DETERMINATION OF AFLATOXINS AND VOLATILE IMPURITIES IN ALCOHOLIC BEVERAGES AND THEIR RAW MATERIALS

BY

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A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF MASTER OF SCIENCE OF KENYATTA UNIVERSITY

JANUARY 1999

DECLARATION

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

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ACKNOWLEDGEMENTS

I wish to extend much gratitude to my supervisors Dr. W. Njue and Dr. R. Musau for their sincere and invaluable assistance throughout this study. Their readiness to read my work, fruitful discussions made the entire work possible.

I would also like to thank G. Ombok and G. N Gikubu, laboratory analysts of Kenya Bureau of standards (KBS) for their tireless effort to see me through in the analysis. I am also thankful to the entire staff of chemistry department for their encouragement and to **Kenyatta University** for offering me a scholarship to carry out this study.

My heartiest thanks are to my parents, brothers, sisters, relatives, and friends Betty, Kirubi, Mildred and sophie for their prayers and persistent encouragement during the hard times of my studies and finally to my fiancé, Ng'ang'a Muigai, for his moral support, deep understanding and encouragement during the entire period.

May God bless everyone who in one way or the other contributed to the success of this work.

DEDICATION TO

my grandmother, Kahaki, parents, Francis Njuguna and Mary Wangari, and my brothers and sisters.

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ABSTRACT

The thesis is in two parts. The first part involved the analysis of aflatoxins in the alcoholic beverages and their raw materials. Thin Layer Chromatography (TLC) was used to determine the concentration of the aflatoxins. This was done by comparing colour intensities with those of the standards of known concentrations using a TLC densitometer. The highest concentration obtained was that of aflatoxin B_1 (52.47 µg/l) and was found in *busaa, a* common local brew prepared from fermented maize flour. Other opaque brews showed lower concentration of aflatoxins in the range of 2.19-42.82 µg/l for B_1, 0.25-2.24 µg/l for B_2 and 0.56-14.63 µg/l for G_1. The clear-looking brews showed the absence of aflatoxins except *kibuku root,* which showed the presence of aflatoxin B_1 in a concentration of 0.46 µg/l.

The raw materials used in the preparation of these brews were found to contain aflatoxins with concentrations ranging from 31.1-169.4 µg/Kg for B_1, 11.3-56.4 µg/Kg for B_2, and 10.7-45.1 µg/Kg for G_1. The concentrations of the aflatoxin B_1 was found to be highest in maize flour (169.4 µg/l) as compared to millet and sorghum flour.

The second part of the thesis involved the analysis of volatile impurities in alcoholic beverages using Gas-Liquid Chromatography (GLC) technique. The brews analyzed showed the presence of n-propanol (4.50-84.88 ppm), methanol (1.37-1798.70 ppm), ethyl acetate (7.59-1176.44 ppm), isobutyl alcohol (4.49-147.64 ppm), butanol (7.54 ppm) and amyl alcohol (32.52-392.55 ppm). *Pineapple wine* was found to have the highest concentration of methanol (1798.70 ppm)

CHAPTER 1

PART 1

1.0.0. INTRODUCTION AND LITERATURE REVIEW

1.1.0. MYCOTOXINS

Mycotoxins are secondary metabolites produced by fungi. They are toxic and cause unnatural biological changes in the consumer. They are potentially harmful to humans and domestic animals because of the adverse effects induced in the biological systems[1] .The occurrence of mycotoxins in foodstuffs depend on the presence of certain strains of fungi and is influenced by environmental factors such as humidity and temperature[2]. Survey of foods and feeds around the world have revealed that the problem of mycotoxicoses is not limited to a single geographical area but a problem in all areas where moulds grow[3]. This is more prevalent in tropics and sub-tropics (temperate regions). These areas have high humidity and temperature which are favourable conditions for the toxin formation[3].

Mycotoxicoses have been recorded in several human disease outbreaks and in numerous animal poisoning. For example, in 1952 an acute haemorrhagic hepatitis of swine and cattle that had eaten mouldy corn was reported in

Georgia, U.S.A[4]. The outbreak was followed by a report of dogs that had died from acute hepatitis after consuming food prepared from mouldy corn[5]. The outbreak of ergotism that killed thousands of people was reported in France[6]. This affliction was characterised by necrosis in affected limbs followed by the development of gangrene and eventual sloughing off of the limbs. The cause of the disease was as a result of a group of alkaloid compounds produced by the fungus *Claviceps purpurea.*

Another human disease known as Alimentary Toxic Aleukia (ATA) was reported in Russia[7]. This disease was caused by consumption of overwintered mouldy grain where several moulds were shown to be involved in the etiology of the disease. This included *Fusarium poae, Fusarium sporotrichoides* and several *Clandosporium* species. The symptoms of the disease were severe dermal necrosis, haemorrhaging, leucopenia (abnormal disease in leucocytes) and bone marrow degeneration. About the same time a disease of horses known as *stachybotryotoxicoses* was reported in Russia. The disease was caused by feeding the horses with the mouldy hay containing the mouldy *Stachybotrys alternan*[8].

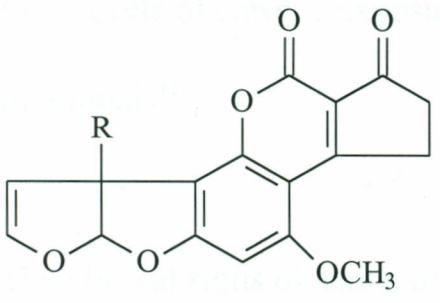
There are different classes of mycotoxins but the four major classes include aflatoxins, ochratoxins, zearalenone and trichothecenes. Out of these classes, aflatoxins are of major concern due to their potent carcinogenic nature and high frequency of occurrence under natural conditions. These characteristics have made aflatoxins to be extensively studied[5,9].

1.2.0. Aflatoxins

The outbreak of "Turkey X" disease in England in 1960 led to the discovery of aflatoxins in the mouldy peanut meal supplement imported from Brazil[10]. The disease was characterized by loss of appetite, lethargy and weakness of wings. The affected birds died within a week.

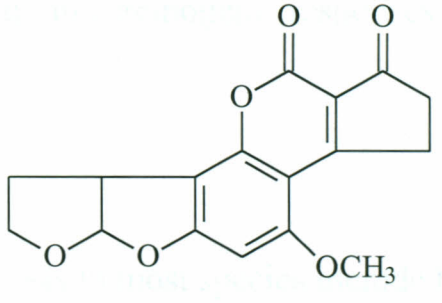
Aflatoxins are a group of acutely toxic and highly carcinogenic mould metabolite produced by a few strains of *Aspergillus flavus* and *Aspergillus parasiticus*. They are chemically related toxins possessing a bisfuran-coumarin structural backbone as shown in **figure 1.** The major toxins in this class are aflatoxins **B_1, B_2, G_1,** and **G_2.** The **B** and **G** notations refer to the fact that aflatoxins B_1 and B_2 fluoresce blue while G_1 and G_2 fluoresce green

when exposed to ultra violet radiation. The various subscripts 1 or 2 relate to the relative chromatographic mobility referred to as retardation factor (R_f).

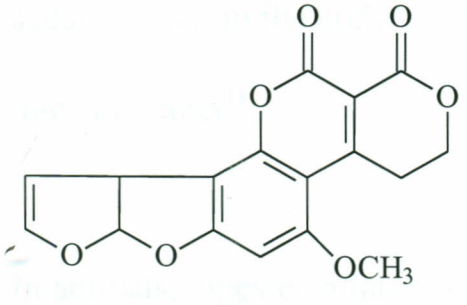


Aflatoxin B_1 R = H

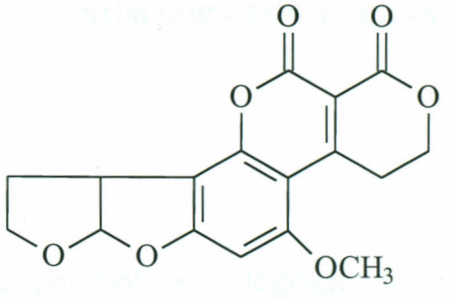
Aflatoxin M_1 R = OH



Aflatoxin B_2



Aflatoxin G_1



Aflatoxin G_2

Figure 1: **Chemical structures of aflatoxins**

Aflatoxins are both potent hepatoxins and carcinogens[11]. Among all the aflatoxins, B_1 has been reported to be the most toxic. Effects of aflatoxins in

vivo vary with dose, duration of exposure, animal species, breed and diet or nutritional status of the animal affected. These toxins are acutely toxic when administered in large doses while sub-lethal doses produce chronic toxicity. Low levels of chronic exposure result in carcinogenic responses in a number of animals[12].

The clinical signs of acute aflatoxicosis in most species include lack of appetite, neurological abnormalities, jaundice of mucous membranes, discoloured liver with necrosis, fat accumulation, convulsions and finally death[13]. Gross liver damage is also evident. There may also be fluid accumulation in the body cavity and haemorrhaging of the kidneys and intestinal tract[13].

In animals, ingested aflatoxins may be metabolically degraded to less toxic metabolites. For example when cattle are fed with aflatoxin B_1 contaminated feed, it is converted to aflatoxin M_1 which is a hydroxylated form of B_1 and occurs in milk, urine and in the excreta. The aflatoxin M_1 retains the toxicity of aflatoxin B_1 but is less carcinogenic[14-15].

1.2.1. Occurrence of aflatoxins

The incidence of aflatoxin contamination is closely associated with climatic conditions and it takes place during the growth, harvesting and storage of various crop[16]. Grains, especially corns, are subject to aflatoxin contamination. The contamination may occur in the field during harvest or in storage. Field fungi include *Fusarium* species, and contamination with these can occur if certain high moisture conditions exist. Contamination by *Aspergillus flavus* which produce aflatoxin can occur in the field as well as during storage[17]. *Fusarium* species can also infect corn in the field through silks and there is experimental evidence that *A. flavus* can also invade corn in this way[18].

Aflatoxin contamination of corn is a world problem and appears to be most severe in the Philippines, Thailand and Uganda where the incidence of aflatoxin ranges from 35% to 97% of the corn supply. The aflatoxin contamination of maize is regularly associated with low-grade cereals[19]. These low quality grades are normally used as animal feeds or for beer brewing. The incidence of occurrence varies with climatic conditions prevailing at the time of harvest and during transportation and later storage.

Contamination of sorghum with aflatoxin has also been reported. Analysis for aflatoxins in sorghum grain has been reported in India, Uganda, Columbia and United States with concentration of aflatoxins ranging from 6-1000 µg/kg [19]. Other grains such as wheat, barley, rye, oats, millet and rice appear to be less susceptible to aflatoxin contamination provided they are properly stored and handled[18]. Aflatoxins have also been detected in crude oils extracted from aflatoxin contaminated oil seeds including groundnuts, coconut oil and olives[20]. Analysis of groundnut oil samples collected from 17 mills in Taiwan showed aflatoxin B_1 concentrations in the range of 10 - 17 µg/kg. Analysis of 25 samples of coconut cooking oil in the Philippines showed that 60% contained aflatoxin B_1 at an average concentration of 3 ppb[20]. Other crops such as cassava, sweet potatoes, tree nuts, pistachio nuts and also green coffee beans have been found to be contaminated with aflatoxins and other mycotoxins[19]. The maximum tolerated levels of aflatoxins especially AFB_1 in different foods and feeds for various countries is shown in **table 1**[21]. It is unfortunate that here in Kenya aflatoxin limits in foods and feeds are not yet established.

Table 1: Maximum tolerated aflatoxin B_1 levels in foods and feeds in various countries.[21]

Country	Commodity	Established tolerance (µg/Kg)
Belgium	All foods	5
Japan	All foods	10
South Africa	All foods	5
China	Rice, peanuts, oats, corn, sorghum, bean, wheat, barley	50
Malawi	All	5

1.2.2. Aflatoxin in indigenous beer and in wines made with yeast

Analysis on alcoholic beverages for aflatoxin contamination revealed the presence of this carcinogen. This was mostly attributed to the raw materials that had been used to prepare these drinks. For example, Alozie[22] studied traditional alcoholic beverages prepared from sorghum and millet substrates in Nigeria and noted aflatoxin contamination for all the samples analyzed. A study by Cook and Collins[23] found no statistical or epidemiological evidence relating the cancer of the oesophagus in East Africa and the consumption of

beer made from maize contaminated with aflatoxin. Despite this, a possible relationship of *Fusarium* toxins in alcoholic drinks with disorder and tumours in man and animals was reported by Schoental[24].

In a study done in Kenya by Peers and Linsell[25] in 1973, out of 304 local beers tested for aflatoxins, only 16 were found to contain aflatoxins in a range of 1 to 25 µg/l. The study showed that the maize used to prepare the local brew was of very low quality and this was cited as the source of the aflatoxins. In a similar study carried out in Zambia to determine the levels of aflatoxins in the commercial opaque maize beers, only one sample of maize malt was found to contain aflatoxin (1.71 µg/l), although the beer made with this malt was aflatoxin free[25]. However, zearalenone was present in a concentration of 4.6 mg/l and was detected in the maize and the maize malt used in making the beer[26]. In Lesotho, out of 151 samples of the local alcoholic beer analyzed, only 17 were contaminated with zearalenone[27]. Malted maize or sorghum malt was used for the brewing and no aflatoxin was detected in the samples. Although aflatoxin contamination was detected in very low quantities in these drinks it is important to note that even low doses are lethal and their effects are cumulative.

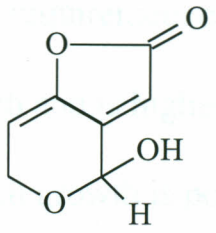
1.2.3. FACTORS INFLUENCING AFLATOXIN PRODUCTION

The development of *aspergillus flavus* has been studied extensively on various agricultural commodities[28-30]. Its growth and thus the production of aflatoxin on or in various agricultural commodities depends upon several factors. These include substrate, moisture and relative humidity, time, temperature and storage conditions. The environmental conditions under which the agricultural commodities are harvested, transported, handled, stored and marketed greatly determine the extent of aflatoxin contamination in these commodities.

1.2.3.1 Substrate

The nutritional requirement for mould growth and for aflatoxin production may be different. Early investigation with synthetic media reported relatively low yield of aflatoxin in the laboratory. Natural materials proved suitable substrate for producing large quantities of aflatoxin[31]. All agricultural commodities do not support aflatoxin production equally. Cereal grains, in general appear to be good substrates for toxin production than oil seeds[19]. This may be due to high proportion of carbohydrates in cereals that may be metabolized by fungi more easily than fats. Moulds are

also frequent contaminants of fresh fruits and vegetables. Apples in particular are susceptible to a rot caused by *Parasiticus expansium* which produces Patulin[32] .



Patulin

Patulin has also been reported to be present in commercial apple juice[33]. It has been found that apples used for preparing juice and wines are sometimes of poorer quality than apples used for direct consumption[34]. Low levels of aflatoxin contamination were also detected in fig fruits[35]. Only limited incidences of aflatoxin in wines have been detected with the average content being lower than 1μg/l[19]. Other substrates such as apricot, grape, grape fruit, mixed vegetable juice, orange, peach, pears, pineapple and tomato juice drinks support production of aflatoxin in the range of 12 to 44 μg/ml[36].

1.2.3.2. Moisture and relative humidity

Moisture and relative humidity are the most important factors in the growth and production of *A. flavus*. Moisture requirements depend on the

temperature as well as nutrients. *Aspergillus flavus*, a mesophyte has a minimum moisture requirement for growth between 80 and 90% relative vapour pressure[37] and the minimum for sporulation is 85% relative humidity[38]. Minimum moisture requirement of mould spore is lowest at the optimum temperature for growth and is highest near the minimum and maximum temperature for which growth is possible. The minimum moisture content also depends on the substrate. For instance, *Aspergillus flavus* has been found to be the predominant mould species on maize stored at relative humidity of 80% and above and at 16.2% to 24.4% moisture content[39]. Lopez and Christener[40] reported that *Aspergillus flavus* did not invade maize samples below 17.5% moisture whereas extensive growth appeared at 18.5% moisture. Little development of *aspergillus flavus* occurred at 14% moisture content, although other aspergillus strains could develop at this moisture level[40]. Similar observations have been made on other cereal grains and it has been established that they should be dried to a moisture content of 13% or less for safe storage[19]. Safe moisture content, however, varies with the temperature at which the commodity is stored and duration of storage. It is important to ensure that the moisture content of stored products is not allowed to increase to levels favourable for mould growth. Products stored

in open containers may also increase the moisture content when exposed to an atmosphere of high relative humidity, eventually attaining moisture levels favourable for mould growth.

1.2.3.3. Temperature

The growth of mould is temperature dependent with maximum growth occurring at optimal temperature. *Aspergillus flavus*, a mesophyllic fungus has minimum, optimum and maximum growth temperatures as 6-8 °C, 36-38 °C, and 44 °C respectively[41]. The psychrophiles have an optimum temperature of below 10 °C. The thermophiles grow at temperatures above 40 °C and even higher than 50 °C. Some members of the latter 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 zero to as high as 50 °C or above. It is of important to note that the optimum temperature for growth may not be the same as the optimum temperature for the production of different aflatoxins[42]. The maximum temperature for growth of *Aspergillus flavus* has also been found to be higher on natural substrates than on synthetic media[43].

1.2.3.4. Time

The significance of time as a factor in aflatoxin production is demonstrated by the lag between mould growth and aflatoxin production. Maximum production of aflatoxin occurs several days after germination and this time period depends on the strain, temperature and substrate[44]. Dickens and Pattee [45] reported that aflatoxin developed rapidly in groundnut samples of 15-30% moisture held at 30 °C during 10 days. The minimum time for aflatoxin production was 2.5 days after inoculation. Schroeder and Hein also showed that aflatoxin production by *A. flavus* reached a peak within 2 days at 35 °C in cotton seeds and at 30 °C in groundnuts and rice[46]. Reducing the temperature from 28 °C to 15 °C resulted in progressively less aflatoxin and only 1 μg/g of B_1 was detected in cultures incubated at 11 °C for 3 weeks on rice. It is worthy noting that the knowledge of the time period between germination and maximum aflatoxin production would be valuable in providing an estimate of the toxic potency of a given mouldy product.

1.2.3.5. Damage

Damage to shell or kernel increases the possibility for direct invasion of kernel and aflatoxin formation. Damage also increases nutrient availability

to its infected fungi. Thus the extent of fungus growth and aflatoxin formation at minimal temperature and relative humidity will be as a result of damage. Bampton[47] reported that the testa damage especially before drying to safe moisture levels increases fungi invasion and toxin formation in kernels of groundnuts. Observation in corn has also shown that more ears are contaminated with aflatoxin when damaged mechanically after silking. Aflatoxin contamination is especially more serious following damage to protective coverings of foodstuffs in places where there is a high *A. flavus* spore load[16].

Insects may carry saprophytic fungi in their gastrointestinal (GI) tract[48]. These insects may infect and breed on cereal grains and become a source of mould contamination. 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[49]. Infection of corn kernels by *Fuserium, Penicillium* and *aspergillus* moulds, particularly *A. flavus*, is more prevalent when the kernels are damaged by *Helminthosporium maydis* which causes corn blight[50].

1.2.3.6. Aeration

Fungi are highly aerobic organisms. The minimum amount of oxygen required for spore germination, vegetative growth and sporulation may be variable[51-52]. Oxygen availability exerts dramatic effects on the catabolic system of *A. flavus*. Reiss[53] showed that the growth of *A. flavus* and production of aflatoxin on sliced bread largely depends upon oxygen permeability of the packaging foil. The lower this factor the weaker the growth of moulds and production of aflatoxin.

Landers *et al*[54] observed that increasing carbon dioxide concentration from 0.03 to 20% (in air) did not reduce the development of *A. flavus* on groundnuts. Aflatoxin elaboration was progressively inhibited with increase in carbon dioxide concentration from 0.03 to 100% and with reduction of oxygen level from 5% to 1%. No growth and aflatoxin production occurred in 100% carbon dioxide concentration. Examination of aflatoxin production on solid substrates has shown that culturing on a rotary shaker considerably increases yield of aflatoxin B_1 by 3-100 folds in cultures of maize, groundnuts, rice, sorghum and wheat[55].

1.2.3.7. Microbial interaction

Production of aflatoxin by *A. flavus* may be affected by the presence of other fungi which is the usual situation in nature for all agriculture commodities. *A. flavus* is frequently found associated with numerous other micro-organisms particularly on stored grains. Thus microbial competition between fungi for substrate under favourable environmental conditions will restrict or reduce the amount of aflatoxin formation. Burmeister and Hesseltine[56] found that *Bacelli, Clostridium* and *Streptomycete* inhibited aflatoxin formation in the substrate by 30 μg/ml of crude aflatoxin.

Schroeder and Boller[57] found that rice inoculated with the combination of *A. flavus* and another aspergillus species found in stored grains, produced little aflatoxin, when incubated at moisture levels suitable for mould growth. In another study Ciegler *et al*[58] observed that *Flavobacterium aurantiacum* **NRRL 3-184** removed aflatoxin B_1 irreversibly from nutrient solution. They also reported that this bacterium completely detoxified aflatoxin contaminated milk, maize oil, groundnuts butter, maize, groundnuts and partially detoxified soyabeans experimentally.

Ashworth *et al*[59] reported *A. niger* and *Rhizoctania solani* as competing fungi causing limited development of *A. flavus* and aflatoxin production in groundnuts. Little or no aflatoxin was found when aflatoxin producing strain of *A. flavus* was inoculated onto moist maize and allowed to grow along with other fungi naturally present on the grain[60].

1.2.4. STABILITY OF AFLATOXINS IN FOODS AND DRINKS

Aflatoxins are generally thought to be stable in most food products. Thermal processing of food, other than roasting of nut is not likely to reduce the aflatoxins content, whereas roasting may cause a reduction of 40 to 60% of the aflatoxin present[61].

Aflatoxins are destroyed almost completely by microwave roasting of contaminated peanuts[62], but this is not a commercial process yet. Roasting soyabeans at 180°C reduced the aflatoxins content by 40% to 73%, but aflatoxin in non-malted beans were more affected than in malted beans and aflatoxin B_1 was more abundant than aflatoxin G_1[63].

As for the grains, corn is a significant source of aflatoxin contamination[64]. Physical cleaning methods, dry cleaning, wet cleaning, density separation and preferential fragmentation have been found to be generally ineffective in lowering the aflatoxin B_1 content of naturally contaminated corn[65]. Laboratory wet milling of inoculated corn showed that aflatoxin B went primarily into sweep water (39% to 42%) and fibre (30% to 38%) with the remainder found in gluten (13% to 17%), germ (6% to 10%) and starch (only 1%)[66]. In dry milling of naturally contaminated corn, the highest levels of aflatoxin B_1 occurred in the germ and hull fractions but distribution varied with the contamination levels; the grits, low-fat meal and low-fat flour (the prime products) contained only 6% to 10% of the aflatoxin B_1[67]. The concentration of aflatoxin B in artificially contaminated rice was greatly reduced by milling, with more than 95% in the bran and polish fraction[68]. As in the case of nuts, aflatoxins are only partially destroyed on heating during processing to grain products. Roasting corn at 145 °C to 165 °C reduced aflatoxin B concentration by 40% to 80%[69]. When contaminated corn grits with aflatoxins were boiled, 25% of the aflatoxin B was destroyed. When the boiled grits were fried, 53% of the aflatoxins was lost with increasing moisture content of the boiled grits. Similarly, normal cooking destroyed

about 49% of aflatoxin B but no difference was observed when naturally contaminated rice or rice which contained added aflatoxin B, was used[70]. Pressure cooking and cooking with excess water destroyed none of the aflatoxin B_1 in the rice (73% and 82% respectively). Boiling contaminated maize (corn) flour in water to make ugali, a traditional African dish, destroyed only 15% and 17.6% of aflatoxin B and G respectively[71]. Eighty three per cent of aflatoxins present in contaminated aleppo pine nuts remained after cooking a pudding made from these nuts, which is widely consumed in Tunisia.

Aflatoxin is not completely removed during the brewing of beer. The contamination is carried forward and passed on to the final product. For example, 18% and 27% of aflatoxin B_1 remained during beer making by using starting materials that had contamination levels of 1 and 10 ppm, respectively[72]. The toxin was relatively stable in the cooker mash treatment, but was significantly lost during the wort boiling and in the final fermentation steps. Dam *et al*[73] found that the amount of aflatoxin B_1 was reduced by 47% after cooking and fermentation of corn during the production of distillers protein concentrate. Low concentrations of aflatoxin

B_1 were present in the wet solids (dry weight basis) after distillation than in the starting corn[74]. However, aflatoxin from contaminated corn was absent in the alcoholic distillate because aflatoxins are not volatile. Accumulation of aflatoxins in spent grains is a potential problem in using this material as an animal feed as aflatoxins would appear in the animal tissue though in a less toxic form. A study by Gitu[75] reported the presence of high levels of aflatoxins in most of the animal feeds analyzed. Fermentation of Mexican corn dough by naturally present micro-organisms did not degrade aflatoxins[74]. However treatment of corn with lime water in the manufacture of *tortillas* considerably reduced the levels of aflatoxins[76]. A study done in Nigeria, showed that 30% of aflatoxin B_1 was present in orgi porridge (fermented sorghum made into porridge) and 24% remained after further fermenting of the grain contaminated with pure aflatoxin B_1[77]. The decrease was attributed to the fermentation process.

1.2.5. Other carcinogens found in alcoholic beverages

Alcoholic beverages contain numerous chemicals that may contribute to the carcinogenicity of the beverages. These include traces of acetaldehyde, N-nitroso compounds (e.g. N-nitrosodimethylamine) and urethan (ethyl

carbamate)[78]. Urethan has been detected in the highest concentration in "stone fruit" (i.e. cherry, plum, apricot etc.), brandies, sake and rice wine. It has been detected at low levels in distilled spirits, wines and beers. Although the carcinogenicity risks from urethan appear to be extremely low, and probably negligible, it may be somewhat increased among those who frequently consume alcoholic beverages with urethan[79,80].

Ethanol as a carcinogen or a co-carcinogen for the liver is, and has been, a subject of great interest because risk for hepatocellular carcinoma has been associated with the alcohol intake[81]. It is believed that ethanol is a promoting agent in rat liver and evidence suggests that it may act in the same manner in the human as well[82]. Ethanol administration prior to and concurrent with aflatoxin administration resulted in an enhanced carcinogenicity in rat liver[83]. A demonstration on the co-carcinogenic effect of ethanol and aflatoxin in animal models suggested that ethanol alters the toxification /detoxification ratio in favour of an increased carcinogenicity of aflatoxin[84]. But when ethanol was administered after the aflatoxin, there was no promotion of the carcinogenicity[85]. Another study showed that ethanol potentiated the toxic effects of aflatoxin B_1 in female rats[86]. However, pre-

treatment with Phenobarbital prevented the induction of liver lesions in rats by aflatoxin B_1[87].

Several studies have been carried out to establish the relationship between alcohol and the increased hepatocarcinogenesis but no clear experimental demonstration has been made[80]. Misslbeck *et al*[84] for instance, fed 35% of calories as ethanol in a fully nutritious diets to rats after they had been given a carcinogenic dose of aflatoxin B_1 and found no effect of ethanol on a number of enzyme-altered foci (EAF). On the other hand, Toskulkao *et al*[87] reported increased hepatotoxicity of single doses of aflatoxin B_1 of 0.75-2 mg/Kg in winstar male rats pre-treated with 4 doses of ethanol of 4 g/Kg between 48 and 21 hr earlier. No long-term studies were performed. Based on these studies it is difficult to make a conclusive relationship between aflatoxin carcinogenesis and the alcohol intake in man.

1.2.6. Relation of aflatoxin to human health

The relationships of aflatoxins to human health as the cause of human disease syndromes are difficult to determine because there is no direct evidence of such involvement in terms of controlled experiments with

man[88]. This is partly understandable because of the limitations of human experiments, the nature of carcinogenic process involving prolonged latency and the difficulty of directly connecting aflatoxins as the causative agents in human poisoning following the consumption of mouldy foods.

Table 2 below indicates the carcinogenicity of aflatoxin in different animal species[89]. The fact that many different animals species are susceptible to both acute and chronic effects of aflatoxins is strong and it is an indirect evidence that man too would most likely be adversely affected in a similar manner[89]. This is particularly so with toxins that affect animals which are physiologically similar to man such as primates and swines. The incidence of mycotoxin diseases appears to be higher in animals than in man, since animals are more likely to be exposed to aflatoxins than man, with respect to the quality of their feeds and their way of feeding.

Table 2. Carcinogenicity of aflatoxin

Species	Dose (LD_{50})	Duration of observation
Duck	30 μg/kg	14 months
Trout	8 μg/kg	1 year
Tree shrew	24-66 mg total	3 years
Marmoset	5.0 mg total	2 years
Monkey	100-800 mg total	Over 2 years
Rat	100 μg/kg	54-88 weeks
Mice	150 μg/kg	80 weeks

Adapted from Linsell,1982 [89].

In the absence of definitive data to interpret the role of aflatoxins in human disease, one must turn to less direct data. These include animal studies, epidemiological data and reports of isolated incidences of human diseases which are thought to be related to aflatoxins. Based on these considerations, the types of diseases that may be caused in man by aflatoxins can be divided into acute toxicity, chronic toxicity and /or carcinogenicity.

1.2.6.1. Acute toxicity

There are reports in the literature that associates aflatoxins with acute poisonings in humans[90-91]. These reports mostly involve children as there are more susceptible to the poisoning. For example, 26 persons in Taiwan were reported ill after consuming mouldy rice for a period of three weeks and experience intoxication[90]. Symptoms of the diseases were oedema of the legs, abdominal pain, vomiting, damaged liver and no fever. Assay of the rice revealed the presence of aflatoxin B_1 in two samples at levels of about 200 μg/ kg.

Another suspected case of fatal aflatoxin poisoning occurred in Uganda and involved a 15-year old boy[92]. Upon admission to the hospital, the boy had a history of abdominal pain, oedema of the legs, damaged liver and no fever, symptoms very similar to the Taiwan cases. The boy's diet consisted mainly of cassava which was found to be mouldy and contaminated with 1.7 mg aflatoxin / kg. Extrapolation of data obtained with monkeys, on the calculation of the amounts of cassava consumed by the boy over a period of several weeks revealed that a fatal dose could easily have been consumed. Two younger children in the same family were ill with the similar symptoms

but recovered. It was postulated that because the younger ate less, they had been exposed to sub-lethal dose levels.

Studies in Thailand show that Reye's syndrome occurs in epidemic proportions in Northeast Thailand[93]. Reye's syndrome occurs in children and is characterised by vomiting, hypoglycaemia, convulsions, coma, and usually death. Pathological examination revealed several cerebral oedema and fatty degeneration of the liver, kidneys and heart. In one fatal case involving a 3-year old boy, it was found that the boy had eaten only leftover of boiled rice for 2 days before becoming ill[93]. Examination of rice revealed that it was mouldy and contained 10 mg of total aflatoxins/kg.

Here in Kenya, Ngindu *et al*[94] reported an outbreak of aflatoxin poisoning in Makueni division of Machakos district. In this incidence, 20 cases of aflatoxicosis occurred after consumption of aflatoxin contaminated maize. Analysis on the maize consumed showed that it had as much as 12,000 ppb aflatoxins. Liver post-mortem contained upto 89 ppb of aflatoxin B_1.

1.2.6.2. Carcinogenicity and chronic toxicity

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. For example, in Uganda where the incidence of liver cancer is high, food crops are also found to be highly contaminated with aflatoxins. In addition, there is a geographical distribution of aflatoxin contamination of food that corresponds to the geographical incidence of liver cancer within the country[95]. The Cancer Registry for 1964-1966 in this country showed a high incidence of hepatoma[96]. Samples of foods collected from village markets or home granaries were assayed for aflatoxin contamination. The results indicated that 44% of 105 food samples in Karamoja district contained more than a 100 μg/Kg; while in other districts where hepatoma incidence was lower, the food samples were less contaminated. The daily total aflatoxin consumption was estimated to be 20 μg to 2 mgs which would be extremely high.

The same type of correlation between aflatoxin in peanuts and liver cancer was also found in Swaziland. Samples of groundnuts were collected from various parts of the country and analyzed for aflatoxins. The frequency of

contamination was high in those areas where there was a high frequency of liver cancer[97].

Studies in Kenya correlated the incidence of liver cancer and aflatoxin contamination of food supplies with altitude. As altitude increased and temperatures became more moderate, the levels of aflatoxins in the food decreased as did the incidence of liver cancer[98]. This study revealed that the mean total aflatoxin intake ranged from 3.46 ng/kg body weight for women and 14.8 ng/kg body weight for men.

It is important to note that the association between liver cancer incidence and aflatoxin intake for any given year does not closely reflect a direct correlation. This is due to the fact 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 correlation is based.

Though 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 suggest a casual relationship.

1.2.7. Aflatoxin analysis

Since the discovery of aflatoxins in 1960, a great deal of research has been devoted to the development of methods for analysing aflatoxins[6]. Due to the variance of chemical properties, no single analytical technique can possibly quantitate more than a few closely related mycotoxins at one time. However, multi-toxin screening methods are available to test for the presence of a range of mycotoxins[88]. These analytical methods must be extremely sensitive because the presence of even minute amounts of aflatoxins in food may have toxicological implications to humans and animals. Each food 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[6]. The accurate monitoring of low levels of aflatoxins in the complex chemical matrix that makes up food is therefore a difficult task. Hence sensitive and accurate methods for analysis of aflatoxins in foods are essential for decreasing the risk of human exposure to aflatoxins.

Generally, all chemical analytical methods for the determination of aflatoxins include extraction, clean-up, separation, detection, quantization and confirmation of identity.

1.2.8. Sample Extraction and Clean-up

The detection of minuscule quantities of aflatoxins in food dictates that the toxin be isolated from interfering compounds present in the sample while retaining a high recovery of the toxin. An initial extraction is performed to remove the toxin from the food matrix, resulting in the co-extraction of a wide range of other compounds. These compounds are likely to interfere with any subsequent analytical procedures, and thus further clean-up steps are usually necessary.

The procedure for the initial extraction of toxin from a food sample depends on the physico-chemical properties of the food as well as those of the toxin. In general, the finely ground food sample is subjected to high-speed blending or mechanical shaking in the presence of the extracting solvent systems. The most efficient solvents for extracting aflatoxins are relatively polar solvents such as methanol, acetone and chloroform[99]. These solvents are combined with small volumes of water to increase penetration of the solvent system into the hydrophilic food tissue. The aqueous phase can be an acid solution designed to break interactions between the toxins and food constituents such as proteins. The slurry is then filtered and ready for subsequent purification procedures[100]. Diatomaceous earth is sometimes included in the solvent

system to speed up this process.

The clean-up techniques are 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 procedures may depend on the method used for detection and determination, the required limit of detection, the speed of analysis and the recovery. High levels of lipids present in certain commodities such as peanuts and cocoa beans will interfere with subsequent analytical procedures[99]. For these foods, non-polar solvents such as hexane can be included in the original solvent system, or they can be added after the homogenization and filtration steps to remove lipid constituents. 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, lead acetate is used to precipitate proteins in powdered egg extracts[100]. Because the fluorescence of aflatoxins contributes to the selectivity and sensitivity of the methods used to analyze them, the removal of the interfering fluorescent compounds is

essential. For example silver nitrate is used to reduce the concentration of theobromine in cocoa beans extracts[101]. The use of the chromatographic columns for the purification of aflatoxin extracts has gained widespread popularity, particularly for samples which tend to form emulsions during solvent extraction[102]. These columns can either be packed in the laboratory or purchased as pre-packaged cartridges. 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 poured onto the column and a more non-polar solvent is added to elute interfering sample components such as lipids. The solvent system is then made more polar, resulting in the elution of a more pure aflatoxin extract. For example, the Contaminants Branch (CB) method[102] for the analysis of aflatoxins in peanut products involves the addition of chloroform:water (10:1) extract to a silica gel chromatographic column. Lipid constituents are removed with hexane and diethyl ether. The toxin is then eluted with a chloroform:ethanol rinse (97:3). Prepacked columns using silica have also been utilised to obtain a crude aflatoxin extract from milk[103].

Other adsorbents that are employed in the purification of mycotoxins are florish R, Sephadex R., aluminium oxide, cellulose, and polyamide[104] . The

pre-packaged cartridge offers the advantage of more uniform packing, and their use frequently results in savings of time and solvents. However, they are less versatile, in that the make-up of the column cannot be easily adjusted for the sample. The final step prior to analysis of the sample involves concentration of the cleaned-up extract. This task is best performed using a rotary evaporator operating under reduced pressure and slightly elevated temperatures.

1.2.9. Chromatographic Techniques

Although final extracted samples have been subjected to clean-up procedures they will still normally contain other co-extracted substances. Chromatographic separation is thus needed to isolate the toxins from compounds of like chemical properties. The isolation and analysis of aflatoxins from a complex food matrix is facilitated by the unique spectral and fluorescent properties which they possess. The chromatographic methods most widely and routinely used are thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC).

1.3.1. Thin-layer Chromatography

Thin-layer chromatography (TLC) is the most widely used analytical method

for separating and identifying aflatoxins from concentrated extracts. This is a form of chromatography that is performed on a thin layer of adsorbent supported by either an inert glass, metal or plastic plate. A wide range of adsorbents can be used in TLC. For aflatoxin analysis, silica gel TLC plates are most often used as they generally offer the best possibility of separating the toxin of interest from matrix compounds. Both pre-coated and self-coated plates can be used. Self-coated plates allow a free selection of adsorbent and a free selection of additives. Pre-coated plates are ready to use, possess a more uniform and rigid layer and do permit a certain choice of support. The mobile phase in TLC is the solvent system that is placed in the bottom of a development tank along with the plate carrying the spotted mixture of compounds. The separation of the different compounds depends on their affinity for the stationary and mobile phases. Tentative identification of the toxins can be done by computing the R_f value, that is the distance the compound migrates divided by the distance the solvent travels. This value is compared to the R_f value determined for a standard toxin run on the same plate.

It is apparent that some methods call for the development of the plate in one direction, whereas other suggests two. One-dimensional TLC is usually

performed on a sample which has undergone extensive clean-up. In two-dimensional TLC, the sample extract is spotted at a corner of a TLC plate and two developments are carried out successively parallel to the two sides of the plate. Two different developing solvents which must be compatible and interdependent are used. For example, in the determination of aflatoxin B in feeds the plate is developed in the first direction with a mixture of dimethyl ether, methanol and water (94:4.5:1.5), dried, and turned 90 °C. It is then developed in the second direction with a mixture of chloroform and acetone (9:1). The standards must be arranged in an anti-diagonal pattern so as not to interfere with the migration of the sample. The anti-diagonal spotting of various concentrations of the standard near the sample results in the migration of the standards in two directions and accounts for the diffusion of the sample toxin during development of the plate for quantification.

Aflatoxins emit light in visible energy region when exposed to UV radiation at 366 nm. For these toxins, the visualization of the separated spots simply involves placing the plate under UV light and marking the location of the toxin with a pencil. The R_f 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.

Approximate aflatoxin concentrations are determined by comparing colour intensities with those 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 utilised to mark off lanes on the cover plate so that the spots are scanned in the direction of long wavelength. 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 samples and standards does not provide conclusive proof as to the identity of the isolated compound. Other techniques must be employed for confirmation.

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. Partial confirmation of identity is obtained if the suspected toxin and internal standard remain superimposed after development of the plate. The derivatization of the suspected sample toxin after isolation by TLC also may provide partial confirmation of toxin identity[105]. The alteration of aflatoxin fluorescence from blue to yellow following treatment of the plate with

sulphuric acid is an example.

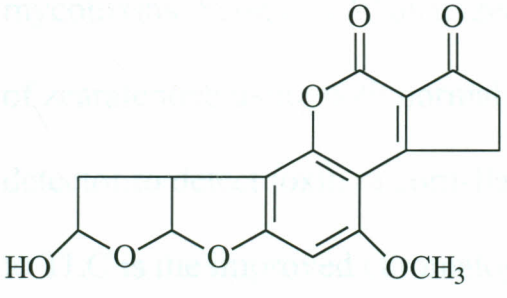
A more definite confirmation of aflatoxin identity is secured by matching R_f values of a suspected aflatoxin and a standard, derivatizing both compounds, and matching the R_f values of the derivatives. This sequence of steps can be performed on a single plate as in a method described by stubblefield *et al*[105] for the isolation of aflatoxin M_1 and aflatoxin B_1 from beef livers. The plate was spotted with the aflatoxin M and B standards in the outside lanes for two-dimensional TLC. The plate was 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 followed by heating the plate in an oven. This treatment of aflatoxins possessing unsaturated terminal furan ring, namely B_1, G_1 and M_1, causes the addition of water across the double bond. After cooling, the hemiacetals of the toxin thus created are developed in the second direction. The matching of R_f values for the sample toxin and the derivatized toxin with their respective standards constitutes positive identification. The derivatization of toxin can also be carried out in a test tube prior to separation by TLC. Bijl *et al*[106] 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

then separated by two-dimensional TLC.

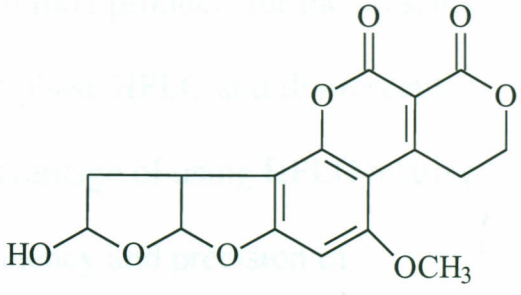
1.3.1. High performance liquid chromatography.

High performance liquid chromatography (HPLC) is a separation technique that has become increasingly useful for the analysis of aflatoxins because it offers increased sensitivity and improved accuracy over TLC methods. For HPLC, the mobile phase is placed under high pressure generated by pumps and is forced through a steel column containing the stationary phase. Initial studies using HPLC for the analysis of aflatoxins utilised silica gel columns and chloroform / methylene chloride solvent systems (normal phase chromatography) and instruments equipped with UV detectors[107]. The lack of selectivity of this detector resulted in significant interference from sample contaminants and poor sensitivity. The adaptation of fluorescent detectors to HPLC increased both the selectivity and sensitivity to this technique. One drawback to this system is that aflatoxins B_1 and B_2 fluoresce weakly in the non-polar solvents with normal phase Chromatography whereas aflatoxin G_1 and aflatoxin G_2 are highly fluorescent. The observation that aflatoxin B_1 and B_2 exhibit an increased fluorescence when absorbed to silica gel led to the advent of fluorescent detectors with silica gel packed flow cells for normal

phase HPLC analysis of mycotoxins. The detection limit achieved was 0.1 - 0.2 (nanograms) ng of all aflatoxins[107]. Aflatoxins can also be separated using reverse phase HPLC but aflatoxin B_1 and G_1 fluoresce poorly in the polar solvent systems, whereas B_2 and G_2 are highly fluorescent. One option is to convert aflatoxin B_1 and G_1 to their hemiacetal derivations, B_{2a} and G_{2a} respectively by reacting the sample extract with trifluoroacetic acid.



Aflatoxin B_{2a}



Aflatoxin G_{2a}

The presence of the hydroxyl group makes them fluoresce strongly. In reverse phase liquid chromatography, a non-polar octadecylsilica stationary phase is utilised while a polar solvent system composed of water, acetonitrile and methanol is typical. Another derivatization technique for the analysis of aflatoxins by reverse phase HPLC involves the post column reaction of toxins with iodine to increase the fluorescence of aflatoxin B_1 and aflatoxin G_1. After eluting the column, the toxins are reacted with iodine saturated with

water, resulting in derivatives of aflatoxin B_1 and aflatoxin G_1 having an equal fluorescence to aflatoxin B_2. Because this reaction is automated in the system, this technique offers the advantages of being faster and giving greater precision than trifluoroacetic acid derivatization[108]. In addition, the identities of aflatoxins B_1 and G_1 can be easily confirmed by uncoupling the iodonation system, resulting in drastic loss of fluorescence of these compounds. The HPLC methods proved to be extremely useful for the analysis of a variety of mycotoxins. Scott *et al*[109] analyzed corn-based food products for the presence of zearalenone using both normal and reverse phase HPLC and fluorescent detector.to detect toxin in corn-flakes. The advantage of using HPLC relative to TLC is the improved chromatographic efficiency and precision of quantification obtained. Nevertheless, the method has its own disadvantages such as: expense of the instrument and the level of expertise required to operate and maintain the instrument, requires relatively larger time for the analysis of a single sample and generally, the samples require more extensive clean-up prior to analysis.

1.4.0. Immunological techniques

With the availability of different types of antibody against aflatoxins, many types of immunoassays including Radioimmunoassay (RIA) and Enzyme-

linked immunosorbent assay (ELISA), as well as several novel immunochemical screening tests, have been developed for aflatoxin analysis. Most of these methods are very sensitive, specific, and simple to perform. Specific antibodies also have been used as immunohistochemical reagents and in affinity columns that are used as clean-up tools for analysis of aflatoxins by other methods. For immunoassay of aflatoxins, several general criteria must be considered[110].

(i) Since most immunoassays are based on the competition of binding between the unlabelled toxin in the sample and the labelled toxin in the assay system for the specific binding sites of antibody molecules, a good marker aflatoxin derivative is needed in addition to the specific antibody in the assay system.

(ii) For accurate quantitation, a good method for the separation of free and bound forms of toxins is important.

(iii) Depending on the approaches that have been used for raising antibodies, the degree of cross-reactivity of these antibodies with their respective structure analogs varies considerably.

1.4.1. Radioimmunoassay (RIA)

The RIA procedure involves incubation of specific antibody simultaneously with a solution of unknown sample or a known standard and a constant amount of labelled toxin. After separation of the free and bound toxin, the radioactivity in these fractions is determined. The toxin concentration of the unknown sample is determined by comparing the results to a standard curve, which is established by plotting the ratio of radioactivities in the bound fraction and the free fraction against the log concentration of the unlabelled toxin.

In practice, improved sensitivity in the RIA can be achieved by a more extensive clean-up of the sample extract. However because of the need for radioactive toxin, RIA can only be used in a laboratory which has facilities for determining radioactive ligands.

1.4.2. Enzyme-linked immunosorbent assay (ELISA).

Two types of ELISA have been used for the analysis of aflatoxin; direct ELISA and indirect ELISA. Both types are heterogeneous competitive assays. Direct ELISA involves the use of aflatoxin-enzyme conjugate. In this competitive assay, specific antibodies are first coated to a solid phase such as

polystyrene beads, polystyrene tubes, nylon beads and to a microtiter plate[111]. The sample solution or standard toxin generally is incubated simultaneously with enzyme conjugate or incubated separately in two steps. After appropriate washings, the amount of enzyme bound to the plate is determined by incubation with a substrate solution containing hydrogen peroxide and appropriate oxidizable chromogen. The resulting colour is measured spectrophotometrically or by visual comparison with the standard. In this assay, toxin in the sample and toxin-enzyme conjugate compete for the same binding site with the antibody coated to the solid phase. Since the toxin-enzyme and antibody concentrations are constant, the colour intensity as a result of enzyme reaction is inversely proportional to the toxin concentration in the testing sample.

In general, ELISA is approximately 10-100 times more sensitive than RIA when purified mycotoxins are used. As little as 2.5 picograms (pg) can be measured. The direct competitive ELISA for aflatoxin generally can be completed in less than 1 hr after extraction of the toxin from the sample[112]. Like that of RIA, the sensitivity of ELISA is improved when a clean- up treatment is included.

Indirect ELISA involves the use of an aflatoxin-protein conjugate and a secondary antibody to which an enzyme has been conjugated. In the indirect ELISA, an AF-protein (or polypeptide) conjugate is prepared and coated onto the microtiter plate before assay. The plate is incubated with specific rabbit (or other type) antibody in the presence or absence of the homologous aflatoxin. The amount of antibody bound to the plate coated with aflatoxin-protein conjugate is determined by reaction with goat anti-rabbit (or anti-other type) IgG-enzyme complex, which is generally commercially available, and by subsequent reaction with the substrate. Thus, toxin in the samples and toxin in the solid phase compete for the same binding site with the specific antibody in the solution. The indirect ELISA also has been used for the analysis of a number of mycotoxins[113]. This type of ELISA requires fewer antibodies and does not require preparation of toxin-enzyme conjugate. However, it requires more analytical time.

In this work, TLC has been employed for the analysis of aflatoxins. A scanning densitometer was used to quantify the amount of the toxins. This method is relatively inexpensive and reliable. It is also an ideal and universal technique for the separation and identification of variety of compounds through a judicious choice of mobile phase.

PART 11

1.5.0. VOLATILE IMPURITIES IN ALCOHOLIC BEVERAGES

1.5.1. INTRODUCTION

All alcoholic beverages are derived either directly or indirectly from fermented products. These include fermented beverages (sour porridges), products of mixed alcohol-lactic acid fermentations, beverages made by distillation and by fortification. Distilled beverages are derived from fermented grains and potatoes (whiskies and vodka), sugarcane by-products (rum), fruits (brandies), and other plants such as mezeal (tequila). Liqueurs are distilled beverages that have been flavoured and sweetened. Fortified beverages require the addition of alcohol during production[114]. Fruits containing high sugar concentrations at maturity and nutrients at levels sufficient to support growth of fermenting yeasts, primarily *Saccharomyces cerevisae*, have traditionally been the raw materials from which wines are made. Beer and sake are produced from barley and rice respectively. Unlike wines, these beverages are derived from carbohydrates that are not initially fermentable. Conversion of carbohydrates into fermentable sugars requires the action of amylases

produced by barley in case of beer, and the fungus *Aspergillus oryzae* during sake production.

The main constituent of the alcoholic beverage is ethanol. Ethanol is a clear, colourless, flammable liquid, miscible with water and many organic solvents in all ratios[114]. It is hygroscopic and relatively non-toxic, exhibiting an oral LD_{50} in rats of 13.7 g/Kg (grams of ethanol per kilogram of body weight required to kill 50% of the animals[115]. Ethanol has a slightly sweet taste and a characteristic aroma[114]. At high concentrations, it causes a burning sensation in the mouth. Addition of sugar to ethanol-water solution increases the threshold for ethanol, indicating that sugar masks its taste and /or aroma. The ethanol content in the alcoholic beverages ranges from 2% to 6% in beers, 10% to 20% in wines, 40% and 50% in spirits such as gin, whisky and brandy. In addition, other agents which may arise naturally from the manufacturing and storing process, or are deliberately added give the distinctive scent and flavour to the product[116].

Because alcohol is an excellent extractant, it exhibits low toxicity, possesses antimicrobial activity and also enjoys wide use as a solvent for food ingredients such as spices and flavours[117].

1.6.0. Alcoholic impurities

Although ethanol is a major component in the alcoholic beverage, other volatile impurities such as methanol, propan-1-ol, acetaldehyde, ethyl acetate and amyl alcohols may also be produced as a result of the fermentation process.

The conditions under which fermentation is carried out may also result to the production of other non-volatile impurities. The threshold- limit Values (TLV) for some of these volatile impurities are given in **Table 3** below. The Threshold limit value is the amount of impurity a person can be exposed to every day without any adverse effects on the person's health[118].

TABLE 3. THRESHOLD LIMIT VALUES (TLV)[118]

Alcohol	Amount (ppm)
Ethanol	1000
1-propanol	200
Ethyl acetate	400
1-Butanol	50
Acetaldehyde	100
2-Methyl propanol	50
3-Methyl butan-1-ol	>500
Methanol	200

The health hazards associated with each of these organic compounds are diverse and have been discussed below.

1.6.1. Methanol

Methanol is a clear, colourless, flammable liquid with a mild odour. It is miscible in water and it is a highly inflammable liquid. The primary routes of methanol to the body are inhalation and ingestion but regardless of the exposure, methanol distributes readily and uniformly to all organs and tissues in direct relation to their water content [118-119].

Ingestion of adulterated alcoholic beverages containing methanol has resulted in innumerable loss of human lives throughout the world. For instance Bennett[120] *et al* described a case in which within 5-day period, 323 people consumed bootlegged whiskey contaminated with 35-40% methanol and 41 of them died. Kane[121] *et al* also reported the poisoning of 18 individuals, of which 8 died, when a diluted paint thinner containing approximately 37% (by volume) methanol was used as an alcoholic beverage. Similarly an outbreak of acute methanol intoxication involving 28 young men resulted in all becoming hospitalized within 8-36 h due to acute metabolic acidosis, severe visual impairment and acute pancreatitis. Four of them died within 72 h after hospitalization[122].

Several case studies of methanol poisoning have also been reported. For example, a case study of acute methanol poisoning in a 27-year old man was reported in 1992[123]. The patient exhibited significant neurological and physical impairment, including trauma to the vocal cords and hypophonic along with cognitive defects. Another case of severe methanol poisoning was reported in the same year of a 33-year old man with a history of

alcoholism[124]. He was put on 21 h of dialysis to bring the serum methanol level down to a level non-toxic level.

In the body, methanol is oxidised to formaldehyde and formic acid. The toxicity of methanol is attributed to its metabolic products in the body. Ingestion of methanol in large amounts affects the brain, lungs, gastrointestinal tract, eyes and respiratory system and can cause coma, blindness and eventually death. Prolonged skin contact causes dermatitis and scaling [119].

Acute methanol toxicity evolves in a fairly defined pattern[126]. A toxic exposure results in a transient mild depression of the central nervous system, similar to that of ethanol, but to a much lesser degree. The initial depressant period is followed by an asymptotic latent period which occurs about 8-24 hours after ingestion of the alcohol but may last from several hours to 2 or more days. The latent is followed by a syndrome that consists of an uncompensated metabolic acidosis with superimposed toxicity to the visual system. Physical symptoms typically may include headache, dizziness, nausea and vomiting. This is followed in more severe cases by

abdominal and muscular pain and difficult periodic breathing, which may progress to coma and death usually from respiratory distress. Death may occur if patients are not treated for metabolic acidosis and blindness may result even if treatment for metabolic acidosis is performed[127–128]. The neurotoxic effects of methanol on the visual system can involve transient abnormalities such as peripapillary oedema, optic disc hyperaemia, diminished pupillary reactions to light and central scotomata. Pallor of the optic disc is an end stage sign of irreversible effects of the visual system and may appear 1-2 months after an acute methanol dosage or possibly following chronic occupational exposure to methanol vapour[128].

1.6.2. Propan-1-ol

Propan-1-ol is a highly flammable, volatile, colourless liquid at room temperature and standard pressure. Its odour is described as alcohol-like, sweet and pleasant[128]. Continuous exposure can result in loss of sensitivity to the odour[129]. The compound is completely miscible with water and with most organic solvents.

Propan-1-ol is rapidly absorbed and distributed throughout the body following ingestion[130]. Data on the absorption rate following inhalation and dermal exposure are lacking[132]. It is metabolized by alcohol dehydrogenase (ADH) to propanoic acid via the aldehyde and may enter the tricarboxylic acid cycle[131]. This oxidation is a rate-determining step of propan-1-ol metabolism acid[132]. *In vitro*, rats and rabbits oxidases are also capable of oxidising propan-1-ol to propionic acid. The relative affinity of ADH and the microsomal oxidising systems for propan-1-ol is much higher than that of ethanol; therefore propan-1-ol is rapidly eliminated from the organism[133].

After ingestion, propan-1-ol causes headache, drowsiness, abdominal cramps, gastrointestinal pain, ataxia, nausea, and diarrhoea. Also, eye contact produces irritation while repeated skin contact causes dermatitis. Although the toxicity of propan-1-ol is low, at high concentration it may produce a narcotic effect, as well as eye, nose and throat irritation[134].

There are no reports of adverse effects in the general population or in occupational groups[128]. In the only fatal poisoning reported, it was

recorded that a woman was found unconscious and died 5 hours after ingestion[128]. Autopsy revealed a swollen brain and lung oedema. In another group of 12 volunteers, erythema lasting for at least 60 minutes was observed in 9 individuals following a five minute application of filter papers containing 0.025 mls of a 75% solution of propan-1-ol in water on the fore arms[132].No epidemiological studies are available to assess the long term effects, including the carcinogenicity of propan-1-ol in human beings[132] . Exposure of human beings to 1- propanol may also occur through ingestion of food or beverages containing it.

1.6.3. Acetaldehyde

Acetaldehyde is a colourless, volatile liquid with a pungent, suffocating odour that is fruity in dilute concentrations. The odour threshold for acetaldehyde has been reported to be 0.09 mg/m^3 (0.05 ppm). Acetaldehyde is a metabolic intermediate in humans and in higher plants. It is also a product of alcohol fermentation [135]. Most exposure of people to acetaldehyde occurs through the consumption of alcoholic beverages[131]. These beverages contain ethanol which is metabolised to acetaldehyde by

alcohol dehydrogenase (ADH). Acetaldehyde has also been detected in a wide range of foodstuffs though few quantitative data is available[137-139].

Acetaldehyde is moderately toxic through inhalation and ingestion routes. Ingestion can resort in conjunctivitis, central nervous system depression, eye and skin burns as well as dermatitis. Large doses can be fatal and because of its metabolic link to ethanol, its intoxication consequences are similar to those of chronic ethanol intoxication [136].

The main pathway for the metabolism of acetaldehyde is by rapid metabolism to acetate, which enters the citric acid cycle in an activated form as acetyl-CoA and is metabolized to carbon dioxide and water. Although catalase and other oxidases may contribute to metabolism[140], because of its high affinity, at least 90% of acetaldehyde is oxidised to mitochondrial acetaldehyde dehydrogenase (ALDH)[141] reducing nicotinamide adenine dinucleotide (NAD) to NADH in the process. Acetaldehyde is a highly reactive molecule that can react with many other large or small molecules by addition, condensation or polymerization. The functional groups - NH_2, - OH and -SH in the three-dimensional protein

molecules are susceptible to - CHO attack. Acetaldehyde can therefore bind to protein and haemoglobin to form stable adducts. Such covalent binding probably alters the biological functions of protein and haemoglobin and thus contributes to its toxicity. These pathways may have little quantitative significance in acetaldehyde metabolism, but the by-products may have biological significance [142]. Rats subjected to inhalation of acetaldehyde for 21 days showed the presence of such `bound' adducts in their intracellular medium[143]. A control experiment on unexposed rats, however, showed similar adducts but at low concentrations. This could have probably formed from trace acetaldehyde generated from intestinal microbial fermentation of alcohols[144].

Acetaldehyde is the primary metabolic product of ethanol oxidation. Since the exposure to exogenous acetaldehyde is small, then endogenous acetaldehyde resulting from the metabolism of ingested ethanol is likely to be the most important source of exposure for most people[136].

There is no direct data to link acetaldehyde with liver injury. However, data obtained from animal models and human subjects suggest that

acetaldehyde may play a role in liver damage. On the basis of this indirect evidence, acetaldehyde has been implicated as the putatively toxic metabolite in the induction of alcohol-associated liver damage, facial flushing, and development effects [145].

1.6.4. Ethyl acetate

Ethyl acetate is a volatile liquid with a pleasant fruity odour. It has a pleasant taste when diluted, moderately soluble in water and miscible with most organic solvents. Ethyl acetate vapour forms explosive mixtures with air in the range of 2 to 11.5% by volume in air. The acute toxicity of ethyl acetate is low in test animals. Inhalation of its vapours can cause irritation of the eyes, nose and throat. Exposure to a concentration of 400 to 500 ppm in air may produce mild eye and nose irritation in humans[146]. Its odour threshold is 50 ppm[147]. Ethyl acetate in the alcoholic beverage is brought by a reaction of ethanol (main constituent) and acetyl Co-enzyme A (CoA) molecule. This reaction is thought to be mediated by the yeast during the fermentation process [148]

1.6.5. Amyl alcohols

Amyl alcohols occur in several isomeric forms, most of which are flammable and slightly toxic. Some of the isomers include pentan-1-ol (active sec-amyl alcohol), 3-pentanol, 3-methyl-2-butanol (fermentation amyl alcohol) and 2-methyl-2-butanol. These are colourless liquids with the characteristic odour of alcohol. They are slightly soluble in water and mix readily with most organic solvents. Inhalation of 3-methyl-2-butanol can cause coughing and irritation to the eyes, nose and throat. Ingestion may produce narcosis, headache and dizziness[118]. Cracking of skin may result from the contact. Prolonged contact with amyl alcohol may cause dysuria, nausea and diarrhoea. The toxic effects of other isomers are not known[149]. Based on animal studies, it appears that at high concentrations all these isomers could be narcotic and can cause eye irritation.

1.7.0. Objectives of the study

The cost of industrially produced alcoholic beverages has considerably gone up. Consequently, the demand for locally brewed alcoholic beverages has increased particularly within the liberalised beer market. Most of these local brews are prepared from raw materials of very low quality, some of which are liable to infection by mycotoxins such as aflatoxins. The conditions under which these brews are prepared are also unhygienic. The high consumption of the many varieties of the local brews has led to numerous incidents of fatal intoxication and other related health complications to the consumers. This, therefore, amplified the need to carry out this study.

The objectives of this study were two-fold.

(i) To determine and compare the levels of aflatoxins in different types of local brews and their raw materials.

ii) To identify and determine the concentration of the volatile impurities present in the alcoholic brews

CHAPTER 2

2.0.0. EXPERIMENTAL TECHNIQUES

2.1.0. Cleaning Apparatus

The glassware were cleaned with soapy water and rinsed three times with tap water. They were then soaked overnight in 5% w/v of sodium hypochlorite. The glassware were rinsed with distilled water and then dried at 110 °C in the oven for at least four hours.

The plastic containers were first washed with a detergent, rinsed with tap water and then filled with 3 M nitric acid solution and left over night. The containers were rinsed with distilled water, air-dried for a day and put in polythene bags.

At the end of each analysis, the used glassware were soaked in 5% w/v sodium hypochlorite for at least 24 hours. This was to decontaminate them from aflatoxins by breaking down the coumarin rings. After that an amount of acetone equal to 5% of the total volume of sodium hypochlorite solution used was added to the washing solution and the glassware were soaked in it for a further 1 hour. This was to destroy any carcinogenic

derivative of aflatoxin B_1, B_2, G_1 and G_2 that might have formed as a result of sodium hypochlorite treatment.

2.1.1. Purification of solvents.

The organic solvents used for this work were dichloromethane, chloroform, ethyl acetate, diethyl ether, methanol, hexane, toluene, formic acid, acetic acid, acetone, benzene and acetonitrile. These solvents were of analar grade obtained from Aldrich and Alpha Chemical companies. The solvents used were dried with anhydrous magnesium sulphate, double distilled and stored in 2.5 L bottles. The aqueous solutions were prepared from distilled de-ionised water.

2.1.3. Chemicals and Instrumentation

The silica gel used for chromatography was Merck silica gel 60, size 0.063-0.2 mm, 70 - 230 mesh.

Hyflo - super cell (celite) was used as a filtering aid.

Anhydrous sodium sulphate was dried for 6 hours at 600 °C before use.

The potassium dichromate and sulphuric acid used were of Analar grade.

Aflatoxin standards were purchased from Sigma Chemicals as dry-films

UV Spectrophotometer 20 (Roy Company) was used to determine the concentration of the aflatoxin reference standards.

UV lamp (Wavelength 254 & 366 nm) was used to visualize the sample and the aflatoxin spots on the TLC plates.

TLC was performed on aluminium sheets pre-coated with silica gel F_{254} (Art. 5735). The concentration of aflatoxin in the extract was determined using a TLC densitometer (Model CD Desaga 60). The weighing was done using an Electronic Top balance model AC 100

2.1.4. Calibration of the UV Spectrophotometer.

9 mMol/L of sulphuric acid was prepared by dissolving 1 ml of concentrated sulphuric acid in 2 litres of distilled-deionised water. The following solutions of potassium dichromate were then prepared in 9 mMol/l sulphuric acid.

(a) **0.25 mMol/l potassium dichromate**

This was prepared by weighing accurately 78 mg of potassium dichromate and dissolving it in a litre of 9 mMol/l sulphuric acid.

(b) **0.125 mMol/l potassium dichromate.**

This was prepared by diluting 25 ml of the solution in (a) to 50 ml using 9 mMol/l sulphuric acid.

(c) **0.0625 mMol/l potassium dichromate**

This was prepared by diluting 25 ml of the solution in (b) to 50 ml using 9 mMol/l sulphuric acid. The absorbance (A) of the three standard solutions of potassium dichromate was determined at maximum absorption near 350 nm against 9mMol/l sulphuric acid as the blank. The molar absorption coefficient ($\in$) at each concentration was then calculated using the following equation.

$$\in = \frac{A}{CL}$$

Where

C = concentration in mMol/l

A = absorbance

L = path length in m

The three values of ϵ_i were averaged to obtain ϵ. The correction factor (CF) for the instrument and the cells was determined by substituting in the equation: $CF = 316/\epsilon$, where 316 is the value for ϵ of potassium dichromate (ϵin m^2\mol) .The sensitivity of the analytical microbalance was 0.001 mg.

2.1.5. Preparation of aflatoxin standards

10 mls of benzene: acetonitrile (98:2) was added to the containers of dry aflatoxins B_1, B_2, G_1 and G_2 to obtain stock solutions with concentrations of about 80-100 mg/l. The label statement of the aflatoxin weight was used as a guide. The solutions were agitated for 1 minute on a Vortex shaker and then transferred without rinsing to convenient size glass stoppered flasks.

2.1.6. Determination of aflatoxin concentrations

The UV spectrum of the aflatoxin standard stock solutions was recorded across the wavelength of maximum absorption (330 to 370 nm) against the solvent used for solution in the reference cell. The concentration of the

aflatoxin solutions was determined by measuring the absorbance (A) at the wavelength of maximum absorption at 350 nm and by using the equation

$$\mu g \text{ aflatoxin / ml} = \frac{A \times Mw \times CF}{L\epsilon}$$

Where

CF = correction factor

Mw = Molecular weight

ϵ = Molar absorption coefficient

L = Path length

For the aflatoxins B_1, B_2, G_1 and G_2, the Mw and E values are as follows.

Aflatoxins	Molecular weight (Mw)	ϵvalues (Benzene: Acetonitrile)
B_1	312	1980
B_2	314	2090
G_1	328	1710
G_2	330	1820

To prepare working primary reference standards, aflatoxin solutions of known concentrations were diluted with benzene: acetonitrile (98:2)

solvent system to give 0.5 μg/l for aflatoxins B_1 and G_1 and 0.1 μg/l for aflatoxins B_2 and G_2. The working solutions were transferred to a 1 ml Microflex tube for convenience of storage and dispensing. A mixed standard of aflatoxin B_1, B_2, G_1 and G_2 was also prepared by mixing appropriate volumes of solutions from the stock solutions to obtain same final concentrations in benzene: acetonitrile (98:2) as for the individual working standards. The flasks containing aflatoxin standard solutions were wrapped tightly with aluminium foil to eliminate light and stored at 0 °C.

2.1.7. Sampling

The local brews were randomly sampled from the local bars in the outskirts of Nairobi like in Kibera, Korogocho, Mathare North, Githurai and Kiwanja. Some of the brews were collected directly from the brewers while some samples which were not available in Nairobi were collected from up-country bars like in Embu, Githunguri (Kiambu) and Maragwa (Muranga).

The raw materials were collected from the brewers where possible while others were bought from their likely sources. The brews were collected in

plastic containers while others were bought as packed by the brewer. The raw materials were collected in polythene bags. All the samples collected were wrapped with aluminium foil, then transported to the University and immediately stored in the deep freezer. Extraction of aflatoxins in brews was done immediately. The extracts were stored in vials at 0 °C awaiting further clean-up.

2.2.0. Analytical procedures

2.2.1. Extraction of aflatoxins in the raw materials

50 g of a homogenized sample of the raw materials was weighed in a 500 conical flask using an electronic balance. 20 g of celite was added and mixed well. 250 ml of chloroform and 25 ml of distilled-deionised water was added to the mixture in the conical flask. The mixture was shaken vigorously on a mechanical shaker for 30 minutes. It was then filtered and 100 ml of the filtrate transferred to a 250 ml round bottom flask. This was evaporated nearly to dryness on a rotary evaporator at 50 °C. The residue was redissolved in 5 mls of chloroform and transferred to the column.

2.2.2. Column preparation

A piece of glasswool was put loosely in the bottom of the chromatographic tube. The column was then filled with chloroform to two-thirds of its height. Then 5 g of anhydrous sodium sulphate was added as the base of silica gel. 10 g of silica gel was added followed by 15 g of anhydrous sodium sulphate. Another piece of glasswool was pushed into the column until it reached the anhydrous sulphate on top. The chloroform was drained until the level reached the glasswool at the top. The tap was opened until the flow rate was 1-2 drops per second.

2.2.3. Column clean-up

The chloroform residue was transferred to the prepared column, rinsing the flask twice with 5 ml of chloroform. The chloroform extract mixture was allowed to drain into the adsorbent. 150 mls of hexane was then added to the column and allowed to flow. The eluate was discarded. This was followed by washing the column with 150 mls of diethyl ether. The eluate was also discarded.

Aflatoxins B_1, B_2, G_1, and G_2 were eluted with 150 mls of a mixture of chloroform and methanol (97:3). This fraction was collected in a round-bottomed flask wrapped with aluminium foil. The eluate was evaporated to dryness at 50 °C using a rotary evaporator. The residue was quantitatively transferred to a 5 mls flask by several washing steps using chloroform. This was wrapped with aluminium foil and preserved in the refrigerator to be used for TLC analysis.

2.2.4. Thin layer chromatography

2.2.4.1. TLC plates

The pre-coated TLC plates used in this work were commercially obtained and needed no drying.

For the analysis, a line was scribed 2 cm from the bottom edge of the plate where the volumes were spotted. Two other lines 0.5 cm from each end were scribed to prevent edge effects. The spots were at intervals of 1 cm from each other. The following volumes were applied on the plate using a 10 µl Hamilton syringe; one spot of 2 µl, 5 µl and two spots of 10 µl of the extract. Then one spot of 2 µl, 5 µl, and 10 µl of each of the aflatoxin

working standard solutions was applied on the same plate. A 10 µl aliquot of the mixed reference standard was spotted to check whether adequate resolution was obtained. Then 5 µl of the mixed reference standard was placed on top of one of the 10 µl sample spots to serve as the internal standard. This was done in triplicates.

The spotted plates were developed in the dark in a solvent tank containing a mixture of toluene: ethyl acetate: chloroform and formic acid (35:25:30:10). The plates were allowed to develop until the solvent reached the pre-drawn solvent front (about 45 min). They were then removed from the tank and air dried until all the solvent evaporated and then covered with a clean glass plate. The plates were observed using long-wave ultraviolet light at 366 nm in a dark room. The pattern of the four fluorescent spots of the mixed reference standard was observed. In order of decreasing R_f values, they were B_1, B_2, G_1 and G_2. The fluorescing spots from the sample were examined to identify those having R_f values and similar appearances close as to those of the standards.

Identification of the aflatoxin B_1, B_2, G_1 and G_2 in the extract was done by comparing the fluorescent intensities of the extract spots with those of the standard spots. The concentrations were determined by the use of a densitometer. In the densitometric determination, the sample plates were covered with clean glass plates edged with tape so that the silica gel was not disturbed. The masking tape was used to mark off the lanes on the cover plates so that the spots could be scanned in the direction of long wavelength at excitation wavelength of 365 nm. Standard spots of variable concentrations were scanned in a similar manner and the response was plotted against the concentration for the standard curve preparation and the quantization of the sample. The peak areas for the sample spots that had the same fluorescence were compared with those of the standard spots.

2.2.5. Confirmation of the Identity

The identity of the aflatoxins was confirmed by spraying the plates with 50% sulphuric acid in water. The blue and the green colour of the aflatoxins B and G turned yellow under ultra violet irradiation[92].

2.2.6. Extraction of aflatoxins in alcoholic beverages

2.2.6.1. Opaque Brews

The extraction was done in portions. The opaque-looking brew was decanted to separate the residue from the solution. 50 mls of each of the portion was mixed with 20 g of diatomaceous earth (celite), and 250 mls of ethyl acetate in a 500 ml flask. The mixture was shaken vigorously for at least 15 minutes, filtered under suction and the residue washed with 35 ml of ethyl acetate. The filtrate was transferred to a separatory funnel and allowed to settle. The organic layer was separated from the aqueous layer, dried with anhydrous sodium sulphate and filtered.

The combined filtrate was transferred into a 250 ml round bottom flask. This was evaporated to 2 ml on a rotary evaporator at a maximum temperature of 35 °C. 200 mls of dichloromethane and 20 mls of water were then added to the 2 mls extract, transferred into a 500 mls separating funnel and the mixture shaken for a about a minute. The two layers were separated and the dichloromethane layer filtered through a filter paper covered with anhydrous sodium sulphate. The filtrate was transferred into

a 250 mls flask, concentrated to about 1 ml and kept for further subsequent clean-up.

2.2.6.2. Clear Brews

The method for extracting aflatoxins in the clear brews was similar to that of opaque brews except that the brews were not decanted.

2.2.6.3. Column clean-up

A column length of about 200 mm, internal diameter 15-20 mm was filled with 3 cm of silica gel 60 (about 3 g) and 2 g of sodium sulphate powder. The concentrated extract was transferred into the column and the flask washed with maximum 5 ml chloroform. The column was then washed successfully with 60 mls toluene: acetic acid (9:1), 75 mls diethyl ether: n-hexane (3:1) and the washes discarded.

Aflatoxins were eluted with 100 ml chloroform: methanol: acetone (18:1:1) mixture and the eluate collected into a 250 mls round bottomed flask wrapped with aluminium foil. The eluate was evaporated in a rotary

evaporator to near dryness and transferred into a vial with chloroform rinses. This was kept for TLC analysis.

2.2.6.4. TLC analysis of aflatoxins

The aflatoxin residue from the vial was dissolved in 100 µl of benzene: acetonitrile (98:2). 10 µl aliquot of sample extract and 2,4, 6, 8, and 10 µl aliquots of the mixed reference standards were spotted using a Hamilton syringe on a line 4 cm from the bottom edge of the plate. Another 8 µl of the sample extract was superimposed with 10 µl of the resolution reference standard to act as an internal standard.

The spotted plates were developed in a tank containing toluene: ethyl acetate: chloroform: formic acid (35:25:30:10) as the solvent system until the solvent front had ascended to about 16 cm. The developed plates were air-dried in the hood and observed under long-wave ultraviolet light at 366 nm in a dark room.

2.3.0. DETERMINATION OF VOLATILE IMPURITIES IN ALCOHOLIC BEVERAGES

2.3.1. Instrumentation

A Gas chromatography Varian 3400 coupled with a flame ionization detector was used for this work. This was interphased to an integrator Model Chrom Jet-CH. The GC was programmed and the following parameters were employed for the analysis.

Injector temperature 150 °C

Initial temperature 50 °C

Initial hold time 1 minute

Heating rate 4 °C per min

Final hold time 20 min

Chart speed 0.25 cm per min

Final column temperature 140 °C

Attenuation 128

Detector sensitivity 10^{-11} AUFSD

Head pressure for the carrier gas 37.5 PI

Flow rate 43 ml/min

Stainless steel column packed with 10 % didecyl phthalate on chromosorb.

2.3.2. Sample preparation.

The opaque brews were first centrifuged and then filtered to obtain a clear liquid. The clear brews were injected neat.

2.3.3. Preparation of the standards

The following volatiles of known purity were used as the standards for this work; methanol, acetaldehyde, propan-2-ol, propan-1-ol, ethyl acetate, isobutyl alcohol, butan-2-ol, butan-1-ol, 3-methyl-2-butanol (amyl alcohol) and n-butyl acetate. 10 % ethanol was used as the solvent as most of the brews contained less than 10 % ethanol. 0.1% of each standard was prepared in 10 % ethanol. 5 µl of each sample was injected at a time into the GC using a Hamilton syringe. The retention time of each was noted. Then a 0.1% mixture of all the standards was prepared in 10 % ethanol and 5 µl of the mixture injected into the GC. The peaks were identified with their retention times and the resolution noted. Other concentrations of the mixed standards were prepared in order to obtain a calibration curve.

2.3.4. Analysis of the sample

5 µl of each sample was injected into the GC and the instrument was allowed to run for 20 minutes. This was done in triplicates. After each injection of the sample, a blank (10% ethanol) was injected. This was to make sure that no impurities were retained in the column. This was followed by the injection of the standard. The retention times of the peaks for the sample were compared with those of the authentic samples (standards). This was done so as to identify the volatiles present in the sample. The peak areas of the standards and the samples were compared and used in the quantification.

CHAPTER 3.

3.0.0. RESULTS AND DISCUSSIONS

3.1.0. Aflatoxin concentrations in alcoholic beverages

Twenty-four different alcoholic beverages were analyzed for aflatoxins. The alcoholic beverages were classified into two groups: opaque-looking and clear-looking. The results of the analysis for the brews from different places are tabulated in **table 4** below.

Table 4: Aflatoxin concentration in brews from different sources.

		Aflatoxins (µg/l)		
Alcoholic Beverage	**Source**	**AFB_1**	**AFB_2**	**AFG_1**
Kibuku root (clear)	Githurai	0.61	ND	ND
"	Mathare North	0.34	ND	ND
"	Kiwanja	0.44	ND	ND
Kibuku Sake (opaque)	Githurai	3.57	ND	0.76
"	Mathare North	1.82	ND	ND
"	Kiwanja	1.18	ND	0.93
Sorghum (opaque)	Kibera	21.89	0.75	ND
"	Mathare North	12.17	ND	ND
"	Githurai	17.90	ND	ND
Nyati (opaque)	Maragwa	1.97	2.47	ND
"	Kibera	2.11	1.09	ND
"	Githunguri	1.26	ND	ND

Table 4 continued...

Mkomboti (opaque)	Kibera	36.24	ND	12.52
"	Korogocho	49.40	ND	6.12
Macore (clear)	Embu	39.16	ND	14.63
Busaa (opaque)	Githurai*	39.59	ND	23.56
"	Githurai**	27.16	ND	3.81
"	Githurai***	17.27	6.72	5.26
Busaa (opaque)	Kibera*	60.47	ND	ND
"	Kibera**	44.86	ND	ND
"	Kibera***	52.08	ND	ND
Ndume (opaque)	Githunguri	18.15	ND	ND

Key

***, ** and *** indicate different brewers from the same locality.**
ND means that no aflatoxin was detected

The most common aflatoxin detected in the alcoholic brews was B_1, followed by G_1 then B_2. Busaa, a common local brew prepared from fermented maize flour had the highest concentration of aflatoxin B_1 (52.47 µg/l) on average followed by mkomboti (42.82 µg/l) also an opaque brew prepared from fermented maize flour. The concentration of aflatoxin B_1 in busaa samples collected from different brewers were 39.59 µg/l (Githurai), 27.16 µg/l (Githurai), 17.27 µg/l (Githurai), 60.47 µg/l (Kibera), 44.86 µg/l (Kibera) and 52.08 µg/l (Kibera). A difference in concentration of AFB_1 in busaa samples

collected from brewers of the same locality was noted. This difference in concentration of AFB_1 was attributed to the different methods of brewing employed and the quality of the raw materials used by the brewers. Only one busaa sample collected from Githurai had aflatoxin B_2 (6.72 µg/l) while aflatoxin G_1 was detected in the samples collected from Githurai with a concentration of 23.56 µg/l, 3.81 µg/l and 5.26 µg/l. Neither aflatoxin B_2 nor G_1 was detected in the busaa samples collected from Kibera. The average concentrations of aflatoxin B_1, B_2, and G_1 are illustrated in **figures 2-4**.

Figure 2: Average concentration of aflatoxin B_1 in alcoholic beverages

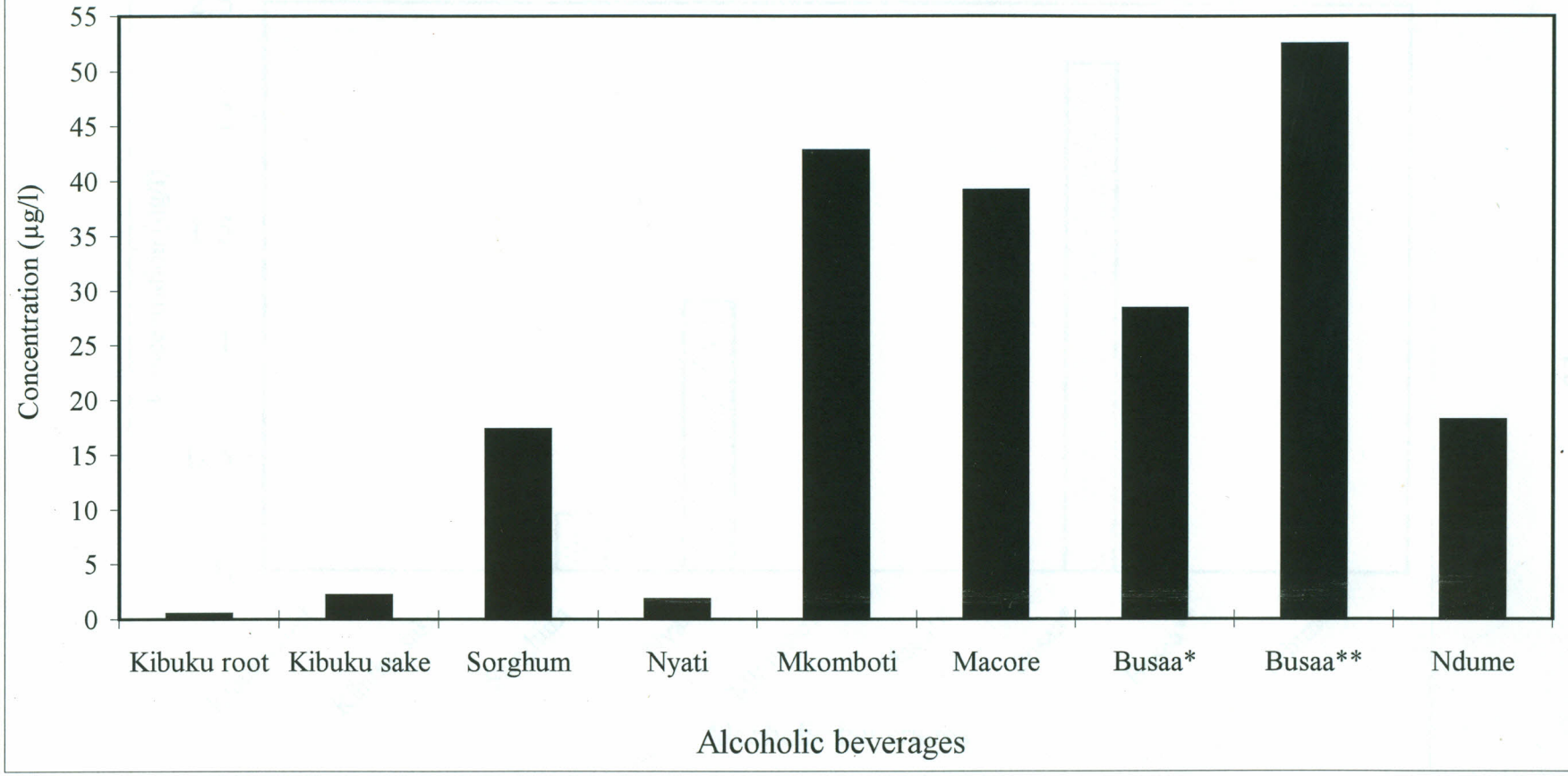


Figure 3: Average concentration of aflatoxin B_2 in alcoholic beverages.

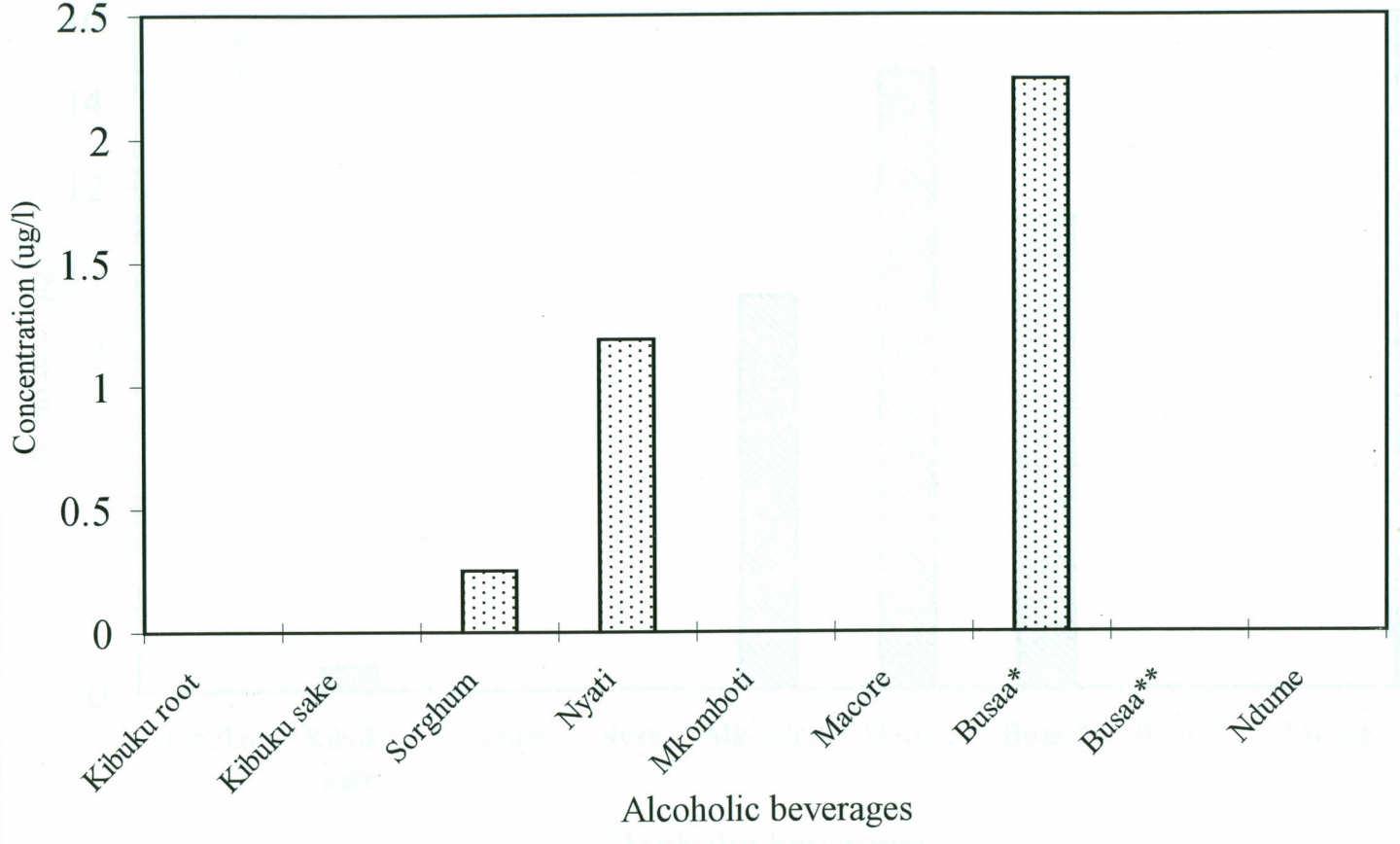
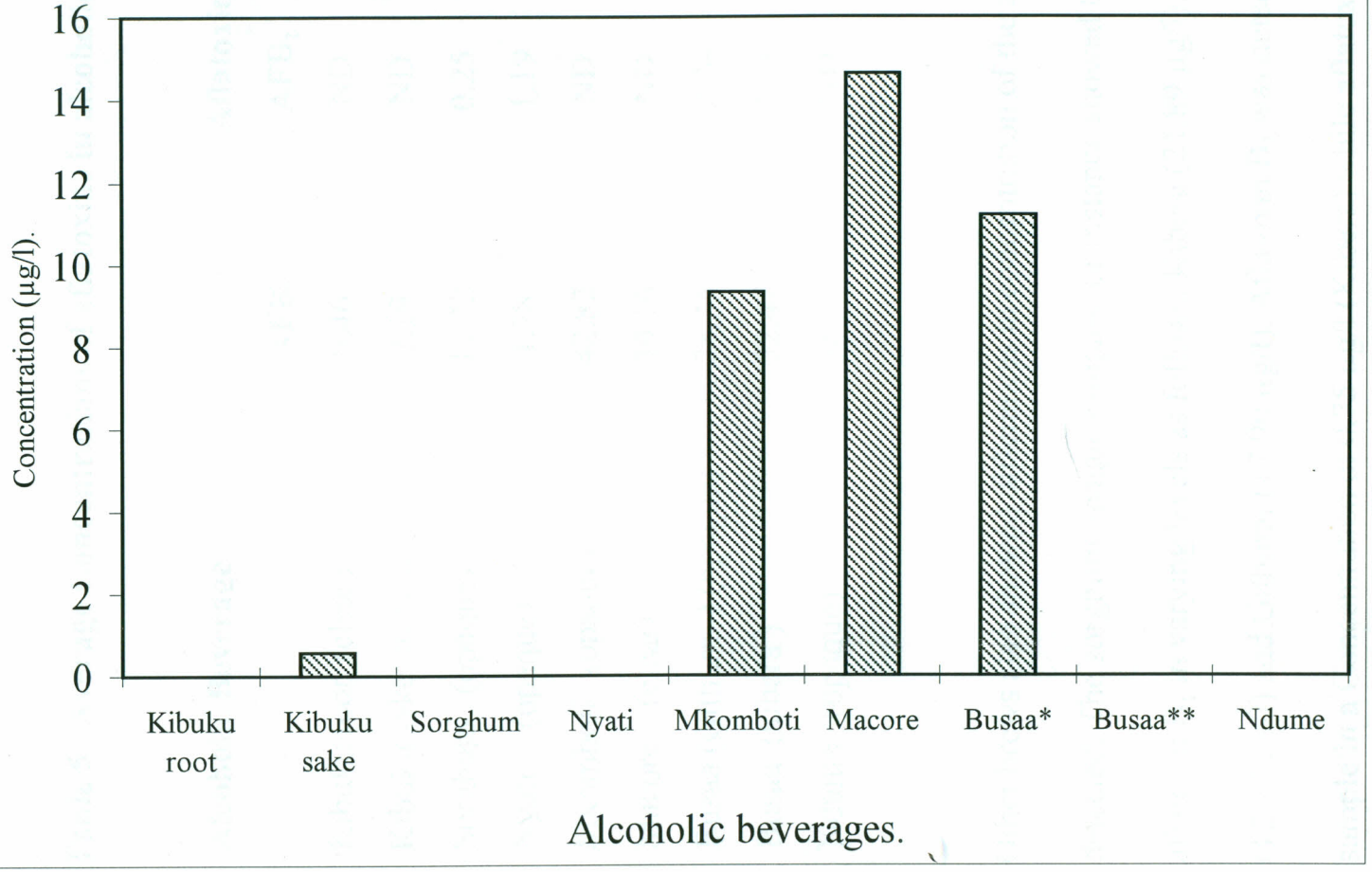


Figure 4: Average concentration of aflatoxin G_1 in alcoholic beverages.



The average aflatoxin concentrations for the alcoholic beverages produced by the same brewer are tabulated in **table 5**.

Table 5. Average Concentrations of aflatoxins in alcoholic beverages

Alcoholic Beverage	Aflatoxin (µg/l)		
	AFB_1	AFB_2	AFG_1
Kibuku root (clear)	0.46	ND	ND
Kibuku sake (opaque)	2.19	ND	0.56
Sorghum (opaque)	17.32	0.25	ND
Nyati (opaque)	1.78	1.19	ND
Mkomboti (opaque)	42.82	ND	9.32
Macore (clear)	39.16	ND	14.63
Busaa (Githurai) (opaque)	28.34	2.24	11.21
Busaa (kibera) "	52.47	ND	ND
Ndume (opaque)	18.15	ND	ND

Other brews also showed differences in concentration of the aflatoxins detected. The sorghum opaque samples for instance showed the presence of aflatoxin B_1 in varying levels as follows; Kibera (21.89 µg/l), Mathare North (12.17 µg/l) and Githurai (17.90 µg/l). Aflatoxin B_2 was detected in only one sample in a Concentration of 0.75 µg/l (Kibera) while aflatoxin G_1 was not detected in any of the sorghum opaque samples. Kibuku sake also showed the

presence of aflatoxins in varying levels; Githurai (3.57 µg/l), Mathare North (1.82 µg/l) and Kiwanja (1.18 µg/l). No AFB_2 was detected while AFG_1 was detected in only two samples with concentrations of 0.76 µg/l (Githurai) and 0.93 µg/l (Kiwanja).

Other opaque-looking brews such as nyati, mkomboti, and ndume showed the presence of aflatoxin B_1 in the following concentrations: nyati (1.97 µg/l, 1.26 µg/l and 2.11 µg/l), mkomboti (36.24 µg/l and 49.40 µg/l), and ndume (18.15 µg/l). Only in nyati brew was aflatoxin B_2 detected in a concentration of 2.47µg/l. Aflatoxin G_1 was detected only in mkomboti in a concentration of 12.52 µg/l, and 6.12 µg/l. No aflatoxin G_2 was detected in all the different alcoholic brews analyzed.

Out of 15 clear brews analyzed, only kibuku *root* and macore showed the presence of aflatoxin B_1. The concentrations were as follows; kibuku root (0.61 µg/l, 0.34 µg/l and 0.44 µg/l) and macore (39.16 µg/l). Aflatoxin $G_1$was also detected in macore in a concentration of 14.63 µg/l. The clear brews that showed absence of aflatoxins were kulta special, kibuku tarzan, medusa, nyuki, karubu from Embu, kiboko punch, chang'aa, karubu from Kiwanja, simba wine, afriwine, paris alcoholic, sun wine, pineapple wine, and vienna

wine. Chang'aa, a distilled brew from busaa did not have any of the aflatoxins. The reason given for this observation was that aflatoxins are not volatile and thus could not be retained in the final product even if the raw materials were contaminated with aflatoxins. It is worth noting that some of the alcoholic beverages were only found in certain areas and it was difficult to get many samples as they were sold in "hideout" places.

Commercially available beers like tusker, pilsner, citizen, guiness and tusker malt lager showed no aflatoxins. The absence of aflatoxins may be attributed to the fact that these beers are prepared from raw materials of high quality, which are dried to a moisture content that does not encourage the growth of the mould. The storage facilities of the raw materials were found to be well built, aerated and free from moisture. These conditions discourage the growth of the fungi and as such the products prepared from these raw materials are not expected to be contaminated with aflatoxins.

The source of the aflatoxins detected in the alcoholic beverages was generally attributed to raw materials used for preparing the brews. Most brewers prepare alcoholic beverages from low quality raw materials that have been rejected for human consumption. This is with an aim to minimise the cost of

production. These raw materials are normally mouldy thus providing suitable conditions for the fungi to grow and produce aflatoxins. It was also noted that most of the local brewers prepare their brews on and off within some simple makeshift `breweries'. This method of preparation pays no attention to the critical issue of quality control either for the raw materials used or for the end product. Furthermore, the brewers did not have appropriate storage facilities for the raw materials, a factor that was found to encourage the growth of mould on the raw materials, thus the high production of aflatoxins.

From the tabulated results, it is clear that opaque-looking brews had the highest concentrations of aflatoxins as compared to the clear-looking brews. These brews were qualitatively similar to fermented porridge and contained a lot of suspended solids that would have retained the aflatoxins[77]. Another factor that could explain the differences observed in the aflatoxin concentration in different brews is the dilution factor. As a way of increasing the volume of the already prepared brew, it is normally diluted with a certain volume of water. The higher the volume of the water used for the dilution, the higher the dilution factor and the lower the concentration of the aflatoxin present.

The presence of aflatoxins in the alcoholic beverages poses a great danger to the consumer as their effects are cumulative and may take a long time before they are recognised. The real danger must be seen in the context of their extreme carcinogenic potency, especially for aflatoxin B_1. For instance, a dose of 1 ppb of this aflatoxin is carcinogenic[150], and also a dose of 0.1 ppb was found to produce tumour in rats when fed for 20 months[151]. It is important therefore for the brewers to ensure that high quality raw materials are used in brewing. This would in turn reduce the numerous cases of fatal intoxication caused by drinking these contaminated brews.

3.2.0. Aflatoxin concentrations in raw materials

Only 17 samples of the raw materials were analyzed for the aflatoxins. The reason being that it was extremely difficult to collect the raw materials from the brewers as they feared being intimidated. Some of the brewing sites could not be traced, as most local brews were prepared in "makeshift" breweries. It was also difficult to establish the likely sources of the raw materials used by these brewers. However, the raw materials analyzed showed the presence of the aflatoxins. The results of the analysis are tabulated in **tables 6-8** .The aflatoxin concentration variations are also presented in **figures 5-7**.

Table 6. Aflatoxin concentration in raw materials obtained from brewers in Kibera

Raw material	Aflatoxin (µg/Kg)		
	AFB_1	AFB_2	AFG_1
Brown sorghum	70.92	56.44	ND
Malted brown sorghum	5.38	22.81	ND
Finger millet	43.72	ND	ND
Maize flour	169.40	ND	45.18
Fermented maize flour	68.70	ND	ND

Table 7. Aflatoxin concentration in raw materials obtained from brewers in Githurai

Raw material	Aflatoxin (µg/Kg)		
	AFB_1	AFB_2	AFG_1
Brown sorghum	52.38	19.71	ND
Finger millet	38.90	11.32	ND
White maize flour	110.72	ND	18.72
Fermented maize flour	54.61	ND	ND

Table 8. Aflatoxin concentration in raw materials obtained from brewers in Korogocho

Raw material	Aflatoxin (µg/Kg)		
	AFB_1	AFB_2	AFG_1
Brown sorghum	45.77	28.72	ND
Finger millet	31.14	ND	ND
White maize flour	156.87	17.45	10.79
Fermented maize flour	62.47	ND	ND

Figure 5: Aflatoxin concentration in raw materials from a brewer in Kibera.

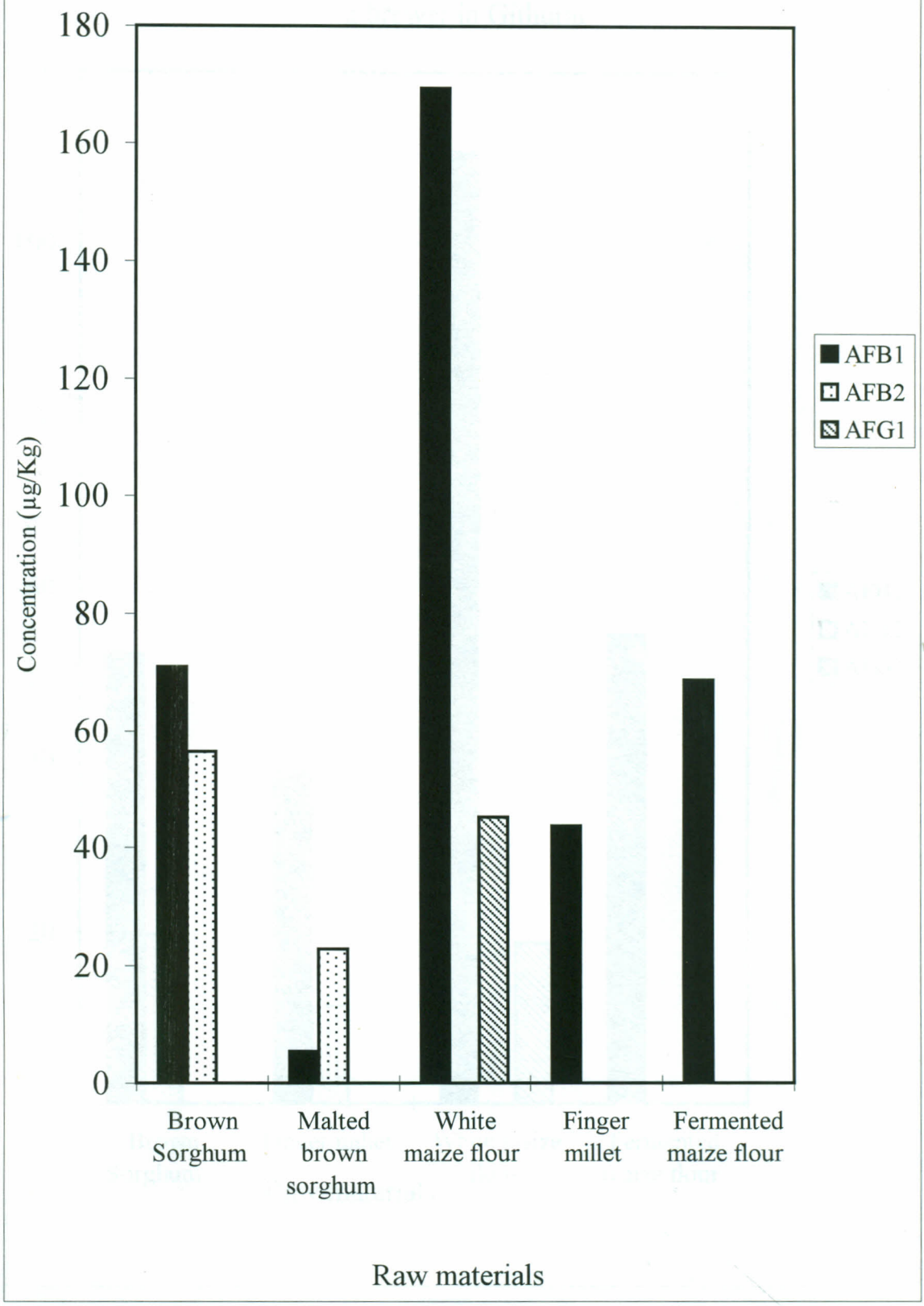


Figure 6: Aflatoxin concentration in raw materials from a brewer in Githurai

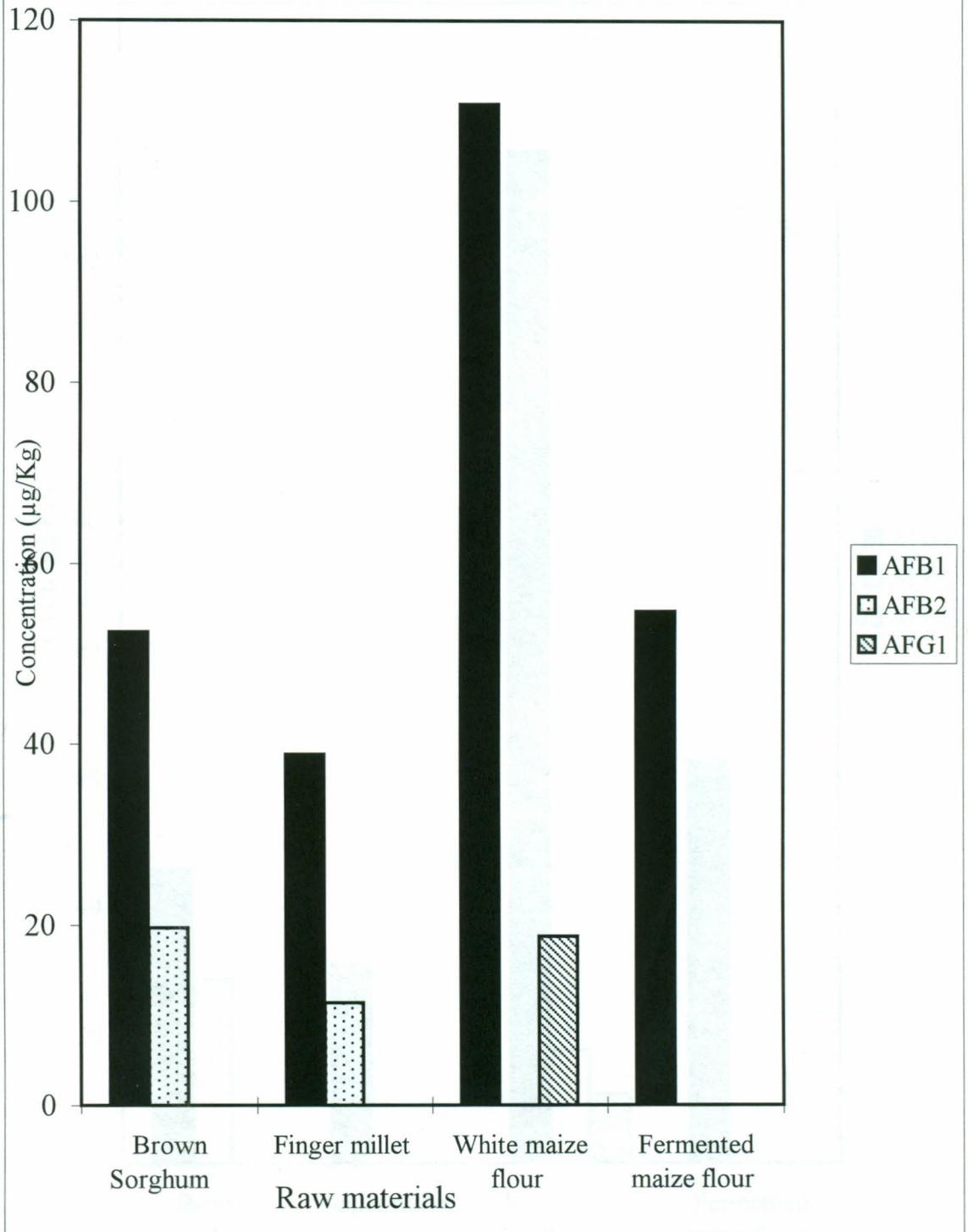


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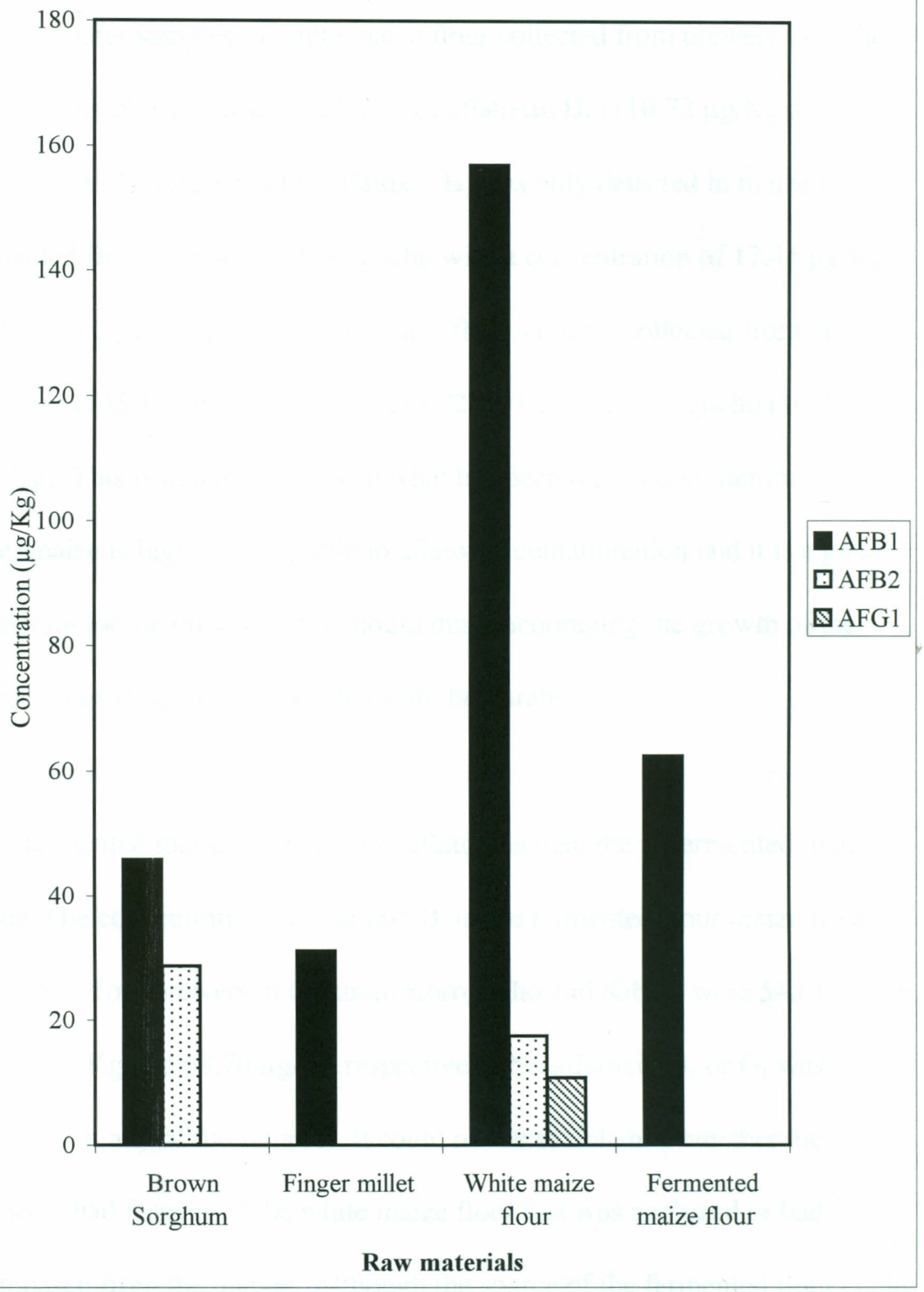
Figure 7: Aflatoxin concentration in raw material from a brewer in Korogocho



The highest concentration of the aflatoxin determined was that of aflatoxin B_1 (169.40 µg/Kg) found in white maize flour collected from a busaa brewer in kibera. Other samples of white maize flour collected from brewers in Githurai and Korogocho also had high levels of aflatoxin B_1 (110.72 µg/Kg and 156.87 µg/Kg) respectively. Aflatoxin B_2 was only detected in maize flour collected from a brewer in Korogocho with a concentration of 17.45 µg/Kg. Aflatoxin G_1 was detected in the maize flour samples collected from brewers in Kibera (45.18 µg/Kg), Githurai (18.72 µg/Kg) and Korogocho (10.79 µg/Kg). This is in agreement with what has been reported in literature[18-19] that, maize is highly susceptible to aflatoxin contamination and it is a good substrate for the growth of the mould thus encouraging the growth of the *Aspergillus flavus* if the conditions are favourable.

The fermented maize flour had less aflatoxins than the unfermented maize flour. The concentration of aflatoxin B_1 in the fermented flour maize flour collected from brewers in Githurai, Korogocho and Kibera were 54.61 µg/Kg, 62.47 µg/Kg and 68.70 µg/Kg respectively. No aflatoxin B_2 or G_1 was detected in any of the samples. It could not be established whether the brewers had fermented the white maize flour that was analyzed or had purchased from the market. Although the source of the fermented flour could

have been different, the results still showed lower values. This difference in concentration between the fermented and the unfermented flour was attributed to the fermentation process. It was noted also that some brewers buy the already fermented flour from the market to avoid waste of time and to reduce the cost of production. This was found to be detrimental, as the source of the flour could not be easily established. It was possible that the flour was prepared from low quality maize that was contaminated with aflatoxins as was evidenced from its price.

The sorghum and millet samples collected from different brewers had lower aflatoxin levels than maize flour. The concentrations of aflatoxin B_1 detected in brown sorghum collected from brewers were 70.92 µg/Kg (kibera), 52.38 µg/Kg(Githurai) and 45.77 µg/Kg (Korogocho). The concentrations of aflatoxin B_2 in the same samples were 56.44 µg/Kg (Kibera), 28.72 µg/Kg (Korogocho) and 19.71 µg/Kg (Githurai). Aflatoxin G_1 was not detected in any of the brown sorghum samples. Only one sample of malted brown sorghum was analyzed for aflatoxins and was found to contain aflatoxin B_1 and B_2 in a concentration of 5.38 µg/Kg and 22.81 µg/Kg respectively. The lower concentration of aflatoxins may be attributed to the germination process that could have degraded the aflatoxins. The concentration aflatoxin

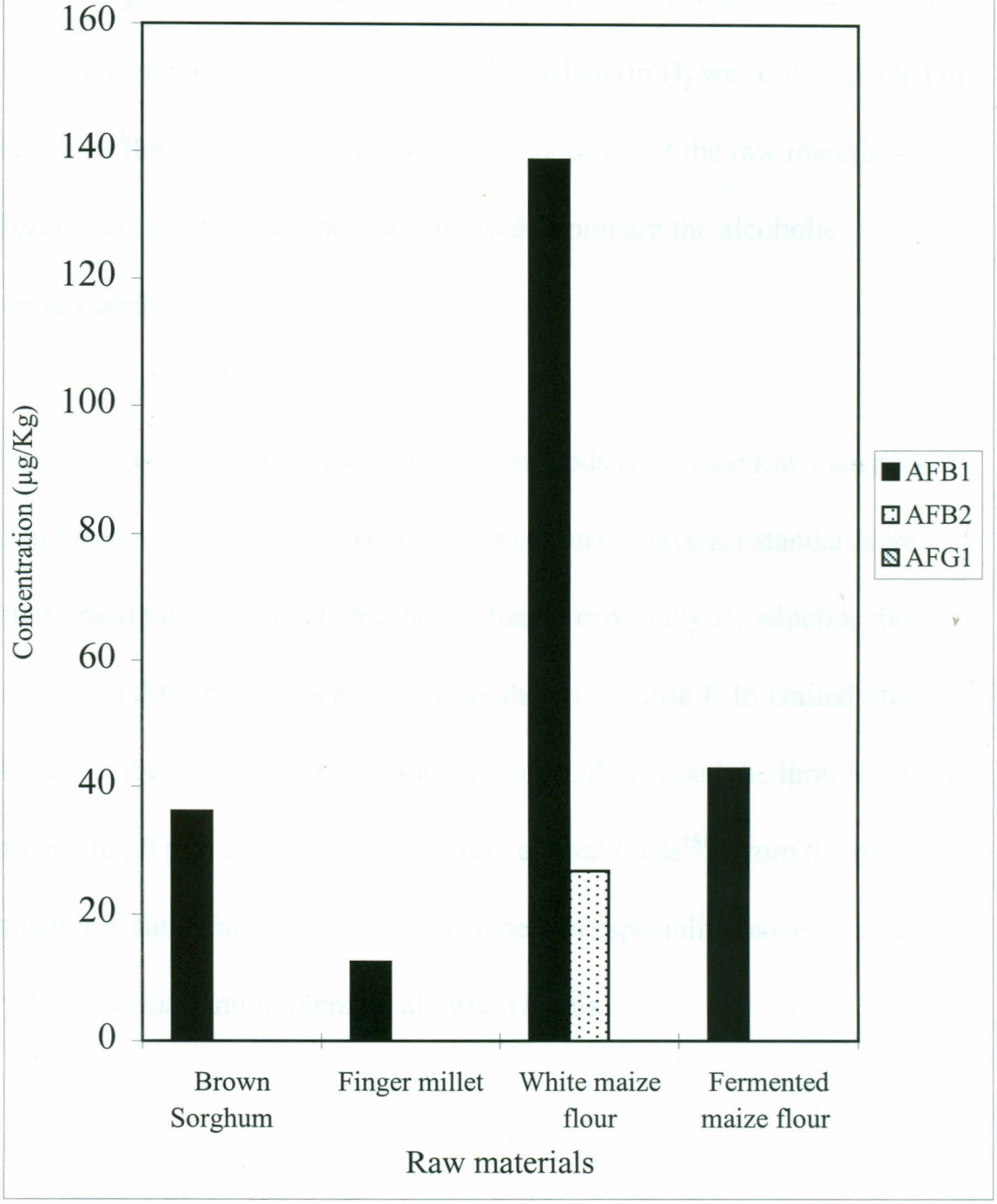
B_1 in finger millet obtained from brewers was found to be 43.72 µg/Kg (Kibera), 38.90 µg/Kg (Githurai) and 31.14 µg/Kg (Korogocho). Aflatoxin B_2 was only detected in a sample of finger millet obtained from a brewer in Githurai in a concentration of 11.32 µg/Kg. Neither aflatoxin G_1 nor G_2 was detected in finger millet. This is actually in agreement with what has been reported in literature that small grains such as sorghum, millet, oats, rye, barley and rice unless poorly handled in storage, they are less susceptible to aflatoxin contamination than are large grains such as maize[19].

The average of aflatoxin concentrations in raw materials obtained from the open-air markets in Githurai, Kibera and Korogocho are tabulated in **table 9** and illustrated in **figure 8.**

Table 9. Average concentration of aflatoxin in raw materials obtained from open-air markets in Nairobi

	Aflatoxin (µg/Kg)		
Raw material	AFB_1	AFB_2	AFG_1
Brown sorghum	36.12	ND	ND
Finger millet	12.17	ND	ND
White maize flour	138.71	26.79	ND
Fermented maize flour	42.84	ND	ND

Figure 8: Average aflatoxin concentrations in raw materials obtained from open-air markets in Nairobi.



The highest average concentration detected was of aflatoxin B_1 detected in white maize flour (138.71 μg/Kg). The average concentrations of aflatoxin B_1 in brown sorghum, millet and the maize fermented flour were 36.12 μg/Kg, 12.47 μg/Kg and 42.84 μg/Kg respectively. Aflatoxin B_2 was only detected in white maize flour (26.79 μg/Kg). It is worth noting that the raw materials analyzed were not the ones necessarily used to prepare the alcoholic beverages analyzed in this work.

There are no set limits for aflatoxin levels in foods and in the raw materials used in brewing in Kenya. However, other countries have set standards for the maximum tolerated levels for the aflatoxins especially B_1 which is the most toxic in different foods and feeds as shown in **table 1**. In United States for instance, the Food and Drug Association (FDA) revised the limit levels of aflatoxins to 20 ppb for all foods including animal feeds[151]. From the results obtained it is clear that most of the raw materials especially maize flour had more than the maximum tolerated aflatoxin levels.

3.3.0. Volatile impurities in the alcoholic beverages

The results for the analysis of the volatile impurities in the alcoholic beverages are tabulated in **table 10**. These results have also been presented in form of bar graphs in **figure 10.** The analysis showed the presence of n-propanol in almost all the alcoholic beverages. The highest (84.88 ppm) and the lowest (4.50 ppm) concentration of n-propanol were detected in afriwine and in karubu (from Embu) respectively. The concentrations of n-propanol in the clear looking brews were as follows; kulta special (54.70 ppm), kibuku tarzan (67.01 ppm), kibuku root (70.70 ppm), vienna wine (82.24 ppm), simba wine (70.68 ppm), medusa (15.33 ppm), pineapple wine (62.81 ppm), sun wine (15.70 ppm), nyuki (66.07 ppm), paris alcoholic (61.90 ppm), karubu from Kiwanja (16.68 ppm) and macore from Embu (12.94 ppm). In the opaque looking the concentrations were : kibuku sake (56.35 ppm), sorghum opaque (10.58 ppm), nyati (47.53 ppm), busaa (4.62 ppm), mkomboti (5.21 ppm) and ndume (11.54 ppm).

The origin of n-propanol in the alcoholic beverages is attributed to the fermentation process. It is normally synthesised from the oxo-acids produced by the deamination of the amino acids. These oxo-acids are decarboxylated to yield carbon dioxide and an aldehyde. The aldehyde is then reduced to an alcohol.

Table 10. Volatile impurities in alcoholic beverages (ppm)

Alcoholic Beverage	Propanol	Ethyl Acetate	Methanol	Butanol	Isobutyl Alcohol	Amyl Alcohol
Kulta Special*	54.70	14.68	ND	ND	ND	ND
Afriwine*	84.88	8.19	ND	ND	ND	ND
Kibuku Tarzan*	67.01	9.39	ND	ND	ND	ND
Kibuku Root*	70.70	ND	ND	ND	ND	ND
Vienna Wine*	82.24	ND	ND	ND	ND	ND
Simba Wine*	70.68	ND	ND	ND	ND	ND
Medusa*	15.33	18.12	ND	ND	164.17	287.38
Pineapple Wine*	62.81	ND	1798.70	ND	ND	ND
Sun Wine*	15.70	44.94	ND	ND	114.48	170.74
Nyati	47.53	42.58	ND	ND	38.18	118.61
Chang'aa*	ND	132.86	ND	7.54	147.64	392.55
Munazi*(Mombasa)	ND	1176.44	ND	ND	18.61	ND
Nyuki*	66.07	ND	ND	ND	ND	ND
Kibuku Sake	56.35	11.29	ND	ND	11.16	ND
Sorghum Opaque	10.58	46.44	ND	ND	13.40	69.11
Karubu* (Kiwanja)	16.68	81.04	ND	ND	13.60	50.30
Mkomboti	5.21	27.40	ND	ND	9.86	32.52
Kiboko Punch*	ND	59.5	ND	ND	ND	ND
Paris Alcoholic*	61.90	7.59	ND	ND	ND	ND
Karubu* (Embu)	4.50	13.08	ND	ND	20.51	99.86
Macore*	12.94	32.46	17.64	ND	55.67	133.93
Busaa	4.62	ND	1.37	ND	10.58	138.75
Ndume	11.54	ND	1.74	ND	4.49	34.63

ND means no volatile impurity was detected and * indicates clear brews.

Ethyl acetate was also detected as an impurity in varying concentrations. The highest concentration (1176.44 ppm) was found in munazi from Mombasa and the lowest concentration (7.59 ppm) was found in paris alcoholic wine. In other clear-looking brews, the concentration detected were kulta special (14.68 ppm), afriwine (8.19 ppm), kibuku tarzan (9.39 ppm), medusa (18.12 ppm), sun wine (44.94 ppm), *chang'aa* (132.86 ppm), karubu from Kiwanja (81.04 ppm), kiboko punch (59.50 ppm), karubu from Embu (13.08 ppm) and macore from Embu (32.46 ppm). The concentrations of ethyl acetate in the opaque-looking brews were as follows; kibuku sake (11.29 ppm), sorghum opaque (46.44 ppm), nyati (42.58 ppm) and mkomboti (27.40 ppm).

The amount of ethyl acetate present in the brew depends on the amount of oxygen present during the fermentation process. Oxygen deficiency results in poor fermentation and probably results in an increased level of acetyl coenzyme A in the yeast cells. This in turn produces an elevated amount of ethyl acetate. Also the concentration of ethanol in the brew may determine the amount of the ester present. The higher the ethanol content in the brews, the higher the concentration of the ethyl acetate obtained. For example, chang'aa, an illicit local

Figure 9: Amount of volatile impurities in different alcoholic brews.

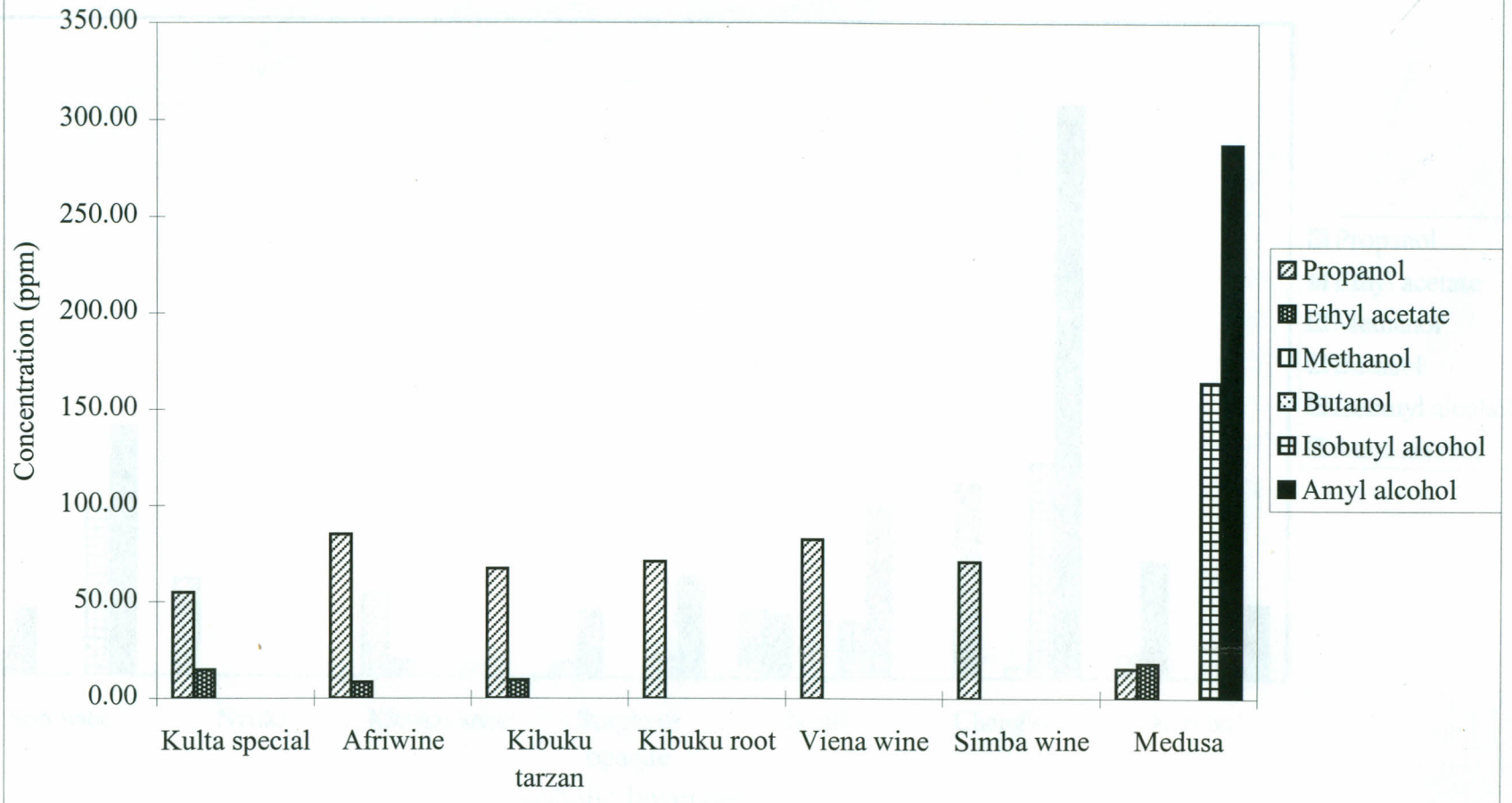


Figure 9: Continued

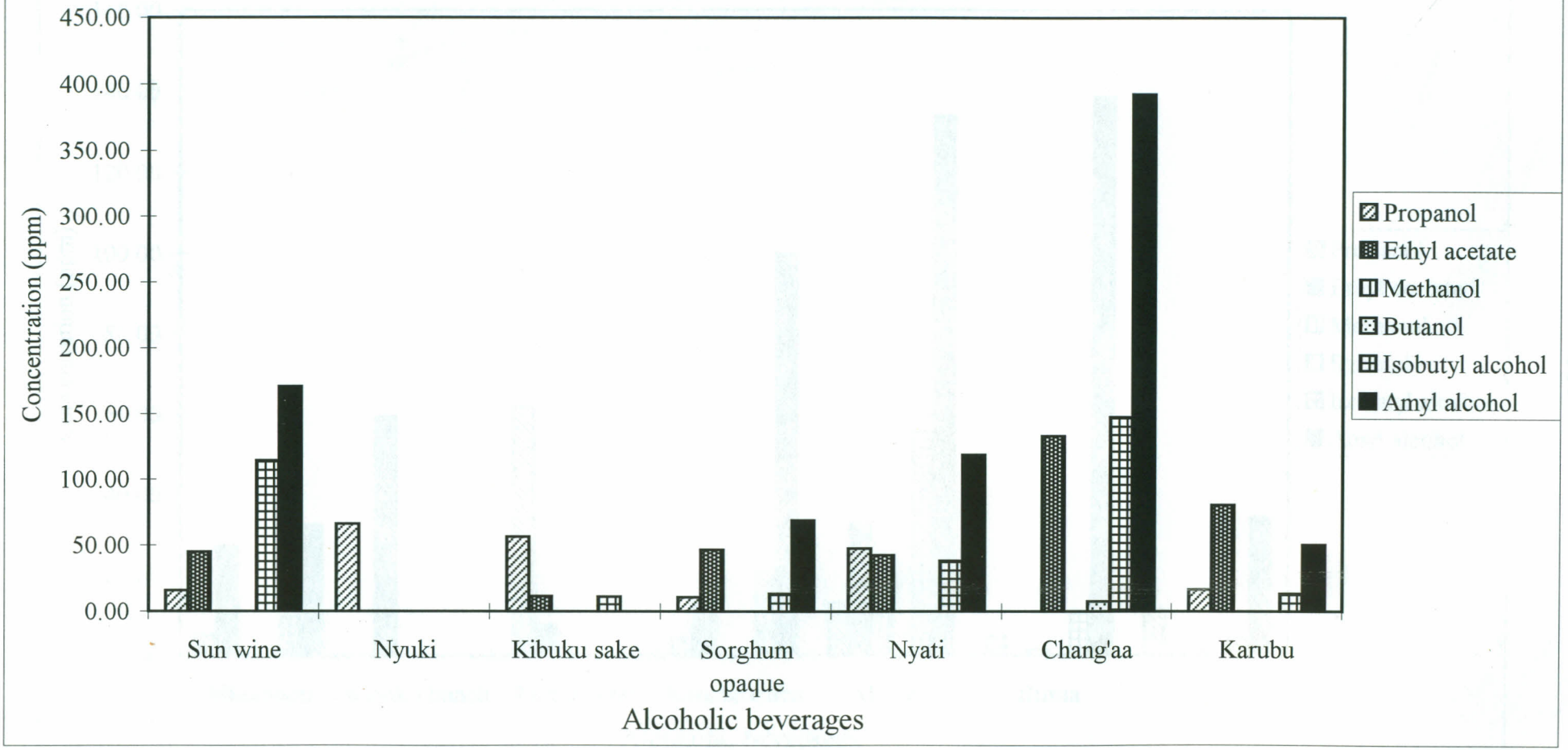


Figure 9: Continued

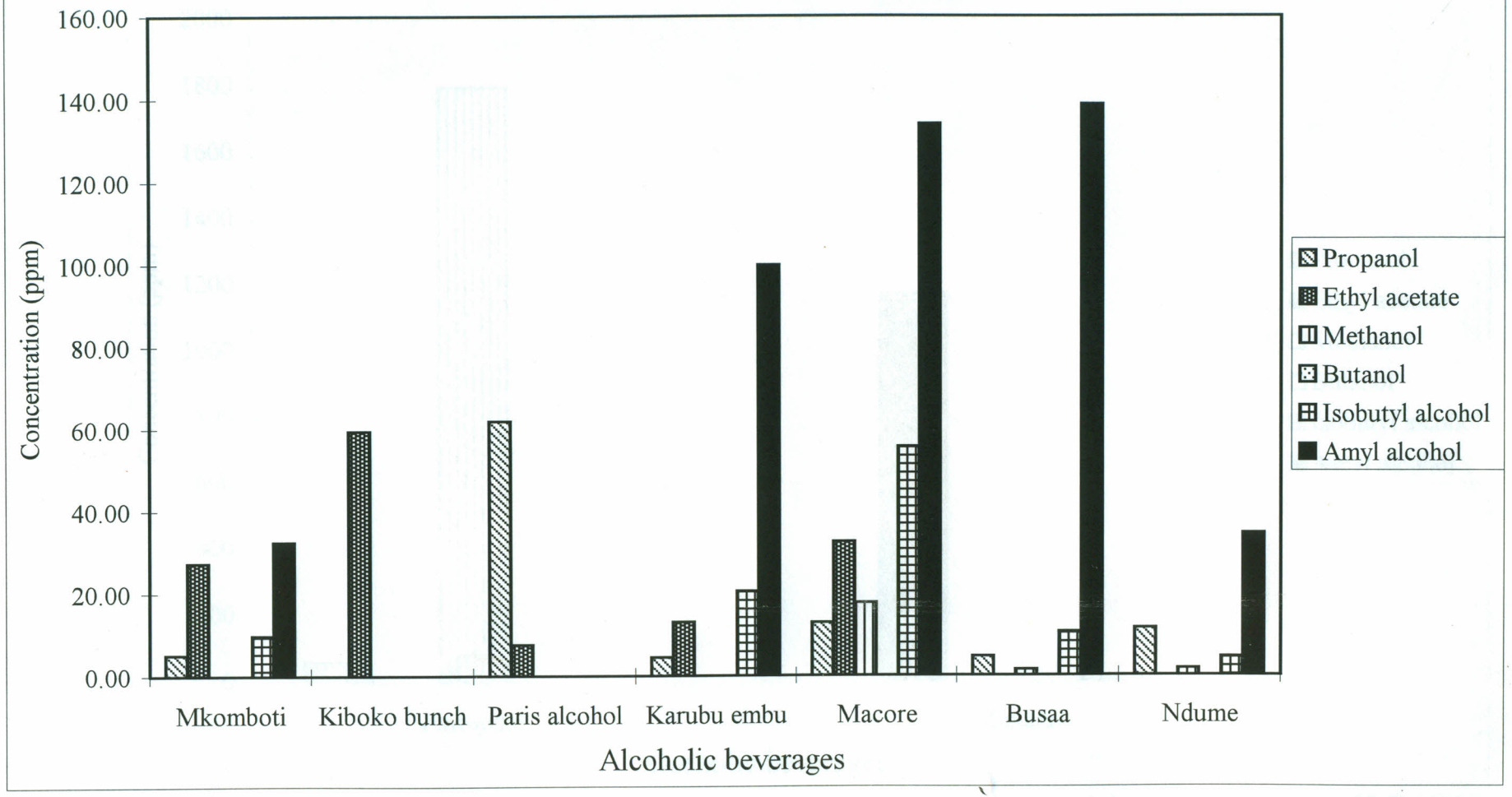
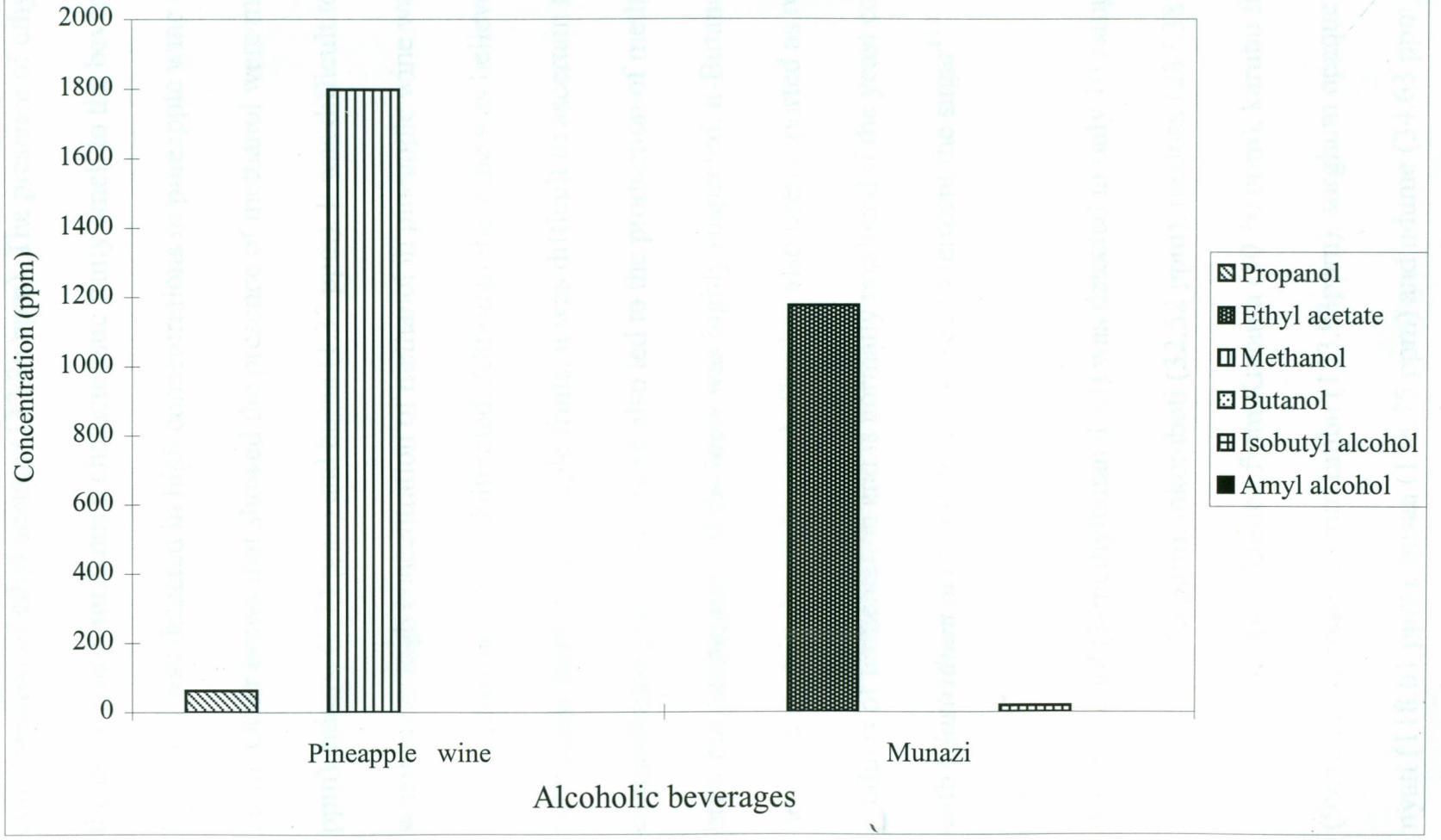


Figure 9: Continued



spirit had a high level of alcohol content and as a consequence showed a high concentration of ethyl acetate (132.86 ppm). The presence of ethyl acetate in the brew also contributes a characteristic fruity smell to the beverage. Methanol was detected in high concentrations in pineapple wine (1798.70 ppm). Other brews that showed the presence of methanol were macore (17.64 ppm), ndume (1.74 ppm) and busaa (1.37 ppm). It was difficult to establish whether the high concentration of methanol in pineapple wine was a result of fermentation or it was adulterated. Although the wine was believed to have been made from the pineapple fruits, it was difficult to ascertain this. Use of contaminated yeast could have also led to the production of methanol in this brew but adulteration of the brew was highly suspected. n-Butanol was only detected in chang'aa (7.54 ppm). This has also been reported as one of the by-products of fermentation and is normally produced if the yeast contaminated with *Clostridium acetobutylicum* is used to ferment the sugar[148].

Amyl alcohol (3-methylbutan-1-ol) was detected in only 10 samples namely chang'aa (392.55 ppm), mkomboti (32.52 ppm), medusa (287.38 ppm), sun wine (170.74 ppm), karubu from Kiwanja (50.30 ppm), karubu from Embu (99.86 ppm), macore from Embu (133.93ppm), sorghum opaque (69.11 ppm), nyati (118.61 ppm), busaa (138.75 ppm) and ndume (34.63 ppm). Amyl

alcohol is also a by-product of fermentation and is synthesized from oxo acids just like n-propanol.

Isobutyl alcohol (2-methyl propanol) was found in 13 brews. The highest concentration was recorded in medusa (164.17 ppm) and the lowest in ndume (4.49 ppm). The concentration in other brews were as follows; sun wine (114.48 ppm), chang'aa (147.64 ppm), munazi (18.61 ppm), karubu from Kiwanja (13.60 ppm), karubu from Embu (20.51 ppm), macore (55.67 ppm), kibuku sake (11.16 ppm), sorghum opaque (13.40 ppm), nyati (38.18 ppm), mkomboti (9.86 ppm), busaa (10.58 ppm) and ndume (4.49 ppm). Isobutyl alcohol is also a by-product of fermentation.

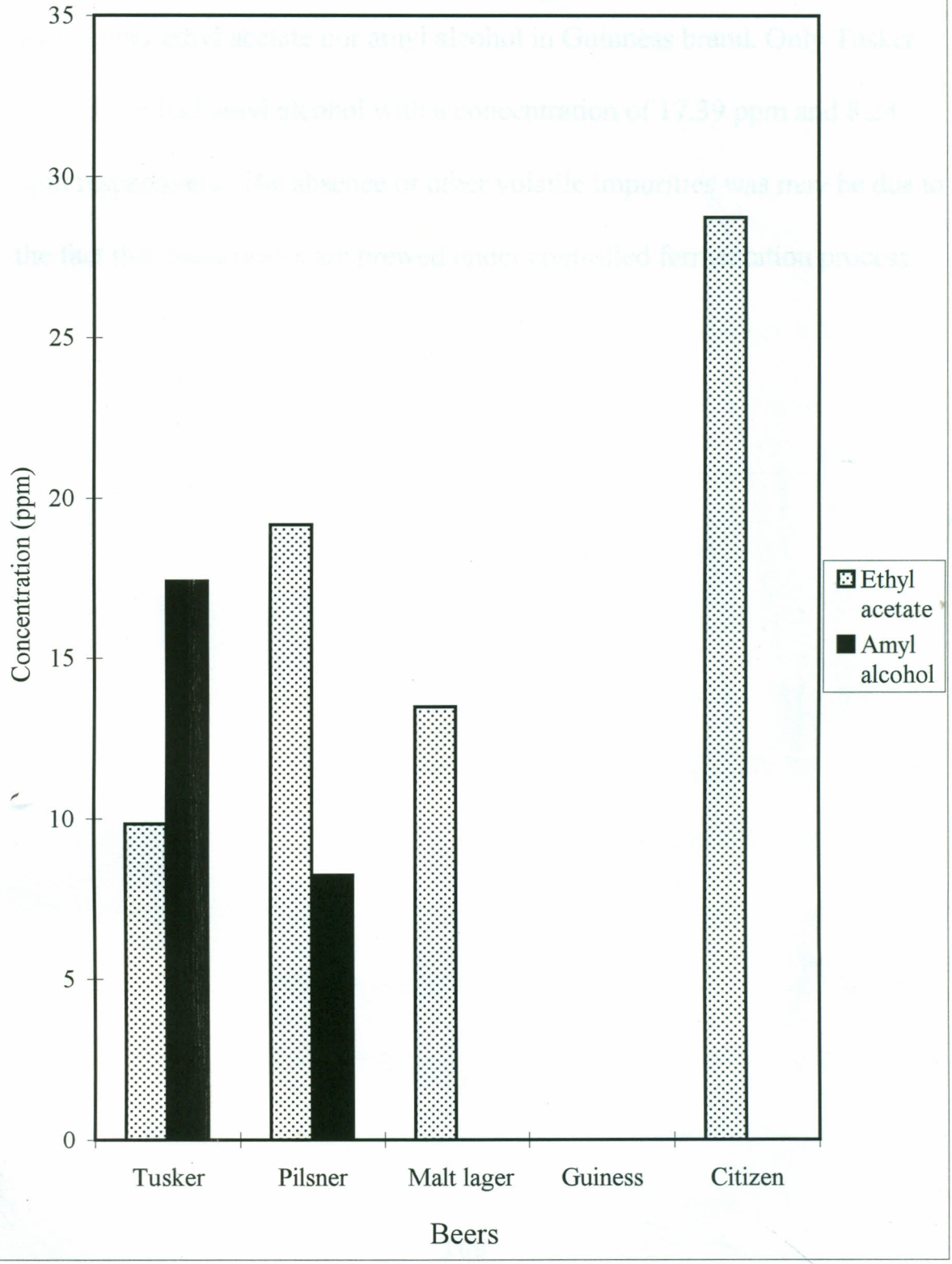
Analysis for volatile impurities in commercial beers like tusker, pilsner, guiness and citizen showed the presence of ethyl acetate and amyl alcohols. The concentrations of these impurities were low as compared to the other local brews and have been tabulated in **table 11** and illustrated in **figure 10**.

Table 11. Volatile impurities in commercially available beers (ppm)

Beer	Ethyl acetate	Amyl alcohol
Tusker	9.85	17.39
Pilsner	19.16	8.24
Tusker malt lager	13.48	ND
Guiness	ND	ND
Citizen	28.71	ND

ND means not detected.

Figure 10. Volatile impurities in commercially available beers



The concentrations of ethyl acetate obtained in different beers were Tusker (9.85 ppm), citizen (28.71 ppm) and tusker malt lager (13.48 ppm). There was neither ethyl acetate nor amyl alcohol in Guinness brand. Only Tusker and Pilsner had amyl alcohol with a concentration of 17.39 ppm and 8.24 ppm respectively. The absence of other volatile impurities was may be due to the fact that these brews are brewed under controlled fermentation process.

3.4.0. CONCLUSION AND RECOMMENDATIONS

This study has found that the cheaply brewed alcoholic brews contain volatile impurities whose concentrations are more than the Limit Threshold Value (TLV). The presence of methanol in high levels in pineapple wine should be noted with great concern, since methanol is highly toxic. The physiological effects of the impurities as earlier discussed are diverse and pose a great danger to the consumer. These brews especially opaque were also found to contain high levels of aflatoxins especially B_1 which is highly toxic and carcinogenic. The most contaminated brew with aflatoxin was found to be busaa irrespective of its origin.

It has also revealed that these alcoholic beverages are brewed from raw materials of very low quality contaminated with aflatoxins. The use of raw materials that had been rendered unfit for human consumption for brewing was also evidenced. Due to high levels of toxic contaminants, these brews are therefore unfit for human consumption. It is of absolute importance therefore that the brewers and the consumers be sensitized on the dangers posed by these brews.

This study therefore recommends the following.

(i) All local brews should be thoroughly tested by Kenya Bureau of Standards and other reputable agencies before they are released to the market.

(ii) The raw materials should also be analyzed for aflatoxins before they are used for brewing

(iii) Determination of non-volatile impurities in the alcoholic brews such as heavy metals and sulphur compounds should be carried out.

(iv) Use of other techniques like high performance liquid chromatography (HPLC) and enzyme-linked immuno-sorbent assay (ELISA) for the comparison of results is recommended for further studies.

(v) Public to be sensitized on the dangers of consuming locally made brews.

(vi) The selling of the fermented flour in open-air markets should be prohibited.

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