DETERMINATION OF AFLATOXIN LEVELS IN STORED WHITE MAIZE (*Zea mays L.*) AND FLOUR IN KITUI, MWINGI AND MAKUENI DISTRICTS IN KENYA

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DECLARATION

March, 2011

I declare that the work presented in this thesis is my original work and has not been presented for a degree in any other university or any other award.

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DEDICATION

This thesis is dedicated to my mother, Felistus Mulau, My father Francis K. Kioko and to the love of my family Veronica, John, Joshua, Laura, Ann and Peter Mutisya. This far the Lord has brought us.

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LIST OF ABBREVIATIONS AND ACRONYMS

AFB_I	Aflatoxin B_I
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
Ag	Antigen
ALT	Alanine transferase
AOAC	Association of Official Analytical Chemists
AST	Asperte amino transferase
HBsAg	Hepatitis B Surface Antigen
CAC	Codex alimentarius commission
CCFAC	Codex committee for food additives and contaminants
CAST	Council for agriculture science and technology
CBS	Central Bureau of statistics
CHS	Chlorohydrocarbon
CI	Confidence interval
EHP	Environmental health perspective
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food Agriculture Organization
FDA	Food and Drug Administration
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HPTLC	High performance thin layer chromatography

HRP	Horseradish peroxidase
ICRISAT	International crop research for semi arid Tropics
LC	Liquid chromatography
LD_{50}	Lethal dose 50 %
N	Normal sample population
n	Sample size
ng	nanogram
NIEHS	National institute of environmental health sciences
NPLC	Normal phase liquid chromatography
OTA	Ochratoxin A
PBS	Phosphate buffered saline
PHLC	High performance liquid chromatography
ppb	Parts per billion
ppt	Parts per trillion
RPLC-BCD	Reverse phase liquid chromatography – pre column delivertisation
RPLC-PCD	Reverse phase liquid chromatography-post column delivertisation
sIL-2R	Surface interleukin 2 receptors
SFE	Supercritical fluid extraction
Sc - Co_2	Supercritical carbon dioxide
SPE	Solid phase extraction
TLC	Thin later chromatography
TMB	Tetramethyl benzydine
UK	United Kingdom
USDA	United States department of agriculture
UV	Ultra violet light

WHO	World health organization
µg	Microgram

ABSTRACT

Aflatoxins are fungal metabolites which are toxic, mutagenic and carcinogenic and thus cause undesirable effects on animal and human health when ingested with feed or foodstuffs. Aflatoxin producing fungi may occur in certain food products in form of spores, and thus when conditions are favourable, the fungi germinate and may produce aflatoxin in varied amounts. Ingestion of higher dose of aflatoxin (Codex Commission upper limit is 10ppb), may result in aflatoxicosis which manifests as hepatoxicity or in severe cases, fulminant liver failure. The fungi grow on starchy foodstuffs such as groundnuts, pearl millet, finger millet, maize grain and maize flour, oats and sorghum in certain conditions. Maize is a staple foodstuff for over 90% of the Kenyan population. Outbreaks of aflatoxin poisoning and aflatoxicosis associated with contaminated maize grain has been reported over the years in Kitui, Mwingi and Makueni districts in Kenya. Despite this no study to date has been carried out to exactly define the prevailing circumstances that may favour occurrence of this problem in the affected districts. A study to determine and correlate aflatoxin levels, storage temperature and moisture content in stored maize grain and grain flour was done in the three districts. The sample size was determined to be 130. A stratified random sampling technique was used, where 14 strata were identified. One (1) kg sample each of maize grain and flour, was collected from each store in the strata. Storage temperature and moisture contents were determined *in situ*. Sample aflatoxin levels were determined by the ELISA method. Data analysis was done by a computer software, SPSS® version 11.51, lead technology 2001, USA. In maize grain overall mean storage temperature was 29.6^{o}C, mean moisture content 12.57% and aflatoxin level 13.17 ppb. A statistically significant ($p<0.01$), positive correlation (+ 0.954) was found between grain moisture content and aflatoxin levels. The correlation between grain storage temperature and aflatoxin levels was + 0.115, while that between the temperature and moisture content was statistically significant ($p<0.01$), at +0.05. A highly significant non association ($X^2 = 2.525$; $p < 0.05$) was found between district of origin and aflatoxin contamination of grain. In maize flour, a highly significant non association ($X^2 = 0.696$; $p < 0.05$) was found between the district of origin and aflatoxin contamination. The correlation between flour storage moisture content and aflatoxin level was + 0.642. That of temperature and moisture content in flour + 0.224. The values were statistically significant ($p<0.01$). Storage moisture content and temperature level in maize grain and maize flour favour production of aflatoxins, however the effect of moisture content is higher than that of temperature. The prevalence of aflatoxin contamination was higher (84.6%) and mean lower (13.17ppb), in maize grain than in flour (79.5%) and higher mean (21.34 ppb) from the same market stores. Mwingi district had lower aflatoxin mean (9.67 ppb), moisture content mean (12.17%), and storage temperature mean at 26.43^{o}C. Makueni had higher mean values at 16.67 ppb, 12.55%, and 31.47^{o}C, respectively. Consequently by inference, maize grain and flour should be stored at 26.4^{o}C or less, and dried down to moisture content of 12.2% or less before storage. The risks of aflatoxin contamination is higher in flour than in maize grain (13.17 ppb <21.54 ppb> 10.00 ppb). Thus, milling grain to flour should be done at moisture content of 12.2% or less, to discourage growth of aflatoxin producing fungi. This information will be submitted to Ministry of Agriculture and used to advice on correct storage temperature and moisture for maize grain and flour.

Aflatoxins have assumed significance due to their deleterious effects on human beings and animals such as livestock, poultry and cattle. The aflatoxin problem was first recognized in 1960 when severe outbreak of a disease referred to as "Turkey 'X' disease" occurred in UK. In that epidemic, 100,000 turkey poults died. The course of the disease was shown to be due to toxins in peanut meal infected with *Aspergillus flavus* and the toxins were named as aflatoxins (Asao *et al.*, 1963; Seargent *et al.*, 1961).

Naturally, aflatoxin producing fungi may occur in certain food products in form of spores, and when conditions are favourable the fungi may produce aflatoxins in high amounts. These foods include maize, sorghum, pearl millet, rice, wheat, groundnuts, soybeans, sunflower seeds, cotton seedcake, chilies, coriander, turmeric and ginger. Tree nuts, including almonds, pistachio, walnuts and coconut are also attacked. Other than aflatoxin M_1 found as a metabolite of B_1 in animal milk products, powdered milk can also be attacked directly by aflatoxin producing moulds. (Deveci *et al.*, 2005).

Aflatoxin B_1 has been found to be dominant in amount in cultures as well as in food products compared to all the other aflatoxins. The presence of these fungal toxins reduces the value of grain as animal feed and devalues it as a commodity for human consumption (Nichols, 1983). Because of the hepatoxicity of these aflatoxins, the duration of exposure is in particular a public health concern. The Food and Agricultural Organization (FAO), estimates that mycotoxins contaminate 25% of agricultural crops worldwide (Smith *et al.*, 1994; CAST, 1998).

Aflatoxin contamination of food stuff such as maize, tree nuts and cotton seeds is a continuing world wide problem. Contamination may occur during pre-harvest period or post-

harvest during storage, transportation and processing (Lopez – Garcia & Park, 1998). After harvest, temperature, moisture content and insect activity are major factors influencing aflatoxin contamination of foodstuffs (Coumbe *et al.,* 1993). *Aspergillus flavus* is also an opportunistic pathogen causing invasive and non-invasive aspergillosis in humans, especially in immune compromised individuals (Cleveland *et al.,* 2004). Continued dietary exposure to aflatoxin is a major risk factor for hepatocellular carcinoma particularly in areas where hepatitis B virus infection is endemic. Ingestion of higher doses of aflatoxins can result in acute aflatoxicoses which manifests as hepatoxicity or in severe cases fulminate liver failure (Fung and Clark, 2004). To reduce the risk to human and animal health, FDA has regulated that corn or corn products intended for animal feeds or human consumption should have aflatoxin levels ≤ 20 ppb (FDA, 2000), while the WHO/FAO Codex Alimentarius Commission has regulated that the levels be ≤ 10 ppb (Codex Commission, 2008).

Aflatoxins have varying molecular weights with B_1 having the lowest followed by B_2, G_1 and G_2 hence they can be separated into individual components by thin layer chromatography. Aflatoxin fluoresce strongly in the ultra violet light (ca 365 mm), with B_1 and B_2 producing blue fluorescence, whereas G_1 and G_2 produce green florescence (Pomeranz, 1992).

1.2 Statement of the problem and justification

In Kenya, maize is a staple foodstuff and over 90% of the population relies on maize grain and maize products for food (FAO, 1997). In Kitui, Mwingi, Makueni and other arid districts in the eastern part of Kenya, maize, the staple food crop grown is harvested and stored in traditional granaries. There has been reported outbreak of aflatoxin poisoning associated with consumption of contaminated maize grain and maize meal in the district for the last 30years (Lewis *et al.,* 2005). Aflatoxin producing fungi, including those of the genus *Aspergillus*

grow on dump starchy foodstuffs such as groundnuts, pearl millets, maize grains and maize flour, oats and sorghum when temperature is favourable. The medical personnel in the three districts suspect that residents have consumed aflatoxin contaminated maize for years and the accumulation of aflatoxins in the liver has been causing serious conditions including vomiting, gastro-intestinal problems, fulminant liver diseases, hepatocellular carcinoma and jaundice in the general population.

In April 2004, an outbreak of acute hepatoxicity was identified among people living in Kenya's eastern and central provinces. Epidemiologic investigations determined that the outbreak was a result of aflatoxins poisoning from ingestion of contaminated maize. As of 20th July 2004, 317 aflatoxicosis cases and 125 deaths had occurred in the region making it one of the largest and most severe outbreaks of acute aflatoxicosis documented world wide (CDC, 2004). The outbreak covered more than seven districts encompassing an area approximately 40,149 km^2. Of the 317 case-patients, 89% resided in four districts including Makueni, Kitui, Machakos and Thika. The estimated total population of these four districts was 2.8 millions by the time (Central Bureau of Statistics, 1999).

Comparing the four districts, Makueni and Kitui was heavily affected and represented 47% and 32% of case-patient respectively. This was followed by Machakos, 6% and Thika 4 % (CDC, 2004). A study of hospital data, (Ministry of health: Kitui district health report 1997-2006) showed that between 1997 and 2006, the total admission in Kitui district hospital alone due to consumption of aflatoxin contaminated maize grain and maize meal was 670 out of which 400 died representing a case fatality of 59.7 %. Thus there is an urgent need to evaluate the role of storage temperature and moisture content in growth of aflatoxin producing fungi and hence aflatoxin production in maize grain and maize flour.

1.3 Hypothesis

Storage of maize grain and maize flour at certain moisture content and temperature does not encourage production of aflatoxin.

1.4 Research questions

i) Does moisture content in maize grain and flour bolster production of aflatoxin by fungi?

ii) What is the role of temperature in production of aflatoxins in stored grain and grain products?

iii) Is there any correlation between moisture content, temperature and aflatoxin production?

1.5 Objectives

1.5.1 General objective

To determine the relationship between storage temperature, moisture content levels and aflatoxin contents of maize grain and flour in Kitui, Mwingi and Makueni districts.

1.5.2 Specific objectives

i) To determine the moisture contents in maize grain and maize flour in various food stores in Kitui, Mwingi and Makueni districts.

ii) To determine storage temperatures of maize grain and maize flour in various stores in Kitui, Mwingi and Makueni districts.

iii) To determine aflatoxin level in maize grain and maize flour from various stores in Kitui, Mwingi and Makueni districts.

1.6 Significance of the study and the anticipated outcome

The data generated from this study will provide crucial information on the minimum moisture content and the temperature range under which growth of aflatoxin producing fungi will be curtailed. The data will be availed to stakeholders in the agricultural sector and will be used to advice farmers on the correct storage conditions. It will go a long way to forestall both health and economic losses due to aflatoxicosis in the districts and the country.

CHAPTER TWO

LITERATURE REVIEW

2.1 Chemical properties of aflatoxins

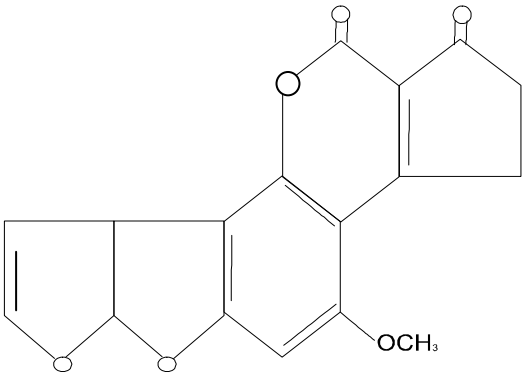
Aflatoxins belong to the group of difuranocoumarins and are classified into two broad groups according to their chemical structure. These are difurocoumarocyclopentenone series (which include AFB_1, AFB_2, AFB_{2A}, AFM_1, AFM_{2A} and aflatoxicol) and the difurocoumaro lactone series (which consist of AFG_1, AFG_2 AFG_{2A}, AFG_2M_1, $AFGM_2$, $AFGM_{2A}$ and AFB_3) (Figure 2). The compounds are usually soluble in methanol, chloroform, acetone and acetonitrile (Scott *et al.,* 1993; ICRISAT, 2000), which are slightly polar but insoluble in non polar solvents.

Aflatoxins react with alkali solutions causing the hydrolysis of the lactone moiety. This hydrolysis is reversible since it has been shown that recyclization occurs following acidification of basic solution containing aflatoxin. At higher temperatures above 100^oC, ring opening followed by decarboxylation occurs and the reaction may proceed further, leading to the loss of methoxy group from the aromatic ring. In the presence of mineral acids aflatoxins B_1 and G_1 are converted into aflatoxin B_{2A} and G_{2A}, due to acid catalyzed addition of hydroxyl group across the double bond in the furan ring. In the presence of acetic anhydride and hydrochloric acid, the reaction proceeds further to acetoxy derivative. Similar adducts of aflatoxin B_1 and G_1 are formed with formic acid-thionyl chloride and trifluro acetic acid. Many oxidizing agents, including sodium hypochlorite, potassium permanganate, chlorine, hydrogen peroxide, ozone and sodium perborate, react with aflatoxin molecule in some way as indicated by the loss of fluorescence in ultraviolet light at 365nm (Healthcote *et al.,* 2005).

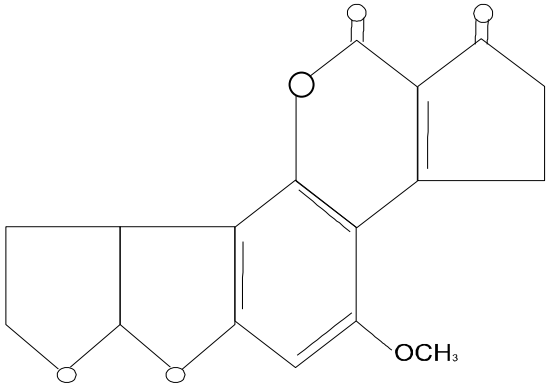
Sodium hypochlorite degradation of aflatoxins is either by chlorination or oxidation depending on the pH. At low pH, chlorination predominates over oxidation and aflatoxins are converted to their dichloro, and di-hydroxy derivatives through an addition reaction across the terminal difuran ring of which the chloro-aflatoxin derivative exhibits high residual toxicity (Samarajeewa *et al.,* 1990). Under high pH, however formation of water soluble β-keto acid derivatives of aflatoxin, dichloro aflatoxin and dehydroxy aflatoxin is known to dominate through opening of the lactone ring (Tabata *et al.*, 1994).

Hydrogenation of aflatoxin B_1 and G_1 yields aflatoxins B_2 and G_2 respectively. Further reductions of aflatoxin B_1 by three (3) moles of hydrogen yields tetrahydroxyaflatoxin. Reduction of aflatoxin B_1 and B_2 with sodium borohydride yields aflatoxin R-B_1 and R-B_2 respectively. These arise as a result of opening of the lactone ring (Figure 1) followed by reductions of the acid group and reduction of the keto group in the cyclopentene ring (Waliyar *et al.*, 2003). The double bond of the difuro terminus of each type of aflatoxin compound and the termini of some analogous having a 2,3, double bond is essential for carcinogenicity of aflatoxins. If the bond is single, carcinogenicity is usually lost but toxicity is retained (Healthcote & Hibbert, 1978). By contrast, if water is added across the bond, the resulting 2-hydroxy compound is non-toxic (Healthcote & Hibbert, 1978).

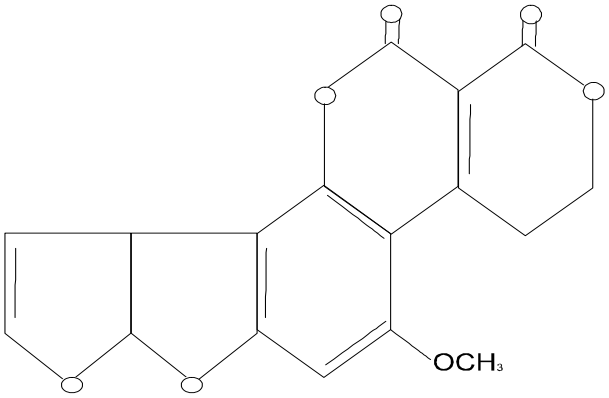
Figure 1: Chemical structures of aflatoxin B_1, B_2, G_1, and G_2



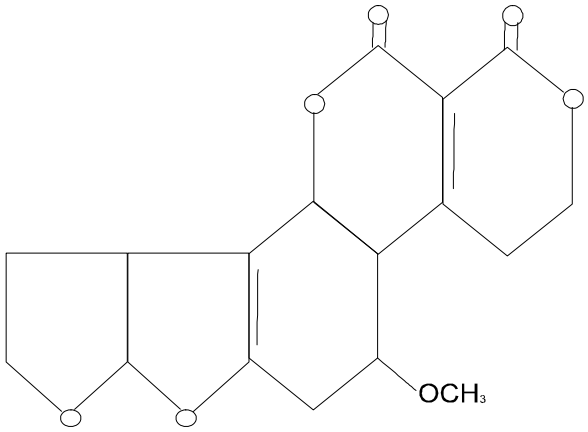
B_1



B_2

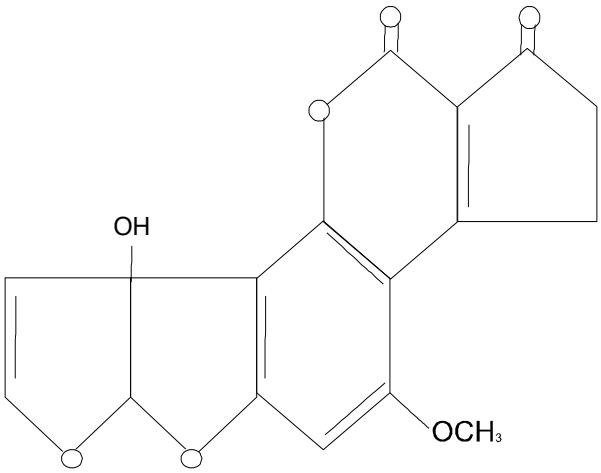


G_1

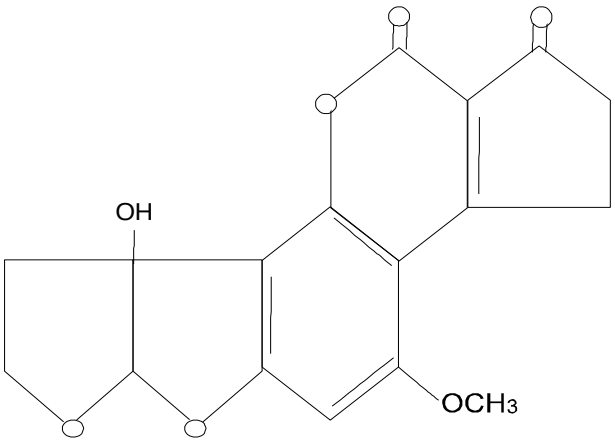


G_2

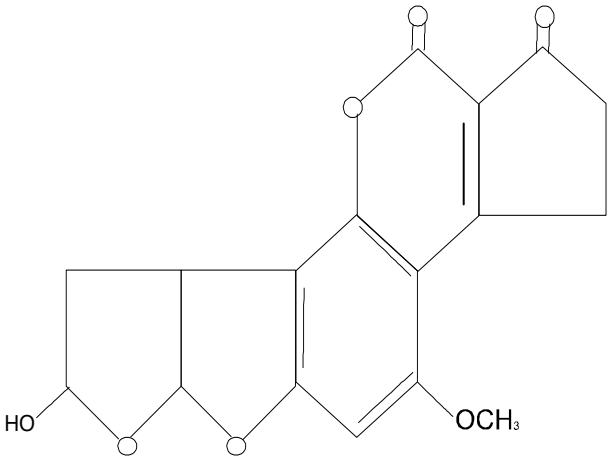
Figure 2: Chemical structures of aflatoxins M_1, M_2, B_{2A} and G_{2A}



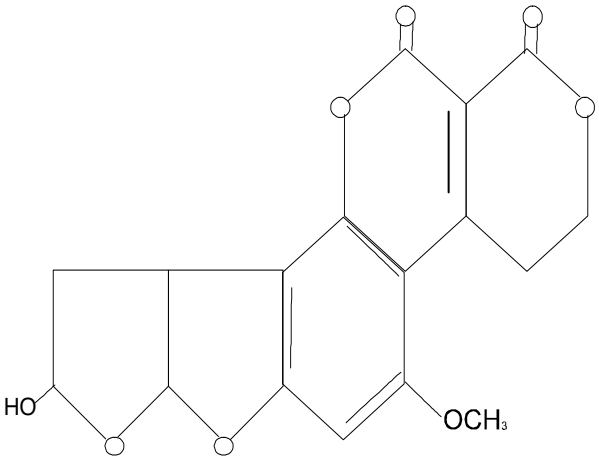
M_1



M_2



B_{2A}



G_{2A}

2.2 Physical properties of aflatoxins

Aflatoxins are crystalline odourless solids when isolated and the colour range from pale white to yellow. The melting points range from 268^{o}C for B_1 down to 190^{o}C for G_{2A} (Table 1). The molecular weights range from 312g per mole for aflatoxin B_1 upto 346g per mole for aflatoxin G_{2A} (Waliyar & Reddy, 2003).

The effect of physical conditions and reagents on aflatoxins have been studied extensively because of the possible application of such reactions to the detoxification of aflatoxin contaminated material (Waliyar & Reddy, 2003). Aflatoxins in dry state are very stable to heat up to the melting point. However, in the presence of moisture and at elevated temperatures, there is destruction of aflatoxins over a period of time. Such destruction can occur to aflatoxin in oil seeds meals, roasted peanuts or aflatoxin in aqua's solution at pH 7. Although the reaction products have not been examined in details, it is likely that such treatment leads to opening of the lactone ring with the possibility of decarboxylation at elevated temperatures (Healthcote & Hibbert, 1978).

Table 1: Molecular weights, formula and melting points of some aflatoxins

Aflatoxin	**Molecular formula**	**Molecular weight g/mole**	**Melting point ^{O}C**
B_1	$C_{17}H_{12}O_6$	312	268-269
B_2	$C_{17}H_{14}O_6$	314	286-289
G_1	$C_{17}H_{12}O_7$	328	244-246
G_2	$C_{17}H_{14}O_7$	330	237-240
M_1	$C_{17}H_{12}O_7$	328	299
M_2	$C_{17}H_{14}O_7$	330	293
B_{2A}	$C_{17}H_{14}O_7$	330	240
G_{2A}	$C_{17}H_{14}O_8$	346	190

Source: ICRISAT strategy

2.3 Aflatoxin toxicity and symptoms in animals

Aflatoxicosis is a term referring to aflatoxin poisoning. Aflatoxin display a potency of toxicity, carcinogenecity, mutagenicity in the order of $AFB_I > AFG_1 > AFB_2 > AFG_2$ as illustrated by their LD_{50} values for day old ducklings and selected data on other animals (Table 2). Structurally the dihydrofuran moiety containing double bonds and the constituents linked to the coumarin moiety (Figure 2) are of importance in producing toxic effects (Eaton & Groopman, 1994).

Table 2: Selected data on aflatoxin toxicity values (LD_{50}) in mg/kg for some animal species

Species	Sex	Weight	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Acute Necrosis	Bile duct hyper plasia
Ducks	M	50g	0.73	1.76	1.18	2.83	+	+
Duck	F	50g	0.36	0.78	1.70	3.44	+	+
Rat	M	200g	1.16	>200	1.75	>200	+	+
Mouse	M-F	40g	1.5	250	1.90	250	+	-
Turkey	M	300g	3.2	35.0	38.0	62.0	+	+
Chicken	F-M	200g	18.0	2.0	19.5	33.5	+	+

Source: Toxicology of aflatoxin by Eaton & Groopman

No animal species is resistant to the acute toxic effects of aflatoxins. Wide variations of LD_{50} values had been obtained in animal species tested with simple doses of aflatoxins (Table 2). For most species the LD_{50} values ranges from 0.5 to 10 mg /kg body weight. Animal species

respond differently in their susceptibility to chronic and acute toxicity of aflatoxins. In each species the liver is the primary target of acute injury. Toxicity can be influenced by environmental factors, level of exposure and duration of exposure, age, health and nutritional status of diet. Metabolism plays a major role in determining the toxicity of aflatoxins B_1. Studies show that this aflatoxin requires metabolic activation to exert its carcinogenic effect (Pomeranz *et al.,* 1992).

Aflatoxins elicit a variety of biological effects in humans and animals which include liver and kidney toxicity, genotoxicity, suppression of the immune system and aggravation of kwashiorkor in children (Hall & Wild, 1994). In humans, enlarged fatty livers are common at low doses of aflatoxin poisoning (Raisback *et al.,* 1991). Aflatoxin ingestion may result in acute hepatitis that presents either as jaundice or elevated liver enzymes, usually preceded with a prodrosomal illness. The clinical features give little indications as to the likely etiological agent. Photophobia, headache and cough may also be dominant in aflatoxin induced hepatitis. A serum sickness like illness occurs in about 60% of patients with acute aflatoxin induced hepatitis and is characterized by urticarial or maculo popular rash and arthralgia, typically affecting the wrist, knees, elbows and ankles. This is due to immune complex formations. Rheumatoid factors are frequently positive. These are almost always self limiting and usually settles rapidly after the onset of jaundice. Aflatoxin induced hepatitis may produce other sub clinical problems like *tinitis* (ringing inside ears) and blurred vision (Weber *et al.,* 1995).

Elevated liver enzymes have been found to be a major indicator of liver fibroids in aflatoxin induced hepatitis. Aspartate amino transferase (AST) higher serum levels predict progression of liver disease than alanine amino transferase (ALT) serum levels. Higher (AST) serum

levels can therefore be used as biochemical indicator for advanced fibrosis or progression of liver diseases in aflatoxin poisoning which may induce liver cirrhosis, steatosis and fibroids (Stransky *et al.*, 2004).

2.4 Association of aflatoxins, hepatitis B virus and hepatocelullar carcinoma

The most frequent complications of aflatoxicosis in human are hepatocellular carcinoma, nephritis, endocarditis, hepatomegally, splenomegaly and suppressed immunity. The carcinogenic effects of aflatoxins seem to be enhanced by underlying viral liver infection usually hepatitis B virus. In a study done in Taiwan (Wang *et al.*, 2002) to determine the carcinogenic effect of environmental aflatoxins exposures, 56 cases of hepatocellular carcinoma diagnosed between 1991 and 1995 where identified and individually matched by age sex, residence and date of recruitment to 220 healthy controls from the same large cohort in Taiwan. Blood samples were analyzed for hepatitis B and C viral markers and aflatoxin albumin adducts, while urine was tested for aflatoxins metabolites. Hepatitis B virus surface antigen carriers had a significantly increased risk for hepatocellular carcinoma. After adjustment for hepatitis B virus, virus antigen serostatus, the matched odds ratio was significantly elevated for subjects with high levels of urinary aflatoxin metabolites. HBsAg sero positive subjects with high aflatoxins exposure had a higher risk than subjects with high aflatoxins exposure but with HBsAg sero-negative blood.

In a more recent study by Fu-sun Yeh & Brian (1989), the role of the hepatitis B virus and aflatoxin B_1 in the development of primary hepatocellular carcinoma (PHC) in a cohort of 7917 men aged 25 to 64 years in southern Guangxi province (China), was determined. The incidence of PHC in Guangxi is among the highest in the world. After accumulating 30,188 man - years of observation, 149 deaths were observed, 76 (51%) of which were due to PHC.

Ninety one (91%) of PHC were hepatitis B surface antigen (HBsAg) positive at the enrollment time, in contrast to 23% of all members of the cohort (RR=38.6). Three of the four patients who died of liver cirrhosis also were HBsAg positive at enrollment. There was no association between HBsAg positivity state and other causes of death. When estimated aflatoxin B_1 levels in the sub population were plotted against the corresponding mortality rates of PHC, a positive and almost perfectly linear relationship was observed (Fu-sun yeh *et al.,* 1989). On the other hand no statistically significant association was observed when the prevalence of HBsAg positivity in the sub populations was compared with their corresponding rates of PHC mortality (Fu-sun Yeh *et al.,* 1989).

These results suggest that environmental aflatoxins exposure enhances the hepatic carcinogenic potential of hepatitis B virus in carriers more than healthy individuals and is a major risk factor (Wang *et al.,* 2002). Elevated liver enzymes have been found to be a major indicator of liver fibrosis in aflatoxin induced hepatitis. These liver enzymes can therefore be used as biochemical indicators for fibrosis or progression of liver diseases in aflatoxin poisoning (Wang *et al.,* 2002).

2.5 Occurrence of aflatoxicosis in Kenya

The food and agriculture organization (FAO) estimates that aflatoxins contaminate 25% of agricultural crops world wide (Smith *et al.,* 1994). Outbreaks of acute aflatoxicosis from highly contaminated food stuffs have been documented in Kenya, Gambia, India and Thailand (CAST, 2003). In an aflatoxicosis outbreak in Kenya, (Ngindu *et al.,* 1981), it was found that Makueni and Kitui had the highest market maize aflatoxin levels, (11.2% and 9.3% prevalence rates) respectively and had also the highest number of aflatoxicosis cases, with incidence rate of 16.7% and 17.1% respectively, out of the of districts surveyed.

Divisions within the districts with one or more aflatoxicosis case patient had significantly ($p = 0.0002$), higher levels of aflatoxin in market maize, (GM = 27.70), than did market maize from divisions with no aflatoxicosis patients, (GM = 6.14), (CDC, 2004). Even though the aflatoxin levels in maize grain were used as surrogate for exposure to aflatoxins, rather than actual biomakers, aflatoxin levels in maize grain provided a good indication of aflatoxicosis in that study since maize was stable food in the population (Moss, 1998).

Lauren *et al.*,(2005), in a study on aflatoxin contamination of locally grown and commercial maize in Eastern and Central provinces, found that aflatoxin levels in market maize indicated widespread aflatoxin contamination. Of the 350 market maize samples collected, 192 representing 55% had levels greater than the WHO (2005), aflatoxin regulatory limit of 20 ppb. One hundred twenty one, (35%) of the maize samples had aflatoxin levels greater than 100 ppb and 24 which is 7% had levels greater than 1000 ppb. Aflatoxin levels ranged from 1ppb, the lower limit of detection, to values as high as 46, 400 ppb. Each of the four study districts (Table 3) had a substantial proportion of market maize with aflatoxins levels greater than 20 ppb. Makueni and Kitui had the highest proportions of samples with aflatoxins levels greater than 20 ppb with 65% and 62% respectively, followed by 51% of maize from Machakos markets and 34% from Thika. The acceptable upper limit for aflatoxins for that particular study was 20 ppb (FDA, 1997 & KBS, 1988). Aflatoxin levels in market maize mirrored the geographic distribution of aflatoxicosis associated with the 2004 outbreak (CDC, 2004).

Table 3: Distribution of aflatoxins in maize products (Lauren *et al.*,2005), collected from agricultural markets in the study districts

			Maize aflatoxin>20ppb → [n (%)]		
Study district	**No. of Maize products**	**Aflatoxin in maize ≤ 20ppb [n (%)]**	**21-99**	**100-1000**	**1000ppb**
Makueni	91	3 (35)	12 (13)	36 (40)	11 (12)
Kitui	73	28 (38)	15 (21)	23 (32)	7 (10)
Machakos	102	50 (49)	26 (25)	23 (23)	3 (3)
Thika	76	50 (66)	13 (17)	10 (13)	4 (4)
TOTAL	**342**	**160 (47)**	**66 (19)**	**92 (27)**	**24 (7)**

Source: Lauren *et al.*,(2005), in Environmental health perspective

A survey done in Western Kenya (Mutegi *et al.*, 2009) to obtain baseline data on levels of aflatoxins in peanuts revealed an aflatoxins range of (0 - 2688) µg/kg and (0 - 7525) µg/kg, in Busia and Homabay respectively. These results were obtained from 384 samples from Busia and 385 samples from Homabay. Of the 769 samples, 7.54% exceeded the Kenyans regulatory limit of 20 µg/kg which is 20ppb (FDA, 1997 and KBS, 1988). There was a highly significant (X^2 = 14.17; $p < 0.0002$), association in between district of origin and samples aflatoxin levels. These analyses quantified the association between concentrations of

aflatoxins in food, exposure history and acute aflatoxicoses in that part of Kenya (NIEHS, 2005).

2.6 Worldwide occurrences of aflatoxicosis

Aflatoxicosis is a world wide problem with a tendency to be more common in countries with tropical climate that have extremes ranges of rainfall, temperature and humidity, including tropical west African countries, south western USA and India. Contamination of maize and other food commodities with aflatoxins is a public concern because of the ability of aflatoxins to cause human and animal diseases, including fulminant liver disease.

In a study in Gambia (Perz *et al.,* 2006), it was found that the overall incidence of aflatoxins associated hepatitis and hepatocellular carcinoma was 15%. The relative contribution and possible mechanism of interactions between aflatoxins and hepatitis B virus in development of hepatocellular carcinoma was investigated in Gambia, using markers developed for exposure to both factors. In this study blood samples were collected over a month period from 117 children aged 3 to 4 years in Kuntain, in the upper Niumi district of Gambia, West Africa. Samples were analyzed for aflatoxins albumin adducts, using markers of hepatitis B virus and liver enzymes ALT, as markers of liver damage and glutathione-s-transferase M_1 genotype. All children except two showed detectable serum aflatoxin albumin with levels ranging from 2.2 to 250.4g aflatoxin B_1- lysine equivalent/mg of albumin.

There was a significant positive correlation between aflatoxins albumin concentration and alanine transferase (ALT). Hepatitis B virus carriers showed moderately higher levels of aflatoxins albumin concentration than non carriers. The null glutathione transferase M_1 genotype was infrequent (17.7%) in this population and was not associated with any

difference in aflatoxin albumin adduct levels compared to glutathiones transferase M_1 positive individual. This suggested a relationship between liver diseases, hepatitis B virus and aflatoxin exposure in Gambia. The high use of groundnuts which may contain aflatoxins B_1 may explain this high incidence of aflatoxicosis (Perz *et al.*, 2006).

An outbreak of acute aflatoxin associated hepatitis occurred in India in 1974. Investigations showed that consumption of aflatoxin contaminated maize with levels as high as 1560 ppb was responsible (Mall *et al.*, 1983). In a study by Misra (1977), 35.5 % (n=48) of 135 maize grain samples from Namital, had aflatoxin B_1 range of 8 ppb to 1850 ppb, while 25% (n=34) of samples had all the four types of aflatoxins B_1, B_2, G_1 and G_2. Other Indian regions found to have aflatoxin contaminated maize grains were Bhagalpur, Lucknow, Madras and Coimbatore (Sinha, 1980).

2.7 Biological control of aflatoxin production in food stuffs

The method used in bio-control of native strains of *Aspergillus flavus* that do not produce aflatoxins (atoxigenic strains) can be applied in order to alter the fungal community on crops through out an area, thereby making maize become less contaminated with aflatoxins. When applied appropriately these native atoxigenic strains competitively exclude aflatoxins producers during colonization of grain. These competitive exclusion principles of biological control have been used as a new method of aflatoxin intervention strategy to mitigate the negative effects of aflatoxin on human health in areas where aflatoxin poisoning is endemic. In peanuts and cotton, siginificant reductions in aflatoxin contamination in the range of 70% - 90% have been observed consistently with non toxigenic *Aspergillus* strains (Dorner, 2004; Pitt & Hocking, 2006; Dorner, 2008). In field trials, it was found that the bio control method

in Zaria, Ikeme, Mokwa, and Ibadan in Nigeria reduced aflatoxin contamination by between 50% and 90% (Ramefit *et al.,* 2004).

2.8. Chemical methods of aflatoxin control in maize grain

Alkaline chemical compounds have been found to degrade aflatoxins. These include, potassium hydroxide, sodium hydroxide, carbonate salts of both potassium and sodium, bicarbonate salts of potassium and sodium, ammonium carbonates, ammonium hydroxide, and ammonium phosphate (Samarajeewa *et al.,* 1990).

Ammonium persulphate probably induces oxidative hydration of aflatoxin across the olefenic bond of the terminal difuran ring to form hydroxy-dihydro aflatoxin (aflatoxin $B_{2\alpha}$) that is 200 times less toxic and 1000 times less mutagenic than the parent molecule (Ciegler & Peterson, 1968). In a study by Tabata *et al.,* (1994), treatment of whole - grain maize spiked with aflatoxin, and treated with 0.5% and 1.0% w/v ammonium persulphate, at 40^{o}C for 16 hours reduced aflatoxin concentration by 63% and 66% respectively. The same study showed 90% degradation of pure aflatoxin treated with 1% w/v ammonium persulphate at 20^{o} C for 14 hours. Sodium hypochlorite, was shown to reduce aflatoxin concentration (Table 4) in whole grain maize and dehulled maize (*Muthokoi*) by as much as 62% (Mutungi *et al.,* 2006).

Anhydrous ammonia (NH_3) gas has been found to effectively inactivate much of the aflatoxin in peanut, cottonseed cakes, and maize grain in a much larger scale than other methods of detoxification (Gardner *et al.,* 1971; Prevot, 1974; Brekke *et al.,* 1978). In a particular study, it was found that treating 29 metric tonnes of corn in a grain storage drying bin with anhydrous ammonia, at a temperature range of 32^{o}C to 43^{o}C for 13 days, reduced aflatoxin B_1 content from 750 ppb to 7 ppb (Brekke *et al.,* 1977a).

Table 4: Effects of some chemical compounds on aflatoxin levels[a] of whole grain maize (Mutungi *et al.*, 2006)

Chemical additive	Conc. % w/v	Aflatoxin content (ng/g)			% decrease[b]	
		Initial	After 6hrs	After 14hrs	After 6hrs	After 14hrs
Water	-	317	278	260	12.2 ± 2.7	15.2 ± 5.8
Ammonium Persulphate	0.2	415	328	184	20.8 ± 5.5	55.6 ± 7.2
	0.5	415	264	166	36.4 ± 4.5	59.5 ± 6.2
	1.0	415	234	143	43.4 ± 9.1	65.4 ± 9.3
Sodium hypochlrite	0.2	359	270	223	24.8 ± 6.2	34.9 ± 7.0
	0.5	359	247	151	31.2 ± 6.4	57.9 ± 6.7
	1.0	359	235	132	34.6 ± 5.8	63.2 ± 8.4
Mineral Salt	0.2	264	233	191	11.6 ± 5.0	27.7 ± 7.8
	0.5	264	219	151	16.9 ± 6.5	42.6 ± 9.5
	1.0	264	220	150	16.5 ± 7.6	42.9 ± 7.5

[a] In ng/g

[b] mean aflatoxin decrease (n = 5) ± standard deviation

Source : Food control 19 (2008)

Whereas, the moderate solubility of aflatoxins in water caused the leaching of the toxins from whole grain to soak water (Cole & Cox, 1981), reducing aflatoxin concentration from 278 ng/g in 6 hrs to 260 ng/g in 14 hrs (Table 4), alkaline mineral salt was found to degrade and reduce aflatoxin concentration in whole grain maize by as much as 72% in 14hrs time (Mutungi *et al.,* 2006). Similar observations were made by Abbas *et al.* (1988); Price &

Jorgensen (1985), in a nixtimalization process in preparation of tortillas, underscoring the use of these chemical compounds in degradation and control of aflatoxins in whole grain maize.

2.9. Physical methods of aflatoxins control in maize grain

Physical methods including physical cleaning, mechanical separation, heat treatment, solvent extraction, density segregation and γ-ray irradiation have all been found to reduce the content of aflatoxin in food stuffs (Rustom *et al.,* 1997). A particular effective physical method was found to be dehulling of maize into a Kenyan food *muthokoi*. In a study by Mutungi *et al.,* (2006), the decrease in aflatoxin levels as a result of dehulling was investigated. This was determined by calculating the difference between the aflatoxin content (ng/g) of whole grain maize and the content in dehulled maize (*muthokoi)* then expressing as percent fraction of the aflatoxin content (ng/g) of the whole maize grain. In that study, aflatoxin content of 48 whole grain maize samples selected for dehulling ranged from 10.7 – 270 ng/g with a mean of 97.3 ng/g (Mutungi *et al.,* 2006). The dehulling ratio, was used to estimate the effectiveness of aflatoxin decontamination of the grains.

Dehulling of the whole grain maize was found to significantly ($p<0.0001$), decrease the aflatoxin levels to between 6.8 ng/g to 182 ng/g, with a mean value of 57.3ng/g. (Mutungi *et al.,* 2006). Aflatoxin contents in the by products which consisted of hull and fines was 2 to 7 times higher than the levels in the whole maize grain and ranged from 103 ng/g to 613ng/g. Aflatoxins are lost by extraction through removal of the impermeable testa during dehulling (Cole & Cox, 1981), since the pericarp (hull), hilium, aleurone layer and the germ of maize grain are more prone to contamination by aflatoxins than other parts of a whole grain maize, dehulling is emerging as an effective control technique (Brekke, Pepliski & Griffin, 1975; Brekke *et al.,* 1975b).

2.10. Methods of sample extraction for aflatoxin analysis

Aflatoxins have been extracted using a mixture of organic solvents such as acetone chloroform or methanol in combination with small amount of water (Bullerman, 1987). Due to the diverse nature of likely contaminated commodities, no single method of extraction is adequate for all products (Ellis *et al.,* 1991).

2.10.1 Supercritical fluid extraction method

Super critical fluid extraction (SFE) which is a process of separating one component (extractant) from another (matrix), using super critical fluids as the extracting solvent (Tenaka & Takeshi, 2004). The ideal fluid, super critical Co_2 (Sc - Co_2), has been used as an extraction method for partially removing aflatoxin B_1 from maize grain (Selim & Dharwan, 1991) and peanut meal (Hass & Engelharcht, 1992). SFE method has been found to be an alternative to conventional organic solvent extraction because of its combination of gas like mass transfer and liquid like solvating properties (King *et al.,* 1992). It has been found to be rapid and quantative method for extracting of polar and non polar liphophilic components from a variety of sample matrices (Lopez Avila *et al.,* 1990; Wheeler & McNelly, 1989). While solvent based extraction methods require the use of 50g samples (AOAC, 1984a), SFE extraction technique require ground maize samples of 3.0 to 3.5g (Scott *et al.,* 1993).

2.10.2 Solid phase extraction method

Solid phase extraction (SPE) is an efficient separation process by which compounds are dissolved or separated from other compounds in a mixture according to physical and chemical properties (Hennion, 1999).

The apparatus for SPE, have been simplified to a tube 2 - 4mm in diameter and 2 - 4cm long, while stainless steel or inert polymer has been the material mostly used (Scott, 2000). The extraction tube is packed with an appropriate bonded phases, depending on whether the normal phase, reversed phase or ion exchange phase procedures are in use for a particular liquid mixture (Thurman *et al.*, 1998).

SPE have been used to purify and isolate analytes from various matrices including methanol and water mixtures in extraction of aflatoxin analytes (Sulpelco, 1998). The technique has been found effective with an efficiency of 90% in a particular study (Scott, 2000).

2.11. Methods of aflatoxin detection and quantification

The following methods have been frequently used to detect aflatoxins in human food stuffs and animal feeds.

2.11.1 Thin layer chromatography

Thin layer chromatography also known as flat bed chromatography is a widely used separation technique in aflatoxin analysis. This method was evaluated in collaborative study conducted by the Association of American Chemists (AOAC) and has since been the official method of analysis (AOAC, 1990). It has been the method of choice to identify and quantitate aflatoxins at levels as low as 1ng/g. The TLC method is often used to verify findings obtained with newer, more rapid methods (Whitaker *et al.*, 1983).

2.11.2 High performance liquid chromatography

Liquid chromatography is similar to TLC in many aspects including analyte application. Stationary phase and mobile phase LC and TLC compliment each other. Liquid

chromatography methods for determination of aflatoxins in food include normal phase liquid chromatography and reversed phase liquid chromatography with pre column delivertisation (BCD) or with post column delivertisation (PCD). In all these techniques, detection of aflatoxin is by use of fluorescence set at Ex 360nm, Em > 420nm and ultraviolet detectors (Park *et al.*, 1995).

2.11.3 ELISA technique

Enzyme linked immunosorbent assay is an immunochemical method whose technique depends on affinities of monoclonal or polyclonal antibodies against aflatoxins (antigens). ELISA is based on competition between unlabelled aflatoxins in the test and the labeled aflatoxins in the assay system for the specific bindings sites of antibody molecules in micro titre wells. An aflatoxin enzyme conjugate is used as a ligand in the ELISA. The technique consists of a two step process as follows:- The competitive reaction between antibody and the toxins both in test sample and the conjugate, and the measurement of the reaction of the substrate with the enzyme attached to the toxin.

Immunochemical methods are quite specific and can be used to screen aflatoxins in grain and grain products (Trucksess *et al.*, 1991). The kit consist of polystyrene microtitre plates which are coated with antibodies specific for aflatoxins, calibrated aflatoxins standards, aflatoxins enzyme conjugate solution and an enzyme substrate solution. Aflatoxins are extracted from test samples with a methanol water solvent (mixed at a ratio of 55:45 respectively), defatted with hexane and diluted to 10% methanol content. Aliquots (50 µl) of this sample extract and those of calibrated standard aflatoxins solution are incubated simultaneously with aflatoxin enzyme conjugate solution in wells of the coated microtitre plate (Trucksess *et al.*, 1990).

Binding of the aflatoxin enzyme conjugate to antibody plate is inhibited by the presence of free aflatoxins in the standard or sample extract solution. Since only a fixed number of antibody binding sites are available on the coated microtitre plates, the amount of aflatoxins enzyme conjugate bound is inversely proportional to the amount of free toxin in standard or sample extract solution. After appropriate washing steps, the amount of aflatoxin enzyme conjugate bound to the antibody is determined by incubation with an enzyme substrate solution. The resultant colour may be evaluated visually or measured with a spectrophotometer (ELISA reader). The intensity of the colour formed is inversely proportional to the amount of aflatoxins in standard and extract sample. The aflatoxin contents of samples extract dilutions are semi - quantitatively deduced by visual comparisons. Alternatively, absorbance values of aflatoxin standard dilutions are measured with an ELISA reader and used to construct a standard curve. On the basis of this curve the aflatoxin contents of sample extract dilutions are quantitatively determined in parts per billion.

ELISA technique is more simple and cost effective than physico-chemical analytic methods. In one comparative study (Waliyar & Reddy, 1998), where aflatoxin estimation was done using TLC, HPLC and ELISA techniques, the results were highly comparable to those done by HPLC method alone.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Area of study

The study was conducted in Kitui, Mwingi and Makueni districts (Appendix I), which are arid and semi arid areas. The study area was classified into 14 strata as per administrative centres (locations) whose distribution is as detailed below (Table 5).

Table 5: Distribution of the strata within the study area

KITUI DISTRICT	MWINGI DISTRICT	MAKUENI DISTRICT
Kalundu	Nguni	Kibwezi
Wikililye	Mwingi	Emali
Kisasi	Masavi	Sultan Hamud
Mutomo		Mtito Adei
Ikutha		Kambu
Kabati		

3.2 Inclusion and exclusion criteria

All the grain stores including supermarkets within Kitui, Mwingi and Makueni districts and whose owners gave an informed consent (Appendix II) to participate in this study were included while those who did not consent were excluded.

3.3 Study design

This was an analytical cross sectional study in which maize grain and flour samples, were collected from the study area and taken for aflatoxin analysis.

3.4 Sample size

The study population (N=270) comprised of all grain stores in Kitui, Mwingi and Makueni districts as was extracted from Kitui, Mwingi and Makueni county Council trading licenses records as at 17th May, 2008. The minimum sample size (n), was determined by using Fisher *et al.,* (1998), formular as detailed here, $n \geq z^2 \times p \times q / d^2$. Where n was the minimum sample size required, $q = (1 - p)$, $z = 1.96$, the standard error, p = prevalence of condition under study, which was aflatoxin contamination of maize grain in the study area, and $d = 0.05$, the absolute precession required for the study at 95% confidence level and 5% significance level. The mean prevalence rate of aflatoxin contamination at study area was 9.3%, (Ngindu *et al.*, 1981; Moss, 1998; Lauren *et al.,* 2004; CDC, 2004), and was used to determine the sample size. Factoring in the value of $q = (1 – p)$, as 0.907, and $p = 0.093$, then $n \geq (1.96)^2 (0.093) (0.907) / (0.05)^2 = 129.61$. The minimum sample size was approximated to be 130.

3.5 Sampling method, collection and storage

Stratified random sampling technique was used. This was done by identifying the strata (centers) as per administrative division and a total of 14 strata were identified. The number of samples for every stratum was determined by dividing total number of stores in the strata by the total number in the study area and the result multiplied by the required sample size. The stores for every stratum were then randomly selected. The names of all stores in a stratum were written on pieces of papers and then put into a container. One paper was picked at random and the name on it recorded. The paper was returned before another one was picked. This was repeatedly done until the required number of stores was obtained.

From each of the sampled stores, one (1) kg each of maize grain and maize flour were collected. An automatic spear type sampler (Pneumac®, Agri. Service Suppliers, UK) primed

to scoop 1kg was used. To avoid contamination, samples were put in sealed khaki (Mafuko®, Mafuko Industries, Kenya) paper bags separately, and transported to the laboratory (Bora®, Biotech Laboratories, Kenya), for quantitative analysis of the aflatoxins. All the samples were stored in a dry conditions and at a temperature range of $15^{o}C$ to $20^{o}C$ before analysis.

3.6 Determination of sample temperature and moisture content

3.6.1 Determination of sample temperature

This was done *in situ* as the grain and flour samples were collected from the stores. A high precision infrared thermometer (Check temp® I, Model HI – 98509, Hanna Instruments, USA), with a temperature range of - $50^{o}C$ to 150^{o} C, and an error margin of ± $0.3^{o}C$, was used. The thermometer probe was pushed into the grain bag and temperature in ^{o}C read on the LCD display. For each sample, the corresponding sample storage temperature was recorded, (Appendix VII and VIII).

3.6.2 Determination of moisture contents.

Moisture content of the samples was done *in situ* as the grain was collected from the stores. A high precision digital probe type moisture meter, (Digital probe®, Model Mc – 7825G, India), with a humidity range of 0% to 30% and an error margin of ± 0.5% was used. The hand held moisture meter probe, was pushed into the grain bag and held in place for between 0.5 min to 1 min, (Model Mc-7878G use manual). The moisture contents were then read on the LCD display and recorded. This procedure was repeated for all the maize grain and flour samples (Appendix VII and VIII).

3.7 Sample preparation and processing

For every maize grain sample, out of the 130 samples, 1kg was coarsely and entirely ground using a hammer mill (Condux D 6450, Germany), then fine milled using a laboratory hammer mill (Cullati®, Type DFH 48, Switzerland), filleted with a 1mm sieve. The sieved material was mixed for 5minutes using a kitchen blender. A sub sample of 5g was drawn from each sample and submitted for aflatoxin analysis. The rest of the ground maize sample was stored at -15^{o}C as an analytical reserve.

3.7.1 Aflatoxin extraction

Aflatoxin extraction was done using the AOAC official method 990.32, with modifications (AOAC, 1995). The ground samples (5g) were mixed with 25 ml of methanol water (1:1 ratio), in a 50 ml conical flasks covered with aluminium foil and stirred for 15mins using a magnetic stirrer, (MR Hei – MixD®, Heidolph, Germany). The mixture was allowed to stand for 5mins and the supernatant filtered through a whatman No. 1 filter paper to 10 ml test tube and a stopper put on to prevent accidental spill.

3.7.2 Preparation of ELISA reagents and standards for analysis

A calibrated aflatoxin B_1 standard (Boratest® Bora Biotech, Kenya) whose concentration was 10 µg/ml, was used to prepare diluted aflatoxin standards of 0 ppt, 37 ppt, 111 ppt, 333 ppt and 1000 ppt, for ELISA analysis, by dilution of the calibrated standards in methanol: PBS (10:90) solution as follows. Six test tubes were arranged in a test tube rack and marked neat (N), S_1, S_2, S_3, S_4 and S_5, with sticker labels. Ten (10 µl), of calibrated aflatoxin standard, (Boratest® Bora Biotech, Kenya), whose composition is aflatoxin B_1 in methanol, was pipetted into the neat test tube and mixed with 1000 µl of (10:90) methanol phosphate buffered, saline (PBS) solution. In S_1, 2000 µl of 10% methanol in PBS, was pipetted and

20 µl of the aflatoxin standard solution in neat (N) added. In S_2, 1000 µl of 10% methanol in PBS was pipetted, and 500 µl of S_1 added. In S_3, 1000 µl of 10% methanol PBS was pipetted, and 500 µl of S_2 added. Similarly, in S_4, 1000 µl of 10% methanol in PBS was pipetted and 500 µl of S_3 added. In S_5 only 1000 µl of 10% methanol in PBS was pipetted. No aflatoxin standard solution (Boratest®), was added.

3.7.3 Preparation of aflatoxin enzyme conjugate

A working dilution of aflatoxin B_1 enzyme conjugate was prepared by diluting a neat aflatoxin B_1 horseradish peroxidase (Boratest® Bora Biotech, Kenya), by methanol in phosphate buffered saline (PBS) at a ratio of 1:10,000.

3.7.4 Preparation of an enzyme substrate

A working dilutions of enzyme substrate solution was prepared by mixing (1:1) portion of citric acid buffered solution (pH 3.8) containing 325 µl of 30% hydrogen peroxide per litre of solution and one portion of a solution of 50.4mg tetra methyl benzidine (TMB) in an acetone – methanol (1:9) solution.

3.7.5 Sample analysis by ELISA method

A direct competitive ELISA kit used was for the detection of the total aflatoxins (B_1, B_2, G_1 and G_2), content. The kit manufacturer (Boratest® Bora Biotech, Kenya), had put the aflatoxin detection efficiency at 88 – 100% with maize products (Gathumbi *et al.*, 2001). Extracts were diluted in methanol PBS (10:90) solution. This dilution brought the sample extract aflatoxin concentration to the sensitivity range ($1 \leq X \leq 100$) of the ELISA assay.A kit with sensitivity range ($100 \leq X \leq 1000$) was used for any sample which registered aflatoxin level of 100 ppb. Fifty (50 µl), micro litres of diluted aflatoxin standards were

pipetted in duplicate to the antibody coated micro titre wells of the assay in the order S_5, S_4, S_3, S_2, S_1. Similarly, 50 µl of sample extracts were pipetted into adjacent wells of coated micro titre wells. Aliquots (50 µl), of diluted aflatoxin B_1 horseradish peroxidase conjugate (enzyme conjugate) was added to all the wells of both the aflatoxins standards and the sample extracts, covered with an aluminum foil and incubated at room temperature (28^oC) for two hours. The plates were then emptied and washed with saline tween solution (8.55gm sodium chloride, dissolved in 1000 µl distilled water, added with 0.25 ml poly oxyethelene sorbitan mono hydrate) and dried by tapping with a blotting paper. An enzyme substrate solution (HRP and TMB) was added and the plates incubated in the dark for 10 mins. The enzyme reaction was stopped by adding a 100 µl of 1M sulphuric acid simultaneously into all the micro wells.

The intensity of colour in both standards and sample extract wells was determined by reading the absorbance at 450nm, using an ELISA reader (Uniskan II®, Labsystems, Finland). The absorbance value data for standards and sample extracts were entered into a computer soft ware (R-ridasoft win® version 1.60, R-biopharm, Germany), which uses percentage absorbance values against known standard aflatoxins concentration to draw a curve. The concentration of aflatoxin in ppt was deduced from the standard curves (Fig 3 & Fig 4). Aflatoxin concentration in parts per billion (ppb) was calculated as follows:-

$$\text{Sample aflatoxin (ppb)} = \frac{\text{Sample extract aflatoxin ppt } \mathbf{x} \text{ sample extract dilution}}{1000}$$

3.8 Data recording

After determination of temperature and moisture data *in situ*, aflatoxin values were recorded against the samples data (Appendix VII and VIII). The data values were analysed for means, ranges, chi squares (X^2), significance levels (p) and the difference between means. Some of the data was presented in form of graphs and tables. Correlation coefficients were determined between moisture, temperature and aflatoxin levels.

3.9 Statistical methods of analysis

Temperature, moisture and aflatoxin levels data were entered into computer software (SPSS, Version 11.51, Lead technology 2001, USA) for analysis into, correlation co-efficient, significance levels (p), Chi square (X^2), means, ranges, prevalence rates (%), and confidence intervals (CI). Bar graphs on temperature, moisture contents and aflatoxin levels were designed using Microsoft office, excel (2003). The p value represented the probability that the results in this study could not be reproduced. The highest value adopted was $p = 0.05$. If $p < 0.05$, then this was considered significant, while $p > 0.05$, was insignificant. Since the data was parametric, Pearson correlation coefficients was the statistical test adopted to determine the influence temperature, moisture contents and aflatoxin levels had on each other. Aflatoxin prevalence rates at various centres were determined as follows:-

$$\frac{\text{Number of samples contaminated with aflatoxin}}{\text{Total number of samples collected from the centre within the period of study.}} \mathbf{x} \; 100\,\%$$

CHAPTER FOUR

RESULTS

4.1 Descriptive results

4.1.1 Distribution of samples within the strata

The strata (Table 6) with the highest number of maize stores (N = 31) and the highest number of samples availed for analysis (n = 15), was Kalundu in Kitui district. Masavi in Mwingi district had the lowest number of maize stores (N =11) and the sample availed for analysis (n = 5).

Table 6: Distribution of samples per strata

Strata	Total per stratum (N)	Samples for analysis (n)	Sampled (%)
Emali	19	9	6.9%
Ikutha	18	9	6.9%
Kabati	14	7	5.4%
Kalundu	31	15	11.5%
Kambu	23	11	8.5%
Kibwezi	25	12	9.2%
Kisasi	12	6	4.6%
Masavi	11	5	3.8%
Mtito Adei	21	10	7.7%
Mutomo	21	10	7.7%
Mwingi	21	10	7.7%
Nguni	23	11	8.5%
Sultan Hamud	18	9	6.9%
Wikiliye	12	6	4.6%
TOTAL	270	130	100%

4.1.2 Samples with highest and lowest aflatoxin levels

The maize grain sample with the higher aflatoxin levels (97.90 ppb) deduced from the standard curves (Fig. 3 and Fig. 4), was collected from Kalundu, in Kitui district. The sample had a moisture content of 21.60% and had been stored at a temperature of 31.5^{o}C (Appendix VII). Maize flour samples with the maximum aflatoxin level (58.20 ppb) was found to have moisture content of 12.80%, while the storage temperature was 30.4 ^{o}C (Appendix VIII).

Overall maize grain had the lowest number of samples 15.4% (n = 20), with no (0 ppb) aflatoxin content. Maize flour was found to have a higher number 20.8% (n = 27), of samples with no (0 ppb) aflatoxin content (Appendix VII and VIII).

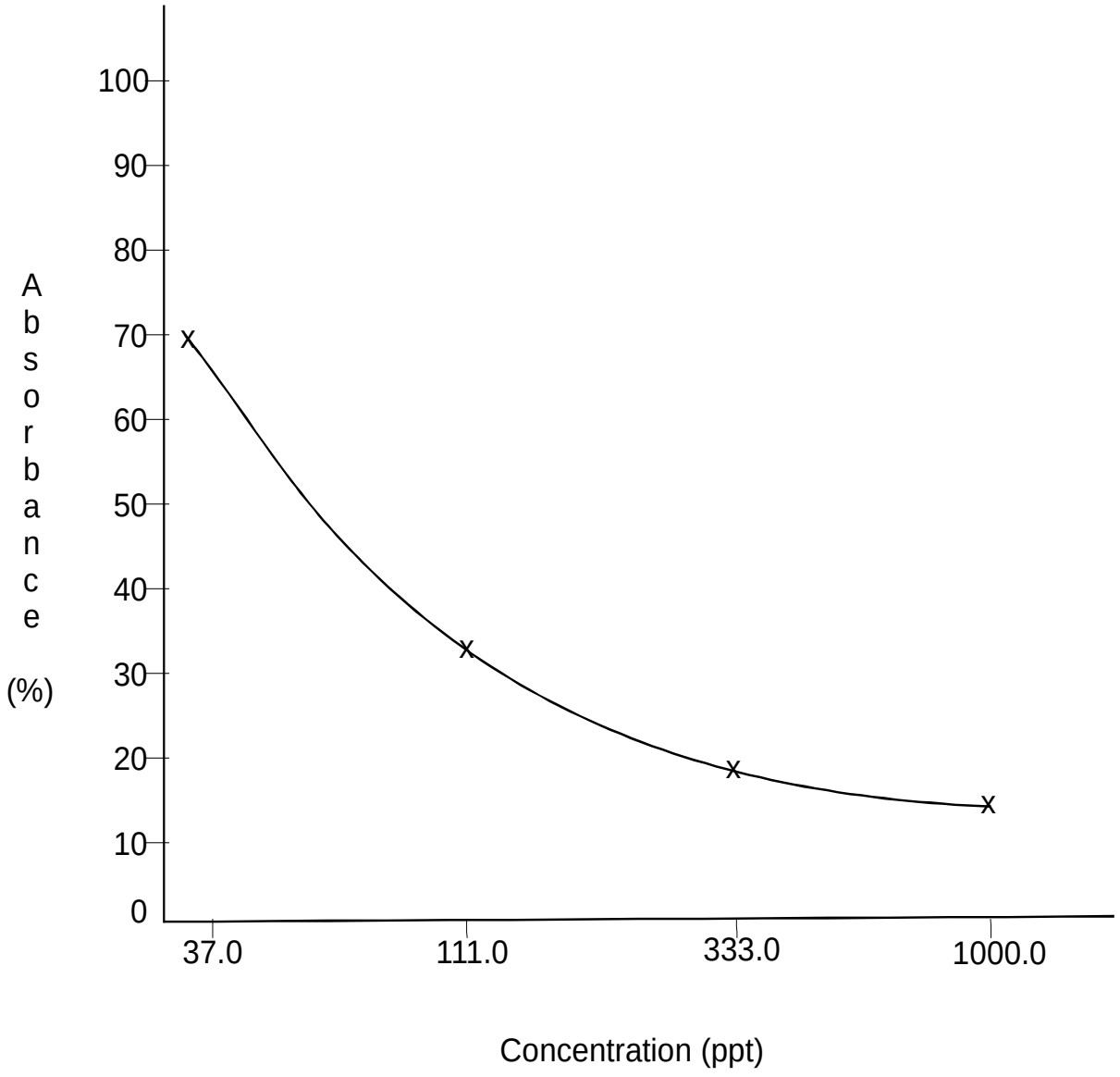


Figure 3: A sample standard curve for maize grain

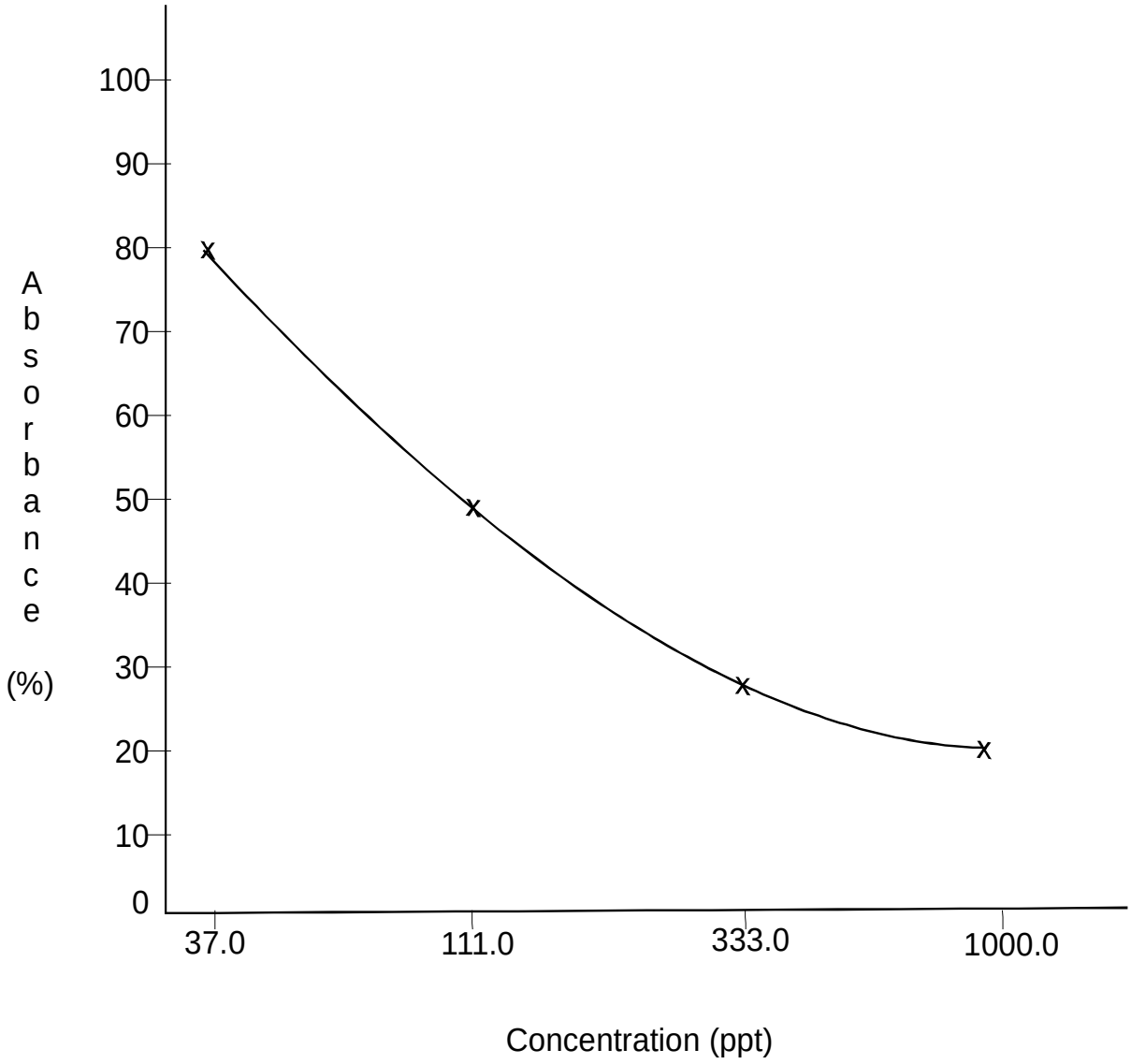


Figure 4: A sample standard curve for maize flour

4.2 Analytical results for maize grains

4.2.1 Temperature

The temperature of the samples ranged from 21.8^{0}C to 39.9^{0}C, while mean temperature of samples at centres ranged from 25.0^{0}C (Nguni) to 32.1^{0}C (Kambu). Overall mean temperature was 29.6^{0}C (CI = 29.07^{0}C — 30.12^{0}C) at 95% confidence and 5% significance levels, while mean temperature standard deviation was 3.04. Figure 5 shows the mean temperatures (^{0}C) of maize grains recorded at various sampling centers.

4.2.2 Moisture content

Moisture content in grains varied from 10.6% to 21.6%. The mean moisture content ranged from 10.8% for Emali to 14.8% for Wikililye. Overall mean moisture content was 12.57% (CI = 12.23%— 12.90%) at 95% confidence and 5% significance levels, while mean moisture standard deviation was 1.95. Figure 5 shows the mean moisture content (%) of maize grains at various sampling centres.

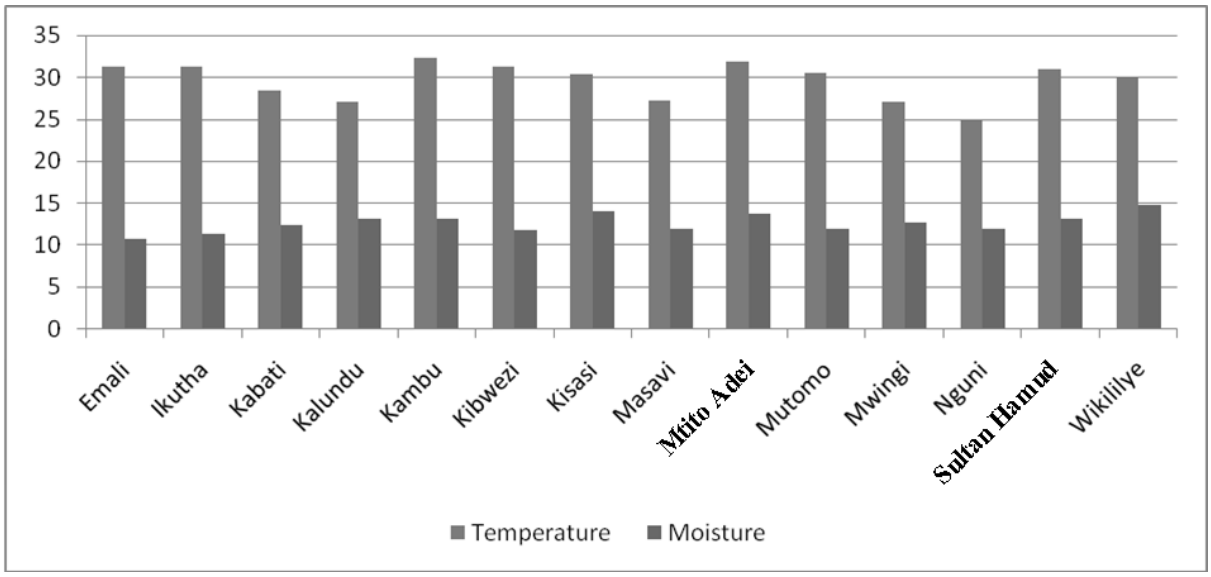


Figure 5: Mean temperature (^{0}C) and moisture content (%) in maize grains at various sampling centres

4.2.3 Aflatoxin levels in grains

Out of 130 grain samples, 84.6% (n = 110) were contaminated with aflatoxins. The level of aflatoxin in the samples ranged from 2.0 ppb to 97.9 ppb. The mean aflatoxin levels per centre ranged between 2.1 ppb (reported at Ikutha) and 35.9 ppb (reported at Wikililye). The overall mean aflatoxin level in grain samples was 13.17 ppb (CI = 12.8 ppb - 13.3 ppb) at 95% confidence level, while the mean aflatoxin standard deviation was 19.90. Figure 6 shows the mean aflatoxin levels in grains at various sampling centres.

Out of the samples contaminated with aflatoxin, 35.45% (n = 39) had levels above the Codex Alimentarius Commission (2005) maximum allowable limit of 10 ppb.

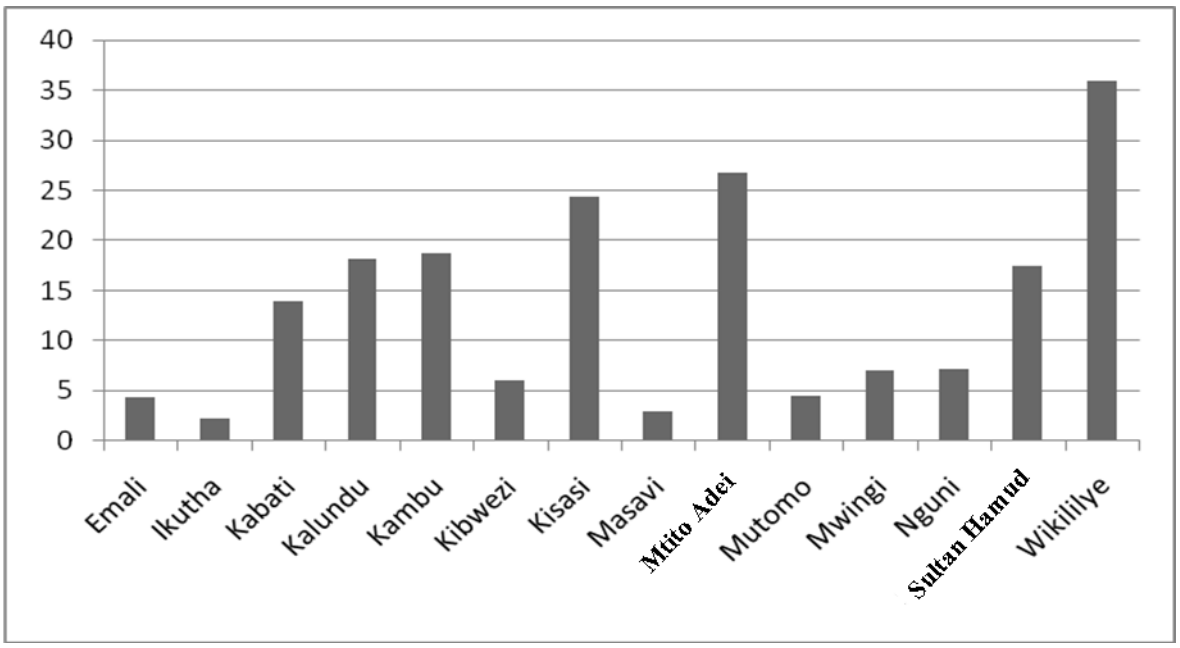


Figure 6: Mean aflatoxin levels (ppb) in maize grains at various sampling centres

Table 7: Geographic distribution of aflatoxin infested maize grain by district during the period of data collection

District	Mean temp level (^oC)	Mean moisture content (%)	Aflatoxin range (ppb)	Mean aflatoxin level (ppb)	Prevalence (%)
Makueni	31.47	12.55	2.0 - 87.6	16.67	86.79
Mwingi	26.43	12.17	2.0 - 54.5	9.67	84.51
Kitui	29.64	12.90	2.0 - 97.9	17.48	82.35

There was a highly significant non association (X^2= 2.525; $p < 0.05$) between the district of origin (Table 7) and aflatoxin contamination even though Makueni had 41.8%, Kitui 38.2% and Mwingi 20% of aflatoxin contaminated samples. In addition 66.7% of samples collected from Kisasi, 83.3 % from Wikililye and 50% from Mtito Adei had aflatoxin contents beyond 10 ppb, Codex Commissions upper aflatoxin limit for human food. Table 8 shows the percentage of maize grain samples for each sampling centre that had aflatoxin levels above 10 ppb.

Table 8: Percentage (%) of maize grain samples per strata that had aflatoxin levels above 10 ppb

Strata name	Range of aflatoxin (ppb)	Overall number of samples exceeding 10 ppb	Percentage of samples exceeding 10 ppb per centre
Emali	2.5-21.5	1	1.10%
Ikutha	2.0-3.2	0	0.00%
Kabati	2.0-15.8	2	33.3%
Kalundu	2.0-97.9	6	42.8%
Kambu	2.2-68.7	5	38.5%
Kibwezi	2.2-46.2	1	8.30%
Kisasi	3.2-65.9	4	66.67%
Masavi	2.0-4.1	0	0.00%
Mtito Adei	2.4-87.6	5	50.0%
Mutomo	2.0-14.8	1	11.1%
Mwingi	2.2-54.5	3	30.0%
Nguni	2.5-31	2	18.2%
Sultan Hamud	2.3-55.7	4	44.4%
Wikililye	2.5-77.8	5	83.3%

4.2.4 Correlations of temperature, moisture content and aflatoxin levels in maize grains

A highly significant ($p < 0.01$), positive correlation (+ 0.954) was found between moisture content of grains and aflatoxin levels. The correlation between the temperature of grains and the aflatoxin level was found to be + 0.115, however the correlation between temperature and moisture content was + 0.05.

4.3 Analytical results for maize flour

4.3.1 Temperature

Maize flour sample temperature ranged from 23.8^{o}C to 34.8^{o}C, while the mean temperature of samples per centre ranged from 26.5^{o}C for Sultan Hamud to 31.35^{o}C for Mutomo. Overall mean temperature was 29.54^{o}C (CI = 29.06^{o}C - 29.59^{o}C) at 95% confidence and 5% significance levels, with a mean temperature standard deviation of 2.78. Figure 7 shows the mean temperatures (^{o}C) of maize flour recorded at various sampling centres.

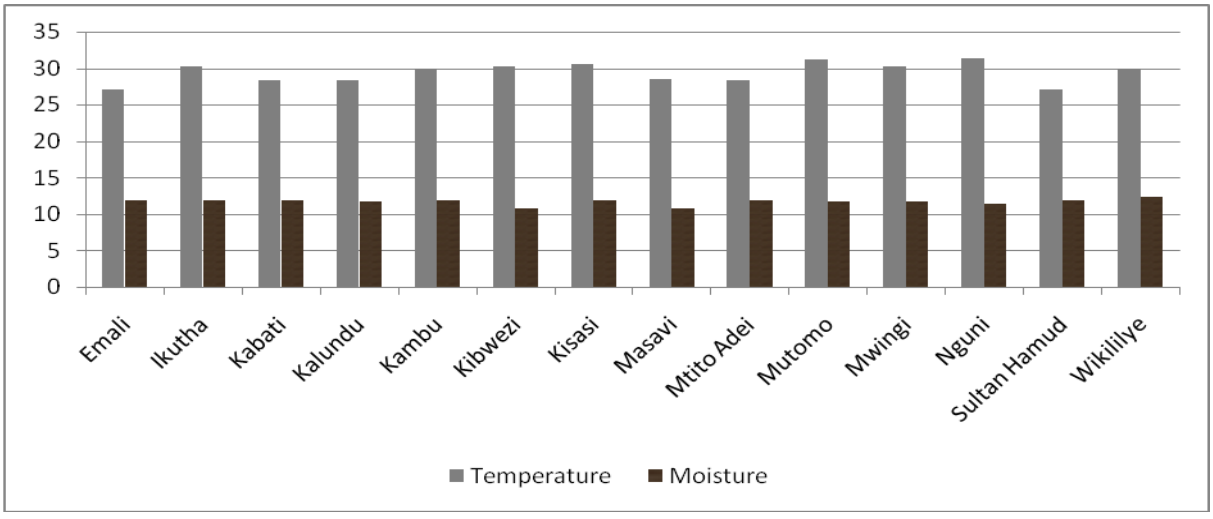


Figure 7: Mean temperature (^{o}C) and moisture content (%) in maize flour at various sampling centres

4.3.2 Moisture content

The moisture content of the samples varied from 9.9% to 13.5%. The mean moisture content per centre ranged from 10.8% (Nguni) to 12.1% (Wikililye). Overall mean moisture content was 11.33% (CI = 11.18% - 11.48%) at 95% confidence and 5% significance levels, with a mean moisture standard deviation of 0.89. Figure 7 shows the moisture content (%) in maize flour at various sampling centres.

4.3.3 Aflatoxin levels in flour

Out of 130 flour samples, 79.23% (n = 103) were contaminated with aflatoxins. The level of aflatoxin varied from 2.0 ppb to 58.2 ppb. The mean aflatoxin level per centre ranged from 2.4 ppb in Sultan Hamud to 43.1 ppb in Wikililye. The overall mean aflatoxin level in flour was 21.34 ppb (CI = 18.12 ppb - 24.56 ppb) at 95% confidence and 5% significant levels, while mean aflatoxin standard deviation was 18.72. Figure 8 shows mean aflatoxin levels per centre.

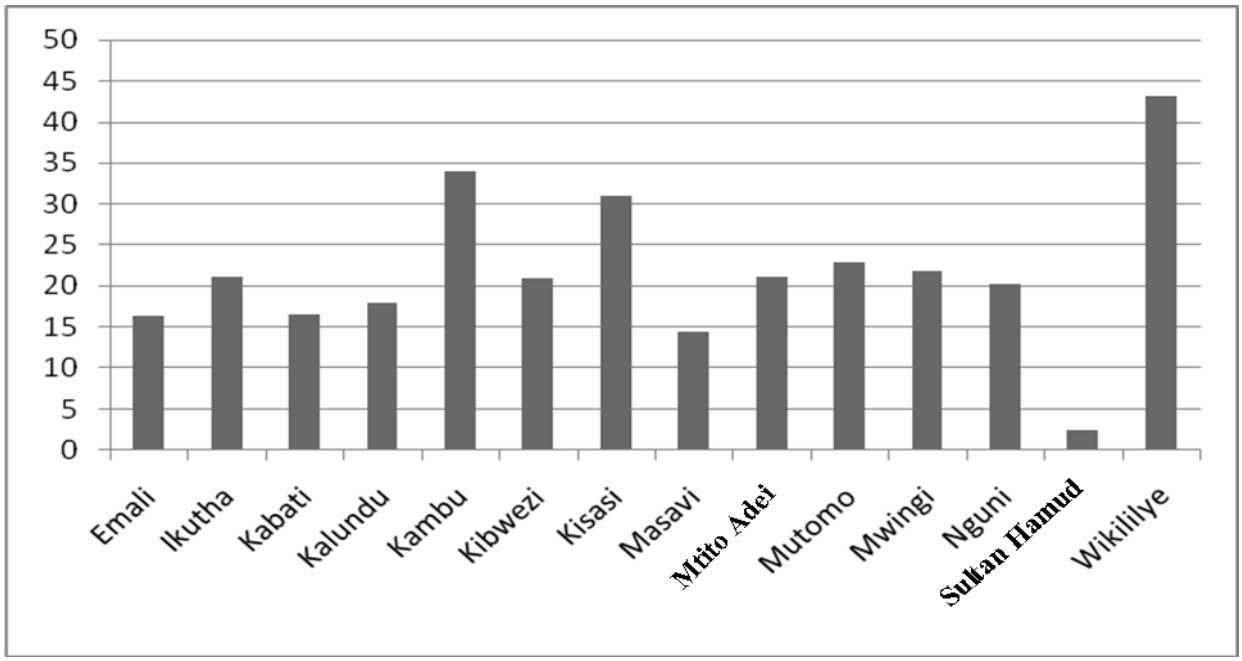


Figure 8: Mean aflatoxin levels (ppb) in flour per centre

There was a highly significant non association ($X^2 = 0.696$; $p < 0.05$) between the district of sampling (Table 9), and aflatoxin contamination in flour even though Makueni and Kitui accounted for 39.8% each, while Mwingi had 20.4% of the total contaminated flour samples.

Table 9: Geographic distribution of aflatoxin contaminated maize flour by district during the period of data collection

District	Mean temp (^oC)	Mean moisture (%)	Aflatoxin range (ppb)	Aflatoxin mean (ppb)	Prevalence (%)
Makueni	28.29	11.30	2.5 - 58.2	25.08	77.35
Mwingi	30.30	10.94	2.0 - 54	24.45	80.76
Kitui	30.09	11.55	2.0 - 55	30.00	80.39

Out of the samples contaminated with aflatoxin, 78.6% (n = 81) had aflatoxin levels above 10 ppb. Only two centers, Sultan Hamud and Kabati, had less than 50 % of the samples not exceeding 10 ppb. Table 10 shows the percentage of maize flour samples for each sampling centre that had aflatoxin levels above 10 ppb. Those levels were above Codex Alimentarius Commission (1995a), allowable upper grain contamination limit of 10 ppb.

Table 10: Percentage (%) of maize flour samples that had aflatoxin levels above 10 ppb

Strata name	Range of aflatoxin (ppb)	Overall number of samples exceeding 10 ppb	Percentage of samples exceeding 10 ppb per centre
Emali	13.2-49.5	6	66.67%
Ikutha	3.0-52.7	6	60.0%
Kabati	3.3-53.8	3	42.8%
Kalundu	2.1-51.5	7	46.6%
Kambu	6.0-58.2	10	83.3%
Kibwezi	3.0-56.6	8	66.7%
Kisasi	13.4-50.2	6	100%
Masavi	3.6-34.6	3	60.0%
Mtito Adei	3.5-56.1	6	60.0%
Mutomo	2.0-46	5	55.6%
Mwingi	2.0-54	6	60.0%
Nguni	11-35.8	9	81.8%
Sultan Hamud	2.5-7.1	0	0.00%
Wikililye	20.5-55	6	100%

4.3.4 Correlations of temperature, moisture content and aflatoxin levels in maize flour

The correlation between moisture content of flour and aflatoxin levels was + 0.642 and was found to be highly significant ($p < 0.01$). The correlation between temperature of flour and aflatoxin levels was also significant ($p < 0.01$) at a value of + 0.384. Similarly, a correlation of + 0.224 was found between temperature and moisture content of maize flour.

4.4 Comparison between aflatoxin levels in maize grain and maize flour

The mean aflatoxin level in maize grain was 13.17 ppb while that in flour was 21.34 ppb. The difference between the two means was statistically significant ($p < 0.05$), at 8.17 ppb, (CI= 3.47 ppb - 12.87 ppb) at 95 % confidence and 5% significance levels (SPSS® , version 11.51, lead technology 2001, USA).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The findings of this study indicate that storage temperature and moisture content promoted production of aflatoxin in maize grain and flour by aflatoxin producing fungi. The findings were found consistent with a study by Lacey (1991), Coumbe *et al.* (1993) and Christensten *et al.* (1997). Coumbe *et al.* (1993), found that after harvest, temperature, moisture content and insect activity were major factors influencing aflatoxin contamination of food and feed grains for animals. He determined that fungi of the genus *Aspergillus* growth was favoured by temperature range of 10^{o}C - 40^{o}C and moisture above 12%. Lacey (1991), also determined that molds of the genus *Aspergillus* grew on food and grain feeds with a minimum moisture content of 12% - 13% while, in another study by Whitlow & Hagler (2002), heat and drought stress, associated with warmer climates was found to favour the growth of *A. flavus* and *A. parasiticus* in grain and hence produced varied amounts of aflatoxin as a metabolite. In a similar study, fungi of the genus *Aspergillu*s was considered a storage fungi since they most oftenly grew at moisture content of above 12% and temperature range of 10^{o}C - 40^{o}C (Christensten *et al.,* 1977). Those findings compared favourably with this study where the sample storage temperature and moisture content range was (21.8^{o}C - 39.9^{o}C), and (9.9% - 21.6%), respectively.

A statistically significant ($p<0.01$) positive correlation (+0.115), between temperature and aflatoxin concentration in grains indicated that production of aflatoxin was promoted by elevated room temperature. It has been shown that temperatures below 8^{o}C and above 40^{o}C discourages aflatoxin production by fungi, (Coumbe *et al.,* 1993; Hassan & Aziz, 1998; Whitlow & Hagler, 2000). This is expected as production of aflatoxin is a metabolic process,

driven by enzymes which operate best between 8^{o}C and 40^{o}C (Cowan, 1997; Daniel *et al.,* 2010). Similar results were obtained by Hassan & Aziz (1998).

In this study a highly significant ($p< 0.01$) positive correlation between moisture content in both maize grain (+0.954), flour (+0.642) and aflatoxin contamination was noted. This indicated that moisture content favoured the growth of aflatoxin producing fungi hence grain and flour contamination with aflatoxins. It also implied that grains could be contaminated with aflatoxin during storage when moisture content was above the recommended level. Similar findings were reported by Lillehoj & Fenell (1975); Shotwell *et al.* (1975) and Coumbe *et al.,* (1993). This could be explained by the fact that water is a constituent of all living organisms including fungi and since fungi do not have a water proof outer covering, their growth in a substrate is limited when exposed to dry conditions.

Grains usually will contain a percentage of moisture even when dried. If the moisture content is too low, then moulds will not grow while a higher moisture content will provide water required by aflatoxin producing moulds for growth (Christensten *et al.*, 1977; Trenholm *et al.*, 1998).

However, when the correlation of temperature to aflatoxin, and correlation of moisture content to aflatoxin, for both grains and flour was compared, moisture content was found to have more positive effect on aflatoxin than was the positive effect of temperature even though both factors were found crucial for growth of aflatoxin producing fungi.. These observations were consistent with findings by Joffe (1986) and Trenholm *et al.* (1988). Rusell *et al.* (1991) found that temperature and humidity variations affected mold aflatoxin production where, 8% of corn grain samples in mid western U.S.A., from 1988 drought season were found to contain aflatoxin. Similar findings were reported by Ashworth *et al.* (1969).

In Wikililye and Kasasi, all the flour samples (100%), had aflatoxin levels above 10 ppb, the upper limit allowed by the Codex Commission. This could be due to stress and shock on the aflatoxin causing fungus (*Aspergillus*) in flour due to extremes of temperature in Kitui Central. This observation was in agreement with studies by Joffe (1986), Boyacioglu *et al.* (1992) and Gareis *et al.* (1994).

There was a highly significant non association (X^2=2.252; $p<0.05$ and X^2=0.696; $p<0.05$), between the district of sampling and aflatoxin contamination in both maize and flour samples respectively. In addition, a proportion of maize grain samples collected from Kisasi, Wikililye and Mtito adei, had aflatoxin content beyond 10 ppb, but this could be attributed to maize grain storage conditions. The strata is characterized by high temperature and humidity range which could have influenced higher storage temperature and moisture content noted in this strata. This could have promoted the occurrence and growth of aflatoxin producing fungi, an observation consistent with aflatoxicosis report in Eastern and Central Kenya (Ngindu *et al.*, 1981; CDC, 2004; Lauren *et al.*, 2005).

There was a higher aflatoxin prevalence in maize grain (84.58%), than flour (79.5%), even though the proportion of maize flour samples exceeding 10 ppb, Codex Commissions upper limit was higher, at 62.3% (n = 81), while that of maize grain was 30% (n = 39), indicating that most of maize grain samples though contaminated, had aflatoxin levels below 10 ppb (Table 7 & 8).

Higher aflatoxin prevalence in maize could have occurred due to higher mean moisture content of 12.57% while flour had 11.33% an observation consistent with results of similar studies by Whitlow *et al.* (1998) and Hassan & Aziz (1998).

Another factor could have been that, during milling of grain, the outer husk, the hilum and germ may be removed. The later are usually the parts of the grain heavily attacked by the hyphae and the mycelia of the aflatoxin producing fungi due to their hygroscopic and hydrophopic nature, which permits some moisture activity even when the overall moisture content of the grain is low (Keller *et al.*, 1994; Brown *et al.*, 1995). Dehusking reduces the chances that a flour sample will test positive for aflatoxin. This observation was in agreement with a study by Mutungi *et al.* (2007), which showed that processing of contaminated grain through dehusking of maize grain greatly reduced the aflatoxin levels in the product.

While the overall mean temperature for maize grain (29.6^{o}C), and flour (29.54^{o}C), was almost equal, the mean moisture content at 12.57% for maize grain and 11.33% for maize flour were remarkably diverse. The overall mean aflatoxin levels for maize grain was lower (13.17 ppb), than that of flour at 21.34 ppb, a statistically significant ($p<0.05$), difference(Table 7&8). From various studies (Christensten *et al.*, 1977; Lacey 1991; Coumbe *et al.*, 1993), on effects of moisture content, humidity and temperature on aflatoxin producing fungi, it was expected that, maize grain samples would have a higher mean aflatoxin level by virtue of a higher mean moisture content. This was not the case.

This could be due to the fact that during milling the grain is crushed into powder form, which increases the surface area of individual particles of flour on which the aflatoxin producing fungi would grow. The amount of air, hence aeration in a unit volume of flour increases, compared to a similar volume of grain, leading to a more fungal sustained growth and a higher aflatoxin concentration (Sihna & Sihna, 1991). In maize grain, the outer covering (testa), is hard, thus limiting the development of fungal rhizoids and hyphea only to the germ and the hilum of grain (Brown *et al.*, 1995), hence milling exposes more nutrients to aflatoxin

producing fungi. The endosperm is richer in carbohydrates than the testa and with a higher supply of nutrients the fungi could multiply more efficiently (Keller *et al.,* 1994; Brown *et al.*, 1995).

Since most fungi species are aerobic, including *Aspergillus* species (Robbin *et al.,* 2000), increased availability of air would increase oxygen supply favouring multiplication of aerobic fungi and hence more aflatoxin production in flour (Lisker *et al.*, 1989; Whitlow & Hagler Jr, 2002).

Silhna & Sihna (1991), in monitoring and identification of aflatoxin in wheat, grain and maize flour in Bihar state India, noted a similar trend where most samples had aflatoxin levels above 20ppb. All these factors could account for the higher mean aflatoxin level in flour, than maize grain.

5.2 Conclusion

- High Storage moisture content and temperature in maize grain and flour favour production of aflatoxin by aflatoxin producing fungi. However the effect of temperature is less than that of storage moisture as indicated by their respective positive correlation coefficient values.

- Milling of grain into flour, though beneficial within a shorter storage period, is a risk to aflatoxin contamination even when the flour is stored at the recommended moisture and temperature longer.

- Since Mwingi among the three districts had the lowest mean aflatoxin level 9.67ppb (Codex Commission upper limit is 10.0 ppb), at lower mean moisture content (12.17%) and a temperature mean of 26.43 ^{o}C, it could be inferred that the safe storage temperature and moisture content for maize grain and flour in that region, was 26.4 ^{o}C and 12.2 % respectively(Table 7 & 8).

- The positive correlations between temperature, moisture and aflatoxin levels in maize grain (+0.115, +0.954) and flour (+0.384, +0.642), respectively showed that there was a near linear relationship between the variables and aflatoxin levels, indicating an increase of moisture and/or temperature increased aflatoxin production by fungi to a certain level.

5.3 Recommendations

- Maize grains should be dried down to a moisture content of 12.2 % or less before storage or milling to discourage growth of aflatoxin producing fungi species.

- Both maize grain and flour should be stored at a temperature range of 26.4^{o}C and below. This would discourage growth of aflatoxin producing fungi species and avoid further aflatoxin contamination.

- Maize grain should only be milled into flour only when necessary as milling changes the physical properties of grains promoting growth of aflatoxin producing fungi.

5.4 Recommendation for further work.

- A longitudinal study to determine the stage at which aflatoxin producing fungi start to attack maize in developing kernels is required in lower Eastern region of Kenya

- A controlled study to pin point the exact storage moisture content (%) and temperature level (^{o}C) at which aflatoxin producing fungi start to attack stored maize grain in lower Eastern region, of Kenya will enable farmers to be more effective in storage of their maize grain and grain products including flour.

- The dynamics of contamination of maize grain, including isolation and characterization of the organism involved in aflatoxin production in stored maize grain and flour in the region of current study is required.

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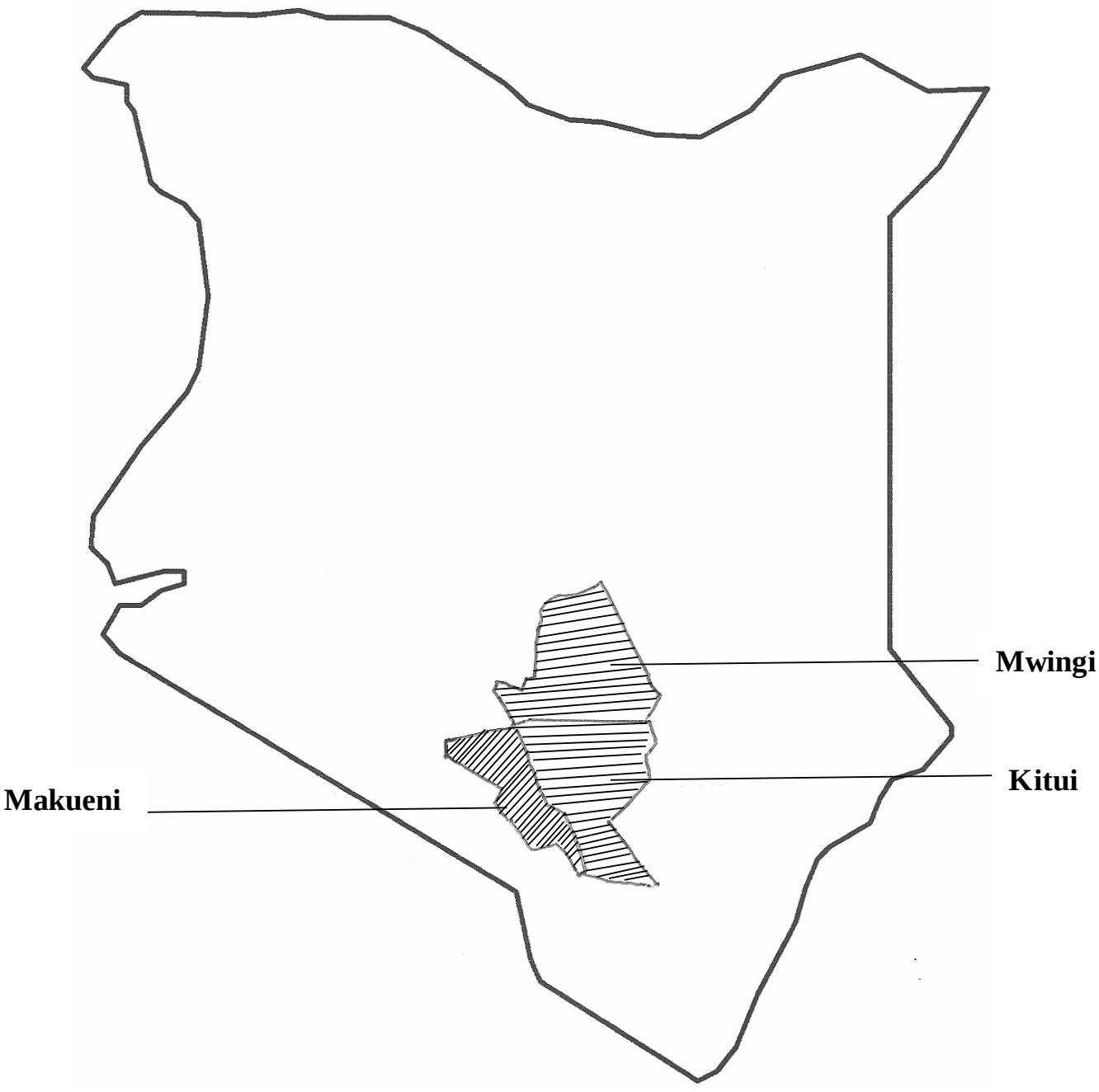
APPENDICES

APPENDIX I: MAP OF THE STUDY AREA (MAKUENI, MWINGI AND KITUI DISTRICTS) IN KENYA



APPENDIX II : INFORMED CONSENT

My name is Pius Kimani, a Masters of Science student at Kenyatta University. I am Carrying out a study on evaluation of parameters associated with growth of moulds (fungi) in maize grains during storage. In this study I need your cooperation by allowing me to pick samples of grain and maize flour in your stores.

This study is expected to provide information on the best storage temperatures for maize grains and flour that do not favour growth of mould. This information will help reduce or eradicate aflatoxins growth and the associated cases of aflatoxicosis. If you agree to participate in this study, the following will be expected of you.

i) Sign the consent form.

ii) Give samples of maize grain and maize flour in your store.

iii) You may sell or donate the samples.

iv) You will not be charged any cost incurred on analysis of the samples.

v) You will not be penalised or held liable in the event that any sample collected from your store is found to be contaminated.

All information on analysis result will be treated with strict confidence and under no circumstances will they be attributed to any particular store.

You are free to withdraw from this research study at any time before the samples are analysed.

Thank you

CONSENT FORM

I Mr. / Mrs. / Miss …….. am the legal owner of maize store known as ……

I hereby freely agree to participate in this study which has been explained to me. I understand that my participation in this study will not affect my business in any way. I also understand that all information about me and my store will be treated with the strictest confidence.

Name of the store owner ……..…….

Sign…….Date……

Witness ……..

Sign…… Date……...

APPENDIX III : PERMISSION

KENYATTA UNIVERSITY
SCHOOL OF PURE AND APPLIED SCIENCE
DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

P.O. Box 43844
Nairobi-Kenya
TEL: 810901–12/811622
Ext. 456

5th December, 2008

The Permanent Secretary,
Ministry of Higher Education
P.O. Box 30040
NAIROBI

Dear Sir,

RE: PERMISSION TO CONDUCT RESEARCH – KIMANI PIUS MUTISYA
REG.NO. I56/CE/12511/04

The above named is a student in the Department of Biochemistry & Biotechnology in Kenyatta University. He is undertaking a Masters course in Infectious Disease Diagnosis.

He has finished his coursework and is now engaged in research, data collection and analysis.

The purpose of this letter is to seek for your approval for the student to conduct research in Aflotoxin in maize in Eastern Province specifically Makueni, Kitui and Mwingi Districts.

Any assistance given will be highly appreciated.

Thank you.

CHAIRMAN
DEPARTMENT OF BIOCHEMISTRY & BIOTECHNOLOGY
KENYATTA UNIVERSITY
P. O. Box 43844 - 00100,
NAIROBI.

Dr. J.J.N. Ngeranwa
Chairman, Department of Biochemistry & Biotechnology

APPENDIX IV : AUTHORIZATION

NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Telegrams: "SCIENCETECH", Nairobi
Telephone: 254-20-241331, 241349,
254-20- 311761, 241376,
Fax: 254-20- 213215

P. O. Box 30623 –00100
NAIROBI- KENYA

When replying please quote

REF: NCST/5/002/R/136/2

10th March 2009

Pius Kimani Mutisya
Kenyatta University
P.O. Box 43844
NAIROBI

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on, ***'Determination of Aflatoxins Levels in Maize Grain and Flour at Different Storage Conditions in Kitui District''***

I am pleased to inform you that you have been authorized to carry out research in Kitui District for a period ending 30th November 2009.

You are advised to report to the District Commissioner, the District Education Officer, the District Agricultural Officer, and the District Public Health Officer Kitui District before embarking on your research.

On completion of your research, you are expected to submit two copies of your research report to this office.

SAID S. HUSSEIN
FOR: EXECUTIVE SECRETARY

Copy to:

The District Commissioner
Kitui District

The District Education Officer
Kitui District

The District Public Health Officer
Kitui District

The District Agricultural Officer
Kitui District

APPENDIX V : CLEARANCE

CONDITIONS

1. You must report to the District Commissioner and the District Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit.
2. Government Officers will not be interviewed without prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two(2)/four(4) bound copies of your final report for Kenyans and non-Kenyans respectively.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice

REPUBLIC OF KENYA

RESEARCH CLEARANCE PERMIT

GPK 6055—3m—10/2003

(CONDITIONS—*see* back page)

APPENDIX VI : CLEARANCE PERMIT

PAGE 2

THIS IS TO CERTIFY THAT:

Prof./Dr./Mr./Mrs./Miss PIUS KIMANI MUTISYA

of (Address) KENYATTA UNIVERSITY

has been permitted to conduct research in

Location,

KITUI District,

EASTERN Province,

on the topic DETERMINATION OF AFLATOXINS LEVELS IN MAIZW GRAIN AND FLOUR AT DIFFERENT STORAGE CONDITIONS IN KITUI DISTRICT KENYA

for a period ending 30TH NOVEMBER, 20 09

PAGE 3

Research Permit No. NCST 5/002/R/136

Date of issue 10.3.2009

Fee received SHS.1000

Applicant's Signature

SAID S. HUSSEIN

FOR Permanent Secretary
Ministry of
Science and Technology

APPENDIX VII: TEMPERATURE, MOISTURE CONTENT AND AFLATOXIN LEVELS IN MAIZE GRAIN SAMPLES

Sample no	Temperature *in situ* (^{0}C)	Moisture *in situ* (%)	Aflatoxin levels (ppb)
1	28.30	12.00	2.50
2	26.90	12.00	2.00
3	26.90	11.75	3.20
4	27.60	12.10	4.10
5	26.40	11.90	2.80
6	26.30	12.00	3.50
7	28.80	11.20	3.60
8	26.90	11.40	4.20
9	24.00	14.90	31.00
10	25.70	11.80	2.50
11	26.70	11.20	3.20
12	21.80	11.00	0.0
13	24.50	12.00	3.40
14	22.50	12.20	4.00
15	22.50	11.50	0.0
16	25.60	12.00	23.30
17	24.50	12.20	11.00
18	24.10	11.00	0.0
19	24.00	12.10	3.30
20	24.70	17.60	80.50

21	24.00	15.10	33.10
22	24.00	14.10	21.30
23	24.80	13.30	14.50
24	31.30	11.30	3.20
25	30.40	21.60	97.90
26	30.00	15.60	38.40
27	29.60	11.60	2.50
28	29.80	15.90	47.00
29	29.90	12.80	10.50
30	30.30	15.50	39.20
31	30.70	17.20	77.80
32	29.50	13.20	13.20
33	29.90	15.20	34.30
34	30.40	12.20	4.20
35	30.50	17.20	65.90
36	30.80	14.20	24.70
37	30.40	11.50	3.20
38	29.40	11.20	0.0
39	29.30	12.60	8.00
40	30.60	13.20	14.80
41	30.80	11.00	2.00
42	30.50	11.80	2.50
43	29.10	11.80	3.20

44	31.20	12.40	6.10
45	29.90	11.30	0.0
46	33.70	12.00	3.00
47	33.40	12.40	6.50
48	33.10	11.60	2.20
49	32.10	11.80	2.80
50	29.60	11.50	0.0
51	31.70	11.20	3.20
52	30.40	11.00	2.00
53	30.00	10.90	0.0
54	31.10	11.20	0.0
55	31.30	11.20	2.30
56	30.40	11.00	2.00
57	31.00	10.60	0.0
58	31.00	10.70	0.0
59	31.00	12.10	3.60
60	29.80	11.70	2.90
61	28.80	10.90	0.0
62	32.20	15.80	46.20
63	31.70	11.50	3.90
64	32.00	11.20	3.50
65	29.80	11.40	3.70
66	30.50	11.80	3.40

67	33.60	11.90	2.20
68	33.40	12.00	3.00
69	31.10	11.20	2.50
70	31.20	11.00	0.0
71	33.10	11.80	3.20
72	33.50	11.50	21.50
73	30.20	10.00	0.0
74	31.20	10.20	5.40
75	29.70	10.20	.00
76	30.10	11.00	3.70
77	30.40	10.20	0.0
78	30.20	12.50	8.40
79	30.60	11.50	2.30
80	30.20	13.00	23.50
81	30.80	13.30	24.60
82	29.00	16.90	55.70
83	36.40	15.20	34.80
84	31.50	12.10	3.90
85	30.40	11.00	4.50
86	29.10	12.30	5.30
87	30.00	10.80	0.0
88	30.30	11.20	2.30
89	26.60	12.50	7.00

90	27.20	11.80	3.20
91	26.60	11.70	2.20
92	25.70	11.40	0.0
93	25.10	12.20	4.30
94	26.60	14.10	23.50
95	26.20	13.90	21.20
96	26.30	16.90	54.50
97	26.00	12.40	6.30
98	26.90	12.20	4.20
99	29.00	13.00	12.60
100	29.20	13.30	15.80
101	28.50	11.50	2.00
102	30.60	11.70	2.80
103	31.20	12.20	4.00
104	29.50	11.80	2.40
105	30.00	10.60	0.0
106	29.50	11.50	2.00
107	28.20	10.80	0.0
108	30.20	10.90	0.0
109	31.20	11.50	2.50
110	33.80	12.10	5.50
111	27.30	15.10	30.80
112	36.90	12.30	8.90

113	30.70	11.10	2.20
114	33.80	11.50	3.00
115	39.90	17.00	68.70
116	32.60	15.80	40.80
117	32.60	12.30	5.90
118	28.50	13.10	13.40
119	32.50	15.20	36.20
120	27.20	12.40	6.30
121	32.60	10.80	3.10
122	36.70	12.40	8.20
123	30.30	16.30	52.80
124	28.40	12.50	7.30
125	33.70	14.20	25.70
126	33.00	15.80	43.50
127	33.80	17.60	87.60
128	28.30	15.10	33.90
129	28.60	11.50	2.40
130	32.50	11.50	2.80

APPENDIX VIII: TEMPERATURE, MOISTURE CONTENT AND AFLATOXIN LEVELS IN MAIZE FLOUR SAMPLES

Sample no	Temperature *in situ* (^{0}C)	Moisture *in situ* (%)	Aflatoxin levels (ppb)
1	28.50	11.80	0.0
2	33.10	10.60	13.90
3	30.40	10.00	3.60
4	30.30	11.40	34.60
5	24.00	10.80	20.20
6	30.80	10.90	22.00
7	29.80	11.20	28.00
8	31.30	11.20	33.40
9	31.00	11.40	35.80
10	33.10	10.80	0.0
11	32.50	9.90	0.0
12	28.30	10.20	18.42
13	32.90	10.40	11.00
14	33.60	11.40	23.30
15	32.40	11.10	29.60
16	29.20	10.60	20.90
17	33.20	11.20	30.90
18	33.90	11.20	31.50
19	28.10	12.40	49.20
20	30.20	11.10	29.20

21	27.60	12.50	0.0
22	29.20	11.40	19.30
23	28.40	11.80	0.0
24	30.00	11.20	0.0
25	29.20	12.40	2.10
26	28.40	13.00	54.00
27	29.70	12.40	50.10
28	29.40	12.90	55.00
29	30.00	11.90	42.90
30	31.40	10.80	20.50
31	30.00	11.50	36.30
32	29.30	10.70	18.30
33	30.00	12.40	50.20
34	31.70	11.80	41.50
35	28.40	10.50	13.40
36	34.50	11.60	15.50
37	33.50	12.10	47.00
38	34.80	11.70	42.80
39	31.50	11.90	43.00
40	33.10	12.10	46.20
41	29.80	11.50	9.80
42	27.90	12.00	2.00
43	34.00	10.20	32.00

44	28.00	11.90	0.0
45	28.20	10.90	0.0
46	34.80	11.10	29.00
47	32.10	11.70	40.10
48	31.80	11.10	33.40
49	29.90	11.30	0.0
50	29.40	11.00	4.50
51	31.00	12.60	52.70
52	29.50	10.00	3.00
53	31.40	11.60	37.00
54	29.90	11.30	21.90
55	29.90	11.30	19.00
56	31.40	12.20	0.00
57	29.30	11.00	27.30
58	30.60	10.60	0.0
59	31.90	11.90	43.00
60	30.50	11.50	12.10
61	28.40	11.50	0.0
62	33.00	10.60	14.90
63	32.00	10.70	18.10
64	34.00	10.20	6.00
65	30.40	11.60	25.00
66	33.00	13.00	56.50

67	24.50	10.00	3.00
68	28.00	12.10	44.30
69	31.20	12.40	49.50
70	30.00	11.10	29.20
71	25.00	11.20	19.50
72	27.00	10.50	0.0
73	29.90	10.80	13.80
74	26.30	10.60	0.00
75	24.00	11.30	13.20
76	24.50	12.20	21.50
77	24.50	10.30	0.00
78	23.80	12.50	3.00
79	32.50	12.90	0.0
80	31.00	10.12	2.50
81	27.80	10.10	0.00
82	26.00	11.40	7.10
83	24.50	10.70	0.0
84	24.00	12.30	6.30
85	24.20	10.00	0.0
86	25.00	10.90	3.00
87	33.40	10.50	13.00
88	30.50	12.80	54.00
89	30.50	11.00	27.00

90	32.60	12.80	51.20
91	29.90	11.30	30.50
92	30.50	10.80	2.00
93	31.00	9.90	0.0
94	31.80	9.90	2.00
95	29.50	11.70	39.20
96	28.50	10.00	0.00
97	32.60	12.60	9.80
98	32.50	12.70	12.00
99	25.00	9.90	0.0
100	25.00	10.10	3.30
101	27.80	11.60	29.60
102	27.00	10.30	7.00
103	31.00	13.50	53.80
104	29.90	12.90	51.50
105	31.00	12.40	42.00
106	24.50	10.00	0.0
107	26.00	9.90	0.0
108	26.50	10.50	0.0
109	25.00	10.50	12.20
110	29.90	12.50	48.60
111	29.80	11.20	31.20
112	28.00	10.20	6.00

113	29.00	10.70	18.00
114	29.50	10.40	10.20
115	27.60	11.50	0.0
116	31.50	12.80	58.20
117	32.00	11.70	39.00
118	32.50	12.60	52.00
119	28.50	12.10	47.30
120	30.00	11.80	39.80
121	30.00	13.00	56.10
122	31.50	12.30	49.00
123	33.00	10.90	22.00
124	29.00	12.00	45.00
125	27.00	11.20	30.20
126	27.90	11.00	25.60
127	27.50	10.30	0.0
128	25.50	11.10	3.50
129	25.00	10.50	6.10
130	34.50	12.00	9.60