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Evaluation of aflatoxin-related products from ozonated corn

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EVALUATION OF AFLATOXIN-RELATED PRODUCTS FROM OZONATED CORN

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ABSTRACT

This study assessed the efficacy of the ozonation process in degrading aflatoxin in corn, and investigated the chemical reaction between aflatoxin and gaseous ozone. Ozonation (12-13 wt%) totally degraded aflatoxin B_1 in a model system. Conversion of aflatoxin into polar compounds was observed during ozonolysis of 100 µg aflatoxin B1 in an aqueous environment and in solid form. Seven intermediate reaction products were separated by two-dimensional thin layer chromatography. HPLC analysis of ozonated AFB_1 revealed the presence of six major peaks. MALDI-MS analysis detected compounds that have higher molecular weights than AFB_1. The dichloromethane fraction contained compounds with molecular ion peaks at 459 and 439 m/z while the water fraction contained compounds with molecular ion peaks at 475 and 494 m/z, after ozonation for 50 sec and 60 sec, respectively.

Biosynthesis of [14-C]-labeled aflatoxin B_1 by *Aspergillus flavus* (A53, C50Aa) and sodium acetate-1,2-[14C] as a precursor yielded 339 µg of [14C]-AFB_1 with a specific activity of 1.06 µCi/µmol (7548 dpm/µg). Corn kernels inoculated with A*spergillus flavus* (A53, C50Aa) resulted in the production of grains contaminated with aflatoxin B1 (7452 ng/g) and aflatoxin B2 (704 ng/g).

Modification of AFB_1 after treatment with gaseous ozone was determined using [14C]-labeled AFB_1. Ozonated and non-ozonated corn spiked with [14C]-AFB_1 were evaluated and compared through a series of extraction, partition, and digestion procedures. Ozonation (9-10 wt%) resulted in 74% and 44% reduction of AFB_1 and AFB_2 levels, respectively. Radioactivity measured by liquid scintillation counting showed an increase in the percentage of radioactivity in more polar and aqueous

solvents from ozonated corn compared with non-ozonated corn. These results suggested the formation of more polar and/or water soluble aflatoxin-related compounds from the reaction of ozone with AFB_1. Based on these results, it is postulated that ozone attacked the double bond in the C8-C9 position and converted aflatoxin B_1 into an aldehyde.

CHAPTER 1. INTRODUCTION

Mycotoxins are secondary metabolites produced by molds in food and feed commodities. Production of mycotoxins can occur in the field before harvest, postharvest, during storage, processing, and feeding under a wide range of climatic conditions. They are produced primarily by molds of the genus *Aspergillus*, *Fusarium*, and *Penicillium* (Council for Agricultural Science and Technology, 1989). Mycotoxins have been reported to be carcinogenic, teratogenic, tremorgenic, and dermatitic to a wide range of organisms, and known to cause hepatic carcinoma in humans (Wary, 1981, Refai, 1988, Kumar *et. al.*, 2008). Human exposure to mycotoxins can be from direct consumption of contaminated commodities, or consumption of foods from animals previously exposed to mycotoxins through feeds. The toxicity syndrome associated from intake of mycotoxins by man and animals are generally known to as "mycotoxicoses".

Mycotoxicoses have been known for a long time and evidence can be traced back to ancient times and the Middle Ages (ergostism) (Zollner and Mayer-Helm, 2006). However, not until the discovery of aflatoxins in the 1960's were mycotoxins recognized as a potential health hazard to both humans and animals. At the present time, some 400 compounds are now recognized as mycotoxins, of which approximately a dozen groups regularly receive attention as threats to human and animal health (Cole and Cox, 1981). The most important groups of mycotoxins that occur quite often in food are aflatoxins and ochratoxins (produced mainly by *Aspergillus* spp.), trichothecenes, zearalenone, and fumonisins (produced by *Fusarium* spp.), and patulin (produced by *Penicillium* spp.). They received by far the most attention due to their frequent

occurrence and their negative effect on human and animal health (D'Mello and MacDonald, 1997; Bennett and Klich, 2003).

Methods for controlling mycotoxins are largely preventive. They include good agronomic practices such as using sound, fungus-free seeds for planting, controlling insects and plant diseases, and proper irrigation practices (Ellis *et al*., 1991). In addition, aflatoxin production also can be successfully prevented by good harvesting, drying and storage practices (Lisker and Lillehoj, 1991). These approaches include developing host resistance through plant breeding, genetic engineering, use of biocontrol agents, and targeting regulatory genes in mycotoxin development (Brown *et al*. 1999; Magan and Aldred, 2007). However, prevention is not always possible under certain agronomic and storage practices (Samarajeewa, *et al*., 1990). Once the contamination has occurred, other control measures must be established and applied to reduce the risk of exposure to this toxin. Necessary approaches include physical, chemical or biological removal, or use of chemical or physical inactivation. In order for these procedures to be acceptable, they must meet certain criteria such as, (1) inactivate, remove or destroy the toxin, (2) not leave or produce toxic residues, (3) retain the commodity's nutritive value, (4) not alter technological properties, and (5) destroy, if possible, fungal spores (Park *et al*., 1988; Park and Lee, 1990).

One method of decontamination for aflatoxin-affected commodities that has been a focus of attention is ozonation, a physical/chemical oxidation method. Several studies undertaken previously had established the effectiveness of ozonation as a decontamination process. It has been found to be effective in reducing aflatoxin levels by as much as 95%. A previous study has proven the effectiveness of ozonation in

degrading aflatoxin in contaminated corn (Prudente and King, 2002). The result of the study also showed that fractions from ozonated contaminated corn had less mutagenic potential in the Ames assay. However, some findings from the study also suggested a possible formation of fat-soluble mutagen.

In view of these findings, the current study aimed to continue the safety evaluation of the ozonation process in reducing the risk associated with aflatoxin contamination. Specifically, the study aimed to determine possible reaction product/s from degradation of aflatoxin by ozone.

The succeeding sections give a brief overview of 5 major mycotoxins: aflatoxins, ochratoxins, trichothecenes, zearalenone, and fumonisins, that are of great concern because of their high incidence in food and feedstuff; and their negative health implications. This also includes some recent publications on the use of ozone in mycotoxin prevention and control.

CHAPTER 2. LITERATURE REVIEW

2.1 Mycotoxins

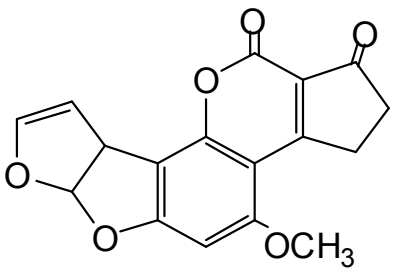
2.1.1 Aflatoxins

Aflatoxins are a group of closely related bis-dihydrofurano secondary fungal metabolites that have been epidemiologically implicated as environmental carcinogens in humans. They are produced primarily by *Aspergillus flavus* and *A. parasiticus* growing on agricultural commodities in the field and/or while the products are stored. Historically, the aflatoxins were discovered as a consequence of an epizootic outbreak of hepatic necrosis, resulting in the deaths of 100,000 turkey poults in England in 1960 and 1961 (Busby and Wogan, 1981). Presently, 18 different types of aflatoxins have been identified, with aflatoxins AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2 being the most common (Beuchat, 1978). Their chemical structures are shown in Figure 2.1. Of these, B_1 and G_1 occur most frequently, with aflatoxin B_1 (AFB_1) being the most potent toxin and carcinogen of the group. The letters B and G refer to the strong fluorescence colors, blue and green under long-wave ultraviolet (UV) light, while the subscripts 1 and 2 noted their position relative to the solvent front on a thin layer chromatographic plate (Bullerman, 1979). The letter M for M_1 and M_2 refer to the milk where these toxins were primarily identified (Bhatnagar *et al.*, 1994).

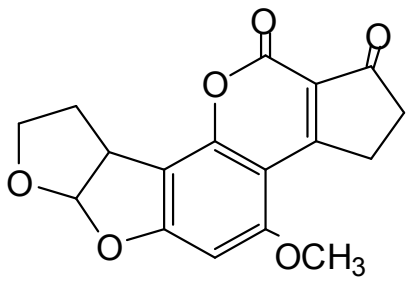
The aflatoxin that has caused the most concern is AFB_1 due to its widespread occurrence, its prevalence among the four naturally occurring aflatoxins, and its acute toxicity and carcinogenicity (McKenzie, 1997). The liver is considered the primary target organ for aflatoxin toxicity. Since its characterization in the early 1960's, acute structural and functional damage to the liver has been reproduced in a wide variety of

species (Lopez-Garcia, 1998; Syed, 1999). Hepatic necrosis, fatty infiltration, bile duct proliferation, and hepatic failure were observed in turkey poults, ducklings, chickens, and pigs fed with feed contaminated with aflatoxins (Newberne and Rogers, 1981). In some cases, fatty acid infiltration and focal necrosis occur in the heart and kidney; necrosis of the spleen and pancreas; cerebral and gall bladder edema; and hemorrhage were observed (Newberne and Rogers, 1981).

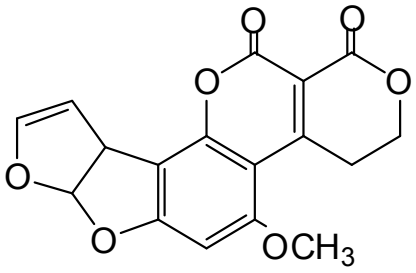
While the acute toxicity of the aflatoxins is noteworthy, it is the carcinogenic potential of AFB_1 that has been the focus of considerable research and regulation (Wogan *et al*., 1971; IARC, 1987; McKenzie, 1997). The delayed results of a single large or repeated lower doses of aflatoxins include hepatocyte regeneration, bile duct proliferation, and fibrosis; however, the major late effect is development of hepatocarcinoma or occasionally, renal, colon, or other carcinomas (Newberne and Rogers, 1981). Evidence that aflatoxin may be carcinogenic to man arises from epidemiological studies and from reports of cases of primary liver cancer (PLC) in primates such as Rhesus monkeys (Ellis *et al*., 1991). Although a direct cause/effect relationship has not been confirmed, the association between mycotoxin exposure and PLC is suggested by correlation of exposure to aflatoxins and PLC incidence rates in some areas of Africa and Asia (Shank *et al*., 1972; Peers and Linsell, 1973; Peers *et al*., 1976; Van Rensberg *et al*., 1985; Hsieh, 1986; Peers *et al*., 1987; Groopman *et al*., 1988; Yeh *et al*., 1989). In 1987, the International Agency for Research on Cancer (IARC) concluded that there was sufficient evidence to classify aflatoxin as a group I carcinogen. The FDA has action levels for aflatoxins regulating the levels and species to which contaminated feeds may be fed (CAST, 2003) (Table 2.1).



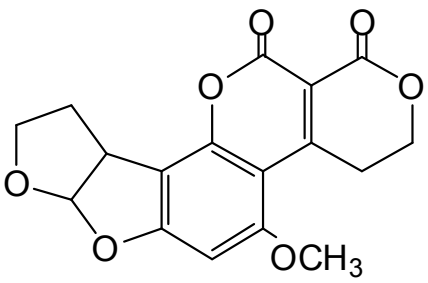
Aflatoxin B_1



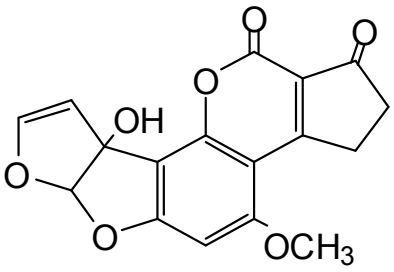
Aflatoxin B_2



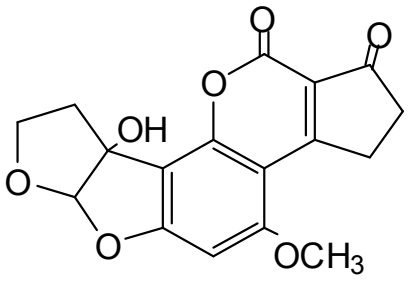
Aflatoxin G_1



Aflatoxin G_2



Aflatoxin M_1



Aflatoxin M_2

Figure 2.1 Chemical structures of most common aflatoxins.

Table 2.1. U.S. Food and Drug Administration action levels for total aflatoxins in food and feed (μg/kg).

Commodity	Concentration
Cottonseed meal as a feed ingredient	300
Corn and peanut products for finishing beef cattle	300
Corn and peanut products for finishing swine	200
Corn and peanut products for breeding beef cattle, swine and mature poultry	100
Corn for immature animals and dairy cattle	20
All products, except milk, designated for humans	20
All other feedstuffs	20
Milk	0.5

Table adapted from Richard (2007).

2.1.2 Ochratoxins

Occhratoxin A, B, and C (OTA, OTB, OTC) are toxins naturally produced by several species of *Aspergillus* and *Penicillum* (Figure 2.2). These mold species are capable of growing in different climates and on different plants thus, contamination of food crops can occur worldwide (Aish *et al*., 2004). OTA attracted by far the most attention since it is distinctly more toxic and prevalent than OTB and is rapidly formed from OTC (Zollmer and Mayer-Helm, 2006). OTA is a fluorescent compound produced primarily by *Aspergillus ochraceus* and *Penicillium verrucosum* (CAST, 2003).

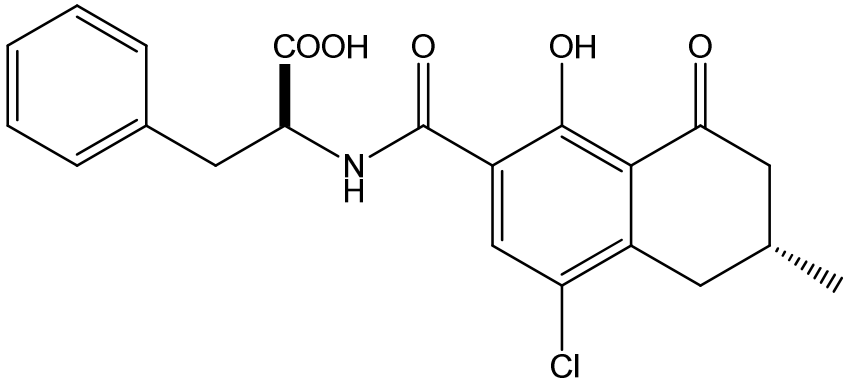


Figure 2.2 Chemical structure of ochratoxin A.

Ochratoxin A is primarily a kidney toxin but in sufficiently high concentrations, it can damage the liver as well (Richard, 2007). It is also found to be carcinogenic in rats and mice and suspected as a contributory agent in some human diseases. One such disease is the Balkan Endemic Nephropathy, a kidney disease associated with upper urinary track urothelial cancer in humans, which is considered by some to be caused by ochratoxin (Pfohl-Leszkowicz *et al*., 2002, Pfohl-Leszkowicz and Manderville, 2007). OTA can cause immunosuppression in animals that may include depressed antibody responses, reduced size of immune organs, changes in immune cell number and function, and altered cytokine production. In addition, it can cause immunotoxicity probably caused by cell death following apoptosis and necrosis, in combination with slow replacement of affected immune cells (Al-Anati and Petzinger, 2006). OTA occurs in a wide variety of commodities such as cereals and cereal products, beer, wine, cocoa, coffee, dried fruits, grape juices, and spices in varying amounts but at relatively low levels (Sforza *et a*l., 2006; Zollner and Mayer-Helm, 2006; Richard, 2007). The International Agency for Research on Cancer in 1993 classified OTA as possible carcinogenic in human. The World Health Organization (WHO) has set a provisional

tolerable daily intake level for OTA of 14 ng/kg body weight (WHO, 1995). Regulations for ochratoxin A are present in the European Community (FAO, 2004) but none have been established in the United States (Table 2.2)

Table 2.2. European Union regulations for ochratoxin (µg/kg)

Product	Concentration
Raw cereal grains	5
All products derived from cereals intended for direct human consumption	3
Dried vine fruit (currants, raisins and sultanas)	10

Table adapted from Richard (2007).

2.1.3. Trichothecenes

The Trichothecenes are a group of around 190 different sesquiterpenoid metabolites (Zollner and Mayer-Helm, 2006) produced by a number of fungal genera, including *Fusarium, Myrothecium, Phomopsis, Stachybotrys, Trichoderma*, and *Trichotecium* (Kumar *et a*l., 2008). The trichothecenes are comprised of four basic groups, with types A and B representing the most vital ones. Type A trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol, and diacetoxyscirpenol (DAS), while type B trichothecenes include deoxyvalenol (DON and its derivatives), nivalenol (NIV), and fusarenon-X (D'Mello, 2003). T-2, DON, and DAS are the most studied toxins among the trichothecenes. Trichothecenes are commonly found as food and feed contaminants. Corn, oats, barley and wheat, which are infected by *Fusarium* fungi, are the main source of trichothecene contamination in food and feedstuff. They have been reported to contain types A and B toxins (CAST, 2003).

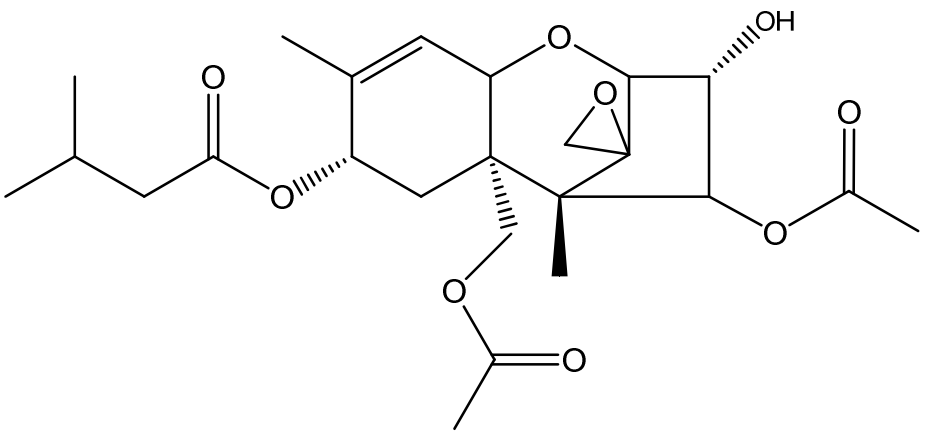


Figure 2.3. Chemical structure of Trichothecenes.

Trichothecene mycotoxins are potent inhibitors of eukaryotic protein synthesis (Nicholson, 2004). These toxins act by inhibiting either the initiation or the elongation process of translation, by interfering with peptidyl transferase activity (Wannemacher and Wiener, 1997). Trichothecene mycotoxins also disrupt the synthesis of DNA and RNA. These mycotoxin-related inhibitions were suggested to be a secondary effect of protein synthesis inhibition. It affects dividing cells such as those lining the gastrointestinal tract, skin, lymphoid and erythroid cells. It can decrease antibody levels, immunoglobulins and certain other humoral factors such as cytokines (Richard, 2007). Ingestion of high doses by farm animals causes nausea, vomiting and diarrhea; and at lower doses, some farm animals i.e. pigs, exhibit weight loss and food refusal (Rotter *et al*., 1996). Several diseases have been directly correlated with trichothecene intoxication, such as the outbreak of alimentary toxic aleukia (ATA) in Russia in 1913 and 1944 (Zollner and Mayer-Helm, 2006). This disease was characterized by severe hemorrhage, extreme leucopenia, agranulocytosis, necrotic angina, and exhaustion of

the bone marrow (Ueno, 1987). Due to its frequent occurrence and toxicity, several countries have established legal regulations or recommendations for DON, HT-2 toxin, and T-2 toxin (FAO, 1997). Currently, trichothecenes are not regulated by the FDA, or by the European Union. However, the FDA set up an advisory level of 1000 µg/kg in cereal products intended for human consumption (FAO, 1997). Some European countries recommend maximum levels of DON between 100 and 1000 µg/kg for human consumption and 400 and 5000 µg/kg in feeding stuff (Codex Alimentarius, 2002).

2.1.4. Zearalenone

Zearalenone (ZEN, Figure 2.4) is a nonsteroidal estrogenic mycotoxin with a phenolic resorcyclic acid lactone structure (Zollner and Mayer-Helm, 2006). It is produced by certain strains of various species of the Genus *Fusarium*, including *F. culmoron*, *F. equiseti*, *F. graminearum*, and *F. moniliforme* (Chelkowski, 1998). Grains infected with this organism may exhibit a pink color associated with the production of a pink pigment simultaneously produced with ZEN. Most often, this mycotoxin is found in corn. It can also be present in bread (Aziz *et al*., 1997) and in others grains such as oat, barley, wheat, and sorghum under prolonged cool and wet weather conditions in temperate and warm regions (Kuiper-Goodman *et al*., 1987; Tanaka *et al*., 1988). ZEN is not acutely toxic. Based upon several animal studies, there is limited evidence of carcinogenity of ZEN (Stolof, 1976). Conversely, it has been the point of study because of its estrogenic effect on mammals. ZEN is the primary toxin causing infertility, abortion or other breeding problems, especially in swine (Alldrick, 2004).

Recommended levels of ZEN in animal feed are imposed by only a few countries. The levels of ZEN are often tested to prevent losses in animal husbandry.

The United States has no regulations imposed on the occurrence of this mycotoxin; however, as shown in Table 2.3, regulations exist from the European Union (FAO, 2004; Richard 2007).

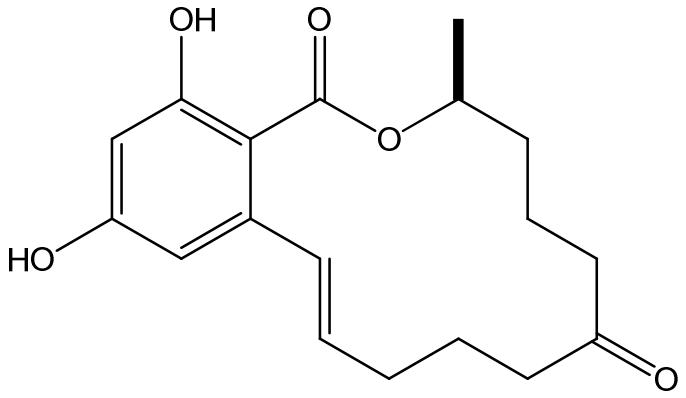


Figure 2.4. Chemical structure of zearalenone.

Table 2.3. European Union regulations for zearalenone (µg/kg).

Product	Concentration
Unprocessed cereals other than maize	100
Unprocessed maize	200
Cereal flour except maize flour	75
Maize flour, meal, grits and refined maize oil	200
Bread, pastries, biscuits, other cereal snacks and breakfast cereals	50
Maize snacks and maize-based breakfast cereals	50
Processed maize-based foods for infants and young children	20
Processed cereal-based foods for infants and young children and baby food	20

Table adapted from Richard (2007).

2.1.5. Fumonisins

The fumonisins are a group of non-fluorescent mycotoxins produced primarily by *Fusarium verticillioides* (formerly F. moniliforme, F. nygamal, and *F. proliferatum* (Marasas, *et al.*, 2001; Rheeder *et al.*;2002; CAST, 2003). The major entities of fumonisins are FB1, FB2 and FB3 (Figure 2.5). Corn is the major commodity affected by this group of toxins. Other commodities such as sorghum, wheat, rice and oat were reported to have been affected by fumonisins (Lopez-Garcia, 1998).

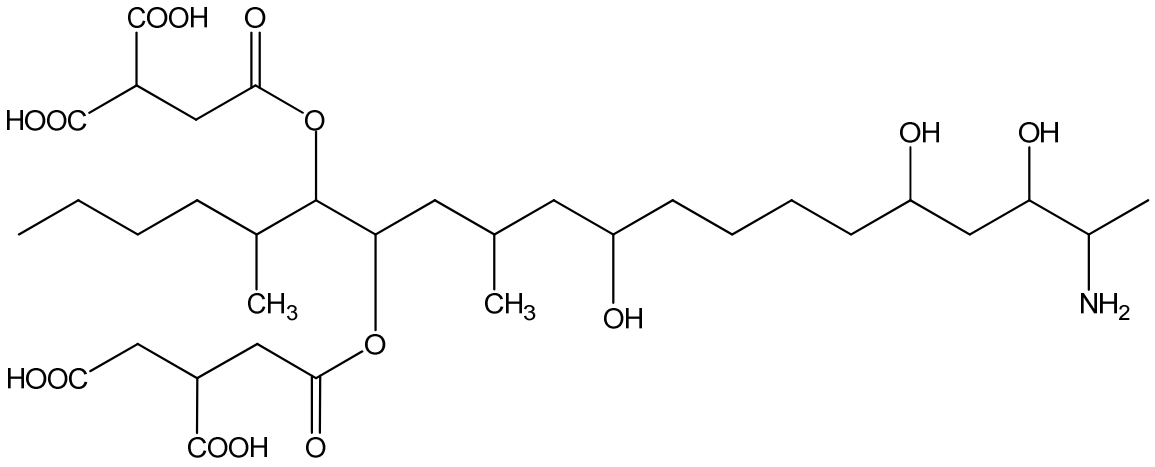


Figure 2.5. Chemical structure of fumonisin B_1.

Fumonisins are obviously disease-causing group of toxins. Numerous instances of animal diseases caused by fumonisins have been discovered and reported. For example, a major disease of horses that includes a softening of the white matter in the brains (leukoencephalomalacia) is caused by the fumonisins (Marasas *et al.*, 1988). Swine lung edema is also caused by the fumonisins (Colvin and Harrison, 1992). Other illnesses caused by fumonisins include liver and kidney tumors in rodents and esophageal tumors in certain human populations (Marasas, 1993 and Marasas, 1995).

The fumonisins usually interfere with sphingolipid metabolism in animals resulting to liver toxicity. Carryover of fumonisins into milk in cow has not been detected and little absorption in tissues has been observed (Richard *et al*., 1996). The guidance levels for total fumonisins (including FB_1, FB_2 and FB_3) in human foods and animal feed proposed by the FDA and European Community are shown in Table 2.4 and Table 2.5, respectively (FAO, 2004).

Table 2.4. U.S. Food and Drug Administration guidelines for fumonisins in human foods and animal feeds (µg/g).

	Concentration Total Fumonisins
Human foods	
Degermed dry milled corn products	2
Whole/partially degermed dry milled corn product	4
Dry milled corn bran	4
Cleaned corn intended for mass production	4
Cleaned corn intended for popcorn	3
Corn and corn byproducts for animals	
Equids and rabbits	5 < 20% diet
Swine and catfish	20 < 50% diet
Breeding ruminants, poultry, mink, dairy cattle, laying hens	30 < 50% diet
Ruminants > 3 mos. before slaughter and mink for pelts	60 < 50% diet
Poultry for slaughter	100 < 50% diet
All other livestock and pet animals species	10 < 50% diet

Table adapted from Richard (2007).

Table 2.5. European Union regulations for fumonisins (μg/kg).

Product	Concentration
Unprocessed maize	2000
Maize grits, meal and flour	1000
Maize-based food for direct consumption except maize grits, meal, flour and processed maize-based foods for infants and young children and baby food	400
Processed maize-based foods for infants and young children and baby food	200

Table adapted from Richard (2007).

2.2 Recent Studies On The Use of Ozone in Mycotoxin Prevention and Control

The effects of ozone gas in reducing aflatoxin concentration in aflatoxin-contaminated agricultural products have been evaluated and the results of the studies appeared to be promising. Dwarakanath *et al*. (1968) reported that ozone (25 mg/minute) reduced aflatoxins in cottonseed meal and peanut meal. In cottonseed meal, 91% of the total aflatoxin content was destroyed by ozone in two hours; this represents a decrease from 214 to 20 ppb. In peanut meal, 78% of aflatoxin was destroyed (a decrease from 82 to 18 ppb) in one hour. In both studies, AFB_1 was completely inactivated after prolonged exposure to ozonation. In a similar study on peanut meal by Dollear *et al*. (1968), results of TLC analysis and feeding experiments in rats showed that ozone (25 mg/minute) was effective in either destroying aflatoxins or significantly reducing the aflatoxin levels. Similarly, results of a study by Maeba and co-workers in 1988 showed that ozone (1.1 mg/L, 5 minutes) inactivated pure aflatoxins in

a model system. In the same study, they found subsequent reduction of mutagenic activities in the Ames assay. Furthermore, no harmful effect of ozone-treated AFB_1 in chicken embryo and rats was detected. In 1993, Chatterjee and Mukherjee studied the impact of ozone on the immunity-impairing activity of AFB_1. Phagocytosis by rat peritoneal macrophages, which was found to be suppressed in the presence of aflatoxin, remained unimpaired when the applied AFB_1 was pretreated with 1.2 mg/L ozone for 6 minutes at a flow rate of 40 ml/min. In 1997, McKenzie developed a novel and continuous source of O_3 gas through electrolysis. He treated corn spiked with aflatoxins and/or naturally contaminated rice powder with ozone. He reported a rapid degradation of AFB_1 and AFG_1 using two wt. % ozone, while AFB_2 and AFG_2 were more resistant to oxidation and needed higher levels of ozone. Total degradation was obtained after 15 seconds using 20 wt. % ozone. Moreover, he reported that the toxicity of aflatoxin was reduced based on a hydra bioassay. In a similar study in 1998, McKenzie found that aflatoxins could be reduced by 95% in corn samples treated with 14 wt % ozone for 92 hours at a flow rate of 200 mg/min. Turkey poults fed with ozone-treated contaminated corn did not show harmful effects as compared to turkey poults fed with untreated contaminated corn (McKenzie *et al*., 1998).

In continuing both the studies done by McKenzie, Prudente and King (2002) reported that ozonation (10-12 wt. %) reduced the level of aflatoxin in contaminated corn kernels (587 ppb) by about 92%. The result of the study also showed that the degraded aflatoxin did not revert back to its original form indicating permanency of the ozonation process. In subsequent mutagenicity evaluation using the Ames assay, crude extracts from ozone-treated and untreated contaminated corn kernels did not

show mutagenic potential. This result confirmed the presence of compounds in corn that interfere with the mutagenicity assay. In addition, it was observed that the extract from ozone-treated contaminated corn kernel had less inhibitory effect compared with the other extracts. This result suggested that the ozonation process might have produced reaction products that have mutagenic potential or the ozonation process destroyed the natural mutagen inhibitor present in corn. Other tests showed that the ozonation process significantly reduced the percentage of unsaturated fatty acids in contaminated corn kernels compared with that of clean corn kernels.

Proctor et al. (2004) used the ozonation process to evaluate the effectiveness of ozonation and mild heat in breaking down aflatoxins in peanut kernels and flour. Ozonation was also used to quantify aflatoxin destruction compared with untreated samples. Peanut samples were mixed with known concentrations of aflatoxins B_1, B_2, G_1 and G_2; and subjected to gaseous ozonation (4.2 wt.%) at various temperatures (25, 50, 75ºC) and exposure times (5, 10, 15 min). Ozonated and non-ozonated samples were extracted in acetonitrile/water, derivatized in a Kobra cell and quantified by high-performance liquid chromatography. Results showed that ozonation efficiency increased with higher temperatures and longer treatment times. The ozonation process resulted in 56-77% reduction of AFB_1 and 61-80% reduction in AFB_2. On the other hand, they observed a of 51% degradation of both AFB_2 and AFG_2 in peanut kernels. For peanut flour, 20% and 30% degradation was observed for AFB_2 and AFG_2, respectively. Regardless of treatment combinations, aflatoxins B_1 and G_1 exhibited the highest degradation levels. Moreover, higher levels of toxin degradation were achieved in peanut kernels than in flour. The temperature effect decreased as the exposure time

increased. This suggests that ozonation at room temperature for 10–15 minutes could yield degradation levels similar to those achieved at higher temperatures while being more economical.

A study on the use of aqueous ozone to degrade trichothecene mycotoxins was reported by Young *et al*., (2006). The degradation of ten trichothecene mycotoxins by aqueous ozone was monitored by liquid chromatography–ultraviolet–mass spectrometry (LC–UV–MS). Results of the experiment showed that saturated aqueous ozone (25 ppm) degraded these mycotoxins to materials that were not detected by UV or MS. In addition, it was observed that intermediate products are present when treated with lower levels (0.25 ppm) of aqueous ozone. Based upon the UV and MS data, it was proposed that the degradation begins with attack of ozone at the C9–10 double bond with the net addition of two atoms of oxygen with the remainder of the molecule left unaltered. The oxidation state at the allylic carbon 8 position was observed to have a significant effect on the ease of reaction, as determined by moles of ozone required to effect oxidation. The amount of ozone required to effect oxidation to intermediate products and subsequent degradation followed the series allylic methylene (no oxygen) < hydroxyl (or ester) < keto. Based on the results of the mass spectrometry, it was proposed that an aldehyde was formed with the reaction of ozone and trichothecenes.

Akbas and Ozdemir (2006) evaluated the efficiency of ozone for the degradation of aflatoxins in pistachio kernels and ground pistachios. Pistachios were contaminated with known concentrations of aflatoxin (AF) B_1, B_2, G_1 and G_2. Pistachio samples were exposed to gaseous ozone at 5.0, 7.0 and 9.0 mg/L ozone concentrations for 140 and 420 min at 20 °C and 70% RH. Aflatoxin degradation was determined by high

performance liquid chromatography (HPLC). They found that the ozonation process reduced total aflatoxin and AFB_1 by 24% and 23 %, respectively, for pistachio kernels and only 5% for ground pistachios. No significant change in the fatty acid compositions of pistachios after the ozonation treatments was observed. Likewise, no significant changes were found between sweetness, rancidity, flavor, appearance and overall palatability of ozonated and non-ozonated pistachio kernels.

Ozone was used in the detoxification of aflatoxin B1 in red pepper (Inan *et al.* 2007). Flaked red pepper with moisture content of 12.6% and containing 20 ppb of aflatoxin B_1 was treated with ozone gas of various concentrations (16, 33, 66 mg/l) for 7.5, 15, 30, and 60 minutes. The ozone gas was generated from pure oxygen through corona discharge type generator (Fischer Ozone 502 Generator). The results of the study showed that the efficiency of the ozonation process was affected by ozone concentration and exposure time. The process reduced the aflatoxin B1 content in flaked red pepper by as much as 80% after ozonation for 60 min. Further, the level of aflatoxin B1 in crush red pepper with moisture content of 12.7% and initial aflatoxin B1 level of 32 ppb, was reduced by as much as 93% after exposure to gaseous ozone for 60 minutes. In addition, no significant change in color between ozonated and non-ozonated samples was observed using the Hunter color parameters (L, a and b).

2.3. Mycotoxin Analysis

Many factors affect mycotoxin analytical techniques including the chemical nature of the target mycotoxins, the molecular weight, and the functional groups. These factors determine the mycotoxin's volatility and solubility. The selection of analytical method for a certain toxin or group of toxins is also influenced by the above-mentioned

factors. Current analytical techniques involve three steps: extraction into a solvent, partial purification or cleanup, and quantitation (Wilson *et. al*., 1998).

A review of current techniques for mycotoxin analysis indicated that an analytical procedure can be devised using different approaches. Results of surveys indicated that there is no best technique for mycotoxin analysis; however, there are a significant number of methods that can be used or modified to satisfy certain analytical requirements. The analyst's preference, the sample matrix, the target mycotoxin, and the availability of supplies and equipment, must be taken into consideration when choosing a procedure for the analysis (Wilson *et. al*., 1998).

There are chemical and immunochemical methods for specific applications. These methods include: 1) thin layer chromatography (TLC), 2) HPLC, 3) GC, 4) mass spectral (MS) techniques and 5) immunochemical methods. For almost all mycotoxins, TLC can be used as a separation technique. However, with this separation technique, the procedure is variable resulting to a large coefficient of variation and poor precision. HPLC and GC are more precise separation techniques because there is less variation related to these procedures (Wilson *et. al*., 1998).

HPLC is recommended for quantitation of the aflatoxins, fumonisins, ochratoxins, patulin, and citrinin. Results of a recent survey showed that GC methods are preferred for DON determinations. For Zearalenone, either a HPLC or GC method can be used. For trace analysis and chemical confirmation of mycotoxins, mass spectral techniques can be applied (Wilson *et. al*., 1998).

The evaluation of mycotoxin contamination for humans and animals depends upon its identification and accurate quantification in food and feedstuffs (Zollner and

Mayer-Helm., 2006). Currently, other methods have been reported to successfully quantify aflatoxins in various food matrices. For example, aflatoxins B_1 and B_2 in pistachio samples were determined using corona discharge ion mobility spectrometry (IMS). Standard aflatoxins or an extracted sample in methanol was introduced into the IMS. The experimental analysis resulted in linear calibration curves with two orders of magnitude and a relative deviation (RSD) of less than 10%. For both aflatoxins, the limit of detection (LOD) was observed to be 0.25 ng. The LOD was improved when ammonia was added to the carrier gas as the dopant. The detection limit for the IMS method was higher compared to other methods; however, IMS has a fast response time, low cost, and the instrument is portable (Shelbani *et al*., 2008).

Another reported technique, the use of internal standards labeled isotope is one approach to quantify aflatoxin levels in certain food matrices. In this study, levels of aflatoxins in peanuts, nuts, grains, and spices were determined using LC-MS/MS stable isotope dilution assay (SIDA). Aflatoxins B_1, B_2, G_1, and G_2 in the food samples were quantified using isotope labeled (deuterated) aflatoxins B_2 and G_2. The limit of detection was 0.31 µg/kg for aflatoxin B_1, 0.09 µg/kg for aflatoxin B_2, 0.38 µg/kg for aflatoxin G_1, and 0.32 µg/kg for aflatoxin G_2. The aflatoxins levels in the samples ranged from 0.5 to 6 µg/kg (Cervino *et al*., 2008).

Beginning in the mid 1990s, the use of atmospheric pressure ionization (API) interfaces began. Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) LC/MS have become the most widely used techniques in environmental and food analysis because of their robustness, easy handling, high sensitivity, accuracy,

and analyte selectivity. The techniques are compatible with almost the whole range of compound polarities (Careri and Corrandi, 2002).

Zollner and Mayer- Helm (2006) reviewed the application of LC- (API) MS in the analysis of frequently occurring and highly toxic mycotoxins, such as trichothecenes, ochratoxins, zearalenone, fumonisins, aflatoxins, enniatins, moniliformin and other mycotoxins. The introduction of atmospheric pressure ionization (API) techniques has made the LC/MS a routine technique in food analysis. This technique surmounts the disadvantage of GC/MS regarding volatility and thermal stability.

The degradation kinetic of type A and B trichothecenes in aqueous ozone and the structure of the main degradation products were determined by LC/MS/MS (Young et al., 2006). Generally, LC/MS can quantify trichothecenes to a low ppb level in several different biological matrices with recovery rates ranging between 70 and 108% (Klotzel et al., 2005). LC/MS is also used to confirm OTA positive samples that have been analyzed using HPLC-FL or ELISA techniques (Ventura *et al*., 2003).

Results of scientific reviews on mycotoxin analyses techniques conducted by Zollner and Mayer-Helm (2006) revealed that LC/MS methods are used for all important mycotoxin groups. MS/MS experimental results indicated mycotoxin quantification with improved sensitivity and accuracy. These methods are also capable of multi-mycotoxin analysis. Moreover, LC/MS/MS mycotoxin analysis can be used as a multi-analyte methodology.

Contrary to the results of the review by Zollner and Mayer-Helm in 2006, Sforza *et al*. (2006) reported that based upon the results of their review on mycotoxin determination in food and feed by hyphenated chromatographic techniques mass

spectrometry, the sensitivity issue is a real problem. This is because with the LC/MS method, different ionization techniques such as ESI, APCI, and APP can have different responses. Hence, the review indicated that LC/MS seems to be an excellent confirmatory technique only when other methods such as fluorescence or UV absorbance can be used to quantify mycotoxins. A problem arises when GC/MS and LC/MS methods are used for exact quantitative determination of mycotoxin in food because the matrices significantly vary. Such problems can be addressed only by using isotopically labeled internal standards or by using ionization interfaces that can reduce matrix effects and ion suppressions. This will result in a simpler sample preparation procedure, and cleanup procedures can be avoided. Finally, Sforza *et al* (2006) concluded that the use of isotopically labeled internal standards or ionization interfaces coupled with MS detectors can be an accurate and precise method of mycotoxin analysis and it is cost effective.

Conventional analytical techniques currently use HPLC or GC in combination with different detectors such as fluorescence detection (FLD) with a pre- or post-derivatization step, UV detection, flame ionization detection (FID), electron capture detection (ECD) or mass spectrometry (MS), to quantitatively determine regulated mycotoxins including fumonisins, aflatoxin and ochratoxin A. Recent developments focus on the LC-MS/MS and other rapid screening methods for mycotoxin determination. LC/MS/MS method is now used to determine and identify multiple and large numbers of mycotoxins. A recently updated report revealed that the method can analyze and identify 87 different mycotoxins simultaneously (Krska *et al*., 2008). Fast screening methods are classified into immunochemical and non-invasive techniques.

Immunochemical techniques such as ELISAs do not require any cleanup or analyte enrichment steps (Gilbert and Anklam 2002; Fremy and Usleber 2003). Non-invasive techniques are optical methods that are fast and non-destructive. New screening methods include FLDs, biosensors, and IR-screening techniques. These methods are fast and cost effective (Krska *et al*., 2008).

CHAPTER 3. MATERIALS AND METHODS

3.1 Study 1: Distribution of Aflatoxin in Ozonated and Non-ozonated Corn

Chemical analyses have shown that ozonation can effectively reduce aflatoxin levels in contaminated corn. In previous studies conducted by Prudente (2001) and Prudente and King (2002), gaseous ozone reduced the aflatoxin level in naturally contaminated corn by about 92%. On the other hand, results of the Ames mutagenicity assay on fractions collected from different solvent extraction procedures suggested the possible formation of reaction products with slight mutagenic potential against tester strain TA 98. Based on these results, additional studies were required to further assess the suitability and safety of the ozonation process to degrade aflatoxin in corn.

The present study was conducted to investigate the formation of ozone and aflatoxin reaction products in corn. Ozonated and non-ozonated ground corn (Batches 1 and 2) from the previous study of Prudente (2001) were used in the present study. Sequential fractionation of ground corn samples was performed to determine the distribution of aflatoxin-related decontamination by-products. Extracts collected were evaluated by thin layer chromatography and reverse phase high performance liquid chromatography analyses.

3.1.1 Chemicals

Standard aflatoxins (B_1, B_2, G_1, and G_2) and Pronase E were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Chloroform, dichloromethane, acetonitrile, petroleum ether, diethyl ether, benzene, methanol, hexane, trifluoroacetic acid, and water were HPLC-grade and were purchased from Fisher Scientific, Raleigh, NC.

3.1.2 Corn Samples

Two batches (Batch 1 and Batch 2) of corn kernel samples provided by Dr. Kenneth S. McKenzie of Lynntech, Inc., College Station, Texas were used in the preliminary studies. Corn samples (10 kg) with and without aflatoxin contamination were treated with gaseous ozone. Each corn sample was placed in a 30-gallon polyethylene reactor with false bottom. A 10-15" headspace was allowed to achieve even ozone dispersion though the corn. The reactor lid was fitted with ¼" Teflon bulkheads. Ozone gas, 10-12 wt%, was flowed in through the top at approximately 2 L/min. A 2.5 L/min vacuum was placed at the bottom. All corn samples were treated for 96 hours with mixing occurring every 30 hours. The treatment protocol included untreated clean corn (control), ozone-treated clean corn, naturally contaminated corn and ozone-treated naturally contaminated corn. This allowed determination of the efficacy of the ozonation process to degrade aflatoxin and to determine the effect of ozone on the quality of the corn from a safety perspective. Corn samples (10 kg) from each treatment were ground using a Romer Hammer Mill and was ground further using a Brinkmann mill to pass a 1.0 mm sieve. Samples were transferred to clean plastic bags, labeled and stored at 4°C.

A third batch of corn was kindly provided by Dr. Manjit Kang of the LSU-Agronomy Department. Freshly harvested corn ears were manually shelled and sound kernels were separated from damaged or visibly contaminated kernels. Two 5-kilogram damaged/contaminated corn kernel samples were prepared. One was for ozonation and the other was the untreated control. The initial moisture content of corn was 11.65% and was adjusted to ~13% by adding the required amount of water and mix-

tumbled overnight to ensure even incorporation of water into the corn kernels. Five hundred grams of kernels were randomly drawn from the sample to determine the concentration of aflatoxins. The remaining kernels were treated with 17.17 wt % ozone gas for 96 hrs at a flow rate of 175 ml/min and mixed every 12 hours. Figure 3.1 shows the set-up of the ozonation process. After ozonation, the samples were air-dried overnight inside the fumehood. Treated and untreated kernels were ground using a Brinkman mill and kept at 4°C. Additional corn kernels (Batches 4 and 5) provided by Dr. Kenneth Damann of the LSU-Department of Plant Pathology and Crop Physiology were used to produce artificially-contaminated corn.

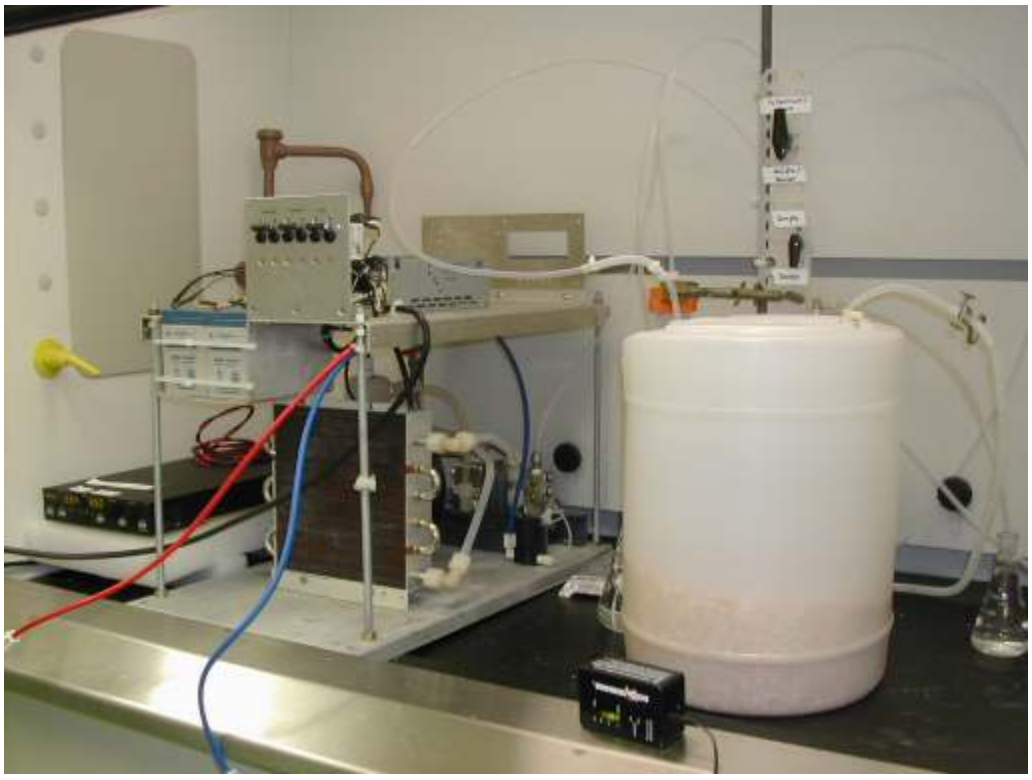


Figure 3.1. Set-up of the ozonation process.

3.1.3 Analysis of Aflatoxins

Aflatoxin determination in samples was carried out using the AOAC approved Multifunctional Column (Mycosep) method (AOAC Official Method 994.08, 2005). Fifty grams of ground sample were combined with 100 ml acetonitrile-water (9:1) solution and blended for 2 minutes at high speed. After blending, the extract was filtered through Whatman No.1 filter paper under vacuum. Fifty ml of the filtrate was collected in a 50-ml disposable centrifuge tube. A 3 ml aliquot of the filtrate was applied onto the Mycosep multifunctional cleanup (MFC) column and was collected in a 20-ml scintillation vial. Two hundred µL of the purified extract was transferred into a derivatization vial and 700 µl of derivatization solution (trifluoroacetic acid + glacial acetic acid + water, 20:10:70) were added. The vial was heated in a 65°C water bath for 8.5 minutes to complete derivatization of aflatoxin B_1 and/or G_1. The vial was then transferred to a Waters 717+ auto-sampler.

Aflatoxin concentrations were determined using a Waters HPLC System equipped with Waters 600E system controller, Waters 717+ autosampler, Waters 486 tunable absorbance detector set at 365 nm, and Waters 470 scanning fluorescence detector using excitation and emission wavelengths of 360 nm and 440 nm, respectively. A Microsorb-MV C-18, (4.6 x 150 mm, Rainin, Woburn, MA) reverse phase column with water-acetonitrile (8:2 v/v) as the mobile phase at a flow rate of 2 ml/min was used to separate the compounds. Thin layer chromatographic analysis was performed on a 20 x 20 cm or a 10 X 20 cm general purpose silica gel plate (Sigma). Mobile phases used were ether-methanol-water (96:3:1) and/or chloroform-acetone (9:1). Plates were examined under long wave (365 nm) UV light.

3.1.4 Sequential Fractionation of Corn

To trace the fate of aflatoxin after ozonation and to investigate the distribution of the ozonated aflatoxin by-products, sequential fractionation of corn samples from batches 1 and 2 was performed. A modified procedure of Park *et al*. (1984) and Martinez *et al*. (1994) was used. Figure 3.2 shows the series of extraction, partition and digestion procedures used in separating and monitoring the aflatoxin-related decontamination by-products.

3.2. Study 2: Evaluation of Ozone and Aflatoxin B_1 Reaction Products in a Model System

Lee *et al*. (1974) utilized a model reaction system to study the chemistry of the ammoniation process in decontaminating aflatoxin B_1. In their study, aflatoxin B_1 was reacted with ammonium hydroxide at 100°C in a Parr bomb. Results of the study identified the major component of the chloroform-soluble fraction of the crude ammoniation product as aflatoxin D_1 (AFD_1). Aflatoxin D_1 is a non-fluorescent phenol with molecular weight of 286 in which the lactone carbonyl moiety characteristic of aflatoxin B_1 was lacking. In study 1 of the present research, it was not possible to identify or observe ozone-aflatoxin reaction products due to possible interferences from the corn matrix and the inability to obtain a concentrated sample.

Therefore, this study was undertaken to better understand the chemistry behind the ozonation process in degrading aflatoxin B_1. Model reactions were conducted using pure standard aflatoxin B_1, in an aqueous solution and in solid form, and treated with gaseous ozone at different time intervals. The primary objective of this study was to

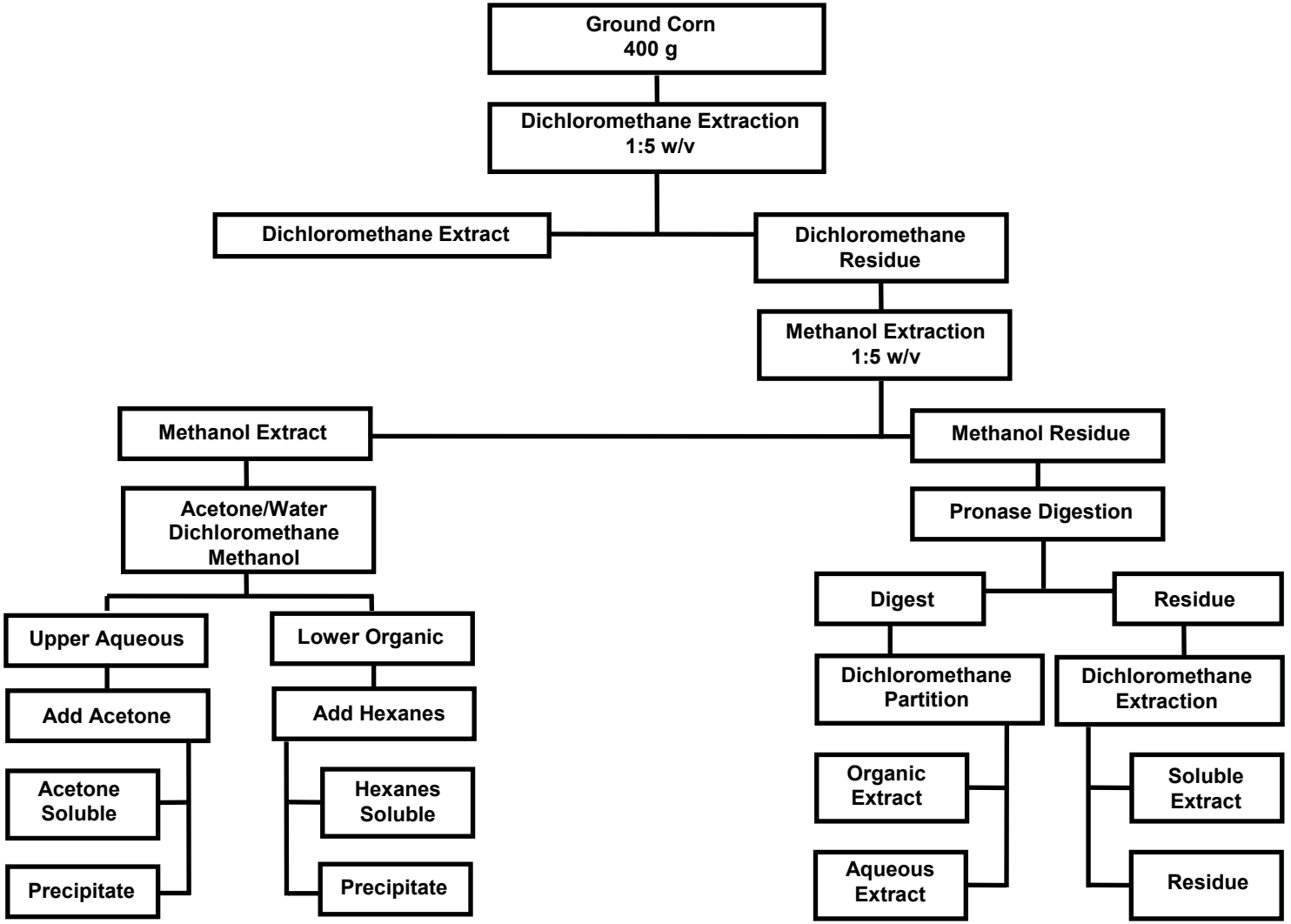


Figure 3.2. Flow diagram of the separation of AFB_1-related decontamination by-products in corn following ozone treatment.

isolate and characterize the by-products to assist in determining the aflatoxin-related products from ozonated corn.

Trial 1. A standard solution of AFB_1 was prepared by dissolving 1 mg of AFB_1 (Sigma, A6636) with 1 ml acetonitrile to give a concentration of 1mg/ml. One-hundred µl of the standard solution containing 100 µg AFB_1 was added to 9.9 ml HPLC grade water in a vial and sealed with a septum. Treatment protocol included ozonation (12-13 wt% at ~150 ml/min) for 0, 10, 20, 30, 40, 50, and 60 seconds. The same procedure was done using a standard mixture of AFB_1, AFB_2, AFG_1, and AFG_2. After ozonation, each solution was transferred into a separatory funnel and aflatoxins were extracted with 10 ml dichloromethane. The dichloromethane layer was carefully collected and transferred into a scintillation vial and evaporated to dryness under nitrogen gas.

The extracts were re-diluted with 1 ml dichloromethane and 20 µl of each was spotted into a TLC plate. The plate was developed with ether-methanol-water (96:3:1) and viewed in a UV cabinet. RP-HPLC (Waters Alliance 2690 Separation Module, Waters Corp., Milford, MA) analysis using a Waters 996 photodiode array detector (210 ~ 500 nm) was done for all the extracts. The same extracts were dried and re-diluted with 2 ml acetonitrile. Ten-µl each of the extracts was injected and passed through a reverse phase column (Microsorb-MV, C18, 4.6 x 150 mm.). The extracts were eluted with acetonitrile-methanol-water (1:1:4) at a flow rate of 1 ml/min.

Trial 2. Five-hundred µL of a standard solution containing 500 µg of AFB_1 were transferred into scintillation vials and evaporated to dryness. Dry materials were suspended in 10 ml distilled water and treated with 12-13 wt.% ozone at a flow rate of ~150 ml/min from 0 to 60 sec at 10 seconds intervals. After ozonation, each solution

was transferred into a separatory funnel and aflatoxins were extracted with 10 ml dichloromethane. The dichloromethane portion was collected and dried under nitrogen. The water portion was transferred to a glass Petri dish, freezed overnight at -80°C and lyophilized. The dried material was re-dissolved in methanol, transferred into vial, and dried under a stream of nitrogen. Both extracts were evaluated with single and 2-dimensional TLC using ether-methanol-water (96:3:1) and chloroform-acetone (9:1) as developing solvents. Sample extracts were submitted for MALDI-MS analysis in the Department of Chemistry Texas A & M University, College Station, TX to partially identify reaction products using 2,5-dihydroxybenzoic acid (DHB) as a matrix.

Trial 3. Aflatoxin B_1 was treated in solid form. Briefly, 100 µl of the standard solution containing 100 µg AFB_1 was transferred into each of the 7 vials and evaporated to dryness under stream of nitrogen gas. The vial was sealed with a septum after drying. The solid standard AFB_1 was ozonated from 0 to 60 sec at 10 seconds intervals. After ozonation, each sample was reconstituted with 500 µl acetonitrile and evaluated by TLC. Two sets of 10 µl of each sample were spotted separately on a 10 cm x 20 cm general purpose TLC plate. For the first set of samples, 5 µl of mixed standard aflatoxins was spotted on top of each original spot and served as internal standard. The plate was developed with ether-methanol-water (96:3:1) and viewed in a UV cabinet.

RP-HPLC analysis of non-ozonated and ozonated pure aflatoxin B_1 was performed. The system consisted of a reverse phase Rainin column (Microsorb-MV, C18, 4.6 x 150 mm), a Waters 600E system controller, a Waters 717+ autosampler, and a Waters 486 Tunable Absorbance Detector set to read at 365 nm. Ten µl of extracts

were injected and eluted with acetonitrile-methanol-water (7:1.5:1.5). at a flow rate of 1.5 ml/min.

3.3 Study 3. Distribution of Ozone-Aflatoxin Reaction Products in Corn After Ozonation

Based on the results of Study 2, the present study was undertaken to determine the fate of aflatoxin after ozonation of contaminated corn kernels. The chemical reaction between aflatoxin and ozone may be different in a meal matrix as compared to a model system because of the presence of other compounds. Radiolabeled aflatoxin B_1, [14C]-AFB_1, was added to artificially contaminated corn kernels prior to ozonation. The distribution of the radiolabeled compounds was used to trace the modification of aflatoxin B_1 after treatment with ozone. The fate of aflatoxin-related reaction products was monitored and isolated through a series of sequential extraction, fractionation, and digestion procedures as described by Park *et al.* (1984) and Martinez *et al*. (1994). The isolation and separation scheme is presented in Figure 3.3.

3.3.1 Chemicals

Standard aflatoxins (B_1, B_2, G_1, and G_2), Pronase E, sodium hypochorite, ammonium molybdate tetrahydrate, ferric sulfate hydrate, zinc sulfate heptahydrate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Chloroform, dichloromethane, acetonitrile, petroleum ether, diethyl ether, benzene, methanol, hexane, trifluoroacetic acid, and water were HPLC-grade and were purchased from Fisher Scientific (Raleigh, NC). Glucose, potassium dihydrogen phosphate, magnesium sulfate heptahydrate, and cupric sulfate pentahydrate were from Fisher Scientific (New Jersey). Ammonium sulfate, sodium tertaborate decahydrate, and manganese sulfate

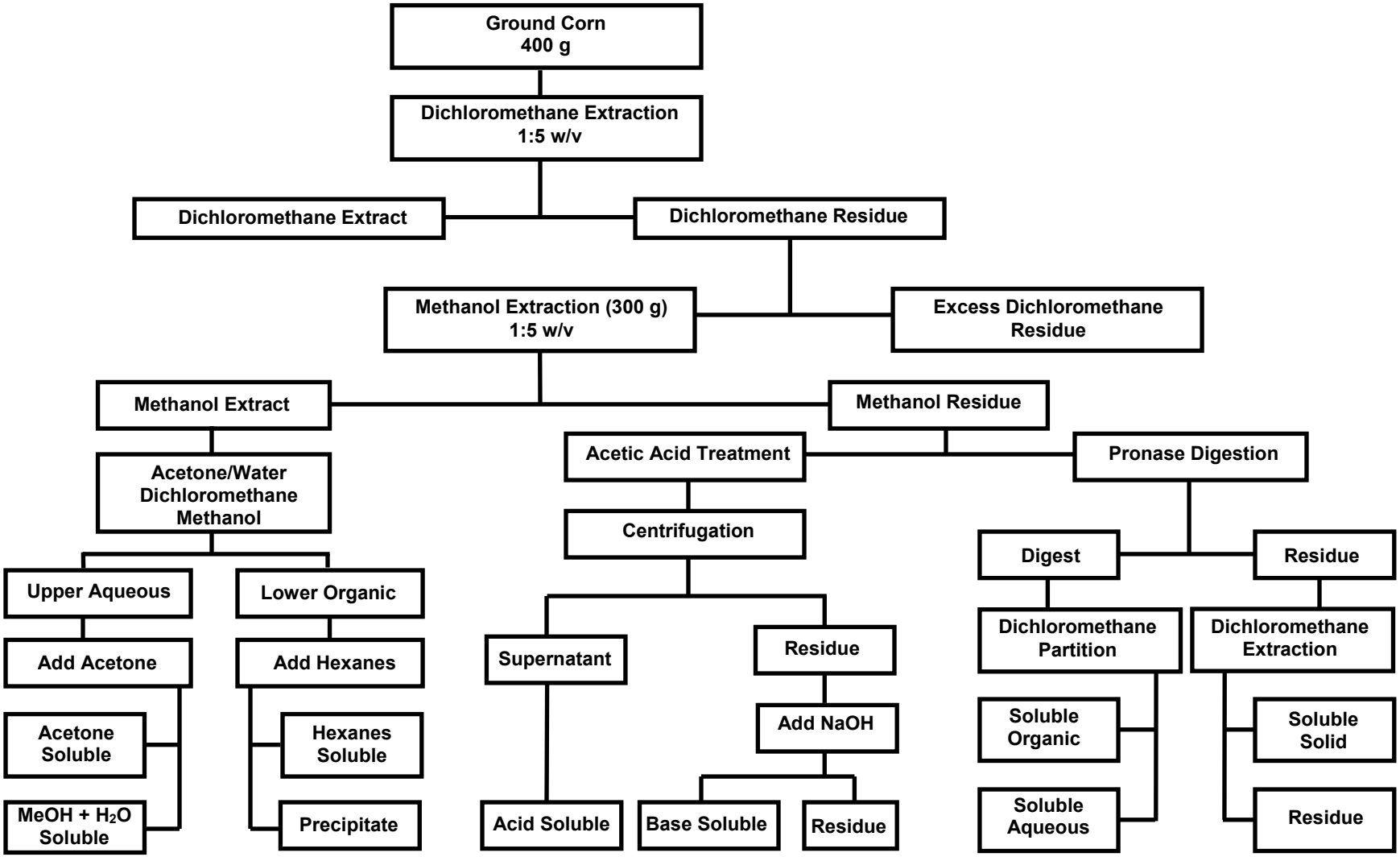


Figure 3.3 Flow diagram of the sequential extraction, fractionation, and digestion procedures used in the separation and isolation of aflatoxin B_1-related products in corn.

monohydrate were purchased from EM Science (New Jersey), Baker Chemicals (New Jersey, and MCB (Ohio), respectively. Hionic fluor, Insta Gel, and Soluene 350 were purchased from PerkinElmer (The Netherlands). .Radiolabeled 14C-acetate-1,2 was purchased from Moravek (Brea, CA).

3.3.2 Sample and Sample Preparation

Artificially contaminated corn was prepared by inoculating kernels with spores of *Aspergillus flavus*. Conidial suspensions of *A. flavus* were prepared by following the method used by Tubajika and Damann (2001). Briefly, conidia of *A. flavus* (A53, C50Aa) suspended in 0.01% Triton X-100 were streaked on a V8 juice agar plate (5% V-8 juice and 2% agar) and incubated for 10 days at 38°C. After incubation, the conidia were scraped-off and washed several times with 0.01% Triton X and transferred to scintillation vials. The concentrations were determined using a counting chamber (2/10 mm depth, 1/16 sq. mm, Speirs-Levy Eosinophil, Hausser Scientific, PA). The concentrations were calculated to be 9.65×10^7 and 9.45×10^7 cells/ml.

Approximately 10 kg of corn with an initial moisture content of ca. 13% was first sterilized for 15 min at 121°C and then transferred into a 5-gal capacity Nalgene container. The moisture content of the corn was adjusted to ca. 20% by adding an appropriate amount of sterile distilled water. Six ml of conidial suspension (9.45×10^7 cells/ml) was added and the corn sample was tumbled overnight to ensure even distribution of conidia and even re-hydration of corn. The inoculated corn kernels were transferred into an autoclavable biohazard bag and incubated at 30°C for 10 days. A pan filled with distilled water was placed inside the incubator to maintain 100% relative humidity. The corn was mixed everyday to avoid an increase in grain temperature and

to make sure that *A. flavus* cells were well distributed. The corn was removed from the incubator after 10 days and placed in a 60°C oven overnight to kill the fungi. A 500-g aliquot was used to determine the aflatoxin content.

3.3.3 Analysis of Aflatoxins

Determination of aflatoxins (B_1, B_2, G_1, and G_2) levels in corn samples was carried out before and after ozone treatment using the AOAC approved Multifunctional Column (Mycosep) method (AOAC Official Method 994.08, 2005) as described in Section 3.1.3. Aflatoxin levels were determined using a Waters HPLC System equipped with Waters 600E system controller, Waters 470 scanning fluorescence detector, Waters 486 tunable absorbance detector, and Waters 717+ autosampler. Fifty µl of each derivatized standard working solution and extract was injected and aflatoxins were separated in a Microsorb-mv C-18 reverse phase column using water-methanol-acetonitrile (700:150:150 v/v) as mobile phase at a flow rate of 1 ml/min. The fluorescence detector was set with operating conditions of 360 nm and 440 nm excitation and emission wavelengths, respectively.

3.3.4 Preparation of [14C]-Labeled Aflatoxin B_1 from Acetate-1,2-[14C]

The preparation of [14C]-labeled aflatoxin B_1 was performed by following and combining the procedures described by Adye and Mateles (1964); Detroy and Ciegler (1971); Ayres *et al*. (1971); Jackson and Ciegler (1972); Mabee *et al*. (1973); Schoenhard, *et al*., (1973); and Floyd and Bennet (1981).

Primary culture stock solution containing each of the following per liter was prepared: glucose (50 g), ammonium sulfate (3 g), potassium dihydrogen phosphate (10 g), magnesium sulfate heptahydrate (2 g), sodium tertaborate decahydrate (0.7 mg),

ammonium molybdate tetrahydrate (0.5 mg), ferric sulfate hydrate (8.2 mg), cupric sulfate pentahydrate (0.3 mg), manganese sulfate monohydrate (0.11 g), zinc sulfate heptahydrate (17.6 mg), and HPLC grade distilled water (1 L).

Two Fernbach flasks each containing 500 ml of the primary stock solution were loosely capped with gauze-wrapped cotton plugs, covered with aluminum foil, and sterilized for 15 min at 121°C and 15 psi. After cooling, 0.1 ml of *A. flavus* (A53, C50Aa) conidial suspension (4.1 X 10^8 spores/ml) obtained from Dr. Kenneth Damann's laboratory (Department of Plant Pathology and Crop Physiology, LSU) was inoculated into each flask. The flasks were incubated in a 30°C rotary shaker water bath at 144 rpm for the first 24 hrs and at 200 rpm for the next 24 hours. After 2 days, the mycelial growth was filtered through sterile cheesecloth and washed with sterile distilled water. The collected mycelia were transferred into a sterilized Waring blender jar. One hundred ml sterilized distilled water was added and blended for 10 sec. The suspension was re-filtered using fresh sterile cheesecloth and rinsed with distilled water.

The collected mold pellets were carefully transferred into a rubber-stoppered Fernback flask containing 500 ml of sterilized resting culture stock solution prepared per liter with the same amount of salts and minerals that were used to prepare the primary culture. The only difference is the amount of glucose added. For the resting culture 3.6 g of glucose was used. The rubber stopper was outfitted with two rubber tubes; one tube was from a positive pressure diaphragm-type aquarium pump and the other tube was to a CO_2 trap used to collect CO_2 produced by the culture. The CO_2 scrubber was prepared by mixing calcium hydroxide (~75%), water (~20%), sodium hydroxide (~3%), and potassium hydroxide (~2%) (http://en.wikipedia.org/wiki/soda_lime). One mCi of

sodium acetate-1,2-[14C] with a specific activity of 100-120 mCi/mmol (Moravek) was dissolved in 1 ml methanol and added to the resting culture. The flask was incubated between 24 and 48 hours in a 30°C rotary shaker water bath at 200 rpm.

After incubation, mycelia were filtered out under vacuum through cheesecloth in a Buchner funnel fitted to a 1 L filtration flask. The mycelial pellets were rinsed slowly with 200 ml chloroform to extract residual aflatoxins. The filtrate and the chloroform extract were both transferred into a 2 L glass separatory funnel. The mixture was extracted three times with 500 ml chloroform to separate the aflatoxin from the aqueous portion. The chloroform portion was dried by passing through a bed of anhydrous sodium sulfate prior to evaporating to dryness in a vacuum by rotary evaporator. Residual solids were re-dissolved in 10 ml chloroform and transferred into a 350 ml-capacity glass chromatography column packed with silica gel in chloroform. The labeled material in the column was eluted with 1 L chloroform-methanol (98:2) at a flow rate of 1 ml/min. The eluate was evaporated to dryness by vacuum rotary evaporation. The residue was re-dissolved in chloroform, transferred into a scintillation vial, and evaporated to dryness under stream of nitrogen gas. The dry materials were reconstituted with 1 ml benzene-acetonitrile (98:2) and spotted on preparative silica gel and/or general purpose silica gel plates. The plates were developed with chloroform-acetone (9:1) and viewed in a UV cabinet. The region where AFB_1 was present was marked, scraped off, and transferred onto a chromatography column packed with 5-10 g anhydrous sodium sulfate. The [14C]-labeled AFB_1 was eluted from the silica gel with 1 L chloroform-methanol (98:2) and the subsequent eluate was dried by rotary

evaporation. These procedures were exhaustive and repeated numerous times to ensure high purity of the labeled material.

The chemical purity and concentration of the labeled AFB_1 was determined by spectrophotometry as described in AOAC Official Method 971.22 (2005). Briefly, dried residue of [14C]-AFB_1 was dissolved in benzene-acetonitrile (98:2) and transferred into a 10-ml capacity glass-stoppered volumetric flask. An aliquot of the stock solution was transferred into a quartz cuvette and the UV spectrum recorded from 200 to 500 nm using a Genesys 21 spectrophotometer. The concentration of aflatoxin B_1 was determined by measuring absorbance (*A*) at wavelength of maximum absorption close to 350 nm and calculated with the following equation:

$$\text{Concentration, } \mu g/ml = \frac{A \times MW \times 1000}{\varepsilon}$$

where MW is the molecular weight of AFB_1 (312 g/mole) and ε is the molecular absorptivity of AFB_1 (19800) in benzene-acetonitrile (98+2). (AOAC Official Method 971.22).

The specific activity of [14C]-AFB_1 was measured with a Beckman LS 6000 Liquid Scintillation Counter and/or Packard (Perkin-Elmer) Tri-Carb 2900TR Liquid Scintillation Counting System. Briefly, 200 µl and 400 µl aliquots of stock solution were transferred into glass scintillation vials. Fifteen ml of toluene-based scintillation liquid (PPO 100 g/L + POPOP 1.25 g/L in toluene, Sigma) (PPO, 2,5-diphenyloxazole;

POPOP, 1,4-di-(2-(5-phenyloxazolyl))benzene) was added and specific activity was counted for 1 min.

3.3.5 Spiking of Contaminated Corn with [14C]-Aflatoxin B_1

The stock solution of [14C]-AFB_1 was dried under a stream of nitrogen and re-dissolved in 10 ml of methanol. The whole amount was distributed into 3.7 kg of aflatoxin-contaminated corn using a 1-ml glass syringe and was air-dried under the fumehood to remove residual methanol. After air-drying, the spiked corn was mixed overnight with a mechanical mixer/tumbler to ensure equal distribution of [14C]-labeled AFB_1. Homogeneity was checked by taking six 10-g portions randomly from the lot followed by the Mycosep extraction and purification method. One-ml each of the collected extracts was transferred into a scintillation vial, mixed with ca. 15 ml of Hionic Fluor (Packard, The Netherlands), a scintillation cocktail suitable for aqueous and non-aqueous solutions, and total [14C] specific activity was counted after the disappearance of chemiluminiscence.

3.3.6 Ozonation of [14C]-AFB_1 Labeled Corn

Radiolabeled corn samples were divided into two portions. Of these, 1.2 kg served as non-ozonated control and 2.5 kg served as ozone-treated sample. Corn sample for ozonation was placed in a 10-gallon carboy container fitted with two ¼“ Teflon tubes. Ozonation was performed with an ozone generator (Lynntech, Inc. College Station, Texas). Ozone gas (9-10 wt %) was flowed in from the bottom of the container at approximately 150 ml/min. Corn samples were treated for 96 hours with mixing occurring every 12 hours. After treatment, the ozonated corn was air-dried and ground using a coffee grinder and was passed through a No. 20 mesh sieve. A coffee

grinder was used to avoid radioactive contamination of the Brinkmann mill. Three 50-g test portions were taken randomly from the entire lot for aflatoxin determination. The remaining ground samples were divided into five 400-g portions for the fractionation study. The excess portion was kept for aflatoxin content determination and for other analyses. Untreated corn sample was ground as well and divided into three 400-g portions. Two portions were used for the fractionation study while the remaining portion was used for aflatoxin content determination (Multifunctional column method) and for other analyses. All samples were transferred into clean HDPE centrifuge bottles, labeled and were stored at ~4°C until further analysis.

3.3.7 Fractionation of Ozonated Corn

3.3.7.1 Dichloromethane Extraction

Four hundred grams of ground corn sample were extracted with dichloromethane (CH_2Cl_2) using a 1:5 (w/v) ratio. The mixture was shaken for 30 min using a modified water bath shaker and filtered using a Buchner funnel with Whatman No.1 filter paper under vacuum. The extract was concentrated to about 500 ml (volume recorded) by rotary evaporation and stored at ~4°C until further analysis. The residue was air-dried overnight in a chemical fumehood to remove residual solvent and weighed.

3.3.7.2 Methanol Extraction

Three hundred grams of the corn meal remaining after dichloromethane extraction was extracted with methanol (1:5 w/v). The rest of the residue from dichloromethane was kept for aflatoxin content determination and for other analysis. The mixture was shaken for 30 min using a modified water bath shaker and filtered using a Buchner funnel with Whatman No.1 filter paper under vacuum. The residue

was air-dried and the weight recorded. The methanol extract was concentrated by rotary evaporation and the volume was adjusted to 500 ml. A 25-ml aliquot was transferred to a pre-weighed glass vial (25-ml) and evaporated to dryness under a stream of nitrogen gas. The remaining extract was stored until further analysis. After drying, the weight of the dried material was recorded and samples were stored at ~4°C until further analysis.

3.3.7.3 Acetone-Hexane Partition

A 50-ml aliquot of methanol extract was transferred to a separatory funnel. Fifty ml acetone-water (3:7), 100 ml dichloromethane, and 40 ml of methanol were added to the separatory funnel, shaken, and allowed to equilibrate. The aqueous phase (upper layer) was removed and transferred into another separatory funnel. Fifty ml of acetone was added into the aqueous phase, shaken, and filtered under gravity with Whatman No. 1 filter paper. The filtrate was evaporated to dryness by rotary evaporation. Dry film of the extract was first extracted three times with 10 ml acetone and the acetone soluble extracts was transferred into a pre-weighed vial. Subsequently, material remaining in the flask that was not dissolved by acetone was extracted three times with 10 ml methanol–water (98:2) and transferred into pre-weighed vial. Both extracts were evaporated to dryness under a stream of nitrogen gas and their weights were recorded.

The organic phase (lower layer) from the first separatory funnel was concentrated to ca. 20 ml by rotary evaporation. One hundred ml of hexane was added and the solution was mixed and filtered. The filtrate was evaporated to dryness, transferred with hexane to a pre-weighed vial, and dried under a stream of nitrogen gas. The precipitate, if present, was air-dried in a chemical hood and then oven-dried

overnight at 60°C. Weights of corresponding soluble fractions were recorded and samples were stored at ~4°C until further analysis.

3.3.7.4 Acid and Base Treatment

Following methanol extraction, a 50 g portion of the residue was transferred to a 500-ml cap centrifuge bottle. Two hundred ml of 0.1 N acetic acid was added and the mixture was placed in a water bath for 2 hours at 90°C. An additional 200 ml 0.1 N acetic acid was added and the mixture was kept in the water bath for another hour. After incubation, the mixture was centrifuged at 10,000X *g* for 30 minutes. The supernatant was poured into pre-weighed 150 mm x 20 mm glass Petri dishes and kept overnight in a -80°C freezer. The residue from the acid treatment was exposed to an alkaline treatment by adding 200 ml of 0.2 N NaOH and shaken vigorously. The pH was adjusted, when necessary, to ca. 10~11 by the addition of 0.2 N NaOH. One hundred ml of distilled water was added into the mixture to make a smooth slurry. The mixture was shaken and then centrifuged at 10,000X g for 30 min. The supernatant liquid was transferred into pre-weighed 150 mm x 20 mm glass Petri dishes and kept overnight in a -80°C freezer. The residue was transferred into a glass beaker, oven-dried at 60°C, and weighed. After freezing, both acid and base extracts were lyophilized and their weights were recorded.

3.4.7.5 Enzymatic Digestion

After methanol extraction, 25 g of residual meal was subjected to enzymatic digestion using Pronase E (Sigma Chemical Co., St. Louis, MO) according to the procedure described by Park *et al*. (1981 and 1984). One hundred mg Pronase E was mixed with 200 ml water to form a slurry (pH = 7.0) and held at 37°C for 2 hours.

Twenty-five grams of the residual meal was added and the mixture was incubated at 37°C for 24 hours with periodic shaking. After digestion, the aqueous soluble portion and precipitate were separated by vacuum filtration. The precipitate was transferred into a glass beaker and dried in an oven at 82°C. One hundred ml dichloromethane was added to the precipitate, shaken, and filtered. The filtrate was evaporated to almost dryness by rotary evaporation, transferred into a pre-weighed glass scintillation vial, and evaporated to dryness under a stream of nitrogen gas. The residue after dichloromethane extraction was transferred into a pre-weighed glass beaker then dried in an oven and weighed. The aqueous portion from the vacuum filtration step was transferred into a 250 ml capacity separatory funnel and partitioned with 100 ml dichloromethane to yield aqueous/organic phases. The aqueous portion was collected, transferred into glass Petri dishes, and kept overnight in a -80°C freezer. After freezing, the extract was dried by lyophilization and the residue was weighed.

The organic portion was evaporated to almost dryness under vacuum by rotary evaporation. The dried material was re-dissolved with ~20 ml dichloromethane and carefully transferred into a pre-weighed glass scintillation vial then evaporated to dryness under a stream of nitrogen gas. Weights of the dried materials from both portions were recorded and extracts were kept at ~4°C until further analysis.

3.3.7.6 Radioactivity Measurements

Radioactivity of various extracts and residues from non-ozonated and ozonated corn was measured by Liquid Scintillation Spectrometry using a Packard (Perkin-Elmer) Tri-Carb 2900TR Liquid Scintillation Counting System located in the Department of Environmental Quality Bldg., Baton Rouge, LA.

Dry films of extracts collected from various steps in the fractionation procedure were re-dissolved with appropriate solvents, i.e. dry materials collected from methanol extraction were re-dissolved in methanol. An aliquot of either 100 µl or 200 µl from each extract was transferred into a glass scintillation vial and counted for radioactivity using 15 ml Hionic Fluor. For solid samples, a modified method was developed to prepare the sample for radioactivity determination. This method was based on procedures described by Porter (1980), Fuschs and De Vries (1985), and Smith and Lang (1987) as noted by Thomson and Burns (1996). Briefly, test portions weighing ca. 200 mg were transferred into glass scintillation vials. Five hundred µl of sodium hypochlorite (NaOCl, 10-15% available chlorine, Sigma) was added and swirled gently to wet the sample completely. The vial was capped tightly and placed in a 60°C water bath to incubate for 1-2 hours. This digestion step solubilized and partially decolorized the sample. Additional 500 µl of NaOCl was added and the vial was returned in the water bath to incubate for another hour to further decolorize the sample. Completeness of digestion was indicated by removal of pigmentation and/or when the solution became clear. After incubation, the vial was cooled down at room temperature and vented under fumehood. Remaining chlorine was blown out with a gentle stream of nitrogen gas or air. Fifteen ml of Hionic fluor was added into the mixture and thoroughly mixed with a Vortex machine. The vial was kept in the dark at room temperature. This allowed the solution to adapt to dark condition (exposure to light excites the fluor in the solution) and temperature before counting thereby minimizing problems associated with chemiluminiscence.

CHAPTER 4. RESULTS

4.1 Study 1: Distribution of Aflatoxin in Ozonated and Non-ozonated Corn

4.1.1 Aflatoxin Content in Corn Samples

Results of the HPLC analysis showed that aflatoxin B_1 and B_2 were present in all contaminated samples except for Batch 3. Table 4.1 summarizes the amount of aflatoxins in each batch. Thin layer chromatographic analysis of samples from Batch 3 showed the presence of aflatoxin, however, further analysis using HPLC did not show the presence of aflatoxins.

4.1.2 Sequential Fractionation of Corn

Table 4.2 summarizes the results of the sequential fractionation procedure of corn samples from Batch 1. The presence or absence of residual aflatoxins was evaluated by thin layer chromatography.

4.1.2.1 Dichloromethane Extract

Extracts were diluted with 5 ml of dichloromethane. Ten and 20 µL of each extract were spotted on the TLC plate. Ten, 20 and 30 µL of mixed standard were also spotted as a reference. After development, the presence of a very intense blue fluorescent spot/band was observed in untreated contaminated samples. These spots had Rf's close to that of the reference standard. A faint blue fluorescent band was also observed in treated contaminated corn. The intensity of the spots was less than those of the standard. No blue fluorescent spots/bands were observed in untreated clean and treated clean corns. The presence of the blue fluorescent spots/bands indicated the presence of aflatoxin in the sample.

Table 4.1. Aflatoxin content in corn samples.

Corn	AFB_1 (ppb)	AFB_2 (ppb)
Batch 1	644	38
Batch 2	140-143	23-25
Batch 3	ND	ND
Batch 4	572	58
Batch 5	8151	871

Table 4.2. Presence of residual aflatoxin B_1 and aflatoxin B_2 in fractions collected from the 1st batch of corn samples.

Extract	Corn Samples							
	Clean Treated		Clean Untreated		Contaminated Treated		Contaminated Untreated	
	AFB_1	AFB_2	AFB_1	AFB_2	AFB_1	AFB_2	AFB_1	AFB_2
Dichloromethane	-	-	-	-	+	+	+	+
Methanol	-	-	-	-	+	+	+	+
Acetone	-	-	-	-	+	+	+	+
Pronase Soluble	-	-	-	-	+	+	+	+
Pronase Organic	-	-	-	-	+	+	+	+
Hexanes	-	-	-	-	-	-	-	-

4.1.2.2 Methanol Extract

Twenty mL of methanol extract from each treatment was transferred into a scintillation vial and evaporated to dryness under a stream of nitrogen. The dried extract was re-dissolved with 2 mL of methanol. Ten µL of each extract was spotted on the TLC plate and developed first with petroleum ether and then with ether-methanol-water (96:3:1). Results showed the presence of numerous fluorescent bands in all of the samples. Bands were observed between the origin and AFG_2, between AFB_1 and AFG_2, and between AFB_1 and solvent front. A very intense blue fluorescent spot with an Rf close to that of AFB1 was observed in untreated contaminated corn extract. A less intense blue spot with an Rf close to that of AFB_1 was also observed in treated contaminated corn extract.

4.1.2.3 Acetone Extract

Acetone extracts were diluted with 5 ml acetone. Twenty µL of the extract and 10 µL of mixed aflatoxins standard were spotted on the plate. The plate was developed first with petroleum ether and then with ether-methanol-water (96:3:1). Results showed the presence of a faint blue fluorescent band in untreated contaminated corn and treated contaminated corn extracts. No fluorescent band was observed in both the treated and untreated clean corn. Fifty µL of extracts from untreated and treated contaminated corn were re-spotted to confirm the presence of AFB_1. Results showed very intense blue fluorescent spots with Rf values close to that of standard AFB_1 in untreated contaminated corn extracts. For the treated contaminated corn, the intensity of the blue fluorescence did not change.

4.1.2.4 Hexane Extract

Extracts were diluted with 1 mL hexane. Twenty µL of the extract and 10 µL of mixed aflatoxins standard were spotted on the plate. After development with petroleum ether and ether-methanol water (96:3:1), no fluorescent spots/bands were observed in all sample extracts.

4.1.2.5 Pronase Soluble Solid Fraction

Sample extracts were diluted with dichloromethane to give a final concentration of 10,000 µg/ml. Ten µL each of the extracts was spotted on two separate TLC plates. Ten µL of mixed standard was spotted as an external standard. Plates were first developed with petroleum ether until it reached the top edge of the plate to elute oil and non-polar compounds. One plate was developed with ether-methanol-water (96:3:1) and the other plate with chloroform-acetone-water (88:12:1.5). Results of the first plate showed that the Rf's for AFB_1, AFB_2, AFG_1 and AFG_2 were 0.88, 0.77, 0.68, and 0.55. Blue fluorescent spots with an Rf of 0.88 were observed in extracts from untreated contaminated corn indicating the presence of AFB_1. No fluorescent spots were observed for other samples. Yellowish streaks were observed in the paths of all samples. This could be due to the pigment of corn that was extracted by dichloromethane. For the second plate, the Rf's were 0.81, 0.76, 0.71 and 0.67 for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. A blue fluorescent spot with an Rf of 0.86 was observed in untreated contaminated corn extract which was similar to AFB_1. For treated contaminated corn, a faint blue fluorescent spot was observed that had an Rf close to that of standard AFB_1. No blue fluorescent spots were observed for treated

and untreated clean corn. The Rf was greater than the standard due to uneven solvent migration.

4.1.2.6 Pronase Soluble-Organic Fraction

Trial 1. The same procedure as above was done except that only ether-methanol-water was used as developing solvent. Ten µL each of the extracts and standard were spotted on the TLC plate. The plate was developed with petroleum ether and ether-methanol-water. Results showed that Rf's for AFB_1, AFB_2, AFG_1, and AFG_2 were 0.92, 0.82, 0.74 and 0.6, respectively A faint blue fluorescent spot with an Rf similar to that of AFB_1 was observed in untreated contaminated corn but none were observed in other samples.

Trial 2. The same procedure as above was followed but the amount of sample spotted was increased to 20 µL. Results showed that Rf's were 0.82, 0.72, 0.64 and 0.51 for AFB1, B2, G1 and G2, respectively. Blue fluorescent spots with Rf's of 0.85 and 0.75 were observed in extracts from untreated contaminated corn. The intensities of the spots were similar with that of the standard. Similar results were observed for ozonated contaminated corn. Faint blue spots similar to the Rf's of AFB_1 and AFB_2 were observed for ozonated contaminated corn revealing the presence of residual aflatoxins. No fluorescent spots were observed in both ozone-treated and non-treated clean corn samples.

Trial 3. The same procedure was followed. Twenty µL of samples and 10 µL of standard were spotted on the plates. The plate was first developed with petroleum ether then with ether-methanol-water. Results showed that Rf's for the standard were 0.75, 0.66, 0.58, and 0.49 for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. Yellow

streaks were observed in all of the samples. Dark, yellowish spots were also observed between Rf 0.23 and 0.34 in all of the samples. These were not investigated further since they were present in all corn sample extracts. Blue fluorescent spots with Rf of 0.75 and 0.66 were observed in untreated contaminated corn extract indicating the presence of aflatoxin B_1 and B_2. The aflatoxin B_1 in sample was more intense than the standard while the B_2 was less intense compared to that of the standard. For contaminated treated corn, faint blue fluorescent spots were observed with Rf's close to those observed from untreated contaminated corn indicating presence of residual aflatoxins.

The results of these experiments supported the findings from the previous work of Prudente (2001). The presence and absence of aflatoxin(s) in the extracts supported the observations in the Ames mutagenicity assay in which extracts from methanol and acetone showed slight mutagenic potentials against TA 98. On the contrary, fraction from dichloromethane did not show mutagenic potential from the previous study even though residual aflatoxin was found present in the current study. This could be due to the presence of materials in corn that interfered with the mutagenicity assay. On the other hand, hexane portions showed slight mutagenic potential in the previous study although no residual aflatoxin was observed in the present study. This result suggested the possible formation of product that is not related to aflatoxin that has mutagenic potential. Noteworthy is the result for the pronase soluble and organic fractions. Prudente (2001) showed that extracts after enzymatic digestion followed by dichloromethane extraction showed strong mutagenic potential against tester strain TA 98. Results of the current study showed a positive correlation between the presence of

residual aflatoxin in the extract and the mutagenic response of tester strains in the Ames assay. It was not possible to determine if ozone-aflatoxin reaction products were formed due to matrix interferences and lack of purified, concentrated products. Therefore a model system was used in Study 2.

4.2 Study 2: Evaluation of Ozone and Aflatoxin B_1 Reaction Products in a Model System

Trial 1. Results showed that AFB_1 was not present in extracts ozonated for 30 seconds or longer. No visible blue fluorescent spots close to the Rf of AFB_1 were observed. Similar results were obtained for the mixed aflatoxins. However, aflatoxins B_2 and G_2 were not affected by ozonation since visible bluish and greenish spots close to the Rf 's of B_2 and G_2 were observed in all extracts. HPLC analysis showed that no peaks were present in all extracts. This may have been due to the small amount of aflatoxins present in the extracts or the small amount of sample injected. It could also be due to HPLC conditions that were used for this particular experiment.

Trial 2. Analysis of dichloromethane extracts showed the presence of AFB_1 after ozonation for 50 sec and AFB_1 was totally degraded after 60 sec. Conversely, analysis of the water portion extracts showed the presence of seven compounds having Rf values of 0, 0.07, 0.07, 0.14, 0.25, 0.39 and 0.5, after ozonation for 60 sec. In comparison, Rf values for AFB_1, B_2, G_1, and G_2 were 0.71, 0.62, 0.56, and 0.46, respectively (Figure 4.1). Results of the study suggested the formation of more polar compounds. Results of MALDI-MS analysis showed the presence of compounds that have higher molecular weights than AFB_1 (Figure 4.2). Mass spectra of water soluble extracts from samples ozonated for 50 and 60 sec showed molecular ion peaks with

molecular weights of ca 475 and 494, respectively (Figures 4.3 and 4.4). On the other hand, extracts from dichloromethane portions showed molecular ion peaks with molecular weights of ca 459 and 439, respectively (Figures 4.5 and 4.6).

Moreover, the mass spectra of dichloromethane portion revealed that aflatoxin B1 at molecular mass of 313 g/mole, was still present after ozonation for 50 seconds and was totally degraded after ozonation for 60 seconds. No residual aflatoxin was detected in the water portions. In addition, it was observed that a compound present in the water portion with a molecular mass of 413 increased in intensity after longer exposure to ozone treatment. Conversely, the same compound which is also present in dichloromethane extract after ozonation for 50 seconds was notably reduced after prolonged exposure to ozone. However, this compound may not be an aflatoxin-related by-product since this was also found in the spectra of pure AFB_1. Nevertheless, the results generated by this study provided additional information that could be used in evaluating the suitability and acceptability of ozonation as a decontamination process.

Trial 3. Results of the TLC analysis of ozonated pure dry standard AFB_1 at different times are shown in Figure 4.7. It was observed that pure AFB_1 was totally degraded after treating with gaseous ozone even just for 10 sec. Results also revealed that another compound was formed that was more polar than AFB_1. The compound was not one of the three other aflatoxins since its Rf value was lower than that of AFG_2. Furthermore, it was also noted that the longer the treatment time, the new compound became more polar based on the decrease in its Rf values. This observation was confirmed when the same samples were re-spotted on another plate and a similar result was achieved (Figure 4.8).

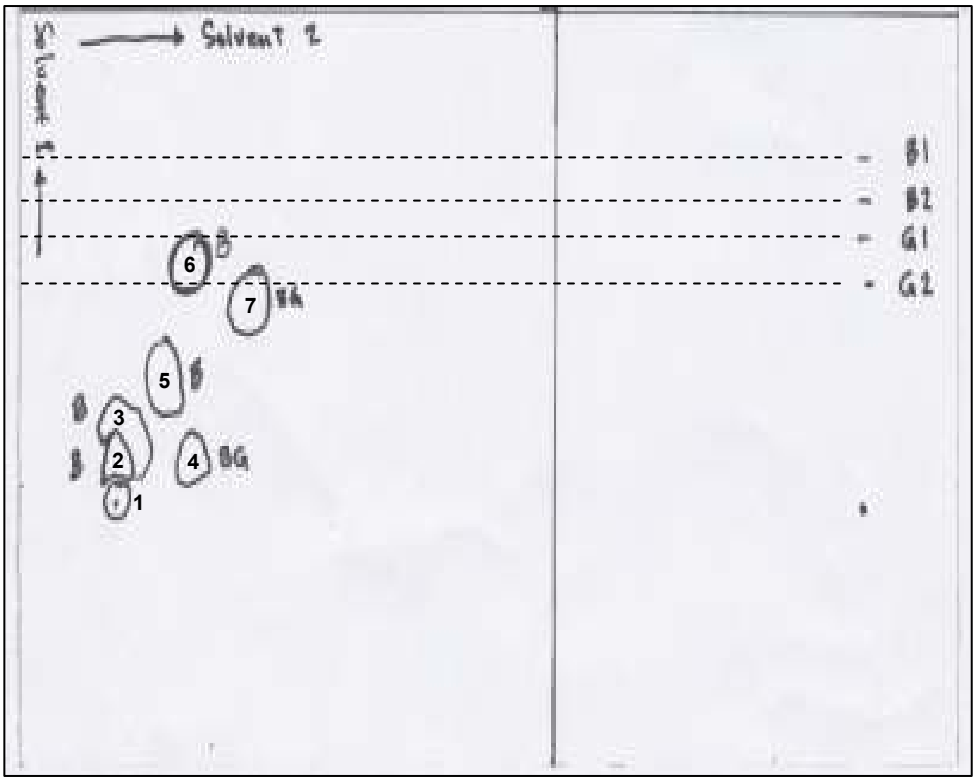


Figure 4.1. Traced image of the two-dimensional thin layer chromatogram of water fraction collected after treating aflatoxin B1 with ozone for 60 seconds. Rf's of AF-B1, B2, G1 and G2 were 0.71, 0.62, 0.56 and 0.46, respectively. Rf's for spots 1 to 7 were 0.0, 0.07, 0.14, 0.07, 0.25, 0.5 and 0.39, respectively.

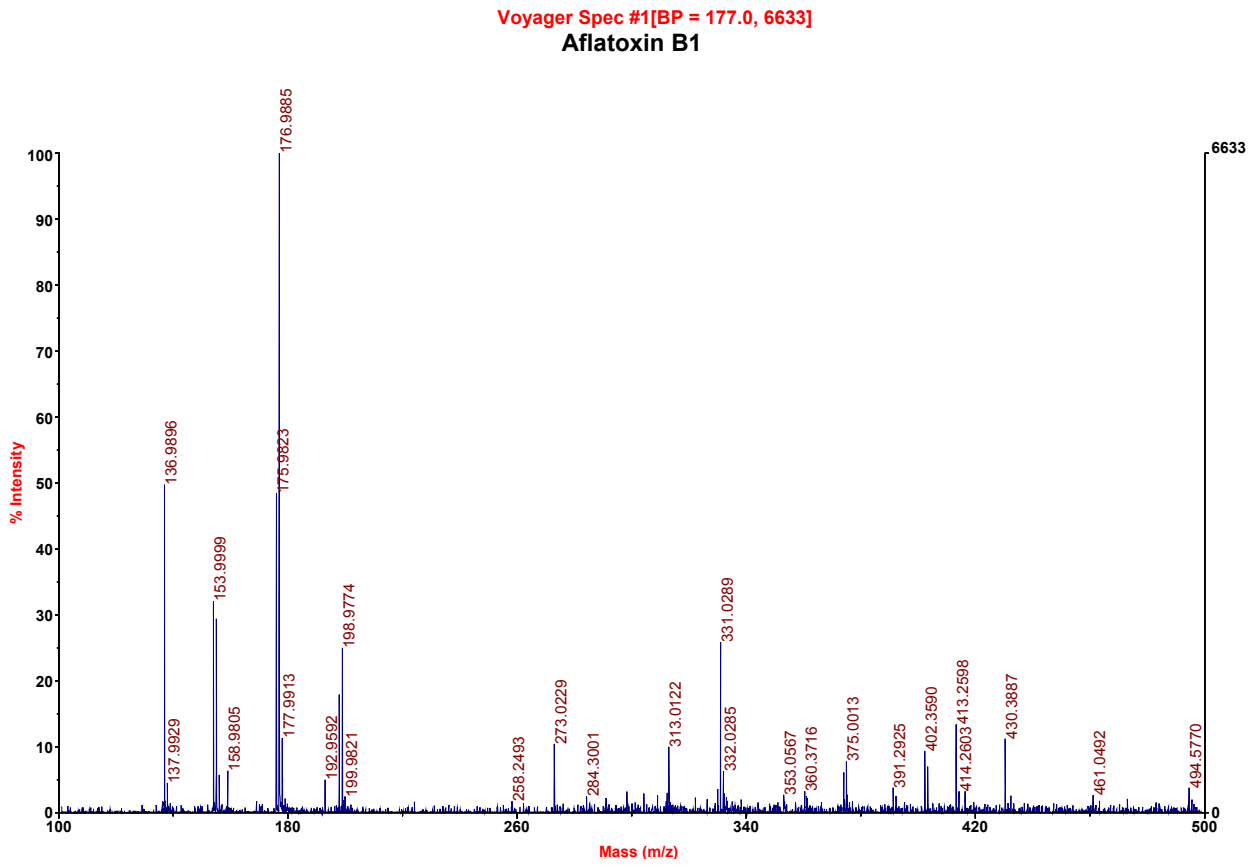


Figure 4.2. MALDI-MS spectra of non-ozonated aflatoxin B_1

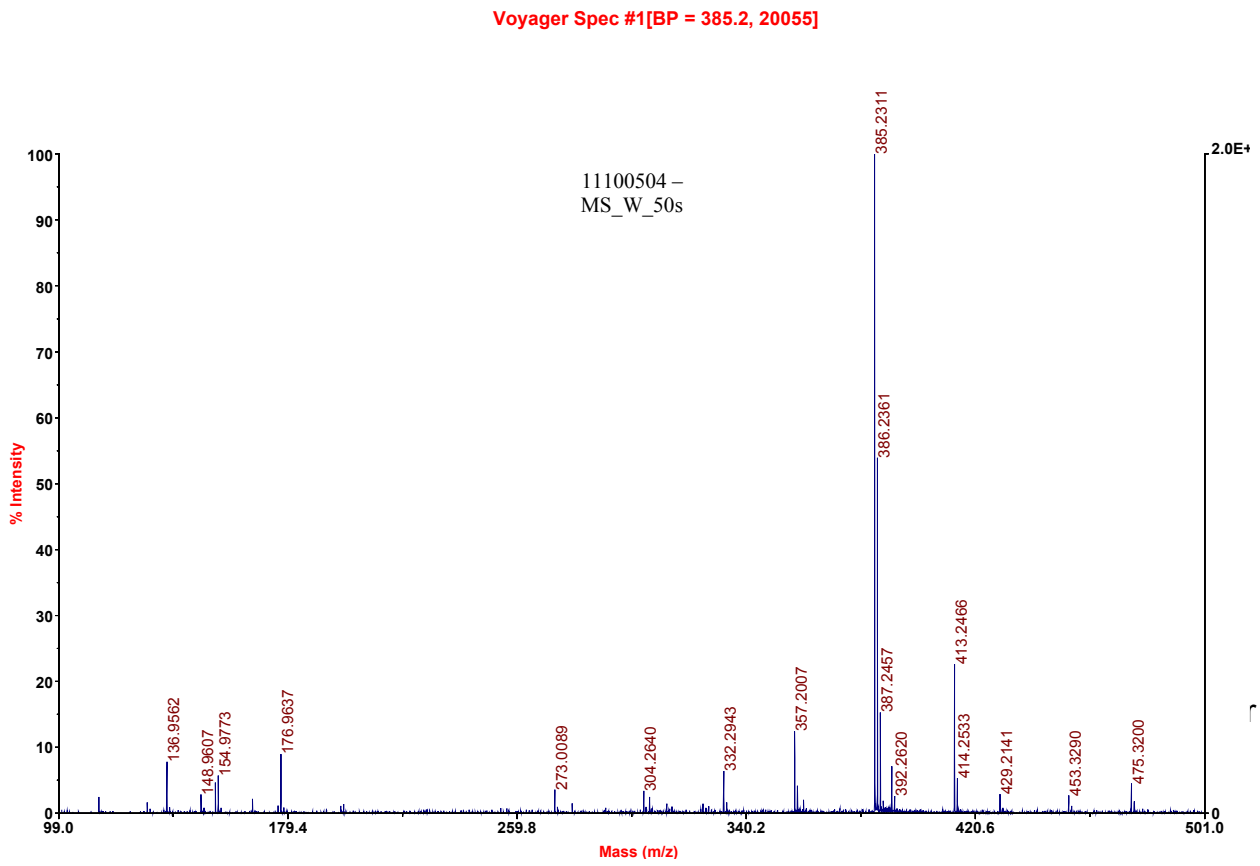


Figure 4.3. MALDI-MS spectra of water fraction collected after ozonation of AFB1 for 50 seconds

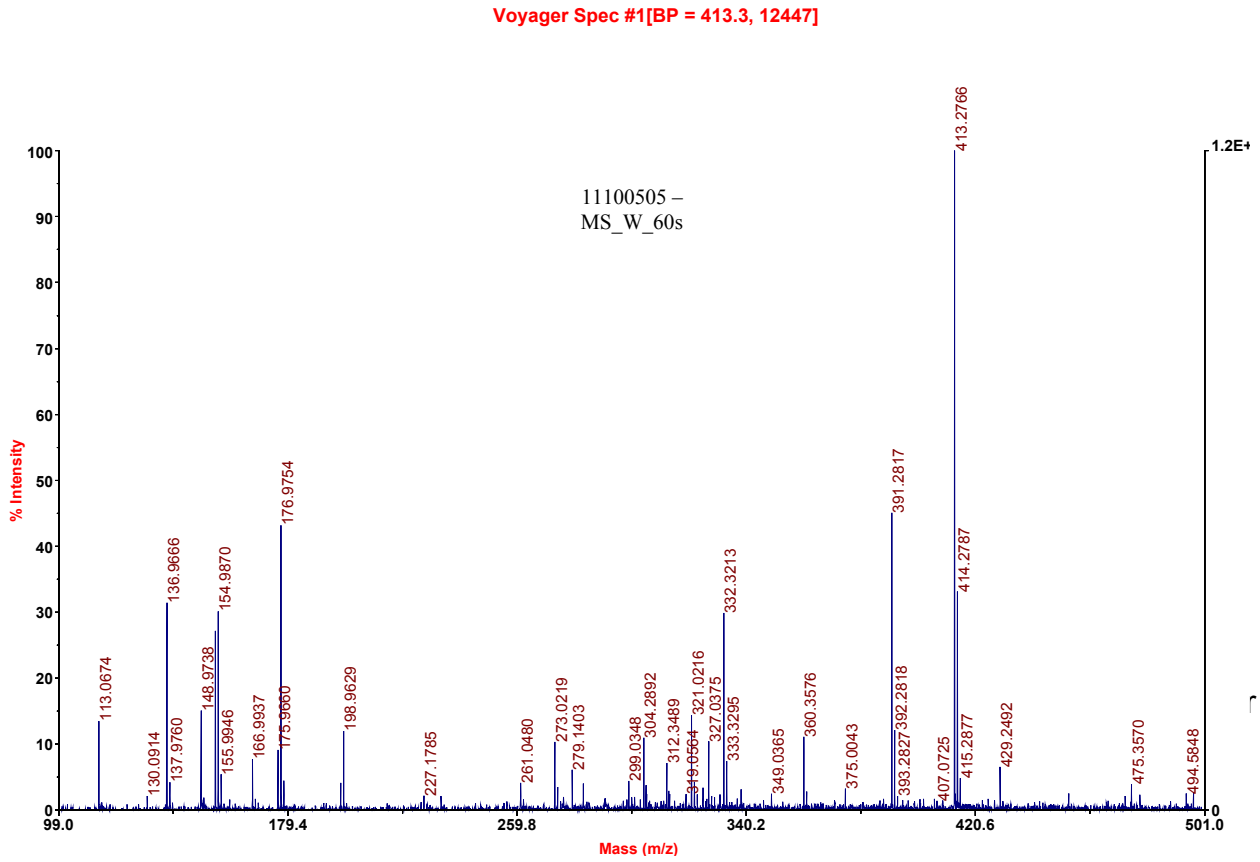


Figure 4.4. MALDI-MS spectra of water fraction collected after ozonation of AFB1 for 60 seconds

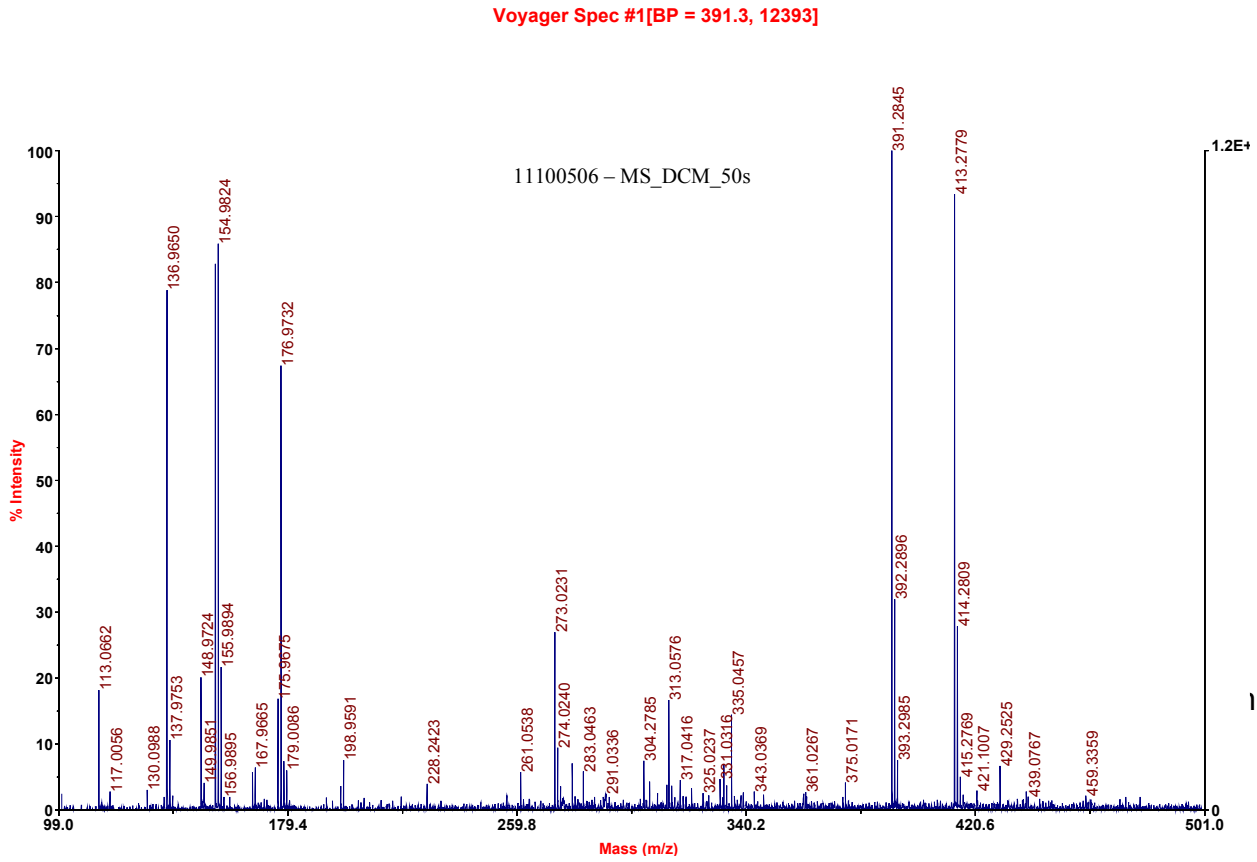


Figure 4.5. MALDI-MS spectra of dichloromethane fraction collected after ozonation of AFB1 for 50 seconds

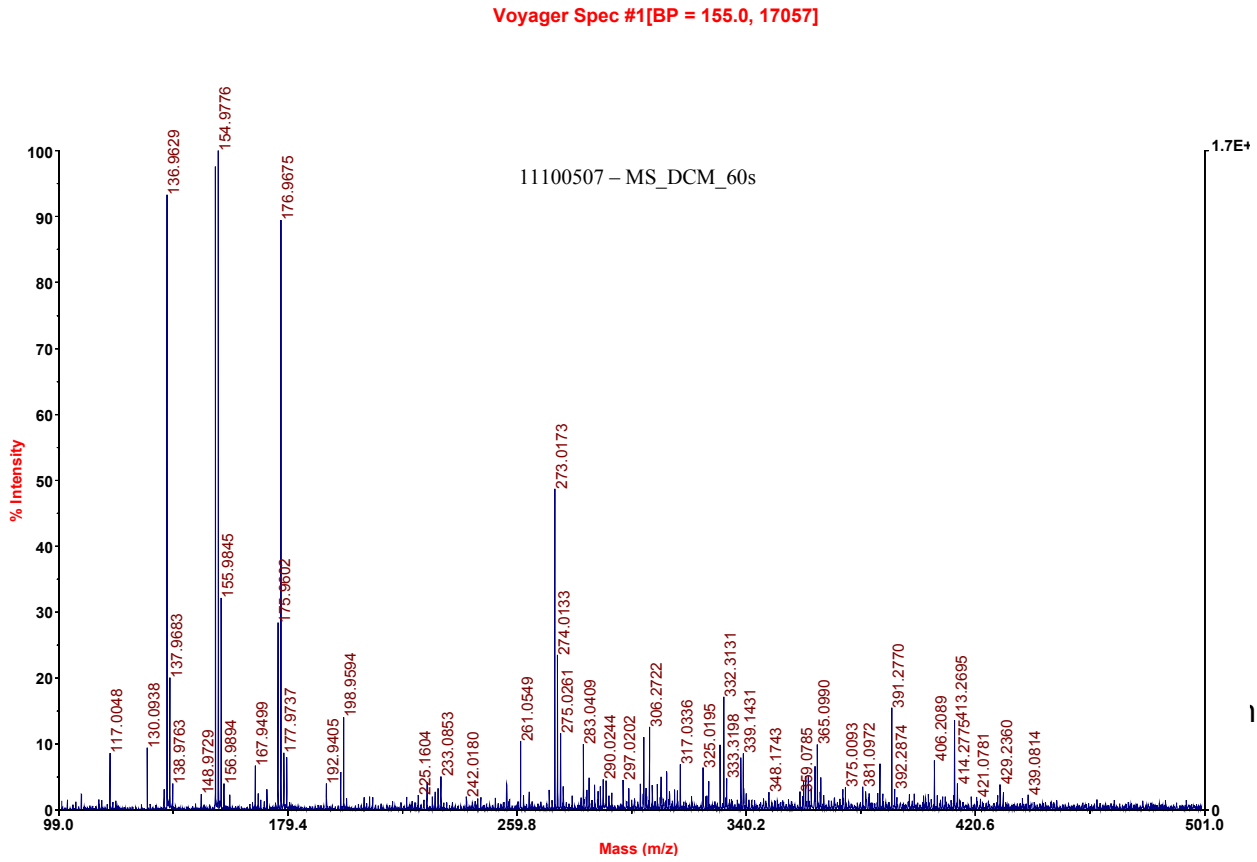
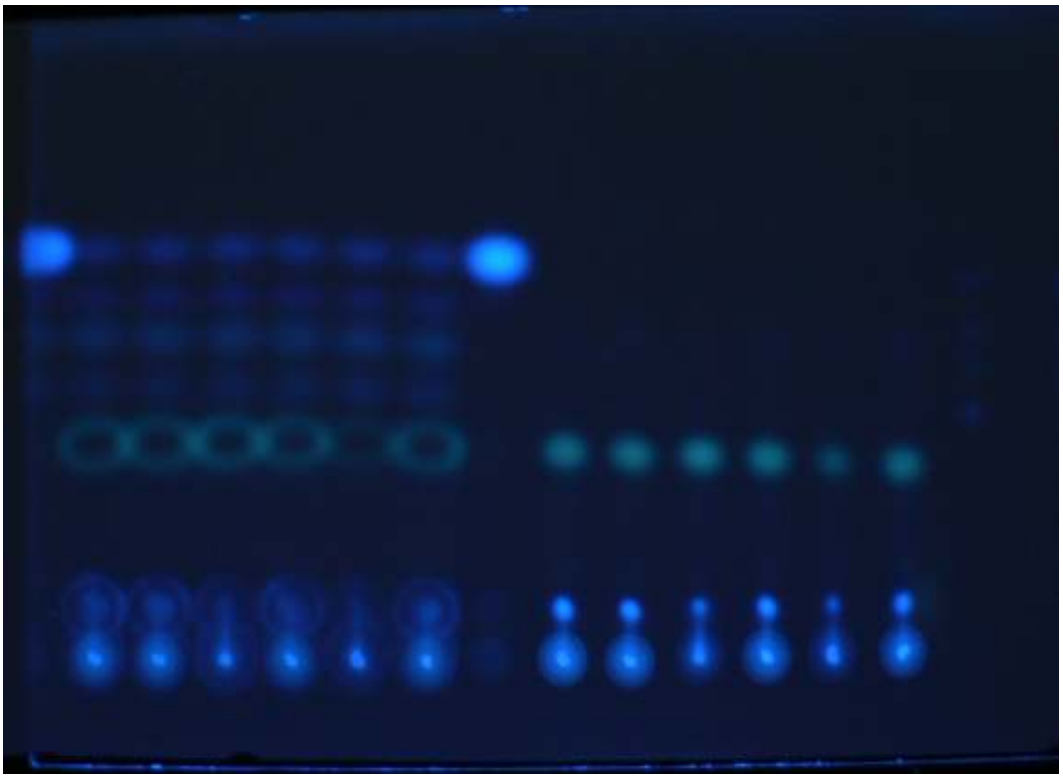


Figure 4.6. MALDI-MS spectra of dichloromethane fraction collected after ozonation of AFB1 for 60 seconds.

To check the purity of this compound, a two-dimensional TLC was performed on samples ozonated after 60 sec. The plate was first developed with ether + methanol + water (96:3:1) and then with chloroform + acetone (9:1). Results revealed the presence of about 8 different spots (Figure 4.9). HPLC analysis of the ozonated samples was performed in an attempt to separate these individual compounds. These compounds could be intermediate degradation products from the reaction of ozone and aflatoxin.

Results of the HPLC analysis confirmed the presence of six peaks with retention times of 1.26, 3.42, 4.19, 6.15, 8.18, and 11.85 minutes (Figure 10). In comparison, HPLC analysis of mixed standard aflatoxins showed retention times of 9.32, 12.43, 14.16, and 19.08 minutes for AFG_2, AFG_1, AFB_2, and AFB_1, respectively (Figure 11). Isolation of individual peaks was attempted using a fraction collector. However, subsequent TLC and HPLC analysis of collected fractions did not show any positive result. This may be due to the small amount of materials collected. No further attempt was made because of the difficulty in concentrating the fraction collected. In addition, numerous TLC and HPLC analyses were conducted to determine if these compounds are present in contaminated treated corn. Materials collected from the sequential fractionation were examined but no positive result was obtained. This could be due to the presence of other materials from corn that interferes with the analysis. It is suggested that further clean-up be conducted on the extracts. Due to lack of purified, concentrated products, Study 3 was implemented using radiolabeled aflatoxin to follow the distribution and confirm the presence of more polar ozone-aflatoxin products in corn.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 4.7. TLC chromatogram of standard AFB1 ozonated at different times and developed with ether+methanol+water (96:3:1): (1) 0 sec + mix standard, (2-7) 10 to 60 sec + mix standard, (8) 0 sec, (9-14) 10 to 60 sec., (15) mixed standard aflatoxins (Rf: B1>B2>G1>G2).

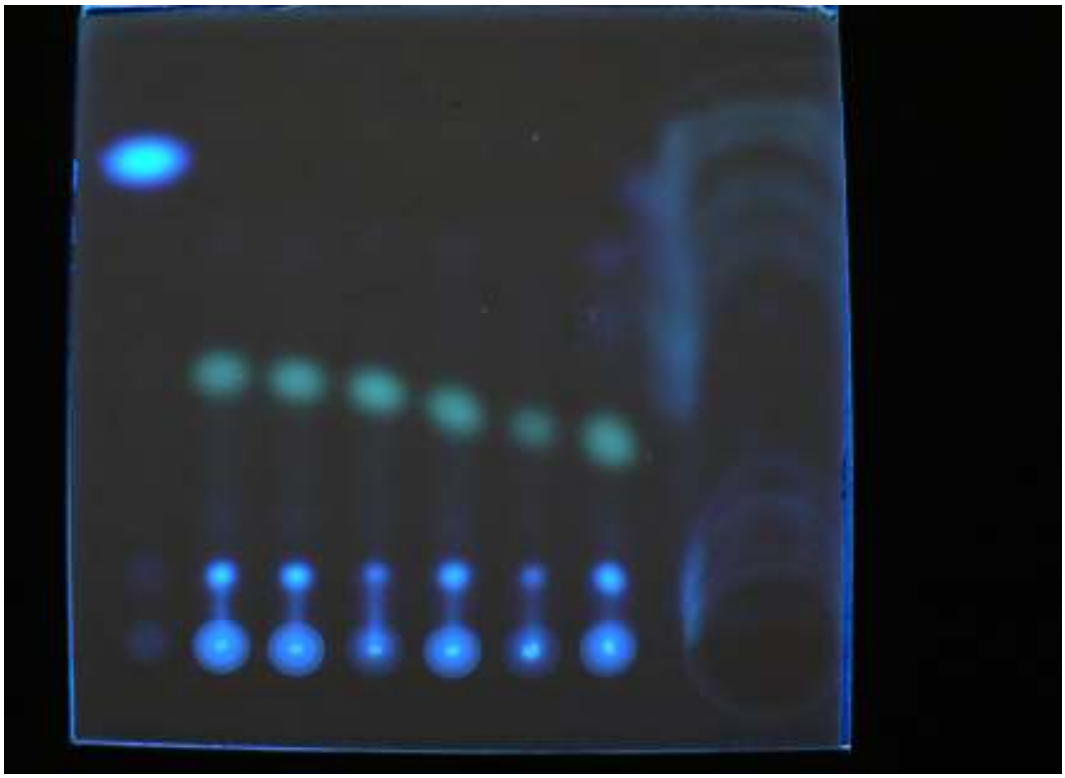


Figure 4.8. TLC chromatogram of standard AFB_1 ozonated at different times and developed with ether+methanol+water (96:3:1): (1) 0 sec, (2-7) 10 to 60 sec, (8) mix standard and (9) standard AFB_1 + trifluoroacetic acid.

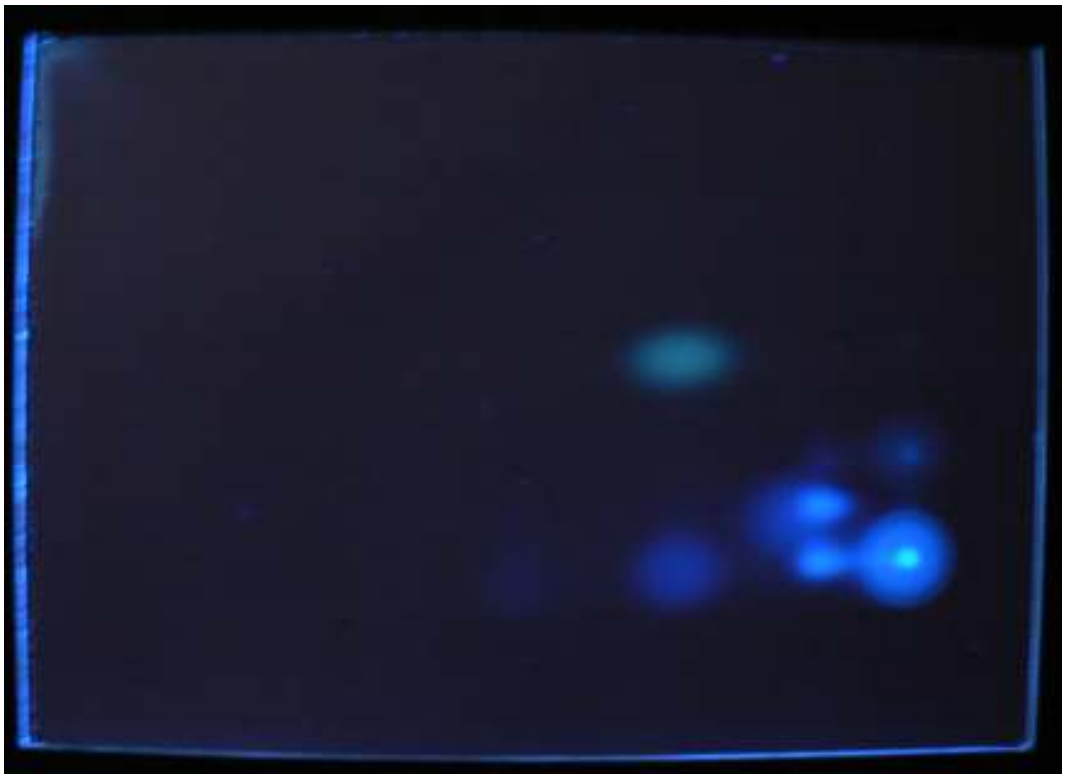


Figure 4.9. Two-dimensional TLC chromatogram of 60-sec ozonated standard AFB_1 developed first with ether+methanol+water (96:3:1) from right to left and with chloroform+methanol (9:1) from bottom to top.

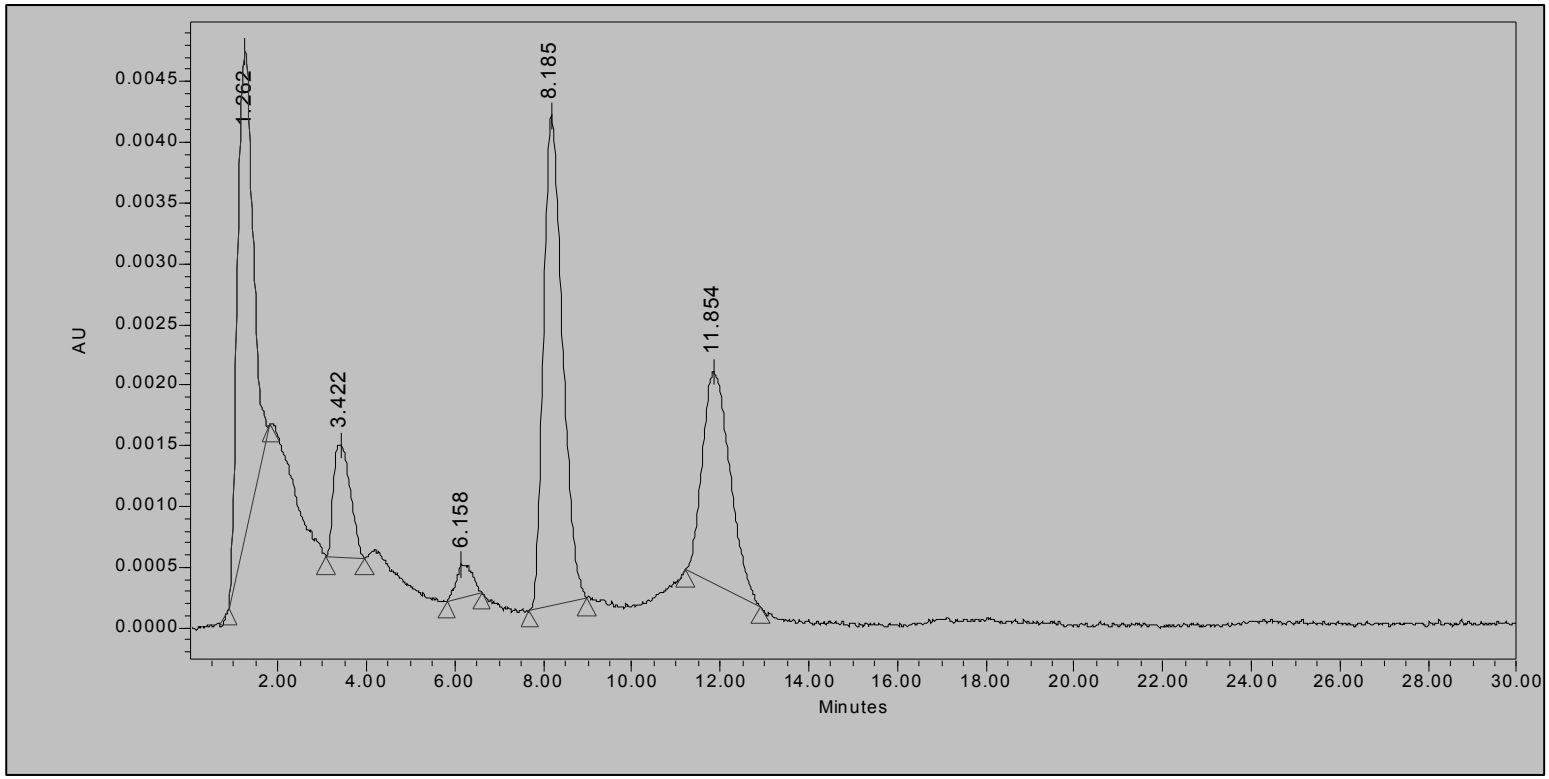


Figure 4.10. RP-High performance liquid chromatogram of pure AFB1 after treating with ozone for 60 seconds using UV detector set at 365 nm.

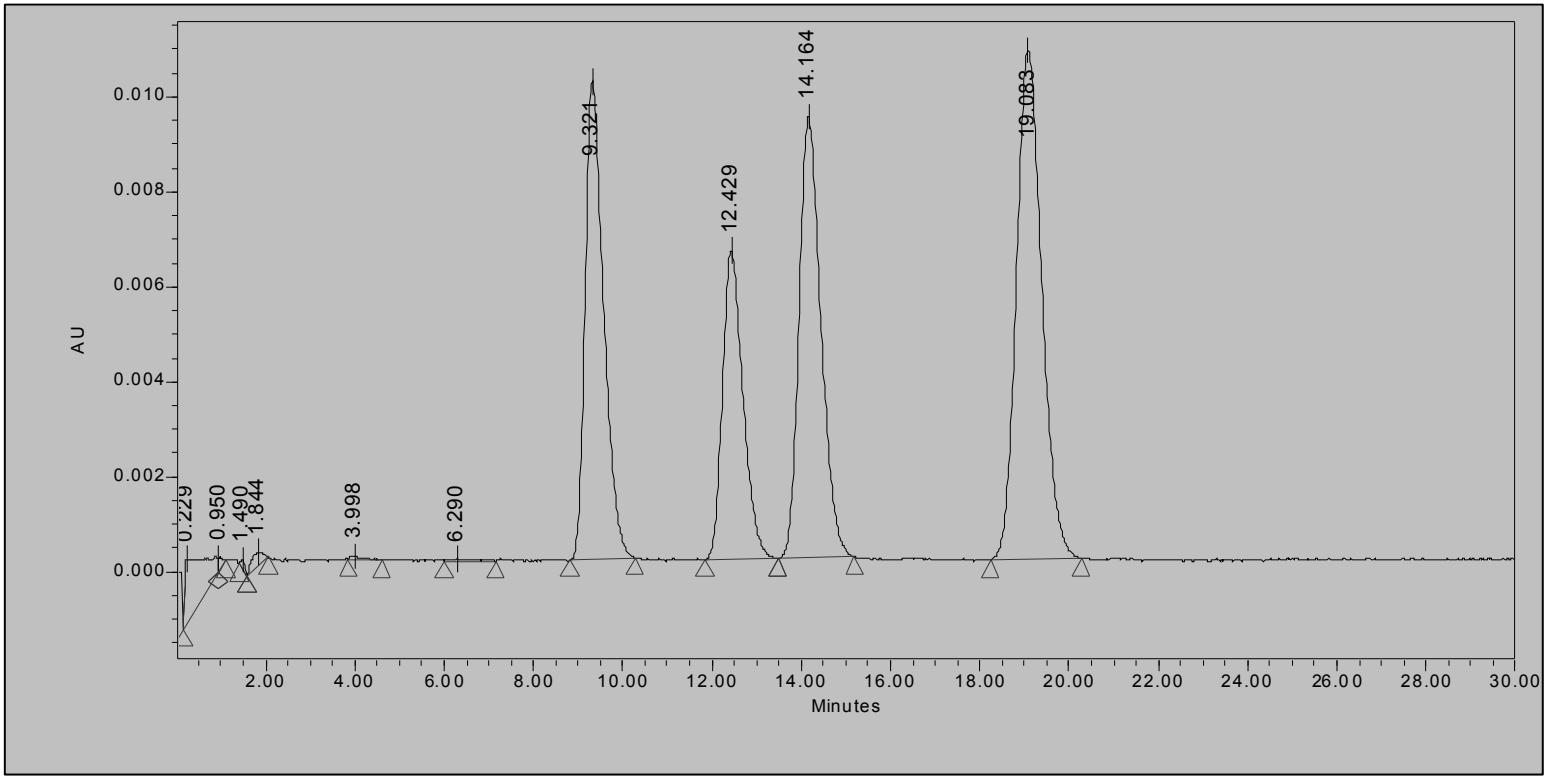


Figure 4.11. RP-High performance liquid chromatogram of non-derivatized standard aflatoxins eluted in the order of AFG2, AFG1, AFB2, and AFB1. UV detector was set at 365 nm.

4.3 Study 3. Distribution of Ozone-Aflatoxin Reaction Products in Corn After Ozonation

4.3.1 Production of Artificially-Contaminated Corn

The inoculation of corn with *A. flavus* spores resulted in the production of heavily contaminated corn kernels (Figure 4.12). The presence of moss green mold/fungal growth was observed all throughout the grains. Although not identified, this mold growth is assumed to be *A. flavus*. Subsequent aflatoxin analysis showed that aflatoxin levels in corn were extremely high. Results of the HPLC analysis showed that corn samples contained 7,452 ppb AFB_1 (n=3) and 704 ppb AFB_2 (n=3) with retention times of about 5.6 and 13.1 min, respectively (Table 4.3). The presence of aflatoxins G_1 and G_2 were not observed in samples confirming that *A. flavus* produces mainly AFB_1 and AFB_2 as noted by Pitt (1989). Figures 4.13, 4.14, and 4.15 show the chromatograms of aflatoxins in mixed standards and in sample extracts before and after ozonation.

Table 4.3. Aflatoxin contents in corn samples before and after treatment with 9-10 wt% ozone gas at a flow rate of ~150 ml/min.

Sample	Aflatoxin Content, ppb (n=3)			
	B1	B2	G1	G2
Non-ozonated Contaminated Corn	7452 ± 272	704 ± 31	nd	nd
Ozonated Corn Contaminated Corn	2010 ± 44	391 ± 5	nd	nd
Percent Reduction	73 %	44 %	n/a	n/a

nd = not detected, n/a = not applicable

Figure 4.12. Appearance of artificially-contaminated corn after inoculation with *A. flavus* (A53, C50Aa).

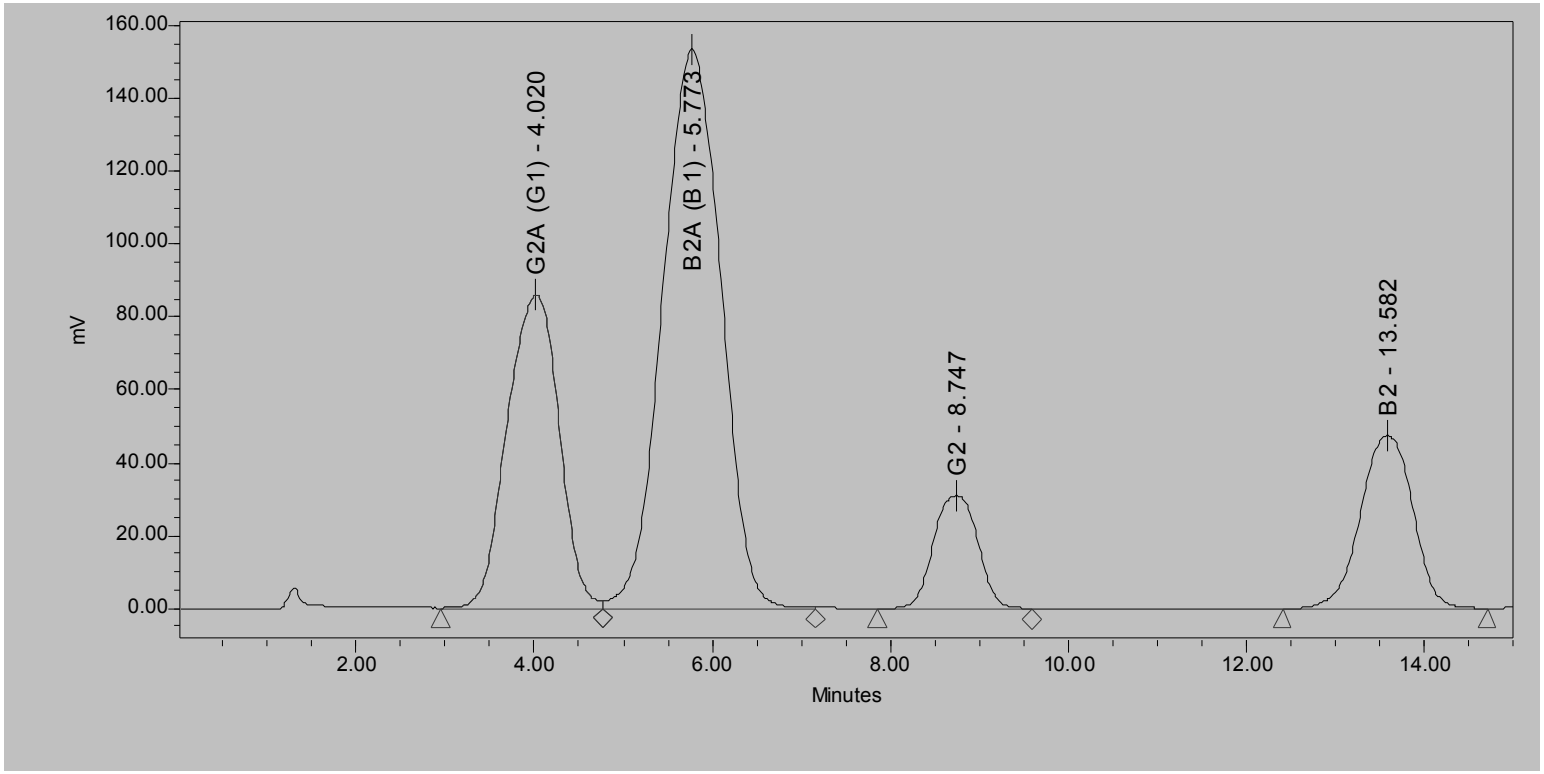


Figure 4.13. HPLC chromatogram of mixed aflatoxin standards.

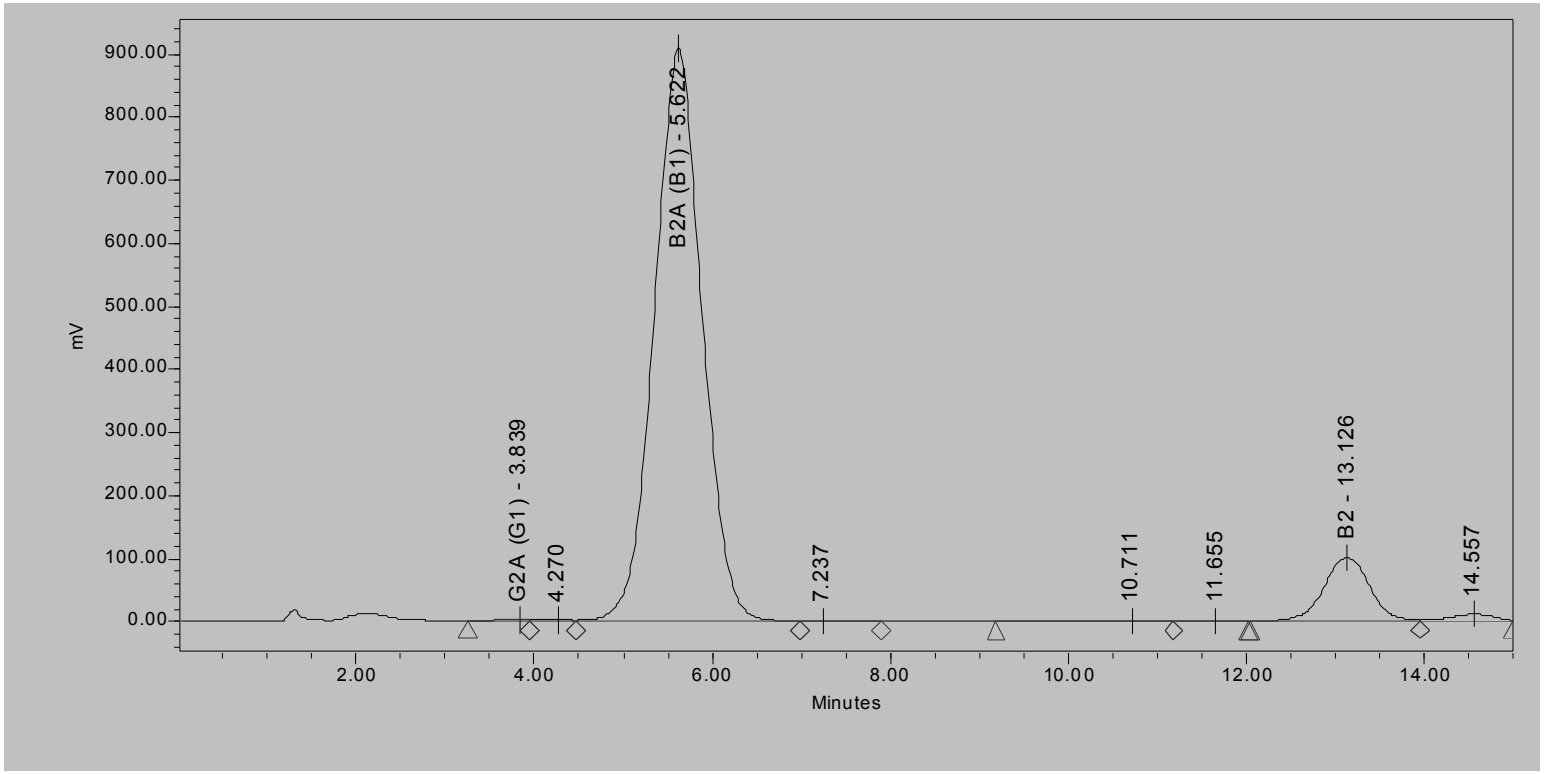


Figure 4.14. HPLC chromatogram of non-ozonated contaminated corn extracts.

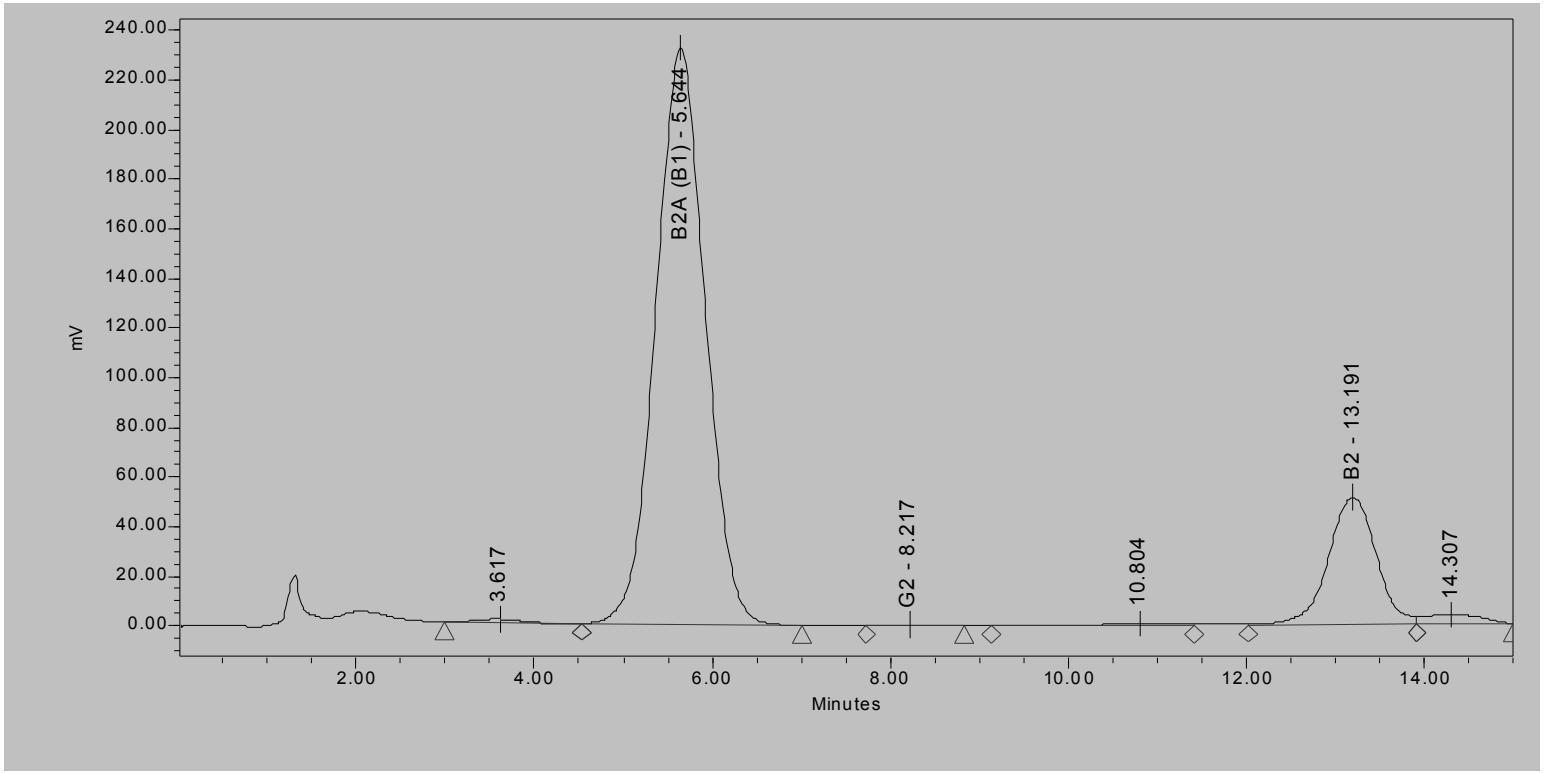


Figure 4.15. HPLC chromatogram of ozonated contaminated corn extracts.

4.3.2 Biosynthesis of [14C]-labeled Aflatoxin B_1

Radiolabeled aflatoxin B1 was obtained by the addition of labeled precursor to mold mycelia in nitrogen-free resting culture. Initial observation on the synthesis of aflatoxin B1 in the primary synthetic medium showed the formation of a small cotton ball-like mass within 24 hours of spore germination (Figure 4.16)

Close examination under the microscope revealed that these cotton balls-like masses were the hyphal form of *A. flavus* clumped together (Figure 4.17). It was also observed that the color of the resting culture changed from clear to yellowish after incubation for 24 hours. In addition, the pH of the solution remained at 5 before and after incubation. These observations are similar to published papers by Detroy and Ciegler (1971) and Jackson and Ciegler (1972).

Initial column chromatography clean-up and subsequent thin layer chromatographic analysis of the [14C]-labeled AFB_1 collected from *A. flavus* mycelia revealed the presence of 8 different compounds when viewed under UV light (Figure 4.18) These were comprised of 3 blue fluorescent spot between the solvent front and the largest blue fluorescent spots (this spot was similar to standard AFB1 in another plate) and 4 blue fluorescent spots below.the largest one. The region containing AFB_1 was stripped from the plates and eluted with chloroform-methanol (98:2) in a glass column chromatography.

Re-chromatography of this portion by TLC showed the presence of a region where blue fluorescent spots of AFB1 are present and another region of blue fluorescent spots lower than that of AFB1 (Figure 4.19). These spots are neither AFB1

Figure 4.16. Cotton-ball like appearance of mycelia collected after incubation for 24 hrs.

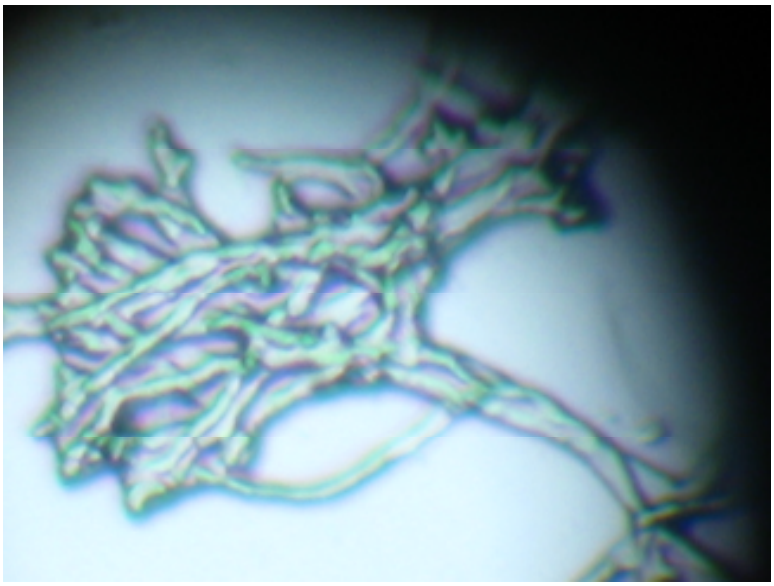


Figure 4.17. Microscopic image of the hyphal form of *A. flavus*. mycelia.

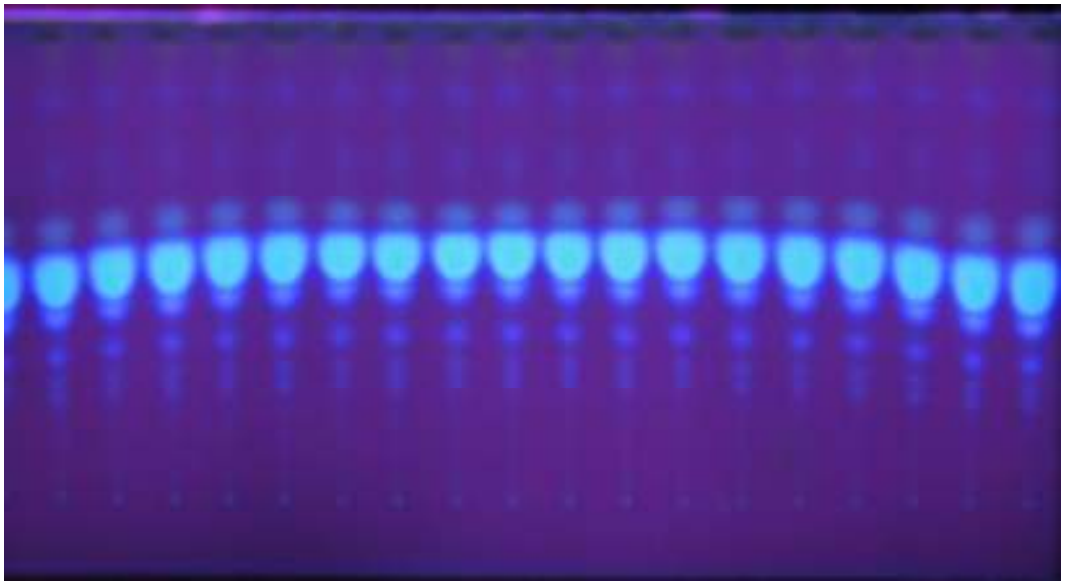


Figure 4.18. Thin layer chromatogram of initial extract collected from synthesis of [14C]-AFB1. No standard aflatoxins are shown.

Figure 4.19. Thin layer chromatogram of relatively purified [14C]-AFB1. No standard aflatoxins are shown.

nor AFB_2 based on comparison with Rf's of standard aflatoxins. The process of purification was repeated numerous times in an attempt to produce pure [14C]-AFB_1. However, the results were the same and no single band was achieved. The purification method was abandoned to avoid further loss of labeled material and the purity of the remaining material was checked.

Spectrophotometric analysis of the relatively purified extract showed a single major peak with maximum absorbance of 0.746 at 348 nm. In comparison, the standard solutions of AFB_1, AFB_2, AFG_1, and AFG_2 had maximum absorbance of 0.841, 0.635, 0.428, and 0.635 at 348, 350, 354, and 356 nm, respectively. These results show the high purity of labeled material. The preparation technique yielded 339 µg of [14C]-labeled AFB_1 with specific activity of 1.06 µCi/µmol or 7548 dpm/µg. The relative isotopic content (RIC) and the percentage of incorporation (PI) were calculated to be 9.09×10^{-3} and 0.094%, respectively. These are according to the following equations adapted from Mabee *et. al*. (1973):

$$RIC = A2 / A1$$

where *A2* and *A1* are the specific activities of the labeled product and sodium acetate, respectively, expressed in µCi per µmole, and

$$PI = (100)\ (RIC)\ (X) / F$$

where *X* and *F* are amounts of labeled product and precursor, respectively, expressed in µmoles.

The concentration and specific activity of [14C]-AFB1 produced were relatively low compared to what other workers; Adye and Mateles (1964); Detroy and Ciegler (1971); Ayres *et al*. (1971); Jackson and Ciegler (1972); Mabee *et al*. (1973);

Schoenhard *et al*., (1973); and Floyd and Bennet (1981) had produced. The efficiency of sodium acetate-1,2-[14C] incorporation is also lower than those previously reported. These differences may be attributable to the species of microorganism used in the present study, the precursor, and the length of incubation period.

4.3.3 Analysis of Ozonated and Non-ozonated Contaminated Corn Spiked with [14C]-AFB1

Radioassays on three aliquot portions each from non-ozonated and ozonated corn showed uniform distribution of [14C]-labeled AFB_1 ($27.44\pm3.67 \times 10^4$ dpm and $27.24\pm3.02 \times 10^4$ dpm, respectively). Results also show that the concentration of the radioactivity in corn sample was more than 99% of that initially added. The distribution of radioactivity in the various fractions is summarized in Table 4.4. The distribution of radiolabeled material n the non-ozonated corn is presented in Figure 4.20.

Only 11.41% of the added labeled material could be extracted by dichloromethane. The material remaining (non-extractable) in the residue after dichloromethane extraction was shown to contain most of the [14C]-labeled AFB_1, which accounted for about 93.2% of total radioactivity. Since AFB_1 was the only radiolabeled material added into the corn sample, the distribution of the radioactivity in the different fractions is relative to the amount of [14C]-AFB_1 present. Succeeding extraction of 300 g, which represents 77.32% of the residue recovered, of dichloromethane residue with methanol resulted in the distribution of 25.3%, 16.8%, and 9.5% of labeled material in methanol, acetone, and methanol-water extracts, respectively.

Table 4.4. Radioactivity distribution in corn residues from non-ozonated corn and ozonated corn following sequential fractionation procedure.

Sample / Extract	Before Ozonation		After Ozonation	
	Total Radioactivity (dpm x 10^4)	Radioactivity Concentration (%)	Total Radioactivity (dpm x 10^4)	Radioactivity Concentration (%)
Initial Corn (400 g)	27.44±3.67	100	27.24±3.02	100
CH_2Cl_2 Residue	25.57±0.67	93.2	25.29±2.20	92.8
CH_2Cl_2 Extract	3.13±0.01	11.4	2.66±0.46	9.8
CH_2Cl_2 Residue *	19.18 (300g)	69.9	20.11 (300g)	73.9
CH_3OH Residue	12.21±2.17	44.5	10.57±1.59	38.8
CH_3OH Extract	6.95±0.28	25.3	5.08±0.53	18.6
Acetone Extract	4.60±0.07	16.8	1.63±0.47	6.0
CH_3OH - Water Extract	2.60±0.04	9.5	3.13±0.85	11.5
Hexanes Extract	Not detected	-	Not detected	-
Acetic Acid Extract	0.91±0.38	3.3	1.39±0.34	5.1
NaOH Extract	4.05±0.35	14.8	4.72±0.56	17.3
Acid-Base Residue **	9.39±1.06	(26.4)	11.50±3.40	(16.4)
Pronase Residue	11.58±1.59	42.2	9.85±2.69	36.2
Soluble Aqueous	0.34±0.36	1.2	0.51±0.16	1.9
Soluble Organic	0.70±0.08	2.6	0.39±0.15	1.4
Solid Soluble	0.16±0.01	0.6	0.06±0.07	0.2

* expected radioactivity in 300 g of CH_3OH Residue; **expected values in parenthesis.

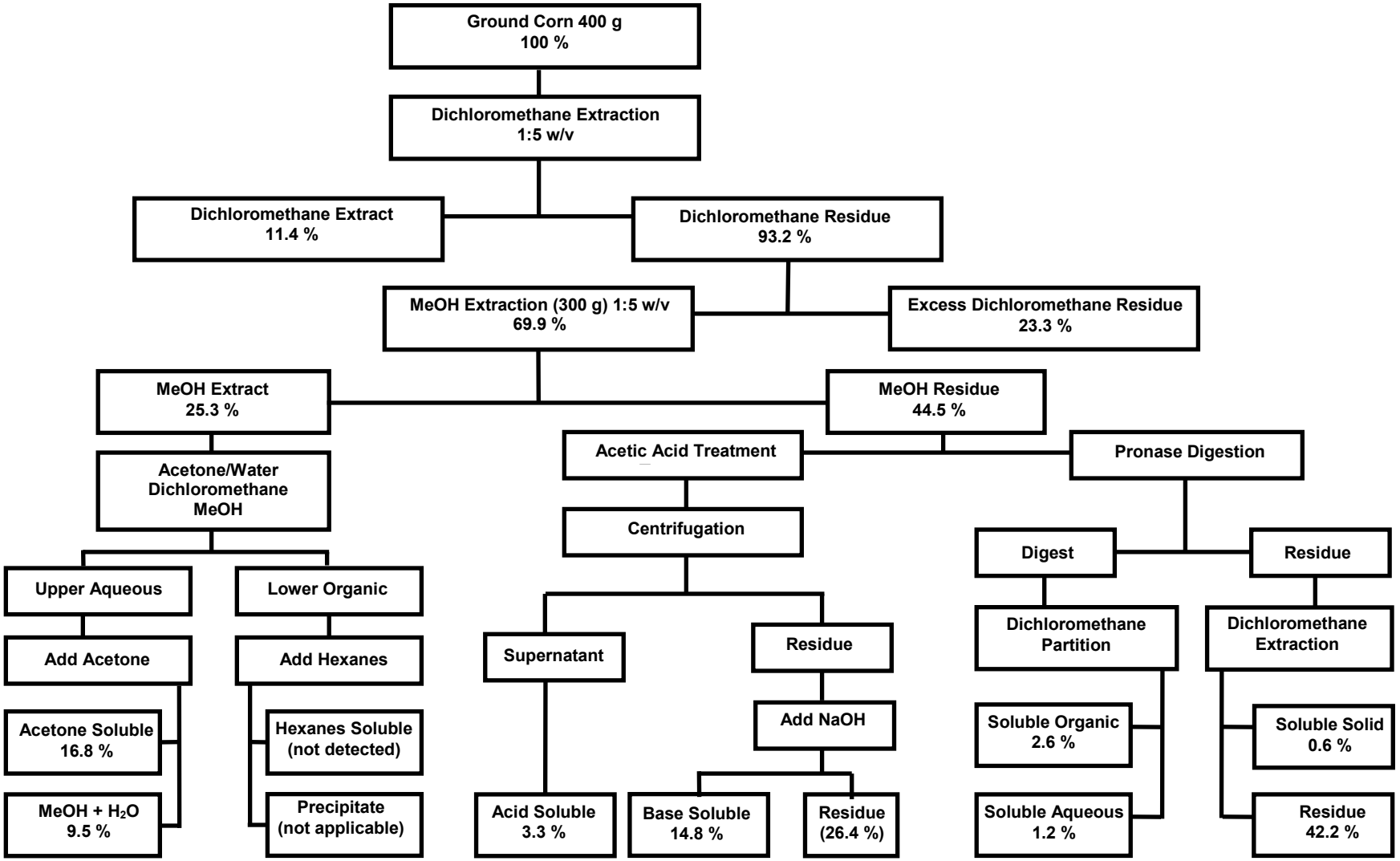


Figure 4.20. Percentage distribution of [14C]-AFB_1 related products from non-ozonated contaminated corn kernels.

On the other hand, no radioactivity was detected in the hexane extract after partition with acetone. An additional 18.1% of the radioactivity was measured after treatment with 0.1 N acetic acid and 0.1 N NaOH. Enzymatic digestion of residue from methanol extraction with Pronase E increased the amount of dichloromethane-extractable aflatoxin. Following enzymatic digestion, 4.4% of the total radioactivity was released. Of this, 3.2% and 1.2% were measured in organic soluble and aqueous soluble fractions, respectively. The rest of the labeled material added remained in the acid-base residue (predicted to be 26.37%) and in the Pronase residue (42.15%).

For ozone-treated contaminated corn, results of the HPLC analysis showed that ca. 2010 ppb AFB_1 and 391 AFB_2 remained, showing 73% and 44% reduction after ozone treatment. These values, especially for AFB1, are below what was reported in previous studies on ozonation by Dollear *et al*, 1968; Dwakanarath *et al*, 1968; Maeba *et al*, 1988; Samarajeewa *et al*., 1990; Ellis et al, 1991; McKenzie *et al*, 1998; Prudente and King, 2002; Proctor *et al*, 2004; Inan *et al*., 2007. They observed reductions in AFB_1 contents of contaminated commodities ranging from 78% to 95%. This difference could be due to the concentration and volume of gaseous ozone used in the present study. In a brief comparison, Prudente and King (2002) used 10-12 wt% ozone with a flow rate of 2L/min to treat contaminated corn, while in the present study 9-10 wt% ozone with a flow rate of 150 ml/min was used.

The distribution of radioactivity in ozone-treated contaminated corn is also presented in Table 4.4 and shown in Figure 4.21. Approximately 92.8% of aflatoxin-related radiolabeled compounds remained in the corn residue after extraction with dichloromethane. Succeeding extraction of 300g of dichloromethane residue (containing

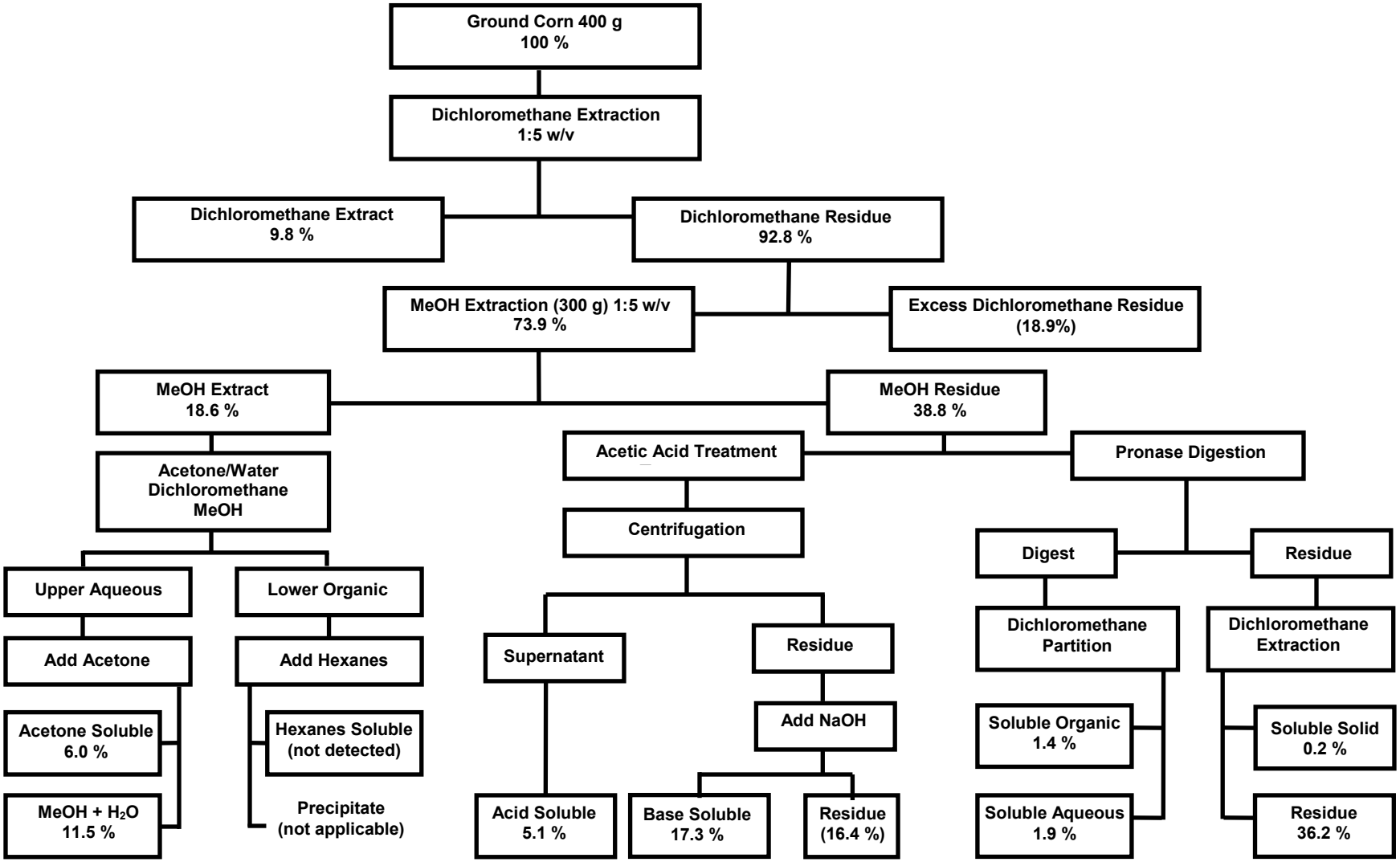


Figure 4.21. Percentage distribution of [14C]-AFB_1 related products from ozonated contaminated corn kernels.

ca. 73.9% of total radioactivity) with methanol showed that 38.8% remained in the residue and 18.6% was extracted. On the contrary, about 16.5% of added radioactivity was lost or volatilized in the extraction process.

A subsequent acetone-hexane partition process resulted in the distribution of 6% and 11.5% of radioactivity in acetone and methanol-water extracts, respectively. Similar to non-ozonated corn, no radioactivity was measured in the hexane fraction. Enzymatic digestion of the treated corn also increased the amount of dichloromethane-extractable compounds. Following enzymatic digestion, 2.6% was extracted by dichloromethane, 1.9% was present in the aqueous portion, and 36.2% of the total radioactivity remained in the residue. For acid and base treatment, 5.1%, 17.3%, and 16.4% (predicted) of aflatoxin-related compounds were found present in acetic acid extract, NaOH portion, and acid-base residue, respectively.

Noteworthy about the results of this study is the observed increase or formation of more polar aflatoxin-related compounds. Comparison of the percentage distribution of radioactivity in the methanol extract following partition with acetone and hexane shows that 66.4% of radiolabeled materials present in methanol extracts from non-ozonated corn were soluble in acetone and 37.4% were soluble in methanol-water (more polar than acetone) (Table 4.5).

Conversely, for methanol extracts from ozonated corn, it was observed that more aflatoxin-related compounds were present in the methanol-water portion (61.8%) compared with the acetone extract (32.3%). These results demonstrate that the reaction of ozone with AFB_1 produces reaction product/s that is/are more polar than theparent compound. The same result was observed in the percentage distribution of

Table 4.5. Percentage distribution of radioactivity in methanol extract following partition with acetone, methanol-water, and hexane.

Sample / Extract	Before Ozonation		After Ozonation	
	Radioactivity Concentration (%)	Radioactivity Distribution In Extract (%)	Radioactivity Concentration (%)	Radioactivity Distribution In Extract (%)
CH_3OH Extract	25.3	100	18.6	100
Acetone Extract	16.8	66.4	6.0	32.3
CH_3OH - Water Extract	9.5	37.4	11.5	61.8
Hexanes Extract or loss	-	-	-	-

radioactivity in methanol residues from treated and non-treated corn following acetic acid and NaOH treatment as shown in Table 4.6. Exposure of residue to acidic and basic conditions increased the amount of aflatoxin-related compounds released that are bound to the corn matrix. After ozonation, the radioactivity present in both extracts increased by more than 50%. The result also suggest the possible formation of an alcohol or a carbonyl compound, or even possibly an aldehyde or a carboxylic acid, that resulted in the reaction between [14C]-AFB1 and ozone. (Razumovski and Zaikov, 1984). A similar trend was observed in the distribution of radioactivity in fractions collected from the methanol residue after Pronase E digestion (Table 4.7). Results show that 94.8% of the radioactivity that was present in the methanol residue from non-

Table 4.6. Percentage distribution of radioactivity in methanol residue following acid and base treatment.

Sample / Extract	Before Ozonation		After Ozonation	
	Radioactivity Concentration (%)	Radioactivity Distribution In Residue (%)	Radioactivity Concentration (%)	Radioactivity Distribution In Residue (%)
CH_3OH Residue	44.5	100	38.8	100
Acetic Acid Extract	3.3	7.4	5.1	13.1
NaOH Extract	14.8	33.3	17.3	61.8
Acid-Base Residue	(26.4)		(16.4)	

Table 4.7. Percentage distribution of radioactivity in methanol residue following pronase digestion.

Sample / Extract	Before Ozonation		After Ozonation	
	Radioactivity Concentration (%)	Radioactivity Distribution In Residue (%)	Radioactivity Concentration (%)	Radioactivity Distribution In Residue (%)
CH_3OH Residue	44.5	100	38.8	100
Pronase Residue	42.2	94.8	36.2	93.3
Soluble Aqueous Extract	1.2	2.7	1.9	4.9
Soluble Organic Extract	2.6	5.8	1.4	3.6
Solid Soluble Extract	0.6	1.3	0.2	0.2

ozonated corn remained after digestion with Pronase E while 93.3% remained in ozonated corn. Subsequently, it was shown that there was an increase in the radioactivity level in the aqueous soluble extract from ozonated corn (4.9%) in comparison with non-ozonated corn (2.7%). Conversely, the amount of aflatoxin-related compounds soluble in dichloromethane decreased after ozonation. These results further show that water-soluble or more polar compounds than the parent are being formed between the reaction of ozone and AFB_1.

CHAPTER 5. DISCUSSION

The results obtained from the present studies demonstrated the degradation of aflatoxin B1 by ozonation and the possible formation of more polar or water-soluble reaction product/s that might be responsible for the decrease in the mutagenic potential and toxicity of AFB_1. Previous research on the evaluation of ozone gas in reducing aflatoxin levels in contaminated commodities did not find any deleterious effects. (Dwarakanath *et al*., 1968; Dollear *et al*., 1968; Maeba *et al*., 1988; Chatterjee and Mukherjee, 1993; McKenzie, 1997; Mckenzie, 1998; Prudente and King, 2002).

Determination of aflatoxin-related products from ozone–treated corn was performed by evaluating ozone-treated corn samples from the previous study of Prudente (2001). However, isolation of the reaction products was not successful probably due to the current methods used. The current protocol used in isolating the reaction products by thin layer chromatography and HPLC may not be efficient enough to isolate these compounds. The presence of other materials from the meal matrix could have affected the efficiency of the process. The attempt to isolate possible reaction products using a series of extraction and digestion procedures produced similar results as no reaction products were able to be isolated. On the other hand, the effort resulted in showing the presence of residual aflatoxin in different fractions collected from the isolation procedure. This information is valuable since it supported the results of the previous mutagenicity assay conducted (Prudente, 2001) wherein some of these fractions exhibited slight mutagenic potentials.

The evaluation of the formation of aflatoxin-related by-products in a model system provided a better understanding of the chemistry of the ozonation process in

degrading aflatoxin B_1. Results of the study revealed the conversion of slightly polar aflatoxin B1 into more polar or water soluble compounds. This information is important since it provided an idea on how to approach the objective of isolating the reaction products between aflatoxin and ozone. In addition, the results provided an idea of what compounds to look for. The determination of fate of aflatoxin in contaminated corn after ozonation using radiolabeled aflatoxin B_1 further proved the formation of more polar or water soluble compounds. There were increases in the radioactivity present in more polar solvent used in the fractionation procedure for ozone-treated contaminated corn compared with that of non-ozonated contaminated corn. This was demonstrated during the extraction and partition of methanol extracts with acetone, dichloromethane, and water. A higher percentage of radioactive material was present in the acetone portion compared with that of the methanol-water portion in non-ozonated corn. This result is expected since unreacted radiolabeled aflatoxin in methanol extract has greater affinity to less polar solvent (acetone and dichloromethane) than to a more polar solvent (methanol-water). On the other hand, the degradation and conversion of radiolabeled aflatoxin by ozonation into more polar compounds resulted in a higher percentage of radioactive material present in the methanol-water portion than in the acetone portion. The same result was observed in the acid and base digestion. Residual radiolabeled aflatoxin in methanol residue from non-ozonated corn was hydrolyzed first by the acetic acid accounting for a higher percentage of radioactivity present whereas, for ozone-treated corn, less intact aflatoxin B_1 were hydrolyzed by the acid. Polar compounds formed by the ozonation process was readily soluble in acetic acid and NaOH solutions.

The olefinic position is one of the most reactive sites for reaction of ozone with organic compounds (Bailey, 1982; Razumovski and Zaikov, 1984; Young *et. al*, 2006). Aflatoxin B_1 contains a double bond in the C8 and C9 position. This position of the double bond is widely recognized as the most reactive site in the aflatoxin structure. Aflatoxin B_1 by itself is not particularly genotoxic. Most of the mutagenic and toxic properties of aflatoxin B_1 are attributed to its reactive metabolite, the *exo*-8,9-epoxide. They are produced via oxidation by cytochrome P450 3A4 and cytochrome P450 2A5 (predominant catalysts in the human and mouse family, respectively) (Pelkonen *et al*., 1997). The *exo*-8,9-epoxide can also be formed by prostaglandin synthase or lipoxygenase. The *exo* isomer of the epoxide is considered a strong electrophile that can form covalent adducts with macromolecules such as proteins, RNA and the N-7 position of guanine residues in DNA (Foster *et al*., 1983; Miller, 1991). Only the *exo* isomer is genotoxic because of the apparent requirement for an SN^2 reaction with the guanyl N^7 in DNA, and the favorable geometry imparted by intercalation between base pairs (Guengerich *et al*., 1998; Njapau, 1999).

Based on these facts, the reaction between ozone and AFB1 is more likely to occur in the C8 and C9 positions of the double bond. Following the Creegie mechanism for this reaction, it is postulated that it could involve a 1,3 cycloaddition of O_3 in the C8-C9 double bond leading to the formation of an unstable intermediate molozonide (Bailey, 1982). This product may rearrange via 1,3 cycloaddition to produce a more stable AFB_1 ozonide.. Further reaction with O_3 or hydration could lead to the opening of the terminal furan ring and formation of a dialdehyde.

The possibility that AFB_1-dialdehyde was produced by the ozonation of aflatoxin B1 could explain the reason why slightly higher percentage of radioactivity was observed in aqueous soluble fraction from ozonated corn compared with non-ozonated corn. The dialdehyde could be bound to the protein in corn and was released during digestion with Pronase.

The opening of the terminal ring and the slight solubility of the aldehyde in an aqueous environment could mitigate the binding capability of the parent aflatoxin to form a DNA adduct that leads to cancer formation. On the other hand, although AFB_1-dialdehyde does not bind to DNA, it can react with protein lysine groups and this adduct may be responsible for the acute toxicity of AFB_1 (Guengerich *et al*., 2001).

CHAPTER 6. SUMMARY AND CONCLUSION

The aflatoxin that has caused the most concern is AFB1. It has been a focus of considerable research since its discovery. Exposure to aflatoxin B1 is generally considered to be a major factor in the high incidence of hepatocellular carcinoma, a malignant neoplasm of hepatic cells, commonly referred to as primary liver cancer. Apart from its effect on health, aflatoxin contamination also impacts the agricultural economy through the loss of produce and the time and cost involved in monitoring and decontamination efforts. In an effort to limit human exposure to these toxins, prevention and control programs have been continuously being studied and established. Methods to decontaminate aflatoxin-affected foods and feed are constantly being studied and evaluated in order to optimize those that already exist, or to obtain more efficient and safer methods.

The use of chemical treatments to decontaminate aflatoxin-containing commodities is currently the most practical approach. Although these chemical treatments are effective, through their direct and indirect interaction with either mold or aflatoxins, concerns about decontamination products are still the points of contention and are undergoing extensive investigations. One method of decontamination for aflatoxin-affected commodities that has been a focus of attention is ozonation, a physical/chemical oxidation method. Several studies undertaken previously had established the effectiveness of ozonation as a decontamination process. It has been found to be effective in reducing aflatoxin levels by as much as 95%. However, few or limited studies have been done on the potential toxicity and possible carcinogenicity of

ozone-aflatoxin reaction products. These aspects are very important in assessing the suitability and acceptability of the ozonation process.

The current study addressed these concerns by evaluating the possible formation of reaction products from ozonation of contaminated corn. Results on the evaluation of the distribution of reaction products in the current study revealed that the ozonation process degrades AFB1 to more polar or water-soluble compounds. Isolation of seven intermediate products by thin layer chromatography and the fractionation process supported and confirmed these findings. The results generated by the current study are encouraging because they supported the claim that ozonation converts AFB_1 to less toxic or mutagenic metabolite/s. In addition, these results further support the claims of other researchers on the safety of the ozonation process as it did not produce deleterious effects. In this study, although the degradation products of the aflatoxins were not identified chemically, the results of the MALDI-MS analysis and the theory of an AFB1-dialdehyde as a possible aflatoxin-related reaction product generated an idea for further evaluation and investigation. Further study should include mutagenicity assays on the products to determine if they are less toxic. Identification of the products should be made with suitable methods for concentration and analysis.

In conclusion, the discovery of more polar and water soluble compounds from the reaction between aflatoxin and ozone provided additional information that could be used to further assess the suitability and acceptability of ozonation as a decontamination process for aflatoxins.

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VITA

Alfredo Domingo Prudente, Jr. was born in Manila, Philippines, on September 4, 1964. He is the eldest child of Mr. Alfredo Echon Prudente, Sr. and Mrs. Adoracion Ilustre Domingo. He earned a Bachelor of Science degree in chemistry in 1986 from the Central Luzon State University in Munoz, Nueva Ecija, Philippines. In 1987, he began his career as a research specialist at the Bureau of Post Harvest Research and Extension (BPHRE). He has been involved in the conceptualization, implementation, and extension of studies related to post-harvest chemistry and entomology.

In 1999, he left BPHRE to pursue his graduate studies in the U.S. He completed a Master of Science degree in the Department of Food Science at Louisiana State University in December, 2001. He continued to work on his graduate degree and was hired as a research associate while pursuing his doctoral degree in food science, which he expects to receive in August 2008.

He is married to Jacqueline Avellanoza Prudente, an environmental scientist at the Louisiana Department of Environmental Quality. Alfredo and Jacqueline have two lovely children, Alyzza-Joshua and Alfred-Joseph.