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COLLEGE OF SCIENCE
DEPARTMENT OF FOOD SCIENCE AND TECHNOLOGY

AFLATOXIN M1 CONTAMINATION OF RAW COW MILK, MILK PRODUCTS AND DIETARY EXPOSURE

A Thesis submitted to the Department of Food Science and Technology in Partial fulfillment of the requirement for the degree of Master of Science in Food Quality Management

By
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DECLARATION

Except for references to other people's work, which have been appropriately acknowledged, I hereby declare that, this thesis, submitted to the School of Research and Graduate Studies, KNUST, Kumasi is the result of my own original research and that this thesis has not been presented to any other institution for award of a degree.

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ABSTRACT

Globally, the occurrence of Aflatoxin M1 in milk and milk products has been reported in many countries and therefore a thorny issue especially for developing countries. Despite the health effects of exposure to Aflatoxin M1 such as acute liver damage, cirrhosis of the liver and tumor induction, studies on occurrence and dietary exposure is generally lacking in Ghana. This study therefore sought to determine the incidence and dietary exposure to Aflatoxin M1 in three selected suburbs in the Greater Accra Region of Ghana.

Thirty (30) samples of raw cow's milk, 30 samples of Burkina drink and 23 locally made cheese (*wagashi*) were randomly purchased from the three suburbs (Ashaiman, Madina, Nima) in the Greater Accra region and analysed for the presence of aflatoxin M1 (AFM1). Solid phase extraction was used for the extraction and clean-up of samples and subsequently analysed using a high-performance chromatography coupled with fluorescence detector and Pyridinium Bromide Perbromide (PBPB) as a post column derivatization agent for detection and quantification. Using a food frequency questionnaire to obtain food consumption estimates and mean body weights, a deterministic approach was used to calculate the estimated daily intake (EDI) of AFM1 through raw cow's milk, burkina drink and wagashi. Five of the thirty milk samples (16.67%) were positive with mean AFM1 levels of ±0.25 ug/l. Seven of the thirty Burkina samples (23.33%) showed presence of AFM1 with mean concentration of ±0.09 ug/l. Five of the cheese samples showed presence of AFM1 with mean concentration of ±0.04 ug/kg. The concentrations of AFM1 were lower in the locally made cheese than in the raw cow's milk and Burkina drink. All the **individual** results for each product was above the EU limit of 0.05 ug/kg. For EDI, the results obtained showed that infants recorded the highest mean AFM1 across the three food types and therefore the age group exposed to significant risk. AFM1 intake through wagashi was relatively lower across all age groups compared to burkina drink and raw cow's milk **TABLE OF CONTENTS**

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LIST OF ABBREVIATIONS

ADI Acceptable Daily Intake

AF Aflatoxin

AFs Aflatoxins

AFB1 Aflatoxin B1

AFB2 Aflatoxin B2

AFG1 Aflatoxin G1

AFG2 Aflatoxin G2

AFM1 Aflatoxin M1

AFM2 Aflatoxin M2

ANOVA Analysis of Variance

AOAC Association of Official Analytical Chemists

ARfD Acute Reference Dose

BW Body Weight

C Concentration

CR Contact Rate

EC European Commission

EDI Estimated Daily Intake

EFSA European Food Safety Authority

ELISA Enzyme-linked Immunosorbent Assay

EU European Union

FAO Food and Agricultural Organization

FFQ Food Frequency Questionnaire

FUM Fumonisins

GSS Ghana Statistical Service

HPLC High Performance Liquid Chromatography

IAC Immunoaffinity Column

IARC International Agency for Research on Cancer

IDF International Dairy Federation

IFST Institute of Food Science & Technology

IPCS International Programme on Chemical Safety

ISO International Organization for standards

JECFA Joint Expert Committee for Food Additives

NOAEL No Observable Adverse Effect Level

PBPB Pyridinium Bromide Perbromide

PBS Phosphate Buffered Saline

SPE Solid Phase Extraction

SPSS Statistical Package for the Social Sciences

TLC Thin Layer Chromatography

TMDI Theoretical Maximum Daily Intake

UK United Kingdom

UV Ultraviolet

WHO World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background

Milk is sometimes referred to as nature‘s single most complete food because milk and milk products have been used by man since primitive times O‘Connor, (1994) and most importantly because nearly all the constituents of milk are very essential for life. It has an extremely high nutritional quality, performing numerous vital functions, including growth, supply of energy,

reproduction, maintenance and repairs, and appetite satisfaction (O'Connor, 1994). Amongst human population, children and especially infants who require milk for proper development during growth, usually consume milk in high quantities. Notwithstanding the health benefits associated with milk, it could also be a potential source of natural food contaminants that may cause ailment and pose adverse health effects. One group of such contaminants is mycotoxins. Contamination of milk and milk products with AFM1 (a mycotoxin) has been known worldwide for close to twenty years. This phenomenon is therefore an important problem across the globe and particularly so for developing countries (Iqbal *et al*., 2015).

―Aflatoxins are naturally occurring mycotoxins produced mainly by moulds (*Aspergillus flavus* and *Aspergillus parasiticus*)‖ (Kim *et al*., 2000). Aflatoxin M1 (AFM1) a metabolite of Aflatoxin B1 (AFB1), has been found to be a major excretory product in the milk of lactating livestock and humans exposed to dietary AFB1. In livestock, this is mainly as result of feeding on contaminated food products.

The International Agency for Research on Cancer (IARC) until the year 2002 classified Aflatoxin M1 as class 2B carcinogen, which meant the toxin was a possible carcinogen to humans. It had been shown experimentally that AFM1 presents high hepatotoxic and mutagenic risk (FAO/WHO, 1999). The IARC however reviewed the classification of AFM1 from class 2B to group 1, meaning AFM1 is carcinogenic to humans after genotoxicity and carcinogenetic of AFM1 was observed in vivo, although lower than those of AFB1, and its cytotoxicity demonstrated (Caloni *et al*., 2006; IARC 2002). Aflatoxins are both acutely and chronically toxic for animals and humans and can produce adverse health effects including acute liver damage, liver cirrhosis and tumor induction (Simon et al., 1998). According to Egal *et al*. (2005), there are some evidence for associations with health problems such as Reye's syndrome, kwashiorkor, and acute hepatitis. Young children and infants have a higher risk of exposure to AFM1compared to adults within the human population. This is mainly so

because infants and young children are known to consume relatively higher quantities. Evidence of AFM1 contamination of milk is well documented. The results of a survey conducted in Tehran, Iran, indicated that Aflatoxin M1 (AFM1) was found in 78% of commercial liquid milk, 33% of milk-based weaning foods analyzed. The levels were found to be higher than the maximum tolerance limit accepted by the European Union (Oveisi *et al*., 2007). A similar survey conducted in India by Rastogi *et al*. (2004), showed the occurrence of AFM1 in infant milk products and milk products. Of the samples analyzed, 87.3% were found to be contaminated with AFM1. The outcome of the study showed that infant milk products were much contaminated at higher levels relative to liquid milk.

It has also been reported that Aflatoxin M1 contamination has been found in milk and milk products in Portugal (Duarte *et a*l., 2013), Serbia (Kos *et al*., 2014), South Africa (Dutton et al., 2012) and Syria (Ghanem and Orfi, 2009). There is also evidence of the occurrence of AFM1 in biological fluids of humans such as urine and breastmilk. Makun *et al*. (2016), detected AFM1 in breast milk of breastfeeding mothers. The results of the study showed that for human breastmilk, the incidence of AFM1 occurred at 77.5%. Also, out of 40 samples, 15 representing 37.5% were contaminated at levels that were above both EU and the Nigerian permitted level of 0.05µg/l.

The global occurrence of Aflatoxin M1 in foods has necessitated the need for a public health intervention to safeguard the health of consumers from the potential adverse health effects of this toxin. One of such interventions is the setting of allowable limits. Thus, the regulatory or governing goal is that the allowable limits implemented by countries as standards will not be exceeded.

The international regulations for the maximum limit for AFM1 in milk and dairy products vary among countries especially within the European Union and the United States of America

(USA). ―According to the United States regulations, the AFM1 levels should not exceed 0.5 µg/kg. However, the Codex Alimentarius set 50 ng/kg as the regulatory limit and for infant milk and follow-on milk, no more than 0.025 µg/kg is allowed‖ (EC, 2006). Similarly, in Austria and Switzerland, the maximum level is only 10 pg/mL for infant food (FAO, 1997). Most studies conducted on milk and milk products across the globe have shown occurrence of AFM1 in dairy products and milk. (Iqbal *et al.,* 2015). Monitoring via regular surveillance studies and analysis of commercially available milk on the market also continues to be one key measure of controlling or eliminating this toxin from human diet and thereby preventing the potential adverse health effects that are associated with it.

1.2 Problem Statement

Milk continues to be a nutritious food and a source of both macro and micronutrients for the development, growth and maintenance of good health. Generally, almost all age categories of the human population consume milk and milk products on a regular basis as part of their diet

(Fallah *et al.,* 2009). In Ghana, especially on the major streets, there is increase sale of raw cow milk product popularly called ―Burkina drink‖. At food vending joints where porridge (locally called ―koko‖) is sold, there is also increase patronage of another product made from raw cow milk, known as ―wagashi – which is a local cheese. In most peri-urban areas in Accra, the fresh/ raw cow milk is also sold and consumed or used in preparing foods.

AFM1 contamination of milk and milk products at the global level is well established and reported in many countries (WHO, 2010). Milk and dairy products contamination with aflatoxin M1 is therefore an important problem worldwide especially for developing countries. It is therefore hypothesized that fresh/ raw cow milk and milk products in Ghana are contaminated with AFM1 and therefore pose health risk to consumers. Despite the health effects of exposure to AFM1, studies on levels in milk and milk products are generally limited

in Ghana. It is therefore imperative to assess the levels and dietary exposure to this human carcinogen. Kumi *et al*. (2015) reported significant levels of AFM1 in urine samples of children (6 to 2years) in three communities in Ashanti region. This study was unfortunately only limited to cereals and legumes fed to children. Studies on levels in milk and milk products are generally lacking. There is therefore an urgent need to determine the levels of AFM1 and estimate dietary exposure. The outcome will contribute significant information to risk management and policy making in Ghana.

1.3 Aims and Objectives

1.3.1 Aim

The main aim of this study is to determine the occurrence and levels of Aflatoxin M1 in commercially available fresh/ raw cow milk and milk products in Ghana and potential adverse effect of consuming these locally manufactured fresh milk and milk products.

1.3.2 Specific objectives

To meet the main aim of the study, below specific objectives have been set out to be achieved;

- To determine the Aflatoxin M1 contamination levels in selected locally produced fresh/ raw cow milk and milk products sold on the Ghanaian market.
- To conduct a dietary exposure assessment for the locally produced fresh cow milk in the selected areas in Accra.

CHAPTER 2

LITERATURE REVIEW

2.1 Mycotoxins

It is widely known that many crops including cereals are prone to fungi occurrence either during storage or whiles on the field. The fungi, once it has attacked the crop, may produce secondary metabolites which belongs to a group of chemicals known as Mycotoxins (Magan and Oslen. 2004).

Getting to the latter part of the log growth phase of fungi, metabolites which are secondary and lethal in nature are then produced. There is no established function between the metabolites and growth of fungi and their metabolism. The genera *Penicillium, Fusarium and Aspergillus* when they are in conducive environment yield the metabolites in foods and other farmed produce (Jay, 2000; Razavilar, 2003).

Once mycotoxins are ingested or contact is made with it, it has an injurious effect and may result in mycotoxicosis disease. One main route by which mycotoxins enter the food chain is by humans directly ingesting a contaminated food or animals feeding on fungal contaminated feed. This can lead to bioaccumulation of the toxin in many organs and tissue of the body. Eggs, milk and meat are main transport route for the toxin to enter the food chain **(Mari**n *et al*., 2013).

The chemical composition of mycotoxins was only recently established though its existence is deemed to have been as far back as crops have existed. There has been evidence to prove their existence even before in 1960s, aflatoxins were recognized by scientist (Richard, 2007)

While there are over 300 known mycotoxins, currently merely 5 or 6 are acknowledged to be of importance and studied. Researchers over the world, have primarily been united in

considering aflatoxins, ochratoxins, trichothecenes, zearalenone and fumonisins (FUM) to be mycotoxins of great importance (Heperkan, 2006).

2.1.1. Aflatoxins

Aflatoxins were discovered in the 1960s when there was an outbreak of a disease called the turkey _X‘ disease in the United Kingdom. This disease claimed the lives of many farm animals especially birds including thousands of turkeys. The loss of these farm animals was ascribed to the fact that an animal feed had been heavily contaminated with a particular mould - *Aspergillus flavus.* When the contaminated feed was subsequently examined, it revealed chemical compounds that were fluorescent in nature and subsequently became known as AFLATOXINS. The fluorescent compounds (AFLATOXINS) were deemed to be responsible for the turkey disease (Sargeant *et al.,* 1961; Davisand Diener, 1979; Bash and Rae, 1969).

AFLATOXIN is an acronym from an amalgamation of the following words: A‘ representing the mould genus *Aspergillus,* followed by, _FLA‘, representing the flavus species, and lastly the word _TOXIN‘ denoting the poisonous nature of this metabolite (Ellis *et al.,*1991).

Aflatoxins are structurally-related compounds produced as secondary metabolites by toxigenic strains of *Aspergillus flavus, A. parasiticus* and *A. nomius* (Betina, 1989; Martin *et al.,* 2001).

Aflatoxins can be found worldwide in the soil, air and are found to infest both living and dead plants and animals. (Rustom, 1997) Most experimental animals have shown that these secondary metabolites (Aflatoxins) are carcinogenic, teratogenic as well as mutagenic (Adejumo *et al*., 2013).

The most studied group of mycotoxins worldwide happens to be aflatoxins which is known to contaminate wide range of both animal feed and human food. In both tropical and subtropical areas, aflatoxin contamination of crops is predominantly through the *Aspergillus* species (Ardic, *et al*., 2008; Decastelli *et al.,* 2007). Aflatoxins (AF) can be grouped into AF B1, AF

B2, AF G1 and AF G2 (Akiama *et al*. 2001). Aflatoxins have been classified using their fluorescence nature when viewed under ultra-violet light and how they separate by chromatography. Aflatoxin B1 and B2 show blue fluorescence under UV whereas aflatoxin G1 and G2 fluoresce as green. (Bennett and Klich, 2003). Aflatoxin B1 is known to be more potent cancer-causing mycotoxin (IARC, 1993) and believed to be the precursor for the other aflatoxins such as AFM1 (Nuryono, *et al.* 2009).

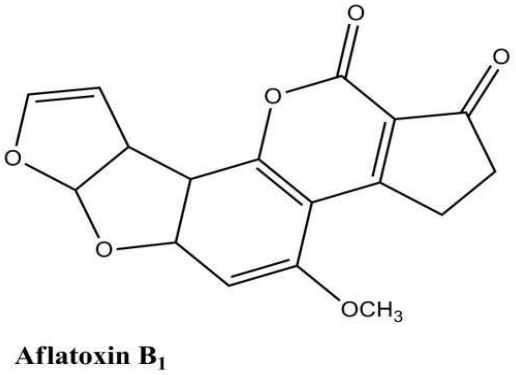
2.1.1.1 Classification of Aflatoxins

Aflatoxin B1

Amongst the four aflatoxins that occur naturally (AF B1, AF B2, AF G1 and AF G2), aflatoxin B1 is typically predominant in crops, animal feeds and more toxic compared to the others in both animals meant for laboratory studies and humans (Newberne and Butler, 1969; Eaton and Gallagher, 1994; Lopez *et al.,* 2002). AFB1 fluoresce blue colour under UV light (Bennett and Klich, 2003).

According to Horn *et al*. (2009) the four naturally occurring aflatoxins are produced by *Aspergillus parasiticus* whereas *Aspergillus flavus* produces only aflatoxin B aflatoxins (B1 and B2). It has a chemical formula as $C_{17}H_{12}O_6$ (Fig. 1) and a molecular weight of 312 g/mol.

Aflatoxin B1 in the liver of mammals are hydrolysed into aflatoxin M1 and expelled from the body through the mammary glands when they end up in the milk of both lactating animals and humans that have ingested feed or food contaminated with aflatoxin B1 (Fallah *et al*. 2009)

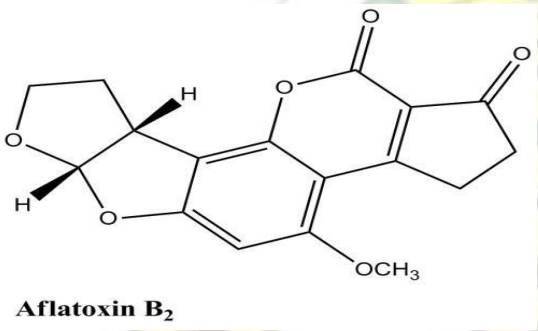


Aflatoxin B_1

Fig.1: Aflatoxin B1 chemical structure (Santini and Ritieni, 2013)

Aflatoxin B2

One of the secondary metabolites produced by *Aspergillus flavus* is aflatoxin B2 (AFB2). Like all aflatoxins, AFB2 is a low molecular weight (314 g/mol.) metabolite with chemical formula $C_{17}H_{14}O_6$ (Fig. 2). Under ultraviolet light, AFB2 to emits blue fluorescence



Aflatoxin B_2

Fig.2: Aflatoxin B2 chemical structure (Santini and Ritieni, 2013)

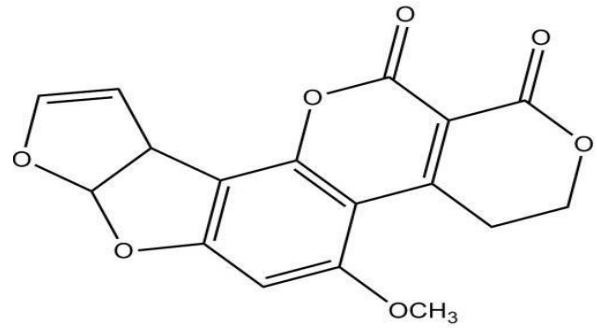
Aflatoxin G1 and G2

Aspergillus parasiticus in addition to producing aflatoxin B1 and B2 also chiefly produce G1 and G2 of the aflatoxin group. *Aspergillus flavus* does not produce this metabolite (Xu *et al*.,

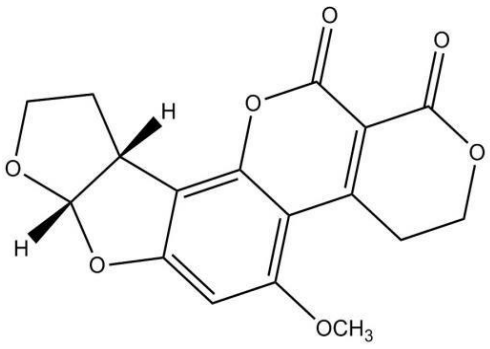
2000). Under ultraviolet light, both AFG1 and AFG2 emit green fluorescence. The IARC has classified AFG1 and AFG2 as group 2B carcinogens (IARC, 1993) which means they are considered possible carcinogens to humans. The molecular weight and other physical properties of AFG1 and AFG2 are shown in Table 1.

Table 1: Chemical properties of the major aflatoxins (O'Neil *et al.*, 2001)

Type of Aflatoxin	Mol. Wgt	Chemical Formula	Melting point (°C)	IUPAC name
B1	312.28 g/mol	$C_{17}H_{12}O_6$	268–269	2,3,6a,9a-tetrahydro-4methoxycyclopenta(c)furo(3',2':4,5)furo(2,3-h)(1)benzo-pyran-1,11-dione
B2	314.29 g/mol	$C_{17}H_{14}O_6$	286–289	2,3,6aa,8,9,9aa-Hexahydro-4 methoxycyclopenta(c)furo(2',3': 4,5)furo(2,3-h)chromene-1,11-dione
G1	328.28 g/mol	$C_{17}H_{12}O_7$	244-246	7AR,cis)3,4,7a,10a-tetrahydro-5-methoxy1H,12H-furo(3',2':4,5)furo(2,3-h)pyrano(3,4-c)chromene-1,12dione
G2	330.29 g/mol	$C_{17}H_{14}O_7$	237–240	1H,12H-furo(3',2':4,5)furo(2,3-h)pyrano(3,4-c)(1)benzopyran-1,12-dione
M1	328.28 g/mol	$C_{17}H_{12}O_7$	299	(6AR-cis)-2,3,6a,9a-tetrahydro-9a-hydroxy-4-methoxycyclopenta(c)furo(3',2':4,5)furo(2,3-h)(1)benzopyran-1,11-dione
M2	330.29 g/mol	$C_{17}H_{14}O_7$	293	2,3,6a,8,9,9a-Hexahydro-9a-hydroxy-4-methoxycyclopenta(c)furo(3',2':4,5)furo(2,3h)(1) benzopyran-1,11-dione



Aflatoxin G_1



Aflatoxin G_2

Fig.3: Chemical structure of aflatoxin G1 and G2 (Santini and Ritieni, 2013)

Aflatoxin M1 and M2

Aflatoxin M1 (AFM1) is a derivative (mono-hydroxylated) of aflatoxin B1 produced in liver when enzymes such as cytochrome p450 and related enzymes are activated. Animals, especially mammals ingesting diets contaminated with AFB1, excrete AFM1 (principal 4hydro-xylated metabolite) into milk (Prandini, 2009). When aflatoxin M1 (AFM1) quantity is usually expressed as a percentage AFB1 which is the mother compound, as reported by Forbisch *et al*. (1986), it is usually between 1-3%. Meaning 1 – 3% of the toxin (AFB1) ingested, gets bio-transformed in the liver to AFM1. Other studies however have reported higher percentages i.e. 6% (Veldman *et al*., 1992). The IARC initially classified AFM1 as a group 2B possible carcinogen) but in 2002, based on available data and research, the classification was moved to

group 1, meaning it is a human carcinogen though its genotoxicity, carcinogenicity and cytotoxicity are lower than those of AFB1 (IARC, 2002).

Furthermore, EFSA (2009), reported that AFM1 acute toxicity appears to be comparable to or slightly less and not as much as that of AFB1 though its carcinogenicity strength is possibly about 1 - 2 orders of magnitude lesser than that of aflatoxin B1.

According to Iqbal *et al*. (2015); Duarte *et al*. (2013) at high temperatures, AFM1 is stable and therefore cannot be removed from milk by heat treatment such as pasteurization and ultra-high temperature. AFM1 can therefore be found in milk and infant foods making AFM1 levels very important regardless how small it may be. AFM1 has a molecular weight of 328 g/mol and chemical formula of $C_{17}H_{12}O_7$. Under ultra-violet light, AFM1 emits blue–violet fluorescence (IARC, 2002). AFM2 is also a metabolite of aflatoxin B2 (AFB2) which is found in milk of lactating mammals. It has chemical formula of $C_{17}H_{14}O_7$ (Fig. 4) and a molecular weight of 330 g/mol.

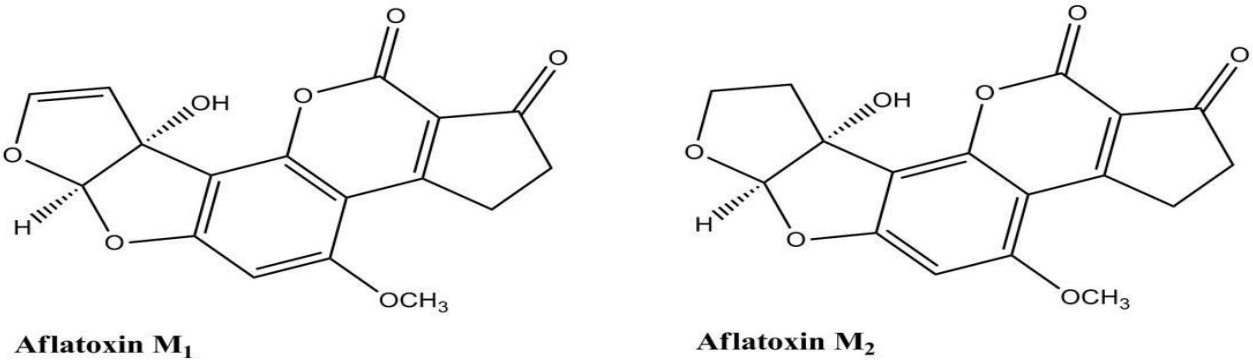


Fig.4: Chemical structure of aflatoxin M1 and M2 (Santini and Ritieni, 2013)

2.2 Production and Stability of Aflatoxins

Occurrence of Toxicogenic molds occur ubiquitously. Their vegetative spores which are usually produced in large numbers and can be in a latent state for a very longtime, easily spreads

hence contaminating agricultural products even before harvest (Northolt and Bullerman, 1982). The invasion and colonization and production of mycotoxins can take place either during pre-harvest or post-harvest stages (Storm *et al*., 2008; Scudamore and Livesey, 1998; Coulombe, 1993). Aflatoxin contamination during pre-harvest of most crops is linked to drought and/or high temperature stresses, with insect impairment occurring during the period of crop maturation (Payne and Widstrom, 1992).

Storage Conditions and climatic conditions are closely related to fungal growth and mycotoxin contamination and therefore will vary with agricultural, locations and manufacturing practices

2.3 Effects of Aflatoxin (Aflatoxicosis)

Aflatoxin poisoning (Aflatoxicosis) mainly affect animals in the liver and causes liver dysfunction, reduced milk, egg production and reduced immunity. Young animals are more susceptible to aflatoxins with clinical manifestations including; digestive disorders, reduced fertility, reduced feed efficiency and anemia (Dhanasekaran et al., 2011). In human exposure, two main pathways have been identified. Firstly, through direct consumption of aflatoxins (mainly AFB1) in contaminated foods such as nuts and ingestion of aflatoxins carried over from animal feed into milk and milk products (Dhanasekaran et al., 2011). ―Acute aflatoxicosis results in death; chronic aflatoxicosis results in cancer, immune suppression, and other "slow" pathological conditions‖ (Hsieh, 1988). Mycotoxins impact on the human immunity system is a well investigated area which is documented or reported (Jiang et al., 2005). Aflatoxins have been implicated in conditions such kwashiorkor especially in Africa in children. This disease which occurs mainly in children because of protein malnutrition (Adhikari et al., 1994) has some association with aflatoxin exposure. A study conducted by Gong et al. (2004) also seems to implicate aflatoxicosis in weakened growth in infants who are exposed to aflatoxins post weaning. In Kenya, contamination of maize with aflatoxins led to many deaths in an epidemic. In 2004, an outbreak of acute aflatoxicosis claimed about 125

lives in 2004 because of maize contamination with aflatoxins (Probst *et al*., 2007). The most powerful carcinogen amongst the aflatoxin group is Aflatoxin B1. Results from human studies, have shown that aflatoxins are major contributing factor that leads to hepatocellular carcinoma and hence as a result, its classification has been put as Group 1 (IARC, 2002).

2.4 Global Occurrence of Aflatoxin M1

The existence of AFMI in dairy products as well as milk is a pertinent issue globally and particularly so for emerging nations (Prandini et al., 2009). Generally, in European countries, milk and related products that gets contaminated with AFM1 is fewer, this is because of strict regulations adopted to control this mycotoxin in both animal feed and dairy products as well as proper storage conditions in Europe (Iqbal et al., 2015). The presence of AFM1 in breastmilk and urine samples studied in Africa, is evidence of Aflatoxin M1 contamination in human diets. A study conducted by Kumi et al. (2015), detected AFM1 in urine samples collected from children weaned on locally prepared food in Ghana. In Nigeria, according to a study conducted by Makun et al. (2016), AFM1 was detected in breastmilk of breastfeeding mothers in Minna. The results obtained from the study indicated high (77.5%) incidence of AM1 contamination in human breastmilk with 37.5% of the samples being above the Nigerian and EU regulated limited (0.05 µg/l). The study also revealed that lower income earners as well as mothers who subsisted mainly on cereals had significantly higher levels of AFM1 compared to middle to higher income earners and mothers who subsisted more on other food source. Table 2 shows some documented case studies of AFM1 contamination of milk & dairy products across the globe.

Table 2: Global overview of aflatoxin M1 occurrence in milk.

Reference	Country, Year	Milk Type	Method of Analysis	Incidence rate (%)	AFM1 mean Conc.

Ghanem *et al.* (2009)	Syria, 20052006	Raw cow milk	ELISA	70/74 (95%)	143 ± 53.2 ng/l
		Raw sheep milk		13/23 (57%)	67 ± 18.43 ng/l
		Raw goat milk		7/11 (64%)	19 ± 13. 8 ng/l
		Pasteurized milk		10/10 (100%)	492 ± 212.56 ng/l
		Milk powder		1/8 (13%)	12 ng/l
Cano-sancho *et al*. (2010)	Catalonia, Spain, 2008	UHT Milk	ELISA	68/72 (94.4%)	9.69 ± 2.07ng/l
Duarte *et al*, (2013)	Portugal, 2011	Pasteurized and UHT Milk	ELISA	11/40 (27.5%)	23.4 ± 24.0 ng/l
Gizachew *et al*, (2016)	Ethiopia, 2015	Raw cow milk	ELISA	110/110 (100%)	0.41ug/l
Rastogi *et al*, (2004)	India, 2002	Infant milk food	ELISA	17/17 (100%)	350 ± 48 ng/kg
		Infant formula	`	17/18 (94%)	326 ± 45 ng/kg
		Milk based cereal weaning food		38/40 (95%)	267± 29 ng/kg
		Liquid milk		4/ 12 (33%)	86± 35ng/kg
Fallah *et a*l. (2009)	Iran, 2007 - 2008	White cheese	ELISA	93/116 (80.1%)	198.6 ± 17.0ng/kg
		Cream cheese		68/94 (72.3%)	166.4 ± 18.6ng/kg

2.5.0 Available Methods for Aflatoxin M1 & B1 Analysis

The methods available for analysis of aflatoxins is expected to meet expectations of regulation, survey work, and research work. The method of analysis selected therefore should meet

appropriate criteria which include but not limited to accuracy, applicability, detection limit, accuracy, ability to be repeated, recovery, selectivity, as well as sensitivity, and reproducibility (IFST, 2009). Consequently, in recent years, different extremely complex and efficient methods have come on board for the analysis of aflatoxins in many food items.

High-Performance Liquid Chromatography (HPLC), thin-layer chromatography (TLC) and Enzyme-linked Immunosorbent assay (ELISA) are known to be the popular methods employed in recent times for analysis and detection of aflatoxins (Lee *et al*., 2009).

According to Hansen (1990), fluorometric method is also used for the detection of aflatoxins.

These analytical methods (HPLC, TLC, ELISA etc.) for aflatoxin analysis are essentially the same with little adjustments to make it more specific for measurement in certain food items. The difference comes about due to the solvents employed in extracting the toxin and the procedure employed to estimate the fluorescence strength of the toxins being analyzed. All the methods principally used for AF analysis have steps that are similar. These include the sampling step, followed by extraction and clean-up. Once clean-up is done, detection of the toxin, confirmatory analysis and subsequent quantification is done (Sinha, 1999).

2.5.1 Sample Preparation Techniques

In analytical identification of aflatoxins, sampling and sample preparation are of greatest importance as it finally affects the final results. In case of solid samples, it is necessary to ground the entire primary sample and mix to ensure homogeneity. Whereas for liquid samples such as milk, because of the homogeneous nature of the food item and hence the toxins, certainty in measuring the toxin is improved. Extraction and clean-up steps are done once sampling is done. These two steps (extraction and clean-up) sometimes can be one step.

During extraction the toxin is released into the solvent used for the extraction and any other unwanted substance present is taking care of by getting rid of it using the clean-up stage. The

main techniques used for toxin extraction are liquid-liquid, immunoaffinity, and solid-phase extraction technique (Hussain, 2011)

2.5.1.1 Separation by Liquid-Liquid Technique

Liquid-liquid separation technique involves separation of compounds which are organic in nature amid an aqueous phase and a solvent which is organic nature and does not mix with the aqueous phase and might be either polar or non-polar. An example of liquid-liquid technique applied to extraction of aflatoxin M1 is a study conducted by Stubblefield (1979) when extraction of aflatoxin M1 was done using chloroform in a separating funnel. The clean-up of the extract was done over column packed with silica gel and subsequently, TLC was used for the separation and detection made using fluorescence.

2.5.1.2 Solid-Phase Separation Technique

For a sample that is liquid in nature, the solid phase extraction technique is appropriate for toxin extraction. There are columns for this purpose i.e. sample purification which has proven not to be only efficient but quick and cost-effective. In the solid phase extraction (SPE) columns or cartridges, the use of substances such as silica gel and florisil is very common. The mechanism is such that the column retains the toxin whereas the non-toxin substances are washed off and subsequently, the toxin is eluted. In a one-step clean-up, sample extract is added to the sample reservoir and by use of a rubber syringe, the sample extract is pushed through the SPE column to purify the sample. The extract that has been decontaminated is then fetched at the base of the conduit which will have the analyte or toxin of interest, and can then be derivatized almost immediately and analyzed using appropriate equipment. SPE unfortunately is not like immunoaffinity columns which can concentrate the toxin through clean-up step. Recovery may also differ depending on type and complexity of sample (Zheng *et al*., 2006). Manetta *et a*l. (2005), in their study, documented the use of solid phase extract as a means to get extract for

AFM1 determination and quantification using high performance liquid chromatography (HPLC).

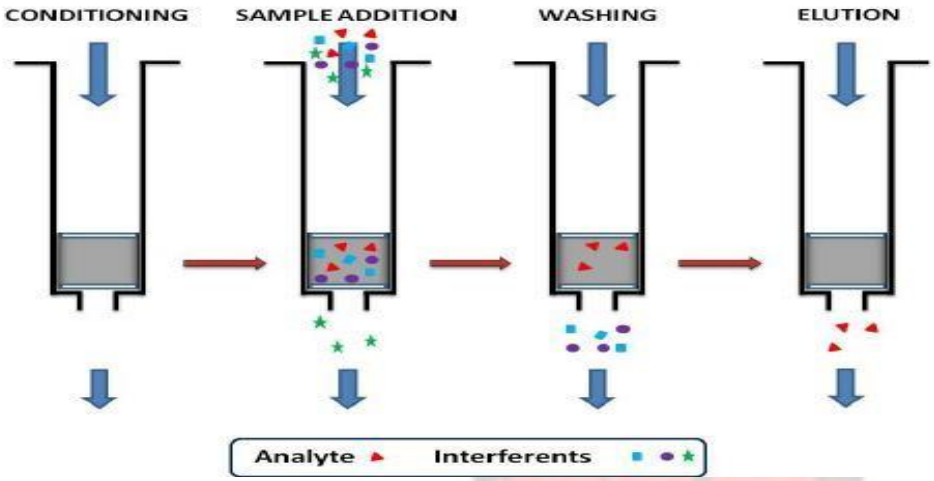


Fig. 5: Schematic diagram of SPE clean-up procedure. (Lucci *et al*., 2012)

2.5.1.3 Immunoaffinity Column (IAC)

International Organization for standards (ISO) method (ISO 14501:2007) and International diary federation method (IDF 171:2007) which involves the analysis of AFM1 in milk, rely on the use of Immunoaffinity columns (IAC) during clean-up step. IACs offers optimum selectivity and very easy to use as well. With this clean up method, the analyte (mycotoxins) are bound selectively to antibodies which is restrained against a compact platform like agarose gel in a phosphate buffer after a pre-conditioning step. A subsequent washing step and pouring an appropriate solvent e.g. methanol into the column, causes denaturation of antibodies thereby releasing the toxin to be eluted. An advantage for using IAC clean-up is the fact that the mycotoxin can be concentrated in the column hence the sensitivity of fluorometric assay is increased. Nonetheless, there is a limit to which IACs can be loaded in terms of size, also the procedures for clean-up using the IACs is not as simple as SPE etc.

(Zheng *et al*., 2006).

2.5.2 Detection Methods

Once the analyte (toxin) has been eluted and cleaned up, the next procedure is identification and quantification. In recent years, lot of methods used for analyzing and quantifying aflatoxins in diverse foodstuff. Key amongst the available methods are high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and enzyme-linked immunosorbent assay

2.5.2.1 Use of high-performance liquid chromatography (HPLC)

When substances are mixed together, the use chromatography can be employed to separate the substances. Almost every type of chromatography operates on identical principle. They have two major phases i.e. stationary and mobile. The stationary phase is usually solid or liquid that is reinforced on a solid whereas the mobile phase is usually a gas or liquid. ―The HPLC system of aflatoxin estimation has high precision, sensitivity, and high automation. This method retains two phase systems: normal phase (liquid/solid, polar stationary phase) and reverse phase (liquid/liquid, polar mobile phase) in conjunction with UV absorption and fluorescence detection‖ (Sinha, 1999). HPLC happens to be the most reliable method employed for ultimate separation and toxin detection when it comes to aflatoxin analysis however prior to use of the HPLC, extraction and clean-up techniques must be applied to the analyte.

Use of HPLC for determination and quantification of mycotoxins especially aflatoxins have been demonstrated by many authors such as Manetta *et al*. (2005); Elgerbi *et al*. (2004) and Chavarría *et al*. (2015) during their work on aflatoxin M1 determination in milk and dairy products.

Commonly used HPLC systems are Shimadzu from Japan and Agilent from USA. All HPLC systems have many components, which includes a liquid pump, column oven, system controller

detectors (fluorescent detector, ultra violet (UV) detector, diode array detector), a software and a communication bus.

Derivatization

Derivatization is applied in the analysis of aflatoxins when enhancement of the detection at minimal concentration is desired, in this regard a fluorophore is usually added to achieve this aim by improving the normal fluorescence of the toxin.

Pre-or post-column derivatization is therefore done to enhance detection. Derivatization of aflatoxins is usually done using strong acids or oxidants e.g., Br_2, I_2. Manetta *et al*. (2005) developed a post-column derivatization method coupled with Hplc for the analysis of AFM1 and found that use of Pyridinium Bromide Perbromide (PBPB) as a post column derivatization agent improved the analysis of aflatoxin M1. The method was simple, quick and easy to automate as well as enhancing reproducibility.

2.5.2.2 Use of thin-layer chromatography (TLC)

Thin layer chromatography (TLC) is one other important method for the analysis of mycotoxins. It is falls under chromatographic procedures employed in identification and quantification of aflatoxins. An important advantage of this method is that it detects and measures toxins at very low concentrations (up to 1ng/g).

The TLC just like the other separation techniques, entails both stationary and mobile phase. In most cases, the mobile phase is a solvent. The analyte, is introduced via the stationary phase and would have initialed been liquified using volatile solvent. The technique depends on capillary action to achieve results. The AOAC has adopted use of thin layer chromatography as the official method for detection and quantification of aflatoxins.

The offline operating principle TLC is one key feature of this technique. The TLC plate can sometimes be used to both isolate impurities from mixture of interest and at the same separation of compounds of interest (Lin *et al*., 1998).

The use of TLC for the determination of AFM1 has been demonstrated by Sessou *et al*. (2013). The study was conducted in Benin where AFM1 was detected in wagashi i.e. a local cheese. In Iran, Kamkar (2006) determined AM1 in Feta cheese using TLC. The range of detection was between 0.15 – 2.41µg/kg.

2.5.2.3 Use Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is now extensively employed in the analysis of aflatoxins because it is simple, sensitive and can easily be used almost everywhere. They have been available for mycotoxin assay for more than a decade (Zheng *et al*., 2006). Two types exist i.e. direct and indirect competitive enzyme-linked immunosorbent assay. The difference between the two methods is that, on the solid phase for the direct method, a specific antibody is coated and for the indirect method, a conjugate made of a toxin and a protein is used. The direct method is commonly used in Aflatoxin analysis. According to Aycicek *et al*. (2005), the EISA method is basically a principle of antigen-antibody reaction.

For qualitative analysis of AFM1, the wells in the micro titre strips are coated with specific antibodies against AFM1. A conjugate is added after a washing step. Free AFM1 and AM1conjugate compete AFM1 binding sites. During a second washing step, any un-bound enzyme conjugate washed. Typically, a color change indicates presence of an analyte or antigen. A specific substrate solution is subsequently added and color measured using a spectrophotometer or visual comparison with standard toxin (Sinha, 1999)

Rastogi *et al*. (2004); Oveisi *et al*., (2007) and Duarte *et al*. (2013) analysed aflatoxin M1 in milk and dairy products using the enzyme-linked immunosorbent assay method.

2.6 Global Regulatory Limits for Aflatoxins

Most international organizations concerned with food safety especially the world health organization (WHO), Codex Alimentarius and European Food Safety Authority (EFSA) are regularly involved in the assessment of mycotoxins across the globe. Coupled with that, most countries especially the developed and advanced countries have government regulatory authorities clothed with the powers to set maximum permissible limits for mycotoxins especially Aflatoxins in food commodities and regulate same. Particularly, for aflatoxin M1 limits to be achieved, regulatory bodies in addition, also set maximum allowable limits for Aflatoxin B1 in feed. The international regulations for the maximum limit for AFM1 in milk and some dairy products are shown in Table 3 below.

Usually in practice, the AFM1 regulatory limit is defined as the total concentration in milk which is equivalent to 1.7% (range from 0.8 – 2.0%) of the concentration of the total AFs in dry matter. This implies that cattle consuming AFs above 30 μg/kg will excrete AF residue in milk above 0.5 μg/kg (EFSA, 2004).

Table 3: Specific countries and regulatory limits for AFM1 (Iqbal et al. 2015)

Nation	Limit for milk (ppb)	Diary product (ppb)
USA	0.5	0.5
EU	0.05	0.05
Austria	0.05, 0.01 (pasteurized infant milk)	0.02 (butter) 0.25 (cheese) 0.4 (powdered milk)
France	0.05 0.03 (for children < 3yrs)	
Switzerland	0.05	0.025 (milk whey and products) 0.25 (cheese) 0.02 (butter)
Bulgaria	0.5	0.10 (powdered milk)

Brazil		0.50 (liquid milk 5.0 (powdered milk)
Check Republic	0.05	
Romania	0	0
Turkey	0.05	0.25 (cheese)
Argentina	0.05	0.5 (milk products)
Honduras	0.05	0.25 (cheese)
Egypt	0	0
Nigeria	1	
Iran	0.5	

As indicated in Table 3, the EU has set the total AF maximum levels in milk as 0.05 µg/kg whereas the Food and Drugs Administration of the USA, has set the maximum AF levels in ilk as 0.5 µg/kg. Yet, the Codex Alimentarius set the regulatory limit as 50 ng/kg and that of follow-on milk as well as infant milk not more than 0.025 µg/kg (European Community, 2006). For feed, a limit of 5 µg/kg destined for dairy cow and 20 µg/kg for cattle is applied by the European community (EC, 2006 whereas in the USA, the FDA has prescribed 20 µg/kg and 100 µg/kg for diary feed and cattle feed respectively (FDA, 2009).

2.7 Exposure Assessment under risk analysis

Risk Analysis comprises of, risk assessment, risk communication and risk management (Figure 8). The Risk assessment section is made up of: hazard identification, hazard characterization, risk assessment, and risk characterization (WHO, 2014). The risk assessment gives a systematic assessment of identified or probable wellbeing concerns ensuing because of human contact with food hazards via ingestion.

At the global level, risk assessment helps with assigned bodies to come out with guidelines, Standards ensuring food safety requirements are acceptable and suitable for adoption in global trade with the aim of protecting consumers.

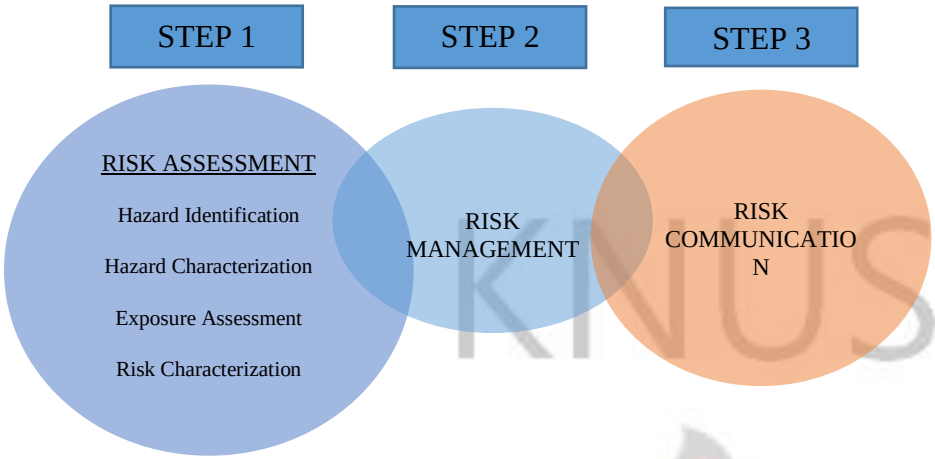


Figure 6: Schematic diagram of Risk Analysis Components

2.7.1 Hazard Identification

Hazard identification is the first step in the risk analysis process and the first of four steps in risk assessment. It involves the identification of biological, chemical and physical agents capable of causing adverse health effects and may be present in a food or group of foods. Thus, effects considered as adverse are identified regardless of the dose and mechanisms required to elicit the effect.

Most studies for hazard identification are designed to investigate both effect and no-effect dose levels as well as no observed adverse effect level (NOAEL) which is helpful in arriving at acceptable or tolerable daily intakes.

2.7.2 Hazard Characterization

The second stage of the risk assessment is Hazard characterization (IPCS, 2004) which looks at the qualitative and/ or quantitative evaluation of the inherent property of an agent (biological, chemical, physical) to cause an adverse effect. A dose response relationship where applicable, is established at this stage which is the principal aim of this step. Also, a central theme is the identification of mode of action and their relevance for human situations as mostly animals are experimentally used to predict potential toxic effects in humans.

For carcinogens including metabolites, the presumption of no threshold is appropriate (Klaunig and Kamendulis, 2008). It is therefore assumed that above zero, there is no safe dose for carcinogens. Aflatoxin B1 from which Aflatoxin M1 is derived, therefore has no exposure threshold below which cancer would never occur. IARC has classified AFM1 as Group 1 carcinogen -meaning it is carcinogenic to humans (IARC, 2002). No safe exposure level has been established for AFB1 due to its genotoxic carcinogenic potential (JECFA, 2007).

2.7.3 Exposure Assessment

Exposure assessment is the aspect of the risk assessment process that estimates or measures the magnitude, frequency and duration of exposure to an agent. It takes into consideration the characteristics of the population exposed and describes the sources, pathways and any uncertainty that may be associated with the assessment. Dietary exposure assessment makes use of both food consumption data and data on concentrations of the chemical in the food.

Ingestion of contaminated food is considered the major route of exposure to many contaminants either caused by industrial or environmental contamination or as result of production processes during human health risk assessment (Santini and Ritieni, 2013). Several methods exist for the estimation food chemical intake however the choice of the method depends on the information available and how accurate the data needs to be.

2.7.3.1 Approaches Available for Conducting Exposure Assessment

Exposure assessment can be done using two main approaches i.e. direct and indirect. The direct also sometimes called biologic, uses biological monitoring techniques to estimate exposure to an agent or toxin in a medium over a stipulated time. Biological fluids such as urine (Kumi *et al*., 2015) and breastmilk (Makun *et al*., 2016; Adejumo *et al*., 2013) have been used for biomonitoring of Aflatoxin M1.

Biological monitoring however has its limitation due to lack of validated biomarkers for most toxicants and most importantly the invasive nature of toxicants. This approach however has an advantage over the environmental approach as concentration of the toxicant measured in the biological fluid gives an indication of the cumulative effect from all possible exposure routes (WHO, 2006)

2.7.3.2 Food consumption Data

Food consumption is usually expressed as per gramme of food consumed per person per day. Since different population groups show variability in food consumption pattern, to assess dietary exposure to food contaminants, different consumption data and approach may be employed. Vulnerable groups such as infants and children usually have higher estimated exposure level as their food consumption per kilogram body weight is higher (EFSA, 2009).

Assessment of food consumption can be done using two major methods i.e. Prospective methods and Retrospective methods. The Prospective methods such as dietary records and recalls, record food consumption data at the time the food is being consumed and the retrospective such as diet history and Food Frequency Questionnaire (FFQ) make use of data over a specified period. It comprises of a structured or pre-determined list of items with aim to assess the frequency with which the listed food items are consumed over a specified period. The period normally varies from daily, monthly, yearly etc. FFQs are intended to collect dietary information from huge numbers of individuals (100 individuals and above) and are normally self-administered, however trained interviewer administered is also possible including telephone interviews as demonstrated by Haraldsdottir *et al*. (2001).

2.7.3.3 Estimating Dietary Exposure

To estimate actual exposure, several methods exist that is used to combine both consumption estimates and chemical contamination of food. The selection of method however depends on

number of factors including nature of population, purpose of the assessment, ease of availability of information and desired accuracy of outcome. Two major methods usually used to combine consumption data and chemical contamination to estimate exposure include; (I) deterministic approach, and (ii) probabilistic or stochastic approach.

2.7.3.3.1 Deterministic Method

A deterministic approach to estimate dietary exposure can simply be said as a value that is characteristic of an exposure of a consumer. Mean dietary exposure, can be estimated by multiplying mean consumption and average residues of the substances in the food medium. Such single value estimation is fundamentally not accurate. The method of estimation of a single value has a lot of conservatism and assumptions.

In Catalonia in Spain, Cano-Sancho *et al*., (2010) used a deterministic approach alongside probabilistic approach to estimate the exposure of Catalonians to AFM1. In both approaches, they arrived at similar values for exposure. Similarly, in Pakistan, Ismail *et al*. (2016) used the deterministic approach to estimate dietary exposure of Pakistanis to AFM1 through milk and dairy products. The study found the exposure to be between 0.22 - 5.45 ng/kg/day with children being the most at-risk group and adults being the least.

2.7.3.3.2 Probabilistic Method

For the purposes of safety of food, an accurate measure is usually required in order not to disregard this concern during initial assessment. The use of improved methods enhances the accuracy of exposure estimates. Probabilistic or stochastic method, gives improved estimation but not necessarily a lesser dietary exposure value compared to the deterministic method. Conceptually, population exposure is a series of values, but not a sole value, since individuals experience different exposure levels in a population. Age, sex, nationality and region are factors that influence variability.

A probabilistic model is like the deterministic however an important differentiation factor is that a variable is represented as a distribution function and not a single value for probabilistic method. Two approaches to obtaining distributions for a stochastic assessment, is parametric and non-parametric. Non-parametric technique is usually applied once actual data sets are obtainable for a parameter, in this case, the data is considered to represents distribution of interest. Parametric techniques interpolate the data points and extrapolate beyond them by using a particular distribution form. Cano-Sancho *et al*. (2013) and Cano-Sancho *et al*. (2010) used both probabilistic and deterministic approach to estimate exposure to aflatoxins Catalonia, Spain.

2.7.4 Risk Characterization

Risk characterization ends the risk assessment process. The IPCS (2004) defines risk characterization ―as the qualitative and, wherever possible, quantitative determination, including attendant uncertainties, of the probability of occurrence of known and potential adverse effects of an agent in a given organism, system, or (sub)-population, under defined exposure conditions‖.

Evidence from exposure assessment and the hazard characterization, during risk characterization, is put together into an advice suitable for decision making in risk management Advice provided to risk managers may be qualitative or quantitative (FAO/ WHO, 2009). The qualitative information may include; providing evidence that the chemical is not toxic even when one is exposed to it at high concentrations, and evidence that the chemical is safe when used as recommended. Quantitative information may also include; estimation at diverse levels, risk associated with dietary intake as well as making comparison of dietary exposure and guidelines based on health

Finally, the risk characterization step involves comparing the EDI or TMDI with the healthbased toxicological value such as ADI, ARfD etc. as the case may be

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIAL

The matrices selected for this work includes raw cow milk and raw cow milk products *("wagashi", "Burkina drink")* due to their susceptibility to aflatoxin M1 contamination. An aflatoxin M1 standard obtained from Sigma-Aldrich was used for this study.

3.2 Equipment and Instrument

Instruments and equipment used for this study included a centrifuge, Metler Toledo 10035 analytical balance, vacuum system, analytical column, and High-Performance Liquid Chromatography (HPLC), Adept Cecil CE 4800 coupled with an automatic sampling system unit and Shimadzu RF-10AXL Fluorescence detector (FD). Determination and quantification of AFM1 of the raw milk, *wagashie* and burkina drinks were done at the Mycotoxin laboratory facility at Kwame Nkrumah University of Science and Technology (KNUST).

3.3 Reagents and Chemicals

HPLC grade acetonitrile, Vicam HPLC grade methanol, n-hexane, 20% acetonitrile, Pyridinium Bromide Perbromide (PBPB), analytical grade sodium chloride, acetone and dichloromethane. SPE -C18 cartridges were supplied by Waters Corporation, USA.

3.4 Study Area

This work was performed in the Greater Accra region, Accra, which is Ghana's capital town. The choice of Accra is mainly due to the populous and cosmopolitan nature of the city. There is also an increasing trend of sale of raw cow milk and its products i.e. ―*Burkina drink*‖ and *"wagashi*‖ in Accra. According to the Ghana Statistical service (GSS) (2010), Accra is 3,245sq/km and a total population of 4,010,054 and intercensal growth rate of 3.1%. The three specific areas used for this study include Ashaiman, Nima and Madina. These areas are known to have high patronage of raw cow milk, *Burkina drink* and *wagashi*. The Ashaiman municipal district has a population of 190,972, Madina previously under the Ga East

Municipal district has a population of about 259,668 and Accra Metropolis which includes Nima, has a population of about 1,848,614 (Ghana Statistical Service, 2010). Figure 7 below shows the map of Greater Accra region, with arrows demonstrating specific districts within which the work was carried out.

Figure 7: Map of Greater Accra Region indicating Districts.

Source: https://en.wikipedia.org/wiki/Districts_of_Ghana

3.5 METHOD

3.5.1 Sampling

Commercially available raw cow milk, *Burkina* and *wagashi* were purchased from the local market in three districts within the greater Accra region for Aflatoxin M1 analysis by HPLC. The districts comprised of Ashaiman municipal, La Nkwantanang-Madina Municipal Assembly and Nima located in the Accra metropolitan Area. At each sampling area, samples were collected making use of random sampling method. In all, 83 samples were sampled from the three districts, which included 30 raw cow milk samples, 30 *wagashi* samples – a soft local cheese made from raw cow milk, and 23 *Burkina* drinks – a local drink made using raw cow milk and millet.

The samples collected were transported in ice-coolers from Accra to the Mycotoxin Laboratory, KNUST, Kumasi. On arrival, the purchased samples in the ice-coolers were transferred and refrigerated (stored) at temperatures below 0°C until extraction and analysis were performed.

3.5.2 Sample Preparation

3.5.2.1 Raw Cow Milk

The raw cow milk samples were homogenized and centrifuged at 3000 x g for 15 - 20 minutes by measuring 0.30 l of the raw milk into a 0.50 l polypropylene centrifuge tube. Since aflatoxins are water soluble, the supernatant was then collected and transferred into capped tubes. 1 0ml of the aqueous phase was diluted with 10 ml of de-ionized water for extraction and clean up (Manetta *et al.*, 2005).

3.5.2.2 Burkina Drink

Same sample preparation done for raw cow milk was applied to the Burkina drink. The supernatant thereof was used for extraction and subsequent aflatoxin M1 analysis.

3.5.2.3 Wagashi (local cheese)

The *wagashi* samples were prepared using the method demonstrated by Manetta *et al*. (2005) and making minimal adjustment to the method. 10 g of the *wagashi* (local cheese) was cut into small pieces and 50ml dichloromethane/acetone (1:1, v/v) and 10 g of sodium chloride were added. This was subsequently centrifuged at 3000 x g for 15 - 20 minutes. Once centrifugation was done, the extract was dried using nitrogen. The amount of extract used for the drying was 0.01litres. 0.5ml of methanol was then used to dissolve the residue followed by 0.01mol/l of sodium phosphate -buffered saline (PBS). 10ml of *n*-hexane were added and shaken. The aqueous phase was collected for clean-up.

3.5.3 Aflatoxin M1 Standard Stock Solution

Using acetonitrile solution of 0.1 µg/l, a stock solution of Aflatoxin M1 (standard) was prepared stoppered and wrapped in aluminum foil. This was stored in the refrigerator at temperatures between -1°C to 5°C. Subsequent Aflatoxin M1 standard solutions used for this work were done by appropriately diluting the standard solution in 10% acetonitrile solution. Concentrations of 0.05 ng/ml, 0.01 ng/ml, 0.02 ng/ml and 0.04 ng/ml of AFM1 were prepared and used to construct a standard calibration cure which was liner.

3.5.4 Extraction and Purification of Samples

The method used for extraction and clean-up is as described by Manetta *et al*. (2005) with minimal alteration. SPE -C18 cartridges were prepared using 5ml acetonitrile, followed by 10ml deionized water. The sample that had been diluted was then introduced onto the conditioned SPE- C18 cartridge using a vacuum pump at a flow rate of 2-3 ml/min. The SPE

cartridge was then washed with 10 ml of deionized water and subsequently using 20 ml of acetonitrile/water (20:80, v/v) and n-hexane (10ml). Afterwards, the SPE cartridge was dried for 10-20 min at 40-50 °C or overnight at ambient temperature (Bottcher and Monks, 2017). After drying, the sample was finally eluted with 3 ml of acetonitrile for HPLC analysis.

3.5.5 Quality Control

To determine the efficacy of the HPLC equipment in determining AFM1 levels in the raw cow milk and milk products, commercially available branded processed milk was spiked at a specified concentration and a sample with no artificial contamination (i.e. acetonitrile) – a blank, was also used to perform a recovery test.

3.5.6 Analysis of Aflatoxin M1 using HPLC

An eluent (25% acetonitrile solution) was pumped through the HPLC column at a constant flow rate. The optimal condition was determined by making use of an extract in combination with AFM1 standard working solution injected separately. The suitability of the chromatographic conditions was determined using a specific quantity of Aflatoxin M1 solution and frequently introducing it into the HPLC until a peak area and height that were unchanging were obtained. Subsequent introductions were stable and showed a maximum of 5% difference in both peak area and height. Suitable volumes of the AFM1 standard solution (serial dilutions) was then injected into the HPLC apparatus via the injection loop. A calibration curve was then prepared. A similar volume of the eluate to that used for the standard working solutions was subsequently injected into the HPLC apparatus via the injection loop. AFM1 present was separated using same conditions as used for the standard solutions. The area or height of the AFM1 peak of the sample eluate was then determined.

The final concentration of the eluate was calculated from the standard curve.

3.6 Analysis of Obtained Data

Results of the HPLC analysis was analyzed making use of Excel 2013 and SPSS (Statistical Package for Social Sciences. One-way ANOVA (confidence interval of 95%) was performed to determine statistical difference between the groups as well as general descriptive statistics such as Mean, Standard Deviation, and standard error were calculated.

3.7 Exposure Assessment

A deterministic approach was used for the exposure assessment during this study.

3.7.1 Food Consumption

Since average milk consumption statistics in Ghana are generally lacking, to calculate average raw cow milk intake for people in selected areas (Nima, Madina, Ashaiman), a similar approach adopted by Cano-Sancho *et al*. (2010) for AFM1 exposure in Catalonia (Spain) was used. A food frequency questionnaire capturing food items known to be consumed with raw cow milk or uses raw cow milk in its preparation was administered in the selected communities. About 300 respondents of different age categories (1-5, 6-10, 11-19,
20-45, 46-60 and >60 years) were interviewed on their milk consumption habits. Using mean Aflatoxin M1 concentration from the HPLC analysis, average consumption of milk and the average body weight for the various age categories considered under this study, the average ingestion of Aflatoxin M1 per body weight per day was calculated.

3.7.2 Body Weight Estimation

The average body weight of the different age groups as mentioned above, were estimated from the feedback from questionnaire. Individuals were asked for their body weight and recorded. For those who did not know their body weight, a calibrated portable scale was provided to check body weight

3.7.3 Consumption Rate Estimation

The average consumption of raw cow milk & products for each age group was calculated using formula I below;

Consumption Rate (kg/day) = Weight of milk (per serving) x Meal frequency (per day)

Formula I

The mean values of ingestion for each age group was calculated using above formula.

3.7.4 Dietary Exposure Estimation

The exposure level sometimes referred to as Estimated Daily Intake (EDI) for the toxin (AFM1) contamination of the raw cow milk & products was calculated for each age category using formula II.

$$EDI = \frac{C \times CR}{BW} \quad \text{..... Formula II}$$

Where EDI is the estimated daily intake (ng/kg/d), C represents mean concentration of AFM1 detected in the milk samples (ng/kg), CR denotes consumption rate (kg/d) and mean body weight in Kg represented by BW.

CHAPTER 4

RESULTS

4.1 Quality Control

To assure quality of analysis, samples were analyzed in triplicates as well as solvent blanks and artificial contamination (spikes) were performed. Spiking was done to ensure there was accuracy as well as efficiency in the HPLC method adopted. The solvent blanks were run to eliminate any interferences from reagents. Triplicate samples were to assure precision of the

analysis. The calibration curve obtained was linear with equation, y = 0 +3.47332 x Area (As) and 0.998 as the correlation coefficient (r^2) (Fig. 8). Percentage recovery of Aflatoxin M1 range between 70 – 80% for the raw cow milk.

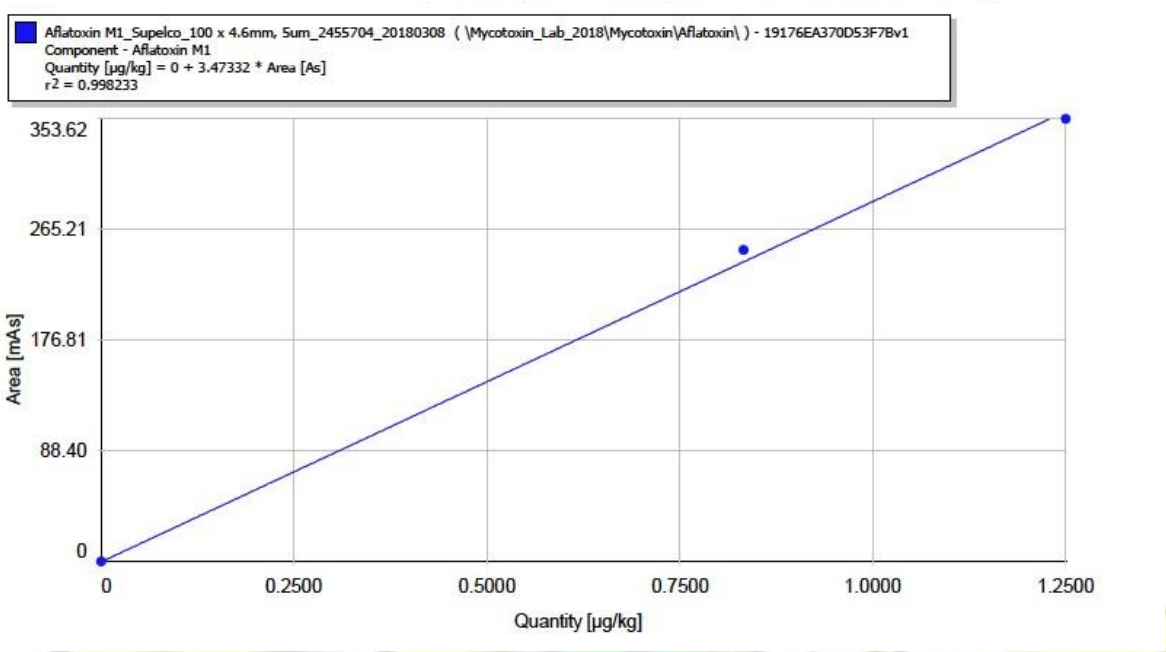


Fig.8: Aflatoxin M1 standard calibration curve

4.2 Aflatoxin M1 in raw cow milk

Commercially available raw cow milk samples were purchased from selected markets for analysis. SPE, C-18 cartridges were used for the extraction and cleanup of the raw cow milk samples. After the clean-up, by use of HPLC coupled with fluorescence detector and PBPB as a post column derivatization agent, Aflatoxin M1 contamination was confirmed.

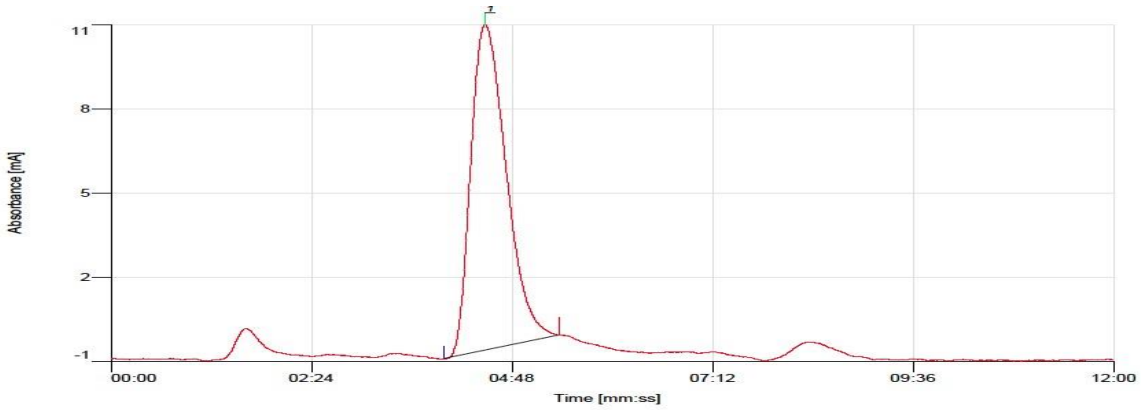


Fig. 9: Chromatogram of Aflatoxin M1 standard using HPLC coupled with fluorescence detector and PBPB as post column derivatization agent.

The above chromatogram (Fig. 9) depicts peaks devoid of interferences in the region where AFM1 was eluted illustrating efficiency of the method used. The retention time of almost 5 minutes though short did not allow un-retained samples to interfere with elution. The outcome is comparable to chromatograph described by Manetta *et al*. (2005).

The shape and peak which is characterized by perfection and clarity may have been because of the post column derivatization agent known to help make fluorescence of aflatoxins more noticeable regardless of the concentration levels.

The chromatograms represented in Fig.10 and 11 show sample of raw milk analyzed for aflatoxin M1 which was *positive* and *negative* respectively.

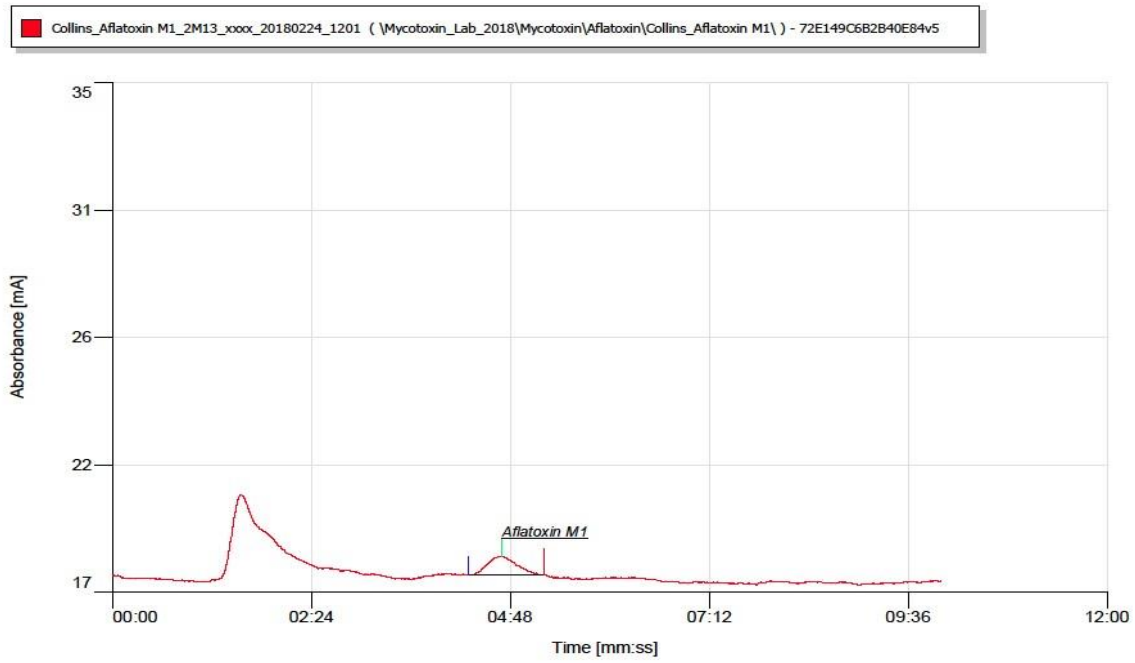


Fig 10: Chromatogram of Aflatoxin M1 of raw cow milk sample using HPLC coupled with fluorescence detector showing positive detection.

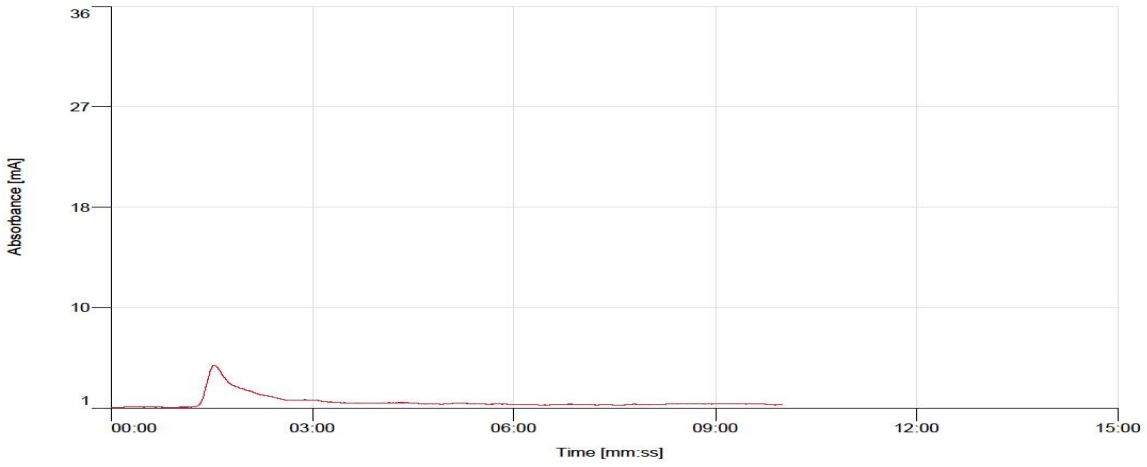


Fig 11: Chromatogram of Aflatoxin M1 of raw cow milk sample using HPLC coupled with fluorescence detector showing no detection.

The peak shown in Fig. 9 occurs at same time as that of the aflatoxin M1 standard chromatogram indicating presence of aflatoxin in the raw milk sample. However, in Fig. 10,

the chromatogram shows no peak at the expected retention time indicating absence of aflatoxin M1 in that sample.

A total of thirty (30) raw cow milk samples were tested for Aflatoxin M1 contamination. The quantification figures obtained are presented in Table. 4.

Table 4: Aflatoxin M1 quantification values obtained for raw cow milk using HPLC coupled with fluorescence detector and using PBPB as post derivatization agent.

Sample No.	Triplicate analysis (µg/l)			Average (µg/l)
	R1	R2	R3	
S1	2.0646	2.0646	2.1186	2.0826
S2	3.7788	3.7806	3.7278	3.7624
S3	0.8784	0.9072	0.8772	0.8878
S4	0.546	0.546	0.546	0.546
S5	0.3396	0.3738	0.3486	0.354
S6	NI	NI	NI	0
S7	NI	NI	NI	0
S8	NI	NI	NI	0
S9	NI	NI	NI	0
S10	NI	NI	NI	0
S11	NI	NI	NI	0
S12	NI	NI	NI	0
S13	NI	NI	NI	0
S14	NI	NI	NI	0
S15	NI	NI	NI	0
S16	NI	NI	NI	0
S17	NI	NI	NI	0
S18	NI	NI	NI	0
S19	NI	NI	NI	0
S20	NI	NI	NI	0
S21	NI	NI	NI	0
S22	NI	NI	NI	0
S23	NI	NI	NI	0
S24	NI	NI	NI	0
S25	NI	NI	NI	0
S26	NI	NI	NI	0
S27	NI	NI	NI	0
S28	NI	NI	NI	0
S29	NI	NI	NI	0
S30	NI	NI	NI	0

NI = Not identified

Five (5) raw cow milk samples representing 16.67% were contaminated with aflatoxin M1. The least and highest contamination respectively, reported from the HPLC analysis are 0.35 µg/l and 3.76 µg/l. Both the least and highest incidence exceeded 0.05 µg/kg which is the EU permitted level.

As indicated in Table 5, the mean of the positive samples (1.53±1.42) µg/l aflatoxin M1 occurrence per results from the analysis using HPLC shows that the levels of aflatoxin M1 in the raw cow milk samples exceeds the permitted levels within the EU i.e. 0.05ppb. A summary of the results are shown in Table 5 as well as Fig. 11.

Table 5: Summarized results of aflatoxin M1 analysis of raw cow milk using HPLC coupled with fluorescence detector.

Sample Type	Analytical sample	Percentage positive	Mean con. (µg/l)	SD	Maximum	<LOD
Raw cow milk	30	5/30 (16.67%)	1.53	1.42	3.76	25/30 (83.33%)

SD = Standard deviation, LOD = Limit of detection

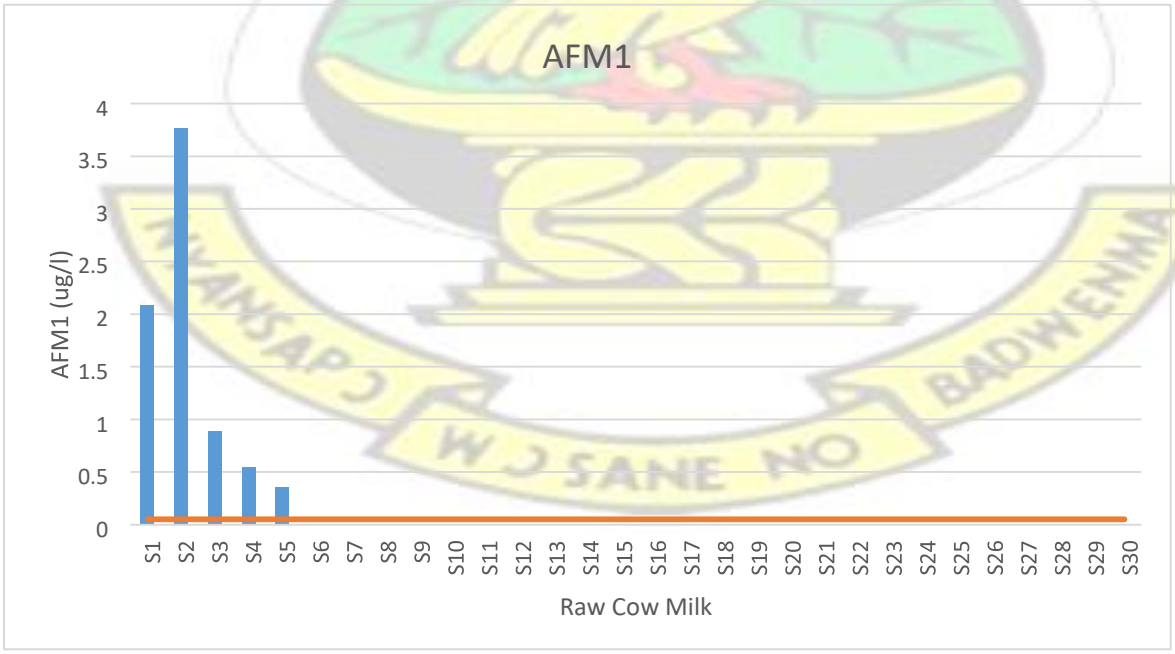


Fig.12: Aflatoxin M1 quantification in raw cow milk samples with permitted EU limit

4.3 Aflatoxin M1 in Burkina drink

Burkina drink samples were purchased from selected markets for AFM1 analysis. SPE, C-18 cartridges were used for the extraction and cleanup. This was followed by confirmatory analysis using HPLC coupled with fluorescence detector using PBPB as a post column derivatization agent.

Fig. 12 and 13 respectively show positive and negative chromatogram for Burkina drink analyzed for aflatoxin M1.

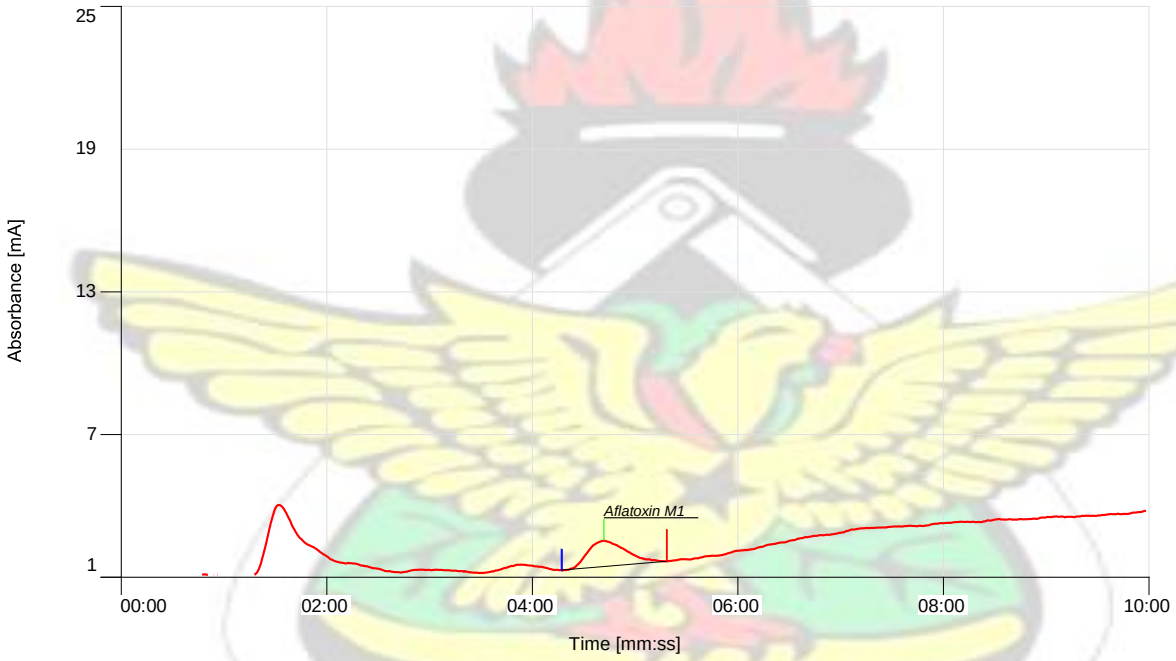


Fig. 13: Chromatogram of Aflatoxin M1 of Burkina drink sample using HPLC coupled with fluorescence detector showing positive detection.

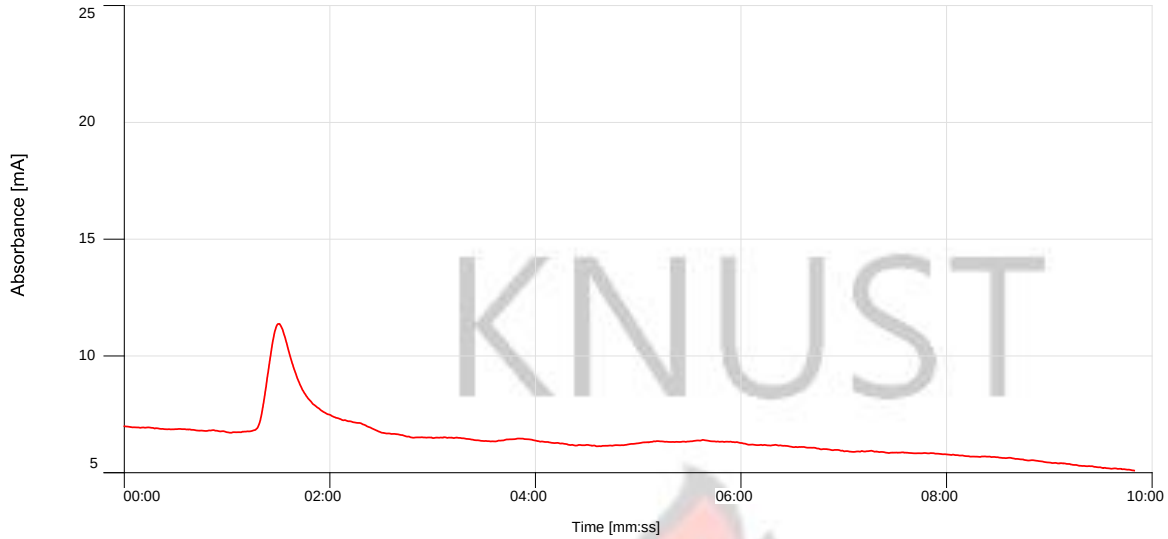


Fig. 14: Chromatogram of Aflatoxin M1 Burkina drink sample using HPLC coupled with fluorescence detector showing no detection.

The peak shown in Fig. 13 occurs at same time as that of the aflatoxin M1 standard chromatogram indicating presence of aflatoxin M1 in the *Burkina drink*. However, in Fig. 14, the chromatogram shows no peak at the expected retention time indicating absence of aflatoxin M1.

A total of thirty (30) *Burkina drink* samples were tested for Aflatoxin M1 contamination. The quantification figures obtained are presented in Table 6.

Table 6: Aflatoxin M1 quantification values obtained for Burkina drink using HPLC coupled with fluorescence detector and using PBPB as post derivatization agent.

Sample No.	Triplicate analysis (µg/l)			Average (µg/l)
	R1	R2	R3	
S1	NI	NI	NI	0
S2	NI	NI	NI	0
S3	NI	NI	NI	0
S4	NI	NI	NI	0
S5	NI	NI	NI	0

S6	0.6552	0.6552	0.6738	0.6614
S7	NI	NI	NI	0
S8	NI	NI	NI	0
S9	NI	NI	NI	0
S10	NI	NI	NI	0
S11	NI	NI	NI	0
S12	NI	NI	NI	0
S13	NI	NI	NI	0
S14	0.2952	0.3204	0.3006	0.3054
S15	0.5886	0.5898	0.5826	0.58
S16	0.256	0.252	0.252	0.25
S17	0.3264	0.366	0.327	0.3398
S18	0.2838	0.2838	0.2844	0.284
S19	0.363	0.3672	0.3654	0.3652
S20	NI	NI	NI	0
S21	NI	NI	NI	0
S22	NI	NI	NI	0
S23	NI	NI	NI	0
S24	NI	NI	NI	0
S25	NI	NI	NI	0
S26	NI	NI	NI	0
S27	NI	NI	NI	0
S28	NI	NI	NI	0
S29	NI	NI	NI	0
S30	NI	NI	NI	0

NI = Not Identified

Seven (7) Burkina drink samples representing 23.33% were contaminated with aflatoxin M1. The highest Aflatoxin M1 contamination for *Burkina drink* reported from the HPLC analysis was 0.66 µg/l whereas the least contamination recorded was 0.25 µg/l. All the samples that showed positive including the least and highest incidence were above the EU permitted limit of 0.05 µg/kg.

As indicated in Table 7, the mean of positive samples (0.40 ±0.16) µg/l aflatoxin M1 occurrence obtained from results of the HPLC analysis shows concentrations of Aflatoxin M1 in the

Burkina drink samples were not below the EU acceptable limit i.e. 0.05 ppb. The results are summarized in Table 7 and Fig. 15.

Table 7: Summarized results of aflatoxin M1 analysis of burkina drink using HPLC coupled with fluorescence detector.

Sample Type	Analytical sample	Percentage positive	Mean con. (µg/l)	SD	Maximum	<LOD
Burkina drink	30	7/30 (23.33%)	0.40	0.16	0.66	23/30 (76.67%)

SD = Standard deviation, LOD = Limit of detection

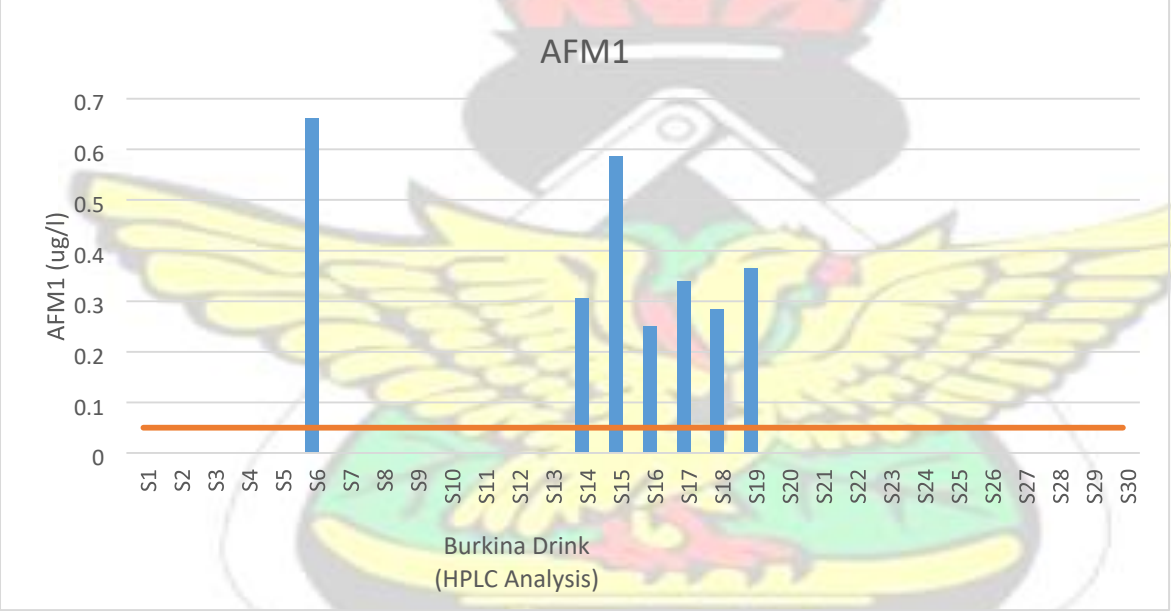


Fig. 15: Aflatoxin M1 quantification in Burkina drink samples with permitted EU limit.

4.4 Aflatoxin M1 in *wagashi* (local cheese)

The *wagashi* samples were purchased from selected open markets for AFM1 analysis. After SPE, C-18 cartridges were used for the extraction and cleanup, confirmatory analysis using HPLC-FD and PBPB as a post column derivatization agent was done.

Fig. 16 illustrates a positive chromatogram for *wagashi* samples with peak occurring within retention time range for AFM1 standard.

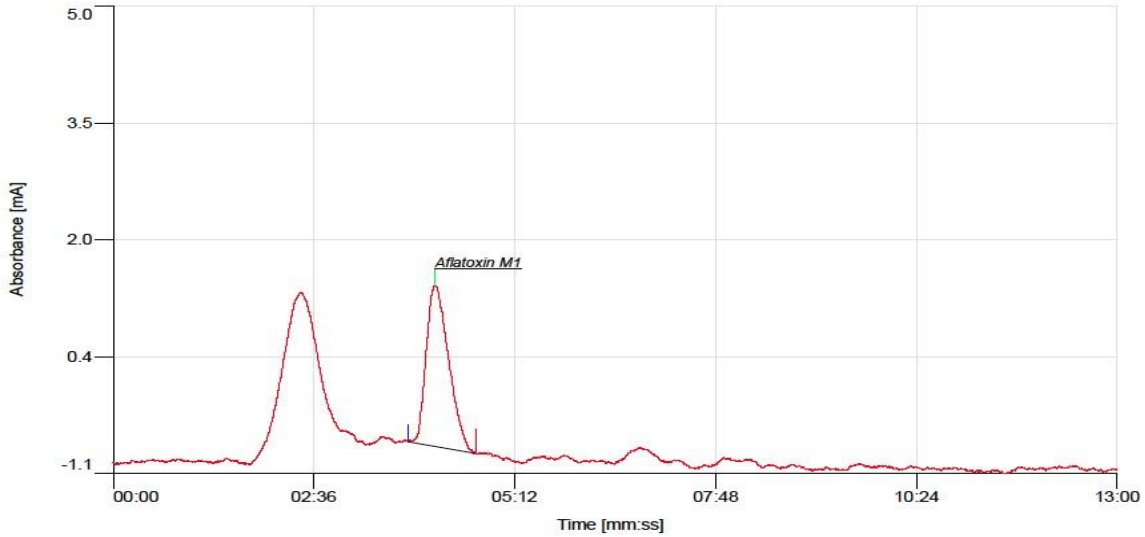


Fig. 16: Chromatogram of Aflatoxin M1 of *Wagashi* sample using HPLC-FD showing positive detection.

A total of thirty (23) *Wagashi* samples were tested for contamination of Aflatoxin M1. The quantification figures obtained are presented in Table. 8.

Table 8: Aflatoxin M1 quantification values obtained for *Wagashi* using HPLC-FD using PBPB as post.

Sample No.	Triplicate analysis (µg/kg)			Average (µg/kg)
	R1	R2	R3	
S1	NI	NI	NI	0
S2	NI	NI	NI	0
S3	NI	NI	NI	0
S4	0.006	0.006	0.006	0.006
S5	NI	NI	NI	0
S6	0.8874	0.8874	0.8568	0.8872
S7	NI	NI	NI	0
S8	NI	NI	NI	0
S9	NI	NI	NI	0
S10	NI	NI	NI	0
S11	0.006	0.006	0.006	0.006
S12	NI	NI	NI	0
S13	NI	NI	NI	0
S14	NI	NI	NI	0
S15	NI	NI	NI	0

S16	0.006	0.006	0.006	0.006
S17	NI	NI	NI	0
S18	NI	NI	NI	0
S19	NI	NI	NI	0
S20	NI	NI	NI	0
S21	0.015	0.018	0.015	0.016
S22	0	0	0	0
S23	NI	NI	NI	0

NI = Not Identified

Out of the twenty-three (23) *wagashi* samples analyzed, only five (5) samples representing 21.74% were contaminated with aflatoxin M1. The highest AFM1 level recorded was 0.8872 µg/kg and least value of 0.006 µg/kg. All positive samples had concentrations exceeding EU tolerable limit (0.05 µg/kg). As indicated in Table 9, the mean concentration of positive samples (0.18 ±0.39) µg/kg of AFM1 recorded for *wagashi* was within the EU permitted levels of 0.05 ppb. The summarized results are in Table 9 and Fig. 17.

Table 9: Summarized results of aflatoxin M1 analysis of *wagashi* using HPLC-FD.

Sample Type	**Analytical sample**	**Percentage positive**	**Mean con. (µg/kg)**	**SD**	**Maximum**	**<LOD**
Wagashi (local cheese)	23	5/23 (21.74%)	0.18	0.39	0.88	23/30 (78.26%)

SD = Standard deviation, LOD = Limit of detection

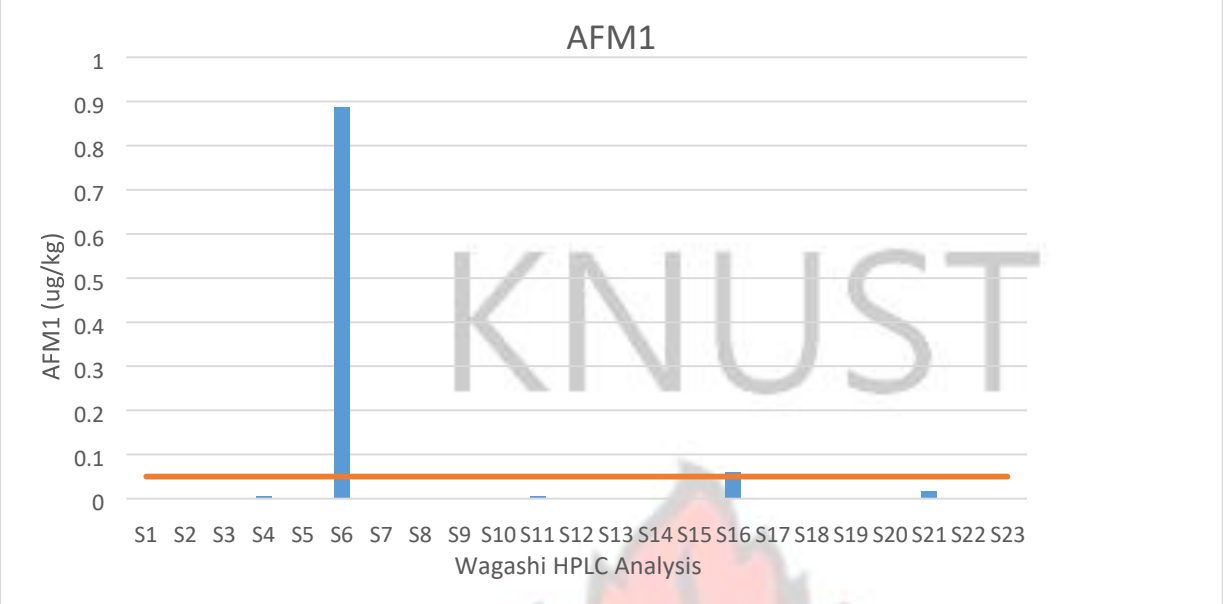


Fig. 17: Aflatoxin M1 quantification in *wagashi* samples with permitted EU limit

4.5 Estimation of Food Consumption

4.5.1 Background Information of respondents

The study was conducted in the Nima, Ashaiman and Madina, which are suburbs in the Greater Accra region. The sample size for this study was 295. The age and sex distribution is shown in Fig. 18 and Fig 19. About 61% of the respondents were males and 39% were females. Majority of the respondents were between the ages 20 – 45years.

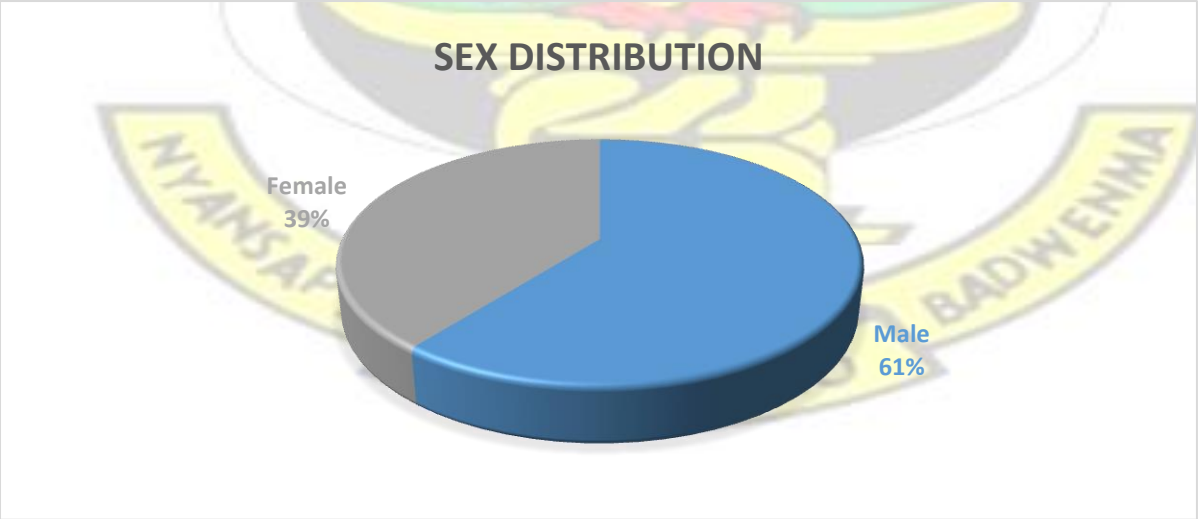


Fig.18: Sex distribution of respondents

The age category with least respondents were 1-5 years followed by >60 years. The age distribution therefore captured infants, adolescents, adults and the elderly giving a good representation of the areas studied.

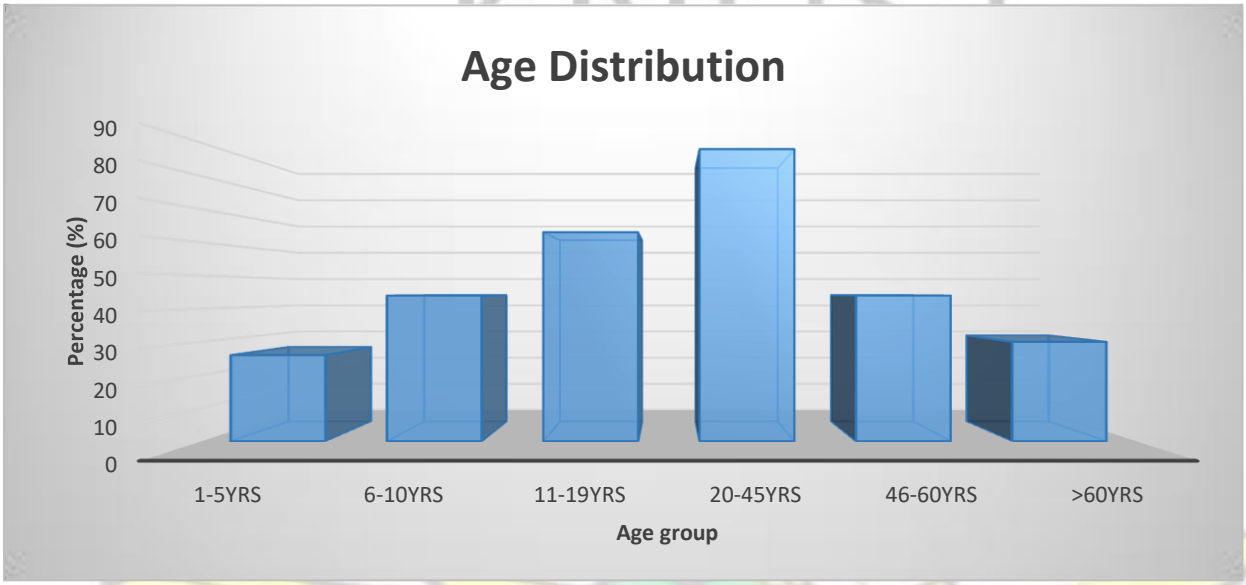


Fig. 19: Age distribution of respondents

4.5.2 Mean body weight and consumption estimation

Food frequency questionnaire was used for gathering data. Respondents were made to recollect the frequency with which they had consumed the food items listed on the questionnaire as well as the quantity each time they took the food item. The recollection was done for the recent past 12 months. Using the mid-point value stated on the questionnaire, the frequency of consumption was captured. Summary of the consumption estimation was done as follows; 4 weeks represented a month, 7days was equated to a week. For example, 1-3 times in a month, per the above conversion, the mid-point is 2 hence this was summarized as 2 times in a month and subsequently converted to 0.5 in a week i.e. (2/4=0.5). To convert the amount per week to per day, 0.5 was divided by 7 (0.5/7 = 0.71).

The mean daily consumption of each listed food (kg) and drink (l) was estimated by multiplying the intake frequency per day and weight of portion size per each time of consumption.

The average daily intake for raw cow milk, *Burkina drink* and *wagashi* for each age category is shown in Table 10.

Calibrated scales were provided to capture the body weight of respondents. Computation of mean consumption and standard deviation was done using SPSS version 20. As shown in Table 8 above, for raw cow milk consumption, the highest occurred amongst females within 11-19 years (adolescent) age category with a mean consumption of 0.41±0.27 l/day. The least average consumption of raw cow milk occurred amongst males of the age category, 6-10 years, with mean consumption of 0.11±0.09 l/day. None of the age categories recorded significant ($p<0.05$) mean consumption with respect to gender.

Table 10: Average intake of raw cow milk and Burkina drink by age categories

Age category	No. of Consumers	Average body weight (kg)	Average ***raw cow's milk*** intake (l/day)	Average ***Burkina drink*** intake (l/day)	Average ***Wagashi*** intake (kg/day)
1 - 5 years					
Male	14	21.50	0.32±0.19	***0.11±0.10***	***0.07±0.04***
Female	12	22.67	0.22±0.16	***0.35±0.14***	***0.12±0.06***
6 - 10 years					
Male	22	35.32	0.11±0.09	0.15±0.13	0.15±0.19
Female	22	36.82	0.17±0.12	0.10±0.13	0.08±0.13
11 - 19 years					
Male	36	55.03	0.35±0.29	0.20±0.15	0.07±0.06
Female	27	57.30	0.41±0.27	0.25±0.21	0.10±0.15
20 - 45 years					
Male	64	69.39	0.26±0.33	0.19±0.23	0.08±0.16
Female	24	66.46	0.25±0.25	0.27±0.25	0.08±0.14

46 - 60 years					
Male	26	75.54	0.31±0.31	0.14±0.23	***0.04±0.04***
Female	18	81.67	0.30±0.22	0.25±0.25	***0.10±0.15***
> 60 years					
Male	18	76.89	0.13±0.11	0.24±0.21	0.04±0.03
Female	12	64.42	0.17±0.17	0.16±0.20	0.06±0.04

Bolden & italic = significant difference ($p<0.05$)

For consumption of *Burkina drink*, the highest mean consumption occurred amongst females within 1-5 years (infants) age category, recording a mean consumption of 0.35±0.14 l/day. The least mean consumption occurred amongst the age category (6-10 years) with a mean consumption of 0.10±0.13 l/day. For age 1-5 category, there was statistically significant differences ($p<0.05$) between sex for Burkina drink consumption.

Generally, the consumption of *wagashi* was low among all the age categories compared to raw cow milk and *Burkina drink* consumption. However, comparing consumption amongst the age groups, the elderly (>60 years) recorded the least mean consumption. Mean levels of 0.04±0.03 kg/day and 0.06±0.04 for males and females respectively were recorded. Infants (1-5 years) recorded statistical difference ($p<0.05$) for consumption between sex. Males within this age group recorded mean consumption of 0.07±0.04 kg/day whereas females recoded 0.12±0.06 kg/day as mean. Another age group that recoded statistical difference ($p<0.05$) between sex for *wagashi* consumption is 46-60 years with mean consumption of 0.04±0.04 kg/day and 0.10±0.15 kg/day for male and female, respectively.

4.6 Exposure assessment to AFM1 using deterministic method

To estimate Aflatoxin M1 dietary intake, a deterministic approach was used by combining mean daily intake of raw cow milk, *Burkina drink* or *wagash*i (taking into consideration individual weight) and average Aflatoxin M1 concentration using the following equation:

individual AFM1 exposure (µgAFM1/kg bodyweight/day) = (food consumed daily /bodyweight) x (average AFM1 concentration (present in drink or food). Exposure to AFM1 through raw cow milk is presented in Table 11.

Table 11: Exposure level/ EDI of AFM1 - raw cow milk consumption

Age category	No. of Consumers	Average body weight (kg)	Average ***raw cow milk*** intake (l/day)	AFM1 intake (**µg**/kgbw/day)	AFM1 intake (**ng**/kgbw/day)
1 - 5 years					
Male	14	21.50	0.32±0.19	0.0036±0.0022	3.679±2.213
Female	12	22.67	0.22±0.16	0.0024±0.0020	2.445±2.001
6 - 10 years					
Male	22	35.32	0.11±0.09	0.0008±0.0006	0.792±0.633
Female	22	36.82	0.17±0.12	0.0012±0.0010	1.199±1.017
11 - 19 years					
Male	36	55.03	0.35±0.29	0.0016±0.0014	1.602±1.384
Female	27	57.30	0.41±0.27	0.0019±0.0014	1.852±1.362
20 - 45 years					
Male	64	69.39	0.26±0.33	0.0009±0.0013	0.982±1.281
Female	24	66.46	0.25±0.25	0.0009±0.0008	0.953±0.888
46 - 60 years					
Male	26	75.54	0.31±0.31	0.0010±0.0010	1.029±1.020
Female	18	81.67	0.3±0.22	0.0009±0.0007	0.996±0.767
> 60 years					
Male	18	76.89	0.13±0.11	0.0004±0.0003	0.442±0.356
Female	12	64.42	0.17±0.17	0.0007±0.0007	0.664±0.675

Mean AFM1 = 0.25µg/kg

The highest intake of AFM1 (ng/kgbw/day) through raw cow milk was recorded amongst 1-5 years (infants) age category with mean values of 3.679±2.213 and 2.445±2.001 for males and females, respectively. This was followed by adolescents (11-19 years). This age category recorded a mean intake value of 1.602±1.384 for males and 1.852±1.362 for females. The

elderly (>60 years) on the other hand had the least AFM1 intake (ng/kgbw/day) with mean intake values of 0.442±0.356 and 0.664±0.675 for males and females, respectively.

There was however no significant difference ($p<0.05$) between sex for all the age categories.

Table 12: Exposure level/ EDI of AFM1 - Burkina drink consumption

Age category	No. of Consumers	Average body weight (kg)	Average ***Burkina drink*** intake (l/day)	AFM1 intake (**µg**/kgbw/day)	AFM1 intake (**ng**/kgbw/day)
1 - 5 years					
Male	14	21.50	0.11±0.10	0.0005±0.0004	***0.459±0.439***
Female	12	22.67	0.35±0.14	0.0014±0.0005	***1.379±0.597***
6 - 10 years					
Male	22	35.32	0.15±0.13	0.0003±0.0003	0.373±0.332
Female	22	36.82	0.10±0.13	0.0002±0.0003	0.247±0.329
11 - 19 years					
Male	36	55.03	0.20±0.15	0.0003±0.0002	0.333±0.272
Female	27	57.30	0.25±0.21	0.0004±0.0003	0.411±0.331
20 - 45 years					
Male	64	69.39	0.19±0.23	0.0002±0.0003	0.253±0.325
Female	24	66.46	0.27±0.25	0.0004±0.0003	0.371±0.347
46 - 60 years					
Male	26	75.54	0.14±0.232	0.0002±0.0003	0.169±0.266
Female	18	81.67	0.25±0.25	0.0003±0.0003	0.296±0.334
> 60 years					
Male	18	76.89	0.24±0.21	0.0003±0.0003	0.292±0.270
Female	12	64.42	0.16±0.20	0.0002±0.0003	0.232±0.296

Mean AFM1 = 0.09µg/kg, Bolden & italic = significant difference (*$p<0.05$*)

AFM1 intake through Burkina drink as shown in Table 12 indicates that the highest intake occurred amongst 1-5 years age category with males recording mean intake of 0.459±0.439 ng/kgbw/day and females, a mean intake of 1.379±0.597 ng/kgbw/day. There was statistical significance (P<0.05) for mean AFM1 intake between males and females within this age category (infants). The least mean intake of AFM1 was recorded amongst the males of 46-60 years age category. Their mean consumption was 0.169±0.266 ng/kgbw/day followed by females >60 years recording mean AFM1 intake of 0.232±0.296 ng/kgbw/day. Except for consumers within the age group of 1-5 years (infants) none other age group recorded statistical significance between sex.

Table 13: Exposure level/ EDI of AFM1 - *wagashi* consumption

Age category	No. of Consumers	Average body weight (kg)	Average ***Wagashi*** intake (kg/day)	AFM1 intake (**ng**/kgbw/day)
1 - 5 years				
Male	14	21.50	***0.07±0.04***	0.124±0.088
Female	12	22.67	***0.12±0.06***	0.209±0.133
6 - 10 years				
Male	22	35.32	0.15±0.19	0.177±0.222
Female	22	36.82	0.08±0.13	0.092±0.055
11 - 19 years				
Male	36	55.03	0.07±0.06	0.051±0.056
Female	27	57.30	0.10±0.15	0.078±0.118
20 - 45 years				
Male	64	69.39	0.08±0.16	0.047±0.099
Female	24	66.46	0.08±0.14	0.051±0.096
46 - 60 years				
Male	26	75.54	***0.04±0.04***	0.019±0.022
Female	18	81.67	***0.10±0.15***	0.051±0.069
> 60 years Male	18	76.89	0.04±0.03	0.021±0.021

Female	12	64.42	0.06±0.04	0.034±0.031

Bolden & italic = significant difference (*p<0.05*)

AFM1 intake through wagashi as shown in Table 13 indicates that the highest intake occurred amongst infants with mean intake of 0.124±0.088 and 0.209±0.133 ng/kgbw/day for males and females, respectively. There was statistically significance difference (P<0.05) for mean AFM1 intake between males and females within this age group (infants). The least mean intake of AFM1 was recorded amongst adults and elderly ranging from 0.019 to 0.034 ng/kgbw/day. Except for consumers within the age group of 1-5 years (infants) and 46 – 60 years, none other age group recorded statistically significance between sex.

CHAPTER 5

DISCUSSION

5.1 Concentration of Aflatoxin M1 in raw cow milk

Thirty commercially available raw cow milks were sampled from three locations within the Greater Accra region for Aflatoxin M1 analysis. Aflatoxin M1 is likely to be present in raw cow milk if the feed for the cow are contaminated with Aflatoxin B1. Raw cow milk contamination with Aflatoxin M1 is therefore an indication of lactating animal (mammals) fed with AFB1 contaminated diet. As reported by Duarte *et al*. (2013), several factors influence the quantity of AFB1 that may be initially present in a feed and what is finally converted to AFM1 and excreted through milk. The animal strain, type of feed and frequency of exposure through feeding are examples of such factors.

As per the results obtained, the mean value of Aflatoxin M1 contamination for raw cow milk was recorded at 1.53±1.42 µg/l. The range of aflatoxin M1 contamination was reported to be 0.35 to 3.76 µg/l. Per the outcome of this work, the occurrence of AFM1 was 16.67% (5 out of 30 samples were positive) indicating low incidence but very high levels of AFM1 for positive samples. The five samples that tested positive as well as the mean concentration reported for AFM1 were above the EC permitted level of 0.05 µg/kg (EC, 2006)
demonstrating unsafe levels. This result seems to be consistent with similar studies conducted in other countries to investigate presence of aflatoxin M1 contamination in raw cow milk.

Aflatoxin M1 has been detected in raw cow milk samples in other African countries. In Tanzania, studies conducted on raw cow milk samples, 83.3% (31/37) using cows from families that were feeding sunflower cake, revealed aflatoxin contamination in the range of

0.026 µg/kg to 2.007 µg/kg exceeding both Tanzania's and EC allowable value which is 0.05 µg/kg (Mohammed et al. 2016).

Makun et al. (2016) in Nigeria also reported contamination of raw cow milk with aflatoxin M1 at levels higher than the EU permitted levels. In their study, contamination of raw cow milk (from nomadic cow) with aflatoxin M1 ranged from 0.0109 to 1.3543 µg/l with and an average concentration of 0.5308 ± 0.0938 µg/l. For commercial cows' milk, aflatoxin M1 contamination ranged between 0.0464 - 0.0992 and 0.0584 ± 0.0052 µg/l as the mean.

A similar trend i.e. levels of aflatoxin M1 contamination above 0.05 µg/ kg has also been reported in a study conducted in Brazil. Goncalves et al. (2017) reported aflatoxin M1 levels in fresh bovine milk to be in between the range of 0.09 µg/l to as high as 3.385 µg/l per their work.

As already mentioned above, the amount of AFB1 from the animal feed converted to aflatoxin M1 is largely dependent on factors such as type of feed, animal strain, well-being of the animal, feeding habits as well as assimilation and absorption (Duarte et al., 2013). It is therefore possible that the high levels of aflatoxin M1 reported per the study, is because of high levels of AFB1 contamination initially present in the feed of the lactating cows thereby leading to more AFM1formation. High consumption rate of the contaminated feed is another possibility accounting for high levels of AFM1. The absence of strict regulation for monitory and regulating type and contamination levels in feed for cows is also implicated in the high levels.

Low levels of aflatoxin M1 has been reported especially in Europe where there is regulation to monitor and regulate feed especially on aflatoxin B1 levels. According to European regulation, all feed materials should be tested for AFB1 and levels detected must not exceed
20 µg/kg whereas complete feedstuff for dairy animals has a limit for AFB1 at 5 µg/kg.

Results of a study on milk conducted in Spain by Cano-Sancho et al. (2010) showed all samples tested did not exceed 0.05 µg/kg. The mean value from the study was 9.69±2.07 ng/kg, which was much lower than the permitted level within the EU i.e. (0.05 µg/kg). In

Greece, results from a work done on aflatoxin M1 contamination of milk revealed that out of 30 samples analyzed in the year 2000, only one sample exceeded 0.05 µg/kg which is the permitted levels within the EU (Roussi et al. 2002). In Croatia, in 2009, a similar study showed 98.4% of raw milk sampled for aflatoxin M1 contamination had concentrations below the EU permitted levels (Bilandzic et al. 2010).

Other studies have also shown that aflatoxin M1 levels is associated with the season the sampling is done. Winter season (cold season) usually has high levels of aflatoxin M1 due to long storage of feed compared to summer (hot seasons), which has relatively low levels as natural forage are usually available for grazing. Blanco et al. (1988), noticed this seasonal trend with lower AFM1 levels occurring in summer months. A study on seasonal prevalence level of aflatoxin M1 in Pakistan by Ismail et al. (2016), showed that milk samples analyzed during different seasons (Autumn, Winter, Spring, Summer), winter recorded the maximum mean AFM1 level (0.14±0.082 µg/l. During the summer however, the mean AFM1 concentration recorded was 0.06±0.034 µg/l and happened to be the minimum recordings.

From above seasonal trend reported by other studies, it is possible that the high levels of aflatoxin M1 recorded in this present study could have been due to the period within which the samples were picked. The samples for this study were picked in December which is relatively a cold season with mean temperature range of 27ºC recorded. Lack of fresh forage as feed might have led to long storage of hay or feed leading to contamination of *Aspergillus s*p. leading to AFB1 contamination.

5.2 Concentration of Aflatoxin M1 in Burkina drink

Burkina drink is a local drink originally made by the people from the northern part of Ghana. However today on the streets of Accra it is very common to see bottled Burkina drink made using millet and fresh cow milk. The source of aflatoxin M1 contamination of this local drink is therefore from the milk used in its preparation.

Thirty (30) *Burkina drinks* were purchased from three markets within the Greater Accra region for aflatoxin M1 analysis. According to the statistical results obtained, the mean value of aflatoxin M1 contamination was recorded at 0.40±0.16 µg/l. The results showed relatively higher incidence (23.33%) of AFM1 contamination though mean concentration levels was lower than that of raw cow milk. All the samples that tested positive were above 0.05 µg/kg, the permitted limits within EU for milk (EC, 2006). The results are comparable to the trend of high concentration of aflatoxin M1 i.e. above the EU limit.

High levels of AFM1 recorded could possibly be because manufacturers of the local drink used contaminated raw cow milk for the preparation of the Burkina drink. Such milks must have come from cows feeding on AFB1 contaminated feed. The relatively high incidence (23.33%) compared to that of raw milk (16.67%) could be attributed to the varied and numerous brands and vendors of Burkina drink, possibly sourcing their raw milk from different sources covering a wider scope.

The lower concentrations compared to that of raw cow milk not used for Burkina drink could be that, the raw milk, when purchased from source is diluted before using to prepare the Burkina to increase volume and maximize profit.

The only treatment during preparation of Burkina drink that can influence AFM is heating, however in general, as indicated by Prandini et al. (2009), aflatoxins are known not to be influenced when heated. Other studies however suggest reductions in AFM1 close to 32%

when milk is heat-treated Kabak (2012). It has also been reported that heat treatment such as pasteurization of milk can lead to loss of AFM1 concentration between 32-64% (Purchase, 1967). The relatively low concentrations of AFM1 observed in Burkina drink compared to the raw cow milk might probably be due to losses during heat treatment in the process of making the Burkina drink.

5.3 Concentration of Aflatoxin M1 in *wagashi* (local cheese)

Wagashi which is a local cheese is made from raw cow milk hence the source of AFM1 contamination. Twenty-three (23) *wagashi* samples commercially available, were purchased from three different markets within the Greater Accra region for AFM1 analysis.

Statistical results obtained from the analysis showed a mean contamination level of 0.18±0.39 µg/kg. Aflatoxin M1 contamination was reported to be 0.006 to 0.8872 µg/kg. Results from this study showed that incidence of AFM1 in *wagashi* was low 21.74% (5/23 samples were positive) but high levels of AFM1 for positive samples. Two of the five samples that tested positive recorded values above 0.05 µg/kg (EC, 2006) demonstrating unsafe levels. Elkak et al., (2012) in a study indicated that cheese contamination with AFM1 is most likely because of AFM1 contamination of source of milk used in making the cheese.

No research work in Ghana on AFM1 contamination of *wagashi* was found. However, Sessou et al. (2013) from Benin, investigated contamination of cheese made locally (*wagashi)* with aflatoxin M1 using TLC. The study found that none of the *wagashi* samples were contaminated with AFM1. In Iran, Bahrami et al. (2016) determined AFM1 in traditional cheese and reported a high incidence of 52% (13/25) of AM1 contamination with a mean level of 181.97±15.8 ng/kg using HPLC-FD. In Minna, Nigeria, Makun et al. (2016) determined AFM1 in traditional cheese and reported mean levels of 0.588±0.1296 µg/l with
40% (8/20) incidence.

The levels reported in this study could be because of contamination of raw cow milk used for making the cheese and probably the process of *wagashi* preparation. The concentrations were however lower than that recorded for both raw milk and *burkina* drink which is in consonance with results of a study by Elgerbi et al. (2004) which indicated that cheese samples and raw cow milk samples analyzed in North Africa (Libya) showed AFM1 contamination levels in raw cow milk to be higher than that of cheese. This is most likely because of varied AFM1 contamination levels of the source milk (raw cow milk) used in making each category of product assessed.

All *Wagashi* samples that tested positive had values higher than of 0.05 µg/kg which is the allowable limit for the EU.

5.4 Risk Assessment

5.4.1 Exposure Assessment

Estimation of food consumption was done for the different age groups i.e. from infants to the elderly as illustrated in Table 10. Making use a food frequency questionnaire. The consumption rate for raw cow milk for infants recorded a mean value of 0.32±0.19 l/day and 0.22±0.16 l/day for males and females respectively. The highest mean consumption was recorded among adolescents (11 -19 years) with mean consumption values of 0.35±0.29 l/day for males and 0.42±0.27 l/day for females.

In calculating the EDI for each age category, the individual body weight was used instead of the mean consumption. A deterministic approach which is a quicker approach, providing a point estimate was used for the exposure assessment for this work. The highest EDI of AFM1 through raw cow milk was recorded amongst infants (Table 9). The EDI for males (infants) was 3.679±2.213 ng/kgbw/day and females (infants) was 2.445±2.001 ng/kgbw/day. The least EDI was however recorded amongst the elderly (>60 years) with values of 0.442±0.356 and

0.664±0.675 ng/kgbw/day for males and females, respectively. As per the results obtained, infants were the group with high exposure to AFM1.

No research work on AFM1 levels in raw cow milk as well as exposure assessment or AFM1 intake conducted in Ghana was found. However, research work on AFM1 intake through milk in other countries such as Spain is well documented. Cano-Sancho et al. (2010) in an exposure assessment to AFM1 in Catalonia in Spain, using a deterministic approach reported that infants were the most exposed age group to AFM1 with mean intake of 0.182±0.109 ng/kgbw/day and 0.236±0.103 ng/kgbw/day for males and females, respectively with statistically significance difference between sex. Adults (20- 65 years) recorded mean AFM1 intake at 0.305 ng/kgbw/day for both males and females. All the age groups however recorded intake values below 1 ng/kgbw/day.

In Serbia, Kos et al. (2014) reported mean AFM1 intake by adults between 0.42 to 1.26 ng/kgbw/day. In Iran, Bahrami et al. (2016) reported 0.17 ng/kgbw/day as EDI of aflatoxin M1 in the summer period whereas during the winter the EDI reported was 0.242 ng/kgbw/day. The EDI calculated for both infants and adults for this work was higher than amounts stated in studies conducted in other countries such as Serbia, Iran and Spain amongst their adult populace. The high values may be due to high mean concentrations of AFM1 in raw cow milk determined by HPLC-FD.

Exposure assessment through a deterministic approach was also done for Burkina drink which is a local drink peculiar to Ghana. Again, infants recorded the highest AFM1 intake with mean values of 0.971±0.928 and 2.912±1.260 ng/kgbw/day respectively for males and females.

The EDI of AFM1 through *wagashi* (local cheese) for infants, was recorded as 0.124±0.088 and 0.209±0.133 ng/kgbw/day for males and females respectively. This was the highest intake

amongst all the age groups. The least intake (0.019 ng/kgbw/day) occurred amongst males within 40-60 years age group.

5.4.2 Hazard Assessment

AFs are known to be carcinogenic but FAO/WHO joint Expert Committee on Food Additives (JECFA, 2007) does not have tolerable daily intake (TDI) stipulated for AFM1.The joint expert committee however concluded that even an exposure to AFM1 below 1 ng/kgbw daily exposes one to the risk of developing cancer especially that of the liver (JECFA, 2001). The recommendation therefore is that for the concentration of AFs in food commodities should be as low as practically possible.

Considering the EDI obtained in this study for infants i.e. 3.679±2.213 and 2.445±2.001 ng/kgbw/day, which are far above 1 ng/kgbw/ day, indicates serious risk to AFM1 through raw cow milk consumption for this age category.

The EDI values obtained for Burkina drink are indication of risk with infants being the most at risk. Adult population recorded mean values ranging between 0.357 to 0.784 ng/kgbw/day. Though these values are less than 1 ng/kgbw/day and relatively lower than that recorded for infants (0.971 and 2.912 ng/kgbw/day), they still give indication of risk.

The EDI values obtained for *wagshi* was generally lower across all age groups compared to raw cow milk and *burkina drink*. Adults 20 years and above recorded intake values between 0.019 to 0.078 ng/kgbw/day. Leblanc et al. (2005), estimated AFM1 that is being ingested amongst the French populace and reported mean intake of AFM1 through cheeses as 0.02 ng/kgbw/day for both adults (15 years and above) and children (3-14 years). The values recorded for this study though higher compared to similar studies, shows the risk to AFM1 posed by consuming *wagashi* is relatively lower compared to consuming raw cow milk and *burkina drink.*

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

This study has provided valuable information on incidence of aflatoxin M1 contamination of raw cow milk, *burkina drink* and local cheese *(wagashi).* The results obtained from this study shows a worrying phenomenon with respect to AFM1 contamination of raw cow milk and its processed products. From the study, aflatoxin M1 was detected in raw cow milk, *burkina drink* and *wagashi* samples randomly purchased from three different markets in Greater Accra region. Mean concentrations were 1.53±1.42 µg/l, 0.40±0.16 and 0.18±0.39 µg/kg for raw cow milk, *burkina drink* and local cheese (*wagashi)* respectively.

The incidence of AFM1 were 16.67% for raw cow milk, 21.74% for *wagashi* and 23.33% for Burkina drink, individual results were all above the EU limit of 0.05 µg/kg except for three samples of *wagashi*. Since milk is a source of important minerals and are regularly consumed especially by mothers and vulnerable groups such as infants, contamination with AFM1 is of great concern.

Health risk assessment conducted for aflatoxin M1 in these foods indicate each food item (raw cow milk, *burkina drink* and *wagashi* pose a health risk with infants being the most at risk. Of the three food types analyzed, raw cow milk posed the highest risk and *wagashi* posed the least risk as milk was consumed in large amounts and more frequently compared to Burkina and *wagashi*. The consumption of *wagashi* and concentrations were relatively low across all age categories compared to raw cow milk and *burkina drink*.

6.2 Recommendations

In this study, raw cow milk, *burkina drink* and *wagashi* were purchased from the open market for laboratory determination of AFM1 using HPLC. The sampling was also done only during one season, it is therefore recommended that a considerable amount of research be done to establish seasonal variations of AFM1 concentration in cow milk i.e. sampling done both rainy season and dry season. It is also recommended to have the Food and Drugs Authority of Ghana to mount surveillance and regular monitoring of AFM1 levels in these products which are already sold openly on the markets. Processed and branded milk and milk products must also be sampled and analyzed for AFM1 levels.

AF B1 content of feed including forage being used in feeding cows should also be carried out to ascertain the levels. The Ministry of Food and Agriculture should also intensify training programs with cattle farmers on good agricultural practices. For *wagashi* and Burkina, a standardized preparation method by sellers spearheaded by FDA, GSA and Ministry of Agriculture will help minimize contamination levels.

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APPENDIX

DEPARTMENT OF FOOD SCIENCE AND TECHNOLOGY
KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

RAW COW MILK/ MILK PRODUCTS CONSUMPTION QUESTIONNAIRE

Questionnaire No _____ Date: _____

Location: 1.Ashaiman 2. Nima 3.Madina _____

BACKGROUND INFORMATION

1. Name/ Initials…… **2. Age (yrs)** 1. 15 - 19 2. 6 - 10

3. 11 - 19 4. 20 - 45 5. 46 - 60 6.> 60

3. Sex of respondent: 1. Male 2. Female

4. What is your Marital status 1.Single 2. Married 3. Divorced

5. What is your educational level 1. None. 2.Primary 3.JHS 4. SHS 5.Tertiary

6. What is your **Weight (Kg)** _____

RAW COW MILK/ MILK PRODUCTS CONSUMPTION

7. Do you consume raw/fresh cow milk & it's products?

1. Yes ☐ 2.No ☐

8. How do you use the raw cow milk? 1. Straight drink ☐ 2. In food preparation ☐ 3. Both ☐

9. Do you consume wagashie 1.Yes 2. No ☐

10. Do you consume Burkina drink? 1. Yes ☐ 2.No ☐

RAW COW MILK & PRODUCTS CONSUMPTION IN SELECTED AREAS OF ACCRA - FOOD FREQUENCY QUESTIONNAIRE

11. Over the past **12 months (1 year)**, how often did you drink **raw cow milk** as a beverage (NOT in food)?

1. Never ☐
2. 1-3 times per year ☐
3. 1-3 times per month ☐
4. 4-6 times per month ☐
5. 1-3 times per week ☐
6. 4-6 times per week ☐
7. 1-3 time per day ☐
8. 4-6 times per day ☐
9. 7-9 times per day ☐

12. Each time you drank **raw cow milk** as a beverage, how much did you usually drink/ consume?

1.less than Half a glass (62.5ml)
2. Half a glass (125ml)
3. Full glass (250ml)
4. One and half glass (375ml)
5. Two glasses (500ml)
6. None

13. Over the past 12 months (1 year), how often did you drink ***Hausa Koko***?

1. Never
2. 1-3 times per year
3. 1-3 times per month
4. 4-6 times per month
5. 1-3 times per week
6. 4-6 times per week
7. 1-3 time per day
8. 4-6 times per day
9. 7-9 times per day

14. Each time you drank ***Hausa koko***, how much raw cow milk did you usually add?

1. 1-3 teaspoons
2. 4-6 teaspoons
3. 7-9teaspons
4. 1-3 tablespoons
5. 4-6 tablespoons
6. less than half glass
7. Half glass
8.None

15. Over the past 12 months (1 year), how often did you drink ***Fula?***

1. Never

2. 1-3 times per year
3. 1-3 times per month
4. 4-6 times per month
5. 1-3 times per week
6. 4-6 times per week
7. 1-3 time per day
8. 4-6 times per day
9. 7-9 times per day

16. Each time you drank ***Fula***, how much raw cow milk did you usually add?

1. 1-3 teaspoons
2. 4-6 teaspoons
3. 7-9teaspons
4. 1-3 tablespoons
5. 4-6 tablespoons
6. less than half glass
7. Half glass
8. None

17. Over the past 12 months (1 year), how often did you drink ***Tea***?

1. Never
2. 1-3 times per year
3. 1-3 times per month
4. 4-6 times per month
5. 1-3 times per week
6. 4-6 times per week
7. 1-3 time per day
8. 4-6 times per day
9. 7-9 times per day

18. Each time you drank ***Tea***, how much raw cow milk did you usually add?

1. 1-3 teaspoons
2. 4-6 teaspoons
3. 7-9teaspons
4. 1-3 tablespoons
5. 4-6 tablespoons
6. less than half glass
7. Half glass
8.None

19. Over the past 12 months (1 year), how often did you drink ***Coffee***?

1. Never
2. 1-3 times per year
3. 1-3 times per month
4. 4-6 times per month
5. 1-3 times per week
6. 4-6 times per week
7. 1-3 time per day
8. 4-6 times per day
9. 7-9 times per day

20. Each time you drank ***Coffee***, how much raw cow milk did you usually add?

1. 1-3 teaspoons
2. 4-6 teaspoons
3. 7-9teaspons
4. 1-3 tablespoons
5. 4-6 tablespoons
6. less than half glass
7. Half glass
8.None

21. Over the past 12 months (1 year), how often did you drink ***Oats***?

1. Never
2. 1-3 times per year
3. 1-3 times per month
4. 4-6 times per month
5. 1-3 times per week
6. 4-6 times per week
7. 1-3 time per day
8. 4-6 times per day
9. 7-9 times per day

22. Each time you drank ***Oats***, how much raw cow milk did you usually add?

1. 1-3 teaspoons
2. 4-6 teaspoons
3. 7-9teaspons
4. 1-3 tablespoons
5. 4-6 tablespoons
6. less than half glass 7. Half glass
8. Full glass
9.None

23. Over the past 12 months (1 year), how often did you drink ***tom brown***?

1. Never
2. 1-3 times per year
3. 1-3 times per month
4. 4-6 times per month
5. 1-3 times per week
6. 4-6 times per week
7. 1-3 time per day
8. 4-6 times per day
9. 7-9 times per day

24. Each time you drank ***tom brown***, how much raw cow milk did you usually add?

1. 1-3 teaspoons
2. 4-6 teaspoons
3. 7-9teaspons
4. 1-3 tablespoons
5. 4-6 tablespoons
6. less than half glass
7. Half glass
8. Full glass
9. None

25. Over the past 12 months (1 year), how often did you drink ***Gari soakings***?

1. Never
2. 1-3 times per year
3. 1-3 times per month
4. 4-6 times per month
5. 1-3 times per week
6. 4-6 times per week
7. 1-3 time per day
8. 4-6 times per day
9. 7-9 times per day

26. Each time you ***Gari soakings***, how much raw cow milk did you usually add?

1. 1-3 teaspoons
2. 4-6 teaspoons
3. 7-9teaspons
4. 1-3 tablespoons
5. 4-6 tablespoons
6. less than half glass
7. Half glass
8. Full glass
9. None

27. Over the past 12 months (1 year), how often did you eat ***WAGASHIE***?

1. Never
2. 1-3 times per year
3. 1-3 times per month
4. 4-6 times per month
5. 1-3 times per week
6. 4-6 times per week
7. 1-3 time per day
8. 4-6 times per day
9. 7-9 times per day

28. Each time you ate ***WAGASHIE***, how much did you usually consume?

1. 1-3 balls
2. 4-6 balls
3. 7-9 balls
4. 10-12 balls
5. None

29. Over the past 12 months (1 year), how often did you drink ***BURKINA DRINK***?

1. Never
2. 1-3 times per year
3. 1-3 times per month
4. 4-6 times per month
5. 1-3 times per week
6. 4-6 times per week
7. 1-3 time per day
8. 4-6 times per day
9. 7-9 times per day

30. Each time you drank ***Burkina drink***, how much did you usually consume?

1.less than Half a bottle (62.5ml)	
2. Half a bottle (125ml)	
3. Full bottle (250ml)	
4. One and half bottle (375ml)	
5. Two bottles (500ml)	
6. None	