

DECLARATION

ANALYSIS OF MYCOTOXINS IN FOODS IN
NAIROBI AND ITS SURROUNDINGS

BY

LEONARD MWANGI GITU

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE

REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE

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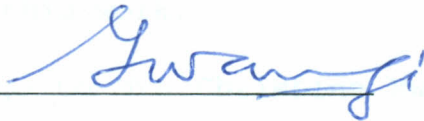
Gitu, Leonard Mwangi
*Analysis of
mycotoxins in foods*



1996/223777

DECLARATION

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




LEONARD MWANGI GITU

This thesis has been submitted for examination with our approval as University Supervisors.



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My thanks also go to Mrs Christine Mwangi of National Public Health Laboratories - Mycotoxin Section, whose help made these studies possible. I also wish to acknowledge with thanks all members of Chemistry Department, Kenyatta University for their assistance in the course of this work. I am also indebted to Kenyatta University for offering me the scholarship for my study.

Lastly, I wish to express my heartiest thanks to my wife Wanjiru for her deep understanding and encouragement throughout the course of this work.

DEDICATION

This thesis is dedicated to my mother, the late
Mrs. Ruth Wathiha Gitu.

ABSTRACT

Animal feeds samples were collected from feeding troughs and analyzed for levels of aflatoxin B₁, a toxic and carcinogenic mycotoxin. When aflatoxin B₁ is consumed by dairy cattle, some of it is hydroxylated to form aflatoxin M₁ which can appear in milk. Since aflatoxin M₁ is also toxic and carcinogenic, it was also determined in liquid milk. The analytical techniques used to carry out the determinations were Thin-Layer Chromatography (TLC), which utilizes the fluorescent properties of these compounds, and Differential Pulse Stripping Voltammetry, a technique that utilizes the electrochemical behaviour of aflatoxin B₁. Some of the feeds, particularly brewers grain, barley husks and used poultry feeds, were found to generally contain high concentrations of aflatoxin B₁ that were above the maximum tolerated levels in foods and feeds in various countries.

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LIST OF SYMBOLS AND ABBREVIATIONS

μg	Microgram
ng	Nanogram
mg	Milligram
Kg	Kilogram
μl	Microlitre
ml	Millilitre
mV	Millivolts
V	Volts
ε	Molar Absorptivities
Mw	Molecular weight
ppb	Parts per billion
ppm	Parts per million
yr	Year

LD ₅₀	Median lethal dose
TLC	Thin-Layer Chromatography
DPSV	Differential Pulse Stripping Voltammetry
NMR	Nuclear magnetic resonance
AFB ₁	Aflatoxin B ₁
AFM ₁	Aflatoxin M ₁
BR	Britton-Robinson buffer
NBS	National Bureau Standards
DNA	Deoxyribonucleic Acid
rRNA	Transfer Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide-2-phosphate
AOAC	Association of Official Analytical Chemists

LIST OF TABLES AND FIGURES

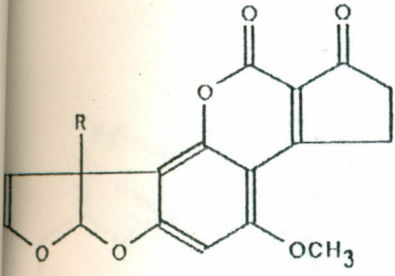
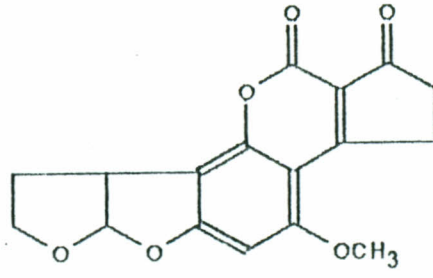
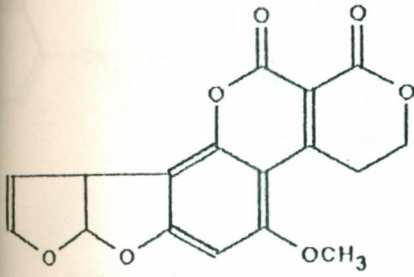
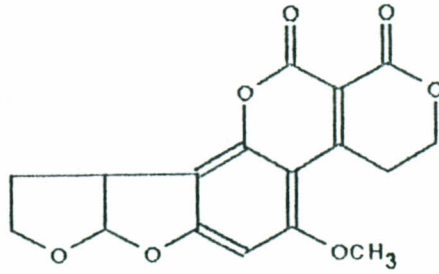
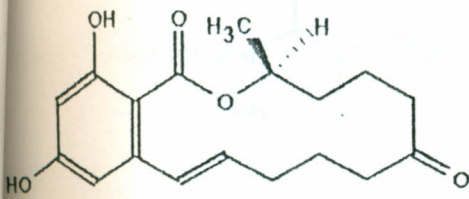
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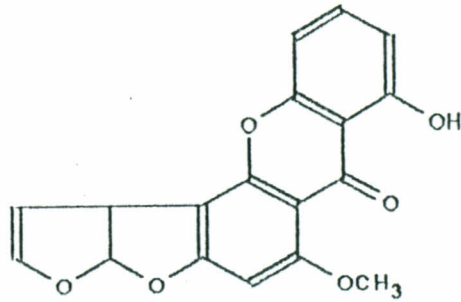
CHAPTER 1

1.0.0 INTRODUCTION

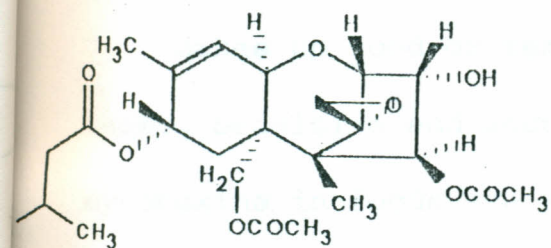
Mycotoxins are toxic secondary metabolites of fungi that represent numerous and diverse chemical classes. Fungi from important genera, including *Penicillium*, *Aspergillus*, and *Fusarium* are capable of producing these toxins in food and feed commodities during all stages of production[1]. Because of the variance of chemical properties, no single analytical technique can possibly quantify more than a few closely related mycotoxins at one time. However, multitoxin screening methods are available for detecting presence of a range of mycotoxins. Analytical methods must be extremely sensitive because the presence of even minute amounts of mycotoxins in foods and feeds may have serious toxicological implications for humans and animals. Each food or feed is composed of compounds which may interfere with the analysis, and thus a method must be specific for a certain class of toxin present in a particular commodity. The accuracy of monitoring low levels of mycotoxins in the complex chemical matrix that

Aflatoxin B₁ : R = HAflatoxin M₁ : R = OHAflatoxin B₂Aflatoxin G₁Aflatoxin G₂

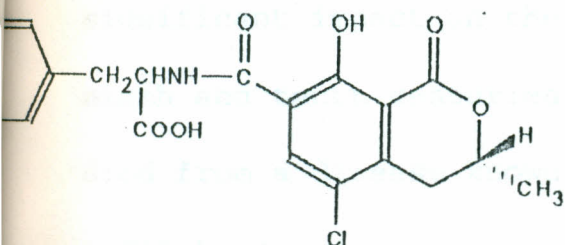
Zearalenone



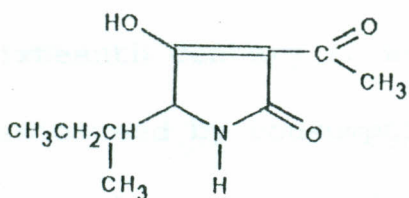
Sterigmatocystin



T-2 toxin



Ochratoxin A



Tenuazonic Acid

Figure 1:- Mycotoxins

makes up food or feed is therefore a difficult task. Sensitive and accurate methods for analysis of mycotoxins in foods are essential for decreasing the risk of human exposure to mycotoxins [2,3].

The contamination of food with mycotoxins has a significant impact on the history of mankind. In the ninth and tenth centuries tens of thousands of people died from a disease known as St. Anthony's fire. This affliction was characterised by necrosis in the affected limbs followed by the development of gangrene and eventual sloughing off of the limbs. In the mid-sixteenth century it was discovered that this disease was caused by consumption of rye contaminated with fungus later to be identified as *Claviceps purpura*[4].

1.1.0 AFLATOXINS

Aflatoxins were first detected in Brazilian groundnut (peanut) meal which contained the etiological agent of the outbreak of "Turkey X" disease that occurred in 1960 in England [5]. Since then, aflatoxins have been found in many other foods and feeds.

The aflatoxins are produced by only a few strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Given suitable environment, these moulds are very efficient at producing aflatoxins. These mycotoxins are a class of chemically related toxins possessing a bisfurano-coumarin structural backbone. The major toxins in this class are aflatoxins B₁, B₂, G₁ and G₂. The B and G notations refer to the fact that these compounds fluoresce blue and green respectively when exposed to long-wave length ultra-violet radiation [6,7]. The various subscripts refer to the corresponding chromatographic Rf values in silica gel under thin layer chromatographic systems with chloroform : methanol (97:3) as the developer.

When aflatoxin B₁ occurs in feeds and is consumed by dairy cattle, the hepatic microsomal mixed-function oxidase system of the cow converts some of it into hydroxylated aflatoxin B₁ which has been designated aflatoxin M₁. The latter retains the toxicity of aflatoxin B₁ but it is less carcinogenic[8]. Thus aflatoxin M₁ is of significance in terms of food safety because milk is a major part of human diet.

Aflatoxins have been found in peanuts and peanut products, maize, cereal grains, cottonseed and in other products that support growth of the aspergilli.

1.1.1 TOXICITY AND CARCINOGENICITY OF AFLATOXINS

The toxicity of aflatoxins has been demonstrated in many domestic and experimental animals. A common feature is its potent hepatotoxicity. The liver lesions occurring following aflatoxin poisoning are quite similar to those produced by the pyrrolizidine alkaloids from Senecio plants [9].

Results by several workers indicate that aflatoxin B₁ is probably the most potent hepatocarcinogen known, at least in experimental animals. It is also known to induce cancer in other tissues. Aflatoxin is a liver carcinogen not only in the rat, but also in rainbow trout [10], ducks [11], ferrets [12], mice [13], guinea pigs [14,15] and monkeys [16].

Most studies on aflatoxin carcinogenesis involve the rat. A demonstration of the extreme carcinogenicity of aflatoxin B₁ has been reported by Wogan and Co-workers [17] using Fischer rats fed an aflatoxin - containing

purified diet. In their experiment a diet containing even 1ppb of the carcinogen produced, out of 22 animals surviving longer than 50 weeks, two animals with confirmed liver carcinomas, six with hyperplastic foci, and one with foci of transitional cells. The authors regarded the hyperplastic foci and transitional cells based on their extensive experience with this experimental model, as early and intermediate stages respectively in the development of liver cancer induced by aflatoxin.

The rainbow trout (*Salmo gairdnerii*) is similar to the rat in its sensitivity to the carcinogenic effects of aflatoxin. Halver [12] estimated that by continuous feeding for 20 months, a minimum effective dose of 0.1 ppb would produce a 10% tumour incidence in this species. Like the rat, this fish will develop liver cancer with an exposure as brief as 2 weeks.

The mouse, duck and ferret are also quite sensitive to aflatoxin carcinogenicity when treated early in life.

Thus, eight of 11 khaki Campbell ducks fed on 30 ppb of aflatoxin B₁ beginning at 7 days of age for 14 months developed liver cancer [11], 100% of inbred mice

injected during the first 10 days after birth with a dose as low as 1.25 μ g of aflatoxin B₁/g body weight showed liver tumours when examined after 82 weeks [13].

Thus, the extreme potency of aflatoxin as a liver carcinogen is well documented. However while the liver is the primary site for carcinogenic induction with this mycotoxin other tissues are also susceptible; for example, the glandular stomach of the rat [18], rat colon [19], rat kidneys [20] and mouse lung [21]. The tumour in the latter was a typical primary adenocarcinoma.

1.1.2 FACTORS THAT AFFECT AFLATOXIN TOXICITY AND CARCINOGENICITY

Species, sex and age of the host influence the toxicity and carcinogenicity of aflatoxin.

Species:- In very susceptible species, aflatoxin B₁ has an LD₅₀ of 1mg/kg body weight or less. These include the dog, duckling, guinea pig, neonatal rat, rabbit, turkey, poult and rainbow trout. The chick and certain types of chickens, cow, ferret, hamsters, mink, monkey, pheasant, pig, coturnix quail, rat and coho salmon are moderately

susceptible having an LD₅₀ of up to 10 times the dose of the first group of very susceptible animals. The mouse and sheep constitute the very resistant species to the acute effects of the mycotoxin being able to withstand large doses of mycotoxin with no apparent ill effects [15].

Nevertheless, these seemingly resistant animals will also develop tumours of the liver and other tissues. For example, sheep fed 1 to 1.75 ppm aflatoxin in the form of toxin peanut meal will develop hepatic and nasal neoplasms [22]. Mice show no ill effects even when fed contaminated peanut meal containing 4.5 ppm of aflatoxin B₁ and G₁ and 0.6 ppm aflatoxin B₂ and G₂ [23].

This dose of aflatoxin B₂ is greater than the dose which would be effective in other animals [24].

Differences in susceptibility to aflatoxin toxicity among strains within a species are also evident from several reports. For example, the Rhode Island Red [25] and the Arbor-Acres broiler type [26] chicks are remarkably more tolerant to aflatoxin than the Pilch strain White Rocks chicks [27]. There are also strain differences in susceptibility among rats. Fischer rats

appear more susceptible than the Sprague-Dawley rats[16].

Sex:- Male rats appear more susceptible to the effect of aflatoxin than females [19]. This difference among Fischer male and female rats is shown in Table 1. Liver tumours appear among the males much earlier, 35-82 weeks following treatment in the males as compared to 82 weeks or more in females. There is evidence that this difference may be hormonal since by simultaneous feeding of diethylstilbestrol (DES), Newberne and Williams[28] reduced the cancer incidence among male Charles River rats from 71 to 20%. Liver nodules still formed as without DES but fewer nodules became malignant.

Table 1 Susceptibility of Male and Female Fischer Rats to the Carcinogenic Effects of Aflatoxin B₁

Dose, µg/Kg diet	Latency	
	Male	Female
15	68 weeks	80 weeks
1000	35-41 weeks	64 + weeks

Source: Wogan and Newberne [19]

Age:- The effect of age on sensitivity toward aflatoxins was demonstrated in a number of animal species. Young animals appear more susceptible to the toxic effects of aflatoxins than adults [15]. Pigs over 8 weeks old may tolerate as much as 150 ppb of aflatoxin in the diet, whereas younger pigs may not tolerate even very low levels of the toxin[22]. The weight gain and feed conversion among growing 18 to 64 Kg pigs are significantly affected with 410 ppb of aflatoxin in the feed but the same level has no effect in older pigs [29]. Similarly turkey poults appear more susceptible than the older birds [30]. A similar observation was seen in trout [17]. Cattle show increasing resistance to aflatoxin toxicity as they grow older[31]. Young rats are extremely sensitive to the carcinogenic effects of aflatoxins compared to adults [32].

1.1.3 THE EFFECTS OF AFLATOXINS IN HUMANS

Because of the toxic and carcinogenic effects of aflatoxins as established in many animal species, it is highly probable that similar effects occur in human

beings. However, as expected, direct evidence of aflatoxins effects in humans is not available. This is understandable partly because of (i) the limitations of human experimentation (ii) the nature of the carcinogenic process involving prolonged latency and (iii) the difficulty of directly connecting aflatoxins as the causative agents in human poisoning following the consumption of mouldy foods. This is because of the presence of several mycotoxins in these foods.

Nevertheless, there is strong circumstantial evidence linking aflatoxins to the symptoms of poisoning in humans consuming mould-contaminated foodstuffs. This is particularly true in those cases in which serious liver pathology is involved.

In many parts of the world, particularly in the tropics, many cases of human poisonings strongly implicate aflatoxin consumption. In Taiwan, 26 persons were poisoned following consumption of mouldy rice for up to 3 weeks. Two samples of mouldy rice contained approximately 200 $\mu\text{g}/\text{Kg}$ aflatoxins. Three poisoned children died. The victims suffered edema of the legs,

abdominal pain, vomiting and palpable liver but no fever[33].

A case in Uganda involved a 15 year old boy who died of symptoms resembling the cases in Taiwan following consumption of diet which included mouldy cassava [34]. The cassava sample contained 1.7 ppm aflatoxin. Two other siblings were likewise affected but recovered. The autopsy finding revealed liver necrosis and mild fatty liver in addition to other pathological changes. Based on monkey studies, a lethal amount of aflatoxin in the cassava might have been consumed if the victim ate the mouldy cassava for a longer period [35].

A case of Reye's syndrome reported from Thailand may have involved aflatoxin poisoning [36]. This case involved a three year-old boy who died of Reye's syndrome following consumption of mouldy rice containing as much as 10 ppm total aflatoxins. Reye's syndrome is characterized by vomiting, hypoglycaemia, convulsions, hyperammonemia, coma and other acute symptoms. Autopsy revealed cerebral edema and fatty accumulation in the liver cells, tubular epithelium and the myocardial

fibres. The similarity between Reye's syndrome and acute aflatoxicosis in the macaque monkey is striking [36]. In the monkey suffering from aflatoxicosis, aflatoxin B₁ was present in tissues up to 6 days after administration of the mycotoxin [37]. In Reye's syndrome, one or more autopsy specimens from victims also contained aflatoxin B₁ and in two cases, the level of aflatoxin was comparable to that in monkeys given an LD₅₀-dose of the mycotoxin [38]. Two additional cases of Reye's syndrome in which aflatoxin was found in the tissues were reported from New Zealand [39].

A case can be made regarding the association between liver cancer in man and the consumption of aflatoxin containing foodstuffs based on surveys from many parts of the world. In northern Thailand there appears to be a correlation between the incidence of liver cancer and Reye's syndrome [40]. In India, aflatoxin consumption may be a factor in the etiology of prevailing childhood cirrhosis, characterised by fatty infiltration of the liver cells, leading to cellular degeneration fibrosis and hepatomegaly [41]. Kwashiorkor patients in India who were unintentionally

fed peanut protein supplements contaminated with up to 300 ppb aflatoxin developed symptoms similar to those in childhood cirrhosis [42].

In certain countries in the tropics, analyses have shown significant aflatoxin contamination of foodstuffs consumed. For example, Campbell [43] found that locally produced peanut butter in the Philippines contained significant amounts of aflatoxin (median level, 500 ppb) and aflatoxin M_1 was found in the urine of children consuming 60g or more of contaminated peanut butter. Since peanut butter consumption by a significant portion of the Filipino population is a recent adaptation, assessment of liver cancer incidence with aflatoxin consumption can only be made several years hence.

In Swaziland, Keen and Martin [44] found an association between the crude liver cancer incidence and the frequency of contamination of peanuts samples. Thus, in the high, middle, and low veld regions where the rates of detectable aflatoxin contamination of peanut samples were 20, 27 and 60%, respectively the corresponding crude liver cancer incidence were 2.2, 4.0, and 9.7 cases/100,000/yr. In Uganda, the highest

incidence of liver cancer (15 cases/100,000/yr) was found to be in the tribal regions where the frequency of aflatoxin contamination of staple foodstuffs was also the highest (44% of the samples collected) [45]. About 3.7% of 480 samples contained as much as 1 ppm aflatoxin. Studies of aflatoxin consumption and liver cancer incidence in Kenya also have shown a positive correlation [46].

In three regions of Kenya, the calculated daily intake of aflatoxin, based on estimated consumption of contaminated food and beverages, was 4.9, 7.8, and 14.8 ng/Kg body weight [47]. The corresponding liver cancer incidence in these regions were 3.1, 10.8, and 12.1 cases/100,000/yr. These results are essentially in agreement with similar studies conducted in Thailand[40,48]. They found that many food products in Thailand which appeared wholesome and fit for human consumption contained relatively high levels of aflatoxin. For example, the aflatoxin content of dried fish, dried chili peppers, corn, and peanuts was shown to be 772, 996, 2700, and over 12,000 ppb, respectively. In some regions, as much as 16% of the samples of

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foodstuffs were contaminated with aflatoxins. In three areas, the calculated average aflatoxin intakes on a family basis were 73 to 81 ng, 45 to 77 ng and 5 to 8 ng/Kg body weight/day. The liver cancer incidence in these regions range from two cases/100,000/yr for the lowest aflatoxin intake to six cases/100,000/yr for the highest intake.

It should be emphasized that the association between observed liver cancer incidence and aflatoxin intake for any given year may not closely reflect a direct correlation. The reason is that the present cancer rate may be the result of aflatoxin consumption several years previously which may be greater or less than the present consumption on which the correlation is based. However, it is reasonable to assume that the food pattern, fungal flora, and conditions of fungal growth in the regions under study are more or less constant, so that mycotoxin consumption of the affected populations then is very similar.

Although the actual intake of aflatoxin appear to be quite small, one should remember the extreme carcinogenic potency of aflatoxin B₁. As already

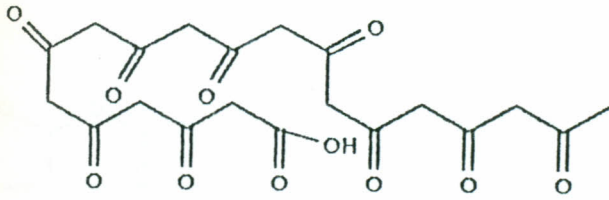
mentioned, a dose of 1 ppb of aflatoxin is carcinogenic[16] and the highest individual intake of aflatoxin in Thailand was 55 ng/Kg body weight/day. This level of intake is comparably a carcinogenic dose for the rat [48].

While the above studies do not warrant a definite conclusion regarding the carcinogenicity of aflatoxin in humans, the consistency of the results in diverse locations and different populations suggests a causal relationship. Because of the extreme, carcinogenic potency of aflatoxin in animal species, the widespread distribution of aflatoxin producing moulds and their great ability to grow in different foods grown or stored under various conditions, it is highly probable that this mycotoxin poses a serious carcinogenic hazard to humans.

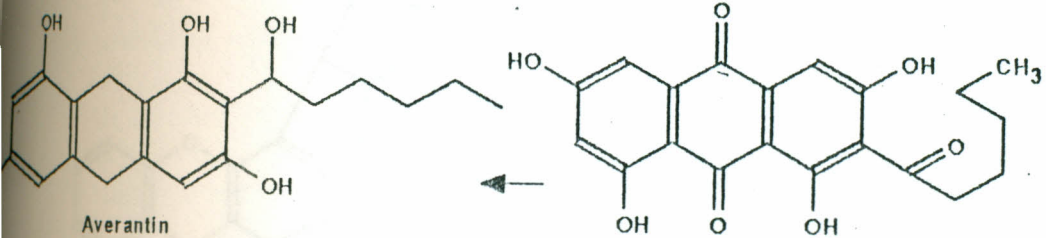
1.2.0 BIOSYNTHESIS OF AFLATOXIN B₁

The biosynthesis of the aflatoxins, specifically AFB₁, has been the subject of conflicting speculation and numerous reviews [49-52]. Studies on biosynthesis of AFB₁, the major metabolite of most aflatoxigenic moulds, have shown that the basic skeleton of the toxin molecule is derived entirely from acetate units via the polyketide pathway, and that methionine contributes the methoxy-methyl group [50,53-55]. The number of acetate units comprising the polyketide precursor of aflatoxin has been controversial. However, several authors support a biosynthetic scheme based on a C₂₀ polyketide (decaetide) precursor [50,56,57]. Figure 2 illustrates, in biosequential order, the five polyhydroxy-anthraquinones believed to be involved in the biosynthesis of AFB₁.

The first anthraquinone for which experimental proof exists is norsolorinic acid [58]. Hsieh et al. [58], working with ¹⁴C - labelled norsolorinic acid produced from 1- ¹⁴C acetate by a deficient mutant of an AFB₁ producer, found that the label from norsolorinic acid was in the AFB₁ molecule. Bennett et al. [59]

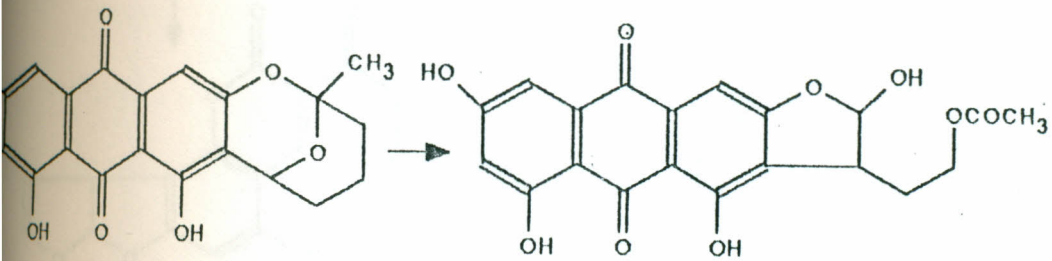
10 CH₃COOH →

Cyclized decaetide



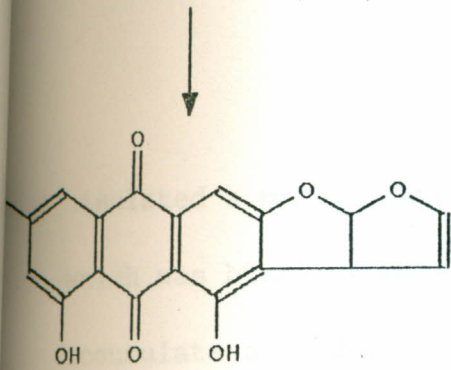
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Norsolorinic Acid

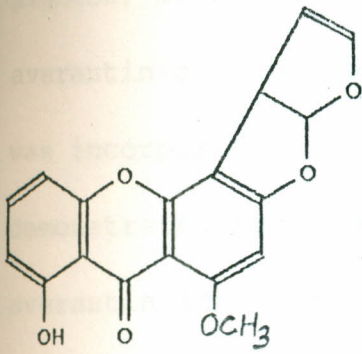


Averufin

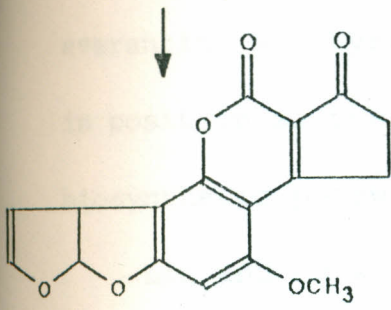
Versiconal hemiacetal acetate



Versicolorin A



Sterigmatocystin

Aflatoxin B₁Figure 2:- Proposed pathway for biosynthesis of aflatoxin B₁

isolated a mutant strain of *Aspergillus parasiticus* which was blocked in aflatoxin production, yet able to accumulate a number of polyhydroxyanthraquinones. One of the polyhydroxyanthraquinones they identified was the pigment, averantin[59]. Radiotracer studies with ^{14}C -averantin showed that 15.3% of the label from averantin was incorporated into AFB_1 . Additional experiments demonstrated the transformation of label from ^{14}C averantin into averufin, but not into norsolorinic acid.

The transformation of ^{14}C - norsolorinic acid into averantin, and averantin into averufin places averantin in position as the second intermediate compound in the biosynthetic pathway leading to AFB_1 .

Lin and Co-workers [60] using labelled averufin provided experimental evidence for its role as an intermediate compound. Two observations[60] supporting this role are:

(a) accumulation and excretion of averufin by mutants blocked in the synthesis of aflatoxin and (b) rapid uptake of averufin by resting mycelium of the parent fungus and its conversion into AFB_1 . Gorst-Allman and Co-workers [61], using ^{13}C NMR, provided data that permitted

predicting the mode of folding of averufin and other related C₂₀- anthraquinones. Conversion of averufin to versiconal hemiacetal acetate, as proposed by Gorst-Allman and Co-workers [61], involves an epoxide intermediate.

Using the insecticide dichlorvos to treat cultures of *A. parasitus*, Yao and Hsieh [62] induced an impairment in AFB₁ production and a concurrent increase in formation of versiconal hemiacetal acetate. Fitzell and Co-workers [63] prepared the orange pigment, versiconal hemiacetal acetate, from 1-¹³C-, 2-¹³C-, 1, 2-¹³C- labelled and unlabelled sodium acetate, using dichlorvos-treated cultures of *A. parasiticus*. Results of the assignments made from the spectra (fourier transform NMR analysis) indicated acetate as its sole carbon precursor and secured its biogenetic relationship to aflatoxins. In addition, Singh and Hsieh[64] found that dichlorvos inhibited conversion of versiconal hemiacetal acetate into AFB₁, but not of versicolorin A or sterigmatocystin. These studies implicate versiconal hemiacetal acetate as a pivotal intermediate compound in the biosynthetic sequence of AFB₁.

The trihydroxyanthraquinone, versicolorin A, contains the unique difurano group present in aflatoxins and has been proposed as an intermediate substance in the biosynthesis of these compounds [60,64,65].

Sterigmatocystin is the final intermediate compound (for which experimental evidence exists) on biosynthesis of AFB₁. Singh and Hsieh[66], using a cytoplasmic fraction from the mycelium of *A. parasiticus*, demonstrated the enzyme activity involved in the conversion of this precursor to AFB₁.

This brief discussion has dealt exclusively with biosynthesis of AFB₁. Studies on the latter stages of aflatoxin biosynthesis in *A. flavus*, using biosynthetically ¹⁴C-enriched compounds, indicate that AFB₁ is converted into most of the other aflatoxins either by oxidation and/or hydroxylation [67,68]. The exception is the hydroxylated form of AFB₁, the AFM₁. Heathcote et al. using the technique of feeding labelled aflatoxins and related metabolites to cultures of aflatoxin-producing moulds, found that AFB₁ could be converted into other aflatoxins, such as B₂, B_{2a}, G₁, G₂ and G_{2a} [67].

1.3.0 AFLATOXIN METABOLISM

Aflatoxins are primarily metabolized by the microsomal mixed-function oxidase system, a complex organization of cytochrome - coupled, O^2^- , and NADPH - dependent enzymes located mainly on the endoplasmic reticulum of liver cells but also present in kidney, lungs, skin, and other organs. These enzymes oxidatively metabolize a wide variety of foreign or Xenobiotic compounds with the net result being the detoxification of the parent compound by the formation of various hydroxylated derivatives, which, in turn, are conjugated with sulphate or glucuronic acid to form water-soluble glucuronide or sulphate esters. These conjugates may then be readily excreted in the urine or bile. During the course of metabolism, certain highly reactive metabolites may be generated that have the capacity to react covalently with various nucleophilic centres in cellular macromolecules such as DNA, RNA, and protein. This "activation" reaction poses a biological hazard to the cell and constitutes a plausible theory by which certain compounds exert toxic and carcinogenic effects[69].

Some of the established pathways of AFB₁ metabolism are discussed below. Many of these reactions share a commonality with mechanisms by which polynuclear aromatic hydrocarbons, a large class of lung and skin carcinogens, are metabolized [70,71].

1.3.1 AFLATOXIN METABOLITES AND PATHWAYS

(i) **AFM₁**:- Ring hydroxylation of AFB₁ at the 4th position forms AFM₁. This metabolite was first detected in the milk of cows ingesting AFB₁ [72,73]. It was produced *in vitro* from AFB₁ by a variety of species including humans [74,75] and excreted in the urine of AFB₁ - treated sheep [76] and monkeys [77]. AFM₁ has also been detected in the urine of humans consuming AFB₁ - contaminated peanut butter [78]. AFM₁ is both toxic and carcinogenic.

(ii) **AFB_{2a}**:- This hemiacetal metabolite is produced from AFB₁ by hydration of the 2,3-vinyl ether double bond resulting in hydroxylation at the 2 position. It was readily formed from AFB₁, under mildly acidic conditions [79] and *in vitro* with chick, duckling, guinea pig, and mouse microsomes [80,81]. AFB_{2a} was apparently unstable

under physiological conditions (pH 7.4) and bound to protein and hepatic microsomes [80,82]. The AFB_{2a} was thought to cleave to form dialdehyde derivatives that then covalently bonded to the amino groups of proteins by Schiff base formation [83].

(iii) AFB₂:- Roebuck et al [84] reported the formation of AFB₁ when AFB₂ was incubated with duckling liver postmitochondrial supernatant. Swenson et al [85] noted that the same aflatoxin derivative (2,3-dihydro-2,3-dihydroxy aflatoxin B₁) was released by acid hydrolysis from the nucleic acids of both AFB₁ and AFB₂- treated rats. On this basis they postulated an AFB₂ to AFB₁ conversion by unsaturation of the 2,3 carbons of AFB₂. This pathway could account for the weakly toxic and carcinogenic properties of AFB₂.

1.3.2 MACROMOLECULAR BINDING OF AFLATOXIN

The covalent incorporation of aflatoxin into nucleic acids and proteins is now considered to be an important mechanism by which AFB₁ initiates its toxic and carcinogenic effects.

It was recognized early that AFB_{2a}, formed by metabolic hydroxylation of AFB₁, could spontaneously bind covalently to proteins at physiological conditions pH 7.4 [80,82]. AFB_{2a} was essentially nontoxic, therefore, this reaction seemed to lack relevance to the disruption of cellular function and has not been investigated further.

In vitro incubation of AFB₁, rat liver microsomes, and appropriate cofactors with RNA, DNA and polynucleotides resulted in AFB₁ binding to these nucleic acids at levels as high as one aflatoxin residue per 30 nucleotides [86,87]. Swenson et al [88] isolated 2,3-dihydro-2,3-dihydroxy-aflatoxin B₁ by mild acid hydrolysis of RNA adducted *in vitro* with liver microsomes and AFB₁. No binding was observed with AFB₂, demonstrating that 2,3-unsaturation of the terminal furan ring was a prerequisite for activation. This information suggested the hypothesis that, aflatoxin B₁-2,3-oxide was the reactive precursor and probably the ultimate carcinogenic metabolite of AFB₁. The 2,3-dihydro-2,3-dihydroxy aflatoxin B₁ was also isolated from liver DNA and rRNA of rats dosed with AFB₁ [89]. Although

binding of AFB₁ to liver proteins was observed, the level was only 4 to 7% that observed with nucleic acids.

Attempts at isolating the highly reactive 2,3-oxide have been unsuccessful. Swenson et al [90] synthesized a more stable model compound, aflatoxin B₁-2,3-dichloride. This electrophilic analog of the epoxide was considerably more potent than AFB₁ in the Ames mutagenesis assay and in the induction of various rat and mouse tumours. The AFB₁ dichloride also reacted spontaneously with DNA, RNA, protein and amino acids. The AFB₁ dichloride was hydrolysed in aqueous solutions to yield the 2,3-dihydro-2,3-dihydroxy aflatoxin B₁, which proved especially reactive towards protein presumably via dialdehyde derivatives forming Schiff bases with the primary amino groups of protein [90]. The major DNA adduct formed by AFB₁ *in vitro* (approximately 90% of the AFB₁ bound to DNA) has been isolated and identified as 2,3-dihydro-2-(N⁷-guanyl)-3-hydroxyaflatoxin B₁[91]. This adduct is also the major product formed *in vivo* in rat liver[92,93].

The relative biological hazard posed by the formation of aflatoxin adducts with different classes of

dose was excreted or present in intestinal tract contents within 24 hours. Faeces represented the principal excretory route accounting for up to 75% of the dose, with urine containing an additional 20%. Only 0.3 to 0.5% of the dose was recovered as carbon dioxide.

Maximum levels of aflatoxin radioactivity occurred in tissues (mainly the liver) 30 minutes after dosing. After 24 hours approximately one-half of the total retained AFB₁ dose was still present in the liver.

Urinary excretion patterns were studied in male Rhesus monkeys given a single ip injection of ring-labelled ¹⁴C-AFB₁ [77]. A maximal rate of excretion was attained within 1 hour and 35% of the total dose was recovered in the urine after 4 days. Approximately 15% of this activity was chloroform soluble and included AFM₁, AFB₁ and several unidentified metabolites. Aflatoxin residues were primarily retained by the liver (6% of the original dose after 4 days) with much lower levels detectable in other tissues. Similar experiments using orally administered ¹⁴C-AFB₁ adsorbed on casein showed that 80% of the total dose was equally excreted in the urine and faeces after 1 week [98]. This pattern

was noted with both high (0.4 mg/Kg) and low (0.15 mg/Kg) AFB₁ doses. Detectable radioactivity was still present in the urine and blood after 5 weeks. The proportion of chloroform soluble metabolites in the urine was initially high (74% in the first day) but decreased rapidly to 10% or less of the total urinary metabolites by 3 days.

1.4.0 FACTORS THAT AFFECT MOULD GROWTH, MYCOTOXIN PRODUCTION AND INFECTION OF FOODS AND FEEDS

A fungal species may require specific conditions for growth, but in general, moulds from among many genera characteristically share similar conditions for growth, development and mycotoxin production. The conditions for growth and mycotoxin production of *Aspergillus flavus* have been thoroughly studied and many of the factors obtained from such studies can be also applied to other moulds, particularly those of the aspergilli group [99].

1.4.1 MOISTURE

The requirement of most, if not all, moulds for spore germination and growth is moisture, and there is a wide range of moisture requirements among moulds.

Moisture content of substrates in relation to mould growth is more accurately expressed as the water activity [99].

$$a_w = V_{ps}/V_{pw}$$

where

a_w = Water activity

V_{ps} = Water vapour pressure of substrate

V_{pw} = Vapour pressure of pure water at the

temperature and atmospheric pressure as the substrate.

Since V_{ps} varies with the substrate, then the percent gross moisture content of each foodstuff on which moulds grow will vary. In terms of a_w , many moulds have similar values for optimum growth at optimum temperature. Table 2 shows Ayerst's data of a_w requirements of number of *Aspergillus*, *Penicillium* and *Strachybotrys atra* moulds.

Table 2: Optimum water Activity Requirements of
Selected Moulds [100]

Mould	a_w		Optimum temp ^a
	Optimum	Minimum	
<i>Aspergillus flavus</i>	0.98	0.78	33°C (12-43°C)
<i>A. fumigatus</i>	0.97	0.82	40°C (12-53°C)
<i>A. chevalieri</i>	0.93	0.71	33°C (10-42°C)
<i>Penicillium cyclopium</i>	0.98	0.82	23°C (<5-32°C)
<i>P. martensii</i>	0.98	0.79	23°C (<5-32°C)
<i>P. islandicum</i>	0.97	0.83	31°C (10-38°C)
<i>Strachybotrys atra</i>	0.98	0.94	23°C (7-37°C)

^a Values in parentheses are the temperature ranges of growth.

NB: At optimum temperature, the water "activities" required for optimum growth by each mould are remarkably similar; all these values approach unity. With the exception of *A. chevalieri* and *S. atra*, the minimum water activity requirement is around 0.80. *A. chevalieri* can grow in a much drier condition than *A. flavus*. *S. atra* requires a much wetter condition. In general, the storage moulds, represented by *A. flavus* and other aspergilli will grow in substrates at moisture

contents between 13 and 18% according to Jarvis[101]. The decay moulds, represented by *S. atra*, require a moisture content of 22-25%. Again, the minimum moisture content depends on the substrate. For example, when growing on peanuts, *A. flavus* requires a moisture content of only 9%.

At higher moisture content, for example, between 40 and 45% in cereal grains, many storage moulds fail to develop[102]. In this moisture range, penicillic acid production in poultry feed by *A. ochraceus* at 22 to 30°C has been observed to begin to drop significantly, but ochratoxin production by the same mould has been reported to begin to drop only when the moisture content was 52% or above[103].

The rate of drying can affect mycotoxin formation. This is because of the fact that mycotoxin biosynthesis does not occur simultaneously with mould growth. For example, *A. flavus* does not produce aflatoxin until about 48 hours after germination [104]. Thus, a product may be rendered safe if it can be dried rapidly to below 13% water moisture, within 48 hours after germination.

1.4.2 TEMPERATURE

The growth of most moulds is temperature dependent. Most moulds are mesophiles; i.e., growing best between 10°C and 40°C. The psychrophiles have an optimum temperature below 10°C. The thermophiles can grow at temperatures above 40°C and even higher than 50°C. Some members of this last group can exist at a wide range of growth temperatures, some extending as low as 20°C. Thus, foodstuffs may be contaminated by a whole spectrum of mould species over a wide range of storage temperatures from several degrees below 0°C to as high as 50°C or above. There are toxicogenic species in all three classes, but most of these are found among the mesophiles.

Psychrophilic moulds are found among species of *Fusarium*, *Cladosporium*, *Trichoderma*, and *Strachybotrys*. These moulds are able to thrive on specific substrates, at near or below freezing temperatures. Many of these moulds require low temperatures for mycotoxin production. Based on a survey of over 300 samples of frozen packed fruits and vegetables, Smart [106] reported many species of *Aspergillus*, *Penicillium*, *Rhizopus*,

Mucor and *Cladosporium* able to survive at -10°C for over a year.

Studies on *A. flavus* [107] have shown that spores survived at temperatures of -45°C or lower, provided the freezing medium did not freeze. However, the percentage survival decreased drastically under three conditions: (1) rapid freezing of spores, (2) freezing of the suspending medium and (3) slow warming of the spores after rapid freezing. The effects of slow cooling and rapid thawing with respect to the increased survival of spores have been observed in other moulds; e.g.

Alternaria, *Aspergillus*, *Fusarium*, *Gliocaldium*, *Penicillium* and others [108,109]. Some mesophilic moulds can grow below 10°C , for example *P. cyclopium* and *P. martensii* can thrive at 5°C or below [100].

There is also an optimum temperature for mycotoxin production, for example mycotoxin production of *F. tricinctum* requires a temperature just below 0°C , a few degrees above the growth minimum. This mould as well as *Cladosporium spp.* grows well between -1 and -7°C [110]. Actually, alternate thawing and freezing temperatures intensify mycotoxin production. *F. raseum* produces more

zearalenone when stored at 12°C even though the mycotoxin may be produced at 25°C [111]. Aflatoxin B₁ production is maximum at 24°C [100,112]. The maximum production of G₁ occurs at 29°C [112] and no mycotoxin is produced by *A. flavus* below 13 or above 42°C [112]. Sorenson et al [113] reported no aflatoxin production above 37°C. The mould was observed to grow between 6 and 45°C. Maximum yields were observed after 15 days at 20°C or 11 days at 30°C [114].

Thermophilic moulds are found in *Mucor*, *Chaetomium*, and a few in *Penicillium* and *Aspergillus*. Their importance lies in their ability to survive sterilization temperatures of 50°C or higher. Since they grow little at ordinary temperatures and can compete with other moulds only at extraordinarily high temperatures [115], they probably pose little or no toxicological significance.

1.4.3 MECHANICAL, INSECT AND MOULD DAMAGE

Growth of moulds require contact with substances. Natural products generally have protective outer tissues or layers which are resistant to mould attack. Damage

or removal of these protective coverings results in easy access of mould spores to the nutrient-laden internal tissues; or else nutrients from these tissues ooze to the outside, whereupon the spores germinate rapidly. Thus, as observed in peanuts, germination of the *A. flavus* spores in the geocarposphere (the surrounding soil layer affected by the developing pod) occurs rapidly when the peanut pod is mechanically injured [116]. Observations on corn also have shown that more ears are contaminated with aflatoxin when damaged mechanically after silking. Fewer undamaged ears are contaminated. Aflatoxin contamination is especially more serious following damage to protective coverings of foodstuffs in places where there is a high *A. flavus* spore load [117]. Similar observations have been reported by Anderson et al [118], who noted increased aflatoxin contamination of corn kernels following mechanical and insect damage.

Insects may carry saprophytic fungi in their gastrointestinal (GI) tract. Ragunathan et al [119] found that grubs, pupae and adults of the rice weevil carry *A. flavus*, *A. ochraceus* and other moulds in their

GI tract. These insects may infect and breed on cereal grains and become a source of mould contamination. In the studies by Anderson et al [118] aflatoxin contamination of corn in the field was reduced by up to 100% (average 96.2%) following insecticide application, resulting in reduced insect and worm damage. Studies in Iowa also pointed to the important role of insect damage in aflatoxin contamination of corn before harvest [117]. This study showed that *A. flavus* infection and aflatoxin contamination were associated with kernel injury caused by second generation European corn borers.

Damage by one mould can facilitate the infection of a foodstuff by another. For example, damage of peanut pods before harvest by the root rot pathogen, *Rhizoctonia solani*, facilitates entry of *A. flavus* and *A. niger* to the peanut kernels [120]. Infection of corn kernels by *Fusarium*, *Penicillium* and *Aspergillus* moulds, particularly *A. flavus*, is more prevalent when the kernels are damaged by *Helminthosporium maydis*, the mould that causes corn blight [121].

1.4.4 TIME

The significance of time as factor in mycotoxin production is demonstrated by the lag between mould growth and mycotoxin production. Maximum production of aflatoxin occurs several days after germination and this time period depends on the strain, temperature and substrate [114]. The maximum aflatoxin production by *A. parasiticus* occurred in one experiment, between the fourth and seventh day of germination and beyond this period mycotoxin production dropped rapidly depending on the amount of sucrose present [101]. Thus, knowledge of the time period between germination and maximum mycotoxin production would be valuable in providing an estimate of the toxic potency of a given mouldy product.

1.4.5 TYPES OF SUBSTRATE AND NUTRITIONAL FACTORS

The nutritional requirements for mould growth and those for mycotoxin production may be different. When three strains of *A. parasiticus* were grown on peanuts, soyabeans, corn, wheat, rice and sorghum, the moulds grew well in all. When tested for aflatoxin production, however, one strain produced very little aflatoxin. The

mould produced relatively greater amounts of aflatoxins on sorghum. Similarly, the other strains produced the lowest amount of the mycotoxin on soyabean, rice appeared to give the most yield, followed by peanuts. The other grains supported production of moderate levels of the mycotoxin [122]. While peanuts may be very susceptible to *A. flavus* infection, the ability of the mould to infect this legume varies with the plant varieties. For example, some low-yielding varieties are resistant to *A. flavus* invasion provided the seed coat is intact [123]. This resistance is likely due to the small and closed hilum, while in the susceptible varieties, the hilum is longer and more accessible [124].

A. flavus prefers glucose, sucrose, or fructose for maximum aflatoxin production [125], whereas mannose and xylose increase aflatoxin production by *A. parasiticus*. These sugars inhibited aflatoxin production by *A. flavus*. Corn steep liquor yeast extracts, peptone and casein hydroxylate stimulate aflatoxin production by *A. parasiticus* or *A. flavus* [125-129]; glycine, glutamic acid and to a moderate degree, alanine, aspartic acid,

and glutamine stimulate aflatoxin production by *A. flavus* [129]. The production of aflatoxin has also been reported to be stimulated by inorganic nitrogen sources such as $(\text{NH}_4)_2\text{SO}_4$ and KNO_3 [125,130].

1.4.6 ATMOSPHERIC OXYGEN AND CARBON DIOXIDE LEVELS

Moulds are highly aerobic and need a minimum amount of atmospheric oxygen for growth and efficient mycotoxin production. Thus, the amount of aflatoxin produced by *A. flavus* diminishes as the atmospheric oxygen concentration falls below 20% [131] and high rates of agitation and aeration in culture systems favour higher production of aflatoxin [132]. A comparison between shaking and stationary cultures has shown that under the former conditions, higher yields of aflatoxin are obtained [128]. However, in some cases, the opposite effect has been obtained with patulin or rubatoxin biosynthesis by the corresponding fungi [133]. An increase in carbon dioxide concentration to 20 or 40% prevented aflatoxin formation at 17°C when the relative humidity was reduced to 86%. At 60% carbon dioxide

concentration growth or spore formation of *A. flavus* can be observed [105].

1.4.7 CHEMICAL TREATMENT

Treatment of cereal grains with fumigants such as phosphine and other chemicals in controlling of insects and other pests may affect the production of mycotoxins. A study by Vandegrift et al [134] on the effect of prior treatment of wheat with phosphine and tetrachloromethane : chloroform (80:20) has shown that the biosynthesis of aflatoxin B₁ by *A. flavus* and *A. parasiticus* and of ochratoxin by *A. ochraceus* and *P. viridicum* is stimulated or inhibited depending on the chemical and fungal species or strain. Thus, phosphine treatment increased aflatoxin B₁ production by *A. flavus* from 10 to 37% depending on the strain. This treatment reduced aflatoxin production by *A. parasiticus* by 12 to 20%, again depending on the strain increased by 17% but decreased by 3% with *P. viridicatum*. With the tetrachloromethane : chloroform mixture, production of these mycotoxins by corresponding mould species and strains was increased; the percentage increase ranged

from 5 to 113% for aflatoxin for both *A. flavus* and *A. parasiticus*. The percentage increase of ochratoxin was around 3% for *A. ochraceus* and 257% for *P. viricatum*.

1.5.0 OTHER MYCOTOXINS

Although aflatoxins are the most widely studied mycotoxins, other mycotoxins have also been detected in various agricultural products. Examples of these mycotoxins are zearalenones, ochratoxins, trichothecenes, sterigmatocystin and *alternaria* metabolites.

Zearalenones are produced by several moulds of genera *Fusarium* and *Gibberella*. They have been detected in crops such as corn, wheat, barley, oats, rice and sorghum. Their biological effects in animals are estrogenic in nature [135].

Ochratoxins are produced by moulds of the genera *Penicillium* and *Aspergillus*. Ochratoxin A is thought to be the most toxic of this family of mycotoxins whose deleterious effects are directed primarily against the kidneys. It has been detected in corn, wheat, barley and coffee beans [136]. Ochratoxins have been suspected of

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being implicated in Balkan endemic nephropathy, a fatal disease that afflicted the inhabitants of rural areas of Bulgaria, Romania and the then Yugoslavia[137].

Trichothecenes are produced on grains by fungi belonging to the genera *Fusarium*, *Acremonium*, *Trichoderma*, *Trichothecium*, *Myrothecium*, *Strachybotrys* and *Cylindrocarpon*. While there are over a hundred toxins in this class which have been identified, the most important relative to contamination of agricultural products are T-2 toxin, HT-2 toxin, deoxynivalenol and nivalenol [4].

Sterigmatocystin is a toxic metabolite produced by various species of *Aspergillus*, *Bipolaris* and *Chaetomium*. It has been shown to be carcinogenic to rats[138].

Alternaria is a genus of moulds found in a variety of field crops such as tomatoes, apples, sorghum and rice. These moulds produce several metabolites of diverse chemical structure, the major ones being tenuazonic acid, alternariol methyl ether and alternariol. Tenuazonic acid causes haemorrhage in the gastrointestinal tract as well as other organs when

administered orally or intravenously to experimental animals. It has been demonstrated to inhibit protein and DNA synthesis[139]. In humans, this toxin has been implicated in Onyalaï, a haematological disorder observed in regions of Africa south of Sahara[4].

CHAPTER 2

2.0.0 LITERATURE REVIEW

2.1.0 MYCOTOXIN ANALYSIS

A wealth of information about mycotoxins and mycotoxicoses has been produced since the early 1960's when the outbreak of "Turkey X" disease was reported in England. The availability of methods of analysis has played a key role in the development of mycotoxin survey and research programmes. The fact that mycotoxins are usually present in agricultural products in minute concentrations means that the possibilities to determine mycotoxins are limited to certain analytical methodologies. Since the initial discovery of the aflatoxins, biological and chemical procedures have been developed for the detection and separation of aflatoxins. Bio-assays, minicolumn chromatography, thin layer chromatography, high performance liquid chromatography, gas-liquid chromatography, voltammetric methods and immunological techniques have been developed.

It is worth noting that in most of these techniques, the toxin must be isolated from interfering

compounds present in the sample while retaining a high recovery of toxin. The general basic steps in all chemical analytical methods are outlined below.

Sampling → Sample preparation → Extraction →

Clean-up → Concentration → (Derivatization) →

Ultimate separation → Detection →

Quantization → Confirmation

2.1.1 SAMPLING AND SAMPLE PREPARATION

In any analytical problem, the results of the most sophisticated analytical procedure mean little if a representative sample of the agricultural product has not been obtained. For the analysis of mycotoxins in agricultural products, the importance of good sampling techniques is amplified because the contamination of agricultural products such as grain or nuts is most likely to occur in isolated "hot spots" of toxin. These pockets of toxin may be due to mould proliferation and contamination of a few plants suffering from the stress of unfavourable conditions in a small portion of a field. Alternately, these pockets may be due to localized conditions such as isolated areas of high

moisture in a warehouse due to a leaky roof. With this pattern of contamination, it is critical to obtain as large a sample as possible because the variance of the estimated concentration is inversely proportional to sample size [140]. The limiting factors regarding sample size are the increased cost and impracticality of handling a large sample. Generally, it can be stated that more heterogeneous samples with larger particles will require larger sample sizes. Thus, peanuts require a relatively large sample size, whereas progressively smaller sample sizes are needed for corn, wheat, rice and millet products [141].

The sampling procedure employed for any study depends upon the design of the experiment. A study investigating the presence of a mycotoxin on crops in a field requires the geometric division of the field and the acquisition of representative samples from each sector. With a commodity such as corn, the sampling must be coordinated with harvesting so as to obtain kernels from a large number of ears [140]. Sampling of stored crops with probes will only result in representative samples if the lot has been mixed by

harvesting or some other mechanical operation. If mycotoxin production has occurred during storage in isolated areas of a bin due to water condensation, for example, it is unlikely that the use of probes will result in obtaining a representative sample. The most representative sample is secured by cross-cut sampling of a continuous stream of the lot as it is unloaded over time from the bin. The increments taken from the lot should be mixed and the entire gross sample ground to reduce particle size and heterogeneity[142].

2.1.2 **EXTRACTION**

Extraction involves the separation of the component of interest from the bulk of the matrix components to obtain the materials of interest in a manageable form. Contact between solvent and solid substrate is accomplished either for a short period (1-3 min.) in a high speed blender, or for a longer period (30 min.) by shaking in a flask. Liquids may be extracted in a separatory funnel or absorbed to a hydrophilic matrix which is pre-packed in a column, after which extraction

is accomplished by eluting the column with an extraction solvent.

Examples of solvent systems utilized in the literature for isolating a variety of mycotoxins are presented in Table 3. The choice of the solvent depends on the physico-chemical properties of the matrix as well as those of the toxin. The most efficient solvent for extracting mycotoxins are the relatively polar solvents such as methanol, acetone and chloroform. These solvents are combined with small volumes of water to increase penetration of the solvent system into the hydrophilic matrix tissue. The aqueous phase can be an acid solution designed to break interactions between the toxins and matrix constituents such as proteins. The slurry is then filtered and is ready for subsequent purification procedures. Diatomaceous earth is sometimes included in the solvent system to speed up this filtration step.

TABLE 3 EXAMPLES OF SOLVENT SYSTEMS UTILIZED FOR THE
EXTRACTION OF MYCOTOXIN FROM FOOD [4]

Mycotoxin	Food	Solvent System
Aflatoxins B ₁ , B ₂ , G ₁ , G ₂	Almonds, corn, peanuts, peanut butter.	Acetone : water (85:15)
	Peanut and peanut products	Chloroform : water (91:9)
Aflatoxin M ₁	Milk	Chloroform : 40% salt solution (92:8)
	Cheese	Acetone : water (86:14)
Trichothecenes nivalenol and deoxynivalenol Deoxynivalenol	Cereals	Acetonitrile : water (85:15)
	Corn and wheat	Methanol : water (50:50)
Zearalenone	corn	Chloroform : water (91:9)
Sterigmatocystin	Barley and wheat	Acetonitrile : 4% potassium chloride (9:1)
	Cheese	Acetonitrile : 4% potassium chloride (85:15)
Ochratoxins	Barley	Chloroform : 0.1M phosphoric solution (91:9)
Alternaria metabolites Tenuazonic acid and alternariol methyl ether	Tomato products	Chloroform : water (18:82)
Patulin	Apple juice	Ethyl acetate

2.1.3 CLEAN UP

Clean-up techniques are separation procedures in which groups of substances with certain physico-chemical properties can be separated from one another. In this way the greater part of the co-extracted material can be removed. The choice of the clean-up procedure may depend on the method used for detection and determination, the required limit of detection, the speed of analysis and the recovery. Since mycotoxins are normally only present at very low levels, a strong concentration of the extract is necessary to make detection possible. The frequent presence of lipids and other substances that may interfere with subsequent analytical procedures make it necessary to clean-up the extract prior to concentration. For instance, the high levels of lipids present in certain commodities such as peanuts and cocoa beans can be removed by including non-polar solvents such as hexane in the original solvent system [143]. Salts like sodium chloride are often included in the aqueous phase to minimize the formation of emulsions during the extraction. Certain heavy metal-containing compounds are used to precipitate interfering

constituents in the crude extract. For example, Trucksess and Co-workers [144] used lead acetate to precipitate proteins in powdered egg extracts. Because the fluorescence of most mycotoxins contributes to the selectivity and sensitivity of the methods used to analyze them, the removal of interfering fluorescent compounds is essential. Silver nitrate is used to reduce the concentration of theobromine in cocoa bean extracts [145].

Several column chromatographic clean-up procedures are possible with materials such as silica gel, modified silica gel, Florisil R, sephadex R, aluminium oxide, cellulose and polyamide. Silica gel is most frequently used. Columns can either be packed in the laboratory or purchased as prepackaged cartridges. The prepackaged cartridges offer the advantage of more uniform packing, and their use frequently results in saving time and solvents and eliminating variations in preparation of columns between analysts. They are less versatile however, in that the make-up of the column cannot be easily adjusted for the sample [146].

While the type of adsorbent used depends upon the chemical properties of the toxin, the basic premise of these clean-up procedures remains the same. The sample extract is usually added to the column in an appropriate solvent, after which the column is washed with one or more solvents in which the toxins are soluble or less soluble than the impurities. Then the solvent composition is changed in such a way that the toxins are selectively eluted from the column and the eluate is collected. Liquid-liquid extraction may also be carried out in separatory funnels, for instance pentane against methanol water. Since most mycotoxins are not lipophilic, fats can be removed in this way without loss of toxin.

Stack used the Contaminants Branch (CB) method for the analysis of aflatoxins in peanut products which involves the addition of the filtered concentrated chloroform : water (10:1) extract to a silica gel chromatographic column[147]. Lipids constituents are removed with hexane and diethyl ether washes and the toxin is eluted with a chloroform : methanol (97:3) rinse.

The final step prior to analysis of the sample involves concentration of the clean-up extract. This task is usually performed by evaporating the solvent in a rotary-evaporator under reduced pressure, or by using a steam bath, while keeping the extract under a stream of nitrogen. The residue is redissolved in a small volume of solvent, quantitatively transferred in a small vial and brought to a specified volume.

Depending on the toxin and the ultimate separation and detection step to be used, derivatization of the mycotoxin of interest may be necessary to make it measurable or to optimize its chromatographic behaviour.

2.1.4 METHODOLOGY

2.1.4.1 BIOLOGICAL ASSAYS

Bioassays utilize a variety of biological systems to test the presence of mycotoxins. While these analyses are semiquantitative at best, they can be an effective method for the screening of mycotoxins. The only bioassay accepted by the AOAC Official Methods of Analysis is the chicken embryo bioassay[142].

Other biological systems that have been employed for mycotoxin assays include brine shrimp larvae, trout, rats, mice, fish eggs, ducklings, day-old chicks, bull spermatozoa, cell cultures and microorganisms. Although these methods are generally simple and inexpensive, most of them suffer from several disadvantages, including lack of specificity, long analysis times, imprecision and less sensitivity than chemical and immunological assays.

2.1.4.2 CHROMATOGRAPHIC TECHNIQUES

While the initial extraction and clean-up procedures remove the chemically dissimilar material from the sample, chromatography is needed to isolate the toxins from compounds of like chemical properties. The isolation and analysis of many mycotoxins from a complex product matrix is facilitated by the unique spectral and fluorescent properties which many of these compounds possess. Examples of these compounds are aflatoxins, sterigmatocystin, zearalenones and ochratoxins. Rubratoxins and trichothecenes are a more difficult

analytical problem owing to the absence of unique sharp absorption bands.

In the determination of mycotoxins, adsorption chromatography and partition chromatography are the most important types of chromatography. Normally, the phenomenon on which the separation is based is a combination of both types which can be sub-divided into:

- (i) open column chromatography
- (ii) thin layer chromatography
- (iii) high performance liquid chromatography
- (iv) gas liquid chromatography

2.1.4.2.1 OPEN COLUMN CHROMATOGRAPHY

Open column chromatography is often used in clean-up procedures. However, a special design - glass minicolumn with an internal diameter of about 5mm - can be used for the ultimate separation and detection of some mycotoxins in certain commodities. In a method described by Romer[148], the minicolumn is packed with successive zones of adsorbents such as alumina, silica gel and florisil with calcium sulphate dryer at both ends and held in place with glass wool. A chloroform

extract is applied on the top of the column, and drained by gravity or mild vacuum prior to the addition of an elution solvent of chloroform : acetone (9:1). The aflatoxins are trapped in a tight band at the top of the florisil layer and are examined under ultraviolet (UV) light for fluorescent material. By comparing sample column with a column containing a known amount of aflatoxins, it is possible to judge whether the sample contains more or less aflatoxins than the standard. Contrary to other chromatographic techniques the mini-column method does not distinguish between the different aflatoxins and can only provide an approximate total mycotoxin concentration.

2.1.4.2.2. THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography (TLC) is a form of chromatography that is performed on a thin layer of adsorbent supported by either an inert glass, metal or plastic plate. The plates can be coated in the laboratory or purchased precoated. For mycotoxin analysis, the adsorbent is usually fluorescent silica gel. The mobile phase in TLC is the solvent system that

is placed in the bottom of a development tank along with the plate. The solvent is allowed to migrate up the plate, carrying the mixture of compounds to differing degrees depending on their affinity for the stationary and mobile phases. Tentative identification of toxins can be performed by computing the Rf value, that is the distance the compound migrates divided by the distance the solvent travels. This value is compared to the Rf value determined for a standard toxin run on the same plate.

Solvent systems used for the separation of a variety of mycotoxins are shown in Table 4. It is apparent that some methods call for the development of the plate in one direction, whereas others suggest two. One-dimensional TLC is usually performed on a sample which has undergone extensive clean-up. The analysis of aflatoxin M₁ in milk, for example, can be performed using either technique.

Table 4 Various Solvent Systems Used in the Separation of Mycotoxins by TLC[4]

Mycotoxin	Solvent system	
	1st direction	2nd direction
Aflatoxin	Chloroform:acetone (90:10)	
Aflatoxin	Ether:methanol: water (96:3:1)	Chloroform:acetone (9:1)
Trichothecenes	Pet ether:diethyl ether:acetic acid (70:29:1)	Toluene:ethyl acetate:formic acid (50:40:10)
Ochratoxin	Benzene:methanol: acetic acid (90:5:5)	
Zearalenone	Chloroform: alcohol (95:5)	
Sterigmatocystin	Benzene:methanol: acetic acid (85:10:5)	
Patulin	Toluene:ethyl acetate:90% formic acid (50:40:10)	

Most mycotoxins, including aflatoxins emit light in the visible energy region when exposed to UV radiation, i.e. they fluoresce. For these toxins, the visualization

of the separated spots simply involves placing the plate under UV lights and marking the location of the toxin with a pencil. The Rf values of the sample spots can be compared to those of the standard toxins for tentative identification. The fluorescence colour also can contribute to the identification of the toxin.

Aflatoxins B and M fluoresce blue, whereas the G aflatoxins fluoresce green. Whereas ochratoxin A appears as a greenish-blue spot, zearalenone fluoresce bluish-green and sterigmatocystin emits a brick-red colour.

The sensitivity of TLC analysis for the detection of weakly fluorescing toxins like sterigmatocystin can be greatly increased by derivatizing the toxin[149]. The derivatization is performed by either incorporating aluminium chloride into the silica gel or spraying the developed plate with an aluminium chloride solution, followed by a heating step in either case. Additional spraying of the plate with a silicone : ether mixture prevents the bright yellow spots from fading[150]. Zearalenone can also be more easily visualized after treatment with an aluminium chloride spray, whereas the

fluorescence of ochratoxins is enhanced after exposure to ammonia fumes. The trichothecenes are not fluorescent, and hence they must be derivatized to be visualized on a TLC plate. Derivatizing agents for some or all of the trichothecenes include aluminium chloride, sulphuric acid, nicotinamide : 2-acetylpyridine, p-anisaldehyde, and 4-(p-nitrobenzyl)pyridine [151].

Approximate mycotoxin concentrations are determined by comparing colour intensities with those of standards of known concentration either visually or by using a TLC scanning densitometer. For the latter technique, the sample plate is covered with a clean glass plate edged with tape so that the silica gel is not disturbed. Masking tape is utilized to mark off lanes on the cover plate so that the spots are scanned in the long direction. Standard spots of variable concentrations are scanned in a similar manner and the response is plotted against concentration for standard curve preparation and quantization of the sample.

The matching of R_f values between sample spots and standards does not provide conclusive proof as to the identity of the isolated compound. Other techniques

must be employed for confirmation, ranging from relatively simple methods to more sophisticated analyses. One simple approach is to rechromatograph the sample and standards using a different solid support and solvent system. Another technique involves the use of an internal standard that is spotted directly on top of the sample spot. After development of the plate, partial confirmation of identity is obtained if the suspected toxin and internal standard remain superimposed. The derivatization of the suspected sample toxin after isolation by TLC also may provide partial confirmation of toxin identity. The alteration of aflatoxin fluorescence from blue to yellow following treatment of the plate with sulphuric acid is an example. Scott et al [152] utilized a Fast Violet B Salt spray to derivatize zearalenone isolated from corn-based food products. The plate was then sprayed with a 50% sulphuric acid solution and heated, resulting in zearalenone appearing as a mauve spot. This technique is effective in distinguishing between two toxins that have similar Rf values and fluorescent properties prior

to derivatization, such as zearalenone and alternariol methyl ether.

A more definite confirmation of mycotoxin identity is secured by matching Rf values of a suspected mycotoxin and a standard, derivatizing both compounds and matching the Rf values of the derivatives. The sequence of steps was performed on a single plate by Stubblefield et al [153] in the isolation of aflatoxins M₁ and B₁ from beef livers. The plate was spotted with standard M₁ and B₁ toxins in the outside lanes for two-dimensional TLC and developed in the first direction and dried. A trifluoroacetic acid : hexane solution was sprayed along the bottom of the plate encompassing the sample toxins and the second pair of standard toxins and the plate was heated in an oven. This treatment of aflatoxins possessing unsaturation in the terminal furan ring, namely B₁, G₁ and M₁ caused the addition of water across the double bond. After cooling, the hemiacetals of the toxins thus created were developed in the second direction. The matching of Rf values for the sample toxin and the derivatized toxin with their respective standards constituted positive identification. The

derivatization of toxin can also be carried out in a test tube prior to separation by TLC. Bijl et al [154] treated a dried extract of cheese containing aflatoxin M_1 and a toxin standard with acid anhydride and pyridine. The acetyl derivative of aflatoxin M_1 was separated by two-dimensional TLC.

The most sophisticated and credible method of confirming the identity of mycotoxins is through the use of High Resolution Mass Spectrometry (HRMS). HRMS in combination with TLC however is rather time-consuming, expensive and requires highly trained personnel. Therefore it is not widely utilized.

Thin-layer chromatography has been the method of choice for this work since it is a relatively inexpensive, universal and sensitive technique for mycotoxin analysis.

2.1.4.2.3 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) has gradually gained in popularity and has become one of the most widely used techniques for analyzing mycotoxins. It is a form of liquid chromatography in which the

mobile phase is placed under high pressure generated by pumps and is forced through a steel column containing the stationary phase. The advantages of using HPLC relative to TLC are the improved chromatographic efficiency and precision of quantization obtained with the former method. However, the method has disadvantages such as the relatively long analysis time for a single sample, the expense of the instrument and the level of expertise required to operate and maintain the equipment. In addition, samples generally require more extensive clean-up prior to analysis.

2.1.4.2.4 GAS-LIQUID CHROMATOGRAPHY

The use of gas-liquid chromatography (GLC) in the analysis of mycotoxins has been limited as most of the mycotoxins are not volatile and must therefore be derivatized before they can be gas chromatographed. In addition, the fact that many of the mycotoxins are readily detected and determined at low levels of concentration using TLC and HPLC techniques has not stimulated the development of gas chromatographic assays. However, GLC has been used to analyze

trichothecenes, a class of toxins which has no fluorescent properties and do not absorb appreciably in the ultraviolet range.

2.1.4.3 VOLTAMMETRIC METHODS

Voltammetric methods refer to electrochemical techniques in which a potential is imposed upon an electrochemical cell and the resulting current is measured. A variety of such methods have been developed and they differ in the type of potential waveforms impressed on the cell, the type of electrode used, and the state of the solution in the cell[155]. Voltammetry has proved to be very useful in the determination of organic compounds in biological materials[156].

Traditionally, the adsorption of organic compounds onto electrode surfaces has been regarded as a problem that limits voltammetric measurements. However, recent work has illustrated that controlled adsorptive accumulation of important compounds on the electrode surface can be used to enhance the sensitivity and selectivity of their measurement. Organic compounds such as heme[157], chlorpromazine[158], dopamine[159],

codeine, cocaine and percaine[160] have been determined at the submicromolar and nanomolar concentration levels. The hanging mercury drop electrode has been used for measuring reducible species, whereas carbon paste, wax-impregnated graphite and platinum electrodes have been used for oxidizable species. In all cases, the adsorbable species has been quantified, following the preconcentration period, using differential pulse or linear scans in anodic or cathodic directions depending on the redox properties.

Aflatoxins were first identified using voltammetric techniques by Gajan et al[161], but no attempt was made to explain the complicated electrochemical behaviour of these compounds. Smyth et al[162] studied these compounds in greater detail and found that their electrochemical behaviour closely parallels that reported for coumarin[163]. They thus explained the electroactivity of the aflatoxins to be likely associated with the coumarin moiety in their molecular structure. The main difference in behaviour lies in the fact that the reduction of aflatoxins occurs at a potential of about 300 mV more positive than that for

coumarin. This is due to increased conjugation caused by the keto group in the neighbouring cyclopentanone (B_1 and B_2) or lactone (G_1 and G_2) rings. The slight difference in the structures of these compounds is responsible for the slight difference in their reduction potentials, i.e. in solutions of pH 8-10, the half-wave reduction potentials of the various aflatoxins are $B_1 = -1.26$, $B_2 = -1.27$, $G_1 = -1.21$ and $G_2 = -1.23$ V (all versus the standard calomel electrode, S.C.E.).

Smyth et al [162] postulated the mechanism of reduction of these compounds to be as shown in Figure 3.

2.1.4.4 IMMUNOLOGICAL TECHNIQUES

The use of immunological techniques in the determination of mycotoxins have been limited to date to radio-immuno-assay (RIA) and enzyme-linked immuno-sorbent assay (ELISA).

Radio-immuno-assay (RIA)

The operating principle of a radio-immuno-assay is based on the competition between unlabelled mycotoxin from a sample and radioactively labelled toxin in the assay system for a limited number of sites on the

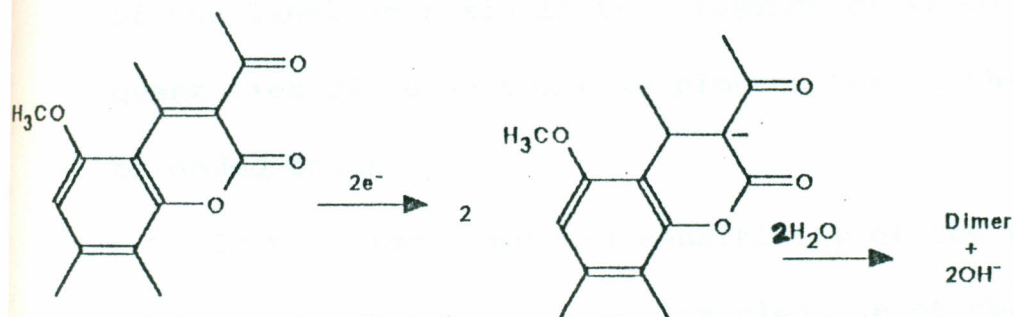
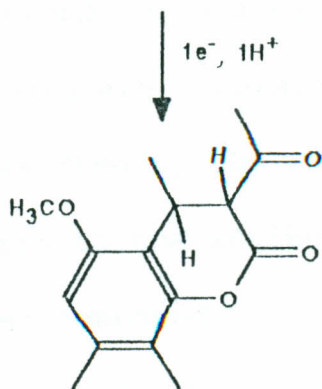
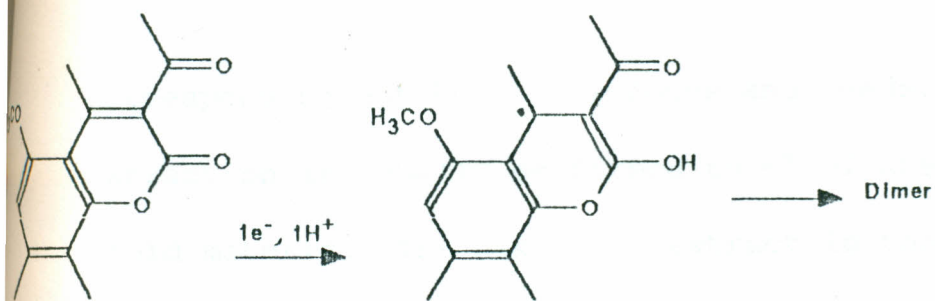


Figure 3.-Mechanism of electrochemical reduction of aflatoxin B₁

corresponding antibody. For foods and feeds, a crude extraction is usually performed to eliminate protein and lipid material. The resulting extract is then incubated with the corresponding antibody and labelled toxin. Free and bound toxin are separated and the level of inhibition exerted by the sample on the binding of labelled toxin to the antibody can be determined. This value is then compared to a standard curve prepared by incubating variable concentrations of the toxin standard with antiserum and labelled toxin. The percent binding of the labelled toxin in the presence of known quantities of added toxin is plotted versus the amount of added toxin.

In practise, improved sensitivity of the assay can be achieved by a more extensive clean-up of the sample extract. Additional clean-up of extracts from corn and wheat using a reverse phase Sep Pak cartridge was shown to be essential to detect T-2 toxin below 5.0 and 2.5 $\mu\text{g}/\text{Kg}$, respectively [164].

Enzyme-Linked Immuno-sorbent Assay (ELISA)

In recent years, the analysis of mycotoxins by immunoassays has widely been practised, largely owing to

the increase in popularity of the enzyme-linked immunosorbent assay (ELISA), which has largely surpassed the RIA in popularity. While both techniques are based on the competitive binding of sample toxins with marker toxins for sites on a corresponding antibody, direct ELISA utilizes an enzyme-toxin conjugate for the marker compound rather than a radioactively labelled one. The amount of isolated enzyme-toxin complex bound to the antibody is then determined by adding a substrate for the enzyme. The enzyme is selected so that the product of the reaction it catalyses can be quantified spectrophotometrically, thus indirectly determining the amount of bound enzyme-toxin conjugate and the amount of sample toxin.

The immunoassays offer some clear advantages over other techniques in terms of simplicity, assay time and specificity. The ELISA is especially well suited for performing mycotoxin analyses of large numbers of samples such as in the large-scale screening of agricultural products. The assay time can be shortened by leaving out sample extract clean-up steps and using direct competitive ELISA for analyses in which the

detection limits need not be in the low microgram/kilogram range [165]. The shortcoming of immunoassays is that the precision is not as high as with the chromatographic methods. In addition, extensive collaborative studies must still be performed to standardize these methods. The specificity of the immunoassays can also be a disadvantage in the case where the analyst desires a mycotoxin profile.

2.2.0 OBJECTIVE OF STUDY

Due to economic liberalisation, many small-scale manufacturers of animal feeds have entered the market and the quality of these feeds may not be of prime consideration. With this view in mind, this project was designed to investigate the levels of aflatoxin B₁ in animal feeds used by zero grazing farmers in Nairobi and its surroundings. This was coupled with an investigation of aflatoxin M₁ levels in liquid milk.

CHAPTER 3

3.0.0 EXPERIMENTAL TECHNIQUES

3.1.0 CLEANING OF GLASSWARE AND PLASTIC CONTAINERS

Glassware was cleaned with liquid detergent and tap water. The glassware was then filled with freshly prepared chromic acid and kept overnight. The chromic acid was removed and glassware rinsed several times with tap water followed by distilled water. It was then dried at 130°C in an electric oven for at least two hours. All the solutions used in this work were stored in plastic containers which had been cleaned with detergent rinsed with concentrated analar grade nitric acid and finally with distilled-deionised water.

At the completion of each analysis the glassware was decontaminated by immersing them in a 5% w/v solution of sodium hypochlorite for 2 hours. After this time an amount of acetone equal to 5% of the total volume of sodium hypochlorite used was added to the wash solution and the glassware was soaked for a further 30 minutes. This was to destroy any carcinogenic derivative of aflatoxin B₁ which might be formed as a result of sodium hypochlorite treatment.

3.2.0 CHEMICALS AND SOLVENTS

All the reagents used were of analar grade or high purity chemicals. Solvents were distilled before use and all aqueous solutions were made in deionised distilled water.

3.3.0 PREPARATION OF AFLATOXIN STANDARD SOLUTIONS [142]

Aflatoxin standards (AFB₁ and AFM₁) were obtained as dry crystals from Sigma Chemical Company. A known volume of benzene : acetonitrile (9:1) was added to the container of dry aflatoxin to prepare stock solutions of concentration 1mg/ml (AFB₁) and 0.05mg/ml (AFM₁), where the label statement of aflatoxin weight was used as guide. The solutions were agitated vigorously for 1 minute and then transferred to glass-stopped flasks.

The concentration of each aflatoxin solution was determined by first diluting the stock solution to prepare a concentration of 8 - 10µg/ml and then measuring absorption at 348nm and using the equation :-

$$\mu\text{g aflatoxin/ml} = A \cdot \text{Mw} \cdot 1000 / \epsilon$$

where Mw and ϵ are as follows,

Aflatoxin	Mw	ϵ
B ₁	312	19 800
M ₁	328	18 815

The flasks containing the solutions were wrapped tightly with aluminium foil and stored at 0°C. Working standard solutions of 5.0ml were prepared by diluting the solutions with benzene : acetonitrile to obtain about 0.2µg aflatoxin/ml for TLC analysis.

3.4.0 SAMPLING

Animal feeds and liquid milk were randomly sampled in Nairobi and its surroundings in the months of May to November 1994. Animal feeds were collected from the feeding troughs and placed in dry polythene bags which were tightly closed. They were then transported to the laboratory and immediately stored in the deep freezer. Liquid milk was also collected and placed in sample bottles which were then tightly closed. The samples were transported to the laboratory and immediately extracted with chloroform. The extracts were wrapped tightly with aluminium foil and stored at 0°C.

3.5.0 ANALYTICAL PROCEDURES

3.5.1 TREATMENT PRIOR TO ANALYSIS (ANIMAL FEEDS)

Ground sample(50g) was weighed in a 500ml conical flask using an electronic Top balance Model AC 100. Diatomaceous earth (25g), 25ml of distilled-deionised water and 250 ml of chloroform were added to the content of the conical flask. The mixture was shaken vigorously for 30 minutes on a shaking apparatus, filtered and 50ml of filtrate collected.

Column Clean-up

A mixture of 50ml of filtrate and 100ml of n-hexane in a 250 ml conical flask was quantitatively transferred to a prepared column, rinsing the flask twice with 10ml n-hexane. The tap of the column was opened and the liquid allowed to flow at a rate of 10ml per minute until it was level with the upper surface of the sodium sulphate layer. The eluate was discarded. This was followed by washing the column with 100ml diethyl ether.

Aflatoxin B₁ was eluted with 150ml of chloroform: methanol (97:3) mixture and the eluate was evaporated using a rotary evaporator. The residue was quantitatively transferred to a 10ml graduated tube,

using chloroform and evaporated on a water bath not exceeding 50°C, under a stream of oxygen-free nitrogen and the volume was adjusted to 2.0 ml with chloroform. This was retained for TLC and voltammetric analysis.

3.5.2 THIN-LAYER CHROMATOGRAPHY (TLC)

Preparation

Clean glass plates (20 x 20 cm) were fitted into TLC plate spreading apparatus and wiped with acetone. Silica gel G (MERCK) (40g) was weighed into a wide-mouthed 500ml conical flask with a stopper. 80ml of distilled water was added into the flask and shaken vigorously for about 3 minutes. The silica gel slurry was then poured into a plate spreader which had been adjusted for a coating of 0.25 mm thickness. The plates were uniformly spread and allowed to stand for about 2-3 minutes or until the coating had set and then activated at 105°C for at least 1 hour. Plates were then removed and stored in a desiccator cabinet.

Aflatoxin analysis

The following were the volumes of standard solution of aflatoxin B₁ and of extract applied 20 mm from the lower edge of the TLC plate and at intervals of 20 mm :-

10, 15, 20, 30 and 40µl of the standard solution of aflatoxin B₁;

10 µl of extract with 20 µl of B₁ standard solution superimposed;

10 and 20 µl of extract.

This was done in triplicates.

The spotted plates were developed in the dark in a solvent tank containing chloroform : methanol (94:6) and allowed to develop until the solvent front had ascended to about 15 cm. The developed TLC plates were air-dried in the dark and then observed under long-wave ultraviolet light (366nm) in a dark viewing cabinet.

Determination of the quantity of AFB₁ in the extract was done by comparing the intensity of fluorescence of the extract spots with that of the standard solution spots. Interpolation was done where necessary.

Confirmation of Identity

The identity of AFB₁ in the extract was confirmed by spraying sufficient 50% sulphuric acid in water on the chromatogram to wet the aflatoxin area. The fluorescence of AFB₁ spots thus treated turned from blue to yellow under ultraviolet irradiation.

3.5.3 EXTRACTION OF LIQUID MILK [166]

Liquid milk (50 ml) was shaken vigorously with 10ml saturated sodium chloride solution and 120 ml of chloroform (at about 35°C) in a 250ml separatory funnel for 60 seconds. After the phases separated (about 2 min.) the chloroform layer was drained into a 125ml Erlenmeyer flask and dried over 10g sodium sulphate. This was then filtered into a 100ml graduated cylinder.

Column clean-up

The entire milk filtrate was drained through a prepared column by gravity into a 150ml beaker. The graduated cylinder and finally the inside of the column was rinsed with chloroform and drained through the column. The column was then washed successively with 25ml toluene : acetic acid(9:1), 25ml hexane and 25ml

hexane : ether : acetonitrile (5:3:2) and the washes were discarded. Aflatoxin M_1 was eluted with 60ml chloroform : acetone (4:1) into a 125ml conical flask and the eluent was evaporated to near dryness on a steam bath. The extract was transferred quantitatively with chloroform rinses to a vial and the solvent evaporated to dryness under a stream of nitrogen.

TLC analysis of aflatoxin M_1

Aflatoxin residue from the vial was dissolved in 100 μ l of benzene : acetonitrile (9:1). A 20 μ l aliquot of the sample extract and 2, 4, 6, 8 and 10 μ l aliquots of AF M_1 working standard solution were spotted using the Hamilton microsyringe on a line 4cm from bottom edge of a prepared 20x20 cm TLC plate.

The spotted plates were developed in chloroform : acetone : isopropanol (85:10:5) as the solvent system, until the solvent front had ascended to about 12 cm. The developed TLC plates were dried in a fume cupboard and then observed under long-wave ultraviolet light (366 nm) in a darkened room.

3.6.0 VOLTAMMETRIC STRIPPING ANALYSIS OF AFLATOXIN B₁

The analysis work was carried out using Princeton Applied Research Model 303A analyzer. This model is a trimode electrode which is able to function as either a dropping mercury electrode, (DME) or a hanging mercury drop electrode, (HMDE).

The model 303A was then connected to the Polarographic Analyzer/ Stripping Voltammeter Model 264A which is an electric polarographic instrument capable of performing dc, sampled dc, normal pulse, cyclic voltammetry and differential pulse polarographic analysis, as well as dc and differential pulse stripping analysis.

The model 264A was then interfaced with a personal computer (a Tatung model No: CM 14 SBS) for recording. The software programme, POLR4 was used to do the voltammetric recordings.

3.6.1 STRIPPING VOLTAMMETRIC WORK PREPARATIONS

The supporting electrolyte

Britton-Robinson (BR) buffer solution (0.04M in glacial acetic acid, orthophosphoric acid, and boric

acid) was used as the supporting electrolyte. The buffer was prepared every day of analysis according to the description of Frugoni[167]. The pH of the buffer solution prepared was then adjusted to 9.0 by addition of 0.2M Sodium hydroxide solution. This is the best pH that gives well defined waves for analytical purposes[162]. pH measurements were done using a Jenway pH meter Model 3020, which was always calibrated using N.B.S. buffers before use.

Voltammetric cell-cups

The cell-cups were soaked in 6M analar nitric acid overnight prior to analysis. They were then rinsed several times with a lot of distilled-deionised water before use.

Working electrode

The working electrode was properly cleaned prior to any analysis. This was necessary to get rid of dirt and clogging, which prevents production of mercury drops at the capillary tip. It also safe guards breaking of the mercury thread in the capillary. Before cleaning, the remains of mercury from the previous filling were blown into a container, using pressurized nitrogen. The

capillary bore was cleaned by aspirating several millilitres of 1M analar nitric acid, followed by rinsing with distilled deionised water. This was finally followed by methanol rinse and air drying of the capillary at 65°C for one and a half hours. To prevent the test solution from penetrating the capillary, the capillary bore was siliconized by placing the tip opposite the ferrule in a fresh vial of siliconizing fluid. The excess siliconizing fluid from the capillary was removed from the opposite end of ferrule. This process was completed by air drying the capillary in an oven at 65°C .

In filling the valve body with mercury, a clean hypodermic syringe (10 ml) with a needle was used, by filling it with mercury and injecting the mercury into a small hole located in the reservoir directly above the valve assembly. This process was repeated several times before all the entrapped air was completely eliminated.

Reference electrode

The reference electrode was a simple silver/silver chloride electrode that made contact with the analyte via a porous vycor frit. It was necessary from time to time

to replace the frit due to either its contamination after prolonged use or when the frit material thinned or had a visible crack. The glass sleeve of the reference electrode was filled with the filling solution (saturated with AgCl) making sure that it was free of bubbles. This was done from time to time since when the electrodes are stored in water for days, shifting of peak potentials occur due to dilution of the filling solution by water diffusing into the reference electrode.

Counter electrode

The counter electrode for the model 303A was a teflon sheathed platinum wire instilled at the factory, and did not require any maintenance. However, this was washed frequently with analar nitric acid followed by rinsing with large amounts of distilled deionised water.

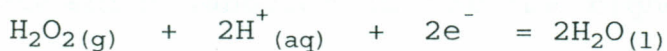
Gas Scrubbing

Aqueous solutions exposed to air contain concentrations of dissolved gaseous oxygen as high as 10^{-3} M at room temperature and pressure. Dissolved oxygen interferes in stripping analysis as it does in classical polarography. Depending upon the pH, oxygen undergoes reduction in two steps:

Step 1



Step 2



The half-wave potentials of these steps are approximately -0.05 and -0.9V versus the saturated calomel electrode. These reduction steps result in an increased background current that obscures the stripping peaks of interest. Hence, dissolved oxygen had to be removed from the sample solutions prior to any stripping analysis. To achieve this, pure nitrogen was bubbled through the solution to remove oxygen immediately before the experiment. Also during the experiment, the nitrogen flow was maintained above the solution to avoid oxygen being redissolved.

Since ordinary tank nitrogen contains traces of oxygen, an oxygen scrubbing system was necessary to

remove the oxygen. This was based on vanadium (II) chloride solution. The required solution was prepared by boiling two grams of ammonium metavanadate with 25ml of concentrated hydrochloric acid, and diluted with water to 250ml. The solution produced was blue or green and contained vanadium in various higher oxidation states. The solution was then transferred to a gas washing tower, and 10g of amalgamated zinc added to it, to reduce all the vanadium to the +2 oxidation state. The amalgamated zinc was prepared by placing 13g of powdered zinc in a beaker, covering it with distilled deionised water, and adding three drops of concentrated HCl. Amalgamation occurred on addition of mercury. The vanadous chloride solution turns from its original colour to purple due to reduction of vanadium to its +2 oxidation state by the zinc amalgam. The blue or green colour returns at exhaustion and rejuvenation was accomplished by adding some more amalgamated zinc or few drops of concentrated hydrochloric acid.

With a scrubbing tower ready, white spot nitrogen gas supplied by BOC Kenya Limited was connected to the tower input, while the output of this tower was connected

to the input of a second tower containing the same electrolyte as that in the analysis cell. The output of the second tower was connected to the nitrogen input part of the Model 303A. The purpose of the second tower was to remove any vanadous chloride solution picked up by the gas and saturate the gas exiting the second tower with electrolyte. The removal of vanadous chloride prevents contamination of the analyte while the saturation with electrolyte prevents sample concentration changes because of evaporation.

3.6.2 DETERMINATION OF AFLATOXIN B₁

For each run, 0.5 ml of the sample extract was pipetted into a 5ml calibrated flask and then diluted to the mark with BR buffer solution (pH 9). After thorough mixing, the resulting solution was placed in the voltammetric cell, with the SCE and the HMDE positioned in the cell, and white spot nitrogen was passed through the solution for 4 minutes in order to remove oxygen from the solution. The sample was then blanketed with an atmosphere of nitrogen as analysis was being carried out using the following conditions:

mode - differential pulse stripping voltammetry;
accumulation potential - -1.0V versus SCE; accumulation
time - 6 minutes; scan rate - 20mVs^{-1} ; pulse amplitude -
25mV; scan direction - negative.

Working standards of AFB₁ were prepared by first diluting the stock solution to prepare a solution of concentration 2.8×10^{-6} M. This was done by putting 5.0ml of 8.74 $\mu\text{g/ml}$ solution into a 50ml volumetric flask and made to the mark using BR buffer solution. Other solutions were prepared by diluting this solution as shown in Table 5. 5.0ml of each of the standard solutions was put in the voltammetric cell, electrolysed and the peak currents recorded. A calibration curve (Figure 4) was made from which the sample reading was read off to give the aflatoxin concentration.

Table 5 AFLATOXIN B₁ STANDARD SOLUTIONS FOR DPSV
ANALYSIS

Vol. of 2.8×10^{-6} M solution (ml)	Vol. of BR solution (ml)	Total volume (ml)	Conc. ($\times 10^{-7}$ M)
5.0	0.0	5.0	28.0
4.5	0.5	5.0	25.2
4.0	1.0	5.0	22.4
3.5	1.5	5.0	19.6
3.0	2.0	5.0	16.8
2.5	2.5	5.0	14.0
2.0	3.0	5.0	11.2
1.5	3.5	5.0	8.4
1.0	4.0	5.0	5.6
0.5	4.5	5.0	2.8
0.25	4.75	5.0	1.4

4.0.0 RESULTS AND DISCUSSION

4.1.0 PEAK CURRENTS OF THE STANDARD SOLUTIONS

The results for the peak currents of the aflatoxin standards are presented in Table 6. The calibration curve shown on Figure 4 was drawn using these values.

Table 6 PEAK CURRENTS OF STANDARD SOLUTIONS

Concentration of AFB ₁ ($\times 10^{-7}$ M)	Peak Currents, I _p (μA)
28.0	0.6126
25.2	0.5953
22.4	0.5754
19.6	0.5468
16.8	0.5644
14.0	0.4541
11.2	0.4377
8.4	0.3618
5.6	0.3072
2.8	0.1653
1.4	0.0812

4.2.0 CONCENTRATIONS OF AFLATOXINS B₁ AND M₁

Tewnty samples of animal feeds and liquid milk were collected from Nairobi and its surroundings in the months of May to November 1994 and analyzed for aflatoxins B₁ and M₁ respectively.

Animal Feeds

The concentration of aflatoxin B₁ in animal feeds varied from 38.2 to 279.5µg/Kg. The results for TLC and DPSV analysis are presented in Tables 7 and 8 while the average concentrations are shown in Table 9. Most of the animal feed samples were found to have concentrations of aflatoxin B₁ above the maximum tolerated in foods and feeds in various countries as shown in Table 10. Aflatoxin limits for animal feeds are not available for Kenya. However, according to the Food, Drugs and Chemical Substances Act, 1978, the maximum tolerated level for aflatoxins B₁, B₂, G₁, G₂ is 20µg/Kg for peanuts, peanut products and other vegetable oils[168].

The generally high concentrations for aflatoxin B₁ may be due to poor feed management during storage and when it is in the feeding troughs. Farmers, and may be

manufacturers, lack the knowledge of feed storage. Thus mould growth and hence aflatoxin contamination is not controlled or prevented. It was also noted that the feeds stayed in the feed troughs for several days and that the troughs were rarely cleared before more feeds were added. The presence of water for feeding the animals close to the feed troughs may also mean that the dairy cows would take the water and then continue to eat the feeds. This may lead to presence of moisture which is favourable for mould growth and hence aflatoxin production.

Most of the zero grazing farmers also practise poultry farming and thus they feed the dairy cows with used poultry feeds. This increases the chances of the feeds getting contaminated because of the time they stay in the feed troughs. The relatively high levels of aflatoxin in barley husks may mostly be due to the way the feeds are handled in the market. The husks are stored in the open where they easily get soaked in water particularly during the wet seasons. These conditions may be favourable for aflatoxin contamination.

Ingestion of aflatoxin B₁ contaminated feeds by dairy cows is of principle concern because of the insidious

abnormalities that are produced in the animals and because the hydroxylated form of aflatoxin B₁ (AFM₁) can appear in their product, milk.

Liquid Milk

In all the samples of liquid milk analyzed, aflatoxin M₁ was not detected. This can be attributed to the fact that aflatoxin M₁ may have been excreted through urine and faeces. Studies by Lynch[169] also indicate that the toxin disappear from milk in 3 to 4 days after elimination of aflatoxin-contaminated diet. This means that milk produced by cows that have consumed aflatoxin-contaminated feed would be essentially free of aflatoxin M₁ within a short time after contaminated feed was no longer consumed. It is also worth noting that milk samples were not necessarily taken from the same places where the feeds were sampled. This was because the farmers were very sensitive with milk and thus presented a barrier to the work.

4.3.0 CORRELATION COEFFICIENT (r)

Results of TLC and DPSV analysis were correlated by product-moment correlation coefficient (r) to test whether the corresponding readings got for each sample by the two

methods were comparable. The value of r was found to be 0.98, and therefore the degree of correlation between the two methods was treated as good because it lies in the range $0.95 < r < 0.99$. The concentrations of aflatoxin B_1 were taken to be the average of the two methods as shown in Table 9.

Sample No.	Method 1 (ppm)	Method 2 (ppm)	Average (ppm)
1	1.2	1.1	1.15
2	2.5	2.4	2.45
3	0.8	0.9	0.85
4	3.1	3.0	3.05
5	1.5	1.6	1.55
6	2.2	2.1	2.15
7	0.5	0.6	0.55
8	4.0	3.9	3.95
9	1.8	1.7	1.75
10	2.8	2.7	2.75
11	0.9	1.0	0.95
12	3.5	3.4	3.45
13	1.1	1.2	1.15
14	2.0	1.9	1.95
15	0.7	0.8	0.75
16	3.8	3.7	3.75
17	1.4	1.5	1.45
18	2.6	2.5	2.55
19	0.6	0.7	0.65
20	4.2	4.1	4.15

Table 7 CONCENTRATIONS OF AFLATOXIN B₁ (µg/Kg) USING

Area	Farmer/ site	Type of feeds	Manufac- turer**	Conc. Mean±std.
Ruiru	1	Cattle	Unga Feeds	38.9±1.0
..	2	46.0±2.0
..	3	61.5±1.0
Wangige	1	poultry*	Milling Corp. of Kenya	99.8±4.0
..	2	124.1±2.0
..	3	cattle	Unga Feeds	67.9±1.8
Gikuni	1	cattle	Unga Feeds	56.8±4.0
..	2	poultry*	Moore Ind.	42.3±1.0
..	3	133.4±2.0
Kiserian	1	cattle	Goldstar Feeds	42.9±1.0
..	2	poultry*	..	109.0±4.0
..	3	brewers grain	KBL by- product	133.4±6.0
Githurai	1	cattle	Unga Feeds	39.4±4.0
..	2	44.0±1.0
..	3	55.7±3.4
Kwamaiko	1	brewers grain	KBL by- product	120.6±2.0
..	2	126.4±4.0
Thika Road	Ruaraka	barley husks	KBL by- product	169.4±4.0
..	Allsopps	213.4±8.0
..	Survey of Kenya	271.4±7.0

Table 8 CONCENTRATION OF AFLATOXIN B₁ (µg/Kg) USING DPSV

Area	Farmer/ site	Type of feeds	Manufac- turer**	Conc. Mean±std.
Ruiru	1	cattle	Unga Feeds	37.4±6.2
..	2	52.0±3.2
..	3	59.5±3.0
Wangige	1	poultry*	Milling Corp. of Kenya	75.7±4.4
..	2	112.6±14.0
..	3	cattle	Unga Feeds	67.1±10.4
Gikuni	1	cattle	Unga Feeds	55.6±3.2
..	2	poultry*	Moore Ind.	41.3±4.2
..	3	124.5±21.2
Kiserian	1	cattle	Goldstar Feeds	41.3±4.2
..	2	poultry*	..	96.2±17.8
..	3	brewers grain	KBL by- product	92.6±46.0
Githurai	1	cattle	Unga Feeds	38.0±2.0
..	2	47.6±10.8
..	3	49.9±5.4
Kwamaiko	1	brewers grain	KBL by- product	109.5±16.4
..	2	108.7±5.8
Thika Road	Ruaraka	barley husks	..	143.3±10.6
..	Allsopps	194.5±31.0
..	Survey of Kenya	287.6±26.5

Table 9 AVERAGE CONCENTRATIONS OF AFLATOXIN B₁

Area	Farmer/ site	Type of feeds	Conc. (µg/Kg)
Ruiru	1	cattle	38.2
„	2	„	49.0
„	3	„	60.5
Wangige	1	poultry*	87.8
„	2	„	118.4
„	3	cattle	67.5
Gikuni	1	cattle	56.2
„	2	poultry*	41.8
„	3	„	129.0
Kiserian	1	cattle	42.1
„	2	poultry*	102.6
„	3	Brewers grain	113.0
Githurai	1	cattle	38.7
„	2	„	45.8
„	3	„	52.8
Kwamaiko	1	brewers grain	115.1
„	2	„	117.6
Thika Road	Ruaraka	barley husks	156.4
„	Allsopps	„	204.0
„	Survey of Kenya	„	279.5

** Animal feeds were collected from the feeding troughs.

* Poultry feeds had first been fed on chicken and then given to dairy animals.

Table 10 MAXIMUM TOLERATED AFLATOXIN B₁ LEVELS IN FOODS AND FEEDS IN VARIOUS COUNTRIES [157]

Country	Commodity	Established tolerance (µg/Kg)
Belgium	All foods	5
Japan	All foods	10
	Peanuts	10
	Peanut meal feed (import)	1000
South Africa	All foods	5
Malawi	Peanuts (export)	5
European Communities	Straight feeding stuffs	50
	Complete feeding stuffs not for dairy cattle, calves, lambs	50
	Complete feeding stuffs for pigs and poultry	20
	Other complete feeding stuffs	10
	Complimentary feeding stuffs for dairy cattle	20
China	Rice, peanuts, oats, corn, sorghum, beans, wheat, barley	50

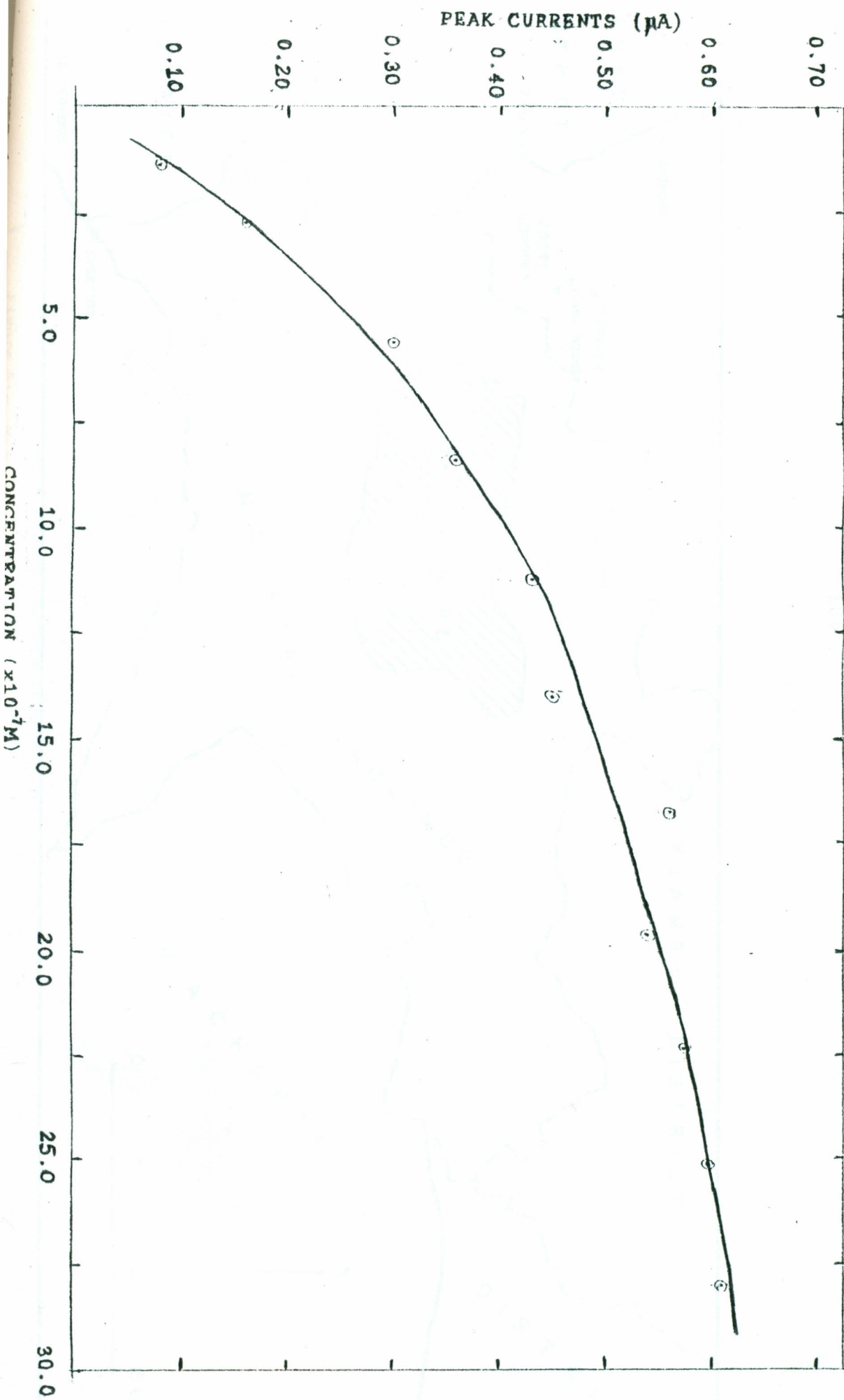
4.4.0 RECOMMENDATIONS

The results indicate that farmers, and may be manufacturers, should be educated on how to handle and store the animal feeds so that mould growth and hence aflatoxin-contamination may be controlled or prevented. The feeds, particularly local feeds, should always be taken for analysis in government laboratories.

The results suggest areas that need further investigations as follows :-

- (i) The short and long term effects of aflatoxin-contaminated feeds on dairy cows.
- (ii) Since this study was on a general survey of the levels of aflatoxin M_1 in milk, further work may be done on determination of the toxin in milk of individual dairy cows consuming aflatoxin-contaminated feeds. This should be done in collaboration with a government enforcing officer to ensure that the farmers do not hinder the intended research.

- (iii) Determination of aflatoxins in beer and other local brews since barley husks and brewers grain were found to be contaminated.
- (iv) General survey of aflatoxins in peanuts and peanut products.
- (v) Other methods of analysis such as high performance liquid chromatography and immunological techniques may also be used to compliment TLC and DPSV.



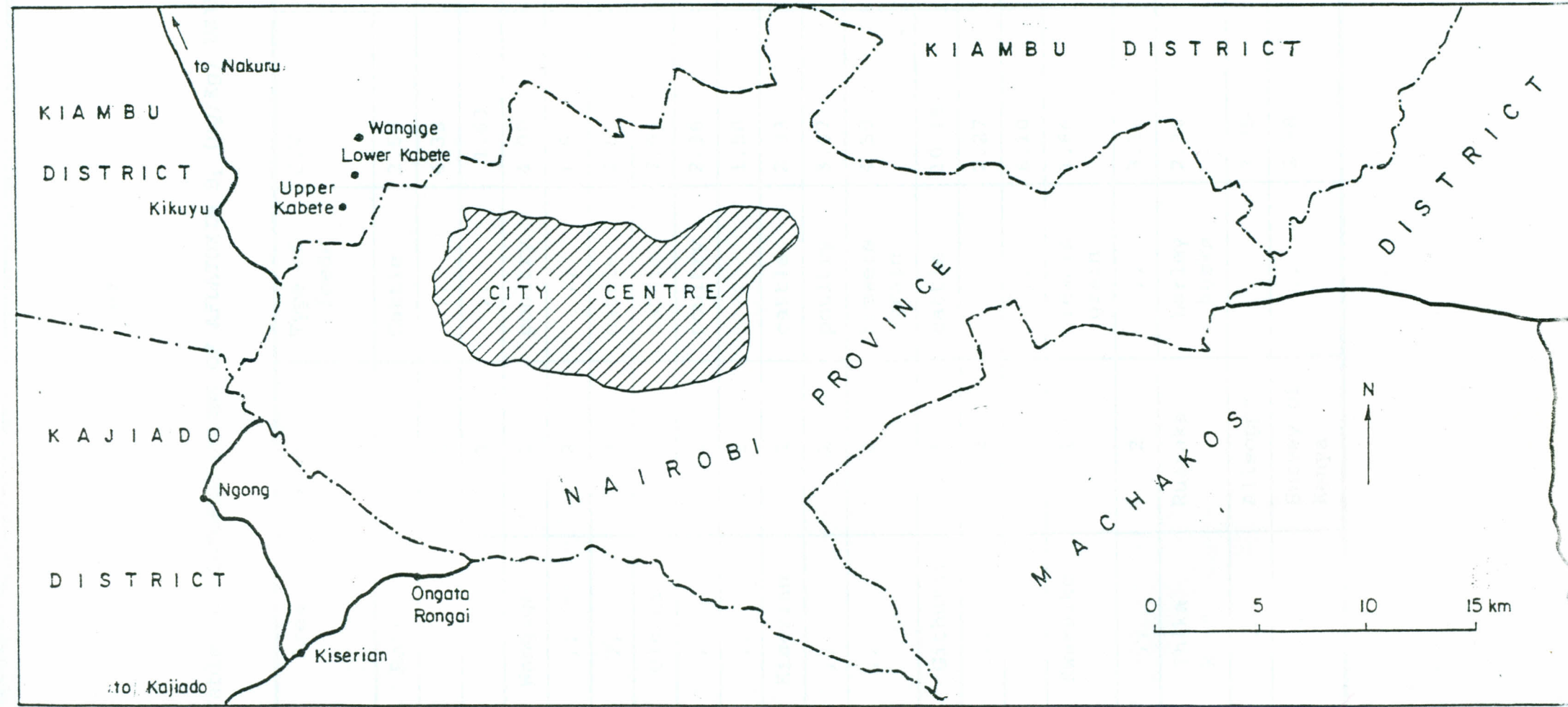


Figure 5:- Map showing the area of study

Table 7A CONCENTRATIONS OF AFLATOXIN B₁ (µg/Kg) USING TLC

Area	Farmer/ site	Type of feeds	C.V.	C.L. (95% Confidence level)
Ruiru	1	Cattle	2.57	38.9±2.48
..	2	..	4.35	46.0±4.97
..	3	..	1.63	61.5±2.48
Wangige	1	poultry	4.00	99.8±9.94
..	2	..	1.61	124.1±4.97
..	3	cattle	2.65	67.9±4.47
Gikuni	1	cattle	7.04	56.8±9.94
..	2	poultry	2.36	42.3±2.48
..	3	..	1.50	133.4±4.97
Kiserian	1	cattle	2.33	42.9±2.48
..	2	poultry	3.67	109.0±9.94
..	3	brewers grain	4.50	133.4±14.9
Githurai	1	cattle	10.15	39.4±9.94
..	2	..	2.27	44.0±2.48
..	3	..	6.10	55.7±8.45
Kwamaiko	1	brewers grain	1.66	120.6±4.97
..	2	..	3.16	126.4±9.94
Thika Road	Ruaraka	barley husks	2.36	169.4±9.94
..	Allsopps	..	3.75	213.4±19.8
..	Survey of Kenya	..	2.58	271.4±17.3

Table 8A CONCENTRATION OF AFLATOXIN B₁ (µg/Kg) USING DPSV

Area	Farmer/ site	Type of feeds	C.V.	C.L. (95% Confidence level)
Ruiru	1	cattle	16.58	37.4±15.40
..	2	..	6.15	52.0±7.95
..	3	..	5.04	59.5±7.45
Wangige	1	poultry	5.81	75.7±10.93
..	2	..	12.43	112.6±34.7
..	3	cattle	15.50	67.1±25.84
Gikuni	1	cattle	5.76	55.6±7.95
..	2	poultry	10.17	41.3±10.43
..	3	..	17.03	124.5±52.6
Kiserian	1	cattle	10.17	41.3±10.43
..	2	poultry	18.50	96.2±44.22
..	3	brewers grain	4.97	92.6±11.43
Githurai	1	cattle	5.26	38.0±4.97
..	2	..	22.69	47.6±26.83
..	3	..	10.82	49.9±13.42
Kwamaiko	1	brewers grain	14.98	109.5±40.7
..	2	..	5.34	108.7±14.4
Thika Road	Ruaraka	barley husks	7.40	143.3±26.3
..	Allsopps	..	15.94	194.5±77.0
..	Survey of Kenya	..	9.21	287.6±65.8

APPENDIX

Statistical Treatment of Data

(See Tables 7A and 8A)

Mean

The mean value of aflatoxin B₁ in each animal feed sample was carried out in triplicate determinations. This was done to ensure accuracy in the actual value of the aflatoxin. The mean was calculated using equation (C-1) below.

$$\bar{x} = \frac{\sum_{i=1}^{i=n} x_i}{n} \dots \dots \dots (C-1)$$

where

\bar{x} is the mean

x_i is the *i*th term of the three determinations

n is the total number of determinations (3)

Standard deviations and relative standard deviation (rsd)

This was used to measure dispersion of the obtained concentrations about the mean. The following equation (C-2) was used to calculate the standard deviation.

$$S = \sqrt{\frac{\sum_{i=1}^{i=n} (x_i - \bar{x})^2}{n-1}} \quad \dots \dots \dots (C-2)$$

It was also used to indicate the precision of the method of determination. Precision was clearly reflected by the value of coefficient of variation (rsd) that was calculated using equation (C-3).

$$C.V. = \frac{100 \times std}{\bar{x}} \quad \dots \dots \dots (C-3)$$

Confidence Limit (C.L.)

95% confidence limit of the aflatoxin concentrations was calculated so as to estimate the range within which the true value might fall. Equation (C-4) was used to calculate the confidence limit.

$$C.L. = \bar{x} \pm ts/\sqrt{n} \quad \dots \dots \dots (C-4)$$

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