



Full Length Research Paper

# Rapid assessment of aflatoxin contamination of groundnuts by thin layer chromatography and competitive enzyme linked immunosorbent assay from selected divisions of Busia county in Kenya

David Ondieki<sup>1,2</sup>, Samuel T. Lutta<sup>2</sup>; Maurice O. Okoth<sup>2</sup>, Paul Kering<sup>2</sup>

<sup>1</sup>Directorate of Occupational Safety and Health Services, Kenya.

<sup>2</sup>Department of Chemistry and Biochemistry, University of Eldoret, Kenya.

Corresponding author email: [rnyukuri@yahoo.com](mailto:rnyukuri@yahoo.com)

## ABSTRACT

Aflatoxins are naturally occurring mycotoxins that are common contaminants of groundnuts thereby presenting a health concern in particular where proper storage of agricultural products is difficult. In this study, groundnut samples were collected from farmers and traders in Busia County. The samples were ground to fine powder and subjected to a preliminary Thin Layer Chromatography (TLC) analysis for detection of contaminated samples. In TLC analysis, the aflatoxin types B1, B2, G1 and G2 were identified with 25.6% of the samples found to be contaminated. The positive samples were further subjected to Enzyme-Linked Immunosorbent Assay (ELISA) test using Stat Fax<sup>®</sup> 3200 ELISA machine for the purpose of cumulative quantification. AF-B1 (aflatoxin B1) was the most dominant contaminant of both market and farm samples with 73% of the contaminated samples found to contain AF-B1. Market samples were more contaminated than farm samples. Contamination levels ranged from a minimum 6.9 ppb to a maximum of 88.7 ppb giving a range of 81.8 ppb, a mean of 44.9 ppb and a standard deviation of 21.78. Contamination exceeded the WHO and Kenya Bureau of Standards (KEBS) maximum allowed limit of 20 ppb and 10 ppb respectively and is therefore a hazard to consumers

**Keywords:** Aflatoxin Concentration (ppb), Aflatoxin B1, TLC, ELISA.

## INTRODUCTION

Aflatoxins are secondary metabolites of mainly produced by *Aspergillus, flavus* and *Aspergillus, nomius*, frequently isolated from groundnut seeds especially in tropical and subtropical regions during the storage period (Das and Mishra, 2003). In Nyanza province of Kenya groundnuts form an important part of the diet and studies have pointed out at a high level of malnutritional and nutritional disorders associated with aflatoxin exposure (Ohingo and Okoth, 2004). Several epidemiological studies have shown a definite connection between areas of high aflatoxin content and a high occurrence of human hepatocellular carcinoma (HCC) (Cardwell *et al.*, 2004).

Studies have revealed that there are four major classes of aflatoxins, i.e. B1, B2, G1 and G2 plus two additional metabolites, M1 and M2 of B1 and B2 respectively. AF-B1 has been shown to be the most

potent and mostly contributes more than half the total aflatoxin content. AF-M1 and AF-M2 were first isolated from milk of lactating animals fed aflatoxin contaminated feed, hence the M designation. The B designation of AF-B1 and AF-B2 resulted from the exhibition of blue fluorescence under ultraviolet light, while the G designation refers to the yellow-green fluorescence of the relevant structures of AF-G1 and AF-G2 (Kaaya and Warren, 2005).

Aflatoxins are very slightly soluble in water (10–30 µg/mL); insoluble in non-polar solvents and freely soluble in moderately polar organic solvents like chloroform and methanol (Cole and Cox, 1981). They have a low molecular weight, are poorly soluble in water, and highly soluble in moderately polar solvents such as chloroform, methanol and dimethylsulfoxide. Aflatoxins are stable at

high temperatures, with the fusion point of AF-B1 being 269 °C.

Groundnut production in Kenya is common in western and Nyanza provinces (Ministry of Agriculture, Nairobi; 2004). It forms a significant part of the communities' diets and is mainly consumed as a sauce, for most of the starch diets or boiled on roasted (Angulo *et al.*, 2009). Groundnut contamination with aflatoxins is one of the main factors that compromise their quality and that of the products.

Busia County is one of the largest groundnut producing counties in Kenya and consequently with a population that consumes more groundnuts in their diets than Kenya's per capita consumption. This is further enhanced by groundnut importation from Uganda which is a larger producer. Busia County borders Lake Victoria to the south and therefore most of this area forms part of the Lake Victoria basin which is hot and humid during most parts of the year. These conditions are known to enhance the growth of *A. flavus* and *A. nominus* which are the major aflatoxin producing fungus.

Aflatoxin contamination is likely to occur in the farm while the groundnuts are still developing, after harvesting when the farmer is drying and preparing for sale and well as storage. Contamination could also occur during the handling by traders in their stores or cereal shops and kiosks. During all this stages, groundnuts could be exposed to physical conditions that have been known to promote growth of fungal species that produce aflatoxins.

The objective of this study was to quantify aflatoxin contamination levels, establish the distribution of aflatoxins in Busia County, and to investigate how handling and storage methods influence aflatoxin contamination of groundnuts.

## MATERIALS AND METHODS

### Sampling

Groundnuts were purchased at random irrespective of their varieties from farmers and market centres in Nambale, Matayos, Samia, Butula and Bunyala Divisions. The samples were immediately stored in sample bags that neither encouraged moisture retention nor absorption so as to ensure that the status of the samples were maintained after collection before analysis. The sampling was designed to represent as much as possible the supply channels of groundnuts to consumers which in turn determines aflatoxin intake. Market centres from which samples were collected were chosen based on their importance on the distribution of groundnuts to the consumers and therefore all major market centres were considered in the sampling stage. These centres were distributed within the divisions where sampling was done and all market vendors with a substantial stock, in this case more than 25 kilograms were considered for

sampling as long as the stock had not originated from the same sack held by a wholesaler. All chemicals used in this study were of analytical grade.

### Sample size

Samples were collected from both the market vendors and farmers. Stocks from which samples were taken were carefully mixed before a 500 gram of sample was extracted. Only one sample was taken per lot and if a trader had more than one lot from different wholesalers then a sample was extracted from each. Samples were extracted from sources that were more than the recommended 25 kilograms. A total of 117 samples were collected.

### Survey questionnaire

A questionnaire was used during sample collection so as to obtain important information which was used in the interpretation of the results during analysis. The questionnaire was used to interrogate the following:

1. Weather patterns in the period preceding harvest (dry spell preceding harvest).
2. Time of harvest (If the crop was harvested in the required/expected time).
3. Postharvest handling practices (drying method, storage).

### TLC Confirmatory Test (Association of Official Analytical Chemists (AOAC) Official Methods of Analysis 200- Ch.33)

#### Sample preparation

The 500 g sample was ground into fine powder using a Ramtons blender so as to pass through a 0.85 mm sieve. 20 g of the ground sample was weighed to the nearest 0.01 g then transferred into a 500 mL wide mouth flat bottom flask. To this 10 g of hyflo super-cel (silica gel 60) and 100 mL chloroform and 20 mL were added. The flask was then covered with aluminium foil and the contents shaken on a mechanical shaker for 30 minutes then filtered through a Whatman Filter No. 254. Only the first 100 mL was collected and transferred into a round bottomed flask for concentration on a rotary evaporator at 40 °C to near dryness.

#### TLC Analysis

This was the preliminary stage and acted as a screening stage. Here the identification of aflatoxins was performed by thin-layer chromatography on precoated silica gel plates (Merck) with dimensions of 20cm × 20cm. Plates were prepared by punching holes at equal intervals of 10 mm using a needle. A constant volume of 20 µL of the sample extract was spotted in the holes made and a

**Table 1.** Aflatoxin distribution by type in Busia County

Division	Total number of samples	Number of contaminated samples	Number of samples contaminated By			
			AF-G2	AF-G1	AF-B2	AF-B1
Nambale	25	5	0	3	2	2
Matayos	25	7	1	0	5	5
Samia	23	4	0	2	1	4
Butula	33	10	2	4	4	8
Bunyala	11	4	3	2	1	3

varying volume of 2 $\mu$ l, 4 $\mu$ l, 8  $\mu$ l & 10 $\mu$ l of the prepared mixed aflatoxin standard was also spotted alongside the samples for comparison. The micropipette was washed with chloroform and rinsed between the spotting of the standard and the samples. The plates were dried in air then inserted in a tank of hexane for defatting.

The plates were developed in a chamber with chloroform, ethyl acetate, toluene and formic acid in the ratio of 30:25:35:10 respectively until the mobile phase reached the limit line.

The TLC plates were removed to dry in air on a rack. Aflatoxin spots were observed under ultraviolet light ( $\lambda=360$  nm) and determined by visual comparison with AF-B1, AF-B2, AF-G1 and AF-G2 standards.

#### **ELISA analysis (AOAC Official Methods of Analysis (2000), Ch.49.2.13 Method, 993.16)**

##### **Sample extraction and analysis**

The ground sample was thoroughly mixed using a high-speed laboratory blender. 10 g of ground sample was weighed to nearest 0.01g on a piece of aluminium foil and transferred into a 50 ml beaker. Then 50 mL of methanol/water mixture (50:50) and 10 mL hexane was added and mixed thoroughly for 30 minutes using a magnetic stirrer. 10 mL of the mixture was centrifuged at 1500 K for 10 minutes. 3 mL of the lower methanol/water layer was recovered and mixed thoroughly on a vortex. 400  $\mu$ L sample extract was pipette into a mixture of 1600  $\mu$ L PBS (Phosphate Buffer Saline) and 2000  $\mu$ L methanol: water (10:90) in a mixing vial.

The required number of ELISA micro-strips (sourced from Bora Biotech Ltd), enzyme conjugate and enzyme substrate were removed from the refrigerator and placed at room temperature for 30 minutes. Aflatoxin standards (0 ppb, 5.6 ppb, 16.7 ppb and 50 ppb) were prepared by serial dilution of the calibrated standard provided.

Then 10  $\mu$ L of enzyme conjugate was added to 10 mL of PBS and mixed gently on a vortex. ELISA micro-strips were washed two times with washing solution and semi-dried. 50  $\mu$ L of the above standards and sample extracts was transferred in duplicates into designated wells. Then 50  $\mu$ L of the diluted enzyme conjugate was added to all

wells. ELISA micro-strips were incubated for 60 minutes at room temperature. ELISA micro-strips were emptied, washed three times and semi-dried. 100  $\mu$ L substrate solution was added to each well then left in the dark for the colour to develop for 7-10 minutes. Colour reaction was stopped by adding 100  $\mu$ L of stopping solution (1 M H<sub>2</sub>SO<sub>4</sub>) to each well.

The aflatoxin concentrations in the contaminated samples were then compared to the set standards of 10 ppb and 20 ppb by KEBS and WHO.

##### **Data analysis**

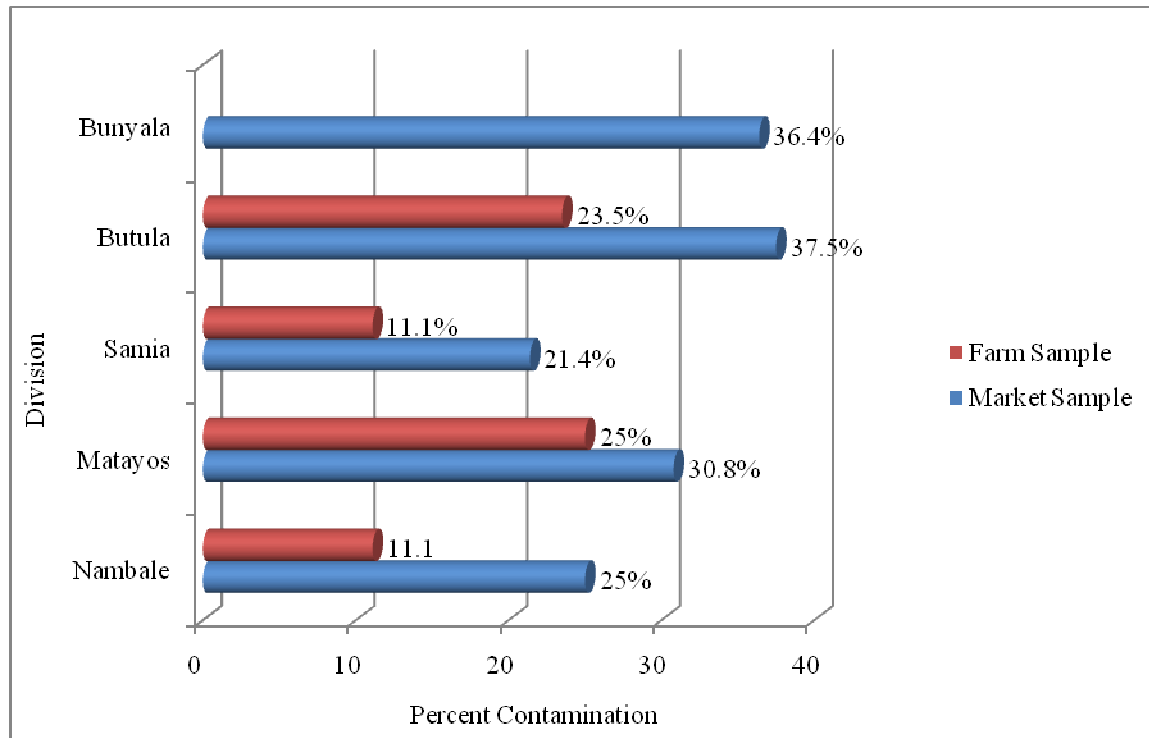
Data was analyzed using the Statistical Package for Social Scientists (SPSS) Statistics 17.0 software. The questionnaire was used and variables generated were used to interrogate aflatoxin contamination levels. An investigation was done to establish whether there was a relationship between aflatoxin contamination and the set variables. Conditions in the divisions were compared to check to what extent they contributed towards fungal growth and subsequent aflatoxin contamination. Analysis of variance (ANOVA) was done. Chi-Square method was used to determine whether there was a relation between the variables and the concentration of aflatoxins in the contaminated samples.

## **RESULTS**

Table 1 gives a summary of the findings of a aflatoxin types distribution in the divisions where the study was done.

##### **Descriptive Results**

A total of 117 samples were subjected to ascending TLC (AOAC Official Methods of Analysis 200- Ch.33) 71 of these samples were from markets while the other 46 samples were from farmers in the five divisions of Busia County where the study was carried out, these represented 61% and 39% respectively. The market samples could have included large imports from Uganda which is an important source of groundnuts for the Kenyan market.



**Figure 1.** Comparison of percentage contamination of market and farm samples per division

In this study, 25 samples were sourced from farmers who reported a dry spell in the period preceding harvest while 21 samples were sourced from farmers who did not report a dry spell. 29 of the samples sourced from farmers who reported to have harvested at the required time while 17 samples were from farmers who reported delayed harvesting. The average time in harvest delay reported by farmers was one month. All Farmers involved in this study also reported the use of PVC bags for groundnut storage.

The minimum concentration recorded for a contaminated sample was 6.9 ppb while the maximum recorded value was 88.7 ppb. The mean concentration of the contaminated samples for Busia County was 44.9 ppb and the median value was 42.3 ppb. The standard deviation was 21.78, which can be explained in terms of the varied environmental conditions which in turn cause huge growth variations in aflatoxin producing fungi.

More market samples were found to be contaminated than farm samples. In Nambale 25% of market samples were found to be contaminated compared to 11% of the farm samples. 25% of farm samples from Matayos division were contaminated compared to 30% of market samples from the same division. Samia Division recorded 11% contamination of farm samples and 21% contamination of market samples while Butula Division recorded 24% and 38% contamination of farm samples and market samples respectively. In Bunyala division where only market samples were collected, 36% contamination was recorded.

This study established that AF-B1 was the most important cause of contamination of groundnuts in Busia County. AF-B1 was found to be present in 73% of all the contaminated samples as shown in Figure 1. AF-G1 was found in 36% of the contaminated samples while AF-B2 and AF-G2 were responsible for the contamination of 33% and 20% of the samples respectively.

Four deductions can be concluded from the results in Table 2:

First, there was no statistically significant difference between the samples collected from the different divisions as determined by one-way ANOVA ( $F(4,112) = 0.648, p = 0.630$ ).

Secondly, there was no significant difference on the levels of aflatoxins between market samples and farm samples with  $F = 2.028, p < 0.157$ . In the third place, there was no significant difference on the levels of aflatoxin of groundnuts that experienced a dry spell during the period preceding harvest and those that experienced adequate moisture in the same period with  $F = 1.910, p < 0.170$ . Lastly, time of harvest (timely/late) did not contribute towards a significant difference on the levels of aflatoxin of groundnut samples  $F = 2.199, p < 0.141$ .

### Chi-Square Test

Table 3 shows a count of samples collected in the farms and markets. Test results indicated that there was no significant difference in the means of aflatoxin

**Table 2.** ANOVA results for samples basing on the area (division), market and farm samples, soil conditions in the period preceding harvest and the time of harvest

		Sum of Squares	df	Mean Square	F	Sig.
<b>Division</b>	Between Groups	0.505	4	0.126	0.648	0.630
	Within Groups	21.803	112	0.195		
	Total	22.308	116			
<b>Sample collected from</b>	Between Groups	0.490	1	0.490	2.028	0.157
	Within Groups	27.817	115	0.242		
	Total	28.308	116			
<b>Dry conditions in the period preceding harvest Season</b>	Between Groups	1.350	1	1.350	1.910	0.170
	Within Groups	81.283	115	0.707		
	Total	82.632	116			
<b>Late or timely harvest</b>	Between Groups	1.614	1	1.614	2.199	0.141
	Within Groups	84.386	115	0.734		
	Total	86.000	116			

**Table 3.** Crosstab of samples collected in the farms and markets

Sample collected from	Market	Count	Test Result		
			Positive	Negative	Total
		Count	21	48	69
		% within Sample collected from	30.4%	69.6%	100.0%
		% within Test Result	70.0%	55.2%	59.0%
		% of Total	17.9%	41.0%	59.0%
	Farm	Count	9	39	48
		% within Sample collected from	18.8%	81.2%	100.0%
		% within Test Result	30.0%	44.8%	41.0%
		% of Total	7.7%	33.3%	41.0%
<b>Total</b>		Count	30	87	117
		% within Sample collected from	25.6%	74.4%	100.0%
		% within Test Result	100.0%	100.0%	100.0%
		% of Total	25.6%	74.4%	100.0%

**Table 4.** Crosstab of Soil Moisture conditions in the period preceding harvest

Harvest Season	Dry spell	Count	Test Result		
			Positive	Negative	Total
		Count	5	23	28
		% within Harvest Season	17.9%	82.1%	100.0%
		% within Test Result	55.6%	59.0%	58.3%
		% of Total	10.4%	47.9%	58.3%
	No Dry spell	Count	4	16	20
		% within Harvest Season	20.0%	80.0%	100.0%
		% within Test Result	44.4%	41.0%	41.7%
		% of Total	8.3%	33.3%	41.7%
<b>Total</b>		Count	9	39	48
		% within Harvest Season	18.8%	81.2%	100.0%
		% within Test Result	100.0%	100.0%	100.0%
		% of Total	18.8%	81.2%	100.0%

contaminated samples between market and farm samples since  $p=0.155>0.05$ .

Table 4 gives results of moisture conditions of the soils in the period preceding harvest. There was no significant difference in the means of aflatoxin

concentrations in the groundnuts that experienced a dry spell and those that did not experience a dry spell in the period preceding harvest since  $p=0.851>0.05$ .

Table 5 shows farm samples harvest timing in the four divisions where samples were collected. Chi-square

**Table 5.** Crosstab of farm samples harvest timing

			<b>Test Result</b>		
			<b>Positive</b>	<b>Negative</b>	<b>Total</b>
<b>Harvest Time</b>	Timely	Count	5	25	30
		% within Harvest Time	16.7%	83.3%	100.0%
		% within Test Result	55.6%	64.1%	62.5%
	Late	Count	4	14	18
		% within Harvest Time	22.2%	77.8%	100.0%
		% within Test Result	44.4%	35.9%	37.5%
<b>Total</b>	Count		9	39	48
	% within Harvest Time		18.8%	81.2%	100.0%
	% within Test Result		100.0%	100.0%	100.0%
	% of Total		18.8%	81.2%	100.0%

**Table 6.** Crosstab of drying method employed in farms

			<b>Test Result</b>		
			<b>Positive</b>	<b>Negative</b>	<b>Total</b>
<b>Drying method</b>	Sheet	Count	9	39	48
		% within Drying method	18.8%	81.2%	100.0%
		% within Test Result	100.0%	100.0%	100.0%
		% of Total	18.8%	81.2%	100.0%
<b>Total</b>	Count		9	39	48
	% within Drying method		18.8%	81.2%	100.0%
	% within Test Result		100.0%	100.0%	100.0%
	% of Total		18.8%	81.2%	100.0%

**Table 7.** Crosstab of storage method

			<b>Test Result</b>		
			<b>Positive</b>	<b>Negative</b>	<b>Total</b>
<b>Storage Method</b>	PVC Bag	Count	30	87	117
		% within Storage Method	25.6%	74.4%	100.0%
		% within Test Result	100.0%	100.0%	100.0%
		% of Total	25.6%	74.4%	100.0%
<b>Total</b>	Count		30	87	117
	% within Storage Method		25.6%	74.4%	100.0%
	% within Test Result		100.0%	100.0%	100.0%
	% of Total		25.6%	74.4%	100.0%

results showed that there were no significant differences between the means of the concentrations of the contaminated samples between samples harvested late and those harvested in time since  $p = 0.633 > 0.05$ .

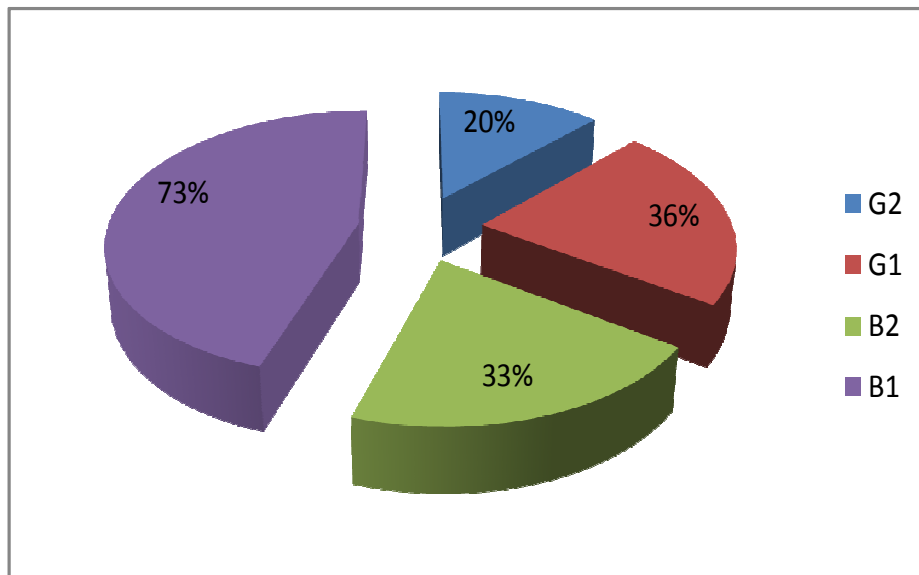
Table 6 shows the drying method used by farmers which was by drying on surfaces that had ground cover and since the method was the same no chi-square result could be obtained.

Table 7 shows storage method employed by both farmers and traders the same and therefore no comparison of means was done.

## DISCUSSION

From the statistical results of both ANOVA and Chi-Square, it can be deduced that the systematic errors (experimental conditions), namely the division from which the sample was collected, the timing of harvesting, moisture content of the soil in the period preceding harvest, and whether a sample was from the market or farm did not influence in a significant way the concentration of aflatoxin in the contaminated samples. This means they could have contributed towards a larger





**Figure 2.** Aflatoxin detection prevalence by type in Busia County

number of contaminated samples because they were enough to cause aflatoxin producing fungi to grow and produce these aflatoxin but not drastic enough to cause a difference in aflatoxin concentrations.

Rapid population increase in Kenya and the entire East African region has created demand for food in communities that cannot produce enough. The advent of free trade in this region has in turn enabled the movement of farm produce across communities and international boundaries. Produce such as groundnuts form a significant part of this trade. This kind of trade in the region involves a significant number of middlemen who out of neglect or ignorance handle groundnuts in conditions that favour the germination of aflatoxin producing fungi therefore exposing them to contamination. This problem has further been compounded with increased dry weather conditions which cause water stress to groundnuts (Blankenship *et al.*, 1981).

Market samples had a higher rate of contamination compared those from farms all the four divisions where farm and market samples were picked from as illustrated in Figure 1. Christie *et al.*, 2010 observed that the fungi *A. flavus* and *A. parasiticus* require certain ecological conditions that are best fulfilled by the poor handling methods that are repeated high up the commodity chain. This brings out the importance of proper post harvesting practices such as processing, handling and storage as well as transportation as they have a bearing of the quality of groundnuts on the consumers table. More should therefore be done by government agencies and nongovernmental organizations through education and policy to address the problem of losing food quality after a painstaking production process.

The basic advice for handling groundnuts after harvesting is to dry them rapidly and completely and to keep them dry and must be kept free of insect infestations or water ingress or heating and cooling gradients which will cause migration of moisture. PVC bags used by all the farmers and traders for groundnut storage restrict air circulation, preserve moisture within thereby raising the temperature of the stored groundnuts hence favouring fungal growth.

Studies carried out have shed light on the ecological environments under which fungal growth is supported. This can be effectively managed if farmers and traders can ensure that groundnuts are kept in aerated environments with moisture levels below what can support fungal growth of any sort over a normal storage life, about one year, i.e. at a water activity below 0.65 (IARC, 1987). Gettinby *et al.*, 1997, reported growth to occur at water activity as low as 0.82 but is very slow below about 0.90 and optimal above 0.99. This corresponds to a moisture content of 8% for groundnuts. According to Hocking and Pitt, 1997, it is evident that reduction of water activity of fresh commodities to below 0.80 will positively prevent aflatoxin production.

The high aflatoxin contamination could have been compounded further by little effort by both farmers and traders sampled to sort and grade their groundnuts. As a result, stocks contained broken, shriveled and rotten groundnuts that acted as precursors in aflatoxin infection of good groundnuts.

## CONCLUSION

In this study 25.6 % of the samples were found to be contaminated by either one or more of the aflatoxins B1,

B2, G1 and G2. In this study all but three of the contaminated samples had aflatoxin levels exceeding the 10 ppb set by KEBS. Results also showed that contaminated samples easily exceeded the maximum limit of 20 ppb set by the WHO. The order of aflatoxin prevalence by type was in the order AF-B1>AF-G1>AF-B2>G2. Aflatoxin B1 was the most predominant type and was found to be present in 73% of the contaminated samples as shown in Figure 2.

Table 1 shows the distribution of aflatoxins B1, B2, G1 and G2 as found in this study. Aflatoxin B1 is important due to its highly carcinogenic nature. Eriksson and Guedes, 2006 have conducted experiments that have shown that B1 can easily ionize due to its low ionization potential. From this contamination levels and such a high prevalence rate of aflatoxin B1, it is safe to state that the consumers of groundnuts sourced from markets and farms in the divisions surveyed within Busia County are exposed to a great public health problem.

In April 2004, one of the largest aflatoxicosis outbreaks occurred in rural Kenya in four districts namely: Makueni, Kitui, Machakos and Thika, resulting in 317 cases and 125 deaths. Fifty five percent of maize products had aflatoxin levels greater than the Kenyan regulatory limit of 20 ppb, 35% had levels > 100 ppb, and 7% had levels > 1,000 ppb (Backer *et al.*, 2005). Between March and June 1981, 20 patients (8 women and 12 men aged 25 to 45 years old) were admitted to three hospitals in the Machakos district of Kenya with severe jaundice. 8 of the patients recovered while 12 died of aflatoxicosis (Kenya *et al.*, 1982). Analyses of breast milk in Ghana, Nigeria, Sierra Leone and Sudan showed primarily AF-M1, AF-M2 and aflatoxicol. Aflatoxin exposure pre- or post-natally at levels  $\geq 100$  ng/L was very often associated with illness in the child (Maxwell, 1998).

Beginning in the 1960's and throughout the 1980's, a large number of ecological correlation studies were carried out to look for a possible correlation between dietary intake of aflatoxins and risk of primary liver cancer. Most of these studies were carried out in developing countries of sub-Saharan Africa or Asia, where liver cancer is common. With some notable exceptions, and despite the methodological limitations of these studies, they tended to show that areas with the highest presumed aflatoxin intake also had the highest liver cancer rates (IARC, 1993). The presence of aflatoxins in human body fluids and tissues in relation to child health in the tropics has been observed Ghana, Kenya, Nigeria and Sierra Leone where 25% of cord blood samples contained aflatoxins, primarily AF-M1 and AF-M2, as well as others in variable amounts (range: 1 ng AF-M1/Litre to 64.97ng AF-B1/Litre) (Maxwell, 1998).

The results show substantial contamination of groundnuts in Busia County which pose a danger to public health to groundnut and groundnut products consumers. To stem this problem aflatoxin contamination which occurs during post harvest processes should be

eliminated through use of good and recommended methods that avoid fungal growth on harvested groundnuts. Fungal growth and development can be eliminated by moisture reduction in harvested groundnuts by simple procedures such as drying them in the sun, sorting and removal of rotten and shriveled nuts. To achieve this, stakeholders both in government through its agencies and nongovernmental organization should work toward dissemination of these simple methods to farmers, traders and consumers.

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