OCHRATOXIN A LEVELS IN COCOA NIBS FROM WESTERN NORTH, WESTERN SOUTH, ASHANTI AND BRONG AHAFO, COCOA GROWING REGIONS OF GHANA

BY

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DECLARATION

I hereby declare that this submission is my own work towards the MPhil 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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DEDICATION

This dissertation is dedicated Mrs. Ophelia Jonfia – Essien for the love and support. I love you mummy, you are the best

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ABSTRACT

Ochratoxin A (OTA), a potent toxin is a secondary metabolite produced by filamentous fungi of the genera *Aspergillus* and *Penicillium* present in a wide variety of foodstuffs. Ochratoxin A can contaminate a wide variety of foods as a result of fungal infection in crops, in the field during growth, at harvest, in storage and in shipment depending on environmental conditions especially when they are not properly dried. As these toxicants can never be completely removed from the food supply, many countries have defined levels in food that are unlikely to be of health concern. 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 conform to international standards. Fifty seven (57) cocoa beans samples obtained from selected districts in four regions of Ghana were analysed using HPLC with a fluorescence detector. The range of concentrations obtained were 0.06 to 2.193 μg/kg (mean- 0.698) for Ashanti; 0.261 to 1.859 μg/kg (mean- 0.933) for Brong Ahafo; 0.186 to 1.557 μg/kg (mean- 0.928) for South western and 0.393 to 4.650 μg/kg (mean- 1.802) for North Western regions. From the results of the study, 93% of the samples had OTA concentrations below the draft standard of 2 μg/kg proposed by EU for cocoa beans and 7% had concentrations above the draft standard of 2 μg/kg proposed by EU. North Western region recorded the highest OTA level of 4.650 μg/kg for samples from Sefwi Kaase from North Western region and the lowest OTA concentration of 0.063 μg/kg was recorded for samples from Forikrom from the Ashanti region. The low levels of OTA detected in this study suggest that exposure of OTA to humans through consumption of cocoa beans from the areas under study is unlikely to be of health concern.

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ABBREVIATIONS

OTA	Ochratoxin A
AFs	Aflatoxins
WHO	World Health Organisation
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 onContaminants 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	Internationa Agency for Research On Cancer
HPLC	High Performance Liquid Chromatography
PLD	Fluorescence Detector
IAC	Immunoaffinity Column
SPE	Solid Phase Extraction

CA	Codex Alimentarius
ND	Not Detected
FAO	Food and Agricultural Organization
ICCO	International Cocoa Organization
GAP	Good Agricultural Practices
LOD	Limit of Determination
LOQ	Limit of Quantification

CHAPTER ONE

1. INTRODUCTION

1.1 The role of cocoa in Ghana's economy

Cocoa is an important cash crop in many tropical countries including Ghana. Ghana is the second largest producer of cocoa beans in the world. As at 2005, about 60% of the country's foreign income came from export of cocoa beans. Export prices of cocoa beans are dependent upon quality. High quality cocoa beans give Ghana a premium price on the world market and it is important to obtain high quality of the final cocoa product. Indications of good quality is whole, well ripe, well fermented and well dried beans (Mikkelsen, 2010).

The cocoa industry support education and human resource development and is essentially a continuing process. Cocoa serves as the major source of revenue for the provision of socio-economic infrastructure in the country (Anon, 1995). The industry employs about 60% of the national agricultural labour force in the country (Appiah, 2004) and is a source of income for approximately 1.5 million people in the country, including 800,000 to 1,000,000 small holder farmers. To these people, cocoa contributes about 70 to 100% of their annual household incomes. Cocoa Farmers Scholarship Trust has been established. The Trust awards 2,500 scholarships annually to children/wards of cocoa farmers in second-cycle institutions. In the last decade, at least 26,830 children have received scholarship awards from the Trust (Fofie, 2010).

Cocoa is a very important ingredient in a number of foods such as cakes, biscuits, child-foods, ice-creams and sweets. Cocoa beans, originating as seeds in fruit pods of the tree *Theobroma cacao,* are source of cocoa powder and come from Africa, Central and South America. Neither storage nor processing conditions of cocoa are strictly

controlled in these tropical countries, thus fungi contamination is possible at many critical points in the cocoa production chain (Aldred and Magan, 2004).

1.2 Mycotoxins in food

The increasing occurrence of mycotoxins in agricultural commodities and the subsequent impact on consumer health as well as on national and global trade is of major concern in both the developed and developing countries (Wu, 2005).

Mycotoxins are secondary metabolites of moulds and are of public health significance (Varga *et al.,* 2005). The prevention and control of mycotoxins is of utmost importance to farmers to reduce losses in sales, exporters to reduce consignment rejection and the teeming population to reduce food shortage. Mycotoxins of major relevance include fumonisins by *Fusarium* species, Aflatoxin B1 (*Aspergillus flavus*) and Ochratoxin A from *Penicillium* and *Aspergillus ochraceus* species (Tagne *et al*., 2000; Tjamos *et al.,* 2004; Varga *et al.,* 2005). Ochratoxin A (OTA) has been reported to be a dangerous, nephrotoxic, and carcinogenic mycotoxin (Vrabcheva *et al.,* 2004). Coffee, cocoa, tea, wine, barley and groundnuts are considered to be potential sources of OTA (Tafuri *et al.,* 2004). A major part of the OTA in cocoa beans is found in the shells (Bonvehi, 2004).

1.3 Ochratoxin A in food

Ochratoxin A has been reported in cocoa powder from Ivory Coast, Guinea, Nigeria and Cameroun up to 4.4mg/kg higher than the EU regulatory level Bonvehi, (2004), and Tafuri *et al*., (2004) established that about 22% of cocoa powder sold in Italian shops contained OTA. Undoubtedly prevention of contamination at the source is the best procedure of controlling the contamination of cocoa beans and most effective public health measure in accordance with the hazard analysis –critical control point

(HACCP) system (Bonvehi, 2004). Experimental indications show that OTA is formed mainly in the initial stage of sun drying (Bonvehi, 2004).

Ochratoxin A (OTA) was first isolated in 1965 in South Africa from a strain of *Aspergillus Ochraceus* (Van der Merwe *et al.,* 1965) which usually occurs as a trace contaminant in many agricultural products. Since its discovery, OTA has been reported to be produced by a few moulds belonging to the *Aspergillus* and *Penicillium* genera (Rizzo *et al*., 2002; Ciegler, 1976; Elbanna *et al*., 1987). The family of Ochratoxins consists of three members, A, B, and C which differ slightly from each other in chemical structures. These differences, however, have marked effects on their respective toxic potentials. Ochratoxin A is the most abundant and hence the most commonly detected member but is also the most toxic. It is a potent toxin affecting mainly the kidney. As in other mycotoxins, Ochratoxin A can contaminate a wide variety of foods as a result of fungal infection in crops, in the field during growth, at harvest, in storage and in shipment under favourable environmental conditions especially when they are not properly dried. Ochratoxin A may be present in a foodstuff even when the visible mould is not seen (Van der Merwe *et al*., 1965, Li *et al*., 1997).

These fungi are ubiquitous and can occur in tropical and temperate climates. Besides the presence of nutrients, the most important factors for growth and mycotoxin production are temperature, water activity (aw) and oxygen. Often contamination of food by fungi may vary due to different origins of contamination, especially storage buildings, bins or underground pits (Christensen and Sauer, 1982). Often, fungi invade only a minor fraction of feed particles with appropriate condition for a growth such as enough water content, aeration, etc. Substrates differ in their ability to support

fungal growth due to differences in their physical and chemical characteristics, which include water activity, oxygen availability and surface area, while chemical characteristic include carbohydrates, fat, protein, trace elements and amino acid composition (Russell *et al.,* 1991).

Cocoa beans are normally placed in heaps and fermented prior to drying and then transported for processing. Thus during the fermentation phase the cocoa beans can become colonized by *A. ochraeus* resulting in OTA contamination. Because cocoa beans are highly hygroscopic they can absorb moisture during storage and transportation. The critical moisture content range is 6–8% which is equivalent to about 0.75–0.85 aw. These conditions can allow mould growth although they are marginal for growth of *A. ochraceus* and OTA production. Since they are rich in oils they tend to deteriorate slower than some carbohydrate rich products.

A recent study has analyzed cocoa beans and hand shelled (coffee nib) beans. This showed that during the shelling process there was a decrease of between 65–95% in OTA contamination (Amezqueta *et al.,* 2005). Thus, OTA may be predominantly present in the shells but care is needed during the shelling process to minimize the contamination of cocoa derived products with OTA. Thus management of the transport phase is critical for conserving quality and preventing OTA contamination.

Fungal species belonging to the genera *Aspergillus, Mucor, Penicillium* and *Rhyzopu*s have been observed on mishandled or improperly dried fermented beans (Roelofsen, 1958; Broadent and Oyeniran, 1968). More recently, *Aspergillus* species have been found to be the most frequently isolated fungi from samples of ground cocoa-based beverages (Oyetunji, 2006). Many fungi, especially species from the genera *Aspergillus* and *Penicillium*, produce mycotoxins that can cause acute or chronic

intoxication and damage to humans and animals after ingestion of contaminated food and feed (Marasas and Nelson, 1987; Moss, 1996). Among the mycotoxins, Aflatoxins (AFs) and Ochratoxin A (OTA) are of special interest, given their high occurrence and toxicity. All AFs are regulated in different products in most countries worldwide (European Communities, 2001). Recently, the European Commission has established 2 μg and 1 μg as the maximum level of OTA in raw material for manufacturing cocoa products and consumer products, respectively (Anonymous, 2007).

1.4 Monitoring of Ochratoxin A in the environment

In recent years, the general concern about the potential effects of mycotoxins on the health of humans and animals has been increasing. Measures have been set up by authorities in many countries to monitor and control mycotoxin levels. Various factors play a role in decision making processes focused on setting limits for mycotoxins. These include scientific factors to assess risk (such as the availability of toxicological data), food consumption data, knowledge about the level and distribution of mycotoxins in commodities, and analytical methodology. Economic factors, such as commercial, trade interests and food security

Throughout the world there are many advisory bodies concerned with food safety, including the World Health Organisation (WHO), Codex Alimentarius, Joint Expert Committee for Food Additives and Contaminants (JECFA) and the European Food Safety Authority (EFSA), and they regularly assess the risk from mycotoxins and advise on controls to reduce consumer exposure.

In 2002, an international inquiry on mycotoxins was initiated by the National Institute for Public Health and the Environment. As part of this inquiry, the Agricultural

Services in Dutch Embassies around the world were requested to gather up-to-date information on the situation regarding mycotoxin regulations from local authorities in as many countries as possible. At least 99 countries had mycotoxin regulations for food and/or feed in 2003, an increase of approximately 30 percent compared to 1995. The total population in these countries represents approximately 87 percent of the world's inhabitants. In 1995, 23 percent of the world's inhabitants were living in a region where no known mycotoxin regulations were in force. This percentage had decreased to 13 percent in 2003, due to a slight increase in coverage in Latin America and Europe, and more significant increases in Africa and Asia/Oceania (FAO, 2003).

In 2008, the 31st session of the Codex Alimentarius Commission adopted guidelines for the maximum levels for OTA in raw wheat, barley and rye, as well as maximum levels for aflatoxins in almonds, hazelnuts and pistachios (for further processing and ready-to-eat) (Codex, 2008). The Codex Committee on Food Additives and Contaminants (CCFAC) at its 38th Session in The Nederlands (2012) discussed a paper on OTA in Cocoa leading to the following broad conclusions and recommendations for consideration at the Sixth Session of the Codex Committee on Contaminants in Foods: The CCCF may consider commencement of new work for the development of a Code of Practice for the Prevention and Reduction of OTA in Cocoa, This code, subject to the approval of new work by the Commission, should be developed along similar lines as the current Code of Practice for the Prevention and Reduction of Ochratoxin A Contamination in Coffee (CAC/RCP 69-2009)**.** The proposed outline of the Code may also be used as a basis agreed on necessity of setting a maximum level for OTA in cocoa and should be assessed after the development and implementation of the Code of Practice and consider the significant differences between the level of OTA in the shells, unroasted beans, roasted beans,

roasted nibs and chocolate & cocoa products containing additives from industrial processing. The implementation of the Code of Practice by all producing countries the necessity of obtaining reliable data on worldwide exposure develop a discussion paper on Ochratoxin A (OTA) in cocoa (Codex, 2012).

1.5 Justification

In today's changing world, safety and security have generally remained basic human needs. Ensuring the safety of food has been a major focus of international and national action over the last years. Both microbiological and chemical hazards are of concern. Among chemical hazards, the contamination of food and feed by mycotoxins (toxic metabolites of fungi), fishery products by phycotoxins (toxins produced by algae) and edible plant species by their plant toxins have been recently characterized by the World Health Organization (WHO) as significant sources of food-borne illnesses (WHO, 2002). Of these three categories of natural toxins, most attention has been directed to mycotoxins until now.

The presence of OTA in food is of great concern due to chronic effects at low levels of exposure, in humans; severe dietary exposure to Ochratoxin A has been associated with chronic, progressive, Balkan endemic nephropathy which is a kidney disease (Ogunledun 2007; Chukwuka 1997; Badru 2005).

Cocoa beans are of great economic importance and in order to maintain the quality, they should be stored under controlled environment that would not be favourable for the growth of fungal flora thereby preventing deterioration of the stored cocoa bean and reduction in the chemical composition. However, apart from good hygiene, proper handling and processing, good agricultural practice should be employed to reduce the contamination of stored cocoa bean.

Consumer exposure to OTA is reported to be increasing gradually and in order to protect consumers the European Union has drawn up a standard to define tolerable contamination limits (European Commission, 1995). As these toxicants can never be completely removed from the food supply, 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).

In 2003, the Italian Ministry of Health, claiming the precautionary principle, set a legal limit of 2.0 μg/kg and 0.5 μg/kg for cocoa powder and chocolate products respectively. On the basis of the risk assessment carried out by Brera *et al*., (2011), which showed no health concerns, and to align with the EU regulation, the Italian Superior Council of Health decided to remove the Italian legal limit for OTA in cocoa and chocolate based products.

Brazil has established 5 μg/kg as maximum level for OTA in cocoa products, including chocolate (ANVISA Resolução nº7/2011). Health Canada is currently in the process of proposing maximum limits for OTA in a variety of foodstuff, as a result of a conducted health risk assessment (Health Canada, 2010). At this time, maximum limits for OTA in cocoa are not being considered. The US FDA has not set advisory limits or action levels for Ochratoxin A in any commodity.

OTA occurrence in cocoa, cocoa powder and cocoa marketed products has been reported in different countries (Tafuri *et al*., 2004; Burdaspal and Legarda 2003; Amezqueta *et al*., 2005) but there is scanty information on OTA contamination of cocoa products from Ghana. Currently there are no standard (maximum limits) for OTA in cocoa or chocolate products in Ghana which directly relates to consumer safety and is a current interest in many countries around the world. The lack of data

pertaining to OTA intakes from cocoa products makes it difficult to assess the health risks to consumers, thereby crippling governmental and international agencies regulatory safety measures.

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.

1.6 Objectives of the study

The objectives of this study are to

i. determine the levels of OTA in dry cocoa beans from the main cocoa growing districts in the North Western, South Western, Ashanti and Brong Ahafo Regions of Ghana
ii. compare the OTA levels with the proposed standard set by EU for cocoa beans in order to determine their suitability for consumption.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 History of cocoa

The story of cocoa begins with a fermented alcoholic drink, discovered in the Ulua Valley of present day Honduras, which dates somewhere between 1400 BC and 1100 BC (Maugh, 2007). Cocoa made its way to Mexico where it became an integral part of Aztec culture – epitomized by the consumption habits of Aztec King Montezuma who allegedly drank 50 cups of *xocoatl* a day.

The Aztecs believed that the cocoa tree had been brought to them by "the god of air, Quetzalcoatl, after man had been driven from the equivalent of the Garden of Eden. Having helped man, Quetzalcoatl departed, leaving the 'quachahuatl' tree." (Dand, 1997).

Consequently, the Aztecs attributed religious significance to cocoa and used it in religious ceremonies. The Aztecs consumed cocoa as a drink, to which they added spices, honey, and sometimes maize –one method involved fermenting the maize, which made the drink alcoholic. The beans were also extremely valuable as currency. The Mayans also enjoyed the chocolaty drink and by AD 600 they had commenced agricultural production by establishing cocoa plantations. For the next two millennia, the rest of the world remained oblivious to the existence of cocoa until 1502.

On his fourth voyage to America, Columbus discovered a canoe off the Yucatan Peninsula laden with fruit and cocoa beans. Columbus presented the cocoa beans to King Ferdinand of Spain, however, the King showed no interest in them. Another Spaniard Hernán Cortés arrived at the court of the Aztec King Montezuma in 1519 and was struck by the popularity and religious significance attached to the xocoatl. He

found that the Aztecs, Olmec and the Mayas valued them so much that they used them both as means of payment and as the source of a beverage drunk at court and religious ceremonies. The Spanish refined the recipes adding sugar and heating the ingredients to improve the taste. By 1828, the cocoa press was developed, allowing the extraction of cocoa butter. Later on, the Swiss developed both milk chocolate and solid chocolate. Although its exact origins are not known, the Cacao tree was then exclusive to the Americas. The closest estimates put the area of origin in and around the valleys of the Amazon and Orinoco Rivers (ADM, 2009).

2.2 The Origin and Spread of Cocoa cultivation in Ghana.

Basel Missionaries, who worked under the aegis of the Danish government, first introduced cocoa to Ghana in 1857 when they planted seeds that they had received from Surinam on their land in Akropong. However, the seedlings died the following year so they tried again, this time with seeds brought from Cape Palmas. By 1861, the plantings had turned into ten little cocoa trees, yet by 1863, only one survived, beetles or worms had destroyed the rest. The missionaries distributed pods from the one remaining tree in Akropong to other Basel Mission stations at Aburi, Mampong and Krobo Odumase, where some of the plants survived (Grossman-Greene and Bayer, 2009). While not the first to bring cocoa to Ghana, Tetteh Quashie, a native played an instrumental role in its dissemination and subsequent development in Ghana (Grossman-Greene and Bayer, 2009).

History attributes the commercial cultivation of cocoa to Tetteh Quarshie, who had travelled to Fernando Po and returned with Amelanodo cocoa pods (Canatus and Aikins, 2009). Upon arrival, Quashie established a cocoa nursery in Mampong Akwapim and when his crop had matured, sold pods and seedlings to local farmers.

Those trees are purported to be the parent trees of Ghana's cocoa industry. From Akwapim, cocoa farming spread to the Ashanti, Brong Ahafo, Central, and Western regions. The first documented shipment of beans from Ghana was in 1891, when 2 bags were sent from Accra to Hamburg, and since then, cocoa has been the main export crop and a major source of foreign exchange and domestic income earner (Canatus and Aikins 2009). Until 1977, Ghana was the world's leading producer of cocoa with the market shares ranging from 30-40%. Records indicate that production increased from a level of 36.3 metric tonnes in 1891 to an all time peak of about 557,000 metric tonnes in 1965 giving Ghana a global output share of about 33% and the leading cocoa producer. Thereafter, production continued to drop and reached the lowest level of 158,956.00 metric tonnes in 1984, which constituted about 9% of world's production (Joy, 2010).

Consequently, Ghana lost her position as the world's number one producer. As part of efforts to arrest the decline in cocoa production, the Government of Ghana through Cocoa Board initiated a National Cocoa Diseases And Pest Control (CODAPEC) programme, popularly known as "Mass Spraying" to assist all cocoa farmers in the country to combat the Capsid/Mirid and the Black Pod disease. Under this programme, cocoa farms across the country were sprayed with insecticides and fungicides at no cost to the farmers. Also introduced is the Hi Tech programme. These programmes have resulted in tremendous increases in cocoa bean production from 340,562 metric tons in the 2002 season to 496,846 metric tons in 2003 and 736,000 metric tons in the 2004 seasons, respectively (Appiah, 2004).

The percentage of locally processed beans has also jumped from 20% to 35% with further re-capitalization and expansion programs underway to reach a target of 50% in

the near future. It has therefore been the intention of government, which is committed to reaping the maximum benefit from the cocoa sector, to ensure that the country increases its cocoa production and also processes more of the beans into downstream products for both the local and export markets (Awuah, 2002). However, along with the positive effects of the CODAPEC programme, some negative impacts on the environment have also been caused.

For instance the use of pesticides on the farms can lead to the destruction of part of the soil flora and fauna through both physical and chemical deterioration. While the Government of Ghana stresses the need for diversifying the nation's economic structure, it also emphasizes the important role of the cocoa sector and has set the target of achieving one million metric tons of cocoa output by 2010 (NDPC 2006). QCC has been able over the year's maintained Ghana's cocoa quality that meets strict specifications in commodity exchanges with price quotations for future delivery, and consistently fetches premiums on world market prices. (ICCO, 2000)

2.3 Classification of Cocoa

Cocoa has many varieties that were originally split into two large groups (subspecies). Traditionally, two main genetic groups, "Criollo" and "Forastero", have been defined within cacao based on morphological traits and geographical origins (Cheesman, 1944). A third group, "Trinitario", has been recognized and consists of "Criollo - Forastero" hybrids (Cheesman, 1944). For other authors, "Criollo" and "Trinitario" should be considered as traditional cultivars rather than genetic groups (Motamayor *et al*., 2002). Two other traditional cultivars have been described: Nacional and Amelonado (Motamayor *et al*., 2002). All these divisions were based on differences

in pod and bean characters. Today there are so many “families” of cocoa that differentiation is made via specific variety names or numbers (Wick, 2003).

Genetic improvement of cocoa through breeding has focused on increasing yield and disease resistance. To increase yield, breeders have capitalized on heterosis that occurs in crosses between trees from different genetic groups (Warren, 1992).

Criollos are light coloured with a mild, nutty character and complex, fruity flavour. It is a flavoured cocoa bean grown mainly in Latin America. Its susceptibility to disease and low productivity, however, means many cacao farmers have traded its rich flavour for hardier plants. Trinitario is a hybrid of the two strains and it is believed to combine the best of both good flavour and hardiness. Forastero cocoas are dark brown, strongly flavoured, slightly bitter, and have a higher fat content. Forastero, the main bulk cocoa bean, accounts for about 90 percent of all beans. It has a clean chocolate flavour with low acidity and is prized for its disease resistance and consistent performance.

Cocoa trees usually commence flowering 22 months from seed or 18 months from potted seedlings. However, first harvests do not usually occur until 18 months after planting. The time taken from flowering until pod maturity is temperature dependent but usually takes 4-6 months. Most mature cocoa plantings have near continuous harvesting with one major pick per year. There are management systems that reduce this continual flowering as competition between flowers and pods can reduce yields (Wick, 2003).

2.4 Factors affecting cocoa production

Cocoa is highly susceptible to drought and the pattern of cropping of cocoa is related to rainfall distribution. Significant correlations between cocoa yield and rainfall over varying intervals prior to harvest have been reported. It was found that in Ghana a year with high rainfall is followed by a year with a large crop, though the correlation was not applicable in all years (Smellie, 1925; Skidmore, 1929, Brew, 1991). Ali (1969) reported both positive and negative correlations between rainfalls in certain months with the yield of the main crop in Ghana.

The annual total rainfall in the cocoa growing regions of Ghana is less than 2000 mm. The rainfall distribution pattern is bi-modal from April to July and September to November. There is a short dry period from July to August during which the relative humidity is still high with over cast weather conditions. There is a main dry season from November to March. The four to six months of dry weather results in soil water deficit and since irrigation is not part of the farming system, cocoa seedling mortality is high during the establishment phase. In bearing plants, the existence of the short dry season during main crop pod filling can affect bean size if it is sufficiently severe. In adult plantings, water deficits result in lower yields and an increase in the level of mirid damage. Values defining the limits or adequate soil moisture capacities or available moisture contents for cocoa cultivation during the dry season in Ghana were found to be variable and under field conditions depend on many factors such as, shade, air movement, soil texture and structure, age and vigour of the cacao, volume and distribution of active roots and root depth. In considering the suitability of a soil for cocoa in relation to soil moisture, it is not the quantity of available soil moisture which is important; it is rather the rate of release of the available water from the soil to the tree which matters (Wessel, 1971; Ahenkorah, 1981).

Cocoa trees grow under shaded conditions with a climate characterized by relatively high temperatures (between 18-32 ºC) and plentiful rainfall. Cocoa production also depends heavily on the pattern of rainfall; the average distribution of monthly rains throughout the year is more important than the annual total. Annual rainfall in excess of 2500 mm may lead to a higher incidence of fungus diseases, the most common known as *phytophtora* pod rot which causes the black pod disease, and the cocoa swollen shoot virus (ICCO 2000; Wood and Lass, 1985). Rainfall must be well distributed across the year, with a minimum of 1,000 mm. Meanwhile, yearly variation in the yield of cocoa is affected more by rainfall than any other climatic factors. Cocoa prefers calm conditions and persistent moderate wind can cause severe damage to yield. The trees must be protected from strong winds, soils must be well aerated, and pests and diseases must be carefully controlled (ADM, 2009).

The successful cultivation of cocoa requires a special climate that is mostly found within the area bounded by the Tropics of Cancer and Capricorn. The majority of the world's crop is now grown within 10° North and South of the equator. It will grow from sea level up to a maximum of some 1,000 meters, although most of the world's crop grows at an altitude of less than 300 meters (ADM, 2006).

Daily, seasonal, or annual variations in the values of the climatic element are of greater importance in determining the efficiency of crop growth (Ayoade, 2004). A number of factors have an interrelated impact on the growth of cocoa plant. This factor ranges from the weather element of rainfall, temperature, sunlight and humidity to others such as, pest and diseases as well as soil nutrient status. Cocoa is highly sensitive to changes in climate, particularly to temperature due to its effects on evapotranspiration (Anim-Kwapong and Frimpong, 2005). The mean minimum

temperature is between 20^{o}C to 22^{o}C while the mean maximum during the dry and wet season varies from 31^{o}C to 32^{o}C and 27^{o}C to 29^{o}C, respectively (Wood and Lass., 1987). It is observed that the higher the temperature (maximum of 32^{0}C), the higher the yield, while the lower the relative humidity, the better the yield. Total annual rainfall of the cocoa growing areas of these countries ranges from 1200 mm to 3000 mm. Cocoa is known to produce well with minimal but sustained water availability throughout the year (Obatolu *et al*., 2003). Rainfall must be well distributed across the year, with a minimum of 1,000 mm. Meanwhile, yearly variation in the yield of cocoa is affected more by rainfall than any other climatic factors. Cocoa prefers calm conditions and persistent moderate wind can cause a severe damage to yield. The trees must be protected from strong winds, soils must be well aerated, and pests and diseases must be carefully controlled (ADM, 2009).

2.5 Harvesting of cocoa beans

First step in the processing of cocoa beans is harvesting of the pods. Harvesting involves removing ripe pods from the trees and opening them to extract the wet beans. Ripe pods are easy to identify by having another colour than the immature pods. For instance, Amelonado turns from green to yellow when ripe. The ripening process is slow, and a mature pod will remain suitable for harvesting for two or three weeks. It is important that only well ripe pods are taken. Unripe pods will not undergo fermentation, and over ripe pods often become dry (Barclays Bank, 1970). The pods are harvested manually by making a clean cut through the stalk with a well sharpened blade. For pods high on the tree, a pruning hook type of tool is used, with a handle on the end of a long pole. By pushing or pulling according to the position of the fruit, the upper and lower blades of the tool enable the stalk to be cut cleanly without damaging the branch that bears it (Figure 2.1).

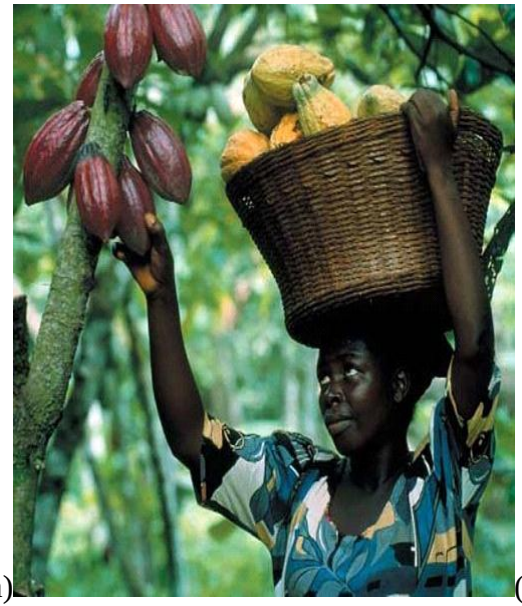
(a)  (b) 

Figure 2.1 Harvesting of cocoa pods (a & b) (AusAid, 2010)

It is important that the harvesting tools are sharp, so the cushions of the trees are not damaged since they are a potential point of entry for fungi. When cutting, the pod stem should be cut as close to the tree as possible, and the thickened joining portion should be left attached to the cushion. It will fall of later and leave a well healed scar that is impermeable to fungi (Barclays Bank, 1970). While harvesting, the farmer may spread fungal diseases from contaminated hooks, knives or shoes (Vos *et al.,* 2003). Unharvested pods turn black and the beans begin to rot or germinate. This may also spread diseases among cocoa trees (Amoa-Awua *et al.,* 2007).

The pods are opened to remove the beans within a week to 10 days after harvesting. In general, the harvested pods are grouped together and split either in or at the edge of the plantation. Sometimes the pods are transported to a fermentary before splitting. If the pods are opened in the planting areas, the discarded husks can be distributed throughout the fields to return nutrients to the soil.

The best way of opening the pods is to use a wooden club which, if it strikes the central area of the pod, causes it to split into two halves and making it easy to remove the wet beans by hand. A cutting tool, such as a machete, is often used to split the pod, though this can damage the beans (Figure 2.2). Some machinery has been developed for pod opening, but smallholders in general carry out the process manually (ICCO, 2011).

(a)  (b) 

Figure 2.2 Breaking of cocoa pod (a & b)

2.6 Fermentation of cocoa

Fermentation can be carried out in a variety of ways, but all methods depend on removing the beans from the pods and piling them together or in a box to allow micro-organisms to develop and initiate the fermentation of the pulp surrounding the beans. The most common are box, heap, tray, and basket fermentation.

2.6.1 Type of fermentation

Box fermentation is a system of three wooden boxes, each having a capacity of 1,000 kg cocoa beans. Box number one is placed two box-sizes above the ground, box number two in the middle, and box three is on ground level. The beans start in box

number one and ferment for two days under a cover of banana leaves. To mix the beans, upper half of the first box is poured into the bottom of box number two, followed by the lower half. The beans will ferment for another two days under banana leaves (Figure 2.3). To make sure that no beans are left in the middle of the boxes during the whole process, the beans are split vertically and then poured into box number three. After two days more under banana leaves, the cocoa beans are properly fermented (Asare, 2010; Are & Gwynne-Jones, 1974).

(a)  (b) 

Figure 2.3 Box fermentation of cocoa beans (a & b) (AusAid, 2010)

The baskets fermentation method is preferable for smaller amount of beans, the basket are of different sizes, but generally hold 30 – 40 kg beans, sometimes up to 90 kg. They are made of weaved plant materials and therefore, they are not airtight. Before the fermentation begins, the insides of the basket are covered with banana leaves, but the bottom remains uncovered to let the sweating drain away (Figure 2.4). The beans are turned on the second and the fourth day, and after six days, the beans are properly fermented (Asare, 2010; Mossu, 1992).

Figure 2.4 Basket fermentation of cocoa beans (AusAid, 2010)

Heap fermentation is carried out on a bed of banana leaves on the ground, and the heap of cocoa beans is covered with banana leaves and sticks (Figure 2.5). It is a cheap method that produces well fermented beans, when it is done properly (Are & Gwynne-Jones, 1974). Among small holders in Ghana, heap fermentation is a popular method. It does not require permanent equipment, and therefore, it suits well to family holders with small production.

Figure 2.5 Heap fermentation of cocoa beans (AusAid, 2010)

Gilmour and Lindblom (2008), experimented in large commercial farms in Cote d'Ivoire and indicated that very little OTA was produced during well controlled fermentations. The fermentation was conducted in bags placed in the middle and on the top of wooden fermentation boxes. The boxes had no visible mould contamination when the fermentation was complete; the content of the boxes was dried at two different depths (3 and 8 cm) in drying beds. No OTA was detected in any of the samples (Codex, 2012).

A fourth method to ferment cocoa beans is the tray fermentation. One tray measures 120 x 90 x 10 cm and holds about 90 kg wet beans. The trays are stacked in piles, 3 - 12 trays high. Less than three trays will not produce enough heat. Higher piles do not affect the fermenting process, but it will be too hard work to handle the trays at this height. The bottom tray is placed on a wooden platform to avoid the ground absorbing the produced heat, to allow the sweating to drain away, and to promote air circulation. Tray fermenting takes four to five days without turning the beans (Asare, 2010).

2.6.2 Biochemical reactions in fermentation

During the last decade knowledge about the cocoa fermentation process has been increasing (Ardhana and Fleet 2003, Camu *et al.*, 2007, Nielson *et al.*, 2007, Schwan and Wheals 2004). Clearly, the different stages of fermentation are essential in the creation of the complex organic components essential to the final taste and enjoyment of cocoa. The micro biota involved in natural cocoa bean fermentation process reflects the environmental factors pH, oxygen tension and temperature, and the metabolism of substrates of the cocoa bean pulp. This results in production times 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.,* 2007, Schwan 1998, Schwan and Wheals, 2004, Schwan *et al.,* 1995).

In the first phase the conditions are more or less anaerobic. During early and mid-time spontaneous fermentation of freshly harvested pulp and cocoa beans, piled into a heap, yeasts produce ethanol under anaerobic conditions causing depectinization of the pulp enabling the pulp to flow away and air ingress (Schwan and Wheals, 2004, Schwan 1995). Citrate and pulp sugars are converted to acetic acid, lactic acid and mannitol by yeasts enabling a slight increase in pH of the pulp (Camu *et al.,* 2007). Changes in concentration of amino acids, pyrazines, peptides, polyphenols and alkaloids during cocoa bean fermentation occur mainly through endogenous enzymatic activities, microbial consumption and conversion, or physical diffusion (Camu *et al.,* 2007, Hansen *et al.,* 1998, Hashim *et al.,* 1998, Jinap *et al.,* 2003, Jinap and Zeslinda 1995, Jinap *et al.,* 1995, Voigt *et al.,* 1994).

Lactic acid bacteria and pectins are broken down by pectinases, which results in liquefaction of the pulp and starts the second aerobic phase of fermentation. Acetic acid produced by acetic acid bacteria is a key metabolite of the cocoa bean fermentation process. The volatile short chain fatty acid diffuses into the bean and this combination together with the heat produced by the exothermic conversion of the ethanol into acetic acid, causes the death of the seed embryo, the disruption of the internal cellular structure of the beans and the end of fermentation (Camu *et al*., 2007, Nielsen *et al.,* 2007). In turn, biochemical changes in the beans are initiated, leading to the enzymatic formation of precursor molecules that are necessary for the development of the characteristic aroma, flavour, and colour of the beans (de Brito *et al*., 2000, Hansen *et al.,* 1998, Holm *et al*., 1993). These properties are developed

further during drying, roasting, and final processing of well-fermented cocoa beans (Thompson *et al.*, 2001) (Figure 2.6).

Figure 2.6 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

2.7 Drying of cocoa beans

Cocoa beans are dried after fermentation in order to reduce the moisture content from about 60% to a maximum of 7.5%. Drying must be carried out carefully to ensure that off-flavours are not developed. Drying should take place slowly. If the beans are dried too quickly some of the chemical reactions started in the fermentation process are not allowed to complete and the beans become acidic, with a bitter flavour. However, if the drying is too slow, moulds and off- flavours could develop. Various research

studies indicate that bean temperatures during drying should not exceed 65ºC (ICCO, 2011).

There are two methods for drying beans - sun drying and artificial drying. Fermentation is followed by sun drying for 7 to 8 days (Figure 2.7). Cocoa beans should preferably be sun-dried to a moisture content of 7.5%. When artificial drying is used, the process should mimic sun drying, using low temperature/ambient air for the initial drying with higher temperatures only for the final stage. Because of the high rainfall and cloud cover in Brazil and Malaysia, other techniques are more popular. In Brazil the beans are typically laid out on broad mats on stilts above ground level to dry. In Malaysia widespread use is made of mechanical rotary driers (ADM 2006).

(a) 

(b) 

Figure 2.7 Fermented cocoa beans being dried (sun drying – a & b)

2.8 Categories of Cocoa Beans

The world cocoa market distinguishes between two broad categories of cocoa beans, fine or flavour cocoa beans, and bulk or ordinary cocoa beans. As a generalisation, fine or flavour cocoa beans are produced from Criollo or Trinitario cocoa-tree varieties. While bulk cocoa beans come from Forastero trees. There are, however, known exceptions to this generalisation. Nacional trees in Ecuador, considered to be Forastero-type trees, produce fine or flavour cocoa. On the other hand, Cameroon cocoa beans, produced from Trinitario-type trees and whose cocoa powder has a distinct and sought-after red colour, are classified as bulk-cocoa beans. The share of fine or flavour cocoa in the total world production of cocoa beans is just under 5% per annum. Virtually all major activity over the past five decade has involved bulk cocoa.

2.9 Mycotoxins

Moulds can grow and produce mycotoxins in plant material during the whole chain from field to table. Some crops, preservation systems and feedstuffs are more susceptible to mould growth and mycotoxin production than others. There are

numerous mycotoxins, some of which are very toxic to farm animals and may cause acute mycotoxicoses. It is, however, more common for mycotoxins to be consumed by animals in lower quantities over a longer time period, causing chronic or more diffuse toxicoses (Pettersson, 2004).

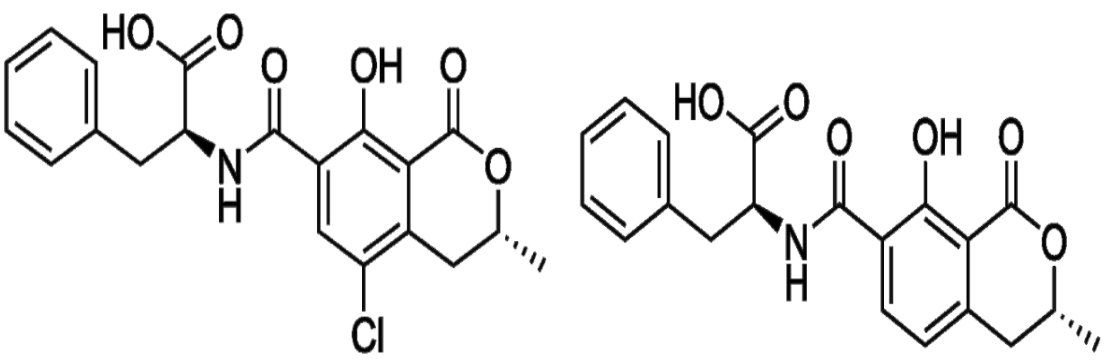
The term mycotoxin is derived from the Greek word ‘mycos’ meaning mould, and the Latin word ‘toxicum’, which means poison. Mycotoxins are highly toxic secondary metabolic products of various moulds, mainly those belonging to the genera *Fusarium, Aspergillus* and *Penicillium* (Heidler and Schatzmayr, 2003). Different mycotoxins are more commonly found in or associated with certain, feedstuffs. Some develop in the growing crop due to its being susceptible to certain toxigenic fungi, while infection and toxin production by others is facilitated by the preservation and storage system used if insufficient care is taken to prevent this. Some mycotoxins are associated only with crops from a certain region, due to the climatic or ecological conditions being suitable for the mycotoxin producing fungi (Pettersson, 2004).

It has been estimated that at least 300 of these fungal metabolites are potentially toxic to animals and humans. However, the most extensively investigated mycotoxins are aflatoxin B1, zearalenone, deoxynivalenol (DON, “vomitoxin”), T-2 toxin, ochratoxin A and fumonisin B1. Their global occurrence is considered to be a major risk factor. According to the Food and Agriculture Organization (FAO) as much as 25 % of the world’s crops are affected annually. The toxicity of mycotoxins depends on the physical and chemical properties of each toxin, level of intake and duration of exposure. Mycotoxins are reported to be carcinogenic, genotoxic, teratogenic, dermatotoxic, nephrotoxic and hepatotoxic (Heidler and Schatzmayr, 2003).

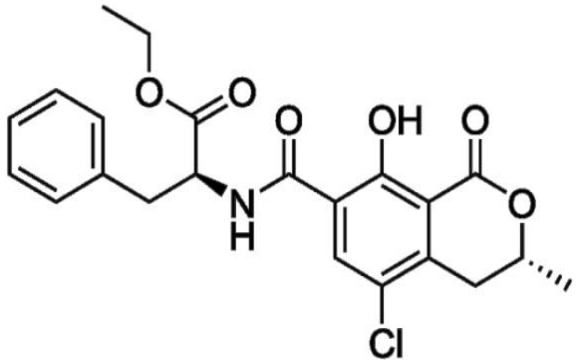
2.10 Ochratoxin A

The discovery of aflatoxins in the 1960s (Sargeant *et al*., 1961), with approximately 100,000 turkey poultry deaths in England, was the seminal event that made the scientific community realize that mould secondary metabolites could be responsible for food and feed safety problems. Several other mycotoxins were identified when fungi in food were more fully investigated. Ochratoxins are mycotoxins produced by two main genera of fungi, *Aspergillus* and *Penicillium.*

Chemically, Ochratoxins are described as weak organic acids consisting of a dihydroisocumarin moiety joined by a peptide bond to 1-phenylalanine (O'Brien and Dietrich, 2005). There are three generally recognized Ochratoxins, designated A, B and C. Structurally, these three toxins differ only very slightly from each other; Ochratoxin A (OTA) is chlorinated, which is unusual for natural products, Ochratoxin B, which is not chlorinated, and Ochratoxin C, the ethyl ester of OTA, are less toxic and less common (Figure 2.8). Most studies on Ochratoxins have therefore focused on OTA. Ochratoxin A (OTA) was purified and characterized from *Aspergillus ochraceus* Wilherm strain K-804 (Van der Merwe *et al*., 1965) isolated from sorghum grain, and proved to be acutely toxic to Pekin ducklings, mice and rats (Hutchison *et al*., 1971). Nowadays, OTA is one of the most relevant mycotoxins, with its presence in food and feed products being regulated in many countries.



Ochratoxin A Ochratoxin B



Ochratoxin C

Figure 2.8 Chemical structures of Ochratoxin A, B & C

2.11 Occurrence of Ochratoxin A (OTA)

OTA is found mainly in cereal and cereal products. This group of commodities has been reported to be the main contributors to Ochratoxin A exposure, in exposure assessments carried out by the European Commission, accounting for 50% of total dietary exposure of OTA in European countries (Miraglia and Brera, 2002). Besides cereals and cereal products, Ochratoxin A is also found in a range of other food commodities, including coffee, cocoa, wine, beer, pulses, spices, dried fruits, grape juice, pig kidney and other meat and meat products of non-ruminant animals exposed to feedstuffs contaminated with this mycotoxin. Ruminant animals such as cows and sheep are generally resistant to the effects of Ochratoxin A due to hydrolysis to the non-toxic metabolites by protozoa in the stomachs before absorption into the blood.

2.12 Human exposure of OTA

Mycotoxins can affect human and animal health. In general, animals are directly exposed to mycotoxins through the consumption of mouldy feedstuff. Human exposure can be via one of two routes; direct exposure due to the consumption of mouldy plant products, or indirect exposure through the consumption of contaminated animal products, containing residual amounts of the mycotoxin ingested by the food producing animals (Boutrif and Bessy 2001). However, animal derived food products contribute to a lesser 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).

2.13 Physico-Chemicals Properties of Ochratoxin A

OTA is a weak organic acid with a pKa value of 7.1 (Miller and Trenholm, 1994, Bredenkamp *et al.*, 1989) and a molar mass of 403.8 $g.mol^{-1}$. OTA is composed of a 7-carboxy-5-chloro-8-hydroxy-3, 4-dihydro-3-*R*-methylisocoumarin moiety and L-*β*-phenylalanine molecule, which are linked through the 7-carboxy group by an amide bond. The IUPAC nomenclature is L-phenylalanine-*N*-[(5-chloro-3, 4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl)carbonyl]-(*R*)-isocoumarin (Abrunhosa *et al*., 2010).

With crystalline structure varying from colorless to white, this molecule possess an intense green fluorescence under UV light in acid medium and blue fluorescence in alkaline conditions (Budavari, 1989). In acid and neutral pH, OTA is soluble in polar organic solvents (alcohols, ketones, chloroform), slightly soluble in water and insoluble in petroleum ethers and saturated hydrocarbons. While in alkaline conditions, this molecule is soluble in aqueous sodium bicarbonate solution and in all

alkaline solutions in general. It has a melting point of about 90 °C when crystallized from benzene as a solvate. However, non-solvated crystals of melting point 169 °C have been obtained from xylene, which are suitable for X-ray structural analysis.

OTA is optically active and the particularity of OTA is due to its high stability. It has been shown that it possesses a resistance to acidity and high temperatures. Thus, once foodstuffs are contaminated, it is very difficult to totally remove this molecule. In 1982, Müller showed that OTA is only partially degraded at normal conditions of cooking. Moreover, this molecule can resist three hours of high pressure steam sterilization of 121 °C (Ciegler, 1972), and even at 250 °C its destruction is not complete (Boudra *et al*.,1995). Gamma irradiation (up to 7, 5 Mrad) of OTA in ethanol does not cause any degradation. However, degradation is observed at low moisture level when OTA has been treated with an excess of sodium hypochlorite (NaOCl) (Castegnaro *et al.*, 1991). Moreover, exposure to fluorescent light is a factor of degradation.

2.14 Biosynthetic pathway of OTA

Although much information exists concerning 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 formed from acetate and malonate *via* a polyketide synthesis pathway (Niessen *et al*., 2005, Edwards *et al*., 2002, Moss 1998). Thus, a polyketide synthase (PKS), which is considered as key enzyme, is involved in the OTA biosynthesis in a similar way of other polyketide mycotoxins such as fumonisins (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 mechanistical model according to the structure of OTA. The heterocyclic portion of OTA is structurally similar 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 non ochratoxigenic species such as *Pezicula spp*. According to Huff and Hamilton (1979) three distinct steps occur in OTA biosynthesis: the first part is polyketide synthesis of ochratoxin A *via* mellein involving a polyketide synthase. The second step includes acyl activation: mellein is methylated and oxidized to 7-carboxy-mellein, this component is then transformed to a mixed anhydride, an activation reaction using ATP. The second precursor phenylalanine is synthesized *via* the shikimic acid pathway, followed by ethyl ester activation so that it can participate in the subsequent acyl displacement reaction. In the third step, linkage of those activated precursors *via* a synthetase takes place, generating OTC, an ethyl ester of OTA. De-esterification by an esterase or transesterification is the last step in this postulated biosynthetic pathway (Figure 2.9).

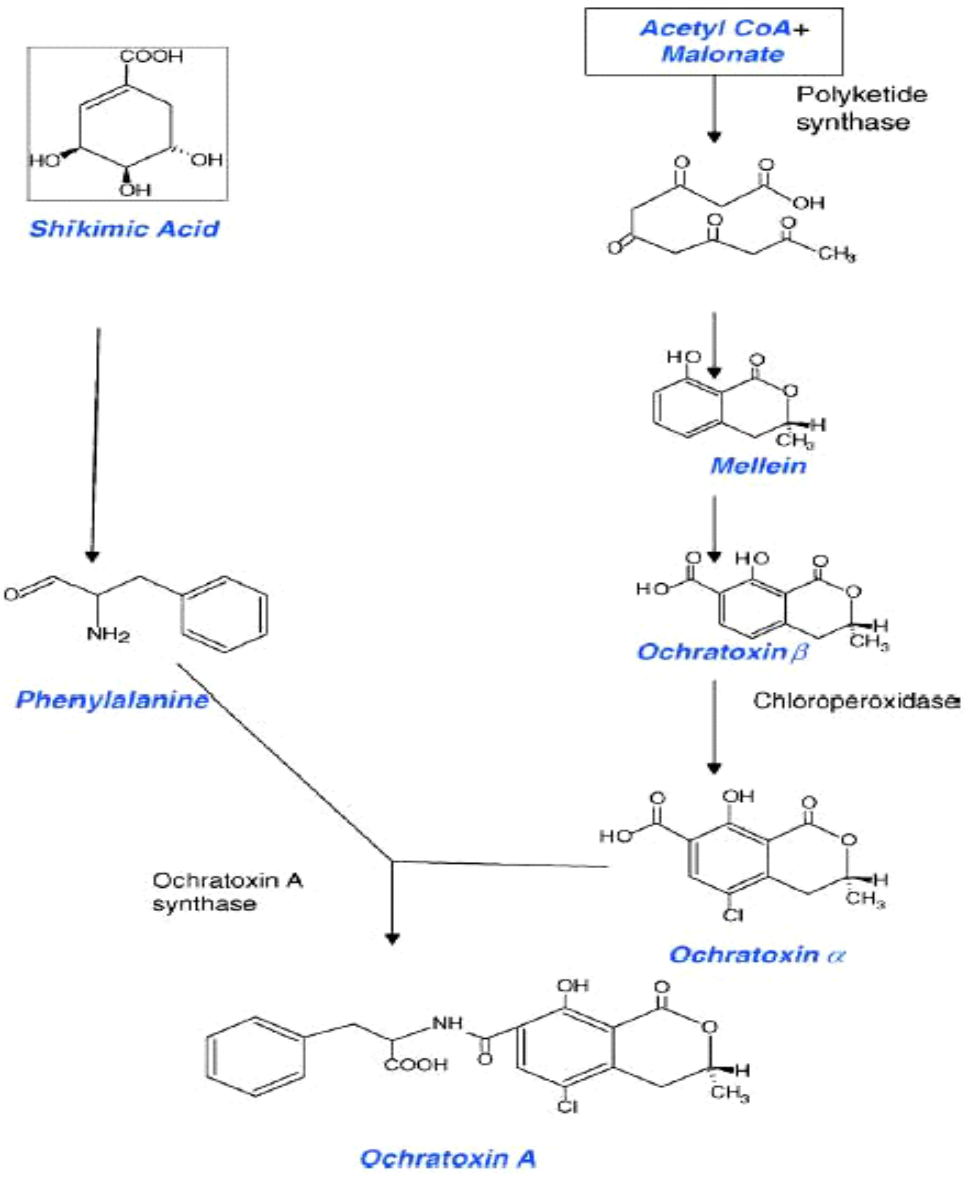


Figure 2.9 Schematic representation of the hypothetical OTA biosynthetic pathway as proposed by Huff and Hamilton, 1979

2.15 Ochratoxin contamination and prevention

The most important abiotic factors which influence the growth and OTA production by such spoilage fungi include water availability, temperature high moisture and gas composition (Magan and Aldred 2005). The interaction between these variables primarily determine whether mould growth will occur and if so the relative development of the fungal community. An accurate determination of the marginal

conditions for growth and OTA production by species such as *P. verrucosum* and *A. ochraceus* is important as it can be used to provide guidelines of the level of risk of contamination of the grain through the food chain. However, this requires detailed information on the ability of isolates of these species to colonize grain matrices over a range of interacting conditions.

Gilmour and Lindblom (2008) reported a study conducted between 1999 and 2004 in West Africa. The objective of the study was to identify critical control points in the cocoa chain intended to form the basis for the formulation of prevention strategies to be instituted in a Hazard Analysis Critical Control Point (HACCP) framework to minimize consumer exposure. The conclusions were that, contamination starts between the on-tree/harvesting to pre-fermentation stages and damaged pods are a major part of the problem indicating that the initial inoculation occurred before or during the fermentation. Drying procedure for cocoa beans may play a role in OTA development, but does not seem to be the main source of contamination. Poor drying allow further increase in toxin levels in already contaminated beans. OTA levels may vary within the cropping season. Further increases in OTA levels are not found in samples of cocoa beans taken at stages later in the supply chain and 50% of the contaminating toxin is physically removed when the shells are removed from the beans (Codex, 2012).

Recent studies by Cairns-Fuller *et al.*, (2005) have shown the general relationship between water availability (water activity, aw), temperature, growth and OTA production for *P. verrucosum* and *A. ochraceus*. Rapid growth occurs at 0.98–0.99 aw (27–30% m.c.) over the temperature range 10–25ºC and is almost completely inhibited at about 0.80–0.83 aw (17.5–18% m.c.). No OTA was produced at 0.80 aw,

although some were produced at 0.85 aw (19%) at 15 and 20ºC. Optimum conditions were at 0.93–0.98 aw (23.5–27.5%) at 10–25ºC on wheat grain incubated for up to 56 days.

The temporal production of OTA by strains of *P. verrucosum* showed that on wheat grain between 7–14 days was required for significant OTA to be produced at levels above the legislative limit (Cairns-Fuller *et al*., 2005). Contour maps of the optimum and marginal conditions of water and temperature for growth and OTA production have been constructed (Cairns-Fuller *et al*., 2005). This study showed that approximately 17–18% moisture content (ca. 0.80–0.83 aw) is the limit for any potential growth or OTA production in wheat grain. Thus, it is essential that grains are dried to lower moisture contents as quickly as possible regardless of the drying system employed. To avoid initiation of mould growth by xerophilic Eurotium species, drying up to 14.5% m.c. 0.70 aw; (Magan and Aldred, 2004) is essential. This has to be maintained during storage and transport to effectively prevent contamination with OTA.

Studies using cocoa media demonstrated the potential of *A. ochraceus* and other moulds that can be isolated from cocoa (*A. carbonarius, A. niger, A. tubingensis)* to grow and produce OTA. Ochratoxin A production was shown to strongly depend on temperature, pH and water activity of the substrate. Water activity of 0.97 was found to be optimal for OTA biosynthesis. *A. Niger* showed highest OTA production at 30ºC on malt glucose agar whereas *A. carbonarius* produced more OTA on cocoa agar at 25ºC (COCOQUAL, 2007).

2.16 Decontamination of OTA

Once OTA has been formed in food it is difficult to remove by most forms of food processing (Moss 1996). A number of these processes have been examined in detail although much remains to be done.

Chemical methods consist of the utilization of compounds to destroy OTA. Some processes use ammonium (ammoniation), alkaline hydrolysis (nixtamalization), bisulphites and ozone (ozonation). Hypochlorite (Castegnaro *et al*., 1991), ammoniation (Chelkowski *et al*., 1982), ozone (MacKenzie *et al*., 1997), alkaline hydrogen peroxide (Fouler *et al*., 1994) and gamma irradiation (Refai *et al*., 1996) treatments, have shown different degrees of success for detoxification of OTA in animal feed. Boudra *et al*., (1995) showed that even at as high a temperature as 250 ºC, complete destruction of OTA in wheat was not achieved. However, none of these physical and chemical processes were recommended for practical detoxification of OTA-contaminated grains and feeds. Scudamore *et al.,* (2004) found a significant reduction on the OTA content of wheat wholemeal by extrusion cooking at the highest temperature and initial moisture content of the samples. The effect of this procedure on the reduction of other mycotoxins content in cereals has been reviewed (Castells *et al*., 2005). A recent study about OTA reduction in artificially contaminated barley meal showed up to 86 % of reduction after extrusion cooking the samples (Castells *et al.,* 2005b). In general, the degree to which OTA is destroyed will further depend on other parameters such as pH, temperature, contamination levels, etc.

Physical methods consist of segregation, sorting, cleaning, peeling and shelling processes that aim to remove the most contaminated fractions of the commodities.

They also may involve the utilization of sorbents as nutritional additives that absorb OTA hence reducing bioavailability. These are reported generally as effective in the elimination of OTA and other mycotoxins (Riley and Norred 1999). However, the toxicological safety of the final product is not always guaranteed since some chemical residues may remain in products and the toxicity of the reaction products formed is not usually studied. Furthermore, there is a significant reduction in palatability and nutritive quality of treated products.

Biological methods use microorganisms, which can decompose, transform or adsorb OTA to detoxify contaminated products or to avoid the toxic effects when mycotoxins are ingested. These are the technologies of choice for decontamination because they present several advantages from being mediated by enzymatic reactions. For example, they are very specific, efficient, environmentally friendly, and they preserve nutritive quality. However, the non-pathogenicity of the microorganism and the non-toxicity of the reaction products formed are essential (Karlovsky, 1999). More research is needed to render these methods practical, effective and economically feasible. Scientists are working to better understand the conditions under which OTA degrades or remains intact throughout food processing.

Abrunhosa *et al*., 2002, isolated 51 strains (67 % of the strains tested) of filamentous fungi from grapes, with ability to degrade more than 80 % of OTA added to a culture medium, being black aspergilli, *A. clavatus*, *A. ochraceus*, *A. versicolor* and *A. wentii*, the most effective species. Furthermore, several reports have described the OTA degrading activities of the microbial flora of the mammalian gastrointestinal tract, including rumen microbes of the cow and sheep (Galtier and Alvinerie, 1976; Hult *et al*., 1976; Pettersson *et al*., 1982; Kiessling *et al*., 1982; Xiao *et al*., 1996; Özpinar *et*

al., 1999). The velocity of the degradation of OTA increased with concentration of starch in the animal diet and the resulting higher number of protozoa, while an influence of the pH-value was not apparent (Özpinar *et al*., 1999). It is reported that the human intestinal microflora can also partially degrade OTA (Akiyama *et al*., 1997).

2.17 Toxicology of OTA

The toxicological status of OTA has been examined 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).

2.18 Regulation and Legislation

In recent years, the general concern about the potential effects of mycotoxins on the health of humans and animals has been increasing. Measures have been set up by authorities in many countries to monitor and control mycotoxin levels. Various factors play a role in decision-making processes focused on setting limits for mycotoxins.

These include scientific factors to assess risk (such as the availability of toxicological data), food consumption data, knowledge about the level and distribution of mycotoxins in commodities, and analytical methodology. Economic factors, such as commercial, trade interests and food security issues, also have an impact. Weighing the various factors that play a role in the decision making process to establish mycotoxin tolerances is therefore of crucial importance. Despite the difficulties, mycotoxin regulations have been established in many countries during the past decades (FAO, 2004) and newer regulations are still being issued.

The Joint FAO/WHO Food Standards Programme and the *Codex Alimentarius* Commission was set-up to protect the health of consumers and ensure fair practices in the food trade. It was initially believed that, if all countries harmonized their food laws and adopted internationally agreed standards, such issues would be dealt with naturally. Through harmonization, the founders envisaged fewer barriers to trade and more freedom of movement among countries, which would be to the benefit of farmers and their families and would also help to reduce hunger and poverty (Codex 2012).

The European Commission Regulation (EC) No. 472/2002 of 12 March 2002 amended the Regulation (EC) No. 466/2001 setting maximum levels for certain contaminants in foodstuffs. This regulation limits OTA contamination in unprocessed cereals, including rice and buckwheat, up to 5 μg/kg. However, concerning cereal derived products, OTA contamination was fixed to 3 μg/kg. This regulation fixes also the contamination of dry grapes to a limit of 10 μg/kg. Moreover, the regulation (EC) No. 683/2004 of April 13 2004 (European Commission, 2004) amended the regulation (EC) No. 466/2001 (European Commission, 2001) including a directive

limiting the OTA contamination to 0.5 µg/kg in all food preparations for babies and in diet foods for special medical purposes intended specifically for infants. Added to that, the regulation (EC) No. 123/2005 (European Commission, 2006) highlights the contribution of many food products such as wine, grape juices and coffee in human OTA exposure. This regulation did not modify the maximum contents established previously on cereals or grapes. However, it includes a new directive limiting OTA contamination in grape juices, wines (red, white and rosé) to 2 µg/L.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Sampling

Dry cocoa beans from selected districts in Ashanti, Brong Ahafo, North Western and South Western regions of Ghana were sampled at random from takeover centre being the main growing cocoa regions in Ghana. The consignment was divided into smaller lots of about 30 bags and the split wire was applied as widely as possible to detect foreign matter. The split wire is made up of copper metal of dimensions 50 mm long x 30 mm wide. The sampling horn made of aluminum metal was used to draw samples from all sides of each cocoa bag and bulked into a container. The dimensions of the sampling horn is 100 mm long x 15 mm internal diameter. The bulked sample was thoroughly mixed and quartered. Two quarters of the opposite sides were rejected. The process was repeated until a final sample of 300 beans was obtained. The sample was then transported to the laboratory for analysis (Quality Control Company Division, 1997).

In total, fifty seven (57) samples were obtained, comprising sixteen (16) from Ashanti Region, nine (9) from Brong Ahafo, eighteen (18) from North Western and fourteen (14) from South Western Regions.

3.2 Reagents and Material

Table 3.1: Identification of reagents and materials

REAGENT	**GRADE**	**SOURCE**
Sodium bicarbonate	AnalaR	Labort, India
Methanol	HPLC	Park scientific limited, UK
Acetonitrile	HPLC	Park scientific limited, UK
Acetic acid	AnalaR	Labort, India
Phosphate buffer saline	AnalaR	R-Biopharm Rhone Ltd, Scotland
Imunoaffinity column	AFLAOCHRA PREP	R-Biopharm Rhone Ltd, Scotland
Filter Paper, No. 4	Whatman	Whatman Int. Ltd England

3.3 Instrumentation and Apparatus

Table 3.2: Equipment Identification

EQUIPMENT	TYPE
HPLC	Shimadzu HPLC—2010 with SIL-10AF Autosampler RF-10AXL Flourescence Detector
Analytical Column	Mediterranea SEA 18 5µm (25 cm x 0.46 mm)
Centrifuge	Centurion Scientific K3 Series
Vacuum Manifold	Supelco, USA
General Laboratory Glassware	Round bottom flask, volumetric flasks, centrifuge bottles, glass funnels, measuring cylinders.
Rotary Evaporator	Buchi Rotary Evaporator, German
Recirculating chiller	Buchi (German)
Ultrasonic Bath	Grant, Sanyo Company
Mercerator	IKA Ultra Turrax Homogenizer

3.4 Preparation of standard solution

An already prepared Ochratoxin A standard solution of 1000 ng/ml was available from R-Biopharm Ltd. Standard working 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 glacial acetic acid : acetonitrile (2:98). Working solutions from standard solution of 100 ng/ml were prepared according to the following table:

Table 3.3: Preparations of standard calibration standards

Standard N°	Concentration (ng /ml)	Volume taken (ml)	Final volume(ml)	Standard concentration (ng/mL)
1	10	2	10	2.0
2	10	4	10	4.0
3	10	6	10	6.0
4	10	8	10	8.0
5	100	1	10	10.0

3.5 Sample Preparation

3.5.1 Analytical Procedure

In the HPLC method, the essential steps are extraction of the analyte from the sample, clean up (or purification) and the chromatographic analysis.

3.5.2 Extraction

The OTA extraction was performed in alkaline conditions in accordance with the method described by Tafuri *et al*., 2004, Amézqueta *et al*., 2005. Approximately 200 g of cocoa beans were weighed and deshelled (Fig. 3.1a). The hammer mill was used in milling the cocoa beans sampled for Ochratoxin A analysis. Exactly 15 g of each sample was weighed into a 250 ml beaker (Figure 3.1b). Then 150 ml of an aqueous solution of a mixture (50:50 v/v) of methanol/ sodium bicarbonate 3% (m/v) was

added (Fig. 3.1c) and the mixture homogenized using the ultraturax for 2 minutes (Figure 3.1d). The mixture was decanted and filtered using the Whatman filter paper no. 4 into a 250 ml conical flask (Fig. 3.1e). Then 11ml aliquot of the filtrate was pipetted into a 100 ml beaker and an equivalent volume of Phosphate Buffered Saline (PBS) was added ready for clean up using immunoaffinity column.

Figure 3.1a

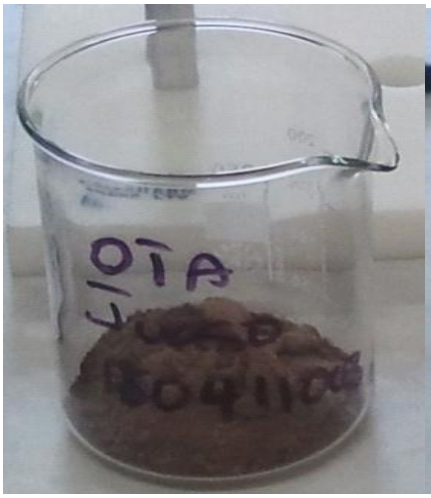


Figure 3.1b

Figure 3.1c

Figure 3.1d **Figure 3.1e**

Figure 3.1 The stages of OTA extraction in cocoa bean sample with methanol and sodium bicarbonate 3% (m/v). (a) Deshelled 200g of dry cocoa beans (b) milled cocoa beans (c) 15g of cocoa powder with methanol and sodium bicarbonate 3% (m/v). (d) Extraction of cocoa powder (e) filtration of the cocoa extract

3.5.3 Clean-Up Using Immunoaffinity Column (IAC)

The immunoaffinity column which was specific for OTA containing antibodies was placed on an SPE manifold (Supelco) and conditioned with 5 ml of PBS at approximately one to two drops per second, maintaining approximately 1 cm of solvent above the IAC antibodies at all times. A receiving flask was placed under the column to collect the eluate (Figure 3.2). Then 20 ml of the sample mixture was taken and loaded onto an empty glass column connected to the immunoaffinity column and released onto the column at a flow rate of 1-2 ml/min. The immunoaffinity column was washed with 10 mL of distilled water and then with 20 ml of PBS to remove non specific components.

OTA was slowly eluted using 1.5 ml of a mixture of acetic acid and methanol (2:98, v/v) at a rate of 1-2 drops /sec. Then the column was washed with 1.5 ml of distilled water and added to the mixture to obtain a final volume of 3.0 ml. This was then analysed using HPLC with Fluorescence Detector.

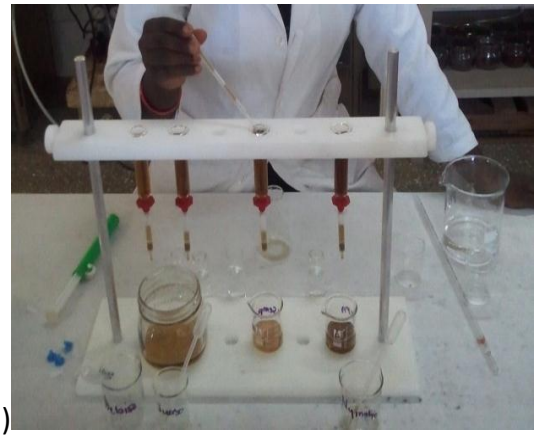
(a)  (b) 

Figure 3.2 Clean-Up of cocoa bean extracts using Immunoaffinity Column

3.6 Recovery test

Recovery test was performed by spiking cocoa samples with 0.5 ng/ml, 0.2 ng/ml and 0.1 ng/ml OTA standards. Exactly 15g of the milled cocoa samples were spiked with 1ml of the OTA standards. The samples were extracted after standing for 15 minutes. The spiked samples and blank samples without standard OTA were then extracted and analyzed by HPLC. Recovery was calculated using the formula below

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

3.7 Instrumental Conditions

Concentrations of OTA were determined with Shimadzu HPLC 2010 with a SIL-10AF Autosampler and Fluorescence Detector RF-10AXL. The separation was performed using isocratic mode at excitation and emission wavelengths of 333 mm

and 460 nm respectively. A 100 ul of the extract was injected into the HPLC and the column held at a temperature of 40^{o}C. The mobile phase was a mixture of acetonitrile/water/glacial acetic acid (55:43:2, v/v) and the column was a Mediterranea SEA18 5um (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 and 10 ng/ml concentrations. 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 concentrations were obtained by extrapolation from the calibration curve and calculated in µg/kg using the formula below

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

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 V_3Volume of test solution in ml,

V_4 is the Injection volume of test solution in ml,

D is the Dilution Factor.

CHAPTER FOUR

4. RESULTS AND DISCUSSION

4.1 Introduction

Fifty seven (57) samples from Ashanti Region, Brong Ahafo Region, Western North and Western South were analysed for Ochratoxin A in cocoa beans. HPLC chromatogram showing the retention time of the standard OTA and the calibration curve of the standard OTA are presented in Figure 4.1 and 4.2 respectively. Retention time for OTA was 7.17 min, the limit of detection (LOD) was 0.1 µg /kg and the limit for quantification (LOQ) was 0.4 µg /kg. Percentage recoveries of the various analyte ranged from 84 to 95%.

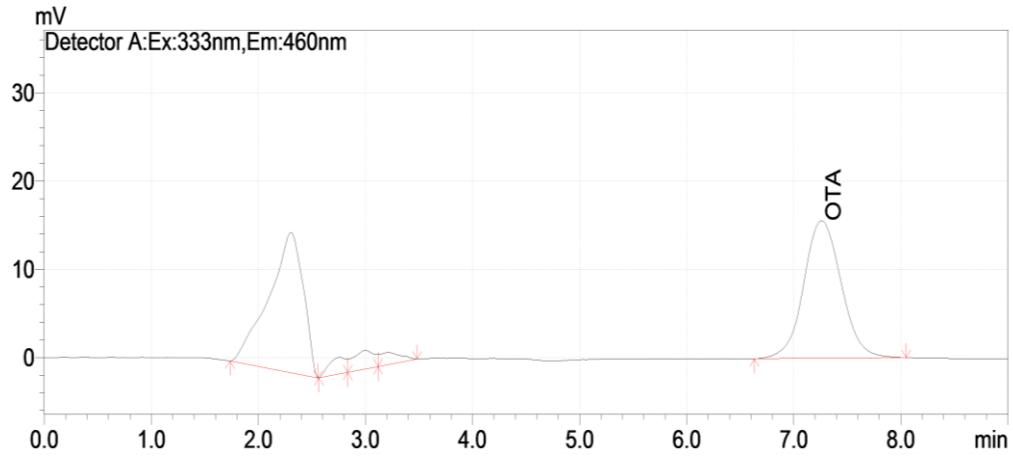


Figure 4.1 HPLC chromatogram showing the retention time of the standard OTA

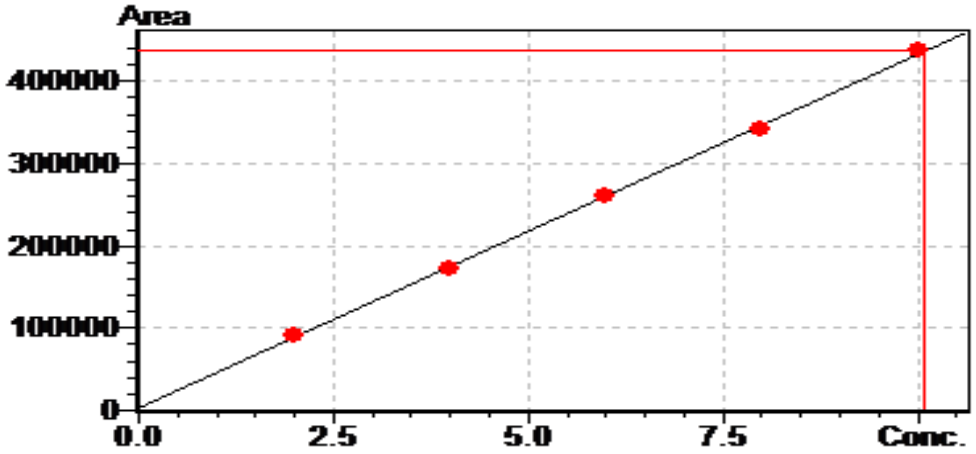


Figure 4.2 Calibration curve of OTA

4.2 Trends in OTA Concentration in cocoa beans sampled from the Regions

OTA was detected in samples from all the regions. The graphical representation showed some variation in the OTA content of cocoa beans samples. The concentration ranged from 0.698 µg/kg to 1.802 µg /kg.

A total of twenty seven (27) samples out of the fifty seven (57) cocoa beans samples contained OTA levels below the European Union (EU) permissible limits of 2 µg /kg and four (4) other samples representing (7%) (EU) were above.

The mean levels of OTA recorded are summarized in Figure 4.3

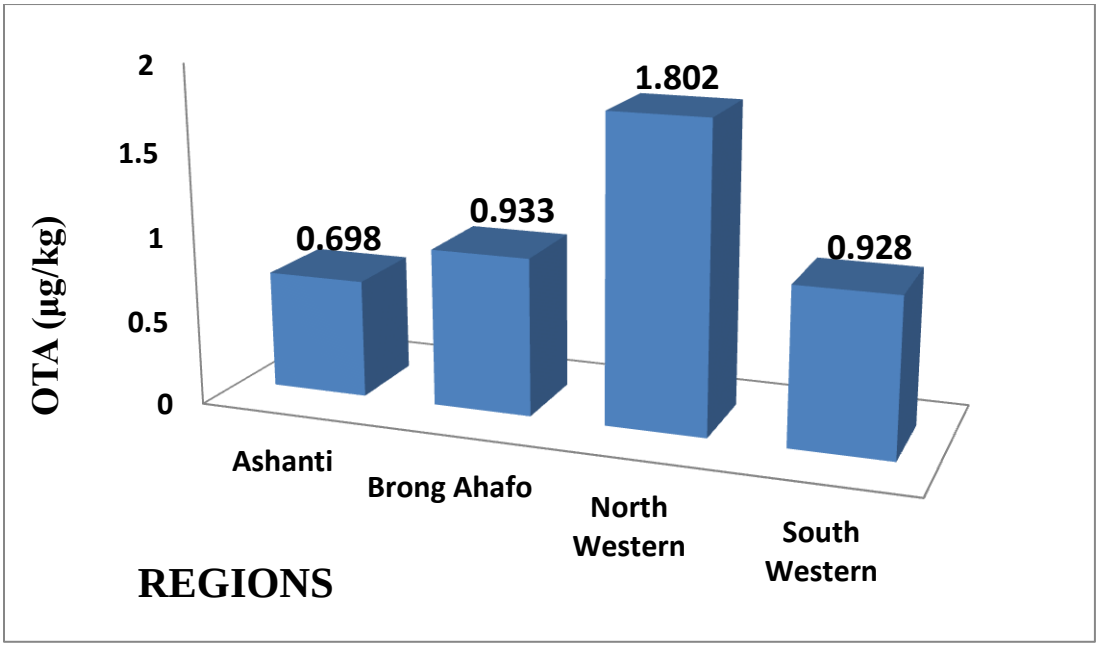


Figure 4.3: Mean concentration levels of OTA found in samples of beans from the regions

North western region recorded the highest mean concentration of 1.802 µg/kg followed by Brong Ahafo region (0.933 µg/kg), South Western region (0.928 µg/kg), Ashanti region recorded the least mean concentration of 0.698 µg/kg (Figure 4.3).

4.2.1 Samples from Ashanti Region

Sixteen (16) samples of dried cocoa beans were analyzed from the Ashanti region and OTA was detected in nine (9) of the samples. The highest concentration of OTA (2.193 µg /kg) was found in cocoa beans sampled from Bekwai and the lowest (0.063 µg /kg) was found in cocoa beans sampled from Forikrom. The levels ranged from ND to 2.193 µg /kg and the mean was 0.698 µg /kg (Table 4.1). All samples analyzed were below EU permissible limit of 2 µg /kg for cocoa beans except the sample from Bekwai which was above the proposed EU level.

Table 4.1 Levels of OTA in Cocoa Beans from Ashanti Region

Districts	Concentration (µg /kg) of OTA in cocoa beans	Districts	Concentration (µg /kg) of OTA in cocoa beans
Agona	ND	Effiduase	ND
Bekwai	2.193	Tepa	0.333
Obuasi	ND	Nkawie	1.275
Nyinahin	0.372	Antoakrom	ND
New Edubiase	ND	Mankraso	0.564
Juaso	ND	Konongo	0.369
Ampenim	0.468	Nsokote	ND
Offinso	0.642	Forikrom	0.063

Mean OTA concentration in cocoa beans from Ashanti Region = 0.698 µg /kg

*ND – Not Detected

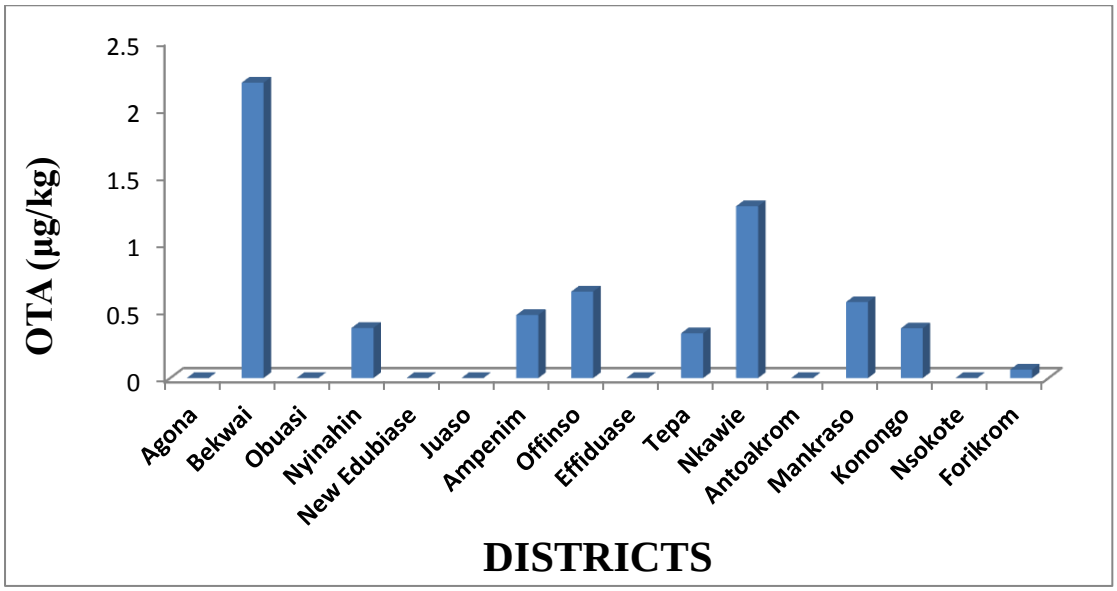


Figure 4.4: Concentration levels of OTA found in samples of beans from the Ashanti region

4.2.2 Samples from Brong Ahafo Region

Nine (9) cocoa beans samples from Brong Ahafo region were analyzed for OTA. OTA was detected in three (3) of the samples. The highest concentration of OTA

1.859 µg /kg was found in cocoa beans sampled from Kasapin and the lowest 0.261 µg /kg was found in cocoa beans sampled from Sankore (Figure 4.5). Levels of OTA for cocoa beans from Brong Ahafo Region ranged from ND to 1.859 µg /kg and the mean concentration was 0.933 µg /kg (Table 4.2). All samples analyzed were below the proposed EU level for cocoa beans of 2 µg /kg.

Table 4.2 Levels of OTA in Cocoa Beans from Brong Ahafo Region

Districts	Concentration (µg/kg) of OTA in cocoa beans	Districts	Concentration (µg/kg) of OTA in cocoa beans
Asumura	ND	Dormaa Ahenkro	0.678
Sunyani	ND	Kasapin	1.859
Nkrankwanta	ND	Goaso	ND
Kukuom	ND	Hwidiem	ND
Sankore	0.261		

Mean OTA concentration in cocoa beans from Brong Ahafo = 0.933 µg/kg

*ND – Not Detected

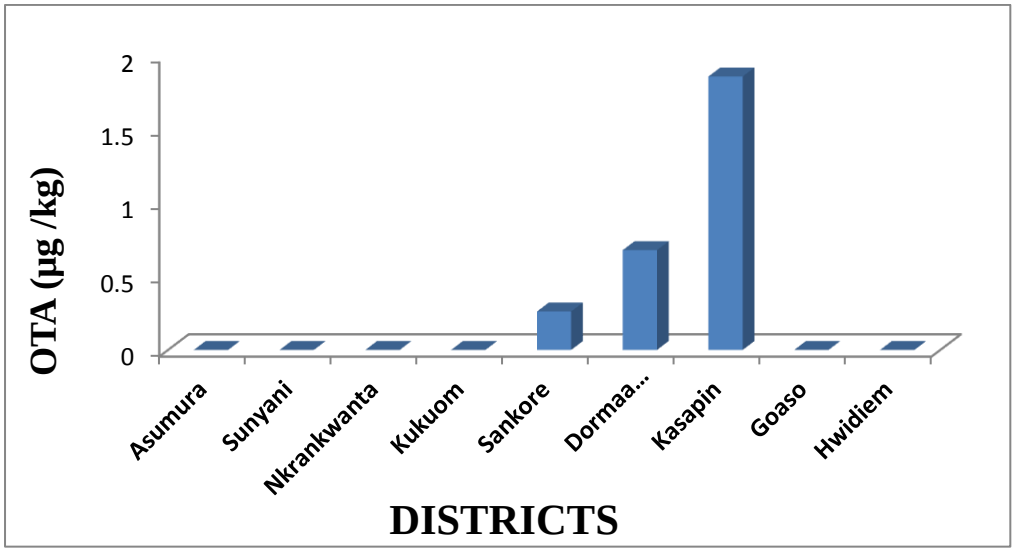


Figure 4.5: Concentration levels of OTA found in samples of beans from the Brong Ahafo region

4.2.3 Samples from North Western Region

OTA was detected in ten (10) out of the eighteen (18) samples analysed from the North Western Region. The highest concentration of OTA (4.650 µg /kg) was found in cocoa beans sampled from Sefwi Kaase and the lowest (0.393 ug/kg) was found in cocoa beans sampled from Debiso 'A' (Figure 4.6). The levels ranged from ND to 4.650 µg /kg and the mean concentration was 1.802 µg /kg. All samples analysed were below EU permissible limit of 2 µg /kg except for samples from Bonso Nkwanta 'A', Sefwi Kaase, Sefwi Asawinso which were 4.221, 4.650 and 2.595 µg /kg respectively.

Table 4.3 Levels of OTA in Cocoa Beans from North Western Region

Districts	Concentration (µg /kg) of OTA in cocoa beans	Districts	Concentration (µg /kg) of OTA in cocoa beans
Fosukrom	ND	Anhwiaso	ND
Juaboso	ND	Debiso 'A'	0.393
Akontombra	0.438	Debiso 'B'	1.970
S/Asawinso	2.595	Adabokrom	ND
Bonsu Nkwanta 'A'	4.221	Sefwi Kaase	4.650
Bonsu Nkwanta 'B'	0.906	Sefwi Wiawso	0.723
Asempaneye	1.331	Sefwi Essam	ND
Essam	ND	S/Tanoso	0.794
Awaso	ND	Bodi	ND

Mean OTA concentration in cocoa beans from Western North Region = 1.802 µg/kg

*ND – Not Detected

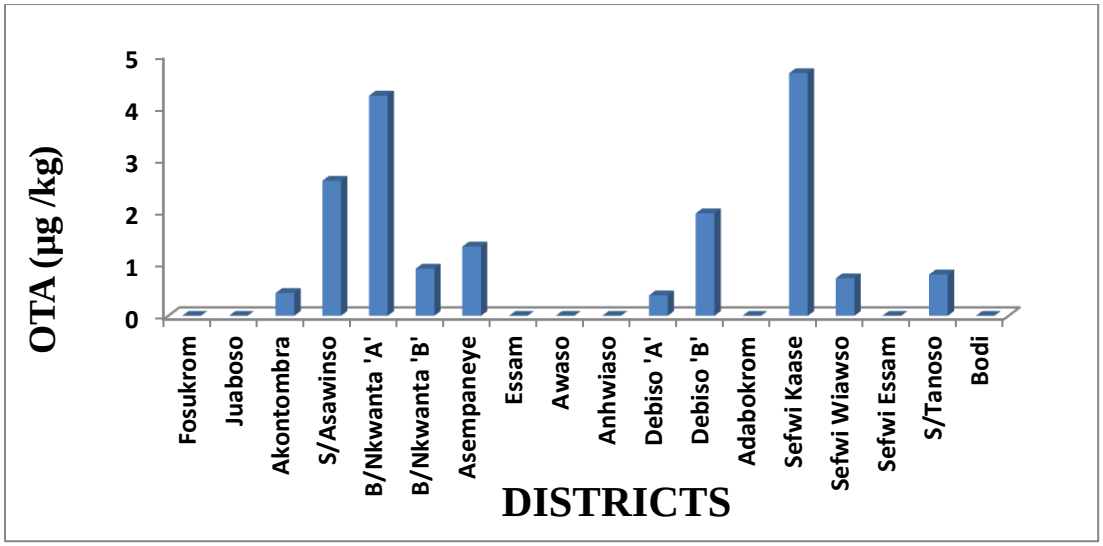


Figure 4.6: Concentration levels of OTA found in samples of beans from the North Western region

4.2.4 Samples from South Western Region

OTA was detected in five (5) out of the fourteen (14) samples analyzed from the South Western region. The highest concentration of OTA (1.557 µg /kg) was found in cocoa beans sampled from Tarkwa and the lowest (0.186 µg /kg) was found in cocoa beans sampled from Manso Amenfi (Figure 4.7). The levels ranged from ND to 1.557 µg /kg and the mean concentration was 0.928 µg /kg (Table 4.4). All samples analysed were below proposed EU level of 2 µg /kg for cocoa beans

Table 4:4 Levels of OTA in Cocoa Beans from South Western Region

Districts	Concentration (µg/kg) of OTA in cocoa beans	Districts	Concentration (µg/kg) of OTA in cocoa beans
Wasa Akropong	ND	Manso Amenfi	0.186
Tarkwah	1.557	Takoradi	0.210
Enchi ‘A’	ND	Dadieso ‘A’	ND
Enchi ‘B’	ND	Dadieso ‘B’	0.902
Asankragwa	1.539	Samreboi	ND
Dunkwa	ND	Nsawore	ND

Bogoso	ND	Diaso	ND
Agona Amenfi	ND		

Mean OTA concentration in cocoa beans from Western South Region = 0.928 µg/kg

*ND – Not Detected

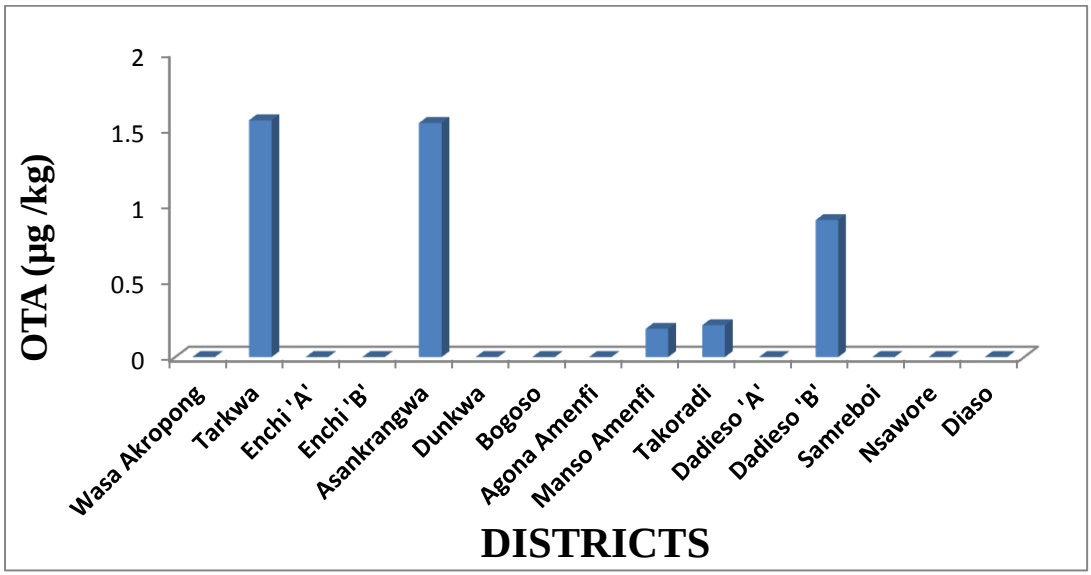


Figure 4.7: Concentration levels of OTA found in samples of beans from the South Western region

In a study carried out in Ghana (Abrokwa and Sackey, 2010), three types of cocoa fermentation were undertaken at three ecological locations using pods classified as healthy, diseased, diseased and damaged and damaged/broken. Several of the samples were positive for OTA at all the three ecological stations but the levels were generally very low ranging from 0.00 to 0.57 ppb (µg/kg). A prior assessment survey of OTA contamination conducted on 298 samples of cocoa beans (10kg each), collected from the commercial ports of Abidjan (151 samples) and San Pedro (147 samples) in Cote d'Ivoire, showed that mean contamination did not exceed 2 µg /kg. The contamination levels were 1.30 ± 0.02 µg /kg and 0.70± 0.02 in Abidjan and San Pedro respectively. The levels of OTA contamination observed in this survey mainly concerned raw

materials selected and destined for export (Dembele, 2009). Results from this study compare with the other results.

Few studies have been carried out on the incidence of OTA in cocoa beans. Miraglia and Brera (2002) reported a study in which none of their 96 cocoa bean samples had OTA levels exceeding 2ug/kg. Tafuri *et al.,* (2004) found OTA contamination of between 0.22 and 0.77 μg/kg in 10 samples of cocoa powder found on the Italian market. In a study conducted in 2005 in Japan, 14 of the 41 retail chocolate samples analyzed had OTA levels ranging from <0.10 μg/kg to 0.94 μg/kg (MHLW, 2006). Ten cocoa (10) powder and nine (9) chocolate samples from the open Belgium market were analyzed in 2005, (Vinkx, 2007). Five cocoa powder samples had levels below the LOQ (0.3 μg/kg) and the remaining 5 samples had OTA levels from 0.60 to 0.81 μg/kg. All the 9 chocolate samples contained OTA levels below the LOQ. Findings from analysis of cocoa beans from Brong Ahafo and South Western Region support this study where levels of OTA were below the proposed EU limits

High contamination frequencies have been found in cocoa samples and by-products. Burdaspal and Legarda (2003) showed 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). The report on the Task for Scientific Cooperation 3.2.7 showed that 81.3% of the cocoa-derived products analyzed were contaminated with OTA. This means out of the 547 cocoa product samples analysed 445 were positive. The contamination level varied from 0.01 to 3.8 μg/kg, with 0.23 μg/kg average (Miraglia and Brera, 2002). Amezqueta *et al.*, (2004) analyzed OTA in 46 cocoa bean samples of different origins and batches. A total of 63% of the samples

were contaminated (LOD of 0.04 μg/kg), with levels from 0.04 to 14.8 μg/kg, mean and median of 1.71 and 1.12 μg/kg respectively. These reports support the findings of analysis of cocoa beans sampled from Ashanti, Brong Ahafo, South Western and North Western region, where some of the cocoa beans were contaminated with high OTA concentration above the proposed EU permissible limit while some were below the limit.

Ochratoxin A contamination appears to be associated with the integrity (intactness) of the pods from which they were obtained, duration and conditions of drying, farm to farm practices and season of primary processing. Ghana Cocoa Board recommendation to farmers is that damaged and diseased pods should not be fermented with wholesome pods, and that beans should be dried to completion in maximum of seven days. The study shows that if these conditions are adhered to, contamination of Ghana's Cocoa beans by Ochratoxin A can be entirely prevented (Abrokwah *et al*., 2013).

The low levels of Ochratoxin A reported in this study indicated that the quality of cocoa in most of the districts are good. The low incidence in Ochratoxin A contamination suggested a low rate of infection of the Ochratoxin A producing fungi with subsequent production of toxin which may be due to good storage and weather conditions in the food supply. Since Ochratoxin A is stable and generally resistant to heat and processing, control of Ochratoxin A contamination lies in the control of the growth of the toxin producing fungi. Effective prevention of Ochratoxin A contamination therefore depends on good farming and agricultural practices. Good Agricultural Practices (GAP) including methods to reduce fungal infection and

growth during harvest, storage, transport and processing provide the primary line of defense against contamination of crops with Ochratoxin A.

It is known (Esteban *et al*., 2006) that drying and storage conditions play a major role in the presence of OTA. The results show that contamination prior to processing also greatly influences end-quality and that good pod condition and immediate pod opening can partly reduce the risks.

The average concentrations of OTA found in cocoa beans of different grade (exportable, non-exportable and total) are below the maximum residue of OTA (2 µg/kg), proposed by the regulation of the European Communities (CA, 2007). This low rate of OTA indicates the application of good production practices and marketing of cocoa beans.

CHAPTER FIVE

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The results from the study indicated that, out of the fifty seven (57) samples analysed OTA was detected in 27 samples accounting to 45 % and four other samples had levels above EU proposed limit of 2 µg/kg representing 7%.

North Western region recorded the highest OTA level of 4.650 µg/kg for samples from Sefwi Kaase followed by Ashanti region which recorded 2.193 µg/kg for samples from Bekwai. Brong Ahafo also recorded a level of 1.859 µg/kg g for samples from Kasapin and South Western also recorded 1.557 µg/kg from Tarkwa. The lowest OTA concentration of 0.063 µg/kg was recorded in cocoa beans sampled from Forikrom from the Ashanti region.

The concentration of Ochratoxin A in 93% of the cocoa beans samples analysed were all below the proposed limit of 2 µg /kg set by EU for cocoa beans and 7% of the cocoa beans samples had levels above the EU limit.

5.2 Recommendations

- Cocoa farmers should be educated on the need to observe good agricultural practices as these practices have been shown to have effect on mycotoxins.

- The results call for more future research work on OTA in cocoa beans from Sefwi Kaase, Sefwi Asawinso and Bonsu Nkwanta A from the Western North Region Ghana

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APPENDIX

TABLE 1: Levels of Ochratoxin A in Cocoa Beans from Ashanti Region

District	Mass in gram	Concentration from HPLC (ppb)	Actual Conc. (ppm)
Agona	15.02	0.000	0.000
Bekwai	15.00	0.731	2.193
Obuasi	15.01	0.000	0.000
Nyinahin	15.01	0.124	0.372
New Edubiase	15.01	0.000	0.000
Juaso	15.02	0.000	0.000
Ampenim	15.00	0.156	0.468
Offinso	15.01	0.214	0.642
Effiduase	15.01	0.000	0.000
Tepa	15.00	0.111	0.333
Nkawie	15.00	0.425	1.275
Antoakrom	15.02	0.000	0.000
Mankranso	15.00	0.188	0.564
Konongo	15.00	0.123	0.369
Nsokote	15.01	0.000	0.000
Forikrom	15.01	0.021	0.063

TABLE 2: Levels of Ochratoxin A in Cocoa Beans from Brong Ahafo Region

District	Mass in gram	Concentration from hplc (ppb)	Actual Conc. (ppb)
Asumura	15.00	0.000	0.000
Sunyani	15.02	0.000	0.000
Nkrankwanta	15.00	0.000	0.000
Kukuom	15.01	0.000	0.000
Sankore	15.00	0.087	0.261
Dormaa Ahenkro	15.01	0.226	0.678
Kasapin	15.01	0.620	1.859
Goaso	15.02	0.000	0.000
Hwidiem	15.00	0.000	0.000

TABLE 3: Levels of Ochratoxin A in Cocoa Beans from Western South Region

District	Mass in gram	Concentration from hplc (ppb)	Actual Conc. (ppb)
Wasa Akropong	15.01	0.000	0.000
Tarkwa	15.00	0.519	1.557
Enchi A	15.02	0.000	0.000
Enchi B	15.00	0.000	0.000
Asankragwa	15.00	0.513	1.539
Dunkwa	15.01	0.000	0.000
Bogoso	15.00	0.000	0.000
gona Amenfi	15.01	0.000	0.000
Takoradi	15.01	0.070	0.210
Dadieso A	15.01	0.000	0.000
Dadieso B	15.02	0.301	0.902
Samreboi	15.00	0.000	0.000
Nsawore	15.01	0.000	0.000
Manso Amenfi	15.01	0.062	0.186

TABLE 3: Levels of Ochratoxin A in Cocoa Beans from Western North Region

District	Mass in gram	Concentration from HPLC (ppb)	Actual conc.
Fosukrom	15.02	0.000	0.000
Juaboso	15.01	0.000	0.000
Akontombra	15.01	0.146	0.438
S/Asawinso	15.01	0.865	2.595
Bonsu Nkwanta B	15.00	0.302	0.906
Asempaneye	15.01	0.444	1.331
Essam	15.00	0.000	0.000
Awaso	15.00	0.000	0.000
Anhwiaso	15002	0.000	0.000
Debiso A	15.01	0.131	0.393
Bonsu Nkwanta A	15.00	1.407	4.218
Adabokrom	15.02	0.000	0.000
Sefwi Kaase	15.00	1.550	4.650
Debiso B	15.01	0.657	1.970
Sefwi Wiawso	15.01	0.241	0.723
Sefwi Essam	15.00	0.000	0.000
Bodi	15.01	0.000	0.000
S/Tanoso	15.01	0.262	0.794