EFFECTS OF DEHULLING MAIZE GRAINS AND TREATMENT WITH CHEMICAL ADDITIVES ON THE LEVELS OF AFLATOXINS DURING *MUTHOKOI* MAKING AND PREPARATION

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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To my beloved parents Gabriel and Priscilla, brothers and sister

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS / DEFINITIONS

AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFB_{2a}	Aflatoxin B_{2a}
AFB_{1S}	Aflatoxin B_1 sulphonate
AFD_1	Aflatoxin D_1
AFG_1	Aflatoxin G_1
AFM_1	Aflatoxin M_1
AFP_1	Aflatoxin P_1
AFQ_1	Aflatoxin Q_1
TLC	Thin layer chromatography
RIA	Radioimmunoassay
ELISA	Enzyme Linked Immunosorbent Assay
SDA	Sabourauds Dextrose Agar
AFPA	*Aspergillus flavus* and *parasiticus* agar
YES agar	Yeast Extract Sucrose agar
FCS	Foetal calf serum
PBS	Phosphate buffered saline
ng/g	parts per billion (ppb)
ng/ml	parts per billion (ppb)
Iati	A local alkaline mineral salt used in households as cooking aid
Muthokoi	A traditional dish among the Akamba community prepared by boiling dehulled maize
dSm^{-1}	deciSiemens per metre
$Meql^{-1}$	Mili-equivalents per litre

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ABSTRACT

Exposure of human populations to acute or sub-acute levels of aflatoxins in contaminated maize and maize products is a major food safety concern in Kenya, particularly among the Kamba community. In an attempt to address this concern, a study was conducted to investigate the effect of dehulling and treatment of maize grains with chemical additives on the levels of aflatoxins in naturally contaminated maize during the traditional *muthokoi* making and preparation process.

Seventy-two (72) samples of maize contaminated with aflatoxins to varying levels were taken from naturally contaminated lots sampled from Makueni, Kitui and Machakos Districts during an aflatoxicosis outbreak. In the first part of the study, 48 of these samples were each de-hulled separately by pounding in a wooden mortar accompanied by intermittent wetting of the grain with either distilled water, or 1 % (w/v) solution of *iati* (a local alkaline mineral salt traditionally used as a cooking aid), or 1 % (v/v) sodium hypochlorite or 1 % (w/v) ammonium persulphate. For each dehulling trial, the whole-grain maize sample, *muthokoi* and by-product, were analysed for aflatoxin levels, total mould counts and *Aspergillus flavus* counts. Aflatoxin reduction was calculated from the difference between aflatoxin levels in whole-grain maize and aflatoxin levels in *muthokoi* expressed as a percentage of the aflatoxin levels in whole-grain maize.

In the second part of the study, samples of dehulled and whole-grain maize were treated with *iati*, by soaking in graded solutions of the salt at room temperature for 6 and 12 hours. The effect of this salt on the levels of aflatoxins was compared with that of sodium hypochlorite and ammonium persulphate, chemical additives that have already been reported to degrade aflatoxins under similar conditions. Other samples of dehulled and whole-grain maize were boiled in dilute solutions of *iati*.

The percentage aflatoxin reduction due to dehulling ranged from 5.5 % to 70.0 % with a mean of 46.6 %. Aflatoxin reduction during dehulling was dependent on the aflatoxin contamination level of the raw-material whole-grain maize and the extent of dehulling achieved. Total mould and *Aspergillus flavus* counts were significantly lower in *muthokoi* compared to whole-grain maize only when chemically active dehulling aids were used. Recovery of moulds was dependent on the grain wetting agent but none of the agents used exhibited ability to completely inhibit mould recovery on *muthokoi*.

During treatment with chemical additives, aflatoxin reduction was higher in dehulled maize than in whole-grain maize. The effects of ammonium persulphate and sodium hypochlorite were high, reaching percentage reduction of 73.2 % and 72.2 % respectively, during overnight soaking of dehulled maize in 1 % concentration solutions. Loss of aflatoxins following soaking treatments of contaminated maize in solutions of *iati* was lower than that attained following treatment with ammonium persulphate and sodium hypochlorite. Overnight soaking of *muthokoi* in 0.5 % (w/v) solution of the salt resulted in mean aflatoxin reduction of 49.7 % without loss of original colour of the maize. A further aflatoxin reduction of 86.1 % was attained after boiling *muthokoi* in 0.5 % (w/v) solution of *iati* for 150 minutes.

The results of this study indicate that dehulling of maize grains to make *muthokoi* substantially reduces the levels of aflatoxins in naturally contaminated maize. Overnight soaking of *muthokoi* in a dilute solution of *iati* can be used to further reduce aflatoxin carry-over into the edible portion while cooking in a dilute solution of the salt can significantly reduce toxicity associated with aflatoxin-contaminated maize. The process of *muthokoi* making, however, does not destroy or inhibit mould recovery and hence the risk of aflatoxin build-up exists in case this product is stored under conditions that favour growth of aflatoxigenic moulds.

CHAPTER ONE

1.0 INTRODUCTION

Maize and maize products are a major source of food for over 85 % of the Kenyan population. The per capita daily consumption of maize meals is estimated at 0.4 Kg (Siboe and Muriuki, 1995). Production of maize for domestic consumption has thus continued as a dominant agricultural activity in order to satisfy the demand for this dietary staple. Quite often, maize is also imported or received as food aid to supplement the locally produced quantities during times of drought.

Contamination of maize and other food commodities with aflatoxins, a group of toxic fungal metabolites has however been an issue of great public health concern since early 1960s. Numerous incidences of aflatoxin poisoning in man and animals, and epidemiological data on the effects of aflatoxins clearly indicate the importance of aflatoxin exposure in the aetiology of human disease (Eaton and Groopman, 1994). In Kenya, several severe outbreaks of human and animal aflatoxicosis have been documented (Price and Heinonen, 1978; Nagindu *et al.*, 1982; Mbugua and Etale, 1987). Comprehensive surveys conducted following these outbreaks revealed presence of high levels of aflatoxins in both human and animal food samples especially maize and maize products (Muraguri *et al.*, 1981; Muriuki, 1992; Gathumbi *et al.*, 1995; Siboe and Muriuki, 1995). More recently, severe outbreaks of acute aflatoxicosis in Makueni and other neighbouring districts of Eastern and Central provinces of Kenya, in May-June 2004 and May 2005, resulted in 157 deaths. The later two incidences were traced to consumption of maize that was heavily contaminated with aflatoxins (MoH, 2005). Besides acute poisoning which occurs due to acute exposure, epidemiological evidence indicates that chronic exposure to low levels of aflatoxins leads to more devastating adverse health effects in the long term (Roebuck and Maxuitenko, 1994).

While prevention of contamination of food commodities by aflatoxin-producing fungi is considered the most rational approach to controlling aflatoxin build-up in the food chain, these fungi are ubiquitous in nature and therefore infestation of host plants in the field is unavoidable. Similarly, absolute control of factors that promote fungal infestation and aflatoxin production such as inoculums availability, weather conditions and pest infestation throughout the period of crop growth, maturation, harvesting and storage is untenable (Lopez-Garcia and Park 1998). Poor pre- and post-harvest grain care practices also promote fungal growth on stored grain and thus a significant amount of grain is eventually contaminated with aflatoxins. Accordingly, low-cost post harvest processing technologies that are capable of removing or degrading aflatoxins in contaminated food commodities, and are applicable at both household and/ or industrial scale remain vital in the minimisation of consumer exposure to aflatoxins (Philips *et al.*, 1999).

In Kenya, maize is processed and prepared in different ways by different communities. It may be consumed as either roasted green maize, boiled whole-grain maize (*Githeri*), gelatinised flour (*Ugali / Uji),* or boiled dehulled grits (*Muthokoi*). *Muthokoi* making is indigenous and common practice among the Kamba community, and to a lesser extent, among the Kikuyu (*Njenga*) and Meru (*Muthikore*) communities living in Central and Eastern provinces of Kenya respectively. The traditional method of making *muthokoi* involves pounding of maize grains in a mortar accompanied by intermittent wetting of the grains with water to soften the grain pericarp (hull). Usually the large grains are preferred hence, the undersized grains are sometimes segregated manually from the initial lot before de-hulling. Separation of the hulls fraction from the de-hulled product is done by winnowing after drying in the sun. In recent years though, mechanical de-hullers have largely replaced the hand de-hulling method in urban and semi urban areas. During preparation, the dehulled maize may be softened by soaking for sometime in water or in some instances in dilute

solution of *iati*, a local alkaline mineral salt, prior to boiling in either fresh water or dilute solution of the salt, in order to shorten the cooking time.

In a review of aflatoxin reduction effects of various conventional maize processing methods, Brekke *et al.* (1975b) reported that the highest levels of aflatoxin occurred in the germ and hull fraction of contaminated maize kernels. Similarly, Tabata *et al.* (1994) reported that treatment of aflatoxin-contaminated foods with alkali increased the solubility of aflatoxins in water. *Muthokoi* processing may thus have certain aflatoxin reduction effects through partial or full removal of some of the highly contaminated parts of the grain, and the extraction or degradation that follows during soaking or cooking in dilute solutions of the local alkaline mineral salt, *iati*.

The aim of this study was to investigate the effect of *muthokoi* making process on the levels of aflatoxins in naturally contaminated maize. Specific objectives were to:

1. Determine the partitioning of aflatoxins, typical aflatoxigenic moulds and total moulds in *muthokoi* and by-product.
2. Determine the aflatoxin-decontamination effect of dehulling of naturally contaminated maize grains during *muthokoi* making process.
3. Investigate the effect of various chemical additives on aflatoxins during *muthokoi* preparation.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 AFLATOXINS

Aflatoxins are a group of 18 variedly toxic, carcinogenic and genotoxic secondary fungal metabolites whose basic structure comprises of a dihydrofurano or tetrahydrofurano moiety fused to a coumarin moiety. The lactone ring of the coumarin moiety is fused to a cyclopentenone ring in the B aflatoxins and to an additional lactone ring in the G aflatoxins. Four types of aflatoxins: AFB_1, AFB_2, AFG_1, and AFG_2 are produced naturally by strains of *A. flavus, A. parasiticus* and *A.nomius* (Hassan and Llyod, 1995; Kurtzman *et al.*, 1987). *Aspergillus flavus* produces primarily AFB_1 and AFB_2 while *A. parasiticus* is capable of producing all four major types of aflatoxins (Dierner *et al.*, 1987). However, not all strains of these species are aflatoxigenic. The other aflatoxins such as AFM_1, AFM_2, AFP_1, and AFQ_1 and aflatoxicol are metabolic derivatives of microbial and animal systems, while others such as AFB_{2a}, AFG_{2a} and AFD_1 are formed spontaneously in response to certain chemical environments (Cole and Cox, 1981). Aflatoxin B_1 is the most toxic and the most commonly found aflatoxin in foods.

The four major aflatoxins: - AFB_1, AFB_2, AFG_1 and AFG_2 are named according to the fluorescent colours when thin layer chromatographic preparations are viewed under long wavelength ultraviolet light (365nm). Aflatoxin B_1 and AFB_2 show bright blue fluorescence while AFG_1 and AFG_2 fluoresce green. The subscripts 1 and 2 represent their relative chromatographic mobility. The chemical structures of some aflatoxins are shown in Figure 2.1.

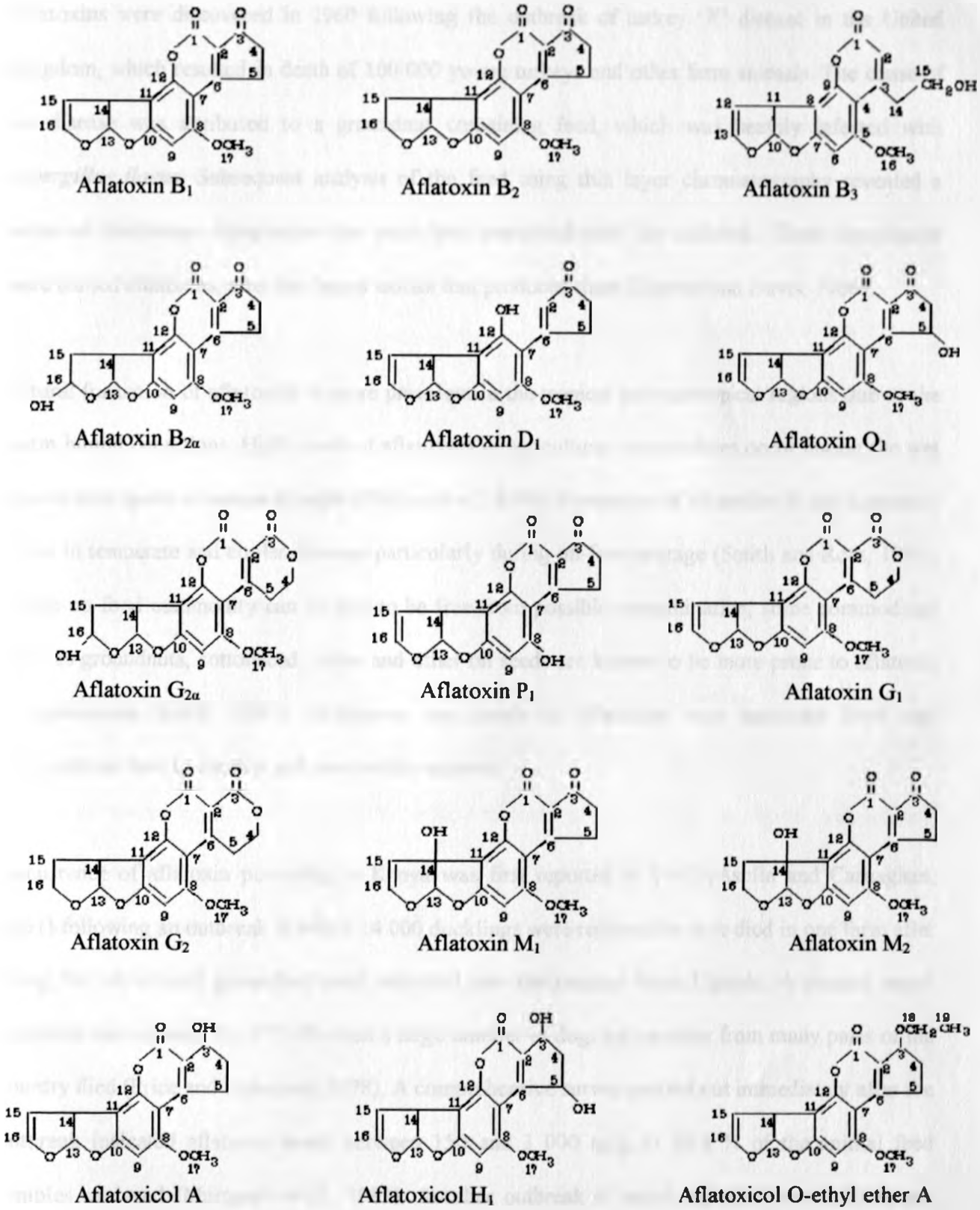

Figure 2.1: Chemical structures of some aflatoxins (Cole and Cox, 1981)

Aflatoxins were discovered in 1960 following the outbreak of turkey 'X' disease in the United Kingdom, which resulted in death of 100 000 young turkeys and other farm animals. The cause of the disease was attributed to a groundnut containing feed, which was heavily infested with *Aspergillus flavus*. Subsequent analysis of the feed using thin layer chromatography revealed a series of fluorescent compounds that were later associated with the outbreak. These compounds were named aflatoxins, after the fungal isolate that produced them (Dierner and Davis, 1966).

Natural formation of aflatoxins is more prevalent in the tropical and subtropical regions due to the warm humid conditions. High levels of aflatoxins in agricultural commodities occur during the wet season after spells of severe drought (Philips *et al.*, 1994). Formation of aflatoxins is also known to occur in temperate and cooler climates particularly during on-farm storage (Smith and Ross, 1991). While no food commodity can be said to be free from possible contamination, some commodities such as groundnuts, cottonseed, maize and other oil seeds are known to be more prone to aflatoxin contamination (Robb, 1993). Incidences and levels of aflatoxins vary markedly from one geographical area to another and even within seasons.

Occurrence of aflatoxin poisoning in Kenya was first reported in 1960 (Asplin and Carnaghan, 1961) following an outbreak in which 14 000 ducklings were reported to have died in one farm after being fed on a toxic groundnut meal imported into the country from Uganda. A second major outbreak was reported in 1977-78 when a large number of dogs and poultry from many parts of the country died (Price and Heinonen, 1978). A comprehensive survey carried out immediately after the outbreak indicated aflatoxin levels between 150 and 3 000 ng/g in 34.8 % of the animal feed samples analysed (Muraguri *et al.*, 1981). Another outbreak of acute hepatitis due to aflatoxins killed 12 people in Machakos district in 1981. Samples of maize and food of maize origin analysed contained 150 to 12 000 ng/g of aflatoxin (Nangindu *et al.*, 1982). Reports of aflatoxicosis in poultry

(Mbugua and Etale, 1987) and dogs (Mitema, 1986) gained prominence in the drought years of 1984 and 1985. In this outbreak, imported maize was found to be the source of aflatoxins (Manwiller, 1987). A study carried out at about the same time of the outbreak found 12.6 % of all persons in various parts of the country to be positive of aflatoxin exposure. In a survey by Muriuki (1992), 8.9 % of 214 samples of maize and maize products collected from Nairobi city were contaminated with aflatoxins.

The 2004 and 2005 outbreaks of acute human aflatoxicosis in Makueni and other neighbouring districts that caused over 157 deaths were traced to consumption of maize heavily contaminated with aflatoxins. Samples of maize analysed contained aflatoxin levels as high as 46 400 ng/g (Lewis *et al.*, 2005). In a follow-up countrywide survey, 26.2 % (132 / 503) of sampled maize contained aflatoxins above the maximum permissible level of 20 ng/g (MoH, 2005).

2.2 BIOLOGICAL EFFECTS OF AFLATOXINS

The adverse health effects of exposure to acute or chronic levels of aflatoxins in the diet relate to the ability of aflatoxins to react with cellular and nuclear proteins thus interfering with protein formation and maintenance of cellular integrity and function (Hall and Wild, 1994). The liver is the principal target organ for aflatoxins in the body. However, aflatoxins have also been reported to affect adversely other vital body organs such as lungs, kidneys, myocardium, and brain in various animal species.

2.2.1 Acute aflatoxicosis

Acute aflatoxin toxicity has been demonstrated in a wide range of mammals, fish and birds. Incidences of acute human aflatoxicosis have been reported mainly in Africa and Asia following ingestion of highly contaminated maize (Krishnamachari *et al.*, 1975; Shank, 1981; Nangindu *et al.*,

1982; Tandon *et al.*, 1987; MoH, 2005). The clinical symptoms of acute aflatoxicosis include jaundice, nausea, vomiting, diarrhoea, headache, abdominal pains, and death. Aflatoxin induced liver damage has also been shown to cause Reye's syndrome in children, a condition characterised by encephalopathy and degeneration of the fat tissue in the liver (Hall and Wild, 1994). The severity of acute aflatoxicosis is influenced by age, sex, nutritional status, alcoholism, smoking, immune status of the body and incidences of primary infectious agents.

2.2.2 Chronic toxicity

(1) Immunosuppression

The immuno-suppressive effect of chronic exposure to aflatoxins is largely attributed to reduction in T-cell population, lymphokine production and immunization failure (Pier and McLoughlin, 1985). This effect may be more devastating among HIV/Aids afflicted populations in most developing countries.

(2) Hepatocellular carcinoma

Numerous studies and reviews on the association between liver cancer and exposure to aflatoxins reveal strong evidence linking dietary aflatoxin intake with the occurrence of primary liver cancer in man (Edmodson and Craig, 1987; Bocsh and Munoz, 1989; Stoloff, 1989).

(3) Genotoxicity

The mutagenic and teratogenic effects arising from ingestion of aflatoxins stem from chromosomal aberrations, and ability of aflatoxins to bind onto cellular DNA (Hall and Wild, 1994).

(4) Kwashiorkor

Reviews in aetiologies of protein – energy malnutrition by Latham (1990) strongly implicate aflatoxins in aetiology of Kwashiorkor. Hendrickse (1991) observed increased incidences of kwashiorkor in the tropics during wet seasons occurring after severe drought, conditions that are associated with high aflatoxin exposures. Studies in Kenya (De Vries *et al.*, 1987) and Sudan (Hendrickse *et al.*, 1982) revealed high levels of urinary excretion of aflatoxin in kwashiorkoric children as compared with normal children.

2.3 CONTAMINATION OF MAIZE WITH AFLATOXINS

In maize, aflatoxin is almost exclusively produced by *Aspergillus flavus*, which produces AFB_1, and AFB_2. Aflatoxigenic strains *A. flavus* are capable of growing on maize in the field and during storage. The optimum conditions for growth and subsequent production of aflatoxins by *A. flavus* include moisture content above 14%, an optimum temperature of 28-30°C and water activity of about 0.83 - 0.97 (Dierner *et al.*, 1987). Oxygen and carbon dioxide ratio, physical integrity of the grain, initial levels of mould infection, pest activity, and genetic properties of the grain also play a vital role (Porter *et al.*, 1986). Immature, broken, undersized, discoloured kernels are most likely to be contaminated with aflatoxin compared to mature sound kernels (Beaver, 1991; Chiou *et al.*, 1994; Rucker *et al.*, 1994). Drought stress also increases the risk of field contamination of maize with aflatoxins (Sanders *et al.*, 1981).

2.3.1 Field contamination of maize with aflatoxins

During the dry hot seasons, *A. flavus* spore population increases on crop debris and on senescent or dormant plant tissues leading to high levels of mould propagules in the air (Wilson and Payne, 1994). Heavy inoculum of *A. flavus* may thus be introduced to the crop as air-borne propagules throughout the period of crop growth and maturation. Insect activity facilitates infection of pre-

harvest maize by enhancing dissemination of inoculum within ears, and creating a favourable habitat for *A. flavus* through injury associated feeding (McMillan, 1983; Payne, 1992). After introduction of inoculum, the environment to which the standing crop is exposed determines the extent of infection. When heavy inoculum occurs on wounded kernels or crop residues, high levels of aflatoxin contamination exceeding 100 000 ppb can result. Extensive colonisation of maize by the fungus, however, does not necessarily lead to significant accumulation of aflatoxin. This is partly because the optimal conditions for growth of *A. flavus* may not favour aflatoxin formation, and the aflatoxigenicity of different strains of *A. flavus* varies widely (Lilleihoj *et al.*, 1987).

2.3.2 Aflatoxin contamination of stored maize

The development of aflatoxigenic and other storage fungi in stored maize is principally a function of inoculum availability, moisture, and temperature of the grain in addition to other factors such as aeration, and pest activity (Wilson and Abramson, 1992). Fungi, including *A. flavus* are generally incapable of growing in water activity below 0.70. The optimum water activity for growth of *A. flavus* group ranges between 0.91 and 0.99. In mature maize kernels, *A. flavus* does not exhibit extensive growth below a_w of 0.85 (Wilson and Abramson, 1992). Spore formation and sporulation however do occur at a_w of 0.81 and 0.83 respectively. At slightly higher a_w levels of 0.87, *A. flavus* grows and produces aflatoxins. Very high water activity however, induces heavy colonization by competing mycoflora such as *A. oryzae*, *A. niger* and *Rhizopus nigricans* that can significantly reduce growth of *A. flavus* (Sauer and Burroughs, 1986).

Moisture distribution within stored maize is critical as high moisture and low moisture grain equilibrate during storage. Depending on the moisture gradient and on whether grains are adsorbing or desorbing, kernels may equilibrate to a moisture level that permits development of *A. flavus* and subsequent production of aflatoxins. Aflatoxigenic moulds can grow and elaborate toxins in

localized spots of high moisture. Even when grain is stored at safe initial moisture content of 13 %, the xerotolerant moulds such as the *A. glaucus* group continue to exhibit slow growth and predominance at 13 to 15 % moisture. At 15 % moisture, the less xerotolerant fungi such as *A. ochraceous*, *A. versicolor*, and *A. flavus* begin to grow. Although the growth ability of *A. flavus* is greatly inhibited at this moisture level, the rapid growth of the xerotolerant fungi creates localised wet spots enhancing conditions that favour growth of *A. flavus* (Wilson and Abramson, 1992).

Establishment and active growth of fungi in the microenvironment of storage not only increases grain moisture but also raises the temperature of stored grain. Temperature differences within stored maize enhance moisture migration from warmer to cooler areas thus raising the moisture content of the entire bulk. Roof leaks and vermin activity in the storage facility also elevate the moisture levels. Significant levels of aflatoxins are formed in a matter of hours when the requisite factors exist within the optimal range. However, subtle environmental changes could dramatically vary the final levels of toxin since the *A. flavus* can simultaneously synthesise and degrade the toxin (Hassan and Lyold, 1995).

2.4 DETOXIFICATION OF AFLATOXIN CONTAMINATED FOODS

Foods contaminated with aflatoxins can be decontaminated by either removal of the toxin through extraction with appropriate food grade solvents, adsorption from aqueous foods using adsorptive substances, physical separation of the highly contaminated fractions or by degradation of the toxin through treatment with physical or chemical agents to less toxic derivatives.

The toxicological activity of aflatoxins is mainly attributed to the ability of aflatoxins to bind DNA and cellular proteins via the double bond of the terminal furan ring. The lactone ring in the coumarin moiety also serves as an auxiliary site for aflatoxin toxicity (Samarajeewa *et al*., 1990).

Physico-chemical treatments for the degradation of aflatoxins target removal of the double bond of the terminal furan ring or opening the lactone ring of the coumarin moiety. Once the lactone ring is opened, further reactions occur that alter the binding properties of the terminal furan ring to DNA and other cellular proteins. Such structural changes could be induced through application of energy in a form absorbable by the toxin or treatment of aflatoxin with chemicals that block or remove the active sites or modify the physico-chemical properties of the molecule (Samarajeewa *et al.*, 1990). Some aflatoxin degradation reactions are shown in Figures 2.2 and 2.3, and the relative toxicity of some of the reaction products given in Table 2.1.

Table 2.1 Relative toxicity of some derivatives of AFB_1 degradation

Aflatoxin	Biological effects
AFB_1	Highly toxic/ carcinogenic to experimental animals; mutagenic to *Salmonella typhimurium*
AFB_2	2.4 times less toxic to ducklings, 500 times less mutagenic
AFB_{2a}	More than 200 times less toxic to ducklings, 1000 times less mutagenic
AFB_{1S}	Unknown toxicity; thought to be non-toxic
AFD_1	18 times less toxic to chick embryo; 450 times less mutagenic
AFG_1	1.6 times less toxic to ducklings; 30 times less mutagenic
AFM_1	3 times less carcinogenic to rainbow trout; same order of toxicity to ducklings as AFB_1, 30 times less mutagenic
AFP_1	15 times less toxic to mice; 1000 times less mutagenic
AFQ_1	18 times less toxic to chick embryos; 83 times less mutagenic

Source: Samarajeewa *et al.*, 1990

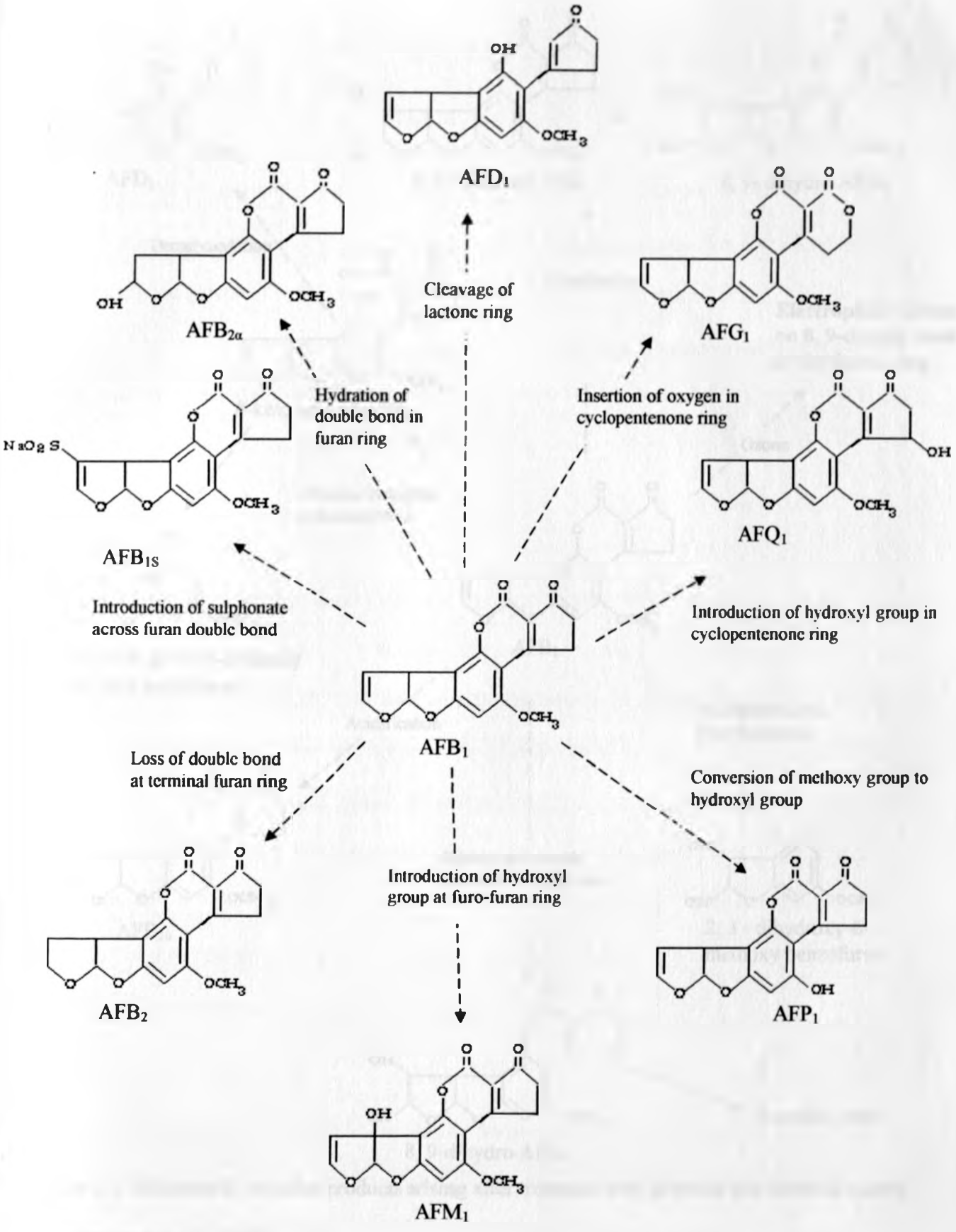

Figure 2.2: Some degradation derivatives of AFB_1 (Samarajeewa *et al.*, 1990)

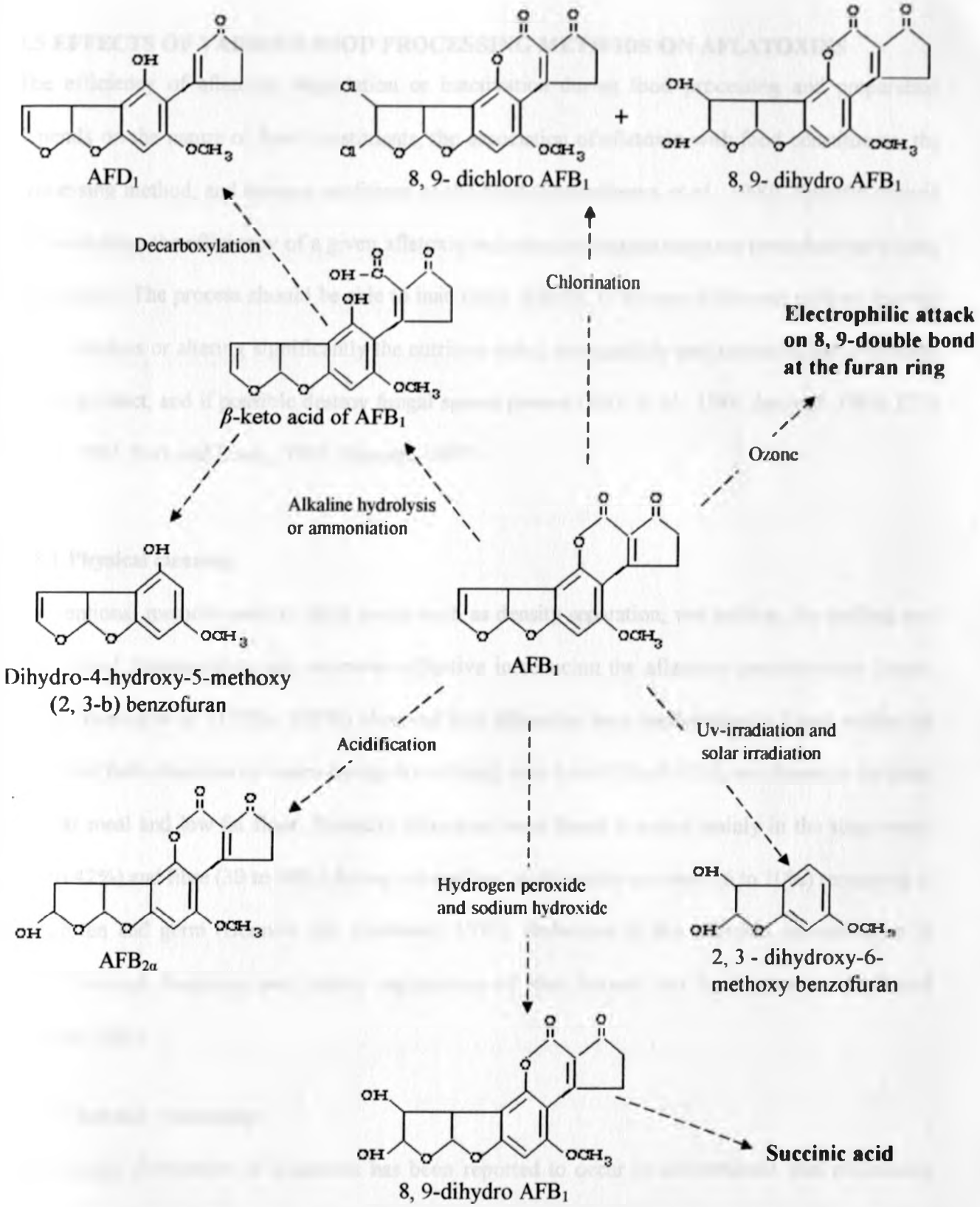

Figure 2.3: Aflatoxin B_1 reaction products arising after treatment with physical and chemical agents (Samarajeewa *et al.*, 1990).

2.5 EFFECTS OF VARIOUS FOOD PROCESSING METHODS ON AFLATOXINS

The efficiency of aflatoxin degradation or inactivation during food processing and preparation depends on the nature of food constituents, the association of aflatoxin with food constituents, the processing method, and storage conditions of the food (Samarajeewa *et al.*, 1990). Specific criteria for evaluating the efficiency of a given aflatoxin reduction or decontamination procedure have been established. The process should be able to inactivate, destroy, or remove aflatoxins without leaving toxic residues or altering significantly the nutritive value, acceptability and technological properties of the product, and if possible destroy fungal spores present (Park *et al.*, 1988; Jemmali 1989; Ellis *et al.*, 1991; Park and Liang, 1993; Rustom, 1997).

2.5.1 Physical cleaning

Conventional methods used to clean maize such as density separation, wet milling, dry milling and preferential fragmentation are somewhat effective in reducing the aflatoxin contamination (Scott, 1984). Brekke *et al.* (1975a; 1975b) observed that aflatoxins were predominantly found within the germ and hulls fractions of maize during dry milling; only 6 to 10 % of AFB_1 was found in the grits, low fat meal and low fat flour. Similarly aflatoxins were found to occur mainly in the steep water (39 to 42%) and fibre (30 to 38%) during wet milling, with smaller amounts (6 to 10%) remaining in the gluten and germ (Bennett and Anderson, 1978). Reduction of the aflatoxin concentration in maize through floatation and density segregation of toxic kernels has been reported (Huff and Hangler 1985).

2.5.2 Thermal processing

Only partial destruction of aflatoxins has been reported to occur in conventional heat processing procedures (Samarajeewa *et al.*, 1990). Aflatoxins are relatively resistant to thermal inactivation; the thermal decomposition temperature of AFB_1 is 269°C (Ciegler and Vesonder, 1983; Cole and Cox,

1981). Temperatures as high as 300 °C were reported to be necessary for degradation of AFB_1 in certain foods (Fischbach and Campell., 1965).

Roasting of maize at 145 to 165 °C was reported to reduce AFB_1 concentration by 40 to 80 % when loss of moisture is not considered (Conway *et al.*, 1978). Twenty-eight percent (28 %) loss of AFB_1 was reported during boiling of naturally contaminated maize grits, while 33 to 53 % aflatoxin reduction was observed when the boiled grits were fried (Stoloff and Trucksess, 1981; Price and Jorgensen, 1985).

Only thirteen percent (13%) loss of AFB_1 was reported when maize meal was made into corn muffins (Stoloff and Trucksess, 1981). Boiling contaminated maize flour in water to make *ugali*, a traditional African dish, destroyed only 11.5 and 17.6 % of the B and G aflatoxins respectively (Seenappa and Nyagahungu, 1982). Considerable reduction in aflatoxins has been associated with treatment of maize with limewater (nixtimalization) in the process of making tortillas (Abbas *et al.*, 1988).

The extent of destruction of aflatoxins by heat is a function of the initial level of contamination, temperature, pressure, time, type of food, type of aflatoxin, moisture content, pH and ionic strength of the food (Rustom, 1997). In the presence of moisture, AFB_1 is thought to undergo hydrolytic opening of the lactone ring to form a terminal carboxylic acid, which then undergoes heat driven decarboxylation (Coomes *et al.*, 1966). Slightly higher sensitivity of AFB_1 to thermal degradation occurs in cooking methods where moisture is not a limiting factor as compared to cooking methods that involve dry heat (Rustom, 1997). Presence of ionic salts such as sodium chloride was reported to increase the extent of aflatoxin degradation by heat (Farah *et al.*, 1983). Likewise, the effect of pH on aflatoxin degradation has also been studied. Aflatoxin B_1 was converted to a less toxic

derivative, AFD_1, during heat treatment in high alkaline pH (Rustom *et al.*, 1993). Similar heat treatment at low pH also resulted in higher aflatoxin degradation as compared to neutral or near neutral pH range. This was attributed to increased hydrolysis of aflatoxins at the lactone ring in alkaline pH and partial hydration of the terminal furan ring in acidic pH, implying synergistic effect of pH and heat in aflatoxin degradation. Microwave heating was reported to have greater potential for degradation of aflatoxin but this was dependent on energy level, treatment time and the type of food (Luter *et al.*, 1982; Pluyer *et al.*, 1987).

The need to use elevated temperatures and high pressure for effective detoxification of contaminated foods, however, hinders exploration of heat treatment as a practical means of aflatoxin detoxification. The impairment of nutritional and organoleptic qualities and doubts concerning the generation of toxic pyrolysates at high temperature further discourage use of heat treatment as a tool for decontamination of aflatoxin-contaminated foods (Samarajeewa *et al.*, 1990).

2.5.3 Irradiation

Aflatoxin B_1 absorbs ultraviolet (UV) radiation at 222, 265, and maximally at 362 nm. Irradiation activates AFB_1 hence increasing its susceptibility to degradation. The effect of UV radiation on AFB_1 occurs at the terminal furan ring (Samarajeewa *et al.*, 1990). Solar irradiation, which possesses energy in the UV and visible spectra, has been shown to have greater efficiency in degrading aflatoxins in edible oils (Samarajeewa *et al.*, 1987). Photo-degradation of aflatoxins in solid foods is minimal as the toxin is bound and protected within the food matrix (Tabata *et al.*, 1992). The possibility of formation of photo-degradation compounds with residual toxicity also limits the prospects in use of UV radiation for degradation of aflatoxins in foods (Nkama *et al.*, 1987).

2.5.4 Chemical inactivation of aflatoxins

Numerous chemicals including chlorinating agents, oxidising agents, acids and alkalis have been screened for their ability to detoxify aflatoxins. Some of the chemicals are already in use in the food industry as food additives or for other purposes.

(1) Chlorine

Aqueous chlorine is used in the food industry to sanitize surfaces and processing equipment and to wash a variety of raw materials including fruits, nuts, meats, and fish, prior to processing. Gaseous chlorine and sodium chlorite have been used as bleaching agents in the flour industry with no signs of hazards (Wei *et al.*, 1985). Sodium hypochlorite is recommended for removing aflatoxins from contaminated surfaces and glassware (Wei *et al.*, 1985). Chlorination with sodium hypochlorite at concentration of 0.2 % (Rhee *et al.*, 1976), 1 % (Mann *et al.*, 1970), 5 % (Fischbach and Campell, 1965) or 11 % (Draughon and Childs, 1982) with 3 % perchloric acid (Chakrabaarti, 1981) or with gaseous chlorine at 10 % (Fischbach and Campell, 1965) or at concentrations of 15 mg chlorine per 100 mg of pure AFB_1 (Sen *et al.*, 1988) was shown to almost totally degrade AFB_1 either in its pure form or in spiked foods. Complete degradation of aflatoxins was also demonstrated when pure aflatoxin and artificially spiked maize were treated at 60 °C with 0.25 % sodium chlorite (pH 4) for 48 h (Tabata *et al.*, 1994). The mode of action of sodium chlorite is considered to be similar to that of sodium hypochlorite; both are either oxidising or chlorinating agents depending on the pH.

(2) Hydrogen peroxide

Hydrogen peroxide is ranked high among possible chemicals for degrading aflatoxins because of its high efficiency, low cost, and ready availability (Rhee *et al.*, 1976; Liuzzo and Ochomogo, 1980). Hydrogen peroxide present in treated foods degrades easily and has no residual toxic effects (Samarajeewa *et al.*, 1990). Different treatments with hydrogen peroxide have been reported to

degrade AFB_1 with little or no effect on food quality. Treatment of groundnut protein isolate (pH 4), maize, and peanut meal (pH 9) with 0.5 %, 3 %, and 6 % hydrogen peroxide respectively were reported to degrade AFB_1 almost completely with a mild effect on food quality (Rhee *et al.*, 1976; Chakrabaarti, 1981). Foods treated with hydrogen peroxide, however, may support fungal growth; low levels of residual hydrogen peroxide in treated foods have been shown to promote fungal growth (El-Gazzar and Marth, 1988).

(3) Bisulphite

Sodium bisulphite, a common food additive in the food industry, is thought to inactivate aflatoxins by causing cleavage of the lactone ring or addition of sulphite group at the terminal furan ring, or both. The dominant inactivation reaction was confirmed to result in formation of the AFB_1 sulphonate (Hagler *et al.*, 1983; Yagen *et al.*, 1989). Concerns about the low efficiency of bisulphite in inactivating AFB_2 and AFG_2 and the paucity of information on possible regeneration of AFB_1 or the toxic entity, mainly the epoxide during metabolism of treated samples limit the progress on industrial exploitation of such treatment (Samarajeewa *et al.*, 1990)

(4) Ozone

Ozone, a powerful oxidising agent reacts across the double bond of the furan ring through electrophilic attack. In 4 % dimethyl sulphoxide at room temperature, ozone was found to degrade pure AFB_1 and AFG_1 effectively within a few minutes (Maeba *et al.*, 1988). The treatment products were confirmed to be non-toxic to experimental rats and chick embryos and non – mutagenic in the Ames test. Ozone was reported to reduce AFB_1 by 19 % in cottonseed meal containing 22 % moisture content within two hrs. However, AFB_2 and AFG_2 were reported to have high resistance to ozone treatment (Samarajeewa *et al.*, 1990).

(5) Ammonium persulphate

Ammonium persulphate is used in the flour milling industry as flour improver and bleaching agent. Tabata *et al.* (1994) demonstrated the potency of ammonium persulphate in degradation of aflatoxins. Complete loss of AFB_1 was reported following treatment of artificially spiked maize with 0.25 % ammonium persulphate at 60 °C for 48 h. Similarly, ammonium persulphate was also shown to degrade pure aflatoxin within 2 h at room temperature (Tabata *et al.*, 1994).

(6) Alkali

Alkaline treatment causes aflatoxin degradation through hydrolysis of the lactone ring. Treatment of vegetable oils with 11 % sodium hydroxide during extraction and processing operations caused partial degradation of AFB_1 (Cruz, 1988). Degradation of aflatoxins in solid foods by alkaline treatment was also demonstrated in cotton meal (Mann et al., 1970), maize (Moerck *et al.*, 1980), distillery protein concentrates and peanut meal. The cooking of peanut meal (containing 30% moisture) with 20 % aqueous sodium hydroxide at 100 °C for 90 min reduced the aflatoxin levels from 111 to 17 ng/g. (84.6 % reduction).

Although aflatoxin degradation by alkaline treatment may be reversible in acidic environments such as in the stomach (Tabata *et al.*, 1994; Samarajeewa *et al.*, 1990), substantive levels of aflatoxins can be removed by steeping contaminated foods in alkaline solutions followed by washing with water (Tabata *et al.*, 1994), since opening the lactone ring increases the solubility of aflatoxins in water.

(7) Acids

Treatment of AFB_1 with acid leads to hydration at the double bond of the terminal furan ring (Dutton and Heathcote, 1968) to form AFB_{2a} which exhibits less toxicity. The use of strong acids is

however discouraged because of their effect on the quality of many foods (Samarajeewa *et al.*, 1990).

2.5.5 Microbiological inactivation

Micro-organisms including yeasts, moulds and bacteria, have been screened for their ability to modify or inactivate aflatoxins. *Flavobacterium aurantiacum* (NRRL B- 184) was shown to remove aflatoxin from a liquid medium significantly without the production of toxic by-products. Hao *et al.*, (1987) reported the removal of AFB_1 from groundnut milk by *F. aurantiacum* without inhibition by presence of the toxin. Certain acid producing moulds have also been reported to catalyse the hydration of AFB_1 to AFB_{2a}, which is 200 times less toxic (Marth and Doyle, 1979).

2.6 ANALYSIS OF AFLATOXINS IN FOODS

2.6.1 Variability in aflatoxin analysis

Estimation of the true concentration of aflatoxins in a contaminated lot with high degree of confidence is difficult. Aflatoxin test results taken from a contaminated lot often show wide variability attributed to errors in sampling, sub-sampling and analytical procedures (Park and Whitaker, 1994).

(1) Sampling variability

The sampling step constitutes the largest source of error in aflatoxin analysis because aflatoxins may be found only in a small percentage of the kernels in a lot, and the concentration of aflatoxins in individual kernels may be extremely variable. Experimentally, the sampling variability associated with maize was estimated as follows by Whitaker and co-workers (1979): -

$V_S = 3.9539[a]/w$

Where:

V_S = sampling variation

[a] = Aflatoxin concentration in the lot in ng/g

w = mass of the sample in Kg

(2) Sub-sampling variability

Since extraction of aflatoxins from a large sample is not possible, extraction is done from a much smaller portion referred to as a sub-sample. The distribution of aflatoxin-contaminated particles in a ground sub-sample is assumed to be similar to the distribution of aflatoxin-contaminated kernels in the sampled lot. However, variability in aflatoxin content also exists among replicated sub-samples but the sub-sampling variance is much smaller compared sampling variance depending on the degree of communition of the primary sample (Park and Whitaker, 1994).

(3) Analytical variability

Considerable variation arises among replicated analyses of the same sub-sample extract because analytical methods for aflatoxins involve several sequential steps including sample extraction, extract cleanup, extract dilution or concentration and quantification (Park and Whitaker 1994). Various workers have described variability associated with various analytical methods for aflatoxins as follows:

Immunoassays: $V_A = 0.01327[a]^{1.5651}/n$ (Park *et al.*, 1991),

High Performance Liquid Chromatography: $V_A = 0.004828[a]^{1.7518}/n$ (Wilson and Romer, 1991),

Thin Layer Chromatography: $V_A = 0.0699[a]^{2}/n$ (Park *et al.*, 1991).

Where:

V_A = Analytical variability

[a] = Aflatoxin concentration in the sub-sample

n = Number of aliquots quantified

In order to obtain a more accurate estimate of lot contamination level, the overall variability associated with aflatoxin analysis is thus minimised by increasing the size of primary sample, the size and degree of communition of sub-sample, and use of a precise method for quantification.

2.6.2 Methods for analysis of aflatoxins in foods

2.6.2.1 Chromatographic methods

(1) Thin Layer chromatography

Thin layer chromatography (TLC) is the most widely used method of aflatoxin analysis. The first analytical method using TLC was published in 1966 and currently numerous TLC methods have been officially adopted by the association of official analytical chemists (AOAC, 1995) for identification and quantification of aflatoxins.

Thin layer chromatographic technique for analysis of aflatoxins involves spotting the sample extract on an adsorbent-coated plate and developing the plate in a glass or metal tank with the appropriate mobile phase, usually chloroform-acetone (88:12) in a non-equilibrated tank. Cleanup and concentration of sample extract is often necessary prior to spotting. The developed plate is then observed under long wavelength UV light and the intensity of fluorescence of the sample spot compared with that of standards visually or using a fluorodensitometer. The need for prior cleanup of sample extract or use of high performance TLC plates coupled with automated instrumentation for reliable quantitative tests makes TLC an expensive and cumbersome method for repeated testing or analyses where many samples need to be analysed (Whitaker *et al.*, 1990).

(2) Liquid chromatography (LC)

Liquid chromatography methods for determination of aflatoxins in foods include normal-phase liquid chromatography (NPLC) and reversed-phase liquid chromatography (RPLC). In NPLC, the

analyte is applied into a column packed with silica gel and toxin concentration determined using a fluorescence detector after elution with a suitable mobile phase. Normal-phase liquid chromatography is however associated with decreased resolution and lowered fluorescence response of toxins caused by the irreversible adsorption of sample matrix onto the stationary phase. In reversed-phase liquid chromatography, the stationary phase is modified with C_{18} while the mobile phase is a mixture of water, methanol and acetonitrile to enhance resolution. In addition, since some aflatoxins such as AFB_1 and AFG_1 do not fluoresce in the aqueous mobile phase, pre-or post-column derivatization procedures are applied to increase sensitivity where fluorescence detectors are to be used; alternatively, electrochemical detector is used (Wood and Trucksess 1994). Although RPLC is among the most effective analytical techniques for aflatoxins, long analytical times, intense labour, expensive instrumentation, and the experience required to solve separation and interference problems are major hindrances to its use in routine aflatoxin analysis.

2.6.2.2 Immunochemical methods

Immunochemical methods for aflatoxin analysis are based on reactions that involve equilibrating aflatoxins (antigens) with their corresponding antibodies (Morgan, 1985). Various types of immunochemical methods are used in aflatoxin analysis including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and immunoaffinity column assay (ICA). Important considerations in these techniques include presence of a good marker aflatoxin derivative, an appropriate technique for separation of the free and bound forms of toxin after toxin-antibody equilibration, good sensitivity and specificity of antibody, and suitable technique for minimising sample matrix interference (Chu, 1994a; 1994b). Numerous sensitive and easy to use immunoassay kits have been developed and adopted for both qualitative and quantitative analysis of aflatoxins.

(1) Radio Immuno - Assay (RIA)

Radio immuno - assay was first developed in 1959 for determination of insulin. In the analysis of aflatoxins, RIA involves comparing the residual radioactivity of the unknown sample with that of known aflatoxin standards after simultaneous incubation with specific antibody and constant amount of radioisotope-labelled aflatoxin marker. The concentration of aflatoxin in the sample is inversely proportional to the radioactivity observed after separation of the unbound aflatoxin and is quantified by plotting a standard curve of the ratio of radioactivities of the bound fraction and free fraction versus log concentration of unlabelled standard toxin.

Although RIA is quite sensitive, the method requires sample cleanup and use of radioactive markers that have high specific-activity for enhanced sensitivity. In addition, elaborate methods involved in separation of the free and bound toxins, and the hazardous nature of radioisotopes such as ^{14}C, ^{3}H, and ^{125}I, which presents disposal difficulties, discourage widespread adoption of RIA for routine analysis of aflatoxins (Chu, 1994b).

(2) Competitive Enzyme Linked Immuno-Sorbent Assay (ELISA)

Enzyme linked immuno-sorbent assay was developed in 1971. Both direct and indirect ELISAs for aflatoxin analysis involve equilibrating aflatoxins (antigen) with specific antibodies (anti-aflatoxins), either of which may be linked to an enzyme marker.

In the direct competitive ELISA, anti-aflatoxin antibodies are first coated to a solid phase such as polystyrene beads, polystyrene tubes, nylon beads, or microtiter wells (Chu, 1991). A diluted test sample extract and aflatoxin standards are then added to the antibody coated solid-phase and incubated simultaneously with aflatoxin-enzyme conjugate, usually, horseradish peroxidase. After appropriate washings to separate unbound aflatoxins, a substrate solution comprising of hydrogen

peroxide and appropriate oxidizable chromogen is added. Intensity of enzyme-mediated oxidation of the chromogen is measured spetrophotometrically or compared visually with the standards. Since aflatoxins in the test extract or standards compete with the aflatoxin-enzyme conjugate for the same sites on the antibody coated solid phase, the intensity of colour resulting from the enzyme reaction is inversely proportional to the concentration of toxin in the test sample extract.

In the indirect competitive ELISA, an aflatoxin-protein conjugate is coated onto the solid-phase such as microtiter wells. Solutions of anti-aflatoxin antibody and known concentration of aflatoxin standards and sample extracts are incubated together. Aflatoxin in the test-sample and the aflatoxin bound onto the solid phase via aflatoxin protein-conjugate compete for the same binding sites with the specific antibody in the solution. The amount of aflatoxin bound to the plate is determined by reaction with an appropriate anti-antibody-enzyme complex and by subsequent reaction with the substrate. Although indirect ELISA requires much fewer antibodies, no preparation of toxin-enzyme conjugate and has been used for the analysis of aflatoxins with sensitivity comparable to or higher than that of direct ELISA, the latter is preferred because of the shorter analytical times needed (Chu, 1994b).

(3) Immunoaffinity Column Assay (ICA)

In the ICA procedure, an affinity column containing specific anti-aflatoxin antibody binds the aflatoxins, which are then eluted from the column using suitable solvent such as methanol for subsequent measurement (Chu, 1991). Immunoaffinity chromatography is used mainly as a cleanup and concentration tool prior to analysis of aflatoxins.

(4) Immunochromatography

This technique involves the separation of aflatoxins in HPLC or TLC followed by analysis of each individual fraction eluted from the HPLC column or TLC plate by ELISA. This approach is used to identify as well as quantify aflatoxins where high degree of specificity is required (Chu, 1994a).

2.6.2.3 Biological methods

These methods are based on observation of toxic effects of aflatoxins such as death, pathological lesions, and biochemical alterations in biological systems and/ or organisms to assess general toxicity of aflatoxins (Yates, 1986). Besides the low specificity and sensitivity of bio-assays, the many parameters that must be controlled in the toxicity test such as the type and amount of delivery reagent, route and time of application and long periods of observation for the events occurring in specific target organs make these methods impracticable for regular surveillance of aflatoxins in foods or feeds (Chu, 1994a).

2.7 METHODS FOR ENUMERATION OF AFLATOXIGENIC MOLDS

2.7.1 Cultural methods

(1) General-purpose media

Although no single medium is available for enumeration of all fungi in foods, most fungi are detected using well-designed high-water activity media. The general-purpose medium used should suppress bacterial growth, be nutritionally adequate, induce compact colony formation and suppress spreading fungi (Hocking and Pitt, 1992).

A wide range of general-purpose media for growth of fungi is available including buffered yeast agar, Czapek's solution agar, malt extract agar, mycological agar, glucose yeast agar, plate count agar modified with antibiotics, Whey agar, Wort agar, Sabouraud's Dextrose agar and Potato

Dextrose agar. The choice of medium used depends on the natural habitat of the fungi and the purpose of culturing.

Czapek's solution agar is widely used for macroscopic identification of moulds because of its ability to enhance growth of aerial mycelia and sporulation (Jarvis, 1987). Colonies of *A. flavus* on Czapek's solution agar are yellow-green in colour with coarsely roughened conidiophores that rise up to 1mm in length. The conidial heads are predominantly biseriate, radiate or columnar. Colonies of *A. parasiticus* are much richer green in colour than those of *A. flavus* with roughened conidiophores, which do not rise beyond 0.4 mm in length. The conidial heads are predominantly uniseriate (Kozakiewicz, 1994).

Both Potato Dextrose agar and Sabouraud's Dextrose agar are generally used for enumeration of total mould counts in foods but the later is considered superior because it contains extra polypeptides that promote mould recovery.

(2) Selective media

General media for mycological examination are made selective by incorporating chemical inhibitors such as some fungicides, antibiotics, acids, adjusting the water activity or by way of limiting nutrient(s). In acidification, media pH is adjusted to the range of 3.5 to 4.5. Organic acids such as tartaric, lactic and citric acid are commonly used. Composition of the media and the type of acid used is critical in determining mould recovery under acid conditions (Jarvis, 1987). The limitations of use of acids to create selective growth conditions for moulds include inability to suppress acid tolerant bacteria and acid enhancement of spreading of fungal colonies, which makes enumeration difficult. Since many fungi are capable of growing well at neutral or slightly alkaline pH values, use of chemical inhibitors is considered superior to acidification in media intended for enumeration of

moulds (Jarvis, 1987). Gentamycin and chloramphenicol are preferred to other antibiotics such as chlortetracycline, streptomycin, oxytetracycline, kanamycin and rose-bengal because of their ability to inhibit a wide range of bacteria and remain stable in the medium without affecting growth of the fungi. Other chemical inhibitors are also used with the advantage of restricting growth of undesired fungal types while promoting the growth of the desired ones. Botran (2, 6-dichloro-4- nitroaniline), a fungicide, is the chemical inhibitor for spreading fungi used in media for selective isolation of *A. flavus* and *A. parasiticus* (Pitt *et al.*, 1983).

(3) Differential Media

(i) Aspergillus Differential Medium (ADM)

Bothast and Fennell (1974) developed Aspergillus differential medium containing 1.5 % tryptone, 1.0 % yeast extract, 1.5 % agar and 0.05 % ferric citrate for presumptive detection of aflatoxigenic fungi. The reaction between ferric citrate and acidic fungal metabolites such as kojic and aspergillic acids gives orange-yellow reverse colouration (Pitt *et al.*, 1983) in agar plate colonies of *A. flavus* and *A. parasiticus*. On this medium, colonies of *fusarium* and *penicillium* are capable of growing although they do not produce the orange-yellow reverse colouration. However, since typical colonies in this medium are detected after 5 days of incubation at 28 °C, other *Aspergillus* group species including *A. ochraceous*, *A. oryzae* and *A.niger* get the chance to grow and produce the pigment (false positives) because of the prolonged incubation time (Pitt *et al.*, 1983).

(ii) Aspergillus Flavus and Parasiticus Agar (AFPA)

The ADM by Bothast and Fennell (1974) was modified by Pitt *et al.* (1983) to *Aspergillus Flavus* and *Parasiticus* agar, containing yeast extract (2 %), bacteriological peptone (1 %), ferric ammonium citrate (0.05 %), agar (1.5 %), chloramphenicol (0.01%), and 0.0002 % Botran (2,6.dichloro-4-nitroaniline). *Aspergillus flavus* and *A. parasiticus* are identified on this medium by

production of typical yellow to olive green spores and a bright orange reverse within 42 h of incubation at 30 °C. On this medium and incubation conditions, *A. niger* produces a yellow reverse colour and after 48 h begins to develop dark brown to black conidia while *A. ochraceous* grows slowly and the yellow colour appears after 48 h thus enabling identification of potentially aflatoxigenic fungi within 42 h of incubation.

2.7.2 Immunological techniques

These techniques are based on detection of specific cell-wall associated antigens through serological techniques. The genus specific antigens are not found in non-mouldy food samples hence this provides potential for use of immunoassays for detection of aflatoxigenic moulds in foods (Kamphis and Notermans, 1992). Although these assays are highly sensitive, easy to perform, and provide an index of the level of mould contamination of the sample, ability to detect moulds with higher specificity beyond the genus level may be limited (Suzhen *et al.*, 2000).

2.8 SCREENING OF *Aspergillus flavus* ISOLATES FOR AFLATOXIGENICITY

2.8.1 Cultural method

In this method, the fungi are allowed to grow on testing media such as aflatoxin producing ability agar (APA) enriched with corn steep liquor or coconut agar medium (CAM) enriched with coconut extract. Aflatoxin producing strains of *Aspergillus spp.* are detected by blue fluorescence of the agar medium surrounding colonies, within 2 to 10 days of incubation depending on their aflatoxigenic potential and incubation conditions (Wei *et al.*, 1984). Though rapid, the detection of aflatoxigenic moulds by way of detecting fluorescence of the culture medium is limiting in that other structurally related substances in the medium may also fluoresce at similar wavelengths as aflatoxins. Further more, strains with low aflatoxigenic ability may not be detected.

2.8.2 Agar plug screening method

In this technique, the presence of aflatoxin in the culture medium or in colony mycelia is detected by spotting eluate of carefully cut agar plugs on TLC plates (Filtenborg and Frisvad, 1980).

The presence of aflatoxin is determined by comparing the relative mobility (r_f values) of fluorescing spot(s) with those of standard toxin spotted along side the agar plug when the chromatogram is developed in a suitable solvent. Presumptive identification of the type(s) of aflatoxins present can also be done on basis of relative mobility of fluorescing compounds in the eluate and by derivatization of eluate with trifluoracetic acid (AOAC, 1995).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 SAMPLING AND SAMPLE PREPARATION

Seventy-two (72) aflatoxin-contaminated maize samples, previously sampled from Makueni, Machakos and Kitui districts during the May-June 2004 foodborne aflatoxicosis outbreak, were obtained from the National Public Health Laboratory Services (NPHLS). Samples that contained aflatoxin levels between 20 and 400 ppb as per the NPHLS analysis done in July and August 2004 were selected for this study.

3.1.1 *Muthokoi* processing

Forty-eight (48) samples, each weighing 1 to 2 kg and having varying levels of aflatoxin-contamination were randomly selected. One-kilogram portion was drawn from each of the selected samples and divided into two equal sub-samples of approximately 500 g each using a 25 g-sampling cup. One sub-sample from each pair was randomly selected and analysed for total aflatoxins and mould contamination (whole-grain maize sample) while the other was set aside for processing into *muthokoi* as per the traditional procedure shown in Figure 3.1.

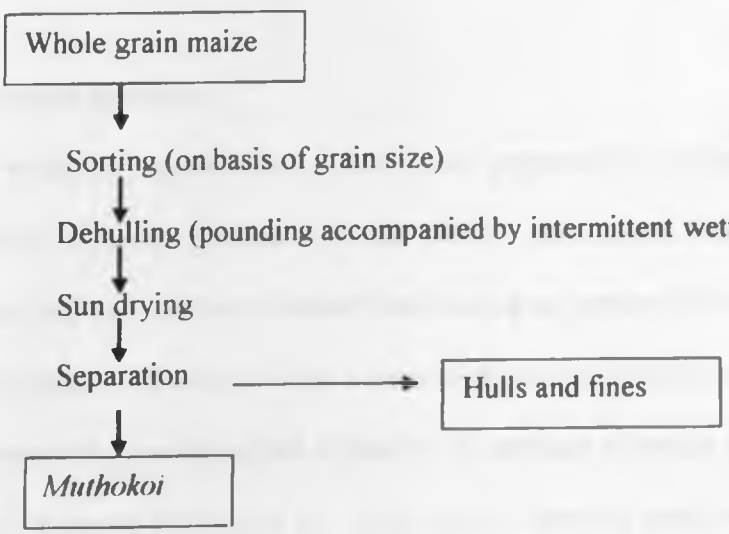

Figure 3.1: Flow chart for traditional *Muthokoi* making procedure

Using similar aflatoxin-free dry maize samples, a pre-trial was conducted to establish the amount of water and duration of pounding needed to attain optimal dehulling of maize grains as practised in the traditional *muthokoi* making process. An amount of water equivalent to 10 % grain weight, added intermittently, and pounding for 30 min was found to be adequate for optimum dehulling of 500 g maize portions in a medium size wooden motor and pestle. This method was adopted for dehulling maize samples.

The sub-samples set aside for *muthokoi* making were randomly divided into four groups of 12 samples each. Wetting of maize grains was done with distilled water in one group (group I), and with aqueous solutions of ammonium persulphate (1 % w/v), sodium hypochlorite (1 % v/v), and local alkaline mineral salt, *iati* (1 % w/v) in groups II, III and IV respectively. The dehulled maize grains (*muthokoi*) together with the hulls (by-product) were spread on sheets of Kraft paper and sun-dried for 2 hours. *Muthokoi* was separated from the by-product (comprising hulls and fines) by sieving through a $2mm^2$ aperture sieve to recover the fines, followed by hand winnowing to recover the hulls. Both *muthokoi* and the by-product were analysed for total and *Aspergillus flavus* mould counts and aflatoxin levels.

3.1.2 Treatment with chemical additives

Reference samples, each weighing approximately 15 kg, were prepared by mixing several of the aflatoxin-contaminated maize samples obtained from the NPHLS in a rotary mixer. Each of the resultant reference samples lots was randomly divided into two equal portions and one portion de-hulled as in the *muthokoi* process. Aflatoxin levels in each of the above dehulled and whole grain reference lots were determined by analysing and obtaining the average aflatoxin content of three 500 g sub-samples drawn randomly from each lot. Experimental samples, each weighing 100 g, were randomly drawn from the reference portions and treated separately in 1000 ml of distilled

water (control) and in 0.2 %, 0.5 %, and 1.0 % solutions of *iati*, sodium hypochlorite, and ammonium persulphate at room temperature for 6 and 12 h. The steep liquor was discarded, samples washed three times in tap water and dried in hot air oven (70 °C) for 12 h. Other 100 g portions were randomly drawn from whole-grain maize and *muthokoi* reference lots and boiled separately in 1000ml of distilled water (control) and 0.2 %, 0.5 %, or 1 % (w/v) solutions of *iati* in open aluminium kettle for 150 min. The boiling solution was drained off and samples dried in hot air oven (70 °C) for 12 h. The treated samples were analysed for residual aflatoxin levels.

3.2 SAMPLE PROCESSING AND AFLATOXIN ANALYSIS

3.2.1 Sample preparation

Entire experimental portions, of either 500 g or 100 g, were coarsely ground using an attrition mill. Sub-samples weighing 50 g were randomly drawn from each of the coarsely ground samples and further ground using a hammer mill fitted with 1mm sieve. Sub-samples were preserved at -15 °C awaiting aflatoxin analysis and mycological examination.

3.2.2 Aflatoxin extraction

Extraction of aflatoxins from samples was done according to the AOAC official method 990.32 (AOAC, 1995) with modifications. Five gram portions were drawn from each of the finely ground sub samples and mixed with 25 ml of methanol-water (50:50). The mixture was stirred for 20 min using a magnetic stirrer and filtered through Whatman No. 1 filter paper. Extracts were stored in the dark at -15 °C and analysed within 24 hours.

3.2.3 Aflatoxin determination

Samples were analysed for total aflatoxin levels using a competitive enzyme-linked Immuno sorbent assay as described by Gathumbi *et al.*, (2001). Ninety-six (96) well microtiter plates (Maxisorp®

F96) were coated by adding 100µl of anti-aflatoxin antibody diluted in bicarbonate buffer (1:15 000) into each well. The plates were incubated overnight at room temperature in a humid chamber. A 200 µl solution of 3 % foetal calf serum (FCS) in phosphate buffered saline (PBS) was added into each well and the plates incubated at room temperature for 30 min to block non-specific binding sites. Plates were then washed three times with washing solution (Appendix 3) and semidried by tapping plate on blotting paper just before use.

Aflatoxin B_1 standards were prepared by diluting a stock solution (15 µg/ml) purchased from Sigma Immunochemicals, in 10 % methanol-PBS to obtain working dilutions of 1 ppb, 0.333 ppb, 0.111 ppb, 0.037 ppb, and 0.012 ppb. A 10 % methanol-PBS solution was prepared and used as a standard blank. Test extracts were prepared for analysis by diluting the methanol content to 10 % using PBS followed by further dilution in 10 % methanol-PBS to reduce the aflatoxin concentration to the sensitivity range of the assay.

Aliquots of 50 µl of toxin standards (4 replicates) and sample extracts (2 replicates) were added in designated wells of the coated microtiter plate (Appendix 4). This was followed immediately by addition of 50 µl of aflatoxin-enzyme conjugate (AFB_1-oxime-horse radish peroxidase) diluted to the ratio of 1:100 000 in PBS into all wells. Plates were rocked gently by hand to mix reagents and incubated at room temperature (23-25 °C) in a humid chamber for two hours. All wells were emptied, washed three times using washing solution (Appendix 3), and semi dried by tapping the plate on blotting paper. An enzyme substrate solution (100 µl) comprising of hydrogen peroxide and tetramethylbenzidine (TMB) was added to each well and reaction allowed to proceed for 10 min in the dark. Enzyme reaction was stopped by adding 100 µl of 1M sulphuric acid into each well. The intensity of colour in each well was determined spectrophotometrically by reading the absorbance at 450nm using Uniskan II® microwell-plate ELISA reader.

3.2.4 Interpretation of results

The average absorbance for each of the standards and sample extracts were transformed to percent colour inhibition values as follows:

$$\% \text{ Inhibition} = B_i/B_o \times 100$$

Where: B_i = Average absorbance for standard or sample extract.

B_o = Average absorbance of sample blank.

A calibration curve for each microtiter plate was constructed by plotting the mean percentage inhibition values for each of the standards against the corresponding aflatoxin concentration expressed as $\log_{10}$ (Appendix 5). A best-fit line was fitted between the points using non-linear regression on Graph pad software version 4.04®.

Aflatoxin concentration in test extracts were obtained by reading the antilog values of ordinates aligned to the respective percentage inhibition values on the calibration curve. Only values within the linear range of the standard curve (20 to 80 % inhibition levels) were considered for accuracy; samples with percentage inhibition above 80 % were considered negative while those with percentage inhibition below 20 % were diluted further. Observed values were converted to the corresponding extract dilution factor (adjusted for sample moisture content) and the aflatoxin levels reported as ng/g of sample.

3.3 MOULD ENUMERATION

Sample homogenates were prepared by diluting 10g portions of the finely ground sub-sample in 90 ml of sterile physiological saline (0.85 % sodium chloride) containing 0.05 % tween-80 in a 250 ml conical flask. The mixture was allowed to stand for 30 min and then stirred at moderate speed using a magnetic stirrer. The homogenate was further diluted decimally up to the 10^{-4} dilution by transferring 1ml aliquots into 9 ml of sterile physiological saline diluent.

3.3.1 Total mould count

Total mould counts were determined by spreading aliquots (0.1 ml) of the 10^{-3} and 10^{-4} homogenate dilutions on duplicate plates of modified Sabouraund's Dextrose agar (Appendix 2) to which 100 mg chloramphenicol per litre had been added. Plates were then incubated upright at room temperature (23-25 °C) for 5-7 days. Duplicate plates containing 10-100 colonies were counted according to the method of Beuchat (1992) and the average count multiplied with the reciprocal of the dilution factor. Total mould count was reported as colony forming units per gram (c.f.u /g) of sample.

3.3.2 *Aspergillus flavus* count

Typical colonies of *Aspergillus flavus* group were enumerated by spreading 0.1 ml aliquots of the 10^{-2} and 10^{-3} sample dilutions on duplicate plates of *Aspergillus flavus* and p*arasiticus* agar (AFPA) (Pitt, *et al.*, 1983; Appendix 2), using a sterile glass rod. Plates were incubated upright at 30 °C for 48 h. Duplicate plates containing 10 to 100 colonies showing the typical bright orange reverse and yellow to olive green spores were counted, the average count computed and counts reported as c.f.u/g of sample.

3.4 SCREENING *Aspergillus flavus* ISOLATES FOR AFLATOXIN PRODUCTION

Thirty (30) AFPA plates with typical 42 h old *Aspergillus flavus* colonies were randomly selected and preserved at 4 °C. Three separately growing typical *Aspergillus flavus* colonies were randomly selected from each of the 30 plates. Using a sterile wire loop, fungal mycelia was cut from each of the selected colonies and sub-cultured separately on Yeast Extract Sucrose (YES) agar (Appendix 2). Yeast Extract agar plates were incubated at room temperature (23-25 °C) for 10 days after which isolates were screened for aflatoxin production using the agar plug screening method (Filtenborg and Frisvad, 1980). Agar plugs were cut out using a sterile open-ended glass tube with an internal

diameter of 8 mm and placed separately at 1.5 cm above the bottom edge of commercially pre-coated Kieselgel 60 E-Merck® TLC plates. The agar plugs were eluted each with 100 µl of chloroform to allow soluble contents to spot on the TLC plate. Three agar plugs from each isolate were eluted onto the same spot. Twenty microlitres (20 µl) of 15 000 ng/ml standard aflatoxin mixture (equivalent to 300 ng of the toxin) was spotted along side the agar spots on the TLC plates. Plates were then developed in unequilibriated chloroform-acetone (88:12) tanks, dried and examined for fluorescing spots under 365 nm ultraviolet lamp. Aflatoxin-producing strains were identified by the presence of bright blue fluorescing spots whose resolution factor (rf) was the same as that of the standard toxin mixture. Aflatoxigenicity of isolates was evaluated based on the intensity of fluorescence of spots and reported as high (about 1000 ng/ml), moderate (below 1000 ng/ml) or low (far much below 1000 ng/ml).

3.5 DATA ENTRY AND STATISTICAL ANALYSIS

For each dehulling trial, reduction in aflatoxin levels was calculated from the difference between aflatoxin levels in whole-grain maize (ng/g) and aflatoxin levels in *Muthokoi* (ng/g) expressed as a percentage of the aflatoxin levels in the whole-grain maize sample (ng/g). Similarly, aflatoxin reduction following treatment with chemical additives was calculated from the difference between aflatoxin content (ng/g) of untreated sample and aflatoxin content (ng/g) of the corresponding treated sample expressed as a percentage of aflatoxin content (ng/g) of the untreated sample. Dehulling ratio was calculated as a ratio of the dry weight of by-product (g) to the corresponding dry weight of *muthokoi* (g) while the ratio of by-product to whole-grain aflatoxin content was obtained by dividing by-product aflatoxin content (ng/g) with the corresponding whole-grain maize aflatoxin content (ng/g).

Data on percentage aflatoxin reduction and data on mould count were transformed to the square root and $\log_{10}$ respectively but results are presented untransformed (Gomez and Gomez, 1984). Paired sample t-test was used to compare aflatoxin levels and mould counts in *muthokoi* and whole-grain maize. Analysis of variance (ANOVA) was used to compare the effect of various grain wetting agents on aflatoxin reduction and mould recovery during dehulling as well as aflatoxin reductions during treatment of dehulled and non-dehulled maize grains with the various chemical additives. Fisher's LSD test was used to separate treatment means while Pearson's product-moment correlation was used to test relationships between aflatoxin contamination level of whole-grain maize, dehulling ratio, ratio of by-product to whole grain aflatoxin content and aflatoxin reduction during dehulling. All statistical analyses were performed on GenStat® statistical package (Sixth Edition).

CHAPTER FOUR

4.0 RESULTS

4.1 DEHULLING PRE-TRIAL

An amount of grain wetting agent equivalent to 10 % grain weight added intermittently, facilitated optimum dehulling of 500 g portions of maize grains within 30 min of pounding in a medium size (1700cm^3 capacity) wooden mortar. The average moisture content was 12.4 ± 0.3 % in whole-grain maize, 14.1 ± 0.3 % in *muthokoi* and 15.3 ± 0.5 % in the by-product. Average moisture contents of *muthokoi* and by-product were significantly higher than the average moisture content of whole-grain maize ($p<0.01$; $n = 3$). A dehulling ratio of 0.18 ± 0.03 ($n = 3$) was attained during the pre-trial.

4.2 EFFECT OF DEHULLING MAIZE GRAINS ON MOULD CONTAMINATION

4.2.1 Mould Counts

The total mould count and *Aspergillus flavus* counts of whole-grain maize, *Muthokoi* and by-product is illustrated in Table 4.1. Total mould and *Aspergillus flavus* counts varied considerably within samples of whole-grain maize. The total mould count ranged from 1.1 x 10^4 to 2.8 x 10^5 c.f.u/g with a mean of 7.2 x 10^4 c.f.u/g while the mean *Aspergillus flavus* count was 1.9 x 10^4 c.f.u/g (range from 1.0 x 10^3 to 1.15 x 10^5 c.f.u/g). Following dehulling, the mean total mould count in *muthokoi* was 1.5 x10^4 c.f.u/g (range from 1.0 x 10^3 to 7.8 x 10^4 c.f.u/g) while the mean *Aspergillus flavus* count was 2.1x 10^3 c.f.u/g (range from 1.0 x 10^2 to 1.5 x 10^4 c.f.u/g). Total mould count in by-product ranged between 1.8 x 10^3 and 1.4 x 10^6 c.f.u/g with a mean of 1.9 x 10^5 c.f.u/g, while *Aspergillus flavus* count ranged between 7.2 x 10^2 to 6.7 x 10^4 c.f.u/g with a mean of 1.6 x 10^4 c.f.u/g.

Generally, total mould and *Aspergillus flavus* counts in *muthokoi* were significantly lower than in whole-grain maize ($p<0.001$) when *iati (*1% w/v*)*, ammonium persulphate (1% w/v) or sodium

hypochlorite (1% v/v) were used as grain wetting agents to aid the process of dehulling (Table 4.2 and Table 4.3). This was however not the case when plain water was used to wet maize grains; *Aspergillus flavus* counts in ***muthokoi*** were not significantly different from *Aspergillus flavus* counts in whole-grain maize ($p=0.095$), implying that the traditional method of dehulling maize grains using plain water to soften grain pericarp may not achieve meaningful reduction of aflatoxin producing moulds on ***muthokoi*** (Table 4.3).

Table 4.1: Mould counts in whole grain maize, *muthokoi* and by-product (c.f.u/g)

	Grain wetting agent																							
	Water						Ammonium persulphate (1 % w/v)						Sodium hypochlorite (1% v/v)						*Iati* (1% w/v)					
	WG		M		BP		WG		M		BP		WG		M		BP		WG		M		BP	
Sample	tmc	afc	tmc	afc	tmc	afc	tmc	afc	tmc	afc	tmc	afc	tmc	afc	tmc	afc	tmc	afc	tmc	afc	tmc	afc	tmc	afc
1	39	19	2	13	228	36	134	2	15	0.1	230	30	20	10	4	0.1	20	5	115	22	78	2	356	11
2	57	1	36	15	168	59	17	6	27	0.2	43	2	90	22	2	0.1	55	10	57	21	9	0.4	395	22
3	88	12	12	13	1418	58	100	15	13	0.1	153	10	54	13	3	0.1	41	3	90	16	35	0.3	229	8
4	86	44	31	8	262	21	86	30	14	0.1	220	21	182	13	4	0.2	13	6	51	8	24	0.4	160	20
5	66	16	14	4	52	33	59	15	8	0.2	78	6	11	2	1	0.1	1.8	3	50	10	14	0.9	216	17
6	54	4	16	2	44	12	185	115	4	1.0	325	14	26	1	2	0.1	148	14	35	5	15	0.2	91	9
7	123	23	51	6	573	27	220	108	9	0.2	729	5	17	7	2	0.1	3	5	77	45	36	0.9	169	26
8	98	14	20	9	256	48	180	13	2	0.1	465	17	18	2	1	0.2	12	0.7	55	16	14	0.4	29	7
9	23	12	14	5	123	67	25	13	1	0.1	92	3	12	8	3	0.1	2	2	72	30	13	4	40	8
10	44	71	46	5	710	19	15	7	2	0.1	61	6	22	17	6	0.1	56	6	73	53	13	0.3	138	13
11	33	11	19	6	34	18	41	4	19	0.1	63	2	13	8	3	0.8	10	3	95	30	18	5	55	15
12	59	2	40	4	28	10	43	3	11	0.1	274	6	275	2	2	0.6	30	5	28	10	18	0.4	81	19

WG – whole grain maize; M – *Muthokoi*; BP – by- product; tmc – Total mould count (x 10^3 c.f.u/g); afc – *Aspergillus flavus* count (x 10^3 c.f.u/g)

Table 4.2: Effect of grain wetting agent on total mould count in *muthokoi* and by-product

Grain wetting agent	Counts (x 10^3c.f.u/g)		
	Whole-grain maize	*Muthokoi*	By-product
Water	$67.80^{g,k}$	24.36^{a}	383.50^{d}
Local alkaline salt	$66.50^{f,k}$	23.92^{a}	227.90^{d}
Ammonium persulphate	$92.08^{h,k}$	10.37^{b}	163.20^{d}
Sodium hypochlorite	$61.67^{i,k}$	2.79^{c}	$32.65^{e,j}$

$^{a, b, c, e, f, g, h, i, k}$ Means across the same row or down the same column sharing same superscript are not significantly different ($p>0.05$)

Table 4.3: Effect of grain wetting agent on *Aspergillus flavus* count in *muthokoi* and by-product

Grain wetting agent	Counts (x 10^2c.f.u/g)		
	Whole-grain maize	*Muthokoi*	By-product
Water	$216.0^{a,k}$	80.7^{a}	$381.6^{d,k}$
Local alkaline salt	$221.7^{g,k}$	11.6^{b}	$150.9^{e,g}$
Ammonium persulphate	$275.8^{h,k}$	2.0^{c}	$99.49^{f,h}$
Sodium hypochlorite	$87.5^{i,k}$	0.1^{c}	$53.32^{f,i}$

$^{a, b, c, d, e, f, g, h, i, k}$ Means across the same row or down the same column sharing same superscript are not significantly different ($p>0.05$)

4.2.2 Aflatoxigenicity of *Aspergillus flavus* isolates

The aflatoxin producing ability of *Aspergillus flavus* isolates randomly selected for screening is summarised in Table 4.4. Eighty-three out of ninety *Aspergillus flavus* isolates (92.2 %) screened for aflatoxin production produced aflatoxin on YES agar. Based on intensity of florescence of sample spots relative to that of the aflatoxin standard, 21.8 % of positive isolates (18 out of 83) were highly aflatoxigenic (about 1000 ng/ml), 55.4 % (45 out of 83) moderately aflatoxigenic (below 1000 ng/ml) and 22.8 % (19 out of 83) low aflatoxin producers (far below 1000 ng/ml). Only two of the positive isolates (2.4 %) produced both AFB_1 and AFB_2 while none produced AFG_1 or AFG_2. It was observed that isolates that produced AFB_2 were also high AFB_1 producers.

Table 4.4: Aflatoxin producing ability of *Aspergillus flavus* isolates

Type of aflatoxin	Number of isolates screened	Number of aflatoxin producing isolates		
		About 1000ng/ml	Below 1000ng/ml	Far below 1000ng/ml
AFB_1	92	19	45	19
AFB_2	92	0	0	2
AFG_1	92	0	0	0
AFG_2	92	0	0	0

4.3 EFFECT OF DEHULLING ON AFLATOXIN LEVELS

4.3.1 Calibration curves for aflatoxin determination

Twenty-four (24) microtiter plates were used to analyse samples for aflatoxins. Table 4.5 shows the summary statistics of the 24 separate calibration curves constructed for aflatoxin quantification. The mean aflatoxin concentrations at the 50 % and 80 % inhibition levels, usually used to characterise sensitivity of the assay (Appendix 4) were 0.071 ng/ml (0.059 - 0.073 ng/ml) and 0.021 ng/ml (0.019 - 0.025 ng/ml), respectively. The percentage coefficients of variation of the means were 10.20 at 50 % inhibition level and 9.52 at 80 % inhibition level.

Table 4.5: Summary statistics for calibration curves used in aflatoxin quantification

Standard curve descriptor	Mean aflatoxin conc. (ng/ml)	n	% c.v
50% inhibition [b]	0.071± 0.007	24	10.20
80% inhibition [a]	0.021± 0.002	24	9.52

[a]: 80% inhibition represents the detection limit

[b]: 50% inhibition represents mid-point of the standard curve

4.3.2 Aflatoxin contamination of whole-grain maize samples

The aflatoxin contamination levels of 48 whole-grain maize samples selected for *muthokoi* making is shown in Table 4.6. Aflatoxin levels ranged between 10.7 and 270.0 ng/g with a mean of 97.3 ng/g. Aflatoxin contaminations of the same samples as determined by the NPHLS ranged between 20 and 400ng/g (Appendix 1), with a mean of 110.0 ng/g. The two means were not significantly different (p=0.4)

4.3.3 Effect of dehulling maize grains on aflatoxin content

The aflatoxin levels in *muthokoi* and by-product recovered after dehulling of whole-grain maize are given in Table 4.6. Aflatoxin levels in two of the 48 samples that were dehulled to *muthokoi* were higher compared to the corresponding whole-grain reference values probably due to sampling or analytical error and these were omitted in the statistical analysis of results. The mean aflatoxin level in dehulled maize was 57.3 ng/g (range from 6.8 to 181.7 ng/g). These levels were significantly lower than the corresponding aflatoxin levels in whole-grain maize ($p<0.001$; $n = 46$) indicating an overall reduction in aflatoxins during the process of dehulling. Percentage reduction in aflatoxin levels was between 5.5 % and 70.0 % with a mean of 46.6 %.

Aflatoxin levels in the by-product comprising of hulls and fines were 1.8 to 7.0 (mean 3.8) times higher than the aflatoxin levels in raw-material whole-grain maize. The actual levels ranged between 103.3 and 613.8 ng/g (Table 4.6). There was significant positive correlation between the ratio of by-product aflatoxin content to whole-grain aflatoxin content and percentage reduction in aflatoxin levels ($r = 0.663$; $p = 0.01$; Appendix 7). The ratio of by-product aflatoxin content to whole-grain aflatoxin content, however, decreased significantly with increasing aflatoxin contamination level of whole-grain maize ($r = -0.699$; $p = 0.01$) as shown in Figure 4.1. Consequently, aflatoxin reduction decreased significantly with increase in aflatoxin contamination of whole-grain maize ($r = -0.671$; $p = 0.01$; Figure 4.3). The effect of dehulling ratio on reduction of aflatoxins was also significant; percentage reduction in aflatoxin levels increased with increasing dehulling ratio as shown in Figure 4.2 ($r = 0.351$; $p = 0.05$). The Effect of various grain wetting agents on aflatoxin reduction is shown in Table 4.7. Mean percentage reductions in aflatoxin levels under the various grain wetting agents ranged between 44.0 and 53.7 % but these were not significantly different at the 0.05 level ($p = 0.826$). The mean dehulling ratios were also not significantly different when different grain wetting agents were used to facilitate dehulling.

Table 4.6: Aflatoxin levels in whole grain maize, *muthokoi* and by-product

	Grain wetting agent																			
	Water					Ammonium persulphate (1% w/v)					Sodium hypochlorite (1 % v/v)					*Iati* (1 % w/v)				
Sample	WG	M	BP	%red	DR	WG	M	BP	%red	DR	WG	M	BP	%red	DR	WG	M	BP	%red.	DR
1	136.7	58.3	365.8	57.3	0.11	65.0	29.7	250.5	54.3	0.20	43.9	13.1	253.8	70.0	0.15	114.7	67.9	335.6	40.8	0.19
2	29.7	12.9	152.9	56.7	0.22	93.5	38.8	283.2	58.5	0.23	270.0	176.7	613.8	34.6	0.20	44.6	15.5	194.2	65.3	0.23
3	63.5	21.8	365.6	65.7	0.26	52.3	17.2	231.4	67.2	0.21	169.6	89.7	463.4	47.1	0.17	182.6	150.0	521.0	17.9	0.11
4	216.8	304.1	603.1	*	0.12	192.4	181.7	403.8	5.6	0.14	86.3	57.0	282.1	33.9	0.16	33.3	16.5	103.3	50.5	0.20
5	27.8	13.1	146.1	53.1	0.19	23.6	10.1	111.3	57.4	0.18	150.9	87.5	543.5	42.1	0.13	51.7	26.9	292.1	48.0	0.12
6	74.9	44.7	308.2	40.3	0.21	47.9	18.6	117.6	61.2	0.28	78.2	40.5	257.2	48.2	0.21	59.5	42.0	155.2	29.4	0.22
7	95.9	47.5	254.8	50.5	0.25	77.5	25.4	312.3	67.2	0.22	17.2	6.8	119.7	60.4	0.12	40.7	16.9	134.2	58.6	0.23
8	151.6	78.7	421.3	48.1	0.22	146.8	79.8	486.5	45.6	0.12	109.5	40.5	357.5	63.1	0.25	61.6	27.7	253.1	55.0	0.14
9	76.7	31.0	274.2	59.6	0.23	21.4	10.8	109.1	49.3	0.12	10.7	6.8	142.8	36.1	0.15	258.4	125.8	548.6	51.3	0.15
10	97.5	61.9	302.1	36.5	0.18	160.0	127.8	317.4	20.1	0.15	96.5	95.9	277.8	0.6	0.16	167.2	101.3	293.6	39.4	0.16
11	44.0	13.6	287.2	69.0	0.13	218.6	174.6	458.7	20.1	0.19	158.9	142.5	286.7	10.3	0.19	86.7	31.8	351.0	63.4	0.20
12	198.9	402.9	198.3	*	0.17	111.7	66.9	474.6	40.1	0.15	95.3	59.6	239.4	37.5	0.13	80.6	30.9	295.1	61.7	0.21

WG – aflatoxin levels in whole grain maize (ng/g); M – aflatoxin levels in *muthokoi* (ng/g); BP – aflatoxin levels in by-product (ng/g); %red – percentage reduction in aflatoxin levels; DR – dehulling ratio; * - no aflatoxin reduction observed due to sampling or analytical error

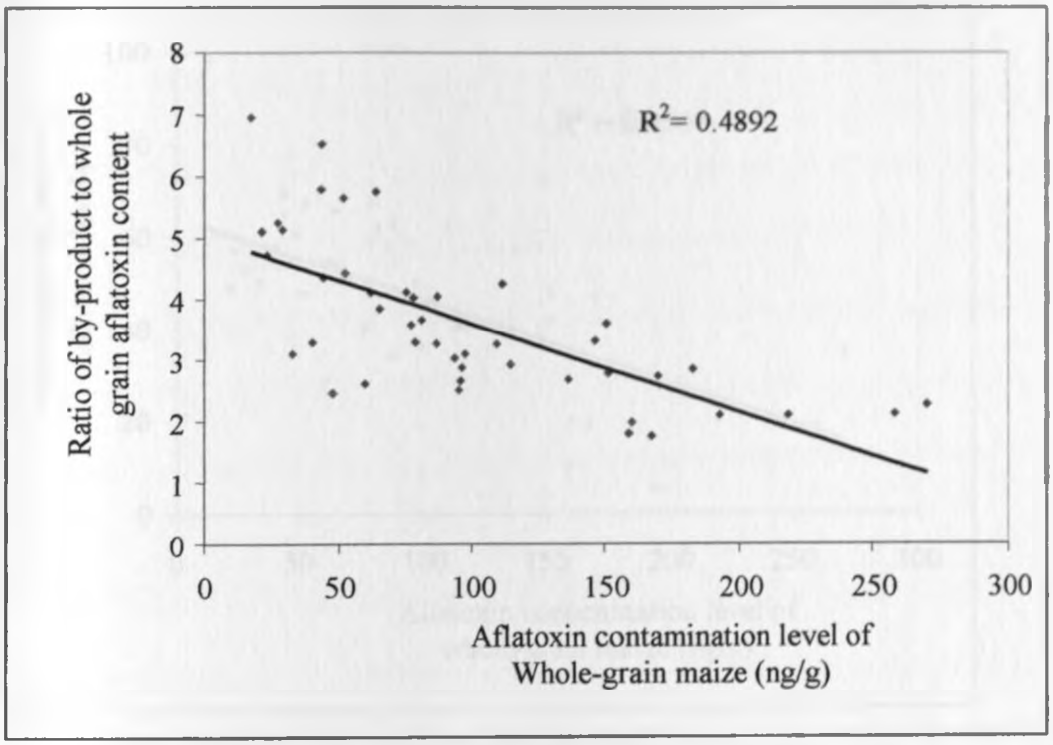

Figure 4.1: Correlation between aflatoxin-contamination level of whole-grain maize and the ratio of by-product aflatoxin to whole grain aflatoxin content.

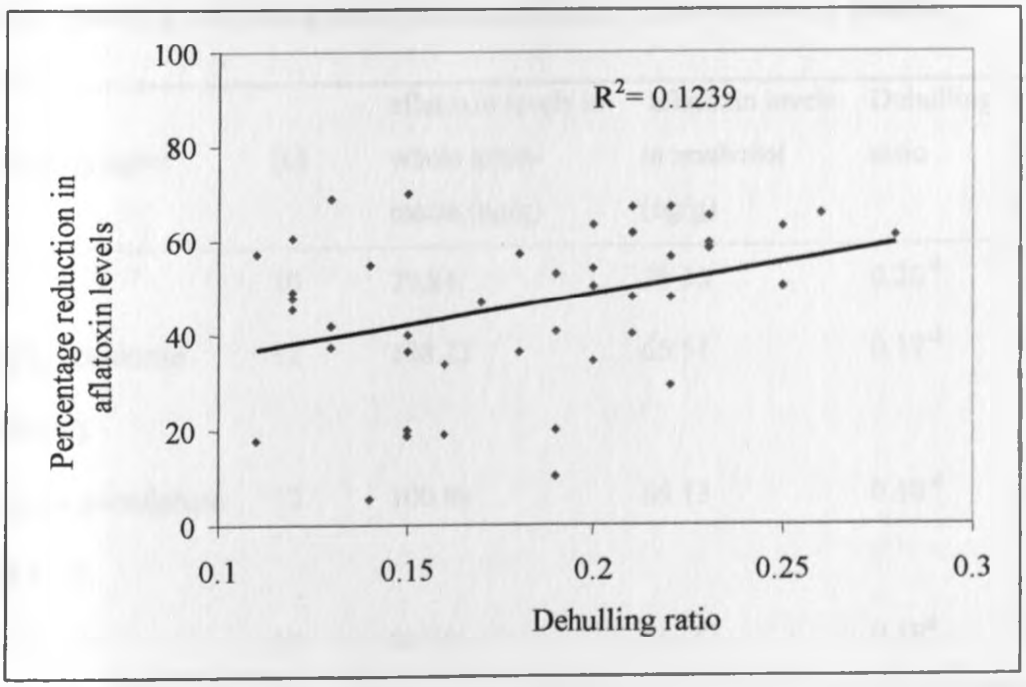

Figure 4.2: Correlation between dehulling ratio and aflatoxin reduction.

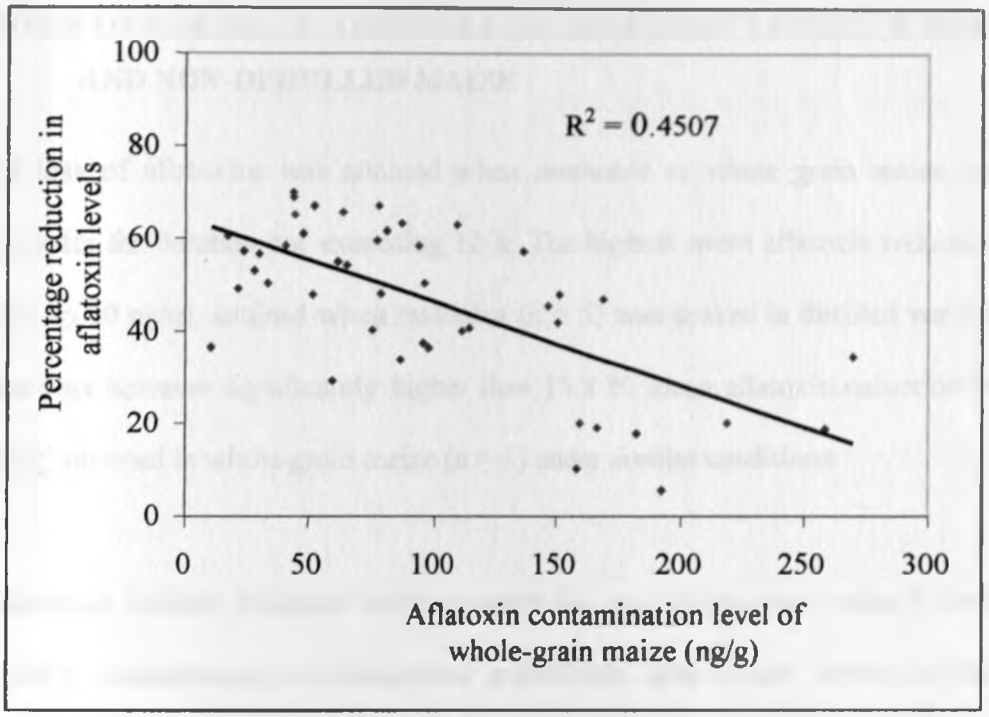

Figure 4.3: Correlation between aflatoxin-contamination level of whole-grain maize and aflatoxin reduction during dehulling.

Table 4.7: Effect of grain wetting agent on mean aflatoxin reduction during dehulling

Grain wetting agent	(n)	aflatoxin levels in whole grain-maize (ng/g)	aflatoxin levels in *muthokoi* (ng/g)	Dehulling ratio	Percentage reduction in aflatoxin levels
Water	10	79.84	38.35	0.20[d]	53.68[a]
Sodium hypochlorite (1% v/v)	12	108.22	65.51	0.17[d]	43.99[a]
Ammonium persulphate (1% w/v)	12	100.89	65.13	0.18[d]	45.54[a]
lati (1% w/v)	12	98.46	64..22	0.18[d]	44.04[a]

[a, d]: Means down the same column having same superscript are not significantly different ($p>0.05$).

4.4 EFFECT OF CHEMICAL ADDITIVES ON AFLATOXIN LEVELS IN DEHULLED AND NON-DEHULLED MAIZE

Minimal loss of aflatoxins was attained when *muthokoi* or whole grain maize were soaked in distilled water for duration not exceeding 12 h. The highest mean aflatoxin reduction was 29.8 % (from 131 to 90 ng/g), attained when *muthokoi* (n = 5) was soaked in distilled water for 12 h. This reduction was however significantly higher than 15.8 % mean aflatoxin reduction (from 317.2 to 275.8 ng/g) attained in whole-grain maize (n = 5) under similar conditions.

The percentage residual aflatoxin levels in *muthokoi* and whole-grain maize following treatment with graded concentrations of ammonium persulphate (pH 2) are shown in Figure 4.4. The respective means of percentage aflatoxin reduction in whole-grain maize and *muthokoi* (n = 5) treated for 6 h were 22.4 % (from 415 to 322 ng/g) and 48.8 % (363.6 to 186.3 ng/g) with 0.2 % ammonium persulphate, 38.2 % (415 to 256 ng/g) and 55.2 % (363.6 to 162.9 ng/g) with 0.5 % ammonium persulphate, and 45.2 % (415 to 227.6 ng/g) and 56.4 % (363.6 to 158.4 ng/g) with 1.0 % ammonium persulphate. Higher aflatoxin reductions of 57.2 % (415 to 177.7 ng/g) and 61.6 % (363.6 to 139.7 ng/g) in 0.2 % ammonium persuphate, 60.5 % (415 to 164.0 ng/g) and 66.1 % (363.6 to 123.1 ng/g) in 0.5 % ammonium persulphate and 66.52 % (415 to 138.9 ng/g) and 73.2 % (363.6 to 97.63 ng/g) in 1.0 % ammonium persulphate were observed during 12 h treatment period. Percentage aflatoxin reduction was significantly higher in *muthokoi* than in whole-grain maize at all levels of concentration of ammonium persulphate during the 6 h treatment regime. This was however not reflected during 12 h treatment period, in which the percentage loss of aflatoxins in *muthokoi* was not significantly different from that attained in whole grain maize ($p>0.05$). Increasing the concentration of ammonium persulphate beyond 0.2 % also, did not significantly increase the loss of aflatoxins in *muthokoi* during both treatment regimes.

Treatment of whole-grain maize and *muthokoi* (n = 5) with sodium hypochlorite (pH 11.5) also resulted in significant aflatoxin loss (Figure 4.5). Mean reductions in aflatoxin levels in whole-grain maize and *muthokoi* during 6 h treatment with 0.2 %, 0.5 % and 1.0 % solutions of sodium hypochlorite were 24.2 % (359.3 to 272.5 ng/g) and 34.5 % (297 to 194.5 ng/g), 30.2 % (359.4 to 250.7 ng/g) and 52.1 % (297 to 142.3 ng/g), and 36.0 % (359.3 to 229.8 ng/g) and 50.5 % (297 to 147 ng/g) respectively. Similarly, the respective aflatoxin reductions following 12 h treatment with 0.2 %, 0.5 % and 1.0 % solutions of sodium hypochlorite were 35.6 (359.3 to 227 ng/g) and 57.1 % (297 to 127.4 ng/g), 57.3 % (359.3 to 153.4 ng/g) and 62.4 % (297 to 111.6 ng/g), and 65.2 % (359.3 to 125.2 ng/g) and 72.2 % (297 to 82.7 ng/g). Loss of aflatoxins was significantly higher in *muthokoi* than in whole-grain maize. Increasing the concentration of sodium hypochlorite beyond 0.5 %, however, did not significantly increase loss of aflatoxins during both treatment regimes.

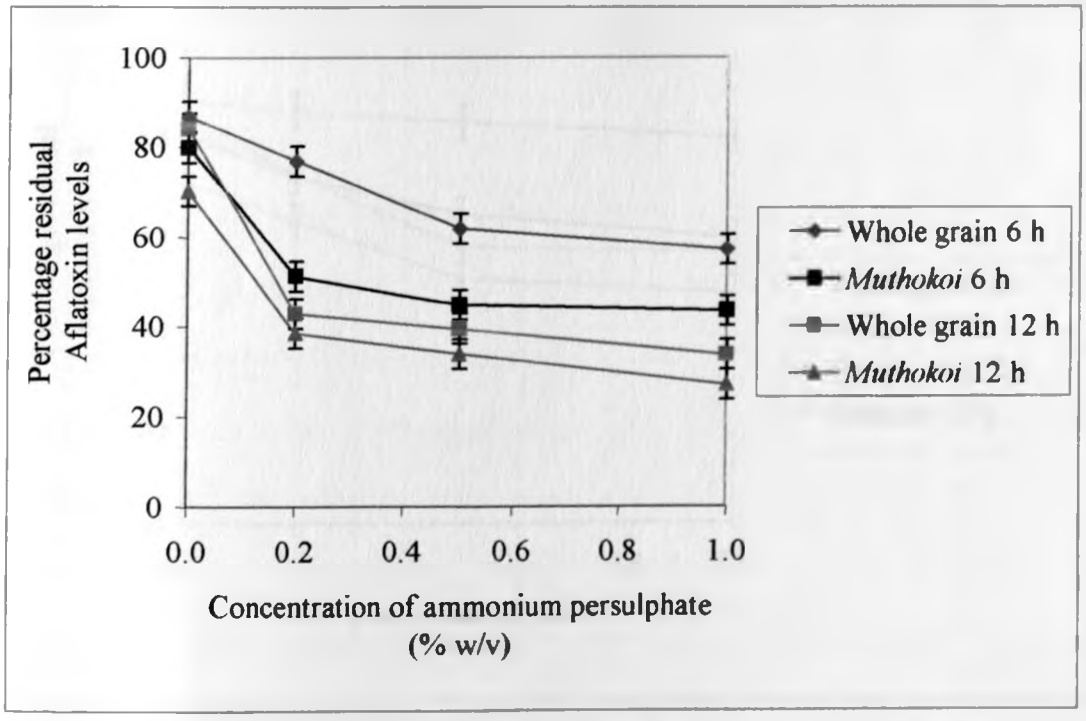

Figure 4.4: Effect of treatment of aflatoxin-contaminated maize with ammonium persulphate

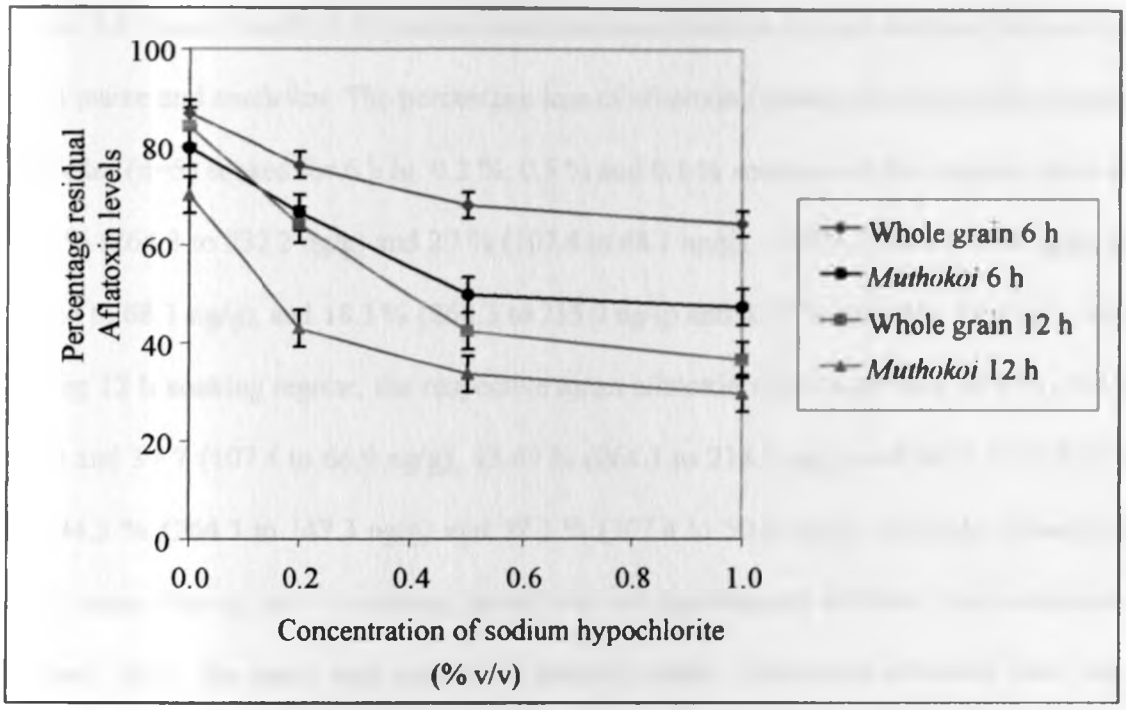

Figure 4.5: Effect of treatment of aflatoxin-contaminated maize with sodium hypochlorite

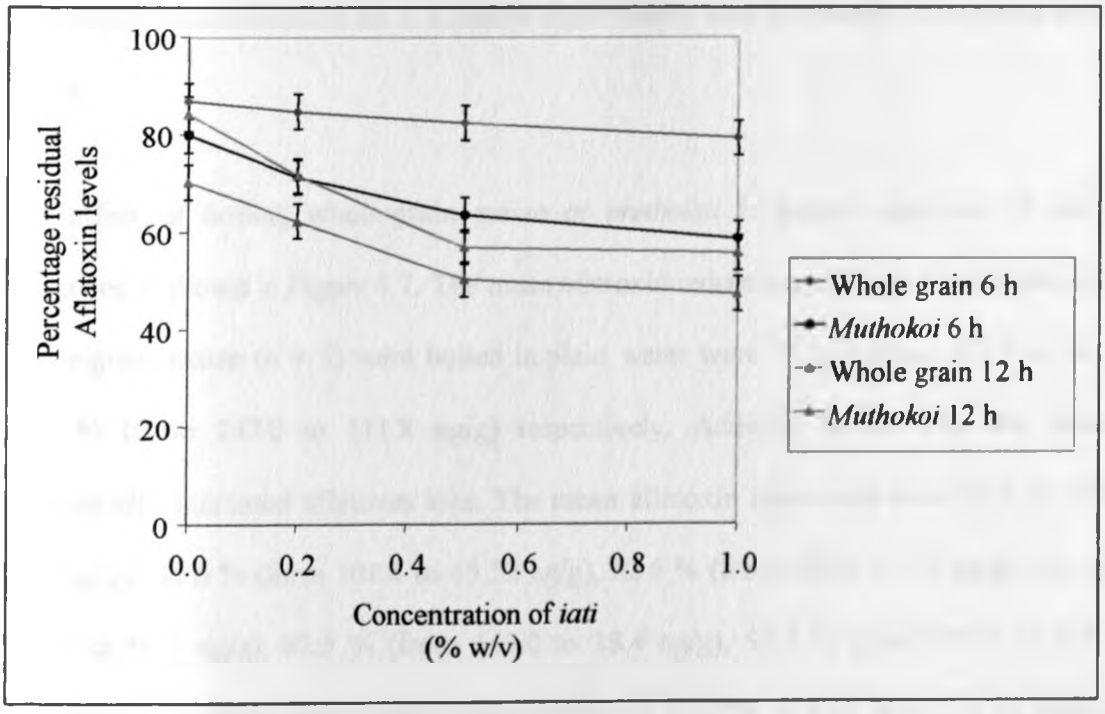

Figure 4.6: Effect of soaking aflatoxin-contaminated maize in solutions of *iati*

Figure 4.6 shows the effect of graded concentrations of *iati* on residual levels of aflatoxins in whole-grain maize and *muthokoi*. The percentage loss of aflatoxin (means) in whole-grain maize (n=5) and *muthokoi* (n=5) soaked for 6 h in 0.2 %, 0.5 % and 0.1 % solutions of the alkaline mineral salt were 12.1 % (264.3 to 232.2 ng/g) and 29 % (107.4 to 68.1 ng/g), 17.4 % (264.3 to 218 ng/g) and 36.6 % (107.4 to 68.1 ng/g), and 18.3 % (264.3 to 215.9 ng/g) and 41.3 % (107.4 to 63.1 ng/g) respectively. During 12 h soaking regime, the respective mean aflatoxin reductions were 28.8 % (264.3 to 188.2 ng/g) and 37.7 (107.4 to 66.9 ng/g), 43.49 % (264.3 to 218.2 ng/g) and 49.71 (107.4 to 54.0 ng/g) and 44.3 % (264.3 to 147.3 ng/g) and 52.3 % (107.4 to 50.8 ng/g). Aflatoxin reduction in whole-grain maize during the 6 h soaking period was not significantly different from aflatoxin reduction attained when the same was soaked in distilled water. Substantial aflatoxin loss was however attained in *muthokoi*. Inclusion of the alkaline salt in the soaking water significantly enhanced aflatoxin reduction during the 12 h soaking period. While aflatoxin reduction in *muthokoi* was significantly higher than in whole-grain maize at all levels of concentration in both soaking regimes ($p>0.05$), loss of aflatoxins did not change significantly with increasing concentration of *iati* beyond 0.5 %.

The effect of boiling whole-grain maize or *muthokoi* in graded solutions of *iati* on residual aflatoxins is shown in Figure 4.7. The mean aflatoxin reductions attained when *muthokoi* (n = 5) and whole-grain maize (n = 5) were boiled in plain water were 21.3 % (from 101.8 to 80.1 ng/g) and 23.9 % (from 147.0 to 111.8 ng/g) respectively. Addition of *iati* into the boiling solution substantially increased aflatoxin loss. The mean aflatoxin reductions were 80.5 % (from 101.8 to 19.8 ng/g), 84.8 % (from 101.8 to 15.52 ng/g), 92.6 % (from 101.8 to 7.6 ng/g) and 60.1 % (from 147.0 to 56.7 ng/g), 87.5 % (from 147.0 to 18.4 ng/g), 94.0 % (from 147.0 to 8.9 ng/g) when *muthokoi* and whole-grain maize were boiled in 0.2 %, 0.5 % and 1.0 % solutions of *iati* respectively. The concentration of *iati* in the boiling solution significantly influenced loss of

aflatoxins during boiling of maize while dehulling (*muthokoi)* or lack of dehulling (whole-grain maize) was only significant at 0.2 % concentration level and not when higher concentrations of the salt were used (Figure 4.7).

Following these observations on aflatoxin reduction effect of *iati*, chemical analysis was done to establish the ionic composition and ionic strength of the mineral salt (n=3). Flame photometry and titration methods were used to determine the cationic and anionic composition respectively, while the electrical conductivity, a measure of ionic strength of the salt, was determined at room temperature (23-25 °C) using conductivity bridge model 31®. The pH was determined using a pH meter. Results of the ionic composition of *iati* are shown in Table 4.8. Electrical conductivity was $5.6x10^2$ dSm^{-1} and the pH 10.2.

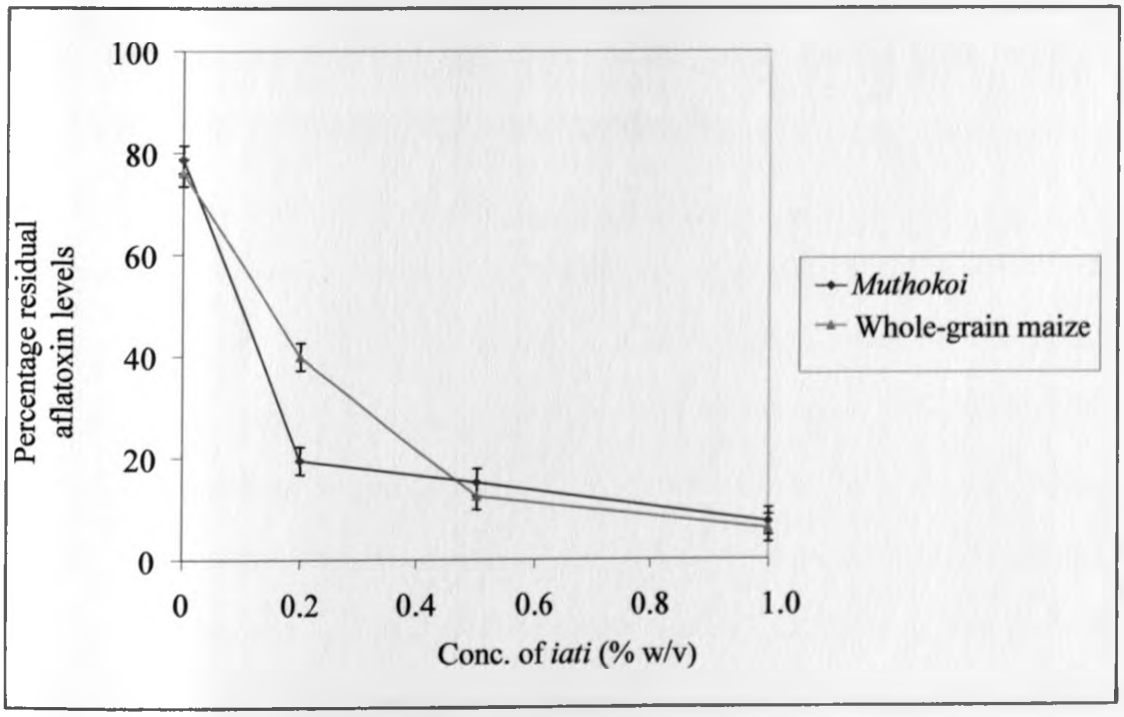

Figure 4.7: Effect of concentration of *iati* on aflatoxin levels during boiling of contaminated maize

Table 4.8: Ionic composition of *iati*

	Ion	Conc. (Meq l^{-1})
Cations	Na^{+}	3 000 ± 278.40
	K^{+}	0.07 ± 0.02
	Mg^{++}	Trace
	Ca^{++}	Trace
Anions	OH^{-}	Trace
	CO_3^{2-}	2 000 ± 265.10
	HCO_3^{-}	956 ± 160.50
	Cl^{-}	22 500 ± 763.80

CHAPTER FIVE

5.0 DISCUSSION

5.1 EFFECT OF DEHULLING ON AFLATOXIN LEVELS AND MOULD CONTAMINATION

Considerable reduction in aflatoxin levels was realized during dehulling of maize grains to make *muthokoi*, as evidenced by the lower levels of aflatoxins in dehulled maize. During the dehulling process, the softened pericarp (hull), the underlying aleuronic layer, the hilum (tip cap) and a sizeable portion of the germ are removed from the grain as by-product. These fractions represent the highly contaminated parts of the maize grain (Brekke *et al.*, 1975a; 1975b). Aflatoxigenic moulds easily invade the hilum because of the high hygroscopic nature of the tip cap. Similarly, the germ is prone to heavy mould infestation due to its hydrophobic nature, which permits high water activity even when the overall moisture content of the grain is adequately low. The soft texture of the germ also enables easy penetration of fungal mycelia into the germ as compared to the endosperm. Keller *et al.*, (1994) and Brown *et al.*, (1995) observed that fungal growth and aflatoxin production was significantly higher in the germ than in other kernel tissues.

The overall aflatoxin reduction effect of dehulling is largely dependent on the level of contamination of the raw material whole-grain maize (Figure 4.3). At higher levels of contamination, the larger proportion of the toxin is probably bound within the internal matrix of the grain as implied by the significant decrease in the ratio of by-product aflatoxin content to whole grain aflatoxin content (Figure 4.1) as the aflatoxin contamination level of whole-grain maize increases. Similar observation regarding the distribution of aflatoxins in the various fractions of naturally contaminated maize has also been reported by other workers (Lopez-Garcia and Park 1998; and Fandohan *et al.*, 2005). The degree of dehulling of maize grains as measured by the ratio of by-product to *muthokoi* (dehulling ratio) was also found to increase the extent of aflatoxin reduction during *muthokoi*

making (Figure 4.2). A low or high degree of dehulling may have been due to the size and physical characteristics of the raw material whole-grain maize. The smaller and shrivelled maize grains are usually difficult to dehull by way of pounding and hence a low dehulling ratio would be expected if a large proportion of the raw material maize consisted of such grains. Since undersized maize grains are also likely to be contaminated with higher levels of aflatoxins (Huff, 1980), this probably explains the lower aflatoxin reduction associated with low dehulling ratio observed in this study. In traditional *muthokoi* making, the practice of segregation of the undersized and shrivelled grains to enhance dehulling may further enhance aflatoxin removal.

Reductions in aflatoxin levels attained with the different grain wetting agents used to facilitate dehulling were not significantly different. However, recovery of mould propagules on both *muthokoi* and the by-product was variably influenced by the grain wetting agent (Tables 4.2 and 4.3) probably due to anti-fungal effect of chemical additives. *Aspergillus flavus* counts in *muthokoi* were not significantly different from the *Aspergillus flavus* counts in whole-grain maize when plain water was used as the grain wetting agent. Since the suitability of any technology adopted for degradation or reduction of aflatoxins in foods is also evaluated based on ability to destroy spores and mycelia of aflatoxigenic fungi (Rustom, 1997), the anti-fungal effect of some grain wetting agents in this study was important. The anti-fungal effect of sodium hypochlorite was highest but none of the grain wetting agents used in this study exhibited absolute ability to inhibit moulds.

5.2 EFFECT OF CHEMICAL ADDITIVES ON AFLATOXIN LEVELS

Maize is commonly soaked in water for some time in order to soften and shorten the cooking time. The 29.8 % mean aflatoxin reduction observed when *muthokoi* was soaked in pure water for 12 h in this study, can be attributed to the moderate solubility of aflatoxins in water (Cole and Cox, 1981).

Similar loss of AFB_1 and AFG_1 was observed (Njapau *et al.*, 1998), when de-hulled maize was soaked in water for 24 h.

The incorporation of some food grade chemical additives in the soaking solution to increase destruction or loss of aflatoxins would be desirable. In acidic ammonium persulphate, AFB_1 is thought to undergo oxidative hydration across the olefinic bond of the terminal furan ring to form hydroxydihydro-aflatoxin B_1 (AFB_{2a}), which is 200 times less toxic compared to AFB_1 (Ciegler and Peterson, 1968). The aflatoxin reductions of 66.54 % and 69.58 % attained in this study following treatment of *muthokoi* in respective solutions of 0.5 % and 1.0 % ammonium persulphate at room temperature for 12 h, were higher than aflatoxin reduction of 65.75 % and 63 % respectively, attained by Tabata and co-workers (1994) following similar treatment of aflatoxin spiked whole-grain maize at 40 °C for 16 h. This difference could be attributed to the fact that dehulled maize *(muthokoi)* exhibits enhanced moisture imbibition owing to exposure of the endosperm, allowing better contact between the toxin and the chemical additive.

In the case of sodium hypochlorite, sodium hydroxide and hypochlorous acid are formed when sodium hypochlorite is dissolved in water (Wei *et al.*, 1985). Hypochlorous acid then ionises to hypochlorite ion, which is an oxidising agent under alkaline conditions and chlorinating agent under acidic conditions. The sodium hypochlorite solution used in this study had a pH of 11.5; commercial solutions of sodium hypochlorite usually contain excess alkali to stabilise the hypochlorite ion. Aflatoxin degradation in alkaline sodium hypochlorite is known to involve formation of water-soluble β-keto acid derivative of aflatoxin through opening of the lactone ring (Samarajeewa *et al.*, 1990) or the formation of a water-soluble β-keto acid derivative of dichloro-aflatoxin and /or dihydroxy-aflatoxin across the double bond of the terminal furan ring of the aflatoxin molecule (Tabata *et al.*, 1994).

The reduction in aflatoxins attained during soaking of *muthokoi* in graded solutions of *iati* can be explained by the proposal by Parker and Melnick (1966) that in alkaline solution, the lactone ring of aflatoxins is opened and aflatoxins then transformed to water-soluble β- keto acid derivative. The ionic composition of *iati* (Table 4.8) revealed high levels of Na^+, Cl^-, HCO_3^- and CO_3^{2-} and a high pH of 10.2. The effects of treatment of pure aflatoxins and aflatoxin-contaminated foods with sodium carbonate and sodium bicarbonate have been reported (Tabata et al., 1994). One hundred percent (100 %) loss of aflatoxins was reported when pure aflatoxin solution was treated with Na_2CO_3 (pH 13) for 16 h at room temperature (Tabata et al., 1994). Similarly, 73 % and 60 % loss of AFB_1 and AFB_2 respectively was observed when pure aflatoxins were treated with $NaHCO_3$ (pH 9), implying that Na_2CO_3 and $NaHCO_3$ were highly capable of degrading aflatoxins in the pure state. In naturally contaminated maize, however, aflatoxins may be protected within the grain matrix (Tabata *et al.*, 1992) hence the much lower loss of aflatoxins observed. Although the reaction involving transformation of aflatoxins to water soluble derivatives by alkaline treatment is reversible in acidic conditions (Tabata *et al.*, 1994), substantial removal of aflatoxins from contaminated foods may be achieved by washing after alkaline treatment.

The increased loss of aflatoxins during boiling in solutions of *iati* can be attributed to increased susceptibility of aflatoxins to hydrolytic opening of the lactone ring followed by heat-induced decarboxylation to form AFD_1, a compound which is 450 times less toxic compared to AFB_1 (Draughon and Childs, 1982; Rustom *et al.*, 1993). In addition, as observed by Farah *et al.* (1983), presence of ionic salts also increases the extent of aflatoxin degradation by heat. The 93.3 % aflatoxin loss in this study was higher than the partial aflatoxin reduction (46 % and 40 %) reported to occur during nixtimalization step (alkaline cooking of maize in calcium hydroxide) in the traditional process of making tortillas in Latin America (Price and Jorgensen, 1985; Abbas *et al.*,

1988). The high ionic strength of *iati* (5.6×10^2 dSm^{-1} as measured using electrical conductivity) and the composite nature of the salt (Table 4.7) probably explain the high percentage loss of aflatoxins observed in this study. Samarajeewa *et al.*, (1990) observed that the relative efficiencies of different alkalis in degrading aflatoxins at 110 °C appeared to take the order potassium hydroxide> sodium hydroxide> potassium carbonate> sodium carbonate> potassium bicarbonate>ammonium hydroxide> sodium bicarbonate> ammonium carbonate.

Because of the toxic nature of aflatoxins, the presence of aflatoxins in foods is limited to the lowest concentration that cannot be removed from the food without having to discard the food commodity and severely compromising availability of major food supplies (FAO/WHO, 1987). The maximum permissible levels for total aflatoxins in maize and maize products is regulated between 10 to 20 ppb (ng/g) by various food safety enforcement agents including the FAO/ WHO joint expert committee on food additives, (JECFA) (Rosner, 1998) and the Kenya Bureau of standards (KBS). Based on the mean aflatoxin reduction of 46.6 % attained during dehulling of maize grains in this study, *muthokoi* processing alone can decontaminate maize at 40 to 50 ng/g aflatoxin contamination level to the permissible levels (20 ng/g). Overnight soaking of the dehulled maize in dilute solution (0.5 %) of *iati*, can further reduce aflatoxins by 50 % hence combined, dehulling and overnight soaking can reduce aflatoxin levels in maize within 100 ng/g level of contamination to the permissible levels of 20 ng/g. Since alkaline cooking does not involve physical removal of the aflatoxins from the grain but instead, degradation of the highly toxic AFB_1 to a less toxic derivative, AFD_1, it would be expected that exposure to acute levels of the highly toxic form would be substantially lowered (by about 86 %) through cooking the dehulled maize in dilute solutions of *iati*. Thus, a combined process involving dehulling, overnight soaking and cooking of the dehulled maize (*muthokoi*) in dilute solutions of *iati* would be able to decontaminate maize within 600 ng/g aflatoxin contamination level.

The levels of chemical additives used in this experiment were considered safe given that considerable amounts would be lost in the discarded soaking solution and residual levels further diluted during subsequent washings after the treatment. According to the United States Food and Drugs Administration (FDA), the maximum levels of sodium hypochorite and ammonium persulphate in dry food starch should not exceed 0.82 % and 0.075 % respectively. An important observation made on maize treated with 1.0 % concentration of *iati* was the development of undesirable yellowing. This effect could be avoided by using a lower concentration of the salt (0.5 %) which has similar aflatoxin reduction effect.

Errors in this study could arise from sampling and replication in the analytical technique. In order to minimise sampling error, experimental units were randomly taken from reference samples whose aflatoxin levels were tentatively known. Sampling and sub-sampling errors were minimised by drawing large primary samples followed by sub-sampling from a large finely ground and thoroughly mixed secondary sample. To minimise errors associated with the assay technique, microtitre wells were coated with highly sensitive antibodies (Gathumbi *et al.*, 2001). The assay was quite sensitive judging from the aflatoxin concentration of 0.071±0.007 ng/g at the 50 % inhibition level of calibration curves constructed to quantify aflatoxin levels in samples. The mean detection limit was 0.021 ng/g (Table 4.1) while the percentage coefficient of variation of the 24 calibration curves constructed for the 24 microtitre plates used in the assay was 10.20 which was within the acceptable range of below 15 % required for minimum inter-assay variation (Heitzman, 1984).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The continued occurrence of acute aflatoxin poisoning in parts of the country and the numerous reports of contamination of human foods with aflatoxins demonstrate the great extent to which unsuspecting consumers are exposed to the toxic compounds in their diets. In Kenya, consumers of maize and products of maize origin in rural and urban areas alike are especially vulnerable to the acute and/ or long-term effects of aflatoxins, which act synergistically with primary infectious agents and malnutrition. Although good crop management and surveillance programmes are essential for control of aflatoxins in foods, they may not resolve the mycotoxins dilemma with certainty because contamination of agricultural produce with aflatoxigenic fungi occurs quite often in the field and during storage. Minimisation of exposure to aflatoxins through post-harvest processing technologies therefore remains vital in the management of the aflatoxin menace. No single strategy, however, can so far be used to fully decontaminate foods that are already contaminated with aflatoxins. Application of low - cost indigenous food processing methods that combine several procedures capable of reducing, inactivating and /or modifying the toxicity of aflatoxins while preserving the nutritional, physical and sensory properties of the food at household level, would offer suitable remedial approach.

In this study, a combination of dehulling and treatment with chemical additives was investigated for the ability to reduce aflatoxins in naturally contaminated maize. The dehulling process was on average found to reduce aflatoxins by 46.6 %. Further reduction in aflatoxins was also attained through treatment of the dehulled maize with chemical additives and although aflatoxin reductions by sodium hypochlorite and ammonium persulphate were comparatively higher than by (*iati*), the latter would preferentially be recommended on basis of its availability and acceptability as

evidenced in its continued use in local household food preparation procedures. The following conclusions can be made from this study: -

1. The traditional process of de-hulling maize to make ***muthokoi*** substantially reduces aflatoxin levels in naturally contaminated maize.
2. The practice of soaking maize in dilute solutions of *iati* for purposes of softening the maize prior to cooking reduces aflatoxin contamination levels.
3. Cooking of maize in dilute solution of *iati* results in remarkable reduction of aflatoxin levels by about 86%.
4. Dehulling of maize to make ***muthokoi*** does not destroy or remove moulds. There is the risk of enhanced mould proliferation and aflatoxin production since the process involves wetting and grain damage. This is particularly important since today ***muthokoi*** is packaged and retailed in shops or in open-air markets.

6.2 RECOMMENDATIONS

1. The effect of soaking de-hulled maize for long hours on nutrient extraction, and the overall nutritive value of maize treated with *Iati* need to be investigated further in order to validate the decontamination procedure.
2. Further research is needed to establish the recovery rate of aflatoxins in maize treated with *Iati* especially under conditions similar to the acidic environment in the stomach.
3. There is increasing evidence that some mycotoxins bind starch and amino acids in the food matrix during food processing, to form complexes that escape analytical detection (Fandohan *et al.*, 2005). Further research is needed to clarify whether aflatoxins apparently lost during alkaline cooking of aflatoxin-contaminated maize in this study, are really hydrolysed or bound to the food matrix to become non-recoverable and whether the binding is reversible with the possibility of releasing the aflatoxins upon consumption of the food.

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APPENDICES

Appendix 1: Aflatoxin contamination levels (ng/g) of whole-grain maize samples as analysed by the NPHLS

Sample	Aflatoxin Levels (ng/g)	Sample	Aflatoxin Levels (ng/g)
wg 1	160	wg 25	95
wg 2	30	wg 26	130
wg 3	120	wg 27	20
wg 4	20	wg 28	170
wg 5	70	wg 29	50
wg 6	40	wg 30	20
wg 7	40	wg 31	100
wg 8	30	wg 32	200
wg 9	300	wg 33	40
wg 10	220	wg 34	180
wg 11	100	wg 35	250
wg 12	120	wg 36	100
wg 13	20	wg 37	180
wg 14	400	wg 38	20
wg 15	110	wg 39	90
wg 16	80	wg 40	50
wg 17	190	wg 41	80
wg 18	80	wg 42	120
wg 19	30	wg 43	190
wg 20	130	wg 44	150
wg 21	30	wg 45	70
wg 22	65	wg 46	100
wg 23	180	wg 47	300
wg 24	90	Wg 48	180

Appendix 2: Composition of Mycological media

1. Sabouraud's Dextrose agar (Difco), modified with antibiotics

Special peptone	10.0g
D (+) Glucose	40.0g
Agar	15.0g
Chloramphenicol	100mg
Distilled water	1000ml
Final pH	5.6 ± 0.1

2. Aspergillus flavus and parasiticus agar (Pitt *et al.*, 1983)

Peptone	10.0g
Yeast extract	20.0g
Ferric ammonium citrate	0.5g
Chloramphenicol	100 mg
Agar	15.0g
Dichloran (2, 6 Dichloro-4-Nitroaniline)	2.0 mg
Distilled water	1000ml
Final pH	6.2

3. Yeast extract sucrose (YES) agar (Dierner and Davis, 1966)

Yeast extract	20g
Sucrose	150g
Agar	15g

Appendix 3: Solutions and buffers for aflatoxin ELISA kit

1. Coating buffer (pH 9.4-9.8)

$NaCO_3$	1.59g
$NaHCO_3$	2.93g
Distilled water	1000ml

2. Washing solution

NaCl	8.55g
Tween 20 (Poly oxyethylene sorbitan monohydrate)	0.25ml
Distilled water	1000ml

3. Phosphate buffered saline (PBS) pH 7.0-7.5

NaCl	6.79g
Na_2HPO_4	1.47g
K_2HPO_4	0.43g
Distilled water	1000ml

4. Substrate mixture (H_2O_2 buffer and Tetramethylbenzidine (TMB) solution in the ratio 20:1 prepared just before use).

H_2O_2 buffer (pH 3.9-4.0)

Citric acid monohydrate	8.81g
1M potassium hydroxide	40ml
Distilled water	160ml
H_2O_2	65µl

TMB solution

Tetramethylbenzidine	50.4mg
Acetone	1ml
Methanol	9ml

5 Stopping reagent: 1M H_2SO_4

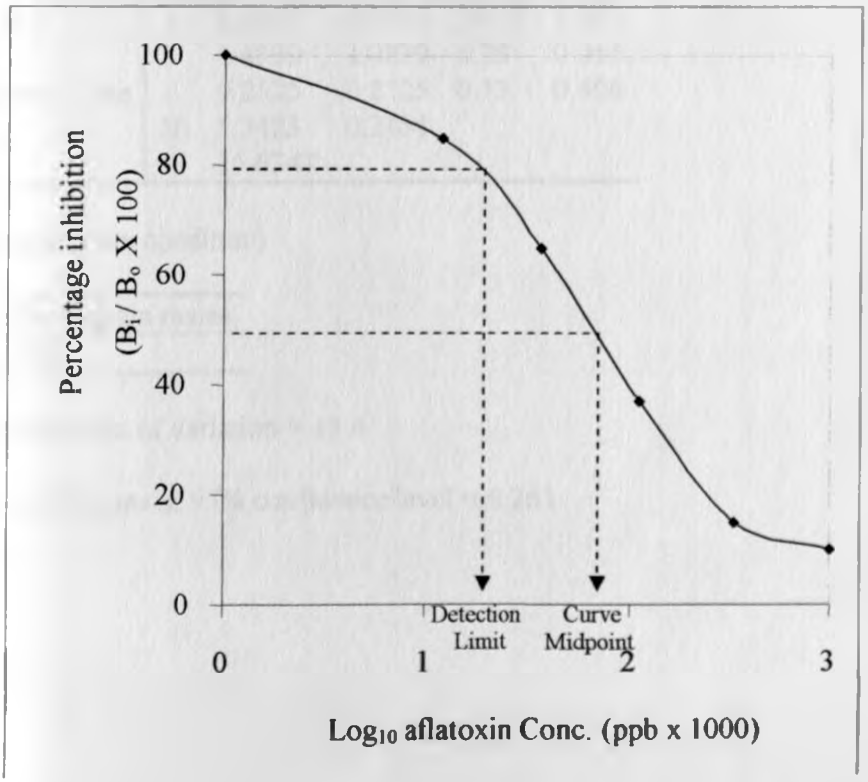
6. Aflatoxin-Enzyme conjugate: AFB_1 Oxime-Horse radish peroxidase

Appendix 4: Titration format on microtitre plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	E	S1	S2	S3	S4	S5	S6	ss1	ss 2	ss 3	ss 4	E
B	E	S1	S2	S3	S4	S5	S6	ss1	ss 2	ss 3	ss 4	E
C	E	S1	S2	S3	S4	S5	S6	ss 5	ss 6	ss 7	ss 8	E
D	E	S1	S2	S3	S4	S5	S6	ss 5	ss 6	ss 7	ss 8	E
E	E	ss 9	ss 11	ss 13	ss 15	ss 17	ss 19	ss 21	ss 23	ss 25	ss 27	E
F	E	ss 9	ss 11	ss 13	ss 15	ss 17	ss 19	ss 21	ss 23	ss 25	ss 27	E
G	E	ss 10	ss 12	ss 14	ss 16	ss 18	ss 20	ss 22	ss 24	ss 26	ss 28	E
H	E	ss 10	ss 12	ss 14	ss 16	ss 18	ss 20	ss 22	ss 24	ss 26	ss 28	E

S – Standards ss- Test samples E- Reagent blank

Appendix 5: Typical calibration curve for aflatoxin quantification



Appendix 6: ANOVA Tables

Appendix 6 (a): Comparison of means of aflatoxin reduction after dehulling maize grains with different grain wetting agents (variate: square root percentage aflatoxin reduction)

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Grain wetting agent	3	4.974	1.658	0.80	0.502
Residual	42	85.259	2.079		
Total	45	90.202			

Table of means

Grain wetting agent			
lati	NaOCl	$(NH_4)_2S_2O_8$)	Water
6.48	6.50	6.52	7.30

Percentage coefficient of variation 21.6%

Standard error of means at 95% confidence level: 0.617

Appendix 6 (b): Comparison of means of aflatoxin reduction after soaking *muthokoi* and whole grain maize in distilled water (Variate: Square root of percentage aflatoxin reduction)

Source of variation	*d.f*	*s.s*	*m.s*	*v.r*	*F pr.*
Grain condition	1	8.3555	8.3555	24.12	<.001
Time	1	1.4839	1.4839	4.28	0.055
Grain condition. Time	1	0.2525	0.2525	0.73	0.406
Residual	16	5.5423	0.3464		
Total	19	15.6341			

Means (effect of grain condition)

muthokoi	Whole-grain maize
5.05	3.76

Percentage coefficient of variation = 13.4

Standard error of means at 95% confidence level = 0.263

Appendix 6 (c): Comparison of means of aflatoxin reduction after treatment of whole-grain maize and *muthokoi* with chemical additives (Variate: Square root percentage aflatoxin reduction)

Source of variation	*d.f*	*s.s*	*m.s*	*v.r*	*F pr.*
Grain condition	1	51.7725	51.7725	156.09	<.001
Chemical additive	2	79.1449	39.5725	119.31	<.001
Conc.	2	38.0712	19.0356	57.39	<.001
Time	1	100.6300	100.6300	303.39	<.001
Grain condition . additive	2	1.2409	0.6205	1.87	0.158
Grain condition . conc.	2	0.7547	0.3774	1.14	0.323
Additive.conc	4	1.2863	0.3216	0.97	0.426
Grain condition. Time	1	11.2942	11.2942	34.05	<.001
Additive. Time	2	0.1232	0.1232	0.19	0.831
Conc .time	2	0.1483	0.0742	0.22	0.800
Grain condition. additive. conc	4	0.6954	0.1738	0.52	0.718
Grain condition. additive. time	2	1.4124	0.7062	2.13	0.123
Grain condition.conc. time	2	0.3801	0.1900	0.57	0.565
Additive.conc.time	4	1.9737	0.4934	1.49	0.209
Grain condition. additive. Conc.time	4	3.3627	0.8407	2.53	0.043
Residual	144	47.7625	0.3317		
Total	179	340.0532			

Means

Chemical additive	Conc. (%)	*muthokoi*		Whole grain maize	
		6h	12h	6h	12h
Ammonium persulphate	0.2	6.973	7.835	4.802	7.549
	0.5	7.419	8.128	6.170	7.770
	1.0	7.511	8.541	6.540	8.139
Sodium hypochlorite	0.2	5.853	7.539	4.883	6.044
	0.5	7.210	7.639	5.468	7.558
	1.0	7.096	8.485	5.987	8.059
lati	0.2	5.354	6.085	3.523	5.325
	0.5	6.035	7.042	4.106	6.562
	1.0	6.384	7.239	4.193	6.634

Standard error of means at 95% confidence level = 0.3642

Percentage coefficient of variation = 8.7

Appendix 6 (d): Comparison of means of aflatoxin reduction after boiling treatment with *lati* (variate square root percentage aflatoxin reduction)

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Grain condition	1	0.0396	0.0396	0.09	0.772
Conc	3	160.8679	53.6226	115.71	<.001
Grain condition. Conc	3	0.3237	0.1079	0.23	0.873
Residual	32	14.8291	0.4634		
Total	39	176.0603			

Means

Conc. (%)	0.00	0.20	0.50	1.00
mean	4.68	8.86	9.28	9.66

Percentage coefficient of variation = 8.4

Standard error of means (95% confidence level) = 0.304

Appendix 6 (e): Comparison of means of *Aspergillus flavus* counts in *muthokoi* (Variate: $\log_{10}$ c.f.u/g)

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Grain wetting agent	3	20.4102	6.8034	57.43	<0.001
Residual	42	4.9755	0.1185		
Total	45	25.3857			

Means

lati	NaOCl	$(NH_4)_2S_2O_8$	Water
2.846	2.173	2.132	3.838

Percentage coefficient of variation = 12.7

Standard error of means (95% confidence level) = 0.1474

Appendix 6 (f): Comparison of means of *Aspergillus flavus* counts in by-product (Variate: $\log_{10}$ c.f.u/g).

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Grain wetting agent	3	5.2454	1.7485	16.10	<0.001
Residual	42	4.5617	.01086		
Total	45	9.8071			

Means

Iati	NaOCl	$(NH_4)_2S_2O_8$	Water
4.205	3.626	3.833	4.525

Percentage coefficient of variation = 8.2

Standard error of means (95% confidence level) = 0.1411

Appendix 6 (g): Comparison of means of total mould counts in *muthokoi* (Variate: $\log_{10}$ c.f.u/g)

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Grain wetting agent	3	6.1543	2.1514	18.67	<0.001
Residual	42	4.6152	0.1099		
Total	45	10.7694			

Means

Iati	NaOCl	$(NH_4)_2S_2O_8$	Water
4.295	3.400	3.865	4.268

Percentage coefficient of variation: 8.4%

Standard error means (95% confidence level) = 0.1419

Appendix 6(h): Comparison of means of total mould counts in by-product (Variate: log_{10}c.f.u/g)

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
Grain wetting agent	3	9.0339	3.0039	14.37	<0.001
Residual	42	8.5608	0.2088		
Total	45	17.4393			

Means

Iati	NaOCl	$(NH_4)_2S_2O_8$	Water
5.092	4.188	5.203	5.216

Percentage coefficient of variation = 9.3%

Standard error of means (95% confidence level) = 0.1957

Appendix 7: Pearson's correlation test for relationship between variables during dehulling of maize grains

Variable	Aflatoxin levels in whole grain maize	Ratio of by-product to whole-grain aflatoxin content	Dehulling ratio	Aflatoxin reduction (%)
Aflatoxin levels in whole grain maize	1.000	-0.699**	-0.187	-0.671**
Ratio of by-product to whole-grain aflatoxin content	-0.699**	1.000	0.277	0.663**
Dehulling ratio	-0.187	0.121	1.000	0.352*
Aflatoxin reduction (%)	-0.671**	0.663**	0.352*	1.000

** Correlation is significant at the 0.01 level, * Correlation is significant at the 0.05 level (2-tailed test)