EXTRUSION AND CHEMICAL TREATMENTS FOR DESTRUCTION
OF AFLATOXIN IN NATURALLY-CONTAMINATED CORN

by
Hossein Ghanim Hameed

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

1993
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Extrusion and chemical treatments for destruction of aflatoxin in naturally-contaminated corn

Hameed, Hossein Ghanim, Ph.D.

The University of Arizona, 1993
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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Hossein G. Hameed entitled Extrusion and Chemical Treatments for Destruction of Aflatoxin in Naturally-Contaminated Corn and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Ralph L. Price, Ph.D. 
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August 24, 1992

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

August 24, 1992

Date

August 24, 1992

Date

August 24, 1992

Date

August 24, 1992
STATEMENT BY AUTHOR

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SIGNED: [Signature]
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ABSTRACT

Naturally-contaminated corn containing 500 ppb total aflatoxins (AF) was ground to pass through a 20 mesh screen. Initial moisture content was determined for each sample and then adjusted to 15-20%. The samples were treated with sodium bicarbonate (NaHCO₃), sodium bisulfite (NaHSO₃), hydrogen peroxide (H₂O₂), calcium hydroxide (Ca(OH)₂), sodium hydroxide (NaOH), azodicarbonamide (Maturox), benzoyl peroxide (Novadelox), ammonium hydroxide (NH₄OH), or ammonium bicarbonate (NH₄HCO₃) in varying concentrations, and then extruded through a Wenger single barrel extruder at a temperature of 105°C. Extrusion conditions were the same for all samples. Prior to analysis the extruded product was dried and ground. All samples were analyzed in triplicate. A second set of samples were analyzed under acidic conditions using a 0.1 N hydrochloric acid (HCl) soak prior to extraction. Protein and amino acid analyses were conducted on extruded samples in which NH₄OH or NH₄HCO₃ were used. The Salmonella microsome mutagenicity assay was used to determine mutagenic potential on both "as is" and acidified extracts of the above samples.

Extrusion without chemical treatment of each meal reduced the AF by 40 to 70%. Ammonium hydroxide and NH₄HCO₃ were the only chemicals which reduced AF to below the United States Food and Drug Administration (FDA) action level of 20 ppb. Protein and amino acids were not significantly changed, and mutagenicity tests were inconclusive.
CHAPTER 1

INTRODUCTION

Aflatoxin (AF), a group of hepatocarcinogenic mycotoxins, is produced under favorable environmental conditions by the molds *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin has received attention because of its hepatotoxic, carcinogenic, mutagenic and teratogenic nature. Aflatoxin is metabolized by the cytochrome-P-450 enzyme system of the liver, and it is during this metabolism that AF becomes carcinogenic as the aflatoxin-8,9-epoxide. This form has been termed the "ULTIMATE CARCINOGEN".

These mycotoxins are protein binding in the commodities which renders those proteins unusable. Aflatoxins are also found in the free form, that is unbound. It is the unbound form which causes health problems.

The relationship between AF and protein nutrition is a complicated one. Not only does AF render the protein to which it is bound useless, but the free form binds to protein in the body. Aflatoxin has been shown to inhibit enzyme and protein synthesis by interfering with both DNA and RNA by binding the N-7 in guanine (Figure 1). Kwashiorkor and marasmus are forms of protein malnutrition.

Malnutrition is recognized in many forms of which marasmus and kwashiorkor are a major manifestation. When deficiency occurs in all nutrients, the result is recognized as marasmus. Kwashiorkor is thought to be the result of chronic protein-deficient food intake. Some literature claims that it is usually an abundance of
Figure 1. Structure of aflatoxin (AF)
carbohydrate energy simultaneous with protein-energy deficiency. Hence, the widely-used term: protein-energy deficiency. Aflatoxin has not been proven to cause these diseases, but it has been shown that the presence of AF can complicate these conditions. The reverse of this is also true, these two conditions can also effect the way AF is metabolized.

Although the chemical structure of AF is changed by treatment with alkali, recent evidence has indicated that AF may reform under acidic conditions such as that which may occur in the stomach (Price and Jorgensen, 1985). Also it is necessary to test for toxicity of any biologically active by-products which may result from processing. Many experiments using ammonia for destruction of AF have been successfully completed (Park and Pohland, 1989). However, due to the toxicity of ammonia these treatments have not been used for human food. Corn is used for animal feeds, and processed corn products such as corn grits, cereal, tortillas, and more recently, corn snacks are for human consumption. It is also a protein source in some developing countries.

Food extrusion has been practiced for over 50 years. Initially its role was limited to mixing and forming macaroni and ready-to eat cereal pellets. Now the food extruder is considered a high-temperature short-time reactor that transforms a variety of raw ingredients into modified intermediate and finished products. The impetus for these development has been the following requirement of food processing: 1) continuous high-throughput processing; 2) energy efficacy; 3) processing of relatively dry viscous
material; 4) improved textural and flavor characteristics of food constituents; and 5) control of the thermal change of food constituents, and used of unconventional ingredient.

The single-screw cooking extruder, used in this research to affect the AF in naturally contaminated corn, was developed in the 1940's to make puffed snacks from cereal flours or grits. The machine is capable of processing dry granular cereal ingredients (up to 20% moisture) for making of cereals.

The objective of this project is to determine the effects of chemical additives and extrusion on AF in naturally-contaminated corn.
CHAPTER 2

LITERATURE REVIEW

Aflatoxins (AF), a group of mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*, have been identified as causing substantial health and economic concerns (Purchase, 1972). Aspergillus are naturally occurring soil and airborne molds which contaminate agricultural commodities while growing in the fields. Once the mold is present, if the moisture of the crop in the field or during storage is high, the mold can continue to grow and produce toxin during storage. These toxins are known to be hepatotoxic and carcinogenic (Polan, Hayes and Campbell, 1974).

In 1961 United States Food and Drug Administration (FDA) scientists were notified by colleagues from England about the toxin found in groundnut (peanut) meal. The molds were first isolated in the 1960s as causing "Turkey X" disease in England which resulted in the death of 100,000 young turkeys (Blount, 1961). The cause was determined to be a shipment of contaminated nutmeal from Brazil. Investigation has shown that these toxic compounds are carcinogenic and will provoke liver cancers in laboratory animals such as rats and rainbow trout. Salmon, mice, among others are somewhat resistant. Although all AF are both toxic and carcinogenic to animals, aflatoxin B₁ (AFB₁) is the most common and most potent form (Wogan, Paglialungas and Newberne, 1974). Animal products can become contaminated if animals are fed AF-contaminated feed. Aflatoxin toxicity is related to its negative interactions with nucleic acid and protein metabolism (Moreau and Moss, 1979).
It should be emphasized that this is a group of chemical compounds, produced by a mold, just as penicillin is produced by *Penicillium notatum*, another mold. It is not a living organism. Aflatoxin-contaminated foodstuffs and cases of aflatoxicosis have been reported in the United States and some European countries (Thean et al., 1980). However, most reports of AF contamination have originated from tropical or semi-tropical countries of Africa, Asia and South America where the temperature and relative humidity are favorable for the growth of molds and production of these toxic metabolites.

Epidemiological studies which correlated average AF intake by humans with official liver cancer mortality statistics in Kenya (Peers and Linsell, 1973), Mozambique (van Rensburg et al., 1974), Swaziland (Peers, 1976; Peers et al., 1987) and China (Yeh et al., 1989) have strongly linked the frequency of primary liver cancer to the consumption of AF-contaminated peanuts and peanut products. Other studies have established a relationship between the consumption of AF-contaminated foods and the onset of kwashiorkor (Hendickese, 1983; Hendickese et al., 1983).

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, Hesseltine and Mehlman, 1977). Among the Bantu populations of Africa, for example, over 100 cases of liver cancer per 100,000 inhabitants are reported annually, while less than one case per year may be found among the same number of people of Holland, Norway or Canada (FDA, 1978; Moreau and Moss, 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 cancers seen today, hepatic
toxins (such as AF) 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 AF is responsible for the exceptionally high rates in parts of Africa and Asia (van Rensburg et al., 1985). A recent finding is that AF can worsen the nutritional disease, kwashiorkor (Hendickese, 1983; Hendickese et al., 1983). Children in Sudan suffering from this condition had much higher levels of AF 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 AF is found in the food supply but is rare or absent in areas with AF-free food, even though the protein-calorie intake of individuals in the two areas may be similar.

The AF fungi, 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 and Moss, 1979). Many believe that AF is produced in these seeds during storage under conditions of high humidity. With certain exceptions, this is not true. Most of the AF is produced in the field when the plant is under some sort of stress such as drought, insect or mechanical damage (Marasas and Nelson, 1987). Another misconception is that AF is produced only on the surface of the seed or that the mold only grows on the surface. In reality, because the mold grows before the seed is dry, when it is still immature, AF usually contaminates the entire seed, from the inside to the outside (Marasas and Nelson, 1987).

Another factor which must be taken into consideration, especially when sampling for analysis, is that only one seed in several hundred may be contaminated.
One very highly contaminated seed may condemn an entire lot, while a small sample may exclude any contaminated seeds and give the analyst a false sense of security. Sample sizes must be somewhat large, on the order of 10 kg of sample, to give confidence in the analytical results (Bauwin and Ryan, 1982; Hurburgh and Bern, 1983).

In 1964 the United States Department of Agriculture (USDA) and the United States peanut industry made a marketing agreement to analyze peanuts for AF, and anything above 100 ppb was to be destroyed. However, for human food better analytical techniques allowed the action level of 20 ppb total AF, with the exception of milk which has an action levels of 0.5 ppb for aflatoxin M₁, a metabolite of AFB₁ (FDA, 1978). In Senegal, AF levels higher than 2,000 ppb have been found in market samples of peanuts (Peers and Linsell, 1977). To make things worse, most African countries have no agencies with enforcing powers similar to the FDA.

Aflatoxin in Milk

Several studies have been done in this area especially upon the relationship between ingested AF and extraction of aflatoxin M₁ (AFM₁) in milk. Allcroft et al. (1966) studied the occurrence of AF in tissue, urine, and milk of sheep.

When AF is fed to animals, it is metabolized in the liver to other forms. One, the epoxy, is extremely reactive, and is the source of the toxicity and carcinogenicity of the compound. Other metabolites are hydroxylated to be excreted from the body as conjugates with glucuronides and sulfates (Wong and Hsieh, 1976). About 1.6% of the original amount of the injected AFM₁ appears in the milk of dairy animals as the
hydroxylated metabolite AFM\textsubscript{i} which is about 1/10 as potent (Price, 1980a,b; Frobish et al., 1986). Masri, Vix and Goldblatt (1969) showed a general correlation between the dosage of AFB\textsubscript{i} and the level of AFM\textsubscript{i} in milk. No AFM\textsubscript{i} could be detected in milk obtained from cows receiving 10 ppb of AF in concentrate diets over 14 days period (Polan et al., 1974).

If the AF is removed from the dairy ration, after 4 days AF will not be detected (Price, Lough and Brown, 1982). Very small amounts of several metabolites are found in the liver and muscles of highly dosed animals if they are killed with AF in their systems. A withdrawal of 2-3 days is sufficient to allow all AF to be metabolized. It is not maintained in the fat systems as are many pesticides (Allcroft et al., 1966).

**Aflatoxin Toxicity**

The LD\textsubscript{50} for AFB\textsubscript{i} for one day-old ducklings is 0.3-0.5 \(\mu\text{g/kg body weight} \) (Purchase, 1967). The LD\textsubscript{50} for the more resistant animals, such as the mouse, is 9 mg/kg body weight. It has been reported that in mice a daily dose of 1 \(\mu\text{g/kg body weight} \) over the course of their lifetime will result in a 50% incidence of tumors. With saccharin, one of the weaker carcinogens for example, the dose required for a similar response is over a million times greater. Aflatoxin can cause serious biological effects when it is consumed regularly at high levels (Ames, Lee and Durston, 1973). Some of the effects are: 1) inhibition of protein synthesis; 2) inhibition of enzyme induction; 3) inhibition of lipid synthesis and transport; 4) immunosuppression; 5) fatty degeneration of the liver leading to bile duct proliferation and fibrosis; 6) depression of nucleic acid
synthesis; and 7) cellular level inhibition of oxidative phosphorylation, which induces the
degradation of polysomal profile and nuclear segregation and causes loss of lysosome
integrity (Hsieh, 1983). It has also been reported that AFB₁ can increase the
susceptibility to hepatitis which could increase liver damage (Ciegler and Bennet, 1980).

Aflatoxin Metabolism and Carcinogenicity

Aflatoxin is metabolized primarily by the cytochrome-P-450 isozyme (C-P-450) and the mixed function oxidase system of the liver. Aflatoxin B₁ is activated by the C-P-450 and converted to the AFB-8,9-epoxide (Figure 2). This epoxide can bind covalently to nucleic acids such as DNA, RNA and other macromolecule 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-AFB₁) which is then excreted or it can undergo reduction, hydroxylation, hydration or o-demethylation (Figure 2) (Fukayma and Hsieh, 1985). It has been mentioned that the high level of water soluble metabolites in the urine and feces of AFB₁ derivatives, at earlier stages after AFB₁ treatment indicate increased levels of metabolism and detoxification of AFB₁. The compounds resulting from detoxification of the AFB₁-epoxide by binding with glutathione are found in the feces.

The AFB₁-8,9-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
Figure 2. Metabolism of Aflatoxin B₁ (from Busby and Wogan, 1981)
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.

Detoxification of Aflatoxin

The strategy for AF control is to prevent its formation in crops during the stages of growing, harvesting, and storage (USDA, 1968a,b; Goldblatt, 1969; Goldblatt, 1971; Anderson, 1983). Since this is difficult, several physical, biological and chemical methods have been used to detoxify AF-contaminated agricultural products (Goldblatt and Dollear, 1977a,b; Diener et al., 1979; Marth and Doyle, 1979; Anderson, 1983; Palmgren and Hayes, 1987).

Chemical Treatment for Detoxification

Detoxification can be accomplished by changing the structure of the AF molecule. The lactone ring is most susceptible to attack by chemical agents. The lactone ring can be easily opened by strong alkalis like sodium or calcium hydroxide (Figure 3; Park et al., 1988). Many chemicals including oxidizing agents and various gases, have the ability to structurally degrade and inactivate AF (Goldblatt and Dollear, 1979; Anderson, 1983). Opening of the lactone ring reduces the toxicity, mutagenicity and carcinogenicity of the AF molecule. Research in the area of detoxification has been extensive. Many chemicals and processes have been researched. Treatment of the contaminated commodities with chemicals such as ammonia, hydrogen peroxide ($\text{H}_2\text{O}_2$), sodium hypochlorite and calcium hydroxide ($\text{Ca(OH)}_2$) are just a few (Goldblatt and
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).
Dollear, 1979; Anderson, 1983). Processes such as tortilla making (Price and Jorgensen, 1985), roasting, microwaving (Feuell, 1966), extrusion (Gréhaigne et al., 1983; Martinez and Monsalve, 1989), biological methods (Ciegler et al., 1966), and solvent extraction (Shantha, 1987) have been studied. Some of these processes have been tested in combination with others.

**Use of Bisulfite.** Treatment with bisulfite resulted in a marked reduction in AFB₁ and aflatoxin G₁ (AFG₁) (Doyle and Marth, 1978). Treating food with up to 2000 ppm of sulfate (SO₄) ion gave significant reduction in AFG₁ which is more rapidly reduced than AFB₁. For every 10°C increase in temperature, there was a two times decrease of AFB₁ and AFG₁ levels.

Treating naturally contaminated corn with sodium bisulfite (NaHSO₃) or sodium hydroxide (NaOH) at 2.0% concentration significantly reduced AFB₁ and AFB₂ (Moerck et al., 1980); NaHSO₃ was more effective than NaOH.

**Use of Acid.** Strong acids can decrease biological activity of AF by changing its structure. Ciegler et al. (1966) reported that AF can be degraded by using strong acid treatment which catalyzes the addition of water to the dihydro-furan moiety group of AF structure. These treatment convert them to the AFB₂a and AFG₂a forms which are not readily converted to the carcinogenic form (Hsieh and Ruebner, 1984). Pons et al. (1972) reported that 95% of 50 mg of AFB₁ was converted to AFB₂a when treated for six hours at pH 3.0 and 100°C. When the pH was reduced to 1.0 and the temperature maintained at 100°C, treatment of AFB₁ for 10 min converted an equal amount of AFB₁ (95%) to AFB₂a. Aflatoxin B₂a is much less potent than AFB₁.
However, the use of strong acid is not practical because of the strength of the acid and time and temperature required. Hence, it is not likely that the use of the acid solution is valid alternative for detoxifying agricultural commodities. Additional work has shown that relatively small amounts of AFB$_2$ and AFG$_2$ in commodities are affected by this treatment.

**Base Treatment.** Different organic and inorganic bases have been evaluated for their effect on AF (Marth and Doyle, 1979). Base treatment with alkali has shown encouraging results in the decontamination of feeds and food. Norred (1979) observed a 99% destruction of AF by base treatment of corn naturally contaminated at 1000 mg AF/kg corn. Dollear (1969) reduced the level of AF in cooked peanut meal from 113 to 18 mg/kg by treating it with 2% NaOH and 22% moisture. When the moisture was increased to 30%, only traces (ca. 5 mg/kg) of AF remained in the treated material.

**Use of Calcium Hydroxide.** Aflatoxin in peanut meal with 15% moisture was reduced from 570 to 26 ppb by treatment with 2% Ca(OH)$_2$ and formaldehyde at 117°C for 1 hour (Condifer, Mann and Dollear, 1976). Price and Jorgensen (1985) showed that the tortilla making process using Ca(OH)$_2$ significantly decreased the AF levels and the mutagenic potential of the products made from naturally contaminated corn. However, they also showed that acidification of the final product caused a reformation of the changed AF indicating that the resulting compound was an intermediate and not a stable product. The acidification was done to simulate the acidic conditions of the stomach.

Camou-Arriola and Price (1989) showed that deep fat frying of corn previously treated with NaOH not only decreased the AF levels but also the mutagenic potential of
products made from naturally contaminated corn. This study also showed a complete degradation of the AF since there was no reformation of the AF when acidified. The main difference between these two studies was the strength of the alkali used and higher temperature of deep frying. Sodium hydroxide is much stronger than Ca(OH)$_2$.

Park et al. (1981) reported that treatment with 2% Ca(OH)$_2$ plus monomethylamine at an atmosphere of 24% moisture and 100°C for 1 hr would reduce AF in peanuts about 94%. This reduction was dependent upon the level of contamination and chemical structure of the AF. Dollear et al. (1968) reported that AF-contaminated peanut meal treated with NaOH and increased temperatures decreased the AF.

**Ammoniation.** Various chemical treatments for the reduction of AF in a product have been studied since AF is relatively resistant to heat (Goldblatt and Dollear, 1979; Anderson, 1983). These treatments are applicable when AF contamination is less than 500 μg AF/kg. The most successful treatment has been using ammonia. Research has shown that not only is the AF degraded, but that the mutagenic potential of the resulting compounds is significantly less than that of the AFB$_1$ (Price and Jorgensen, 1985; Camou-Arriola and Price, 1989). A review by Park et al. (1988) shows that the different ammonia procedures have yielded products that would be safe to the animals consuming the ammoniated AF-contaminated commodity. Jorgensen et al. (1990) showed that the mutagenic potential of the milk from cows fed ammoniated cottonseed was not significantly different from the milk from cows fed non-contaminated cottonseed.

Gaseous ammonia has been used under two sets of conditions: 1) high temperature/high pressure, and 2) ambient temperature/ambient pressure. Many different
agents of ammonia have been used in combination with other chemicals such as NH₄OH, methylamine, NaOH and formaldehyde to achieve a 95-99% reduction in AF, ammonia was used at concentration of 1.4-3.5%, for 15 to 30 min, at a temperature of 70-120°C, and 10-15% moisture content.

A technique was developed to detoxify AF in cottonseed contaminated with 400 to 7000 ppb of AF in multi-ton lots (Price et al., 1982). The contaminated cottonseed was treated with 1.5% ammonia, packed into plastic bags, held for 21 days and then fed to lactating dairy cattle at a level of 3.5 kg per day for 19 days. Less than 0.1 ppb of AF was found in milk, a reduction of 99%.

The regulatory status for ammoniation is pending. Ammoniation of animal feed is allowed to increase the protein value as non-protein nitrogen, but it is not currently allowed for the purpose of AF degradation (Lancaster and Bothast, 1976; Conkerton et al., 1981). Several states and foreign countries, however, do allow ammoniation of AF-contaminated products to decrease the levels of AF (Park and Pohland, 1986). These ammoniated products cannot be shipped across state lines (FDA, 1983).

Physical Methods of Separation

Complete physical separation of contaminated products is difficult to achieve because one cannot always tell by looking at a product whether or not it is contaminated. Residual contamination, at a low level, is usually present in final product. It has been reported that electronic and hand sorting of contaminated peanuts resulted in reduction of the AF level (Dickens and Whitaker, 1975; Natarajan et al., 1975). Huff (1980) found
a significant decrease in the total AF concentration by flotation of AF-contaminated corn in water and sucrose solution.

Thermal Inactivation

Heating AF-contaminated commodities gave varying results. Feuell (1966) said that the use of heat for destruction of AF was not successful, but pure AF melted at 250°C. Baking temperatures did not significantly change AF levels in dough (Reiss, 1978). The toxicity of AF was not reduced by heating the meal at 150°C for several hours. Aflatoxin M₁ was stable in raw milk during storage under refrigeration and resistant to pasteurization and processing (Stoloff et al., 1975; Stoloff, 1980).

Mann, Codifer, and Dollear (1967) found that the degradation of AF was a direct function of temperature, heating time, and moisture content. Aflatoxins are heat-stable and can not be totally destroyed by boiling, autoclaving, or by usual food and feed processing procedures (Christensen, Mirocha, and Meronuck, 1977). Aflatoxin in nuts, oilseed meals and corn can be decreased somewhat by roasting (Conway, Anderson, and Bagley, 1978; Marth, and Doyle, 1979).

Irradiation

Feuell (1966) found that gamma irradiation (2.5 mrad) did not degrade AF in contaminated groundnuts. Shantha and Sreenivasa (1977) found that when contaminated peanut oil was exposed to shortwave and long wave UV light the level of AF was significantly reduced.
Biological Methods of Detoxification

Microorganisms such as yeasts, mold and bacteria have the capability to change or inactivate the AF molecule. The application of microbial detoxification has been reviewed (Ciegler, 1978; Marth and Doyle, 1979). Ciegler et al. (1966) used *Flavobacterium aurantiacum* to remove AF from peanut milk and found no toxic by-products in the decontaminated material. Hao, Brackett and Nakayama (1987) also reported successful removal of AFB₁ from peanut milk using *F. aurantiacum*.

Dam, Tam and Satterlee (1977) degraded AF by fermenting contaminated grain. Normal fermentation of corn to produce ethanol does not degrade AF. It remains in the distiller's residue (Bothast et al., 1982).

Extrusion

Extrusion is the process which unites the operations of mixing, working, forming, cooking and transport. The extrusion process has been described as a low moisture, continuous flow process capable of changing the molecular and physical structure of a product. There is an increasing use of extrusion in food processing (Harper, 1981). Little research has been done to determine the effects of extrusion of food products containing chemical additives. This would determine if chemical reaction(s) take place between the additive and any constituent of the product.

Physical Effects

Colonna and Mercier (1982) showed that extrusion of commercial manioc starch using a twin-screw extruder at 2 speeds (40 and 80 rpm), moisture content of 22%, 24
kg/h feed rate and temperatures of 125, 150, 175 and 200°C resulted in macromolecular degradation of the starch.

Davidson et al. (1984) studied the effect of using a single screw extruder on molecular weight distribution of wheat starch. They found mechanical degradation of the amylopectin. These structural changes were effected by shear stress and time in the extruder. Davidson et al. (1984) continued to work in this area to determine the nature of the degradation. They found that the structural changes in the extruded product effected the physical characteristics of the product, for example, density and elasticity.

Giddey, Brandt and Bunter (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. Gréhaigne et al. (1983) 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 30%. 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. Martinez and Monsalve (1989) found the extrusion cooking process of naturally-contaminated AF (20.8 ng/g) of Venezuelan white corn resulted in a 79% decrease in AF.
Effects on Nutrients

Recently, reviews have been done on the effects of extrusion cooking on specific nutrients of foods. These reviews deal with effects on starch, fiber, fat, protein, vitamins and minerals, and evaluated the changes from a nutritional viewpoint.

Starch. Starch granules undergo gelatinization and melting by the action of heat and moisture on hydrogen bonding. Among lightly packed polysaccharides, lipid has an influence on the behavior of the starch. While short chain polar lipids may actually accelerate the rate of gelatinization, the medium and long chain compounds inhibit the swelling of granules and uptake of water.

Colonna et al. (1984) studied changes in the molecular weight of amylopectin after extrusion at 11% moisture at both 130 and 180°C. A extruder screw speed of 270 rpm was used and amylopectin molecular weight dropped from 58 million to 2.7 million after extrusion. The mechanism responsible for the decrease of molecular weight is not currently known, but the authors believe shear to be a major factor. Davidson et al. (1984) reported degradation of amylopectin by a single screw extruder to a molecular weight average of 500 KDa.

Protein. Faubion and Hoseney (1982a,b) reported that protein isolate and wheat gluten had opposite effects in HTST extrusion. Soy protein isolate added at 7.5% to wheat starch caused a 25% increase in expansion, with similar levels of added gluten causing a 14% reduction in expansion.

The chemical reaction between a reducing sugar, such as glucose, fructose, lactose, or maltose, and a free amino group on an amino acid, usually the epsilon-amino
group of lysine, has important nutritional and functional consequences. The reaction, known as non-enzymatic browning, is actually a series of reactions with a wide variety of compounds produced as a result (Waller and Feather, 1983). Feather (1985) reported that lysine in food did not react with a reducing sugar during extrusion.

**Other Compounds.** Sugar may decrease gelatinization by increasing the temperature. Ionic species, depending on the electrolyte, had the opposite effect.

Evans and Haisman (1982) indicated there is a relationship between water activity and the effect of the solute. Sodium chloride produced an increase in gelatinization temperature up to 1.5 M; lower concentrations reduced the gelatinization temperatures. Calcium chloride reduced gelatinization temperatures initially, but increased it as concentration increased beyond 3 M. Sodium acetate also decreased gelatinization temperature.

**Ames Test**

Aflatoxins are well-characterized toxic and carcinogenic fungal metabolites produced by *Aspergillus parasiticus* and *Aspergillus flavus*. Their synthesis is through precursors of hydroxyanthraquinones, including norsolorinic acid and biological activity (Hsieh et al., 1976), averufin (Lin et al., 1973), versicolorin (Lee, 1989), and averantin (Bennett et al., 1980).

A human cancer can be caused by exposure to a carcinogenic chemical. These chemical carcinogens are usually mutagenic. It is suggested that damage to DNA is a primary event in both carcinogenesis and mutagenesis (Ramsdell and Eaton, 1988).
is very important to protect humans from exposure to these compounds by identifying them and discovering their potencies.

Ames, McCann and Yamski (1975) discovered a simple and sensitive test for detecting chemical mutagens. A thin layer of agar containing about 100 bacteria of an especially constructed tester strain of *Salmonella* is placed on a Petri dish. The inoculated bacteria is unable to grow because the essential histidine is absent from the media. Mutation of one of the genes of the bacteria prevents biosynthesis of this amino acid. Adding a mutagen to the plate may result in a new mutation and reverse the bacteria to the original so that histidine can be synthesized again and growth will occur.

Bjeldanes, Chang and Thomson (1978) have shown by the Ames mutagenicity test that mutagenic compounds are produced by wide range of fungi. Wong and Hsieh (1976) have found that AFB₁ has the strongest mutagenic potential of all AF.

Price and Jorgensen (1985) tested naturally-contaminated corn and tortillas for mutagenic potential using the Ames assay. They found that treating the tortillas with acid resulted in an increase in the mutagenic potential when compared to those tortillas which were not treated with acid. This was done in order to simulate the monogastric digestive system. The results of this study showed that the tortilla making process was insufficient to eliminate AF from contaminated corn.

Camou-Arriola and Price (1989) found that using lye and high cooking temperatures of deep fat frying not only decreased the AF levels in naturally-contaminated corn, but also resulted in a significant permanent decrease in the mutagenic potential something which was not seen with the tortilla study.
Jorgensen et al. (1990) studied the mutagenic potential of milk from cows fed naturally-contaminated cottonseed and ammoniated-naturally-contaminated cottonseed. The results of this study showed that there was no difference in the mutagenic potential when comparing the ammoniated milk to the milk from cows fed clean cottonseed. This confirmed that not only does ammonia result in permanent degradation of AF but that it does not increase the mutagenic potential of the milk.
CHAPTER 3

MATERIALS AND METHODS

Ground white corn contaminated with AF (500 ppb) was extruded through a Wenger X-5 extruder at temperatures exceeding 100°C. Various FDA approved food additives were added in concentrations up to 1% of the weight of the corn. After extrusion, AF analysis was performed on the extruded product to determine the extent of AF destruction. The extruded product was acidified to simulate conditions in the monogastric stomach and analyzed to determine the extent of permanent AF destruction.

Extruded corn from treatments showing the most promise was analyzed for changes in mutagenicity using the Ames Salmonella mutagenicity test. Amino acid analyses were also run on these selected samples to determine changes that may have been caused by heat treatment of corn under alkaline conditions.

Source of Chemicals

All chemicals, unless otherwise noted, were of reagent grade and were obtained from Sigma Chemical Co., St. Louis, MO. S9 extracts were from Moltox, Molecular Toxicology, Inc., Annapolis, MD. Agar was from DIFCO Laboratories, Detroit, MI. Petri dishes were from Becton Dickinson Labware, Lincoln Park, NJ. Sodium bisulfite (NaHSO₃) and 30% hydrogen peroxide (H₂O₂) were from Fisher Scientific, Fairlawn, NJ. Sodium metabisulfite (Na₂S₂O₅) and calcium hydroxide (Ca(OH)₂) were from
Mallinckrodt Chemical Works, St. Louis, MO. Azodicarbonamide (Maturox) and benzoyl peroxide (Novadelox) were from Pennwalt Corp., Buffalo, NY.

**Source of Contaminated Corn**

Arizona white corn, naturally contaminated with 500 ppb total AF was obtained from a feed mill in Tolleson, Arizona. It was cleaned and stored at ambient Arizona temperatures (15 - 50°C) for 1 to 12 months before treatment.

**Use of the Extruder**

A Wenger X-5 single screw extruder with a fluted stainless steel barrel (Wenger Co., Sabatha, KN) was used for all extrusions. Extrusion conditions were established for added water, temperature, pressure and raw material input velocity so that a product was produced with as little variation and as much expansion as possible.

**Preparation of the Product**

Approximately 50 kg of contaminated corn was ground to pass through a 20 mesh screen. One kg sample was used for each treatment. The moisture of the corn flour (10%) was determined by drying in a vacuum oven (Assoc. Offic. Anal. Chem. (AOAC), 1984; method 14.003). The amount of moisture to give maximum expansion of the product was determined by varying the amount of water added directly to the corn flour from 5 to 15% immediately before extrusion. All additives, except H₂O₂ and ammonium hydroxide (NH₄OH), were added dry to the flour prior to addition of 10%
water. Sufficient NH₄OH (29%) or H₂O₂ (30%) were added to the water to provide the indicated addition percentage.

After addition of the fluid(s), the meal was mixed for 20 min with the whip beater of a Hobart mixer (Hobart, Inc., Troy, OH). The corn meal was then added directly to the hopper for feeding the extruder. No additional water was added through the extruder and the steam to the extruder barrel was on full. The product was further mixed under pressure inside the extruder, and extruded at a temperature of 105°C (research papers done where temperatures were measured indicate that temperatures may be close to 150°C). Temperature at the extruder head and within the extruder barrel could not be monitored continuously because there was no temperature monitoring device. A mercury-in-glass thermometer was taped to the outside of the extruder barrel and covered with insulation. A steel barrel, hand held thermometer was placed into the product immediately as it left the extruder die. Upon leaving the 1 mm extruder die, the product expanded with an immediate release of steam, formation of solid structure, and subsequent cooling due to the latent heat of evaporation. Between samples, the extruder was not stopped nor cleaned; but the hopper was allowed to empty, a small portion of the next sample provided a purge, and the next sample was added to the hopper.

The extruded product with the most favorable physical characteristics (approximately 2 cm in diameter) was produced with 10% added water. About 4 kg of extruded product was obtained every hour with the extruder motor speed set at 4 (120 rpm), and the hopper screw speed set at 8 (25 rpm). After a desirable product was
obtained from the extruder, all conditions leading to that product were maintained. Only the addition of the chemicals varied.

Between treatments, sample collection stopped for 2 min until all of the previous sample had been expended. Extruded products were dried at 25°C. Samples were ground to pass a 20 mesh screen and were analyzed for AF. Selected treatments were analyzed by the Ames test for mutagenicity, and for amino acids and protein.

**Aflatoxin Analysis**

All corn samples were analyzed before and after extrusion for AF according to the method of Thean et al. (1980) using visualization and comparison with standards (Sigma Chemical Co, St. Louis, MO) on 25 μ silica gel thin layer chromatographic (TLC) plates (Uniplate, Analtec, Inc, Newark, DE). The application solvent was benzene:acetonitrile (98:2, v/v) and the developing solvent was chloroform:acetone (90:10, v/v). All samples were analyzed in triplicate.

A second set of samples were similarly analyzed with soaking the product in a 0.1 N HCl prior to the extraction. When AF is treated with alkali, an intermediate compound is formed, which cannot be detected by regular analysis, but which may revert to the original compound upon entering the stomach. Soaking with acids allows an evaluation of that reversion (Figure 3).

**Mutagenic assay**

Each sample of extruded corn was extracted with acetone in the ratio of 25 g/125 ml according to Felton et al. (1981). For acidification of the sample, 25 g of corn
was agitated with 125 ml acetone:0.1 N HCl (85:15, v/v) using a wrist type shaker for 30 min. After filtering and drying (25°C), samples were reconstituted with 1 ml dimethylsulfoxide (DMSO). Each sample was diluted five times by a factor of 100 with DMSO to yield five test solutions with concentrations ranging from 1:100 to 1:10^10.

The tester strain of *Salmonella typhimurium* (TA. 100) was supplied by Dr. Douglas L. Park, Food Toxicology Research Group, Department of Nutrition & Food Science, The University of Arizona. The strains were stored following procedure of Ames et al. (1975) and Maron and Ames (1983). Preliminary checks were run with the Ames test to be sure the bacteria had not been altered during storage (Appendix A).

The S9 mix was prepared just prior to use and kept on ice. To 2 ml of sterile top agar in a 5 ml sterile test tube, 0.1 ml of each sample, 0.1 ml bacterial culture, and 0.5 ml of S9 mix were added. The tubes were agitated by a vortex mixer and were then poured on the surface of the MGA plates. The plates were rotated immediately to ensure that the media completely covered the plates. After the top agar hardened the plate was placed upside down for 48 hr in an incubator at 37°C.

Another plate without the S9 mix was made to test mutagenicity without liver enzyme transformation. Aflatoxin standards (5, 10, 25, 50 and 100 µg) were run as controls. A media control without added chemicals for spontaneous reversion was plated.

**Amino Acid Analysis**

One hundred mg of each corn sample was weighed in duplicate and hydrolyzed with 6 N HCl in an autoclave (16 to 18 h, 121°C) in the presence of sodium thioglycolate
(mercaptoacetic acid, sodium salt, w/w) to preserve methionine (Veronese et al., 1974),
(designated amino acid A), or with HCl in the presence of performic acid to oxidize
cystine to cysteic acid (Schram, Moore and Bigwood, 1954; Hirs, 1956) prior to the acid
hydrolysis described above (designated amino acid B cysteic acid sample). Both samples,
A and B, were diluted to 10 ml with 0.1 N HCl, filtered through Whatman No. 5 filter
paper and the filtrate collected in small vials (Appendix B).

Amino butyric acid (2.5 μmole/ml) was added to each sample vial as an internal
standard. A Spectra Physics high performance liquid chromatograph (HPLC, model
SP8000B, Spectra-Physics, Mountain View, CA) equipped with a fluorescence detector
(Model 157, Beckman Instruments, Inc., Berkeley, CA), with automatic injection (Model
725, Micromeritics Instrument Corp., Norcross, GA), and a reversed phase 3 μm C-18
spherical silica (10 cm × 4.6 mm I.D.) column (Rainin Short One; Rainin Instrument
Co., Inc., Emeryville, CA) at room temperature was used in the determination of amino
acids (Lindroth and Mopper, 1979; Jones, Pääbo and Stein, 1981; Appendix C).

Protein Analysis

Protein was determined using the micro-Kjeldahl method (AOAC, 7.015; 1984)
as protein nitrogen by removing all carbon and oxygen and trapping the nitrogen during
digestion. Nitrogen was then determined by titration.

TREATMENT 1: Sodium Bisulfite and Calcium Hydroxide

Two levels (0.1 or 0.25%, w/w) of NaHSO₃ and 0.1% Ca(OH)₂ were added
dry to corn previous to extrusion. Experimental design is shown in Table 1.
Table 1. Addition of sodium bisulfite and calcium hydroxide to aflatoxin-contaminated ground corn for extrusion with 10% added moisture.

<table>
<thead>
<tr>
<th>% NaHSO₃</th>
<th>% Ca(OH)₂</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0.25</td>
<td>0.1</td>
</tr>
</tbody>
</table>

TREATMENT 2: Sodium Bisulfite and Dilute Hydrochloric Acid

Sodium bisulfite (NaHSO₃) and Na₂S₂O₅ were added alone or as 0.1 and 0.25% (w/w) to the corn in mixture as a powder along with water or 0.1 N HCl previous to the extrusion. Water which was normally added to the corn meal previous to the extrusion was replaced by the same amount 0.1 or 0.25% of 0.1 N HCl to complete the final percentage of the moisture contained in the corn (15 - 20%) as a treatment. To the acid-treated corn meal, NaHSO₃ or Na₂S₂O₅ were added (0.1 - 0.25%) and the whole was mixed for 20 min before extrusion. Experimental design is shown in Table 2.

Table 2. Addition of sodium bisulfite or sodium metabisulfite with dilute hydrochloric acid or water to aflatoxin-contaminated ground corn for extrusion with 10% added moisture.

<table>
<thead>
<tr>
<th>% NaHSO₃ or Na₂S₂O₅</th>
<th>% Ca(OH)₂</th>
<th>HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>0.1 N</td>
</tr>
<tr>
<td>0.25</td>
<td>0.1</td>
<td>0.1 N</td>
</tr>
</tbody>
</table>
TREATMENT 3: Sodium Bicarbonate and Oxidizing Agents

Sodium bicarbonate (NaHCO₃) and three oxidizing agents, H₂O₂ (0.1 or 0.2%), azodicarbonamide (Maturox, 0.0025 or 0.005%), and benzoyl peroxide (Novadelox, 0.015 and 0.03%) were added to the corn previous to extrusion. Each level of each oxidant was added to corn with and without 0.5 or 1.0% NaHCO₃. Both dry and wet additives were thoroughly mixed for 20 min with the whip beater previously described. The corn meal was then added to the extruder. Control corn meal without additives and with 0.5 or 1.0% NaHCO₃ was extruded as separate batches. Experimental design is shown in Table 3.

Table 3. Addition of sodium bicarbonate and hydrogen peroxide or Maturox or Novadelox to aflatoxin-contaminated ground corn for extrusion with 10% added moisture.

<table>
<thead>
<tr>
<th>% NaHCO₃</th>
<th>% H₂O₂</th>
<th>% Maturox</th>
<th>% Novadelox</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>2.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
<td>2.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

TREATMENT 4: Hydrogen Peroxide and Ammonium Hydroxide

Hydrogen peroxide (0, 1.0 or 5.0%) and NH₄OH (0, 0.5 or 1.0%) were added alone or in combination to the ground corn with the conditioning water during the 20 min mixing period previous to extrusion. The experimental design is shown in Table 4.
Table 4. Addition of ammonium hydroxide and hydrogen peroxide to aflatoxin-contaminated ground corn for extrusion with 10% added moisture.

<table>
<thead>
<tr>
<th>% NH₄OH</th>
<th>% H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>1.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**TREATMENT 5: Ammonium Hydroxide**

Concentrated NH₄OH (29 N) was mixed along with water, to give a 0, 0.1, 0.4, 0.7 or 1.0% concentration in the corn meal. The liquid and meal were mixed for 20 min just previous to extrusion. Samples were taken before and after the extrusion. This experiment was repeated with exactly the same experimental conditions. No samples of unextruded corn were collected the second time.

**TREATMENT 6: Ammonium Bicarbonate**

Powdered ammonium bicarbonate (NH₄HCO₃) was added to the dry ground corn to give final concentrations of 0, 0.1, 0.4, 0.7 or 1.0% on a weight basis. Prior to mixing for 20 min, 10% water was added to the meal. of NH₄HCO₃ added directly to 90 to 100 ml of water during the blending prior to extrusion.

**TREATMENT 7: Concentrated Caustic Soda and Hydrogen Peroxide**

Calculations were made whereby all of the moisture added to the ground corn previous to extrusion would consist of equal amounts of 1% sodium hydroxide (NaOH)
and 1 or 2% H$_2$O$_2$. They were sprayed together into the stainless steel mixing bowl onto the ground corn meal during the mixing period. In addition, a portion of the corn was heated in an oven at 70°C for 30 min previous to mixing to destroy any catalase that might be present in the grain. A portion of each sample was further heated at 70°C for 30 min after mixing to allow the chemicals to react further with the AF. Half of the corn was then extruded; the other half provided an extrusion control. Experimental design is shown in Figures 4-6.

**Statistical Analysis**

Statistical analysis was performed using ANOVA for multifactorial design. Differences between means were separated by least significant differences at $p<0.05$. 
Figure 4. Schematic representation of the extrusion of aflatoxin-contaminated corn
Figure 5. Schematic representation of the extrusion of aflatoxin-contaminated corn
EXTRUSION OF CORN MEAL
Hydrogen Peroxide and Sodium Hydroxide

Figure 6. Schematic representation of the extrusion of aflatoxin-contaminated corn meal
CHAPTER 4

RESULTS AND DISCUSSION

Ground white corn contaminated with AF (500 ppb) was extruded through a Wenger X-5 extruder at a temperature of 105°C. Various FDA approved food additives were added in concentrations up to 1% of the weight of the corn. Aflatoxin analysis was performed on the extruded product to determine the extent of destruction of AF. Analysis was also performed on the acidified, extruded product to simulate conditions in the monogastric stomach and to determine the extent of permanent destruction of AF. Extruded corn from treatments showing the most promise was analyzed for changes in mutagenicity using the Ames *Salmonella* mutagenicity test. Amino acid analyses were also run on these selected samples to determine changes that may have been caused by heat treatment of corn under alkaline conditions.

**Addition of Food Grade Approved Additives**

Previous experiments have shown the possibilities of destroying AF with certain food additives (Stoloff, 1980). Ammonia has shown the most promise in feed for animals, but thus far has not been used to decontaminate food intended for human use because of its possible toxicity and strong smell (Park et al., 1988). Wang (1982) and Ito (1982) used Ca(OH)$_2$, potassium bisulfite, sodium hypochlorite, and H$_2$O$_2$ alone or in several combinations to destroy AF in moistened, contaminated peanut meal. These experiments indicated that increasing the time, temperature, additive concentration, or
amount of moisture of the reaction increased the destruction of AF. Unfortunately, none of the treatments was successful in permanent reduction to less than 20 ppb even at 60°C, the highest temperature used (Ito, 1982; Wang, 1982).

If one of the treatment parameters could be increased enough, the others could be decreased or at least maintained. Increasing the concentration of chemicals might leave residues; increasing the time or amount of moisture would leave the product subject to microbial attack. The parameter most easily increased would be temperature, but the maximum temperature possible under atmospheric conditions would be 100°C.

The extrusion process offers several advantages. Temperatures within the extruder barrel exceed 105°C. The time of extrusion is much less, in the order of one to a few minutes. The moisture of the meal to be extruded is about 20% instead of the 30 to 40% of previous experiments. Added moisture is mostly removed at the extruder head as it vaporizes into steam while puffing the product; thus, there is need for minimal drying of the product. In some cases the food additive is also removed as a vapor. In all, the food extruder shows promise for increasing the heat treatment of the product and decreasing the other treatments.

Higher temperatures for treatment of the corn meal, as are commonly used to inactivate trypsin inhibitor in soy meal before it is fed to livestock (Stephenson, 1972; Scott, Sandholm and Hochstetler, 1976), could be employed with base and/or oxidizing agent included, because they are easily attainable in both commercial and domestic applications. Early studies of AF breakdown by H₂O₂ and base in peanut meal required the meal to be defatted and suspended in water (10% solids) for 30 min at 80°C
(Sreenivasamurthy et al., 1967). The method required removal of the water afterward by filtration and then dehydration; the 30 min residence time delays the process.

It is our premise that AF is relatively resistant to breakdown by common methods of food preparation, but is susceptible to ring opening by alkaline conditions and cleavage of the open ring by oxidizing agents. This decomposition is probably increased by heat, pressure and added moisture during reaction. This reduction should have minimum losses in nutrititive value without the production of other toxic materials, and the cost for the treatment should be economical.

Stages involved in the corn processes are as follows:

- original samples (cleaned and ground)
- mixed with the treatment (chemicals)
- * extruded
- * dried and ground again
- * analysis

**Preliminary Extrusion Procedures**

In order to choose the extrusion procedures which would be most beneficial, the corn was extruded many times, varying the amount of water added, the speed of the extruder screw, the rate of feed into the extruder, and the amount of steam added to the extruder jacket. The only measurement that we could make to judge the effectiveness of the extrusion process was very subjective. The corn had to leave the extruder cooked and with just the amount of temperature and pressure to allow the superheated steam to
flash off the product. This expanded the product to about 10 mm in diameter forming a solid matrix and allowing the steam to escape into the atmosphere, thus drying and cooling the product. Appropriate extruder conditions have been given in Materials and Methods. Once these conditions were reached, they were not varied throughout the run. It was always necessary to run a preliminary batch or two until the temperatures and pressures within the extruder had stabilized. Only then were the treated batches started.

The first time this extruder was used for this set of experiments, a thermocouple was attached to the extruder close to the exit. Temperatures above 100°C were noted in the first 5 min of the first run. At this point, the high pressure broke the temperature measuring device and it was not possible to monitor the temperatures further. When we used a laboratory thermometer, insulated against heat loss, to measure the outside of the heated barrel, the highest temperature indicated was 95°C, just below boiling. Temperature of the product leaving the extruder die as measured by a hand held, steel dial thermometer was 105°C. This gave an approximation of the temperature, but gave no indication of the product temperature within the barrel. Other studies which monitor the temperature of the product within the extruder barrel have indicated that temperatures may approach 150°C. Because of the rapid volatilization of water and expansion of the corn product, I believe this to be the case here.

Aflatoxin Analysis

The ground corn which was used in these extruder experiments was originally part of a half ton lot which had been separated by the State of Arizona as unsuitable for
feed grain because of excessive AF levels. The original levels had been determined by
the Arizona State Chemist’s laboratory to be about 800 ppb. It is unknown which method
of determination was used for this analysis. Repeated tests on various portions of this
lot using the methanol extraction method of Thean et al. (1980) gave a mean value of 500
ppb total AF. This method uses a minimum of chlorinated hydrocarbons and benzene,
chemicals previously used in AOAC approved methods. Aflatoxin G\textsubscript{1} and AFG\textsubscript{2} were
not present at detectable levels. Because of the many analyses giving 400-500 ppb, it is
likely that the total AF in this naturally-contaminated corn was close to these levels.

In addition, the Thean et al. (1980) method was modified slightly in another
analysis of the same samples to give an idea about how permanent the destruction of AF
was. Mild alkaline conditions open the lactone ring of AFB\textsubscript{1}, make it somewhat more
water soluble and much more difficult to detect by common analytical methods Norred
(1979). This reaction may be accelerated by higher temperatures and pressures. If the
open ring form of AF is then subject to mild acid conditions, such as those which occur
in the monogastric stomach, it can revert to the original structure, with all the same
health implications. Therefore, a 30 min soak of the ground material to be analyzed in
0.1 N HCl previous to analysis, should give an idea about the permanent nature of AF
destruction. A previous study with tortillas indicated that up to 80% of the AF was
reformed by the acid media (Jorgensen and Price, 1985) even though the ground corn
dough was baked on a hot plate. If the temperature of processing was higher, such as
that which occurred during the production of a corn snack by NaOH soak, pressure
cooking, and subsequent frying in hot oil, there was significant destruction and little reformation of AF (Camou-Arriola and Price, 1989).

It was desirable to have a relatively high level of AF in this corn because changes would be easier to follow. There is such a high variation in analysis as the amount of AF approaches analytical zero, that small decreases would become insignificant (Hsieh, 1983). In reality, to produce a commercially viable process for the destruction of AF in feed grain, the process must assure that the total AF will be less than 20 ppb, the FDA action level. It was this goal that we were seeking. A process giving higher levels may be effective but not practical.

**Destruction of Aflatoxin by the Extrusion Process**

Throughout these experiments, each control group gave an indication of the destruction of AF by the extrusion process itself. If each table is examined, it is apparent that the process of extrusion alone reduced AF in this naturally-contaminated corn by about 70% (from an original 500 ppb down to about 150 ppb). This varied from one experiment to another. For an example see the control group in Tables 5 and 6.

Martinez and Monsalve (1989) reported 86.06% of AFB₁ and 97.7% of AFB₂ AF were destroyed in "spiked" contaminated corn by extrusion processing. Gréhaigne et al. (1983) also found similar results. In our experience, it has always been easier to destroy AF which has been "spiked" into a product than AF which is there throughout the matrix. Distribution of AF has been shown to be throughout the grain and not only on the surface (Lee, Lillehoj and Kwolek, 1980).
Extrusion of Corn Meal with Sodium Bisulfite and Calcium Hydroxide

Addition of NaHSO₃ and Ca(OH)₂, acceptable food additives commonly used in a number of food products such as dried fruits, fruit juices, jams, or tortillas, is a procedure which showed some promise in the studies of Wang (1982) and Ito (1982). Both additives were added dry to the corn meal, and then water was added before mixing and extruding. Analysis of the extruded product by the normal method is shown in Table 5. Both chemicals broke down almost 80% of the AF; however, the main effect here is that of extrusion. Neither NaHSO₃ nor Ca(OH)₂ had any significant further effects.

Table 5. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn (500 ppb total AF) after extrusion with 10% added moisture and sodium bisulfite and calcium hydroxide (normal analysis).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control¹</th>
<th>0.1%¹</th>
<th>0.25%¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extruded</td>
<td>117 ± 18c</td>
<td>76 ± 32de</td>
<td>***</td>
</tr>
<tr>
<td>Not extruded</td>
<td>266 ± 18a</td>
<td>***</td>
<td>138 ± 18b</td>
</tr>
<tr>
<td>0.1% Ca(OH)₂</td>
<td>128 ± 6bc</td>
<td>48 ± 27e</td>
<td>96 ± 5d</td>
</tr>
</tbody>
</table>

¹Mean ± standard deviation.
*Means with common letter superscripts are not significantly different (p > .05).
*** Samples not taken.

Effect of Acid Extraction

Aflatoxin in samples which were soaked in dilute acid prior to analysis showed no differences other than those caused by extrusion (Table 6). These results were entirely unexpected. Contrary to expectation, AF levels remaining in the corn were
sometimes higher when the meal was treated with acid before analytical extraction. Other times levels were about the same. This suggests that degradation of AF by extrusion under these conditions is permanent, and AF will not reform in the stomach. All other studies from this laboratory have shown more AF after acid treatment.

Table 6. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn (500 ppb total AF) after extrusion with 10% added moisture and sodium bisulfite and calcium hydroxide (acid analysis).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NaHSO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control¹</td>
</tr>
<tr>
<td>Extruded</td>
<td>117 ± 18c</td>
</tr>
<tr>
<td>Not extruded</td>
<td>266 ± 18ª</td>
</tr>
<tr>
<td>0.1% Ca(OH)₂</td>
<td>117 ± 18bc</td>
</tr>
</tbody>
</table>

¹Mean ± standard deviation.  
ªMeans with common letter superscripts are not significantly different (p > .05). 
*** Samples not taken.

A previous study Park et al. (1981) with peanut meal indicated that Ca(OH)₂ and monomethylamine destroyed up to 94% of AF. Up to 46% destruction was obtained by Price and Jorgensen (1985) using Ca(OH)₂ and boiling whole corn. Doyle and Marth (1978) were successful in using bisulfite to destroy AF in mycelia.

Extrusion with Sodium Bisulfite or Sodium Metabisulfite with or without Dilute Acid

The previous study using NaHSO₃ and Ca(OH)₂ showed some unexpected results. There did not seem to be an advantage to using the alkali and those samples
which were acidified during analysis had slightly lower, although not significantly lower, values. This would seem to indicate that the acid conditions may have contributed to a greater destruction of AF, a result contrary to the majority of previous experiments. Aflatoxin destruction by acid usually requires a much stronger acid than was used here.

Consequently in this extrusion, it was decided to test the hypothesis that dilute acid at high temperatures might influence the destruction of AF by using a 0.1 N HCl solution instead of added water and compare the two. At the same time, the effect of added bisulfite from two different sources, NaHSO₃ and Na₂S₂O₅, at two levels, 0.1 and 0.25%, would be examined. As before, after extrusion, all samples would be analyzed using both the normal method and the acid soak method.

Results are shown in Tables 7 and 8. The effects of extrusion in permanently lowering AF about 70% were most important. Added bisulfite from either of the two sources at either of the two levels gave results not significantly different from the control. Using dilute HCl as a moistening agent did not produce any significant differences from the extruded control samples or those treated with bisulfite. Soaking the samples in acid prior to analysis (Table 8) indicated that the AF destruction was permanent. This experiment showed no benefits from these chemicals during extrusion.

Extrusion with Sodium Bicarbonate and Oxidizing Agents

Sodium bicarbonate was chosen because it causes an alkaline reaction with water, it is inexpensive, it produces carbon dioxide when heated, and it is normally used in foods. The first step, establishing alkaline conditions, was thought necessary to begin
Table 7. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn (500 ppb total AF) after extrusion with 10% added moisture and sodium bisulfite or sodium metabisulfite with or without added acid (normal analysis).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control(^1)</th>
<th>0.1(^{1})%</th>
<th>0.25(^{1})%</th>
<th>0.1(^{1})%</th>
<th>0.25(^{1})%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extrusion with 10% added water</td>
<td>128 ± 64(^{ab})</td>
<td>96 ± 32(^{abc})</td>
<td>106 ± 18(^{abcd})</td>
<td>128 ± 21(^{ab})</td>
<td>149 ± 18(^{ab})</td>
</tr>
<tr>
<td>Extrusion with 10% added 0.1 N HCl</td>
<td>149 ± 18(^{ab})</td>
<td>160 ± 0(^{a})</td>
<td>122 ± 40(^{ab})</td>
<td>64 ± 32(^{cd})</td>
<td>149 ± 18(^{ab})</td>
</tr>
</tbody>
</table>

\(^1\)Mean ± standard deviation.
\(^{a-d}\)Means with common letter superscripts are not significantly different (p > .05).

Table 8. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn (500 ppb total AF) after extrusion with 10% added moisture and sodium bisulfite or sodium metabisulfite with or without added acid (acid analysis).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control(^1)</th>
<th>0.1(^{1})%</th>
<th>0.25(^{1})%</th>
<th>0.1(^{1})%</th>
<th>0.25(^{1})%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extrusion with 10% added water</td>
<td>138 ± 64(^{ab})</td>
<td>141 ± 36(^{ab})</td>
<td>117 ± 36(^{abc})</td>
<td>106 ± 48(^{bcd})</td>
<td>98 ± 18(^{bc})</td>
</tr>
<tr>
<td>Extrusion with 10% added 0.1 N HCl</td>
<td>149 ± 18(^{ab})</td>
<td>117 ± 36(^{abc})</td>
<td>138 ± 18(^{ab})</td>
<td>101 ± 51(^{bcd})</td>
<td>120 ± 52(^{abc})</td>
</tr>
</tbody>
</table>

\(^1\)Mean ± standard deviation.
\(^{a-d}\)Means with common letter superscripts are not significantly different (p > .05).
degradation of AF by opening the lactone ring. Addition of an oxidizing agent, in this case a peroxide, should complete the degradation by cleaving the molecule. Oxidizing agents such as H₂O₂ also meet the above requirements and are often used in the food industry as maturation agents for flour in baking or as a sterilants in aseptic packaging.

The first treatment was 0.5% NaHCO₃ alone and with two levels of either H₂O₂, Maturox, or Novadelox (the latter two are dough maturation agents for the baking industry). In a second treatment the NaHCO₃ was doubled to 1%. A third treatment involved the peroxides only. All additives except the H₂O₂ were added dry to the flour previous to the addition of the 10% water. Sufficient H₂O₂ (30%) was added to the water to provide the indicated addition percentage. This water with peroxide was then added directly to the flour previous to the mixing period.

Results of the studies are shown in Tables 9 and 10. Again it must be emphasized that in each table, the difference between the water extract and the acid extract is that the latter simulates that which happens to AF in the monogastric stomach.

It can be seen that when the ground corn was extruded, the extrusion process itself lowered the AF significantly, but somewhat less than previous extrusions. Some treatments with chemicals added seemed to cause a greater destruction of AF than just extrusion with water alone but none of these were significant, nor did the apparent differences allow any conclusions that one was more effective than another. Use of the peroxides with NaHCO₃ was no more effective than the peroxides alone. In any case, no treatments caused enough destruction to cause the corn to be less than the legal limit, 20 ppb. In theory, additional peroxide should degrade the AF even more or prevent its
Table 9. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn (500 ppb total AF) after extrusion with 10% added moisture and sodium bicarbonate and hydrogen peroxide or Maturox or Novadelox (normal analysis).

<table>
<thead>
<tr>
<th>NaHCO₃</th>
<th>Control¹</th>
<th>H₂O₂</th>
<th>Maturox</th>
<th>Novadelox</th>
<th>NaHCO₃</th>
<th>Means²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2.5%¹</td>
<td>5.0%¹</td>
<td>0.5%¹</td>
<td>2.5%¹</td>
<td>0.5%¹</td>
</tr>
<tr>
<td>0</td>
<td>283 ± 24*</td>
<td>256 ± 55abc</td>
<td>96 ± 16h</td>
<td>149 ± 37gs</td>
<td>171 ± 49df</td>
<td>176 ± 28def</td>
</tr>
<tr>
<td>1.0%</td>
<td>272 ± 16b</td>
<td>213 ± 49abdef</td>
<td>256 ± 32abc</td>
<td>272 ± 16ab</td>
<td>251 ± 94abc</td>
<td>219 ± 72bdef</td>
</tr>
<tr>
<td>5.0%</td>
<td>235 ± 37abcde</td>
<td>62 ± 0h</td>
<td>235 ± 37abcde</td>
<td>256 ± 0abc</td>
<td>208 ± 16cdef</td>
<td>245 ± 49bdef</td>
</tr>
<tr>
<td>Oxidizer</td>
<td>263 ± 32A</td>
<td>177 ± 96C</td>
<td>196 ± 84BC</td>
<td>226 ± 61AB</td>
<td>210 ± 64BC</td>
<td>213 ± 55BC</td>
</tr>
</tbody>
</table>

¹Mean ± standard deviation; n=3.
²Mean ± standard deviation; n=21.
³Mean ± standard deviation; n=9.

*Means with common letter superscripts are not significantly different (p > .05).
A-CMeans with common letter superscripts within the row are not significantly different (p > .05).
Y-ZMeans with common letter superscripts within the column are not significantly different (p > .05).
Table 10. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn (500 ppb total AF) after extrusion with 10% added moisture and sodium bicarbonate and hydrogen peroxide or Maturox or Novadelox (acid analysis).

<table>
<thead>
<tr>
<th>NaHCO₃</th>
<th>Control¹</th>
<th>H₂O₂</th>
<th>Maturox</th>
<th>Novadelox</th>
<th>NaHCO₃ Means²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5%¹</td>
<td>5.0%¹</td>
<td>0.5%¹</td>
<td>2.5%¹</td>
<td>0.5%¹</td>
</tr>
<tr>
<td>0</td>
<td>272 ± 16²</td>
<td>256 ± 85²</td>
<td>139 ± 18³</td>
<td>224 ± 96³</td>
<td>144 ± 28³</td>
</tr>
<tr>
<td>1.0%</td>
<td>145 ± 11³</td>
<td>122 ± 53³</td>
<td>235 ± 18³</td>
<td>235 ± 67³</td>
<td>213 ± 37³</td>
</tr>
<tr>
<td>5.0%</td>
<td>192 ± 32⁴</td>
<td>128 ± 32⁴</td>
<td>139 ± 18⁴</td>
<td>187 ± ⁴</td>
<td>155 ± 91⁴</td>
</tr>
<tr>
<td>Oxidizer Means³</td>
<td>203 ± 81²</td>
<td>168 ± 84²</td>
<td>171 ± 51²</td>
<td>215 ± 63²</td>
<td>171 ± 60²</td>
</tr>
</tbody>
</table>

¹Mean ± standard deviation; n=3.
²Mean ± standard deviation; n=21.
³Mean ± standard deviation; n=9.
⁴Means with common letter superscripts are not significantly different (p > .05).
reformation in acid media. This did not happen here. Applebaum and Marth (1982) showed a significant lowering of AFM₁ in milk by using H₂O₂.

When NaHCO₃ was used, the production of carbon dioxide gas accelerated the puffing process and may have decreased the residence time of the product within the extruder. In this case time at high temperatures would be a more important factor than the chemical itself. Perhaps an alkaline agent such as lime, which does not degrade into a gas, would be preferable. It could be added directly to the water and mixed into the meal previous to the extrusion.

Extrusion with Ammonium Hydroxide and Hydrogen Peroxide

To raise the pH of the reaction within the extruder, consideration was given to adding NaOH or Ca(OH)₂ to the corn meal previous to the extrusion, but since it was desirable to leave the corn with as little residue as possible, NH₄OH was added at various levels. Ammonium hydroxide or aqueous ammonia has previously been used successfully in the treatment of animal feed (FDA, 1976). It has not been considered for human food because of the strong undesirable odor of the ammonia and its toxicity. When NH₄OH at various levels was applied to corn, peanut or cottonseed, the ammonia portion of the molecule somehow impeded the reformation of the lactone ring of AF which was opened by the alkali (Park et al., 1988).

Accordingly, all extrusion conditions were the same as in previous studies except that NH₄OH at 0.1, 0.4, 0.7, or 1.0% of the weight of the meal was added to the meal prior to extrusion. In addition, H₂O₂ at 0.5 or 1.0% was added to the control meal
and to other groups of ammoniated meal. The odor of ammonia was present in the room at the extruder head during extrusion. However, after the extruded product cooled, it had no perceptible ammonia odor; but the color was changed from pale to darker yellow. The yellow color was not objectionable.

Addition of $\text{NH}_4\text{OH}$ significantly decreased AF in extruded corn, especially at 1.0% where remaining AF was less than 20 ppb, the legal limit (Table 11). Similar work done to destroy AF during extrusion at higher levels of ammonia (Gréhaigne et al., 1983) gave comparable results.

Table 11. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn (500 ppb total AF) after extrusion with 10% added moisture and ammonium hydroxide and hydrogen peroxide (normal and acidified analysis).

<table>
<thead>
<tr>
<th></th>
<th>Normal Analysis</th>
<th>Acidified Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{NH}_4\text{OH}$</td>
<td>$\text{NH}_4\text{OH}$</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}_2$</td>
<td>0%$^1$</td>
<td>1.0%$^1$</td>
</tr>
<tr>
<td>0</td>
<td>85 ± 11$^a$</td>
<td>6 ± 4$^c$</td>
</tr>
<tr>
<td>0.15%</td>
<td>45 ± 11$^b$</td>
<td>4 ± 3$^c$</td>
</tr>
<tr>
<td>0.30%</td>
<td>86 ± 10$^a$</td>
<td>6 ± 1$^c$</td>
</tr>
<tr>
<td></td>
<td>75 ± 11$^a$</td>
<td>6 ± 1$^c$</td>
</tr>
<tr>
<td></td>
<td>48 ± 40$^b$</td>
<td>3 ± 2$^c$</td>
</tr>
<tr>
<td></td>
<td>70 ± 7$^a$</td>
<td>0.2 ± 0$^e$</td>
</tr>
<tr>
<td></td>
<td>6 ± 1$^c$</td>
<td>0.2 ± 0$^e$</td>
</tr>
</tbody>
</table>

$^1$Mean ± standard deviation.
$^a$$^c$Means with common letter superscripts are not significantly different ($p > .05$).

Addition of $\text{H}_2\text{O}_2$ to the ammoniated samples did not significantly change the results. There was some destruction as previously reported with $\text{H}_2\text{O}_2$ alone, but no greater destruction was noted over that caused by the ammonia additives alone. This
could be due to the fact that some catalase was present which destroyed the H₂O₂ before it could react with the AF. This supposition is still to be tested. The lack of destruction could also be due to the fact that only a small amount of H₂O₂ was used for this test.

Results of the acidified analysis (Table 11) indicate that the degradation of AF by the extrusion alone and with added NH₄OH and H₂O₂ is permanent.

Extrusion with Ammonium Hydroxide Alone

Because there was a significant decrease of AF to below 20 ppb in the preceding experiment with no apparent benefit from adding H₂O₂ to the reaction, it was decided to test NH₄OH alone at levels ranging from 0.1 to 1.0% on a per weight basis. As before the NH₄OH was mixed in the appropriate concentrations into the moistening water prior to the 20 min mixing before extrusion. Samples were taken on the mixed material before extrusion; their analysis gave an indication of the effect of NH₄OH alone.

The odor of ammonia was again evident at the extruder die and was greater at the higher concentrations. In practice, it would be both economically and environmentally necessary to remove the ammonia from the atmosphere around the extruder die. The yellow color of the extruded corn also became more pronounced as concentration increased.

Results of treating of contaminated ground corn with low levels of NH₄OH during extrusion are shown in Table 12. Ammonium hydroxide significantly decreased AF in extruded corn, especially at 1.0% in the second repetition, where no AF could be detected. Several treatments succeeded in lowering AF below the 20 ppb action level.
Table 12. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn (500 ppb total AF) before and after extrusion with 10% added moisture and ammonium hydroxide (normal and acidified analysis).

<table>
<thead>
<tr>
<th>NH$_4$OH</th>
<th>Normal Analysis</th>
<th>Acidified Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extruded$^1$</td>
<td>Not Extruded$^2$</td>
</tr>
<tr>
<td>0</td>
<td>109 ± 28$^a$</td>
<td>480</td>
</tr>
<tr>
<td>0.1%</td>
<td>70 ± 51$^b$</td>
<td>160</td>
</tr>
<tr>
<td>0.4%</td>
<td>64 ± 16$^b$</td>
<td>160</td>
</tr>
<tr>
<td>0.7%</td>
<td>17 ± 15$^c$</td>
<td>142</td>
</tr>
<tr>
<td>1.0%</td>
<td>6 ± 4$^c$</td>
<td>128</td>
</tr>
</tbody>
</table>

$^1$Mean ± standard deviation.
$^2$Samples not extruded had no replicate analysis.
$^a$, $^b$, $^c$Means with common letters are not significantly different (p > .05).

This experiment was repeated, mostly to obtain a repetition for statistical analysis. Even though each of the sample analyses were triplicated to provide statistical analysis, the individual processing steps were not. In this case samples of corn with just NH$_4$OH added were not collected since it was obvious from the preceding experiment that extrusion was necessary for maximum destruction of AF. Results of the second replication are essentially the same as in the previous experiment (Table 13).

Extrusion with Ammonium Bicarbonate

Although extrusion with 1% added NH$_4$OH was very successful in permanently destroying the majority of the AF, handling the NH$_4$OH was difficult because of the
Table 13. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn (500 ppb total AF) after extrusion with 10% added moisture and ammonium hydroxide (normal and acidified analysis).

<table>
<thead>
<tr>
<th>NH₄OH</th>
<th>Normal Analysis¹</th>
<th>Acidified Analysis¹</th>
<th>NH₄OH Means²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>127 ± 31</td>
<td>133 ± 42</td>
<td>130 ± 33*</td>
</tr>
<tr>
<td>0.1%</td>
<td>107 ± 12</td>
<td>107 ± 12</td>
<td>107 ± 10*</td>
</tr>
<tr>
<td>0.4%</td>
<td>53 ± 42</td>
<td>73 ± 23</td>
<td>63 ± 32b</td>
</tr>
<tr>
<td>0.7%</td>
<td>47 ± 12</td>
<td>40 ± 20</td>
<td>43 ± 15cbe</td>
</tr>
<tr>
<td>1.0%</td>
<td>12 ± 8</td>
<td>20 ± 0</td>
<td>16 ± 7c</td>
</tr>
<tr>
<td>Analysis Means³</td>
<td>69 ± 48</td>
<td>75 ± 48</td>
<td></td>
</tr>
</tbody>
</table>

¹Mean ± standard deviation; n=3.
²Mean ± standard deviation; n=6.
³Mean ± standard deviation; n=15.

*Means with common letter superscripts are not significantly different (p > .05).

strong odor and caustic effects on the respiratory system. It was desirable to find a product with a similar action which would be easier to handle which would not affect the product. A leavening agent which is used in the baking industry which would seem to have the desired characteristics is NH₄HCO₃. It has many of the advantages mentioned previously. When NH₄OH is heated, it is converted to carbon dioxide, ammonia, and water, all of which are gases at extrusion temperatures. There would be no residue in the extruded product. In addition, an alkaline media is created when NH₄HCO₃ reacts with water in the product. An added advantage is the reaction of ammonia with AF which results in its permanent destruction. Ammonium bicarbonate is easily handled,
does not have a strong odor until mixed with water, is a common ingredient in the baking industry, and is relatively inexpensive.

It was added in quantities equal to 0.1 to 1.0\% of the ground corn meal immediately prior to the 20 min mixing process. As before, during extrusion the odor of ammonia was noted; but both the odor and the yellow color were much less evident. At the higher levels (0.7 and 1.0\%) puffing and popping of the corn as it left the extruder die were greatly increased, so much so that at the highest level the corn was forcefully expelled at a high velocity from the extruder. This was obviously a result of the great volume of the three gasses that were formed by the heat of the extruding process. Samples taken after mixing but without extrusion showed effects of the chemicals alone.

Results are seen in Table 14. Addition of NH₄HCO₃ to corn meal was very successful in destruction of AF, especially at the lower levels (0.1 and 0.5\%), and the destruction was permanent. At the highest levels (0.7 and 0.1\%), destruction was not as great. The probable cause was that the formation of gases quickly pushed the corn out of the extruder; therefore, the AF did not have enough time to be destroyed during extrusion processing. This is indicated most clearly at 1\% added NH₄CO₃. When these results are compared with those of NH₄OH (Table 14), they are somewhat similar but much less bicarbonate had nearly the same effect as 1\% hydroxide, whereas increasing the bicarbonate to above 0.7\% was counterproductive. Each chemical probably has its own application.
Table 14. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn (500 ppb total AF) before and after extrusion with 10% added moisture and ammonium bicarbonate (normal and acidified analysis).

<table>
<thead>
<tr>
<th>NH₄CO₃</th>
<th>Normal Analysis</th>
<th>Acidified Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extruded¹</td>
<td>Not Extruded²</td>
</tr>
<tr>
<td>0</td>
<td>127 ± 14c</td>
<td>480</td>
</tr>
<tr>
<td>0.1%</td>
<td>27 ± 5a</td>
<td>***</td>
</tr>
<tr>
<td>0.4%</td>
<td>55 ± 8ab</td>
<td>114</td>
</tr>
<tr>
<td>0.7%</td>
<td>58 ± 5ab</td>
<td>112</td>
</tr>
<tr>
<td>1.0%</td>
<td>88 ± 8bc</td>
<td>160</td>
</tr>
</tbody>
</table>

¹Mean ± standard deviation.
²Samples not extruded had no replicate analysis.
***Samples not taken.

Extrusion with Caustic Soda and Hydrogen Peroxide

It was suggested by chemists from ELF Aquitaine, who have a great deal of experience in the use of H₂O₂ in bleaching paper and beet pulp, that optimum conditions were not established in the initial peroxide experiments. They suggested that the H₂O₂ may have been destroyed by catalase in the ground corn, and that the optimum conditions for reaction of H₂O₂ are those where significant amounts of concentrated peroxide and caustic soda solution are sprayed together on the material. Accordingly an experiment was devised to compare results from corn which had been heated to 90°C to destroy catalase previous to peroxide and base addition. It also tested the effect of heating the chemicals together for 30 min after the mixing period. Although the experimental design
is shown in Materials and Methods, it is repeated here with the results so that they can be compared (Figure 7).

During the experiment, the observations differing from normal extrusion was the strong soapy odor present as the peroxide and caustic soda were sprayed together in the mixing bowl. The only other difference is that the samples which were held at 70°C for 30 min after mixing were very dry and extrusion was difficult. Nevertheless, only that corn which had the desired physical characteristics previously described were collected for analysis. Both normal and acid analysis were performed.

Results are extremely confusing. As expected, the original meal and meal which had been heated for 30 min without addition of chemicals had the original levels of 500 ppb. In all other cases, including those extruded without chemicals, those extruded with chemicals, those with chemicals but not extruded, and all combinations of heating had the same statistical levels after treatment. About 80% permanent destruction was achieved, but in no case was the target level of 20 ppb attained. This must be a subject for further research.

Amino Acid and Protein Analyses

Heat treatment of food under alkaline conditions is likely to cause changes in the nutrients of the food. Vitamins and proteins are alkali and heat sensitive and changes in lysine, tryptophan, and water-soluble vitamins would be expected. An attempt was made to try to evaluate changes in proteins and amino acids of ground corn extruded under these conditions. Ammonium treatment can, itself, alter the protein comparison
Figure 7. Aflatoxin (ppb) remaining in aflatoxin-contaminated ground corn after extrusion with 10% added moisture and hydrogen peroxide and sodium hydroxide (normal and acid analysis)
(Park et al., 1988), along with heat and pressure of the extrusion processing. Protein and amino acid analysis of extruded and non extruded corn are shown in Table 15. Only the samples which had been extruded with 1.0% NH₄OH were chosen for analysis because those were the most stringent conditions applied to the corn and they were the conditions leading to nearly complete destruction of AF. Table 15 shows a 14% increase in protein as non-protein nitrogen (NPN) in the corn receiving an ammonium treatment of 1%. This is expected because of the added ammonia. Figure 8 shows the comparison between the amino acids, lysine, cysteine and methionine, at the various levels of treatment. The study indicated there was 11.3% decrease in the cysteine during extrusion with 1% ammonium (Figure 8). Gréhaigne et al. (1983) found an 11% decrease in lysine and a

<table>
<thead>
<tr>
<th>NH₄OH</th>
<th>% Moisture</th>
<th>% Protein</th>
<th>% Total Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>0</td>
<td>10.0</td>
<td>9.0</td>
<td>8.68</td>
</tr>
<tr>
<td>0.1%</td>
<td>***</td>
<td>***</td>
<td>8.68</td>
</tr>
<tr>
<td>0.4%</td>
<td>***</td>
<td>***</td>
<td>8.68</td>
</tr>
<tr>
<td>0.7%</td>
<td>***</td>
<td>***</td>
<td>8.68</td>
</tr>
<tr>
<td>1.0%</td>
<td>***</td>
<td>***</td>
<td>8.68</td>
</tr>
</tbody>
</table>

*** Samples not taken
Figure 8. Cysteine, lysine and methionine in extruded, ammoniated corn.
smaller decrease of other amino acids. Lysine and methionine did not change in this study. Park et al. (1988) indicted that there may be a significant reduction under some conditions of ammonia treatment of AF contamination.

Ames Test

One of the most controversial areas in the degradation of AF in feed or feed is the question of the fate of the AF. Should this material be used for consumption by animals or humans? Under what conditions should it be used? Even though AF is completely destroyed, are there not some compounds resulting from its fragmentation which are equal or even greater in potency? These are questions which are being studied now by researchers using chemical and biological tests. In this case, the Ames test for mutagenicity was chosen. The Ames test has been invaluable throughout the world in the attempt to predict carcinogenicity by subjecting microbes to chemicals and extracts which have the potential of changing genetic material. Noting these changes and preparing dose-response curves can give a rough estimate of potency of a given chemical or extract.

Accordingly, appropriate extracts of extruded corn meal were made. The specific ammoniation treatment for the meal was again the most stringent which gave the best results, 1% added NH₄OH. The dose response curve of AFB₁ shown in Figure 9 illustrates mutagenic activity using the Ames test with S. typhimurium TA 100, after naturally contaminated corn was processed with 0.1 to 1.0% NH₄OH and NH₄HCO₃. None of the samples showed mutagenic activity at any treatment level with or without S9.
Figure 9. Ames test results from acetone extracts with and without S9 fraction from corn meal treated with 0 to 1% ammonium hydroxide and then extruded at 105°C die temperature
It was hoped that there would be large significant differences seen in the results of this involved, time-consuming test. Although the dose-response curve shows positive results, first an increase in microbial numbers as mutagenicity increases and then a decrease as increasing AF kills the microbes, there was absolutely no correlation with microbial number and amount of residual AF. There were no significant differences between extracts from the control corn which had 500 ppb and the treated corn which had less than 20 ppb. Indeed there were no significant differences between the control plates which had no added chemicals and the plates from any corn samples. As a result, no conclusions concerning the presence or absence of reactive fragments from the AF ammonia treatment can be made.

Summary

Aflatoxin in peanuts and corn of tropical Africa, Latin America, and Southeast Asia is of great economic and public health significance. When these products cannot be sold for export, at least two problems are created: one, money which would be coming into the country is lost to the country and the agriculture interests, and two, the contaminated feed or food is usually not destroyed, but rather enters into the food chain and becomes an even greater public health problem. Aflatoxin has caused human deaths in these areas of the world, and it, along with hepatitis B, has been implicated as causing or contributing to liver cancer, a common cause of death in these areas. It definitely is hepatotoxic, and in smaller doses causes a decreased function of the immune system. In this case, diseases which are normally mild become virulent. Aflatoxin has also been
associated with worsening the effects of kwashiorkor. This fact has been well established in the Sudan.

A simple, inexpensive method to destroy the AF in these commodities on an industrial and domestic scale and render the product nutritious, flavorful and desirable to human beings is needed, especially in the case of the poorer countries. Studies in this laboratory have shown that treating peanut meal with alkali and then with an oxidizing agent will destroy a portion of the AF. The reaction is temperature dependent. Hydrogen peroxide and NH$_2$OH would seem to have promise in commercial utilization. Raising the temperature of the reaction mixture by using a food or feed extruder would also be desirable. In this manner, the water level of the feed would not have to be excessive, the reaction would occur at temperatures above the boiling point of water and the water vapor and residual peroxides and/or ammonia would be removed naturally by expansion from the product immediately upon its leaving the extruder.

The development of such a detoxification of AF-contaminated human foods will provide nutritional, health, and economic benefits to African, Asian and South American populations. Corn is a staple food in most developing countries and is also one of the main sources of protein.

Conclusions

1. The extrusion process at a temperature of 105°C was effective in significantly lowering AF levels in ground corn with 10% moisture added from 50 to 80%. The
fate of the destroyed AF is unknown. Extrusion alone was not effective in lowering AF to below 20 ppb, the FDA action level.

2. Calcium hydroxide, alone or in combination with bisulfite, was no more effective in AF destruction by extrusion alone. The same was true with NaHCO₃ alone or in combination with H₂O₂, Novedelox or Maturox; the latter two are oxidizing agents commonly used in the baking industry.

3. Extrusion of ground corn with strong solutions of H₂O₂ or caustic soda had no added benefits over extrusion alone; extrusion alone was no more beneficial than use of the above solutions. Heating corn meal previous to addition of H₂O₂ to destroy catalase had no beneficial effects.

4. Use of ammonia, either as NH₄ at 0.7 and 1.0% or NH₄CO₃ at 0.4% was effective in lowering AF to below 20 ppb. The extruded product was slightly more yellow but had no odor of ammonia after drying.

5. There was more non-protein nitrogen in these samples than there was in the non-ammoniated, extruded samples, but the amino acid profile appeared unchanged.

6. The Ames test, designed to detect increased mutagenicity in extracts from the above samples was non-conclusive.
APPENDIX A: AMES SALMONELLA ASSAY

The Ames Salmonella microsome mutagenicity assay is a short-term assay used to detect chemically induced mutagenesis caused by two types of DNA alterations. These are the base-pair substitution mutagen and the frame-shift mutagen. Both of these alterations cause changes in the reading frame of the DNA and result in three kinds of coding errors:

1. Sense: The codon has been altered but still reads for the same protein.
2. Non-sense: The codon has been changed and makes no sense and reading stops.

The assay uses histidine-requiring strains of Salmonella typhimurium. Mutagenicity is determined by the reversion of the Salmonella from histidine-requiring to histidine independent (wild type). For this reason the assay is a reverse mutation assay. This type of assay takes a shorter period of time to run and is also more sensitive. Because of this sensitivity, it is important to note that the bacteria are very sensitive and subject to alterations by mishandling or improper storage. They are also subject to outside sources of contamination. This can cause major problems in both the master culture and any subsequent cultures. It is very important that work areas are kept clean and are sterilized both before and after running the assay. Contamination can result from the cultures, the work area, the stock solutions, any equipment used or from the incubator itself. Once contamination has occurred the entire assay must be discarded and repeated. This causes not only a loss of time, but also sample which in many cases is very limited.

Improper handling of the bacteria can also cause problems. These bacteria are mutant strains of Salmonella typhimurium which has been known to cause food borne infections. Therefore, these organisms must be handled with care in order to prevent laboratory or personnel contamination. It is also important to take extreme care not to contaminate someone else’s research with these organisms. Carelessness with these or any other organism can cause more problems than imaginable.
Preparation of Materials

Agars and Mediums:

1. **Vogel-Bonner (VB) Medium**

   **Use:** Mutagenicity Assay

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium sulfate</td>
<td>1 g</td>
</tr>
<tr>
<td>Citric acid monohydrate</td>
<td>10 g</td>
</tr>
<tr>
<td>Potassium phosphate, dibasic, anhydrous</td>
<td>50 g</td>
</tr>
<tr>
<td>Sodium ammonium phosphate</td>
<td>17.5 g</td>
</tr>
</tbody>
</table>

   Autoclave at 121°C for 20 min, cool and store in the refrigerator after use.

2. **Minimal Glucose Plates**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per 1 liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>930 ml</td>
</tr>
<tr>
<td>VB Medium</td>
<td>20 ml</td>
</tr>
<tr>
<td>40% Glucose solution</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

   Autoclave agar-water solution separately from VB medium and glucose at 121°C for 20 min. After autoclaving add VB medium and glucose solution and swirl carefully. Pour plates, 30 ml medium per plate. Allow plates to harden, then invert to allow excess moisture to escape.

3. **Top Agar**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per 200 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>1.2 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

   Autoclave at 121°C for 20 min. Cool and store in the refrigerator until ready to use.
4. **Nutrient Broth Agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount per 200 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxoid Nutrient Broth No. 2</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Bacto Agar</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

Autoclave at 121°C for 20 min. Pour plates, 10 ml/plate. After plates have hardened, invert.

5. **Nutrient Broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxoid Nutrient Broth No. 2</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Autoclave at 121°C for 20 min. Cool to room temperature before adding bacteria.

6. **0.5 mM histidine/biotin solution**

Use: Mutagenicity Assay (add 10 ml to 100 ml top agar)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount per 200 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-biotin</td>
<td>24.7 mg</td>
</tr>
<tr>
<td>L-histidine</td>
<td>19.2 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

Autoclave at 121°C for 20 min. Store in glass at 4°C (in the refrigerator).
7. **Master Plates (TA 97, 98, 100 and 102)**

Use: Master storage plates and ampicillin and tetracycline resistance

Minimal glucose plates with the following added after medium has been allowed to cool slightly:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per 1 liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile L-histidine (0.5 g/100 ml)</td>
<td>10 ml</td>
</tr>
<tr>
<td>Sterile 0.5 mM D-biotin</td>
<td>6 ml</td>
</tr>
<tr>
<td>Sterile ampicillin (8 mg/ml sterile 0.02 N NaOH)</td>
<td>3.15 ml</td>
</tr>
</tbody>
</table>

For TA 102 Only - Tetracycline
(8 mg/ml 0.02 N HCl)

After medium has been mixed, pour plates, let harden then invert and store in the refrigerator.

**Solutions:**

1. **Salt Solution**

   **Use:** S9 mix for assay

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per 250 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium chloride</td>
<td>30.75 g</td>
</tr>
<tr>
<td>Magnesium chloride, hexahydrate</td>
<td>20.35 g</td>
</tr>
</tbody>
</table>

   Dissolve in distilled water and autoclave at 121°C for 20 min. Store in glass in refrigerator or room temperature.

2. **2 M Sodium phosphate buffer, pH 7.4**

   **Use:** S9 mix for assay

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per 200 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dihydrogen phosphate</td>
<td>5.52 g</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>5.68 g</td>
</tr>
</tbody>
</table>

   Autoclave at 121°C for 20 min. Store at room temperature or in refrigerator.
3. **0.1 M NADP solution**

 Use: S9 mix for assay

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per 5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP (F.W. 765.4 g)</td>
<td>383 mg</td>
</tr>
<tr>
<td>Sterile water</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

4. **1 M Glucose-6-phosphate (G-6-P)**

 Use: S9 mix for assay

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per 1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate</td>
<td>282 mg</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

5. **S9 Mix**

 Use: Mutagenicity Assay

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Liver S9 fraction</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Salt solution</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>G-6-P solution</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>NADP solution</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>19.75 ml</td>
</tr>
</tbody>
</table>

After mixing, keep on ice. The mix should be prepared just prior to using. Discard any mix leftover - NEVER REFREEZE S9.
Bacterial Tester Strains

The bacteria used for this assay are mutant strains of *Salmonella typhimurium*. The bacteria has been altered genetically depending on the type of mutation it is designed to detect. Strains TA 97 and TA 98 are used to detect frame-shift mutagens and strains TA 100 and TA 102 are used for detecting base-pair substitution mutagens. Strains TA 97 and TA 102 are more sensitive than are TA 98 and TA 100. Strains TA 97a and TA 102 have double error prone DNA repair systems. This makes these strains much more sensitive and therefore much more prone to alterations if they are not handled properly. All 4 strains are normally used in combination when initially running the assay on substances which have not been tested.

**THESE BACTERIA ARE MUTANT STRAINS AND VERY SENSITIVE.
THEY MUST BE HANDLED WITH CARE TO PREVENT ANY ALTERATIONS.**

Growing Cultures:

For initial cultures use Oxoid Nutrient Broth No. 2. Inoculate the sterile broth with bacteria from either a frozen culture or Master Plate. Incubate at 37°C for 10-16 hr and make sure the flask is agitated. Oxygen incorporation is crucial for the bacteria to grow. After incubating remove the culture and check turbidity using a spectrophotometer at 650 nm. The reading should be 0.8 which indicates a density of $1-2 \times 10^8$ cells per ml. This is the minimal reading if the culture is to be used for the assay. If the reading is lower, then the amount of bacteria is less and this results in an inaccurate test results. Keep cultures at room temperature if they are to be used the same day, if not keep them refrigerated (not longer than 3 days) prior to use.

Confirming tester stains:

1. Histidine requirements - Use Vogel-Bonner (VB) minimal glucose plates one with the histidine/riboflavin solution and one without. Label plates with strain, and whether or not it includes histidine/riboflavin solution. Incubate at 37°C for 48 hr; however, growth can be seen in 24 hr. The results should be: a) growth with histidine and b) no growth without.

2. Crystal violet (Rfa) - Use nutrient broth plate. Streak bacteria onto plate then place a disk containing crystal violet on the plate, incubate at 37°C for 24 hr. The results should be a zone of inhibition around the crystal violet disk.

3. Ampicillin resistance (R-factor) - Use nutrient broth plate streaked with ampicillin. Steak bacteria onto plate, incubate at 37°C for 24 hr. Results should be bacterial growth. For strain TA 102 you must also test for tetracycline resistance which is done in the same manner as the ampicillin.
APPENDIX B: AMINO ACID HYDROLYSIS

Principle:

A proteinaceous sample is subjected hydrolysis with 6 N HCl either in the presence of sodium thioglycolate to preserve methionine or in the absence of sodium thioglycolate following the performic acid oxidation of cystine to cysteic acid (HOOCCH(NH₂)CH₂SO₃H) and methionine to methionine sulfoxide (HOOCCH(NH₂)CH₂CH₂SO₂CH₃C).

Reagents:

1. Sodium Thioglycolate: (NaC₂H₃O₂S) (Sigma T-0632)
2. 6 N Hydrochloric Acid:
   Dilute 960 ml concentrated hydrochloric acid (HCl) to 2 L with deionized H₂O and mix.
3. .1 N Hydrochloric Acid:
   Dilute 16 ml concentrated hydrochloric acid (HCl) to 2 L with deionized H₂O and mix.
4. 30% Hydrogen Peroxide: (H₂O₂)
5. 80% Formic Acid: (CH₂O₂)
6. Performic Acid:
   a) Add 5 ml 30% H₂O₂ to 45 ml 80% CH₂O₂ in a 125 ml Erlenmeyer flask.
   b) Heat in a water bath at 50°C for 3 min.

Procedure:

1. Weigh duplicate 100 mg samples into 250 ml round bottom flasks.
2. Add ~100 mg sodium thioglycolate to one replicate (a) and set aside.
3. a) Add 4 ml performic acid to the second replicate (b).
   b) Heat in a water bath at 50°C for 15 min.
   c) Evaporate to dryness under vacuum using a rotary evaporator.
4. Add ~25 ml 6 N HCL to each flask (a + b) and cover by inverting a small beaker over each.

5. Autoclave for 16-18 hr at 121°C (15 psi).

6. Evaporate samples to dryness under vacuum using a rotary evaporator.

7. Dissolve residues by pipetting 10.00 ml .1 N HCl into each flask and mixing well.

8. Filter samples using Whatman 5 paper and collect filtrate in 4 dr screw-cap vials.
   NOTE: Samples must be clear.

9. Store samples in refrigerator.
APPENDIX C: AMINO ACID ANALYSIS

Principle:

In the presence of 2-mercaptoethanol, o-phthalaldehyde (OPA) forms highly fluorescent adducts with primary amines, but not with secondary amines. The fluorophors have excitation and emission wavelengths of 360 and 455 nm, respectively. Amino acids may be detected at the picomole level. The low fluorescence yield of the lysine adduct is improved by the presence of Brij 35 in the OPA reagent solution. The low fluorescence yield of cystine may be overcome by performic acid oxidation of cystine to cysteic acid.

Reagents:

1. **.5 M Potassium Borate Buffer, pH 10.4:**
   Dilute 950 ml 1 M potassium borate buffer, pH 10.4 (Pierce 27035 - Fluorophosphate Reagent Diluent) to 1900 ml with deionized H₂O. Mix well.

2. **30% Brij 35 (w/w):** (Pierce 20150)

3. **2-Mercaptoethanol:** (C₂H₆S)

4. **Methanol:** (CH₃OH)

5. **o-Phthalaldehyde (OPA):** (Pierce 26010 - Fluoropa)
   a) Weigh 10 mg o-phthalaldehyde (C₆H₆(CHO)₂) into a 10 ml volumetric test tube.
   b) Dissolve using ~3 ml potassium borate buffer, pH 10.4.
   c) Add 250 μl methanol.
   d) Add 37.5 μl 30% Brij 35.
   e) Add 25 μl 2-mercaptoethanol.
   f) Dilute to 10.0 ml with potassium borate buffer, pH 10.4. Mix well.
   g) Store refrigerated in a 4 dr screw-cap vial.
   NOTE: OPA solution requires a 24-hr aging period.
      Prepare fresh daily and 1 day prior to use.

6. **.1 N Hydrochloric Acid:**
   Dilute 16 ml concentrated hydrochloric acid (HCl) to 2 L with deionized H₂O and mix.
7. **a-Amino Butyric Acid (2.5 μmole/ml):**
   a) Weigh exactly 25.78 mg α-amino butyric acid (CH₃CH₂CH(NH₂)COOH) and quantitatively transfer to a 100 ml volumetric flask.
   b) Dissolve and dilute to mark with .1 N HCl and mix.
   c) Store refrigerated.

8. **Amino Acid Standard (.1 μmole/ml):**
   a) Pipet 2.00 ml prepared amino acid standard solution containing 2.5 μmole/ml (Pierce 20089) into a 50 ml volumetric flask.
   b) Add 2.00 ml α-amino butyric acid (2.5 μmole/ml) as an internal standard (IS).
   c) Dilute to mark with .1 N HCl. Mix well.
   d) Store refrigerated.

9. **Cysteic Acid (2.5 μmole/ml):**
   a) Weigh exactly 42.29 mg cysteic acid (HOOC(NH₂)CH₂SO₃H) and quantitatively transfer to a 100 ml volumetric flask.
   b) Dissolve and dilute to mark with .1 N HCl and mix.
   c) Store refrigerated.

10. **Amino Acid Standard + Cysteic Acid Standard (.1 μmole/ml):**
    a) Pipet 2.00 ml prepared amino acid standard solution containing 2.5 μmole/ml (Pierce 20089) into a 50 ml volumetric flask.
    b) Add 2.00 ml cysteic acid (2.5 μmole/ml)
    c) Add 2.00 ml α-amino butyric acid (2.5 μmole/ml) as an internal standard (IS).
    d) Dilute to mark with .1 N HCl. Mix well.
    e) Store refrigerated.

11. **Glacial Acetic Acid:** (CH₃COOH)

12. **Tetrahydrofuran:** (C₄H₈O)

13. **.1 M Sodium Acetate Buffer, pH 6.2 + 1% Tetrahydrofuran:**
    a) Weigh 16.4 g sodium acetate (NaC₂H₃O₂) into a 2 L mixing cylinder.
    b) Dissolve and dilute to ~1800 ml with deionized H₂O.
    c) Adjust pH to 6.2 with glacial acetic acid.
    d) Dilute to 2 L with deionized H₂O. Mix well.
    e) Filter through .5 μ filter.
    f) Add 20 ml tetrahydrofuran. Mix well and filter.
**Instrument:** Spectra-Physics Model 8000B HPLC

**Procedure:**

A. **Sample Preparation:**

Hydrolyzed .1 g samples either sodium thioglycolate-containing (designated "amino acid" or "a") or performic acid-oxidized (designated "cysteic acid" or "b") which have been diluted to 10.00 ml with .1 N HCl and filtered are diluted with α-amino butyric acid (2.5 µmole/ml) as an internal standard (IS) as follows:

- 100 µl hydrolysate + 40 µl IS → 1 ml (1:10 dilution)
- 100 µl hydrolysate + 80 µl IS → 2 ml (1:20 dilution)

**NOTE:** Internal standard (IS) must be added at a rate of .4 ml for each 10 ml of final solution.

B. **Sample Derivatization:**

Immediately prior to loading the injection loop, combine 100 µl OPA solution and 100 µl sample or amino acid standard solutions containing IS in a small test tube and mix.

C. **Sample Injection:**

1. Fill 50 µl syringe with sample or standard solution containing IS and OPA. Avoid air bubbles.

2. a) Insert syringe into injection port.
   b) At keyboard type: SO (ENTER)
   c) Expel syringe contents.
   d) At keyboard type: SK (ENTER)
   e) Remove syringe from injection port.
D. High Pressure Liquid Chromatography (HPLC):

1. Conditions:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>RP-18, 3µ, Spherical (Rainin Short One)</td>
</tr>
<tr>
<td>Detector</td>
<td>Fluorometric (with OPA filters)</td>
</tr>
<tr>
<td>Temperature</td>
<td>25 - 27°C</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1.0 - 1.2 ml/min</td>
</tr>
<tr>
<td>Solvent Mode</td>
<td>QG</td>
</tr>
<tr>
<td>Run Time</td>
<td>25 - 30 min</td>
</tr>
<tr>
<td>Loop Fill Time</td>
<td>2 min</td>
</tr>
</tbody>
</table>

Set PW 015 and PT 400 and MA 4000 initially.

2. Mobile Phase File:
Solvent A = Methanol
Solvent B = .1 M Sodium Acetate Buffer, pH 6.2 + 1% Tetrahydrofuran

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Amino Acid &quot;a&quot;</th>
<th>Cysteic Acid &quot;b&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% A</td>
<td>% B</td>
</tr>
<tr>
<td>0.0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>5.0</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>8.0</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>10.0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>16.0</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>18.0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>22.0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>23.0-24.0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>30.0</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

Solvents must be degassed with He for at least 20-30 min prior to running through column.
Samples being chromatographed together must be either all sodium thioglycollate-containing (designated "amino acid" or "a") or all performic acid-oxidized (designated "cysteic acid" or "b") due to differences in the mobile phase profile.

3. Elution Order and Retention Times (RT):

<table>
<thead>
<tr>
<th>Elution Order</th>
<th>Amino Acid</th>
<th>~RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 &quot;b&quot;</td>
<td>Cys</td>
<td>2.1</td>
</tr>
<tr>
<td>1 &quot;a&quot;</td>
<td>Asp</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>Glu</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>Ser</td>
<td>6.5</td>
</tr>
<tr>
<td>4</td>
<td>His</td>
<td>7.5</td>
</tr>
<tr>
<td>5</td>
<td>Gly</td>
<td>10.3</td>
</tr>
<tr>
<td>6</td>
<td>Thr</td>
<td>10.8</td>
</tr>
<tr>
<td>7</td>
<td>Arg</td>
<td>12.0</td>
</tr>
<tr>
<td>8</td>
<td>Ala</td>
<td>14.6</td>
</tr>
<tr>
<td>9</td>
<td>Tyr</td>
<td>15.4</td>
</tr>
<tr>
<td>10 &quot;a&quot;</td>
<td>IS</td>
<td>17.4</td>
</tr>
<tr>
<td>10 &quot;b&quot;</td>
<td></td>
<td>17.5</td>
</tr>
<tr>
<td>11</td>
<td>Amm</td>
<td>18.6</td>
</tr>
<tr>
<td>12</td>
<td>Met</td>
<td>19.0</td>
</tr>
<tr>
<td>13</td>
<td>Val</td>
<td>19.3</td>
</tr>
<tr>
<td>14</td>
<td>Phe</td>
<td>19.7</td>
</tr>
<tr>
<td>15</td>
<td>Ile</td>
<td>20.5</td>
</tr>
<tr>
<td>16</td>
<td>Leu</td>
<td>20.8</td>
</tr>
<tr>
<td>17</td>
<td>Lys</td>
<td>22.1</td>
</tr>
</tbody>
</table>
4. Useful HPLC Keyboard Commands:

<table>
<thead>
<tr>
<th>Command</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>F- (ENTER)</td>
<td>Displays flow rate</td>
</tr>
<tr>
<td>T- (ENTER)</td>
<td>Displays column chamber temperature</td>
</tr>
<tr>
<td>P- (ENTER)</td>
<td>Displays column pressure</td>
</tr>
<tr>
<td>DIL (ENTER)</td>
<td>Displays detector baseline</td>
</tr>
<tr>
<td>E- (ENTER)</td>
<td>Displays run time</td>
</tr>
</tbody>
</table>

Calculations:

1. Calculate $K_{Fi}$ for the response of each amino acid in the standard in relation to the internal standard response:

$$K_{Fi} = \text{Conc}_i \times \frac{\text{Area}_{IS}}{\text{Area}_i}$$

where:

- $\text{Conc}_i$ = μmole amino acid/ml standard
- $\text{Area}_i$ = area under the curve for amino acid $i$ of standard
- $\text{Area}_{IS}$ = area under the curve for IS

2. Calculate each amino acid content of the sample using $K_{Fi}$ as follows:

$$\text{Conc}_i = K_{Fi} \times \frac{\text{Area}_i}{\text{Area}_{IS}} \times XF$$

where:

- $\text{Conc}_i$ = μmole amino acid/g sample
- $K_{Fi}$ = response constant for amino acid $i$
- $\text{Area}_i$ = area under the curve for amino acid $i$ of sample
- $\text{Area}_{IS}$ = area under the curve for IS
- $XF$ = dilution factor for sample
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