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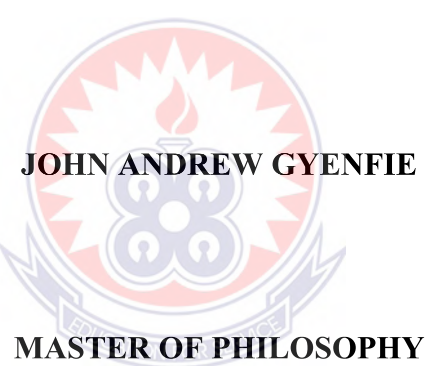
DETERMINATION AND ANALYSIS OF AFLATOXINS IN COMMERCIAL DOG FOODS ON THE GHANAIAN MARKET



JOHN ANDREW GYENFIE

MASTER OF PHILOSOPHY

2023

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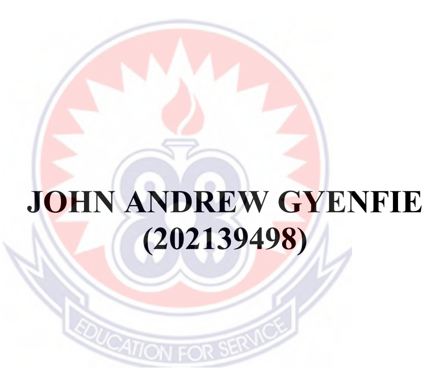

JOHN ANDREW GYENFIE
(202139498)

A Thesis in the Department of Chemistry Education, Faculty of Science Education, submitted to the School of Graduate Studies, in partial fulfilment of

the requirements for the award of the degree of Master of Philosophy (Chemistry) in the University of Education, Winneba

SEPTEMBER, 2023

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DECLARATION

Student's Declaration

I, **JOHN ANDREW GYENFIE** declare that this thesis, except quotations and references contained in published works which have all been identified and duly acknowledged, is entirely my original work, and it has not been submitted, either in part or whole, for another degree elsewhere.

SIGNATURE:…………………………..…………..

DATE: ……………………………………………..

Supervisor's Declaration

I hereby declare that the preparation and presentation of this thesis were supervised in accordance with the guidelines for supervision of thesis as laid down by the University of Education, Winneba.

NAME OF SUPERVISOR: PROF. EMMANUEL K. OPPONG

SIGNATURE:……………………..……..……..

DATE: ………………………….……..……..

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DEDICATION

To my loving wife, Linda Mawuena Anyigba, grandparents, parents, and brothers, I say thank you for being the pillars in my life.

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Finally, I would like to express my gratitude to my family for their endless support, resources, and prayers.

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TABLE OF CONTENTS

Content **Page**

DECLARATION iii
DEDICATION iv
ACKNOWLEDGEMENTS v
TABLE OF CONTENTS vi
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF ABBREVIATIONS xi
ABSTRACT xiii

CHAPTER ONE: INTRODUCTION 1
1.0 Overview 1
1.1 Background to study 1
1.2 Statement of the problem 4
1.3 General and Specific Objectives 4
1.4 Relevance and Justification 5
1.5 Scope of Work and Delimitations 6
1.6 Structure of the Dissertation 6
CHAPTER TWO: LITERATURE REVIEW 8
2.0 Overview 8
2.1 Commercial Pet Food 8
2.2 History of commercial pet food and formats 8
2.3 Formats of pet food 14
2.3.1 Dry food 14
2.3.2 Semi-moist foods 15
2.3.3 Moist or canned foods 16
2.4 History of aflatoxins 17

University of Education,Winneba http://ir.uew.edu.gh

2.5 Aflatoxins 18
2.5.1 Physical and chemical properties of aflatoxins 21
2.6 Source of Aflatoxin contamination in commercial dog food 22
2.7 Aflatoxin contamination in commercial dog food 24
2.8 Mechanisms of toxicity in dogs 26
2.9 Outbreaks of aflatoxicosis in dogs 28
2.10 Pet Food Regulation 31
2.11 Economic impact of Aflatoxin contamination 32
2.12 Methods for Detection and Quantification of Aflatoxins 33
2.12.1 Methods used for extraction and clean-up of aflatoxins 33
2.12.2 Liquid-liquid partitioning 34
2.12.3 Solid phase extraction 34
2.12.4 Immunoaffinity columns 35
2.13 Analytical Methods for Aflatoxins 36
2.13.1 Thin Layer Chromatography (TLC) 37
2.13.2 High-Performance Liquid Chromatography (HPLC) 39
2.13.3 Gas Chromatography (GC) 40
2.14 Enzyme-Linked Immunosorbent Assay (ELISA) 42
2.15 Cultural methods for detection of Aflatoxins 43
2.16 Isolation of aflatoxigenic fungi and media for aflatoxin production 44
2.17 Types of cultural methods 44
CHAPTER THREE: METHODOLOGY 47
3.0 Overview 47
3.1 Study Area 47
3.2 Sampling 48
3.3 Materials for microbial culture 49
3.4 Materials for HPLC 50
3.5 Media Preparation 51
3.6 Isolation of pathogens from samples and sub-culturing 52

University of Education,Winneba http://ir.uew.edu.gh

3.7 Chemical extraction of Aflatoxin 52

3.8 High-Performance Liquid Chromatography (HPLC) Determination 53

CHAPTER FOUR: RESULTS AND DISCUSSIONS 55

4.0 Overview 55

4.1 Pathogen identification 55

4.2 Frequency and Percentage Occurrence of Pathogens 60

4.3 Linearity of the system (calibration curves) 63

4.4 Aflatoxin contamination levels in the commercial dog foods 66

CHAPTER FIVE: SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATION 72

5.0 Overview 72

5.1 Summary of Findings and Conclusion 72

5.2 Recommendations 74

5.2.1 Scientific Community 74

5.2.2 Dog food sellers 74

5.2.3 Regulatory bodies 75

REFERENCES 76

APPENDICES 87

EDUCATION FOR SERVICE

University of Education,Winneba http://ir.uew.edu.gh

LIST OF TABLES

Table **Page**

2.1: Physical Properties of Major Aflatoxins 22

2.2: Some Aflatoxicoses outbreaks in dogs 30

3.1: Major constituents of the brands sampled. 49

4.1: Pathogens recorded on samples 56

4.2: Pathogen Description 57

4.3: Frequency and percentage occurrence of pathogens 60

4.4: Fungal mycoflora detected in the commercial dog food compared to other published works 62

4.5: Limits of Quantification (LOQ) of aflatoxins AFB1, AFB2, AFG1, AFG2 66

4.6: Results of the Aflatoxin contamination levels detected by HPLC in the commercial dog food 68

4.7: Comparison of mean aflatoxin concentrations of Aflatoxins B1, B2, G1 and G2 in commercial dry dog food in the study area and published data 70

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LIST OF FIGURES

Figure	Page
2.1: Extrusion cooking system	13
2.2: Chemical structures of aflatoxin B (AFB1 and AFB2), aflatoxin G (AFG1 and AFG2) and Aflatoxin M1	20
2.3: Structures of other metabolites and degradation products of aflatoxins	20
2.4: Biotransformation reactions of aflatoxin B1 in poultry and mammals, including Humans	28
2.5: Schematic overview of the immunoaffinity clean-up	36
2.6: Vertical development TLC	39
2.7: HPLC instrumentation (with two coupled columns)	40
2. 8: Gas chromatograph	42
2.9: Principle of a competitive ELISA to screen mycotoxin	43
2.10: A blue fluorescence surrounding aflatoxigenic colonies under UV-light	45
3.1: Map of Ghana showing the regions where sampling was done	48
3.2: Chart model of the experimental procedures	51
3.3: A picture of the 1260 Infinity II Agilent UPLC with fluorescence detector system.	54
4.1: Percentage Occurrence of Pathogens	61
4.2: HPLC chromatogram of the Aflatoxin standards	63
4.3: Calibration curve for Aflatoxin B1	64
4.4: Calibration curve for Aflatoxin B2	64
4.5: Calibration curve for Aflatoxin G1	65
4.6: Calibration curve for Aflatoxin G2	65

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LIST OF ABBREVIATIONS

AFs	Aflatoxins
Aft	total aflatoxin
AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFG1	aflatoxin G1
AFG2	aflatoxin G2
AFL	aflatoxical
AAFCO	Association of Feed Control Officials
EMA	Economically motivated adulteration
EU	European Union
FDA	Food and Drugs Authority
LOQ	limit of quantification
LOD	limit of detection
ppm	parts per million
ppb	parts per billion
LLE	liquid-liquid extraction
IAC	Immunoaffinity columns
TLC	Thin-layer chromatography
HPTLC	High-performance thin-layer chromatography
HPLC	High-performance liquid chromatography
GC	Gas chromatography
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
ELISA	Enzyme-Linked Immunosorbent Assay

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UPLC	Ultrahigh-pressure Liquid Chromatography
a_w	water activity
IARC	International Agency for Research on Cancer
GST	Glutathione S-transferases
RI	refractive index
rpm	revolutions per minute
NMR	Nuclear Magnetic Resonance
RP-HPLC	reversed-phase High-performance liquid chromatography
FLD	fluorescence detector
FEDIAF	European Pet Food Industry Federation

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ABSTRACT

Aflatoxins are a group of highly toxic mycotoxins. They can contaminate a variety of feed products, including commercial dog food. This study investigated aflatoxin contamination in various commercial dog food brands in Ghana. A total of 18 samples of commercial dog food were collected from two major cities, Kumasi and Accra. The samples were analyzed for the presence of toxic fungi, particularly *Aspergillus spp.*, using a standard cultural method. The levels of aflatoxins B1, B2, G1, and G2 in the samples were also determined using High Performance Liquid Chromatography. *Aspergillus spp*. was found in 65 % of the samples, with *Aspergillus flavus* being the most prevalent species. This suggests that commercial dog food in Ghana is at a high risk of aflatoxin contamination. The average concentrations of aflatoxins B1, B2, G1, and G2 in the samples were 8.58±1.68 ng/g, 53.24±0.43 ng/g, 53.30±0.49 ng/g, and <0.20 ng/g, respectively. The predominant aflatoxin was aflatoxin B1, which is known to be mutagenic and carcinogenic. Also, a total of 16.7 % of the samples had total aflatoxins that exceeded the FDA(USA) mandated limit of 20 ng/g (20 ppb) for animal feeding. The findings of this study suggest that aflatoxin contamination is a serious problem in commercial dog food in Ghana. This poses a significant health risk to dogs, as aflatoxins can cause a variety of health problems, including liver damage, immunosuppression, and cancer. The study also highlights the importance of routine monitoring for aflatoxin contamination in commercial dog food, especially in repackaged commercial dog food. Good hygiene standards should be maintained during storage and repackaging to prevent the development of aflatoxins.

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CHAPTER ONE

INTRODUCTION

1.0 Overview

This opening chapter is a brief introduction to the study that is presented in this thesis. This chapter explores the background context of the study, identifying gaps in the literature into which this original research fits. The general objective and specific objectives are outlined in addition to the relevance of this study. Following on from this, this chapter will then conclude with an outline of the structure of the thesis.

1.1 Background to study

Commercial dog foods are made from a variety of ingredients, such as meat meals, soy, oatmeal, nuts, and cereals (corn, rice, wheat, barley, and sorghum), derivatives of meat from pigs, chicken, or fish, dairy products to create a balanced diet that provides the right amount of calories for protein, fat, fibre, carbohydrates, vitamins, and minerals needed to maintain and promote a healthy life (Tahira et al., 2015). All of these ingredients are prone to aflatoxin contamination, including cereals, which can result in aflatoxin poisoning and adversely affect the health of dogs (Wouters, et al., 2013).

Dog diets may now be tailored to suit the breeds, ages, and activities of dogs due to a greater understanding of their nutritional needs (Martínez-Martínez, et al., 2021). Due to the close bond between humans and their dogs, where dogs are even regarded as members of the household, dog owners are now prioritizing the diet of both their pets and themselves (Martínez-Martínez, et al., 2021). The majority of families in Ghana have one or more dogs, which generates a sizable market for the pet food sector. Dog breeding has drawn a lot of attention in Ghana, where foreign breeds are bred and sold

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for excessive rates. All of these have helped to significantly increase the demand for commercial dog food.

After an unusual veterinary crisis near London, England, in which more than 100,000 turkeys on several poultry farms perished in the course of a few months from an alleged new disease known as "turkey X disease," which is characterized by loss of appetite, lethargy, and weakness of the wings, the term "mycotoxin" was coined in 1962 (Wogan & Goldblatt, 1969). When the unexplained turkey X sickness was later determined to be caused by peanut (groundnut) meal contaminated with *Aspergillus flavus* secondary metabolites (aflatoxins), it made researchers more aware of the possibility that other possible mould metabolites might also be fatal (Zain, 2011). Pathogenic fungi of all kinds, including *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*, naturally produce mycotoxins, that are either phytotoxic or harmful to human and animal health. Currently, mycotoxins are thought to be the most harmful and pervasive pollutants in food and animal feed. Mycotoxins are poisonous fungi that can cause both acute and chronic toxicity, which can have fatal outcomes as well as detrimental effects on the central nervous system, cardiovascular, respiratory, and digestive systems. They can also cause cancer, cause teratogenic effects, and suppress the immune system (Valladares-Carranza, et al., 2018). The fungi *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* are the main producers of aflatoxins, which are mycotoxins. The primary aflatoxins produced by these toxigenic fungi in feedstuffs are aflatoxins B1, B2, G1, and G2, with B1 being the most pathogenic of the four. Aflatoxin formation is encouraged by several favourable environmental factors, including warm temperatures (36 to 38°C) and high humidity (over 85%). Even though the presence of these moulds in food does not always indicate dangerous amounts of aflatoxin, it may nevertheless pose a serious risk (Khorrami et al., 2022). The pet food business is seriously threatened

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by mycotoxin contamination, and aflatoxins are typically to blame for acute mycotoxicosis (Aflatoxicosis) outbreaks linked to commercial dog food (Wouters, et al., 2013). Cereal products are the usual sources of aflatoxins in these cases (Valladares-Carranza, et al., 2018). Additionally, it is important to be aware that meat, dairy, and egg products may contain the fungus *Aspergillus spp*., aflatoxins, or some of their secondary metabolites (Martínez-Martínez, et al., 2021).

The word "aflatoxicosis" is used to describe a particular type of animal intoxication that is typically characterized by bleeding, hepatic necrosis, and bile ductile growth (Eaton & Groopman, 1994).

Aflatoxin exposure can result in pathological diseases that are latent, acute, or chronic in both people and animals. In cattle, guinea pigs, dogs, rabbits, trout, laboratory animals, and swine, numerous investigations have demonstrated the link between aflatoxicosis and reduced humoral and cellular immunity (Eaton & Groopman, 1994). In addition to the usual anorexia, depression, and icterus that aflatoxins cause in dogs, they can also cause haemorrhages, melena, and pulmonary oedema (Eaton & Groopman, 1994). Additionally, the International Agency for Cancer Research has classed several aflatoxins as being very toxic and potentially carcinogenic to animals (International Agency for Research on Cancer [IARC], 2012). Aflatoxins are therefore a major health danger to pets when present in pet food. They can result in immediate death when concentrations are high (Tahira et al., 2015; Weidenbörner, 2007). They can also result in lowered weight, weakened immunity, and other symptoms at lower concentrations, which could result in expensive costs for the pet owner (Weidenbörner, 2007). Animals' diets should be examined for mycotoxin contamination in cases of unexplained diseases and/or syndromes.

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Over the past few years, there have been numerous aflatoxin outbreaks reported in commercial pet food across the globe. In up to 50% of samples of commercial dog foods tested in several countries, aflatoxins with considerable amounts have been found (Wouters, et al., 2013). The majority of pet mycotoxicosis outbreaks are rarely reported and may result in the death of hundreds of animals (Boermans & Leung, 2007).

1.2 Statement of the problem

Several types of imported dog and cat foods were recalled from the Ghanaian market in February 2021. According to the FDA(Ghana), the recall was necessary due to significant aflatoxin contamination, which resulted in the deaths of 70 dogs and other illnesses in an additional 80 in the United States of America (Food and Drugs Authority, 2021). As the imported contaminated pet food was not registered, the implicated pet food importer was unable to conduct a full recall (Food and Drugs Authority, 2021). To ensure the safety of pet foods imported into the country, there is a need for continuous mycotoxin surveillance of pet foods at various ports and harbours. There have been numerous studies on the presence of aflatoxins in food consumed by the Ghanaian population, but there is limited information on aflatoxins contamination of pet food or the potential health effects on pets. This study aims to assess commercial dog foods for aflatoxin contamination that can pose a health risk to dogs in Ghana.

1.3 General and Specific Objectives

The main goal of this study is to assess the prevalence of fungi that produce aflatoxins and determine the levels of aflatoxins present in commercial dog foods in Ghana.

To achieve the general objective, this study aims to address the following specific objectives:

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i. To isolate and identify pathogens responsible for aflatoxin contamination in different brands of commercial dog food.
ii. To determine the level of Aflatoxins, present in the different brands of commercial dog foods.
iii. To compare the estimated aflatoxin concentrations with international recommended limits as well as similar studies carried out in other countries.

1.4 Relevance and Justification

There is limited data on the aflatoxin levels in commercial dog food, even though contaminated dog food is a primary cause of aflatoxicosis, which kills hundreds of dogs every year. Awareness among the many stakeholders is necessary for the successful management of aflatoxins, in addition to the implementation of various control techniques. Due to the absence of regulatory rules governing the minimum Aflatoxins requirements for pet food in Ghana, industries rely on worldwide standards set by agencies like the FDA(USA) and the American Association of Feed Control Officials (AAFCO). This study seeks to evaluate the current state of aflatoxin contamination, including the type and amount, as the aflatoxin content is not typically taken into account in the manufacture of dog food. It will also add to the general body of knowledge and research work on aflatoxins in commercial pet food by providing useful data in observing possible health risks to pets. Additionally, these data may be relevant for designing local regulations for importation and the local manufacturing of commercial pet foods. The data collected will form the basis for future research on the toxicological effects of contaminated pet food consumed by other pets in Ghana.

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1.5 Scope of Work and Delimitations

A survey was undertaken to identify the popular types of commercial dog foods on the Ghanaian market. This was done in the Greater Accra and Ashanti Regions where more than one-third of persons in Ghana live (Ghana Statistical Service, 2021). Therefore, the sampling was also done in the Greater Accra (Accra) and Ashanti Regions (Kumasi). Due to their ease of feeding and storing, dry commercial dog foods make up by far the largest portion of the pet food market. Only commercial dry dog food widely marketed and sold was sampled. Additionally, commercial dog foods were selected depending on whether they had any ingredients that could potentially contain aflatoxin, such as cereals.

1.6 Structure of the Dissertation

The work is organized into five (5) chapters:

Chapter 1 is the introduction which comprises the background, statement of the problem being investigated, justification and relevance of this work, and the objectives and scope of the work.

Chapter 2 contains a literature review on pet food, aflatoxin contamination of pet food, effects of aflatoxins on pet species, regulations of pet food, works already done and gaps being filled by this research work.

Chapter 3 describes the study area, sampling procedures and analytical methods used for the isolation and identification of aflatoxins as well as in the measurement of the concentrations of aflatoxins in the samples.

Chapter 4 presents and discusses the results obtained from the data collected, including analytical techniques employed and the evaluation of the findings.

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Finally, in Chapter 5, the study results are summarized, which incorporates the conclusion of the study, recommendations and suggestions in some areas for further research.

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CHAPTER TWO

LITERATURE REVIEW

2.0 Overview

This chapter reviews relevant literature on similar works, carried out on the study of aflatoxins, their existence, sources and levels in commercial dog food. This chapter also focuses on the mechanism of toxicity of aflatoxins in dogs and outbreaks of aflatoxicoses in dogs. Also, methods that can be used for the detection and quantification of aflatoxins in food matrixes are extensively discussed. Finally, the regulation of the pet food industry as well as the economic impact of aflatoxin contamination of pet food is also discussed.

2.1 Commercial Pet Food

Any product that satisfies a pet's nutritional and metabolic demands is referred to as pet food. Any product made by a pet food company that is meant to be consumed by pets (including dogs) after going on the market is considered commercial pet food (European Pet Food Industry Federation [FEDIAFb], 2021). This includes products that have been processed, partially processed, or not at all (European Pet Food Industry Federation [FEDIAFb], 2021). Commercial pet food is a term used to describe pre-packaged foods that include grains, ingredients originating from animals, vegetables, fruit, water, vitamins, minerals, and other additives that are "required" to preserve the quality of commercial pet foods, such as binders, humectants, preservatives, and so on.

2.2 History of commercial pet food and formats

Table leftovers are still fed to animals in the majority of underdeveloped nations today. Feeding table scraps to pets could provide possible food safety hazards due to lack of preservation, especially if the scraps are not consumed right away after preparation

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(White et al., 2018). Since at least the early 18th century, there have been treats made specifically for dogs and, to a lesser extent, cats. Dogs were the primary target of the formulation of early-prepared pet food. The first commercially manufactured dog food is widely credited to an Englishman named James Spratt (Olson, 2023). The first commercial manufacturing of James Spratt's pet biscuits, Spratt's Dog Cakes, began in the United States in 1895 for affluent American dog owners (White et al., 2018). With the assistance of Mr. Walker, a baker from London, Spratt created Spratt's Patent Meat Fibrine Dog Cake (Wrye, 2012). The dog cakes were prepared using wheat meals, vegetables, beets, and meat products in the belief that he might make something better for animals to eat, particularly during lengthy travels (Olson, 2023). The readymade pet food market was therefore established. Pets with chronic illnesses could now be fed medicated dog bread thanks to the invention of new pet food companies like Boston's A.C. Daniels Company in 1901 (Grier, 2006). Furthermore, Bennett and Co. introduced the Milk-Bone dog biscuits in 1908; these biscuits were shaped like bones (Wortinger & Burns, 2015). Being the first items to be offered for sale as a source of nutrition for dogs, these pet foods were unique on the market. Clarence Gaines established Gaines Food Co. in the middle of the 1920s, which later developed a pelleted type of dog food known as Gaines Krunchons (White et al., 2018; Wortinger & Burns, 2015). Gaines is also recognized for developing the first food that made the claim of providing complete and balanced nutrition, which added nutritional adequacy to the concerns about food safety related to pet food. Complete pet food is a type of pet food that, according to its content, is adequate for a daily ration (European Pet Food Industry Federation [FEDIAFa], 2021). As food science, food safety technologies, and knowledge of the specific nutritional requirements of pets continued to advance, pet food manufacturers added new forms to better satisfy the needs of pets and their owners. These formats

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included canned "wet" food, extruded dry diets, and moist semi-solid foods (FEDIAF, 2019). The Chappel Brothers produced the first batches of canned dog food in the early 1920s, in an effort to create a market for horsemeat, popularized the product (Wortinger & Burns, 2015). A daily supply of 500,000 dogs' worth of "balanced" canned food was being sold by Chappel Brothers (Wrye, 2012). Despite this, demand for canned pet food grew, peaking in 1941. As a result, dog food manufacturers were compelled to employ any inexpensively available meat or byproduct material, but they were also compelled to add more grains to their products. Companies used byproducts and meat from a wide range of domesticated and wild species, including reindeer, buffalo, salmon and other fishes, cattle, swine, and even whales. The Chappel Brothers brand was the first to advocate combining dry and wet pet food feeding in the 1930s. Up to 91 per cent of pet food sold at that time was canned food, which eventually came to dominate the industry (White et al., 2018). By 1946, wet pet food accounted for only 15% of total sales, and the pet food market had gone back to being dominated by dry foods (White et al., 2018). Compared to wet food, which frequently had a strong odour, dry pet food was simpler to handle, generally less expensive, more practical, and less intrusive. A more consistent dry pet food had to be created, so various strategies were developed through various studies. Earlier, industrial baking was used to make dry pet food. The ingredients are combined to form a dough, which is then spread out and baked on sizable trays. After cooling, the cooked product sheets are divided into pieces (if not already pre-shaped), and then packaged. In 1947, General Foods introduced the first hard, dry dog food created with pelleting technology. This method allowed for a more reliable and technologically advanced cooking procedure by replacing baking. Pelletizing is the process of preparing food by putting finely powdered components through a mechanical action along with heat, pressure, and moisture (Muramatsu et. al,

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2015). Compared to baking, this method of dry food preparation is simpler, faster, and more practical, and it usually produces more consistent results (Wrye, 2012). Dry pet food is more cost-effective than wet canned pet food since it includes significantly less water. In addition, dry pet food may be left out in a bowl for an animal to consume whenever they want without spoiling or hardening, which is quite convenient for pet owners. Furthermore, because of their low moisture content, they also had a substantially longer shelf life than their predecessors.

Initially, canned wet pet foods were hermetically sealed in steel cans that had been soldered together; however, over time, the can design changed to include drawn and ironed steel cans, and more recently, aluminium and plastic components (White et al., 2018).

Following World War II, veterinary science and animal medicine expanded rapidly because of government financing schemes and university animal research programs. The physiological responses of animals to vitamins, minerals, and other supplements were of great interest to scientists (Wrye, 2012). In 1955, pet food manufacturers started incorporating synthetic vitamins, minerals, and macronutrients into pet food formulae in addition to naturally occurring ones. This represented a huge improvement in the production of pet food. Pet food had changed from being a place to dispose of human food waste to a standalone item.

The introduction of extruded pet food, which almost immediately became the top brand in the United States of America, marked a significant shift in the industry in the 1950s. The new era of pet food production was ushered in by the important technological advancement of extrusion by researchers of Purina laboratories (Wortinger & Burns, 2015). All of the ingredients are combined in the extrusion process to create dough,

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which is then heated to a high pressure and temperature (80 to 200°C) (Case et al., 2010). The machine that is used to cook and shape expanded foods is called an extruder (Fig. 2.1). The dough is further combined as it passes through the extruder, moving very rapidly. Increased digestibility and palatability are the result of the dough's starches being cooked quickly during extrusion (Case et al., 2010). Once the kibbles have cooled, they are typically sprayed with fat or another enhancer to improve their palatability. The product's overall moisture content is reduced to 10% or less through hot-air drying (Case et al., 2010). In the pet food industry, extrudates are produced in a variety of shapes and sizes using basic materials such as fish, meat, grains, and other vegetable products. A flighted screw that spins inside a tightly fitting cylindrical barrel makes up a food extruder (Quang, 2008). Raw materials are pre-ground and mixed before being put into the extruder's feeding mechanism. In comparison to their baked or pelleted counterparts, extruded pet foods contained more animal-derived meal content in the 1950s and 1960s, though still significantly less than most do now. In other words, modern extruded pet diets contain a significant amount of carbohydrates from cereal foods (Quang, 2008).

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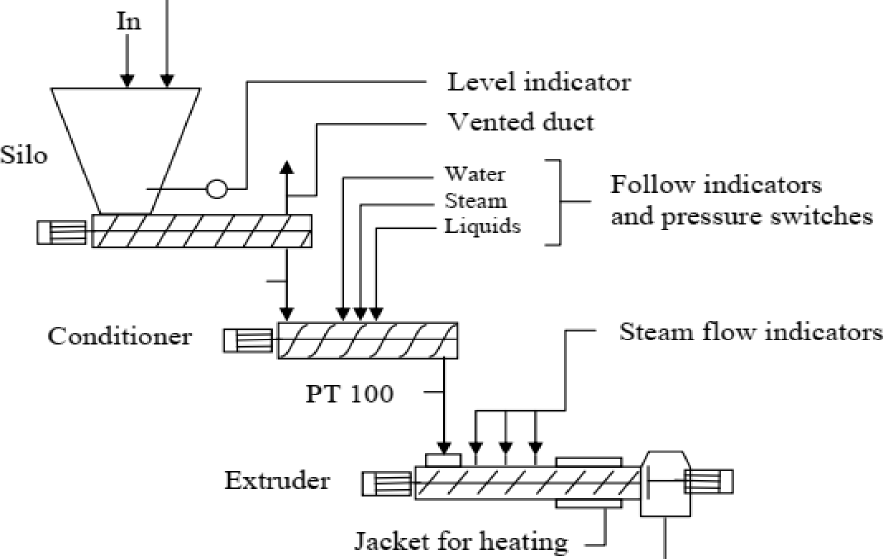

Figure 2.1: Extrusion cooking system

Source: (Quang, 2008)

In the mid-1970s, companies developed premium pet food brands targeting different dog life stages, exclusively sold through pet supply stores, feed stores, and veterinarians (Case et al., 2010).

The 1980s brought about the addition of chunk-in-gravy style canned food, whereas the loaf format formerly predominated. To account for chunk binding and gravy viscosity, several adjustments to the formulation were necessary. Products featuring real meat as the number one ingredient in a dry, extruded formulation began to be produced in the 1990s (White et al., 2018).

The development of mink diet feeding, and soft moist food formulation needed considerable application of hurdle technologies to maintain food safety at greater moisture levels. A brand-new pet food format known as soft dry that combined dry and soft wet ingredients into a single product was introduced in 1981 (White et al., 2018). The problem of preventing moisture migration from the soft, moist element to the dry component arose as a result, and this brought new difficulties.

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Freeze drying has become more popular since 2011, growing at a rate of 45.5% annually. One type of pet food manufacturing that has been mostly used for treat items is injection moulding, which has its roots in the plastics sector (White et al., 2018). Greenies, an injection moulded treat introduced in 1998 by S&M NuTec, LLC, is of particular importance from a food safety aspect. In terms of value sales, Greenies have exceeded all other treats by 2003 (White et al., 2018). After numerous allegations of choking and digestive problems with the treat, the company terminated the brand in 2006. Another sort of pet treat that has developed recently and raises serious questions about food safety is dehydrated jerky treats. The FDA(USA) received 4500 reports of pet illness and 580 reports of pet death as a result of consuming Chinese jerky treats between 2007 and 2013 (White et al., 2018). Despite extensive investigations, no exact cause was ever identified. But after the state of New York discovered that jerky treats made in China contained traces of an antibiotic that were not permitted for consumption, numerous brands voluntarily recalled their Chinese-made products, leaving a gap on the shelves that was eventually filled by jerky treats made in the United States (White et al., 2018).

2.3 Formats of pet food

There are three (3) different types of commercial pet foods based on their moisture content (Niamnuy & Devahastin, 2010). They are Dry, semi-moist and moist (canned) pet foods.

2.3.1 Dry food

Dry pet foods contain between 6% and 10% moisture and 90% or more dry matter (Case et al., 2010). It is generally sold in the form of kibbles, biscuits, meals and expanded extruded pellets and also tends to be the most economical to feed (Wortinger & Burns,

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2015). Due to its ease of storage and feeding, dry food makes up the majority of the pet food market. Dry pet food can be produced in a variety of ways, including baked, air- or freeze-dried, and extruded products, the latter of which make up the majority of dry pet foods now on the market (Watson et al., 2023). The majority of ingredients in dry pet food, which is a nutritionally complete feed, include meat, fish, animal derivatives, cereals, cereal byproducts, vegetables, fruit, and even a few herbs (Yang, et al., 2023). In comparison to other pet food types, dry pet meals typically contain higher levels of carbohydrates (such as corn, wheat, and soybean) (Niamnuy & Devahastin, 2010). Dry pet foods, due to their low water content, are protected from spoilage, allowing some dog owners to keep them in their pet bowls throughout the day (Jacobs, 2005). Ingredients chosen based on the formulation are combined and homogeneously blended before being extruded to create dry foods. Dry foods contain a greater concentration of nutrients and energy per unit weight than foods of higher moisture content and because of this, relatively small amounts are needed to provide a particular quantity of nutrients (Wortinger & Burns, 2015).

2.3.2 Semi-moist foods

Semi-moist foods are the second type of pet food format, and they account for a sizable share of the produced pet food market. Foods that are semi-moist are produced similarly to extruded foods, but the water content is kept higher. As the ultimate moisture level is between 15 and 30 per cent, mould and spoilage are more likely to occur, but these issues can be mitigated by using mould inhibitors (Yang et al., 2023). Humectants such as sugars, salts and glycerol are included in the foods to decrease the availability of water for use by invading microorganisms (Wortinger & Burns, 2015). In addition, small amounts of organic acids can be included to decrease the pH and further help in inhibiting bacterial growth (Aquino & Benedito, 2011; Wortinger & Burns, 2015). The

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main ingredients of semimoist pet foods are meat or meat by-products, or a combination of meat and vegetable proteins (Niamnuy & Devahastin, 2010). Semi-moist pet foods have a softer texture which makes them more palatable, well-liked by pets, and satisfy their caloric needs (Watson et al., 2023). Some owners prefer semi-moist diets due to the relative lack of odour and mess associated with moist foods and because they come in shapes similar to the foods that they eat (Wortinger & Burns, 2015).

2.3.3 Moist or canned foods

Historically, a far larger portion of the manufactured pet food market was made up of moist or canned foods, but their use has declined. Nowadays, you can find moist diets in foil containers, plastic trays, and pouches instead of just cans (Wortinger & Burns, 2015). Pet foods with a meat-like texture and a minimum moisture content of 50% by weight and a maximum permitted moisture content of 78% by weight are typically classified as moist pet foods (Niamnuy & Devahastin, 2010). In comparison to dry pet foods, canned pet foods often have a higher percentage of animal-based components. This means that their protein and fat concentrations are higher and their carbohydrate and fibre contents are lower (National Research Council, 2006). Canned foods are primarily composed of fresh or frozen meat, poultry, fish, and animal by-products (Girginov, 2007). To kill food-borne germs, all canned or moist foods are sterilized using steam and heat, as such no other preservatives are needed (Aquino & Benedito, 2011). This makes them ideal for a client concerned about the use of preservatives in their pets' food (Wortinger & Burns, 2015). Many owners do not like the smell or mess associated with moist diets or the fact that unused portions need to be stored in the refrigerator.

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2.4 History of aflatoxins

Aflatoxin contamination in groundnut meal led to an outbreak of turkey "X" illness in England in the late 1950s, which resulted in a large number of mortalities of turkey poults, ducklings, and chicks. This outbreak led to the discovery of aflatoxins (Richards, 1972; Wogan & Goldblatt, 1969). In 1960, a seemingly new ailment known as "turkey X disease," which is marked by lack of appetite, lethargy, and weakness of the wings, caused almost 100,000 turkeys to perish on poultry farms in the south and east of England over the course of a few months (Wogan & Goldblatt, 1969). As a result of consuming such contaminated rations, other domestic animals such as ducklings, chickens, cattle, and swine also died (Richards, 1972; Wogan & Goldblatt, 1969). Additionally, reports of substantial losses of ducklings due to a similar sickness came from Kenya and Uganda (Wogan & Goldblatt, 1969). The toxic substances detected in the groundnut meal were made by a fungus called *Aspergillus flavus*. The toxic substances induced acute liver illnesses in ducklings and liver cancer in rats when fed with extracts contaminated with *A. flavus* (Babu, 2010). As a result, the toxin was given the name aflatoxin (*A. flavus* toxin) and it was discovered that it was to blame for the death of turkey poults via liver cancer. The outbreak of turkey "X" illness was accompanied by massive liver necrosis, parenchymal cell degeneration, and bile duct proliferation in infected poults (Babu, 2010). Early detection techniques revealed that there were two forms of aflatoxins: blue (aflatoxin B) and green (aflatoxin G) that generate high fluorescence in UV light (Babu, 2010). Various aflatoxins and their metabolites were found, identified, and confirmed as a result of subsequent research. The toxins were categorized into four chemical components, aflatoxins B1, B2, G1, and G2, as well as a metabolite of aflatoxin B1 known as aflatoxin M1.

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2.5 Aflatoxins

Aflatoxins (AFs) are secondary metabolites that chemically correspond to a bisdihydrodifuran or tetrahydrobisfuran bound to a coumarin substituted by a cyclopentanone or lactone (Fuentes, et al., 2018). Aflatoxins form colourless to pale-yellow crystals which are intensely fluorescent in ultraviolet light, emitting blue (aflatoxins B1 and B2) or green (aflatoxin G1) and green-blue (aflatoxin G2) fluorescence, from which the designations B and G were derived, or blue-violet fluorescence (aflatoxin M1) (IARC, 2012). The chemical structures of aflatoxin B (AFB1 and AFB2), aflatoxin G (AFG1 and AFG2) and Aflatoxin M1 are shown in Fig.2.2.

Aspergillus flavus and *A. parasiticus* are the major fungi that produce AFs, though not all strains of these species do. Other species produce aflatoxin, although they are less prevalent, including *Aspergillus bombycis*, *Aspergillus ochraceoroseus*, *Aspergillus nomius*, and *Aspergillus pseudotamari* (Zain, 2011). One of the most researched and earliest-named genera of fungi is *Aspergillus*, which has been described as a diverse group of moulds in terms of metabolism and ecology (Babu, 2010). While *A. parasiticus* produces aflatoxins B1, B2, G1, and G2, *A. flavus* only produces aflatoxins B1 and B2. (Yu, 2012). The oxidative metabolic byproducts of aflatoxins B1 and B2, aflatoxins M1 and M2 are formed by animals after consumption and can be found in their milk, urine, and faeces (Aycicek et al., 2005; Popescu et al., 2022). Another reductive metabolite of aflatoxin B1 is aflatoxicol (AFL) (Aycicek et al., 2005). Structures of other metabolites and degradation products of aflatoxins are also shown in Fig.2.3.

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Aflatoxin-producing *A. flavus* and *A. parasiticus* strains can infect plants in the field before colonizing harvested or stored plant products. As a consequence, aflatoxins have the potential to build up in many essential agricultural products. These products may come from tropical and subtropical regions and include maize and other cereal grains, groundnuts and other legumes, fresh and dried fruits, spices, herbs, vegetables, cottonseed and other oilseeds, cassava and other roots and tubers. Therefore, aflatoxins may contaminate many processed foods and feeds. Aflatoxins have a wide spectrum of toxic activities that are connected to their reactivity with nucleic acids and cell nucleoproteins and the subsequent impact of these events on protein synthesis and cellular integrity in living organisms (Logrieco et al., 2003). They can affect farm animals in two different ways: as an acute poisoning that causes fatal liver tumours and immune system suppression, as well as a chronic aflatoxicosis characterized by cancer and other slow-moving pathological diseases (Logrieco et al., 2003; Kumar, 2018). According to the International Agency for Research on Cancer (IARC), aflatoxins are categorized under Group 1 as compounds that are carcinogenic to humans and animals due to their ability to cause mutagenesis, teratogenicity, and cancer (IARC, 2012).

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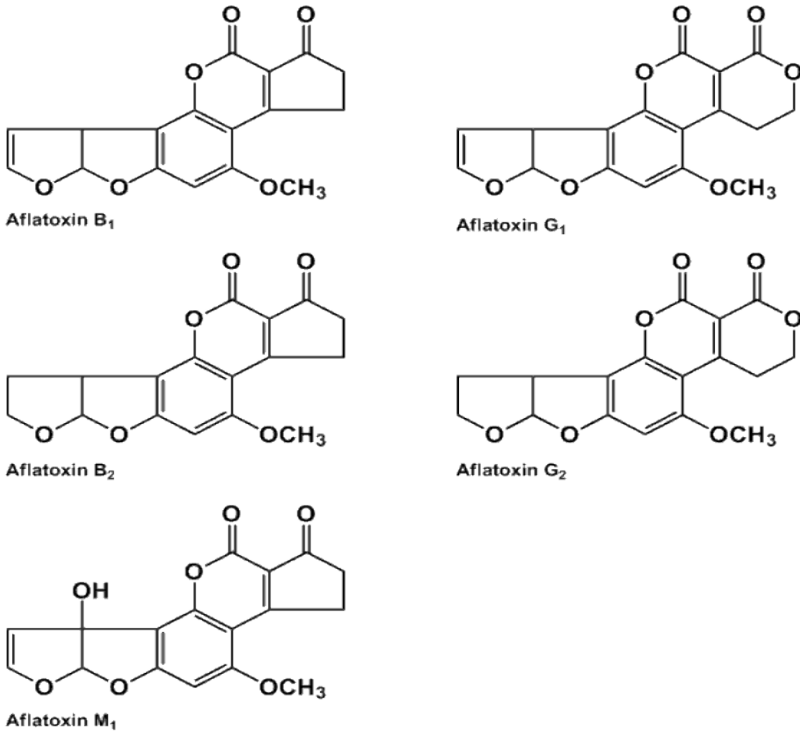

Figure 2. 2: Chemical structures of aflatoxin B (AFB1 and AFB2), aflatoxin G (AFG1 and AFG2) and Aflatoxin M1

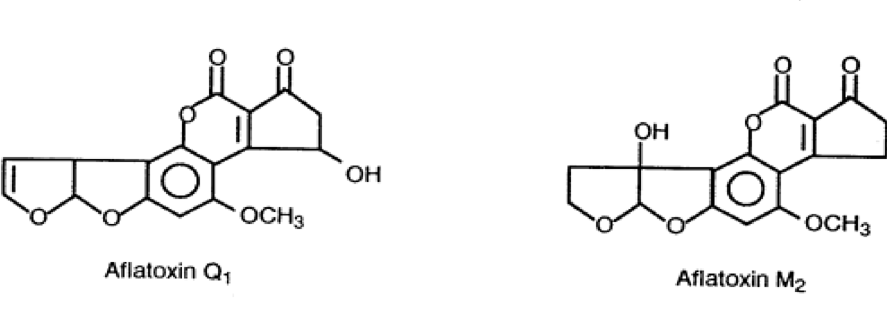

Figure 2. 3: Structures of other metabolites and degradation products of aflatoxins

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2.5.1 Physical and chemical properties of aflatoxins

Aflatoxin produces colourless to light yellow crystals that glow when exposed to UV light as shown in Table 2.1. In addition, Table 2.1 also describes other physical properties of major Aflatoxins which include their molecular weights and melting points. Aflatoxins are soluble in moderately polar solvents such as chloroform, methanol, and dimethyl sulfoxide, as well as water (10-20 mgL^{-1}). In the presence of oxygen, UV radiation makes them unstable, and high pH (<3 or >10) makes them unstable as well (Kumar, 2018).

Under the catalytic effect of a strong acid, aflatoxin B1 has also been shown to react additively with a hydroxyl group (Wogan, 1966). During ozonolysis, aflatoxin B1 is broken up, resulting in the byproducts levulinic, succinic, malonic, and glutaric acids (Wogan, 1966). Also, under alkaline conditions, the lactone ring opens, destroying the aflatoxins, although this reaction is reversible with acidity. Ammonia promotes decarboxylation of aflatoxins by opening the lactone ring at high temperatures, which is an irreversible process (Kumar, 2018).

The aflatoxins appear to partially degrade when left in a methanolic solution, and this process is significantly sped up in the presence of light or heat (Dhanasekaran et al., 2011).

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Table 2.1: Physical Properties of Major Aflatoxins

Property	AFB1	AFB2	AFG1	AFG2
Molecular formula	$C_{17}H_{12}O_6$	$C_{17}H_{14}O_6$	$C_{17}H_{12}O_7$	$C_{17}H_{14}O_7$
Molecular weight	312	314	328	330
Crystals	Pale yellow	Pale yellow	Colourless	Colourless
Melting point (° C)	268-269 *	287-289 *	244-249 *	237-240 *
Fluorescence emission(nm)	425	425	450	450
Fluorescence under UV light	Blue	Blue	Green	Green

* **Decomposes, Source:** (Kumar, 2018; Wogan, 1966)

2.6 Source of Aflatoxin contamination in commercial dog food

A. flavus and *A. parasiticus* can infect almost any foodstuff and have been found on most agricultural products. As a result, aflatoxins have the potential to occur in a variety of foods. The quality and safety of the entire feed are compromised by the presence of these toxic *Aspergillus spp*. in cereals or other dog food ingredients. Dog food typically contains cereals, particularly corn, sorghum, rice, wheat, oats, barley, and millet, which are good sources of carbohydrates, fibre, protein, fat, minerals, and vitamins (Martínez-Martínez, et al., 2021).

It is usual for a mixture of aflatoxins B1 and B2 perhaps together with aflatoxins G1 and G2 to be present in animal feed. Once formed, aflatoxins are quite stable and are likely to persist during storage and processing.

According to recommendations from the Association of American Feed Control Officials (AAFCO), the majority of produced pet foods are prepared to satisfy specified nutrient requirements to support growth, maintenance, or gestation/lactation. The nutrients that are targeted include the calories, protein, fat, carbohydrates, vitamins, and minerals required to sustain life and, where possible, optimize performance. Sorghum, maize, soya, rice, cereals, meal of meat and bones, by-products of birds, fish, and

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chicken, and derived product of egg and milk were the main ingredients of pet food (Aquino & Benedito, 2011).

Aflatoxin contamination of commercial dog food has many causes, but they all start with the components. Both in the pre-harvest stage and during storage, cereals and other substances make suitable substrates for the formation of fungal microflora. There have been reports of aflatoxin contamination in a variety of products, including nuts, rice, cottonseeds, spices, and figs, as well as grains like corn, soya, wheat, rice, cottonseed, and dairy products.

Aflatoxins often occur in crops in the field before harvest. Post-harvest contamination can happen if crop (cereals and nuts) drying is delayed, and agricultural storage can become contaminated if water levels are allowed to rise above those necessary for mould growth. Mold growth is facilitated in some stored goods by insect or rodent infestations (Dhanasekaran et al., 2011). Therefore, aflatoxin contamination is more likely to occur in pet food raw materials with high carbohydrate and fat content.

Aflatoxins are primarily found in temperate and tropical climates, which are present in parts of North and South America, as well as South Asia and Africa. Ingredients included in feed such as corn, peanuts, cottonseed, tree nuts, wheat, and rice are the main route via which mycotoxins affect food in these areas.

Due to a greater understanding of the nutritional requirements of dogs based on their breeds, ages, and activities engaged in, there is an increase in the variety of dog food formulas. The growing availability of agro-industrial ingredients with a variety of bromatological compositions is another factor that influences the supply of commercial dog food. This facilitates the diversification of suitable feed formulas for dogs with

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various nutrient profiles, digestive requirements, and metabolic requirements (Dhanasekaran et al., 2011).

2.7 Aflatoxin contamination in commercial dog food

According to several studies, *Aspergillus spp*., *Mucor spp*., *Penicillium spp*., and *Rhizopus spp.* are the most frequent fungi found in commercial dog foods. The health of dogs is at risk since several of the genera and species that were isolated and identified are mycotoxigenic. Although information regarding the presence of *Aspergillus spp*. and other fungal microflora in various processed food products for human consumption in Ghana is extensive, studies of this contamination in commercial dog foods are limited, despite being made with similar ingredients.

Akinrinmade and Akinrinde (2012) used High-performance liquid chromatography to quantify aflatoxins B1, B2, G1 and G2 in commercial dry dog foods in the city of Ibadan. Results indicate that aflatoxins B1, B2, G1 and G2 were detected in all the samples investigated, with B1 being the most abundant. The range of concentration of total aflatoxins was 7.76 to 11.93 µg/kg with an average of 9.61 µg/kg across the brands.

Valladares-Carranza et al. (2018) assessed 20 samples of different brands of commercial dog food in Toluca City, in the central Valley of Mexico through the qualitative thin layer chromatography test (utilizing the Stoloff method) and a positivity of 80% (16/20) of aflatoxins G1 and G2 was detected.

Fuentes et al. (2018) reported the average Aflatoxins (µg/kg) contamination in industrialized dry dog foods to be AFB1 (1.6), B2 (0.1), AFG1 (28.2), AFG2 (1.3), AFM1 (1.8), AFM2 (0.2), P1 (1.7), Aflatoxicol (28.6) and Total aflatoxins (59.1). In

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canned dog foods, the averages were AFB1 (14.2), AFB2 (2.3), AFG1 (60.4), AFG2 (4.5), AFM1 (2.1), AFM2 (4.6), AFP1 (18.4), AFL (13.1), and total aflatoxins (119.5), and the average of all of the samples was 15.3 µg/kg. They found that the dry food croquettes for dogs had 51.6% less aflatoxins, with an average of 7.9 µg kg^{-1} total aflatoxins, under the tolerable legal limit, and the canned food, more contaminated (15.3 µg kg^{-1}), and surpassed the tolerable limit for Codex Alimentarius.

A study by Tahira et al. (2015) in Pakistan analyzed imported dog food (solid = 150 and semi-solid = 30) using chromatographic methods (HPTLC). It revealed the incidence of AFB1 in solid types of pet foods i.e.,18 % (range, 0.5-8 ng/g; mean 4.83±1.01 ng/g) for dog foods. However, for the semi-solid type, 6.66% (range, 0.5-9 ng/g; mean, 2.80±1.45 ng/g) were recorded for dogs' pet foods. The imported dog foods were safe for consumption, as the observed mean level of aflatoxin B1 recorded was below the regulatory limit i.e. 20 ng/g.

Singh and Chuturgoon (2017) investigated mycotoxin contamination of supermarket and premium brand pelleted dog food in Durban, South Africa. A total of 20 samples were analyzed with the results showing the mean level of Aflatoxins (AF) to be 44.17 µg/kg in standard brands and 20.17 µg/kg in premium brands with the level of AFB1 in some of the samples exceeding the concentration limit of 10 ng/g limit as prescribed by the South African Government.

Bruchim et al. (2011) analysed 10 food samples of aflatoxin-contaminated commercial canine diet (Nutra Nuggets, Diamond Pet Foods, USA) and their aflatoxin concentration ranged between 80 and 300 ng/g exceeding the upper concentration limit of 20 ng/g as permitted by the US Food and Drug Agency regulations.

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Arnot et al. (2012) using the Veratox Aflatoxin Quantitative Test analysed 124 dog food samples for total Aflatoxin concentration. The concentrations ranged from below the limit of quantification (LOQ < 5 μg/kg) to 4946 μg/kg. Additional analysis using HPLC of six Hi-PRO® dog food samples showed very high Aflatoxin B1 concentrations (> 90 μg/kg) in four of the six samples.

2.8 Mechanisms of toxicity in dogs

Aflatoxicosis is a general term used to describe illnesses brought on by aflatoxin exposure. Animal liver damage is the main clinical impact of aflatoxins; aflatoxin B1 is the most lethal, followed by aflatoxins G1, B2, and G2 (Benkerroum, 2019).

One of the animal species most susceptible to aflatoxins is the dog, with young dogs more susceptible to the toxicity of aflatoxins than older dogs (Rumbeiha, 2001). Aflatoxin toxicity varies depending on the species, age, sex, length of exposure, and nutritional state (Sarma et al., 2017).

When dogs consume food containing aflatoxins, the toxins are taken up by the intestine and transported to the liver (Lizárraga-Paulín et al., 2011). AFB1, AFG1 and AFM1 are converted to their respective epoxides, which can bind covalently to both DNA, RNA, and protein enzymes (Aquino & Benedito, 2011; Martínez-Martínez, et al., 2021; Kumar, 2018). AFB1-8,9-exo-epoxide and AFB1-endo-8,9 epoxide are the stereoisomers that result from the hepatic metabolism of aflatoxins, particularly AFB1, by cytochrome P450 (CYP450) microsomal enzymes; the former is more reactive and toxic than the latter by a factor of >1000 (Bbosa, 2013). High affinity for guanine (G) bases in DNA makes AFB1-8,9-exo-epoxide a highly unstable molecule that covalently binds to DNA to generate DNA adducts, predominantly AFB1-N7-guanine in the target cells (Kumar, 2018; Kidanemariam & Fesseha, 2020). The primary mechanism by

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which these adducts cause DNA mutations is a transversion of G (guanine) to T (thymine) in the DNA (Kidanemariam & Fesseha, 2020). Figure 2.4 summarizes the different toxicity mechanisms of AFB1 involving AFB1-exo-8,9 epoxide. AFG1, AFQ1, and AFP1, three major hydroxylated metabolites, as well as aflatoxicol, which is excreted in urine, are produced by a class of enzymes called glutathione S-transferases (GST), which is important in stabilizing and inactivating the epoxide in many animal species (EFSA CONTAM Panel, 2020; Lizárraga-Paulín et al., 2011).

When animals like dogs consume high doses of aflatoxin (>0.5-1 mg/kg pet food) either once or repeatedly over a brief period, they develop acute aflatoxicosis. This condition causes typical symptoms like oedema, jaundice, nausea, lethargy, hemorrhagic necrosis of the liver tissues and bile duct hyperplasia, which ultimately leads to death (10–60 per cent) from severe liver damage (Benkerroum, 2019). Anorexia, lethargy, jaundice, intravascular coagulation, and death within two to three weeks are the symptoms of sub-acute aflatoxicosis (0.5 to 1 mg aflatoxin/kg pet food) (Fuentes et al., 2018). Chronic aflatoxin exposure with 0.05-0.3 mg aflatoxin/kg pet food over 6–8 weeks can likewise result in similar hepatotoxic effects (Boermans & Leung, 2007). Reduced animal weight growth and reduced feed conversion efficiency were the earliest symptoms of chronic aflatoxicosis (Eaton & Groopman, 1994). Extensive hepatic fibrosis, immunosuppression, and increased susceptibility to bacterial, fungal, viral, or parasitic infections are observed in chronic instances (Rumbeiha, 2001). With chronic exposures, the real number of dogs impacted by aflatoxins may be larger because it may involve consuming low doses of aflatoxin over a long period (Wouters et al., 2013).

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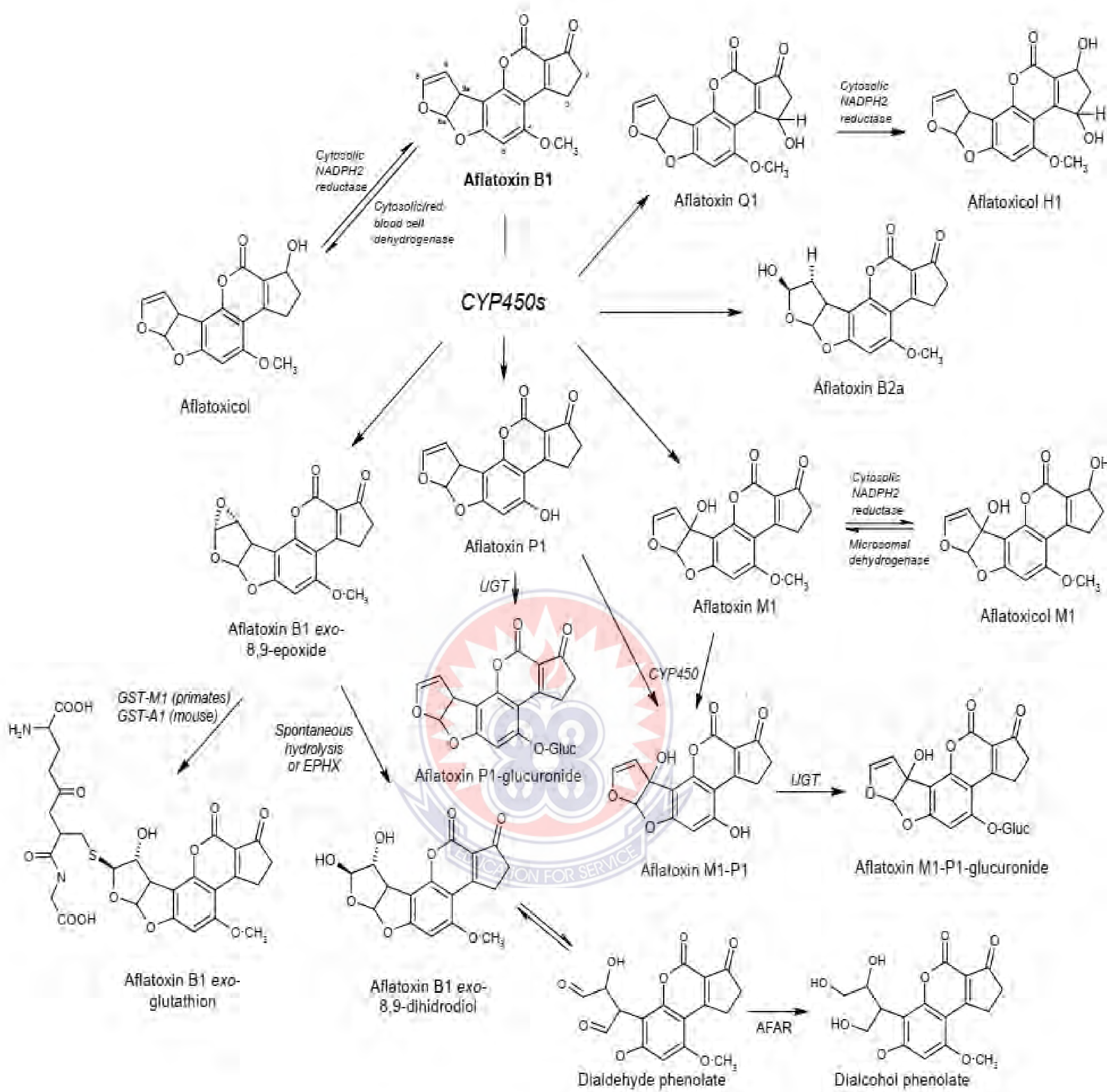

Figure 2. 4: Biotransformation reactions of aflatoxin B1 in poultry and mammals, including Humans

Source: (Diaz & Murcia, 2011).

Definitions: CYP450- Cytochrome P450, UGT- UDP-glucuronosyltransferase, NADPH2-nicotinamide adenine dinucleotide phosphate, EPHX- Epoxide hydrolase

2.9 Outbreaks of aflatoxicosis in dogs

Several aflatoxicosis outbreaks in dogs have been reported worldwide as listed in Table 2.2. Aflatoxins were also found in food for horses, rats, birds, fish, and cats, with

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varying prevalence across different locations. However, due to the small number of reported cases of food poisoning, these findings of acute aflatoxicosis do not fully depict the mycotoxin issue related to pet foods. Veterinarians frequently overlook or fail to recognize mycotoxins as the root cause of chronic conditions such as liver and kidney fibrosis, infections brought on by immunosuppression, and cancer (Akinrinmade & Akinrinde, 2012). These findings imply that mycotoxin contamination in pet food poses a major health risk to several pet species, particularly dogs (Boermans & Leung, 2007).

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Table 2. 2: Some Aflatoxicoses outbreaks in dogs

Year	Location	Scale	Diet	Reference
1951	Southeastern United States	71 food poisoning cases of dogs (with several dead)	A brand of commercial dog food suspected to be made with contaminated corn	(Bailey & Groth, 1959)
1987	Pretoria, South Africa	10 dogs died with 1 acute, 7 subacute and 2 chronic cases	A brand of contaminated commercial dog food	(Bastianello et al., 1987)
1998	United States	55 dogs died from both acute and chronic cases	17 different formulations of commercial dog food made with two rail cars of nonuniformly contaminated corn in a milling plant in Texas in late-summer	(Garland & Reagor, 2001)
2005	United States	At least 100 dogs dead	19 different formulations of commercial dog food made with contaminated corn in a milling plant in South Carolina in the summer	(Stenske et al., 2006)
2006	Korea	3 dogs died with renal failure	Fungal nephrotoxins in the diet.	(Jeong, et al., 2006)
2011	Israel	50 dogs	Consumption of an aflatoxin-contaminated commercial canine diet	(Bruchim et al., 2011)
2011	Gauteng Province, South Africa	Illnesses in approximately 100 dogs	Low-cost brands of pelleted dog food contaminated with very high concentrations of aflatoxins.	(Arnot et al., 2012)
2011	Southern Brazil	60 dogs dead	Diets with cooked cornmeal as a common ingredient.	(Wouters et al., 2013)
2020	United States	28 deaths and 8 illnesses	Brands of contaminated dog foods	(US FDA, 2021)
2021	United States	70 dogs dead and illnesses in another 80	Brands of contaminated dog foods	(Food and Drugs Authority, 2021)

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2.10 Pet Food Regulation

Public health concerns with aflatoxins and mycotoxins in general have not received much attention in low-income countries. This is a result of a lack of knowledge about mycotoxins, the breadth and depth of their harmful health effects, as well as a failure to raise acknowledged issues with governments in regions where contamination is most prevalent. Regulatory authorities focus on exposure mitigation to manage the risks associated with toxic chemicals by prioritizing strategies that reduce the likelihood of contact with the chemical, thereby protecting animal health (Boermans & Leung, 2007).

It is the responsibility of the FDA(Ghana) to control the production, processing, importation, exportation, packaging, storage, transportation, distribution, and sale of all foods, including pet foods. When it comes to the quantity of certain contaminants in food and feed products, specialized rules may set limits. Food and Drugs Authorities must ensure that the ingredients used in the preparation of animal feed including pet food are safe. The quality control procedures may involve testing for nutrient content, aflatoxins, or other contaminants that could endanger the safety of pets. It is essential to test commercial dog foods carefully for aflatoxins and eliminate contaminated packages (Aquino & Benedito, 2011). The FDA(USA) has set 20 ppb as the action limit for aflatoxin in pet foods (US FDA, 2021).

However, rather than adhering to the scientific approach of risk assessment and safety determination, government regulations of aflatoxin contamination are frequently hindered by analytical methods and detection limits, regional prevalence, as well as trade relationships among different countries.

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The safety of commercial dog foods is of prime interest to manufacturers. Healthy dogs contribute to greater sales, so breakdowns in product quality can have catastrophic effects on profits or even company viability (Boermans & Leung, 2007).

2.11 Economic impact of Aflatoxin contamination

The economic impact of aflatoxins on animals is determined by several different factors. Costs associated with regulation, confiscating contaminated goods, regulatory costs, animal deaths, health care and veterinary care expenses, and research costs aimed at reducing the severity and impact of the aflatoxin problem are all factors to be taken into account (Zain, 2011).

Aflatoxin exposure restrictions have been put in place by numerous countries, and are normally expressed in parts per billion (ppb). Depending on the intended use, certain nations have varying restrictions; the ones that relate to human consumption and exports are the strictest, while those that apply to industrial products are the highest (Partnership for Aflatoxin Control in Africa [PACA], 2012). Due to higher compliance costs, such as the price of testing, shipment rejection, and even the loss of admissibility into foreign markets, these restrictions may result in lost trade income. In the international market, products that do not meet the aflatoxin standards are either rejected at the border, rejected or eliminated in channels of distribution, or assigned a reduced price. Domestic markets may experience comparable economic losses if consumer knowledge of the issue grows, marketing channel leaders start to pay more attention, and/or laws are either tightened or more thoroughly enforced. For a short time, premiums for commodities free of aflatoxin may be realized in any of these situations. As compliance becomes a must for being accepted as a supplier, the premium will inevitably diminish in the long run. While stricter phytosanitary standards may

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come with more costs than benefits, in reality, once suppliers internalize the financial costs of non-compliance and bear them, greater economic benefits for society will materialize. These benefits include expanded and more stable markets and a decreased burden of disease (PACA, 2012).

2.12 Methods for Detection and Quantification of Aflatoxins

For the detection of aflatoxins in food and feed samples, cultural approaches have been widely used. Additionally, the characterization and quantification of aflatoxins are now more accurate as a result of analytical techniques like chromatography, which have been used for both qualitative and quantitative assessment of aflatoxins since they have strong UV absorption and fluorescence capabilities. Sample preparation, which includes extraction and cleanup procedures, is necessary for aflatoxin testing by chromatographic methods. They are equally crucial and cannot be separated.

2.12.1 Methods used for extraction and clean-up of aflatoxins

To achieve satisfactory aflatoxin recoveries, extraction must be applied to samples first, followed by cleanup. Sometimes the steps of extraction and clean-up are combined, and other times they are carried out separately. Organic solvents like methanol, acetonitrile, or acetone are frequently used, depending on the physical properties of the target analyte (Gilbert & Vargas, 2003; Razzazi-Fazeli & Reiter, 2011). The clean-up phase removes the interfering chemicals. More reliable outcomes have reportedly been obtained from extraction processes using methanol (MeOH) and water combinations (Razzazi-Fazeli & Reiter, 2011). The following are some examples of extraction techniques.

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2.12.2 Liquid-liquid partitioning

The conventional liquid-liquid extraction (LLE) method involves partitioning the analytes between two non-miscible solvents and moving them from one phase to the other (Razzazi-Fazeli & Reiter, 2011). Aqueous (hydrophilic) and hydrophobic organic solvents are usually the phases. In most situations, acetone, chloroform, acetonitrile, and methanol are used in the extraction process (Hussain, 2011; Razzazi-Fazeli & Reiter, 2011; Wacoo et al., 2014). Utilizing small amounts of water increases extraction efficiency. Hexane and cyclohexane are frequently employed for aliphatic compounds, while dichloromethane and chloroform are used for medium-polar contaminants (Hussain, 2011).

LLE was the original extraction technique used for the measurement of aflatoxins in samples of feed and food from the beginning of the mycotoxin story in the 1960s, but it has gradually been replaced by various extraction and clean-up techniques. The main limitations of this method include the high amounts of applied organic and previously chlorinated solvents, errors in analyte dilution caused by handling large solvent volumes, and reduced recovery caused by the creation of sample emulsions (Zhang & Banerjee, 2020). This method is now being replaced by solid-phase extraction or immunoaffinity extraction. LLE is still employed, nonetheless, to prepare samples of some mycotoxins. LLE is still utilized as a second cleaning step when evaluating trace mycotoxin concentrations in complex matrices for the analysis of aflatoxins.

2.12.3 Solid phase extraction

Solid phase extraction (SPE), which has a wider variety of adsorbents to choose from than liquid-liquid extraction, is more sample and solvent-efficient, selective, and adaptable than liquid-liquid extraction (Razzazi-Fazeli & Reiter, 2011; Zhang &

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Banerjee, 2020). The major objectives of the targeted sample clean-up procedures are the elimination of matrix interferences and the preconcentration of analytes.

Sorbent particles, which are either based on a silica matrix or polymeric phases, are typically inserted in plastic tubes between porous frits. The analyte is adsorbed to the solid phase due to interactions with the matrix, and after a washing step, the analyte is eluted, most typically using organic solvents. These days, sorbents are widely available, including carbon, porous polymers, diatomaceous earth, and alumina, as well as modified silica (Razzazi-Fazeli & Reiter, 2011; Zhang & Banerjee, 2020). The most popular SPE columns and cartridges contain modified silica such as C18 (octadecylsilane), phenyl or aminopropyl-bound phases, and others (Hussain, 2011; Razzazi-Fazeli & Reiter, 2011; Zhang & Banerjee, 2020).

2.12.4 Immunoaffinity columns

Immunoaffinity columns (IAC) clean-up is based on the specificity of antibody-aflatoxin binding (Hussain, 2011; Zhang & Banerjee, 2020). In the presence of complex food and feed matrices, it has been demonstrated that IACs have a high degree of specificity in binding aflatoxins, indicating that they can be used to purify samples that have been contaminated with diverse aflatoxins as illustrated in Fig.2.5 (Zhang & Banerjee, 2020).

The antibodies against the aflatoxins are packed in a column after being coupled to an agarose gel, sepharose, or dextran carrier (Hussain, 2011). The analyte molecules (aflatoxins) bind to the antibodies in the column in a selective manner. The antibodies do not interact with the matrix elements, and most potential interferences are eliminated by rinsing (washing). To elute the toxin, organic solvents that cause antibody denaturation, such as acetonitrile or methanol, can be utilized (Razzazi-Fazeli & Reiter,

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2011). IACs are more effective than liquid-liquid partitioning in terms of recovery. Major drawbacks include the high costs and the fact that a column can only be used once because of the denaturation of antibodies during the elution process.

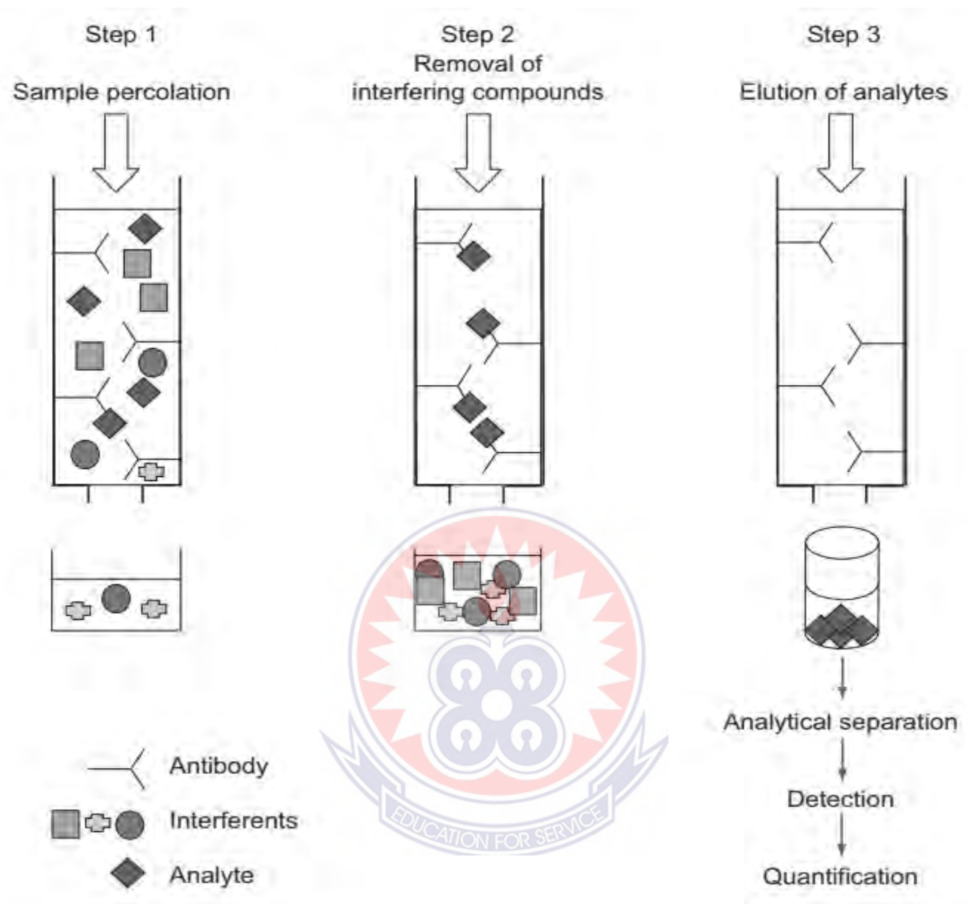

Figure 2. 5: Schematic overview of the immunoaffinity clean-up
Source: (Razzazi-Fazeli & Reiter, 2011)

2.13 Analytical Methods for Aflatoxins

Following the extraction of the analyte (aflatoxin) from the sample and the use of a clean-up procedure to get rid of interferences, the final step of the analytical methodology is identification and quantification.

Chromatography is one of the most often used procedures for analyzing aflatoxins since it can separate them using a variety of chromatographic techniques. The most common

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techniques of chromatography utilized are Thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), high-performance liquid chromatography (HPLC) and Gas chromatography (GC).

Along with chromatographic techniques, the Enzyme-Linked Immunosorbent Assay (ELISA) holds a leading position as a quick test for identifying specific mycotoxins like AFB1, AFB2, AFG1 and AFG2. In terms of speed and sensitivity, ELISA are advantageous screening and quantification method.

2.13.1 Thin Layer Chromatography (TLC)

Both HPTLC and TLC will be referred to as TLC in this section unless it is important to make the difference. TLC separation of aflatoxins provides a foundation for sensitive analytical procedures, delivering a reasonable level of selectivity and sensitivity in the separation of aflatoxins from other interfering chemicals (Hussain, 2011).

It consists of a stationary phase made of either silica (which is an example of a polar, acidic stationary phase), alumina (acidic, neutral, and basic), magnesium oxide, magnesium silicate, cellulose (polymer of D-glucopyranose units), and polyamide (Lundanes et al., 2013). These adsorbents are mainly used for normal-phase TLC.

Also, the use of chemically bonded stationary phases which are based on covalently bound phenyl, C2, C8, and C18 to silica has been utilized. Interactions between the stationary phase and the analytes are based on hydrophobic interactions (Lundanes et al., 2013).

The components of the mobile phase must be miscible and volatile enough to be easily removed from the TLC plate before detection. Typical mobile phases for normal-phase development are composed of two–to five solvents such as diethyl ether, isopropanol,

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acetic acid, dichloromethane, dioxane, toluene, and chloroform (Lundanes et al., 2013). For reversed-phase development, mixtures of water, buffer components, acetonitrile and methanol are common (Lundanes et al., 2013).

The distribution of aflatoxins between the mobile and stationary phases in TLC is principally determined by the analytes' different solubilities in the two phases and their affinity towards the stationary phase. Different analytes either adhere to the stationary phase more or stay in the mobile phase, based on their molecular structures and interactions with the stationary and mobile phases, allowing for efficient separation as illustrated in Fig. 2.6 (Wacoo et al., 2014).

The aflatoxins separated on the stationary phase (e.g. silica gel plates) may be readily visualized under short and long-wavelength UV light. The nomenclature of the aflatoxins as ‘B’ and ‘G’ was derived from their blue and green fluorescence colours observed under such a light. The concentration of analytes can be estimated visually by comparing the size and intensity of the spots on the sample with the spots on the standards (Lundanes et al., 2013).

It should be emphasized that TLC processes lack precision as a result of the introduction of potential errors during the steps of sample application, plate formation, and plate interpretation (Hussain, 2011). By employing high-quality plates coated with stationary phase made of uniformly finer particles, enhanced TLC techniques like HPTLC can therefore enhance the resolution of aflatoxins (Zhang & Banerjee, 2020). HPTLC offers increased sensitivity and more effective separation thanks to automated sample application devices and fluorescence densitometers for improved quantitative analysis (Shephard, 2011).

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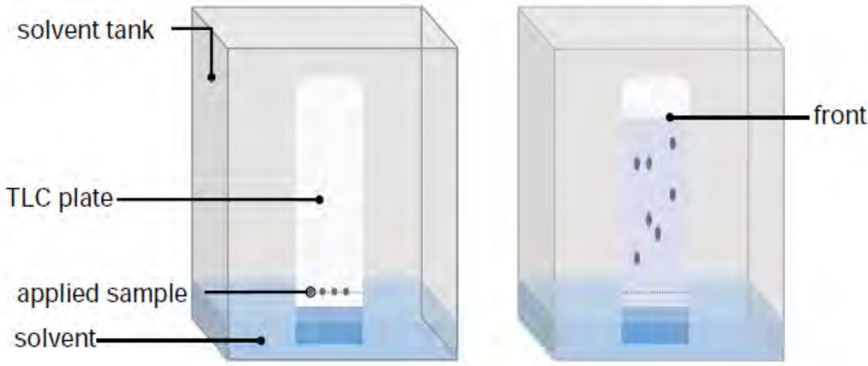

Figure 2.6: Vertical development TLC
Source: (Lundanes, Reubsaet, & Greibrokk, 2013).

2.13.2 High-Performance Liquid Chromatography (HPLC)

HPLC is an analytical technique that involves the separation, detection and quantification of sample constituents. As shown in Fig.2.7, the basic HPLC instrumentation consists of a pump(s), injector (manual or automatic injection), column(s), detector, and a data handling device (Snyder et al., 2011). It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Most conventional pumps operate at pressures up to 400 bar, while some ultrahigh-pressure systems (i.e. Ultrahigh-pressure LC - UPLC) may operate at as high as 1000–1200 bar.

The adsorbent material and each component in the sample interact slightly differently, resulting in various flow rates for the various components and the separation of the components (Carter, 2017). To enable quantitative analysis of the sample components, the components are eluted by connecting tubing to the detector, which produces a signal proportional to the amount of sample components emerging from the column. The most significant detector in HPLC has historically been a UV detector, but benchtop mass spectrometers, fluorescence detectors, light scattering detectors, electrochemical detectors, refractive index (RI) detectors, conductivity detectors, corona discharge

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detectors, as well as detectors for measurement of radioactivity, optical rotation, and Nuclear Magnetic Resonance (NMR), have all steadily gained in practical use (Hussain, 2011; Lundanes et al., 2013). From the detector, signals are sent to a PC, where data handling is done. All the steps can be fully managed by the PC with the right software.

The two most used HPLC methods are normal-phase chromatography and reversed-phase chromatography. A polar stationary phase, such as silica gel, and a non-polar solvent, such as hexane, are used in normal-phase chromatography. Reversed-phase chromatography, on the other hand, uses polar mobile phases like water, methanol, or acetonitrile along with non-polar stationary phases like C-8 or C-18 hydrocarbons.

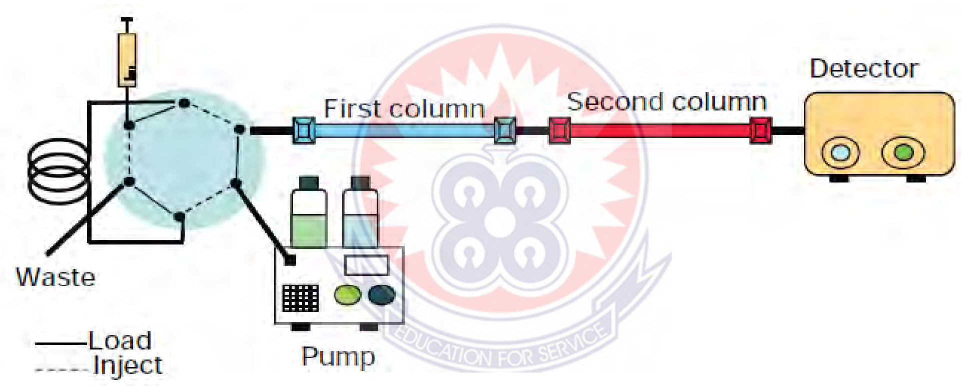

Figure 2.7: HPLC instrumentation (with two coupled columns)
Source: (Lundanes, Reubsaet, & Greibrokk, 2013).

2.13.3 Gas Chromatography (GC)

A popular method of chromatography in analytical chemistry for separating and studying chemicals that may be evaporated without decomposing is gas chromatography (GC) (Carter, 2017).

The mobile phase in gas chromatography (GC) is a gas, and the analytes must be sufficiently volatile to pass through the column (Carter, 2017). Furthermore, the

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analytes must maintain their stability at the temperatures they are exposed to in the injector and/or column (Lundanes et al., 2013).

In GC, separation occurs mainly according to two principles: adsorption and partition chromatography. When the analytes have differing adsorptivities to a solid stationary phase, separation is achieved in adsorption chromatography. The primary use of gas adsorption chromatography is the separation of permanent gases (Lundanes et al., 2013). The stationary phase in partition chromatography is a nonvolatile liquid, and separation is achieved when the analytes have a different distribution between the mobile and stationary phases (Lundanes et al., 2013).

In GC, the separation takes place in a column that is located in a heated compartment (column oven), providing temperature control of the separation (Fig. 2.8). A pressurized gas container releases the mobile phase, or the carrier gas, into the system (gas flask). A reduction valve is fitted to the gas flask to give a suitable gas flow to the column. The injection mechanism, which is temperature controlled, introduces the sample to be analyzed into the column. Temperature control is also used for the detector at the column outlet. The detector is coupled to a data system that handles data and controls the instrument.

It should be emphasized that only samples that are volatile below 300°C can be analyzed by GC (i.e. it is not applicable for very-high-boiling or nonvolatile materials). Consequently, approximately 75% of all known compounds cannot be separated by GC (Snyder et al., 2011). Since there are other less expensive chromatographic techniques, gas chromatography is less frequently used in commercial analyses of aflatoxins (Wacoo et al., 2014).

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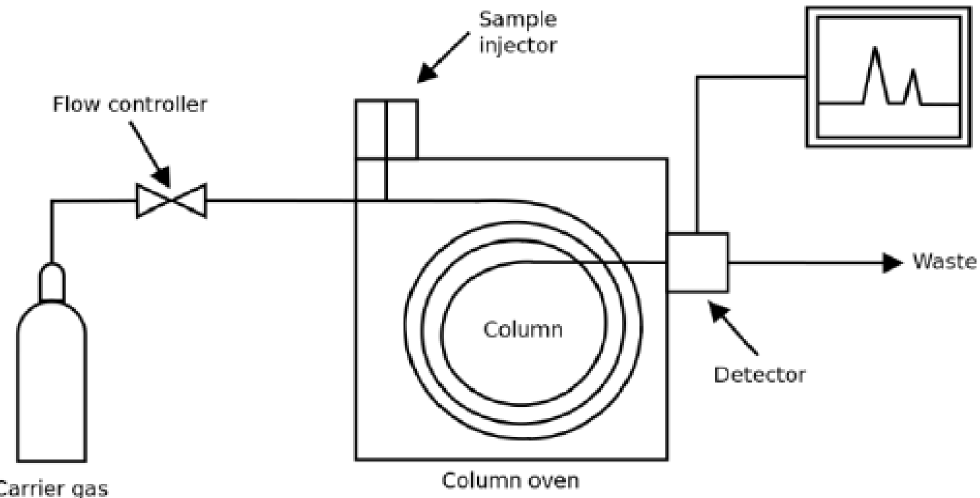

Figure 2. 8: Gas chromatograph

Source: (Carter, 2017)

2.14 Enzyme-Linked Immunosorbent Assay (ELISA)

In most cases, ELISA is used in conjunction with quick and easy extraction, preconcentration, and clean-up techniques, however, these steps are sometimes skipped. Matrix interferences are often sufficiently eliminated by extraction with aqueous methanol or acetonitrile, followed by dilution of the extract with buffers (Goryacheva & De Saeger, 2011). Immunoaffinity columns (IAC) are used for clean-up and analyte preconcentration in situations where matrices are highly coloured or exhibit a considerable influence on the test findings, increasing sensitivity. After the extraction, incubation follows. The commonly used detection system in ELISA is based on the use of colourimetric readers. In the case of on-site use of ELISA test kits, visual colour evaluation could be performed when compared with standard solutions. Fig.2.9 shows how ELISA is utilized to screen for mycotoxins.

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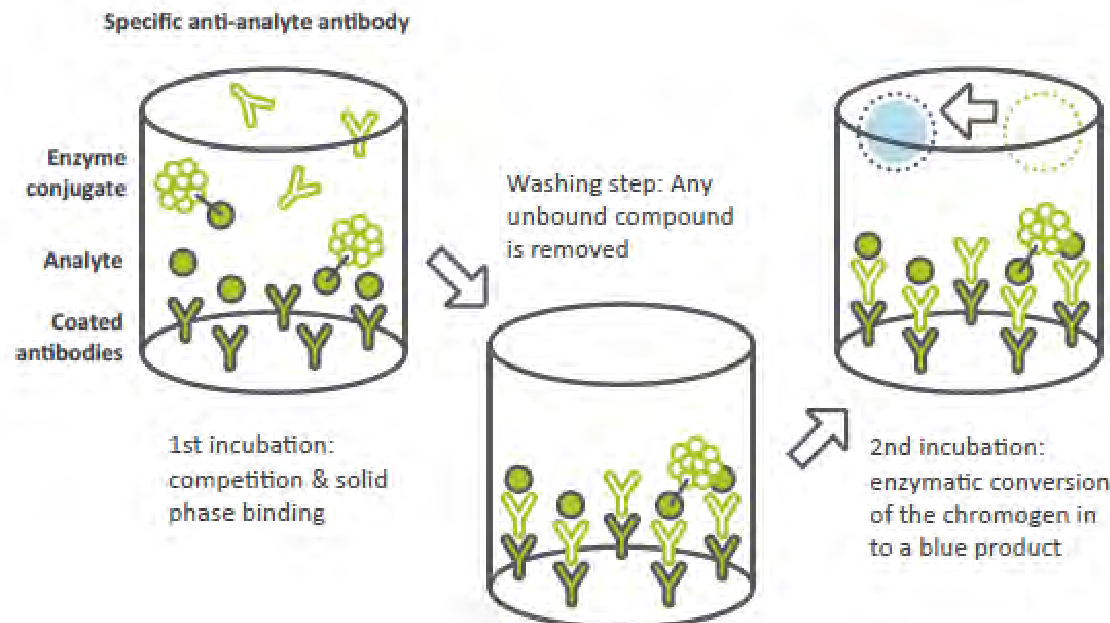

Figure 2.9: Principle of a competitive ELISA to screen mycotoxin
Source: (PACA, 2012)

2.15 Cultural methods for detection of Aflatoxins

Analytical techniques can be used to identify aflatoxins; however, while these techniques are sensitive, quantitative, and reliable, they can be quite expensive in many developing countries. As a result, there has long been a demand for aflatoxin detection methods that may not require expensive equipment investments or high per-unit costs for assay supplies. Consequently, aflatoxigenicity can be assessed utilizing cultural techniques (Abbas et al., 2004). The two primary criteria employed in culture techniques for identifying aflatoxins are either the apparent colour of the pigments that their colonies generate or the fluorescence of the aflatoxins that fungal isolates produce (Abbas et al., 2004; Sudini et al., 2015). The cultural methods include:

a. blue fluorescence
b. cyclodextrin-enhanced blue fluorescence
c. yellow pigmentation;
d. ammonium hydroxide vapour-induced colour change.

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2.16 Isolation of aflatoxigenic fungi and media for aflatoxin production

Almost every commonly used fungi-preparation medium will support the growth of *Aspergillus flavus* and *Aspergillus parasiticus*. Important considerations in the isolation of these fungi on agar media include their ability to grow at relatively high temperatures (37°C), sensitivity to particular antibiotics in comparison to other fungi, and tolerance to low moisture content in the growth medium (Abbas et al., 2004).

Media used for growing aflatoxigenic *Aspergillus spp*. include Aspergillus flavus and parasiticus agar (AFPA), Czapek's yeast extract agar (CYA), yeast extract sucrose agar medium (YES), coconut agar medium (CAM), coconut milk agar (CMA), coconut cream agar (CCA), aflatoxin producing ability medium (APA), Potato dextrose agar (PDA), Malt extract agar (MEA), Sabouraud Dextrose Agar (SDA), Dichloran Rose-Bengal Chloramphenicol (DRBC) (Sudini, et al., 2015; Rafik et al., 2020). The ability of these media to produce toxins varies depending on external factors such as pH, temperature, and duration, which must be noted (Sudini, et al., 2015).

Another reliable substrate for the fast detection of aflatoxigenic fungus is palm kernel media. The yellow colouring of the toxigenic isolates in palm kernel media indicates the presence of aflatoxins (Atanda et al., 2018).

2.17 Types of cultural methods

a) Blue Fluorescence

As shown in Fig. 2.10, the blue fluorescence of aflatoxins has been utilized to develop qualitative culture techniques for determining the generation of aflatoxins by *Aspergillus spp.* grown on suitable media (Sudini, et al., 2015). While some techniques use liquid media, like the aflatoxin-producing ability medium (APA) and a medium containing maize steep liquor, others use solid media, like potato dextrose agar and

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coconut agar (Abbas et al., 2004). Under long-wave UV light, *Aspergillus spp*., which produces aflatoxins, was detected (365nm) (Alkhersan et al., 2016). In the ultraviolet photographs, the isolates that produced aflatoxin showed up as grey or black colonies, while the isolates that did not produce aflatoxin showed up as white colonies (Zrari, 2013). UV light absorption is mainly caused by aflatoxins B1 and G1 (Abbas et al., 2004)

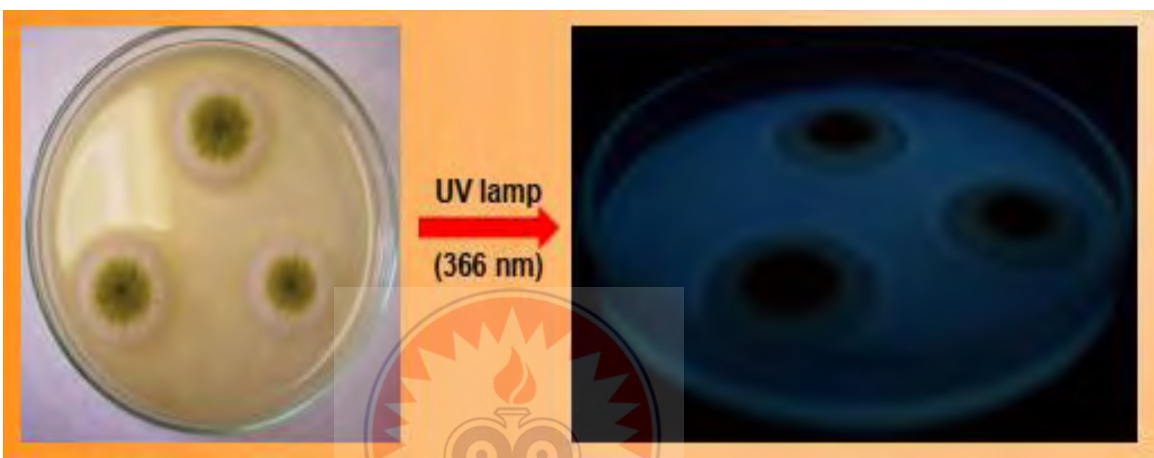

Figure 2. 10: A blue fluorescence surrounding aflatoxigenic colonies under UV-light

Source: (Jefremova, Ostry, Malir, & Ruprich, 2015)

b) Cyclodextrin-enhanced Blue Fluorescence

Known to enhance fluorescence in fungal culture media, cyclodextrins (CDs) are cyclic oligosaccharides made up of multiple glucose subunits arranged in an α (1–4) configuration (Fente et al., 2001; Maragos et al., 2008; Sudini et al., 2015). CDs are classified by the number of subunits (α= 6, β= 7, γ= 8) and by the type and degree of substitution (Maragos et al., 2008). Because of their enhanced fluorescence under UV exposure, beta-cyclodextrins (β-CDs) and their methylated derivatives can be utilized as effective aflatoxin signal enhancers (Szente, 2013; Nikolić et al., 2017). Fungi growth medium such as YES, Sabouraud dextrose and yeast extract (SD-YES) and

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PDA, fortified with 0.3% methyl-β-cyclodextrin (Mβ-cyd) gives an increased fluorescence under UV exposure (Mamo et al., 2017).

c) Yellow Pigment

Without the use of ultraviolet light, aflatoxin-positive isolates can be identified by the formation of yellow pigment in mycelia and medium *(*Lin & Dianese, 1976; Nurtjahja et al., 2019). Comparing *Aspergillus parsiticus* and *Aspergillus flavus* to other related species, these two species differ in that they both produce a bright yellow-orange colour at the base of their colonies (Chandrasekharan et al., 2014). Non-aflatoxigenic isolates do not produce yellow pigments (Nikolić et al., 2017). The level of yellow pigmentation was directly correlated with blue fluorescence for every media that was studied (Okereke & Godwin-Egein, 2018). Nevertheless, Abbas et al.(2004) found that the formation of yellow pigment was not a reliable measure of aflatoxin levels across all media.

d) Ammonium Hydroxide Vapour-Induced Color Change

This method, as reported by Abbas et al. (2004), entails culturing a single colony in the centre of a Petri dish with a medium such as potato dextrose agar. One or two drops of a concentrated ammonium hydroxide solution were applied to the inside of the dish's lid while it was upside down. After the bottom of the Petri dish was inverted over the lid containing the ammonium hydroxide, the undersides of colonies that produced aflatoxin soon became plum-red (Moradi et al., 2017). On the undersides of colonies that are not making aflatoxins, there is essentially no colour change.

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CHAPTER THREE

METHODOLOGY

3.0 Overview

This chapter presents the study area, materials and the analytical method, upon which the study was conducted. The microbial analysis and the analytical technique were carried out at the Department of Agriculture and Horticulture, University of Energy and Natural Resources (Dormaa Campus) and the Mycotoxin and Food Analysis laboratories of the Kwame Nkrumah University of Science and Technology (Kumasi) respectively.

3.1 Study Area

Ghana is located on the West Coast of Africa. It covers about 23,884,245 ha of land and water area between latitudes 4°N and 11°N and longitudes 4°W and 2°E. The country is demarcated into 16 regions and 216 districts with a population of 30,792,608 (Ghana Statistical Service, 2021). The sampling was done in the Greater Accra (Accra) and Ashanti Regions (Kumasi) since more than one-third of persons in Ghana live in the Greater Accra Region or Ashanti Region (Fig. 3.1) (Ghana Statistical Service, 2021).

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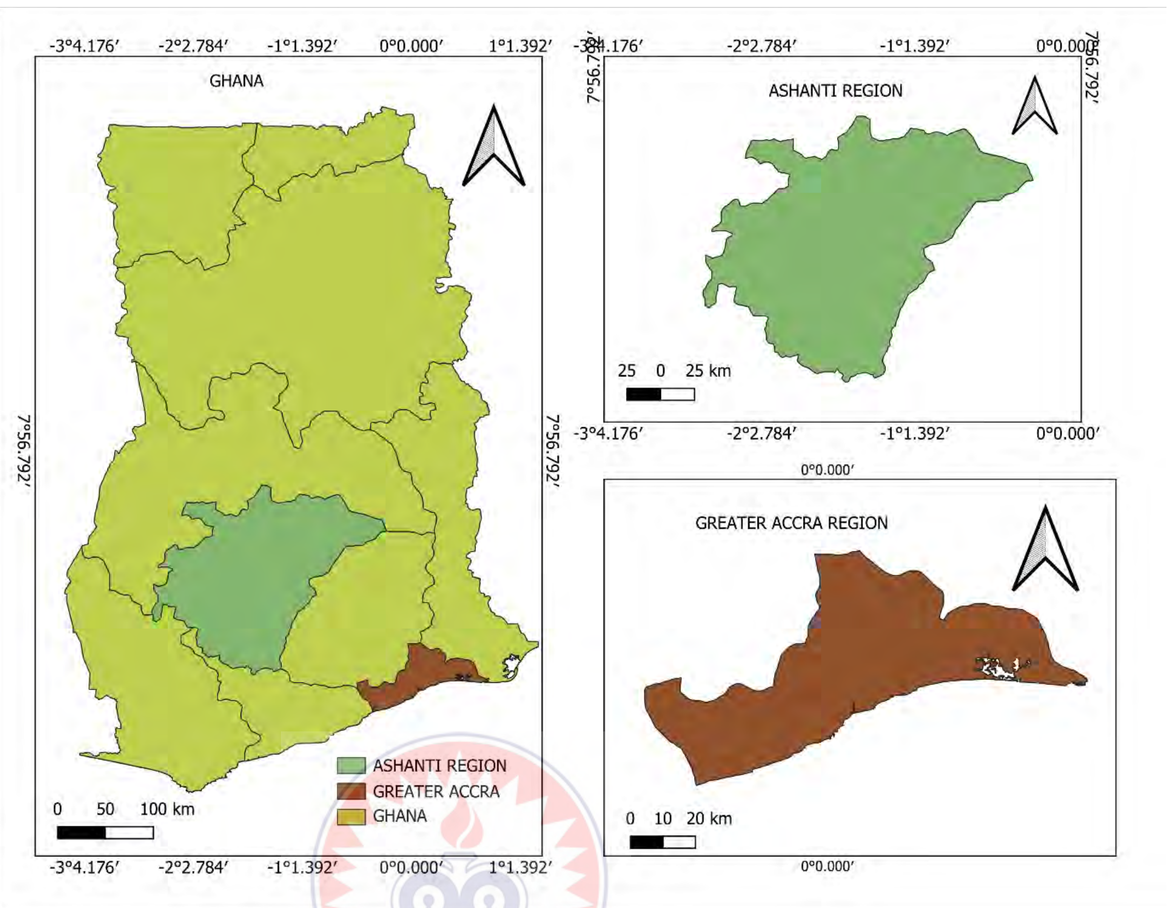

Figure 3.1: Map of Ghana showing the regions where sampling was done

3.2 Sampling

A total of 18 random samples made up of different brands of imported dry commercial dog food were purchased from different pet shops between the months of November and December (2022) in both Kumasi and Accra. A representative sample of 500 g each of the commercial dog food samples was fetched and sealed in a new clean plastic bag and sent to the laboratories where they were stored at -4 °C until ready for analysis. These samples were well-coded and all other relevant information was gathered (Table 3.1). Dry commercial dog food is the most widely sold dog food on the Ghanaian market. This may be because of its ease of storage, feeding convenience and protection against spoilage due to their low water content (Fuentes, et al., 2018).

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Table 3.1: Major constituents of the brands sampled.

Sample ID	Location	Main constituents
DF1	Accra	Unavailable
DF2	Accra	Unavailable
DF3	Accra	Unavailable
DF4	Accra	Unavailable
DF5	Accra	Unavailable
DF6	Accra	Cereals (rice and corn), corn gluten, dried beet pulp, dehydrated animal protein, chicken fat, wheat bran dehydrated lamb protein, vitamins, minerals, liver aroma.
DF7	Accra	Whole grain corn, whole grain wheat, soybean meal meat and bone meal, corn gluten meal, beef fat preserved with mixed-tocopherols, chicken by-product meal, egg and chicken flavour, Vitamins and minerals.
DF8	Accra	Maize, barley, rice, chicken fat, beet pulp, salmon oil, seaweed, dried peas, vitamins, minerals
DF9	Accra	Unavailable
DF10	Kumasi	Unavailable
DF11	Kumasi	Whole grain wheat, chicken, egg and chicken flavour, whole grain corn, meat and bone meal, corn gluten meal, beef fat preserved, pork and poultry digest, minerals, vitamins.
DF12	Kumasi	Whole grain corn, rice, dried poultry protein, beet fibre, dried meat, ground chicory root, poultry fat, vitamins and minerals.
DF13	Kumasi	Whole grain ground corn, wheat flour, chicken by-product meal, chicken fat, egg product, fish meal, salmon oil, vitamins, minerals
DF14	Kumasi	Ground whole grain corn, ground soft wheat, pork meal, chicken fat, rice bran, corn gluten meal, cereal food fines, flaxseed, minerals and vitamins
DF15	Kumasi	Rice, whole grain corn, dried meat, poultry fat, dried poultry protein, beet fibre, ground chicory root.
DF16	Kumasi	Cereals and cereal by-products, chicken and chicken by-products, meat and meat by-products, soybean meal, soybean oil, vitamins, minerals, preservatives, and flavours.
DF17	Kumasi	Chicken by-product meal, ground brewers rice, ground yellow corn, chicken fat, wheat flour, fish meal, dried whole eggs.
DF18	Kumasi	Ground whole grain corn, ground soft wheat, pork meal, chicken fat, rice bran, corn gluten meal, cereal food fines, flaxseed, minerals and vitamins

3.3 Materials for microbial culture

Potato dextrose agar (PDA) (Oxiod Ltd., Basingstoke, England), chloramphenicol, sterile Petri dishes, sterile wash bottles, glass rod, autoclave, incubator, measuring cylinder, inoculating loop, pipette tip, pipette filler, beakers, laboratory weighing

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balance, spatula, filter paper, test tubes, test tubes rack, water bath, distilled water, Bunsen burner, Azio – Lab5 microscope.

3.4 Materials for HPLC

Methanol (HPLC grade), acetic acid (HPLC grade), acetonitrile (HPLC grade), magnesium tetraoxosulphate(VI), sulphatesodium Chloride, filter paper (Whatman no.1), microfilter (0.45 nm), distilled water, chemical balance, Hehich Zemtrifugen Universal-320 centrifuge, Genie Vortex machine, Preethi Mixer Grinder, pipette, 50 mL centrifuge tubes, a 1260 Infinity II Agilent UPLC system, standards of aflatoxins (AFs), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2).

A chart of the experimental procedures to be undertaken has been presented in Fig. 3.2. It gives a brief outline of the various steps to be performed for both the microbial analysis and the Aflatoxin determination using HPLC.

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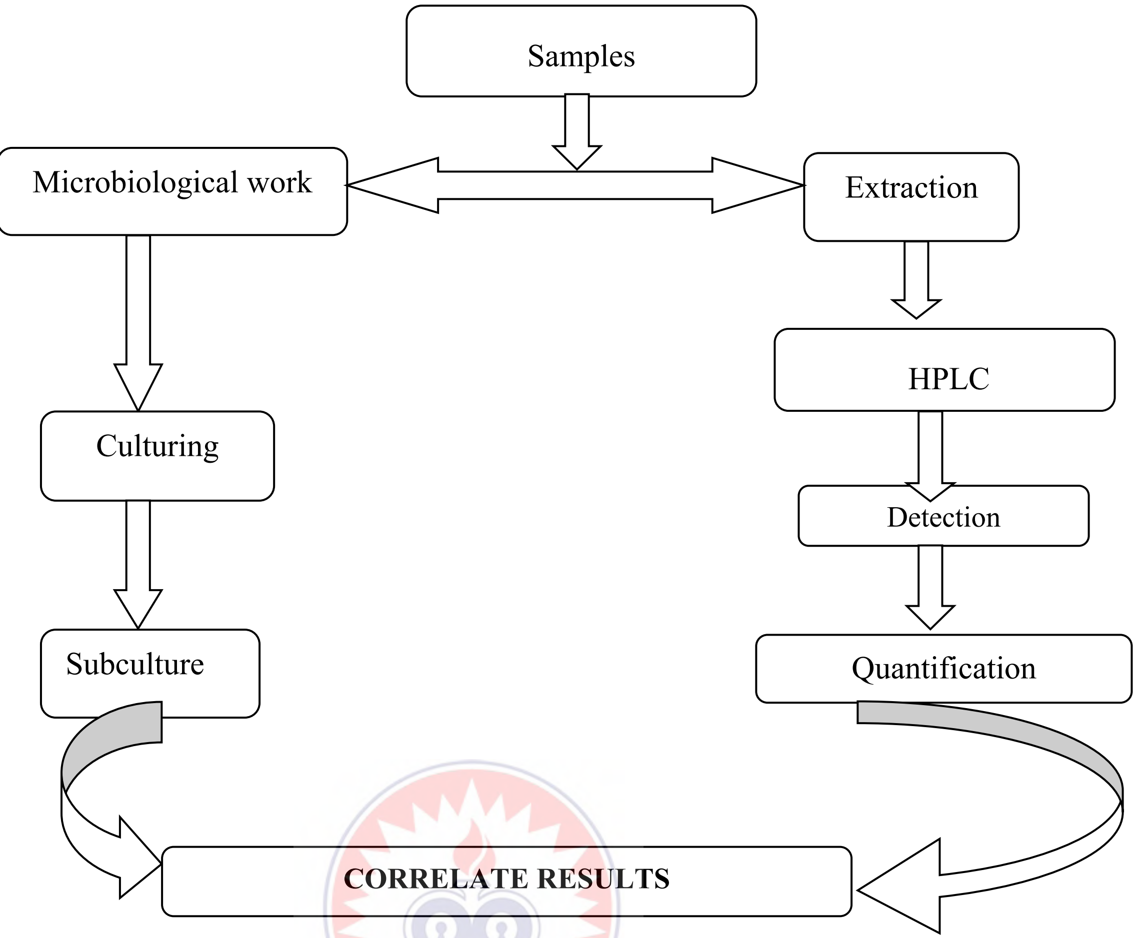

Figure 3. 2: Chart model of the experimental procedures

3.5 Media Preparation

All materials used for the media preparation were sterilized and the media was prepared according to the manufacturer's instructions. Using manufacturer instructions (i.e.: 39 g of PDA: 1 L of distilled water), 21.06 g of potato dextrose agar (PDA) was suspended in 540 mL of distilled water. The mixture was thoroughly mixed by heating until completely dissolved using the magnetic stirrer. The mixture was then sterilized in an autoclave for 15 minutes at 121 °C. and then cooled to 45 °C to 50 °C. 15 mL of the mixture was carefully dispensed into each of the 36 Petri dishes under a controlled condition without infections. The media was left to stand for 2 – 3 hours to solidify.

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Antibiotics (2 drops of Chloramphenicol) were spread on the surface of each media in the Petri dishes to prevent bacterial contamination.

3.6 Isolation of pathogens from samples and sub-culturing

Portions of each sample were isolated and plated directly on the media in two replicates of the Petri dishes in three equidistance. The inoculated Petri dishes were parafilmed and left to incubate for three (3) to seven (7) days in an incubator (28 ± 2 °C) for the observation of the mycelial growth. The grown hyphae were collected from the developed fungal isolates and sub-cultured (pure cultured) by hypha-tip sub-culturing with a sterilized inoculation needle and incubated at the temperature as stated above. Pure cultures of the putative pathogens were established and used for morphological identification (Barnett & Hunter, 1998; Pitt & Hocking, 1997; Samson et al., 1996; Silva et al., 2011).

3.7 Chemical extraction of Aflatoxin

Aflatoxin was extracted using methods as described by Sirhan et al. (2014) with modifications. Samples were milled and homogenized using a Preethi Mixer Grinder. A weight of 10 g of sample was weighed into a 50 mL centrifuge tube, 5 mL of distilled was added and the tube vortex for 1 minute. The solution was allowed to stand for 5 minutes. A volume of 10 mL 1% (v/v) acetic acid in acetonitrile solution was added. The resultant mixture was vortexed using the Genie Vortex machine for 3mins. A mass of 2 g of anhydrous $MgSO_4$ and 0.5 g of NaCl were added to the mixture and the vortexed for 1 min. The tube was centrifuged for 5 min at 4000 rpm and the supernatant was filtered through a 0.45µm nylon syringe before injection. A volume of 50 µL of the filtered extract was injected into the High-performance Liquid Chromatography (HPLC).

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3.8 High-Performance Liquid Chromatography (HPLC) Determination

HPLC Determination was done based on AOAC Official Method 2005.08 with LCTech UVE Photochemical Reactor for post-column derivatization and a 1260 Infinity II Agilent UPLC with fluorescence detector (Ex: 360nm, Em: 440 nm) (Association of Official Analytical Chemists [AOAC], 2006). The stationary phase was Poroshell 120 EC-C18 (2.7 um, 3.0 x 150 mm). The mobile phase used was water: methanol: acetonitrile (60:30:10, v/v) at a flow rate of 0.5 mL/min with column temperature maintained at 40 °C. Aflatoxin Mix (G_1, G_2, B_1, B_2) standards (ng/g) were prepared from Romer Labs® aflatoxin standard of 5.02 ng/µL in acetonitrile. Aflatoxins in samples were detected by using the retentions of the standard solution run and quantification were done using the calibration curves of each respective toxin. The limit of Detection (LOD) and Limit of Quantification (LOQ) of total aflatoxin were established at 0.5 ng/g and 1 ng/g respectively.

Aflatoxin Calculation:

$$Aflatoxin, ng/g = A \times \frac{T}{I} \times \frac{1}{W}$$

where,

A = ng of aflatoxin as eluate injected,

T = final test solution eluate volume (µL),

I = volume eluate injected into LC (µL),

W = mass (g) of commodity represented by the final extract.

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Figure 3. 3: A picture of the 1260 Infinity II Agilent UPLC with fluorescence detector system.

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CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.0 Overview

In the present chapter, the analyzed data from the laboratories are presented as results and are discussed according to the study's objectives. Results and discussions on the microbial analysis of the commercial dog food are presented in section 4.1 Frequencies and percentages are used in the presentation of this result. Also, the results on the calibration of the HPLC system are presented in the first section 4.3 while the results and discussions on the individual aflatoxin concentrations in the commercial dog food are presented in section 4.4.

4.1 Pathogen identification

In all, eighteen samples were cultured for pathogen growth. After the culturing, pathogen growth was observed in some of the samples, whilst a few other samples did not give any growth even after repeated culturing (Table 4.1). In the same vein, other samples recorded more than one pathogen growth. Individual pathogen growth on a single plate was sub-cultured for further identification.

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Table 4. 1: Pathogens recorded on samples

S/N	Sample Code	Name of Pathogen
1	DF1	*Penicillium spp.*
2	DF2	*Rhizopus spp., Aspergillus flavus*
3	DF3	No pathogen growth
4	DF4	*Aflatoxigenic Aspergillus flavus*
5	DF5	*Aflatoxigenic Aspergillus flavus*
6	DF6	*Aspergillus flavus, Rhizopus spp.*
7	DF7	No pathogen growth
8	DF8	*Aspergillus niger*
9	DF9	*Rhizopus* spp., *Aspergillus niger, Aspergillus flavus*
10	DF10	No pathogen growth
11	DF11	*Aflatoxigenic Aspergillus flavus*
12	DF12	*Aspergillus flavus, Aspergillus niger, Rhizopus* spp., *Penicillium* spp.
13	DF13	*Rhizopus spp.*
14	DF14	No pathogen growth
15	DF15	*Aspergillus niger, Aspergillus flavus*
16	DF16	No pathogen growth
17	DF17	*Aspergillus flavus*
18	DF18	*Aspergillus flavus, Aspergillus niger, Penicillium spp.*

The morphological characters of the putative pathogens were identified based on the fungal characters, using fungal identification keys as described by Barnett & Hunter, 1998; Pitt & Hocking, 1997; Samson et al., 1996; Silva et al., 2011. Further identification was made with the help of Azio – Lab5 microscope to know the shape and form of the conidia. Analysis of the different fungal species isolated from the dog food samples for morphological and cultural characteristics as shown in Table 4.2

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indicated that there was variation in the colony colour, margins, and texture and colony reverse colours (Ibrahim et. al, 2016 Al-Hindi et al., 2018).

Table 4. 2: Pathogen Description

Pathogen	Pathogen Description	Colony growth on PDA media	Microscopic Features
Aspergillus flavus	Powdery masses of bright yellow-green mycelia. The conidiophores of the identified pathogen were colourless, roughed, thick-walled and produced vesicles.		
Aspergillus niger	The pathogen growth at the beginning was white, which became black with the production of masses of conidia. The colony growth produced radial fissures on the media. Hyphae were hyaline and septate. The head of the conidial was initially radiated and biseriate, which split into columns when matured. The conidia produced were coloured brown, globose to subglobose in nature surrounded by rough walls.		

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Penicillium spp.	Mycelial growth showed shades of grey to blue-green masses with a condensed felt of conidiophores. The pathogen produced single-celled conidia in chains borne in basipetal sequence from a conidiogenous cell (phialide).		
Aflatoxigenic Aspergillus flavus	Powdery masses of light yellow-green mycelia. The nonseptate conidiophores of the identified pathogen were colourless, roughed, thick-walled and produced vesicles. The conidia were globose.		
Rhizopus spp.	The mycelium was cottony cream to white, which later became grey-brown. It had unbranched sporangiophores with black, rounded sporangia at the terminal end containing spores.		

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Nearly 72% of the tested dog food was contaminated with fungi suggesting that these contaminated foods may present a potential health risk for the dog's health, such as mycotoxicosis and immunosuppression. This was confirmed by the presence of *Aspergillus flavus, Aspergillus niger, Rhizopus spp., Penicillium spp. and Aflatoxigenic Aspergillus flavus* in some of the dog foods as indicated in Table 4.1.

Of the 18 samples, seven (7) were contaminated by the mycotoxigenic fungus, *Aspergillus flavus*, and another three (3) by Aflatoxigenic *Aspergillus flavus*. Other mycotoxigenic fungi that were co-occurring with *A. flavus* were *A. niger* in five (5) samples. The predominance of *A. flavus* could be due to physiological characteristics, which enable it to survive adverse conditions. It is also a rapidly growing, temperature-tolerant fungus that can withstand low moisture levels (Mngadi et al., 2008).

Aspergillus flavus is known to produce aflatoxin B1 and B2. Also, isolates of *Aspergillus niger* can produce other mycotoxins such as ochratoxin A, fumonisin B2, and sterigmatocystin which exhibits acute toxic, carcinogenic, mutagenic, teratogenic, immunotoxic or oestrogenic effects in animals and humans (Al-Hindi et al., 2018).

The constant contact of improperly packaged dog food with air and during re-bagging as well may enable fungi such as *Penicillium spp.*, *Aspergillus spp.*, *Rhizopus spp.* and Fusarium spp. present in the environment to be transferred to the feed. These are airborne agents that can easily colonize the food, especially when humidity and temperature are favourable (Girio et al., 2012).

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4.2 Frequency and Percentage Occurrence of Pathogens

Five (5) different fungal species belonging to four (4) genera were observed including *Aspergillus flavus, Aspergillus niger, Rhizopus* spp., *Penicillium* spp. and *Aflatoxigenic Aspergillus flavus*. The occurrence of isolated fungus was defined as the percentage of samples in which each fungus was present (Table 4.3).

In this work, *Aspergillus* spp. was the most frequent mould of the mycoflora, occurring in 65% of samples, followed by *Rhizopus* spp., which occurred in 22% and *Penicillium*, which occurred in 12%. Only three species, *Aspergillus flavus, Aspergillus niger* and *Aflatoxigenic Aspergillus flavus*, were identified among the *Aspergillus* spp. The predominant was *Aspergillus flavus* followed by *Aspergillus niger* and *Aflatoxigenic Aspergillus flavus* (Fig. 4.1).

Table 4. 2: Frequency and percentage occurrence of pathogens

S/N	Pathogen	No. of Occurrence	Percentage Occurrence (%)
1	*Aspergillus flavus*	7	30
2	*Aspergillus niger*	5	22
3	*Rhizopus spp.*	5	22
4	*Aflatoxigenic Aspergillus flavus*	3	13
5	*Penicillium spp.*	3	13

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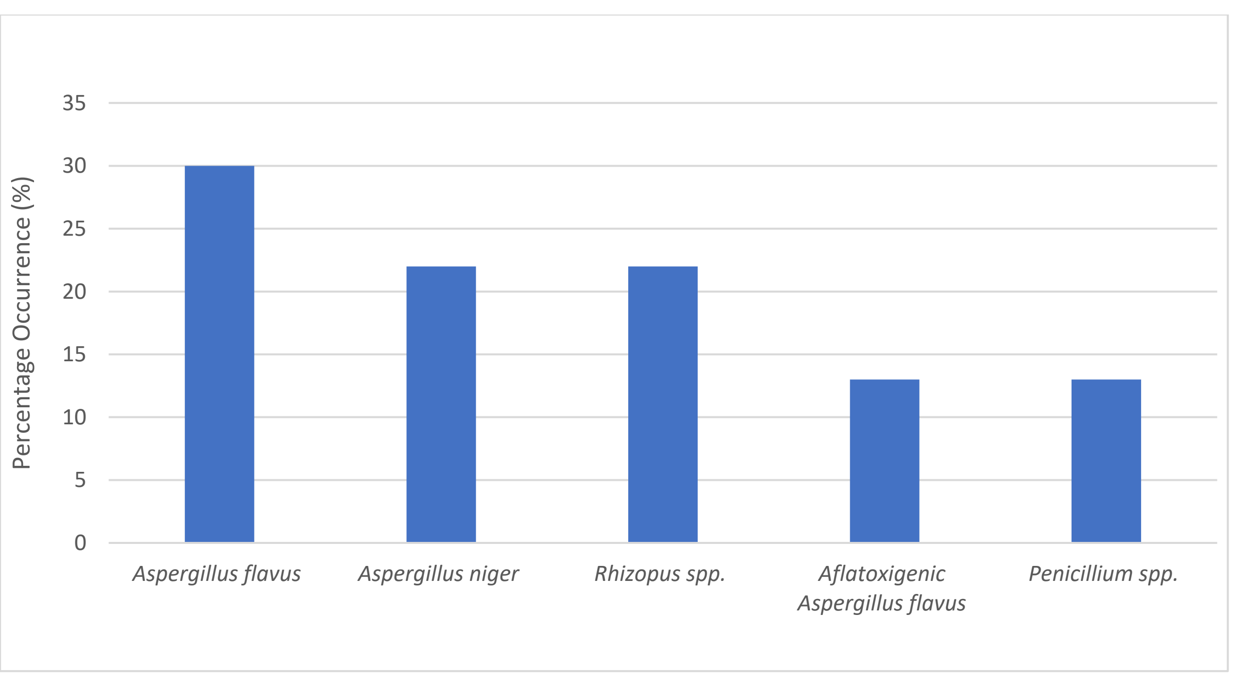

Figure 4. 1: Percentage Occurrence of Pathogens

Research studies on the microbiological quality of commercial dog food have been carried out by several scientists (Table 4.4). Bueno et al. (2001) studied the mycoflora in 12 commercial dog foods and found a predominance of genera *Aspergillus spp.* (67%) in the dog food. Also, among the *Aspergillus spp*., only two species, *A. flavus* and *A. niger* were identified.

Our results presented in Table 4.4 agree with Holda et al. (2017) who identified *Aspergillus*, *Penicillium* and *Rhizopus* species within the observed colonies when a comprehensive microbiological evaluation of dry foods for growing dogs marketed in Poland was performed. Singh & Chuturgoon (2017) detected *Aspergillus spp.*, *Fusarium spp.* and *Penicillium spp.* in 20 bags of pelleted dog food with the fungal isolates of *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus parasiticus* amongst the most prevalent. In a study by Martins et al. (2003), the most frequent mould occurring in dry pet food was *Aspergillus spp.* (58.3%) followed by *Penicillium spp*., which occurred in 38.3 % of the samples. Among the *Aspergillus,* the percentage of

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Aspergillus niger was high (55.0 %) in dog food compared to 22% in this study. In our study, fusarium genera were not identified in any sample in contrast to studies by Witaszak et al. (2019), Tegzes et al. (2019) and Singh & Chuturgoon (2017).

Table 4. 4: Fungal mycoflora detected in the commercial dog food compared to other published works

Location	Number of samples	Major fungi identified	Reference
Ghana	18	*Aspergillus flavus, A. niger, Rhizopus* spp., *Penicillium* spp. and *Aflatoxigenic Aspergillus flavus*.	Present work
Argentina	12	*Aspergillus flavus, A. niger, Penicillium* spp., *Fusarium spp., Mucor globosus, M. plumbeus, M. racemosus, Rhizopus* spp.	(Bueno et al., 2001)
Brazil	180	*Aspergillus flavus, A. parasiticus, Penicillium* spp., *Fusarium spp.*	(Campos et al., 2009)
Poland	25	*Fusarium proliferatum, Fusarium verticillioides, Aspergillus spp., Penicillium* spp.	(Witaszak et al., 2019)
Poland	25	*Aspergillus* spp., *Mucor* spp. And *Penicillium* spp.	(Blajet-Kosicka et al., 2014)
South Africa	20	*Aspergillus flavus, A. fumigatus, A. parasiticus, Penicillium* spp., *Fusarium graminearium, F. verticilliodes*	(Singh & Chuturgoon, 2017)
Poland	20	*Aspergillus spp., Penicillium spp.* and *Rhizopus spp.*	(Holda et al., 2017)
Poland	36	*Aspergillus spp. and Rhizopus spp.*	(Kazimierska et al., 2021)
Portugal	20	*Aspergillus spp., Penicillium spp. and Mucor racemosous*	(Martins et al., 2003)
Venezuela	4	*Aspergillus flavus, Penicillium spp., Fusarium spp., Clasdosporium herbarum*	(Munoz et al., 2015)

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4.3 Linearity of the system (calibration curves)

All analytical methods require calibration for quantitation to get reliable and accurate results. Calibration is a process that relates the measured analytical signal to the concentration of analyte (Beyene et al., 2019). The chromatograms of the Aflatoxins standards used for calibration using the optimized parameters as described in section 3.8 are shown in Figure 4.2. The HPLC system used in this study was calibrated before the analysis of our samples and showed very strong linearity with a correlation coefficient value of $R^2 > 0.999$ (Figures 4.3,4.4,4.5,4.6). The limit of quantification (LOQ) for AFB1, AFB2, AFG1 and AFG2 were 0.10 ng/g, 0.20 ng/g, 0.10 ng/g and 0.20 ng/g respectively (Table 4.5).

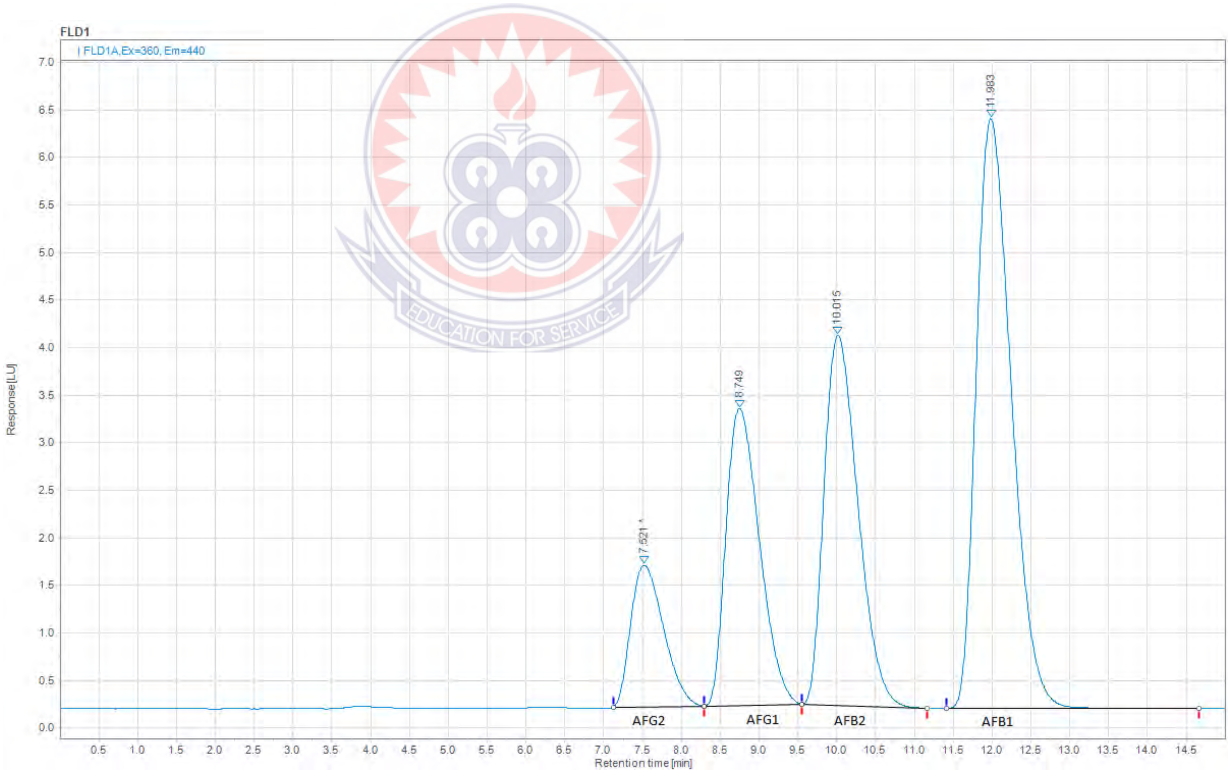

Figure 4. 2: HPLC chromatogram of the Aflatoxin standards

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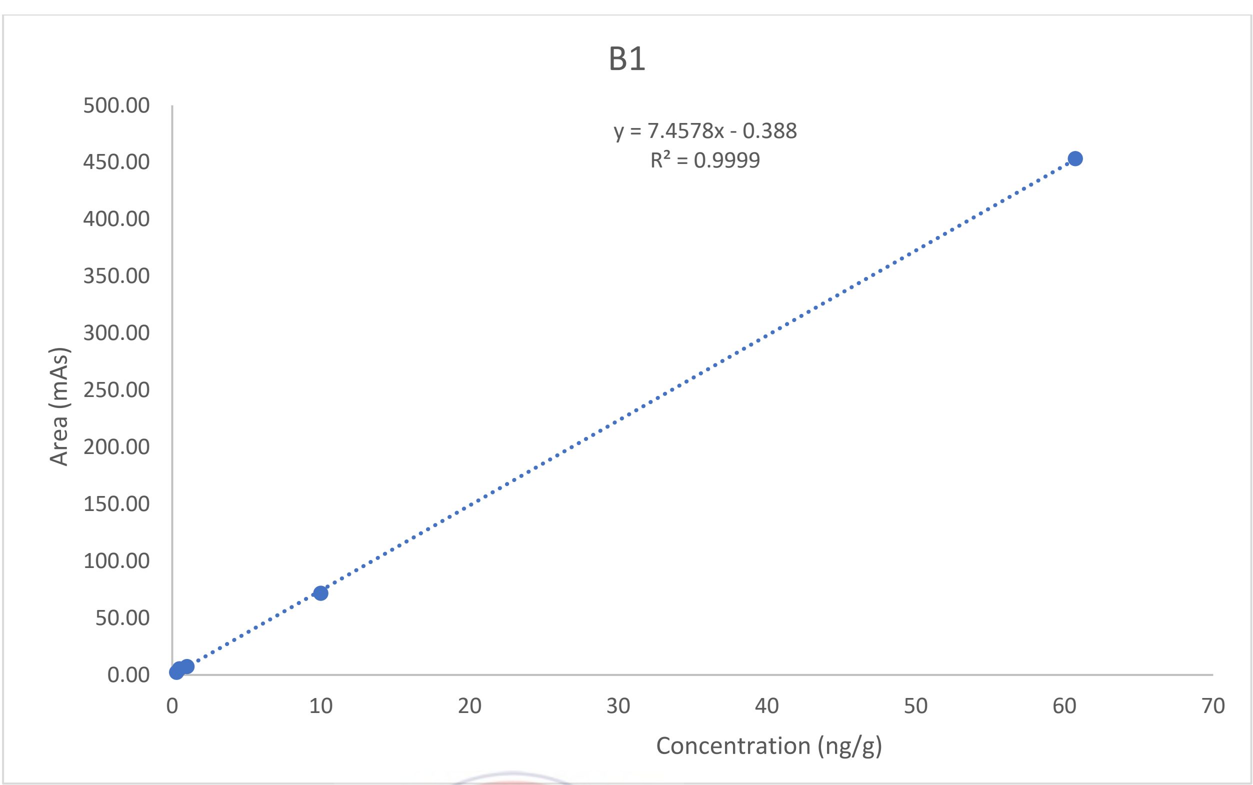

Figure 4. 3: Calibration curve for Aflatoxin B1

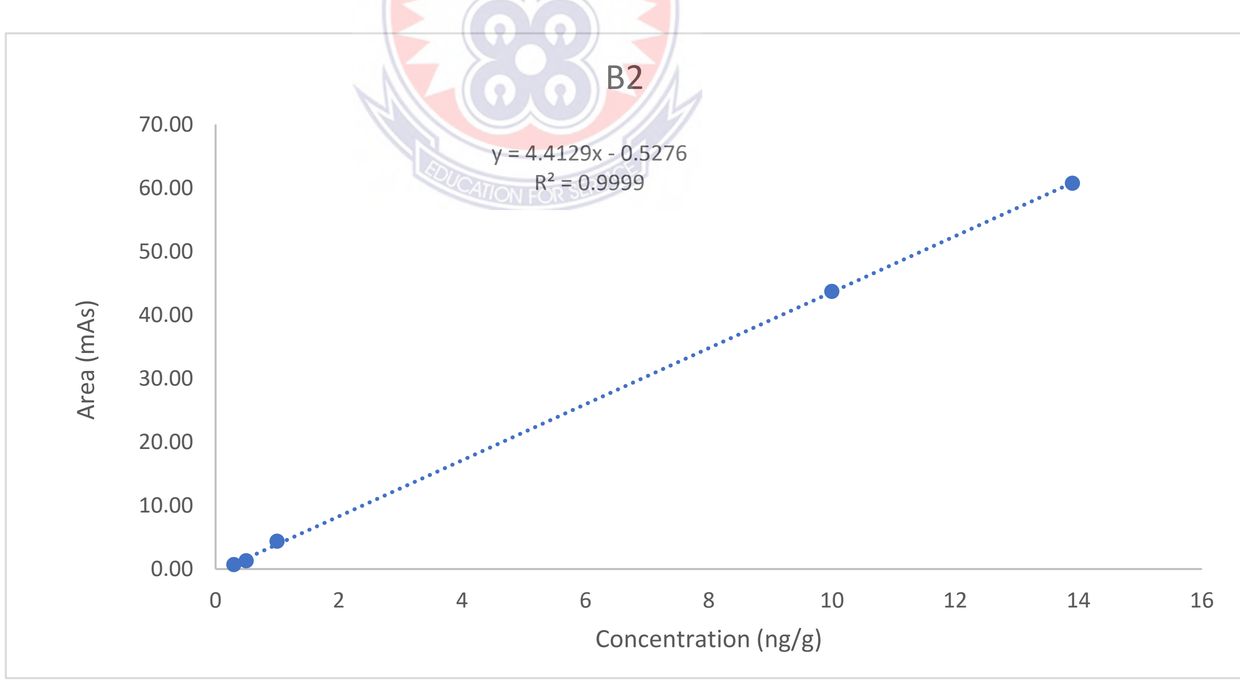

Figure 4. 4: Calibration curve for Aflatoxin B2

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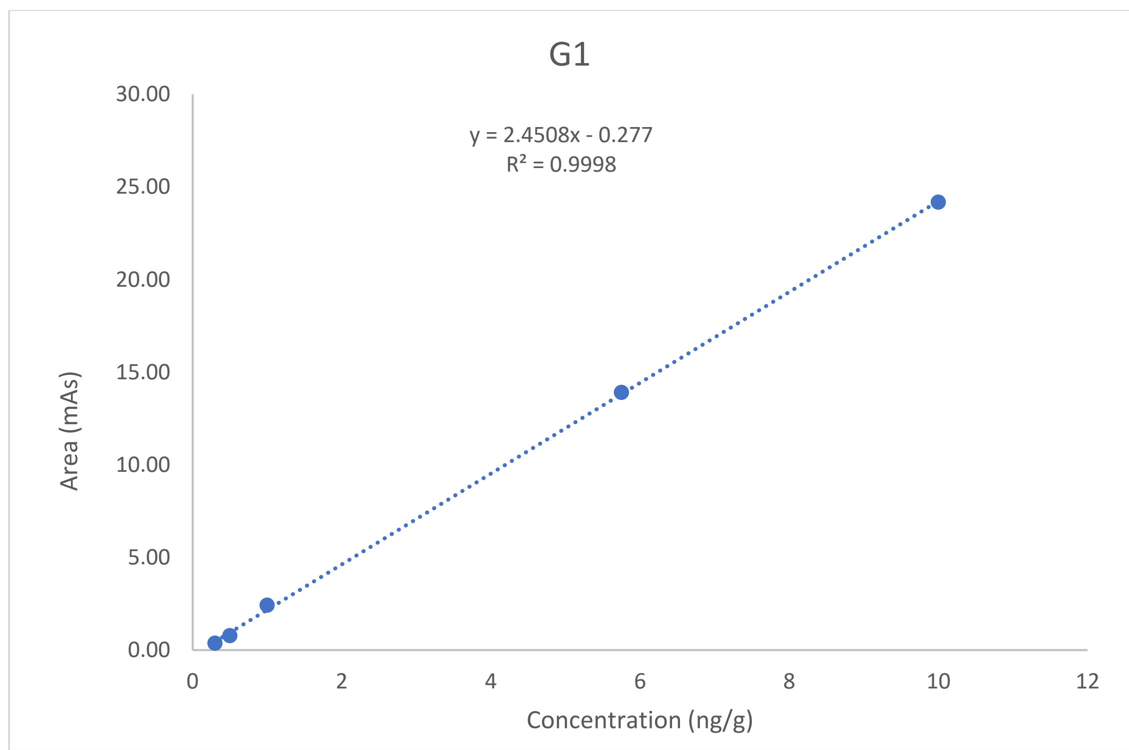

Figure 4. 5: Calibration curve for Aflatoxin G1

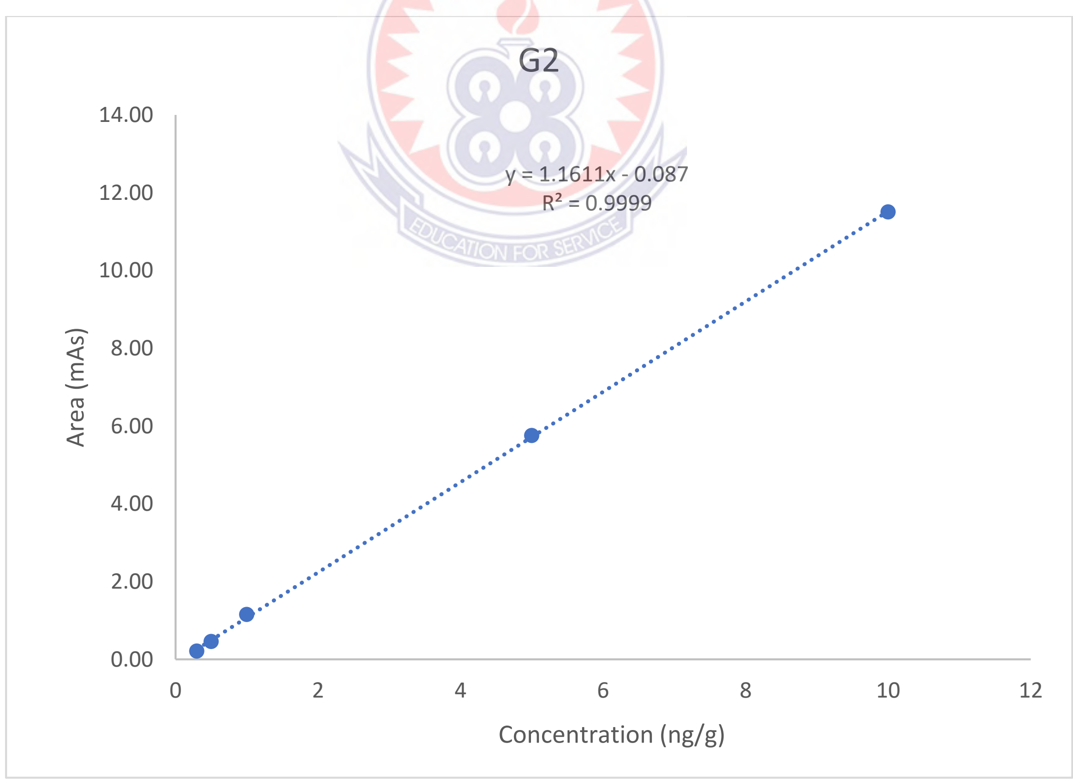

Figure 4. 6: Calibration curve for Aflatoxin G2

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Table 4.3: Limits of Quantification (LOQ) of aflatoxins AFB1, AFB2, AFG1, AFG2

Aflatoxin	Amount (ng/g)
B1	0.20
B2	0.10
G1	0.20
G2	0.10

4.4 Aflatoxin contamination levels in the commercial dog foods

The estimated aflatoxin concentrations of AFB1, AFB2, AFG1 and AFG2 in the commercial dog foods are shown in Table 4.6. In this study, 66.7% of the samples of the dry commercial dog food had non-detected quantitative levels of aflatoxins. This fact may indicate the influence of good manufacturing practices in commercial dog food production on mycotoxin levels (Campos et al., 2009).

The aflatoxin concentration of AFB1 in the dry commercial dog foods ranged from <0.10 - 21.65 ng/g with an average value of 8.58±1.68 ng/g. The highest concentration of AFB1 occurred in DF10 while DF1, DF2, DF4, DF6, DF8, DF9, DF12, DF14, DF16, DF17 and DF18 had their concentration of AFB1 below the limit of quantification (LOQ). DF11 which had an AFB1 concentration of 19.86±0.45 ng/g was the second highest. For the AFB2´s concentration, it varied from <0.20 - 64.60 ng/g with an average value of 53.24±0.43 ng/g. The AFB2 concentration was below the limit of quantification in 88.9% of the samples (DF1, DF2, DF3, DF4, DF5, DF6, DF7, DF8, DF9, DF12, DF13, DF14, DF15, DF16, DF17 and DF18 while DF11 had the highest AFB2 concentration of 64.60±0.38 ng/g. DF10 had the second-highest AFB2 concentration of 41.89±0.21 ng/g. The concentration of AFG2 was below the limit of quantification in all the samples. AFG1 was detected in only DF5 with a concentration

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of 53.30±0.49 ng/g while the rest of the samples recorded AFG1 concentrations below the limit of quantification. Table 4.6 reveals great variations in the concentration of AFB1.

A. flavus was the predominant fungus isolated, and this fungus is known to produce aflatoxin B1 and B2. In this study, the HPLC analysis revealed that aflatoxin B1 was the most abundant in all the dry commercial dog food; present in seven (7) samples, B2 in two (2) samples and G1 in one (1) sample. G2 was not detected in any of the samples. Also, despite the presence of *Aspergillus spp.* in seven (7) commercial dog food samples (DF2, DF4, DF6, DF9, DF12, DF17 and DF18), no toxins were detected in them using HPLC. Therefore, the correlation between the isolated fungi and their respective toxins was very low.

A dog food might contain aflatoxins even though it shows no fungal activity (DF3, DF7 and DF10). In sample DF10, the concentrations of Aflatoxin B1 and B2 were 21.65±1.54 ng/g and 41.89±0.21 ng/g respectively. Ingredients previously contaminated with fungi might have aflatoxins but growth and survival of fungi may have been halted by processing, treatments or other means that kill fungi and fungal spores but do not destroy the aflatoxins already present. These reasons may have accounted for the non-detection of *Aspergillus spp*. in most of the samples during the microbial analysis but their detection during the HPLC analysis.

Also, since all the samples were pelleted commercial dog foods, it meant low moisture content and the presence of preservatives in the samples may have suppressed the production of these toxins. In addition, aflatoxins are known to be susceptible/degraded by heat, physical and chemical methods, therefore, if present initially all or some may have been eliminated during processing (Mngadi et al., 2008).

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The presence of Aflatoxins in some commercial dog foods indicates that improper storage conditions may have occurred before pelleting as heat treatment during pelleting would have destroyed the fungi but may not affect Aflatoxin concentrations already present. Also, samples collected from the various pet shops might have been re-contaminated with the fungi which produce aflatoxins. Dog foods are very expensive in Ghana. As such they might be re-bagged in smaller packs to make it affordable (Figure 3.2). These may then get contaminated if bagging conditions are not sterile such as unclean hands, unclean environment and unclean bag.

Table 4. 4: Results of the Aflatoxin contamination levels detected by HPLC in the commercial dog food

Sample Code	Aflatoxin average (ng/g) in commercial dog food				
	G2	**G1**	**B1**	**B2**	**AFt**
DF1	<0.20	<0.10	<0.10	<0.20	<1.00
DF2	<0.20	<0.10	<0.10	<0.20	<1.00
DF3	<0.20	<0.10	0.93±0.02	<0.20	0.93
DF4	<0.20	<0.10	<0.10	<0.20	<1.00
DF5	<0.20	53.30±0.49	1.18±0.04	<0.20	54.48
DF6	<0.20	<0.10	<0.10	<0.20	<1.00
DF7	<0.20	<0.10	3.54±0.44	<0.20	3.54
DF8	<0.20	<0.10	<0.10	<0.20	<1.00
DF9	<0.20	<0.10	<0.10	<0.20	<1.00
DF10	<0.20	<0.10	21.65±1.54	41.89±0.21	63.54
DF11	<0.20	<0.10	19.86±0.45	64.60±0.38	84.46
DF12	<0.20	<0.10	<0.10	<0.20	<1.00
DF13	<0.20	<0.10	0.82±0.16	<0.20	<1.00
DF14	<0.20	<0.10	<0.10	<0.20	<1.00
DF15	<0.20	<0.10	12.11±0.19	<0.20	12.11
DF16	<0.20	<0.10	<0.10	<0.20	<1.00
DF17	<0.20	<0.10	<0.10	<0.20	<1.00
DF18	<0.20	<0.10	<0.10	<0.20	<1.00
Average	<0.20	53.30±0.49	8.58±1.68	53.24±0.43	36.51
Range	<0.20	<0.10 - 53.30	<0.10 - 21.65	<0.20 - 64.60	

Definitions: DF- Dog food, AFt- Aflatoxin total

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Table 4.7 shows the total aflatoxin (AFt) of the commercial dry dog food in this present work to be about 12 times higher than the mean total aflatoxin in Thailand. Again, the average aflatoxin concentration of AFB2 in the commercial dry dog food in this study is higher than other published works except in the USA (Newman et al., 2007). The average aflatoxin concentration of AFG1 was also found to be higher than reported aflatoxin G1 concentrations from Mexico (28.2 ng/g), Italy (<0.50 ng/g), Pakistan (<0.10 ng/g), USA (<1.0 ng/g) and Thailand (0.05ng/g). Our results are in agreement with Basalan et al. (2004), Castaldo et al. (2019), Tahira et al. (2015) and Tansakul et al. (2014), who found their mean AFB1 concentration to be less than 10ng/g in dry commercial dog foods. In contrast, Newman et al. (2007) and Singh & Chuturgoon (2017) found dry commercial dog food with average AFB1 levels ranging from 18.6 ng/g to 44.1 ng/g.

The mean aflatoxin concentration of AFG2 in the commercial dry dog food in this study was below its limit of quantification (LOQ) as were with those reported by other authors (Tahira et. al 2015; Tegzes et. al 2019; Tansakul et. al 2014), but it was found to be lower than means obtained in Mexico and Italy.

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Table 4.5: Comparison of mean aflatoxin concentrations of Aflatoxins B1, B2, G1 and G2 in commercial dry dog food in the study area and published data

Location	Mean AF (ng/g)	Reference
Ghana	AFB1(8.58±1.68) AFB2(53.24±0.43) AFG1(53.30±0.49) AFG2(<0.20) AFt(36.51)	Present work
USA	AFB1(18.6) AFB2(529.6)	(Newman et al. 2007)
Nigeria	AFt(9.61)	(Akinrinmade & Akinrinde 2012)
Mexico	AFB1(1.6) AFB2(0.1) AFG1(28.2) AFG2(1.3)	(Fuentes et al., 2018)
Italy	AFB1(<0.50) AFB2(5.7) AFG1(<0.50) AFG2(15.8)	(Gazzotti et al., 2015)
Pakistan	AFB1(4.83) AFB2(<0.50) AFG1(<0.10) AFG2(<0.50)	(Tahira et al., 2015)
Turkey	AFB1(6.69)	(Basalan et al., 2004)
USA	AFB1(<1.0) AFB2(<1.0) AFG1(<1.0) AFG2(<1.0)	(Tegzes et al., 2019)
Turkey	(PC) AFt(1.30) (EC) AFt (1.98)	(Kara, 2022)
Thailand	AFB1(2.49) AFB2(0.70) AFG1(0.05) AFG2(<0.80) AFt (3.17)	(Tansakul, et al., 2014)
Italy	AFB1(4.3)	(Castaldo et al., 2019)
South Africa	(SF)AFB1 (44.1) (PF) AFB1 (20.1)	(Singh & Chuturgoon, 2017)

Definitions: AF: Aflatoxin; AFt: Total aflatoxins; AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFG2: Aflatoxin G2, PC: Premium Class, EC: Economy Class, SF: standard feed; PF: premium feed. NB: The aflatoxin concentration values represented in the Table above are for the localities from which the samples were taken but are not representative of their respective countries.

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Pet animals receive less attention than farm animals when it comes to global regulations governing the aflatoxin content of animal feed. AFB1 is the most common toxin found in common foods like cereals, which are important components in the production of pet food. It is also thought to be the most potent mutagenic and carcinogenic of the four naturally occurring types of aflatoxins. (Akinrinmade & Akinrinde, 2012; Tahira et al., 2015; Aristil et al., 2020). As a result, the European Union (EU) (Commission Directive 2003/100/EC) has set a maximum acceptable level of aflatoxin B1 in all products intended for animal feed at 20 ng/g (20 ppb) (European Commission, 2003). The aflatoxin B1 content was below the European limit in 94.4% of the samples suggesting that these samples are safe for consumption by dogs. Notwithstanding that, continuous consumption of such contaminated dog feed over a long period may lead to serious health effects since dogs are among the most susceptible animals to aflatoxins (Wouters et al., 2013). One sample (DF10) recorded an aflatoxin B1 level above the EU's permissible limit. Also, in this present study, the total aflatoxins (B1, B2, G1 and G2) in 16.7% (3/18) of the samples exceeded the action level (20 ng/g) set by the FDA (USA), in animal food (US FDA, 2019).

Due to relevant health concerns related to aflatoxin to pets such as dogs, aflatoxin-contaminated dog foods have repeatedly been rejected by law enforcement authorities such as the FDA(Ghana), which results in high economic losses both for commercial dog food producers and traders (Food and Drugs Authority, 2021).

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CHAPTER FIVE

SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATION

5.0 Overview

The purpose of this study was to assess commercial dog foods on the Ghanaian market for aflatoxin contamination. The summary, conclusions, and recommendations of the study are presented in this chapter. This chapter also discusses the limitations of the study and proposes additional research to be conducted in the future.

5.1 Summary of Findings and Conclusion

The toxigenic fungi occurrence, with particular attention to *Aspergillus spp.* in 18 samples of commercial dog food in two major cities (Kumasi and Accra) was estimated using cultural methods. In addition, the concentrations of Aflatoxins B1, B2, G1 and G2 were measured using high-performance liquid chromatography (HPLC).

The present study has provided information about fungi in commercial dog food, where *Aspergillus* spp. was the most frequent mould of the mycoflora, occurring in 65% of samples, followed by *Rhizopus* spp., which occurred in 22% and *Penicillium*, which occurred in 12%. *Aspergillus flavus*, an aflatoxin-producing fungi was isolated and identified among the genera and species from the commercial dog food analysed implying risk for the dog's health.

The concentrations of Aflatoxins B1, B2, G1 and G2 in the commercial dog food averaged 8.58±1.68 ng/g, 53.24±0.43 ng/g, 53.30±0.49 ng/g and <0.20 ng/g respectively. Aflatoxin B1 was the dominating aflatoxin occurring in approximately 39% of the samples with an average within the acceptable limits, therefore commercial

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dog foods may be safe to consume. Notwithstanding that, Aflatoxin B1 is considered a potential mutagenic and carcinogenic among the four naturally occurring forms of aflatoxins (Martins et al., 2003). This implies that the presence of AFB1 in these samples raises the possibility of a health risk to dogs who might consume it.

The present study also showed the total aflatoxins (B1, B2, G1 and G2) in 16.7% of the samples exceeded the stipulated threshold of 20 ng/g in animal food set by the FDA(USA). These findings suggest that commercial dog food may have a significant role in identifying the cause of aflatoxicosis in dogs that consume it. This can serve as the justification for the Ghanaian market's rejection of commercial dog food of this kind.

Similar risks of aflatoxins are present in the pet food sector for foods made with agricultural raw ingredients. Aflatoxins in the raw ingredients have the potential to persist and end up in the finished pet foods. To prevent aflatoxins from forming during storage as well as during repackaging for sale, it is essential to practice good hygiene standards.

The findings support the issue of pet food safety in a developing country like Ghana about commercial dog food and the need for adopting practical and efficient good practices in a nation with limited facilities.

In conclusion, it is crucial to routinely monitor commercial dog foods, especially those that have been repackaged for sale, for fungal growth and aflatoxin contamination because the presence of aflatoxins in commercial dog food constitutes a major health risk to dogs.

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5.2 Recommendations

The following are some recommendations to relevant stakeholders based on the results of my study.

5.2.1 Scientific Community

This study could be extended to include other pet foods and types. There is also a need for specific research to determine the maximum permissible levels of aflatoxins that should be permitted specifically for commercial dog food. The study might be expanded to take into account food surpluses given to dogs and to calculate the effects of long-term exposure to low levels of aflatoxins in pets. Additionally, alternative detection methods that make use of Species-specific PCR methodologies should be used to detect and identify the presence of aflatoxigenic fungi. It is also recommended that further research be done to determine the presence of other mycotoxigenic fungi, such as ochratoxin A, deoxynivalenol, zearalenone, and fumonisins in commercial dog food.

5.2.2 Dog food sellers

Despite the challenges of quality control, avoiding contamination is more effective than dealing with the myriad of negative effects that toxic fungi have on dog's health. In pet stores and storage facilities, it is important to correctly identify potential points of contamination for commercial dog food and to take precautions to prevent fungal growth by handling and storing pet food properly.

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5.2.3 Regulatory bodies

This study's findings can inform the development of targeted interventions by Ghanaian regulatory bodies, such as the Standards Authority and the Food and Drug Authority. These interventions could include raising awareness among commercial pet food retailers and establishing effective regulations and safeguards to guarantee the safety of pet food throughout Ghana. To reduce exposure, morbidity, and dog mortality, the Foods and Drugs Authority and the Veterinary Association of Ghana should handle incidents involving suspected aflatoxin contamination of commercial dog foods promptly and appropriately.

Governmental agencies, private enterprises, nongovernmental organizations, and national media networks including radio and television shows, as well as articles in newspapers and magazines, can raise awareness about aflatoxins and the risks they pose to pets.

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APPENDIX A:

PICTURES OF SOME PET SHOPS SHELVES

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APPENDIX B:

PREPARATION OF SAMPLES FOR MICROBIAL ANALYSIS

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APPENDIX C:

A PICTURE OF CULTURED SAMPLES

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APPENDIX D:

SOME PICTURES OF CULTURES AFTER 7DAYS