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Is approved by the final examining committee:

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Approved by Major Professor(s): Klein E. Ileleji and Richard L. Stroshine

Approved by: Bernard A. Engel 12/9/2016
Head of the Departmental Graduate Program Date

INVESTIGATION OF METHODS FOR REDUCING AFLATOXIN CONTAMINATION IN DISTILLERS GRAINS

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Hu Shi

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

December 2016

Purdue University

West Lafayette, Indiana

To

My parents and my family

业精于勤，荒于嬉；

行成于思，毁于随。

---韩愈 ·《进学解》

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TABLE OF CONTENTS

Page

LIST OF TABLES....................ix
LIST OF FIGURES....................xii
LIST OF ABBREVIATIONS....................xvii
ABSTRACT....................xviii
CHAPTER 1. INTRODUCTION 1
1.1 Background: Production and importance of distiller grains 2
1.2 Problem Statement: Mycotoxin problem in Distiller Grains 4
1.3 Current Aflatoxin Decontamination Approaches....................5
1.4 Research Hypotheses 6
1.5 Research Objectives 6
1.6 Dissertation outline 8
1.7 References 10
CHAPTER 2. REDUCTION OF AFLATOXIN IN CORN THROUGH SIZE SCREENING AND DENSITY SORTING 12
2.1 Background: Reduction of Aflatoxin in Corn by Sorting 13
2.2 Objectives....................16
2.3 Materials and Methods....................17
2.3.1 Corn Sample 17
2.3.2 Physical Properties Measurement.................... 17
2.3.3 Experiment at the Commercial Inbred Seed Processing Facility 21
2.3.4 Aflatoxin Level Determination.................... 23
2.3.5 Statistical Analysis.................... 24
2.4 Results and Discussion....................25

Page

2.4.1 Difference in Size, Shape and Density of Healthy and Aflatoxin Contaminated Corn Kernels 25
2.4.2 Aflatoxin Reduction by Size and Density Segregation Using a Screen Cleaner and Gravity Table 28
2.4.3 Kernel Densities and Density Distributions in Fractions Separated Using the Gravity Table 32
2.5 Conclusions 34
2.6 References 38
CHAPTER 3. REDUCTION OF AFLATOXIN BY CONVENTIONAL AND MICROWAVE HEATING 41
3.1 Reduction of aflatoxin B_1 by dry and wet conventional heating 42
3.1.1 Background: thermal stability of aflatoxin 42
3.1.2 Material and methods 45
3.1.3 Results and discussion 47
3.2 Reduction of aflatoxin by microwave heating 54
3.2.1 Background: introduction of microwave heating 54
3.2.2 Literature review: Reduction of aflatoxin by microwave heating 55
3.2.3 Material and method 57
3.2.4 Results and discussion 58
3.3 Conclusions 62
3.4 References 64
CHAPTER 4. REDUCTION OF AFLATOXIN BY FOOD ADDITIVES 69
4.1 Literature review of aflatoxin degradation by food additives 70
4.2 Usage, Regulation and guidance levels of Food additive 71
4.3 Problem statement and objectives 73
4.4 Experiment set up 74
4.5 Results 78
4.5.1 Degradation of aflatoxin without substrate by food additives 78
4.5.2 Reaction products and reaction mechanism of aflatoxin by food additives .. 79

Page

4.5.3 Degradation of aflatoxin in DWG and CDS by food additives 83
4.5.4 Effects of concentration of citric acid and heating time on aflatoxin degradation. 85
4.6 Discussion 88
4.6.1 Efficacy and safety of food additives for aflatoxin degradation distillers grains. 88
4.6.2 Applicability of food additives for aflatoxins degradation during ethanol bioprocessing. 91
4.7 Conclusions 92
4.8 References 94
CHAPTER 5. REDUCTION OF AFLATOXIN BY HIGH VOLTAGE ATMOSPHERIC COLD PLASMA (HVACP) TREATMENT...................................... 98
5.1 Introduction of high voltage atmospheric cold plasma (HVACP).........................100
5.2 Characterization and kinetics of gas species generation by HVACP system 104
5.2.1 Background.. 104
5.2.2 Set up of HVACP system 104
5.2.3 Characterization of gas species generation using Optical Emission Spectroscopy.. 105
5.2.4 Kinetics of gas species generation using Optical Absorption Spectroscopy 110
5.2.5 Ozone and NOx concentrations measured using Drager tube: Effect of relative humidity and gas type 117
5.2.6 Conclusions.. 120
5.3 Reduction of aflatoxin in corn by HVACP treatment 121
5.3.1 Background: literature review of reduction of aflatoxin by cold plasma 121
5.3.2 Materials and Methods 122
5.3.3 Results and discussion 126
5.3.4 Conclusions.. 134
5.4 Application of HVACP for reduction of aflatoxin in distiller grains.....................135

Page

5.4.1 Background: Critical parameters on application of HVACP in distiller grains……………………………………………………………………………..135
5.4.2 Experimental Set up and methods 136
5.4.3 Results on ozone concentrations during HVACP treatment of Distiller grains ……………………………………………………………………………….....139
5.4.4 Results on aflatoxin reduction in distiller grains by HVACP..................... 144
5.4.5 Conclusions for application of HVACP to distiller grains 149
5.5 Structures of Degradation Products and Degradation Pathways of Aflatoxin B1 by HVACP Treatment.. 150
5.5.1 Background.. 150
5.5.2 Materials and Methods 150
5.5.3 Results and Discussion 153
5.5.4 Conclusions... 163
5.6 References 164
CHAPTER 6. SUMMARY OF CONCLUSIONS AND DISCUSSIONS 170
6.1 Thesis overview.. 170
6.2 Major findings corresponding to the objectives 171
6.2.1 Objective 1: Reduction of aflatoxin by size screening and density sorting. 171
6.2.2 Objective 2: Reduction of aflatoxin by conventional heating, microwave heating.. 171
6.2.3 Objective 3: Reduction of aflatoxin by food additives 172
6.2.4 Objective 4: Reduction of aflatoxin by high voltage atmospheric cold plasma (HVACP) treatment 173
6.3 Future work 175
VITA……………………………………………………………………………………..178

LIST OF TABLES

Table Page

Table 2.1 Means of dimensions, sphericities and densities of healthy and moldy kernels from the corn lot.......... 26

Table 2.2 Results of K-S test to indicate whether there are differences between healthy and moldy kernels in the distributions of the major, intermediate, and minor diameters, the sphericities and the densities. 26

Table 2.3 Summary of aflatoxin reductions achieved using a screen cleaner with two different round-hole sieves and a gravity table. 31

Table 2.4 Aflatoxin reduction in representative corn sample by passing through 17/64 inch slotted and round-hole sieve in a lab clipper screen cleaner. 32

Table 2.5 Means of kernel densities and moisture contents of the original and sorted samples.......... 33

Table 3.1. Aflatoxin reduction in food, feed, and nuts in food processing thermal operations. 44

Table 3.2. The proposed formula of AFB_1 degradants by dry heating at 150 ºC for 1h... 50

Table 3.3. The proposed formula of AFB_1 degradants by wet heating at 80 ºC for 1h. ... 51

Table 3.4. Summary of literature on reduction of aflatoxin by microwave heating. 56

Table 3.5. Reduction of aflatoxin by dry and wet heating with conventional and microwave heating methods. 60

Table Page

Table 4.1. Literature of selected food additives in degradation of aflatoxin in food and feed products. 71

Table 4.2. Uses and regulations for selected food additives 73

Table 4.3. Effectiveness of food additive on degradation of aflatoxin without substrate.78

Table 4.4. Effects of different food additives on aflatoxin degradation in DWG and CDS. All samples (except the control with no heating) were heated at 90˚C for 1 h..... 85

Table 5.1. Subdivisions of plasma. 100

Table 5.2. Comparison of methods for generation of atmospheric cold plasma 101

Table 5.3. Selected order, wavelength intervals for each species and absorption cross-section reference. 112

Table 5.4. Fitting parameters and ozone concentration for HVACP in different fill gases. 116

Table 5.5 Residual aflatoxin levels in corn treated with HVACP using different gases, RH, and treatment time* 128

Table 5.6 Results on the parameters of the models fitted to degradation kinetics of aflatoxin by HVACP treatment. 131

Table 5.7. Effects of the mode of reaction on aflatoxin degradation in corn by HVACP treatment. 132

Table 5.8. Effects of post-treatment storage on aflatoxin degradation in corn by HVACP treatment 133

Table 5.9. Effects of corn stirring on aflatoxin degradation in corn by HVACP treatment 134

Table Page

Table 5.10. Maximal and final ozone concentration during 10min HVACP. 141

Table 5.11. Ozone concentrations within the package during HVACP treatment for different sample amounts, and surface areas. 143

Table 5.12. Aflatoxin reduction in DDGS by HVACP with varying sample amount, surface area, depth, and treatment time. 146

Table 5.13. The proposed formula of AFB_1 and its degradation products obtained using LC-TOF MS. 156

LIST OF FIGURES

Figure Page

Figure 1.1. Dry-grind ethanol production process and co-products.................................3

Figure 2.1 An image of a corn kernel showing the minor, intermediate, and major diameters. 18

Figure 2.2 Micropycnometer used for measuring the volumes of corn kernels. 21

Figure 2.3 Diagram showing size and density segregation tests conducted at a commercial inbred seed processing facility. 23

Figure 2.4 Cumulative Density distributions for both healthy and moldy kernels of the following attributes: a) major diameter, b) intermediate diameter, c) minor diameter, d) sphericity and e) kernel density. 27

Figure 2.5 Histograms showing the kernel density distribution for: a) original sample, b) higher density fraction from the gravity table, c) lower density fraction from the gravity table, and d) the cumulative density distributions. 34

Figure 3.1. Percent reduction of aflatoxin by dry heating in a forced convection oven with varying heating temperatures. 47

Figure 3.2. Chromatograph of aflatoxin B_1 sample dry heated at 80 ºC for 1 h in a forced convection oven 48

Figure 3.3. Chromatograph of aflatoxin B1 sample dry heated at 150 and 200 ºC for 1 h in a forced convection oven. 48

Figure Page

Figure 3.4. Chromatograph of AFB_1 wet heated at 80 ºC for 1h in a water bath............ 49

Figure 3.5. Proposed structure of AFB_1 degradants by wet heating in a wet bath at 80 ºC for 1 h. 51

Figure 3.6. Degradation pathway of AFB_1 by wet heating. 53

Figure 3.7. Heating temperature during (A) dry heating and (B) wet heating by conventional and microwave heating methods. 59

Figure 3.8. Chromatograph of AFB_1 by microwave dry heating at 80 ºC. 61

Figure 3.9. Chromatograph of AFB_1 by microwave and conventional dry heating at 150 ºC. 61

Figure 3.10. Chromatograph of AFB_1 by microwave and conventional wet heating 80 ºC. 62

Figure 4.1. Chromatogram of AFB_1 heated at 90 ºC for 1 h with 1% sodium bisulfite. .. 80

Figure 4.2. Reaction mechanism of AFB_1 by bisulfite. 80

Figure 4.3. Chromatogram of AFB_1 heated at 90 ºC for 1 h with 1% sodium hypochlorite. 81

Figure 4.4. Chromatogram of AFB_1 heated at 90 ºC for 1 h with 1% citric acid. 82

Figure 4.5. Reaction mechanism of AFB_1 by heating at 90 C for 1 h 82

Figure 4.6. Chromatogram of AFB_1 heated with 1% ammonium persulfate.................... 83

Figure 4.7. Effects of rate of addition of citric acid on aflatoxin reduction in DWG and CDS. 86

Figure 4.8. Effects of heating time on aflatoxin reduction in DWG and CDS. 87

Figure 5.1. Different schematic set-ups of atmospheric cold plasma. 102

Figure Page

Figure 5.2. Set-up of high voltage atmospheric cold plasma (HVACP) system 105
Figure 5.3. Schematic diagram of experimental set-up of optical emission spectroscopy. 105
Figure 5.4 Optical Emission spectra of HVACP for a) Air and b) MA65 at 40% relative humidity 107
Figure 5.5 Emission intensity of HVACP by filled gas (a) Air at wavelength 337.453 nm and (b) MA at wavelength 777.546 nm MA over time...................................... 109
Figure 5.6. Absorption cross-sections of dominant species in humid air plasma and integration intervals. 112
Figure 5.7. Change of the optical absorbance spectra during HVACP treatment. 114
Figure 5.8. Increase of Ozone concentration over time during HVACP treatment for different fill gases (Air, MA, N_2)... 115
Figure 5.9. Increase of ozone concentration during HVACP treatment for air at different relative humidity (RH 5, 40, 80%).. 117
Figure 5.10 Concentration of (a) Ozone and (b) NOx generated by HVACP with different relative humidities and types of working gas. 120
Figure 5.11. Schematic diagram of experimental set-up for HVACP treatment of aflatoxin in corn 124
Figure 5.12 Reduction of aflatoxin level in corn by HVACP treatment with different fill gases, relative humidities, and treatment time. 129

Figure Page

Figure 5.13. Percent reduction of aflatoxin in corn by HVACP treatment in a) Air and b) MA65 at different relative humidities over time. Solid lines represent fitted model 130

Figure 5.14. Ozone concentration with different material inside the package during 10 min HVACP treatment. (filled gas: ambient air) 141

Figure 5.15. Ozone concentration within the package during 10 min HVACP treatment for (a) different sample amount (5, 10, 20g), and (b) different surface area (34, 56, 112 mm^2) 143

Figure 5.16. Relationship of ozone consumption by DDGS with surface area and treatment time during HVACP treatment 144

Figure 5.17. Aflatoxin reduction in different matrix (DWG, CDS, DDG, DDGS) by HVACP treatment. 145

Figure 5.18. Relative importance of treatment time, sample amount, surface area, depth for percent reduction of aflatoxin by HVACP treatment. 148

Figure 5.19. Schematic of experimental set-up for HVACP treatment of aflatoxin in corn 152

Figure 5.20. Chromatograms of AFB_1 untreated (5 µg/ml) in 50% ethanol solution (A) and AFB_1 sample treated by HVACP in ambient air for 5 min. 154

Figure 5.21. Relative change in response value of AFB_1 and degradation products 1 to 6 (P1 - P6) for AFB_1 sample with increasing HVACP treatment time in air. 155

Figure 5.22. Orbitrap MS/MS spectra and proposed fragmentation (insets) of degradation products of AFB_1 by HVACP 158

Figure Page

Figure 5.23. Proposed structure of reaction products (P1 – P6) of AFB1 by HVACP .. 159

Figure 5.24. First degradation pathway of AFB_1 by HVACP 161

Figure 5.25. Second degradation pathway of AFB1 by HVACP 162

LIST OF ABBREVIATIONS

DDGS	Distiller's dried grains with solubles
CDS	Condensed distiller solubles
DWG	Distiller’s wet grains
ppb	Parts per billion
ppmv	Parts per millions by volume
RH	Relative humidity
AFB_1	Aflatoxin B_1
HVACP	High voltage atmospheric cold plasm
MA	Modified atmosphere

ABSTRACT

Shi, Hu. Ph.D., Purdue University, December 2016. Reduction of aflatoxin in distiller grains. Major Professor: Dr. Klein E. Ileleji and Dr. Richard L Stroshine.

Distillers grains with solubles (DDGS) is a coproduct of dry-grind ethanol bioprocessing made by mixing and drying two intermediate products: distillers wet grains (DWG) and condensed solubles (CDS). Distillers grains are valuable nutrient ingredients and are widely used in beef and dairy diets. However, in worldwide surveys 98% of analyzed DDGS samples contained at least one type of mycotoxin. Among the toxins produced by fungi, aflatoxins are the most toxic and have been classified as type one carcinogens. Aflatoxins are carried over into the distillers grains where they can be concentrated to up to 3 times the values in the incoming corn. The presence of aflatoxin in the distillers grains poses a serious risk to the health of animals and makes marketing of distiller grains difficult if not impossible. Methods for aflatoxin reduction in distillers grains need to be developed. Thus the overall goal of this project was to reduce the aflatoxin level in the final coproduct of ethanol bioprocessing--DDGS. This was pursued by examining the effects of reduction of aflatoxin in the incoming corn prior to bioprocessing, the degradation of aflatoxin in the intermediate products, namely DWG and CDS, during processing, and aflatoxin degradation in the DDGS. Segregation

techniques (size screening and density sorting) and detoxification methods (conventional and microwave heating, food additives, and high voltage atmospheric cold plasma) were evaluated for their effectiveness in aflatoxin reduction.

Effectiveness of physical segregation of aflatoxin contaminated corn was investigated by size screening and density sorting in a 737 kg corn lot with an aflatoxin level of 185 ppb. There are statistically significant differences in major and minor diameters, the sphericities and the densities between moldy and healthy corn kernels. The moldy corn kernels had a smaller major diameter, greater sphericity and a lower density. Results indicated that removal of fine material from the corn lot through size screening could significantly reduce aflatoxin in the remaining lot. Further removal of small size kernels through cleaning with a screen cleaner and removal of lower density kernels with a gravity table gave an additional reduction of aflatoxin in the remaining corn lot.

Reductions of aflatoxin achieved by conventional heating (using a convection oven and water bath) and microwave heating to degrade the aflatoxin were also investigated. The presence of water is critical to aflatoxin degradation during heating. Aflatoxin is very stable during dry heating and a temperature of 150 ºC is required to initiate decomposition of aflatoxin. HPLC-MS studies revealed that aflatoxin B_1 was converted into its enantiomer by dry heating. During wet heating for 1 h at 80°C, 73% of the AFB_1 was degraded. Degradation of AFB_1 by wet heating involves hydrolysis of the furofuran moiety and the lactone ring along with further decarboxylation. Microwave heating produced the same degradation products as conventional heating, indicating that degradation during microwave heating is purely due to its thermal effects.

Degradation of aflatoxin by food additives was also investigated. Four selected food additives, i.e., sodium bisulfite, sodium hypochlorite, citric acid, and ammonium persulfate, were able to effectively (>86%) degrade aflatoxin with no substrate by heating at 90 ºC for 1 h with 1% (by weight) food additive solutions. A protective effect of the substrate was found for aflatoxin degradation in DWG and CDS. Citric acid is the most promising additive for degrading aflatoxin since it has been classified as GRAS (generally recognized as safe) by FDA. Degradation of aflatoxin B_1 by citric acid was through acid-catalyzed hydrolysis which converts the AFB_1 to AFB_2 and AFB_1-Citric ($C_{23} H_{19} O_{13}$). Aflatoxin reduction was enhanced by adding more citric acid and prolonging the heating time.

Performance of the HVACP system and generation of reactive species were characterized using optical emission spectroscopy and optical absorption spectroscopy. During the 120 s HVACP treatment, ozone concentrations generated by HVACP follows a logarithmic function for both the gas MA and air ($R^2_{adj} > 0.98$). Ozone generation rate and final ozone was higher when the MA gas was used instead of air, and when the relative humidity was low (5%). Aflatoxin in corn could be degraded by HVACP treatment within minutes. Three kinetic models (a first-order, a Weibull, and a logistic model) were fitted to the aflatoxin degradation data. The logistic model was found to be the best to describe the degradation kinetics of aflatoxin by HVACP with a high coefficient of determination ($R^2 \geq 0.99$). Degradation of aflatoxin by HVACP was influenced by the type of materials treated. It was more readily degraded in DWG and DDG than in DDGS and CDS. A Relative Importance Analysis indicated that sample amount, treatment time, and grain depth were critical parameters that determine percent

reduction of aflatoxin in DDGS by HVACP Treatment. The mechanism whereby AFB_1 is degraded during HVACP treatment involved hydrogenation, hydration, and oxidation of the furan ring. The hydrogen radical, hydroxyl radical, hydroperoxyl radical and ozone were proposed as the major reactive agents for AFB_1 degradation generated by HVACP treatment. Based on the literature, the degradation produced changes in the furofuran and lactone rings, and cyclopantenone and methoxyl structures. These should pose less of a risk to biological activity than AFB_1 according to their structure-bioactivity relationship.

CHAPTER 1. INTRODUCTION

This dissertation presents a study on reducing the aflatoxin levels in distiller dried grains with solubles (DDGS) manufactured by the dry-grind bioprocessing of corn to ethanol. This is pursued by reduction of aflatoxin in the incoming corn prior to bioprocessing, degradation of aflatoxin in the intermediate products during processing, and aflatoxin degradation in DDGS. Sorting methods (size and density) and detoxification methods, i.e. conventional and microwave heating, incorporation of food additives, and High voltage atmospheric cold plasma (HVACP), were evaluated for their effectiveness in reducing aflatoxin outside a food matrix, in corn, in the intermediate products (DWG and CDS), and in DDGS. The influence of process parameters, the reaction mechanisms and the degradation products of the detoxification methods were evaluated and clarified.

This chapter gives an overview of the aflatoxin problem in corn and distillers grains. Section 1.1 introduces the importance of distiller grains to the feed industry, and Section 1.2 summarizes information on the prevalence of mycotoxins in distillers grains, with a focus on aflatoxin as the most critical problem. Section 1.3 gives an introduction to aflatoxin decontamination methods. Sections 1.4 and 1.5 present the research hypothesis and the objectives. And finally, Section 1.6 provides an overview of the remainder of the dissertation.

1.1 Background: Production and importance of distiller grains

Due to the increasing energy demand and the mandate for energy independence, production of biofuels, which are a renewable source of energy, has increased dramatically in the last decade. Under the Energy Independence and Security Act (EISA) of 2007, the total amount of biofuels used in the U.S. needs to be increased to 36 billion US gallons by 2022 from 4.7 billion US gallons in 2007. This goal has been partially fulfilled with a production of 13.3 billion gallons in 2013 (USEIA, 2012). The production of the coproduct: distillers grains with solubles (DDGS), followed a similar trend to that of ethanol. Its productionincreased from 9 million tons in 2005 to 35.5 million tons in 2013.

Corn can be converted into ethanol by two major processes: wet milling or dry-grind processing. The wet milling process separates the kernel into various fractions and produces multiple food and industrial products including ethanol. The dry-grind process does not fractionate the kernel and produces only two coproducts (distiller grains and condensed solubles). Over 80% of U.S. ethanol plants use the dry grind process, because of its lower investment and operational costs, as well as improvements in fermentation technology (Rausch and Belyea, 2006). A series of bioprocess operations for the dry-grind process were shown in Figure 1.1. In this process, the corn is first ground with a hammer mill. Then the corn flour is cooked using a jet cooker, and the slurry is held in a liquefaction tank. Next the mash is transferred to a fermentation tank and goes through saccharification and fermentation processes which involve the addition of yeast and enzymes. The ethanol is distilled from mash using distillation columns. The mash remaining after distillation of ethanol is called whole stillage, which is comprised of

water, protein, fiber and lipids. The whole stillage is centrifuged to separate coarse solids from liquid. The liquid is called thin stillage, which goes through the evaporation process thereby producing condensed solubles (CDS), which contain approximately 30% dry matter and are often called “syrup” in the ethanol industry. The coarse solids are called distillers wet grains (DWG) and contain about 35% dry matter. Distillers wet grains can be sold locally as cattle feed without drying, or they canbe mixed with condensed solubles and dried to produce Distiller's dried grains with solubles (DDGS), which have a moisture content of around 12%.

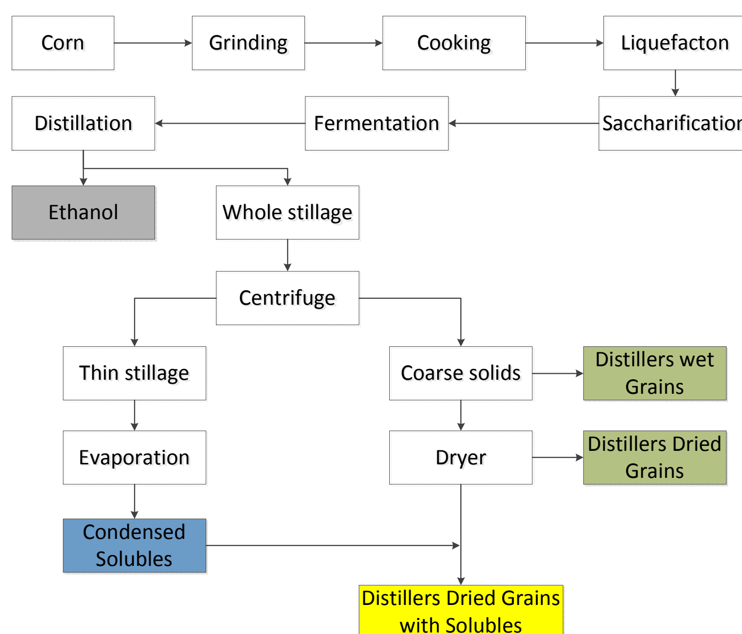

Figure 1.1. Dry-grind ethanol production process and co-products (Erickson et al., 2005)

From a nutrient standpoint, DDGS is a valuable ingredient in animal diets. Eighty percent of DDGS is used in beef and dairy diets. The production and sale of DDGS is essential for economical sustainability and for continued development of the ethanol industry. It generally contributes about 10% to 20% of the revenue to the ethanol plant,

and the contribution can be as high as 40% depending on the its market value (Liu and Rosentrater, 2012).

1.2 Problem Statement: Mycotoxin problem in Distiller Grains

Mycotoxins are toxic secondary metabolites produced by molds that can adversely affect the health, growth and reproduction of animals when mycotoxin contaminated grains or coproducts are ingested. Mycotoxins are unavoidable contaminants in crops. According to the Food and Agriculture Organization of the United Nations (FAO), 25% of the world's crop are contaminated with mycotoxins during growth or storage ("Mycotoxins", 2013;GIPSA, 2006). Major mycotoxins that could be potentially found in corn include aflatoxins, deoxynivalenol (DON), fumonisins, T-2 toxins and zearelenone (ZEA). The mycotoxins found in DDGS originate from those associated with the cornthat is used as the raw material for ethanol production (Whitlow and Hagler, 2006).

The nutrient content of DDGS makes it a valuable ingredient for animal diets. Approximately 80% of DDGS is used in beef or dairy diets (Liu and Rosentrater, 2012) . Multiple studies have reported detectable levels of mycotoxins in DDGS (Zhang and Caupert, 2012;Zhang et al., 2009;Rodrigues and Chin, 2012). Between January 2005 and December 2010, a total of 409 corn and DDGS samples sourced worldwide were analyzed for the 5 major mycotoxins: aflatoxins, zearalenone, deoxynivalenol, fumonisin and ochratoxin, In one study, 98% of the DDGS samples analyzed contained at least one mycotoxin, and 92% of the samples were contaminated with 2 or more mycotoxins

(Rodrigues and Chin, 2012). These studies have raised concerns about the wisdom of using DDGS produced in the U.S. as animal feed.

Among the 5 major mycotoxins, aflatoxins are the most toxic. They have been classified as type one carcinogens by the International Agency for Research on Cancer (Stoloff, 1989). Aflatoxins are toxins produced by the *Aspergillus* species, mainly from *Aspergillus flavus* or *Aspergillus parasiticus*. Aflatoxins can be carried into distillers grains from the incoming corn. One study reported that during ethanol bioprocessing there was very little aflatoxin degradation, and the aflatoxin level in distiller grains was concentrated up to 3 times compared to the content of the incoming corn (Murthy et al., 2005). According to the FDA regulatory levels for aflatoxin in the feed, the maximum allowable aflatoxin levels are 300 μg/kg for finishing cattle, swine and poultry, 100 μg/kg for breeding cattle, swine and poultry, and 20 μg/kg for all other animals (NGFA, 2011). The presence of aflatoxin in the distiller grains poses a serious risk to the health of the animals consuming the feed and makes marketing of distillers grains difficult if not impossible. Therefore, decontamination methods for aflatoxin reduction in distillers grains need to be developed.

1.3 Current Aflatoxin Decontamination Approaches

The only method approved by FDA to detoxify aflatoxin is ammonization, but it is limited to use in cottonseed under the guidance of FDA's compliance policy (CPG Sec. 670.500). Currently there is no FDA approved method for detoxifying aflatoxins in corn or distillers grains. A number of methods for degrading aflatoxin in corn or other grains or nuts have been tested with varying degrees of success. These methods generally fall

into three categories: physical, chemical and biological (Samarajeewa et al., 1990;Piva et al., 1995;Wu et al., 2009). Physical methods include cleaning, density and color sorting, UV, microwave, and gamma irradiation. Chemical methods include treating with various food additives or chemical agents, ammonization, and ozonation. Biological methods include treating with microorganisms, including bacteria, molds and yeasts, or treating with enzymes. The FAO requirement for an acceptable decontamination process involves the following essential features: (FAO, 1977;Piva et al., 1995)

1. It must inactivate, destroy, or remove aflatoxins;
2. It must not produce nor leave toxic/carcinogenic/mutagenic residues;
3. It must not significantly alter the nutrient, sensory of other important attributes of the material;
4. Ideally it must destroy fungal spores or mycelium that could proliferate and produce new toxins.

In a search of the literature, the author did not find studies on aflatoxin degradation in distiller grains or its related coproducts.

1.4 Research Hypotheses

We hypothesize that aflatoxin levels in distiller grains could be reduced by a systematic approach by: reducing the aflatoxin levels in corn prior to, during, and post bioprocessing into ethanol through physical segregation, and thermal or chemical detoxification methods.

1.5 Research Objectives

The overall goal of this project was to reduce the aflatoxin level in the Coproduct DDGS from corn bioprocessing into ethanol. This was pursued by reduction of aflatoxin

in the incoming corn prior to bioprocessing, degradation of aflatoxin in the intermediate coproducts during processing (DWG and CDS), and aflatoxin degradation in DDGS. This research was broken down into four primary objectives, with the following sub-objectives:

1. Determine the effectiveness of size screening and density sorting on aflatoxin reduction in corn:
    1.1. Measurement of kernel size, shape, and density of representative corn samples.
    1.2. Evaluation of the difference in physical properties (kernel size, shape, density) and its distribution between moldy and healthy corn kernels.
    1.3. Determining aflatoxin reduction by size screening using a screen cleaner and density segregation using a gravity table.
2. Understand the thermal stability of "pure" aflatoxin without a food substrate being present, using conventional and microwave heating:
    2.1. Determine aflatoxin reduction by heating both dry and wet samples in a conventional oven.
    2.2. Determine aflatoxin reduction by heating both dry and wet samples using microwaves.
    2.3. Determine the degradation mechanisms for dry and wet heating, and clarify whether there are non-thermal effects from microwave heating.
3. Investigate detoxification of aflatoxin using food additives both without a food substrate and within a food substrate (DWG and CDS):
    3.1. Test the effectiveness of selected food additives on aflatoxin degradation

without substrate, and clarify the reaction mechanism.

3.2. Test the effectiveness of selected food additives on aflatoxin degradation in a substrate (DWG and CDS), and evaluate the influence of the factors: food additive concentration, and treatment time.

4. Reduction of aflatoxin by high voltage atmospheric cold plasma (HVACP) treatment:

   a. Characterization of HVACP with optical emission spectroscopy (OAS) and optical absorption spectroscopy (OAS).

   b. Evaluate effectiveness of HVACP treatment in aflatoxin reduction in corn and DDGS, and understand the influence of the following critical process parameters: gas type, relative humidity, mode of reaction, sample size, surface area, and sample depth.

   c. Clarify the degradation products and degradation mechanisms of aflatoxin by HVACP treatment.

1.6 Dissertation outline

The remainder of this dissertation is divided into five chapters. Chapter 2 reports on studies of the difference in physical properties including shape, size and density between healthy and aflatoxin contaminated corn kernels and investigates the effective of screening and sorting in reduction of aflatoxin in a specific corn lot. Chapter 3 investigates the stability of aflatoxin for dry and wet heating conditions using both conventional and microwave heating. Chapter 4 describes the results of a study on the detoxification of aflatoxin by food additives without food substrate being present and with an substrate (DWG and CDS). Chapter 5 investigates the effectiveness of HVACP

treatments for aflatoxin reduction in corn and in distillers grains, the reaction mechanism caused by HVACP treatment for aflatoxin degradations and it also clarified the degradants produced. Chapter 6 summarizes the findings of this dissertation and includes some suggestion for further work based on this research.

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CHAPTER 2. REDUCTION OF AFLATOXIN IN CORN THROUGH SIZE SCREENING AND DENSITY SORTING

In this Chapter, the difference in physical properties including size, shape, and density between healthy and aflatoxin contaminated kernels was characterized, and their relationship to reduction of aflatoxin levels by size screening and density sorting was studied. The results have been presented at conference and submitted for publication, with the citations shown below:

Journal paper:

Shi, H., Stroshine, R.L., Ileleji, K. Differences in kernel shape, size and density between healthy and aflatoxin contaminated kernels and their relationship to reduction in aflatoxin levels in a sample of shelled corn. Submitted to Applied Engineering in Agriculture. 2016.

Conference presentations:

Shi, H., R.L. Stroshine, and K. Ileleji. 2014. Aflatoxin Reduction in Corn by Cleaning and Sorting. ASABE Paper No. 14-1890901. St. Joseph, Mich. ASABE.

Shi, H., R.L. Stroshine, and K. Ileleji. 2014. Mycotoxin reduction in corn from pre-cleaning and kernel Sorting. Omaha, Nebraska. NC-213 meeting.

2.1 Background: Reduction of Aflatoxin in Corn by Sorting

Aflatoxins are naturally occurring contaminates in corn, produced mainly by three species of Aspergillus, namely *A. flavus, A. parasiticus* and *A. nomius* (Rustom, 1997). Aflatoxin contamination in corn is promoted by high ambient temperatures and drought stress during the growing season, and by improper handling and storage of corn kernels after harvesting. The U.S. Food and Drug Administration (FDA) has set action level limits for aflatoxin as 20 ppb in corn and other grains intended for consumption by humans, poultry, dairy animals or unknown uses (National Grain and Feed Association, NGFA, 2011). The levels were established after tests indicated that aflatoxins may cause liver cancer, affect immune system and reduce growth rate of the human or animals that consume the contaminated grain (Groopman et al. 1988; Virdi et al. 1989; Khlangwiset et al. 2011).

When aflatoxin is present in corn prior to harvest, the most heavily contaminated regions of the ear are often at the tip of the ear where the kernels can be rounder and smaller than kernels from other locations on the ear. Fungi growing on a corn kernel usually consume dry matter from the kernel, potentially lowering the kernel density and making the kernels more susceptible to breakage during handling and during combine harvesting. This hypothesis was at least partially corroborated by the observation of Shotwell et al. (1972) that corn kernels with high levels of aflatoxin are structurally weakened and crumble easily. These observations suggest that there may be pieces of contaminated kernels with differences in kernel size, shape, and density that explain the ability of screening and gravity table density sorting to, in some instances, reduce aflatoxin levels in a corn lot.

Screening is commonly used in the grain industry to remove fines and foreign materials from cereal grains and oilseeds. Several studies have reported that size segregation by screening can reduce aflatoxin levels, although the degree of success varies. Dowell et al (1990) screened 17 loads of farmers' stock peanuts with initial aflatoxin levels of 110.7 ppb over a screen belt with openings of 9.5 mm (24/64 inch). The aflatoxin level was lower in the overs (90.5 ppb) and higher in the throughs (210.6 ppb). The aflatoxin level of shelled peanuts was further reduced from 90.5 to 3.8 ppb by removal of smaller fractions (other edibles, oil stock, loose shelled kernels, and damaged kernels) by passing the peanuts over 6.35 mm (16/64 inch) and 5.56 mm (14/64 inch) screens. Brekke (1975), found that removal of fine and foreign material did not markedly reduce aflatoxin in the remainder of several corn lots, except for one lot which contained a high percentage (16%) of fines and foreign material. These conflicting results regarding the effectiveness of screening suggest that a more thorough study of the relationship between kernel size and shape would provide a better understanding of why screening helps in some instances but not in others.

Density segregation is sometimes used to remove diseased kernels from good kernels before corn is processed for food. It is also used frequently by the seed industry to purify the seed by removing kernels that have undesirable traits. For example, in one study a correlation was found between density and aflatoxin contamination of cottonseed. In general, higher levels of aflatoxin were found among lower density seeds (Koltun et al. 1974). Although factors such as invasion by other fungi, insect damage, or premature death of the kernels can also reduce seed density, studies have demonstrated that aflatoxin-contaminated corn can be segregated from aflatoxin free corn based on density

(Huff and Hagler, 1982). Density segregation of contaminated kernels can be done in a laboratory by floatation in various solutions, including water, sodium chloride, hydrogen peroxide, and saturated sucrose. In several studies, significant reductions were achieved for various types of mycotoxins (Aflatoxins, DON, Zearolenone, Fumonisin) in a variety of grains (Huff and Hagler 1985; Clavero et al. 1993; Rotter et al 1995; Shetty and Bhat 1999). However, the floatation method rewets the kernels and if used in a commercial setting it would introduce an additional cost for drying the rewetted grain. An alternative method of density segregation is to use a gravity table. In one study a gravity table effectively removed lower density wheat kernels, such as those that had sprouted (Tkachuk et al 1991a). Removal of severely infected (thin and shriveled) kernels reduced Trichothecene levels in wheat infected with Fusarium Head Blight (Tkachuk et al. 1991b). In another study, Brekke et al. (1995) reported that the gravity table was not effective in reducing aflatoxin in corn. However, they did not provide data on the density variations among the kernels in the sample. In light of the aflatoxin reduction achieved using the floatation method, and the capability of the gravity table to segregate according to density, a more detailed study of the performance of the gravity table in reducing aflatoxin levels is needed.

If the effectiveness of aflatoxin reduction in contaminated corn using size and density segregation is to be better understood and potentially improved, the differences in size and density between moldy and healthy corn kernels should be studied. The authors did not find research quantifying the size or density distribution of healthy and moldy corn kernels. Moldy kernels tend to have a lower density because of the consumption of nutrients and disintegration of cellular components resulting from fungal invasion.

However, there is also very little literature that quantifies the difference in density between healthy kernels and those invaded by fungi, possibly because of the difficulty associated with accurately measuring individual kernel density. Martin et al. (1998) determined kernel densities of healthy and scab damaged wheat kernels using a micro-pycnometer. The scab-damaged wheat kernels had a mean density of 1.08 g/cm3, compared to mean density of 1.28 g/cm3 for healthy wheat kernels. However, the authors of this paper did not find any similar studies on moldy and healthy corn kernels.

2.2 Objectives

In this study, the authors characterized the kernel size and shape along with the kernel densities of samples of moldy and healthy corn kernels in a lot of shelled corn (737 kg or 1625 lbs) containing high levels of aflatoxin. The overall goal was to gain a better understanding of the differences in these attributes and their relationship to the ability to remove moldy kernels and reduce aflatoxin contamination in the infected lot of corn. The specific objectives were to determine the following for this contaminated shelled corn, which was obtained from a central Indiana farm: (1) the dimensions, sphericity, and density distribution of healthy and moldy corn kernels from a representative sample of the corn; (2) the effectiveness of sieving with 5.16 and 6.75 mm (13/64 and 17/64 in) round-hole sieves, respectively, and how the effectiveness was related to the distribution of the kernel dimensions; and (3) the effectiveness of gravity table as a means of removing aflatoxin contamination and how that was related to the density distribution and presence of broken kernels in the sample.

2.3 Materials and Methods

2.3.1 Corn Sample

A 737 kg (1625 lb.) corn lot was purchased from a farm located in central Indiana near Clayton in November of 2012. The corn was taken from an overhead bin used to load trucks. The bin had been filled by removing corn from the center (core) of bins where the corn contained high levels of aflatoxin. It had a very high percentage of fine material and the initial aflatoxin level in samples taken from the corn lot was 185 ±28 ppb.

2.3.2 Physical Properties Measurement

2.3.2.1 Moisture Content

Moisture contents (MC, wet basis) of all corn samples were determined using the whole kernel oven drying method (ASABE Standard S352.2). About 15 g of sample were dried at 103 °C for 72 h using a forced convection oven (American Scientific Products DN-42, CA, USA).

2.3.2.2 Kernel Size and Shape

The size of corn kernels can be characterized using the major, intermediate, and minor diameters (Figure 2.1). A total of 115 moldy kernels and 131 healthy kernels were selected from subsamples of the original sample for dimension measurement. The values of corn kernel diameters (mm) were determined to the nearest 0.05 mm using a dial calipers.

The Shape of corn kernels was quantified by calculating the sphericity of each of the kernels using the measured diameters. Sphericity is defined as the ratio of volume of

the kernels to the volume of a sphere having a diameter equal to the diameter of a circumscribing sphere (Stroshine, 2014):

$$Sphericity = \frac{(abc)^{1/3}}{a} \quad \text{(Eq. 2.1)}$$

where the major, intermediate, and minor diameters are: 2a, 2b, and 2c, respectively.

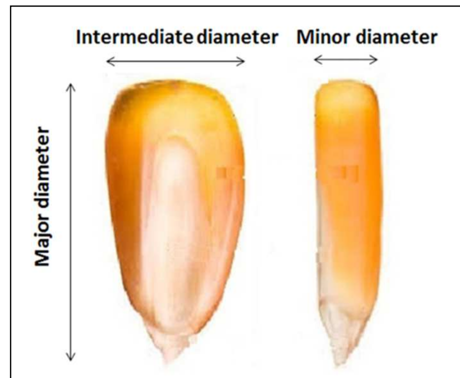

Figure 2.1 An image of a corn kernel showing the minor, intermediate, and major diameters.

2.3.2.3 Kernel Density

A total of 47 moldy kernels and 56 healthy kernels were selected from subsamples of the original sample for measurement of kernel density. Individual corn kernel density was determined using a micropycnometer constructed by the Agricultural and Biological Engineering (ABE) Department's shop personnel (Figure 2.2). This apparatus was similar in construction to a pycnometer used in a study of the density of wheat kernels (Martin et al. 1998). The chamber into which the kernel was placed was a length of glass tubing with an inside diameter of 10.78 mm (0.424 in.). Glass tubing was used because it was easy to see through the glass to view the height of the liquid in the chamber. In initial tests with a plastic chamber, which was not as clear as the glass, it was difficult to see clearly in order to accurately adjust the height of the oil in the kernel chamber. The chamber was partially filled with red gauge oil purchased from Dyer Instruments (Michigan City, Indiana). This was the same fluid used by Martin and coworkers (Martin et al. 1998). It was selected because of its low volatility and because it was not readily absorbed by the corn kernel. It had a relatively low specific gravity of 0.826 g/cm^3. The glass kernel chamber was seated in a plastic cylindrical base that allowed for connection with a smaller diameter cylinder that served as a reservoir for the gage oil. A small brass plunger with an o-ring seal moved up and down in the smaller cylinder allowing gauge oil to be added or removed from the chamber containing the corn kernel. A mechanism attached to the plunger allowed a dial indicator to be used to measure the displacement of the plunger to the nearest 0.001 mm. The volume of fluid added or removed from the kernel chamber by a given distance of movement of the plunger could be calculated using the inside diameter of the cylinder (5.0038 mm or

0.197 in.). The level of fluid in the kernel chamber was adjusted to a consistent height using a removable point gage incorporated into the top of the container and held firmly in place by a spring mechanism.

The volume of the corn kernels was determined using the liquid displacement method. After the kernel chamber was partially filled with gage oil, the point gage was put in place and the plunger was moved upwards until the gage oil came into contact with the tip of the point gage. Contact could be detected by liquid adhesion of the oil to the tip of the point gage. After contact was made, the position of the plunger was noted on the dial indicator and the plunger was moved downwards. The point gage was removed, the kernel was dropped into the gage oil, the point gage was returned to the top of the kernel chamber, and the plunger was moved upwards until the gage oil once again made contact with the tip of the point gage. The kernel volume was calculated from the difference in the distance the plunger moved and the inside diameter of the plunger.

The accuracy of the volume measurements was evaluated using four precision ball bearings. Two had a diameter of 7.935 mm and the remaining two had a diameter of 7.492 mm. These volumes are similar to the volumes of corn kernels. When the volume of the ball bearing was calculated from the diameter and compared to the volume measured using the micropycnometer, the two values agreed to within 1.0%. The coefficient of variation for three replicate measurements was less than or equal to 0.1%. When measurements were made on corn kernels, the weight of each corn kernel was first determined to the nearest 0.001 g using a precision electronic balance (Denver Instruments, NY, USA). Kernel density was calculated by dividing the measured kernel weight by its measured volume.

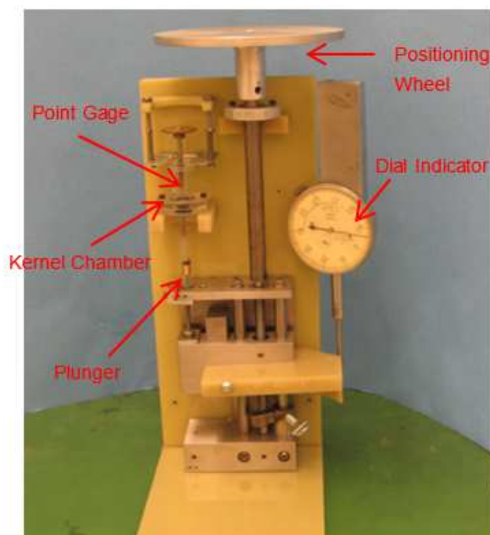

Figure 2.2 Micropycnometer used for measuring the volumes of corn kernels.

2.3.3 Experiment at the Commercial Inbred Seed Processing Facility

The reduction in aflatoxin levels that could be achieved in the 737 kg (1625 lb.) corn lot by means of screening with round hole sieves and by means of a gravity table were determined by cleaning and sorting the corn at a Beck's Hybrids inbred seed processing facility near Sharpsville, Indiana. Figure 2.3 summarizes the cleaning process and the source of samples. Since the corn lot contained a high percentage of fine material, the first step was to remove the fine material by passing the corn through a Clipper screen cleaner (A.T. Ferrell Company, Blufton, IN.) equipped with a 5.16 mm (13/64 in.) round-hole sieve. Normally fines would be removed with a 4.76 mm (12/64 in.) sieve. However, that screen size was not available and the closest available size was used instead. As the corn was being cleaned, a sample of the "overs" was collected by periodically passing a bucket through the grain stream. The "fines" and "overs" that were removed were collected and weighed. Smaller representative samples of both the "fines" and the

“overs” were obtained by repeatedly passing the “fines” or the “overs” through a Boerner divider.

After the fines were removed with the 5.16 mm (13/64 in.) sieve, the remaining whole kernels and larger kernel pieces were again passed through the screen cleaner. However, this time a 6.75 mm (17/64 in.) round-hole sieve was inserted in the cleaner. This separated the corn lot into two streams, one with whole larger kernels and kernel pieces and the other with smaller pieces of kernel and very small whole kernels. The weights of the two streams were obtained and representative samples were collected in the same manner described above. After the first pass through the 6.75 mm (17/64 in.) screen was completed, the two streams were re-combined. Then the composite sample was weighed, the two fractions were mixed using a shovel, and the composite sample was passed through the 6.75 mm (17/64 in.) sieve a second time. As in previous tests, the streams were weighed and representative samples were collected.

After the second screen separation, the two streams were again recombined and mixed, and the composite sample was passed through an LMC gravity table (Lewis M. Carter Manufacturing, Donalsonville, GA.). This separated the corn kernels into two streams, one with a higher density and the other with a lower density. The weight of each stream was determined using an electronic scale. Samples were obtained by periodically sweeping the flowing outlet streams with a bucket and then using a Boerner divider to obtain smaller representative subsamples from each stream. Upon completion of the first pass through the gravity table, the streams were once again combined and the gravity table separation was repeated.

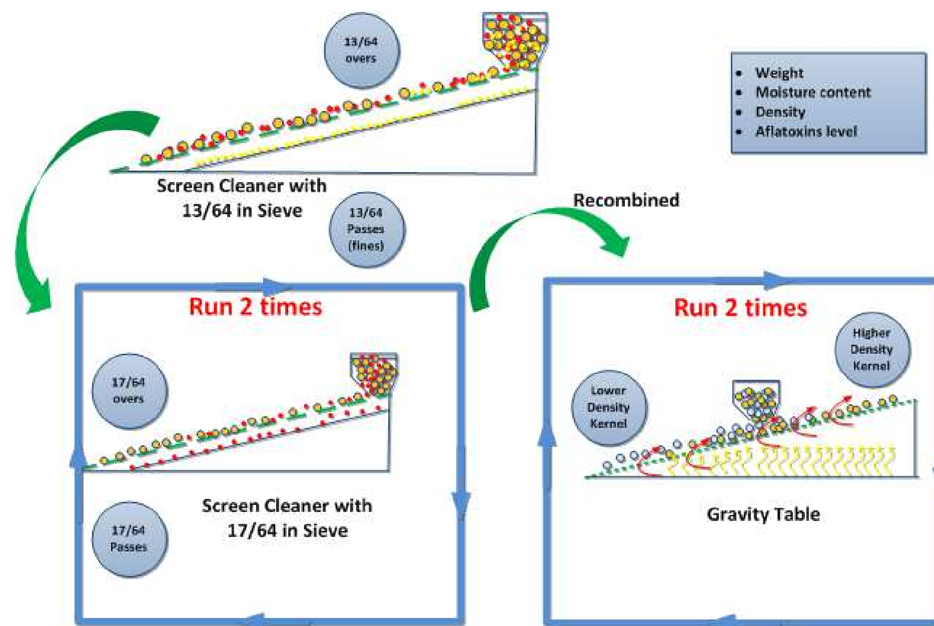

Figure 2.3 Diagram showing size and density segregation tests conducted at a commercial inbred seed processing facility.

2.3.4 Aflatoxin Level Determination

The aflatoxin levels were determined by ELISA assay (Envirologix Inc., USA). A representative sample weighing about 1 kg was taken from the corn lot or from the streams coming from the cleaner or gravity table. The samples were first ground in a Glen Mills Type “C” Table Top Disc Mill (Seedburo Equipment Co., Des Plaines, IL.) using a 20 mesh screen. A 20 g subsample for aflatoxin testing, was obtained by shaking and mixing the bag and then removing 20 g with a spoon. The aflatoxin was extracted by adding 40 ml of 50% ethanol. Ethanol (99% proof) was added to the sample and the mixture was shaken. The particles were allowed to settle until there were two distinct layers (10 to 30 min.). Then 100 µl top layer extract was pipetted and mixed with buffer solution, and dipped with a commercial lateral flow Devices (LFDs) QuickTox test strip

(AQ109BG) for 5 min, aflatoxin levels were then determined from the color change of the test strip using a color reader (Envirologix Inc., USA). The reader has a detection range of 0 to 30 ppb. Aflatoxin levels higher than 30 ppb were determined by dilution. Serial extract dilution factors used were 6, 18, and 54, as recommendation by the manufacturer. AQ109BG test strips (Envirologix Inc., USA) were used for aflatoxin quantification in corn. According to the manufacturer, the COV of this quantification method is around 10%. The CV (RSD) of the this quantification method is around 10% and is certified by the USDA in the Addendum Certificate No. FXIS 2013-047.1.

2.3.5 Statistical Analysis

Statistical analyses were performed using SAS 9.3 (SAS Institute Inc, USA) software. The t-test procedure was used to compare the differences in mean values of physical properties between the moldy and healthy corn kernels (p=0.01). The Kolmogorov-Smirnov (or K-S) test, available in the SAS software's NPAR1WAY procedure, was used to compare the attribute distributions of the healthy and moldy corn kernels. The K-S procedure calculates a test statistic "D" which is a simple measure of whether the difference between two distributions is statistically significant. It is defined as the maximum value of the absolute difference between the two cumulative distribution functions $S_1(\mathrm{x})$ and $S_2(\mathrm{x})$:

$$D = \max_{-\infty < x < \infty} \left| S_1(\mathrm{x}) - S_2(\mathrm{x}) \right| \qquad \text{(Eq. 2.2)}$$

2.4 Results and Discussion

2.4.1 Difference in Size, Shape and Density of Healthy and Aflatoxin Contaminated Corn Kernels

Means of the diameters, sphericity, and density of healthy kernels and kernels invaded with mold are shown in Table 2.1. The differences between moldy and healthy corn kernels were statistically significant for two of the diameters, and for the sphericity and density. For this particular corn lot, the moldy corn kernels had a smaller major diameter and a larger minor diameter. They also tended to have a greater sphericity and a lower density. All the above differences were statistically significant at an alpha level of 0.01 as determined using a 2 tail t-test. However, the difference between the intermediate diameters of the moldy and healthy corn kernels was not statistically significant at an alpha level of 0.01. The observed differences may be the result of the portion of the ear that was most often invaded by the mold. The invasion occurs most often near the tip of the ear where the kernels are often more spherical ("rounds") and smaller. The greatest difference between moldy and healthy kernels was in the major diameter. Therefore, among the three diameters, segregation on the basis of major diameter would give the best results for this particular corn lot. There was also a significant difference in sphericity between the two fractions suggesting that separation according to this attribute may also be effective in reducing the aflatoxin level in the corn lot. Unfortunately, the equipment needed for sorting on the basis of major diameter and sphericity was not available at the seed corn processing facility used for sorting the corn lot. Therefore, in order to test the separation result based on minor diameter, a representative sample

(≈3kg) was pulled and run through a slotted sieve in table top clipper cleaner (Clipper Office Tester, A.T. Ferrell Company, Blufton, IN).

Differences in density were also observed for the sample that was evaluated, the moldy kernels had a lower density than the healthy kernels. Kernel density is lowered when the fungi break down the cellular structure of the kernels and consume nutrients. The observed differences indicate that segregation based on kernel density also has potential.

The distributions of the measurements of the three diameters, the sphericity, and the density of the moldy and healthy corn kernels are shown in Figure 2.4. The results of the K-S tests are summarized in Table 2.2. There is a significant difference (P<0.0001) between the distributions of the moldy and healthy kernels for all physical properties measured, except the intermediate diameter. Kernel density and major diameter had the largest D value (indicating largest difference), followed by sphericity and minor diameter.

Table 2.1 Means of dimensions, sphericities and densities of healthy and moldy kernels from the corn lot.

Corn Sample	Size*			Sphericity	Density (g/cm^3)
	Major Diameter (mm)	Intermediate Diameter(mm)	Minor Diameter(mm)		
Healthy	12.016 ±1.226[a]	7.842 ±0.854[a]	5.337 ±0.891[a]	0.644 ±0.079[a]	1.215 ±0.092[a]
Moldy	10.551 ±1.261[b]	7.963 ±0.928[a]	5.969 ±0.966[b]	0.757 ±0.093[b]	1.147 ±0.101[b]

*means with a different superscript letter indicate significant different at 0.01 probability level.

Table 2.2 Results of K-S test to indicate whether there are differences between healthy and moldy kernels in the distributions of the major, intermediate, and minor diameters, the sphericities and the densities.

K-S test statistics	Size			Sphericity	Density (g/cm^3)
	Major Diameter (mm)	Intermediate Diameter(mm)	Minor Diameter(mm)		
D	0.4989	0.1104	0.3424	0.4643	0.5099
P	<0.0001	0.4445	<0.0001	<0.0001	<0.0001

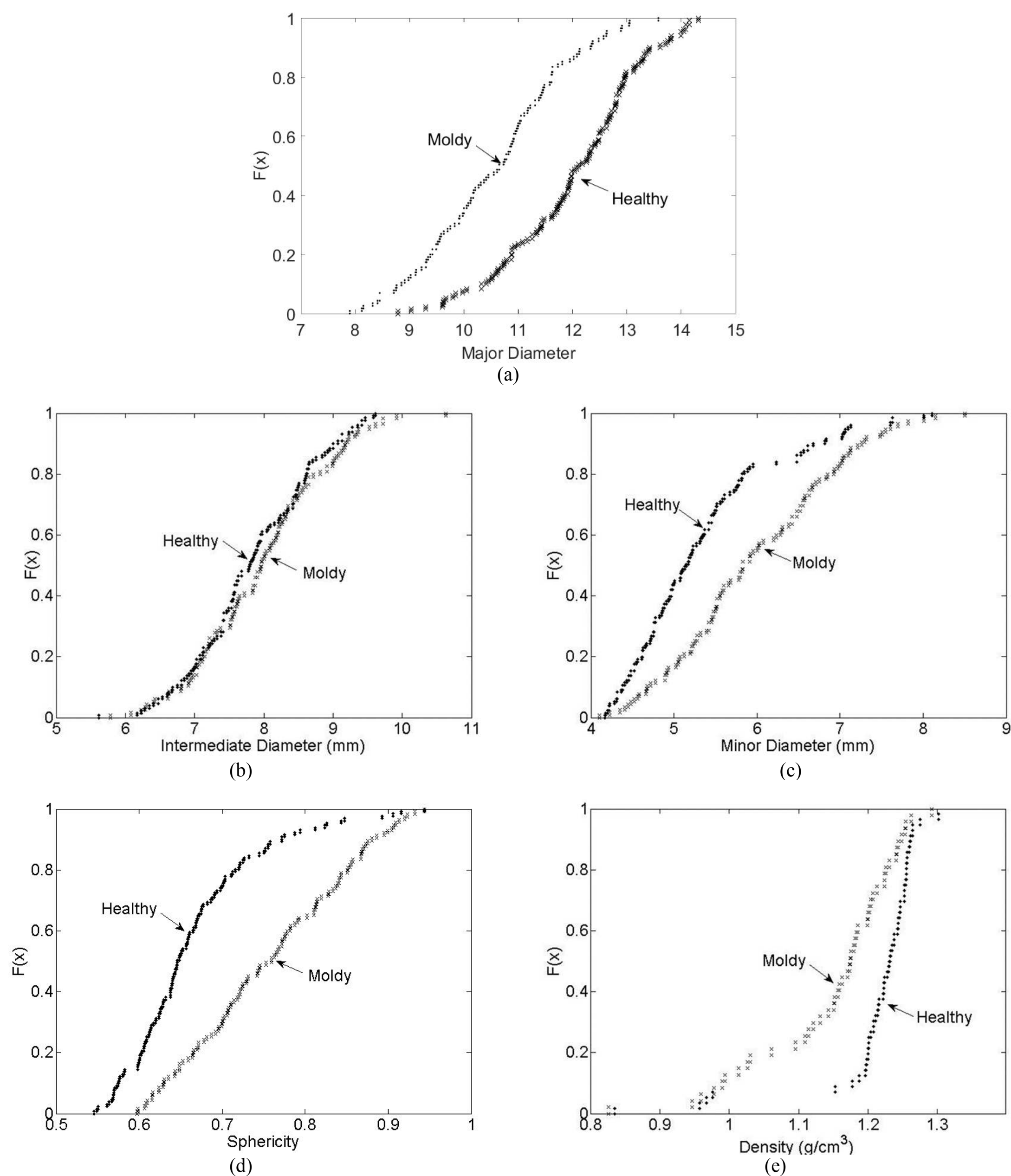

Figure 2.4 Cumulative Density distributions for both healthy and moldy kernels of the following attributes: a) major diameter, b) intermediate diameter, c) minor diameter, d) sphericity and e) kernel density.

2.4.2 Aflatoxin Reduction by Size and Density Segregation Using a Screen Cleaner and Gravity Table

This section reports the results of the tests conducted at the seed corn processing facility. The weight percentages of kernels, kernel pieces, and fine material removed by screening and the gravity table are shown in Table III 3 along with the levels of aflatoxin in each of the samples. The 737 kg corn lot contained approximately 10% fine material, which is an unusually high percentage. This can be explained by the fact that the corn came from a bin that was filled by "coring" the other bins being used for storage of the type of grain being tested. Coring means that corn was withdrawn from the center well of bins. The corn withdrawn from the storage bins came from beneath the spout lines of the bins, where fines tend to collect. The fine material had an extremely high aflatoxin level (1404±143 ppb), which is approximately 46 times higher than the level in the kernels and larger kernel pieces. A possible explanation is that kernels damaged by Aspergillus ear rot tended to break into small pieces (fine material) during harvesting and handling because the invasion by mold weakened them and made them more brittle. Also, broken kernels and fines are more susceptible to invasion by molds than healthy whole kernels with intact pericarps. Removing the fine material (10.1% of the weight of the sample) reduced the aflatoxin levels by 83.8%.

The aflatoxin level was also higher in the small kernels and kernel pieces that were removed with the 6.75 mm (17/64 in.) round hole sieve. It should be noted that, although round-hole sieves can be used to separate according to intermediate diameter, the 6.75 mm (17/64 in) sieve was removing larger kernel pieces and very small kernels because 6.75 mm is more than one standard deviation below the mean minor diameter of

the moldy kernels (Table 2.1). The sieve removed around 3 percent by weight of the shelled corn and reduced the aflatoxin levels by another 1.8 and 9.4% of the original level in the first and second passes, respectively (Table 2.3). If it is assumed the intermediate diameter is normally distributed with a mean of 7.84 mm and a standard deviation of 0.928 mm (Table 2.1), then 10% of the kernels should have fallen through the larger of the two round-hole sieves. The fact that the removal percentage is much lower than 10% may mean that the sieving was inefficient and did not remove all of the kernels that could have fallen through the sieve.

Although there was a noticeable variation in the aflatoxin levels in the two streams from the two passes through the 6.75 mm (17/64 in) screen, in both tests the amount of aflatoxin in the smaller particles was always greater. It was approximately 7 times greater for the first pass and approximately 15 times greater for the second pass through the sieve. Although the aflatoxin level in the healthy corn coming from the screen cleaner on the first pass was 27 ppb, which is above the FDA aflatoxin limit of 20 ppb, the second pass reduced the aflatoxin level to only 13 ppb, which is below the FDA limit of 20 ppb. The differences in the results from the two tests may have been caused by a random sampling error. The presence of a single contaminated kernel can greatly increase the aflatoxin level in a sample.

Screening through round hole sieves is based on intermediate diameter. Although initial evaluation indicated that there is no significant difference in intermediate diameter between moldy and healthy corn kernels, a significant reduction of aflatoxin level was achieved by screening through the 6.75 mm (17/64 inch) round hole sieve. A more careful examination of the material removed by the sieve revealed that it was mainly

(74% by w.t.) composed of pieces of broken corn. The distribution shown in Figure 2.4 is only for whole kernels. Further aflatoxin analysis revealed that the aflatoxin level in the broken pieces (152 ppb) was much higher than the level in the whole kernel fraction (32ppb). A follow up experiment was conducted using the table top Clipper cleaner. A representative sample taken from the corn that was retained by the 5.16 mm (13/64 inch) screen at the seed corn processing facility, was passed over both a 6.75 mm slotted and a 6.75 mm (17/64 inch) round hole sieve. Aflatoxin levels are shown in Table 2.4. The percentages of material retained on top of the Slotted sieve were greater, although comparable in magnitude, than the percentage that passed through the round hole sieve. However, the aflatoxin level of the kernels retained by the 6.75 mm (17/64 inch) slotted sieve was over twice the level in the material that passed through the 6.75 mm (17/64 inch) round hole sieve. Thus sorting the corn kernels by slotted sieve based on minor diameter achieved greater reduction in aflatoxin in corn than sorting by round hole sieve which is based on intermediate diameter. This is in agreement of the results of our initial size evaluation of the corn kernels: the difference in minor diameter is larger than in intermediate diameter between moldy and healthy corn kernels. And based on these results, slotted sieve works better than round hole sieves for sorting this specific corn slot.

The results from the two gravity table tests show that removing the lower density kernels, about 5% percent by weight of the sample, reduced the aflatoxin level by 12.6 and 16.4%. The lower density kernels removed by the gravity table had much higher aflatoxin levels than the higher density kernels (>50 times). Furthermore, for the first pass through the gravity table, the aflatoxin level of the higher density kernels was only 3.8% of the 185 ppb level found in the original corn lot. For the second pass through the

gravity table, higher density kernels had an aflatoxin level below the detection limit of the LFDs test strips.

In both the tests with the 6.75 mm (17/64 inch) round hole sieve and the gravity table, the aflatoxin level in the healthy corn from the second pass was lower than it was for the first pass through the device. In these tests approximately 1kg of moldy kernels containing high aflatoxin levels was removed from the bulk sample for testing. It is possible that removal of these moldy kernels may have influenced the second pass. Another possibility is that when the two fractions that had been separated during the first pass were mixed together prior to the second pass, the two fractions remained partially segregated and that this segregation made the removal process more effective.

Another observation is that the gravity table reduced the aflatoxin levels more than the 6.75 mm (17/64 inch) round hole screen that was used in the screen cleaner. The kernels removed by the gravity table had a higher aflatoxin level than the kernels removed by the screen. The gravity table also removed a larger percentage by weight of kernels. However, the density segregation tests using the gravity table took 1.5 to 2 times longer than the screen cleaning.

Table 2.3 Summary of aflatoxin reductions achieved using a screen cleaner with two different round-hole sieves and a gravity table.

Process	Percent Removed[a] (wt, %)	Percent Reduction[b] (%)	Aflatoxin level[c] (Mean ± Std, ppb)	
			Retained	Removed
Screen 5.16 mm (13/64 in)	10.1	83.8	30 ±8	1404 ±143
reen 6.75 mm (17/64 in) (Pass No. 1)	3.3	1.8	27 ±1	246 ±27
creen 6.75 mm (17/64 in (Pass No. 2)	2.8	9.4	13 ±2	197 ±32
Gravity Table (Pass No. 1)	4.8	12.6	7±1	384 ±27
Gravity Table (Pass No. 2)	5.4	16.4	<LOD[d]	342 ±31

[a] Percentage of material removed is based on weight of original sample
[b] Percentage reduction of aflatoxin is based on initial aflatoxin level in original sample
[c] Aflatoxin level in original sample was 185 ±28 ppb
[d] LOD indicates limit of detection

Table 2.4 Aflatoxin reduction in representative corn sample by passing through 17/64 inch slotted and round-hole sieve in a lab clipper screen cleaner.

Sieve type	Sample fraction	Weight (g)	Weight pert (%)	AF level (ppb)
Slotted sieve	>17/64 in	63.9	4.6	65
	<17/64 in	1302.6	95.4	14
Round hole sieve	>17/64 in	2926.8	98.5	24
	<17/64 in	43.98	1.5	32

2.4.3 Kernel Densities and Density Distributions in Fractions Separated Using the Gravity Table

Table 2.5 summarizes the results of density tests on subsamples taken from the two streams from both the screen cleaner and the gravity table. The mean density of the "overs" from the 6.75 mm (17/64 in.) screen was only slightly higher than the density of the kernels that passed through that sieve (by 0.015g/cm3). This difference in density was not statistically significant. However, there was a substantial difference in density between the two samples separated by the gravity table (0.1g/cm3) and the difference was statistically significant.

The moisture contents of all the samples are also reported in Table 2.5. They were close to each other with the exception of the lower density fraction from the gravity table. One explanation for this lower moisture is that it contained a higher percentage of broken kernels, which had a lower moisture content. The moisture content of fines was 14.81% ±0.04%, which is also lower than that of whole and higher density fractions.

To further characterize the effectiveness of density separation using the gravity table, histograms showing the distributions of the densities were plotted for the original sample, and for the higher density and lower density fractions (Figure 2.5a, b, and c, respectively). Cumulative density distributions for all three samples, which show the total percentage of kernels with a density less than a given value are shown in Figure 2.5d. The

density of the original sample and the higher density fraction from the gravity table were similar to a normal distribution, whereas the density distribution of lower density kernels was skewed toward the left. The cumulative density distribution of the original sample is between those of the higher density and lower density kernels (Figure 2.5d), but is much closer to the distribution of the higher density fraction. This is reasonable, because the light kernels constituted only around 6% of the total weight of the corn lot that was tested. Although there was considerable overlap between density distributions of higher and lower density corn kernels, there are still obvious differences between their cumulative distributions. For the higher density and original samples, the percentage of kernels with a density less than the given density increases dramatically at a kernel density of approximately 1.15 g/cm3. Therefore, this density would potentially give the best separation between moldy corn kernels and healthy corn kernels.

Table 2.5 Means of kernel densities and moisture contents of the original and sorted samples

Sample	Density(*g/cm*^{3})[a]	Moisture Content (Mean± STD, %)[a]
Original sample	1.212 ±0.065[a]	15.11 ±0.04[a]
Screen 5.16 mm (13/64 in.) overs	1.213 ±0.058[a]	15.19 ±0.03[a]
Screen 6.75 mm (17/64 in.) overs	1.221 ±0.063[a]	15.24 ±0.01[a]
Screen 6.75 mm (17/64 in.) passess	1.206 ±0.101[a]	15.03 ±0.04[b]
Gravity table higher density kernels	1.218 ±0.070[a]	15.02 ±0.10[b]
Gravity table lower density kernels	1.118 ±0.162[b]	14.66 ±0.06[c]

[a] Different subscripts in the columns indicate significant difference at alpha level 0.05

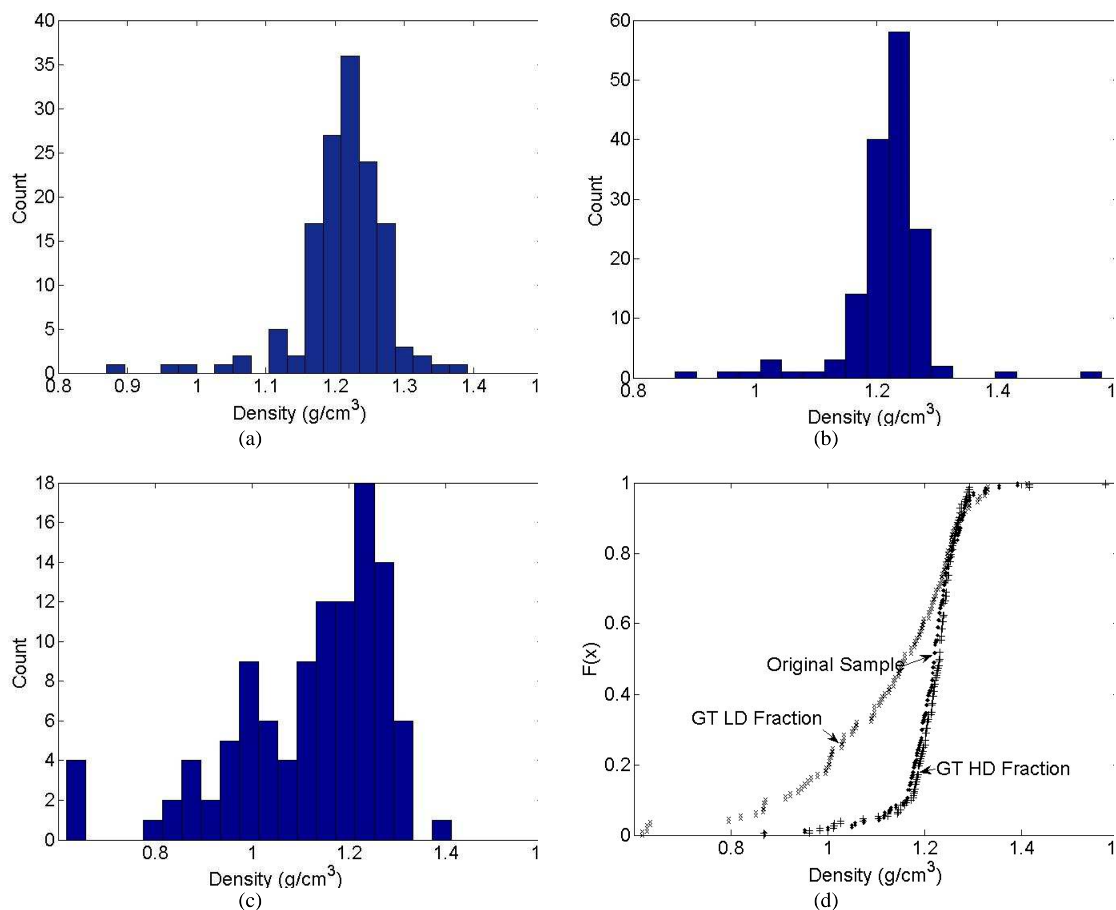

Figure 2.5 Histograms showing the kernel density distribution for: a) original sample, b) higher density fraction from the gravity table, c) lower density fraction from the gravity table, and d) the cumulative density distributions.

Note: In Fig 5d, GT LD Fraction indicates gravity table lower density fraction, and GT HD Fraction indicates gravity table higher density fraction)

2.5 Conclusions

In this study, the size and density distributions of corn kernels in representative samples taken from a 737 kg corn lot were determined. There were statistically significant differences in major and minor diameters, the sphericities and the densities between moldy and healthy corn kernels in this particular corn lot.

The corn lot had a high percentage of fines (around 10%) and removal of the fines using a 5.16 mm (13/64 in) round-hole sieve considerably reduced the aflatoxin levels in the lot. Removal of smaller kernels using a 6.75 mm (17/64 in.) round-hole sieve reduced aflatoxin levels further. When the material that was removed with the 6.75 mm sieve was added back to the sample, density segregation using a gravity table also gave a reduction in aflatoxin levels in the corn lot that remained. In one of the two passes through the 6.75 mm (17/64 in.) round-hole sieve and in both passes through the gravity table, the aflatoxin level was reduced to below the 20 ppb action level limit set by the FDA. Analysis of the kernel dimensions revealed that there were the least differences between the intermediate diameters of the moldy and healthy kernels. However, separation with the larger of the two round-hole sieves, which segregates according to intermediate diameter, made a substantial reduction in aflatoxin levels. Examination of the material that was removed with the sieve revealed that it consisted of pieces of kernels along with very small kernels that probably came from the tips of the ears. Those fractions of the original grain mass contained higher levels of aflatoxin.

The gravity table was more effective in removing moldy corn kernels and reducing the aflatoxin level than was the 6.75 mm (17/64 in.) sieve. The results suggest that it would be possible to reduce the aflatoxin levels in the corn used in this study to acceptable limits using a screen cleaner to remove fine material followed by a gravity table to remove lower density kernels.

There are several factors that could explain the differences in effectiveness of size and density segregation in reducing aflatoxin levels in corn as reported in the literature cited in the introduction to this paper. One factor is aflatoxin contamination level.

Kernels with lower aflatoxin contamination levels are probably less susceptible to breakage during harvesting. In the corn lot tested in this study, there were high levels of aflatoxin in the fine material and larger broken pieces that were removed by the sorting equipment. The highly contaminated kernels present prior to harvest could have been broken by the high velocity impacts that occurred during combine harvesting and subsequent handling. Also, when mold damage is less severe, there will be smaller changes in kernel density. A second factor that could alter the effectiveness of size and density separation is corn hybrid. It is possible that the techniques used in this study would not work as well for corn hybrids that have fewer small round kernels at the tips of the ears where invasion is most likely to occur. Finally, if aflatoxin contamination occurs during storage rather than prior to harvest, size and density separation may be less effective because the contaminated corn may not have been subjected to high velocity impacts that would break severely contaminated kernels. These observations mean that similar tests should be conducted on additional corn lots with thorough documentation of the corn hybrid or hybrids that make up the lot, the growing conditions encountered, and the harvesting, handling, and storage history.

In this study, the discolored corn kernels are considered to be moldy, however in future study it is recommended to further distinguish the moldy corn kernels from the discolored corn kernels based on fungal profiling and color measurement. The authors also recommend that studies of the effectiveness of various segregation techniques be accompanied by analysis of the size and density distribution of the kernels. This will facilitate interpretation of the results. Another implication of this study is that an initial evaluation of the properties of healthy and moldy kernels taken from a given corn lot

could be used to identify the techniques that offer the greatest potential for removal of the moldy kernels and reduction of aflatoxin levels. Furthermore, the same approach could be used to identify the best methods for cleaning shelled corn that is contaminated with species of fungi that produce mycotoxins other than aflatoxin.

2.6 References

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CHAPTER 3. REDUCTION OF AFLATOXIN BY CONVENTIONAL AND MICROWAVE HEATING

In this chapter, thermal stability of aflatoxin B_1 was investigated during conventional heating (convection oven, water bath) and microwave heating. The influence of water on aflatoxin degradation was investigated, and the reaction mechanism of dry heating and wet heating (without presence of water molecule) was clarified. The question of whether there are non-thermal effects from microwave on aflatoxin degradation was answered.

3.1 Reduction of aflatoxin B_1 by dry and wet conventional heating

3.1.1 Background: thermal stability of aflatoxin

Aflatoxins are very thermal stable and Aflatoxin B_1 has a melting point as high as 268 ºC (Haynes, 2010). Decomposition temperatures of aflatoxins range from 237 to 306°C. Under dry heating condition, aflatoxin B_1 is quite stable at temperature below its decomposition point. When heated to the point of decomposition, it emits acrid smoke (Lewis, 2004). Destruction of aflatoxin B_1 is only apparent above temperature of 250°C, at which the isolated aflatoxin breaks down (Feuell, 1966). AFB_1 started to degrade at a temperature of 250°C when cooked in artificially contaminated corn oil or naturally contaminated peanut oil (Peers and Linsell, 1975).

However, there are also several studies reported that aflatoxin was reduced by thermal operations at temperature below 250 ºC. Heating Pure aflatoxin B_1 at 150°C for 1h in furnace reduced 70% of its initial level, a treatment at 180°C for 1h led to complete degradation of aflatoxin B_1 (Raters and Matissek, 2008). Aflatoxin reduction in foods, feeds, and nuts by food processing operations were summarized in Table 3.1. For dry heating operations, including frying, roasting, baking and oven heating, with temperature ranges from 120 to 200 ºC, the percent reduction of aflatoxin was between 30% and 100% complete degradation depending on the food substrate involved, heating temperature, and treatment time applied. Temperatures of 150 ºC are generally required for aflatoxin to start to degrade under dry heating thermal operations (Table 3.1). Moisture content is an important factor for degradation of aflatoxin in thermal operations. Aflatoxin degradation in cottonseed and peanut meal was increased with higher moisture content. Heating cottonseed with moisture content of 6.6, 15.0, 20, 30% at 100 °C for 1h

reduced the aflatoxins (AFB_1+AFB_2) by approximately 33, 45, 50, and 75% (Mann, Codifer et al. 1967). For thermal operation involving wet heating (heating with water), including cooking food with water and boiling, aflatoxin in food was reduced by 10 to 80% with temperatures at or below 100 ºC (Table 3.1). Comparing the temperature required to start the degradation of aflatoxin under dry and wet heating (≥150 and ≤100 ºC respectively), it seems plausible that different mechanisms are involved in dry and wet heating. The mechanism of aflatoxin by dry heating has not been clarified. It has been suggested that during wet heating, the water molecule helps in opening the lactone ring in AFB_1 to form a terminal carboxylic acid. The terminal acid group then undergoes heat induced decarboxylation (Mann, Codifer et al. 1967, Samarajeewa, Sen et al. 1990). However, the exact reaction mechanism and degradation products have not been clarified.

In this part of the study, the goals were to determine the temperature required for aflatoxin degradation by dry heating; to identify the degradation products; and to clarify the reaction mechanism of aflatoxin degradation by dry and wet heating.

Table 3.1. Aflatoxin reduction in food, feed, and nuts in food processing thermal operations.

Treatment methods	Substrate	Degradation (%)	Ref
Frying at 150 ºC for 30 min	Unrefined oil	Half aflatoxin was degraded	(Dwarakanath, 1969)
Baking at 120 C for 30 min	Wheat flour	Degradation during kneading, insignificant degradation during baking	(Reiss, 1978)
Heating at 150°C 30min	Dried wheat	50% degradation	(Hwang and Lee, 2006)
Heating at 200°C 30min	Dried wheat	90% degradation	(Hwang and Lee, 2006)
Oil roasting at 325 °F 6 min	Peanuts	AFB1 83% reduction, AFG1 76%	(Lee et al., 1969)
Dry roasting at 250°F 7 min	Peanuts	AFB1 69% reduction, AFG1 67% reduction	(Lee et al., 1969)
Roasting at 191 °C 15 min	Pecan	80% reduction	(Escher et al., 1973)
Roasting at 191 °C 15 min	Pecan meal	80% reduction	(Escher et al., 1973)
Roasting at 145 to 165 °C	corn	40-80% reduction	(Conway et al., 1978)
Oven roasting 150°C, 30 min	peanuts	30-45% AFB1	(Pluyer et al., 1987)
Roasting at 200° C	Coffee beans	93%-complete degradation	(Micco et al., 1991)
Roasting at 150-180° C for 10-15 min	Green coffee beans	42.2-55.9% reduction	(Soliman, 2002)
Roasting 90-150°C, 30-120 min	Peanuts	Max reduction AFB1 78.4%, AFB2 57.3%, AFG1 73.9% ,and AFG2 75.2%	(Arzandeh and Jinap, 2011)
Roasting 150°C at 30min or 120 C at 120min	Pistachio nuts	63% degradation	(Yazdanpanah et al., 2005)
Normal cooking 30 min	Rice	49% reduction	(Rehana et al., 1979)
Cooking with excess water	Rice	81.6% reduction	(Rehana et al., 1979)
Preparation of porridge, roti, balls and popcorn	corn	About 50% reduction	(Rehana and Basappa, 1990)
boiling	Corn meal grit	28% reduction	(Stoloff and Trucksess, 1981)
Salt boiling at 116 °C, 0.7 bar 30min	Unshelled peanut	80% to complete degradation	(Farah et al., 1983)
Cooking porridge	Corn and adlay	10-30% reduction	(Tabata et al., 1992)
Normal cooking	rice	AFB1 34% reduction	(Park et al., 2005)

3.1.2 Material and methods

3.1.2.1 Chemical agents

Aflatoxin B1 was purchased from Cayman Chemicals Inc. (Ann Arbor, MI, USA). Chloroform and 200 proof Ethanol were obtained from the campus store. AFB_1 powder was dissolved in chloroform and serially diluted to a concentration of 50 µg/ml, and the AFB1 standard solution was stored at -5 °C in a freezer prior to conducting the tests.

3.1.2.2 Treating aflatoxin by dry and wet heating

The AFB_1 samples were prepared by pipetting 100 µl standard AFB_1 solution (50 µg/ml in chloroform) into a small glass bottle. The glass bottle was rinsed 3 times beforehand with 50% ethanol solution to avoid contamination. The sample was then immediately transferred to a fume hood where it was kept for 1.0 h so that the chloroform could completely evaporate.

For the dry heating treatment, AFB_1 samples were placed in a convection oven (American Scientific Products DN-42). The temperature of the convection oven was set as 80, 120, 150, or 200 ºC for different samples. For wet heating, 2 ml distiller water was pipetted into the glass bottle containing AFB_1 and the glass bottle was covered with foil and placed in a water bath **()**. The temperature of water bath was set at 80 ºC and the samples were heated for 1 h.

3.1.2.3 HPLC- MS Analysis.

Wet heated AFB_1 samples were placed in a fume hood for 3 days to fully evaporate the residual water. Then all AFB_1 samples (Dry heated, wet heated, control) in

the glass bottle were carefully rinsed multiple times with 1 ml of a 50% ethanol aqueous solution to extract the AFB1 and degradation products.

The extracts were then transferred to an Eppendorf tube (2.0 ml) and stored at -5°C in a freezer before being used for HPLC MS (mass spectrometry) analysis. HPLC-MS data on degradation products of AFB1 were obtained using a time-of-flight (TOF) instrument system (Agilent Technologies, USA) equipped with an 1100 series binary solvent delivery system and an autosampler. Chromatography was performed using a 2.1×150 mm, 3.5 um particle Waters Xterra C18 column. The injection volume was 10 µL, the flow rate was 300 µL/min. The mobile phase was a gradient prepared from water with 0.1% formic acid (component A) and acetonitrile with 0.1% formic acid (component B). The gradient elution started with 10% B for 1 min, then B was increased linearly to 95% in 20 min, and kept isocratic for 1 min. The proportion of B was then decreased back to 10% in 1 min and kept isocratic for 7 min. The total run time was 30 min. The MS was run with positive electrospray ionization (ESI), and data was collected over the range of 75 – 1000 m/z. High mass accuracy was insured by infusing a lock mass calibrant corresponding to 121.0508 and 922.0098 m/z.

3.1.2.4 Molecular formula proposition and structure identification

Because AFB1 was treated by HVACP in a pure system (without food substrate), AFB1 and its degradation products could only be composed of 3 elements: Carbon, Oxygen, and Hydrogen. The molecular formulas were proposed according to the isotope distribution patterns and exact mass by using MassHunter Qualitive Analysis software (Agilent Technologies, USA). The molecular formula with the highest agreement score is

selected as the molecular formula of the degradation product. The structures of degradation products were proposed taking into account of the molecular formula and structure of aflatoxin, as well as principles of chemical reaction mechanisms.

3.1.3 Results and discussion

3.1.3.1 Thermal stability of aflatoxin B_1 by dry heating in convection oven

Percent reduction of aflatoxin by dry heating at varying temperature is shown in Figure 3.1. AFB_1 was intact by dry heating at temperature 80, and 120 ℃, AFB_1 starts to degrade by dry heating at temperature 150 ℃. AFB_1 was reduced by 32 and 55% with dry heating at temperatures of 150 and 200 ℃ for 1 h.

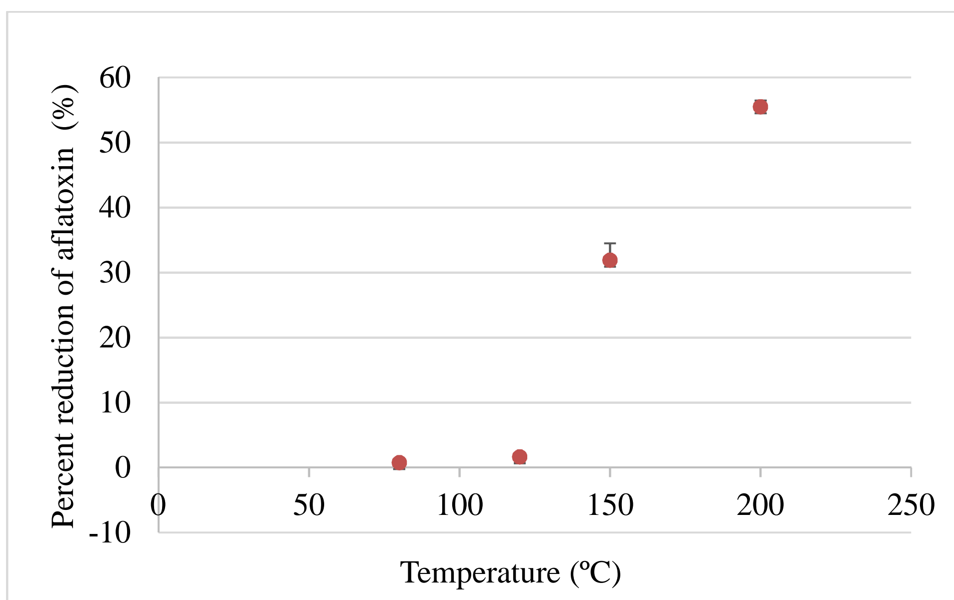


Figure 3.1. Percent reduction of aflatoxin by dry heating in a forced convection oven with varying heating temperatures.

The chromatograph of aflatoxin B_1 dry heated at 80 ℃ for 1h in a convection oven is shown in Figure 3.2. The chromatograph of the AFB_1 sample heated at 80 ℃

follows the chromatograph of AFB_1, AFB_1 was intact after dry heating at 80 C for 1h, as indicated by the absence of peaks for degradation products. The chromatograph of aflatoxin B_1 dry heated for 1 h at higher temperatures of 150 and 200 ºC in a convection oven is shown in

Figure 3.3. One new peak (peak 1) accounting for the degradation product appeared when AFB_1 sample that was dry heated at 150 and 200 ºC.

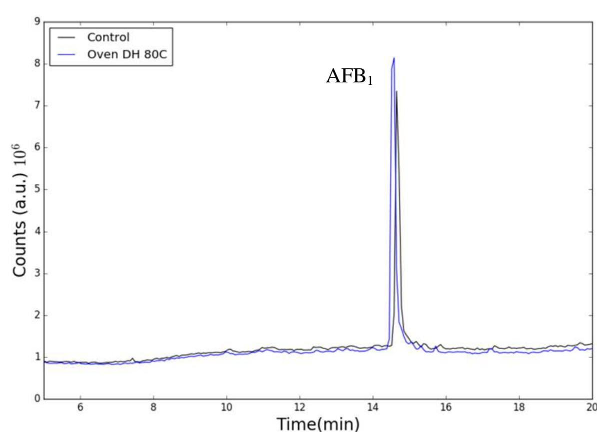


Figure 3.2. Chromatograph of aflatoxin B_1 sample dry heated at 80 ºC for 1 h in a forced convection oven

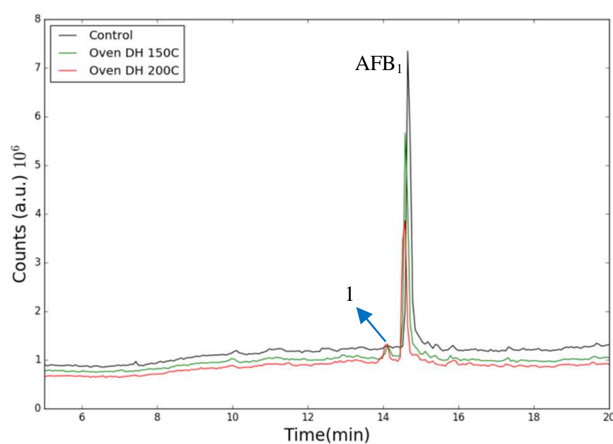


Figure 3.3. Chromatograph of aflatoxin B1 sample dry heated at 150 and 200 ºC for 1 h in a forced convection oven.

3.1.3.2 Thermal stability of AFB_1 by wet heating in water bath

Based on the peak area, about 73% AFB_1 as measured by AFB_1 peak area was degraded by wet heating at 80℃ for 1h. The chromatograph of AFB1 wet heated at 80 ℃ for 1h in a water bath is shown in Figure 3.4. Two new peaks (peak 1 and peak 2), which correspond to new degradation products, appeared in the chromatograph.

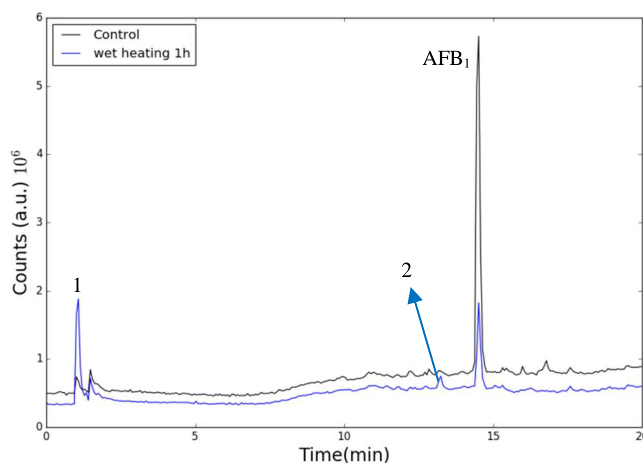


Figure 3.4. Chromatograph of AFB_1 wet heated at 80 ℃ for 1h in a water bath.

3.1.3.3 Molecular formula and structure of degradation products of AFB_1 by dry heating and wet heating

The data of degradation products of AFB_1 by dry heating, including retention time, proposed formula, experimental mass, mass error and agreement score (the overall score is 0- 100%, and the score closer to 100% being better) is listed in Table 3.2. Similarly, the data of degradation products of AFB_1 by wet heating is listed in Table 3.3.

The degradation product of AFB_1 by dry heating was found to have the same chemical formula as AFB_1.This means that the degradation product of AFB_1 by dry

heating is an isomer of AFB_1. In this case, they are enantiomers, which are two stereoisomers that are related to each other by a reflection. In other word, they are mirror image of each other that are non-superimposable (L.G. Wade, 2011). During the dry heating process, racemization of AFB_1 occurred. Racemization is the process in which one enantiomer of a compound converts to the other enantiomer. Racemization is common inorganic compounds to which heat is applied. For example, racemization of glutamic acid by heating at 180 -200 ℃ (Arnow and Opsahl, 1940), the mechanism of racemization of hydroxyl-acid by heat has already been clarified (James and Jones, 1912).

Table 3.2. The proposed formula of AFB_1 degradants by dry heating at 150 ℃ for 1h.

Proposed Products	Retention time (min)	Proposed Formula	Observed mass (m/z) [a]	Diff (ppm)	Score (%)
1	14.1	$C_{17}H_{12}O_6$	313.0697	-2.78	99.2
AFB_1	14.6	$C_{17}H_{12}O_6$	313.0704	-2.32	99.4

There were four major degradation products from AFB_1 treated by wet heating at 80 ℃ for 1 h (Table 3.3). The structure of these degradation products was presented in Figure 3.5. The degradation products are the results of breaking down the structure of the AFB_1. Their structures resemble parts of the structure of AFB_1. Furofuran moiety and lactone ring structure are essential for the toxicity and carcinogenicity of AFB_1 (Lee et al., 1981;Wogan et al., 1971). The degradation products (1, 2, 3) shown in Figure 3.5 are simple molecules that do not possess any of the toxic structure of AFB_1. Degradation product 4 still possess the lactone ring structure but not the furofuran moiety. Therefore, according to above mentioned structures-biological activity relationship from the

literature, the toxicity, carcinogenicity and mutagenicity of the degradation products from wet heating will be reduced substantially compared to AFB_1.

Table 3.3. The proposed formula of AFB_1 degradants by wet heating at 80 ℃ for 1h.

Proposed Products	Retention time (min)	Proposed Formula	Observed mass (m/z) [a]	Diff (ppm)	Score (%)
1	1.1	$C_4H_6O_2$	86.0892	-2.98	95.12
2	1.1	$C_6H_6O_3$	125.9863	-1.82	98.9
3	1.1	$C_9H_{10}O_3$	167.0130	-3.64	93.62
4	13.2	$C_{14}H_{10}O_6$	275.0559	-3.85	94.25
AFB_1	14.5	$C_{17}H_{12}O_6$	313.0716	-2.52	99.1

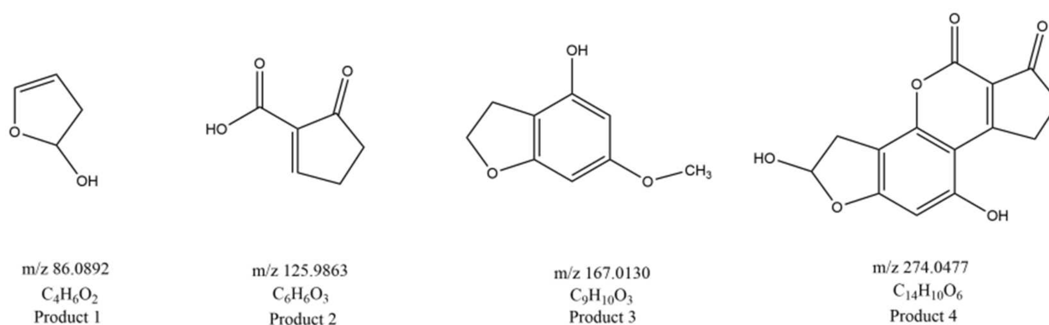


Figure 3.5. Proposed structure of AFB_1 degradants by wet heating in a wet bath at 80 ℃ for 1 h.

3.1.3.4 Degradation pathway of AFB_1 by wet heating

Based on the structure of the four degradation products of AFB_1 by wet heating, degradation pathway shown in Figure 3.6 was proposed. The first and second branch of reaction involves hydrolysis of ether group in the furofuran moiety of AFB_1 and resulted in generation of products 1 and 4. The ether group could go through acid catalyzed hydrolysis by heating (L.G. Wade, 2011). The third brand of reaction starts with hydrolysis of lactone ring of AFB_1 and produced product 2 and an intermediate product

($C_{11}H_{10}O_4$). This intermediate product is further decomposed into product 3 through decarboxylation. Lactone rings are prone to be hydrolyzed by heating, the water molecule was served as base catalyst for opening the lactone ring (Gomez-Bombarelli et al., 2013). Thus our results confirmed that what was proposed by previous research that aflatoxin is reduced by wet heating through hydrolysis of lactone ring and further decarboxylation (Mann et al., 1967;Samarajeewa et al., 1990). In addition, we found that there was hydrolysis of AFB_1 in the furofuran moiety when subjected to wet heating.

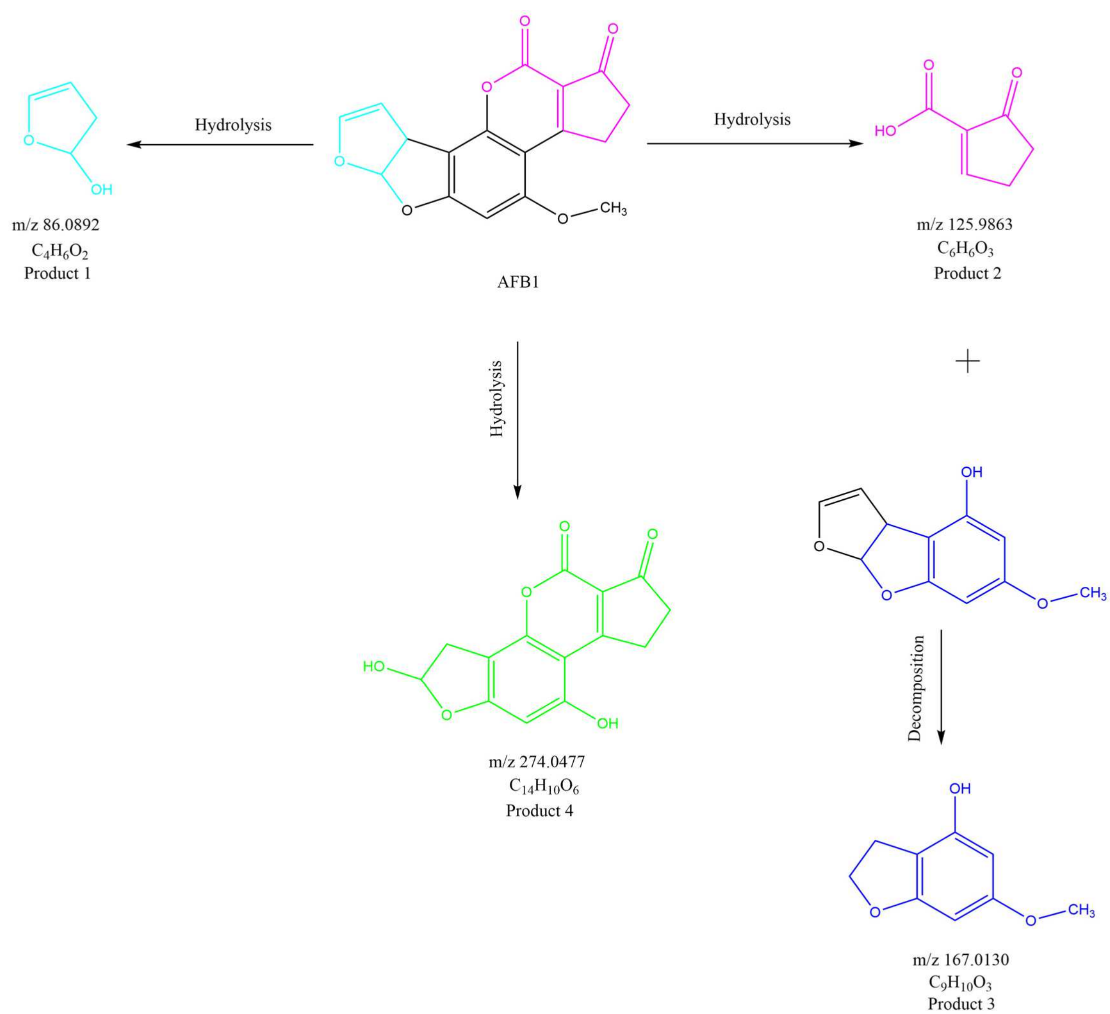


Figure 3.6. Degradation pathway of AFB_1 by wet heating.

3.2 Reduction of aflatoxin by microwave heating

3.2.1 Background: introduction of microwave heating

Microwaves are a form of electromagnetic radiation in frequency ranging from 0.3 GHz to 300 GHz with wavelengths from 1m - 1mm. Microwaves have varieties of applications, such as communication, navigation, radar, and heating. Heating or drying of materials is the most common and important application of microwave. Two frequency bands are allocated in the USA by the Federal Communications Commissions (FCC) for industrial, scientific, and medical applications (ISM). The 915 M band is used for industrial heating only, while 2450 M band is used both in the industry and domestic heating. Microwaves systems between 10 to 200 kW heating capacities are used in the food industries.

Microwaves interact with polar water molecules and charged ions, heat is generated by friction resulting from polar molecules alignment and migration of charged ions in rapidly alternation electromagnetic field. The volumetric heat generation distinguish microwave heating from other surface drying methods, bring about advantages of rapid heating and relatively high drying efficiency. In microwave drying of foods, a reduction of drying time by 25-90% and an increase in drying rate of 4-8 times have been reported compared to traditional heating (Feng et al., 2012). Other advantage includes: improved dried products quality due to less drying time and instantaneous control of turn-on and off. On the other hand, microwave drying alone has some major drawbacks that include non-uniform heating, limited penetration depth of

microwave radiation, and possible textural damage due to difficulty to control the final product temperature in MW drying.

3.2.2 Literature review: Reduction of aflatoxin by microwave heating

Effectiveness of microwave heating on reduction of aflatoxin has been investigated by several studies and a summary of the treatment conditions and aflatoxin reduction is presented in Table 3.4. In these studies, aflatoxins were reduced by microwave heating both in a model system (aflatoxins were coated on a silica gel) and in substrate of corn and peanuts. Percent reduction of aflatoxin is increased with Increasing microwave power, treatment time and heating temperature. The rate of microwave heating has very little or no effect on aflatoxin degradation, and similar degradation of aflatoxin could be obtained with at a higher power level for a shorter treatment time or at a lower power lever for a longer treatment time (Luter et al., 1982). Temperature is a critical factor and internal temperature higher than 150 ºC are generally required in the studies for achieving high percent (>90%) reduction of aflatoxin (Farag et al., 1996;Luter et al., 1982). In another study, microwave heating was found to be not very effective in degrading aflatoxin in poultry feed. The concentrations of total aflatoxin and AFB1 were reduced from 955 to 760 and 890 to 690 µg/kg, respectively, and the percentage reduction of total aflatoxin or AFB_1 was around 20-35%, when the artificially contaminated feed was microwave heated at power level 1.45 KW for 10 min (Herzallah et al., 2008). However, the internal temperature of microwave heating was not measured.

We have investigated the effectiveness of conventional heating methods (dry and wet heating) on aflatoxin degradation as well as its degradation mechanism in section 3.1. One critical question on aflatoxin degradation by microwave heating is “whether there

are any non-thermal effects from microwave heating on its degradation on aflatoxin”. The non-thermal effects of microwave heating were evaluated by comparing the difference in percent of aflatoxin degradation between microwave heating and conventional heating methods at approximately the same heating temperature. Another indicator of non-thermal effect of microwave heating is the appearance of new degradation in microwave heating, but not in conventional heating methods, which suggest difference in degradation mechanism between microwave heating and conventional heating methods.

Sine we have found that the different degradation mechanisms exist in conventional dry heating and wet heating in section 3.1. In this section, we will determine the existence of non-thermal effects of microwave by comparing microwave dry heating to conventional dry heating, and microwave wet heating to conventional wet heating at approximately the same temperature.

Table 3.4. Summary of literature on reduction of aflatoxin by microwave heating.

Contamination Methods	Substrate	Power, time, Temperature	Reduction	Ref
Coated with pure aflatoxin	Silica gel	1.3kw, 9min,100°C	Complete degradation of AFB1 and AFG1. 68, 78% degradation of AFB2 and AFG2	(Farag et al., 1996)
Spiked	corn	1.3kw, 6min, 140°C	98% aflatoxins were degraded	(Farag et al., 1996)
Spiked	Peanuts	1.3kw, 6min, 170°C	97% aflatoxins were degraded	(Farag et al., 1996)
Naturally contaminated	Peanuts	3.2kw, 5 min or 1.6kw 10min 150°C	>95% degradation of aflatoxins	(Luter et al., 1982)
Spiked	Peanuts	0.7 kw, 8.5min, 150°C	30-45% of AFB1	(Pluyer et al., 1987)
Naturally contaminated	Peanuts	0.7 kw, 8.5min, 150°C	48-61% degradation of AFB1 32-40% degradation of AFG1	(Pluyer et al., 1987)
Naturally contaminated	Alkalized maize	1.65 kw, 5.5min	68-84% degradation of aflatoxins	(Perez-Flores et al., 2011)

3.2.3 Material and method

3.2.3.1 Chemical reagents

Aflatoxin standard solution in chloroform (50µg/ml) were prepared using the same procedure as described in section 3.1.2.1.

3.2.3.2 Treating aflatoxin by microwave and conventional heating

Aflatoxin samples were prepared the same way as in section 3.1.2.2. The glass bottle which contains the spiked aflatoxin was placed in a microwave oven (Panasonic microwave oven, model: NN-SN6615). The maximal temperature inside the microwave heated bottle was measured using a thermal camera (SeeK thermal, model: Compact Pro, USA). For microwave dry heating at temperature around 80 ºC, aflatoxin samples were first preheated to a temperature of about 80 ºC with microwave power level 2 for 10 min. Then the aflatoxin sample was heated at microwave power level 2 for another 10min, for every 2 min, the microwave heating was stopped for a period of 1 min. The maximal temperature of the aflatoxin sample was recorded immediately after microwave heating and the next cycle of 2 min microwave heating was started. For microwave dry heating at temperature around 150 ºC, the experimental procedure was the same as described, however the power level was set as 4, aflatoxin was first preheated for 20 min to a temperature of about 150 ºC and then microwave heated for another 10 min. Every 2 min, the microwave heating was stopped for 30s in order to cool down the sample. For microwave wet heating at 80 ºC, pipette was used to introduce 2 ml distilled water to the aflatoxin sample before placing the sample in the microwave oven to heat set at a power level of 2. The sample was first preheated for 6 min to increase the temperature of the

sample to around 80 ºC, and was further microwaved heated for another 10 min. Every 2 min, the microwave heating was stopped for 30 s for cooling down the sample.

For conventional dry heating at 150 ºC, the procedure is described in section 3.1.2.2. Glass bottle containing aflatoxin sample was first preheated in forced convection oven (temperature set as 150 ºC) for 6 min, then the sample was further heated for another 12.5 min, the temperature of the sample was recorded every 2 min using the thermal camera. For conventional wet heating, aflatoxin sample was placed in and heated in a water bath for 12.5 min.

3.2.3.3 HPLC- MS Analysis.

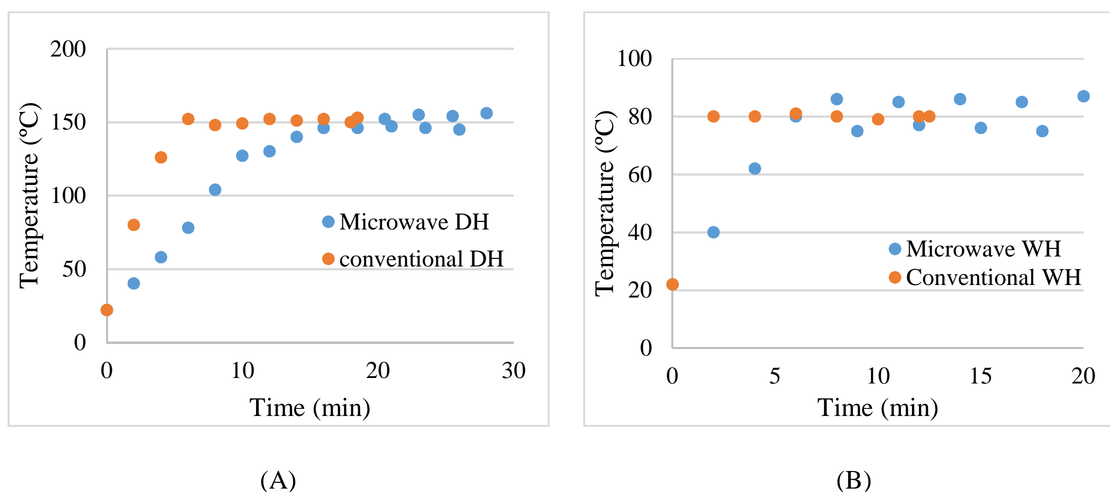
Measurement of aflatoxin levels and identification of reaction products are done through HPLC-MS analysis. This analysis was described in section 3.1.2.3.

3.2.4 Results and discussion

3.2.4.1 Heating temperature during microwave heating and conventional heating

Heating temperature during dry heating and wet heating by microwave and convectional heating are shown in Figure 3.7. For both microwave and conventional heating, aflatoxin was subject to approximately the same thermal condition: the same temperature on average and the same heating time. However, because of the cycle of microwave heating and cooling in this experiment, the temperature varies more during microwave heating than conventional heating. For dry heating at 150 ºC, the temperature range of microwave and conventional dry heating was 145 - 156ºC and 148 - 152 ºC, respectively. For wet heating at 80 ºC, the temperature range of microwave and conventional dry heating was 75 - 87 ºC, and 79 - 81 ºC, respectively. Thus although the

average temperature of microwave heating and conventional heating was controlled, the maximal temperature during microwave heating was slightly higher than that during conventional heating.



(A) (B)

Figure 3.7. Heating temperature during (A) dry heating and (B) wet heating by conventional and microwave heating methods.

3.2.4.2 Reduction of aflatoxin by microwave dry and wet heating.

Reduction of aflatoxin by dry and wet heating with microwave and conventional heating methods is presented in Table 3.5. High percent reduction (around 70%) of aflatoxin was achieved by wet heating at 80 ℃ than dry heating at 150 ℃. This confirmed our previous finding that water molecule plays a critical role in aflatoxin reduction by heating in section 3.1.3.4. Reduction of aflatoxin by microwave heating was comparable to conventional heating for both dry and wet heating type, microwave heating gives an additional 5-8% in percent of aflatoxin reduction compared to conventional heating. We believe that this slight difference comes from higher maximal temperature generated during microwave heating (as mentioned in section 3.2.4.1), rather

than non-thermal effect of microwave heating. Non-thermal effect of microwave heating need to be determined from appearance of new degradation products besides conventional heating.

Table 3.5. Reduction of aflatoxin by dry and wet heating with conventional and microwave heating methods.

Type of heating	Heating methods	Aflatoxin (ug/ml)	Percent reduction (%)
Dry heating at 150 ºC	Microwave	31.3±2.1	37.4
	conventional	35.2±2.8	29.6
Wet heating 80 ºC	Microwave	12.3±1.2	59.2
	conventional	14.8±1.4	54.4
Control	-	50.0	-

Chromatographs of AFB_1 microwave dry heated at 80 ºC, and microwave and conventional dry heated at 150 ºC are shown in Figure 3.8 and Figure 3.9, respectively. Under low impact thermal effects (at relatively low dry heating temperature 80 ºC), microwave heating was not able to degrade aflatoxin (Figure 3.8). Under high impact thermal effects (at high dry heating temperature 150 ºC, aflatoxin was degraded by microwave heating. The chromatograph of aflatoxin dry heated at 150 ºC by microwave heating closely follow that of conventional heating, with the appearance of the same degradation products (peak 1) (Figure 3.9). Chromatograph of AFB_1 wet heated at 80 ºC by microwave and conventional heating was shown in Figure 3.10. The chromatograph of aflatoxin wet heated by microwave heating match that of conventional heating, with the appearance of the same degradation products (peak 1, and 2) (Figure 3.9). Thus dry and wet heating by microwave results in the same degradation products as conventional heating, these degradation products have already been clarified in section 3.1.3.3.

Absence of new degradation products from microwave during both dry and wet heating indicates degradation of aflatoxin by microwave heating is purely a result of its thermal effects, and non-thermal effects of microwave in aflatoxin degradation was not found.

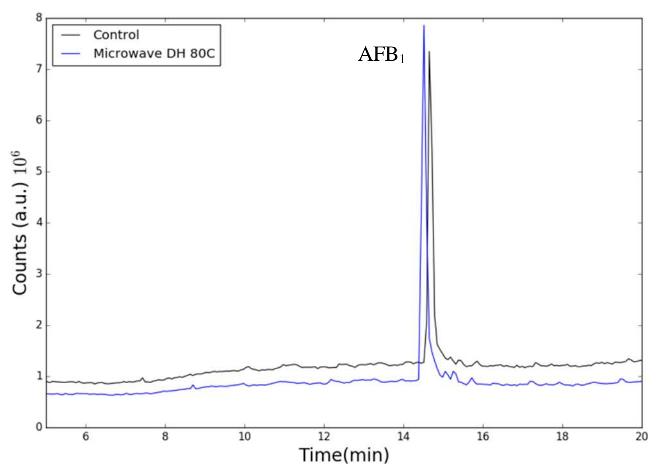


Figure 3.8. Chromatograph of AFB_1 by microwave dry heating at 80 ºC.

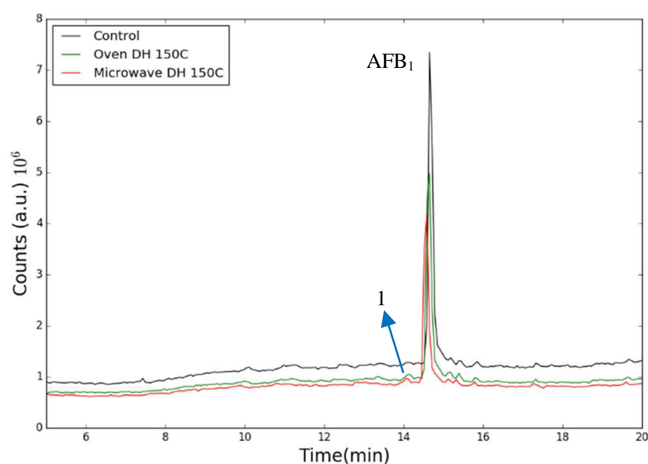


Figure 3.9. Chromatograph of AFB_1 by microwave and conventional dry heating at 150 ºC.

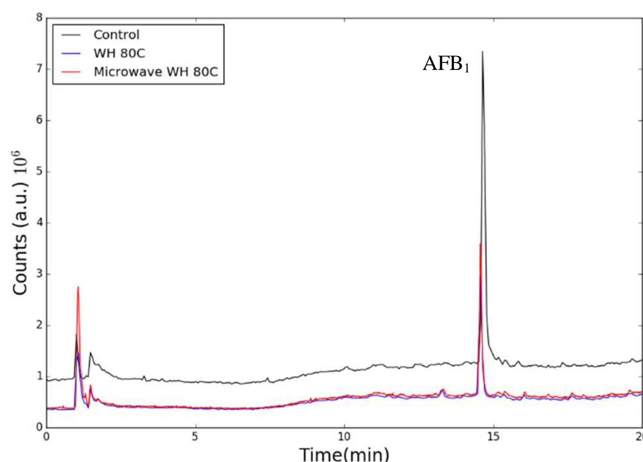


Figure 3.10. Chromatograph of AFB_1 by microwave and conventional wet heating 80 ºC.

3.3 Conclusions

Aflatoxin are very stable to dry heating and temperature of 150 ºC is required for aflatoxin to start decomposition. Aflatoxin B_1 was converted into its enantiomer during the dry heating process. During wet heating, aflatoxin B_1 was reduced at much lower temperature. 73% AFB_1 was degraded by wet heating at 80 ºC for 1 h. Water molecule plays a critical role in degradation of aflatoxin during wet heating. Reaction of Aflatoxin degradation by wet heating involves hydrolysis of furofuran moiety, lactone ring and further decarboxylation, 4 major degradation products of aflatoxin B_1 was found for wet heating at 80 ºC.

Microwave heating was found to be slightly more effective (5-8%) than conventional heating to degrade aflatoxin B_1 in both dry and wet heating condition, under the same average heating temperature for the same time. However, this difference results from higher maximal temperature generated during microwave heating. Dry and wet heating by microwave results in the same degradation products as conventional heating

The absence of new degradation products from microwave during both dry and wet heating indicates degradation of aflatoxin by microwave heating is due to its thermal effects.

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CHAPTER 4. REDUCTION OF AFLATOXIN BY FOOD ADDITIVES

In this chapter, the effectiveness of selected food additives was investigated on aflatoxin degradation without substrate and in intermediate products from ethanol bioprocessing: DWG and CDS, the reaction mechanisms of aflatoxin by food additives were clarified, influence of process parameters were evaluated. The results have been presented and published, with the citations below:

Journal Papers:

Shi, H., Stroshine, R.L., Ileleji, K. 2016. Determination of the Relative Effectiveness of Four Food Additives in Degrading Aflatoxin in Distillers Wet Grains and Condensed Distillers Solubles. Journal of Food Protection. Accepted.

Conference presentations:

Ileleji, K., Shi, H., Stroshine R.L. 2015. “Reduction of Aflatoxin in distiller grain by physical and chemical methods”. NC-213 meeting.

4.1 Literature review of aflatoxin degradation by food additives

In our previous chapter, we have shown that Aflatoxin are very stable when subjected to dry heat thermal processing, but could be partially reduced by wet heating process such as cooking. In this study, we will examine aflatoxin degradation by wet heating with food additives, which were relatively safe compounds at the dose levels used.

A number of chemical reagents have been tested for the detoxification of aflatoxins. Tests with chemical agents such as methylamine, sodium hydroxide, and formaldehyde have shown them to be effective in reducing aflatoxin levels (Piva et al., 1995). A study has systematically studied the effectiveness of food additives on aflatoxin degradation, which showed that the a number of food additives could react with aflatoxin, including acid additives, alkaline additives and neutral food additives that are oxidizers (Tabata et al., 1994). Reaction of aflatoxin by alkaline additives involves hydrolysis of the lactone ring of aflatoxin (Codifer et al., 1976;Dearriola et al., 1988). However, this reaction is reversible, and high percentage of aflatoxin degradation products from alkaline treatment could be reconverted to its original structure upon acidification (Price and Jorgensen, 1985;Mendez-Albores et al., 2004). In addition, alkaline treatment leaves strong off-odor and the products treated were susceptible to recontamination (Park et al., 1981). Thus alkaline food additives were not selected to for this study. Four food additives, i.e., sodium bisulfite, sodium hypochlorite, citric acid, and ammonium persulfate were selected for this study for comparison of their effectiveness and safety evaluation. These fours food additives were selected because of extensive research has shown them to be effective in degrading aflatoxin in food and feed products during

thermal processing, their treatment conditions and degradation percentage were summarized in Table 4.1.

Table 4.1. Literature of selected food additives in degradation of aflatoxin in food and feed products.

Food additives	substrate	Effect	reference
Sodium bisulfite	None	Bisulfite reacts with AFB1 and AFG1 reaction rate is first order with bisulfite concentration	(Doyle and Marth, 1978a)
	Corn	2% sodium bisulfite for 24 h reduced aflatoxin from 235 to below 20 ppb	(Moerck et al., 1980)
	corn	8% sodium bisulfite for 14d for total degradation of aflatoxins	(Hagler et al., 1982)
Sodium hypochlorite	None	5% solution for a few second caused loss of aflatoxin fluorescence	(Fischbac.H and Campbell, 1965)
	None	1.25% solution cause instantaneous reduction of aflatoxin	(Trager and Stoloff, 1967)
	Peanut protein isolates	0.25% solution completely eliminated AFB1 during process of producing peanut protein.	(Natarajan et al., 1975)
Citric acid	Ground corn	1 N aqueous citric acid reduced 96.7% AFB1 (ratio 3ml/g)	(Mendez-Albores et al., 2005)
	Duckling feed	1 N aqueous citric acid reduced 86% AFB1 (ratio 3ml/g)	(Mendez-Albores et al., 2007)
	rice	1 N aqueous citric acid reduced 86% AFB1 (ratio 3ml/g)	(Safara et al., 2010)
	Sorghum	Addition of 1N citric acid degraded aflatoxin in sorghum during extrusion process from 17 to 92% depending on M.C. and temperature	(Mendez-Albores et al., 2009)
Ammonium Persulfate	None	1% ammonium persulfate solution, aflatoxin was completely destroyed in 16h at 40°C and 1 h at 100 °C	(Tabata et al., 1994)
	None	64% reduction of aflatoxins in whole grains maize were degraded when soaked for 14h in 1% ammonium persulfate solutions	(Mutungi et al., 2008)
	Corn grits	Spiked aflatoxins were completely degraded at 60 C for 24 with 10 ml of 1% ammonium persulfate solution to 1 g corn grits	(Tabata et al., 1994)
	corn	Adding 2% ammonium persulfate in the liquefaction process reduced 87% of aflatoxin levels in the final products of ethanol production	(Burgos-Hernandez et al., 2002)

4.2 Usage, Regulation and guidance levels of Food additive

Bisulfite is a highly reactive chemical agent and is a generally accepted food additive. Bisulfite could inhibit enzymatic and non-enzymatic browning, acting as antioxidant and reducing agent. It could also be used for control growth of microorganism and is commonly added to wines, fruit juice, dried fruits as avoid aging,

browning, kill microbes and preserve flavor (Doyle and Marth, 1978a;Mcevily et al., 1992). Sodium hypochlorite is an effective disinfectant, which is produced by combing chlorine with inorganic compounds, such as sodium or calcium (Fischbac.H and Campbell, 1965). Citric acid is one type of dietary organic acids, which could inhibit the microorganism growth in feed, and improve the health and performance of animals by maintain the microbial balance in the gastrointestinal tract (Salgado-Transito et al., 2011). Citric acid is one of the most widely used food additives, which could be used as preservative, antioxidant, flavor enhancer, and pH controller. Ammonium persulfate is a powerful oxidizer as well as a source for ammonia generation, and modifier for food starch (Burgos-Hernandez et al., 2002) .

The use of selected food additives is subjected the FDA regulations, as specified in table. Both Sodium hypochlorite and ammonium persulfate are used as starch modifier. The use of sodium hypochlorite should be less than less than 0.5% as starch modifier, and should be 0.5-1.2g/L as microbial control agent. Both Citric acid and Sodium bisulfite are generally recognized as safe (GRAS), sodium bisulfite should not be used in food as a source of vitamin. Citric acid is most acceptable for the animals and is as dietary organic acids.

Table 4.2. Uses and regulations for selected food additives

Food additives	**Effects, Use**	**Limits and Restrictions**
Sodium Bisulfite $NaHSO_3$	Chemical Preservative	GRAS, Not in meats or foods recognized as a source of Vitamin B1 (REG-182.3739)
Sodium hypochlorite NaClO	Microbial control agent	GMP, used in food: 0.5-1.2g/L (CODEX STAN 192-195)
	Modifier for food starch	GMP, not to exceed 0.5 percent (REG-172.892)
Citric Acid ($C_6H_8O_7$)	Sequestrant, buffer	GRAS/FS
Ammonium Persulfate $(NH_4)_2S_2O_8$	Modifier for food starch	Not to exceed 0.075 percent (REG-172.892)

GRAS, generally recognized as safe
FS, permitted as ingredient in food
GMP, in accordance with good manufacturing practice

4.3 Problem statement and objectives

The presence of aflatoxin in in distiller grains decreases their value, makes marketing of the products more difficult when regulator thresholds are exceeded. Higher levels also jeopardize the health of animals that consume the distillers grain. Therefore, aflatoxin detoxification technologies could provide significant benefit to corn ethanol processors.

In this section, the effectiveness of four food additives, i.e., sodium bisulfite, sodium hypochlorite, citric acid, ammonium persulfate, in degradation of pure aflatoxin without substrate and in substrate: DWG and CDS. The specific objectives of this study were: 1) To determine the percent of aflatoxin reduction by food additives without substrate; 2) Identify the degradation products of aflatoxin by food additives and clarify the reaction mechanism; 3) determine the relative effectiveness of the four food additives in degrading aflatoxin in distiller's grain; 4) evaluate the influence of the concentration of

food additives and treatment time on the effectiveness of the most promising additives in degrading aflatoxin in distiller's grains.

4.4 Experiment set up

4.4.1.1 Chemical agents

Pure Aflatoxin B_1 (AFB_1) powder (5.0 mg) was purchased from Sigma Aldrich and dissolved in 1ml chloroform solution. The food additives sodium bisulfite, 10% (by wt.) sodium hypochlorite, citric acid, and ammonium persulfate were also purchased from Sigma Aldrich. Fresh DWG and CDS samples were obtained from The Andersons Clymers Ethanol LLC (Clymers, Indiana) in Oct, 2014, and stored in a freezer at -20 °C until thawing for experiments.

4.4.1.2 Treating AFB1 without substrate by food additives

Solutions of 1% (by weight) food additive were prepared by adding 1.0 g of additive to 100. ml. of purified water. One hundred µl of AFB1 solution (50 µg/ml in chloroform) was transferred using a pipette into a glass vial (10 ml) that was immediately transferred to a fume hood where it was kept for 1.0 h so that the chloroform could completely evaporate. Next, 2.0 ml of each food additive solution was transferred by pipette into a glass vial and 2.0 ml of purified water was added. The glass vial was covered with foil and placed in a water bath where it was heated at 90 ℃ for 1.0 h.

4.4.1.3 HPLC–MS analysis: Measurement of aflatoxin levels and identification of degradation products for aflatoxin treated with food additives with no substrate.

AFB1 samples (in a glass vial) that was treated by food additives without substrate were placed in a fume hood for 3 days to fully evaporate the residual water. After the water was fully evaporated, then pipette 1 ml of a 50% ethanol aqueous solution in the glass vial to extract the AFB1 and degradation products. The procedure of HPLC-MS analysis for of aflatoxin levels and identification of degradation products was described in detail in section 3.1.2.3.

4.4.1.4 Treatment of AFB_1 in DWG and CDS with food additives.

A 70.0 g sample of DWG was spiked with 100 µl of 100. µg/ml of AFB1 standard solution (in chloroform). A 10.0 g CDS sample was spiked with 100 µl of 40. µg/ml AFB1 standard solution. Thereafter, 35.0 and 5.0 ml of food additive solution were added, respectively, to 70.0 g of DWG and 10.0 g of CDS. This gives a water/material ratio of 0.5 ml/g. The food additive concentration (by wt.) can be calculated. For example, Addition of 0.50 ml 2.0% food additive solution to a 1.0 g sample of dry matter will give 1% (by wt.) food additive to dry matter in the sample. The DWG or CDS mixtures containing food additive solutions were placed in glass jars (250. ml) and covered with a lid. Then the jar containing the sample was placed in a shaker equipped with a heated water bath (Bellco Glass Inc., Vineland, NJ) stabilized at 90°C, and the samples were heated for 1.0 h. Higher concentrations of citric acid solution (1., 2., 2.5% by weight) and longer heating times (2.0, 5.0 h) were used to test for greater reduction of

aflatoxin in DWG and CDS. After treatment the aflatoxin levels were determined using the previously described procedures.

4.4.1.5 Determination of aflatoxin levels in DWG and CDS.

Since no official method has been designated for aflatoxin level determination in DWG and CDS matrices, their AFB_1 levels were determined using a commercial Lateral Flow Device (LFD) QuickTox test stripDDGS (AQ-109-BG3) and a QuickScan reader (Envirologix USA, Inc., Portland, Maine). According to company literature, the CV (RSD) of the AQ109 BG3 test strip is around 10% (Welch., 2013), as certified by the USDA in Addendum Certificate No. FXIS 2013-047.1. The method was first calibrated by measuring the AFB_1 levels in DWG and CDS samples spiked with a series of known AFB_1 standards (0-500 µg/kg, with intermediate levels at 100 µg/kg intervals). Linear regression was used to determine the relationship between the measurements and the levels in the spiked samples ($R^2 \approx 0.99$), The CV's (RSD) of the three measurements at each of the six aflatoxin levels were all less than 10.7% and the relationship was linear for the range of 0-500 µg/ml.

Aflatoxin in DWG was measured by first drying 70.0 g of DWG, spiked with AFB_1, at 60°C for 12h in a forced convection oven to reduce moisture content to around 10%. After drying was completed, the dried DWG sample was ground in a Disc Mill (Seedburo Equipment Co., Des Plaines, Il.) using a 20 mesh screen. A 10.0 g subsample was mixed with 30.0 ml 50% ethanol solution, and the mixture was shaken by hand for 1.5 minutes. After the mixture settled (around 10 minutes) there were two distinct layers.

The top layer was used for aflatoxin determination by means of the ELISA test strips. For aflatoxin measurements on CDS, a 10.0 g sample was mixed with 30.0 ml of 50% ethanol solution. The mixtures were shaken by hand for 1.5 minutes, then filtered by passing them through a funnel lined with Whatman filter paper. The extract that passed through the filter paper was used for the aflatoxin level determination.

The recovery of LFD measurement was also determined by first using the test strips to measure the aflatoxin level in a solution of 50% ethanol to which aflatoxin was added to give 500 ug/ml aflatoxin. Next, same amount of aflatoxin was added to samples of DWG and CDS to give 500 ug/ml of aflatoxin and the 50% ethanol was used to extract the aflatoxin from the matrix. Then the test strips were used to determine the aflatoxin levels in the extracts and these levels were compared to the first measurements, the recoveries of LFDs measurement for DWG and CDS were 69.2 and 76.9% respectively.

Another concern with using an LFD test strip is cross reactivity in which the degradation products could still possibly show up in the test strips. This was tested by comparing the aflatoxin levels measured by both LFDs test trips and HPLC instrument equipped with a Mass Spectrometer after treating aflatoxin with 1% food additives solutions and heated at 90 ºC for 1 h. The two methods measured 5.0 µg/ml aflatoxin for the control. Treatment with the additives reduced the aflatoxin levels in the remainder of the samples. The LFD’s gave 10 and 13.3% higher values for the bisulfite and citric acid treatments, respectively. and 1.9 and 11.4% lower for heated water and persulfate treatments, respectively. For the solution treated with hypochlorite, the LFD apparently reacted with one of the metabolites because the test strips indicated that there was 1.78

μg/ml remaining after treatment whereas the HPLC MS indicated there was no aflatoxin remaining.

4.4.1.6 Data analysis

The effectiveness of each type of food additive on degradation of aflatoxin was evaluated using one-way ANOVA analysis. Duncan's Multiple range test was used to determine the significant differences between different treatments at the $P<0.05$ level (SAS 9.3, SAS Institute Inc., USA).

4.5 Results

4.5.1 Degradation of aflatoxin without substrate by food additives

Effectiveness of food additive on degradation of aflatoxin without substrate when heating at 90 ºC for 1 h is presented in Table 4.3. Heating at 90 ºC for 1 h, water resulted in 59% reduction of aflatoxin, food additives of sodium bisulfite, citric acid, and ammonium persulfate achieved similar percent reduction of aflatoxin (86 ~ 89). Among tested four food additives, the sodium hypochlorite is the most effective and completely degrade the aflatoxin completely following heating at 90 ºC for 1 h.

Table 4.3. Effectiveness of food additive on degradation of aflatoxin without substrate [a]

Treatment [b]	HPLC-MS		Percent Reduction [c]
	Peak Area	Calibrated (μg/ml)	
control	21186180	50.00	-
water no heat	17223790	40.65	18.7
water	8681664	20.49	59.0
Sodium bisulfite	2668736	6.30	87.4
Sodium hypochlorite	3129	0.01	100.0
citric acid	2257252	5.33	89.4
persulfate	2884386	6.81	86.4

[a] All samples (except the control and water with no heating) were heated at 90˚C for 1 hr.
[b] 2 ml 1% (by weight) food additive solution.
[c] Percentage reduction was based on aflatoxin levels of the control (no treatment).

4.5.2 Reaction products and reaction mechanism of aflatoxin by food additives

4.5.2.1 Reaction of AFB_1 by sodium bisulfite

Chromatogram of AFB_1 heated with 1% sodium bisulfite was shown in Figure 4.1Figure 4.3. One new peak (peak 1) which corresponds to degradation products appeared in the chromatograph. The degradation product of AFB_1 treated with sodium bisulfite was proposed as aflatoxin B_1S (molecular formula: $C_{17}H_{18}O_9NaS$) based on the observed Infrared (IR) spectrum (Hagler et al., 1983). However, the exact mass of the degradant was unknown at the time that study was performed since because mass spectroscopy was not readily available. In our study the degradation product of AFB_1 with sodium bisulfite was identified as aflatoxin B_1S ($C_{17}H_{19}O_9S$) using HPLC-MS analysis, which is very similar in structure to aflatoxin B_1S ($C_{17}H_{18}O_9NaS$) and would closely resemble its IR spectrum. The reaction mechanism of AFB_1 with bisulfite is shown in Figure 4.2. The bisulfite group adds to the double bond in the furofuran ring of AFB_1.

Figure 4.1. Chromatogram of AFB_1 heated at 90 ºC for 1 h with 1% sodium bisulfite.

Figure 4.2. Reaction mechanism of AFB_1 by bisulfite.

4.5.2.2 Reaction of AFB_1 by sodium hypochlorite

Chromatogram of AFB_1 heated at 90 ºC for 1 h with 1% sodium hypochlorite was shown in Figure 4.3. AFB_1 was completely degraded by sodium hypochlorite, and no degradation products appeared in the vicinity of AFB_1 peak in the chromatogram. It is expected that The AFB_1 was oxidized into small molecules that did not show up in chromatogram.

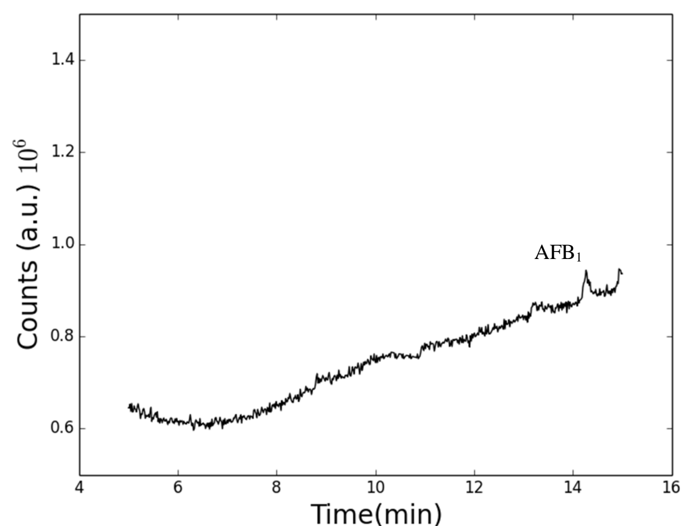

Figure 4.3. Chromatogram of AFB_1 heated at 90 ºC for 1 h with 1% sodium hypochlorite.

4.5.2.3 Reaction of AFB_1 by citric acid

Chromatogram of AFB_1 heated at 90 ºC for 1 h with 1% ammonium persulfate was shown in Figure 4.4. Two new peak (peak 1, and 2) which corresponds to degradation products appeared in the chromatograph. One degradation product is AFB_{2a} as reported by previous investigators through acid-catalyzed hydrolysis which converts the AFB_1 to AFB_{2a} (Pons et al., 1972). The second degradant was identified as AFB_1-Citric ($C_{23}H_{19}O_{13}$), which is produced by adding citric acid to the double bond of the furan ring of AFB_1. The reaction mechanism of AFB_1 with citric acid is illustrated in Figure 4.5.

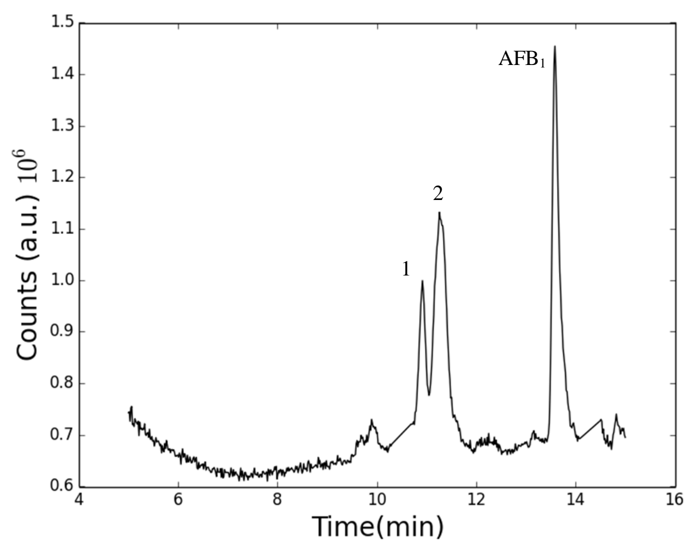

Figure 4.4. Chromatogram of AFB_1 heated at 90 ºC for 1 h with 1% citric acid.

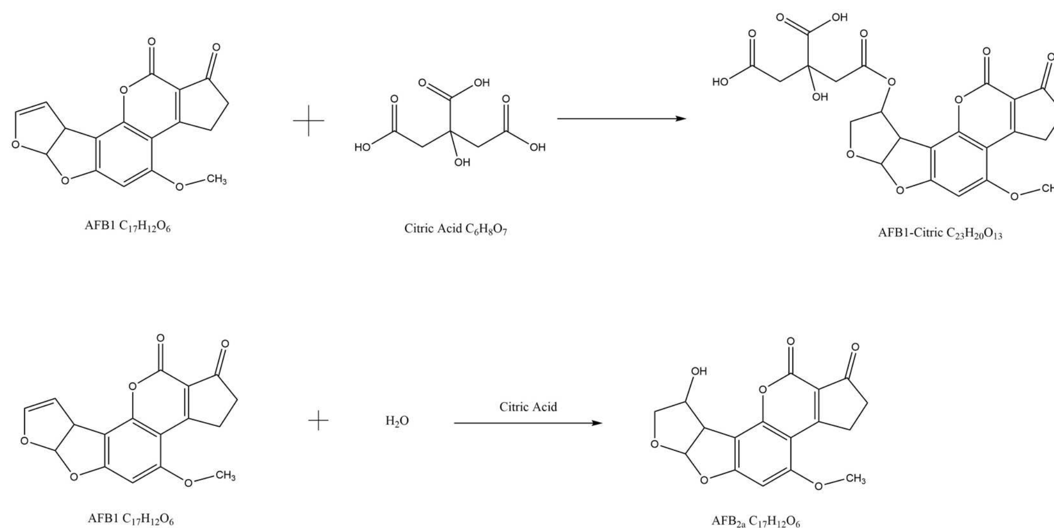

Figure 4.5. Reaction mechanism of AFB_1 by heating at 90 C for 1 h with citric acid.

4.5.2.4 Reaction of AFB_1 by ammonium persulfate

Chromatogram of AFB_1 heated with 1% ammonium persulfate was shown in Figure 4.6. AFB_1 was completely degraded by ammonium persulfate. Similar to reaction with the sodium hypochlorite, no AFB_1 degradation products appeared in the vicinity of AFB_1 peak in the chromatogram. Thus it is also suspected that The AFB_1 was oxidized into small molecules that did not show up in chromatogram.

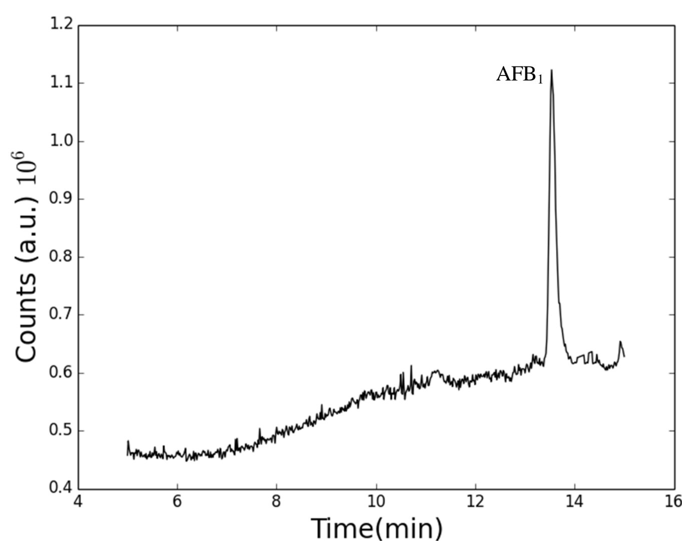

Figure 4.6. Chromatogram of AFB_1 heated with 1% ammonium persulfate

4.5.3 Degradation of aflatoxin in DWG and CDS by food additives

The Effects of different food additives on aflatoxin degradation in DWG and CDS are shown in Table 4.4. The initial concentrations of aflatoxin in DWG and CDS samples (control) are 360 ± 15 and 462 ± 25 µg/kg, respectively. Heating DWG or CDS for 1h at 90 °C without addition of any additives reduced AFB_1 levels in DWG and CDS by 17 and 21% respectively. Adding water (0.5 ml/g of DWG or CDS) to the DWG and

CDS gave a slight improvement in aflatoxin degradation, increasing the degradation from 17-21% to 22-27%. However, this increase was not statistically significant. The food additives selected were generally not very effective in degrading aflatoxin in DWG or CDS at a 0.5% (by wt.) inclusion rate. The most effective agent for aflatoxin degradation was sodium hypochlorite which reduced the AFB_1 levels in DWG and CDS by 42% and 56%, respectively. Adding Sodium bisulfite did not improve degradation of aflatoxin in either DWG or CDS. Citric acid and ammonium persulfate were comparable in efficacy, and reduced the aflatoxin level by 31-51% in DWG and CDS. These four Food additives results in percentage reduction of aflatoxin in DWG and CDS ranging from 24~56%, this is much less compared to their effectiveness to degrade pure aflatoxin without substrate in the same thermal conditions (heating at 90 ℃ for 1 h), the percent reduction of four food additives were 86~100% (Table 4.3). The discrepancy of effectiveness of food additives in degradation of aflatoxin in substrate (DWG and CDS) and without substrate indicates protective effects of substrate from degradation of aflatoxin. This protective effect has been reported during treatment of aflatoxin by chemical methods (Samarajeewa et al., 1990). The protective effect of substrate could be a result of binding of aflatoxin to the substrate or depletion of reactive chemical reagents through reaction with the substrate.

Among the three effective food additives, which were sodium hypochlorite, citric acid and ammonium persulfate, citric acid is the most economical and it is generally regarded as safe (GRAS). Thus it has the greatest potential for use in the ethanol industry for detoxification of aflatoxins in distiller grains. Since a 0.5% inclusion level and heating for 1.0 h only achieved a 31-51% reduction of aflatoxin in DWG and CDS, the

influences of the amount of citric acid included and the heating time were further investigated in the hope of achieving a greater reduction.

Table 4.4. Effects of different food additives on aflatoxin degradation in DWG and CDS. All samples (except the control with no heating) were heated at 90˚C for 1 hr.

Food additives [a]	DWG		CDS	
	AFB_1 level (µg/kg) [b c]	Percent reduction (%) [d]	AFB_1 level (µg/kg) [bc]	Percent reduction (%)[d]
Control	360 ± 15 A	-	462 ± 25 A	-
Heating only	300 ± 10 B	17	363 ± 15 B	21
Distilled water	282 ± 23 BC	22	336 ± 13 BC	27
Sodium bisulfite ($NaHSO_3$)	277 ± 12 BC	24	300 ± 43 CD	35
Sodium hypochlorite (NaClO)	208 ± 22 D	42	201 ± 13 F	56
Citric acid ($C_6H_8O_7$)	249 ± 14 C	31	225 ± 34 EF	51
Ammonium persulfate ($(NH_4)_2S_2O_8$)	198 ± 17 D	44	269 ± 19 DE	42

[a] 1% (by wt) food additive solution, addition ratio: 0.5 ml /g DWG or CDS
[b] Values are mean ± standard deviation (n=3)
[c] Among different treatments, values with different letters are significantly different ($P < 0.05$) as determined using the Duncan's multiple range test.
[d] Percent reduction was calculated by comparing to the control (the initial level of AFB_1).

4.5.4 Effects of concentration of citric acid and heating time on aflatoxin degradation.

Aflatoxin reduction increased in both DWG and CDS when the citric acid inclusion increased and the sample was heated for 1h (Figure 4.7). The percent reduction in DWG increased linearly as the percent by weight of citric acid increased over the range of 0.0 -2.5 % (by wt). However, the reduction of aflatoxin in CDS was less with an incremental increase in citric acid addition rate above 2.0% (by wt.). The greatest reductions of aflatoxin in DWG (65%) and CDS (80%) were achieved at the highest citric acid inclusion rate (2.5% by wt.).

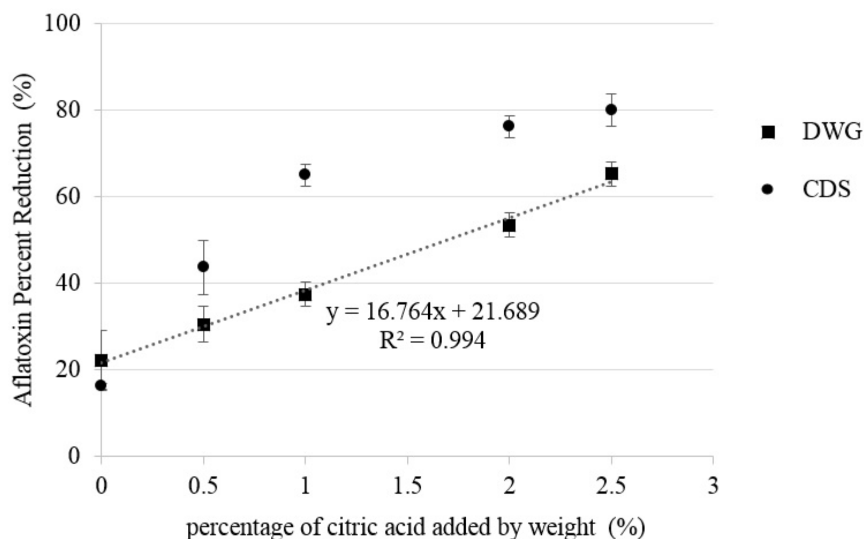

Figure 4.7. Effects of rate of addition of citric acid on aflatoxin reduction in DWG and CDS.

* Heating at 90 °C for 1 h. Initial aflatoxin levels in DWG and CDS were 360 ± 15 and 402 ± 23 µg/kg, respectively. The equation shown is from a linear regression of percent reduction in DWG versus percent citric acid added. The response for CDS becomes non-linear when more than 1% citric acid was added.

Also, aflatoxin reduction in DWG and CDS increased as heating time increased, regardless of the rate at which citric acid was added (Figure 4.8). Since the natural logarithm of AFB_1 concentration varies linearly with time (R^2 =0.79-0.90 for DWG and CDS), the reaction rate decreases with increasing heating time and the degradation of aflatoxin approximates a first order reaction. This is consistent with a previous study that reported the reaction of AFB_1 with sulfuric acid is a first order reaction(Pons et al., 1972). The largest percentages of aflatoxin reduction in DWG (77%) and CDS (86%) were obtained by heating the DWG and CDS for 5.0 hours with the addition of citric acid at 1.0% (by wt.). However even without addition of citric acid, the aflatoxin level gradually decreased during heating. When distilled water was added instead of citric acid, aflatoxin

levels in DWG decreased 53% and levels in CDS decreased 73% when the materials were heated at 90 °C for 5.0 h. Aflatoxin reduction for CDS was greater than it was for DWG for all citric acid inclusion rates and heating times. This indicates that it is easier to degrade aflatoxin in CDS than in DWG. This may be a consequence of the fact that CDS is liquid and the fine solids in CDS provide a larger surface area for interaction between aflatoxin and the food additives that the coarse solids in DWG.

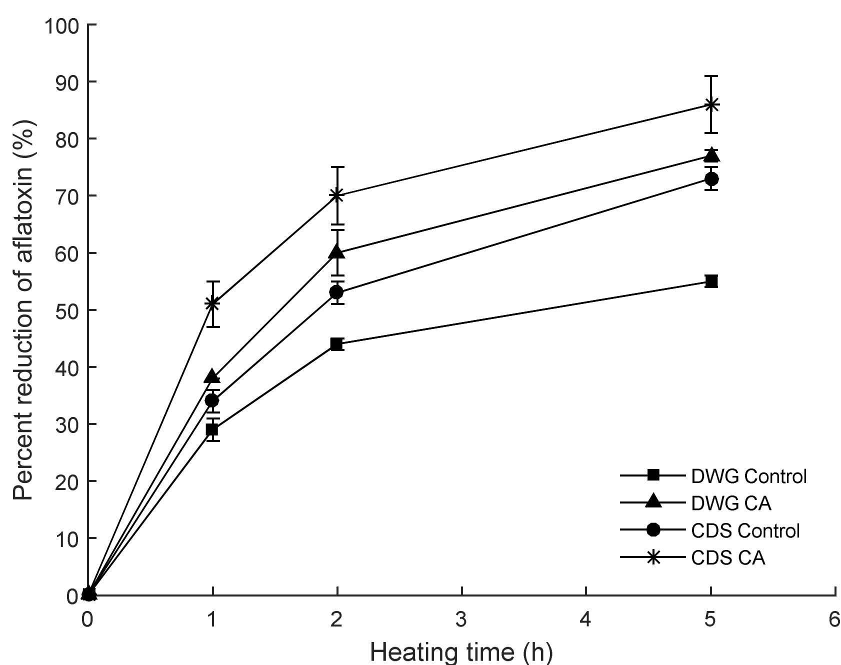

Figure 4.8. Effects of heating time on aflatoxin reduction in DWG and CDS. (Initial aflatoxin levels in DWG and CDS were 283 ± 25 and 335± 15 ppb respectively. Note: CA means 1% citric acid, and the control was prepared with distilled water.)

4.6 Discussion

4.6.1 Efficacy and safety of food additives for aflatoxin degradation distillers grains.

In this study, sodium bisulfite was ineffective in degrading aflatoxins in DWG and CDS. This contradicts several previous studies, one of which reported that sodium bisulfite was able to react with aflatoxin in liquid solution (Tabata et al., 1994). Bisulfite reacts with AFB_1 by adding bisulfite to the double bond between AFB_1 and aflatoxin B_1S (Hagler et al., 1983). Several studies reported that Sodium bisulfite could be used to degrade aflatoxins in corn (Doyle and Marth, 1978a;Doyle and Marth, 1978b;Moerck et al., 1980;Tabata et al., 1994). It completely degraded aflatoxin in highly contaminated corn (2350 µg/kg B_1) when the corn kernels were soaked in sodium bisulfite solution for 72 h (Hagler et al., 1982). However, in this study, it was found that sodium bisulfite is ineffective in degrading aflatoxin in DWG and CDS. The results reported here agree with one study that reported bisulfite was not an effective AFB_1 detoxifier (Trager and Stoloff, 1967). The discrepancy in aflatoxin degradation by sodium bisulfite could be a result of the treatment time. In both this study and the study that reported ineffectiveness of bisulfite, the treatment time was just 1 h. However, in the studies that reported significant degradation of aflatoxin in corn, the treatment time was 24-72 hours (Doyle and Marth, 1978b;Moerck et al., 1980;Tabata et al., 1994). Another important factor that affects aflatoxin degradation by sodium bisulfite is the acidity of the substrate. Both DWG and CDS are very acidic (pH ≈4.5) compared to corn. Acids could retard the reaction of aflatoxin with sodium bisulfite by consumption of the bisulfite ion through the following reaction: $H^+ + HSO_3^- \longleftrightarrow H_2O + SO_2$ (2). The sulfur dioxide (SO_2) produced by this reaction is not an effective aflatoxin detoxifier (Doyle and Marth, 1978b).

Sodium hypochlorite, a common constituent of bleach, has strong oxidizing power and is an effective disinfectant for microorganisms such as bacteria and fungi(Yang, 1972). Sodium hypochlorite could react with aflatoxin in water solutions (Fischbac.H and Campbell, 1965;Trager and Stoloff, 1967). In one study, it effectively degraded aflatoxins when added in the process of preparing peanut proteins isolates and concentrates(Natarajan et al., 1975). Results in this study agree with previous studies, which show that sodium hypochlorite effectively degraded aflatoxin in distiller's grains. In fact, it was the most effective detoxifier among the four food additives tested. However, treating with sodium hypochlorite caused the sample to have a pungent odor and to be lighter in color (bleached) than untreated DWG and CDS. The pungent odor comes from chlorine gas, which is generated by the acid reaction of hypochlorite from the sodium hypochlorite: $2H^{+}+ClO^{-}+Cl^{-} \longleftrightarrow Cl_2+H_2O$ (Wiberg, 2001). Since color is an important quality characteristic of distiller's grains, the lighter color produced by the treatment could lower its market value. Thus sodium hypochlorite is not suitable for aflatoxin degradation.

Citric acid is one of the most widely used food additives. It has been used in food as a preservative, an antioxidant, a flavor enhancer, and to control pH. The US Food and Drug administration (FDA) classifies citric acid as generally recognized as safe (GRAS). Several studies have reported that AFB_1 was degraded by citric acid in various feeds as well as in whole grain. As examples, AFB_1 was reduced by 96.7% in contaminated ground maize (Mendez-Albores et al., 2005), 86% in duckling feed (Mendez-Albores et al., 2007), and 97.2% in rice(Safara et al., 2010) when the mixture was heated at 40°C for 48 h after the addition of 1 N aqueous citric acid solution at a rate of 3 ml of solution/g of material . In those treatments, approximately 90% of the

aflatoxin was degraded at 40°C. However, the high percent reduction was achieved with a very high rate of addition of citric acid (19.2% by wt.). In this study, the aflatoxin level was reduced by 65% in DWG and by 80% in CDS by the addition of 2.5% (by wt.) citric acid and heating at 90 °C for 1 h.

An important consideration is that food additives should be used in accordance with FDA regulations. These state that sodium bisulfite should not be used in meats or food recognized as source of Vitamin B_1 (REG-182.3739); that the concentration of sodium hypochlorite in food should be less than 1.2% (REG-172.892); and that the concentration of ammonium persulfate should not exceed 0.075% (REG-172.892). It should also be noted that heating DWG and CDS for a prolonged time could significantly reduce the aflatoxin level in DWG and CDS by acid-catalyzed hydrolysis. This is the type of reaction that occurs between citric acid and aflatoxin , in which AFB_1 or AFG1 are converted to AFB2a and AFG2a (Pons et al., 1972). Based on an Ames test for mutagenicity and a duckling test for toxicity (Mendez-Albores et al., 2005;Mendez-Albores et al., 2007), the mutagenicity and toxicity of aflatoxin contaminated feeds were both greatly reduced after treatment with citric acid. Therefore, distiller's grains treated with citric acid should be safe for animal consumption. No significant changes in color and odor were observed in DWG and CDS after treatment with citric acid. However, the nutrient value of DWG and CDS needs to be further evaluated.

4.6.2 Applicability of food additives for aflatoxins degradation during ethanol bioprocessing.

A number of food additives could react with AFB_1, including acid, alkaline, and neutral food additives that are oxidizers (Tabata et al., 1994). Since both DWG and CDS are acidic, a significant amount of alkaline must be added to these two materials in order to treat for aflatoxin. Also alkalization leaves an off-odor, making alkaline food additives a nonviable option. Among neutral food additives, sodium bisulfate, which has a relatively low oxidizing power, was not effective in aflatoxin degradation in DWG and CDS because it reacted with the substrate. The additives sodium hypochlorite and ammonium persulfate, with high oxidizing capacity, were able to significantly degrade aflatoxin in DWG and CDS. However, the distiller's grains were bleached by sodium hypochlorite. Their nutrient value could also be affected. A variety of organic materials, including carbohydrates, lipids, and several amino acids in the foodstuff are subject to oxidation and chlorination reactions and result in formation of new chloro-organic compounds (Fukayama et al., 1986). Thus neutral food additives that are strong oxidizers are also not viable options for aflatoxin degradation in distiller's grains.

Citric acid appears to be the most promising food additive for degrading aflatoxin in distiller grains because of its efficacy and safety. During ethanol bioprocessing, the corn goes through several processes including cooking, liquefaction, fermentation, distillation, and drying of coproducts. During liquefaction and fermentation, the pH needs to be stable for maximal yield from the yeast. Therefore, the citric acid would have to be added after the fermentation process. In this study, the addition of 2.5% (by wt.) citric acid along with heating at 90°C for 1.0 hour reduced aflatoxin in DWG by 80% and

in CDS by 61%. Addition of citric acid at a higher rate or a longer heating time would be required for higher degradation of aflatoxins. However, a balance would have to be achieved between aflatoxin degradation efficacy and the cost of treatment. Citric acid is around 3 to 5 times more expensive than DDGS. Adding 2.5% (by wt.) citric acid could therefore increase the cost of distillers grains by around 10%. Nevertheless, addition of citric acid appears to be a promising method of salvaging DDGS when there is a severe aflatoxin outbreak.

4.7 Conclusions

Four selected food additives, i.e., sodium bisulfite, sodium hypochlorite, citric acid, and ammonium persulfate, were able to effectively degrade aflatoxin by heating at 90 ℃ for 1 h with 1% (by weight) food additives solutions. The percent reduction of aflatoxin ranges from 86% to complete degradation, and sodium hypochlorite is the most effective aflatoxin detoxifier among these four food additives.

For the selected food additives treatment by heating aflatoxin in DWG and CDS for 90 ℃ for 1 h with, Sodium bisulfite was not effective, sodium hypochlorite was the most effective. However, it bleached the substrate and left an off-odor. Citric acid and ammonium persulfate reduced aflatoxin levels by 31-51%. Citric acid is the most promising additive for degrading aflatoxin because it has been classified as GRAS (generally recognized as safe) by FDA. Degradation of aflatoxin B1 by citric was through acid-catalyzed hydrolysis which converts the AFB_1 to AFB2a, the second degradant was AFB_1-Citric (C_{23} H_{19} O_{13}), which is produced by adding citric acid to the double bond of the furan ring of AFB_1.

Aflatoxin reduction was enhanced by increasing the citric acid addition level and prolonging the heating time. Reductions of 65% and 80% in DWG and CDS were obtained by addition of 2.5% (by weight.) citric acid and heating at 90 °C for 1 h. Aflatoxin levels in DWG and CDS were gradually reduced with prolonged heating at 90 °C even without addition of food additives. Aflatoxin reductions of 53 and 73% were achieved in DWG and CDS as a result of heating at 90 °C for 5 h.

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CHAPTER 5. REDUCTION OF AFLATOXIN BY HIGH VOLTAGE ATMOSPHERIC COLD PLASMA (HVACP) TREATMENT

In this chapter, we first characterized the generation of gas species by HVACP were characterized via optical emission spectroscopy (OES), optical absorption spectroscopy (OAS) and dragger detection tubes. Then we investigated the effectiveness of High Voltage Atmospheric Cold Plasma treatment on reduction of aflatoxin in corn. The influences of operational parameters (gas type, relative humidity, mode of reaction, stirring of material) on the degradation of aflatoxin by HVACP treatment. HVACP was applied for aflatoxin degradation in distiller grains were investigated. Critical parameters of different material, sample amount, surface area, grain depth, and treatment time were evaluated for their effects on ozone consumption and aflatoxin degradation during HVACP treatment. We further investigated the mechanism of aflatoxin degradation by HVACP treatment, clarified the structure of AFB_1 degradation products, elucidated the degradation pathway of AFB1 through mass spectrometry, and the toxicity of degradation products.

Journal papers:

Shi, H., Stroshine, R.L., Ileleji, K., Keener, K. Reduction of aflatoxin in corn by high voltage atmospheric cold plasma. Journal of Food and Bioprocess Technology. 2016, In review.

Shi, H., Cooper, B., Stroshine, R.L., Ileleji, K., Keener, K. Structure of Degradation Products and Degradation Pathway of AFB1 by High Voltage Atmospheric Cold Plasma (HVACP) Treatment. Journal of Agricultural and Food Chemistry. 2016, In review.

Conference presentations:

Ileleji, K., Shi, H., Strohine, R.L., Kevin, K.M. Reduction of Aflatoxin in Corn by High Voltage Atmospheric Cold Plasma". NC-213 meeting, 2016/02

5.1 Introduction of high voltage atmospheric cold plasma (HVACP)

Plasma is referred as the fourth state of matter. It is generated by supplying energy to a neutral gas. The atoms in the gas molecule will be stripped of electrons in their outer shells, this process is called ionization. When the gas is not fully ionized, plasma is composed of photons, electrons, ions, free radicals and gas molecules (Moreau et al., 2008).

Plasma could be categorized as low or high temperature plasma depending on the temperature of gas molecule, ions, and electrons, and the low temperature plasma could be further divided into thermal and cold (nonthermal) plasma (Table 5.1). Thermal plasma has temperatures higher than ambient (40 ºC) and are in thermal and charge equilibrium. The temperature of electrons in thermal plasma is equal or close to the temperature of neutral gas and ions (Ehlbeck et al., 2011). Thermal plasma is generated at higher pressures and requires high power. Examples of thermal plasma are arc plasma that are applied in cutting, welding and spraying (Wu et al., 2014).

Table 5.1. Subdivisions of plasma.

Low-Temperature plasma (LTP)		High-temperature plasma (HTP)
Cold (Non-thermal) plasma $T_i=T \approx 300$ K $T_e \leq 10^5$ K e.g. low pressure glow discharge	Thermal plasma $T_e=T=T_i < 2\times10^4$ K e.g. arc plasmas	$T_i=T=T_e \geq 10^7$ K e.g. fusion plasma

Note: adapted from (Rutscher, 2008).

On the other hand, cold (nonthermal) plasma has near ambient temperature of 30-60 ºC, and is in non-equilibrium state which the temperature of the electrons is much higher than the temperature of ions and neutral gas. Cold plasma could be obtained at atmospheric or reduced pressures (vacuum). Plasma generation at atmospheric pressure is

of interest for the food industry because this does not require extreme conditions and equipment. Typical approaches for atmospheric cold plasma generation include the cold plasma jet, dielectric barrier discharges (DBD), corona discharge, plasma jet, microwave radio frequency (Misra et al., 2011;Surowsky et al., 2014). The schematic set-ups of these methods for generation of atmospheric cold plasma was illustrated in Figure 5.1. The Characteristics and comparison of advantages and disadvantages of different atmospheric cold plasma (ACP) generation approaches was summarized in Table 5.2.

Table 5.2. Comparison of methods for generation of atmospheric cold plasma

ACP methods	Characteristics	Advantages	Disadvantages
Atmospheric pressure plasma jet (APPJ)	Consist of one needle and one ring electrodes Produce “small flames” using radio frequencies. Ignite voltage is around 100 V	Small dimension, targeted applicability, ability to penetrate into narrow gap	Spatial limitation (an array of discharges needs to be used for large areas)
Dielectric barrier discharge (DBD)/HVACP	Two electrodes separated by a dielectric High ignition voltages (>10 kv)	Great variety of gas could be used Comparably low flow rate Good adaptability with different geometries.	High ignition voltages Small gaps
Corona discharge (CD)	Point to plate geometry Operated in DC or pulsed mode	Little first expenses and operation costs	Small treatment area
Microwave discharge (MD)	Microwave generated by a magnestron (2.45 GHz)	Electrodeless setup and Ability to ignite in air environment	Spatial limitation

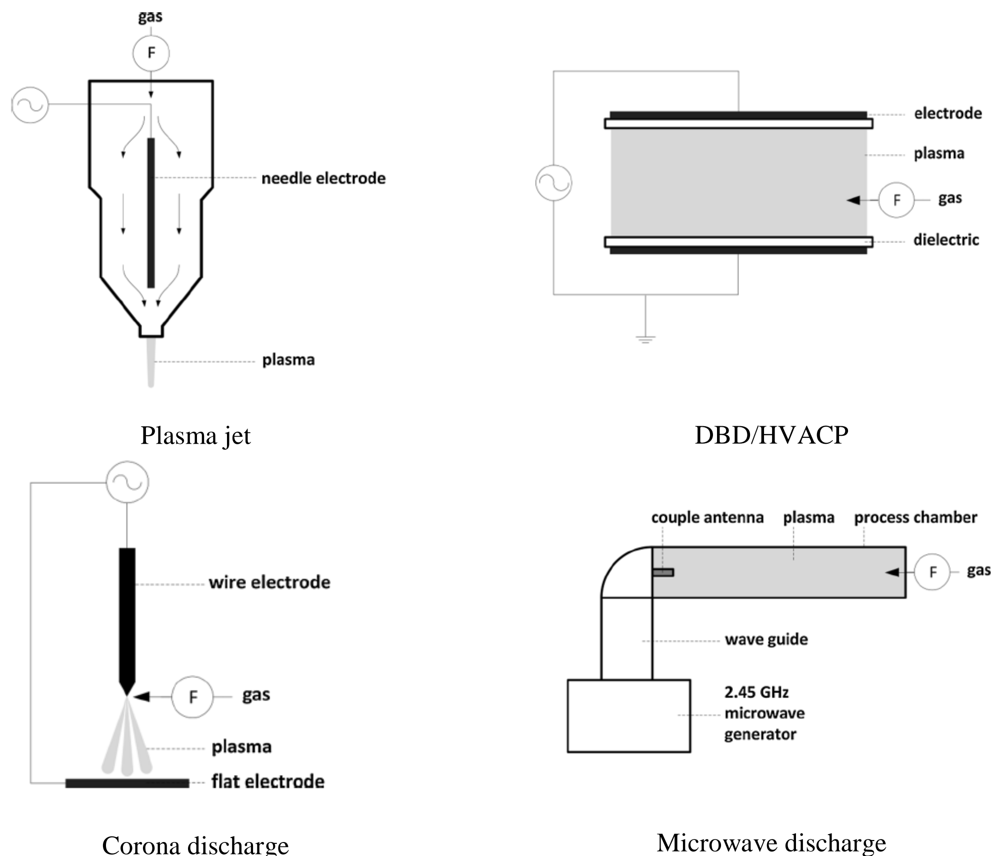


Figure 5.1. Different schematic set-ups of atmospheric cold plasma. Adapted from (Surowsky et al., 2014)

High voltage atmospheric cold plasm (HVACP) is cold atmospheric plasma generated through dielectric barrier discharge (DBD). The HVACP system consists of two parallel aluminum electrodes with a gap space between them, and the current in the electric field breaks down the gas between the plates. High voltage atmospheric cold plasma (HVACP) is a novel nonthermal food processing technology, which has the advantage of high efficiency, short treatment time, low impact on the quality of food products, and leaves no residue (Schluter et al., 2013;Thirumdas et al., 2015;Misra et al., 2011). HVACP treatment is able to effectively inactivate a variety of food microbes such

as *Escherichia coli* O157:H7, *listeria monocytogenes*, and *Staphylococcus aureus* (Niemira, 2012). Fungus species such as *Aspergillus flavus*, *Aspergillus Spp.*, and *Penicillum* could also be destroyed by HVACP treatment (Basaran et al., 2008;Selcuk et al., 2008). One study on the use of HVACP to extend the shelf-life of wet distillers grains with solubles indicated a level of success with a reduction in microbial load, and thus an extension of shelf-life (McClurkin, 2016). The aforementioned study was a precursor to this work on reducing aflatoxin using HVACP treatment.

5.2 Characterization and kinetics of gas species generation by HVACP system

5.2.1 Background

The generation of gas species in different relative humidities (5, 40, 80%) is investigated using optical emission spectroscopy. The concentration of gas species generated in different relative humidity during HVACP were analyzed from optical absorbance spectroscopy, and compared with measurement of ozone and NOx concentration using dragger tubes results.

5.2.2 Set up of HVACP system

Figure 5.2 is the experimental set up for HVACP treatment. HVACP Treatment were conducted utilizing BK 130 HVACP system (Phenix Technologies, Accident, MD), a patented technology developed by Dr. Keener at Purdue University (Keener and Jensen, 2014). The HVACP system was operated at 200 W and 50 H_Z generating 90 kV between the electrodes (4.5 cm gap). The samples were placed in the polypropylene compartment box with a dimension of 4.4 cm Height ×18.4 cm× 27.9 cm (Grainger Inc, USA). Boxes were sealed inside high-barrier Cryovac B2630 film in order to prevent leakage of fill gas as well as generated reactive gas species. Air (78% N_2, 22% O_2) and modified atmosphere gas (MA65) (65% O_2, 30% CO_2, 5% N_2) were purchased from a local gas supplier (American Welding, Lafayette, IN) with a certificate of analysis. The storage boxes and sealed bags containing contaminated corn sample (25 g) were filled with the working gas (air or MA65) and purged multiple times for 2 min to ensure purity of the contained gas.

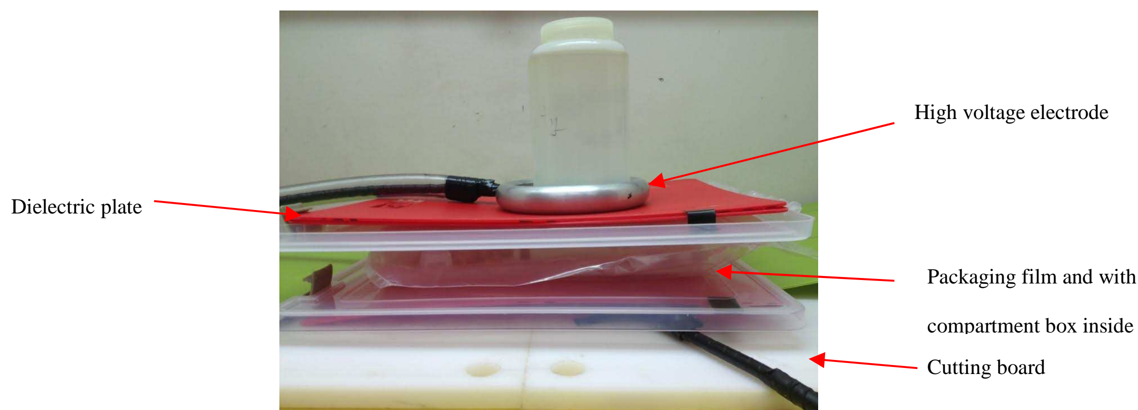


Figure 5.2. Set-up of high voltage atmospheric cold plasma (HVACP) system

5.2.3 Characterization of gas species generation using Optical Emission Spectroscopy

5.2.3.1 Experiment set-up of optical emission spectroscopy (OES)

The Schematic diagram of experimental set-up of OES for HVACP treatment is shown in Figure 5.3. Emission spectra during HVACP with different RH (5, 40, 80%), two filled gas (air, MA65) were acquired by a HR series spectrometer connected with a T300 series optical fiber (Ocean optics, USA).

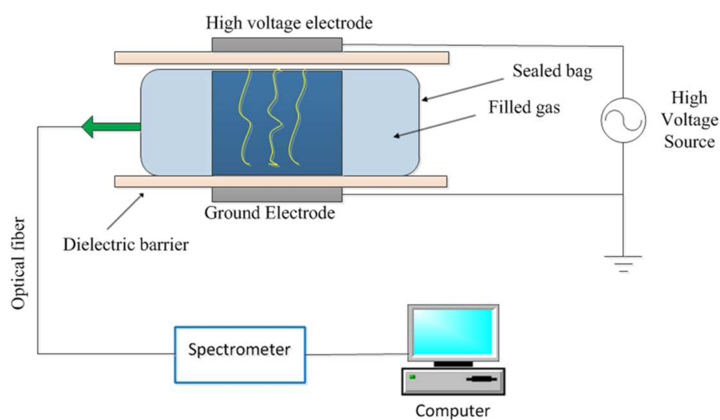
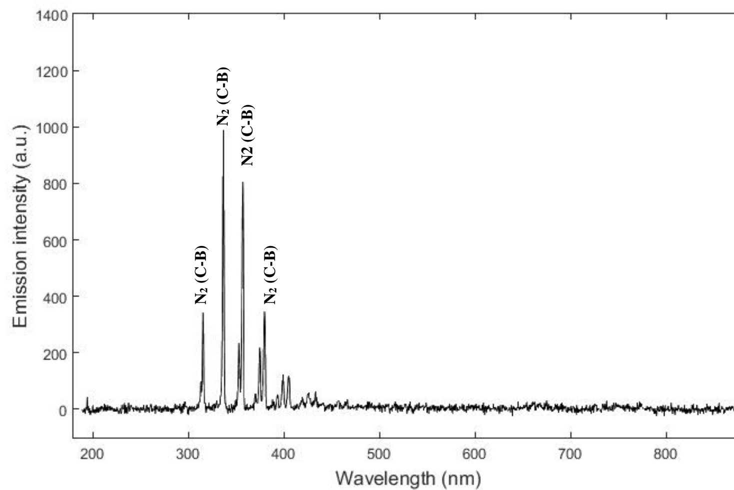


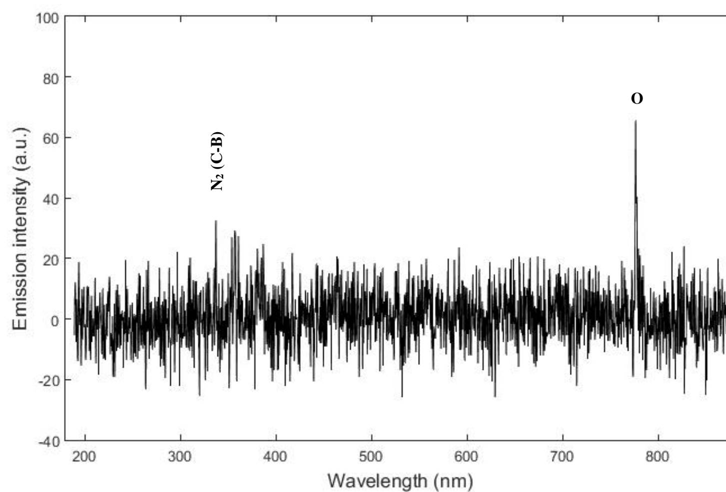
Figure 5.3. Schematic diagram of experimental set-up of optical emission spectroscopy.

5.2.3.2 Optical emission spectra analysis of HVACP

Figure 5.4 shows the emission spectra of HVACP for working gas air and MA65. The emission peaks for HVACP treatment using air was previously reported to be mainly attributed to N_2, O atom, and OH species. For example the transition of N_2 species has been reported at 335.3, 335.7, 357.9 nm (Laux et al., 2003) and at 315.93, 337.13, 353.67, 357.69 nm (Connolly et al., 2013). OH emission band was observed at 306-310, 306-322 nm (Parigger et al., 2003). O atoms emission was observed at 776.06 and 780 nm region (Connolly et al., 2013;Misra et al., 2013). From Figure 5.4(a), the major emission peaks for air during the HVACP treatment were at 316.102, 337.453, and 357.367 nm, which can be attributed to N_2 species transition. No distinct peaks were found for OH radical or O atom for air. The OH radical peak is known to be weak and could be easily overlapped with the N_2 species (Connolly et al., 2013), the emission peak for O atom is also weak for air during HVACP treatments (Misra et al., 2013). To our knowledge, no previous study reported the emission spectra for MA65. The major emission peak for MA65 is at 777.546 nm, which belongs to O atom on transition from O (^1D) to O (^3P). There were also small emission peaks from N_2 species in the near UV region, which were much smaller in MA65 than in air. The differences of emission spectra between MA65 and air are mainly caused by the difference in gas composition, MA65 gas consists of more oxygen and less Nitrogen than air, resulting in major emission peak of O atom and small emission peak of N_2.



(a)

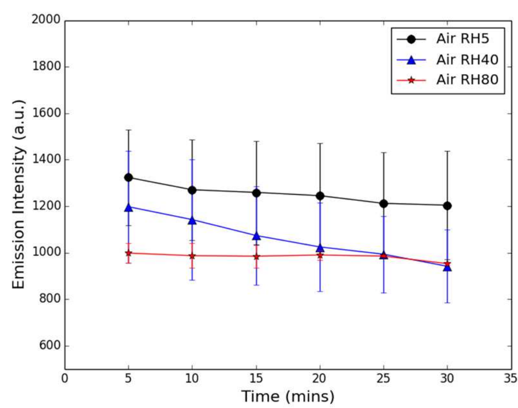


(b)

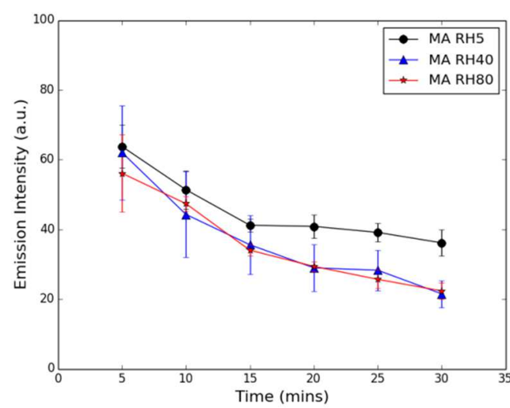
Figure 5.4 Optical Emission spectra of HVACP for a) Air and b) MA65 at 40% relative humidity

The effect of relative humidity (RH) during HVACP treatment was studied by evaluating the emission intensity of the major emission peak of HVACP treatment at wavelength 337.453 nm (N_2) for air and wavelength 777.546 (O) nm for MA65, and is shown in Figure 5.5. The emission intensity of N_2 emission peak (337.453 nm) tended to

decrease with increasing relative humidity and longer treatment time, although the change is not significantly different. The emission intensity of O emission peak (777.546 nm) decreased with increasing relative humidity and longer treatment time. The emission intensity in N_2 and O emission peaks decreased with increasing relative humidity, which may have resulted from competition for dissociation of water molecule. This is similar to one study that reported an increased emission intensity of OH radicals by dissociation of water molecule when the water content in air was increased to 50% and then stayed constant with a further increase in water concentration (Shin, Park et al. 2000). In this study increasing relative humidity to 40% significantly decreased the emission intensity of O atom, and no significant difference was found when relative humidity was further increased to 80%. The descending trend of emission intensity over time could be caused by gradual increase in temperature of filled gases and the discharging electrode (from 22 °C to around 38 °C), or by the decreased availability of diatomic oxygen for dissociation. These results suggested that the continuous application of HVACP is less efficient over long period time in breaking down diatomic Nitrogen and Oxygen for generation of ozone and NOx species. A pulsed HVACP system may be more efficient and may be applied in the future design of the system.



(a)



(b)

Figure 5.5 Emission intensity of HVACP by filled gas (a) Air at wavelength 337.453 nm and (b) MA at wavelength 777.546 nm MA over time.

5.2.4 Kinetics of gas species generation using Optical Absorption Spectroscopy

5.2.4.1 Principle of OAS

Optical absorption spectroscopy has been used to assess the gas composition after plasma exposure, observing the concentration of ozone (O_3) and that of NO_2, NO_3, N_2O_4. Within the applicability limits of Beer-lambert law, the absorbance is an additive quantity that depends linearly on species concentrations.

$$A = Ln\frac{I_{x=0}}{I_{x=L}} = \sum_{i=1}^{n}\sigma_i(\lambda)\cdot C_i \cdot L \qquad \text{Eq. 5.1}$$

The gas species concentration can be derived from the Beer-Lambert law under several conditions. Firstly, the Beer-Lambert law is valid only within a range of absorbance values, the upper limit is A=4.6 (for transmittance T=1%). Since the optical intensity at 250 nm is about 1000 (a.u.), and the detection minimum is 40 (a.u), thus the upper limit for our spectrometer is A= 3.2. Secondly, a direct calculation of species concentration is only valid when the absorption of other species is negligible (due to lower absorption cross-section area or very low concentration) over the considered wavelength interval. The absorption from water is not interfering here since its cross section between 200-600 nm is very low (cross section are 10^{-23}-10^{-25}cm^2/molecule) at about 5 order or magnitude lower than that species assessed in the study. The concentration of gas species are express as parts per million by volume (ppmv) with a conversion coefficient of $2.46*10^{13}$ molecule cm^{-3} for 1 ppmv (for air at atmospheric pressure and room temperature) The gas temperature during HVACP does not exceed 38 °C (measured with IR thermometer) and does not affect the ppmv conversion.

5.2.4.2 OAS experiment set up

The OAS was performed with using a UV-Vis (190-1100 nm) Ocean Optics HR2000+ES spectrometer with 0.9 nm resolution and a deuterium-halogen lamp with emission in the same UV-Vis range. The highly insulated optical probes consists of UV-Vis collimater (Ocean optics 6 nm diameter lens) and. The optical probes aligned by fitting into a notched tube. The optical signal transmitted through optical fibers (OZ optics UV-Vis, 1000 um corn, PVC coated, 30cm long). The integration time was adjusted to make peak intensity of optical signal around 90% from maximum detection limits, data was averaged over 10 measurements. The path length of OAS is the same as length of notches 24 mm.

5.2.4.3 Methods for calculation of gas species concentrations from OAS data

Figure 5.6 shows the absorption cross-sections of dominant species in humid air plasma and integration intervals. The wavelength intervals were selected considering the maximum (or a local maximum) in the absorption cross-sections for each species: NO_2, N_2O_4, O_3 and NO_3, around 398 nm, 346 nm, 253 nm and 525 nm, respectively (Table 5.3). Averaging absorbance along a wavelength interval allows for better accuracy of calculation.

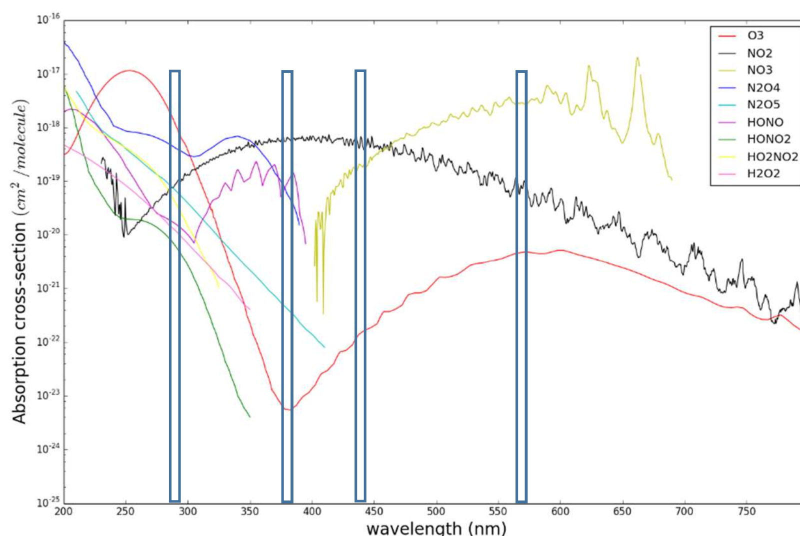


Figure 5.6. Absorption cross-sections of dominant species in humid air plasma and integration intervals.

Table 5.3. Selected order, wavelength intervals for each species and absorption cross-section reference.

i	Species	Wavelength interval (nm)	Absorption cross-sections	
			Refs	T (K)
1	NO_2	394.553-403.273	MPI[1]— Bogumil (2003)	298
2	N_2O_4	340.199-352.205	MPI— HallBlacet (1952) & Schneider(1987)	298
3	O_3	250.101-256.633	MPI—JPL, 2011	298
4	NO_3	515.361-536.151	MPI— IUPAC(2004)	298

MPI: Absorption cross-sections database (The MPI-Mainz UV/VIS Spectral Atlas of Gaseous Molecules of Atmospheric Interest

In order to estimate the concentration of gas species, NO2, N2O4, NO3a and O3 simultaneously across interested interval. Writing linear equations according to the Beer-Lambert law for 4 absorbers at 4 considered intervals.

$$\sum_{k=1}^{4}\sum_{i=1}^{4} C_i \times \sigma_{\lambda i} \times L = A(\lambda_k) \quad \text{Eq. 5.2}$$

Where C_i are the concentrations of each absorber (i), $\sigma_{\lambda i}$ is the absorption cross section area of each absorber (i) at specific wavelength (k), L is the path length and A is the measured absorbance at wavelength λ_k. A Gauss-Seidel successive under relaxation (GS-SUR, relaxation = 0.2) method (Mathews and Fink, 2004) was used to solve the above system of equations with relative error < 10^{-4}. The results from GS-SUR methods could be confirmed by using direct deconvolution methods (DD).

The observed species have absorption cross sections with maxima at intervals where other species have lower absorption therefore enable a direct deconvolution of their absorbance by successively subtracting the absorbance of each species, leading to residual absorbance:

$$A_i(\lambda) = A_{i-1}(\lambda) - C_{i-1} \cdot \sigma_{i-1}(\lambda) \cdot L \qquad \text{Eq. 5.3}$$

For λ in the interval 0-800 nm, i ≥ 1, where σ_i is the absorption cross section of the ith species. The order is based on that species which have least superposition of their absorbance with that of other species on their maximal absorption wavelength interval. Considering the absorption cross section in Figure 5.6, the order is NO2, N2O4, O3 and NO3. The absorbance at first wavelength interval is all attributed the first absorber, the concentration of first absorber is calculated according Beer-Lambert law using equation 1, then the concentration of other absorber are calculated sequentially from residual absorbance (calculated from Eq. 5.3) at their respective wavelength intervals.

5.2.4.4 Optical absorbance spectra analysis: Kinetics of ozone with different gas type and relative humidity.

5.2.4.4.1 Change of the optical absorbance spectra over time

The change of optical absorbance spectra with increasing HVACP treatment time is shown in Figure 5.7. A pronounced absorption over ozone Hartley band (200-300 nm) is observed. The height of peak over ozone Hartley band increases with longer treatment time, indicating higher concentration of ozone concentrations, absorbance at other wavelengths are minimal during the initial 2 min HVACP treatment.

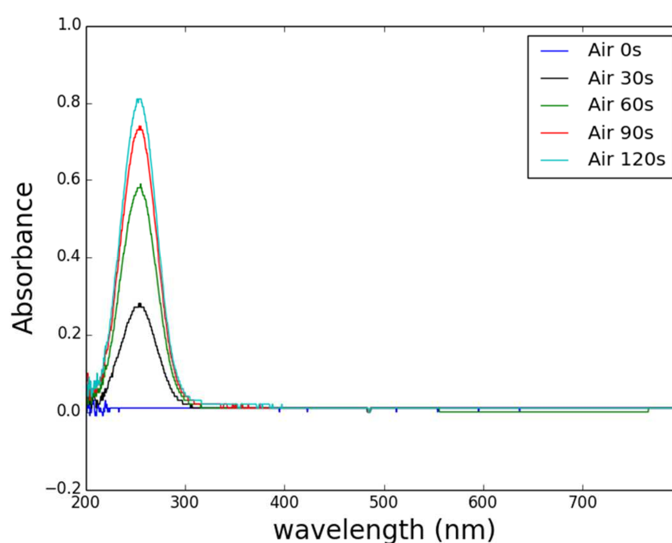


Figure 5.7. Change of the optical absorbance spectra during HVACP treatment.

5.2.4.4.2 Kinetics of ozone generation with different gases as filled gas in HVACP system

The concentration of ozone generated during HVACP treatment for different gases is shown in Figure 5.8. Very low concentration of ozone was generated for Nitrogen gas during HVACP treatment. The generation rate and final concentration of

ozone during HVACP treatment are higher in gas MA than in air. The increase of ozone concentration follows a logarithmic function for gas MA and air (R^2_{adj} >0.98) during the120 s HVACP treatment, the fitting parameters are put in Table 5.4. The generation rate (parameter a) of MA is 2.3 times of Air. Concentrations of generated ozone with 120 s HVACP treatment are 66, 1135, 2872 (ppmv) by gas Nitrogen, Air and MA65 respectively. The difference in generation rate and final concentration of ozone in MA, air, N_2 is mainly attributed to availability of oxygen, percentage of oxygen are 65, 21, and <0.01% in MA, air and N_2 respectively. It should be also noted that the increasing percent of oxygen did not result in the same amount of increase in the ozone generated, the percent of oxygen in MA is 3.1 times in air, however the generation rate and final ozone concentration in MA is 2.3 and 2.5 times the ozone concentration in air.

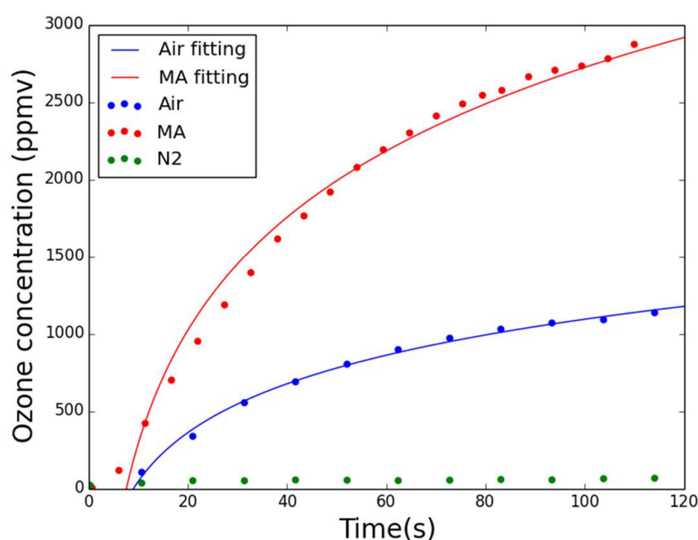


Figure 5.8. Increase of Ozone concentration over time during HVACP treatment for different fill gases (Air, MA, N_2).

Table 5.4. Fitting parameters and ozone concentration for HVACP in different fill gases.

Gas Type	a	b	R^2_{adj}	RMSE	O_3 (ppmv) [a]
Air	455	-1002	0.995	23.92	1135
MA	1057	-2144	0.981	117.1	2872
N2	NA				66

[a] Ozone concentration at 120 s HVACP treatment time.

5.2.4.4.3 Kinetics of ozone generation in air with different relative humidity during HVACP treatment

Increase of ozone concentration during HVACP treatment for air at different relative humidity was shown in Figure 5.9. The increase of ozone concentration follows logarithmic growth at different relative humidity ($R^2_{adj} > 0.98$). The fitting parameters and ozone concentration (at 120 s) for HVACP in air with different relative humidity are summarized in table 2. Higher relative humidity of air resulted in lower generation rate and ozone concentration during HVACP treatment. Generation rate (parameter a) of ozone during HVACP in air at RH 5% is 1.8 and 2.4 times than at RH 40 and 80%. Ozone concentration for 120 s HVACP in air at RH 5% is 1.8 and 2.3 times than at RH 40 and 80%.

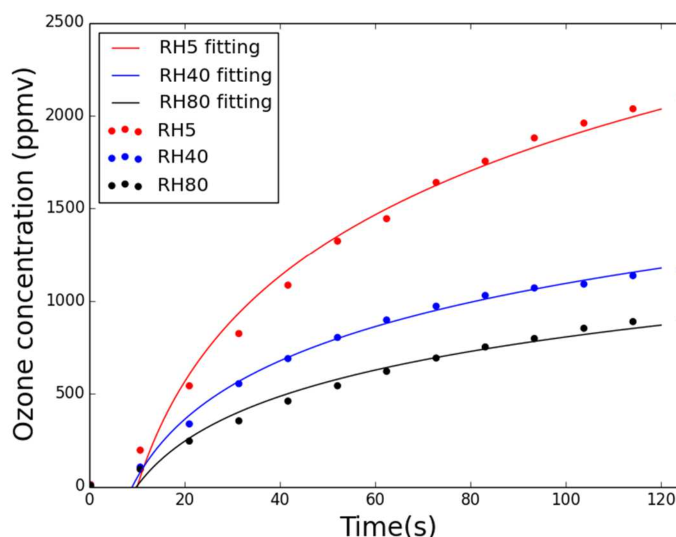


Figure 5.9. Increase of ozone concentration during HVACP treatment for air at different relative humidity (RH 5, 40, 80%)

5.2.5 Ozone and NOx concentrations measured using Drager tube: Effect of relative humidity and gas type

5.2.5.1 Measurement of Ozone and NOx concentrations using Drager tubes

Ozone and NOx concentrations in the sealed bag were measured in separate experiments from HVACP treatment without corn sample inside the bag, using Dragger short term detector tubes (ozone and NOx tube number is CH21001 and CH31001 respectively), the detector tubes gave a precision of ±10 ~15% (Draegerwerk AG, Germany). These tubes are easy to use and could give rapid measurement. Each tube contains colorimetric indicators that change color when coming into contact with specific gas. Each gas was analyzed by detector tube using Accuro Gas detect Pump (Draeger safety AG & Co. KGaA, Germany) with each pump equivalents to 100 ml. For high

concentrations of ozone, smaller gas sample volumes were collected in 5 or 20 ml syringes, and connected to the detection tubes by means of flexible tubing.

5.2.5.2 Concentration of ozone and NOx generated by HVACP with air/MA65 at different relative humidity

The concentration of ozone and NOx generated by HVACP with air/MA65 at different relative humidity are shown in Figure 5.10. The effects of relative humidity, gas type and treatment time are all significant on ozone concentration generated by HVACP. Higher ozone concentration was generated in MA65 than in air. Increasing relative humidity resulted in decreased ozone concentration in air and MA65, ozone concentrations generated by HVACP for 5 min with 5, 40, 80% RH are 5250±354, 4500±441, 3750±768 ppm in air, and 15000±2500, 13125±2652, 11875±2419 ppm in MA65. The reduced ozone concentration with higher relative humidity could be caused by decreased ozone generation and increased ozone decomposition rate. Decreased concentration of generated ozone is due to competing dissociation of the water molecule under high RH conditions. Less diatomic oxygen are broken down as indicated from the reduced emission intensity of O atom at 40 and 80% RH compared to 5% RH (Figure 5.5 b). Also the decomposition rate of ozone is significantly increased with elevated relative humidity (Mueller et al., 1973); thus lower concentration of ozone were left after HVACP treatment at higher relative humidity. Increasing treatment time from 0 to 10 min increased the concentration of ozone concentration, then the ozone concentration reached saturation status when the rate of generation and decomposition of ozone were equal. At 5% RH, the saturated ozone concentration generated by HVACP in MA65 was

17500±1000 pm, which is 3.33 times the saturated ozone concentration in air 5250±354 ppm, the ratio of saturated ozone concentration MA65 to air was 3.3, which is very close to the ratio of percent oxygen 3.1 in MA65 (65%) to air (21%). The increased ozone concentration generated in MA65 than in air could be ascribed to increased availability of diatomic oxygen to be dissociated into oxygen atom and then be recombined to form ozone, as could be confirmed from optical emission spectra. The emission peak of oxygen atom in air is not discernible as compared to obvious emission peaks of oxygen atom in MA65 (Figure 5.4).

For NOx concentration generated by HVACP, the factor effects of gas type, relative humidity, and treatment time were also significant. Higher concentrations of NOx were obtained in MA65 at lower relative humidity over longer treatment time (Fig. 4b). The increased concentration of NOx in MA65 is mainly due to high concentration of O atoms from breaking down diatomic oxygen as indicated in Figure 2. The O atoms are able to recombine with Nitrogen species to form NOx (Moiseev et al., 2014), the concentration of mentioned NOx species decreased with increasing relative humidity, which agrees with the study by (Moiseev et al., 2014). In that study the concentration of the various NOx species (N_2O_4, NO_2, NO_5, NO_3) were deconvoluted from the absorption spectra, decreased NOx concentration with higher relative humidity is attributed to competition of dissociation energy by water molecule (Moiseev et al., 2014). Increasing treatment time from 0 to 10 min increased the NOx concentration to saturation, then the NOx concentration stabilized with further increase in treatment time. NOx concentrations generated by HVACP for 10 min with 5, 40, 80% RH were 1650±400, 625±100, 563±88 ppm in air, and 12250±354, 9000±3000, 8750±2500 ppm in MA65.

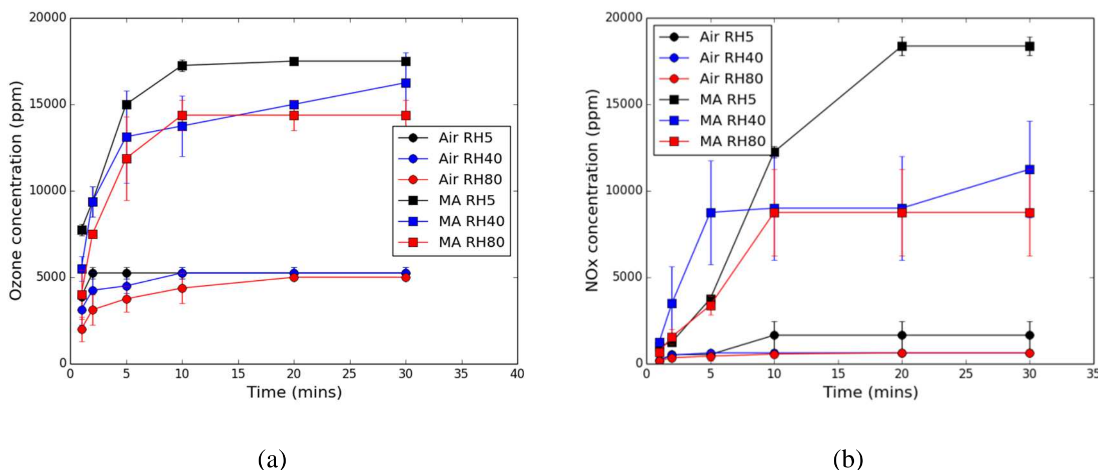


Figure 5.10 Concentration of (a) Ozone and (b) NOx generated by HVACP with different relative humidities and types of working gas.

5.2.6 Conclusions

Optical emission analysis shows that during HVACP treatment, the major emission peaks were N_2 and O atom for air and MA65 emission spectra, respectively. The emission intensity of O atom decreased with increasing relative humidity and longer treatment time. The HVACP system is less efficient in breaking down diatomic nitrogen and oxygen over a longer period of time.

Ozone concentration during HVACP treatment could be successfully deconvoluted through optical absorption spectra analysis. Kinetics of generation of ozone concentration follows a logarithmic function for gas MA and air (R^2_{adj} >0.98) during the120 s HVACP treatment. Ozone generation rate and final ozone is higher in gas MA than air, with lower relative humidity (5%) than with higher relative humidities (40, 80%). Ozone generation rate and final ozone concentration are 2.3 and 2.5 times higher in MA than in air. Generation rate (parameter a) of ozone during HVACP in air at RH

5% is 1.8 and 2.4 times than at RH 40 and 80%. Ozone concentration for 120 s HVACP in air at RH 5% is 1.8 and 2.3 times than at RH 40 and 80%.

Results from measurement of Ozone and NOx using dragger tubes confirmed the finding of OAS study. During 30 min HVACP treatment, Higher concentration of ozone and NOx are generated in MA65 than in air, and with lower relative humidity. The concentration of ozone and NOx increased initially with increasing HVACP treatment time, reached a peak and stabilized after10 min HVACP treatment.

5.3 Reduction of aflatoxin in corn by HVACP treatment

5.3.1 Background: literature review of reduction of aflatoxin by cold plasma

To our knowledge, no research has been conducted to study the effect of HVACP treatment on aflatoxins produced by the fungal species. Two studies that generate cold plasma other than HVACP have demonstrated the potential of cold plasma for aflatoxin detoxification. Pure aflatoxin B_1 on cover glass was deactivated by microwave induced plasma that employ noble gas argon (Park et al., 2007). In another study, 50% reduction of aflatoxin in nuts was achieved by lower pressure cold plasma (LPCP) (Basaran et al., 2008). HVACP system is capable of generating a number of reactive gas species, such as charged particles, ions, radicals, reactive oxygen and nitrogen species. Ozone is one of the major species generated by HVACP treatment (Misra et al., 2013). Ozone has been reported as effective detoxifier of aflatoxin in corn and peanuts (Luo et al., 2014;Chen et al., 2014). Thus HVACP treatment holds great potential for degradation of aflatoxins.

Generation of reactive species by HVACP could be influenced by a variety of parameters, such as the voltage, electrode gap distance, type of filled gas, gas relative humidity, and mode of reaction (direct/indirect) (Keener and Jensen, 2014;Moiseev et al., 2014). The performance of HVACP treatment is also affected by treatment time, post-treatment storage, or stirring of products during experiment (Misra et al., 2011;Ziuzina et al., 2013). This research studied the effect of gas type (air and MA65), relative humidity (5,40 80%), treatment time (0, 1, 2, 5,10, 20, 30 min) on efficacy of aflatoxin degradation in corn by HVACP treatment. The optical emission spectroscopy (OES) is applied to evaluate performance of HVACP system with different gas types and relative humidity. The concentration of ozone and NOx generated are also measured with different working gases at different relative humidities.

The objectives of this section were to investigate the influence of relative humidity, gas type, and treatment time on degradation of aflatoxin in corn by HVACP treatment, to obtain the kinetic model of percent degradation of aflatoxin in corn, and to investigate the effect of other important factors, such as mode of reaction, post-treatment storage, stirring on degradation of aflatoxin in corn by HVACP treatment.

5.3.2 Materials and Methods

5.3.2.1 Aflatoxin contaminated corn sample

Pure Aflatoxin B_1 (AFB_1) powder (5 mg) was purchased from Sigma Aldrich and first dissolved in 1ml chloroform solution, then serially diluted to concentration of 50 ug/ml in chloroform. Dent yellow corn was harvested in Purdue Agronomy Center for

Research and Education (ACRE) farm in 2014 fall, hand shelled and naturally dried to moisture content of 14.7% (w.b.), and confirmed free from aflatoxin contamination. Each aflatoxin contaminated corn sample was then prepared by spiking 100 ul AFB_1 solution in chloroform (50 µg/ml) on 25g corn kernels, and spiked samples were allowed to dry (30s) as the chloroform solution evaporated.

5.3.2.2 HVACP treatment of aflatoxin in corn

Figure 5.11. is the schematic of the experimental set up for HVACP treatment of aflatoxin in corn. 25 g corn sample spiked with AFB1 was placed inside a translucent polypropylene compartment box and sealed inside high-barrier Cryovac B2630 film in order to prevent leakage of fill gas as well as generated reactive gas species. Air (78% N_2, 22% O_2) and modified atmosphere gas (MA65) (65% O_2, 30% CO_2, 5% N_2) were purchased from a local gas supplier (American Welding, Lafayette, IN) with a certificate of analysis. HVACP Treatment were conducted utilizing BK 130 HVACP system and operated at 200 W and 50 H_Z generating 90 kV between the electrodes (4.5 cm gap). The storage boxes and sealed bags containing contaminated corn sample (25g) were filled with the working gas (air or MA65) and purged multiple times for 2 min to ensure purity of the contained gas. The gases in the tank have 5% relative humidity, to increase the relative humidity to 40 and 80%, the working gas are passed through a water bubbler with specific flow rate and water depth, the resultant humidity was measured with a psychrometer (Extech Instruments Inc.). The final relative humidities were ±3% for target relative humidity of 40 and 80%. The samples were HVACP treated for 1, 2, 5, 10,

20, 30 min, either direct or out of field (indirect), with or without post-treatment storage inside the sealed bag for 24 h at room temperature (22 ºC).

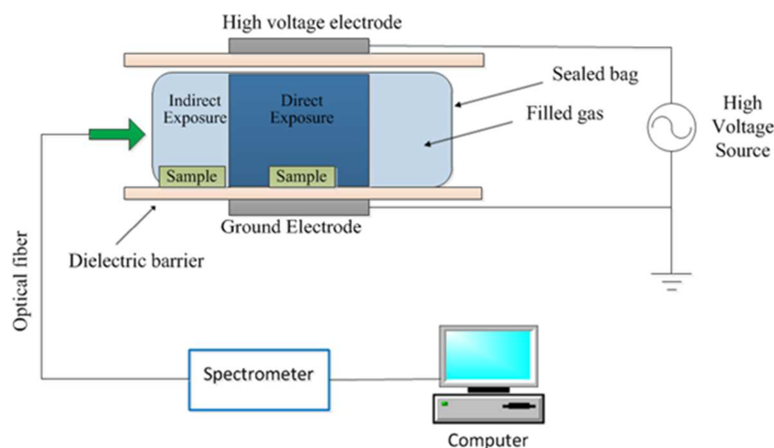


Figure 5.11. Schematic diagram of experimental set-up for HVACP treatment of aflatoxin in corn

5.3.2.3 Temperature measurement

Temperatures of the electrodes and treated samples were measured immediately following treatment using an infrared thermometer (Omega Engineering Inc, NY, USA). The electrodes of HVACP system were cooled to room temperature (around 22 °C) between treatments for uniform treatment temperature conditions.

5.3.2.4 Degradation kinetics of aflatoxin by HVACP

First-order model has been traditionally used to model the inactivation kinetics of microorganism and enzyme by heating and other processing technologies (Schaffner and Labuza, 1997). Experimental data of aflatoxin reduction in corn was modeled using a first-order degradation model:

$$AF = AF_0 \cdot e^{-kt}$$

Where AF is the residual aflatoxin level (ppb), AF_0 is initial aflatoxin level (420 ppb), t is HVACP treatment time, k in the degradation constant, and is obtained by least square nonlinear regression.

However due to significant deviation from first order model have been reported, Weibull model was frequently used to describe destruction kinetics of microorganism and enzymes (Chen and Hoover, 2004;van Boekel, 2002). In this study, Weibull model was fitted the degradation kinetics of aflatoxin:

$$AF = AF_0 \cdot e^{-(t/\alpha)^{\gamma}}$$

Where, α is the scaler factor (min) and γ is the shape parameter (dimensionless) which indicates concavity or convexity of the curve. In addition to the first-order and Weibull model, a three parameter logistic model, was evaluated considering the visual shape of data, this model has been applied to fit the inactivation of enzyme by HVACP treatment (Pankaj et al., 2013). The logistic model for aflatoxin degradation kinetic is as:

$$AF = \frac{AF_0 - AF_{min}}{1 + (t/t_{50})^p} + AF_{min}$$

Where, AF_{min} is the minimum value attained by the logistic function ($AF_{min} \geq 0$ ppb), p is the power term, and t_{50} is the time for half degradation of aflatoxin by HVACP treatment. This equation represents a sigmoidal type of degradation curve. The data of aflatoxin degradation kinetics was fitted using the Curve Fitting toolbox of Matlab R2015a (Mathworks Inc, USA). Two fitting parameters, adjusted coefficient $R^2_{(adj)}$ and root mean

squared error (RMSE), was obtained during curve fitting, and were used for assessment of the goodness of fit.

5.3.2.5 Statistical analysis

Statistical analysis was performed for the data of aflatoxin level, ozone and NOx concentrations, and optical emission intensity using statistical software package (SAS 9.3, SAS Institute Inc., USA). The factor effects were analyzed by running the Proc GLM model and the means were compared using Tukey's test. Significant differences between treatments were obtained at $P < 0.05$ for the analysis.

5.3.3 Results and discussion

5.3.3.1 Effect of RH, gas type on degradation of Aflatoxin

Residual AF levels in corn by HVACP with different gases, relative humidity, and treatment time were listed in Table 5.5 and plotted in Figure 5.12. All the factors: relative humidity, gas type and treatment time have a significant effect on the aflatoxin degradation in corn by HVACP.

MA65 gas is more effective than air in degrading aflatoxin in corn as the fill gas for HVACP treatment with all relative humidity and treatment times. For example, the residual aflatoxin levels in corn were 161±15 and 102±17 ppb after 1 min HVACP treatment with air and MA65 at 40% RH. This difference could be mainly attributed to higher concentration of reactive species generated in MA65 than in air. The generated concentration of ozone and NOx are more than 3 and 10 times higher in MA65 and in air during 5 min HVACP treatment at relative humidity 40% (Fig. 4). However, it should be noted that the difference in aflatoxin degradation between using air or MA65 was

minimized when a longer treatment time was used, the residual aflatoxin in corn after 10 min HVACP treatment with air and MA65 at 40% RH are 65±7 and 53±6 ppb, respectively.

More humid air (40, 80% RH) resulted in higher degradation of aflatoxin in corn by HVACP compared with dry air (5% RH). There was no significant difference in aflatoxin degradation by HVACP between 40 and 80% RH. The optical emission spectra have shown that HVACP treatment breaks down more diatomic oxygen, and that higher concentration of ozone and NOx are generated at lower relative humidity 5% than at 40, 80% RH. Thus, factors other than ozone and NOx concentration are responsible for the higher degradation of aflatoxin in corn at higher relative humidity (40, 80% RH). The increased efficacy in aflatoxin degradation by humidified gas could most likely be attributed to higher concentration of OH radicals either generated by HVACP or through reaction of ozone with water. OH radical is a strong oxidizer and have a stronger oxidizing power than ozone (Diao et al., 2013). The main pathway leading to OH radicals by HVACP is the direct dissociation of water molecule in the gas. Increased emission intensity of OH radicals was observed when RH was increased to 50%, indicating higher concentration of OH radicals generated using pulsed corona plasma by dissociation of water molecules, and then the concentration of OH radicals remained constant with further increase of RH. (Shin et al., 2000). The OH radicals could also be formed from decomposition of ozone by reaction with water and enhance the oxidization power (Staehelin and Hoigne, 1985). Ozone application with wet method (water bubbled) had better efficacy in degrading aflatoxin B_1 than the dry method (dry gaseous) in in wheat, paddy rice, and corn, due to generation of OH radical through reaction of water with

ozone (Wang et al., 2010). Thus, the oxidation power of ozone is enhanced by water-vaporization. This is confirmed by the study that show that humidified ozone is shown to more effective than dry ozone in decontaminating fungi on surface of corn kernels when applied in a modified screw conveyor (McDonough et al., 2011).

Aflatoxin in corn was detoxified very quickly using HVACP treatment; more than half of aflatoxin was degraded in 1 min. Increasing the treatment time further increases aflatoxin reduction in corn; over 70% of aflatoxin was degraded after 10 min of HVACP treatment. However, the increase of aflatoxin degradation is low when the HVACP treatment time is more than 10 min. There exist limiting factors that prevent complete elimination of aflatoxin in corn. The maximum percentage of aflatoxin degradation in corn is around 90% even with 30 min HVACP treatment. The obstacles for complete elimination of aflatoxin could be a result of two factors: lack of stirring of corn to enable kernels to be fully exposed to reactive species, and/or difficulty of penetration of the reactive gas species into corn kernels to react with the aflatoxin.

Table 5.5 Residual aflatoxin levels in corn treated with HVACP using different gases, RH, and treatment time*

Gas Type	Air			MA65		
Time (min) \ RH (%)	5	40	80	5	40	80
1	$187\pm10^{A\alpha}$	$161\pm15^{A\alpha\beta}$	$127\pm21^{A\beta\gamma}$	$143\pm24^{A\beta\gamma}$	$102\pm17^{A\gamma}$	$110\pm10^{A\gamma}$
2	$151\pm14^{B\alpha}$	$132\pm12^{B\alpha\beta}$	$100\pm12^{B\gamma\sigma}$	$111\pm13^{AB\beta\gamma}$	$72\pm5^{B\sigma}$	$92\pm8^{AB\gamma\sigma}$
5	$141\pm8^{BC\alpha}$	$92\pm5^{C\beta}$	$80\pm5^{C\beta}$	$87\pm11^{BC\beta}$	$49\pm2^{C\beta}$	$78\pm5^{B\gamma}$
10	$112\pm8^{CD\alpha}$	$76\pm4^{CD\beta}$	$70\pm5^{CD\beta}$	$80\pm5^{BC\beta}$	$53\pm6^{BC\gamma}$	$46\pm3^{C\gamma}$
20	$89\pm17^{DE\alpha}$	$65\pm7^{D\alpha\beta}$	$59\pm5^{DE\beta}$	$65\pm2^{C\alpha\beta}$	$44\pm5^{C\gamma}$	$49\pm6^{C\beta\gamma}$
30	$81\pm4^{E\alpha}$	$52\pm4^{D\gamma}$	$42\pm2^{E\sigma}$	$67\pm7^{C\beta}$	$42\pm2^{C\sigma}$	$37\pm3^{C\sigma}$

*replicates: 3, the initial AF level in corn is 420 ± 21 ppb, Values with different English and Latin superscripts are significantly different among the specific column and row.

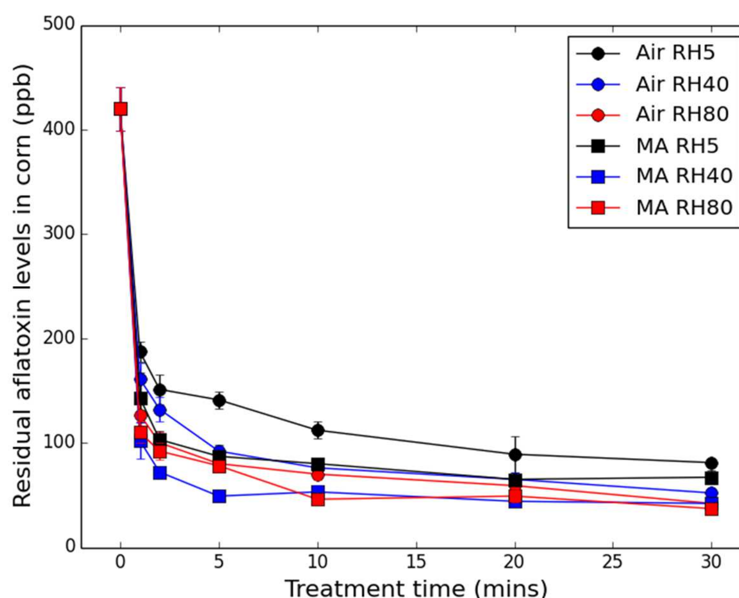


Figure 5.12 Reduction of aflatoxin level in corn by HVACP treatment with different fill gases, relative humidities, and treatment time.

5.3.3.2 Degradation kinetics of aflatoxin by HVACP

Degradation kinetics of aflatoxin by HVACP treatment was fitted using first-order, Weibull and logistic model, results on the parameters of on the parameter of the models were summarized in Table 5.6. From fitting parameter R^2 and RMSE, it can be observed that the first-order model is not satisfactory with low coefficient ($R^2 = 0.48$ ~0.90), and Weibull and Logistic model were comparable with high coefficient ($R^2 \geq$ 0.99). It could be observed that Weibull model could not account for the tailing effect of the aflatoxin degradation data, for example when the AFmin > 0 ppb (For data of Air at 5% RH, and MA at 5 and 40% RH). A higher RMSE was resulted from the tailing effect. Logistic model fitted the degradation kinetics more closely with lower RMSE compared to Weibull model. This result is similar to the inactivation kinetic for tomato peroxidase by HVACP treatment, which is also better described by the logistic model than first-

order, and Weibull models (Pankaj et al., 2013). The logistic model curve fitting for degradation kinetics of aflatoxin in corn by HVACP treatment was plotted in Figure 5.13. The parameter AF_{min} represents the tailing effect of logistic model, which is from incomplete degradation of aflatoxin by HVACP due to inability of penetration of reactive gas species into corn kernels. The parameter t_{50} (time required for half reduction) value from the logistic model ranges from 0.07 to 0.48 min, indicating rapid degradation of aflatoxin by HVACP treatment. Also parameter t_{50} value is smaller for gas MA65 at higher relative humidities (40, 80%), indicating more rapid degradation rate for HVACP in MA65 and at higher relative humidities.

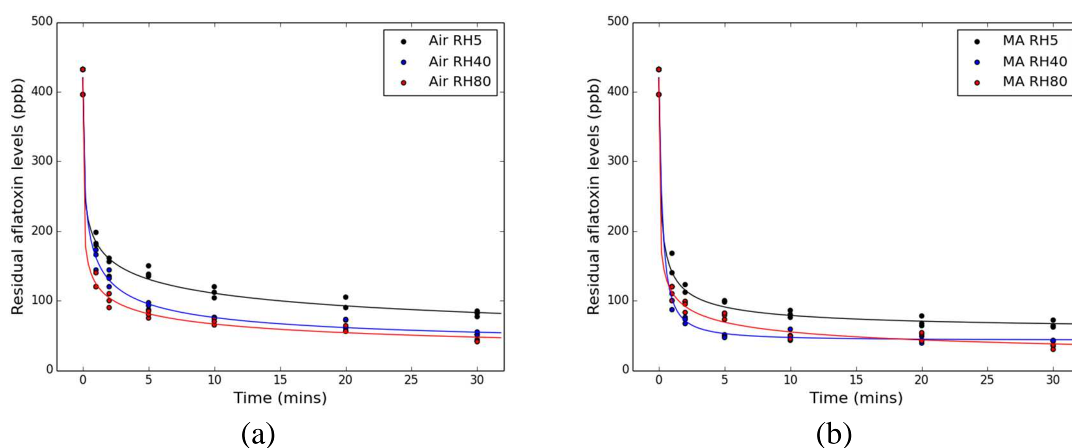


Figure 5.13. Percent reduction of aflatoxin in corn by HVACP treatment in a) Air and b) MA65 at different relative humidities over time. Solid lines represent fitted model

Table 5.6 Results on the parameters of the models fitted to degradation kinetics of aflatoxin by HVACP treatment.

Fitting Model	Parameter	Air[a]			MA65[a]		
		RH 5	RH 40	RH 80	RH 5	RH 40	RH 80
First order	k	0.47±0.11	0.68±0.11	0.94±0.16	0.82±0.16	1.21±0.17	1.07±0.16
	$R^2_{(adj)}$	0.48	0.77	0.82	0.72	0.90	0.86
	RMSE	80.41	58.24	53.47	63.33	40.67	47.25
Weibull	α	2.47±0.32	1.05±0.15	0.30±0.08	0.43±0.153	0.05±0.04	0.20±0.07
	γ	0.20±0.02	0.23±0.02	0.17±0.02	0.15±0.20	0.14±0.20	0.18±0.02
	$R^2_{(adj)}$	0.99	0.99	0.99	0.99	0.99	0.99
	RMSE	12.31	17.80	14.60	23.07	21.95	10.22
Logistic	AF_{min}	0.00±18.82	26.55±22.40	0.00±21.12	54.62±19.38	42.70±5.21	0.00±23.64
	t_{50}	0.48±0.56	0.32±0.06	0.08±0.03	0.16±0.10	0.25±0.07	0.07±0.04
	P	0.34±0.19	0.56±0.22	0.35±0.19	0.64±0.32	1.20±0.39	0.38±0.23
	$R^2_{(adj)}$	0.99	0.99	0.99	0.99	0.99	0.99
	RMSE	12.76	10.20	9.78	12.57	9.78	10.65

[a] fitting parameters (mean ±standard error).

5.3.3.3 Effect of mode of reaction, post-treatment storage, and stirring on aflatoxin degradation in corn

In order to better understand the mechanism of aflatoxin degradation in corn by HVACP, the parameter effect of mode of reaction, post-treatment storage, and stirring were studied. The effect of mode of reaction in corn by HVACP is presented in Table 5.7. No significant difference in aflatoxin degradation in corn was found between direct or indirect HVACP treatment. Generally, under direct HVACP treatment, the sample is exposed to all of the generated reactive gas species, including positive or negative ions, charged particles, excited and non-excited molecules, free radicals, or heat and UV radiation. In contrast, under indirect HVACP treatment, the sample is subject only to long-lived reactive species such as NOx, ozone, peroxide etc. (Ziuzina et al., 2013;Laroussi and Leipold, 2004). Thus, it could also be inferred that the aflatoxin in corn is mostly destroyed by long-lived reactive gas species like ozone or NOx, instead of short-lived species like free radicals, charged particles, ions. Aflatoxin degradation by

HVACP generated by Nitrogen gas is remarkably different from air and MA65, thus the oxygen molecule in air and MA65 plays a vital role in generating reactive oxygen species (e.g. ozone, peroxide), which are likely to be responsible for degrading the aflatoxin B_1. Ozone is long-living and very oxidative species generated during HVACP treatment and peroxide could be produced by the ozone reacting with water molecules (Klockow and Keener, 2009). The exact reaction mechanism of aflatoxin during HVACP treatment and degradation products of aflatoxin still needs to be further investigated.

Table 5.7. Effects of the mode of reaction on aflatoxin degradation in corn by HVACP treatment.

Gas type	Treatment Time (min)	Mode of reaction	AF (ppb) (mean ± std)	AF Percent reduction (%)
Nitrogen	1	indirect	390±15	7
		direct	396±18	6
	30	indirect	330±26	21
		direct	340±16	19
Air	1	indirect	161±15	62
		direct	159±14	62
	30	indirect	52±4	88
		direct	55±12	87
MA65	1	indirect	102±17	76
		direct	138±32	67
	30	indirect	42±2	90
		direct	47±8	89

Note: Initial aflatoxin level in corn: 420±21, 3 replicates, HVACP treatment condition: RH 40%, treatment time 10min.

The effect of post-treatment storage on aflatoxin degradation in corn by HVACP is presented in Table 5.8. Storing HVACP treated corn within the sealed package for 24 h significantly improved the aflatoxin degradation in corn ($P<0.01$). The increase in percent reduction of aflatoxin was 9% for air and 8% for MA65. It should be noted that majority of aflatoxin detoxification happened during the first 10 min of HVACP treatment instead

of the prolonged storage period (24 h). This indicates that the HVACP treatment could detoxify most of the aflatoxin in corn quickly (within minutes), post-treatment storage of the sample is not required, although it could slightly increase the amount of aflatoxin degraded.

Table 5.8. Effects of post-treatment storage on aflatoxin degradation in corn by HVACP treatment [a]

Gas species	Storage time (h)	AF (ppb) (mean ± std)[b]	Percent Reduction (%)
Air	0	113±15 [A]	73
	24h	76±4 [B]	82
MA65	0	103±4 [A]	76
	24h	53±6 [C]	88

[a]Initial aflatoxin level in corn: 420±21, HVACP treatment condition: 40% RH, treatment time 10min,
[b]Different letters in the column mean significant difference between treatments ($P < 0.05$).

The effect of stirring of corn on aflatoxin degradation in corn by HVACP treatment is presented in Table 5.9. Sample stirring significantly increase percent reduction of aflatoxin in corn for both air and MA65 ($P<0.05$). HVACP treatment degraded 82 and 88% of aflatoxin in corn in air and MA65 without stirring. Stirring the corn multiple times increased the percent reduction of aflatoxin to 92% for both air and MA65, this clearly demonstrated that stirring could increase aflatoxin degradation to a certain limit (92% in our study) by fully exposing the outer surface of corn kernels. However complete elimination of aflatoxin in corn is not achieved by stirring, which might result from the lack of penetration of reactive species to detoxify the aflatoxin present inside the corn kernels.

Table 5.9. Effects of corn stirring on aflatoxin degradation in corn by HVACP treatment [a]

Working gas	Stirring/No stirring[b]	AF (ppb) (mean ± std)	Percent Reduction (%)
Air	No-stirring	76±4 A	82
	Stirring	34±5 C	92
MA65	No-stirring	53±6 B	88
	Stirring	35±6 C	92

[a]Initial aflatoxin level in corn: 420±21, HVACP treatment condition: RH 40%, treatment time 10min.
[b]Stirring: shake the corn in the plastic box 30s after every 2.5 min HVACP treatment

5.3.4 Conclusions

Aflatoxin in corn is rapidly degraded by HVACP treatment with increasing HVACP treatment time, aflatoxin in corn was degraded by 62% and 82% after 1 and 10 min, respectively, using HVACP treatment in RH 40% air. Three kinetic models (first-order, Weibull, and logistic model) were fitted to the aflatoxin degradation data, logistic model is found to be the best to describe the degradation kinetics of aflatoxin by HVACP with high coefficient ($R^2 \geq 0.99$). Stirring the corn during HVACP treatment significantly increased aflatoxin degradation to 92%. The inability of reactive species generated by HVACP to penetrate into corn kernels seems to be the limiting factor preventing complete degradation of aflatoxin in corn. MA65 is slightly more effective for HVACP treatment in degrading aflatoxin in corn than air as the working gas because of higher concentration of Ozone and NOx species are generated in MA65. HVACP treatment with humid air/MA65 at relative humidity 40% and 80% was more effective than dry gas (5% RH). Direct or indirect HVACP treatment was equally effective in the degradation of aflatoxin in corn. The majority of aflatoxin degradation happens within minutes during

HVACP treatment, post-treatment storage of corn and further interaction with reactive gas species slightly increase aflatoxin degradation potential.

5.4 Application of HVACP for reduction of aflatoxin in distiller grains

5.4.1 Background: Critical parameters on application of HVACP in distiller grains

Application of HVACP to reduce aflatoxin in corn have been investigated, process parameters for HVACP, such as relative humidity, gas type, direct/indirect treatment, and stirring of samples on the efficacy of HVACP treatment have been investigated in previous sections. Parameters related to material for application could also have great importance. For example, sample amount, grain depth have great influence on the efficacy of HVACP treatment on microbial load reduction in distiller wet grains (McClurkin, 2015).

This section investigated application of HVACP for reduction of aflatoxin in distiller grains. Degradation of aflatoxin by HVACP treatment were compared for three coproducts during ethanol bioprocessing: wet distiller grain (DWG), condensed solubles (CDS), and dried distiller grain with solubles (DDGS). Impact of Parameters, including sample amount, surface area of exposure, depth of sample, treatment time, were investigated for their effects on reduction of aflatoxin in DDGS by HVACP treatment. Effects of parameters of material type, sample amount and surface area on ozone concentration during HVACP treatment was evaluated by monitoring the ozone concentration during HVACP treatment using optical absorption spectroscopy. Relative

importance analysis was performed to identify the critical parameters for the efficacy of HVACP treatment. Stepwise regression model was performed for prediction of percent reduction of aflatoxin in DDGS by HVACP treatment.

5.4.2 Experimental Set up and methods

5.4.2.1 Sample spiked with aflatoxin

DDGS sample were prepared by mixing 90% (by weight) wet distiller grains (WDG) and 10% (by weight) condensed solubles (CDS), and drying the mixed material in air oven at 150 °C for 48 hours to moisture content of around 12%. The DWG and CDS were obtained from Andersons Clymers Ethanol LLC (Clymers, Indiana). Same amount of aflatoxin was spiked to WDG, CDS, DDGS, 100 µl aflatoxin B_1 solution in chloroform (50 µg/ml) into 10g of the samples of WDG, CDs, DDGS.

5.4.2.2 Measurement of aflatoxin in DWG, CDS and DDGS

AFB_1 levels in DDGS were determined using a commercial Lateral Flow Device (LFD) QuickTox test strip for DDGS (AQ-109-BG3) and a QuickScan reader (Envirologix USA, Inc., Portland, Maine). DDGS sample was firstly ground in a Disc Mill (Seedburo Equipment Co., Des Plaines, Il.) using a 20 mesh screen. A 10.0 g subsample was mixed with 30.0 ml 50% ethanol solution, and the mixture was shaken by hand for 1.5 minutes. After the mixture settled (around 10 minutes) there were two distinct layers. The top layer was used for aflatoxin determination by means of the LFD test strips. Measurement of aflatoxin levels in DWG and CDS follows the methods described in section 4.4.1.5.

5.4.2.3 Measurement of ozone concentration using Optical absorption spectroscopy

Ozone concentration generated during HVACP treatment were measured using optical absorption spectroscopy. The specific measurement set up is described in section 5.2.5.2. To investigate the effect of different material on ozone generation during HVACP treatment, different material (water, DWG, CDS, DDGS) were placed in a plastic petri dish (90 * 15 mm) without cover and placed in the box and treated with HVACP. Different amount of DDGS sample (5, 10, 20 g) were placed in the plastic petri dish (90 * 15 mm) to determine effect of sample amount on ozone concentration in the box. DDGS samples were exposed with different surface area by placing in a different container, small petri dish (65 * 15 mm), one standard petri dish (90 * 15 mm), two standard petri dish, and subject to HVACP treatment. The surface area of DDGS placed for small petri dish, one standard petri dish and two standard petri dish are 34, 56, 112 mm^2 respectively. The optical absorption spectra of generated gas species by HVACP were obtained over 10 min HVACP treatment.

5.4.2.4 Treatment of aflatoxin in distiller grains by HVACP

Samples of aflatoxin spiked DWG, CDS, or DDGS were treated by HVAVP in the same way as aflatoxin in corn, and is described in detail in section. The samples (DWG, CDS, and DDGS) in this experiment is treated by HVACP for 2 and 10 min. aflatoxin spiked DDGS were treated with different sample amount (5, 10, 20 g) and varying surface area (34, 56, 112 mm^2) for exposure to the cold plasma field.

5.4.2.5 Data analysis

5.4.2.5.1 Optical absorption spectra analysis

Methods for optical absorption spectra analysis for is presented in section 5.2.5.2. and ozone concentration during HVACP treatment was obtained from the optical absorption spectra analysis.

5.4.2.5.2 Relative importance analysis

Within the context of linear regression, the term *relative importance* refers to the proportional contribution of each predictor variable makes to the total variance, taking into account a variable's contribution by itself and in combination of other predictor variables (Johnson & LeBreton, 2004).

Relative importance refers to the quantification of one individual regressor's contribution to a multiple regression model. In a multiple linear model, when all regressors are uncorrelated, each regressor's contribution is just the R^2 from univariate regression, and all univariate regression adds up to the full model R^2. However, if the regressors are correlated, it is no longer straightforward to break down model R^2 into shares from individual regressor. The R package **relaimpo** offered several methods for assessing relative importance in linear aggression, the metric of First, Last, lmg were selected for evaluation. The metric "first" is the univariate contribution of the regressor to the full model, and metric "last" is determined by the ability of each aggressor to explain the variance in addition to all other regressors. "lmg" metric decompose R^2 into on-negative contributions that automatically sum to the total R^2. Among them "lmg" metric is most computer intensive and is the recommended methods.

5.4.2.5.3 Stepwise regression analysis

A general linear model (lm function) was first run in package R including all of the variables (sample amount, surface area, depth, treatment time) in the model. However, since some variables are correlated, multicollinearity problem could be an issue. A stepwise regression model (step function direction: both) was developed in R to compare the effect of each variable on percent reduction of aflatoxin by HVACP and to choose the best linear model automatically according to mallows's C_p value. The stepwise regression polynomial model is given by:

$$Y = \beta + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_n X_n + \varepsilon$$

When β values were determined from SAS output, and the X values represents the different parameters for each variable that was selected in the final linear regression model. And Y is the percent reduction of aflatoxin.

5.4.3 Results on ozone concentrations during HVACP treatment of Distiller grains

5.4.3.1 Influence of matrix (DWG, CDS, DDGS, distilled water) on ozone concentration

Ozone concentration with different matrix inside the package during 10 min HVACP treatment is presented in Figure 5.14. and listed in Table 5.10. During 10 min HVACP treatment without food matrix (control), the ozone concentration increase rapidly in the initial 2 min, and then the increase rate is slowed and reached a maximal concentration of 1216 (ppmv) at 3-4 min, then the ozone concentration started to decrease gradually over time. Ozone concentration generated by HVACP with matrix follows

similar trend, however the maximal concentration of ozone was lowered compared to the control. The reduced ozone concentration depends on the type of matrix within the package. The maximal ozone concentration generated during HVACP treatment with matrix water, DWG, CDS, and DDGS were 84, 64, 75, 71% the maximal concentration ozone for the control. These matrices have different moisture content and in different forms. Water and CDS are liquid material, whereas DWG and DDGS are bulk materials that have much large surface area than liquid material. DWG and CDS matrix have moisture content of 65% and DDGS moisture content of 12%. By comparing the maximal ozone concentration for liquid material water and CDS, CDS contains a lots of nutrients, around 8% crude fats, 6% proteins, 3% and 4% reducing sugars on dry matter basis (Probst et al., 2013), which could reacts with ozone and results in lower ozone concentration inside the package. By comparing maximal ozone concentration in bulk materials DWG (722 ppmv) and DDGS (866 ppmv), which have similar chemical compositions but different moisture content, the ozone concentration is lower in the DWG and DDGS. Thus influence of food matrix on ozone concentration during HVACP is dependent on the moisture content and forms of matrix, wet and bulk matrix with large surface area lead into lower concentration of ozone.

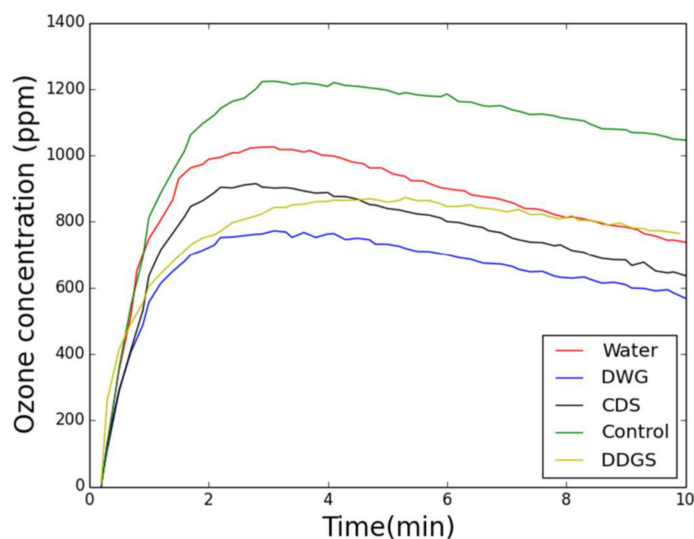


Figure 5.14. Ozone concentration with different material inside the package during 10 min HVACP treatment. (filled gas: ambient air)

Table 5.10. Maximal and final ozone concentration during 10min HVACP.

Matrix [a]	O_3 max			O_3 at (10min)	
	Concentration (ppmv)	Time [b] (min)	Pert of max [c]	Concentration (ppmv)	Pert of max [c]
DWG	772	3.1	64	567	47
CDS	915	2.8	75	636	52
DDGS	866	4.5	71	764	63
Water	1025	3.1	84	738	61
Control	1218	3.6	-	1046	86

[a] The mass of all the matrix is 10 g
[b] Time for ozone concentration to reach maximal concentration
[c] Pert of maximal is ozone concentration relative to maximal ozone concentration of control (1218 ppmv)

5.4.3.2 Influence of sample amount, surface area on ozone concentration during HVACP.

Ozone concentration during HVACP treatment for different sample amount and different surface area was plotted in Figure 5.15, the maximal and final ozone concentration during treatment was summarized in Table 5.11. Increasing sample

amount and sample surface area of exposure, the ozone concentration during HVACP treatment was reduced. With the same surface area 56 mm^2 but increasing sample amount from 5g to 20g, the maximal and final ozone concentration generated by HVACP was reduced slightly by 19 and 12%. With the same sample amount 10g but increasing surface area from 34 to 112 mm^2, maximal and final ozone concentration was reduced by 29 and 23%. Thus surface area of exposure has a greater impact on ozone concentration (for both maximal, final) during 10 min HVACP than the sample amount. Surface area for exposure is especially critical for ozone concentration during the initial period (first 2 min) of HVACP. With sample exposure surface area of 34, 56, and 112 mm^2, the ozone concentration at 2 min HVACP is only 83, 69, and 43% the ozone concentration of control (surface exposure area 0 mm^2). Relationship of ozone consumption by DDGS with surface area and treatment time during HVACP treatment is plotted in Figure 5.16. The ozone consumption by DDGS sample is calculated as the difference in integration of ozone concentration over time between control and DDGS sample. The ozone consumption by DDGS sample is linearly related to the products of surface area and HVACP treatment time (R^2=0.95).

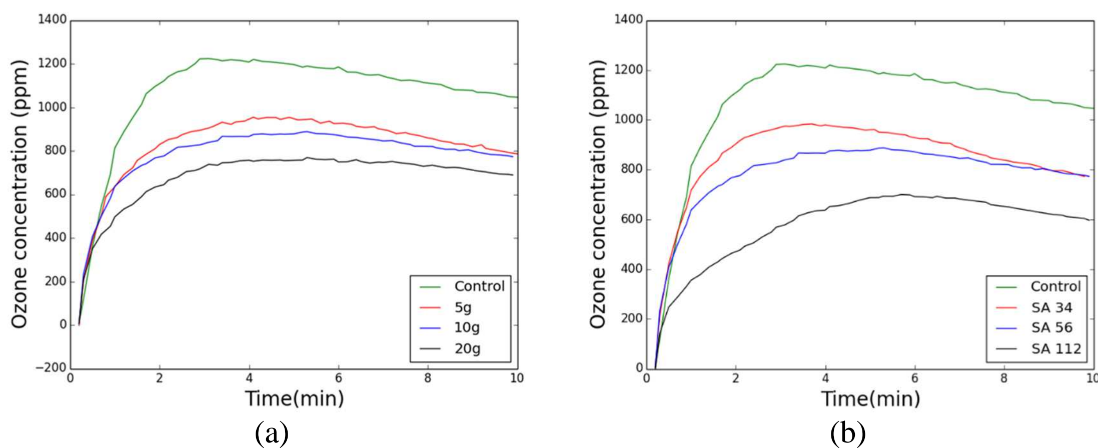


Figure 5.15. Ozone concentration within the package during 10 min HVACP treatment for (a) different sample amount (5, 10, 20g), and (b) different surface area (34, 56, 112 mm^2)

Table 5.11. Ozone concentrations within the package during HVACP treatment for different sample amounts, and surface areas.

Sample amount (g)	Surface area (mm^2)	O^3 max and time (ppmv, min)	O^3 (2 min) (ppmv)	O^3 (10min) (ppmv)
5	56	954, 4.1	829	786
10	56	888, 5.3	776	773
20	56	769, 5.3	644	689
10	34	983, 3.7	928	773
10	112	700, 5.7	477	596
Control	-	1218, 4.1	1121	1046

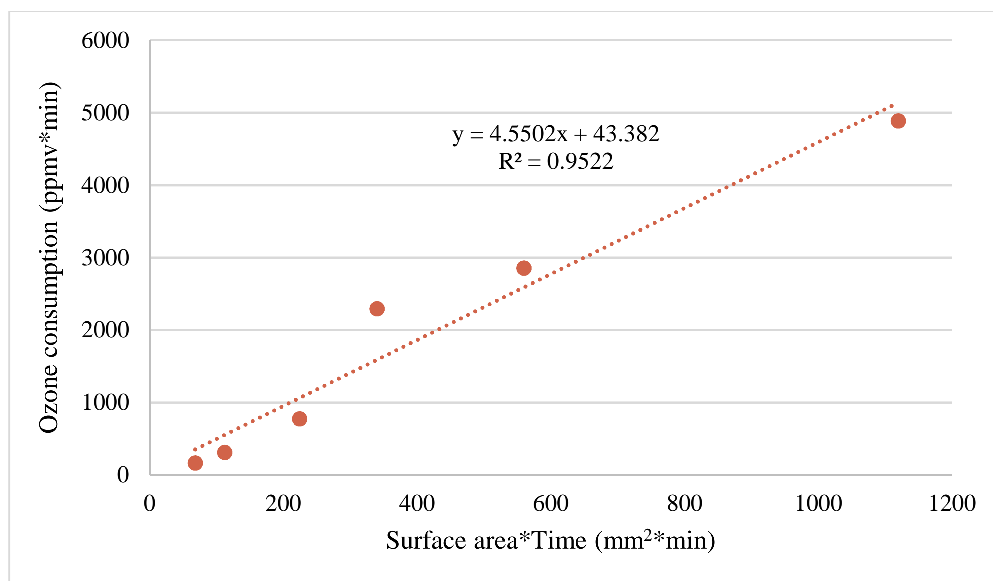


Figure 5.16. Relationship of ozone consumption by DDGS with surface area and treatment time during HVACP treatment

5.4.4 Results on aflatoxin reduction in distiller grains by HVACP

5.4.4.1 Aflatoxin reduction in different matrix (DWG, CDS, DDG, DDGS) by HVACP

Aflatoxin reduction in the matrix of DWG, CDS, DDG and DDGS was plotted in Figure 5.17. By 10 min HVACP treatment, aflatoxin in CDS, DWG, DDG, and DDGS was reduced by 20, 52, 59, 35%. Aflatoxin in CDS is reduced the least by HVACP compared with other matrix, this is mainly due to the fact that CDS is liqueous material have a smaller surface area exposure compared to other matrix which are bulk material. In addition, CDS have high content of protein, lipid and mineral nutrients, which react readily with ozone, thus exerts a protective effect on aflatoxin within in CDS during HVACP treatment. For bulk material of DWG, DDG and DDGS, aflatoxin in DWG and DDG were more readily reduced by HVACP treatment than DDGS. This effect is mainly attributed to fact that DWG and DDGS larger surface area for exposure than DDGS. The

surface is inversely related to bulk density, the bulk density of DWG and DDG is kg/m^3, compare to DDGS. From these results, it is suggested to apply HVACP treatment to degrade aflatoxin in DWG, DDG instead of DDGS and CDS.

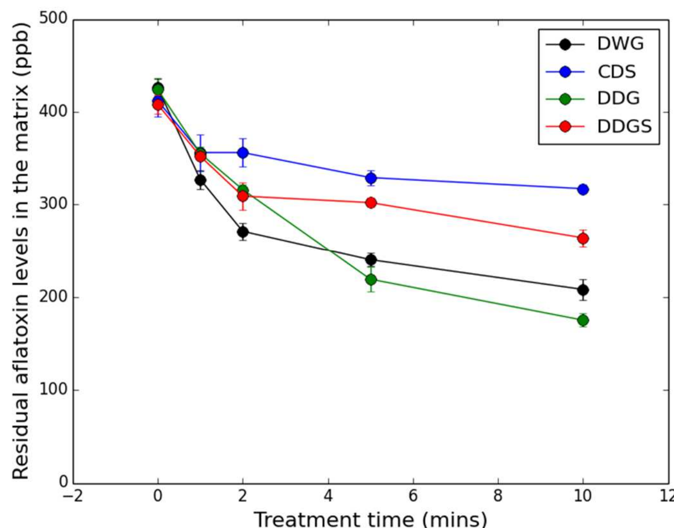


Figure 5.17. Aflatoxin reduction in different matrix (DWG, CDS, DDG, DDGS) by HVACP treatment.

5.4.4.1.1 Influence of sample amount, surface area, sample depth, treatment time on aflatoxin reduction in DDGS by HVACP

The results of aflatoxin reduction in DDGS by HVACP treatment with varying sample amount, surface area, depth, and treatment time are presented in Table 5.12. From analysis of variance (ANOVA), parameter of sample amount, and treatment time are significant factors (at alpha level 0.05) that affect the percent reduction of aflatoxin in DDGS during HVACP treatment. Since collinearity issue exist between surface area and grain depth, the parameters of surface area and grains are found not significant with p value of 0.15 and 0.75. Higher percent reduction of aflatoxin by HVACP was achieved

with less sample amount, larger surface area/lower grain depth, and with longer treatment time. The higher percent reduction of aflatoxin in DDGS was around 45%. When sample amount is small (5g), there are not significant difference in percent aflatoxin degraded by HVACP caused by different surface area, since the grains depth is still low (≤4.9 mm) and DDGS could still have good reaction with generated ozone species.

Table 5.12. Aflatoxin reduction in DDGS by HVACP with varying sample amount, surface area, depth, and treatment time.

Sample amount (g)	Surface area (mm^2)	Grain depth (mm)	2 min treatment		10 min treatment	
			Residual AF levels (ppb)*	Percent reduction (%)	Residual AF levels (ppb)*	Percent reduction (%)
5	112	1.5	308±11e	27	235±14c	44
	56	3	313±12cde	26	235±14c	44
	34	4.9	338 ±11c	20	243±18c	42
10	112	3	305±14e	28	233±11c	45
	56	5.9	309±16de	27	265±14bc	37
	34	9.9	335±14cd	20	288±18b	31
20	112	5.9	380±8b	10	350±14a	17
	56	11.8	394±8ab	6	360±14a	14
	34	19.8	415±10a	1	356±15a	16

*different letter in the column means significant difference at level 0.05 from pairwise t test.

5.4.4.1.2 Stepwise regression model

In order to evaluate the effect of parameters and predict aflatoxin reduction in DDGS by HVACP treatment, a stepwise regression model is performed to explain the contribution of parameters to the variance, the parameters included into models are sample amount, time, and surface area and grain depth. In the best model selected after stepwise regression, the variable of sample amount, surface area, and treatment time were incorporated into regression model.

$$Y=28.8-1.63 X_1+ 0.0745X_2 + 1.76 X_3 \quad (R^2_{adj} = 0.89) \qquad \text{Eq. 5.4}$$

Where X_1 is sample amount (g), X_2 is surface area (mm^2), and X_3 treatment time (min).

From Eq. 5.4, the Percent of aflatoxin reduction increases with longer HVACP treatment time and more surface area for interaction, and decreases with the amount of sample treated. This aflatoxin reduction model agrees with the microbial deactivation model developed by (McClurkin, 2015), which found that the effect of sample amount of treatment time was significant on log reduction of microbial load. In our model, the parameter of surface area was also found to be a significant factor.

Because multicollinearity issue exist between the variables sample amount, surface area, and grain depth, the variable depth is not incorporated in the selected model. However, this does not mean that grain depth is an insignificant parameter in determining the efficacy of HVACP treatment on aflatoxin. In fact, the second best regression model during stepwise regression model incorporates variable grain depth instead of variable surface area, the regression model is

$$Y=33.8-1.27\,X_1-0.582\,X_2+1.76\,X_3\;(R^2adj=0.88) \qquad \text{Eq. 5.5}$$

Where X_1 is sample amount (g), X_2 is depth (mm), and X_3 treatment time (min).
We could see that this model that incorporates grain depth is only slightly inferior to previously selected model, the adjusted residual square for the model with grain depth and the model with surface are 0.88 and 0.89 respectively.

5.4.4.1.3 Relative importance of factors sample amount, treatment time, surface area and sample depth

Results of relative importance analysis were plotted in Figure 5.18. The parameters of sample amount and treatment time are important variables explaining great amount of variance regardless of the metrics used for relative importance analysis, which

agrees with previous stepwise regression model which incorporates the variables sample amount and treatment time (McClurkin, 2015). Grain depth is also found to be an importance parameters explain 43% variance in “first” and 23% in “lmg” metric relative analysis, which is higher than the variance explained by parameter surface area. Thus grain depth is more important variables than the surface area in determining percent reduction of aflatoxin in DDGS by HVACP treatment. In our previous stepwise regression analysis, the best prediction model for percent aflatoxin reduction incorporates variables of sample amount, treatment time, and surface area, and dropped the parameters of sample depth. It should be noted that stepwise model selected the variables basted on metric “last”, and from relative importance analysis by metrics “last”, surface area is slightly more significant than grain depth.

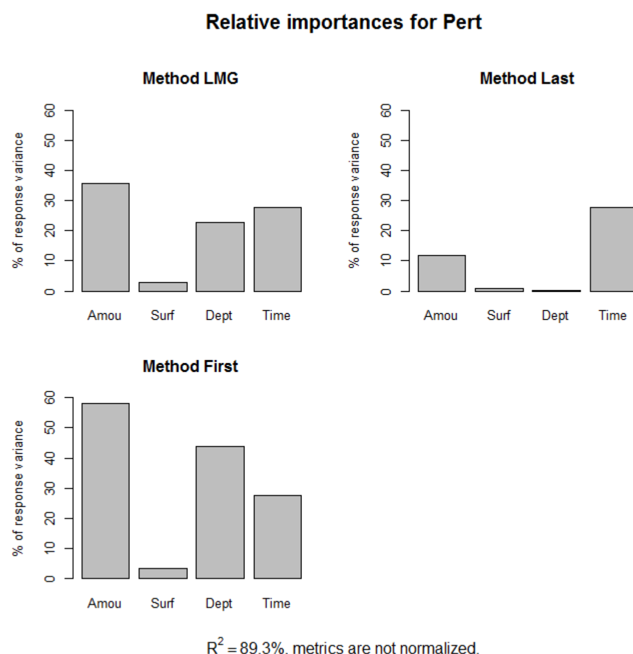


Figure 5.18. Relative importance of treatment time, sample amount, surface area, depth for percent reduction of aflatoxin by HVACP treatment.

5.4.5 Conclusions for application of HVACP to distiller grains

Ozone concentration during HVACP treatment could be measured by optical absorption spectroscopy (OAS). Ozone concentration in the package during HVACP depends on the material types and conditions being treated (moisture content, liquid or bulk material, chemical compositions), sample amount and surface area exposed to treatment are also important factors. Ozone consumption by the DDGS sample during HVACP treatment is linearly related to the products of sample surface area and treatment time. Degradation of aflatoxin by HVACP is influenced by type of materials treated, aflatoxin was found more readily degraded in DWG and DDG compared to DDGS and CDS. From relative importance analysis, sample amount, treatment time, and grain depth are critical parameters that determine percent reduction of aflatoxin in DDGS by HVACP. The percent reduction of aflatoxin by HVACP in DDGS could be modelled through stepwise regression analysis.

5.5 Structures of Degradation Products and Degradation Pathways of Aflatoxin B1 by HVACP Treatment

5.5.1 Background

This section aims to clarify the structure of AFB1 degradation products, elucidate the degradation pathway of AFB1 by HVACP treatment, and identify reactive gas species that are responsible for AFB1 degradation during HVACP treatment. The toxicity of degradants will also be analyzed according to their structures. Pure AFB1 powder spiked on a glass slide was treated by HVACP, the degradation products of AFB1 by HVACP treatment were separated, their chemical formulas were elucidated by liquid chromatograph time of flight coupled with mass spectrometry (HPLC-TOF MS), which has been used as an effective tool in analyzing degradation products of aflatoxin (Diao et al., 2012). The structure of the reaction products was further investigated using Orbitrap Mass spectrometry, a new technology of high resolution mass spectrometry, with miniature design, high speed detection and excellent quantification, which has been used for reaction product identification and molecular structure characterization (Perry et al., 2008;Zubarev and Makarov, 2013).

5.5.2 Materials and Methods

5.5.2.1 Chemicals and Reagents.

Aflatoxin B1 was purchased from Cayman Chemicals Inc. (Ann Arbor, MI, USA). Chloroform and 200 proof Ethanol were obtained from campus laboratory store. AFB1 powder was dissolved in chloroform and serially diluted to a concentration of 50

µg/ml, and then the AFB1 standard solution was stored at -5 °C in a freezer prior to conducting the tests.

5.5.2.2 Treating AFB_1 with HVACP.

Figure 5.19 shows the schematic of experimental set up for HVACP treatment of AFB_1 on glass slide. A 100 µl pipette was used to prepare the standard AFB_1 solution in chloroform (50 µg/ml) on a glass slide with a wait time of 2 h for the chloroform to fully evaporate. The AFB_1 on glass slide was placed inside a translucent polypropylene compartment box with a dimension of 4.4 cm height ×18.4 cm width× 27.9 cm length (Grainger Inc, USA). Boxes were sealed inside a high-barrier Cryovac B2630 film in order to prevent leakage of the filled gas as well as contain the generated gas species. The fill gas air (78% N2, 22% O2) was purchased from a local gas supplier with a certificate of analysis. The gas in the tank had a 5% relative humidity. In order to increase the relative humidity (RH) to 40%, the working gas was passed through a water bubbler, the gas flow rate and water depth was adjusted, and the resultant humidity was measured with a psychrometer (Extech Instruments Inc., USA), the final relative humidity of humidified air as filled gas is 40 ±3%. The storage bags containing AFB1 on glass slide were filled with the working gas (air, 40% RH) and purged multiple times for 2 min to ensure purity of the gas in the bag. HVACP treatment were conducted utilizing the HVACP system (Phenix Technolgies, Accident, MD) shown in Figure 5.19, which is patented technology developed by Dr. Keener at Purdue University. The HVACP system was operated at 200 W and 50 HZ generating 90 kV between the electrodes (4.5 cm gap).

The AFB1 samples were HVACP treated for 1, 2, 5 min. After treatment, the AFB1 samples inside the sealed bag were stored at room temperature for 24 h.

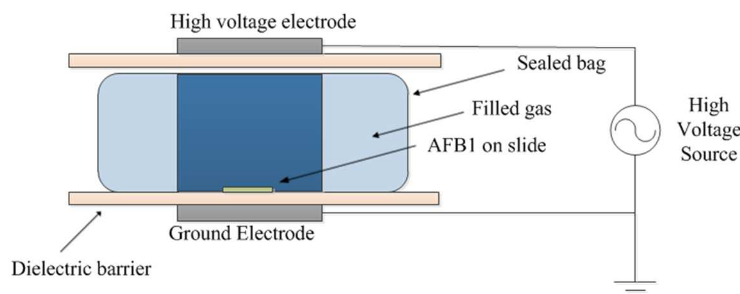


Figure 5.19. Schematic of experimental set-up for HVACP treatment of aflatoxin in corn

5.5.2.3 HPLC- MS Analysis.

The HVACP treated and untreated AFB1 samples on glass slides were carefully rinsed multiple times with 1 ml of a 50% ethanol aqueous solution to extract the AFB1 and degradation products. The extracts were then transferred to an Eppendorf tube (2.0 ml) and stored at -5°C in a freezer before being submitted for HPLC with mass spectrometry analysis. HPLC-MS data of degradation product of AFB1 were obtained on a time-of-flight (TOF) instrument system (Agilent Technologies, USA) equipped with a 1100 series binary solvent delivery system and an autosampler. Chromatography was performed on a 2.1×150 mm, 3.5 um particle Waters Xterra C18 column. The injection volume was 10 µL, the flow rate was 300 µL/min. The mobile phase was a gradient prepared from water with 0.1% formic acid (component A) and acetonitrile with 0.1% formic acid (component B). A gradient elution started with 10% B for 1 min, then B was increased linearly to 95% in 20 min, and kept isocratic for 1 min. The proportion of B

was then decreased back to 10% in 1 min and kept isocratic for 7 min. The total run time was 30 min. The MS was run with positive electrospray ionization (ESI), and data was collected over the range of 75 – 1000 m/z. High mass accuracy was ensured by infusing a lock mass calibrant corresponding to 121.0508 and 922.0098 m/z.

5.5.2.4 HPLC- MS-MS Analysis

The same chromatographic conditions were used for the HPLC/MS/MS analysis as described in the HPLC/MS section above. Work was performed using a Thermo LTQ Orbitrap XL mass spectrometer. The analysis used positive polarity electrospray ionization. High mass accuracy, fragmentation data were acquired using data dependent scanning mode. FTMS resolution of 60,000 with a mass range of 50-1100 was used for full scan analysis and the FTMS was used for MS/MS data acquisition with a resolution of 7500 and collision induced dissociation (CID) mode. The top five most intense ions were acquired with a minimum signal of 1000, isolation width of 2, normalized collision energy of 35, default charge state of 1, activation Q of 0.250, and an activation time of 30.

5.5.3 Results and Discussion

5.5.3.1 Formation of AFB1 degradation products by HVACP with time

Figure 5.20 shows the total ion chromatograms of untreated AFB1 sample and sample treated by HVACP in ambient air for 5 min. Only one peak appeared for AFB1 sample without HVACP treatment, while seven larger peaks (including AFB1 peak) were observed for HVACP treated sample, which indicates AFB_1 was degraded by HVACP treatment. Six major degradation products are shown in Figure 5.20 B. The retention

time and peak shape revealed satisfactory separation effect for the degradation products except products 4 and 5, whose retention times were very close and their peaks were not completely separated.

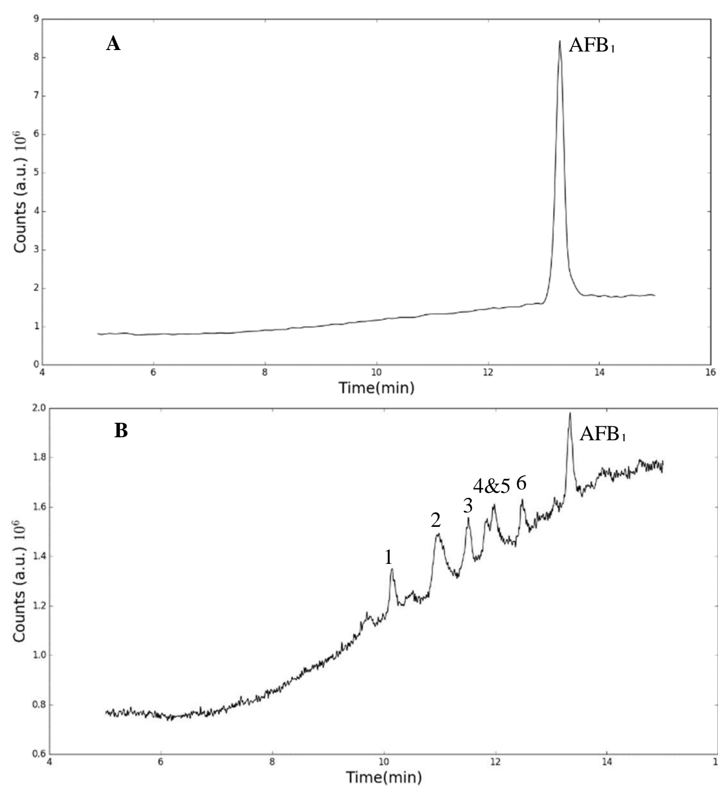


Figure 5.20. Chromatograms of AFB_1 untreated (5 µg/ml) in 50% ethanol solution (A) and AFB_1 sample treated by HVACP in ambient air for 5 min.

Figure 5.21 shows the change in response value for AFB1 and degradation products (P1 - P6) with increasing HVACP treatment time in air. With increasing HVACP treatment time, AFB1 was decomposed gradually, and the degradation products were increasing gradually. About 76% of AFB_1 as measured by peak area was degraded after 5 min HVACP treatment.

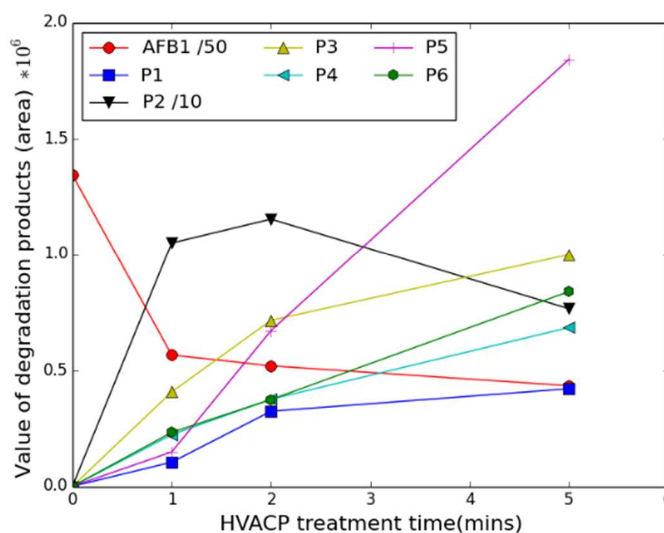


Figure 5.21. Relative change in response value of AFB_1 and degradation products 1 to 6 (P1 - P6) for AFB_1 sample with increasing HVACP treatment time in air.

Note: AFB_1 and P2 are normalized to have the same scale.

5.5.3.2 Molecular formula of degradation products

To identify the molecular formulas of the degradation products of AFB_1 before and after HVACP treatment, the data of degradation products of AFB_1 by HVACP, including retention time, proposed formula, experimental mass, mass error, double bond equivalent (DBE) and score (the overall score is 0-100%, and the score closer to 100% being better) are listed in Table 5.13. Compared with the theoretical mass obtained from the proposed molecular formula, the mass determined by TOF MS under experimental condition had less than a 6 ppm error. The results showed that since the product masses were acutely determined, the elemental composition could be determined by considering the number of proposed elemental compositions. Because AFB_1 was treated by HVACP in a pure system, AFB_1 and its degradation products could only be composed of 4 elements:

Carbon, Oxygen, Hydrogen, and Nitrogen. The molecular formulas were proposed according to the isotope distribution patterns and exact mass by using MassHunter Qualitive Analysis software (Agilent Technologies, USA). As an example, the molecular formula and some possible molecular compositions of product 2 were proposed as $C_{17}H_{14}O_7$, $C_{18}H_{10}N_4O_3$, $C_{16}H_8N_7O_2$ with scores of 95, 89, and 90% respectively. Since $C_{17}H_{14}O_7$ has the highest agreement score, it is more likely to be the molecular formula. Another indicator is the double bond equivalents (DBE) value of the degradation products, which should be similar to that of AFB_1. It is well known that AFB1 consist of 17 carbon atoms and 12 hydrogen atoms with a DBE value of 12. The DBE of the molecular formulae, $C_{17}H_{14}O_7$, $C_{18}H_{10}N_4O_3$, $C_{16}H_8N_7O_2$ are 11, 16, 14.5, respectively. Since the molecular formula and DBE of $C_{17}H_{14}O_7$ are more similar to AFB_1, it is more likely to be the molecular formula compared with other two formula.

Table 5.13. The proposed formula of AFB_1 and its degradation products obtained using LC-TOF MS.

Proposed Products	Retention time (min)	Proposed Formula	Observed mass (m/z) [a]	Diff [b] (ppm)	DBE	Score (%)
1	10.1	$C_{16}H_{16}O_6$	305.1028	-1.98	9	98.12
2	11.0	$C_{17}H_{14}O_7$	331.0820	-1.67	11	98.7
3	11.5	$C_{14}H_{12}O_5$	261.0772	-5.92	9	90.32
4	11.9	$C_{17}H_{12}O_7$	329.0666	-4.01	12	92.00
5	12.0	$C_{14}H_{10}O_6$	275.2560	-3.62	10	96.24
6	12.5	$C_{19}H_{18}O_8$	375.1085	-2.93	11	96.09
AFB_1	13.4	$C_{17}H_{12}O_6$	313.0716	-2.52	12	99.1

[a] The m/z in the table is the m/z of $[M+H]^+$. [b] DBE = double bond equivalents.
[b] Difference between observed and theoretical mass

5.5.3.3 Structure proposition for degradation products

To elucidate the structure of the six degradation products of AFB_1, the degradation products were further analyzed by orbitrap MS/MS to determine the exact masses of the fragmentation ions that enable postulation of their most probable parental structure and the structure of the reaction products of AFB_1. On the basis of the accurate masses of the parent ions and fragments obtained from MS/MS, the analysis of the structure of the six degradation products are shown in Figure 5.22. The structures of the degradation products of AFB_1 by HVACP are summarized in Figure 5.23. The structures of the six degradation products (P1 - P6) are similar to the structure of AFB_1. The degradation by HVACP resulted in the modification of AFB_1 structure of furo-furan ring (Products 1, 2, 3,4,5,6), cyclopentenone (Products 1, 3, 6) and methoxy group (Product 1). Product 1 ($C_{16}H_{16}O_6$) and Product 2 ($C_{17}H_{14}O_7$) have been shown to be ozonolysis products of AFB1 by aqueous ozone,(Luo et al., 2013;Diao et al., 2012) and four other major degradation products were newly identified, which shows that reaction of AFB_1 by HVACP treatment involves new pathways other than ozonolysis as reported by other research.

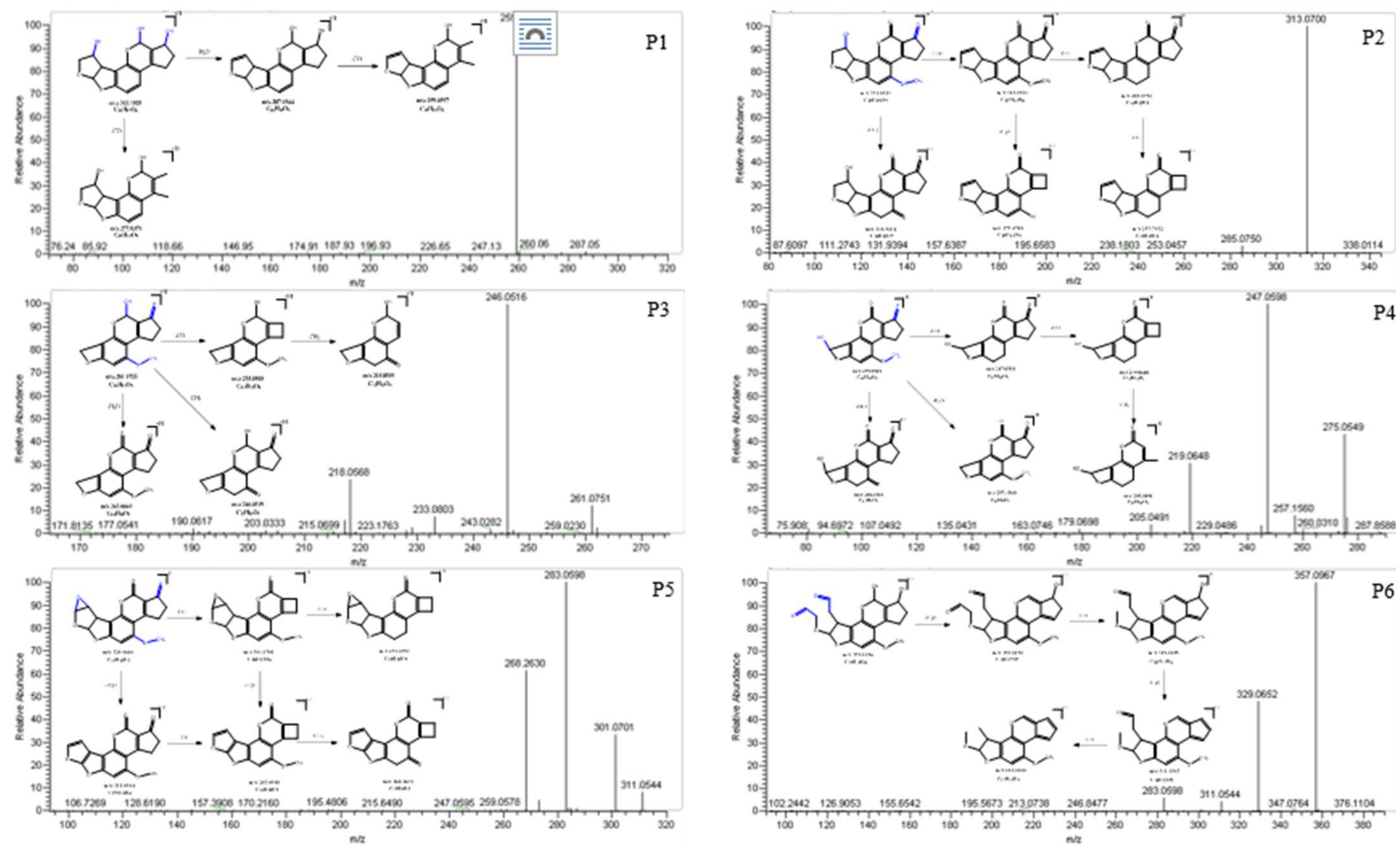


Figure 5.22. Orbitrap MS/MS spectra and proposed fragmentation (insets) of degradation products of AFB_1 by HVACP

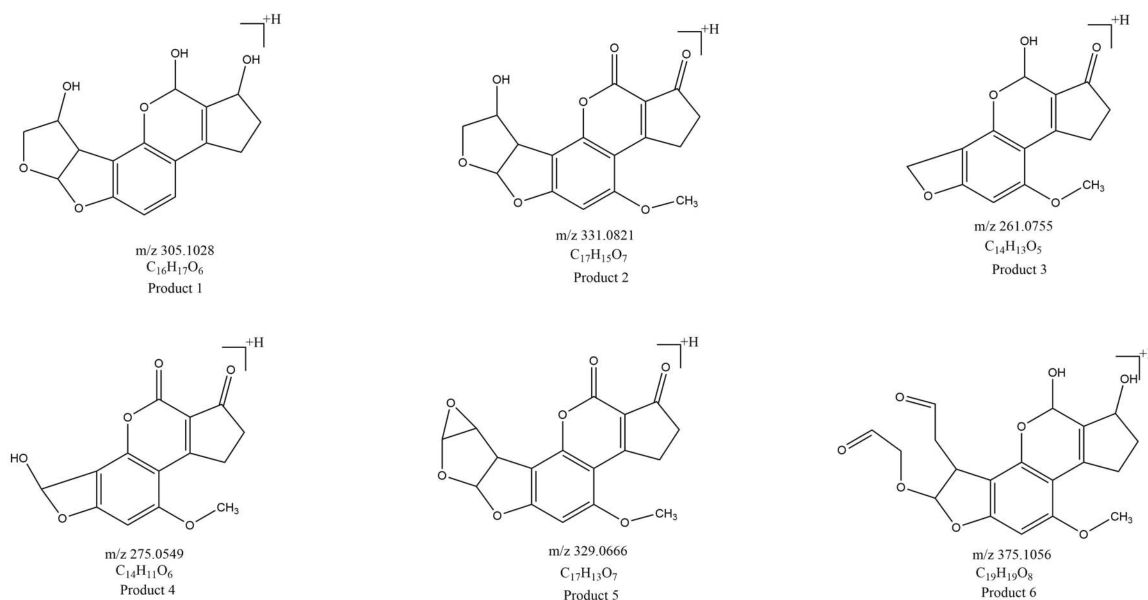


Figure 5.23. Proposed structure of reaction products (P1 – P6) of AFB1 by HVACP

5.5.3.4 Degradation pathway of AFB1 by HVACP

According to the structure of the six degradation products of AFB_1 by HVACP, two degradation pathways were proposed as in Figure 5.24 and Figure 5.25. The first pathway is from AFB_1 to reaction products $C_{17}H_{15}O_7$ (m/z 331.0821), $C_{16}H_{17}O_6$ (m/z 305.1028), $C_{19}H_{19}O_8$ (m/z 375.1056). The second pathway is from AFB1 to reaction products $C_{14}H_{13}O_5$ (m/z 261.0755), $C_{14}H_{11}O_6$ (m/z 275.0549), and $C_{17}H_{13}O_7$ (m/z 329.0666).

The first degradation pathway mainly involves addition reaction by adding water molecule (H_2O), hydrogen molecule (H) or aldehyde group (CHO) to AFB_1. The first branch of reaction starts with the addition of water molecule (hydration reaction) to the C8-C9 double bond at the furan ring of AFB_1, and formed degradation products, $C_{17}H_{15}O_7$ (m/z 331.0821). The methoxy group (-OCH3) of AFB_1 was cleaved to form the

intermediate product $C_{16}H_{13}O_6$ (m/z 301.0712), and the carbonyl groups of the intermediate product $C_{16}H_{13}O_6$ were further hydrogenated to form degradation product $C_{16}H_{17}O_6$ (m/z 305.1028). The second branch of reaction is the addition of an aldehyde group (CHO) to form the intermediate product, $C_{19}H_{15}O_8$ (m/z 371.0767), then again the carbonyl groups in lactone ring and cyclopentanone of this intermediate product were hydrogenated to form the degradation product, $C_{19}H_{19}O_8$ (m/z 375.1056). From the first degradation pathway of AFB_1, the crucial reactive agents are H and OH radicals, which was generated in HVACP system by the breaking down of water molecules by HVACP.(Rodriguez-Mendez et al., 2013) These two radicals are responsible for hydration and hydrogenation to form new degradation products. Another reactive agent generated by HVACP is aldehyde (CHO) radical, which was formed in HVACP system when carbon dioxide (CO_2) was present.(Siow et al., 2006)

The second pathway mainly involves oxidation reactions. The first branch was the formation of degradant $C_{17}H_{13}O_7$ (m/z 361.0560) through epoxidation of the terminal double bond of AFB_1. The epoxidation reaction could be caused by hydroperoxyl radical ($HO_{2\cdot}$) which is generated during HVACP treatment and its concentration increases with higher relative humidity.(Dorai and Kushner, 2003) $HO_{2\cdot}$ is one type of peroxy radical, which reacts with double bonds and leads to epoxide formation.(Koelewijn, 1972) The second branch involves cleavage of furan ring of AFB_1 to form an intermediate compound $C_{14}H_{11}O_5$ (m/z 259.0606), and this compound was either hydrogenated into the degradation product, $C_{14}H_{11}O_5$ (m/z 261.0755), or was hydroxylated into the degradation product $C_{14}H_{11}O_6$ (m/z 275.0549). In the second pathway, the main reactive agents are ozone molecule responsible for addition to the double bond and cleavage to

furan ring of AFB_1, the H and OH radical also play a role in further degradation of the intermediate products.

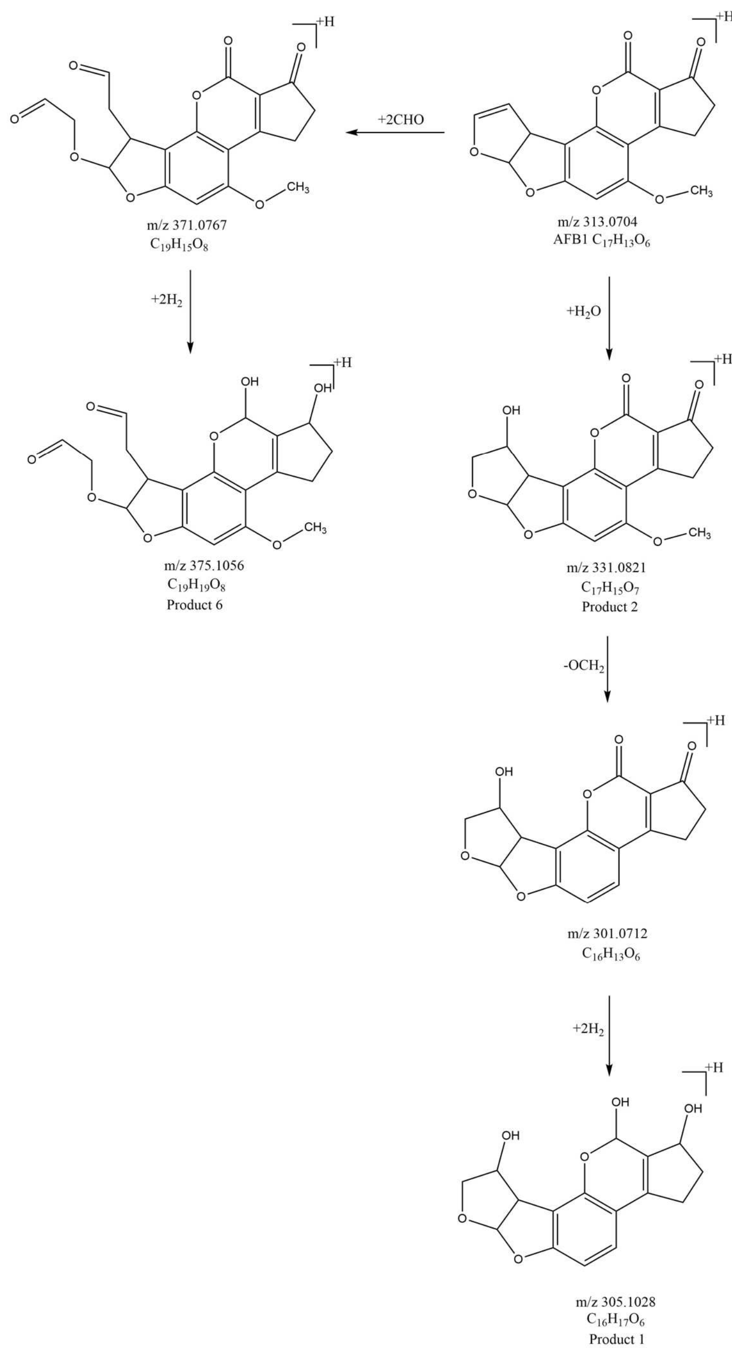


Figure 5.24. First degradation pathway of AFB_1 by HVACP

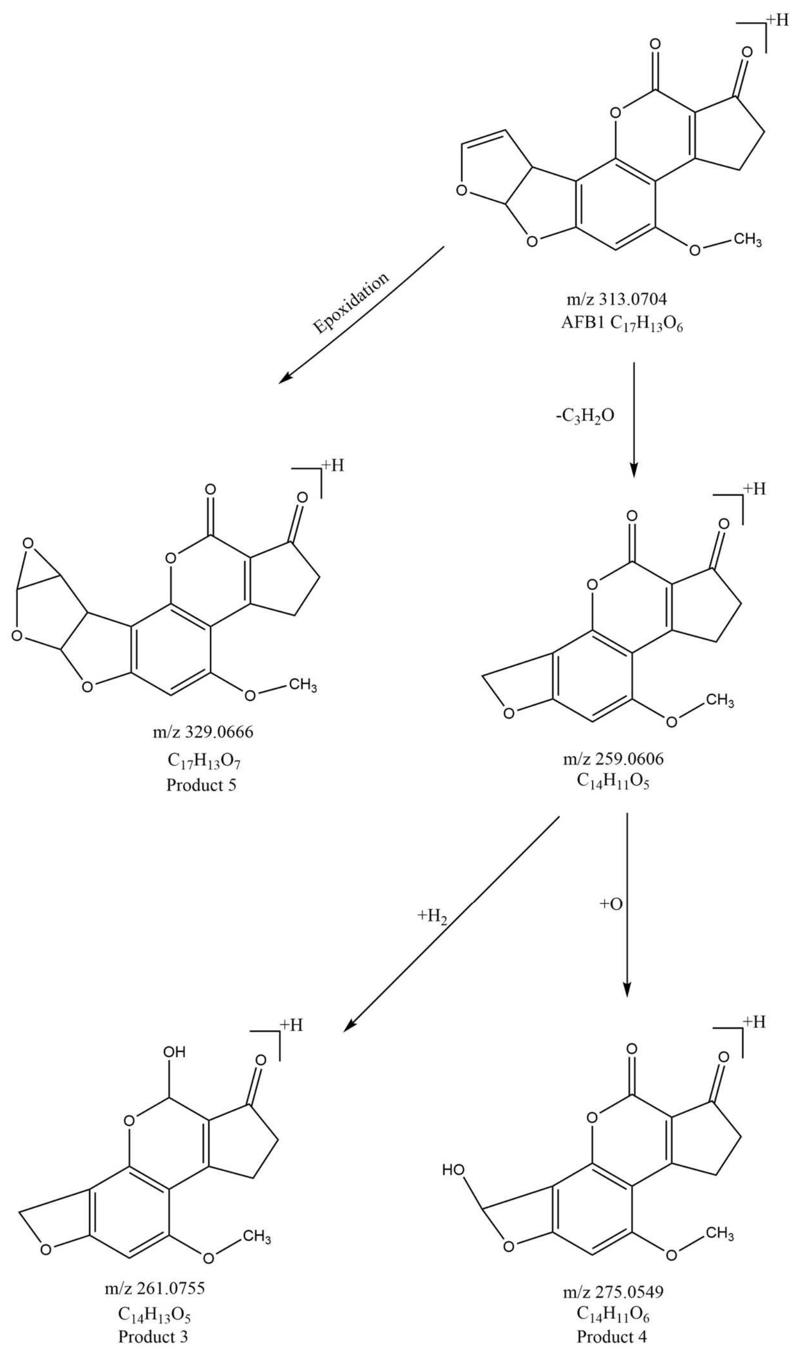


Figure 5.25. Second degradation pathway of AFB1 by HVACP

5.5.4 Conclusions

This study determined and elucidated the structure of the degradation of pure Aflatoxin B_1 (AFB_1) powder treated by a high voltage atmospheric plasma (HVACP) system. About 76% degradation was observed for a 5 min treatment time in air (78% N_2, 22% O_2). Aflatoxin B1 (AFB_1) was degraded into six degradation products, with their formulae and structures elucidated using liquid chromatograph time of flight (TOF) mass spectrometry (HPLC-TOF MS) and orbitrap mass spectrometry. Two degradation pathways were proposed based on the structure of degradation products. Among the six degradants, two degradants were ozonolysis products of AFB_1, appearance of other four degradants indicates that HVACP generated other reactive species besides ozone that reacts with AFB_1. The main degradation mechanism of AFB_1 involves hydrogenation, hydration, and oxidation of the furan ring. Hydrogen radical, hydroxyl radical, hydroperoxyl radical and ozone are proposed as the major reactive agents for AFB_1 degradation generated HVACP treatment. Based on the literature, it appears that the degradation products, which involved changes in the furofuran and lactone rings, and cyclopantenone and methoxyl structures should pose less biological activity risks than AFB_1. However, this needs to be confirmed by bioactivity tests in order for the results to be conclusive.

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CHAPTER 6. SUMMARY OF CONCLUSIONS AND DISCUSSIONS

6.1 Thesis overview

The overall goal of this study was to evaluate several approaches to the reduction of the aflatoxin levels in DDGS, the final product of ethanol bioprocessing. The first approach pursued was to reduce the aflatoxin in the incoming corn prior to bioprocessing. The second was degradation of aflatoxin in the intermediate products of corn ethanol bioprocessing: DWG and CDS. The last was degradation of the aflatoxin in the DDGS using high voltage cold plasma (HVACP) treatment.

With respect to the first approach, two segregation and detoxification methods were evaluated for their effectiveness in reducing aflatoxin. The effectiveness of size screening in reducing aflatoxin was determined and then density sorting using a gravity table was examined. For the second approach, the thermal stability of aflatoxin was evaluated by heating with or without the presence of water (dry or wet heating). Both conventional and microwave heating methods were investigated. At the same time, the effectiveness of the addition of food additives was determined. First the reduction achieved without any substrate present (pure aflatoxin) was evaluated and second and evaluation was made when the aflatoxin was added to DWG and CDS. Finally, treatment with High Voltage Atmospheric Cold Plasma (HVACP) was evaluated as a novel method for degradation of aflatoxin in corn and DDGS.

6.2 Major findings corresponding to the objectives

6.2.1 Objective 1: Reduction of aflatoxin by size screening and density sorting

For a 737 kg corn lot we tested, there were statistically significant differences in major and minor diameters, the sphericities and the densities between moldy and healthy corn kernels. The distribution in size, shape and density between mold and healthy corn kernels were significantly different. The moldy corn kernels had a smaller major diameter, greater sphericity and a lower density. Removal of fine materials mixed in with the corn lot using size screening significantly reduced the aflatoxin level in the remaining lot. Further removal of small size kernels through cleaning through a screen cleaner and removal of lower density kernels using a gravity table brought an additional and considerable further reduction in aflatoxin in the remaining corn lot.

6.2.2 Objective 2: Reduction of aflatoxin by conventional heating, microwave heating

The thermal stabilities of aflatoxin B_1 during dry and wet heating using either conventional or microwave heating were investigated. Aflatoxin is very stable during dry heating and a temperature of 150 ºC is required to initiate its decomposition. HPLC-MS analysis indicated that the aflatoxin B_1 was converted into its enantiomer during the dry heating process. During wet heating, aflatoxin B_1 was reduced at a much lower temperature. Seventy three percent of the AFB_1 was degraded when it was heated wet at 80 ºC for 1 h. The water molecule played a critical role in degradation of aflatoxin during

wet heating. Aflatoxin degradation by wet heating involved hydrolysis of the furofuran moiety and the lactone ring and further decarboxylation.

For a given average heating time and temperature, microwave heating was found to be slightly more effective (5-8%) than conventional heating in degrading aflatoxin B_1 for both dry and wet heating scenarios. However, this difference probably is the result of the higher maximum temperature generated during microwave heating. Dry and wet heating by microwave resulted in the same degradation products as conventional heating. The absence of new degradation products indicates that degradation of aflatoxin by microwave heating is due to its thermal effects.

6.2.3 Objective 3: Reduction of aflatoxin by food additives

In tests on pure aflatoxin, four selected food additives, namely sodium bisulfite, sodium hypochlorite, citric acid, and ammonium persulfate, were shown to effectively (>86%) degrade aflatoxin immobilized in a glass bottle when it was heated at 90 ºC for 1 h with 1% (by weight) food additive solutions. When food additives were added to DWG and CDS and the substrates were heated at 90 ºC for 1 h, there was apparently a protective effect of the substrate because there was less aflatoxin degradation. Sodium bisulfite was not effective, and for the other food additives the aflatoxin reduction was less than 56%. Citric acid was the most promising additive for degrading aflatoxin because it has been classified as GRAS (generally recognized as safe) by the FDA. Degradation of aflatoxin B_1 by citric acid was through acid-catalyzed hydrolysis which converts the AFB_1 to AFB_{2a}. The second degradant was AFB_1-Citric (C_{23} H_{19} O_{13}), which

is produced by adding citric acid to the double bond of the furan ring of AFB_1. Aflatoxin reduction was enhanced by increasing the citric acid addition level and prolonging the heating time. Aflatoxin levels in DWG and CDS were gradually reduced with prolonged heating even without addition of food additives.

6.2.4 Objective 4: Reduction of aflatoxin by high voltage atmospheric cold plasma (HVACP) treatment

Performance of the HVACP system and generation of reactive species were characterized using optical emission spectral analysis. During HVACP treatment, the major emission peaks were from N_2 when the treatment was conducted in air and from O atoms when it was conducted in MA65. The HVACP system is less efficient in breaking down diatomic nitrogen and oxygen over a longer period of time.

Ozone concentration during HVACP treatment was monitored using optical absorption spectral analysis. The kinetics of the generation of ozone concentration follows a logarithmic function for both MA and air ($R^2_{adj} > 0.98$) during the120 s HVACP treatment. Ozone generation rate and the final ozone level were higher in MA gas than in air, and when the relative humidity was lower (5%) as contrasted with higher (40 or 80%). Ozone concentration inside the package during HVACP depended on the type of material being treated and the treatment conditions (moisture content, liquid or bulk material, chemical composition). In addition, Ozone consumption by the DDGS sample during HVACP treatment was linearly related to the product of the sample surface area and the treatment time.

Aflatoxin in corn could be rapidly degraded by HVACP treatment. Incorn it was degraded by 62% and 82% by 1 and 10 min HVACP treatments, respectively. Three kinetic models (first-order, Weibull, and logistic model) were fitted to the aflatoxin degradation data. The logistic model was found to be the best to describe the degradation of aflatoxin by HVACP with a high coefficient of determination ($R^2 \geq 0.99$). Stirring the material during HVACP treatment could further increase aflatoxin degradation. Penetration of the reactive species generated by HVACP into the material being treated is believed to be the limiting factor preventing complete degradation of the aflatoxin. The use of MA65 as the working gas during HVACP treatment was slightly more effective in degrading aflatoxin than the use of air because higher concentrations of Ozone and NOx species were generated when MA65 was used. HVACP treatment with humid air/MA65 at relative humidities of 40% and 80% were more effective than dry gas (5% RH). In addition, degradation of aflatoxin by HVACP was influenced by the type of material being treated. Aflatoxin was more readily degraded in DWG and DDG compared to DDGS and CDS. From relative importance analysis, the sample amount, the treatment time, and the grain depth were the critical parameters that determined percent reduction of aflatoxin in DDGS by HVACP. The percent reduction of aflatoxin by HVACP in DDGS could be modelled using stepwise regression analysis.

As a result of the HVACP treatment, aflatoxin B_1 was degraded into six degradation products. Their formulae and structures were elucidated using liquid chromatography time of flight (TOF) mass spectrometry (HPLC-TOF MS) and Orbitrap mass spectrometry. Two degradation pathways were proposed based on the structure of degradation products. Among the six degradants, two degradants were ozonolysis

products of AFB_1. The appearance of the other four degradants indicates that HVACP generated other reactive species besides ozone that reacts with AFB_1. The main degradation mechanism of AFB_1 involves hydrogenation, hydration, and oxidation of the furan ring. Hydrogen radicals, hydroxyl radicals, hydroperoxyl radicals and ozone are proposed as the major reactive agents for AFB_1 degradation generated using HVACP treatment. Based on the literature, it appears that the degradation products, which involved changes in the furofuran and lactone rings, and cyclopantenone and methoxyl structures, should pose a lower risk to biological activity than does AFB_1.

6.3 Future work

The first approach evaluated was screening and density sorting to remove contaminated kernels and fine material thereby reducing the aflatoxin levels in the corn lot. For future studies of this type, the author recommends that tests to determine the effectiveness of the techniques be accompanied by analysis of the size and density distribution of the kernels. This will help to explain why the sorting technique was or was not effective. In addition, other techniques such as sieving with slotted sieves, and color or NIRS sorting should be evaluated. The same approach used in this study for aflatoxin could be used to determine the effectiveness of various techniques, such as size screening and density sorting, in reducing mycotoxins in shelled corn contaminated with other species of mycotoxin producing fungi.

The second approach that was discussed was to heat the material so that the aflatoxin is broken down into non-toxic or less toxic compounds. In order to better

understand the thermal stability of aflatoxin in a complex food matrix during thermal food processing, a model food substrate consisting of various constituents including starch, lipids, fibers, and proteins, could be used for tests. The tests would be conducted with the addition of different amounts of water, to further evaluate the effect of water within the food substrate on the decomposition of the toxin. The tests could be conducted using varying thermal conditions so that the effect of the interaction of thermal conditions and food substrate on aflatoxin decomposition can be determined.

The HVACP system used for the tests reported in this thesis could only be used to treat small samples (25g). In order to obtain samples with homogenous levels of aflatoxin, the corn samples were spiked with aflatoxin instead of beginning with a sample of wet corn that was inoculated with the Aspergillus species that would then grow on the wet corn and produce the aflatoxin. When the toxin is produced by the fungi growing on the corn, it may be located deeper in the kernel where it would be more difficult for the various decontamination processes to interact with it. The HVACP system should also be scaled up to accommodate a sample of one kilogram or greater of naturally contaminated corn. To achieve better exposure of the corn to reactive species, the HVACP system should be modified so there is continuous treatment. It should also incorporate a method of stirring the sample as it is being treated. Finally, the effect of HVACP treatment on detoxification of other mycotoxins (Deoxynivalenol, Fumonisin, Zearalenone, etc.) could also be investigated.

Another approach that could be evaluated would be the use of a combination of the aflatoxin degradation methods that were tested in this study to achieve a higher level of degradation. For example, first the corn most severely contaminated with aflatoxin

could be removed using size screening and density sorting. Then the segregated highly contaminated corn kernels could be decontaminated using thermal processing, food additives, or HVACP treatments.

The author's final recommendation is that additional Ames tests for mutagenicity and toxicity be conducted using ducklings, which are among the animals most sensitive to aflatoxin. This would reveal the bioactivity of the degradation products of AFB_1 that are produced by thermal processing, food additives, and HVACP treatments. In addition, changes in physical, chemical, sensory properties and palatability of corn treated using the various methods are also recommended for future research.

VITA

VITA

Education

Purdue University, West Lafayette, IN

Ph.D. Agricultural & Biological engineering (ABE) 08/2012-12/2016

China Agricultural University, Beijing, China

M.S. Agricultural and Biological Engineering 09/2010-06/2012

B.S. Mechanical Engineering 09/2006-06/2010

Teaching Experience

Teaching Assistant, Purdue University. 2013, 2014, 2015, 2016 fall

- Course ABE45000: Finite Element Method in Design and Optimization. Grading, held office hours, assisted in lab sessions.

Research Experience

Research Assistant, Purdue University, West Lafayette, IN.

2012 Fall-Present

Thesis project: Investigation of Methods for Reducing Aflatoxin Contamination in Distillers Grains

- Reduction of aflatoxin in corn by size screening and density sorting
- Test thermal stability of aflatoxin by dry and wet heating and microwave heating
- Detoxification of aflatoxin by food additives
- Characterization of high voltage atmospheric cold plasma (HVACP) through optical emission and optical absorption spectra, and test degradation of aflatoxin in corn and DDGS by HVACP treatment.

Research assistant, China Agricultural University. 09/2010-06/2012

MS thesis: Experimental and Finite Element Analysis of the Mechanical Properties of Wheat Straw

- Measured the tensile and shear properties of wheat straw biomass
- Built a porous layer structural model of wheat straw using Pro/Engineer
- Analyzed the stress response of the structural model with ANSYS

BS thesis: Design of refractance window drying equipment for fresh whole foods.

Honors and Awards

Outstanding undergraduate student scholarship, China Agricultural University, Beijing, China, 06/2010.
Distillers Grains Scholarship, Distillers Grains Technology Council, Kansas City, Missouri, 05/2015.

Engagement and Service

Secretary of Graduate association of Agricultural and Biological Engineering, Purdue University, 2014.
Member of American society of Agricultural and Biological Engineers.
Members of American Association of Cereal Chemists.

Presentations

"Reduction of aflatoxin in distillers grains". Purdue ABE GSA symposium, Purdue University, 02/2014.
"Reduction of aflatoxin in corn by cleaning and sorting", ASABE annual conference meeting, Montreal, Canada, 07/2014.
"Reduction of aflatoxin in distiller grains and condensed solubles". 19th Annual Distillers Grains Symposium, Kansas City, Missouri, 05/2015.
"Reduction of Aflatoxin in Corn by High Voltage Atmospheric Cold Plasma". NC-213 meeting, 2016/02.

Manuscripts (published and papers in review)

1. Shi, H., Stroshine, R.L., Ileleji, K. 2014. Aflatoxin reduction in corn by cleaning and sorting. ASABE paper No. 14-1890901. St Joseph, Mich. ASABE.
2. Shi, H., Stroshine, R.L., Ileleji, K. Determination of the Relative Effectiveness of Four Food Additives in Degrading Aflatoxin in Distillers Wet Grains and Condensed Distillers Solubles. Journal of Food Protection. 2017. In press.
3. Shi, H., Stroshine, R.L., Ileleji, K. Differences in kernel shape, size and density between healthy and aflatoxin contaminated kernels and their relationship to reduction in aflatoxin levels in a sample of shelled corn. Applied Engineering in Agriculture. 2016. In review.
4. Shi, H., Stroshine, R.L., Ileleji, K., Keener, K. Reduction of aflatoxin in corn by high voltage atmospheric cold plasma. Journal of Food and Bioprocess Technology. 2016, In review.
5. Shi, H., Cooper, B., Stroshine, R.L., Ileleji, K., Keener, K. Structure of Degradation Products and Degradation Pathway of AFB1 by High Voltage Atmospheric Cold Plasma (HVACP) Treatment. Journal of Agricultural and Food Chemistry. 2016, In review.