KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF SCIENCE

THE EFFECT OF AFRAMOMUM DANIELLI ON OCHRATOXIN A (OTA) PRODUCTION; AN EMERGING HAZARD IN DRY COCOA BEANS

A THESIS SUBMITTED TO THE DEPARTMENT OF FOOD SCIENCE AND TECHNOLOGY, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE IN FOOD QUALITY MANAGEMENT

BY

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DECLARATION

I hereby declare that this submission is my own work towards the MSc and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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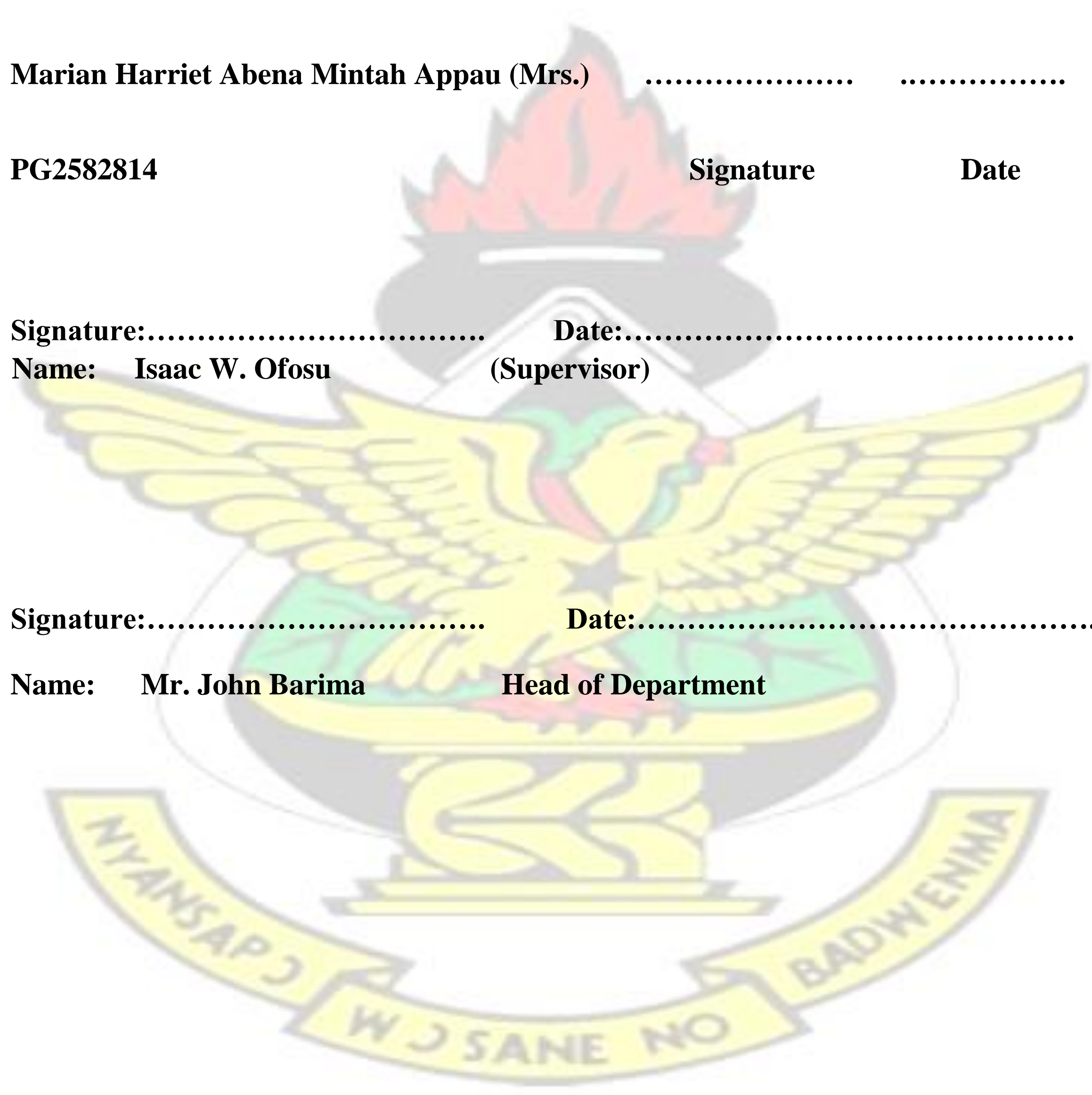

ABSTRACT

Forty-eight cocoa beans samples from bruised cocoa pods were analyzed for Ochratoxin A (OTA). Samples of 5000 g cocoa beans each containing initial OTA concentration of 5.66 ng/g were treated with varying quantities of *Aframomum danielli* spice powder ranging from 5 g to 250 g (w/w). The samples treated were analyzed for OTA concentrations using HPLC with a fluorescent detector. The study was done using response surface design. The effect of the spice powder was evaluated on two (2) quality attribute of cocoa i.e. Free Fatty Acid and Acidity (pH). Results from the study established a clear trend whereby OTA concentrations in treated cocoa samples decreased from 5.66 ng/g to 0.61 ng/g i.e. 89.22% reduction at 65.79 g of *A. danielli* powder. There was a decline in the development of OTA in the treated samples (i.e. samples treated with *A. danielli* powder) as compared to the control samples. Validating the results of the *A. danielli* at 65.79 g within 95% Confident Interval of 0.45 to 0.81 yielded a residual OTA from 5.66 to 0.63 ng/g, Free Fatty Acid from 3.09 to 1.48% and pH from 6.71 to 7.30. In conclusion, A. *danielli* spice powder exhibited a bio-preservative effect on OTA i.e., inhibit and reduce its growth in OTA contaminated cocoa beans at maximum level of 65.79 g per 5000 g of cocoa beans above which the quality of the beans will be affected.

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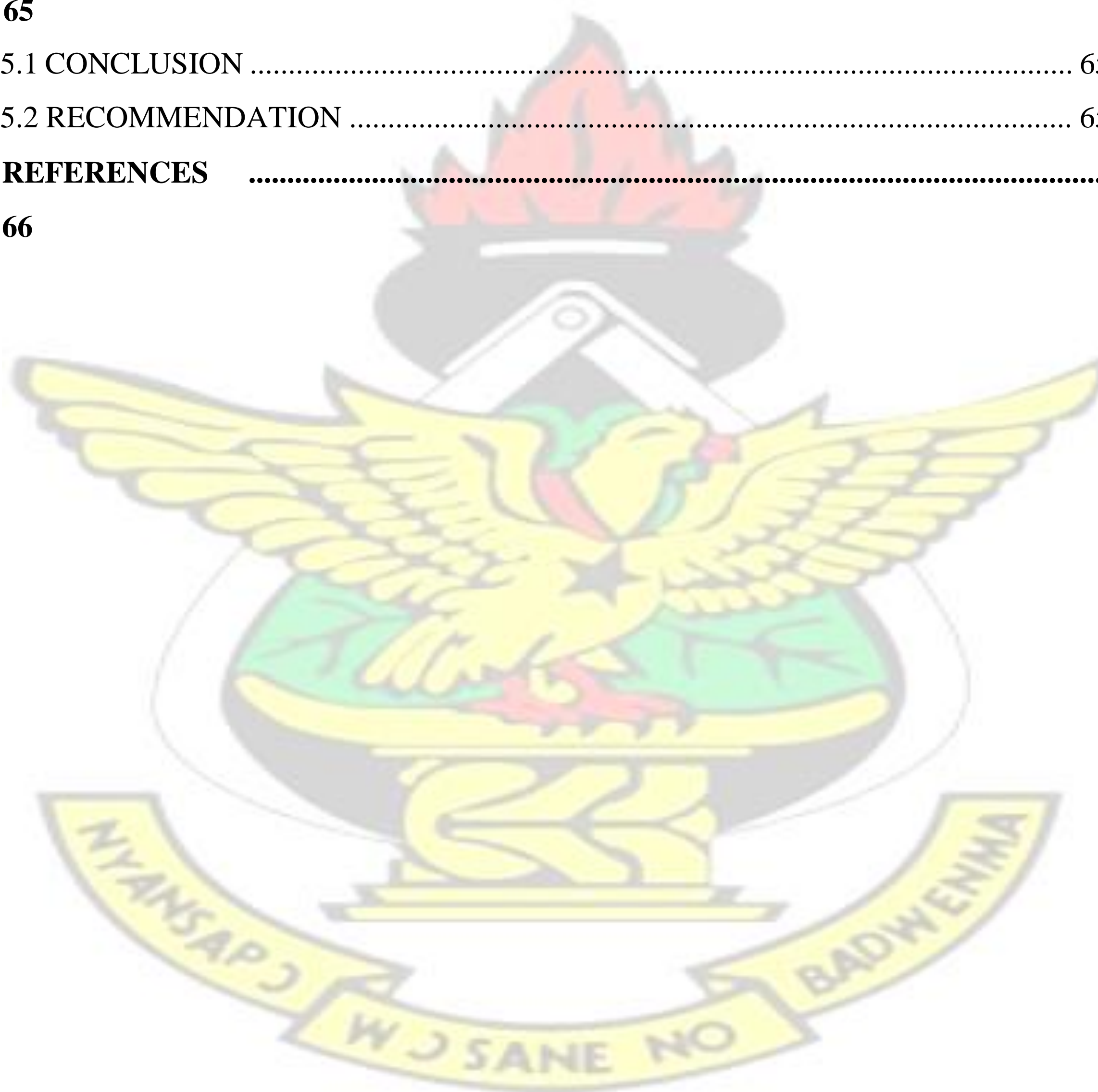

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DEDICATION

This dissertation is dedicated to Samuel Kwadjo Berko Appau and Eugenia Yaa Gyapomaa Appau for their love and support. I love you dearly.

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ABBREVIATIONS

OTA	Ochratoxin A
AFs	Aflatoxins
WHO	World Health Organization
JECFA	Joint Expect Committee for Food Additives and Contaminants
EFSA	European Food Safety Authority
CCFAC	Codex Committee on Food Additives and Contaminants
CCCF	Codex Committee on Contaminants in Food
CAC	Codex Alimentarius Commission
EU	European Union
USFDA	United States Food and Drugs Authority
NDPC	National Development Planning Commission
SCOOP	Scientific Cooperation

PKS	Polyketide Synthase
IUPAC	*International Union of Pure and Applied Chemistry*
UV	Ultraviolet
EC	European commission
HACCP	Hazard Analysis Critical Control Point
MC	Moisture Content
AW	Water Activity
IARC	International Agency for Research on Cancer
HPLC	High Performance Liquid Chromatography
PLD	Fluorescence Detector
SPE	Solid Phase Extraction
CA	Codex Alimentarius
FAO	Food and Agricultural Organization
ICCO	International Cocoa Organization
GAP	Good Agricultural Practices
LOD	Limit of Determination
LOQ	Limit of Quantification
DADP	Defatted *Aframomum danielli* powder

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CHAPTER ONE

INTRODUCTION

In today's changing world, safety and security have generally remained basic human needs. Ochratoxin A (OTA) has been a safety issue that has previously not been considered in years past. However, with the international or European market quest on ensuring adherence to safety standards and specifications in order to protect the safety of consumers, Ochratoxin A in food has come to the fore.

The presence of Ochratoxin A (OTA) in food has become critical due to the chronic effects even at low levels of exposure. In humans, records of severe dietary exposure to OTA have been associated with chronic, progressive, Balkan endemic nephropathy which is a kidney disease (Chukwuka, 1997; Badru, 2005; Ogunledun, 2007). OTA has been reported in several food stuffs including cocoa and its derivatives. The mycotoxin has been discovered in minute levels in some food products such as eggs meat, milk and also in dairy product being the carryover of contaminated feed stuffs fed to animals (Reddy *et al.,* 2010; Majica *et al.,* 2010). The European Union being aware of the gradual increased exposure of consumers to OTA in recent years has proposed standards defining the acceptable contamination levels of consumption (European Commission, 1995).

Products like coffee, cereals, and wine have previously been covered by international regulations whereas cocoa, a major foreign exchange earner, has however not been covered by these regulations. It was estimated that the World's cocoa production was 3592000 tons (ICCO, 2006) and OTA contamination in cocoa and its derivatives has been reported in different countries in the world (Tafuri *et al*., 2004; Burdaspal and Legarda 2003;

Amezqueta *et al*., 2005). The International Agency for Research on Cancer (IARC, 2007) has reported that contamination of foods is of a major health concern for both humans and animals. They can enter into the human food chain through foods of plant origin and indirectly goes through foods from animal products hence; IARC has categorized OTA as a probable carcinogen to humans (group 2B).

Consumer introduction to OTA is reported to be increasing progressively and in order to guard consumers the European Union has drawn up a standard to define acceptable contamination limits (European Commission, 1995). Since these mycotoxins can never be completely removed from the food supply chain, many countries have defined levels in food (tolerances, guideline levels, maximum residue levels) that are unlikely to be of health concern (Stoloff *et al*., 1991). Cocoa growing countries, in view of this hazard, has set for themselves some limits to monitor the prevalence. For instance, in 2003, the health ministry of Italy, the call for the defensive principle has set a legal limit at 2.0μg/kg and 0.5μg/kg for cocoa powder and chocolate products, respectively. Similarly, a risk assessment analysis carried out by Brera *et al*. (2011), showed no health concerns and to support the EU regulation, the Superior Council of Health of Italy decided to get rid of the Italian permissible limit for OTA in cocoa and chocolate based products. Brazil has similarly, established 5μg/kg as maximum level for OTA in cocoa products which includes chocolate (ANVISA Resolução nº7/2011). Whereas, the Canadian Ministry of Health is currently proposing maximum limits for OTA in variety of foodstuff, as a result of an assessment conducted on health risk (Health Canada, 2010). On the contrary, the United States Food

and Drugs Administration (FDA) have not yet set advisory limits or action levels for OTA in any commodity.

Currently, there are no limits set for OTA in cocoa or chocolate products in Ghana which directly relates to consumer safety and is of current interest in many countries around the world. Lack of data pertaining to OTA intake through cocoa products makes it difficult to determine the health risks to consumers, thereby crippling governmental and international agencies interventions. Since Ghana is one of the leading exporters of cocoa worldwide, it is therefore necessary to monitor the levels of OTA in cocoa beans to determine whether the cocoa beans produced in Ghana conforms to international standards and subsequently adopt interventionary actions to reduce the prevalence.

1.1 PROBLEM STATEMENT AND JUSTIFICATION

Research has shown that OTA has been found in human blood, tissue and breast milk in several countries. It is considered to be nephrotoxic and is suspected of being the main etiological agent responsible for human Balkan Endemic Nephropathy (BEN) and associated urinary tract tumors. The mycotoxin has been reported in cocoa and its derivatives. However, very little has been done by way of intervention such as, chemical agents and radiations amongst other preventionary approaches have been employed. The use of natural spices (*Aframomum danielli*) has been known to stall ochratoxigenic growth (Adegoke *et al.*, 1998). Cocoa products are widely enjoyed by consumers all over the world therefore if OTA contamination of cocoa beans is not prevented or minimized, the safety of consumers will be jeopardized. Moreover, cocoa growing countries will lose heavily to

their competitors in the European Union and other markets around the globe who are gearing up to put in more stringent measures to protect the lives of their consumers.

Ochratoxin A (OTA) has been a safety issue that has previously not been considered but with the international or European market quest for ensuring that safety standards and specification are adhered to in order to protect the safety of consumers, the mycotoxin has come to the fore. OTA has been reported in many food commodities including cocoa and its products (Reddy *et al.,* 2010; Majica *et al.,* 2010). The European Union knowing the exposure of consumers to OTA in recent years, which is reported to be gradually increasing, has drawn standards defining the acceptable contamination levels of consumption (European Commission, 1995).

Products, like coffee, cereals and wine have previously been covered by the international regulations; cocoa being a major foreign exchange earner has however, not been covered by these regulations. It is estimated that the World's cocoa production is at 3,592,000 tons (ICCO, 2006). Meanwhile, OTA contamination in cocoa and its products has been reported in different parts of world (Tafuri *et al.,* 2004; Burdaspal and Legarda 2003; Amezqueta *et al.,* 2005).

1.3 MAIN OBJECTIVE

The main objective was to study the effect of defatted *Aframomum danielli* powder (DADP) in the control of Ochratoxin A in cocoa beans and to study the effect of the powder on the Free Fatty Acids and the pH of cocoa bean.

1.4 SPECIFIC OBJECTIVES

The specific objective was to determine the quantitative effectiveness of defatted *Aframomum danielli* powder (DADP) on Ochratoxin A in cocoa beans and the effect on free fatty acids and pH.

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CHAPTER TWO

LITERATURE REVIEW

2.1 COCOA PRODUCTION IN GHANA

Cocoa (*Theobroma cacao* L.), commonly originated from the Central and Southern parts of America, is a vital agricultural export commodity in the world forming the backbone of economies and generating high income to some West African countries, especially Cote d'Ivoire and Ghana. Production of cocoa is done in the country's forested areas; Ashanti, Central, Brong-Ahafo, Eastern, Western and the Volta Regions of the country with rainfall in the ranging between 1,000-1,500 millimeters per year (Wood and Lass, 1985; Fowler *et al.,* 1998).

In Ghana, the crop is grown mostly wholly by small-stake farmers with an average farm size of about 4.0 ha with a production yield of 246.4 kg/ha. The main type cultivated by farmers throughout the country is the Forastero variety with an average size cultivated being Amazonica (34.4%), the Amelonado (13.3%) and the hybrid (52.3%) (Afoakwa, 2010). The cocoa bean is composed of the shells, cocoa powder, the dry powder made of grinding dried cocoa seeds and removing the cocoa butter from the beans and cocoa butter. These are mostly processed into chocolate, confectionery and other cosmetic products (Ntiamoah and Afrane, 2008). Cocoa products using a wide range of intermediate products and by products such as cocoa pulp juice, cocoa liquor, wine cocoa butter, cocoa cake and raw cocoa powder (Afoakwa *et al.,* 2007; Pandey *et al.,* 2011; Jayathilakan *et al*., 2011). Ntiamoah and Afrane (2008) also, researched on the utilization of the pod husks and shells in the preparation of animal feed and fertilizer in Ghana. The production of high quality dried beans is a unique culture in Ghana, as engraved in the traditional farming practices of the peasant farmers, together with rigorous research and quality control programs

embarked upon consistently by successive Governments till now. This has guaranteed Ghana's cocoa its premium status on the international market. The processing of cocoa beans into various cocoa and chocolate products begins with an on-farm fermentation of the beans followed by drying and roasting during industrial processing. These postharvest processes are very crucial to the quality of finished products as they initiate the formation of chocolate flavor precursors and the desired brown color of cocoa products (Schwan *et al*., 1995; Adegoke *et al.,* 2003).

The fermentation process breaks down the mucilaginous pulp surrounding the beans and causes the death of the cotyledon (Sanchez *et al.,* 1985; Gotsch 1997; Afoakwa *et al.,*2008). This initiates biochemical transformation inside the beans, leading to reduction in bitterness and astringency, development of flavor precursors such as, free amino acids, peptides and sugars (Thompson *et al.,*2007; Kratzer *et al.,*2009). The fermentation of cocoa is influenced by many factors such as the type of cocoa, climatic and seasonal differences and disease, (Afoakwa, 2008), turning, batch size (Lehrian and Patterson 1983, quality of beans (Wood and Lass, 1985) and also pulp pre-conditioning.

Pulp pre-conditioning involves changing the properties of the pulp prior to the development of microorganisms in fermentation. The pulp is the substrate metabolized during fermentation by a sequence of bacteria and fungi and since the properties of the substrate determine microbial development and metabolism, changes in the pulp may affect the production of acids by lactic acid bacteria, yeast and acetic acid bacteria. Three basic processes of pulp pre-conditioning have been assessed for the treatment of fresh cocoa beans preceding fermentation- pod storage, mechanical or enzymatic depulping and bean spreading (Rohan, 1963; Wood and Lass, 1985; Schwan and Wheals, 2004).

Traditionally, Ghanaian farmers have accidentally adopted this technique of pod storage by their practice of using family labor to collect the harvested pods piles between 3-5 days before organizing friends and neighbors as labor to help break open the pods prior to fermentation. This method of pod storage appears to have highly useful effect on the chemical composition and subsequent development of chocolate flavor, although the precise chemical and biochemical effects, conditions and processes still remain unknown. With increasing specialty niche products in chocolate confectionery, understanding the factors contributing to variations in the chemical composition and physical qualities of cocoa beans during pod storage and subsequent fermentation processes would have significant commercial implications.

2.2 HARVESTING OF COCOA

Cocoa fruits are harvested when ripe and harvesting is done weekly during the peak periods and once in two weeks in lean periods. Good farm sanitation should be ensured by removing diseased cocoa fruits weekly with a machete, “bolo” or cocoa hook that is used only for that purpose. Diseased or mummified pods must be separated from healthy pods and discarded before transporting from the farm. Harvesting must be carefully done to avoid cuts on the cocoa pods which could serve as a critical point for OTA production. Unripe pods should not be harvested because they have solid pulp with no mucilage and this makes it difficult for the beans to separate from the pod during primary processing.

Moreover, the beans do not ferment properly and become slaty.

2.3 PROCESSING OF COCOA

The cocoa pod is sterile when not affected by disease or attacked by any animal and free from microorganisms specially fungi and hence mycotoxin. However, when the pod is infected with diseases, attacked by animals or bruised on the farm prior to and during harvest, the beans are then exposed to bacterial and fungal contamination (Amezqueta *et al*., 2005). Harvested cocoa pods must be opened within seven days after harvesting in order to prevent the proliferation of fungi. The pods are cut opened with the aid of a sharpened knife-edge. Cocoa beans with its pulp are removed and massed together in baskets, boxes, trays or platforms to permit microbial growth to begin the fermentation process. Precaution should be taken to avoid cocoa beans from getting in contact with water during fermentation. The period of fermentation is commonly 4 to 7 days, which also depend on the method of fermentation. It is however recommended that fermentation beyond 7 days be avoided as this could lead to fungal proliferation and seed germination. Fermentation is recommended to avoid ochratoxigenic fungal growth and Ochratoxin A production because acetic, lactic and citric acid produced by bacteria during fermentation can compete with and inhibit these undesirable fungal species. These processes aredone in attempt of reducing the acidity in dried fermented cocoa beans. Over acidity in processed cocoa beans has been associated with the production of high levels of lactic and acetic acid during fermentation.

After fermentation, the beans are mostly sun dried in an open drying area, on suspended tables and technological inventions such as mechanical dryers. For effective sun drying and mechanical drying could be combined to ensure the beans are appropriately dried to the set moisture levels. A moisture content of less than 8% in cocoa beans is considered

optimal in order to avoid growth of microorganisms and for good storage. The drying area should be located away from contaminant sources and should receive maximum sum exposure and air circulation during most times of the day, to speed up the drying process of cocoa beans. Shady areas should be avoided. However, they must be sorted to remove flat, black, shriveled, germinated, insect damaged and small and fused beans and other defects. A major part of OTA initial presence in cocoa beans is found in the shell of the bean. Therefore, industrial procedure for the removal of cocoa shells as well as dried episperm or integument of the cocoa seed before and after the roasting can help in reducing OTA levels considerably. Considerable number of research has been carried out on the fermentation, drying and processing aimed at solving certain quality and flavor problems.

2.3.1 Biochemical Reactions during Processing of Cocoa

Whilst several studies have been carried out on the evolution of filamentous fungi in coffee and their relation with OTA contents during post-harvest processing (Suàrez-Quiroz *et al.,* 2004; Wilkens and Jörissen, 1999; Studer-Rohr *et al.,* 1995) this is not the case for cocoa. A microbiological analysis on cocoa samples from nine (9) producing countries led to the isolation of *Aspergillus rhizomucorpusillus* and *A. fumigatus*. There is no study on filamentous fungi and OTA-producing species in cocoa depending on the type of postharvest processing. Conversely, there is high contamination records found in cocoa samples and their by-products. Burdaspal and Legarda (2003) reported that OTA was found in 99.7% of chocolate and cocoa powder samples. Contamination of 81.3% was also described in cocoa by-products by Miraglia and Brera (2002). Tafuri *et al.* (2004) found

OTA contamination of between 0.22 and 0.77 µg.
As knowledge increases the process of fermentation in the past decades have improved

(Adhana and Fleet, 2003, Camu *et al.,* 2007, Lagunes *et al.,* 2007, Nielson *et al.,* 2005, Nielson *et al.,* 2007, Schwan and Wheals 2004). Obviously, the different stages in the fermentation processes are essential in the creation of the complex organic components which are essential to the final taste and aroma we enjoy from cocoa. The micro biota activity involved in natural cocoa bean fermentation process shows the effect and the interaction of the environmental factors such as pH, oxygen and temperature with the metabolism of the cocoa bean pulp. This results in the production of significant amounts of ethanol, lactic acid and acetic acid representing a succession of yeasts, lactic acid bacteria and acetic acid bacteria in the cocoa bean fermentation course (Ardhana and Fleet, 2003; Camu *et al.,* 2003, Schwan, 1998; Schwan and Wheals, 2004; Schwan *et al.,* 1995). Re-wetting of cocoa beans should be avoided because cocoa beans with a level of moisture above 8% can allow rapid growth of the mycelium and the possibility of OTA production. Mouldy cocoa bean should be discarded. Protect the cocoa beans during drying from domestic animals, which can be a source of biological contamination. Drying equipment and tools should be cleaned regularly (Wood and Lass, 1985).

2.4 STORAGE, TRANSPORTATION AND TRADING OF DRIED COCOA BEANS

Before storage of dried cocoa beans, they must be sorted to remove flat, slaty, shriveled, black, mouldy, small and/or fused beans, germinated beans and beans with insect damage, etc. Facilitiesand equipment related with sorting process are regularly inspected, maintained and cleaned in order to avoid physical damage to cocoa beans that make them more susceptible to contamination, deterioration and to prevent the introduction of new

contamination and undesirable materials, an appropriate degree of personal hygiene should be maintained by all personnel.

Dried cocoa beans under storage must be properly identified by lots, at the farm level or in out-of-farm warehouses, in bulk or in clean bags under appropriate storage conditions. Bags used in storage and transport of cocoa beans need to be free of noxious substances such as mineral oils. Cocoa beans should be packaged in clean bags which are sufficiently strong and properly sewn or sealed to withstand transport and storage and which are suitable for food contact use and discourage pest infestation.

The vehicles must have floor, sidewalls and ceilings (in closed vehicles) checked for the presence of points where exhaust fumes or water from rain can be channeled into the cocoa cargo. Tarpaulins and plastic canvas used to cover the cargo should also be regularly checked to ensure that they are clean and without holes.

Appropriate place and condition not directly exposed to outside elements should be ensured aboard the ship to store the cocoa to reduce the possibility of undesirable situations mentioned that can lead to OTA contamination. Keep the ventilation holes in the containers free from clogging.

2.5 THE QUALITY OF COCOA BEANS

The quality of cocoa beans and its products depends on numerous factors. One important parameter for defining the quality of a cocoa bean is the bean size. In general, thefact holds that the bigger the bean the better. This is defined by the so called 'bean count', stating the number of beans per 100 grams with a standard of 1 gram per bean likewise, the weight of

the shell around a nib impact negatively on the mass of the cocoa. Thus, the lesser weight of waste the higher the yield (quality) of the bean (Wood and Lass, 1993).

The basic quality check done on cocoa is through sampling. The sampler randomly selects a significant percentage of the bags for inspection and a stabbing iron (horn) is used to draw a number of beans from the selected bags, or if the cocoa is in bulk, samples are taken at random from beans as they go into a hopper and also as they are spread on tarpaulins. Differing level of cocoa beans/ samples may be set by different authorities for inspection; International standards recommend that the samples should not be less than 300 beans for every tonne of cocoa. For bagged cocoa, samples should be taken from not less than 30% of the bags, and for bulk cocoa there should be not less than 5 samplings per tonne.

The cocoa samples are analyzed using the cut test. Most exporting countries' authorities specify standards dependent on the International Standards Organization cut test, as is done on normal physical cocoa contracts. The cut test determines an assessment of the beans from which analysts may infer certain characteristics of the cocoa, which gives an indication of quality.

The cut test involves counting of 300 beans, cut lengthwise through the middle and examined. Separate counts are done on beans which are defective; mouldy, slaty, insect damaged, germinated or flat. The results for each kind of defect are expressed as percentage of the 300 beans examined. The total defective beans discovered in the cut test gives manufacturers an indication of the flavor features of the beans (Wood and Lass, 1993).

Bean count is another measure of quality that producing countries often use, although there is no internationally accepted bean size classification. The Federation of Cocoa Commerce describes the following method for bean counts as a sample of not less than 600 g of whole beans irrespective of size but does not include flat beans, will be counted to obtain the number of beans per 100 g (Wood and Lass, 1993).

Further tests are carried out by chocolate manufacturers and cocoa processors, mostly for beans from origins of inconsistent quality or prone to off flavor. It is difficult for manufacturers to sift out all the defective beans to ensure good quality at the selection stage. Consistency in quality for the production of cocoa mass cannot be achieved when using one source of cocoa beans because of the large natural variability which exists in each lot. The differences can be reduced by using different types and lots of cocoa beans of known quality in stock and making an appropriate blend.

The grading of cocoa beans into its acidity, slatiness, and moldiness are amongst the most noticeable quality parameters for grading cocoa beans. All these methods are time consuming and laborious. Using ergosterol index as to measure the possibility of mycotoxins production in cocoa beans till now has not been reported. Although this method of determination can be a faster and relatively precise method which can be aimed at grading contaminated cocoa beans using fungal determinations, ergosterol index and Ochratoxin A formation. In addition, proper usage of insecticides and fertilizers has become a growing importance in the cocoa industry with stricter regulations in several markets.

Other factors for quality are related to the quality of the processing of the beans after harvesting. High humidity and related mould in the bean, slate-colored beans and the damage by insects are all of negative influence on the value of cocoa beans.

Flavor is very important to the chocolate manufacturer. This assessment is normally carried out by a panel between five and ten experienced tasters. Off flavor can readily be detected by tasting roasted ground nib of cocoa liquor directly or they can be mixed with sugar and water to make a basic dark chocolate before tasting. Moldy and smoky off flavor and excessive bitterness cannot be removed during processing. Acid tastes can be altered in processing through neutralization with an alkaline.

Strict control of the roasting and alkalizing processes is very much required to produce the best quality. For chocolate manufacturing, the yield of nib is very important as well as the amount of cocoa butter in the nib. Higher level of cocoa butter determines lower levels of cocoa butter needed to be added later on in the manufacturing process. Nib yields are determined in the laboratory.

Low grade or substandard beans can be pressed whole to produce expelled cocoa butter which is further refined. High quality beans are de-shelled before pressing to produce pure pressed cocoa butter and subsequently, cocoa press cake (which ultimately becomes cocoa powder). Chocolate producers have a number of requirements to meet with respect to the quality of cocoa butter namely, hardness, melting and solidification feature. Associations and national authorities of Cocoa trade produce standards or grading for cocoa beans covering the bean count per 100g and the percentage of permitted faults, moisture and

foreign matter, and the International Standards Organization (Wood and Lass, 1993) provide specification for cocoa beans.

Molds are common contaminants of agricultural commodities. Fungal development on alimentary substrates can lead to different detrimental effects: alteration of technological properties, decrease of nutritive value and synthesis of mycotoxins (Pitt and Hocking., 1985). Evaluation of molds development is of interest to estimate global quality of raw materials and may be useful to take decision on their possible use. Ergosterol is considered as the principal sterol of fungi and it plays an important role as cell membrane component Therefore, it has been proposed as a global indicator of mycological quality of foods and feeds (Bailly *et al.,* 1999; Cahagnier, 1998; Scnurrer, 1993; Schwardorfk and Muller, 1980; Seitz *et al.,* 1977). Ergosterol levels are commonly used as quality parameters in ecological (Sashdhar *et al.,* 1989), industrial, (Hippelein and Rugermer, 2004), and agronomics environments (Kadakal and Artik, 2004, Sashdhar *et al.,*1989). Moreover, significant correlations were found between ergosterol and the major mycotoxins (fumonisin B1, Zearalenone, Deoxynivalenol, ochratoxin A, patulin) in maize (Peitri *et al.,*2004, rice (Saxena *et al.,*2001) and wheat (Abramson *et al.,* 2005). Therefore, ergosterol determination can be considered as a good index of fungal development on cereals and could be an early indicator of potential mycotoxin production. Its determination can be used in industry to screen productions, prior to mycotoxin analysis. On cereals, according to Cahagnier (1998), 3µg of ergosterol per gram is considered as the maximum acceptable level for maize while for wheat, 8µg of ergosterol per gram is the retained value for certifying correct quality of the grains. On the other hand, when the amounts of ergosterol

are higher than 8µg/g in maize and 12µg/g in wheat, a doubtful quality of grains is suspected (Cahagnier, 1998).

Cocoa beans, a produce of commerce of *Theobroma cacao* is a principal raw material for the chocolate industry. Cocoa of commercial grade should conform to some criteria among which absence from moldiness and mycotoxin production is one (Aroyeum *et al.,* 2007). Grading of cocoa beans into acidity, slatiness, moldiness is among the most prominent quality parameters for grading cocoa beans. All these methods are time consuming and laborious. Using ergosterol index as a measure to the possibility of mycotoxin production in cocoa beans has not been reported. Since the method of ergosterol determination can be a faster and relatively precise method, this study was designed with the aim of grading contaminated cocoa beans using fungal determinations, ergosterol index and Ochratoxin A formation.

2.6 RECOMMENDED PRACTICESIN COCOA PROCESSING

Cocoa beans and the pulp are microbiologically sterile in relation to OTA fungi production while inside the healthy cocoa pod. The contamination of the fungi occurs during the opening process of cocoa pod and in the ensuing processes. Thus, the cocoa plantation should be well maintained to ensure low levels of mould infestation to avoid contamination by OTA producing fungal spores during the opening of the cocoa pod. The following phyto- sanitary practices should be adhered to in order to minimize or prevent OTA contamination in cocoa beans.

The cocoa plant should be kept healthy, through the proper use of good agricultural practices (GAP) namely; weeding, improving soil quality, preventing soil erosion, pruning, fertilizer application, pest and disease control, and irrigation. For establishment of new

cocoa farms, cocoa trees should be planted in the most suitable soil, pattern and density to ensure easy management of the farms.

Overhead irrigation during the flowering and fruit development period must be avoided as it could increase normal spore spreading rates and increase the chance of infection of beans by the OTA fungi. Avoid the disposal of uncomposted organic wastes from cocoa and any other source in and around the cocoa plantation. The cocoa seeds and its associated material, such as dust, earth, and other seed may encourage the propagation of OTA producing fungi.

2.7 OVERVIEW OF MYCOTOXINS

Molds grow and produce mycotoxins in plants during the chain from farm to fork. Some crops, preservation systems and feedstuffs are more susceptible to mould growth and mycotoxin production than others. Many mycotoxins exist, some of which are very toxic to farm animals and may cause severe mycotoxicoses. Animals consume mycotoxins in lower quantities over a long period of time causing chronic or more diffuse toxicoses (Petterssonet *al.,* 1982). Mycotoxin is derived from the Greek word mycos and the Latin word toxicum meaning mold and poison. They are highly toxic secondary metabolites of several molds, mainly belonging to the genera *Fusarium, Aspergillus* and *Penicillium* (Heidler *et al.,* 2003).

Different mycotoxins as described by Pettersson *et al.,* (1982) are more commonly associated with and in certain, feedstuffs. Some develop in growing crops due to their prone to certain toxigenic fungi, whereas some infection and toxin production occur during preservation and storage of these foodstuffs when the system used in the preservation is

unfavorable. Due to crops from certain region, climatic or ecological conditions favorable and suitable environments are created for the mycotoxin production (Pettersson *et al.,* 1982). According to research, it is estimated that over three hundred (300) mycotoxins of fungal metabolites are potentially toxic to both animals and humans are characterized (Adegoke, 2004).

From the viewpoint of trade and health, mycotoxins that are toxic are aflatoxins, Ochratoxins, fumonisin, deoxynivalenol, T-2 and T-2 like toxins (trichothecenes) and alternariol (Thompson and Henke, 2000). Thompson *et al*. (2001) indicated that food crops found in tropical and subtropical areas are more prone to mycotoxins contamination as compared to that in the temperate zones due to high humidity subsequently giving that toxin formation strives with temperature, which provide optimal conditions. Likewise, it has been reported that drought environments can stress plants and render them vulnerable to contamination by *Aspergillus* spp. In the case of some fruits, under the conditions of warm and humid weather, fruit rots in fruits such as blueberries are of great concern. Postharvest rot can occur on healthy fruits at harvest but carry fungal spores that will infect and develop in the fruits during storage and processing. It is therefore important to detect fungal contaminants in fresh fruits, since molds can develop and produce mycotoxins on these commodities (Tournas and Katsoudas, 2005). Their global existence is considered to be a major risk factor. Furthermore, a report from the Food and Agriculture Organization (FAO) indicates that, over 25% of the world ‘s crops are affected by mycotoxins (molds) annually. The effect of these toxins depends on the physical and chemical properties of the toxin, the rate of exposure and level of intake. Mycotoxins are by nature reported to be

carcinogenic, genotoxic, teratogenic, dermatotoxic, nephrotoxic and hepatotoxic (Heidler *et al.,* 2003).

2.7.1 Factors that Contribute to Mycotoxin Production

There are time- bound factors in the food chain that is important to consider in the production of mycotoxins during the pre -harvest and harvest handling activities of agricultural products. These factors are classified into both intrinsic and extrinsic. Examples of intrinsic factors are moisture content, water activity, substrate type, plant type and nutrient composition. Climate, temperature and oxygen level are extrinsic factors which contribute to the production of mycotoxins. Production factors such as drying, blending, addition of preservatives and handling of grains have also been reported to influence mycotoxin production. Fungal strain, insect interactions and microbiological ecosystem which are inherent factors have also been reported.

2.7.2 Prevention and Reduction of Mycotoxins

Despite the stated factors contributing to the growth of mycotoxins there are some activities employed that could prevent contamination. Activities such as during pre-harvest high resistant varieties, good field management practices and the use of biological and chemical agents, similarly, post-harvest practices, improved drying methods, good storage conditions and also the use of natural and artificial agents are some recommended methods used to prevent mycotoxins. Kabak and other also suggested irradiation for some food products (Kabak*et al*., 2006). The inclusion of sorbent materials in feed or addition of enzymes or microorganisms capable of detoxifying mycotoxins has been reported to be reliable methods for mycotoxin prevention in feeds (Jard *et al.,*2011). However, while bentonite and aluminosilicate clays have been used as binding agents for reducing aflatoxin

intoxication in pigs, cattle (Diaz *et al.,* 2008) and poultry without causing digestive problems when mixed with aflatoxin-contaminated feed, care must be taken as the clays canalter nutritional value by binding trace minerals and vitamins and reducing their bioavailability and even produce dioxins (Devegowda and Castaldo, 2000). Preference foresterified glucomannan (a naturally-occurring organic compound in yeast) over clay in reducing the toxicity of aflatoxin has been reported.

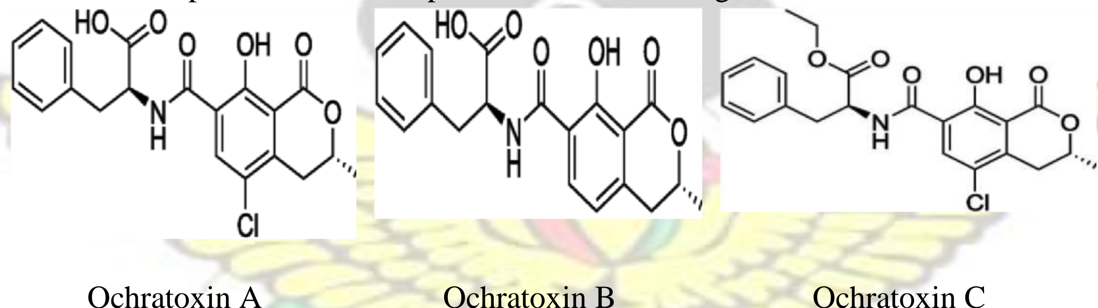
Devegowda and Castaldo (2000) found that using glucomannan supplementation at 0.05% of diet of dairy cows that consumed aflatoxin-contaminated feed; there was a reduction of 58% in aflatoxin in the cow's milk. Although in developing countries prevention of mycotoxins from entering the food chain may not currently be receiving sustainable attention or focus as in developed countries due to different food systems, financial constraints, availability of food policies, levels of food safety education and technological development, nonetheless, methods recommended herein can help curb the prevalence.

2.7.3 Ochratoxin

Simply, Ochratoxins are minor groups of chemically related toxic fungal metabolites (mycotoxins) which are produced by certain molds of the genera *Aspergillus* and *Penicillium* developing on a wide range of raw food commodities. Some Ochratoxins are potent toxins and their presence in food produces adverse effect to consumers. They are also pentaketides made up of dihydro-isocoumarin linked to β-phenylalanine. There are different types of Ochratoxins occurring in nature; these are Ochratoxin A, Ochratoxin B,

Ochratoxin C, α and β (Figure 2.1). The vital and most toxic Ochratoxin found naturally in food is Ochratoxin A (OTA).

Ochratoxin B is the only toxin found also but they are very rare and less toxic. There are other structurally related Ochratoxin which includes Ochratoxins C, α and β. Though they are structurally related they have been isolated from fungal cultures, they do not exist in foods. Ochratoxin B and C as described by Luster and Weidenborner are not chlorinated and are less toxic (Weidenborner, 2001). Whereas OTA is the major compound found as a natural toxin of plant material, the role of OTA in the production of fungus is not yet known. Cocoa contamination by Ochratoxic fungi is mostly chance upon during fermentation of its powder to various products at various stages.



Ochratoxin A Ochratoxin B Ochratoxin C

Figure 2.1 Chemical structures of Ochratoxin A, B & C

OCHRATOXIN A: Ochratoxin A (OTA) being a toxic secondary metabolite produced by several species of *Aspergillus* and *Penicillium* genera. It causes damage to the organism of humans and animals (Abarca *et al.,* 1998). It has nephrotoxic (Mantle and McHugh, 1993), immunotoxic, teratogenic and carcinogenic properties (Kuiper-Goodman and Scott, 1989; Kuiper-Goodman, 1996; Höhler, 1998). It has been associated with Balkan Endemic Nephropathy (BEN) and tumor growth in the urinary tract (Mantle and McHugh, 1993).

This follows the experiments on animals by the International Agency for Research on Cancer (IARC, 2007) classified OTA as a carcinogenic for humans (group 2B). OTA

produced by *Aspergillus carbonarius, A. niger* and *A. ochraceus* in tropical zones, and by *Penicillium verrucosum* and *P. nordicum* in temperate zones (Pitt *et al.,*1985; Abrunhosa, *et al.,* 2001; O'Callaghan *et al.,* 2003). Also, Benford *et al.* (2001) noted that *Aspergillus ochraceus, A. carbonarius, A. melleus, A. sclerotiumand Penicillium verrucosum* are the main producers of OTA.

CONDITIONS THAT PREDISPOSES OCHRATOXIN A PRODUCTION IN FOOD:

Research has shown that, the existence of OTA synthesizing fungi in food does not certainly translate to the production of OTA but the production of OTA by ochratoxigenic fungi is made possible at certain environmental conditions such temperature, oxygen availability, water activity level, pH and presence of certain metal ions (Astoreca *et al*., 2007; Amezqueta *et al*., 2005; Kokkonen *et al*., 2005). Also, these parameters vary between different *Penicillium* or *Aspergillus* strains (Aziz and Moussa, 1997; O'Callaghan *et al*., 2006).

There is available data indicating that OTA production is stimulated by a high-water activity level (Belli *et al*., 2007a; Pardo *et al*., 2004; Kapetanakou *et al*., 2009; Tassou *et al*., 2007; Valero *et al*., 2006). The production of OTA by *A. carbonarius* is highly dependent on water activity unlike A. ochraceus *sp*., which produces OTA even at low water activity levels (Kapetanakou *et al*., 2009). The impact of pH on OTA secretion by OTA producing strain is not well known (Kapetanakou *et al*., 2009).

The composition of substrate determines the rate of OTA production (Pardo *et al*., 2006), sucrose and glucose promoted OTA synthesis (Muhlencoert *et al*., 2004). OTA production

in *Aspergillus* usually occurs in the range between 20 to 30°C, at temperatures a little below favorable temperature for their food is strain specific or the reason for its Ochratoxin production (Pardo *et al*., 2004).

In a research work done by Mortensen in 2006, he indicated that, OTA adsorbs to soil organic matter and is rapidly degraded especially in planted soil due to its rich microflora (Mortensen *et al*., 2006) reducing the risk of OTA being washed into the underground water environment.

Occurrence in Food Commodities: Ochratoxin A is found in several plant raw materials and also in food products (Pohland *et al.,*1992). It is mainly found in cereals and cereal derived products (Duarte *et al*., 2009), corn (Magnoli *et al.,* 2007), coffee (Lombaert *et al.,* 2002), cocoa and cocoa products (*Copetti et al.,* 2011), figs (Iamanaka *et al.,* 2005), chilli peppers (Thirumala-Devi *et al*., 2000), liquorice (Majerus *et al*., 2000), grape juice (Majerus *et al.,* 2001), dried vine fruit (MacDonald *et al.,* 1999), wine (Otteneder and Majerus, 2000), meat products (Castella *et al.,* 2002) and several food commodities available on the market.

Studies by various researchers have indicated the incidence of OTA in different types of food commodities. In a related study in Spain, 90% (19 out of 21) of cereal derived products were contaminated with OTA (Araguas *et al.,* 2005). Other studies, 47% of dried figs were found to be contaminated with appreciable levels of OTA ranging from 0.12-15.31 μg/kg (Karbancioglu-Glurer and Heperkan, 2008), 38% of the commercial chilly (pepper) powder samples purchased from the local markets were contaminated with OTA (Iqbal *et*

al., 2013). Additionally, a study in Brazil, involving grape juice and red wine samples indicated a contamination of OTA of 25% and 28% respectively (Rosa *et al.,* 2003).

Ochratoxin A in Coffee: PossibleOchratoxin A producers in coffee are *A. niger, ochraceus a*nd *A. carbonarius* (Joosten *et al.,*2001, Urbano *et al.,* 2001, Nakajima *et al.,* 1997, Teren *et al.,* 1996). Others are *A. sclerotoniger, A. lacticoffeatus* (Alborch et al., 2011), *A. westerdijkiae* and *A. steynii* (Frisvad *et al.,* 2004). Temperatures of 35-37ºC and 24-31ºC serve as the optimum growth conditions for *A. nige rand A. ochraceus* and water activities of 0.77 and 0.95-0.99, respectively (Pitt and Hocking, 1997).

A survey conducted by Lombaert in Canada, 51% of ground and 67% of instant coffee samples were contaminated with OTA (Lombaert *et al.,* 2002), natural occurrence of OTA is reported in the range of 0.2-360 µg/kg (Joosten *et al.,* 2001). Also in Brazil, about 63%, 31%and 3% of coffee samples surveyed in a study were contaminated with *A. niger*, *A. ochraceus* and *A. carbonarius,* respectively (Taniwaki *et al.,* 2003). Napolitano *et al.* (2007) reported that among samples from seven different geographic regions, Indian and Costa Rican green coffees were the most contaminated samples with 11 and 13µg/kg, respectively, while Ethiopian coffee was the least contaminated having 3.8µg/kg.

Coffee cherries when mixed frequently during the drying process, a significant decrease in fungal contamination was observed. A consistent reduction in OTA levels was observed after roasting the coffee beans during processing (Romani *et al.,*2003). After preparation, reductions of 49.8% of OTA in expressed coffee, 32.1% in mocha brewing, and 14.5% in auto drip were observed (Perez de Obanos *et al.,*2005). The method of coffee preparation also plays a key role in final human exposure to OTA.

Ochratoxin A in Cocoa: Cocoa a cash crop produced in many countries of the world with approximately 71% of the production from West Africa (Codex Alimenatarius Commission, 2008). Itis mostly fermented to produce cocoa powder used for several products in the food, cosmetic and the pharmaceutical industries. Although, over two thirds of world cocoa is produced in Africa, European countries are the major consumers of this product with a percentage of 41.1% as recorded in the year 2006 (Codex Alimentarius Commission, 2008). The major occurring mycotoxins in cocoa are Ochratoxin (Mounjouenpou *et al*., 2007). OTA and thefungi are said to be present at all stages of the cocoa production with the greater percentage associated with cocoa bean shell (Amezqueta *et al*., 2005).

Dongo *et al*. (2008) recorded the presence of OTA in fifty-four (54) out of fifty-nine (59) samples of cocoa products tested from Nigerian ready to be sold with concentrations ranging between 1.0 and 277.5µg/kg. Likewise, evaluation of cocoa samples from ports of Cote d`Ivoire, showed that twenty-three (23) out of one hundred and forty-seven (147) samples from Abidjan and ten (10) out of one hundred and fifty-one (151) samples at San Pedro had over 2.0 µg/kg of OTA (Codex Alimentarius, 2008). Similarly, sampling twentyone (21) cocoa and cocoa products in Brazil yielded one hundred and twenty-three (123) *Aspergillus* toxigenic isolates from 42.9% of the samples. All the *Aspergillus carbonarius* and *A. ochraceus* isolates were of ochratoxigenic while only 18.2% of *A. niger*isolates were OTA positive. Based on the research carried out by the Federal Agency for Food Safety, Belgium in 2006, five (5) out of thirteen (13) cocoa beans samples had OTA levels above

0.4 µg/kg (Codex Alimentarius Commission, 2008). In the investigation of the presence of OTA in cocoa, cocoa bean, cake, nib, powder, shell, butter, chocolate and chocolate cream from different countries showed that 40% of cocoa samples had a high concentration of OTA, whereas OTA levels were low for cocoa products (Bonvehi, 2004). Further studies carried out by Amezqueta *et al*. (2005) showed that by carefully removing cocoa shell, twenty (20) samples out of the twenty-two (22) tested had over 65% less of OTA concentration signifying that OTA is predominantly on the shell. Tabata *et al*. (2008) investigated the occurrence of OTA in one hundred and fifty-seven (157) samples consisting of cereal, fruit, coffee and cocoa products from Japan. Highest incidence of OTA was found in cocoa powder (10/12) and in the third place was cocoa with OTA present in five (5) out of eight (8) samples, co-occurrence of OTA and Aflatoxins was found to occur in cocoa products. Outcomes of a sampling of retailed chocolates in Japan for OTA in 2005 was positive for all the forty-one (41) samples; levels in twenty (20) were above 0.20 µg/kg, seventeen (17) samples were below 0.20 µg/kg while fourteen (14) samples had OTA below 0.10 µg/ kg (Codex Alimentarius Commission, 2008). In a project sponsored by CAOBISCO/ECA/FCC (1999) to decrease impact of OTA in cocoa showed that, a mean OTA level of 1 µg/kg for cocoa samples tested as against 0.26 µg/kg for dark chocolate which had highest value of OTA amongst the products, signifying that only low levels of OTA are present in ′ready to eat′ cocoa products, (Codex Alimentarius, 2008). A study by Tafuri and others on cocoa powder from different sources marketed in Italy indicated that 22% of samples tested exceeded the European limit for cocoa, 50% of samples had OTA level above 0.22-0.77 µg/ kg (Tafuri *et al*., 2004). According to the assessment of dietary intake of Ochratoxin A by the European Union population, 81.3% of the cocoa products

were OTA positive with contamination levels from 0.01 to3.8 μg/kg (Miraglia and Brera, 2002). Between 1997 and 1998 the Ministry of Agriculture, Fisheries and Food (MAFF) presented a result on cocoa powder samples in which 19/20 and 20/20 samples, respectively were OTA positive. Contamination levels had a mean value of 0.68 for 1997 and 1.67 for 1998.

Burdaspal and Legarda (2003) did record the occurrence of OTA in 296 samples of different types of chocolate and cocoa powder purchased from sixteen (16) different countries. Results showed that although 99.7% of the samples were contaminated, OTA levels were low, leading to the conclusion that the consumption of cocoa and its associated products only contributes a minor fraction to the OTA tolerable daily intake. The scientific cooperation on questions relating to foods (SCOOP Task 3.2.7, 2004) results indicated that cocoa contributed only 5% of total OTA intake (Miraglia and Brera, 2002).

There are seasonal variations relating to the levels of cocoa contamination; cultivation practice, phyto-sanitary conditions of damaged cocoa pods are attributed to pest, physically wounded, rotten and mummified. Also, other factors include harvesting and fermentation conditions with prompt removal of cocoa shell which plays a positive role (Mounjouenpou *et al*., 2007). Sanchez-Hervas *et al*. (2008) in his research work showed that the predominant fungal contaminants of the cocoa beans are of the genus *Aspergillus* belonging to section Nigri and Flavi out of which 49.2% of the black *Aspergilla* isolated were ochratoxigenic. Two (2) ochratoxigenic species of *Aspergilla* were found amongst 86 isolates, mostly from West African countries such as Ghana, 16 from Nigeria and 13 from Cote d`Ivoire obtained during cocoa processing (Codex Alimentarius, 2008). Meanwhile, Mounjouenpou *et al*. (2008) also worked on the fungal population of cocoa during

postharvest practices. *A. fumigatus*, *A. tamari*, *A. versicolor*, *P. sclerotium*, *P. paneum* and *P. crustosum* isolated did not produce OTA, OTA producing strains isolated were predominantly *A. carbonarius* (100%) and to a lesser extent *A. niger* (70%).

Common ochratoxigenic species that are present in cocoa beans are *Aspergillus carbonarius, A. niger*, *A. melleus, A. westerdijkiae* and *A. ochraceus*. In a study in Brazil on cocoa beans, there was a strong positive correlation (63% of the OTA positive samples showed thepresence of *A. carbonarius*) between the presence of *A. carbonarius* and contamination with OTA in the cocoa beans (Copetti *et al.,* 2011). In Spain, some of the roasted cocoa powder and chocolate samples surveyed in 2000 were contaminated with Ochratoxin A, with the levels varying from 0.63 to 2.41mg/kg (MAPA, 2000).

Ochratoxin A in Animal Products: Food products from animal; meat and edible tissues do contribute to the total OTA intake through a "carry-over effect" (Gareis, 1996). Buildup of toxin in the animal tissue after the intake of contaminated feed is due to the carry-over effect. Ochratoxin A can also be produced by molds developing on pork products during the ripening process, which are known to give a characteristic flavor to the final product (Gareis, 1996). Many fungal species produce OTA in meat products particularly*P. nordicum* whichhas been mainly isolated from proteinaceous foods, such as cheeses and fermented meats (Castella *et al*.,2002; Lund and Frisvad, 2003). Additionally, *P. nordicum* has been proven to have the ability to grow on meats (Battilani *et al.,* 2003; Sorensen *et al.,* 2008). Some animal products reported by the European Union have been said to be contaminated with OTA, with an average concentration of0.052 μg/kg (Jorgensen, 2005). In a survey to evaluate the occurrence of OTA in meat products from a German market, Gareis and Scheuer (2000) reported a maximum concentration of 0.141 g/kg and also

contamination of 68%, 67% and 77.2% of the liver, bologna and blood sausage samples, respectively. According to Ostry (2001), conditions that favor the production of mycotoxins in meat and meat products are mainly; the presence of oxygen, a temperature of between 4ºC and 40ºC, a pH value between 2.5and 8, minimum water activity of 0.80 and also maximum salt concentration of 14%.

Ochratoxin A in Dried Fruits: Nuts and dry vine fruits are mainly used as ingredients in cereal based foods such as cereal bars, biscuits, puddings, cookies and breads. These ingredients or commodities are mostly attacked by common spoilage fungi namely black *Aspergilli* and the most prominent among them are *Aspergillus niger, A. carbonarius, A. aculeatus, A. ellipticus, A. heteromorphous* and *A. japonicus*. Though *A. niger*and *A carbonarius* are the most common among them, *A. carbonarius* is most likely the potential OTA producer in dried fruits (Romero *et al.*, 2007). Morphological variation between the two species is difficult and hence molecular based techniques are required for their identification (Abraca *et al.,* 1994; Schmidt *et al*., 2004). Several surveys have been conducted in many countries to evaluate the natural occurrence of OTA in dry vine fruits and also to estimate the ability of black *Aspergilla* (*Aspergillus* section *nigri*) isolates to produce Ochratoxin A. In a survey done in Brazil, 15% of black *Aspergilla* isolates from dry fruits were found to produce OTA.Among the dry fruits analyzed, high incidence levels of OTA, 26.3% and 33% were observed in dried figs and black Sultana, respectively (Iamanaka *et al*., 2005). In a survey in Argentina, *A. carbonarius* was the major OTA producer (82.6%) in dried vine fruits (Magnoli *et al*., 2003). In a survey conducted in the United Kingdom, 88% of the dry vine fruit samples had detectable levels of OTA and the highest level found was 53.6 µg/kg (MacDonald *et al*., 1999).

2.7.4 Ochratoxin A Occurrence in Nature

OTA is particularly widespread in the tropical and sub-tropical regions where they are mainly produced by *Aspergillus* species (*A. ochraceus*), but then in the temperate climates in countries such as Canada, Northern Europe and some parts of South America, the main producer is *Penicillium verrucosum*. OTA production by *A. ochraceus* is promoted by relatively high temperatures of 13ºC to 37ºC. Meanwhile, *P. verrucosum* grows and produces their toxins at temperatures as low as 0ºC. *Aspergillus ochraceus* and *P. verrucosum* are able to produce OTA at water activities down to 0.80 and 0.86, respectively. Both are referred to as storage fungi, relatively than field contaminants or plant pathogens. These toxins are produced mainly when commodities that are prone to these microbes are stored under inappropriate conditions mostly at high moisture levels. It has been established that in northern Europe cereal contamination with Ochratoxin (OTA) occurs predominantly during post-harvest and are caused by *Penicillium verrucosum,* due to the cooler temperature conditions that renders the cereals damp (Magan and Olsen, 2004) due to ineffective drying of the grain resulting in pockets of growth by *P. verrucosum* in storage cereals (Magan *et al*., 2007). There has been interest in determining probable treatments which could effectively control this harmful mycotoxigenic species. Whereas moist grain are sometimes treated with aliphatic acid-based treatments which are often fungi stats and hence require very wide exposure to be effective, sub-optimal applications can result in a stimulation of OTA production by *P. verrucosum* strains (Aroyeum*et al.,*

2007).In a subsequent research into the "Inhibition of Ochratoxin A Production of *Aspergillus carbonarius* by Yeast Species" by Kabak (2006)and others recorded high loads of Yeasts and fungi in cocoa powder stored at warehouses. The production of OTA by yeasts and molds are indications that favorable conditions for their growth prevails. Many reports have shown that bad storage conditions such as high temperature with high relative humidity result in moldy cocoa powder in the warehouse which may predispose the powder to toxic secondary metabolites such as Ochratoxin A (Kabak *et al.,* 2006).

2.7.5 Foods Products that are Prone to Ochratoxin A

According to surveys, OTA has been found in a very wide range of raw and processed food products all over the world. The incidence was first reported in cereals, but now has been found in other products, this includes coffee, spices, dried fruits, wine, beer, cocoa, nuts, beans, peas, bread and rice. It has also been detected in meat and animal products, especially pork and poultry, resulting from transfer of contaminated feed.

Ochratoxins have been detected in several agricultural products from temperate and tropical zones. JECFA (2001) found OTA in cassava flour, cereals, fish, peanuts, dried fruits, wine, eggs, milk coffee and cocoa beans. Cereals, wine, grape juice, coffee and pork are the major sources of human Ochratoxin exposure (JECFA, 2001). Aish *et al.* (2004) also noted that Ochratoxin A (OTA) is found in wheat, corn and oats having fungal infection and in cheese and meat products of animal consuming Ochratoxin-contaminated grains. There are varying levels of OTA levels in different food products, nonetheless are generally low in well stored commodities with a mean value less than 1 μg/kg for cereals from temperate regions. The concentration is much higher under inadequate storage

conditions. Canadian wheat and UK barley have reported levels as high as 6,000 μg/kg and 5,000 μg/kg, respectively as compared to the usual concentrations below 50 μg/kg. The major food products that are prone to OTA in the diet in Europe are cereals and wine. Coffee which was supposed to be high in this respect is now considered less significant. Pork products have also been proposed as a significant dietary source.

2.7.6 Stability in Food

OTA is a comparatively heat stable molecule and survives in most cooking processes to some extent, though the reduction in concentration during heating depends on factors like temperature, pH and the components in the product. For instance, heating moist wheat at 100°C for 2.3 h gave about 50% reduction in OTA concentration, whereas dried wheat for 12 h (Scudamore *et al.,* 2004). Processes such as coffee roasting and baking of cereal products and biscuits can produce substantial losses in OTA levels, but processes like pasta production produces little reduction. OTA also survives during brewing and winemaking and can be found in a variety of processed consumer food products. OTA is mostly destroyed by acids, alkaline hydrolysis and also by the action of some oxidizing agents (Scudamore *et al.,* 2004).

2.7.7 Human Exposure to Ochratoxin A

Mycotoxins can affect the health of both human and animal. Generally, animals are directly exposed to mycotoxins through the consumption of moldy feedstuff whereas humans are exposed in two ways; directly in consumption of moldy plant products and indirectly through the consumption of animal products contaminated with the toxin containing residual amounts of the mycotoxin consumed by the food producing animals (Boutrif and

Bessy, 2001). Animals however derived food products contribute to a reduced extent to human OTA exposure, with the exception of babies and infants, due to their high consumption of milk and milk products, and their specific metabolism (Kuiper-Goodman, 1998; Gilbert *et al*., 2001). OTA is also a genotoxic and teratogenic. These characteristics of OTA damage the DNA and the foetus, respectively. Hence it is considered a possible carcinogen. When affected can cause renal carcinoma and other cancers in a number of animal species, though the metabolism for this is uncertain. Also, it is described to have adverse effects on the immune system of some species. Again the evidence for carcinogenicity in humans is not certain but there are records for other mammalian species where the presence of OTA in food when fed is considered undesirable. Toxicologists suspect that OTA may be very significant food contaminant from a public health point of view.

From the reports of the European Food Safety Authority (EFSA), OTA was detected in human blood and breast milk, demonstrating a dietary exposure. Estimated daily intakes fall in the range between 0.2 and 4.7 ng/kg bodyweight. In 2006, the authority derived a tolerable weekly intake of 120 ng/kg bodyweight for OTA in the diet, based on the current scientific evidence.

2.7.8 Biosynthetic Pathway of OTA

Though much evidence exists regarding the various toxigenic properties of OTA, unlike other important mycotoxins, not very much is known about the OTA biosynthetic pathway in any fungal species. It is widely believed that the isocoumarin group is a pentaketide made from acetate and malonate through polyketide synthesis pathway (Niessen *et al.*,

2005, Edwards *et al*., 2002, Moss 1998). Thus, a polyketide synthase (PKS), considered as key enzyme, is involved in the OTA biosynthesis in a related way to other polyketide mycotoxins such as fumonisin (Proctor *et al*., 1999) and Aflatoxins (Bhatnagar *et al.,* 2003, Varga *et al.*, 1996). Huff and Hamilton (1979) proposed a biosynthetic pathway based on a mechanistic model according to the structure of OTA. The heterocyclic portion of OTA is structurally comparable to mellein, a secondary metabolite produced by many OTA producing species such as *A. ochraceus, A. westerdijkiae and A. melleus.* Mellein is also produced by a non ochratoxigenic species such as *Pezicula spp.* According to Huff and Hamilton (1979) three distinct stages occur in OTA biosynthesis: the first part being polyketide synthesis of Ochratoxin A viamellein involving a polyketide synthase.

The second step includes acyl activation: mellein is methylated and oxidized to 7-CarboxyMellein, this part is then transformed to a mixed anhydride, an activation reaction using adenosine triphosphate (ATP). The second precursor phenylalanine is synthesized via the shikimic acid pathway, followed by ethyl ester activation so that it can partake in the subsequent acyl displacement reaction. In the final step, linkage of those activated precursors via a synthetase takes place, generating an ethyl ester of OTA. De-esterification by an esterase or transesterification is the last step in this postulated biosynthetic pathway (Fig.2.2)

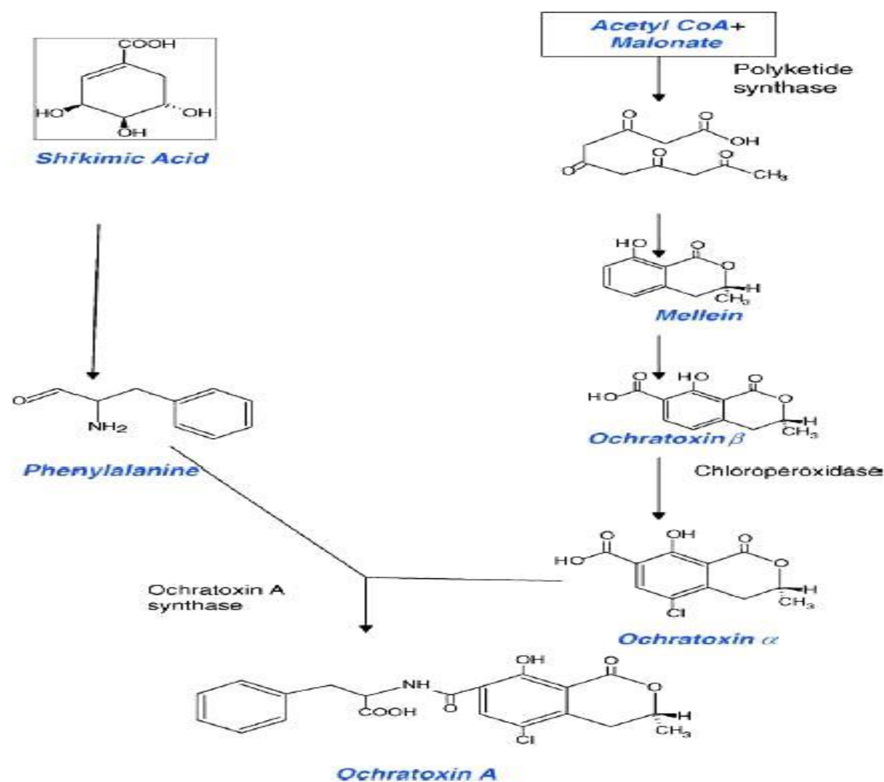


Figure 2.2 Schematic representation of the hypothetical OTA biosynthetic pathway as proposed by Huff and Hamilton (1979).

2.7.9 Toxicology of OTA

The toxicological status of OTA has been studied many times and was the subject of a complete monograph by the International Agency for Research on Cancer (IARC) in 1993 (IARC, 1993). Following the discovery of human and animal spontaneous nephropathies, many experimental studies were carried out in order to show the implication of OTA in these diseases (Zimmerli and Dick 1996; Otteneder and Majerus 2000). These studies showed that this molecule can have several effects such as nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic on several species of animals, and can cause kidney and liver tumors in mice and rats. However, its toxicity varies depending on the sex, the species and the cellular type of the tested animals (O 'Brien *et al.,* 2001). The

genotoxic status of OTA is still controversial, due to contradictory results obtained in various microbial and mammalian tests. However, evidence of DNA-adducts formation was shown following chronic exposure of OTA to rat and sub-acute exposure to pig (Faucet *et al.,* 2004).

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2.7.10 Decontamination of Ochratoxin A

Natural and chemical methods: Fresh farm products dried in a clean, dry setting with mechanized driers as opposed to sun (Suarez-Quiroz *et al*., 2005; Valero *et al*., 2007) in small batches or piles to avoid prolonged drying (Lopez-Garcia *et al*., 2008) ensures decrease in postharvest mold growth and OTA production and recontamination of products. OTA level in wheat bread and artificially contaminated barley meal significantly decreased with the use of extrusion cooking at very high temperatures (Scudamore *et al*., 2004).

The ability of OTA-producing fungi to grow on a wide range of food commodities and the persistence and ubiquity of OTA in the food chain mean that control is best achieved by measures designed to prevent the contamination of foods using HACCP-type techniques. Detection and removal of OTA-contaminated material from the food supply chain is also important for imported products. This is followed by further drying down to a moisture level of 15% (UK Code of Good Storage Practice). Other important cereal storage factors are effective cleaning of grain stores and handling equipment between crops, and fumigation to prevent insect infestation. In tropical regions, the use of controlled atmosphere storage to control insects may also help to inhibit mold growth. Rapid and effective drying is also important in the control of OTA production in other commodities,

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especially coffee. For dried fruits, minimizing mechanical and insect damage during handling and storage helps to prevent the entry of molds into the fruit before drying.

Due to the several harmful effects caused to human health, some physical and chemical approaches such as that carried out by Kabak and others includes heat-treatment, irradiation, extraction with sorbents and chemical agents; these were restricted due to the difficulties regarding the safety issues and, potential losses in the nutritional quality of the treated commodities (Kabak *et al.,*2006). Much work on the prevention Aflatoxins in foodstuffs have been done (Cotty 1994; Dorner *et al.,*2002; Kabak *et al.,*2006) as compared to OTA prevention (Petersson *et al.,*1982; Fuchs *et al.,*2008), deoxynivalenol, fumonisin B1 other toxins. In this quest, these researchers have studied the ability of yeasts isolated from wine grapes in Turkey to prevent the production of OTA by *A. carbonarius*. Further research leading to the adapting of alternative approaches such as the use of biological and chemical agents have been tried to remove or reduce the growth of OTA and other toxins associated with contamination of commodities.

Several strategies have been applied by scientist like Majica and others to prevent and control the growth of OTA producing fungi in grains and other storage foods. In past years, chemical centered control has been the common treatment to inhibit the incidence of postharvest contamination in various food stuffs. Benzimidazoles, aromatic hydrocarbons and sterol biosynthesis inhibitors are some of the common antifungal chemicals being used but however, these chemicals subsequently leave residues which are toxic to humans and animals exposing them to much risk (Majica*et al.,* 2010). Application of these chemicals are mostly done or not done well which leads to fungal resistance. Many efforts have been made in recent years to set limits for use of these chemicals. There is therefore the need to

resort to natural antimicrobial and antifungal agents that will replace these chemicals to reduce or replace the use of synthetic chemicals.

Chemical Control of OTA: Varying the atmosphere during grain storage with CO_2 has been reported for OTA detoxification. However, the success of this method varies with the type of ochratoxigenic species (Magan and Aldred, 2007). Pateraki *et al*. (2007) adding sodium metabisulpite solution at low concentrations of (100 mg/l, 250 mg/l) led to increased OTA production by *A. carbonarius* whereas at 750-1000 mg/lit totally inhibited mycelia growth and OTA production irrespective of the water activity level. Similarly, a field application of cyprodinil and fludioxonil at various concentrations for 21 days before harvest drastically reduced the growth and contamination of grapes by the black *Aspergilla* before harvesting (Belli *et al*., 2007).

A natural polyene, Natamycin that acts by compromising the fungal membrane functions resulting in a leakage of ions and electrolytes; this has been reported as an effective agent in inhibiting the growth of ochratoxigenic fungi (Medina *et al*., 2007). Similarly, Amezqueta *et al*. (2008) reported the use of both sodium carbonate and sodium bicarbonate at different conditions of pressure, temperature and time for decontamination of OTA present on cocoa shells. Aqueous potassium carbonate (2%) at 1,000 lb/in^2 at 90°C for 10 min gave the highest inhibiting value.

It should be noted that the use of inadequate amounts of fungicides create stressful conditions which could stimulate the production of Ochratoxin in some species (Aroyeum *et al.,* 2007; Magan and Aldred, 2007). Fluazinam, procymidone, carbendazim have stimulating effects on OTA production of *A. carbonarius* (Medina *et al*., 2007; Battilani

and Pietri, 2002). Bleve et al. (2006) reported that fungicide which contains sulphur rather enhance the production of OTA.

Studies have also been carried out on the use of adsorbents materials such as activated charcoal, activated carbon, cholestyramine, potassium caseinate, sodium and calcium aluminum silicates, bentonites and wood fragments with variable results. Although the use of charcoal was relatively effective, as compared to others, poor product quality and animal poisoning makes it unfavorable for use (Amezqueta *et al*., 2009; Gambuti *et al*., 2005). Modified zeolites and insoluble vegetable fibers have also been reported as effective adsorbents for OTA decontamination of food (Dakovic *et al*., 2005; Tomasevic-Canovic *et al*., 2003).

In a related research by Jard *et al.* (2011) in Europe, maximum level for OTA in roasted coffee is set at 5µg/kg, an addition of sorbent agents, microorganisms or enzymes in feed is able to detoxify mycotoxins have been reported to be reliable in the prevention of mycotoxins in feeds. Agents such as aluminosilicate clays and bentonite have been used as binding agents in reducing aflatoxin intoxication in pigs (Schell *et al.,*1993), cattle (Diaz *et al.,* 1997) and poultry (Scheideler, 1993) without causing any dietary problems, however, care must be taken when mixing with aflatoxin-contaminated feed as the clays can change the nutritional composition thereby binding with traces of minerals and vitamins to reduce their bioavailability to further produce dioxins (Devegowda and Castaldo, 2000). The use of esterified glucomannan agent, a naturally-occurring organic compound in yeast is quite preferred over clay in reducing the toxicity of Aflatoxin has been investigated.

Moreover, the interaction between microbial species influences the extent of ochratoxigenic fungi and OTA production in a substrate. A report by Lee and Magan (1999) in his research work on the ability of *A. ochraceus* to colonize maize and produce mycotoxins is primarily determined by its competitive abilities relative to other species. According to Valero *et al.* (2006), the reduction of OTA amount may be due to the control of the growth of OTA-producing fungus, hostile fungi consumption of specific nutrients that are required for OTA synthesis and the OTA degradation by other fungi.

Furthermore, some plants have been proven to inhibit fungal growth and the production of OTA; for example, essential oils of thyme and anise (500 ppm), cinnamon (1000 ppm) and spearmint (2000 ppm) are known to inhibit*A. ochraceus* growth. One percent (1%) oils of thyme and anise and 2% oil of cinnamon can totally inhibit OTA production in wheat (Soliman & Badeaa 2002). Fungicidal activity of thyme essential oil against *A. ochraceus* and *P. verrucosum* has also been investigated by Nguefack *et al.* (2009). Cinnamon essential oil was very effective against *A. niger*, likewise garlic bulb extract completely inhibited the growth of *A. ochraceus* (Singh *et al,* 2007*) and* Reddy *et al. (*2007*).*

Natural agents: There have been emerging issues with chemical residues in food products directly and indirectly leading to various diseases that are harmful to both humans and animals. Essential Oils and natural agents may serve as an alternative to chemical control agents because they contribute a rich source of bioactive compounds (Burt, 2004) which is able to reduce the environmental risk, increase the shelf life, safety of food products and satisfy the consumer's request. The 8th Edition of the French Pharmacopeia (1965), discusses that essential oils are products of complex general composition that is composed

of volatile values present in plants during their preparation. Structures of Essential oils comprise of a complex mixture of several compounds. They mostly contain chemical constituents that are terpenoids, as well as monoterpenes, sesquiterpenes and oxygenated products, characterized by low molecular weights. Terpenes are the most active antimicrobial compounds of essential oils among the group (Bakkali *et al.,*2008). Detoxifying contaminated food by microorganisms, several methods have been developed; nonetheless, few of them have been accepted for practical use. Many scientists are of the view that the best method for decontamination should be degradation by particular microorganisms. Significant interest has developed in recent years on the preservation of grains by the use of spice essential oils or spice powder to effectively inhibit the growth and mycotoxin production (Bullerman *et al.,* 1977). Fasoyiro *et al.* (2007) in their study reports the properties of preliminary phytochemical compounds in *A. danielli* seeds and their antimicrobial properties. In a related study, *A. danielli* was used as treatment on soybean and cowpea (Fasoyiro, 2007). These spices are flavor impart plant materials and contribute greatly to the daily antioxidant intake in most foods (Carlsen *et al.,* 2010). They are derived from different parts of specific plants such as the plants' barks, flowers, roots, seeds and fruits. *A. danielli* seeds are smooth, shinning olive-brown in appearance, with a turpentinelike taste and they are used medicinally. This spice has been shown to induce health benefits and have been proven to counter oxidative stress in vitro and in vivo (Adefegha *et al.*,2012).

The nutritional profile of *A. danielli* and the essential oils of the seed had been reported by Adegoke *et al.,* (2004, 2003). Also the antimicrobial activities of the crude extracts of *A. danielli* against a number of micro-organisms have been reported (Fasoyiro *et al*., 2001).

Furthermore, owing to chemical and environmental risk associated with the use of chemicals Aroyeum and others in their study on “Potential of *A. danielli* spice powder in reducing Ochratoxin A in cocoa powder” used *A. danielli* spice powder in reducing and preventing the growth of OTA in cocoa bean. Similarly, boldo, poleo and clove oils have been used to inhibit the growth of *A. niger and A carbonarius* by affecting the OTA biosynthesis pathway. In a related study by Pasteur *et al.* (1995) essential Oils from thyme and oregano have been found to be effective fumigant against fungi that attacks grains (Aroyeum *et al.,* 2007; Pasteur *et al.,* 1995).

Bluma and Etcheverry (2008) screened about forty-one (41) aqueous and ethanolic extracts from plant which has essential oils having a broad spectrum of antifungal characteristic against *Aspergillus* and *Flavi* strains. They further concluded that boldo, poleo, clove, anise and thyme oils could be a possible antifungal agent. In their studies, essential oils selected for antifungal effect has been added to the media followed by diffusion. Furthermore, in contaminated cocoa beans Aroyeum et al., (2007) used essential oils of *A. danielli* to reduce OTA contamination levels in spiked cocoa powder and obtained a decline effectiveness of 64-95%. Aroyeum *et al.* (2007) described the potentials of *A. danielli* spice in reducing OTA in cocoa powder and found that the powder of *A. danielli* is capable to be used as a bio-preservative with a maximum concentration of 60,000 ppm in cocoa powder contaminated with OTA. Also Adegoke *et al.* (2007) stated that during processing, DaniellinTM can completely reduce the level of OTA in non-alcoholic beverage. Postharvest measures for preventing OTA from entering the food chain have been documented. Maintaining the appropriate level of gases in the atmospheres where cereals and other foodstuffs are stored can help prevent OTA production. Paster *et al.* (1995) established that OTA production by

A. ochraceus was totally inhibited by the presence of 30% CO_2. This practice can be adopted by developing countries. Another method is using a combination of cleaning, scouring and removal of the bran and offal fraction as observed by Scudamore *et al*. (2004). An overall decline of about 75% of OTA was realized in white bread. Also, wet- milling, according to Wood (1985), produced 96% and 49% reductions of OTA in the germ and grits of corn, respectively.

The influence of roasting on the reducing OTA levels has been examined by Van der Stegen *et al*. (2001) asserting that OTA was fairly stable in heat processing, subsequently reducing OTA up to 90% found during coffee bean roasting. Nehad *et al*. (2007) similarly found that roasting is able to reduced 30μg/kg of OTA by 31% and filtering reduced OTA by 72% with a final coffee temperature of 204 ^{0}C. In a research conducted by Romani *et al.* (2003) they obtained a reduced percentage of more than 90 % of OTA. Nonetheless direct removal of damaged coffee has also been found to effectively reduce OTA contamination (CIRAD, 2012).

To sustain the management of mycotoxins throughout the food chain, the following procedures have been suggested. These are complying with Hazard Analyses Critical Control Point (HACCP) standards; having good agronomic practices through transportation and up to consumption; best practices for harvesting, drying and storage of agricultural products coupled with effective insect and pest management; adoption and sustainability relevant food safety education and careful and systematic enforcement of legislation on food safety

In a study on the occurrence of OTA in Nigerian kola nuts, the researchers sought to quantitatively obtain data on the OTA concentrations of randomly selected kola nut samples (Dongo *et al.,* 2008). These researchers used ELISA technique for OTA analysis and further analyzing the data analysis using SPSS. They concluded that the samples of kola from the open market in Ibadan, Nigeria were contaminated by OTA. Fasoyiro *et al.* (2007) in characterizing the antimicrobial property of *A. danielli* extract. Using a method of as described by Chang *et al.* (1977) for the extraction using petroleum ether, the extract was then quantified by the thin layer chromatography (TLC) and was further fractionated using vacuum liquid chromatography as described by Odukoya *et al.* (2007). After the study, the findings were able to highlight on some of the phytochemicals present in *A. danielli* spice as alkaloids, carotenoids and polyphenols which could possibly exist as glycosides. In addition, the antimicrobial properties of the extracts and fractions showed higher activities towards gram positive bacteria. They finally concluded that there is possibility of the use of *A. danielli* spice as an agent in reducing the incidence of food spoilage and food toxins. In their further study in the preservative property of *A. danielli* fractions in stored grains (maize, cowpea and soybean), the preservative property with the extract were determined by reduced percentage infestation with increased concentration of the extract. There was a reduction of up to 47% in infestation in maize, 53% in soybean and 57% in cowpea. Adriana *et al.* (2014) used four (4) different commercial essential oils to inhibit mycotoxins in food, in their research they used microbial strains and controlled growth conditions, agar disk diffusion assay to screen the essential oils and later used ANOVA to statistically analyze the results. The results of the study showed that these oils could be used as food preservatives in some food products in which *A. flavus, A. niger and*

A. ochraceus growth and potential production of mycotoxins are considered health hazards. Further research is needed to ascertain the levels of the essential oils needed to inhibit the fungal growth.

In their work, Aroyeum *et al*., (2007) research, *A. danielli* powder was used in the reduction of OTA in cocoa powder, ELISA method according to Teren *et al.* (1996), HPLC isocratic system with C18 column and fluorescence detector was used. The powder was treated with different concentrations of the *A. danielli* spice w/w. From the study, the presence of OTA in cocoa powder was detected at the storage houses at different concentrations. Favorable conditions for growth of OTA producing molds in storage houses were responsible for the prevalence of OTA. This confirms the earlier study in the literature herein about poor storage conditions giving rise to the incidence of OTA in stored food stuffs. With the hygroscopic nature of the cocoa powder tied with high relative humidity and high temperature at the warehouse, which are principal factors responsible for OTA contamination.

Treatments of OTA contaminated cocoa powder with natural spice *A. danielli* significantly reduced OTA to a level accepted at the EU. According to the paper there was a desired positive effect on the nutritional and the sensory values of the cocoa beverage up to 60,000ppm and above which the organoleptic score became lower and acceptability also reduced. It was concluded that treatment of contaminated cocoa powder should not exceed 60,000ppm as quantities above this level will make the cocoa powder give a bad taste and a dull color.

2.8 Future outlook

Physical control of ensuring low moisture content in food products and also maintaining appropriate level of gases in the atmosphere in stored foods inhibit the production of OTA. Similarly, employing HACCP technique can help inhibit the production of OTA. Subsequently, chemical agents such as cyprodinil, fludioxonil, aluminosilicate clay, bentonite, sodium carbonate and sodium bicarbonate have been used at different levels have shown to decontaminate the OTA in cocoa prior to harvesting. On the contrary, research has indicated that sulphur based fungicides should be avoided since they enhance the production of OTA.

Natural spice powder (*A. danielli*) and essential oils are natural agents that contain the complex mixtures of several compounds known as terpenes and monoterpenes which have been reported of its detoxifying ability to inhibit OTA. Previous research has investigated the effectiveness of *A. danielli* in finished and semi- finished products of cocoa at different levels of treatment. Whereas chemical inhibition is done at the pre-harvest stage, prevention at the bean stage has yet not been investigated; rather molded beans are discarded reducing the yield in cocoa production. The gap between post-harvest and initial bean processing stage control activity have been identified. Likewise, the impact of pH on OTA secretion by OTA producing strain is not well known (Kapetanakou *et al*., 2009). In this regard, the current research work is poised at investigating the level of effectiveness of *A. danielli* at the bean stage before processing to reduce waste and subsequently reduce or inhibit prevalence of diseases associated with the consumption of contaminated food products. This paper seeks to report on the findings of the effect of powder extract of *A. danielli* seeds on cocoa beans.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Cocoa Pods: Bruised and damaged cocoa pods were collected randomly from farmers at Akim Tafo in the Eastern Region of Ghana.

Defatted Aframomum danielli powder (DADP)**:** seeds were purchased from a local market in Tema. The seeds were cleaned of the extraneous materials. The seeds were dried in an oven and milled into powder using a hammer mill. The seeds were milled to separate the seed coat and the endosperm. The seeds were later extracted with petroleum ether to obtain the essential oil, using Soxhlet extraction. The residues (powder) after extraction were further air-dried at room temperature and reserved for use after milling. The powder was then sieved with a wire-mesh to obtain fine powder and stored at room temperature for use.

Reagents

Key reagents used for the extraction of OTA and running of its HPLC determination included methanol, magnesium sulphate, acetonitrile, acetic acid and sodium chloride, which were all purchased from Park Scientific Limited(UK). Standard solution of Ochratoxin A at 1000 ng/ml was obtained from R-Biopharm Ltd(UK). A working standard solution of 100 ng/ml was obtained by diluting 10 ml of the stock solution up to the 100 ml mark of the volumetric flask using a solution of glacial acetic acid: acetonitrile at a ratio of 2:98.

3.2 METHODS

3.2.1 Research Design

Statistical Methods: The design was based on one factor response surface quadratic design and the mass of the *A. danielli* was varied from 5 g to 250 g per 5000 g cocoa bean in triplicates. A total of forty- five (45) experimental samples including control were run as presented in the Table 1.

The data obtained was loaded into the statistical tool (Design Expert, 2014) and run to generate regression parameters and Analysis of Variance (ANOVA). Data obtained were initially fitted to models that could explain the behavior of the treatment factor (mass of *A. danielli*) and the responses over the design space. This involved studying coefficients as the regression- R^2, the standard deviation, adjusted regression (adj. R^2), prediction regression (pred R^2) and adequate precision (adeq precision) of the models selected. The p-values for the regression models of the factor of extraction were tested against $p < 0.05$.

The effect of the *A. danielli* was performed to predict the optimum level of effectiveness. Statistical significance of the terms in the regression model was examined by analysis of variance (ANOVA) for each response. To evaluate the goodness-of fit of the models, regression (R^2), F-value, the derived p- values and coefficient of Variance (CV) were evaluated. The lack-of-fit term was also used to judge the adequacy of the model.

Treatment: The cocoa pods were further bruised and exposed to mold growth for seven days. Afterwards, pods were opened using to remove the beans. The beans were mixed together to ensure the spread of the molds. About 5000g of the cocoa beans were weighed in triplicates. The previously defatted *A. danielli* were weighed between 5 g and 250 g

(w/w) and mixed thoroughly with the triplicates of the cocoa beans and fermented using the box fermentation process. After seven (7) days of fermentation, the beans were dried under humid conditions for another seven (7) day. A total of forty- five samples were obtained including the control sample and were analyzed for OTA.

3.2.2 Extraction and Determination of Ochratoxin A

Dried cocoa beans were milled in a hammer mill (AWF23, China) to attain a homogenous (2-micron size) powder. The OTA extraction was performed using the QuEChERS protocol for mycotoxins determination in dry seeds by Yogendrarajah *et al.* (2013). Subsequently 1.0 g accurately weighed cocoa powder sample was transferred (fig 3.1-3.3) into a 50 mL extraction tube. Five (5) ml of deionized water was added and vortex using the vortex mixer (SA7, Stuart) for 1 min. The mixture was allowed to stand for 10 to 15 min (Fig. 3.4). Again 5ml of 1% of acetic acid in Acetonitrile was added and vortexed for another 1min. The mixture was agitated vigorously using a Thomas scientific orbital shaker (SSL2, UK) at 400rpm for 10 min. The mixture was centrifuged at 4000rpm for 10min. The supernatant Acetonitrile layer was taken and ultra- centrifuged at 10000rpm for 3 min. The supernatant was evaporated to dryness at 40 °C using a rotary evaporator (Buchi, German). And also under a stream of N_2, the dry residue was re-dissolved in mobile phase (0.3 mL).The resulting aliquot was dried at 40 °C under vacuum using rotary evaporator or nitrogen gas, it was reconstituted in 2ml of appropriate solution or mobile phase and 100ul of the extract was injected into the HPLC.

The recovery test was performed by spiking cocoa powder samples with 0.5ng/ml, 0.2 ng/ml and 0.1 ng/ml OTA standards. Exactly 1g of the milled cocoa samples was spiked with 1ml of

the OTA standards. The samples were extracted after standing for 15 min. The spiked samples and blank samples without standard OTA were then extracted and analyzed by HPLC as done earlier. Recovery was calculated using the formula below.

$$\% \text{ Recovery} = \frac{\text{Amount of analyte recovered}}{\text{Amount of analyte spiked}} \times 100$$

3.2.3 Determination of OTA

A 100 µl of the extract was injected into the HPLC and the column held at a temperature of 40°C. The mobile phase was a mixture of Acetonitrile /water/glacial acetic acid (55:43:2, v/v) and the column used was a Mediterranean SEA18 5µm (25 cm x 0.46 mm) and the flow rate was 1 ml/min. For creating calibration curve five calibration points were obtained from 2 ng/ml, 4 ng/ml, 6 ng/ml, 8 ng/ml, 10 ng/ml and 20 ng/ml concentrations. The separation was performed using isocratic mode at excitation and emission wavelengths of 333 mm and 460 nm respectively. Standard curve was plotted from the peak areas against concentrations. The peak of OTA was identified by comparison of the retention time with that of the standard and the concentration was obtained by extrapolation from the calibration curve and expressing in µg/kg using the formula below.

$$\text{Concentration in } \mu g/kg = \frac{M_{OTA} \times V_1 \times V_3 \times D}{M_s \times V_2 \times V_4}$$ where,

M_{OTA} is the mass of OTA in aliquot of test solution injected into the column in µg, M_S is the Mass of sample extracted in g, V_1 is the Volume of extraction solvent in ml, V_2 is the Volume of test solution used for purification in ml, V_3 is the Volume of test solution in ml, V_4 is the Injection volume of test solution in ml, D is the Dilution Factor.

Figure 3.1 is an HPLC chromatogram showing the retention time of the OTA standard at 20 ppb while Fig. 3.2 shows the calibration curve of the OTA standard at same concentration. The Retention Time for OTA was 6.20 min while the limit of detection (LOD) and the limit of

quantification (LOQ) were 0.3 ng/g and1.1 ng /g, respectively. Percentage recoveries of the various analyte ranged from 84 to 105%.

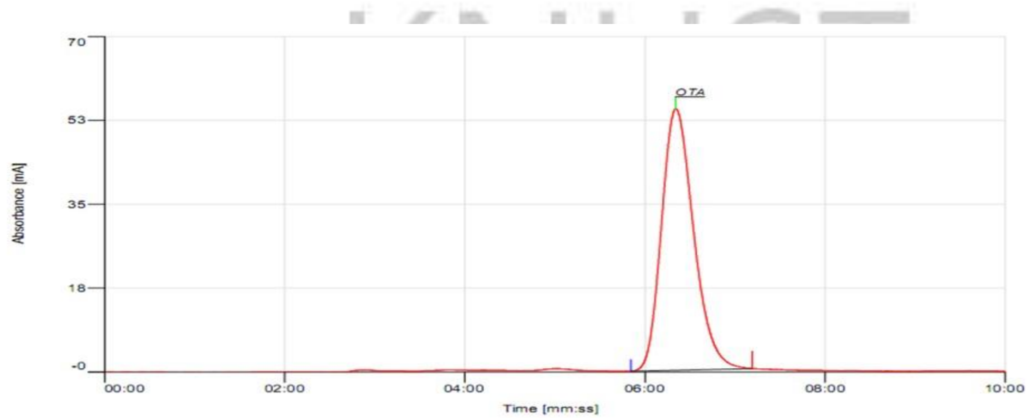

Figure 3.1 Chromatogram showing the retention time of the standard OTA

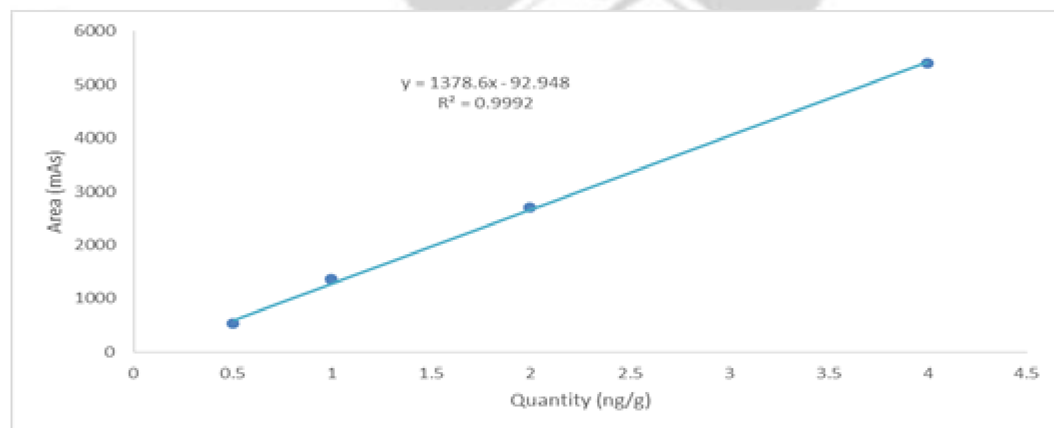

Figure 3.2 Calibration curve of OTA

3.3 DETERMINATION OF FAT CONTENT

A 50g of accurately weighed cocoa beans was peeled and milled using a hammer mill (as above) to 2 microns to attain a greater surface area for extraction. Subsequently, 5g (A) of the grounded cocoa beans (cocoa masse) was weighed into an extraction thimble. An AOAC (2007) method of analysis of extraction of fat was used. A 500ml extraction flask (round bottom flask) was accurately weighed (C) with the flask containing a few glass beads or boiling chips

(carborundum), it was then filled with approximately 300 ml of petroleum ether. The fat was extracted using the sample contained in the thimble with petroleum ether for at least 80 cycles in a minimum of 4 h in a Soxhlet extraction apparatus (Buchi, German). Upon completion of the extraction, the petroleum ether was evaporated using a rotary extractor (Buchi, German). The flask with its content was put in a mechanical convection oven at 100 - 102 °C (Gallenkamp, UK) until a constant weight was obtained. It was subsequently cooled to room temperature in a desiccator (Fisher, UK). The flask with the fat extract was weighed accurately (B).

$$\text{Fat content} = \frac{100(B - C)}{A}$$

A is the sample weight, B is the weight of the Round bottom flask and the extracted fat and C is the empty round bottom flask before extraction

3.4 FREE FATTY ACID (FFA) DETERMINATION

A 2g of the extracted fat (above) was accurately weighed with a digital scale (Mettler Tolledo, UK) and dissolved with 50ml of ethanol/ diethyl ether solution 1/1 (v/v) into a 100 ml beaker. The mixture was titrated with a standardized 0.1M ethanolic sodium hydroxide (NaOH) using phenolphthalein indicator to obtain an end point color pink. The percentage FFA as oleic was then computed.

$$\text{Free Fatty Acid (\% FFA)} = \frac{282 \times V \times C}{10 \times m}$$

V is the volume of standardized NaOH used for titration, m is mass of oil, C is the concentration of NaOH solution, 282 is the molar mass of oleic acid and 10 is the mass of the sample.

3.5 pH DETERMINATION

The pH determination was carried out based on the ICA Analytical Method 15. An amount of 10 g of the previously milled cocoa nibs was weighed into a beaker with a digital scale. A 90 ml of boiling water was added and stirred. The mixture was then filtered. The filtrate was cooled to 20-25 ºC. The pH was immediately determined using a calibrated pH- meter (GONDO Company Ltd, USA).

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CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Effect of Defatted Aframomum danielli powder (DADP) on Residual Ochratoxin A (rOTA) in cocoa beans

There was a reduction in the amount of OTA level in the cocoa beans. From an initial treatment of 5 g (w/w) of DADP to 5000 g cocoa beans with a resultant OTA of 2.43 ng/g to 0.61 ng/g at treatment level of 250 g (w/w) of DADP (Table 4.1). This shows a percentage reduction of approximately 74.90 %. The regression model obtained for the degree of effectiveness of DADP on the rOTA was: $Y_m = \beta_0 - \beta_1 X_1 + \beta_2 X_2$, where β (0-2) were the coefficient of variations and X (1-2). However, the model obtained for the degree of effectiveness of the DADP was significant ($p < 0.05$). This implied that there was a significant reduction in the rOTA amount for the levels of DADP treatments on the cocoa beans. The model employed was significant ($p<0.05$) as far as the inhibition was concerned (Table 4.2).

Table 4.1 Summary of Response surface model experimental design of treatment levels and the corresponding responses.

Run	Treatment factor	Response Variables

	Mass of *A. danielli* (g/5000g)	**OTA (ng/g)**	**%FFA**	**pH**
Control	0	5.66	3.09	6.71
1	5	2.43	2.40	7.10
2	10	2.48	1.97	7.23
3	15	2.05	1.51	7.20
4	20	1.95	1.81	7.06
5	25	1.39	1.91	7.09
6	35	1.23	1.88	7.48
7	40	1.12	1.59	7.52
8	45	0.93	1.51	7.34
9	50	0.95	1.49	7.38
10	60	0.71	1.36	7.01
11	75	0.62	1.45	7.12
12	100	0.61	1.24	6.90
13	150	0.61	1.09	6.93
14	250	0.61	1.26	7.15

Table 4.2 ANOVA for response surface of the model component for the Residual OTA response for the treatment conditions of the cocoa beans

	Sum of Squares	df	Mean Square	F Value	p-value
Model	4.29	2	2.15	111.33	0.0001*
Mass of *A. danielli* (A)	0.25	1	0.25	12.74	0.0091*
A^2	0.72	1	0.72	37.24	0.0005
Residual	0.13	7	0.02		
Cor Total	4.43	9			

Significant *

Table 4.3 Shows the predicted R-square of 0.93 which is in reasonable agreement with the adjusted R-Squared" of 0.96. According to Montgomery and Myres (2002) adequate precision measures the signal to noise ratio and when greater than 4 are desirable. Therefore, a ratio of 26.015 indicates an adequate signal and the model could be used to navigate the design space.

Table 4.3 Summary of the statistics of the analysis of variance for the specific treatment response of the DADP on OTA

Std. Dev.	Mean	C. V. %	PRESS	R-Squared	Adj. Rsquared	Pred. R-Squared	Adeq. Precision
0.14	1.48	9.36	0.30	0.97	0.96	0.93	26.01

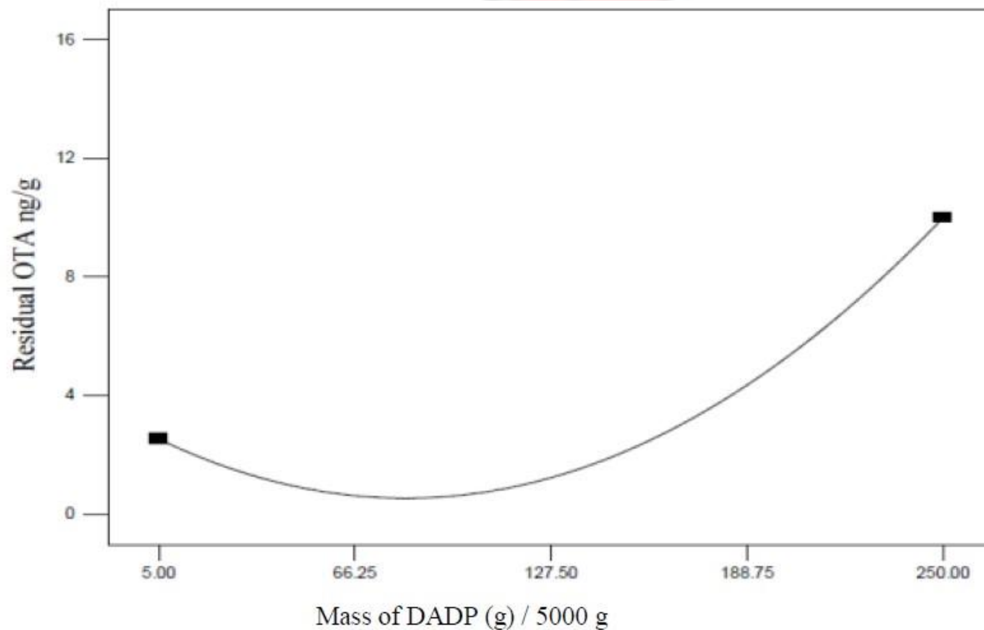

Fig. 4.1 Residual OTA (rOTA) resulting from the impact of defatted A. danielli powder (DADP) on cocoa beans

4.1.2 Effect of DADP on Free Fatty Acids (FFA) and pH in cocoa beans

The regression model obtained for the degree of response of the treatment of DADP on FFA and pH was: $Y_m = \beta_0 - \beta_1 X_1$ and $Y_m = \beta_0 + \beta_1 X_1 - \beta_2 X_1^2$, respectively, where β (0-2) were the coefficient of variations and X (1) is the response factor (DADP) that was varied. The model obtained for the degree of effectiveness of the DADP was significant ($p > 0.05$). This implies that there was a

significant effect of DADP on the FFA and pH for the levels of treatments, indicating that the model employed was significant as far as the inhibition was concerned (Table 4.4). The lack of fit of the model was however significant ($p>0.05$) for the degree of application.

Table 4.4 Summary of p- values of the responses

Response Factors	P- value
FFA	0.01
pH	0.02

Effect of DADP on FFA of cocoa beans

Table 4.1 shows the FFA of cocoa beans with varying DADP treatment. The chart in Fig 4.2 also illustrates the relationship between the level of DADP treatment and the mean FFA observed. The chart indicates decreasing values of FFA of cocoa as there was an increase in DADP treatment. It showed a steady decline in % FFA values with an increase in the mass of DADP. Therefore, an inverse relationship between levels of DADP treatment and FFA of cocoa beans indicating a significant reduction in FFA amount for the levels of treatment. Thus, the FFA content in the control was significantly higher than the treated. (Appendix 7). A percentage reduction of 47.5% was attained in the current research. Table 4.5 shows a predicted R-square of 0.41, measuring that it was in reasonable agreement with the adjusted R-Squared" of 0.57. According to Montgomery and Myres (2002), an adequate precision various (measures the signal to noise ratio) greater than 4 is desirable. Therefore, a ratio of 8.42 indicates an adequate signal and the model could be used to navigate the design space. A mean value of 1.72% was attained which is slightly below the international limit of 1.75% FFA content in cocoa beans.

Table 4.5 Summary of the statistics of the analysis of variance for the specific response of the DADP on FFA

Std. Dev.	Mean	C. V. %	PRESS	R-Squared	Adj. R-squared	Pred. R-Squared	Adeq. Precision

Table 4.6 Predictions

Coefficient Estimate	Standard Error	*df*	95% CI Low	95%CI High	Factor
Intercept	0.94	1	0.23	0.41	1.47
A-Mass AfraD	-1.07	1	0.30	-1.75	-0.38

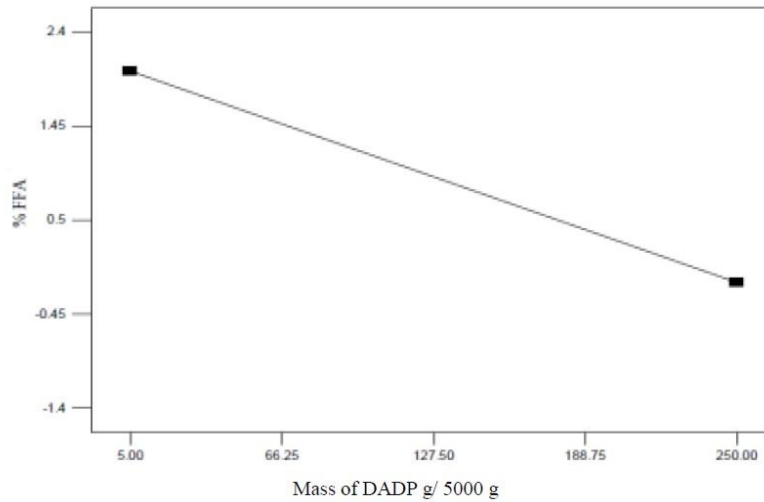

Fig 4.2 Effect of DADP treatment on Free Fatty Acid content of cocoa beans

Effect of DADP on pH of cocoa beans

The chart in Fig 4.3 illustrates the relationship between the level of DADP treatment and the mean pH observed. The chart indicates both increasing and decreasing values of pH of cocoa beans as the amount of DADP increases. An inverse relationship between levels of DADP and pH of cocoa beans therefore exists.

This implied that there was a reduction trend for the levels of treatments; the model employed was significant as far as the inhibition was concerned. The lack of fit of the model was however significant ($p < 0.05$) for the degree of application. A mean 7.21 pH was attained in the study which is higher than the international standard for cocoa bean (between 5.5 and 6.5).

Table 4.7 Summary of the statistics of the analysis of variance for the specific response of the DADP on pH

	Sum of Squares	df	Mean Square	F Value	p-value
Model	0.21	2	0.11	5.32	0.04*
Mass of A. danielli(A)	0.21	1	0.21	10.44	0.001*
A^2	0.19	1	0.19	9.60	0.0174
Residual	0.14	7	0.02		
Cor Total	0.35	9			

Significant *

Table 4.8 shows a predicted R-square of 0.24, which is not as close to the adjusted R-Squared" of 0.49 as one might normally expect. This indicates a large block effect or a possible problem with the model. Things to consider are model reduction, response transformation, outliners etc. However, according to Montgomery and Myres (2002) an adequate precision (measures the signal to noise ratio) value greater than 4 is desirable. Therefore, a ratio of 6.383 indicates an adequate signal and the model could be used to navigate the design space.

Table 4.8 Summary of the statistics of the analysis of variance for the specific effect of DADP on the pH

Std. Dev.	Mean	C. V. %	PRESS	R-Squared	Adj. Rsquared	Pred. R-Squared	Adeq. Precision
0.14	7.21	1.96	0.27	0.60	0.49	0.24	6.42

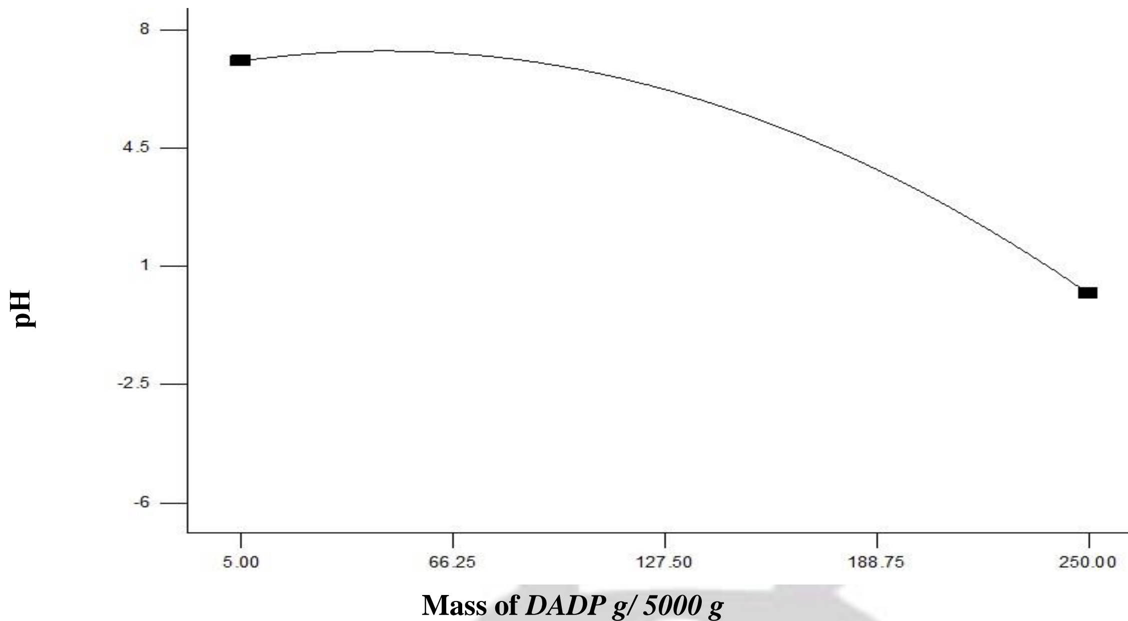

Fig. 4.3 Effect of DADP on pH of cocoa beans

4.2 RESULTS VALIDATION

Table 4.9 indicates a summary of constraints encountered by the model, applying 250 g of DADP to 5,000 g of OTA infested cocoa beans with OTA level of 5.66 ng/g; a resultant response of 0.61 ng/g residual OTA, a percentage free fatty acid of 1.235 and a pH of 6.9 will be attained. However, treating cocoa beans with high dosage of DADP led to a significant increase in OTA level in the cocoa beans as the pH increased as indicated in fig 4.1. Hence, a level of 65.79 g of DADP was selected above which the effectiveness of the DADP is rendered ineffective. Validating these results at 95% confident interval (CI) high and 95% confident interval (CI) low of the mass of DADP per 5,000 g of OTA infested cocoa beans at 5.66 ng/g, an expected results of OTA was 0.63 ng/g, pH = 7.30 and percentage FFA of 1.48 will be acceptable beyond which the quality of the beans will be decreased.

Table 4.9 Summary of Response surface model experimental design of the treatment constraints and possible solution.

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Mass AfraD	is in range	5	250	1	1	3
Res Ochra	minimize	0.61	2.48	1	10	5
FFA	minimize	1.235	2.395	1	10	5
pH	maximize	6.9	7.52	1	1	3

Solutions

Number	Mass AfraD	Res Ochra	FFA	pH	Desirability	
1	65.79	0.63	1.48	7.30	0.25	Selected

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	Mass *A. danielli*	65.79	5	250	0	Actual

Response	Prediction	SE Mean	95%CI low	95% CI high	SE Pred	95%PI low	95%PI high
Res Ochra	0.63	0.08	0.49	0.81	0.16	0.25	1.00
FFA	1.48	0.10	1.25	1.70	0.24	0.92	2.03
pH	7.30	0.08	7.12	7.48	0.16	6.92	7.68

4.2 DISCUSSION

4.2.1 Dynamics of OTA in cocoa beans

Figure 4.1 shows a graph of mass of DADP against residual OTA in cocoa. From the research undertaken, it was observed that the presence of the DADP effectively inhibited the

growth and reduced the OTA prevalence in the cocoa bean. This confirms the antimicrobial property of terpenes and monoterpenes which are in *A. danielli* as reported by Bakkali *et al.* (2008) and Tian *et al.* (2012). Fasoyiro *et al*. (2001), in their research study indicated a similar work on antimicrobial effectiveness of the crude extracts of *A. danielli* against a number of micro-organisms.

In the current research work a treatment level of 5 g (w/w) of the DADP resulted in a residual Ochratoxin A level of 2.43 ng/g, percentage reduction of 74.90% and 89.22% in the control sample as per the untreated cocoa beans. A similar research done by Aroyeum *et al.* (2011) on spiked cocoa powder with samples containing greater than 2ng/g of OTA were treated with powder of *A. danielli* at 0 ppm, 40,000ppm, 60,000ppm and 80,00ppm of the powder (w/w). The samples were later analyzed for OTA using high performance liquid chromatography with fluorescent detector and enzyme linked immune-sorbent assay. Their study established the presence of OTA in 71.7% of all samples examined. In all the samples, OTA of 0.15-0.50ng/g was the commonest followed by 0.51-1.6ng/g. Only four out of five samples had > 2ng/g OTA. Treating cocoa powder with *A. danielli* spice powder resulted in 65% reduction of OTA at 40,000ppm and at 80,000ppm. It further reduced the OTA to1.86 ng/g. At 80,000ppm of the spice powder treatment, the taste became bitter, color became duller and general acceptability became lower. They concluded that *A. danielli* spice powder could be used as a bio-preservative in cocoa powder contaminated with Ochratoxin A at maximum concentrations of 60,000ppm above which the sensory quality decreased. Although they succeeded in reducing the level of OTA in the cocoa powder, the percentage reduction was lower than the results attained in this research work. Also, in the current research the treatment was applied at the cocoa

beans before the fermentation, whereas in the work of Aroyeum *et al.,* (2011) the treatments were done to the semi processed stage. The effectiveness can also be due to the stage at which the treatment was done.

4.2.2 Influence of DADP treatment on FFA in cocoa beans

The effect of DADP as shown in the chart in Fig 4.2 illustrates the relationship between level of DADP treatment and the mean FFA observed. It was observed that there was a considerable reduction in the percentage FFA, indicating a positive effluence of the DADP on the quality attributes of the cocoa beans. This could also be attributed to the presence of the antimicrobial agent (DADP) activity in the sample decreasing the water activity in the sample, hence, preventing the growth of mycotoxins in the sample. As moisture content increases there is a release of carbonyl acids in the triglycerides facilitating the development of lipase or oxidation of the fat leading to free radical of fatty acid formation as reported by Selamat *et al*. (1996).

4.2.3 Effect of DADP treatment on pH of cocoa beans

Figure 4.3 also illustrates the relationship between level of DADP treatment and the mean pH observed. It was observed from the current study that there was no clear relationship between the presence of OTA and pH affirming the research done by Kapetanakou (2009), (Kapetanakou *et al.,* 2009). On the contrary, Bandelin (1958) reported a progressive loss of antifungal activity with increasing pH values to the point that antifungal agents were totally ineffective in the neutral to alkaline range. Upon this background the results attained by this work could be explained that as the pH of the sample increased it rendered the DADP ineffective, hence increasing the residual OTA in the sample as observed in fig. 4.1.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The study determinedthe effectiveness of defatted *A. danielli*powder (DADP) in the treatment of OTA in cocoa beans. Baring the conditions at desirability of 0.25 at an optimum treatment level of 65.79 g (w/w) of *A. danielli* per 5000 g, a residual OTA of 0.63 ng/g, FFA of 1.48% and a pH of 7.30 will be attained.

5.2 RECOMMENDATION

- Cocoa farmers should be educated on the need to observe good agricultural practices. Good Agricultural Practices (GAP) has been shown to reduce the growth of on mycotoxins.

- Also farmers should be educated on the application of *A. danielli* powder on cocoa beans from bruised and molded cocoa pods to help inhibit the growth of OTA.

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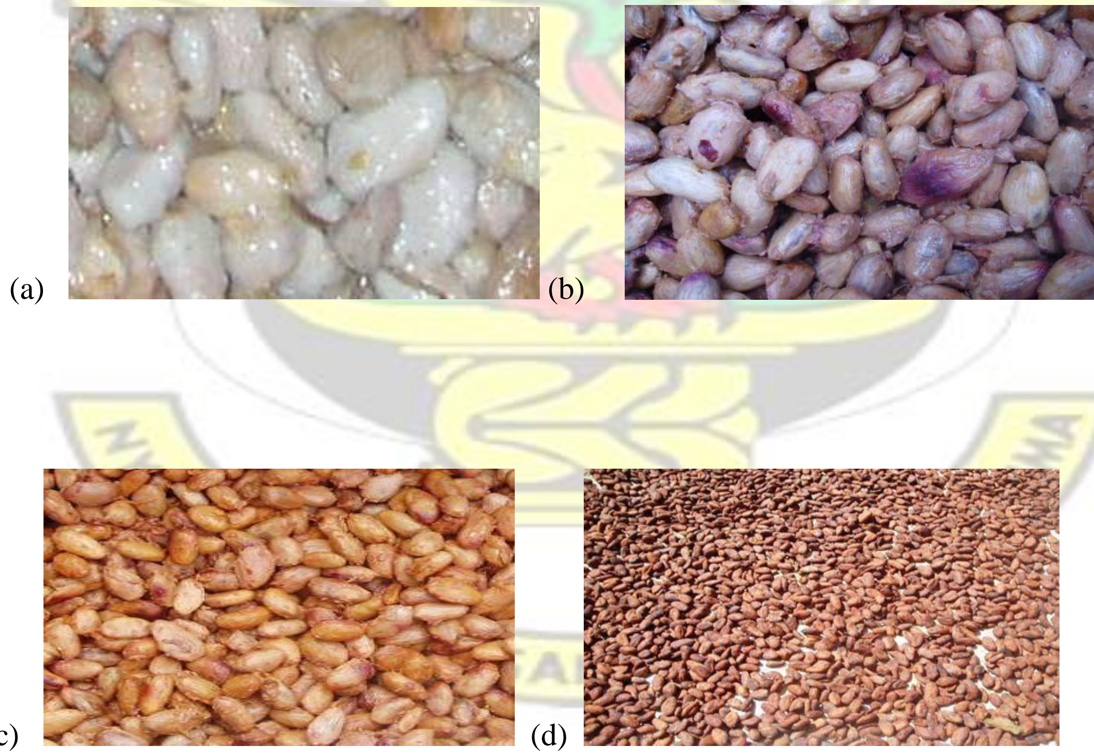
APPENDICES

Appendix 1: Harvesting of cocoa pod

Appendix 2 Bruised cocoa pods



(a) (b)

(c) (d)

Appendix 3: External appearances of cocoa beans before, during and after fermentation (a) Fresh beans; (b) Day 2 of fermentation; (c) Day 5 of fermentation & (d) Dry beans

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Appendix 4 Treated cocoa beans before fermentation

Appendix 5 Drying of Cocoa Beans Appendix

Appendix 6 Treated dried cocoa beans and masse

Appendix 7 OTA Extraction using QUECHERS method of toxin extraction Appendix 8. Effect of A. danielli on Residual OTA

Treatment	Residual Ochratoxin A ng/g			
	A	B	C	Mean
Control	5.73	5.59	5.66	5.66
5g	2.44	2.41	2.43	2.43
10g	2.47	2.49	2.48	2.48
15g	2.06	2.03	2.05	2.05
20g	1.96	1.94	1.95	1.95
25g	1.43	1.36	1.39	1.39
35g	1.26	1.20	1.23	1.23
40g	1.10	1.15	1.12	1.12
45g	0.89	0.98	0.93	0.93
50g	0.93	0.97	0.95	0.95
60g	0.71	0.72	0.71	0.71
75g	0.63	0.61	0.62	0.62
100g	0.61	0.62	0.61	0.61

150g	0.61	0.62	0.61	0.61
250g	0.61	0.61	0.61	0.61

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Appendix 9 Effect of *Aframomum danielli* treatment on the pH

Treatment	pH			
	A	B	C	Mean
Control	6.69	6.71	6.72	6.71
Control 0g	7.10	7.10	7.11	7.10
5g	7.19	7.25	7.26	7.23
10g	7.20	7.20	7.21	7.20
15g	7.06	7.04	7.08	7.06
20g	7.08	7.10	7.08	7.09
25g	7.45	7.50	7.48	7.48
35g	7.52	7.51	7.52	7.52
40g	7.34	7.35	7.35	7.34
45g	7.35	7.30	7.38	7.38
50g	7.00	7.01	7.01	7.01
60g	7.10	7.14	7.12	7.12
75g	7.01	7.01	7.01	7.01

100g	6.92	6.94	6.92	6.93
150g	7.15	7.17	7.16	7.15
250g				

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Appendix 10. Effect of *A. danielli*Treatment on FFA

Treatment	Percentage Free Fatty Acid %			
Control	A	B	C	Mean
Control 0g	3.20	3.10	2.98	3.09
5g	3.60	3.40	3.56	3.52
10g	2.96	2.78	2.91	2.88
15g	2.58	2.72	2.51	2.60
20g	2.05	1.98	1.81	1.95
25g	2.03	1.91	1.91	1.95
35g	2.03	1.78	1.88	1.90
40g	1.43	1.64	1.59	1.55
45g	1.31	1.09	1.57	1.32
50g	1.41	1.40	1.49	1.43
60g	1.54	1.18	1.36	1.36
75g	1.31	1.29	1.25	1.28
100g	1.01	1.25	1.24	1.17

150g	0.98	1.06	1.22	1.09
250g	1.01	0.81	1.06	0.96

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Chromatogram Report

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Run Time [mm:ss]	10:00.0	Sample Rate [point/s]	12.500	Readings	7500
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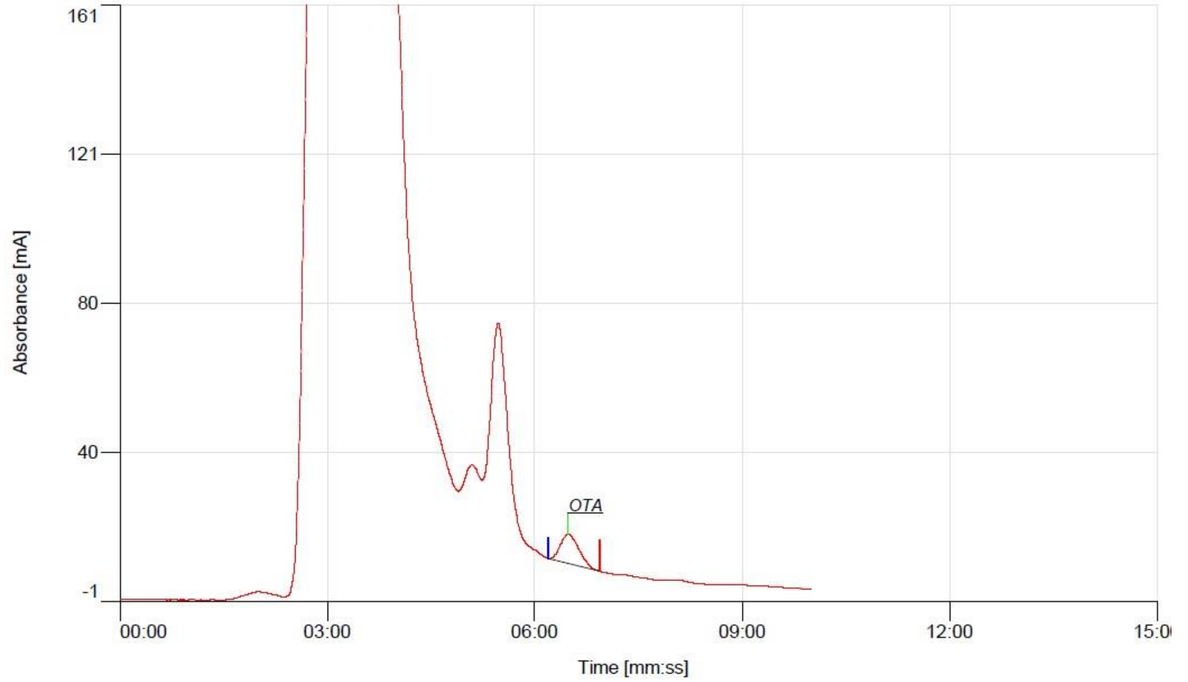
Detector Offset	0.0000	Sample Name	Sample014/1	Method ID	5A8420FDE2403270v53
Method Name	Waters_uBondpack C18_...	Amount / Final Vol.	1.000 / 1.000	ISTD Conc.	1.000



Created: 2/5/2016 7:32 AM, Modified: 2/26/2016 4:06 PM, Printed: 2/26/2016 16:07, User: Mycotoxin Laboratory PowerStream v4.2 (Build 20255)

Chromatogram Report

Run Time [mm:ss]	10:00.0	Sample Rate [point/s]	12.500	Readings	7500
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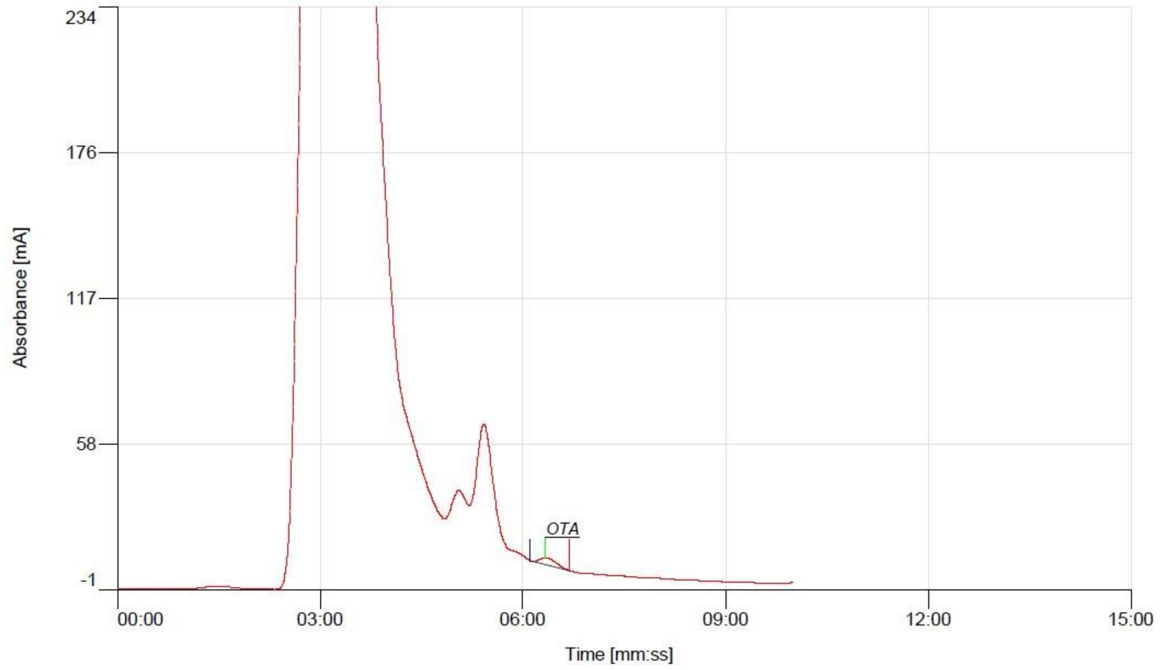
Detector Offset	0.0000	Sample Name	Sample022/1	Method ID	5A8420FDE2403270v46
Method Name	Waters_uBondpack C18_...	Amount / Final Vol.	1.000 / 1.000	ISTD Conc.	1.000

Manual Baseline



Chromatogram Report

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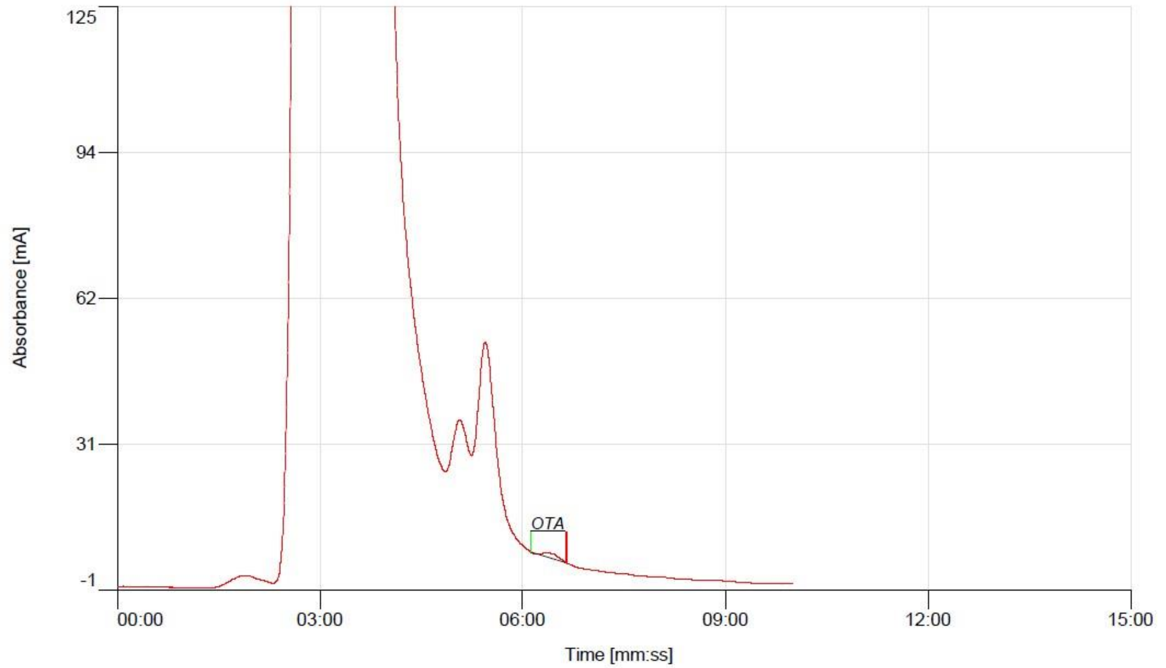
Method Name	Waters_uBondpack C18_...	Amount / Final Vol.	1.000 / 1.000	ISTD Conc.	1.000

Manual Baseline

MC_OTA_250g (\2015 Mycotoxin Lab\Ochratoxin\ ) - C85DAF5727B2C5AB...



Chromatogram Report

Page - 1

Run Time [mm:ss]	10:00.0	Sample Rate [point/s]	12.500	Readings	7500
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Detector Offset	0.0000	Sample Name	Sample004/1	Method ID	5A8420FDE2403270v31
Method Name	Waters_uBondpack C18_...	Amount / Final Vol.	1.000 / 1.000	ISTD Conc.	1.000