982).
Sodium Hypochlorite Detoxification

Sodium hypochlorite has been in use for a long time to detoxify equipment and laboratory wastes contaminated with aflatoxin B₁. The reaction has been shown to produce as a reactant the fluorescent, carcinogenic 2,3-dichloro derivative (Castegnaro et al., 1981). Natarajan (1974) found that sodium hypochlorite was very effective in destroying aflatoxin B₁ and B₂ in protein isolates from both raw and defatted peanut meal. Concentration and reaction pH are important factors in reducing aflatoxin in protein isolates to below detectable quantities, although temperature and extraction time did not affect the inactivation. Color changes were observed with hypochlorite treatment. Tyrosine and tryptophan changes were observed in the amino acid composition of hypochlorite treated meal.

Commercial bleach (5.25% sodium hypochlorite) was used to inactivate aflatoxin in one experiment. "The lowest mutagenic activity was observed in the treatment at 50% moisture, 2% sodium hypochlorite at 62°C. An increase in treatment temperature and moisture significantly decreased mutagenicity as measured by both microbial strains" (Ito, 1982).

Wang (1982) found a variety of treatments with sodium hypochlorite were capable of degrading aflatoxins. "Variations in temperature and concentrations of sodium hypochlorite had a greater effect than variations in moisture content," although elevation of the moisture content in one treatment caused a significantly greater aflatoxin B₁
degradation without affecting the other forms of aflatoxin present.

**Sodium Hypochlorite and Calcium Hydroxide**

The combination of calcium hydroxide with sodium hypochlorite significantly decreased the aflatoxin contamination (Wang, 1982) and mutagenicity (Ito, 1982) when compared with that of sodium hypochlorite alone.

**Detoxification using Extrusion and Chemicals**

Crosslin (1980) found that extrusion cooking with sodium carbonate showed significant indications of detoxification of corn contaminated with 500 ppb aflatoxin. Using weight gain in turkey as an index, he also found that the presence of 3% sodium carbonate in steam pelleted aflatoxin contaminated corn produced similar results. Significant weight gains in comparison to that of control, were also observed with 2% ammonia, 2% sodium hydroxide and 2% sodium carbonate treatments with extrusion cooking (Crosslin, 1980).

Aflatoxin $B_1$ was determined to have been reduced by 40-87% in the presence of ammonium hydroxide in contaminated peanut meal. As mentioned earlier extrusion cooking in the absence of ammonium hydroxide reduced aflatoxin $B_1$ content by 23-66% (Grehaigne et al., 1983). Price found comparable results of destruction in the absence of reagents of 33 to more than 80% aflatoxin in contaminated corn depending on extrusion conditions. The theory behind Price's treatments with reagents followed by extrusion cooking was to first open up the lactone ring of the aflatoxin molecule with an alkali and
then fragment it with an oxidizing agent. The fragmented molecule loses its potency. Two alkaline agents and three oxidizing agents all at different levels and in different combinations were studied. All treatments with chemicals added effected a greater destruction of aflatoxin than just water, but no treatments without ammonia caused enough destruction for the corn to be less than the legal limit of 20 ppb. The corn used was naturally contaminated with 400 ppb total aflatoxin, which is sufficiently high to prevent it from having been used in interstate commerce. After the extruder product cooled, no perceptible ammonia odor remained, but the color was changed from pale to darker yellow. The yellow color was not objectionable. Addition of hydrogen peroxide to the ammoniated samples did not significantly change the results (Price, 1991).
CHAPTER III

MATERIAL AND METHODS

Twelve kilograms of roasted peanuts (*Arachis hypogaca*) naturally contaminated with approximately 600 μg/kg total aflatoxin were obtained from Beatrice/Hunt-Wesson, Inc.- Sylvester, Ga. The peanuts were ground in a mill to pass a 4 mesh screen and mixed in a rotary mixer for 30 minutes. This meal was refrigerated and stored at 4 °C for subsequent use. Various FDA approved food additives (See flow chart, p. 53) were added in concentration at 2% of the weight of the ground peanut.

Source of Chemicals and Solvents

All chemicals, unless otherwise noted, were of reagent grade and were obtained from Sigma Chemical Co., St. Louis, Mo. Hydrogen peroxide (H₂O₂) was from Fisher Scientific, Fairlawn, NJ. Sodium hydroxide (NaOH) was from VWR Scientific, San Francisco, CA. Sodium bicarbonate (NaHCO₃), ammonium sulfate [(NH₄)₂SO₄] and hexane (C₆H₁₄) were from Mallinckrodt Inc., Paris, Kentucky. Ammonium hydroxide (NH₄OH), methylene chloride (CH₂Cl₂), acetone (C₃H₆O), acetonitrile (CH₃CN), methanol (CH₃OH) and Silica Gel 60 were from EM Science, Gibbstown, NJ. Ammonium persulfate [(NH₄)₂S₂O₈] and ethyl ether [(C₂H₅)₂O] were from J.T. Baker Inc. Phillipsburg, NJ.
Preparation of the Samples

Approximately 12 kilograms of naturally contaminated peanut were utilized in the determination of aflatoxins. From that, three 500 gram portions were separated, thoroughly mixed, ground in a Regal La Machine II food processor (model LM 2, KEW, WI) and stored under refrigeration at 0°C in zip-lock plastic bags. For chemical treatment during cooking, 200 gram samples of ground peanuts were used. The aflatoxin extraction and determination were performed using 50 gram samples in triplicates.

Moisture Determination

The moisture content in the peanut was determined by drying a 10 gram sample for 24 hours in a vacuum oven Thelco Precision model 19 made by Precision Scientific, Chicago, IL at 130°C in duplicate. The ratio between the loss of weight and the original sample weight, times one hundred, was reported as percentage moisture content (Assoc. Anal. Chem.- AOAC, 1984; method 14.003). Determination of the moisture content was necessary to calculate the aflatoxin levels on a dry weight basis. The pH was determined according to the Official Methods of Analysis, 14.022, page 252 of the above mentioned AOAC, using a pH meter Orion model SA 520, Boston, MA, with a combination pH electrode model 91-56, standardized by buffer solution of pH 4.01 and of pH 9.18.

Chemical Treatment of the Samples

The reduction of aflatoxin in peanut meal was conducted by chemically treating the samples during the cooking process. This was performed by adding the chemicals at about
5 minutes after the meal started boiling, to avoid the decrease of the internal mass
temperature. The cooking process continued for 25 more minutes at a slow heat. The
process was conducted for all samples as showed in figure 5 and the chemical percentages
were determined, based on the amount of ground peanut meal used.

The peanut sauce was prepared using 200 grams of ground roasted peanuts with
700 ml of water. The mixture was brought to boiling and the additives were added. Then
the mixture was cooked for 30 minutes at slow boil. These different chemical treatments
were performed on separate batches of peanut sauce during their preparation (Figure 4).
The additives, which are all USFDA approved (Furia, 1968), were the following:

TREATMENT 1: Hydrogen Peroxide

Hydrogen peroxide was added in concentrations of 1 or 5% to the peanut meal
during the cooking process.

TREATMENT 2: Sodium Bicarbonate and Hydrogen Peroxide

Two percent sodium bicarbonate (NaHCO₃) was added to the peanut meal during
the cooking process, either alone or combined with hydrogen peroxide (1 or 5%).

TREATMENT 3: Ammonium Bicarbonate and Hydrogen Peroxide

Two percent ammonium bicarbonate (NH₄HCO₃) was added to the peanut meal,
alone and in combination with hydrogen peroxide (1 or 5%) during the 30 minute cooking
period.
FIGURE 4 - Treatment and additives of peanut sauce (Each done in triplicate).
TREATMENT 4: Sodium Hydroxide and Hydrogen Peroxide

Two percent sodium hydroxide (NaOH) was added alone and in combination with one percent hydrogen peroxide to the peanut meal.

TREATMENT 5: Ammonium Persulfate and Hydrogen Peroxide

Two percent ammonium persulfate [(NH₄)₂S₂O₈] was added to the peanut meal during the cooking process, either alone or in combination with hydrogen peroxide (1 or 5%).

TREATMENT 6: Ammonium Hydroxide and Hydrogen Peroxide

Two percent ammonium hydroxide (NH₄OH) was added to the peanut meal, alone or combined with 1% hydrogen peroxide.

Aflatoxin Extraction

Two methods of aflatoxin extraction were used. The first was the CB method described in the Official Methods of Analysis - AOAC, 14th ed., 1984 - Arlington, Virginia, Secs. 26.001 - 26.083, "Aflatoxin in Peanut and Peanut Products" (Figure 5). This method was used for all samples. Sodium hydroxide and ammonium hydroxide produced an stable emulsion, so that the extraction could not be accomplished by this process. The second method was a modified methanol extraction method of Thean et al. (1980) (Figure 6). All samples were extracted in triplicate.
Figure 5. Diagram of the Extraction of Aflatoxin (CB Method) from Peanut Sauce

50 gr. of Sample

250 ml dichloride methane + 25 ml of distilled water
+ 25 gr. diatomatous earth

500 ml flask

Extract for 30 minutes
with a wrist action shaker

Extracted Sample

Filtrate - Whatman #4 (18.5 cm.)

Collect first 50 ml in 250 ml beaker
or in measuring tube.

Prepare the column for chromatography; then:
- Pour 50 ml. collected sample,
- Add 50 ml. of hexane solvent,
- Followed by 50 ml. anhydrous ether,
- Elute aflatoxin with 50 ml of
-3 parts methanol : 97 parts dichloride methane

Collect the aflatoxin, evaporate nearly to dryness on a steam bath, then transfer to a vial containing dichloride methane, evaporate again, seal the vial and save for TLC.
Figure 6. Diagram of the Extraction of Aflatoxin (Methanol Method) from Peanut Sauce

50 gr. of Sample

| 160 ml methanol + 40 ml of distilled water
| 250 ml flask

Extract for 30 minutes
with a wrist action shaker

Filter 100 ml extract through Whatman #4 fluted filter paper

Add 100 ml 8% NH₄SO₄ and stir with 5 g. Celite

Filter 100 ml into separatory funnel

Shake for 1 min with 5m CH₂Cl₂ (methylene chloride)

Collect the methylene chloride fraction over 5 g Na₂SO₄

Repeat the washing 3 more times

Filter and dry samples under nitrogen

Dissolve samples in appropriate volume of benzene /acetonitrile (98/2) for TLC

Or dissolve in acetonitrile for HPLC
Aflatoxin Analysis

Thin-Layer Chromatography

Sensitive thin-layer chromatographic (TLC) methods are available for determining aflatoxins B₁, B₂, G₁ and G₂ in foods and feeds in parts per billion levels. These procedures are based on TLC separation of aflatoxins on silica gel-coated plates, coupled with quantitation, either by visual comparison of the intensity of fluorescence of samples and standard spots or by fluorodensitometric scan of developed chromatograms (Pons and Glodblatt, 1969). Aliquots of the filtrates were chromatographed on silica gel HL (Analtech, Inc., Newark, DE. 19714).

Five and 10 μl aliquots of standards containing 5 μg/kg of aflatoxin B₁ and G₁ and 1.5 μg/kg of aflatoxin B₂ and G₂ per ml of stock solution were spotted in between 5 and 10 μl aliquots of the sample. The plates were developed in CHCl₃/Acetone (9:1 v/v). Aflatoxins were estimated quantitatively by visual comparison of fluorescent intensities to that of a known standard, then calculated according to AOAC Method (Assoc. of Offic. Analy. Chem., 1984).

High Performance Liquid Chromatography (HPLC)

For aflatoxin analysis a High Performance Liquid Chromatograph - Spectra-Physics 8000 A, Piscataway, NJ. with a Partisil 10/25 ODS column for reversed phase partition HPLC and an injector loop of 10 μl was used. The instrument was stabilized at flow rate of 3.0 ml/min and the detector sensitivity of 0.010 absorbance units full scale (AUFS) for 15 minutes before use. The preparation of the standard solution for this analysis was done according to Park et al. (1990), as described: 200 μl hexane, 100 μl of standard solution (5 μg/ml AF-B₁ and G and 1.5 μg/ml AF-B and G - Sigma Co.), 100 μl of trifluoroacetic acid (TFA) and 300 μl of Acetonitrile/water (80:20 v/v). The dry sample extracts were dissolved in 100 μl TFA, 400 μl water/acetonitrile (80:20 v/v) following the addition of 200 μl hexane (Park et al, 1990). Chromatograms of standards and sample
extracts injections (10 μl) were recorded after 12 minutes elution with mobile phase of water/acetonitrile/acetic acid (90:25:10). Aflatoxin content in the samples were calculated in ug/kg as follows:

\[ \text{ug/kg} = \frac{A_x \cdot C_s \cdot V_s \cdot \text{SD}}{A_s \cdot V_x \cdot W} \]

where \( A_x \) = integrator area of AF sample peak; \( C_s \) = concentration of Af in standard μg/ml; \( V_s \) = μl standard injected; \( \text{SD} \) = dilution of sample extract, in μl; \( A_s \) = integrator area count of AF standard peak; \( V_x \) = μl sample extract injected; \( W \) = g sample represented for HPLC (Pons and Franz, 1977).

**Statistical analysis**

Analysis of variance was done using the statistical system (SAS User's Guide: Statistics, 1982). Differences between means were tested using least significant differences at \( P > 0.05 \).
CHAPTER 4

RESULTS AND DISCUSSION

Aflatoxin in peanuts of tropical Africa, Latin America and Southeast Asia is of great economic and public health significance. Epidemiological studies correlated the incidence of human liver cancer to the consumption of aflatoxin contaminated peanuts and peanut products in African countries (Van Rensburg et al., 1985). It was also demonstrated that high aflatoxin levels in children diets could exacerbate the nutritional disease, kwashiorkor (Hendrickse, 1984; Moss, 1989). Aflatoxins M1 and M2 were found in breast milk of African mothers as a result of the consumption of aflatoxin B1 contaminated diets (Liang, 1993). Even though ammoniation was found to be an effective method for aflatoxin decontamination in peanuts and several other agricultural products, a simple, inexpensible method to destroy the aflatoxins in foods and render the product nutritious, flavorful and desirable to humans is needed in African countries.

Ground roasted peanuts (Arachis hypogaca), naturally-contaminated with aflatoxin, were used for the preparation of the African peanut sauce recipes. Various FDA approved food additives were introduced to the reaction system during cooking, 5 minutes after the meal started boiling, in concentration levels up to 2% of the weight of the ground peanut aliquot. Hydrogen peroxide, however, was used in concentration levels up to 5% in order to determine whether higher amounts of this additive would promote greater aflatoxin reduction in the peanut meal. The samples were analyzed in triplicate. Two analytical
procedures were performed, the CB method described in the Official Methods of Analysis (AOAC, 1984) and the modified methanol extraction method of Thean et al. (1980). In some of the most successful treatments, half of the cooked, treated peanut sauces was treated with 1N HCl prior to analysis to measure the amount of reformation of the original aflatoxins from the alkali-opened lactone forms. This is important because it simulates conditions found in the monogastric stomach.

**Controls**

The level of aflatoxin contamination in the ground peanut meal without treatment, cooking or addition of chemicals, was determined by High Performance Liquid Chromatography (HPLC); this fraction was named control uncooked. In order to simulate the conditions which might occur during African food preparation a 200 g aliquot of the peanut meal was cooked for 30 minutes and the aflatoxins content determined by HPLC. This fraction was named control cooked and was considered as reference when comparing the aflatoxin content in the chemically treated samples. The aflatoxin levels in the control uncooked (3% moisture) and in the control cooked (65% moisture) are shown in Figure 7.

Wang (1982) stated that the aflatoxins were heat stable at temperatures up to their melting point (250°C). She also established that when contaminated peanut meal containing 20% moisture is heated at 100°C for 2 hours, 80% reduction in the aflatoxin levels were observed, against no loss if the water content was 6.6%. Doyle et al. (1982)
Figure 7 - Aflatoxin content in original and control samples
found 85% toxin reduction when the peanut meal containing 30% moisture was heated for 2.5 hours at 100 °C and 50% reduction when the moisture was 6.6%. In this study the sauces at 68% moisture were heated for 30 minutes at 80°C. A 42% to 75% reduction was observed in the levels of aflatoxins B₁, B₂ and G₂ respectively, while almost no reduction was noted for aflatoxin G₁, which was not affected by the cooking process. This was relevant considering the short term heating process used in the preparation of the African peanut sauces.

**Hydrogen Peroxide Treatment**

Hydrogen peroxide is an oxidizing agent used in many food products and since the late sixties, it has been used to degrade aflatoxin (Screenivasamurth et al. 1967, Carter et al., 1974). In this study the hydrogen peroxide was used at different levels of concentration. The extraction of aflatoxins were carried out by the CB method (AOAC, 1984) and the methanol method (Thean et al., 1980) and their content in the samples determined by HPLC and TLC procedures. The addition of hydrogen peroxide promoted some degradation of all aflatoxins in cooked peanut meal, independent of the level of the additive used and the method of extraction and analysis employed (Figures 8 and 9). The results for this treatment show that there were no significant differences in the reduction levels of the aflatoxins when the CB method was compared to the methanol method. The values in Figure 8 show that the percent reduction in aflatoxins at the 1% H₂O₂ level varied from 30 to 87% and that at the 5% H₂O₂ level varied from 50 to 98%. Therefore,
Figure 8 - Aflatoxin content of peanut sauce made using $H_2O_2$, analyzed using CB and methanol methods and quantitated using HPLC and TLC. aMethanol extraction
Figure 9 - Aflatoxin content in peanut sauce containing $H_2O_2$, extracted using 80% methanol and quantitated using HPLC and TLC.

*HPLC.
no significant differences were found between the two treatment levels. On the other hand, except for aflatoxin B2 at the 1% H2O2 level, analyzed either by CB or methanol methods with HPLC which did not show great reduction, all the other aflatoxins were significantly reduced.

**Sodium Bicarbonate Treatment**

Sodium bicarbonate (NaHCO3) is normally used in foods and when heated during cooking or baking it promotes an alkaline reaction that leads to the production of carbon dioxide. It is known that alkaline conditions favors the degradation of aflatoxins by opening their lactone ring. Hydrogen peroxide is an oxidizing substance often used in the food industry as a maturation agent for flour in baking or as a sterilant in aseptic packaging. As such could complete the degradation process. Therefore, in this study, sodium bicarbonate was added to the peanut meal during the cooking process, either alone or combined with hydrogen peroxide at 1% and 5% levels. Aflatoxin extraction of the baking soda treated peanuts using the CB method was not feasible because a strong emulsion was formed during the extraction. Extracted by the methanol method, the aflatoxin analysis was performed by HPLC and by TLC and the results are shown in Figs. 10 and 11. The two methods of analysis showed no significant differences between the values obtained for all aflatoxins. The treatment was very efficient for reducing the levels of aflatoxin in the peanut meal since the percent reduction ranged above 70% for all aflatoxins in all levels of combination with hydrogen peroxide. Aflatoxin G1 seemed to be
Figure 10 - Aflatoxin content of peanut sauce using 2% NaHCO₃ with and without H₂O₂, analyzed using the Methanol Method and quantitated using HPLC.
Figure 11- Aflatoxin content of peanut sauce using NaHCO₃ with and without H₂O₂, analyzed using Methanol Method and quantitated using TLC.
the most susceptible to the treatment followed by G2, B2 and B1. No difference in aflatoxin reduction was found between the 1% hydrogen peroxide and the 5% hydrogen peroxide treatments.

**Ammonium Bicarbonate Treatment**

Ammonium bicarbonate (NH₄HCO₃) known as a dough strengthener and leavening agent, dissolves in water producing an alkaline media and reacts when heated to release carbon dioxide which causes dough to rise. It is used in baked goods such as cookies and baking powders. It is used mainly in products baked to a low moisture content as texturizer (Igoe, 1989). Powdered ammonium bicarbonate was added to the peanut meal also during the cooking process, either alone or combined with hydrogen peroxide at 1% and 5% levels. Ammonia is released along with carbon dioxide, giving initially a strong odor to the process.

In this study, the aflatoxins were determined by HPLC and TLC when the extraction was performed by the CB method, while it was determined only by TLC when the methanol method was used. Figures 12 and 13 show the results obtained for the CB method analyzed by TLC and HPLC. Comparing the two methods of analysis it's possible to see that the reduction detected by TLC, 47 to 82% at 0% H₂O₂, 78 to 93% at 1% H₂O₂ and 80 to 91% at 5% H₂O₂ are a little higher than those detected by HPLC, 46 to 90% at 1% H₂O₂ and 22 to 86% at 5% H₂O₂. However there were no significant differences between the values except for aflatoxin B₂ at 5% H₂O₂.
Figure 12 - Aflatoxin content of peanut sauce made using NH₄HCO₃ with and without H₂O₂. Samples were analyzed using both CB and Methanol Method and quantitated using TLC.
Figure 13 - Aflatoxin content of peanut sauce made using NH₄HCO₃ with and without H₂O₂. Samples were analyzed using both CB and Methanol Method² and quantitated using HPLC.
Figures 14, 15, and 16 show the results for the methanol extraction method analyzed by TLC. The levels of aflatoxin reduction were in the same range of those from the CB method, 51 to 80% at 1% $\text{H}_2\text{O}_2$ and 58 to 82% at 5% $\text{H}_2\text{O}_2$. No significant difference was found between the values from the two methods of extraction. This treatment was also very effective in promoting aflatoxin destruction in peanut meal, especially if combined with hydrogen peroxide at the levels used. The susceptibility of aflatoxins to the treatment was $G_2 > G_1 > B_2 > B_1$.

Sodium Hydroxide Treatment

Sodium hydroxide is an alkali, soluble in water, which is used to destroy the bitter chemicals in olives that are to become black olives. It also functions to neutralize acids in various food products. It was used in this experiment at a 2% level and was added to the peanut meal 5 minutes after it started boiling. The cooking process continued for an additional twenty five minutes until the peanut sauce was done and ready to be analyzed.

Similar to one of the previous treatments, aflatoxins could not be extracted in this study by the CB method due to the formation of a very strong emulsion. For this reason a different approach was then determined for aflatoxin analysis using this additive. In one sample, aflatoxins were extracted by the methanol method without any previous changes in the alkalinity of the peanut meal. Another sample had its pH lowered to a mild acidity ($\text{pH}=5.0$) so that the CB method could be used in the aflatoxin extraction step. A third sodium hydroxide sample had its pH extremelly reduced after cooking ($\text{pH}=1.5$) in order
Figure 14 - Aflatoxin content in peanut sauce made with and without NH₄HCO₃ with H₂O₂ and analyzed using the CB Method and quantitaed using HPLC.
Figure 15 - Aflatoxin content in peanut sauce made with and without NH₄HCO₃ with H₂O₂ and analyzed using the Methanol Method and quantitated using TLC.
Figure 16 - Aflatoxin content in peanut sauce made with and without NH₄HCO₃ with H₂O₂ and analyzed using the CB Method and quantitated using TLC.
to verify the reversibility of the aflatoxins structures after being destroyed by the alkaline treatment.

The experiments where hydrogen peroxide was added along with sodium hydroxide did not affect either the reduction of aflatoxins nor the formation of the emulsion on CB extraction. The values obtained for aflatoxins presented in Figure 17 shows that the percent reduction in the aflatoxin levels for the pH=5.0 sample (7% to 100%) and for the alkaline sample (48% to 90%), were very similar and showed no significant differences, except for aflatoxin B$_2$ which was significantly less, especially at pH 5.0. The results for the pH=1.5 sample showed the aflatoxin B$_2$ as the one with the lowest level of aflatoxin reduction (10% reduction) followed by aflatoxin B$_1$ (16% reduction).

From these results its possible to see that acidifying the products prior to analysis caused reformation of much of original aflatoxin as was observed before with treated corn meal (Price et al., 1985). However aflatoxins G$_1$ and G$_2$ practically did not show any reformation (92% and 96% reduction respectively) probably due to irreversible opening of the lactone ring. These results showed that sodium hydroxide is also very effective for the destruction of aflatoxins in peanut meal and that reformation of aflatoxins B$_2$ and B$_1$ occur under more acidic conditions.

**Ammonium Persulfate Treatment**

Ammonium persulfate (NH$_4$)$_2$S$_2$O$_8$ is normally used in food as flour bleaching and oxidizing agent. In this study the action of this additive upon aflatoxins in peanut meal was
Figure 17 - Aflatoxin content in peanut sauce made NaOH and analyzed using both the CB Method and the Methanol Method. Methanol Method*, CB Method**
studied with and without the use of hydrogen peroxide at 1% and 5% levels. It was added five minutes after the peanut meal started boiling and the cooking process, under slow heat, continued for 30 minutes more. The aflatoxins were extracted by the CB method and the analysis was performed by HPLC. The peanut sauce obtained after the cooking process was acidic (pH=3.5). Results seen in Figure 18 show that the additive was more effective when combined with 5% hydrogen peroxide (38% reduction) than with 1% hydrogen peroxide (26% reduction) and without hydrogen peroxide (16% reduction). The low aflatoxin reduction for this treatment may be due to the low pH observed in the peanut sauce that prevents opening of the lactones of aflatoxin.

Ammonium Hydroxide Treatment

The ammoniation process using either ammonia hydroxide or gaseous ammonia has been shown to reduce aflatoxin levels in corn, peanut meal-cakes and whole cottonseed and cottonseed products by greater than 99% (Park, 1993). Aflatoxin in peanut meal has been reduced to nondetectable levels by ammoniation for 30 minutes under 30 pounds pressure at 65°C (Goldblatt et al., 1977).

Ammonium hydroxide is used as a leavening promoting fermentation process agent, pH control agent and as a surface finishing agent in baked foods, cheese, puddings and processed fruits (Igoe, 1989).

In this study the additive was used alone or combined with 1% hydrogen peroxide, added 5 minutes after the peanut meal started boiling. The cooking process continued for
Figure 18 - Aflatoxin content in peanut sauce made with (NH₄)₂S₂O₈ with and without H₂O₂, analyzed using the CB Method and quantitated using HPLC.
an additional 30 minutes. The aflatoxin extraction was carried out mainly by the methanol method and its contents analyzed by HPLC and TLC methods. Also in this treatment the extraction of the aflatoxins using the CB method was not possible, as in the case of treatment with sodium hydroxide, due to the formation of a strong emulsion. Therefore, the method was used to analyze two samples, one strongly acidic (pH = 1.5) and the other mildly acidic (pH=6.0). In both the aflatoxin contents were determined by the HPLC method and the results are shown in Figures 19-20.

The 0% hydrogen peroxide sample (pH=6.0) showed reductions varying from 84% to 87% for aflatoxins B₁, G₁ and G₂ and 4% for aflatoxin B₂. The pH=1.5 sample showed 29% reduction for aflatoxins B₁ and B₂ and for aflatoxins G₁ and G₂, 87% and 94%, respectively. Again here, as was observed with sodium hydroxide, aflatoxin B₂ showed the most reformation at acid pHs. Aflatoxin B₁ showed reformation as well as aflatoxin B₂, but at lower pHs. The samples extracted by the methanol method did not show significant differences when compared either at 0% and 1% H₂O₂ levels or when analyzed by HPLC and TLC and the percent reduction range fell in between 42% and 100%. No significant differences were seen when the 0% H₂O₂ samples CB and methanol extracted method were compared, as well as the 1% H₂O₂ extracted by the methanol method. This treatment was shown to be very efficient in reducing the aflatoxin levels especially when ammonium hydroxide was used in combination with hydrogen peroxide.
Figure 19 - Aflatoxin content in peanut sauce made using NH₄OH with and without H₂O₂, analyzed using the Methanol Method and quantitated using HPLC and TLC.
Figure 20 - Aflatoxin content in peanut sauce made with NH₄OH and analyzed using the Methanol Method.
CHAPTER 5
CONCLUSIONS

Based on the results presented in this study it is possible to conclude that:

1. The cooking of ground peanut meal using the beginning processing steps of a typical African recipe (Appendix B) produced considerable reduction in the content of aflatoxins B_2, G_2 and B_1, in that order, but had very little effect upon aflatoxin G_1.

2. When hydrogen peroxide was added alone it produced little effect on the reduction of aflatoxins B_1 and B_2, however aflatoxins G_1 and G_2 were strongly affected by this treatment.

3. The reduction of aflatoxin was greater when hydrogen peroxide was used in combination with sodium and ammonium bicarbonate, but it showed no difference when combined with sodium hydroxide, ammonium persulfate and ammonium hydroxide. Also, no differences were noted between the 1% and 5% peroxide levels.

4. Sodium bicarbonate was the most efficient of the chemicals used in this study independently of the aflatoxin analyzed, method of extraction or method of analysis. Combined with 1% hydrogen peroxide it reduced the level of aflatoxin B_1 to nearly the 20 ppb FDA action level.

5. Aflatoxin B_2 showed high reformation even at the weakly alkaline pH of 7.6, followed by aflatoxin B_1 at high acidity. The aflatoxins G_1 and G_2 showed very low or no
reformation by acid.

6. Even though the HPLC method of aflatoxin analysis is considered more accurate and precise when compared with the TLC method, no significative difference was found in this study between the two methods. The same was found between the CB and the methanol methods of extraction.

7. Despite the considerable reduction in the aflatoxin content observed in the peanut meal when some of the chemicals were used, it is recommended that high quality peanut with minimal aflatoxin contamination be used in the preparation of the peanut sauces rather than relying on the process for degradation of the toxins. However, in the absence of an effective monitoring system or in the presence of a largely contaminated peanut supply, addition of sodium bicarbonate, a very cheap and common household cooking ingredient, to the peanut cooking water would be beneficial in reducing the total aflatoxin exposure.
SUMMARY

Aflatoxin in peanuts and corn from tropical African and Southeast Asia is of great public health and economic significance. Along with hepatitis B, it has been implicated as causing or contributing to liver cancer, a common cause of death in these areas. Aflatoxin aggravates kwashiorkor because it increases protein requirements. When contaminated products cannot be sold for export, at least two problems are created: one, money which would be coming into the country is lost, and two, the contaminated food or feed is not destroyed but enters into the domestic food chain and is used by the population creating greater public health problem. This study was conducted to develop a method or methods using low cost, low technology to reduce the level of aflatoxins in prepared peanut products in Africa. To accomplish this objective, the extraction of the aflatoxins in the peanut meals, was carried out by the CB method and the methanol method and their content in the samples determined by HPLC and TLC.

Overall, judging from the data, we can conclude that sodium bicarbonate alone or when combined with 1% hydrogen peroxide effectively reduced the level of aflatoxin, especially aflatoxin B1 to nearly the 20 ppb FDA action level. The addition of hydrogen peroxide usually produces a strong smell in the samples, in this treatment however, the meal did not present a residual smell, therefore, the treatment can be applied to edible peanut products.
Appendix A.

An African Peanut Sauce from the Republic of the Ivory Coast

Ingredients:

2. Tomato (whole or paste) 200 grams.
3. 1 tsp. salt  8.92 grams.
4. 1/2 tsp. black pepper  1.83 grams.
5. 2 hot peppers (Jalapeno)  40.15 grams.
6. 2 tbs. vegetable oil (corn)  20 grams.
7. 200 grams of roasted and ground peanuts.
8. 700 ml of water.

Preparation:

Add the oil, onion, tomato, salt, black pepper and chopped hot pepper in a saucepan and cook for 15 minutes. Add a half litre of water and then cook for 20 minutes. Prepare peanut paste by combining the peanuts and the remaining 200 ml of water. Add the peanut paste to sauce pan and cook for 30 minutes.
<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>AFB₁</th>
<th>AFB₂</th>
<th>AFG₁</th>
<th>AFG₂</th>
<th>TREATMENT</th>
<th>AFB₁</th>
<th>AFB₂</th>
<th>AFG₁</th>
<th>AFG₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.00</td>
<td>6.87</td>
<td>16.10</td>
<td>13.20</td>
<td>Control</td>
<td>51.00</td>
<td>6.87</td>
<td>16.10</td>
<td>13.20</td>
</tr>
<tr>
<td>TLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HPLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% H₂O₂  C</td>
<td>38.10</td>
<td>32.71</td>
<td>4.52</td>
<td>24.13</td>
<td>1% H₂O₂  C</td>
<td>48.61</td>
<td>11.91</td>
<td>80.10</td>
<td>83.10</td>
</tr>
<tr>
<td>5% H₂O₂  C</td>
<td>37.12</td>
<td>11.78</td>
<td>-10.18</td>
<td>41.26</td>
<td>5% H₂O₂  C</td>
<td>42.04</td>
<td>45.80</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>1% H₂O₂  C</td>
<td>41.54</td>
<td>19.32</td>
<td>100.00</td>
<td>100.00</td>
<td>NH₄HCO₃  C</td>
<td>43.34</td>
<td>1.95</td>
<td>69.11</td>
<td>79.90</td>
</tr>
<tr>
<td>5% H₂O₂  C</td>
<td>52.72</td>
<td>46.16</td>
<td>100.00</td>
<td>100.00</td>
<td>NH₄HCO₃/H₁ C</td>
<td>63.37</td>
<td>36.83</td>
<td>80.25</td>
<td>86.66</td>
</tr>
<tr>
<td>NH₄HCO₃  C</td>
<td>52.68</td>
<td>46.67</td>
<td>59.85</td>
<td>78.78</td>
<td>NH₄HCO₃/H₅ C</td>
<td>56.04</td>
<td>8.88</td>
<td>82.58</td>
<td>83.28</td>
</tr>
<tr>
<td>NH₄HCO₃/H₁ C</td>
<td>46.70</td>
<td>46.16</td>
<td>65.11</td>
<td>76.75</td>
<td>NaHCO₃  M</td>
<td>51.05</td>
<td>-1.44</td>
<td>61.85</td>
<td>52.68</td>
</tr>
<tr>
<td>NH₄HCO₃/H₅ C</td>
<td>51.00</td>
<td>38.22</td>
<td>57.77</td>
<td>70.01</td>
<td>NaHCO₃/H₁ C</td>
<td>93.31</td>
<td>85.92</td>
<td>97.13</td>
<td>90.48</td>
</tr>
<tr>
<td>NaHCO₃  M</td>
<td>38.26</td>
<td>32.71</td>
<td>4.51</td>
<td>24.13</td>
<td>NaHCO₃/H₅ C</td>
<td>85.85</td>
<td>84.41</td>
<td>86.79</td>
<td>76.12</td>
</tr>
<tr>
<td>NaHCO₃/H₁M</td>
<td>85.56</td>
<td>86.54</td>
<td>77.96</td>
<td>79.43</td>
<td>NaOH/H₁C</td>
<td>92.54</td>
<td>54.09</td>
<td>91.78</td>
<td>57.96</td>
</tr>
<tr>
<td>NaHCO₃/H₅ M</td>
<td>57.02</td>
<td>48.51</td>
<td>100.00</td>
<td>58.40</td>
<td>NaOH C</td>
<td>69.52</td>
<td>64.73</td>
<td>92.35</td>
<td>55.01</td>
</tr>
<tr>
<td>NaOH M</td>
<td>41.54</td>
<td>23.73</td>
<td>100.00</td>
<td>100.00</td>
<td>NaOH C</td>
<td>84.56</td>
<td>7.20</td>
<td>83.72</td>
<td>69.70</td>
</tr>
<tr>
<td>NH₄OH M</td>
<td>65.62</td>
<td>64.11</td>
<td>100.00</td>
<td>75.52</td>
<td>1%H₂O₂  M</td>
<td>56.31</td>
<td>-6.91</td>
<td>69.54</td>
<td>60.92</td>
</tr>
<tr>
<td>H₂O₂  1 M</td>
<td>38.10</td>
<td>32.71</td>
<td>4.51</td>
<td>24.13</td>
<td>NH₄HCO₃/H₁ C</td>
<td>62.63</td>
<td>-12.16</td>
<td>68.32</td>
<td>64.90</td>
</tr>
<tr>
<td>H₂O₂  5 M</td>
<td>37.12</td>
<td>11.77</td>
<td>-10.18</td>
<td>75.86</td>
<td>NH₄HCO₃/H₅ C</td>
<td>66.18</td>
<td>9.05</td>
<td>85.07</td>
<td>70.63</td>
</tr>
<tr>
<td>NH₄HCO₃/H₅M</td>
<td>26.76</td>
<td>5.79</td>
<td>-10.18</td>
<td>41.26</td>
<td>5% H₂O₂</td>
<td>34.89</td>
<td>-0.23</td>
<td>36.43</td>
<td>-36.06</td>
</tr>
<tr>
<td>NH₄HCO₃/H M</td>
<td>26.41</td>
<td>32.71</td>
<td>88.98</td>
<td>26.57</td>
<td>P/H/1CB</td>
<td>7.00</td>
<td>-440.54</td>
<td>-49.75</td>
<td>-455.00</td>
</tr>
<tr>
<td>NH₄OH/H M</td>
<td>78.68</td>
<td>77.57</td>
<td>76.13</td>
<td>75.52</td>
<td>P/H/5CB</td>
<td>17.31</td>
<td>-501.98</td>
<td>9.99</td>
<td>-234.10</td>
</tr>
<tr>
<td>PERSULFATE</td>
<td>-12.62</td>
<td>-629.27</td>
<td>-33.06</td>
<td>-92.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Appendix B - Percent Aflatoxin decrease of each treatment. H₁= 1% H₂O₂; H₅ = 5% H₂O₂; P = Persulfate; M= Methanol Extraction; C=CB extraction.
Appendix C - ANOVA results for each figure.
One Way Analysis of Variance $\alpha = 0.05$; Pairwise Multiple Comparison

**Figure 7** Original 1 vs Control.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>1</td>
<td>66030.4</td>
<td>66030.4</td>
<td>29.3</td>
<td>0.0056</td>
</tr>
<tr>
<td>Residual</td>
<td>4</td>
<td>9018.5</td>
<td>2254.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>75048.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB**<sub>_1</sub> Statistically significant difference $P = 0.0056$

**AFB**<sub>_2</sub> No significant difference $P = 0.781$; **AFG**<sub>_1</sub> No significant difference $P = 0.0676$;

**AFG**<sub>_2</sub> No significant difference $P = 0.698$

**Figure 8** H<sub>2</sub>O<sub>2</sub> Treatment-CB vs Methanol extraction; HPLC and TLC quantitation.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>6</td>
<td>215006.2</td>
<td>35834.4</td>
<td>35.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>14265.3</td>
<td>1018.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>229271.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB**<sub>_1</sub> - Statistically significant difference $P = 0.0000000116$

**AFB**<sub>_2</sub> - Statistically significant difference $P = 0.000215$

**AFG**<sub>_1</sub> - Statistically significant difference $P = 0.00000000526$

**AFG**<sub>_2</sub> - Statistically significant difference $P = 6.20E-013$.  

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>6</td>
<td>71237.1</td>
<td>11872.8</td>
<td>56.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>2948.6</td>
<td>210.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>74185.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG**<sub>_2</sub> - Statistically significant difference $P = 6.20E-013$.  

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>6</td>
<td>18485.1</td>
<td>3080.8</td>
<td>213.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>202.5</td>
<td>14.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>18687.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 9** H$_2$O$_2$ Methanol extraction; HPLC and TLC quantiation.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>98390.3</td>
<td>24597.6</td>
<td>30.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>8015.8</td>
<td>801.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>106406.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB$_2$** No statistically significant difference  

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>54796.5</td>
<td>13699.1</td>
<td>12.4</td>
<td>0.0007</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>11074.1</td>
<td>1107.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>65870.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG$_1$** Statistically significant difference  

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>5132.0</td>
<td>1283.0</td>
<td>11.3</td>
<td>0.0010</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>1138.4</td>
<td>113.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>6270.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG$_2$** Statistically significant difference  

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>3</td>
<td>221992.7</td>
<td>73997.6</td>
<td>60.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>9802.3</td>
<td>1225.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>231795.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 10** 2% NaHCO$_3$ with and without H$_2$O$_2$; Methanol and HPLC.

**AFB$_1$** - Statistically significant difference  

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>3</td>
<td>14350.3</td>
<td>4783.4</td>
<td>9.08</td>
<td>0.0059</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>4212.6</td>
<td>526.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>18562.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB$_2$** - Statistically significant difference  

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>98390.3</td>
<td>24597.6</td>
<td>30.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>8015.8</td>
<td>801.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>106406.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB$_2$** No statistically significant difference  

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>54796.5</td>
<td>13699.1</td>
<td>12.4</td>
<td>0.0007</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>11074.1</td>
<td>1107.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>65870.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 11** 2% NaHCO₃ with and without H₂O₂; Methanol and TLC.

**AFG₁** - Statistically significant difference  \( P = 0.00106 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>3</td>
<td>53855.2</td>
<td>17951.7</td>
<td>15.5</td>
<td>0.0011</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>9238.9</td>
<td>1154.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>63094.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG₂** - Statistically significant difference  \( P = 0.0150 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>3</td>
<td>11322.6</td>
<td>3774.2</td>
<td>6.57</td>
<td>0.150</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>4594.4</td>
<td>574.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>63094.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB₁** - Statistically significant difference  \( P = 0.0000113 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>224710.2</td>
<td>56177.6</td>
<td>32.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>17578.3</td>
<td>1757.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>242288.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB₂** - Statistically significant difference  \( P = 0.00227 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>15765.7</td>
<td>3941.4</td>
<td>9.12</td>
<td>0.0023</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>4323.6</td>
<td>432.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>20089.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG₁** - Statistically significant difference  \( P = 0.0000914 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>74059.6</td>
<td>18514.9</td>
<td>20.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>9238.9</td>
<td>923.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>83298.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG₂** - Statistically significant difference  \( P = 0.00776 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>11944.3</td>
<td>2986.1</td>
<td>6.47</td>
<td>0.0078</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>4618.4</td>
<td>461.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>16562.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 12 NH₄HCO₃ with and without H₂O₂; CB and Methanol using TLC.

AFB₁ - Statistically significant difference P = 0.00000153.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>6</td>
<td>118108.1</td>
<td>19684.7</td>
<td>23.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>11764.1</td>
<td>840.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>129872.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AFB₂ - Statistically significant difference P = 0.00404.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>6</td>
<td>11016.4</td>
<td>1836.1</td>
<td>5.52</td>
<td>0.0040</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>4657.3</td>
<td>332.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>15673.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AFG₁ - Statistically significant difference P = 0.000186.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>6</td>
<td>52916.3</td>
<td>8819.4</td>
<td>10.3</td>
<td>0.0002</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>11986.1</td>
<td>856.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>64902.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AFG₂ - Statistically significant difference P = 0.000883.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>6</td>
<td>15744.9</td>
<td>2624.1</td>
<td>7.62</td>
<td>0.0009</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>4818.4</td>
<td>344.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>20563.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 13 NH₄HCO₃ with and without H₂O₂; CB and Methanol using HPLC.

AFB₁ - Statistically significant difference P = 0.000000188.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>6</td>
<td>161145.4</td>
<td>26857.6</td>
<td>32.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>11525.4</td>
<td>823.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>172670.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AFB₂ - No statistically significant difference P = 0.150.
AFG\(_1\) - Statistically significant difference \( P = 0.0000230. \)

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>6</td>
<td>59604.9</td>
<td>9934.1</td>
<td>14.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>9313.3</td>
<td>665.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>68918.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AFG\(_2\) - Statistically significant difference \( P = 0.000507. \)

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>6</td>
<td>16922.5</td>
<td>2820.4</td>
<td>8.51</td>
<td>0.0005</td>
</tr>
<tr>
<td>Residual</td>
<td>14</td>
<td>4638.7</td>
<td>331.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>21561.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 14. NH\(_4\)HCO\(_3\) with 1\% and 5\% H\(_2\)O\(_2\); CB method using HPLC.

AFB\(_1\) - Statistically significant difference \( P = 0.0000696. \)

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>3</td>
<td>126800.2</td>
<td>42266.7</td>
<td>33.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>10056.3</td>
<td>1257.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>136856.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AFB\(_2\) - No statistically significant difference \( P = 0.335. \)

AFG\(_1\) - Statistically significant difference \( P = 0.00144 \)

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>3</td>
<td>49082.6</td>
<td>16360.9</td>
<td>14.2</td>
<td>0.0014</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>9217.6</td>
<td>1152.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>58300.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 15. NH\(_4\)HCO\(_3\) with 1\% and 5\% H\(_2\)O\(_2\); Methanol method using HPLC.

AFB\(_1\) Statistically significant difference \( P = 0.00132. \)

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>3</td>
<td>66093.2</td>
<td>22031.1</td>
<td>14.6</td>
<td>0.0013</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>12110.6</td>
<td>1513.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>78203.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AFB\(_2\) - No statistically significant difference \( P = 0.409 \);

AFG\(_2\) - No statistically significant difference \( P = 0.360. \)
**AFG**₁ - Statistically significant difference \( P = 0.000793 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>3</td>
<td>70508.8</td>
<td>23502.9</td>
<td>16.9</td>
<td>0.0008</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>11093.2</td>
<td>1386.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>81601.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 16** NH₄HCO₃ with and without H₂O₂; CB method using TLC.

**AFB**₁ - Statistically significant difference \( P = 0.0000472 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>103951.4</td>
<td>25987.9</td>
<td>23.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>11160.2</td>
<td>1116.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>115111.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB**₂ - No Statistically significant difference \( P = 0.0699 \).

**AFG**₂ - Statistically significant difference \( P = 0.00397 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>31488.4</td>
<td>7872.1</td>
<td>7.83</td>
<td>0.0040</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>10052.3</td>
<td>1005.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>41540.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG**₂ - Statistically significant difference \( P = 0.00619 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>13008.3</td>
<td>3252.1</td>
<td>6.91</td>
<td>0.0062</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>4707.4</td>
<td>470.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>17715.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 17** NH₄OH; CB and Methanol method using HPLC.

**AFB**₁ - Statistically significant difference \( P = 0.000000610 \).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>234715.7</td>
<td>58678.9</td>
<td>59.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>9834.2</td>
<td>983.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>244550.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB**₂ - Not a statistically significant difference \( P = 0.179 \).
**AFG₁** - Statistically significant difference ($P = 0.000197$).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>64026.5</td>
<td>16006.6</td>
<td>16.8</td>
<td>0.0002</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>9553.1</td>
<td>955.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>73579.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG₂** - Statistically significant difference ($P = 0.0000123$)

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>19614.2</td>
<td>4903.6</td>
<td>31.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>1563.3</td>
<td>156.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>21177.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB₁** - Statistically significant difference ($P = 0.00132$).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>117937.4</td>
<td>29484.3</td>
<td>10.5</td>
<td>0.0013</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>28046.0</td>
<td>2804.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>145983.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB₂** - Statistically significant difference ($P = 0.000215$).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>82106.2</td>
<td>20526.5</td>
<td>16.4</td>
<td>0.0002</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>12505.5</td>
<td>1250.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>94611.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG₁** - Statistically significant difference ($P = 0.000000205$).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>64026.5</td>
<td>16006.6</td>
<td>74.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>2137.1</td>
<td>213.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>66163.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG₂** - Statistically significant difference ($P = 0.000503$).

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>68143.9</td>
<td>17036.0</td>
<td>13.4</td>
<td>0.0005</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>12723.4</td>
<td>1272.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>80867.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 18** \((\text{NH}_4)_2\text{S}_2\text{O}_8\) with and without \(\text{H}_2\text{O}_2\); CB method using HPLC.
**Figure 19** NH₄OH with and without H₂O₂; Methanol method using HPLC and TLC.

**AFBI** - Statistically significant difference $P = 0.0000000998$.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>5</td>
<td>289340.9</td>
<td>57868.2</td>
<td>52.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>13302.5</td>
<td>1108.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>302643.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB₂** Statistically significant difference $P = 0.00138$.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>5</td>
<td>15607.0</td>
<td>3121.4</td>
<td>8.26</td>
<td>0.0014</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>4532.8</td>
<td>377.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>20139.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG₁** - Statistically significant difference $P = 0.0000368$.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>5</td>
<td>75988.1</td>
<td>15197.6</td>
<td>17.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>10340.8</td>
<td>861.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>86328.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG₂** - Statistically significant difference $P = 0.00609$.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>5</td>
<td>12613.1</td>
<td>2522.6</td>
<td>5.77</td>
<td>0.0061</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>5243.4</td>
<td>436.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>17856.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 20** NH₄OH; Methanol method using HPLC.

**AFB₁** - Statistically significant difference $P = 0.00000262$.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>232719.5</td>
<td>58179.9</td>
<td>43.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>13274.9</td>
<td>1327.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>245994.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFB₂** - Statistically significant difference $P = 0.00356$.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>14429.6</td>
<td>3607.4</td>
<td>8.07</td>
<td>0.0036</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>4469.4</td>
<td>446.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>18899.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**AFG_1** - Statistically Significant Difference P = 0.0002

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>67798.2</td>
<td>16949.6</td>
<td>16.4</td>
<td>0.0002</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>10340.1</td>
<td>1034.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>78138.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AFG_2** Statistically Significant Difference P = 0.0102

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Treatments</td>
<td>4</td>
<td>12379.3</td>
<td>3094.8</td>
<td>5.95</td>
<td>0.0102</td>
</tr>
<tr>
<td>Residual</td>
<td>10</td>
<td>5199.6</td>
<td>520.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>17578.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
REFERENCES


Baur, Fred J. 1974. Effect of storage upon aflatoxin levels in peanut materials. Presented at the AOCS spring meeting, Mexico City, April 1974.


Food and Drug Administration. Assessment of estimated risk resulting from aflatoxins in consumer peanut products and other food commodities. FDA. Jan. 1978.


