UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

EFFECTS OF COLA NUT EXTRACT AND POWDER ON FUNGAL GROWTH, MYCOTOXIN PRODUCTION, PHYSICO-CHEMICAL PROPERTIES AND FAT QUALITY DURING DRYING OF FERMENTED COCOA BEANS

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL IN FOOD SCIENCE DEGREE

JULY, 2019

DECLARATION

I declare that this thesis was undertaken by me in the Nutrition and Food Science Department, University of Ghana, under the supervision of Professor Emmanuel Ohene Afoakwa, Professor Firibu K. Saalia and Professor Agnes S. Budu. All the references made to other research works have been duly acknowledged.

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DEDICATION

I dedicate this thesis to my wife and children, Georgina Kyei-Baffour, Afia Japongmaa Kyei-Baffour and Kwabena Otitiru Kyei-Baffour. I also dedicate it to my entire family especially my parents, Monica and Jacob who have been strong pillars in my life and also to my siblings and all my nieces and nephews.

ACKNOWLEDGEMENT

I want to acknowledge God first and foremost for the gift of life, His mercies and grace which have led me to this point of my life. To my Professors, Professor Emmanuel Ohene Afoakwa, Professor Firibu K. Saalia and Professor Agnes S. Budu, under whose supervision and directions have made this research a success. I say God bless you all and I am very much grateful. I am again grateful to Dr. Edem Kongor of Food Research Institute for his immerse contribution and constructive criticisms.

Again, I would like to express my gratitude to the Council for Scientific and Industrial Research – Food Research Institute (CSIR-FRI) for allowing me to use its laboratories for this research work most especially to the head of Chemistry Division, Mr. George Anyebuno, the Technologist in Charge of the Chemistry Lab, Mr Nelson Amey and also to Miss Emefa Gblende of the Chemistry Lab. Much appreciation also goes to the Head of Microbiology Lab, Dr. Margret Owusu and to Mr. Alexander Appiah and Mr. Yahaya all of the microbiology Laboratory and also to Papa Toa of the Processing Division for their great support and insight given me.

I am very much thankful to my colleagues and friends who helped me in many ways to make this work successful especially Elorm Ofori, Efua Essandoh, Francis, Abigail, Portia, Loretta and Frank Mboom.

Finally, I say a very big thank you to Mr. Danso for allowing me to use his cocoa farm for this research work. I say God bless us all.

ABSTRACT

Cocoa beans are the main ingredient for making chocolate and in Ghana, it is a major contributor to the economy as it generates an annual foreign exchange of about US $2 billion. The ability to produce high quality cocoa beans free from moulds and mycotoxin contamination has therefore become critical due to the impact mycotoxins have on health of consumers and global trade. This study investigated changes in fungal growth, mycotoxin concentrations, physico-chemical properties and cocoa butter qualities as affected by cola nut raw powder and cola nut powdered extract using a 2 x 4 full factorial experimental design with treatment type (cola nut raw powder and cola nut powdered extract) and treatment levels (0 g/5 kg, 50 g/5 kg, 100 g/ 5kg and 150 g/5 kg) as the principal factors. Changes in pH, titratable acidity, fermentation index, cut test scores, free fatty acids, peroxide value, iodine value, mould colony forming unit (*Aspergillus spp, Penicillium spp, Rhizopus* and *Mucor spp*) and mycotoxins concentrations (Aflatoxins and Ochratoxin A) of cocoa beans as affected by cola nut powder and extract were studied using standard analytical methods.

There was a general decrease in acidity of cola nut treated cocoa beans as treatment concentrations increased. The measured pH ranged from 5.39 for control to 5.71 for 150 g/5 kg cola powder treated beans while the titratable acidity ranged from 0.112 (mole of NaOH/g) for 150 g/5 kg cola powder treated beans to 0.122 (mole of NaOH/g) for the control.

The cocoa butter qualities of cola nut treated cocoa beans were better than that of the control which had no cola nut treatment. The 150 g/5 kg cola treated beans recorded the least FFA content of 0.71 % and 0.61 % for cola extract and cola powder treatments, respectively. These were significantly different ($p < 0.05$) from the control which recorded 2.34% free fatty acid content. Peroxide value of 21.6 Meq/kg was recorded for the control which was significantly higher than

15.8 Meq/kg and 14.2 Meq/kg for 150 g/5 kg cola extract and cola powder treated beans, respectively at 95% confidence level. The untreated cocoa beans had the highest iodine value of 35.96 g I/100 g which was significantly different ($p < 0.05$) from 33.63 g I/100 g and 33.21 g I/100 g for the 150 g/5 kg treated beans for cola nut powder and extract, respectively.

Penicillium spp., *Aspergillus spp.*, *Rhizopus* and *Mucor spp.* of moulds were identified in the dried fermented cola nut extract and powder treated cocoa beans. Concentration of 150 g/5 kg cola extract significantly ($p < 0.05$) inhibited growth of *Penicillium spp* from 6.13 log cfu/g to 4.18 log cfu/g which represented 98.9% inhibition while 150 g/5 kg cola powder inhibited growth from 6.13 log cfu/g to 4.7 log cfu/g representing 96.3% inhibition. *Aspergillus spp* had a 100% inhibition for both cola nut extract and powder for the highest treatment concentration of 150 g/5 kg which significantly ($p < 0.05$) reduced growth from 4.88 log cfu/g for control (0 g/5 kg) to no visible growth on OGYEA plate. *Rhizopus* and *Mucor spp* in contrast showed no significant reduction for all cola nut treatment concentrations except for 150 g/5 kg cola extract which significantly ($p < 0.05$) reduced growth of *Rhizopus* and *Mucor* from 4.95 log cfu/g for control to 4.3 log cfu/g.

Aflatoxin levels for all treatment concentrations including the control were below the instrument's limit of detection (0.13 µg/kg for aflatoxins B1 and B2 and 0.15 µg/kg for aflatoxins G1 and G2). With respect to the control (0 g/5 kg), there was significant reduction of OTA ($p < 0.05$) from 8.32 µg/kg to 2.85 µg/kg and 3.02 µg/kg, respectively for the 150 g/5 kg extract and 150 g/5 kg powder concentrations. These represented 65.7% and 63.7% reduction for 150 g/5 kg cola extract and powder, respectively.

Generally, cola nut powder and extract at concentrations of 150 g/5 kg of cocoa beans led to a considerable reduction of mouldiness and associated mycotoxin production in dried cocoa as well as improved the cocoa butter qualities.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Cocoa (*Theobroma cacao L.*) is considered the major ingredient for making chocolate and other cocoa related products (Beckett, 2008). It is a significant agricultural export commodity in the world especially for some developing countries. In Ghana, cocoa is a key contributor to the government income and the Gross Domestic Product (GDP). Ghana Cocoa Board [COCOBOD] (2018) stated that about 70% of cocoa grown in Ghana is exported and this generates an annual foreign exchange of approximately US $2 billion with current production reaching 800,000 tons per annum.

Food safety and security has become a basic requirement and so the ability to produce high quality cocoa beans free from moulds and its associated mycotoxins for chocolate production has become very important. The contamination of agricultural commodities by moulds and associated mycotoxins is of concern to both advanced and developing countries due to its impact on health of consumers and on global trade (Wu, 2005). In cocoa, the mycotoxins of utmost importance include Ochratoxin A produced by *Aspergillus ochraceus* and *Penicillium spp*. and Aflatoxins produced by *Aspergillus flavus* (Tjamos *et al.,* 2004; Varga *et al.,* 2005). Work done by Copetti *et al.* (2011) showed the production of these mycotoxins occurs mainly during the primary stage of processing the cocoa beans particularly during fermentation, drying and storage. Copetti *et al.* (2011) reported the isolation of *Penicillium spp*. at the fermentation phase of cocoa beans, *Aspergillus flavus* and *Aspergilus paraciticus* during drying of cocoa beans whereas *Aspergillus flavus*, *Penicillium* and *Aspergillus ochraceus* have been isolated during storage of cocoa beans. Work done by Copetti *et al.* (2013) found Ochratoxin A in cocoa beans during fermentation with

concentrations below 50 ppb as well as Aflatoxin B1 with concentrations below 20 ppb (Copetti *et al.*, 2011). During storage, the Ochratoxin A levels in cocoa have been found to be above 100 ppb (Copetti *et al*., 2013; Coulibaly *et al*., 2013). Ochratoxin A concentrations in cocoa beans from the Western region of Ghana have also be found to exceed 2 ppb which is the proposed European Union (EU) regulatory limit (Yamoah, 2015).

Earlier works done to control mycotoxin production in cocoa beans have focused on the use of chemical agents, radiations and natural spices after the drying process to lower the levels of Ochratoxin A in cocoa beans (Mintah-Appau, 2016). The possibility of pretreatment of the cocoa beans prior to drying (i.e. after fermentation) with natural alkaloids to control the growth of moulds and associated mycotoxins have not been considered. Naturally occurring alkaloids are known to be very important in protecting plants against pathogenic organisms (Wink, 1998) and they pose no harmful effect on the environment when compared to the alkaloids of synthetic origin (Duke *et al.*, 2008). Example of a naturally occurring alkaloid is caffeine (1,3,7-trimethylxantine) which is found mostly in coffee, cola nut and tea leaves (Smith, 2002). Caffeine has been found to inhibit the growth of moulds, yeast and bacteria (Raut *et al*., 2013). Work done by Park *et al.* (2005) concluded that caffeine causes cell wall changes in fungi by inhibition of the enzyme phosphodiesterase in cell walls ensuing in high intracellular calcium levels. This leads to the antagonistic effect of adenosine receptors thereby leading to the death of cells (Serafin, 1995). The growth of *Aspergillus spp* as well as *Penicillium spp* have also been demonstrated to be inhibited by caffeine (Buchman and Lewis, 1984; Raut *et al.*, 2013). The natural alkaloid has also been found to prevent the production of aflatoxins by *Aspergillus parasiticus* and *flavus* (Maraqa *et al*., 2007). Nonthakaew *et al.* (2015) demonstrated that crude coffee extracts, which is a good source of caffeine, could significantly reduce the fungal load on Areca palm leaf sheath including

Aspergillus spp. and *Penicillium spp*. It is therefore assumed that rich sources of the natural alkaloid (caffeine) could be used to control mould growth in cocoa beans in humid environments where the risk of mould contamination is high.

Cola nut is a major source of caffeine where one nut is known to contain more caffeine than two big cups of coffee in America (Kiple and Ornelas, 2000). In addition to the high caffeine content, cola nut has also been found to contain modest amount of methylxanthine and theobromine with appreciable amounts of anthocyanins and other phenolic compounds (Burdock *et al.*, 2009). Cola nut extract, a rich source of caffeine, has also been reported to hinder the proliferation of some types of *Aspergillus* fungi to about 70% (Kanoma *et al*., 2014). In this study, cola nut extract and powder were used to treat cocoa beans before drying in a humid environment to control mould growth and mycotoxin production.

1.2 Problem statement and justification

Drying of cocoa beans using the sun is an inexpensive and very popular method used by cocoa farmers in Ghana and is regarded the best method for optimal quality (Afoakwa, 2014). Sun drying allows slow movement of moisture throughout the cocoa beans making the transportation of flavour precursors formed during fermentation possible (Sukha, 2003). According to Afoakwa (2014), cocoa beans are ideally dried between 5-7 days. Prolonged drying of the beans (beyond the 7 days) favours the growth of moulds and possible mycotoxin production resulting in low quality cocoa beans. During the raining season, there is not enough sunshine often resulting in prolonged drying period (beyond the 7 days), leading to mould infestation (Afoakwa, 2014). In such weather conditions, it is necessary to find safer ways to mitigate the issue of mould infestation and mycotoxin production. Although some work has been done using chemical agents, radiations

and natural spices to reduce mould infestation and subsequent production of mycotoxins in dried fermented cocoa beans (Mintah-Appau, 2016), none of these earlier works focused on the use of natural alkaloids to effectively reduce mould infestation and subsequent production of mycotoxins prior to the drying process in a safer way. Cola nut is locally cultivated in Ghana and it has high content of the natural alkaloid caffeine and therefore cola nut powder and extract could be used to treat cocoa beans before drying to reduce mould infestation and subsequent production of mycotoxins.

1.3 Main objective

The core aim of this research was to study the effects of cola nut extract and powder on fungal growth, mycotoxin concentrations, physico-chemical properties and fat qualities of dried fermented cocoa beans.

1.3.1 Specific objectives

The specific objectives of the study included the following;

i. To identify and enumerate the growth of moulds on dried fermented cocoa beans as affected by cola nut extract and powder treatments.
ii. To assess the effectiveness of cola nut extract and powder on Ochratoxin A and Aflatoxin reduction in dried fermented cocoa beans.
iii. To determine the effects of cola nut extract and powder on the physico-chemical properties (pH, titratable acidity, fermentation index, cut test) of dried fermented cocoa beans.

iv. To determine the effects of cola nut extract and powder on the fat qualities (free fatty acids, iodine value and peroxide value) of dried fermented cocoa beans.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Cocoa Production

Cocoa (*Theobroma cacao L.*) is part of the Malvaceae family and originated from Central and South America tropical forests. Economically, the cacao specie is amongst the few *Theobroma* genus that has been exploited (Afoakwa, 2014; Alverson *et al.*, 1999). The bulk of global cocoa (80–90%) is produced by about 5–6 million smallholder cocoa growers, with 40–50 million persons worldwide dependent on cocoa for their living (World Cocoa Foundation, 2014). In Ghana, approximately 800,000 farm families are engaged in cocoa farming and they are spread over the six cocoa producing regions of Ghana (Ghana Cocoa Board, 2010). A typical cocoa farm in most producing countries in Africa extends between 2–5 ha of land with each hectare producing about 300–400 kg of cocoa beans (World Cocoa Foundation, 2014).

Ghana has had different administrations including the colonial regime who have been dependent on cocoa as a chief source of public income (Ghana Cocoa Board, 2018). Depending on the global prices of cocoa and macroeconomic circumstances such as inflation, revenue extraction by the government have had changing effects on cocoa cultivation (Herbst, 1993). McKay and Coulombe (2003) noted that data have suggested that since the 1990s, cocoa-farming families in the cocoa cultivating regions of Ghana have experienced massive improvements in their livelihood when compared to farmers involved in general crop farming. Cocoa as a cash crop has therefore helped reduced poverty among cocoa farmers and a household survey has indicated that poverty within cocoa-producing families were approximately 60 percent at the beginning of the 1990's and this dropped to as low as 23.9 percent in 2005 (World Bank, 2007).

2.2 History of cocoa

The name cocoa was adopted from cacao as a corrupted version, which is known to have come from the Amazons in South America (Dillinger *et al*., 2000). The historical story of cocoa is known to have begun with an alcoholic drink which had undergone fermentation that was discovered in the Ulua Valley, present day Honduras dating back to 1400 BC (Maugh, 2007). The Aztecs in Mexico were the first people known to have cultivated the crop when cocoa found its way to Mexico, then to South America, then later spread throughout the Caribbean islands (Umaharan, 2018). The Aztecs attributed religious importance to cocoa and they used cocoa for religious ceremonies and believed that the cocoa tree was given to them by the god of air, Quetzalcoatl (Dand, 1997). Cocoa was consumed as a drink, to which spices and honey were added and sometimes even maize by the Aztecs. The maize when added were fermented to make the cocoa drink alcoholic (Nair, 2010). According to Nair (2010), cocoa beans were also used as money which was considered highly valuable during trade.

By AD 600, the Mayans had started agricultural cultivation of the cocoa tree and had established cocoa farms. Until 1502, the rest of the world was not involved in cocoa production and during the 1520s, Hernán Cortés, a Spaniard, sent cocoa to Spain and Spanish Guinea. Mass production of cocoa begun in the 16th century by the Spanish in Central America (Afoakwa, 2014). It then extended to the British, French and Dutch West Indies (Jamaica, Martinique and Surinam) in the 17th century and also to Brazil in the 18th century. Cocoa found its way to Saõ Tomé and Fernando Pó (currently part of Equatorial Guinea) from Brazil in the 1840s. The cash crop then got to other parts of West Africa, including the Gold Coast, modern day Ghana and the Côte d'Ivoire from Saõ Tomé and Fernando Pó (Afoakwa, 2010). The Swedish botanist Carolus Linnaeus called the cocoa tree *Theobroma cacao*, which is considered as the official botanical

name (Umaharan, 2018). The cocoa press was developed in 1828 which allowed for cocoa fat to be gotten from cocoa beans, whiles the Swiss later developed milk chocolate and solid chocolate both from cocoa beans (Fowler, 2009).

Records available in Ghana according to Ghana Cocoa Board (2010) show that Dutch proselytizers established cocoa in the coastal belt of the then Gold Coast as far back as 1815 and the Basel proselytizers also had planted the plant at Aburi in 1857. However, the crop planted during this period of time did not result in the mass cultivation of the crop in Ghana until Tetteh Quarshie, who hailed from Osu in Accra, went to Fernando Pó and returned home with Amelonado cocoa pods and cultivated the crop at Akwapim Mampong in the Eastern Region of Ghana in 1879. The Ghana government in order to acknowledge the major input of cocoa to Ghana's economy established the Ghana Cocoa Board (COCOBOD) in 1947 which serves as the core institution to develop the cocoa sector in Ghana. The Ashanti, Brong Ahafo, Eastern, Volta, Central and Western regions of Ghana are the main cocoa producing regions in Ghana (Ghana Cocoa Board, 2010).

2.3 Taxonomy and classification of cocoa

The botanical name of cacao (*Theobroma cacao)* was first defined by the father of modern-day plant classification, Carolus Linnaeus. *Theobroma* is exclusively known to be neotropical with respect to its origin and its natural occurrence is in the tropical lowland rainforests (Hebbar *et al.*, 2011). There have not been any known reproductive challenges between cultivated and wild cacao trees. Morphological characteristics of even the earliest cultigens, Criollo cacao from Mesoamerica, are similar to those of their wild counterparts (Iwaro *et al*., 2005). The wild type of cacao can therefore be used for cultivation of cocoa commercially as either progenitors or as clones

(Eskes and Efron, 2006). Five categories of *Theobroma* have been described by Bernoulli (1869) according to their floral morphology and fruit structure. These are:

- Cacao is one of the five categories which has a stipitate petal ligule. The staminodes is erect and subulate with the stamens having 2-antheriferous. The calyx is parted into five with equal laciniae. The cacao also has ovate-oblong fruits and there are four species including *T. cacao L.*, *T. pentagona* (cacao lagarto), *T. leiocarpa*, (cumacaco), and *T. saltzmanniana*.
- Oreanthes has appendix petal being subsessile with the staminodes being erect and subulate. The stamens have three antheriferous and the calyx is parted into five with the laciniae being equal. It includes three species: *T. speciosum, T. quinquenervia* and *T. spruceana*, all from Brazil.
- Rhytidocarpus have their petals being subsessile with appendix and the staminodes are erect and claviform. The stamens have 2-antheriferous and the calyx is parted into five with equal laciniae. The fruits appear woody and they include two species: *T. bicolour Humb*. (synonym, *T. ovatifolia DC*.) and *T. glauca Karst*.
- Telmatocarpus have petal appendix with erect staminodes and the subulate are linear with a broad base. The stamens have 3-antheriferous, the calyx is parted into five, the laciniae are equal, the fruit is ovate and lacunose. This included only one species: *T. microcarpa Mart*.
- Glossopetalum have petal ligule stipitate, staminodes are reflexed and petaloid, stamens have 3-antheriferous, the calyx is irregular with 3-5 fid and foliaceous with a sublignose fruit. These have the largest section, including eight species: *T. augustifolia DC*., *T.*

subincana Mart., T. sylvestris Mart., T. macrantha, T. ferruginea, T. obovate, T. alba and *T. nitida*.

Aside these categories based on their flora morphology and fruit structure, there are 22 recognized species of *Theobroma* and among these species, *Theobroma cacao* is the main specie grown outside its original habitat and also cultivated for commercial purposes (Hebbar *et al*., 2011). These 22 species are also characterized into 6 sections with regards to their structural morphology. These are Andropetalum, Glossopetalum, Oreanthes, Rhytidocarpus, Telmatocarpus and Theobroma (Hebbar *et al*., 2011). According to Hebbar *et al*. (2011), there are four main varieties of the *Theobroma cacao* tree and these are the Forastero, cultivated mainly in West Africa, Criollo which is mostly susceptible to diseases, the Nacional which has fine flavour and it is grown in Ecuador and Trinitario, which is a hybrid of Forastero and Criollo.

The Criollo variety refers to genetically similar trees, believed to have been made fit for cultivation by the Mayan civilization (Hebbar *et al*., 2011). Criollo has its beans typically round and consist of cotyledons that are white or pale purple in nature. This variety exhibit symptoms such as poor productivity and are highly susceptible to diseases (Fowler, 2009). Due to this, they are less widely cultivated (Afoakwa, 2010; Fowler, 2009). However, they are considered to be of high quality due to their less bitter taste and high aromatic nature and hence impact a mild nutty cocoa flavour than other varieties (DeZaan Cocoa Manual, 2009). This makes Criollo cocoa beans highly priced on the cocoa market. Currently, Criollo is not very popular and it is available in Venezuela, Central America, Madagascar, Sri Lanka and Samoa, mostly in old plantation farms (Fowler, 2009). The pods of Criollo normally have a thin husk which is soft in nature with a textured surface and red pigmentation to some extent. Chocolate produced from Criollo has a light dark colour and usually low in basic chocolate flavour (Fowler, 2009).

Forastero in Spanish means 'foreigner' and trees of cocoa that are genetically different from Criollo or a hybrid are referred to as Forastero. Usually, the seeds of Forastero cocoa are deep purple in colour (Hebbar *et al.,* 2011). The Forastero cocoa tree is grown mostly in West Africa and also in Southeast of Asia and it forms the majority of cocoa produced worldwide (Afoakwa *et al*., 2012; Afoakwa, 2010; Fowler, 2009). With respect to fruit and tree morphology, the Forastero show high level of variability. The cocoa pods are hard when ripe with a yellow and more rounded form similar to a melon and the pods may contain 30 or more beans. Comparatively, Forastero is more resistant to diseases including the swollen shoot, the yellow mosaic and the black pod than Criollo and Trinitario cocoa (Afoakwa, 2010). It is also less susceptible to pests including capsids and cocoa pod borer (International Cocoa Organization [ICCO], 2012a). Characterized by darker brown cotyledons, the Forastero variety has slightly bitter taste with a strong flavour. Chocolate produced with this variety is characterized by a high chocolate flavour and low fruity flavour notes (Hebbar *et al*., 2011). The cultivars of Forastero are the Amelonado, Amazonica and the new mixed hybrid which make up 13.3%, 34.4% and 52.3%, respectively (Afoakwa, 2014).

Trinitario is mainly referred to as progenies of hybrids which were originally produced in Trinidad using the Criollo and Forastero varieties (Willson, 1999). Its existence is believed to have come about after a hurricane almost destroyed the local Criollo crops in 1727 according to Wilson (1999). This variety has hard pods and different colours with more than 30 beans per pod having variable colours although white beans are not usually seen (DeZaan Cocoa Manual, 2009). Because Criollo and Forastero were used to produce the Trinitario variety, it expresses traits common to both varieties (Afoakwa, 2014). It can also show traits which are totally different and vary from each other. The Trinitario is hard when matured with high yield, a trait from Forastero

and also has the refined taste of Criollo. Trinitario types have not been found under wild conditions (ICCO, 2012b).

Ecuador is the home for the Nacional and the variety is supposed to have come from Ecuador in the Amazonian region (Fowler, 2009). Its aroma and flavour characteristics are quite different from the other varieties and there are some which have arriba flavour and these are hybrids of Nacional and Trinitario (Afoakwa, 2010; Fowler, 2009). Due to the fact that it is cultivated less, its contribution to global cocoa production is only 5%. A genetic mixture between native Nacional cocoa and foreign germplasm is found in current day Ecuador plantations (Solorzano *et al*., 2009), thus reducing the fine-flavoured cocoa aroma of the variety.

2.4 Cultivation conditions for cocoa

For the successful cultivation of the cocoa crop, certain environmental conditions must be met to ensure proper growth of the cash crop. These environmental conditions include temperature, rainfall patterns and soil types (Afoakwa, 2014).

2.4.1 Temperature

In general, temperatures between 18–21 °C are considered the minimum range for cocoa cultivation whereas maximum temperature required ranges from 30–32 °C. For mass production of cocoa, the average minimum temperature should not go below 13 °C even during the coldest time of the year and when temperatures fall below 10 °C, the yield will be massively reduced (Afoakwa, 2014). According to Afoakwa (2014), at temperatures below 8 °C, defoliation and dieback of the cocoa occurs.

2.4.2 Rainfall

Rainfall just like temperature, affects flowering and pod setting during the growth of the cocoa tree. Rainfall has the maximum influence on the yield of cocoa throughout the year more than any other environmental factor that also influence yield (Afoakwa, 2014). Cocoa cultivation needs rainfall throughout the year ranging between 1,000–4,000 mm per year, but precisely between 1,500 and 2,500 mm are known to be the average range required by the cocoa plant (Afoakwa, 2014). In conditions such as the dry season where there is no rain or little rain below 100 mm per year, cocoa trees may not survive should conditions continue longer than three months (Afoakwa, 2014).

2.4.3 Soil

The cocoa trees require soils with particles considered as coarse so that it can allow for free space for the root to develop. There should also be nutrients available to a surface depth of about 1.5 m to enable the roots access them. Afoakwa (2014) reported that the growth of the cocoa tree is also dependent on the pH conditions of the soil where it may grow well in soils whose pH range is between 5.0 to 7.5 which makes cocoa one of the crops that can withstand both acidic and alkaline soils. However, when the acidity of the soil is below a pH of 4.0 and alkalinity above a pH of 8.0, the cocoa tree might not grow well. The organic content in the soil should also be high enough having about 3.5% organic matter when a depth of 15 centimeters of the top soil is considered. The soil should also have a total cation exchange capacity of about 35% (ICCO, 2005). The best total nitrogen/phosphorus proportion must also be about 1.5 according to ICCO (2005). Approximately 200 kg of nitrogen, 25 kg of phosphorus, 300 kg of potassium and 140 kg calcium

is required for every hectare of land to cultivate the cocoa tree prior to pod production (Afoakwa, 2014).

2.5 Propagation of cocoa

Cocoa is propagated using seedlings and they are nursed before planting on the field to protect them against strong wind and also to provide them with nutrition and irrigation. The seeds from ripe cocoa pods are used and if the seeds are immediately planted upon opening, it results in at least 90% of seedlings germinating within two weeks (Afoakwa, 2014). Mostly when certain desired traits are required, vegetative propagation is also done. Propagation without seeds can be done by cuttings, budding or marcotting. Cuttings comprise making cocoa tree cuttings with a minimum of 2-5 leaves and 1 or 2 buds. The leaves are cut in half and the cutting is put in a pot under polythene until roots sprout (Willson, 1999). Marcotting involves removing a strip of bark from a branch and the stripped area is covered in sawdust and a polythene sheet. The area will shoot roots and the branch can then be cut off and planted (Adu-Ampomah *et al.*, 1987; Willson, 1999). With budding, a bud is taken from a tree and it is put under a flap of bark on another tree. The budding patch is then guaranteed with raffia, waxed tape of clear plastic to prevent moisture loss. When the bud is growing, the old tree above it is then cut down (Adu-Ampomah *et al.*, 1987; Willson, 1999). These desired traits may include special flavour profiles, drought resistance, higher yields, early maturing and disease resistance. The trees grown from vegetative propagation grow uniformly and performs better than the trees grown using the traditional system of planting with seeds. After three to six months, young cocoa plants can be transferred to the field and planted (Afoakwa, 2010).

2.6 Pests and diseases of cocoa

Cocoa trees and its pods are susceptible to some pests and diseases and ICCO (2015) estimates that losses of cocoa resulting from the infestation of pest and diseases can reach between 30% to 40% of global production. According to Nair (2010), more than 1,500 diverse insects have been documented to feed on cocoa trees, however, those which are of economic importance may not exceed 2%. The cocoa pod borer, the cocoa beetle, mirids, thrips, stemborers and the broad mite are considered to be the common insects that feed on cocoa trees.

The infestation of the Cocoa Pod Borer (CPB) is mainly triggered by the presence of the insect *Conopomorpha cramerella*. The major symptoms resulting from CPB attacks include unevenness and early ripening of pods and they attack both young and mature cocoa pods (ICCO, 2015). The Cocoa Beetle (CB) lay eggs at the bark of cocoa trees and the larvae creates a chamber in the cambium and the bark of the tree where it feeds. Shade is key to the attack of this insect where the intensity of infestation increases as shade on the farm is less (Nair, 2010). Cocoa Stemborers come in diverse forms including the larvae of *Z. coffeae* and their activities affect and cause damage to young cocoa trees which lead to breaking off of small cocoa branches (Adu-Acheampong *et al.*, 2004). *Phytophthora palmivora* canker is a disease that may be introduced to cocoa trees by Stemborers aside causing massive damage to the trees by themselves (Adu-Acheampong *et al.*, 2004). Thrips also lay eggs and use fluids to cover their eggs making the eggs look like a dark disc (Astridge *et al.*, 2005). Eggs from Thrips may hatch in four days where the larvae feed on the leaf content by puncturing holes in them (Baah and Anchirinah, 2011). *Distantiella theobroma* and *Sahlbergella singularis* are the most common species of Mirids in many West African countries (Baah and Anchirinah, 2011). According to ICCO (2015), in a time period of three years, Mirid destruction alone can cause a massive yield reduction to as much as

75% if no measures are put in place to control them. Mirids feed on the stems of cocoa trees, branches, and pods by piercing and this leads to the death of cells causing dieback (Baah and Anchirinah, 2011). Broad mites on the other hand feed on young fruits (2.5cm in diameter) to cause scar and expose the tissues. Although most feeding occur on fruits, Broad Mites also feed on young leaves causing the leaves to curl up (Baah and Anchirinah, 2011).

The Witch's Broom (WB), Black Pod, Frosty Pod Rot and Vascular Streak Die back (VSD) are the major diseases known to infest cocoa and they are mainly caused by fungi and viruses. The fungi *Moniliophthora perniciosa* is known to cause the Witches Broom disease (ICCO, 2015). According to ICCO (2015), the witches broom disease within a period of ten years caused losses to as high as 70% at a district in Brazil called Bahia. Young growing tissues are the target of the fungi and so they infest shoots, young pods and flowers. This attack on cocoa trees results in the growth of branches bearing no fruits and so leaves tend to wither on such branches. The pods affected by this disease shows green patches resulting from uneven ripening (ICCO, 2015). *Moniliophthora roreri* is the fungi whose activities cause the Frosty Pod Rot disease in cocoa. Pod tissues which are constantly growing are the ones that get contaminated by these fungi. The major symptom that become visible appear on the pod surface as a white mat (ICCO, 2015). The dry, powdery form of spores also permit the distribution of the spores by either water or wind and also by any physical disturbance of infested pods with the majority of spores being produced during the raining season (ICCO, 2015).

The activities of the *Phytophthora spp* of fungi on cocoa trees result in the Black Pod disease with *P. megakarya, P. palmivora and P. capsica* known as the species responsible for the disease (CRIG, 2008). ICCO (2011) reported approximately 20-30% yield losses of cocoa to *P. palmivora* and the fungi is also responsible for cocoa tree deaths of up to 10% yearly. Infestation

of the pods may occur at any developmental stage with first visible symptom being dark spots at any part of the pod which are usually small and hard. Internal tissues, with the beans inclusive, are also attacked resulting in shriveled mummified dark cocoa pods (ICCO, 2015). During the raining season where there is high relative humidity, up to 4 million sporangia could be produced from infested pods which can infest other cocoa trees upon dispersion (Opoku *et al.,* 2007). *Oncobasidium theobroma* is known to be the main fungus responsible for the Vascular Streak Dieback disease in cocoa (ICCO, 2015). There is the possibility of hyphae growing from infested leaves when they fall during the raining season. These hyphae develop into basidiocarp which becomes visible as a whitish flat covering over the leaf scar (ICCO, 2015).

2.7 Harvesting of cocoa pods

Harvesting of cocoa pods is the foremost step in cocoa beans processing and it is only ripe pods that are harvested since immature pods will not undergo fermentation (Afoakwa, 2014). Cocoa pods that are ripe may be available throughout the whole growing season but many cocoa producing countries have two major harvesting peaks every year. However, there can be dramatic changes in harvest times due to changes in weather patterns which may affect yields (World Cocoa Foundation, 2014). Ripe pods are easily identified from the unripe using the colour of the pods. Some varieties of the cocoa pods turn from green to yellow when matured and so can easily be identified from immature pods (Mikkelsen, 2010). Manual harvesting is done using a sharp blade to make a cut through the stalk for low hanging cocoa pods whereas a pruning hook with a long pole is used for high hanging pods by pushing or pulling based on how the fruit is positioned to permit the stalk to be cut without any damage to the branches. Sharp blades are necessary during harvesting due to the ease with which fungi can infest cocoa trees when damaged during

harvesting. It is imperative that the harvesting blades are sharp, so the cushions of the trees are not injured since they are a possible point for fungi contamination (Mikkelsen, 2010). When fungi are introduced to cocoa tree during harvesting, pods that have not been harvested become black and the beans start to deterioration or germinate, and this may also spread diseases among other cocoa trees (Amoa-Awua *et al*., 2006). Within 3 to 7 days upon collecting the cocoa pods, they are opened to scoop out the beans. Using a wooden club to split open the cocoa pods is the most appropriate way to open the pods which if struck at the center, makes the pod to split into two halves which makes scooping out the wet beans by hand easy. A machete can be used as an alternative to open the pods, though this may cause some damage to the beans (ICCO, 2011).

2.8 Fermentation of cocoa beans

Fowler (2009) stated that cocoa beans that have not been processed have a very bitter taste which is not pleasant. According to Romero-Cortes *et al*. (2013), fermentation, drying and roasting of the cocoa beans give it the desired characteristics of good flavour for cocoa powder and chocolate production. The fermentation is done immediately after the cocoa beans have been scooped from the pods. The beans become unintentionally inoculated with a variety of microorganisms just when they are scooped out from the pods (Thompson *et al*., 2001). Spontaneous fermentation is the foremost step of cocoa processing which can last for 3 to 10 days where heaps, boxes, baskets, or trays are employed. Heap fermentation mostly is practiced and very popular in Ghana (Afoakwa, 2014).

During fermentation, microbial activities in the mucilaginous pulp lead to the production of alcohols and acids by breaking down sugars and this raises the temperature of the beans to kill the cells. The death of the bean results in many changes that are important for flavour development

during the production of chocolate (Romero-Cortes *et al*., 2013). The major acid produced during fermentation is acetic acid and variations in the levels of acetic acid results in the changes in pH observed during fermentation where its movement from fermented pulp to the cotyledon of the bean decreases pH inside the bean (Thompson *et al*., 2001). Ardhana and Fleet (2003) observed that fermentation occurs with microorganisms including yeasts, lactic acid bacteria, and acetic acid bacteria. The yeasts break down the pectin in the pulp to liquify it leading to the reduction in pulp viscosity (Ardhana and Fleet, 2003). Ethanol is produced from the sugars in the pulp with some amount of citric acid under anaerobic conditions. As the pulp drains out, ethanol is produced which increases temperature and pH also increases creating an environment perfect for lactic acid bacteria and acetic acid bacteria to grow (Ostovar and Keeney, 1973).

Thompson *et al*. (2001) reported that the conversion of sugars and organic acids primarily into lactic acid is due to the activities of lactic acid bacteria and as more oxygen becomes available, acetic acid bacteria also grow and oxidizes the ethanol formed by yeasts to acetic acid. The ethanol and acetic acid produced together with the heat generated kills the embryo of the bean as the ethanol and acid migrate into the beans by diffusion (Afoakwa *et al*., 2013). The death of the seed results in many biochemical changes which lead to the development of many flavour precursor molecules for flavour and colour development of the beans (Afoakwa, 2016; Afoakwa *et al*., 2013; Thompson *et al*., 2001). Five major fermentation techniques are mostly used, and they include heap, box, basket and tray fermentations and also curing on drying platforms.

2.8.1 Box fermentation

Afoakwa (2014) stated that among all the various fermentation methods, box fermentation is very popular and it is done across the globe and it is used in West Africa as well. Boxes which are made from wood or concrete are used where wet cocoa beans are poured to undergo fermentation. To make way for the sweatings coming from the pulp to drain off, holes are punched at the bottom end of these boxes and the holes at the same time enable air to enter the box during fermentation. The boxes are at all times elevated above the ground and fixed on drains to make way for the sweating to drain off (Afoakwa, 2014). The boxes could be arranged such that fermenting beans could easily be mixed and turned by transferring beans from a higher box into a lower one after every 48 hours (Opoku-Ameyaw *et al.*, 2010). The boxes are built large enough to enable 25 kg to 1000 kg wet beans (1.2 x 1.2 x 1.2 m) to be fermented. Plantain leaves are used to cover beans in the topmost box where temperature is monitored and the closer the temperature get to 50 °C, the better the fermentation (Afoakwa, 2014; Opoku-Ameyaw *et al.*, 2010).

Figure 2.1: Wooden box fermentation of cocoa beans

Source: AusAid (2010)

2.8.2 Heap fermentation

Heap fermentation is popular among smallholder cocoa growers in Ghana and other West African cultivating countries. This method is the simplest and normally used on small farms and has been recognized to produce good quality cocoa beans when compared to the other fermentation techniques (Afoakwa, 2014, 2010). Heap fermentation is done by putting fresh leaves of plantain in a circular manner on the ground and wet beans heaped on them. Holes are mostly made into the plantain leaves to create drainage holes where the pulp sweatings can drain off. More fresh plantain leaves are used to cover heaps and logs are used to support the leaves in place. The beans are covered to prevent drying at the surfaces, mould growth and also to help retain heat produced inside the heaped beans. The size of the cocoa beans heap varies from 25 kg to 1000 kg of cocoa beans (Afoakwa, 2014; Opoku-Ameyaw *et al.*, 2010).

Figure 2.2: Heap fermentation of cocoa beans

Source: AusAid (2010)

2.8.3 Tray fermentation

Tray fermentation came about as a result of studies done on heap fermentation to come up with improved method of fermentation over the heap fermentation (Afoakwa, 2014). Shallow trays are perforated at the bases and stacked on top of one another in which wet cocoa beans are fermented. Around 90 kg of wet cocoa beans are put in these wooden trays which measure 1.2 x 0.9 x 0.1 m in dimensions for fermentation. About 6 to 12 of such trays may be placed on top of each other with an unfilled tray left at the base to enable air to move into fermenting beans and also to allow for the drainage of pulp sweatings (Opoku-Ameyaw *et al.,* 2010). Beans loaded in the top most tray are covered with banana or plantain leaves and finally with gunny sacks after 24 hours of fermentation. This is to enable the conservation of heat that is produced during fermentation. There is no need for turning and fermentation becomes complete after 3 to 5 days (Afoakwa, 2014; Opoku-Ameyaw *et al.,* 2010).

Figure 2.3: Wooden tray fermentation of cocoa beans

Source: AusAid (2010)

2.8.4 Basket fermentation

The basket fermentation method is somehow comparable to the heap fermentation regarding their simplicity and it is used by small-holder farmers mostly in some part of Ghana as well as other part of the world including Nigeria, the Amazon region, the Philippines (Mossu, 1992). Baskets used are woven and there are no defined sizes for the baskets used. Before wet beans are placed in these baskets, fresh plantain leaves are used to line the inside of the baskets and they usually hold up to 150 kg of wet beans (Opoku-Ameyaw *et al.*, 2010). The baskets are covered with more plantain leaves and logs used to hold the leaves in place. The flanks and the base of the baskets serve as entry points for air as well as exit points for pulp sweatings to drain off. Turning of beans during fermentation is done by transferring them from one basket to another (Afoakwa, 2014; Opoku-Ameyaw *et al.*, 2010).

Figure 2.4: Woven basket fermentation of cocoa beans

Source: AusAid (2010)

2.8.5 Curing on drying platforms

According to Afoakwa (2014), Ecuador is the only country mainly known to practice this method of cocoa fermentation. The pods are split opened and wet beans heaped on drying platforms. During the day, beans are spread on the drying stands and at night, they are heaped to retain heat. Some parts of Ecuador employ an alternate approach where an extended trench is occupied with stones and sand and bamboo mattings used for covering. Wet cocoa beans are heaped in masses on this base to a depth of about 40 cm and leaves are used to shelter the cocoa and the whole also sheltered with a tin roof with mixing after every 24 hours. This method of cocoa fermentation is convenient, but if not managed properly results in under-fermented cocoa with undesirable mould growth. It also results in cocoa with off-flavour if not managed properly (Afoakwa, 2014).

2.9 Drying of cocoa beans

Cocoa beans may be stored for long durations before marketing and processing thus to prevent mould contamination and low-quality beans during storage, the moisture content needs to be reduced to 7%. It is advisable to maintain 7% moisture content of dried cocoa beans because when the moisture content exceeds 8%, the possibility of moulds growing on and within the beans are very high, whereas below 5% the beans also become very brittle (Cunha, 1990). Some of the biochemical changes which occur during fermentation continue during drying and this helps to improve flavour development during chocolate production (Afoakwa, 2014). Knight (2000) reported that the changes aside flavour development also reduces bitterness and astringency of chocolate produced from well dried beans. Various ways are available for drying fermented cocoa

beans and these can be categorized as sun-drying (natural) and mechanical (artificial) drying according to Afoakwa (2014).

With sun drying, a drying area with raised mats in an open space is used where the beans are spread thinly on elevated mats in the open sun. The beans are stirred frequently to pick out germinated, spoilt beans, placenta and any foreign materials. Sun drying is best for good quality beans and this is because as much as it is an inexpensive method of drying cocoa, it also allows for slow movement of water molecules throughout the beans (Sukha, 2003). Sukha (2003) again noted that this makes the transportation of flavour precursors formed during fermentation to all parts of the bean possible. During sun drying, there is a partial transportation of nonvolatile acids (lactic acid) to the shell of the bean whilst volatile acids (acetic acid) evaporates through the shells of the bean. Polyphenols are also oxidized during drying which results in the decrease in astringency and bitterness of the bean (Romero-Cortes *et al.*, 2013). The beans should give a cracking sound when slightly pressed if they are well dried. During sun drying, the beans are dried for a minimum of 7 days when there is continuous sunshine during the day (ICCO, 2011).

Mechanical or artificial drying of cocoa has resulted mainly due to frequent raining and also the frequency with which turning of the bean is required which can be tedious when drying is done manually. Mechanical dryers are used and some of these dryers vent hot air to the drying bed which has the beans by using rocks painted black which accumulate the heat from the sun. Also, they have a roof which is let down whenever the rain falls and also at night. This help to prevent the movement of the cocoa bean around resulting in minimal period and labour needed for the drying of cocoa (Thompson *et al*., 2001). Afoakwa (2014, 2010) stated that heat exchangers are used to construct other artificial dryers and the hot air is separated from the products of combustion which is made to pass through an exhaust to the atmosphere. Knight (2000) also noted that direct

fired heaters may also be used to construct artificial dryers, where the combustion products blend with the hot air and are used to dry the beans. Oil or solid fuels can be used as a source of power for these dryers. Mechanical dryers lead to fast drying of cocoa beans leading to a high acid, low chocolate flavoured cocoa (Afoakwa, 2016, 2010; Knight, 2000; Thompson *et al*., 2001).

2.10 Storage of cocoa beans

After drying, the cocoa beans are cleaned from any foreign debris before they are collected into clean, strong jute sacks. The dried beans are stored in a well-ventilated storage room with relative humidity below 70% to avoid re-humidification of the beans and the bags of cocoa packed on wooden pallets to avoid rodents and insect pests. With a moisture content of 7%, cocoa beans can be kept for 3 to 12 months in warehouses under ideal storage conditions prior to shipment (Afoakwa, 2016; Opoku-Ameyaw *et al.,* 2010).

2.11 Mould growth and mycotoxin production in cocoa

Mycotoxin is a term that originated from the Greek word “mycos” and “toxicum” also derived from the Latin language to mean mould and poison, respectively (Adegoke, 1993). Heidler *et al*. (2003) indicated that the term mycotoxin is used to define a set of secondary products of metabolism formed by some fungal species of several moulds, mainly associated with the genera *Fusarium*, *Aspergillus* and *Penicillium* that when ingested by humans or animals may cause diseases or even lead to death. According to Pettersson (2012), there are countless number of these toxins in existence, some of which are very toxic thus when ingested into the system may cause severe mycotoxicosis. Mycotoxin biosynthesis is directly affected by environmental conditions including temperature, rainfall patterns, humidity as well as certain farm practices including

cultivation and harvesting, storage periods of agricultural produce and some post-harvest activities. Foods that have moulds growing on them are not always contaminated with mycotoxins, even when mould infection of the foods might have occurred, and secondary metabolism might have taken place (de Magalhães, 2010).

Work done by Pettersson (2012) showed that some mycotoxins are more common in certain crops than others due to the susceptibility of the crop to certain toxigenic moulds and so conditions become favourable for the moulds to produce these toxins. Crops found at certain locations and regions are more prone to mycotoxin production than other regions due to the particular climatic or ecological conditions in such places (Pettersson, 2012). According to Adegoke (1993), there are over three hundred (300) mycotoxins of fungal metabolites in existence that are possibly toxic to both animals and humans. However, Thompson and Henke (2000) indicated that those of economic importance in relation to trade and health are aflatoxins, Ochratoxins, fumonisins, deoxynivalenol, T-2 and T-2 like toxins (trichothecenes) and alternariol.

One condition that is very conducive for mycotoxins to be produced is high humidity, thus food crops found in tropical and subtropical zones are highly susceptible to mycotoxins contamination as compared to those in the temperate regions (Thompson *et al*., 2001). Also, work done by Tournas and Katsoudas (2005) showed that plants found in environments with drought can be rendered vulnerable to contamination by *Aspergillus* species. A major risk factor considered is their global existence and over 25% of the world's produce are susceptible to mycotoxin (moulds) contamination annually as stated by the Food and Agriculture Organization (FAO, 1999). Heidler *et al*. (2003) noted, the impact of these toxins depends on the physical and chemical nature of the toxin, the rate of exposure and level of intake. Mycotoxins are by nature known to be

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

According to Fowler (2009), in Ghana, cocoa production is very common in the country's forested zones including the Ashanti, Central, Brong-Ahafo, Eastern, Western and Volta regions which are noted for their high rainfall patterns, ranging between 1,000-1,500 mm per year (Fowler, 2009; Wood and Lass, 1985). Ochratoxin A (OTA) is a mycotoxin that has been reported to contaminate cocoa and its products and with the maximum permitted value of OTA set at 2 µg/kg. Cocoa cultivation in Ghana is dominated by peasant farmers on a low input and technology basis, with a regular farm size of about 4.0 hectares and a cultivation yield of 246.4 kg/ha (Afoakwa, 2014). Bonvehi (2004) has estimated that about 40% of the cocoa which arrives in European countries from West African countries may not be deemed as compliant products. The incidence of toxigenic fungi in dried cocoa beans has become a common observation according to Mounjouenpou *et al.* (2008).

Mounjouenpou *et al.* (2008) showed that filamentous fungi are a major contaminant of cocoa bean throughout the post-harvest processing starting from pod breaking to the drying of the fermented cocoa bean. Guehi *et al*. (2010) stated that fermentation type used during cocoa fermentation is one of the processes that could be used to control fungal contamination of the beans though the growth of the fungal microflora mostly is dependent on relative humidity, temperature, efficiency and rate of the drying step of cocoa beans. The growth of these toxigenic fungi may result in the formation of secondary metabolites including Ochratoxin A (OTA) and aflatoxins which are the major mycotoxins formed mostly by some species of *Aspergillus* and *Penicillium* genera of moulds (Copetti *et al*., 2013; Mounjouenpou *et al*., 2008).

2.11.1 Ochratoxin A

One of the most widespread and carcinogenic substances among mycotoxins is Ochratoxin A. This mycotoxin is a chlorinated isocoumarin compound, with the chemical name [7-(L-*â*-phenylalanylcarbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocoumarin] (Pitt and Hocking, 2009). According to Aydin *et al*. (2003), Ochratoxin A is effectively nephrotoxic, teratogenic, and immunotoxic, predominantly on the renal system. The toxin is formed by some fungal species in the genera *Aspergillus*. However, Aydin *et al*. (2003) stated that *Aspergillus carbonarius* and *Penicillium* v*iridicatum* are most common in cocoa bean at a minimum water activity (*A*w) of 0.85. Under different conditions of moisture, pH and temperature, these fungal species are able to grow on different varieties of substrates but in fruits, they only can appear after they have been contaminated by pathogenic organisms or after the fruit has suffered a physical or physiological damage (Zimmerli and Dick, 1996). Both, fungi and OTA, can exist in all stages of the cultivation chain of cocoa, that is from harvest through to fermentation, drying, storage and transport (FAO, 1999).

Clark and Snedeker (2006) reported the presence of OTA in many food crops from plant origin (cereal grains, grapes, legumes and nuts) whereas Mounjouenpou *et al*. (2008), Copetti *et al*. (2013) and Turcotte and Scott (2011) have also stated the presence of the mycotoxin in cocoa and cocoa products. When products contaminated with Ochratoxin A are ingested, humans and animals take up the toxin through the digestive tract and also by breathing of air contaminated with OTA may pose an added exposure to humans. The major food sources prone to OTA contamination includes cereals, cocoa, wine, spices, coffee, musts, grape juice, dairy products, beer, dried fruits, and pulses (Burdaspal and Legarda, 2003).

There are a diverse range of Ochratoxin byproducts that can be characterized, however, Ochratoxin A and infrequently Ochratoxin B are those that exist naturally in secondary metabolites of moulds (Pitt and Hocking, 2009). Romani (2003) stated that Ochratoxin A is not easily destroyed during food processing and can withstand heating in foods. According to Abrunhosa *et al.* (2010), the genera of moulds usually associated with toxin production, which occur naturally, are *Aspergillus*, *Penicillium* and *Fusarium*. The International Agency for Research on Cancer has characterized Ochratoxin A as a conceivable human cancer-causing agent (group 2B). According to European Commission (2010), in order to preserve public health, prevention of food contamination by OTA and other mycotoxins is very important because of their potential toxicity and possibility of accumulation through chronic exposure. The European Union has therefore set a maximum permitted level for OTA in certain products (including cereals, cocoa, coffee and wine) and is also thinking about its expansion to other foods including meat items.

The International Agency for Research on Cancer expressed that worries have been on the ascendancy about human exposure to Ochratoxin A in the most recent decade and therefore the enthusiasm for studies assessing the sources of this contaminant in harvests has turned out to be essential. Consumer exposure to OTA is reported to be on the ascendancy and so to secure consumers, the European Union has put up a standard to define tolerable limits for regulation (European Commission, 2010). As these toxicants can never be totally expelled from the food supply, numerous nations have characterized levels in food (tolerances, guideline levels, maximum residue levels) that are probably not going to be of well-being concern (Stoloff *et al*., 1991). The regulatory limits in cereals have been set at 5 µg/kg for whole grain and 3 µg/kg for processed products whereas the proposed regulatory limit for cocoa product has been set at 2 µg/kg (European Commission Regulation, 2004).

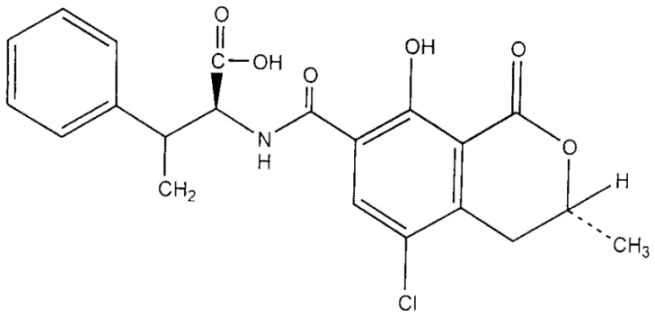

Figure 2.5: Chemical structure of Ochratoxin A

Source: Bennett and Klich (2003)

2.11.2 Aflatoxins

Aflatoxins are secondary products of metabolism biosynthesized by fungi in the genus *Aspergillus*, mainly *A. flavus* and *A. parasiticus* (Makun *et al*., 2010). Council for Agricultural Science and Technology, [CAST] (2003) stated that *Aspergillus* is a name used to describe moulds in a genus that undergo asexual reproduction only. According to Wild and Gong (2010), in *Aspergillus* taxonomy, the morphological structure of the conidiophore, which is the assembly that produces asexual spores, is the main substantial taxonomic character considered. In natural ecosystems and as part of the human economy, *Aspergillus* species which are ubiquitous are part of the major successful class of moulds. *Aspergillus* spores are regular components of aerosols and are carried by air currents to disperse over both near and far depending on environmental conditions. The spores are deposited on either liquid or solid surfaces when they contact such surfaces and with the right conditions of moisture, germination occur, and the presence of these moulds may lead to the biosynthesis of aflatoxins as their secondary metabolite during their metabolic activities (Wild and Gong, 2010).

Aflatoxins exists in 4 main forms; namely aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2 and Eaton and Groopman (2013) indicated that there are more than eighteen structurally similar mycotoxins known to exist in these four types of aflatoxins. The fungi know to produce aflatoxins are mostly found in nature and is capable of growing under varied environmental conditions where organic food sources and moisture are present (Eaton and Groopman, 2013). According to Warburton *et al*. (2009), aflatoxins are odourless, tasteless and colorless, however, *Aspergillus* often look greenish to the naked eyes. Nath and Sarma (2005) stated that aflatoxins are chemically stable and resistant to degradation and destruction under normal cooking temperatures and procedures. Aflatoxins designated by B1 and B2 have been found to give off strong blue fluorescence when viewed under ultraviolet light, whilst the G1 and G2 forms give off greenish yellow fluorescence under ultraviolet light (Guo *et al*., 2008). According to Yu (2004), *Aspergillus flavus* is known to produce aflatoxins B1 and B2 as well as other toxic compounds including cyclopionic acid, kojic acid, aspergillic acid, nitropropionic acid and aspenoxin. Aflatoxin G1 and G2 on the other hand are known to be produced by *Aspergillus parasiticus* in addition to kojic acid, aspergillic acid, nitropropionic acid and aspenoxin but not cyclopionic acid.

Among all types of aflatoxins, the B1 is known to have high levels of toxicity and also considered as the most gangerous hepatocarcinogenic natural compound ever isolated. The B1 is predominant with level of carcinogenicity being B1, G1, B2 and G2 in order of high to low carcinogenicity respectively (FAO, 2007). Aflatoxin M1 and M2 are oxidative forms of aflatoxin B1. They are significant members belonging to the aflatoxin family which can be isolated from milk and are altered in the gastrointestinal tract of humans and some animals (Huang *et al*., 2010). Eaton and Groopman (2013) noted that aflatoxins are not needed by the fungi for growth and came

about as a result of secondary metabolic activities of the fungi and so unlike primary metabolites, these secondary metabolites have survival functions in nature. At high growth rates, there is no occurrence of the expression of secondary metabolite biosynthesis genes. This points to the synthesis of these metabolites occurring during growth repression (Eaton and Groopman, 2013). Despite the low occurrence of aflatoxins in cocoa unlike Ochratoxins A, work done by Copetti *et al.*, (2011) reported levels not exceeding 20ppb in cocoa beans. The European Union has however proposed a maximum permissible limit of 2ppb for Aflatoxin B1 in cocoa products (European Commission Regulation, 2004).

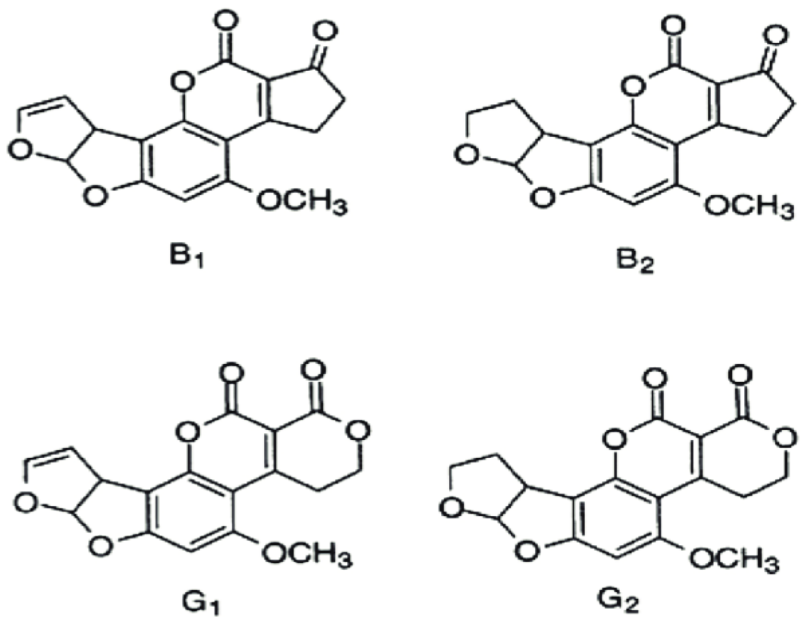

Figure 2.6: Chemical structures of four types of aflatoxins

Source: Guo *et al*. (2008)

2.12 Natural alkaloids

Alkaloids exist in nature as basic nitrogen containing chemical compounds and so the name basically was gotten from the word alkaline and it described any nitrogen containing base (Zhang *et al*., 2013). Many groups of organisms are capable of producing these compounds and they include bacteria, fungi, plants and animals (Dang *et al*., 2012). According to Kakhia (2003), extraction of these natural alkaloids from plant origins are mostly done by extracting with acids generally hydrochloric acid or sulfuric acid, even though organic acids including maleic acid and citric acid can also be used. Dang *et al.* (2012) recounts that natural alkaloids mostly have pharmacological properties and so they are used either as medicines or in medications, as drugs for recreational purposes, or in entheogenic rituals.

Some examples of natural alkaloids include cocaine, used as a local anesthetic and also as a stimulant, the most popular and widely used stimulant caffeine, nicotine, analgesic morphine and quinine which is employed as an anti-malarial drug. Alkaloids are basic on the pH scale and their basicity rest on their lone pair of electrons on their nitrogen atoms (Kakhia, 2003). Kakhia (2003) again stated that alkaloids can form salts with mineral acids mainly because they are organic bases and these mineral acids include hydrochloric acid, sulfuric acid and some organic acids including tartaric acid and maleic acid. Their salts are mostly soluble in water than the free base forms. According to Kanoma *et al*. (2014), cola nuts are sources of natural alkaloids in some pharmaceutical preparations.

2.12.1 Caffeine

Caffeine is a bitter, white crystalline alkaloid that exist in nature and is categorized as a xanthine and can be utilized as a psychoactive stimulant medication (Kakhia, 2003). It forms structures with some chemical compounds and insoluble complexes including guaranine found in guarana, a complex found in mate, mateine, and theine which likewise is found in non-herbal tea. These chemical complexes containing caffeine may likewise contain extra alkaloids, for example, the cardiovascular stimulants theophylline and theobromine, and frequently, they may contain different chemicals, for example, polyphenols which can form insoluble complexes with caffeine (International Food Information Council Foundation [IFIC], 2003). As indicated by Kakhia (2003), caffeine is a white crystallin solid substance that have a density of 1.2 g/cm^3 and it is somewhat soluble in water at room temperature with increase in solubility in water with an increase in temperature, yet it is very soluble in solvents like ethyl acetate, chloroform and pyrimidine. Kakhia (2003) again noticed that caffeine is moderately soluble in alcohol and marginally dissolvable in petroleum ether and benzene. Its melting point is around 235 °C and has a boiling point of 178 °C. Plants that contain this natural alkaloid are noted for their caffeine content and they include coffee, tea, cola and some extent cocoa (Kakhia, 2003).

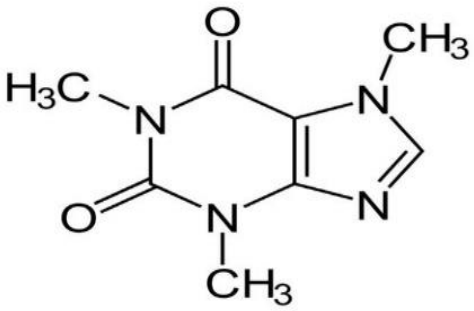

Figure 2.7: Chemical structure of caffeine

Source: Belay (2011)

2.12.2 Effects of caffeine on moulds growth and mycotoxins

Kanoma *et al*. (2014) noted that a natural occurring component and important factor in disease resistance for some higher plants has been the presence of antifungal compounds in them. These compounds have been found to be selective in their toxicity and are therefore regarded as important for controlling some fungal related plant diseases. Kanoma *et al*. (2014) again argued that extracts from these plants might be able to inhibit enzymes produced by invading pathogens and that different phenolic compounds from plants have been known to affect the proliferation and growth of many fungal pathogens. Caffeine can be found in varying concentrations in plants at different parts of the plant including the beans, the leaves, and the fruits. Ali-Shtaye (1999) stated that caffeine can exist in the plant to act as a natural fungicide and pesticide that prevents the germination and growth of moulds and also kills certain insects feeding on the plants. Rao *et al.* (2005) and later Tsirilakis *et al.* (2012) established that about 80–90% of *Aspergillus spp.* chitinases was inhibited in the presence of methylxanthines including caffeine, theophylline and pentoxifylline. Fungal chitinases are crucial for fungal growth, replication, repair and cell wall integrity, therefore the inhibition of chitinase action will lead to the inhibition of fungal growth (Duo-Chuan, 2006). Caffeine is used by humans mostly in infusions extracted from the fruit of the coffee plant, the leaves of tea bush plant and also from the cola nut. Yerba mate, guarana berries, and the yaupon holly are all very good sources of caffeine (Kakhia, 2003).

Ali-Shtaye (1999) noted that in most developing countries, diseases caused by fungi infections in both animals and plants make up a greater proportion of all health problems. Unfortunately, over the period, indiscriminate use of commercial antifungal agents has made microorganisms develop resistance to many synthetic fungicides and antibiotics. This situation forced scientists and researchers around the world to look for alternate antifungal substances from

various springs including medicinal plants. The use of synthetic antifungal substances such as fungicide, bacteriocide and pesticides have been reported by Amusa and Odubaku (2007) to have negative effects on agricultural lands and water. The perceived negative effects have increase public awareness of the risk involved to the health of animals and man that consume the products of these plants. Therefore, focus is being given on alternative, safer and eco-friendly method of controlling these pathogens (Amusa and Odubaku, 2007). As such, some plant extracts have been known to provide that safer alternative to the current use of synthetic fungicides for the control of pathogenic fungi due to their rich sources of bioactive molecules (Wink, 1998).

Raut *et al*. (2013) demonstrated that caffeine, which is a natural alkaloid found in many plants including coffee and cola can inhibit the germination and growth of moulds, yeast and bacteria. Buchman and Lewis (1984) have also demonstrated that caffeine inhibit the proliferation of *Aspergillus spp* and *Penicillium spp* of moulds. It has also been found to inhibit the biosynthesis of mycotoxins such as Aflatoxins secreted by *Aspergillus flavus* and *parasiticus* according to Maraqa *et al*. (2007). Green plants have been known to be a reservoir of compounds that are very effective and can therefore be used for chemotherapeutic purposes. These compounds can also provide useful fungicides and pesticides which largely is non-phytotoxic and more systemic which can be degraded by biological agents more easily. Because of the effectiveness of products of plants origin as chemotherapeutic agents, such plants can provide a renewable source of anti-fungal and anti-bacterial which are biodegradable in nature and do not pose any threat or side effects (Farombi, 2003).

Many works have shown that caffeine (1,3,7-trimethylxanthine) has effects on mould growth and associated mycotoxin production. Kanoma *et al*. (2014) noted that *C. nitida* and *C. acuminata* extracts (rich sources of caffeine) reduced the mycelia growth of test isolates and

impaired the growth of test isolates *Aspergillus niger*, *Aspergillus fumigates*, *Mucor recemosus*, *Rhizopus oryzae* and served as botanical fungicides. Buchanan *et al.* (1982) demonstrated that caffeine could inhibit the germination and growth of polyketide mycotoxins in a number of fungi species from the genus *Aspergillus* and *Penicillium*. Buchanan *et al.* (1982) argued that the inhibitory mechanism of caffeine appears to be extremely specific, and that the process does not appear to involve an inhibition of cyclic AMP phosphodiesterase since the much more related dimethylxanthines, theophylline and theobromine was known to have little effect on mycotoxin formation. Buchanan *et al.* (1978) noted earlier that, potentially, the identification of the caffeine inhibition mechanism of polyketide synthesis could be a very beneficial basis for well understanding the bioregulatory mechanism that control mycotoxin formation. It was also observed that the inhibition mechanism of aflatoxin production may represent separate effects, since the inhibition of growth could be partially overcome by supplementation with adenine or guanine, meanwhile these purines had little effect on aflatoxin synthesis (Buchanan, 1983). Again, other studies done by Nartowicz *et al.* (1979) stated that caffeine plays a vital role in the inhibition of aflatoxins specifically aflatoxin B1 inhibition in coffee. However, Nartowicz *et al.* (1979) again noted that *Aspergillus flavus* colonization of coffee and aflatoxin B1 contamination is not as significant as Ochratoxin A contamination in coffee. Tsubouchi *et al*. (1985) also stated the inhibition of growth and production of Ochratoxin A by *Aspergillus ochraceus*, IFM 0458 in a Yeast Extract Sucrose (YES) medium which contained 1% caffeine. In contrast however, the production of Ochratoxin A was found to be higher in the presence of 5% and 10% caffeine in another strain of *A. ochraceus* IFM S-235.

Caffeine which is about 2% in coffee could have great result on Ochratoxin A buildup. A dose-related reduction in Ochratoxin buildup was obvious, with 3 mg/ml level displaying

approximately 98% inhibition (Buchanan, 1982). It also could reduce the growth rate of *Aspergillus versicolor, Penicillium citril1um* and *Penicillium urticeae* (Buchanan *et al.*, 1983). Nartowicz *et al.* (1979) showed that caffeine could inhibit the production of some mycotoxins such as aflatoxins produce by *Aspergillus parasiticus*, Ochratoxin A produced by *Aspergillus ochraceus* (Buchanan *et al.*, 1982) and Sterigmatocystin produced *Aspergillus versicolor* (Buchanan *et al.*, 1983).

2.13 Cola nut

Cola, classified in the family of *Sterculiaceae*, originates from Africa and the genus comprises of about 125 species (Dah-Nouvlessounon *et al*., 2016). Although few species of cola can grow up to 25 meters, they are mostly small sized trees and evergreen. According to Dah-Nouvlessounon *et al*. (2016), the cola genus has a lot of species that are generally grown in tropical countries, specifically in West Africa with *C. verticillata*, *C. vera*, *C. acuminata* and *C. nitida* being the most common species cultivated. *Cola acuminata* and *C. nitida* however, have the greatest economic importance mainly because of their caffeine content as stated by Yalwa and Bello (2017).

Cola acuminata, one of cola species measures 20 meters high and the leaves are long and ovoid with a texture that may be described as leathery and pointed at both ends. The flowers of the tree are yellow in colour with spots of purple and has a star like shaped fruit. It has about 12 round or square seeds which is enclosed in seed shells with a white colour. The tree is cultivated commercially around the world, most specifically in Nigeria, Sri lanka, Indonesia, Brazil and other parts of South America (Opeke, 2005). *Cola nitida* on the other hand was originally distributed along the West African coast countries from Sierra Leone to the Republic of Benin with the

majority of this distribution and variability occurring in the forest zones of Côte d'Ivoire and Ghana (Opeke, 2005).

Cultivation of cola nut was carried eastwards and countries in West and Central Africa used it many years before the European voyagers arrived in Africa (Yalwa and Bello, 2017). According to Kuoame and Scande (2006), cola nut is extremely popular amongst the people in West Africa, mainly due to its caffeine content that acts as a stimulant. Sweet and rose-like aroma have been associated with the nut and the consumers of this nut describe that it tastes bitter at first but becomes sweet upon mastication (Kuoame and Scande, 2006). The cola nuts could be eaten whole or in the form of a powder and could also be mixed with liquid for a drink thus, used as a flavoring ingredient in beverages (Kuoame and Scande, 2006). Cola nuts are chewed with the aim to bring back vitality and also to ease hunger pangs by the people in West African as a part of their culture and it is an important part of spiritual practice and religion in West Africa (Ratsch, 2005).

In West Africa, the nut is cultivated mainly because it contains two major alkaloids, caffeine and theobromine. Kiple and Ornelas (2000) stated that these alkaloids are powerful stimulants that counter the effects of tiredness, conquer thirst and hunger, and are also believed to boost intellectual capabilities. The cola seed is made up of 13.5% water, 9.5% crude protein, 1.4% fat, 45% sugars and starch, 7.0% cellulose, 3.8% tannin and 3% ash. Caffeine (2.8%) and theobromine (0.05%) are the two alkaloids found in cola (Burdock *et al.*, 2009). Moloney (1887) did some comparative studies on the nutritive composition of cola nuts to other species used as stimulants such as cocoa (Sterculiaceae) and coffee (Rubiaceae). He concluded that cola nut has more caffeine than coffee, and that the nut also has an appreciable amount of theobromine and high glucose content. Its starch content was found to exceed that in cocoa to about three folds but had little oil content compared to cocoa. Atawodi *et al.* (2007) also noted that, the caffeine content

of cola nut could range from 1.5% to 3.8% depending on the variety of cola nut. One cola nut has also been found to contain more caffeine than two large cups of American coffee (Kiple and Ornelas, 2000).

Figure 2.8: Three most common species of cola.

Source: Sanders (2008)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Cola nuts: White matured fresh *Cola nitida* (5 kg) was purchased from the Nima market in the Greater Accra region of Ghana and was identified as *C. nitida* by the presence of two cotyledons of the seeds. The cola nuts were transported to the Council for Scientific and Industrial Research – Food Research Institute of Ghana and stored at room temperature prior to processing.

Cocoa pods: Matured cocoa pods (400 pods) of Forastero variety, the mixed hybrid cultivar was harvested from a cocoa farm at Tutu in the Akuapim north municipality in the Eastern region of Ghana. Identification of the variety and cultivar was done by the cocoa farmer who called it "Akokrabedi" which is the mixed hybrid cultivar of the Forastero variety. Collected cocoa pods were stored for 5 days on the farm at an average temperature of 28.4 °C and a relative humidity above 70% prior to fermentation.

Drying environment for cocoa beans: Normal farm environmental drying conditions were monitored for three days prior to drying of fermented cocoa beans and average temperatures of 27.23, 27.40 and 27.07℃ were recorded for days 1, 2 and 3 respectively. Relative humidity of 76%, 75% and 77% were also recorded for days 1, 2 and 3 respectively. The results showed that the farm environment was ideal for mould growth and so drying of the fermented and treated cocoa beans were done on the farm.

3.1.2 Research design

The experimental design used was a 2 x 4 full factorial design with the principal factors being cola nuts used (cola nut powder and cola nut crude extract) and cola nut mass concentration (0, 50, 100 and 150 g per every 5000 g of fermented beans).

3.1.3 Sample preparations

Cola powder and extract: Dirt and other extraneous materials were removed from purchased *C. nitida* and 5 kg was weighed and cut into small pieces after washing. Cut cola nuts were dried in an oven at 80 °C for 24 hours. Dried nuts were milled into a coarse powder and further dried at 80 °C for 12 hours. Dried coarse powder was again milled into very fine powder and stored at -4 °C prior to use. About 3 kg of fine cola powder was macerated with distilled water in a ratio of one part of cola powder to five parts of distilled water (1:5) for 24 hours on a shaker. The mixture was then boiled on a hot plate at 100 °C for 30 minutes with stirring every 5 minutes. The mixture was then brought to room temperature and strained using glass wool. Crude cola extract (filtrate) was poured into oven trays and water evaporated at 80 °C for 24 hours to get dry cola extract. Dried extract was milled into powder and kept at -4 °C prior to use.

(a) **(b)** **(c)**

Figure 3.1: (a) White variety cut *Cola nitida* (b) Oven dried white cola *nitida* (c) *Cola nitida* powder

Cocoa beans: Harvested cocoa pods were split open with a cutlass and cocoa beans were scooped out into a basin for fermentation. About 40 kg of scooped beans were heaped on plantain leaves for heap fermentation on the farm. Heaped beans were covered with same plantain leaves and kept in place with logs. The temperature in the heap was monitored daily with a delta ohm probe thermometer (HD 2107.2) as fermentation was allowed to occur for 4 days. Fermented beans were separated from remaining chuff and weighed for sample treatments.

Figure 3.2: Heap fermentation of cocoa beans on the farm

Sample treatments: Prior to cola powder and cola crude extract treatments, the moisture contents of the cola extract (11.83%) and powder (4.75%) were factored into treatment concentrations. Thus, 4.75% extra mass of cola powder was added to every treatment concentration using the powder and 11.83% extra mass of cola extract was added to every treatment concentration using the extract. To every 5 kg of fermented cocoa beans, treatment with the cola powder and cola crude extract was done at concentrations of 0 g, 50 g, 100 g and 150 g prior to sun drying. Treated beans were mixed well and spread evenly on drying trays for sun drying. Drying of treated samples was done on the farm for 14 days on a raised platform made from wood and temperature and relative humidity monitored constantly to make sure humidity levels above 70% and temperatures below 30 °C was maintained.

(a) **(b)** **(c)**

Figure 3.3: (a) Cola raw powder and powdered extract used to treat cocoa samples

(b) Process of cocoa bean treatment with various powder concentrations

(c) Treated cocoa beans drying under a shade on the farm

3.2 Analytical Methods

3.2.1 Enumeration and identification of moulds

Enumeration of moulds was done based on the methodology described by Pitt and Hocking (2009) where 10 g of milled cocoa nibs were aseptically weighed into 90 ml of sterile salt peptone solution (SPS) containing 0.1 % peptone and 0.8 % sodium chloride and the pH was adjusted to 7.2. The sample-SPS mixture was homogenized in a stomacher (Model 4001, Seward Medical) for 30 seconds at a speed of 230 rpm. Since cocoa contains high fat content, tween 80 solution was added to the salt peptone solution to ensure uniform mixture of the cocoa to give a 10^{-1} dilution. Using a clean and sterile pipette, 1 ml of the 10^{-1} dilution was pipetted into 9 ml of SPS to obtain 10^{-2} dilution. This was repeated to get 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions. Aliquot (1 ml) of each dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) was inoculated into sterile petri dish plates and oxytetracycline glucose yeast extract agar (OGYEA) was added and carefully swirled to mix. Plates were incubated at 25 °C for 5 days after which 10^{-4} and 10^{-5} dilution plates were selected and counted using the colony counter after identification. The number of colony-forming units per gram (cfu g^{-1}) of sample was calculated.

Penicillium spp was identified by the presence of bluish centered colonies with white margins. For *Aspergillus spp*, the presence of yellow-greenish centered colonies with white margins was used for identification. *Rhyzopus* and *Mucor* were identified with the presence of white colonies, sometimes with black centers. This was confirmed by teasing out colonies on a slide and viewing under the microscope where their morphological characteristics were used to confirm their identifications.

3.2.2 Determination of Ochratoxin (A) and aflatoxins (B1, B2, G1, G2)

Ochratoxin A and aflatoxins (B1, B2, G1 and G2) were determined based on CEN official method EN14123 (2007). About 500 g of cocoa beans were sampled by thoroughly mixing and heaping the whole batch into a cone. Using a cardboard, the heap was divided into four equal parts. Two opposite parts were mixed together, and the remaining two parts packed, and process repeated until a representative 500 g sample was achieved and ground into fine cocoa powder. Exactly 25 g of powdered sample was extracted with 5 g sodium chloride and 200 ml methanol in distilled water in a ratio of (4:1) respectively. Hexane (100 ml) was added to the mixture and homogenized for 3 min at 3000 rpm (2 min) and 3500 rpm (1 min). The mixture was filtered through Whatman number 4 filter paper and 10 ml of filtrate was measured into a beaker and used for aflatoxins and ochratoxin A analyses.

For Ochratoxin A, 150 ml of phosphate buffer saline (PBS) was added to 10 ml of filtrate and mixture stirred. Immunoaffinity columns specific for Ochratoxin A were pre-conditioned and used for solid phase extraction. Columns were fitted to a vacuum manifold and antibodies in the column activated by eluting 10 ml of phosphate buffer saline through it at a flow speed of 3 ml min^{-1}. Exactly 50 ml of the 160 ml filtrate-PBS mixture was loaded onto the pre-conditioned immunoaffinity column specific for Ochratoxin A and allowed to drain by gravity. The columns were washed three times with 5 ml distilled water and allowed to elute at a flow rate of 5 ml min^{-1}. Using a vacuum pump, air was blown through the column to get rid of all wash solvent molecules. Ochratoxin A was eluted in two steps into a 5 ml volumetric flask by first eluting with 1 ml of methanol (highest grade) followed by another 1 ml of methanol after one minute. Air was blown through the column to collect all eluates. Aqueous acetic acid (1%) was used to make up

volume of eluate to 4 ml and eluate vortexed after which 2 ml was pipetted into HPLC vials for quantification.

For aflatoxins, 60 ml of phosphate buffer saline (PBS) was added to 10 ml of filtrate and mixture stirred. Immunoaffinity columns specific for aflatoxins were pre-conditioned and used for solid phase extraction. Columns were fitted to a vacuum manifold and antibodies in the column activated by eluting 10 ml of phosphate buffer saline through it at a flow speed of 3 ml min^{-1}. All 70 ml of the filtrate-PBS mixture was loaded onto the pre-conditioned immunoaffinity column specific for aflatoxins and allowed to drain by gravity. The columns were washed three times with 5 ml distilled water and allowed to elute at a flow rate of 5 ml min^{-1}. Using a vacuum pump, air was blown through the column to get rid of all wash solvent molecules. Aflatoxins were eluted in two steps into a 5 ml volumetric flask by first eluting with 0.5 ml of methanol (highest grade) followed by 0.75 ml of methanol after one minute. Air was blown through the column to collect all eluates. Aqueous acetic acid (1%) was used to make up volume of eluate to 5 ml and eluate vortexed after which 2 ml was pipetted into HPLC vials for quantification.

Agilent high performance liquid chromatography system (HPLC 1260 infinity series) with a quaternary pump and fluorescence detection was used for OTA and aflatoxins quantification. Data acquisition and quantification was done using Chem station (Pro lab edition). The Agilent HPLC equipped with fluorescence detector was used with an excitation wavelength of 360 nm and an emission wavelength of 440 nm and the column compartment temperature regulated at 35 °C. The mobile phase was a mixture of water: methanol: acetonitrile at ratios of (65:20:15) respectively and isocratic delivery mode employed at a flow rate of 1 ml min^{-1} with an injection volume of 10 µl. The run times were set at 10 min for aflatoxins and 5 min for Ochratoxin A. Six-point calibration was made using pure aflatoxin and Ochratoxin standards at concentrations of 5

ppb, 10 ppb, 15 ppb, 20 ppb, 25 ppb and 30 ppb and linearity accepted at 0.99 or 99% for regression line.

3.2.3 Determination of pH and titratable acidity

Determination of pH and titratable acidity were based on AOAC method 970.21 (2005) as described by Afoakwa (2014). About 10 g samples of nibs was homogenized for 30 seconds in 90 ml of hot distilled water and vacuum filtered through Whatman filter paper No. 4. Filtrate was cooled to room temperature and a 25-ml portion was measured into a beaker and the pH determined with a pH meter in duplicates and the mean value recorded. Titratable acidity was determined on the 25 ml aliquot filtrate by titrating with 0.1N NaOH solution using phenolphthalein as indicator to a pink end point. The analysis was done in duplicates and titratable acidity was expressed as acetic acid equivalent and values were recorded as moles of NaOH per grams of sample.

3.2.4 Fermentation index

Fermentation index was done using a method described by Afoakwa (2014). Dried cocoa beans shells were removed to obtain the nibs. The nibs were ground using a knife mill. About 0.5 g of the ground nibs were homogenized in 50 ml methanol: HCl (97:3) solution. The homogenate was incubated at 4 °C overnight, filtered and read in duplicate at 460 nm and 530 nm wavelength. Ratio of absorbance at 460 nm and 530 nm were calculated.

3.2.5 Cut test

Cut test was done as described by Afoakwa (2014). A total of 100 dried fermented beans were taken randomly from 250 g samples derived by conning and quartering technique. The dried

beans were cut lengthwise into halves for maximum surface exposure. Both halves of each surface were inspected under broad daylight categorized into the following: fully brown, partly brown, partly purple, fully purple and mouldy/slaty beans. The number of these types of beans were counted and expressed as percentage of the total number of beans used. The percentage of the beans was converted into Equivalent Percent Fully Brown (EB) score to compare the degree of fermentation. The formula for EB used as described by Bariah (2014) is as follows:

EB = 1 x % fully brown + (0.7 x % purple-brown) + (0.5 x % fully purple) + (0.3 x % slaty/mouldy)

3.2.6 Free fatty acid (FFA) determination

Free fatty acid was determined based on AOAC method (2005) where cold extraction was used to extract the fat in the cocoa beans using petroleum ether. The extracted fat (1.4 g) was dissolved in 15 ml methanol/diethyl ether (1:1) and titrated against a standardized 0.1M ethanolic sodium hydroxide using phenolphthalein as indicator to obtain pink end point. The percentage of free fatty acid as oleic acid was then calculated as:

$$FFA = \frac{\text{V x N x } 28.2}{\text{W}}$$

Where: V = volume (ml) of NaOH solution, N = normality of NaOH solution, W = weight of oil sample, 28.2 = from molar mass of oleic acid (282/10).

3.2.7 Peroxide value

Peroxide value was done as described by Cox and Pearson (1962). One gram of cocoa butter was weighed into a tube and 1 g of powdered KI added with 20 ml of acetic acid in chloroform (2:1). The mixture was allowed to boil for 30 seconds and transferred into a conical flask containing 20 ml of 5% KI solution. The tube was rinsed with 25 ml distilled water and added

to conical flask content. Mixture was titrated against freshly prepared 0.002 N sodium thiosulphate solution until the yellow colour almost disappeared. Half milliliter of 1% starch solution was added and titrated until dark blue colour disappeared. Peroxide value was then calculated.

$ROOH + KI \rightarrow ROH + KOH + I_2$

I_2 + starch + $2Na_2S_2O_3$ (blue) $\rightarrow$ 2NaI + starch + Na_2S4O_6 (colorless)

Peroxide value (mEq / kg sample) = $[(S - B * M * 1000) / (\text{Sample weight (g)})]$

Where; S = sample titre value, B = blank titre value, M = molarity of $Na_2S_2O_3$

3.2.8 Iodine value

Iodine value was determined according to AOCS method (1993) with slight modifications where 0.30 g of cocoa butter was dissolved in 10 ml of carbon tetrachloride and 25 ml of Habul's solution added using a pipette. It was allowed to stand in the dark for 12 hours with occasional shaking. This was followed by the addition of 20 ml 10% KI shaken after which 100 ml of freshly boiled and cooled water was added. It was then titrated against freshly prepared 0.1 N sodium thiosulphate solution until it was pale brown in colour. Few drops of 1% starch solution were added and titrated against the 0.1 N sodium thiosulphate solution until the dark blue colour disappeared and became colourless. The iodine value was then calculated.

Iodine value = (B – S) * N * (12.69 / weight of oil)

Where; B = blank titre value, S = sample titre value and N = normality of $Na_2S_2O_3$.

12.69 g from molecular weight of iodine (126.9/10).

3.3 Statistical analysis

Minitab software version 17 was used to analyze obtained data for microbiological, physicochemical, cocoa fat quality and mycotoxins analyses. Two-way ANOVA was used to determine the significant difference between the treatments and means of responses. Significance was set at 95% confidence level. Regression analysis was used to explain the behavior of treatment factor and the response.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Temperature and relative humidity during the fermentation and drying process

4.1.1 Temperature of fermenting mass during heap fermentation

Cocoa beans are the major raw material needed for chocolate manufacture and raw cocoa beans have an astringent, unpleasant taste and flavour and have to be fermented to obtain the precursors needed for the desired characteristic cocoa flavor and taste for chocolate production (Afoakwa *et al*., 2013). The temperature trends during the four days heap fermentation of cocoa beans is shown in Figure 4.1. An initial temperature of 28.8°C was recorded for the first day of fermentation. There was a rise in temperature within 24 hours of fermentation from 28.8 °C to 41.6 °C on the second day. The temperature kept rising and within 48 hours of fermentation, a temperature of 42.1 °C was recorded. Fermentation temperature however began to drop after 48 hours to a temperature of 38.3 °C on the fourth day after which fermentation was stopped.

The sudden rise in temperature within 48 hours of fermentation was as a result of the activities of microorganisms where yeast breaks down the pectin in the pulp of the beans to reduce its viscosity and as the pulp drains away, exothermic production of ethanol from sugars occur which increases the temperature of the beans (Afoakwa *et al.,* 2013; Thompson *et al.,* 2001). Fermentation temperature began to drop with the death of the cocoa bean after lactic acid bacteria and acetic acid bacteria under aerobic conditions produced lactic acid and acetic acid, respectively from sugars and ethanol which diffused into the bean to kill the cells together with the high temperatures (Afoakwa *et al*., 2013).

Studies carried out by Camu *et al*. (2007) reported similar fermentation temperature trend where there was a rise in temperature from an average of 26.3 °C (26.0 to 30.0 °C) at the start of

the fermentations to a maximum average temperature of 43.5 °C (42.2 to 47.7 °C). Work done in Malaysia by Bariah (2014) also reported similar trend of fermentation temperature where temperature in the fermenting heap exceeded 40 °C within the first 24 hours of fermentation. Afoakwa (2014) remarks that the nearer the maximum fermentation temperature is to 50 °C the better the fermentation process. This is because the high temperature together with the diffusing acids facilitate the death of the cocoa bean embryo allowing for flavour precursors to be formed.

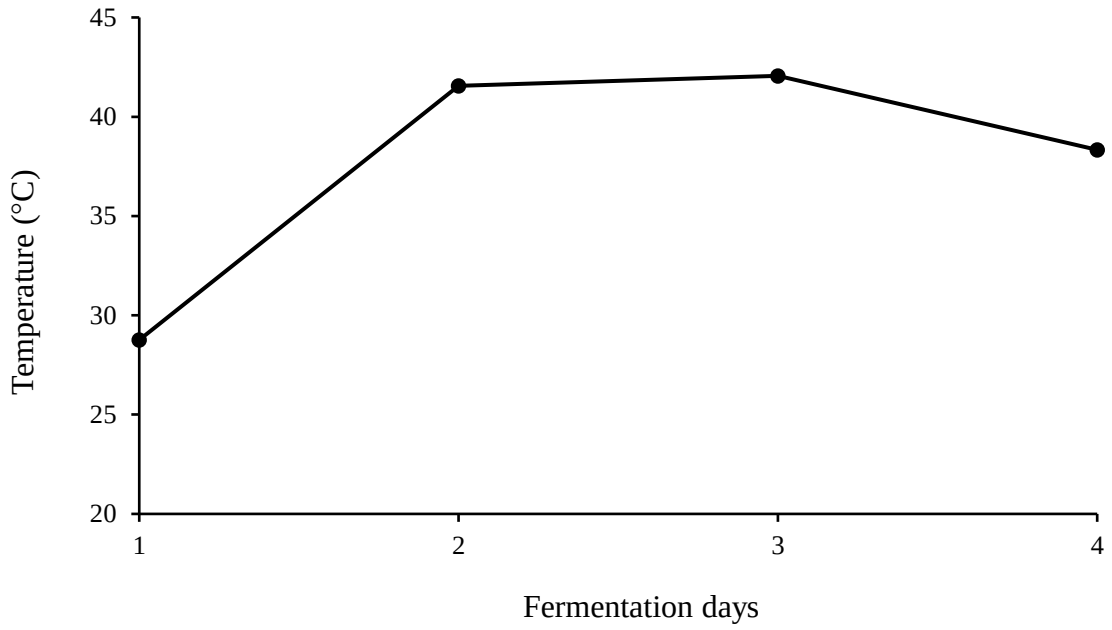

Figure 4.1: Temperature trend during heap fermentation of cocoa beans

4.1.2 Temperature and relative humidity trends during the 14 days drying period

Results from the temperature and relative humidity readings for the 14 days drying period indicated that the environmental conditions were capable of promoting the growth of moulds as shown in Figures 4.2 and 4.3 for temperature and relative humidity, respectively. The lowest temperature was recorded on the 8th day of drying in the morning at 24.9 °C where it rained with

a 100% corresponding relative humidity. The 10th day however, recorded the lowest relative humidity of 70% in the afternoon and the 7th day recorded the highest temperature of 30.6 ℃ in the afternoon. According to Moses (2005), the optimum temperature required for moulds to grow ranges between 15 ℃ to 30 ℃ with a relative humidity above 70%. Despite the variations in temperature and humidity throughout the 14 days drying period, the environment was conducive enough to support and promote the growth of moulds.

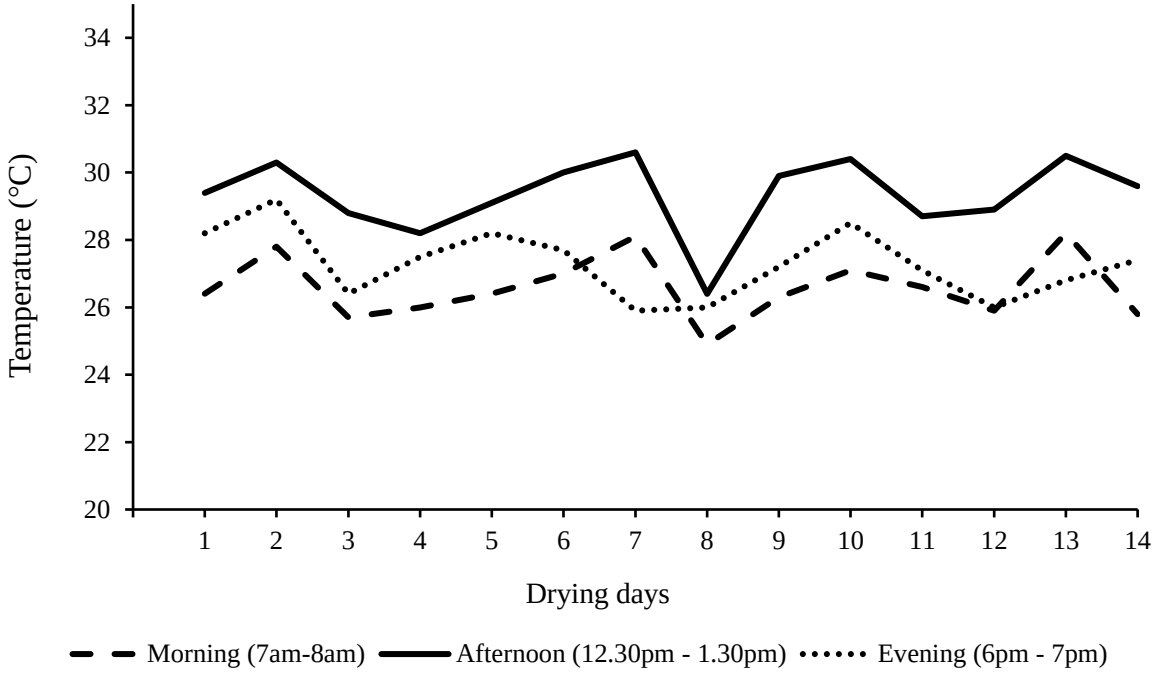

Figure 4.2: Temperature trend of drying environment during 14 days drying of fermented cocoa beans

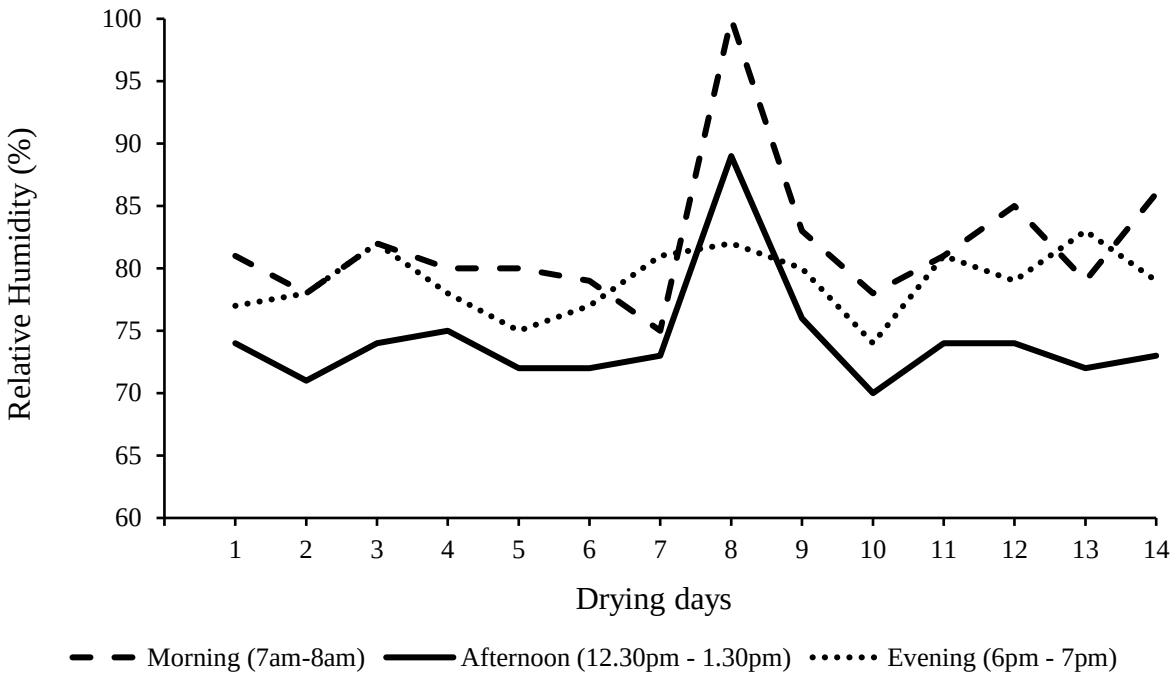

Figure 4.3: Relative humidity trends of drying environment during drying of fermented cocoa beans

4.2 Growth of moulds in treated and untreated cocoa beans

4.2.1 Penicillium spp

According to American Public Health Association [APHA] (2001), moulds are a diverse group of fungi that can grow in foods within a wider range of pH from 2–9. The growth of *Penicillium viridicatum* in cocoa has been reported by Aydin *et al.* (2003) as the most common mould in cocoa bean at a minimum water activity (a_w) of 0.85. There was growth for *Penicillium spp* on oxytetracycline glucose yeast extract agar (OGYEA) plate and this was identified by the presence of bluish centered colonies with white margins as shown in Figure 4.4. The growth of *Penicillium spp* was affected by the addition of cola nut powder and extract to cocoa beans (based on control) prior to sun drying (Figure 4.5). The least concentration (50 g/5 kg) for both cola extract

and powder could not cause any inhibition to the growth of *Penicillium spp* but rather had an increase in growth of 6.82 and 6.87 log cfu/g of sample for cola extract and powder, respectively as compared to 6.13 log cfu/g for the control (0 g/5 kg). There was significant ($p < 0.05$) inhibition of *Penicillium spp* for both samples treated with 150 g cola extract and powder when compared to the control. Concentration of 150 g/5 kg cola extract significantly ($p < 0.05$) inhibited growth from 6.13 log cfu/g to 4.18 log cfu/g which represented 98.9% inhibition while 150 g/5 kg powder inhibited growth from 6.13 log cfu/g to 4.7 log cfu/g representing 96.3% inhibition. There was no significant difference ($p > 0.05$) between cola nut extract and powder for all the other concentrations.

The increase in growth for the least concentration (50 g/5 kg) could be as a result of biotic competitive interactions between the different genera of moulds present. This is because as the 50 g/5 kg concentration suppressed some of the competitive moulds to a smaller extent, the growth of *Penicillium* became much more favoured than that of the control. This competitive phenomenon has been reported by Cvetnic and Pepeljnjak (2007) who confirmed that the presence of *Mucor* could inhibit the growth of *Aspergillus* to cause a 50% reduction in the production of aflatoxins B1. Kumar *et al.* (2017) also reported similar interactions where there were biotic interactions between *Penicillium, Rhizopus, Mucor and Aspergillus spp* and since cola powder and extract were more sensitive to *Aspergillus spp*, their competitive interactions was reduced in cocoa treated with 50 g/5 kg to promote the growth of *Penicillium spp*. However, as concentration increased, the level of antimicrobials in the cola including alkaloids (mostly caffeine) and phenolic compounds also increased which according to Ali-Shtaye (1999), acted as a natural fungicide to inhibit growth of *Penicillium.* Raut *et al*. (2013) also reported that caffeine could inhibit some yeast and moulds.

Similar results were obtained by Buchman and Lewis (1984) who demonstrated that caffeine inhibit the growth of *Penicillium spp.*

The general regression model obtained for the degree of effectiveness of cola concentration on *Penicillium spp* growth is $Y = \beta_0 + \beta_1 X + \beta_2 X^2 + \varepsilon$ (Figure 4.6). The regression model was significant ($p < 0.05$) with an F value of 136.97. Treatment type however was not significant and was eliminated from the final regression model by backward elimination. The adjusted R^2 for the model was 94.8% which was in reasonable agreement with the R^2 of 95.5%. This means that, about 95% of variations in *Penicillium* growth was explained for by changes in concentrations of the cola nut used as treatment. This shows that the cola nut powder and extract were effective in controlling *Penicillium* growth.

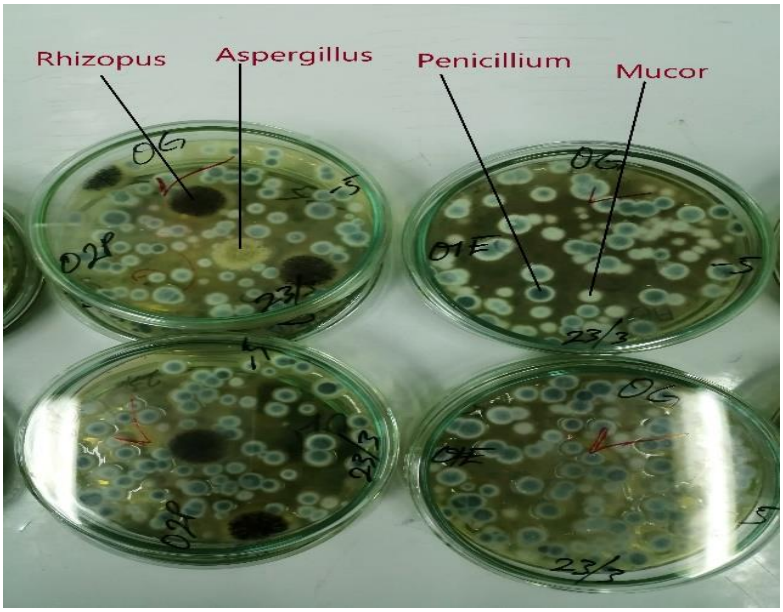

Figure 4.4: Appearance of moulds on (OGYEA) plate based on colour used in mould identification

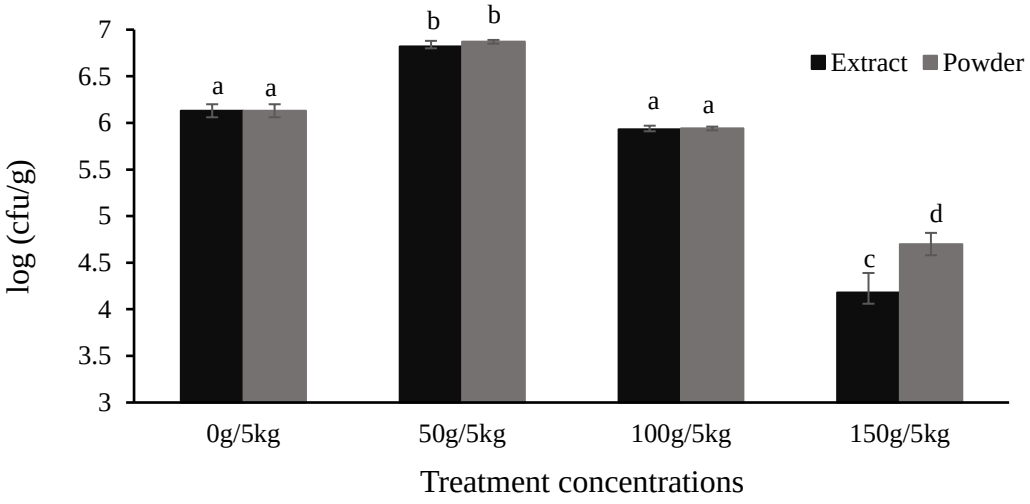

Figure 4.5: Colony forming units (CFU) for *Penicillium spp* as observed on (OGYEA) plate for various cola treatment concentrations in sun-dried fermented cocoa beans (means that do not share a letter are significantly different)

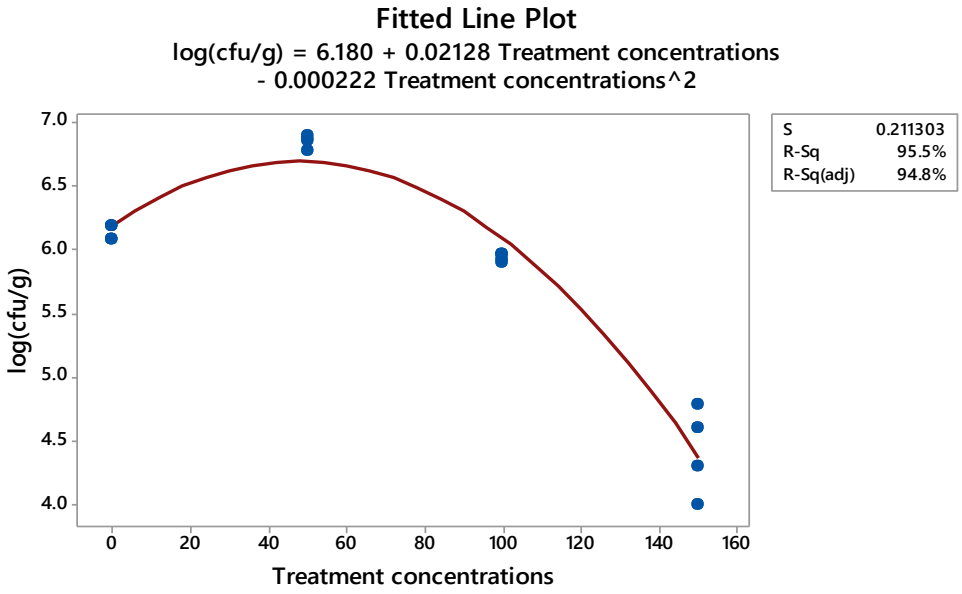

Figure 4.6: A polynomial regression line plot for *Penicillium spp* and cola treatment concentrations including its quadratic terms

4.2.2 Aspergillus spp

The growth of *Aspergillus spp* on oxytetracycline glucose yeast extract agar (OGYEA) plate was identified with the presence of yellow-green centered colonies with white margins as shown in Figure 4.4. The effects of cola nut extract and powder on the growth of *Aspergillus spp* is shown in Figure 4.7. There was a 100% inhibition for both cola nut extract and powder for the highest treatment concentration of 150 g/5 kg which significantly ($p < 0.05$) reduced growth from 4.88 log (cfu/g + 1) for control (0 g/5 kg) to no visible growth on OGYEA plate. Similar trend was observed for 100 g/5 kg cola extract concentration which significantly reduced growth from 4.88 log (cfu/g + 1) to no visible growth. There was a significant reduction ($p < 0.05$) in growth for 100 g/5 kg cola powder from 4.88 log (cfu/g + 1) to 4.00 log (cfu/g + 1). The 50 g/5 kg concentration recorded more growth when compared to the control (0 g/5 kg) though this increase was not significant ($p > 0.05$) for both cola nut extract and powder.

According to Wheeler *et al.* (1991), even though moulds can tolerate a wider range of pH (3-8), *Aspergillus spp* was reported to do well in an optimum pH close to neutral. This may have accounted for the high growth of *Aspergillus spp* in the 50 g/5 kg treated cocoa beans than the control. Kakhia (2003) recounted that alkaloids including caffeine which are considered as the main antimicrobial substance in cola nut are basic and may have interfered with the acidic environment of fermented cocoa beans to raise pH of environment in the 50 g/5 kg than the control to favour the growth of *Aspergillus* much more than the control. However, as concentration increased further (100 g/5 kg and above), the antimicrobials in the cola also increased to have significant inhibitory effect on *Aspergillus spp* when compared to control. There was no significant difference ($p > 0.05$) between cola nut extract and powder for all treatment concentrations except for 100 g/5 kg treatment concentration. The results obtained for concentrations of 100 g/5 kg and

150 g/5 kg agreed with work done by Kanoma *et al.* (2014) who reported that cola nut extracts inhibited the growth of some moulds including *Aspergillus spp* to about 70%. Sivetz and Desrosier (1979) reported that compounds including caffeine and phenolics have some antimicrobial properties. Cola nut is a rich source of these compounds, which might have accounted for the inhibition of *Aspergillus spp* in the cocoa beans at concentrations of 100 g/5 kg and 150 g/5 kg for both cola nut extract and powder.

The general regression model obtained for the degree of effectiveness of cola concentration on *Aspergillus spp* growth is a simple linear regression given by the equation $Y = \beta_0 + \beta_1 X + \beta_2 X^2 + \varepsilon$ (Figure 4.8). The regression was significant ($p < 0.05$) with about 77 % of the variations in *Aspergillus* growth explained for by changes in treatment concentrations. This shows that cola nut powder and extract are effective in controlling the growth of *Aspergillus spp* of moulds in cocoa. The adjusted R^2 (0.73) was also in agreement with the R^2 of 0.77. Treatment type however, was not significant and hence was excluded from the final model by backward elimination.

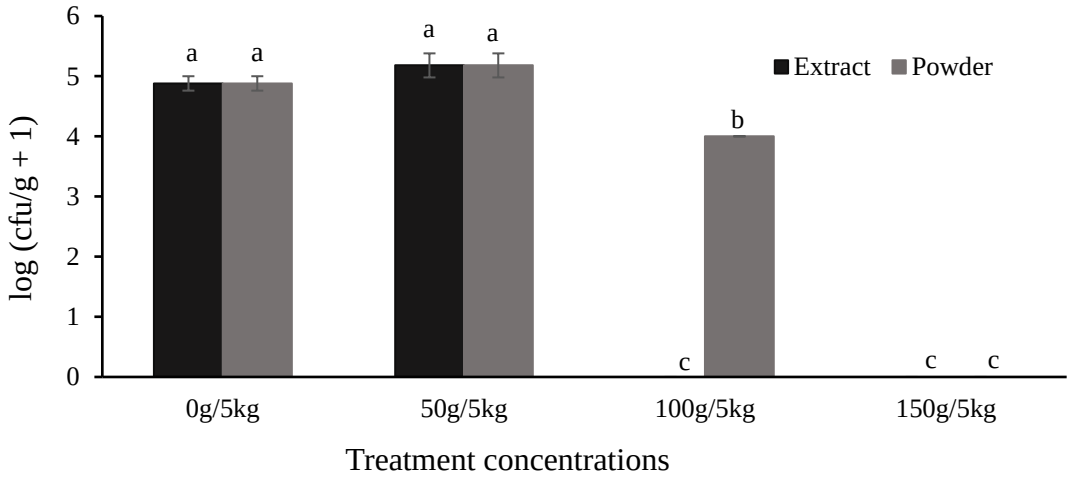

Figure 4.7: Colony forming units (CFU) for *Aspergillus spp* as observed on (OGYEA) plate for various cola treatment concentrations in sun-dried fermented cocoa beans (means that do not share a letter are significantly different)

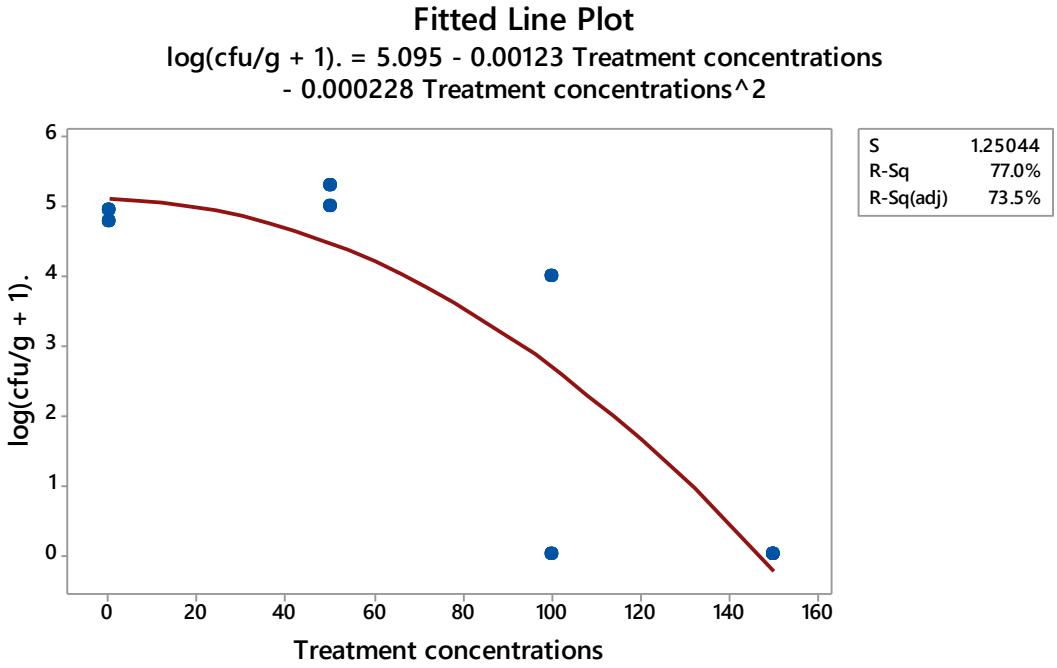

Figure 4.8: A polynomial regression line plot for *Aspergillus spp* and cola treatment concentrations including its quadratic terms

4.2.3 Rhizopus and Mucor

The cola nut powder and extract were found to be less sensitive to both *Rhizopus* and *Mucor* (Figure 4.9). All concentrations did not show any significant reduction for *Rhizopus* and *Mucor* except for 150 g/5 kg cola extract which significantly ($p < 0.05$) reduced growth of *Rhizopus* and *Mucor* from 4.95 log cfu/g for control to 4.3 log cfu/g. Although the 150 g/5 kg cola powder also showed a reduction from 4.95 log cfu/g for control to 4.74 log cfu/g, this reduction was not significant ($p > 0.05$). Both 50 g/5 kg and 100 g/5 kg extract and powder concentrations had more growth when compared to the control though the levels in 100 g/5 kg concentrations were lower than 50 g/5 kg concentration. This result was in contrast to work done by Fardiaz (1995) who confirmed that coffee extract (rich source of caffeine) inhibited *Rhizopus* on agar plate with a minimum inhibition concentration of 2.5 g/100 ml extract. Chuku *et al.* (2015) showed that *Mucor*

mucedo, *Asperillus niger* and *Rhizopus stolonifer* are the principal moulds that are associated with the spoilage of cola nut and this was evident as all concentrations of cola treatment did not significantly inhibit the growth of *Rhizopus* and *Mucor* except for 150 g/5 kg cola extract.

A regression model was used to predict the concentrations of cola nut extract and powder that can give significant reduction of *Rhizopus* and *Mucor* and was given by the general model equation $Y = \beta_0 + \beta_1 X + \beta_2 X^2 + \varepsilon$ (Figure 4.10). The regression model was significant ($p < 0.05$) considering the quadratic term order. The adjusted R^2 which is a true indication of variation in the dependent variable as explained for by the independent variables was 80.1% which was in agreement with the R^2 of 82.8%. Treatment types however was not significant and hence eliminated from the final model.

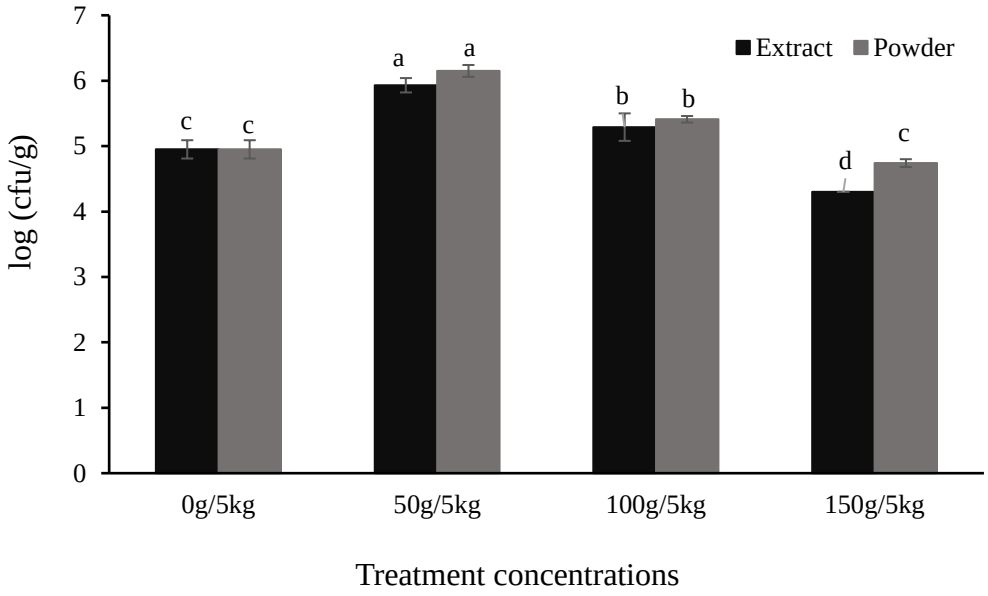

Figure 4.9: Colony forming unit (CFU) for *Rhizopus* and *Mucor* growth as observed on (OGYEA) plate for various treatment concentrations in treated sun-dried cocoa beans (means that do not share a letter are significantly different)

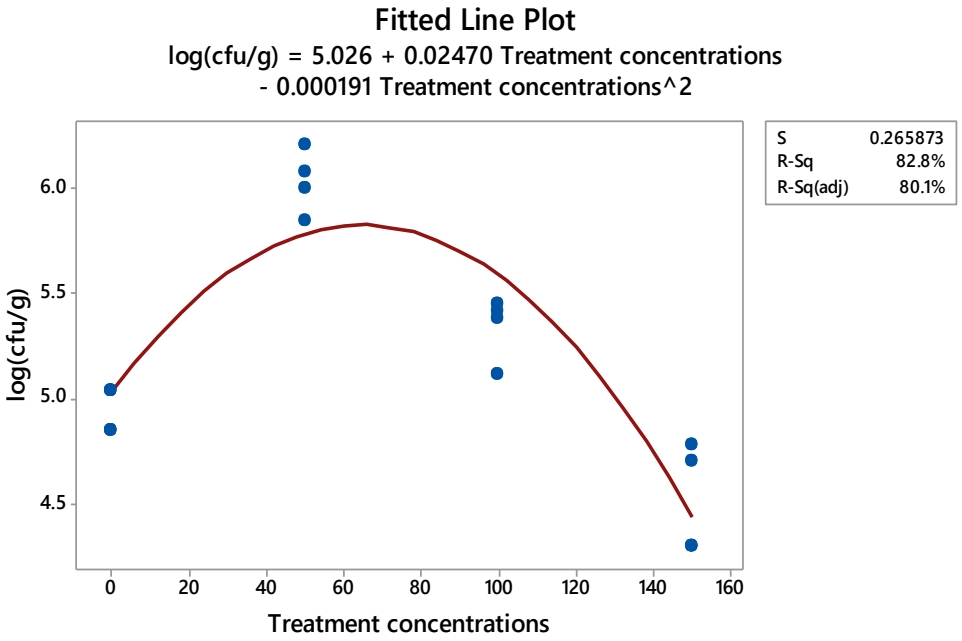

Figure 4.10: A polynomial regression line plot for *Rhizopus/Mucor* and cola treatment concentrations including its quadratic terms

4.3 Mycotoxin production in treated cocoa beans

4.3.1 Aflatoxins

Mycotoxins in cocoa are of major concern due to the impact it has on consumer health and global trade (Wu, 2005). Aflatoxin levels for all treatment concentrations including the control were below the detection limit of instrument used for quantification (Table 4.1). The detection limit of instrument was calculated to be 0.13 µg/kg for aflatoxins B1 and B2 and 0.15 µg/kg for aflatoxins G1 and G2. It can be said that either the aflatoxins were not present in the cocoa beans at all or they were present, but were below instrument detection limit and hence not detected. If the aflatoxins were below the instrument detection limit then they were far below the proposed European union regulatory limit of 2 µg/kg for aflatoxin B1 (European Commission Regulation, 2004). No or low levels of aflatoxins have been associated with cocoa beans including work done

by Asiedu (2017) who did not record any aflatoxin contamination among 60 cocoa beans samples from the major cocoa producing regions in Ghana. This low level of aflatoxins production in cocoa beans have been attributed to the fact that some bacteria involved in cocoa bean fermentation double as inhibitors to the synthesis of aflatoxins by *Aspergillus spp* of moulds (Karunaratne *et al.,* 1990).

Gourama and Bullerman (1995) demonstrated the effect of small molecular weight metabolites formed by *Lactobacillus spp* in the inhibition of aflatoxin B1 and G1. Karunaratne *et al.* (1990) reported that aflatoxin production by *A. parasiticus* was considerably reduced in the presence of cell-free supernatants of *L. bulgaricus*, *L. acidophilus* and silage inoculant cultures and also reported a 100% inhibition of aflatoxin production in the presence of lactic acid bacteria cells. El-Nezami *et al*. (1998) also reported the ability of lactic acid bacteria to remove aflatoxin B1 from artificially contaminated liquid media. The removal was strain dependent and very fast, with two strains of *L. rhamnosus* removing about 80% of the toxin at the beginning of the incubation time.

Table 4.1: Aflatoxin concentrations for various cola nut treatment concentrations in sun-dried fermented cocoa beans

Treatments	Aflatoxin B1 (ppb)	Aflatoxin B2 (ppb)	Aflatoxin G1 (ppb)	Aflatoxin G2 (ppb)
0 g/5 kg	ND	ND	ND	ND
50 g/5 kg extract	ND	ND	ND	ND
50 g/5 kg powder	ND	ND	ND	ND
100 g/5 kg extract	ND	ND	ND	ND
100 g/5 kg powder	ND	ND	ND	ND
150 g/5 kg extract	ND	ND	ND	ND
150 g/5 kg powder	ND	ND	ND	ND

ND* = Below instrument detection limit (B1 and B2 = 0.13 ppb) (G1 and G2 = 0.15 ppb)

4.3.2 Ochratoxin A

Ochratoxin A is one of the most dangerous mycotoxins and it has been reported to be nephrotoxic, hepatotoxic, embryotoxic, teratogenic, neurotoxic, immunotoxic, genotoxic and carcinogenic in many species with species and sex-related differences (Malir *et al*., 2013; Sava *et al*., 2007; Weidenbach *et al*., 2004). Cola powder and extract treatments were observed to have effects on ochratoxin A production in cocoa beans (Figure 4.11). With respect to the control (0 g/5 kg), there was significant reduction of OTA ($p < 0.05$) from 8.32 µg/kg to 2.85 µg/kg and 3.02 µg/kg, respectively for the 150 g/5 kg extract and 150 g/5 kg powder concentrations. These represented 65.7% and 63.7% reduction for 150 g/5 kg cola extract and powder, respectively. Concentrations of 100 g/5 kg cola extract and powder also recorded 40.4% and 41.1% reduction

in OTA levels, respectively which were significantly different ($p < 0.05$) from the control (0 g/5 kg). The 50 g/5 kg concentrations for both cola extract and powder were not significantly different ($p > 0.05$) when compared to the control. There was no significant difference among the different treatment types for cola nut at 95% confidence level (Cola extract and Cola powder). Ochratoxin A levels for all treatment concentrations of cocoa beans however, exceeded the proposed EU regulatory limit of 2 µg/kg for cocoa products for final consumption (European Commission Regulation, 2004).

The significant reduction in OTA levels for 100 g/5 kg and 150 g/5 kg was as a result of the alkaloids present in cola nut (mostly caffeine) which interfered with the biosynthesis of the mycotoxin. Akbar *et al*. (2016) reported that 0.5% caffeine significantly inhibited OTA production while the average levels of OTA production by *A. ochraceus* in caffeinated green coffee beans was reported to be 29.36 µg/g which significantly increased to 49.76 µg/g when the green coffee beans were decaffeinated (Nehad *et al*., 2005). Caffeine again was reported to reduce ochratoxin production as much as 98% while theobromine had relatively little effect on ochratoxin A production (Buchanan *et al*., 1982). The inhibitory effect of cola nut extract and powder on growth of moulds responsible for producing these mycotoxins might have contributed to the reduction in mycotoxins recorded for 100 g/5 kg and 150 g/5 kg treatment concentrations. A lot of work has associated ochratoxin A production in cocoa beans including work done by Yamoah (2015) who reported OTA levels ranging from 0.186 µg/kg to 4.650 µg/kg for cocoa beans coming from Western region of Ghana. Copetti *et al*. (2013) also reported levels reaching 5.131 µg/kg in cocoa powder while Deus *et al.* (2018) also reported levels in cocoa beans reaching 7.1 µg/kg in Brazil. Ochratoxin A was also detected in cocoa beans at levels from 0.1 to 3.5 µg/kg in Spain (Bonvehi, 2004).

The general regression model obtained for the degree of effectiveness of cola concentration on OTA is a simple linear regression given by the equation $Y = \beta_0 + \beta_1 X + \varepsilon$ (Figure 4.12). The regression was significant ($p < 0.05$) with 89.5% of the variations in OTA levels explained for by changes in treatment concentrations. This shows that cola nut powder and extract was effective in controlling the production of OTA in cocoa. The adjusted R^2 (0.895) was also in agreement with the R^2 of 0.902. Treatment type however, was not significant and hence was excluded from the final model.

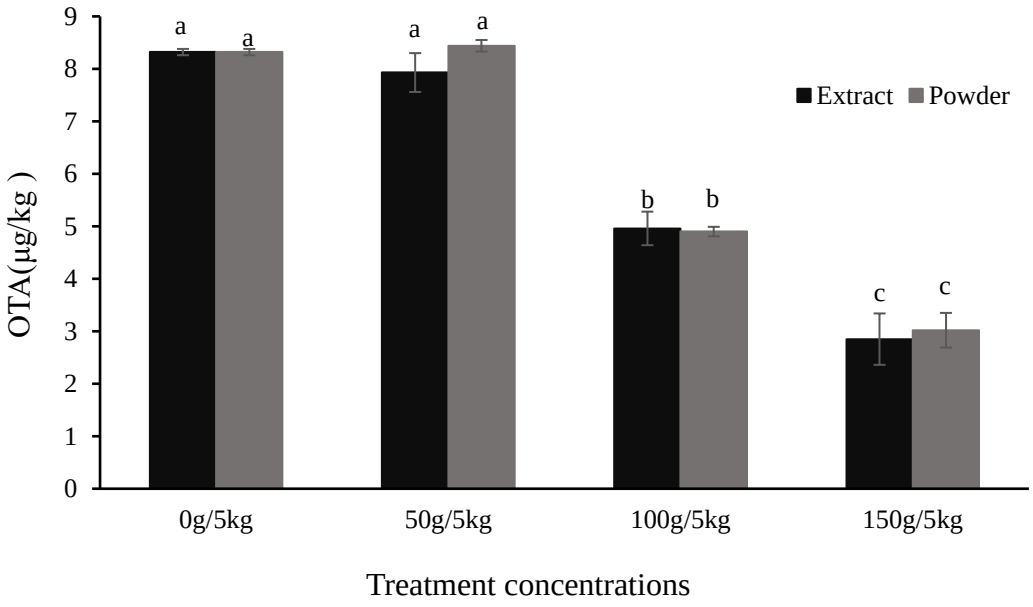

Figure 4.11: Ochratoxin A concentrations for various cola nut treatment concentrations in sun-dried fermented cocoa beans (means that do not share a letter are significantly different)

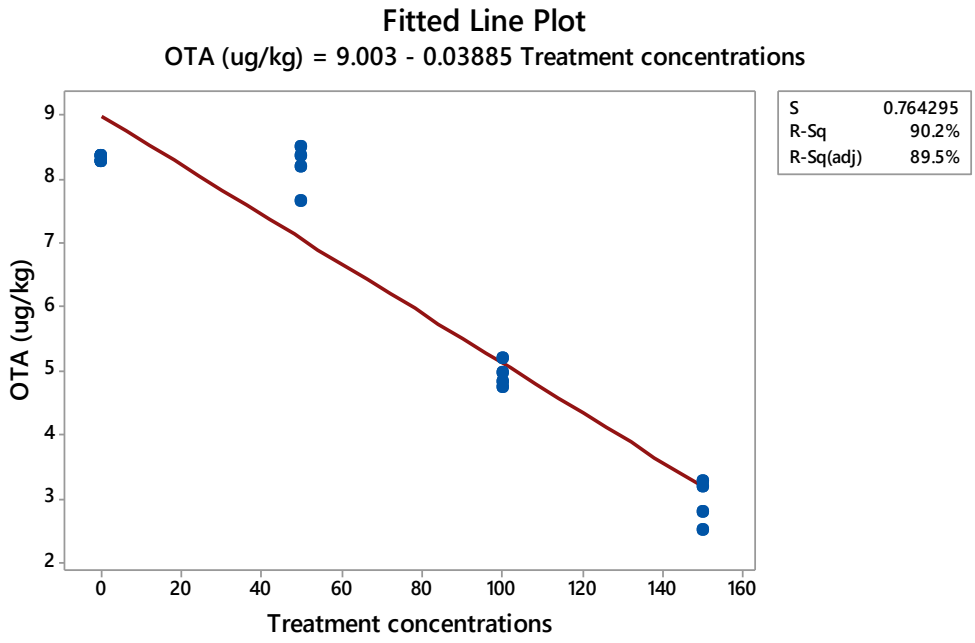

Figure 4.12: A linear regression line plot for OTA and cola treatment concentrations

4.4 Effects of cola treatment on physiochemical properties of sun-dried cocoa beans

4.4.1 Titratable acidity and pH

Cocoa bean quality is indicated by the degree of fermentation, low astringency and bitterness as well as the absence of off-flavours and excessive acidity. Titratable acidity and pH of dried fermented cocoa beans affect these quality indicators (Afoakwa *et al*., 2012). The measured pH and titratable acidity of cola nut treated cocoa beans are shown in Figures 4.13 and 4.14, respectively. There was significant increase ($p < 0.05$) in pH for beans treated with 50 g/5 kg, 100 g/5 kg and 150 g/5 kg concentrations of both cola extract and cola powder when compared to the control. There was no significant ($p > 0.05$) increase in pH of samples treated with 50 g/5 kg cola powder. The measured pH ranged from 5.39 for control to 5.71 for 150 g/5 kg cola powder treated

beans. There was however no significant difference ($p > 0.05$) between cocoa beans treated with cola powder and cola extract for 100 g/5 kg and 150 g/5 kg concentrations.

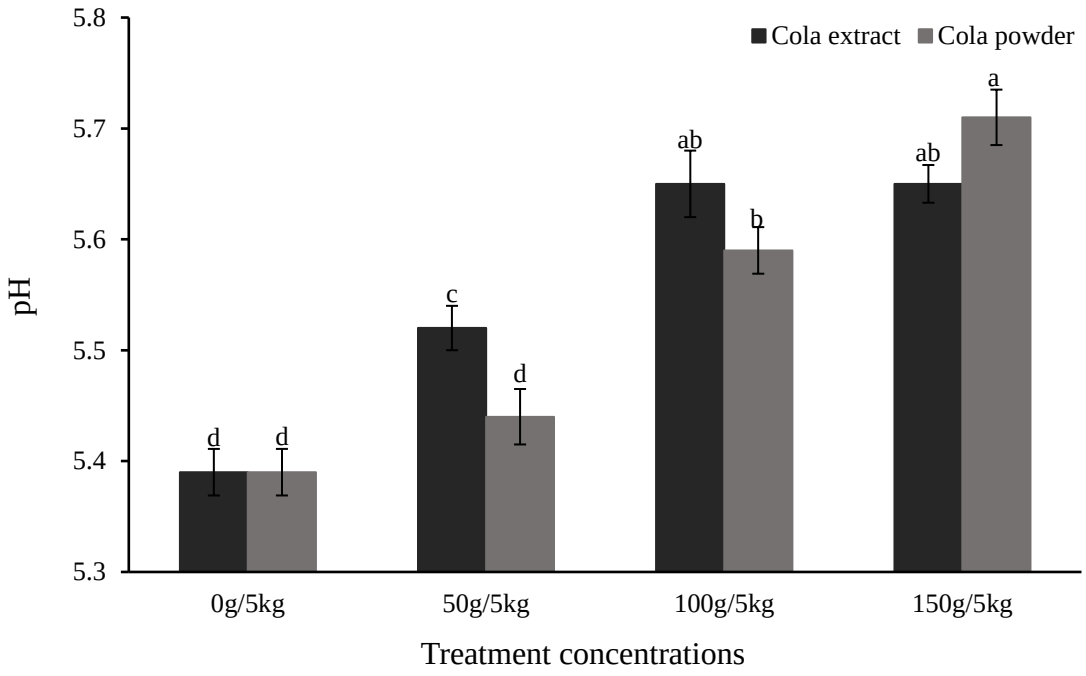

Figure 4.13: Measured pH of cola nut treated sun-dried cocoa beans at various levels of treatment concentrations (means that do not share a letter are significantly different)

Titratable acidity (TA) on the other hand showed trends opposite to the observations made for pH. There was a reduction in titratable acidity values as concentration of cola powder or extract increased in the cocoa beans though the reductions in TA were only significant ($p < 0.05$) for 150 g/5 kg cola powder/extract and 100 g/5 kg cola extract treated beans. The titratable acidity ranged from 0.112 (mole of NaOH/g) for 150 g/5 kg cola powder treated beans to 0.122 (mole of NaOH/g) for the control. There was no significant difference ($p > 0.05$) between cocoa beans treated with cola powder and cola extract for all concentrations.

The results obtained were quite higher as compared to those reported by other studies being 3.8 - 4.5 (Zahouli *et al.*, 2010). The high pH values and low titratable acidity values were however in agreement with work done by Afoakwa *et al*. (2015) who showed that prolong drying time increased pH and decreased titratable acidity of cocoa beans. Franke *et al*. (2008) also observed that natural sun drying of cocoa beans led to less acidic beans and this might be as a result of the continuous slow migration of moisture together with volatile acids (mostly acetic acid) produced during fermentation from the beans without breaking the diffusion path. Kakhia (2003) recounted that alkaloids including caffeine in cola nut are basic and so these alkaloids in cola may have neutralized some of these acids produced during fermentation and this was evident as acidity of cola treated beans further decreased with an increase in cola concentration. According to Nazaruddin *et al*. (2006), titratable acidity is a better indicator of acidity than pH; a trend of decreasing acidity with an increase in cola treatment concentrations was observed though this decrease in acidity was not significant ($p > 0.05$) except for 150 g/5 kg treatment concentrations.

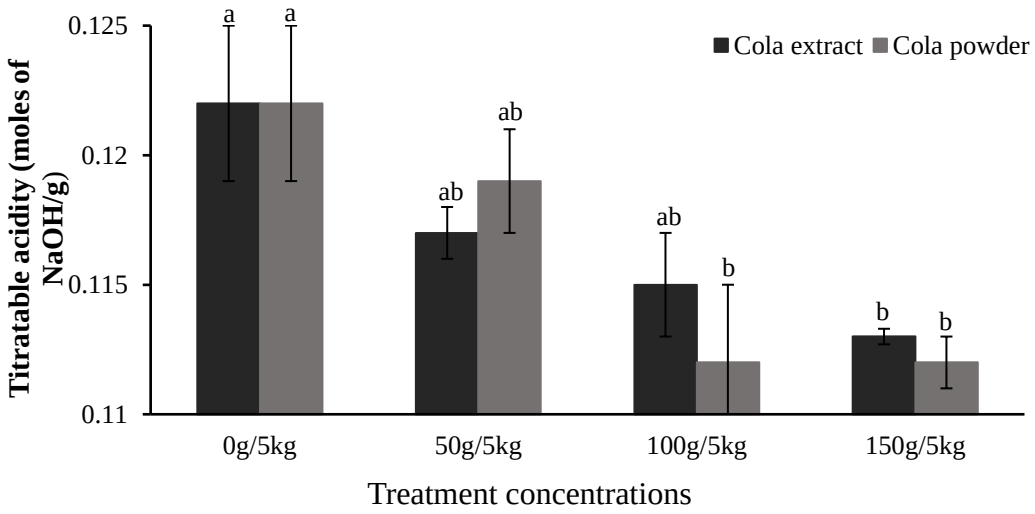

Figure 4.14: Measured titratable acidity of cola nut treated sun-dried cocoa beans at different treatment concentrations (means that do not share a letter are significantly different)

4.4.2 Cut test

The cut test is a standard method of assessing quality of cocoa beans as defined in grade standards and can be used to estimate off-flavours (mouldy), unfermented and under fermented cocoa beans (Afoakwa, 2014). Results from the cut test analysis are shown in table 4.2. There was a decrease of fully brown beans from 71% for control to 57% and 61% for 150 g/5 kg cola extract and powder treated beans, respectively. As a result, the percentage of partly purple and partly brown beans were higher for 150 g/5 kg cola extract and powder treated beans than that of the control. In contrast, there were more mouldy beans in the control (23%) than there were in 150 g/5 kg cola extract and powder treated beans (6% and 7%, respectively). This agreed with the microbiological results obtained for mould growth which recorded lower growth in cocoa beans treated with 150 g/5 kg cola concentrations. Despite the high percentage of fully brown beans for the control than the 150 g/5 kg cola treated beans, the equivalent fully brown score (EB score) which considers both fully brown as well as all defective beans (100 % fully brown, 70 % partly purple/brown, 50 % fully purple and 30 % mouldy/slaty beans) was better for the 150 g/5 kg cola treated beans (Figure 4.15). The high percentage of EB scores of treated beans which indicates well fermented beans contrast the low acidity recorded in terms of pH and titratable acidity. This however suggest that, the long drying duration (14 days) lead to low acid beans as reported by Afoakwa *et al*. (2015) even though the beans were properly fermented. Though none of the cola treated beans could meet the minimum requirement for mouldy beans (3%), the level of moldiness in 150 g/5 kg treated concentrations (6% and 7%) for cola extract and powder respectively, reduced when compared to the control (23%).

Table 4.2: Cut test scores for cola nut treated sun-dried fermented cocoa beans at different treatment concentrations

Treatments	Fully Brown (%)	Partly Brown (%)	Partly Purple (%)	Fully Purple (%)	Mouldy (%)
0g/5kg	71	3	2	1	23
50g/5kg (Extract)	72	10	5	2	11
50g/5kg (Powder)	72	4	8	2	14
100g/5kg (Extract)	69	9	8	5	9
100g/5kg (Powder)	63	11	7	8	11
150g/5kg (Extract)	57	19	11	7	6
150g/5kg (Powder)	61	14	10	8	7

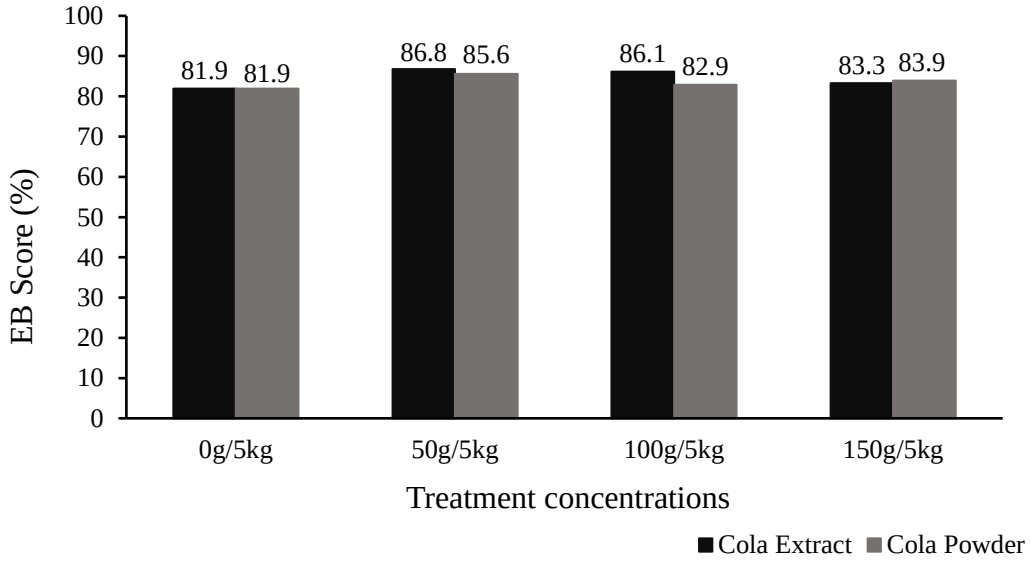

Figure 4.15: Equivalent fully brown score for cola nut treated sun-dried cocoa beans at various treatment concentrations

4.4.3 Fermentation index

Fermentation index, unlike the cut test is an unbiased way of assessing the degree of fermentation of cocoa beans. It measures the degree of hydrolysis of anthocyanins, proanthocyanin and catechins as they break down during fermentation of cocoa beans (Afoakwa, 2014). Figure 4.16 shows the results for fermentation index of cola nut treated cocoa beans. The cocoa beans samples without any treatment (control) recorded the highest fermentation index of 1.116 whereas the 150 g/5 kg cola extract treated beans recorded the least fermentation index of 1.014. There was significant difference ($p < 0.05$) between the control and the 100 g/5 kg and 150 g/5 kg cola treated beans for both extract and powder. There was however, no significant difference in fermentation index for the two treatment types of cola nut extract and cola nut powder.

Regardless of the high fermentation index value for the control than the cola treated beans, they were all within the accepted quality limit of 1.000 - 1.599 (Gourieva and Tserrevitinov, 1979). Cocoa beans with fermentation index less than 1.000 are considered under fermented while beans with fermentation index above 1.599 are considered over fermented. This result was consistent with the cut test scores which had high levels of fully brown beans for the control than the other cola treated cocoa beans. The breakdown of anthocyanins to oxidative products lead to the formation of brown colour in the beans by the action of polyphenol oxidase which act on these oxidative products from the breakdown of anthocyanins thereby reducing purple pigments in the beans (Afoakwa, 2014). The basic components of cola nut (alkaloids) may also have interacted with some of the acidic end product of fermentation (acetic acid) to result in relatively low fermentation index values as the concentration of cola nut increased in cocoa beans.

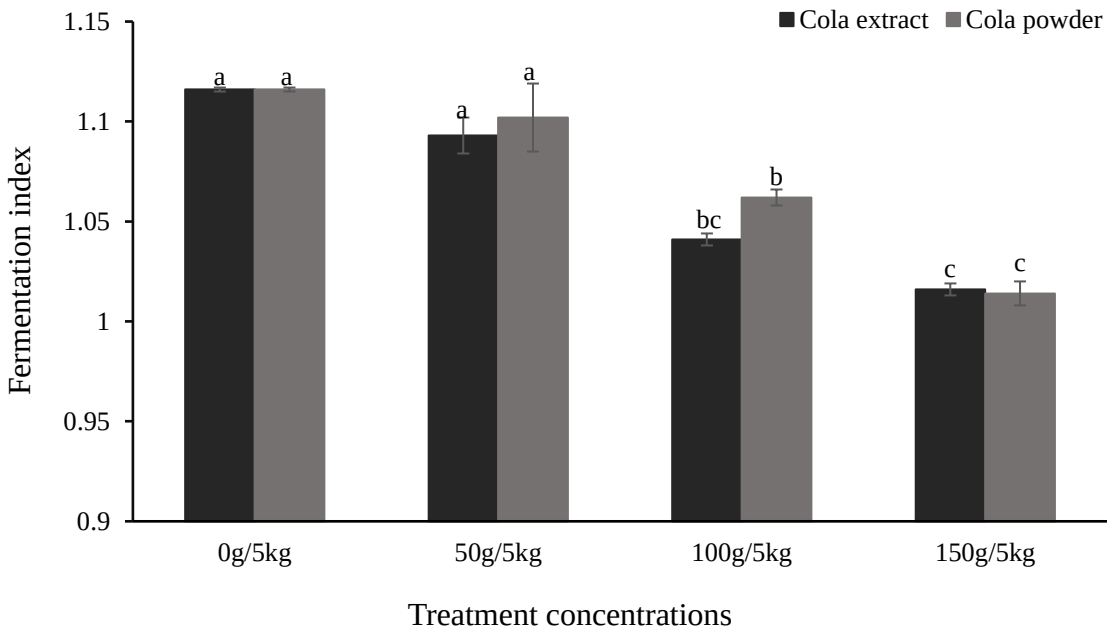

Figure 4.16: Fermentation index results for cola nut treated sun-dried cocoa beans at different treatment concentrations (means that do not share a letter are significantly different)

4.5 Effect of cola nut on cocoa butter quality

4.5.1 Free fatty acids (FFAs)

The physical characteristics of cocoa butter are assessed by measurements of various parameters including free fatty acid content which is a measure of rancidity (Knight, 2000). The results for the cola nut treated cocoa beans are presented in Figure 4.17. There was a continuous decrease in free fatty acid levels as treatment concentrations increased with the 150 g/5 kg cola treated beans recording the least FFA content of 0.71 % and 0.61 % for cola extract and cola powder treatments, respectively. These were significantly different ($p < 0.05$) from the control which recorded 2.34% free fatty acid content. There was also a significant decrease ($p < 0.05$) in percent free fatty acid for 100 g/5 kg treated beans for both cola extract and cola powder when compared to the control. The 50 g/5 kg treated beans however, saw a significant ($p < 0.05$) decrease

for only cola powder treated beans when compared to control. Even though there was a decrease in percent free fatty acid for 50 g/5 kg cola extract treated beans, this decrease was not significant at 95% confidence level. There was however, no significant difference ($p > 0.05$) between cola extract and cola powder treated beans at all concentration levels. Despite the decrease in percent free fatty acid with increase in cola treatment concentrations, it was only the 100 g/5 kg and 150 g/5 kg treated beans which had levels below the codex maximum limit of 1.75% (Krysiak, 2011).

Deus *et al*. (2018) reported a significant reduction in antioxidant activity with reduced phenolics content and methylxanthines during drying of cocoa beans where 0.04 mg/g of catechin before drying reduced to 0.02 mg/g after drying with theobromine levels reducing from 19.44 mg/g before drying to 11.71 mg/g after drying. Similar results were reported by Peláez *et al*. (2016) who found out that polyphenol content of fresh beans reduced from 7.0 g GAE/100 g to 5.05 g GAE/100 g after drying. Since antioxidants scavenge for free radicals like free fatty acids and reduce the generation of these compounds, the reduction in antioxidants activity during drying may have caused the high free fatty acid levels in the control. The 150 g/5 kg treated beans however had low free fatty acid levels due to the presence of the cola nut which have been reported to have high levels of these phytochemicals which may have increased the antioxidant activity in beans treated with the cola nut. Phenolic contents of white *Cola nitida* was reported to be 26.76 mg/g fresh weight with flavonoid levels reaching 803.03 mg/kg fresh weight (Nyamien *et al*., 2014). Dah-Nouvlessounon *et al*. (2015) also reported 679.87 (µgEqGallicAcid/100 g) total phenolics for white *Cola nitida* with 374.60 (µgEqQuercetin/100 g) flavonoid levels. As moisture levels in the cocoa increases, the activities and growth of moulds also increase leading to the release of carbonyl acids in the triglycerides of cocoa butter by lipases from these moulds as well as the endogenous lipase enzyme in the cocoa itself to produce these free radicals of fatty acids (Selamat *et al*., 1996).

This was evident from the microbiological results which agreed with the free fatty acid results where high levels of moulds in control subsequently led to significantly high ($p < 0.05$) free fatty acid levels in the control than the 150 g/5 kg cola treated beans.

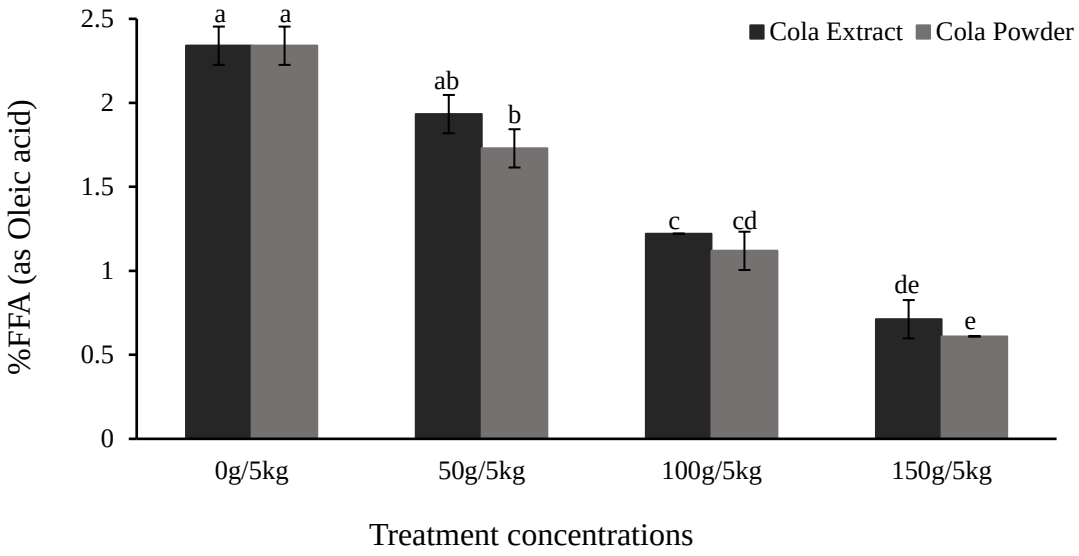

Figure 4.17: Free fatty acid content of cola nut treated sun-dried cocoa beans at different treatment concentrations (means that do not share a letter are significantly different)

4.5.2 Peroxide value

Peroxide value signifies the extent to which the cocoa butter sample has undergone primary oxidation or the extent to which oil has deteriorated (Afoakwa, 2014). Peroxide values for cola nut treated cocoa beans are presented in Figure 4.18. There was a general decrease in peroxide value as cola nut concentration increased. The control recorded a value of 21.6 Meq/kg of beans which was significantly higher than 15.8 Meq/kg and 14.2 Meq/kg for 150 g/5 kg cola extract and cola powder treated beans, respectively at 95% confidence level. Similar trends were observed for 50 g/5 kg and 100 g/5 kg cola treated beans which showed significantly lower peroxide values when compared to the control. There was however no difference ($p > 0.05$) between cola extract treated

beans and cola powder treated beans at all levels. Despite the high peroxide values recorded, they were all below the levels at which rancid taste of oils is most likely to be noticeable between 30 Meq/kg to 40 Meq/kg (Chakrabary, 2003). However, according to Shahidi (2005), a good quality cocoa butter should have peroxide values less than 10 Meq/kg.

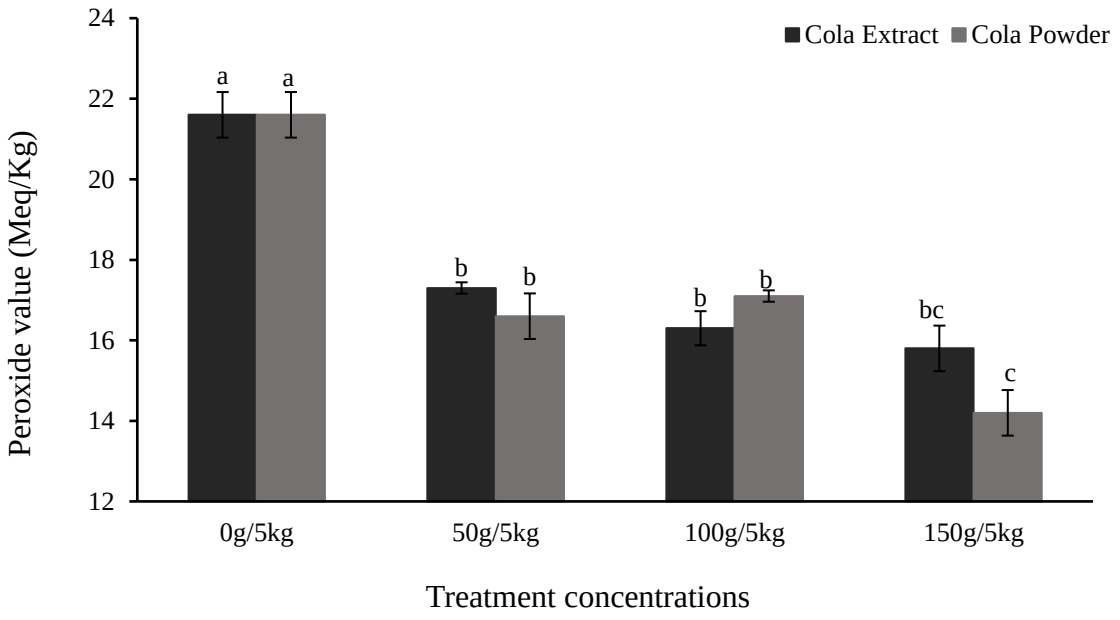

Figure 4.18: Peroxide values of cola nut treated sun-dried cocoa beans at different treatment concentrations (means that do not share a letter are significantly different)

The high levels of free radicals in the control with significant decrease as cola nut concentration increased can be said to be responsible for the high peroxide values recorded in the control and similarly decreasing with increase in cola nut concentration in the cocoa beans. This is as a result of autoxidation of these free radicals leading to the formation of intermediate peroxides which give off flavours to the oil and hence it is used to determine the extent of rancidity of oils (Choudhary and Glover, 2013). The high phytochemical levels in cola nut as reported by

Dah-Nouvlessounon *et al*. (2015) might have contributed to reducing the extent of autoxidation in the cocoa butter from the cocoa treated with high concentrations of cola nut leading to low levels of peroxides in the 150 g/5 kg cola nut treated cocoa beans. The high load of moulds in the control than the 150 g/5 kg cola treated beans may also have contributed to the high peroxide formation in control than the 150 g/5 kg as the lipase activity of the moulds lead to the generation of free radicals leading to rancidity of the oil (Selamat *et al*., 1996).

4.5.3 Iodine value

The iodine value (IV) gives a measure of the average degree of unsaturation of a lipid. The higher the iodine value, the greater the number of C=C double bonds and it is inversely proportional to the melting point of lipids. An increase in iodine value indicates high susceptibility of lipids to oxidative rancidity due to high degree of unsaturation (Otunola *et al*., 2009). The effects of cola nut extract and powder on the iodine value of the cocoa beans is presented in Figure 4.19. The untreated cocoa beans had the highest iodine value of 35.96 g I/100 g which was significantly different ($p < 0.05$) from 33.63 g I/100 g and 33.21 g I/100 g of the 150 g/5 kg treated beans for cola nut powder and extract, respectively. Similar trend was observed for the 50 g/5 kg and 100 g/5 kg cola treated cocoa beans which had iodine values significantly lower than that of the control at 95% confidence level. The high iodine value of control as compared to treated beans at all concentration levels agreed with the peroxide values measured which had high values in the control than the treated cocoa beans. Despite the relatively high iodine value for the untreated cocoa beans, it was still within the acceptable limit of the codex standard of 33 to 42 g I/100 g for cocoa butter (Codex Standard 86, 1981). The results also agreed with work done by Biehl and Ziegleder (2003) who reported iodine values (36.5 g I/100 g) for cocoa butter extracted from

Ghanaian cocoa beans. Afoakwa (2014) reported that cocoa butter from unstored pods had iodine value of 33.97 g I/100 g cocoa fat and those from pods stored for 3, 7 and 10 days had iodine values of 34.27 g I/100 g cocoa fat, 34.07 g I/100 g cocoa fat and 33.79 g I/100 g cocoa fat, respectively.

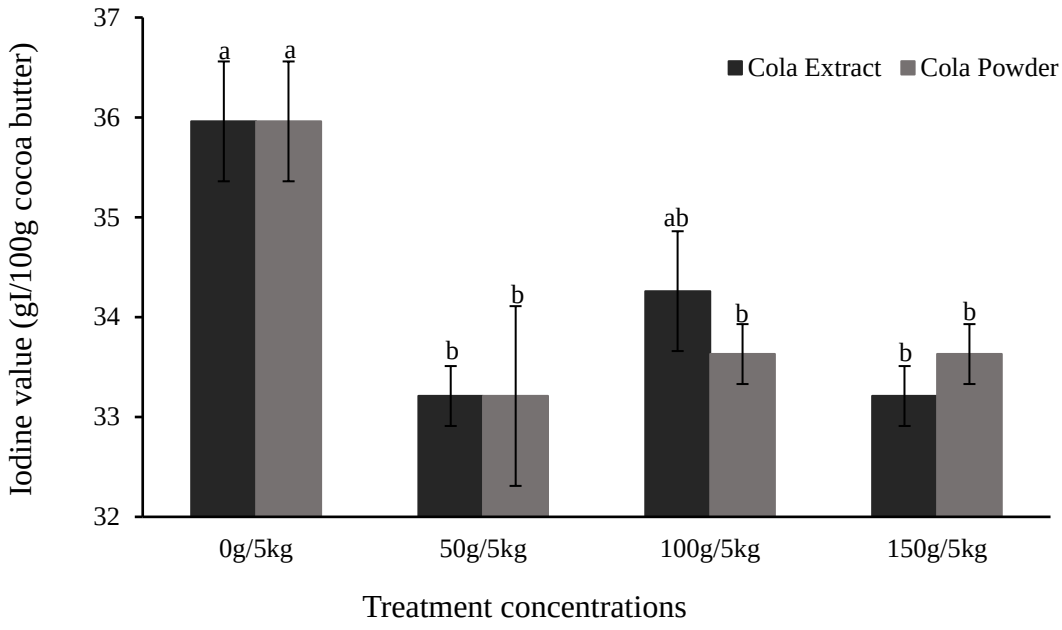

Figure 4.19: Iodine values for cola nut treated sun-dried cocoa beans at different treatment concentrations (means that do not share a letter are significantly different)

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The conclusions drawn from the study are as follows:

i. *Penicillium spp.*, *Aspergillus spp.*, *Rhizopus* and *Mucor spp.* of moulds were identified in the dried fermented cola nut extract and powder treated cocoa beans. Cola nut extract and powder were found to have a significant inhibition effect on the moulds identified. This significant inhibition however was both concentration and mould type dependent. Cola nut concentration of 150 g/5 kg had the highest inhibition effect on the moulds and was very effect against *Penicillium* spp. and *Aspergillus* spp. than it was for *Rhizopus* and *Mucor*.

ii. There were no aflatoxins detected for both cola nut extract and powder treated and untreated cocoa beans. Ochratoxin A however, was observed to decrease with an increase in cola nut extract and powder treatment concentrations. A significant decrease in Ochratoxin A levels was observed for concentrations of 100 g/5 kg cola nut and above.

iii. Cola nut extract and powder had a decreasing effect on the acidity and fermentation index of dried fermented cocoa beans with an increase in cola nut extract and powder concentrations. However, the acidity and fermentation index of dried fermented cocoa beans were acceptable for cola nut concentrations up to 150 g/5 kg of cocoa beans.

iv. The cocoa butter qualities of dried fermented cocoa beans improved for cocoa beans treated with cola nut extract and powder. This improvement however, was concentration dependent and a concentration of 150 g/5 kg cola nut extract and powder treated cocoa beans had the best cocoa butter qualities.

5.2 Recommendations

The following recommendations are made based on the findings of this study:

i. During the raining season where there is not enough sunshine to dry cocoa beans, farmers should be educated on the use of cola nut powder to control mould growth in cocoa beans.

ii. Further studies should be carried out to investigate the effects of cola nut powder and cola nut extract on the fermentative quality and physico-chemical characteristics of dried fermented cocoa beans.

iii. Further studies should also be carried out to evaluate the effects of cola nut powder on the flavour profile of dried fermented cocoa beans for chocolate production.

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APPENDICES

Appendix A: Monitored temperature and relative humidity for selection of drying environment at a specific location

Days	Temperature (°C)			
	Morning	Afternoon	Evening	Mean
1	25.7	29.9	26.1	27.23
2	26.4	30.1	25.7	27.4
3	25.9	29.3	26	27.07

Days	Relative Humidity (%)			
	Morning	Afternoon	evening	Mean
1	80	71	78	76.33
2	79	72	74	75
3	83	71	77	77

Appendix B: Raw measured data for the effect of cola nut on the physicochemical properties of fermented dried cocoa beans

pH

	Replicates			Mean	Stdev
Treatments	1	2	3		
0g/5kg	5.37	5.41	5.38	5.39	0.021
50g/5kg (Extract)	5.5	5.52	5.54	5.52	0.020
50g/5kg (Powder)	5.47	5.42	5.44	5.44	0.025
100g/5kg (Extract)	5.65	5.62	5.68	5.65	0.030
100g/5kg (Powder)	5.6	5.57	5.61	5.59	0.021
150g/5kg (Extract)	5.63	5.66	5.66	5.65	0.017
150g/5kg (Powder)	5.71	5.69	5.74	5.71	0.025

Fermentation index

Treatments	Replicates											
	Absorbance @ 460nm	mean 1	Absorbance @460nm	mean 2	Absorbance @ 530nm	mean 1	Absorbance @ 530nm	mean 2	FI (1)	FI (2)	Mean (FI)	Stdev
0g/5kg	0.467		0.47		0.419		0.421					
	0.466	0.464	0.469	0.469	0.413	0.416	0.42	0.42	1.117	1.116	**1.116**	0.000854
	0.46		0.467		0.415		0.419					
50g/5kg (Extract)	0.596		0.597		0.549		0.538					
	0.59	0.594	0.591	0.594	0.549	0.547	0.54	0.54	1.086	1.099	**1.093**	0.00948
	0.595		0.593		0.542		0.542					
50g/5kg (Powder)	0.551		0.553		0.497		0.511					
	0.556	0.553	0.558	0.555	0.501	0.496	0.509	0.509333	1.114	1.090	**1.102**	0.017386
	0.551		0.554		0.49		0.508					
100g/5kg (Extract)	0.611		0.587		0.588		0.559					
	0.611	0.612	0.585	0.585	0.591	0.589	0.563	0.560667	1.039	1.043	**1.041**	0.002672
	0.615		0.582		0.589		0.56					
100g/5kg (Powder)	0.642		0.644		0.601		0.61					
	0.639	0.642	0.645	0.645	0.606	0.603	0.609	0.608333	1.065	1.060	**1.062**	0.003501
	0.645		0.645		0.602		0.606					
150g/5kg (Extract)	0.619		0.621		0.611		0.609					
	0.621	0.620	0.624	0.622	0.611	0.611	0.61	0.611	1.014	1.018	**1.016**	0.002706
	0.62		0.621		0.612		0.614					
150g/5kg (Powder)	0.62		0.625		0.616		0.613					
	0.625	0.623	0.623	0.624	0.617	0.617	0.612	0.612667	1.010	1.018	**1.014**	0.006204
	0.624		0.624		0.618		0.613					

Titratable acidity

Treatments	Replicate Volumes									
	Replicate 1			Replicate 2						
	Initial vol.	Final vol.	Actual vol.	Initial vol.	Final vol.	Actual vol.	**TA (1)**	**TA (2)**	**Mean TA**	**Stdev**
0g/5kg	0.4	25.7	25.3	0	24.5	24.5	0.124	0.120	0.122	0.0028
50g/5kg (Extract)	0.2	24	23.8	23.9	48	24.1	0.116	0.118	0.117	0.0010
50g/5kg (Powder)	24	48.7	24.7	0.5	24.6	24.1	0.121	0.118	0.119	0.0021
100g/5kg (Extract)	25.7	49	23.3	0	23.9	23.9	0.114	0.117	0.115	0.0021
100g/5kg (Powder)	0	22.5	22.5	24.6	48	23.4	0.110	0.114	0.112	0.0031
150g/5kg (Extract)	22.5	45.6	23.1	24.5	47.5	23	0.113	0.112	0.113	0.0003
150g/5kg (Powder)	2.4	25.2	22.8	22.6	45.8	23.2	0.112	0.113	0.112	0.0014

Appendix C: Raw measured data for the effect of cola nut on the cocoa butter qualities of fermented dried cocoa beans.

Free fatty acids

Treatments	Replicate one (ml)				Replicate two (ml)							
	Initial	Final	Blank	Actual	Initial	Final	Blank	Actual	FFA1	FFA2	Mean FFA	Stdev
0g/5kg	0.7	2	0.1	1.2	6.4	7.6	0.1	1.1	2.441314	2.237871	2.3396	0.144
50g/5kg (Extract)	3.3	4.3	0.1	0.9	8.7	9.8	0.1	1	1.830986	2.034429	1.9327	0.144
50g/5kg (Powder)	2	2.9	0.1	0.8	5.4	6.4	0.1	0.9	1.627543	1.830986	1.7293	0.144
100g/5kg (Extract)	0	0.7	0.1	0.6	10.2	10.9	0.1	0.6	1.220657	1.220657	1.2207	0.000
100g/5kg (Powder)	4.7	5.4	0.1	0.6	7.6	8.2	0.1	0.5	1.220657	1.017214	1.1189	0.144
150g/5kg (Extract)	4.3	4.7	0.1	0.3	8.2	8.7	0.1	0.4	0.610329	0.813771	0.7121	0.144
150g/5kg (Powder)	2.9	3.3	0.1	0.3	9.8	10.2	0.1	0.3	0.610329	0.610329	0.6103	0.000

Peroxide value

Treatments	Replicate one				Replicate two								
	Initial	Final	Blank	Actual	Initial	Final	Blank	Actual		Peroxide value 1	Peroxide value 2	mean Peroxide Value	Stdev
0g/5kg	9.7	21.9	1.2	11	8.1	19.9	1.2	10.6		22	21.2	21.6	0.566
50g/5kg (Extract)	18.8	28.6	1.2	8.6	18.9	28.8	1.2	8.7		17.2	17.4	17.3	0.141
50g/5kg (Powder)	0	9.7	1.2	8.5	19.9	29.2	1.2	8.1		17	16.2	16.6	0.566
100g/5kg (Extract)	9.3	18.8	1.2	8.3	9.7	18.9	1.2	8		16.6	16	16.3	0.424
100g/5kg (Powder)	28.6	38.4	1.2	8.6	0	9.7	1.2	8.5		17.2	17	17.1	0.141
150g/5kg (Extract)	0	9.3	1.2	8.1	29.2	38.1	1.2	7.7		16.2	15.4	15.8	0.566
150g/5kg (Powder)	21.9	30.4	1.2	7.3	0	8.1	1.2	6.9		14.6	13.8	14.2	0.566

Iodine value

Treatments	**Replicates (ml)**									
	Rep 1			Rep 2						
	Final	**Blank**	**Actual**	**Final**	**Blank**	**Actual**	**Iodine value 1**	**Iodine value 2**	**mean iodine Value**	**Stdev**
0g/5kg	38.6	47	8.4	38.4	47	8.6	35.532	36.378	**35.955**	0.60
50g/5kg (Extract)	39.1	47	7.9	39.2	47	7.8	33.417	32.994	**33.2055**	0.30
50g/5kg (Powder)	39.3	47	7.7	39	47	8	32.571	33.84	**33.2055**	0.90
100g/5kg (Extract)	38.8	47	8.2	39	47	8	34.686	33.84	**34.263**	0.60
100g/5kg (Powder)	39.1	47	7.9	39	47	8	33.417	33.84	**33.6285**	0.30
150g/5kg (Extract)	39.1	47	7.9	39.2	47	7.8	33.417	32.994	**33.2055**	0.30
150g/5kg (Powder)	39	47	8	39.1	47	7.9	33.84	33.417	**33.6285**	0.30

Appendix D: Enumeration of moulds growth on dried fermented cocoa beans as affected by cola nut treatment.

Penicillium spp.

Samples	Replicate 1	Replicate 2				
	cfu/g	cfu/g	Log cfu/g rep 1	log cfu/g rep 2	mean log(cfu/g)	stdev
0g/5kg	1200000	1510000	6.079181246	6.178976947	6.13	0.070566
50g/5kg (extract)	5900000	7200000	6.770852012	6.857332496	6.82	0.061151
50g/5kg (powder)	7100000	7700000	6.851258349	6.886490725	6.87	0.024913
100g/5kg (extract)	900000	790000	5.954242509	5.897627091	5.93	0.040033
100g/5kg (powder)	900000	830000	5.954242509	5.919078092	5.94	0.024865
150g/5kg (extract)	20000	10000	4.301029996	4	4.18	0.21286
150g/5kg (powder)	40000	60000	4.602059991	4.77815125	4.70	0.124515

Aspergillus spp.

Samples	Replicate 1	Replicate 2				
	cfu/g	cfu/g	mean log (cfu/g)	rep 1 log (cfu/g)	rep 2 log (cfu/g)	stdev
0g/5kg	90000	60000	4.88	4.954247335	4.778158489	0.124514
50g/5kg (extract)	200000	100000	5.18	5.301032167	5.000004343	0.212859
50g/5kg (powder)	100000	200000	5.18	5.000004343	5.301032167	0.212859
100g/5kg (extract)	0	0	0.00	0	0	0
100g/5kg (powder)	10000	10000	4.00	4.000043427	4.000043427	0
150g/5kg (extract)	0	0	0.00	0	0	0
150g/5kg (powder)	0	0	0.00	0	0	0

Rhizopus and Mucor spp.

Samples	Replicate 1	Replicate 2				
	cfu/g	cfu/g	mean log(cfu/g)	Log (cfu/g) rep 1	Log (cfu/g) rep 2	stdev
0g/5kg	70000	110000	4.95	4.84509804	5.041392685	0.138801
50g/5kg (extract)	700000	1000000	5.93	5.84509804	6	0.109532
50g/5kg (powder)	1600000	1200000	6.15	6.204119983	6.079181246	0.088345
100g/5kg (extract)	260000	130000	5.29	5.414973348	5.113943352	0.21286
100g/5kg (powder)	240000	280000	5.41	5.380211242	5.447158031	0.047339
150g/5kg (extract)	20000	20000	4.30	4.301029996	4.301029996	0
150g/5kg (powder)	60000	50000	4.74	4.77815125	4.698970004	0.05599

Appendix E: Raw data for Ochratoxin A levels of fermented dried cocoa beans as affected by cola nut treatment

	Rep 1	Rep 2		
Treatments	**Ochratoxin A (ppb)**	**Ochratoxin A (ppb)**	**Mean Ochratoxin A (ppb)**	**Standard deviation**
0g/5kg	8.36	8.28	8.32	0.056568542
50g/5kg extract	7.66	8.19	7.93	0.374766594
50g/5kg powder	8.36	8.51	8.44	0.106066017
100g/5kg extract	4.73	5.18	4.96	0.318198052
100g/5kg powder	4.83	4.96	4.90	0.091923882
150g/5kg extract	3.19	2.5	2.85	0.487903679
150g/5kg powder	3.25	2.78	3.02	0.332340187

Appendix F: Summary of ANOVA and Regression results

General Linear Model: Fermentation Index versus Treatment Type, Treatment Concentrations

Factor	Type	Levels	Values
Treatment Type	Fixed	2	Extract, Powder
Treatment Concentrations	Fixed	4	0g/5kg, 100g/5kg, 150g/5kg, 50g/5kg

Analysis of Variance

Source	DF	Seq SS	Seq MS	F-Value	P-Value
Treatment Type	1	0.000204	0.000204	3.56	0.096
Treatment Concentrations	3	0.025041	0.008347	145.54	0.000
Treatment Type*Treatment Concentrations	3	0.000339	0.000113	1.97	0.197
Error	8	0.000459	0.000057		
Total	15	0.026043			

General Linear Model: pH versus Treatment type, Treatment Concentrations

Factor Information

Factor	Type	Levels	Values
Treatment type	Fixed	2	Extract, Powder
Treatment Concentrations	Fixed	4	0g/5kg, 100g/5kg, 150g/5kg, 50g/5kg

Analysis of Variance

Source	DF	Seq SS	Seq MS	F-Value	P-Value
Treatment type	1	0.001837	0.001837	3.53	0.079
Treatment Concentrations	3	0.321713	0.107238	205.90	0.000
Treatment type*Treatment Concentrations	3	0.017813	0.005938	11.40	0.000
Error	16	0.008333	0.000521		
Total	23	0.349696			

General Linear Model: Titratable Acidity versus Treatment Concentrations, Treatment Type

Factor Information

Factor	Type	Levels	Values
Treatment Concentrations	Fixed	4	0, 50, 100, 150
Treatment Type	Fixed	2	Extract, Powder

Analysis of Variance

Source	DF	Seq SS	Seq MS	F-Value	P-Value
Treatment Concentrations	3	0.000227	0.000076	16.83	0.001
Treatment Type	1	0.000000	0.000000	0.06	0.820
Treatment Concentrations*Treatment Type	3	0.000018	0.000006	1.35	0.325
Error	8	0.000036	0.000005		
Total	15	0.000282			

General Linear Model: Free Fatty acid versus Treatment Type, Treatment concentrations

Factor Information

Factor	Type	Levels	Values
Treatment Type	Fixed	2	Extract, Powder
Treatment concentrations	Fixed	4	0g/5kg, 100g/5kg, 150g/5kg, 50g/5kg

Analysis of Variance

Source	DF	Seq SS	Seq MS	F-Value	P-Value
Treatment Type	1	0.04139	0.04139	2.67	0.141
Treatment concentrations	3	6.50842	2.16947	139.78	0.000
Treatment Type*Treatment concentrations	3	0.02069	0.00690	0.44	0.728
Error	8	0.12417	0.01552		
Total	15	6.69467			

General Linear Model: Iodine value versus Treatment Type, Treatment concentrations

Factor Information

Factor	Type	Levels	Values
Treatment Type	Fixed	2	Extract, Powder
Treatment concentrations	Fixed	4	0g/5kg, 100g/5kg, 150g/5kg, 50g/5kg

Analysis of Variance

Source	DF	Seq SS	Seq MS	F-Value	P-Value
Treatment Type	1	0.0112	0.01118	0.04	0.846
Treatment concentrations	3	18.9106	6.30352	22.55	0.000
Treatment Type*Treatment concentrations	3	0.5703	0.19011	0.68	0.589
Error	8	2.2366	0.27958		
Total	15	21.7287			

General Linear Model: Peroxide value versus Treatment Type, Treatment concentrations

Factor Information

Factor	Type	Levels	Values
Treatment Type	Fixed	2	Extract, Powder
Treatment concentrations	Fixed	4	0g/5kg, 100g/5kg, 150g/5kg, 50g/5kg

Analysis of Variance

Source	DF	Seq SS	Seq MS	F-Value	P-Value
Treatment Type	1	0.563	0.5625	2.47	0.154
Treatment concentrations	3	95.947	31.9825	140.58	0.000
Treatment Type*Treatment concentrations	3	3.128	1.0425	4.58	0.038
Error	8	1.820	0.2275		
Total	15	101.458			

General Linear Model: OTA (µg/kg) versus Treatment type, Treatment concentrations

Factor Information

Factor	Type	Levels	Values
Treatment type	Fixed	2	Extract, Powder
Treatment concentrations	Fixed	4	0, 50, 100, 150

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment type	1	0.0961	0.0961	1.25	0.296
Treatment concentrations	3	82.7353	27.5784	357.99	0.000
Treatment type*Treatment concentrations	3	0.1965	0.0655	0.85	0.504
Error	8	0.6163	0.0770		
Total	15	83.6442			

Regression Analysis: OTA (µg/kg) versus Treatment concentrations

The regression equation is
OTA (µg/kg) = 9.003 - 0.03885 Treatment concentrations

S = 0.764295 R-Sq = 90.2% R-Sq(adj) = 89.5%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	75.4661	75.4661	129.19	0.000
Error	14	8.1781	0.5841		
Total	15	83.6442			

General Linear Model: *Aspergillus* versus Treatment type, Treatment concentrations

Factor Information

Factor	Type	Levels	Values
Treatment type	Fixed	2	Extract, Powder
Treatment concentrations	Fixed	4	0, 50, 100, 150

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment type	1	4.0001	4.0001	263.11	0.000
Treatment concentrations	3	72.4293	24.1431	1588.02	0.000
Treatment type*Treatment concentrations	3	12.0002	4.0001	263.11	0.000
Error	8	0.1216	0.0152		
Total	15	88.5513			

Polynomial Regression Analysis: *Aspergillus* versus Treatment concentrations

The regression equation is
log(cfu/g + 1). = 5.095 - 0.00123 Treatment concentrations - 0.000228 Treatment concentrations^2

S = 1.25044 R-Sq = 77.0% R-Sq(adj) = 73.5%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	68.2243	34.1122	21.82	0.000
Error	13	20.3269	1.5636		
Total	15	88.5513			

General Linear Model: *Penicillium* versus Treatment type, Treatment concentrations

Factor Information

Factor	Type	Levels	Values
Treatment type	Fixed	2	Extract, Powder
Treatment concentrations	Fixed	4	0, 50, 100, 150

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment type	1	0.0915	0.09154	9.47	0.015
Treatment concentrations	3	12.4401	4.14671	428.86	0.000
Treatment type*Treatment concentrations	3	0.2027	0.06758	6.99	0.013
Error	8	0.0774	0.00967		
Total	15	12.8118			

Polynomial Regression Analysis: *Penicillium* versus Treatment concentrations

The regression equation is
log(cfu/g) = 6.180 + 0.02128 Treatment concentrations - 0.000222 Treatment concentrations^2

S = 0.211303 R-Sq = 95.5% R-Sq(adj) = 94.8%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	12.2313	6.11566	136.97	0.000
Error	13	0.5804	0.04465		
Total	15	12.8118			

General Linear Model: *Rhizopus* and *Mucor* versus Treatment type, Treatment concentrations

Factor Information

Factor	Type	Levels	Values
Treatment type	Fixed	2	Extract, Powder
Treatment concentrations	Fixed	4	0, 50, 100, 150

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment type	1	0.16235	0.16235	11.91	0.009
Treatment concentrations	3	4.96016	1.65339	121.33	0.000
Treatment type*Treatment concentrations	3	0.09936	0.03312	2.43	0.140
Error	8	0.10902	0.01363		
Total	15	5.33089			

Polynomial Regression Analysis: *Rhizopus* and *Mucor* versus Treatment concentrations

The regression equation is
log(cfu/g) = 5.026 + 0.02470 Treatment concentrations - 0.000191 Treatment concentrations^2

S = 0.265873 R-Sq = 82.8% R-Sq(adj) = 80.1%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	4.41193	2.20597	31.21	0.000
Error	13	0.91895	0.07069		
Total	15	5.33089			

Appendix G: Chromatograms for mycotoxin analysis of cola treated fermented dried cocoa beans

Calibration Report

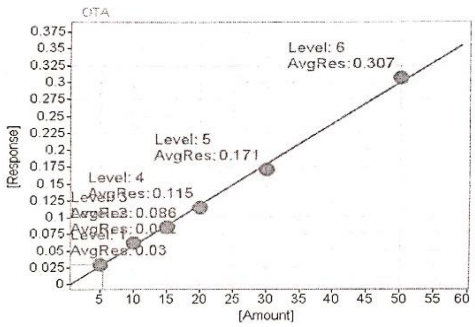
Agilent Technologies

ESTD report based on Area

Name	RT [min]	Area	Height	RF	Amount [ppb (ug/kg)]
OTA	2.121	0.0298	0.0035	179.69457	5.351

Calibration Curves:

Compound:	OTA
Signal:	FLD1A
Exp. RT:	2.114
Corr. Coeff.:	0.998702
Residual:	0.00576
Formula:	y = ax + b
a:	0.00606
b:	-0.00265
c:	0.00000
d:	0.00000



Calibration curve from Ochratoxin A standard used to estimate the concentrations of OTA in cola treated fermented dried cocoa beans

ata File C:\Chem32\...chratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08\Ochra0000010.D
ample Name: Sam 50g/5kgP

```
==========================================================================
Acq. Operator    : Dorothy Narh                    Seq. Line :  10
Acq. Instrument  : HPLC 1260                        Location :   8
Injection Date   : 4/11/2019 6:30:26 PM                 Inj :   2
                                                  Inj Volume : 10.000 µl
Acq. Method      : C:\Chem32\1\Data\OchratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08
                   \OCHRATOXIN A FINAL.M
Last changed     : 4/11/2019 5:49:08 PM by Dorothy Narh
Analysis Method  : C:\Chem32\1\Data\OchratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08
                   \OCHRATOXIN A FINAL.M (Sequence Method)
Last changed     : 4/12/2019 12:36:18 PM by Dorothy Narh
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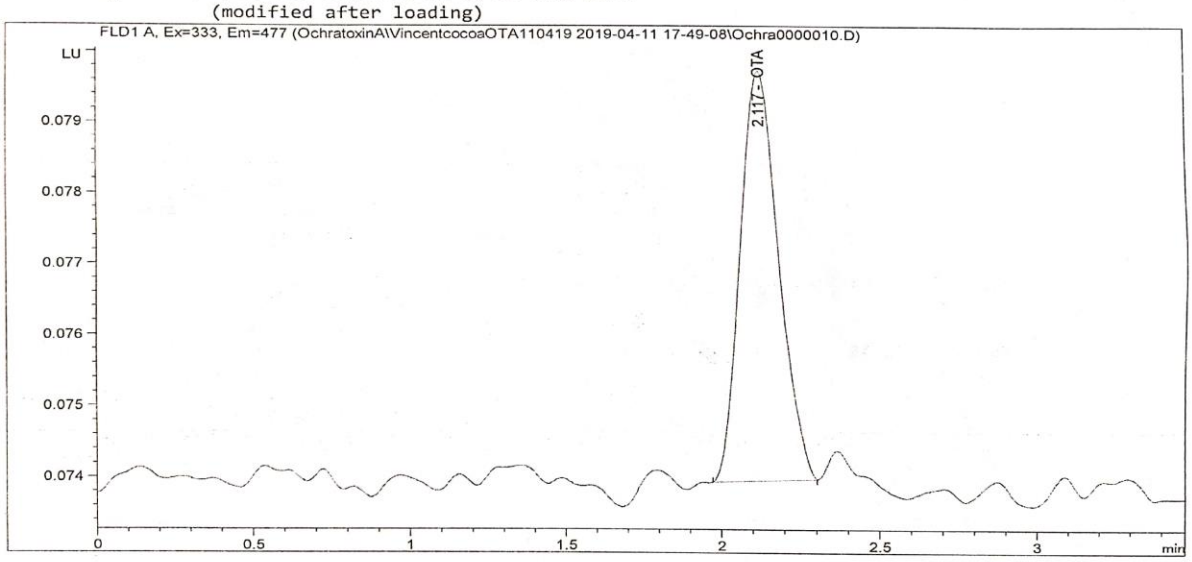


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Sorted By             :      Signal
Calib. Data Modified  :      Friday, April 12, 201912:36:18 PM
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Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex=333, Em=477

RetTime  Type     Area      Amt/Area    Amount   Grp   Name
 [min]          [LU*s]                ppb (ug/kg
-------|------|----------|----------|----------|--|------------------
  2.117 BB     4.88942e-2  173.95277    8.50528    OTA

Totals :                                8.50528

==========================================================================
```

HPLC 1260 4/12/2019 12:40:15 PM Dorothy Narh Page 1 of 2

Chromatogram for 50g/5kg cola powder treated cocoa beans for one replicate

Data File C:\Chem32\...chratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08\Ochra0000013.D
Sample Name: Sam 100g/5kgP

```
Acq. Operator    : Dorothy Narh                    Seq. Line :  13
Acq. Instrument  : HPLC 1260                        Location :  10
Injection Date   : 4/11/2019 6:43:58 PM                 Inj :  1
                                                  Inj Volume : 10.000 µl
Acq. Method      : C:\Chem32\1\Data\OchratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08
                   \OCHRATOXIN A FINAL.M
Last changed     : 4/11/2019 5:49:08 PM by Dorothy Narh
Analysis Method  : C:\Chem32\1\Data\OchratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08
                   \OCHRATOXIN A FINAL.M (Sequence Method)
Last changed     : 4/12/2019 12:36:18 PM by Dorothy Narh
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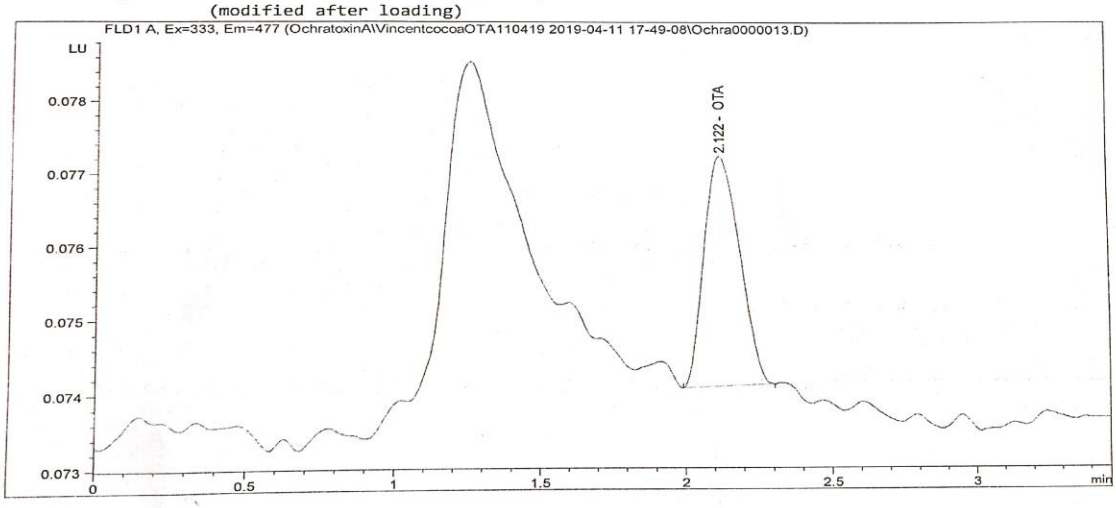

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Sorted By             :      Signal
Calib. Data Modified  :      Friday, April 12, 201912:36:18 PM
Multiplier            :      1.0000
Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex=333, Em=477

RetTime  Type     Area       Amt/Area    Amount    Grp   Name
 [min]          [LU*s]                 ppb (ug/kg
-------|------|----------|----------|----------|--|------------------
  2.122 BB     2.66126e-2  181.44211    4.82865     OTA

Totals :                                 4.82865
```

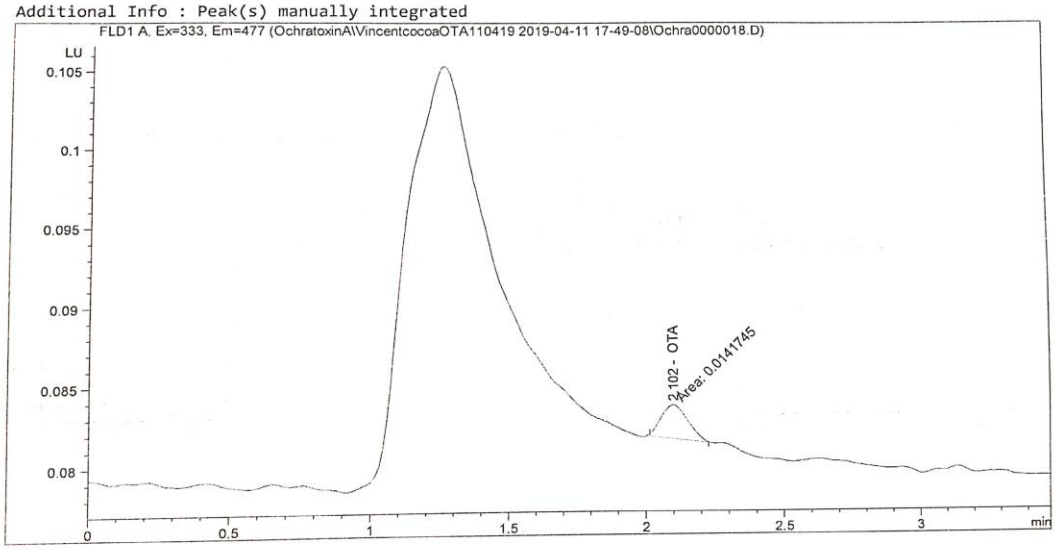
HPLC 1260 4/12/2019 12:42:12 PM Dorothy Narh

Page 1 of 2

Chromatogram for 100g/5kg cola powder treated cocoa beans for one replicate

Data File C:\Chem32\...chratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08\Ochra0000018.D
Sample Name: Sam 150g/5kgP

Acq. Operator	: Vincent Kyei-Bafour	Seq. Line	: 18
Acq. Instrument	: HPLC 1260	Location	: 1
Injection Date	: 4/12/2019 12:04:29 PM	Inj	: 2
		Inj Volume	: 10.000 µl
Acq. Method	: C:\Chem32\1\Data\OchratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08 \OCHRATOXIN A FINAL.M		
Last changed	: 4/12/2019 11:59:09 AM by Vincent Kyei-Bafour		
Analysis Method	: C:\Chem32\1\Data\OchratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08 \OCHRATOXIN A FINAL.M (Sequence Method)		
Last changed	: 4/12/2019 12:36:18 PM by Dorothy Narh (modified after loading)		
Additional Info	: Peak(s) manually integrated		



External Standard Report

Sorted By	:	Signal
Calib. Data Modified	:	Friday, April 12, 201912:36:18 PM
Multiplier	:	1.0000
Dilution	:	1.0000

Do not use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex=333, Em=477

RetTime [min]	Type	Area [LU*s]	Amt/Area	Amount ppb (ug/kg	Grp	Name
2.102	MM	1.41745e-2	195.86327	2.77627		OTA
Totals :				2.77627		

HPLC 1260 4/12/2019 12:44:14 PM Dorothy Narh

Chromatogram for 150g/5kg cola powder treated cocoa beans for one replicate

Data File C:\Chem32\...chratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08\Ochra0000019.D
Sample Name: Sam 0g/5kg

```
==========================================================================
Acq. Operator     : Vincent Kyei-Bafour          Seq. Line :  19
Acq. Instrument   : HPLC 1260                     Location :   2
Injection Date    : 4/12/2019 12:08:58 PM             Inj :   1
                                                Inj Volume : 10.000 µl
Acq. Method       : C:\Chem32\1\Data\OchratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08
                    \OCHRATOXIN A FINAL.M
Last changed      : 4/12/2019 11:59:09 AM by Vincent Kyei-Bafour
Analysis Method   : C:\Chem32\1\Data\OchratoxinA\VincentcocoaOTA110419 2019-04-11 17-49-08
                    \OCHRATOXIN A FINAL.M (Sequence Method)
Last changed      : 4/12/2019 12:36:18 PM by Dorothy Narh
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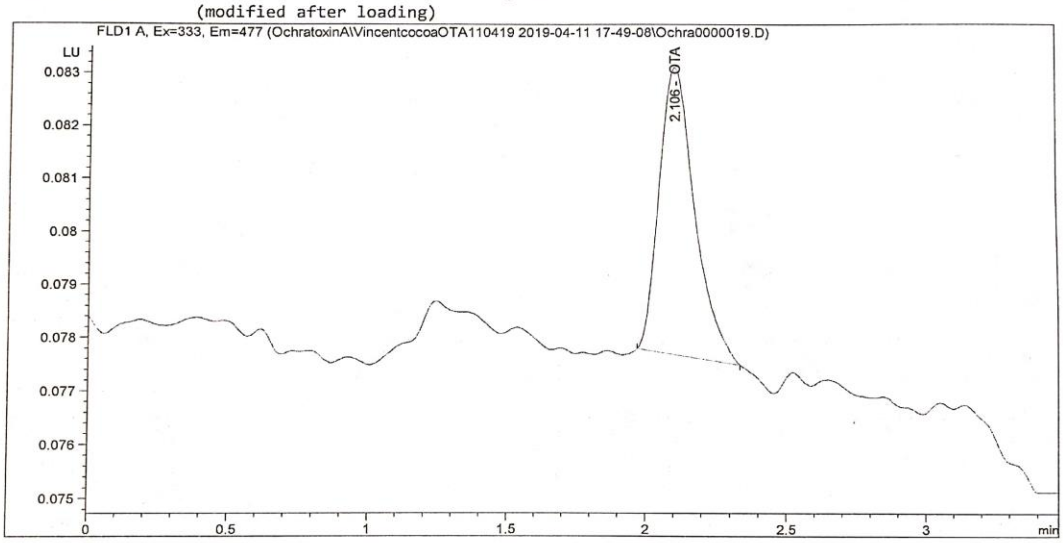


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Multiplier            :      1.0000
Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex=333, Em=477

RetTime  Type     Area      Amt/Area    Amount   Grp   Name
 [min]          [LU*s]                ppb (ug/kg
-------|------|----------|----------|----------|--|------------------
  2.106 BBA    4.80200e-2  174.11561    8.36103    OTA

Totals :                                8.36103

==========================================================================
```

HPLC 1260 4/12/2019 12:44:36 PM Dorothy Narh Page 1 of 2

Chromatogram for fermented dried cocoa beans for one replicate without any treatment

Data File C:\Chem32\1\Data\aflatoxin\90419 2019-04-09 15-52-37\Afla0000019.D
Sample Name: Sam IRS 20

```
==========================================================================
Acq. Operator    : Vincent Kyei-Bafour          Seq. Line :  19
Acq. Instrument : HPLC 1260                      Location :   2
Injection Date  : 4/9/2019 7:17:59 PM               Inj :   1
                                                Inj Volume : 10.000 µl
Acq. Method      : C:\Chem32\1\Data\aflatoxin\90419 2019-04-09 15-52-37\AFLATOXIN FINAL.M
Last changed     : 4/9/2019 4:35:57 PM by Vincent Kyei-Bafour
Analysis Method : C:\Chem32\1\Data\aflatoxin\90419 2019-04-09 15-52-37\AFLATOXIN FINAL.M (
                  Sequence Method)
Last changed     : 4/9/2019 7:43:22 PM by Vincent Kyei-Bafour
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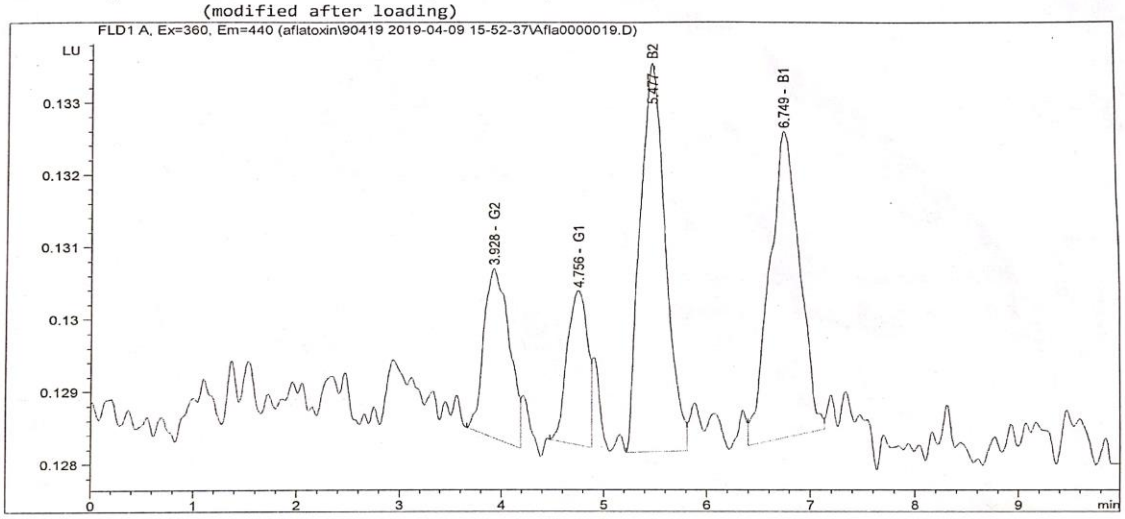


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                        External Standard Report
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Sorted By             :      Signal
Calib. Data Modified  :      Tuesday, April 09, 20197:42:53 PM
Multiplier            :      1.0000
Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex=360, Em=440

RetTime  Type     Area      Amt/Area    Amount   Grp   Name
 [min]          [LU*s]                 [ug/kg]
-------|------|----------|----------|----------|--|------------------
  3.928 BV     4.12865e-2 1196.85980   49.41420    G2
  4.756 BV     2.94066e-2  925.93860   27.22875    G1
  5.477 BV     9.22287e-2  383.00952   35.32447    B2
  6.749 VV     8.45024e-2  364.55373   30.80568    B1

Totals :                              142.77311
```
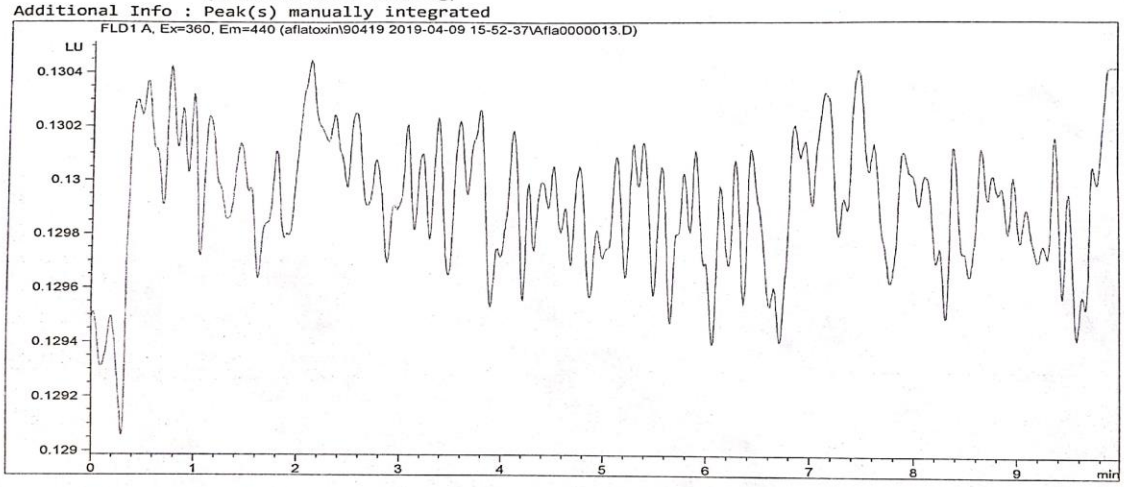


Chromatogram for internal reference sample for aflatoxin analysis

Data File C:\Chem32\1\Data\aflatoxin\90419 2019-04-09 15-52-37\Afla0000013.D
Sample Name: Sam 100P/5kgA

Acq. Operator	: Vincent Kyei-Bafour	Seq. Line	: 13
Acq. Instrument	: HPLC 1260	Location	: 13
Injection Date	: 4/9/2019 6:11:48 PM	Inj	: 1
		Inj Volume	: 10.000 µl
Acq. Method	: C:\Chem32\1\Data\aflatoxin\90419 2019-04-09 15-52-37\AFLATOXIN FINAL.M		
Last changed	: 4/9/2019 4:35:57 PM by Vincent Kyei-Bafour		
Analysis Method	: C:\Chem32\1\Data\aflatoxin\90419 2019-04-09 15-52-37\AFLATOXIN FINAL.M (Sequence Method)		
Last changed	: 4/9/2019 7:43:22 PM by Vincent Kyei-Bafour (modified after loading)		
Additional Info	: Peak(s) manually integrated		



External Standard Report

Sorted By : Signal
Calib. Data Modified : Tuesday, April 09, 20197:42:53 PM
Multiplier : 1.0000
Dilution : 1.0000
Do not use Multiplier & Dilution Factor with ISTDs

Signal 1: FLD1 A, Ex=360, Em=440

RetTime [min]	Type	Area [LU*s]	Amt/Area	Amount [ug/kg]	Grp	Name
3.910		-	-	-		G2
4.741		-	-	-		G1
5.443		-	-	-		B2
6.728		-	-	-		B1
Totals :				0.00000		

HPLC 1260 4/9/2019 7:48:45 PM Vincent Kyei-Bafour Page 1 of 2

Chromatogram for 100g/5kg cola powder treated cocoa beans showing no peak for aflatoxins

Appendix H: Pictures showing cola nut powder and extract preparation as well as fermentation and treated dried cocoa beans on the farm

Freshly cut white *Cola nitida*

Oven dried white *Cola nitida*

Coarsely milled dried cola nut

Finely milled cola nut

Cola nut extract preparation on a shaker

Cola nut extract on a hot plate

Harvesting of cocoa pods

Opening of harvested cocoa pods

Cocoa bean heap fermentation

Fermented cocoa beans

Cola powder and powdered extract

Treatment of cocoa beans with cola nut

Dried cola powder treated cocoa beans (150g/5kg)

Dried cola powder treated beans (100g/5kg)

Dried cola extract treated cocoa beans (50g/5kg)

Dried cocoa beans without treatment