LEVELS OF AFLATOXIN M_1 AND SELECTED HEAVY METALS IN THE BREAST MILK OF LACTATING MOTHERS IN OWERRI, NIGERIA

BY

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CERTIFICATION PAGE

This research project work "Levels of Aflatoxin M_1 and selected heavy metals in the breast milk of lactating mothers in Owerri, Nigeria" has been certified for submission and conclusively completed by Ekeanyanwu, Chidinma Lynda, Registration number 20164994638 of the Postgraduate programme in Biochemistry. We examined and found it acceptable for the award of Master of Science degree in Biochemistry, Federal University of Technology, Owerri.

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DEDICATION

To Almighty God, My creator and who is the fountain of all wisdom.

TABLE OF CONTENTS

Title page - - - - - - - - - - -i

Certification page - - - - - - - - - - -ii

Acknowledgement - - - - - - - - - - -iii

Dedication - - - - - - - - - - -iv

Table of contents - - - - - - - - - - -v

Lists of tables - - - - - - - - - - -viii

List of figures - - - - - - - - - - -ix

Abstract - - - - - - - - - - -x

CHAPTER ONE

1.0 Introduction - - - - - - - - - -1

1.1 Background Information- - - - - - - - - -1

1.2 Problem Statement - - - - - - - - - -2

1.3 Objectives - - - - - - - - - - -2

1.4 Justification of the Study - - - - - - - - -3

1.5 Scope of the Study- - - - - - - - - -3

CHAPTER TWO

2.0 Literature Review - - - - - - - - - -4

2.1 Overview of mycotoxins - - - - - - - - -4

2.2 Overview of Aflatoxin - - - - - - - - -5

2.2.1 Chemistry of Aflatoxins - - - - - - - -9

2.2.2 Absorption, distribution, metabolism and excretion of Aflatoxin - -9

2.2.3 Impact of Aflatoxin in Human health - - - - - -12

2.2.4 Liver Carcinogen - - - - - - - - -12

2.2.5 Immune suppression - - - - - - - - -12

2.2.6 Impairment of Childhood obesity - - - - - -13

2.2.7 Acute toxicity and health - - - - - - - -14

2.3 Occurrence of Aflatoxins in Breast milk - - - - - -14

2.4 Lactational transfer for Aflatoxin B_1 and M_1 - - - - - -18

2.5 Exposure of infants to Aflatoxins - - - - - - -18

2.6 Metal Toxicity- - - - - - - - - -19

2.7 Heavy Metals and their toxicity mechanism - - - - -20

2.7.1 Arsenic - - - - - - - - -20
2.7.2 Lead - - - - - - - - - -21
2.7.3 Mercury - - - - - - - - -22
2.7.4 Cadmium - - - - - - - - -23
2.7.5 Chromium - - - - - - - - -24
2.7.6 Copper - - - - - - - - -26
2.7.7 Zinc - - - - - - - - - -27
2.7.8 Iron - - - - - - - - - -28

CHAPTER THREE

3.0 Materials and Methods - - - - - - - -29
3.1 Materials - - - - - - - - - -29
3.1.1 Chemicals and Reagents - - - - - - -29
3.1.2 Types of Equipments - - - - - - - -29
3.2 Methods - - - - - - - - - -29
3.2.1 Study Area - - - - - - - - -29
3.2.2 Ethical Approval and Consent - - - - - -30
3.2.3 Sample size determination - - - - - - -30
3.2.4 Sampling - - - - - - - - -30
3.2.5 Inclusion criteria - - - - - - - -30
3.2.6 Exclusion criteria - - - - - - - -31
3.2.7 Collection of participants' personal information and food consumption data-31
3.2.8 Determination of AfM_1 in breast milk sample - - - -31
3.2.8.1 Determination of AfM_1 in Breast milk sample - - -31
3.2.8.2 High-performance Liquid Chromatography Analysis - -32
3.2.8.3 Trueness and Repeatability of Measurement - - - -32
3.2.9 Determination of Heavy metals - - - - - -33
3.2.9.1 Digestion of breast milk sample - - - - -33
3.2.9.2 AAS Analysis - - - - - - - -33
3.2.10 Determination of estimated daily intake of AfM_1 -- - - -34
3.2.11 Estimated daily intake (EDI) of heavy metals -- - - -34
3.2.12 Statistical Analysis - - - - - - - -34

CHAPTER FOUR

4.0 Results and Discussion- - - - - - - -36

4.1 Sociodemographic information of infants and breast feeding mothers - -36

4.2 Food consumption determinants data of the study - - - - -38

4.3 Levels of AfM_1 in human breast milk- - - - - - -39

4.4 Estimated daily intake of AfM_1 by the age of infants- - - - -40

4.5 Association between maternal diet, employment status, educational level and AfM_1 in breast milk - - - - - - - - - - - - -40

4.6 Heavy metals in Human Breast milk - - - - - - -43

4.7 Mean daily intake of heavy metals in Breast milk - - - - -44

4.8 Association between daily and weekly maternal diet, heavy metals exposure and heavy metals in breast milk - - - - - - - - -44

4.9 Some reports on the concentrations of toxic heavy metals by country - -51

CHAPTER FIVE

5.0 Conclusion and Recommendation - - - - - - -51

5.1 Conclusion - - - - - - - - - -51

5.2 Recommendation - - - - - - - - -51

References - - - - - - - - - - -52

Appendix I (Informed consent form) - - - - - - - -76

Appendix II (Questionnaire) -- - - - - - - - -79

Appendix III (Ethical Approval) - - - - - - -81

Appendix IV (Aflatoxin Standard Calibration Curve) - - - - -82

Appendix V (Heavy Metals Standards Calibration Curve for AAS) - - - -83

LIST OF TABLES

Table 2.1: Description of certain mycotoxins and their side effects - - - -7

Table 2.2: Suggested daily intake levels for certain mycotoxins - - - -8

Table 3.1: Recommended instruments parameters for AAS - - - - -34

Table 4.1: Participants personal information - - - - - - -36

Table 4.2: Profile of the analysed population of lactating mothers - - - -37

Table 4.3: Occurrence of AfM_1 in breast milk of mothers - - - - -39

Table 4.4: Correlation between maternal diet, employment status, educational level and AfM_1 in breast milk - - - - - - - - - - -41

Table 4.5: Recently reported prevalence and level of AfM_1 in breast milk of mothers in Nigeria and other countries - - - - - - - - -42

Table 4.6: Concentrations of Pb, Cd, Cr, Cu, Zn, As, Fe and Hg in breast milk of mothers and effect on mothers age - - - - - - - - - -43

Table 4.7: Correlations between daily and weekly maternal diet, heavy metal exposure and heavy metals in breast milk - - - - - - - - -45

Table 4.8: Cd, Cr, Cu, Fe, Pb and Zn concentration in the breast milk of mothers from Nigeria and other countries - - - - - - - - -51

LIST OF FIGURES

Figure 2.1: Chemical Structure of Some important Mycotoxins -- - - -6

Figure 2.2: The Chemical Structure of Aflatoxins - - - - - -10

Figure 2.3 Sources of Aflatoxin exposure and its flow in the food chain. - - - - - - -16

Figure 2.3: Factors influencing the exposure of infants to mycotoxins in early life -- -17

Figure 3.1: The Dispersive liquid-liquid microextraction procedure - - - -32

Figure 4.1: Food consumption data - - - - - - - -38

Figure 4.2: Estimated daily intake of AfM_1 in breast milk of mothers by age of infants -40

Figure 4.3: Estimated daily intake of Pb, Cd, Cr, Cu, Zn and Fe from breast milk per kg of infant weight - - - - - - - - - - -44

ABSTRACT

Mother's breast milk which is the basic diet for infants is loaded with proteins, fats, carbohydrates and essential minerals which are necessary for proper nutrition of the infant. Regrettably, breast milk is also a likely source of aflatoxins and toxic metals which are unsafe for the breastfeeding child. In Nigeria, there is a paucity of information on aflatoxin and toxic heavy metals exposure, especially in the southeast region. In the present study, we assessed the level and frequency of breast milk AfM_1 and some heavy metals as biomarkers of maternal exposure. Breast milk samples were collected from a selected group of 40 lactating mothers of infants attending the Federal Medical Centre Owerri, Imo State, between June and August 2019. Some heavy metals (Pb, Cd, Cr, Cu, Zn, Fe, As, and Hg) were analysed using Atomic Absorption Spectrophotometry while AfM_1 levels were assessed by HPLC with fluorescence detection after aflatoxin extraction. The mean (±standard deviation) concentration of AfM_1 in the breast milk samples was 4.02±1.12 ng/L and 100% of all the samples contained AfM_1 at 2.33 – 7.08 ng/L. AfM_1 concentration was positively and significantly ($p<0.01$) associated with the daily consumption of cassava-based foods, groundnut oil, maize, tomatoes and dry fruit ($p<0.05$). No significant association ($p>0.05$) was observed between AfM_1 concentration in breast milk with employment status and educational level in nursing mothers. The mean (±standard deviation) values of these heavy metals were Cd: 0.029±0.013 mg/L, Cr: 0.019±0.011 mg/L, Cu: 0.035±0.013 mg/L, Fe: 0.049±0.039 mg/L, Pb: 0.038±0.013 mg/L and Zn: 0.009±0.008 mg/L. The result of the estimated daily intake of breast milk by the breastfed infants shows that heavy metals such as Pb, Fe, and Cd are ingested more daily than other metals analysed. There was a weak positive but non-significant correlation between heavy metal content and daily intake of maternal diet except for beans where a significant correlation ($p<0.01$) was found with Cr, Cu and Zn. A weak positive but non-significant correlation was also observed between exposure to heavy metals such as Cu, Fe and Pb and maternal diets. None of the samples exceeded the national and international legal regulatory limit for AfM_1 and the selected heavy metals in breast milk except for chromium. Generally, their presence still poses a health risk.

Keywords: Aflatoxin M_1; Heavy metals; Breast Milk; Infants; Exposure; Nigeria.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Several documented reports in the literature have proven that breast milk is the best source of nutrients/diet for newborns/infants, probably because of its reported nutritional characteristics and health beneficial properties (Kunter *et al*., 2017). Important dietary components and balanced nutrients such as carbohydrates, fats, proteins found in breast milk contribute to infant growth, ability to fight infection/diseases and maintenance (Ballard and Morrow, 2013). These assets make the breast milk a distinctive source of food for infants, and nutritionists and medical practitioners have always encouraged and recommended breastfeeding (Kulkarni *et al*., 2013). Despite breast milk's nutritionally and immunologically valuable constituents, it may sometimes contain substantial quantities of toxic chemicals because of exposure of the mother to certain foods (Cherkani-Hassani *et al*., 2016). These contaminants which include heavy metals, pesticides and fungal toxins may affect the health of the infant negatively.

Mycotoxins include a very diverse group of chemicals with a wide spectrum of toxic effects. Mycotoxins are known to occur naturally and they are usually highly toxic fungal metabolic products of secondary metabolism of moulds such as *Aspergillus flavus* and *Aspergillus parasiticus* (Kunter *et al*., 2017). The US Food and Drug administration considers mycotoxins as unavoidable food contaminants (Kim *et al*., 2000; Williams *et al*., 2004). The unavoidable problem is not just peculiar to the western world but heavily predominant in sub-Saharan Africa. In addition to diverse organ-specific actions (liver, kidney, central nervous system and lungs), mycotoxins are known to adversely affect the digestive tract, cause skin irradiation, have haematological effects and reduce growth (Etzel, 2006; Sherif *et al*., 2009). A common type of mycotoxin is Aflatoxin (Kim *et al*., 2000; Williams *et al*., 2004). Humans may be exposed to aflatoxins directly from the ingestion of contaminated foods. When breastfeeding mothers consume contaminated foods containing aflatoxins, the substance and its metabolites may accumulate in breast milk (Prandini *et al*., 2009; Leong *et al*., 2012). Other known health problems associated with aflatoxins include growth impairment (Egal *et al*., 2005), malnutrition (Tchana *et al*., 2010), and reduced immune activities (Turner *et al*., 2003).

Currently, four major types of naturally occurring aflatoxins have been identified; they are aflatoxin B_1, B_2, G_1 and G_2, as well as two other metabolic products, Aflatoxin M_1

and M_2 (Williams *et al*., 2004). Aflatoxin M_1 is the hydroxylated metabolite of aflatoxin B_1 found in animal and human milk and it is considered a biomarker to aflatoxin B_1 exposure to contaminated foods by lactating mothers (Polychronaki *et al*., 2006; Gurbay *et al*., 2010; Williams *et al*., 2004; Prandini *et al*., 2009; and IARC, 2002).

Another important contaminant in breast milk is heavy metals. Breast milk also contains heavy metals such as arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), copper (Cu), zinc (Zn), silver (Ag), chromium (Cr) and platinum group elements. In breast milk, these elements act as an indicator for the presence of environmental and food contaminants and breast milk can also be a pathway of maternal excretion of heavy metals (Mead, 2008). Besides, heavy metals in breast milk are known to have a wide range of toxicity and carcinogenic effects on infants (Kunter *et al*., 2017). It has been reported that the presence of heavy metals such as As, Cd, Hg and Pb affects nervous system development in infants, bone formation processes as well as causing anaemia, rickets and autism (Winiarska-Mieczan, 2014). Breastfeeding infants also ingest these toxic metals such as Cd and Pb in their breast milk (Nishijo *et al*., 2002; Kutlu *et al*., 2006). Infant breastfeeding has been the most desirable way of feeding infants in low-income countries. However, issues of environmental pollution and food contamination are high in some of these countries (Landrigan *et al*., 2002; Bryden 2007).

1.2 Problem Statement

Breast milk may sometimes contain substantial quantities of toxic chemicals because of exposure of the lactating mother to certain foods, and environmental toxicants despite its valuable constituents. These toxic contaminants may affect the health of the infant negatively. There is, therefore, need for the constant monitoring of the Aflatoxins and heavy metals concentration in the breast milk of mothers in Owerri, Imo State and with extension in Nigeria, for the sole purpose of periodically assessing the quality of infant's and mother's nutrition.

1.3 Objectives

The specific objectives of the study included the following:

1. To determine the levels of Aflatoxin M1 in the breast milk of lactating mothers in Owerri, Eastern Nigeria.
2. To determine the levels of some toxic heavy metals (Pb, Cd, Cr, Cu, Zn, As, and Hg) in the breast milk of lactating mothers in Owerri, Eastern Nigeria.

3. To evaluate the main socio-demographical and food consumption determinants of the lactating mothers.

1.4 Justification

Due to the poor development of some protection mechanisms such as blood-brain barrier, enzymatic elimination mechanisms in the liver and kidney, plasma protein binding capacity and immune systems in infants, infants could be vulnerable to the environmental pollutants (Pohl and Hibbs, 1996; Chance, 2001). It is also known that the physiological differences between infants and adults make the former more vulnerable to these environmental contaminants. Therefore it is important to assess the AFM_1 and some heavy metals concentration in breast milk, which may constitute a risk factor for the nutrition of the breastfeeding mothers, and to a greater extent the infants. There is, therefore, need for the constant monitoring of the Aflatoxins and heavy metals concentration in the breast milk of mothers in developing countries such as Nigeria, for the sole purpose of periodically assessing the quality of infant's and mother's nutrition.

1.5 Scope of the Study

This study is focusing on determining the levels of Aflatoxin M_1 (Mycotoxin found in food) and heavy metals (Pb, Cd, Cr, Cu, Zn, As, and Hg) in breast milk of lactating mothers in Owerri, Eastern Nigeria and to evaluate the main socio-demographical and food consumption determinants of the lactating mothers. This study was conducted in Owerri, Imo State which is located in the Southeastern part of Nigeria for a period of six months. Only subjects with evidence of breastfeeding, and who have duly completed the food and sociodemographic questionnaires were selected for the study. A sample size of 225 lactating mothers was selected for the study.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Mycotoxins

Mycotoxins include a very varied group of chemicals with a wide range of toxicity effects. These secondary metabolites are produced by different fungal species, which grow on field crops or during improper storage of grains and other agricultural products (CAST, 2003). The worldwide occurrence of mycotoxins as contaminants in foods and feeds is worrying because they can cause animal and human diseases or disorders. Judgements on which of the many known mycotoxins are the most important are often based upon their frequency of occurrence and/ or the severity of the disease that they can produce (Sherif *et al.,* 2009). Those with carcinogenic properties, namely aflatoxins, fumonisins and Ochratoxin A, are of major concern to humans (Figure 2.1). Chronic exposure, even at low doses, to these toxins is considered a risk (Kuiper- Goodman *et al*., 2010; Wild and Gong, 2010). Certain essential toxins include citrinine, Deoxynivalenol, T-2 mycotoxin, Zearalenone and certain ergot alkaloids (CAST, 2003). Such toxins can have adverse effects on specific organ systems (Table 2.1) after critical dose exposure. In utero exposure can also have teratogenic or developmental effects: fumonisins, for example, can cause neural tube defects. Aflatoxins, ochratoxin and trichothecenes can affect the immune system, and the ZEA for mycoestrogen mainly affects the reproductive system. Besides various organ-specific actions (liver, kidney, central nervous system), mycotoxins can adversely affect the gastrointestinal system, cause skin irradiation, have haematological effects, and decrease growth (Etzel, 2006; Sherif *et al*., 2009).

Human exposure to mycotoxins often occurs while eating infected foods. Many countries have set maximum allowable levels of mycotoxins in different food supplies for minimizing human exposure (Van Egmond *et al*., 2007). Together with TDI values for major mycotoxins (Table 2.2), these regulations provide a basis for protecting consumers from undesirable health effects of mycotoxins in food. The TDI values developed by scientific advisory committees are extracted from non-observed adverse effect levels of a given compound as defined in animal studies and then divided by uncertainty / safety factors (100 or more) to extrapolate to a safe dose for humans that can be consumed throughout a lifetime without any significant health danger. A TDI cannot be derived for carcinogenic compounds without thresholds such as AFB, since any small dose can constitute a risk (Steffer *et al*., 2004).

Although dietary exposure to mycotoxins may be reduced, it cannot be avoided altogether. A strict control of mycotoxins in food is not enforced everywhere. In fact, in some countries with poor food supply and climate stress seasons, parts of the population have no choice but to consume poor quality or starving foods (Wild and Gong, 2010; Kensler *et al*., 2011). The nursing mothers' food deserves special attention since some of the ingested mycotoxins can also appear in the human breast milk. The presence of mycotoxins in babies' main nutrient source is of concern since newborns and infants are considered more susceptible than adults to adverse effects of these toxins (Sherif *et al*., 2009).

2.2 Overview of Aflatoxin

Aflatoxins are highly toxic secondary metabolites which are synthesized primarily by *Aspergillus* spp fungi, mostly *A. flavus,* and *A. parasiticus* (IARC, 1993; Bhat *et al*., 2010) and seldom from *A. nominus* (Olsen *et al*. 2008). The Aspergillus species are prevalent in tropical and subtropical regions of the world where warm temperatures and humid conditions promote fungal growth and subsequent aflatoxin production (IARC, 1993). The term aflatoxin was created by combining a letter "A" for flavus species representing the Aspergillus genus "fla" and poison toxin (Ellis *et al*., 1991).

There are various types of naturally occurring aflatoxins, which have been identified (IARC, 1993; 2002). However, aflatoxins of major importance are aflatoxin B_1 (AfB_1), aflatoxin B_2 (AfB_2), aflatoxin G_1 (AfG_1) and aflatoxin G_2 (AfG_2). AfB_1 and AfB_2 are produced by fungi of *A. flavus*, while *A. parasiticus* produces AfB_1, AfB_2, AfG_1 and AfG_2 (Kurtzman *et al*., 1987; IARC, 1993; Huchchannanavar and Balol, 2011). The letters B and G refers to blue and green fluorescence colours, respectively, produced by the toxins under ultraviolet light on thin layer chromatography, while numbers 1 and 2 represent different homologues (Huchchannanavar and Balol, 2011; Quadri *et al*., 2013).

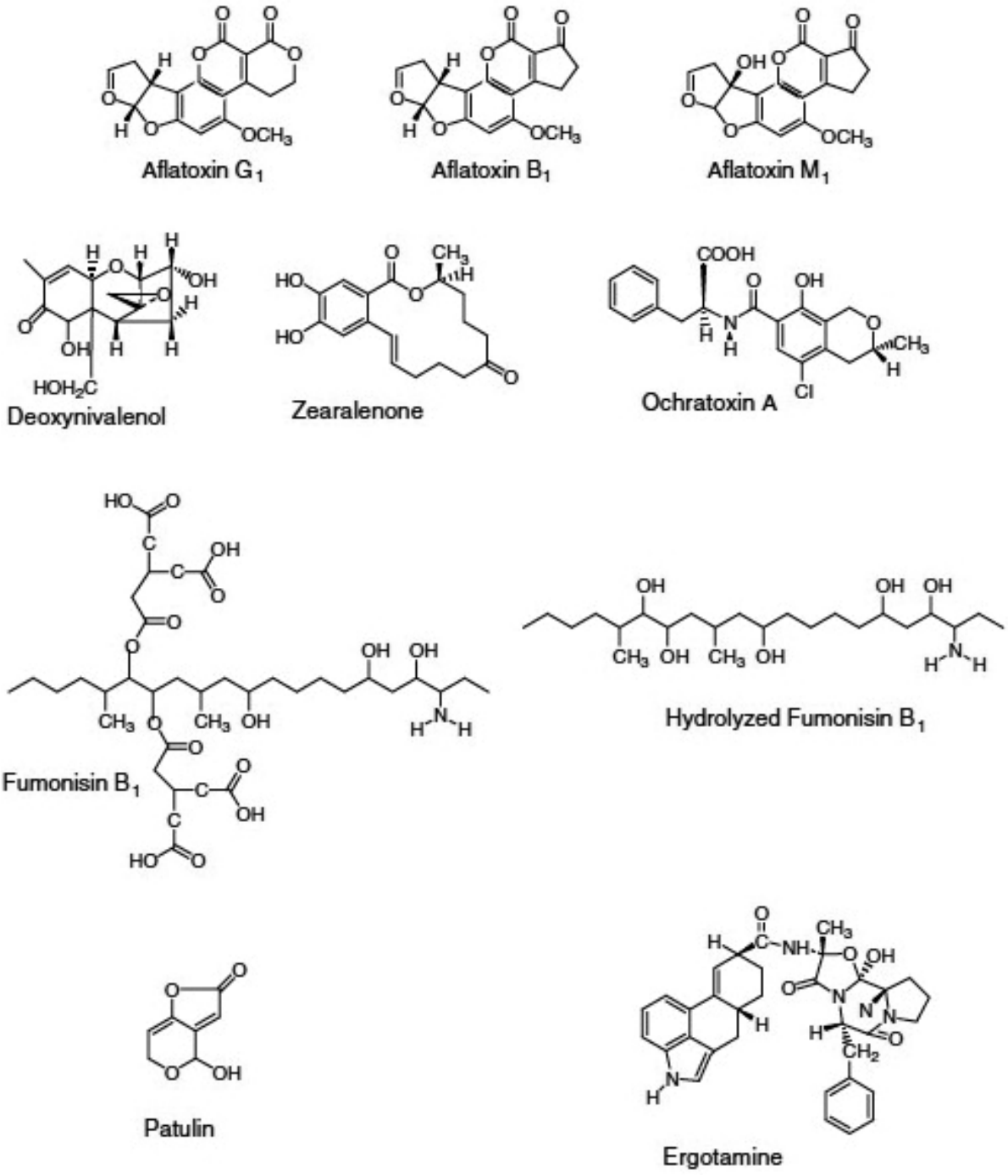


Figure 2.1: Chemical structure of some important Mycotoxins

Table 2.1: Description of certain mycotoxins and their side effects

Mycotoxins	Reported toxicities
Aflatoxin B_1, M_1	• Hepatotoxic and carcinogenic in all species tested except mice. • Genotoxic: adducts formation in human and animals.
Citrinin	• Nephrotoxic in pigs and rodents.
Deoxynivalenol	• Feed refusal and vomiting in domestic pigs • Immunosuppression in mice.
Ergot Alkaloids	• Ergotism with convulsive and/or gangrenous form.
Fumonisins (FB1)	• Leucoencephalomalacia in horses • Pulmonary oedema in pigs • Neural tube defects in mouse embryos. • Rodent carcinogen.
Ochratoxin A	• Nephrotoxic, tetratogenic, immunosuppressant, rodent carcinogen.
Patulin	• Causes damage in intestinal tissues, alterations in renal function.
T-2 and HT-2	• Alimentary toxic aleukia, haemorrhage and vomiting.
Zearalenone	• Oestrogenic effects in pigs and experimental animals.

(Source: Degen *et al.*, 2013)

Table 2.2: Suggested daily intake levels for certain mycotoxins

Mycotoxin	Tolerable Daily Intake
Aflatoxin B_1, M_1 and total	A risk-free intake cannot be identified, but maximum residue levels have been set in foods for direct human consumption*
Deoxynivalenol	1μg/kg b.w
Fumonism B1, B2, B3 alone or in combination	2 μg/kg b.w
Ochratoxin A	5ng/kg b.w
	120 ng/kg b.w/week = 17 ng/kg b.w/day
Patulin	0.4 μg/kg b.w
T-2, HT-2	0.1 μg/kg b.w
Zearalenone	0.25 μg/kg b.w

* These levels are the ones deemed as low as reasonably achievable.

(Sources: EC, 2006; EFSA, 2011a, b; Kuiper-Goodman *et al*., 2010)

In both acute and chronic toxicity, the magnitude order is AfB_1 > AfG_1 > AfB_2 > AfG_2 (IARC, 1993) with respect to the magnitude of the aflatoxin potency. Likewise, in terms of the extent of incidence, AfB_1 accounts for about 70 percent of the total food contents of aflatoxins, although this may also differ, followed by AfG_1, while AfB_2 and AfG_2 occur at significantly lower levels (Horn, 2003). Aflatoxin B1 and B2 can undergo hydroxylation to form metabolites of aflatoxin M_1 and aflatoxin M_2, respectively, which may be excreted in lactating mammals ' milk due to food or feed ingestion contaminated with aflatoxin (Quadri *et al*., 2013). The IARC has classified aflatoxin M_1 as "possibly carcinogenic to humans" (IARC, 2002).

2.2.1 Chemistry of Aflatoxins

Purified aflatoxins are pale-yellow crystalline compounds in colourless form (Pitt *et al*., 2012). These are highly fluorescent in ultraviolet light with blue fluorescence emitting AFB_1 and AfB_2, while green fluorescence emitted by AfG_1 and AfG_2 (Huchchannanavar and Balol, 2011; Pitt *et al*., 2012; Quadri *et al*., 2013). Aflatoxins are marginally water soluble (Nabok *et al*., 2011), insoluble in non-polar solvents, freely soluble in organic polar solvents, and very stable up to temperatures above 1000C (Quadri *et al*., 2013). Aflatoxins are degraded by ammonia or sodium reaction.

2.2.2 Absorption, distribution, metabolism and excretion of Aflatoxins

The principal route of exposure to aflatoxin is the oral consumption of products infected with aflatoxin. Ingestion of aflatoxins transported from feed into animal products such as milk, meat and their products can also result in exposure to aflatoxins via food ingestion (Bhat *et al*., 2010; Völkel *et al*., 2011). In addition, breast-feeding children may be exposed to AfM_1 from breast milk when lactating mothers have consumed food contaminated with aflatoxin (Khlangwiset *et al*., 2011; Magoha *et al*., 2014a; Diaz and Sanchez, 2015). Other important exposure routes are the inhalation of aflatoxin-contaminated dust as a result of occupational exposure, the handling and processing of contaminated crops, in particular during undertakings such as shelling and processing (Bbosa *et al*., 2013a) and direct dermal contact (Zain, 2011). Animal studies found that 50% of AfB_1 ingested is rapidly absorbed into the duodenum and reached the liver through the portal system (Kumagai, 1989; Coulombe *et al*., 1991).

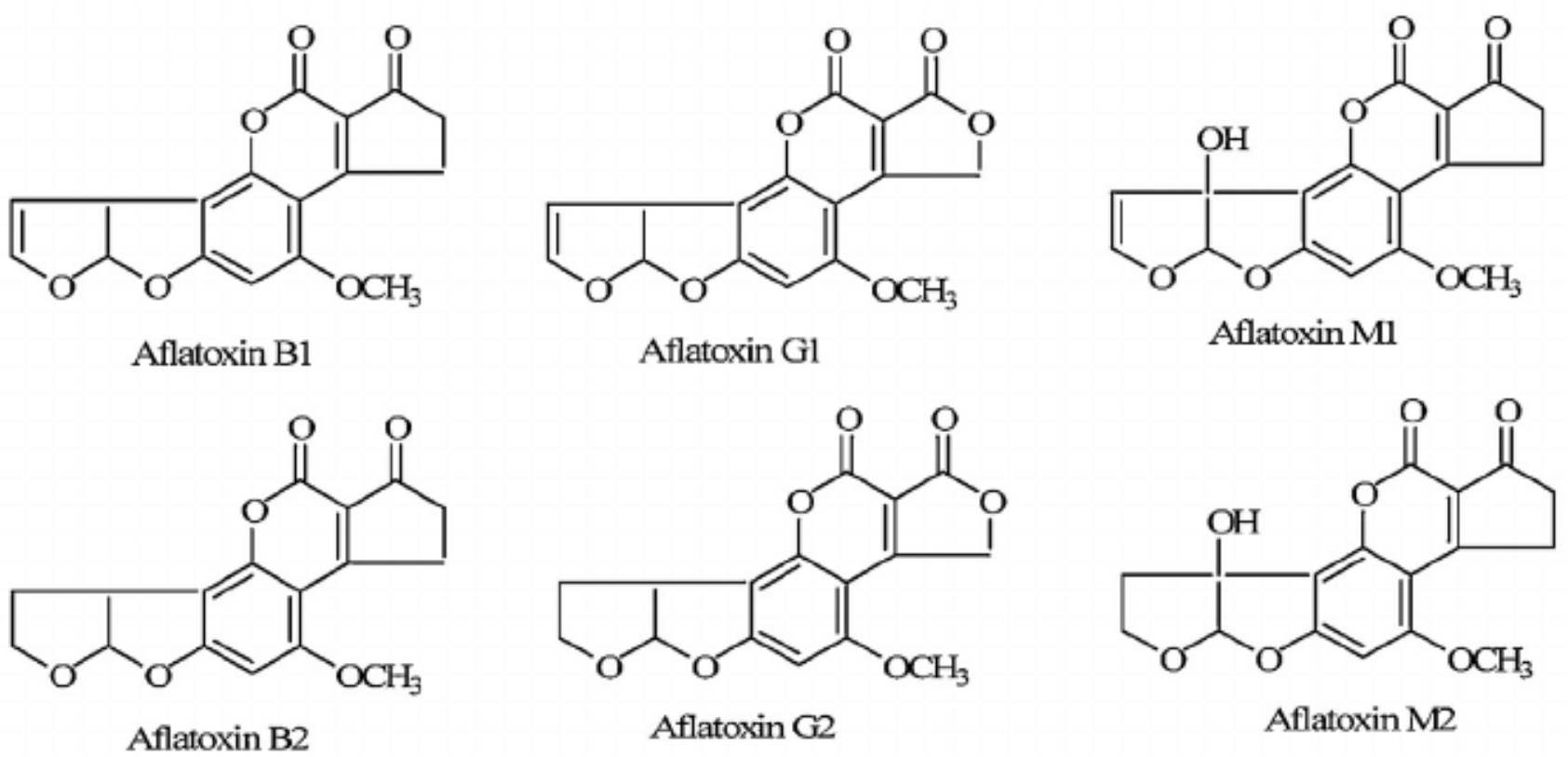


Figure 2.2: The chemical structure of aflatoxins.

Aflatoxin B1 is extremely lipo-soluble and, in the case of occupational airway exposure (Larsson and Tjälve, 2000), is effectively absorbed into the bloodstream through the cell membranes from the duodenal zone of the small intestines (Kumagai, 1989) and the respiratory tract. Aflatoxin B_1 is concentrated in the liver and lower in the kidneys (Wogan *et al*., 1967). The liver is a target site for both acute and chronic toxicity due to exposure to aflatoxin. In the liver, AfB1 is activated by the family of several cytochrome P450 enzymes (CYP450), and biotransformed before excretion into several metabolic products. One of the major AfB_1 metabolites is AfB_1-8, 9-epoxide, a highly active molecule which binds covalently to deoxyribonucleic acid (DNA) and produces AFB_1N7-guanine (Raney *et al*., 1993), as well as other cellular macromolecules. In addition to DNA, the epoxide may also bind to serum albumin to form aflatoxin albumin (Af-alb) or lysine adducts that then enter the circulation of the system (Essigmann *et al*., 1982; Forrester *et al*., 1990; Sabbioni and Wild, 1991).

It is believed that the mechanism for the formation of AfB_1-8, 9-epoxide and subsequent binding to DNA and proteins to form adducts accounts for the biological effects and damage of cell targets (i.e. DNA and proteins) (Wild and Turner, 2002; Bbosa *et al*., 2013b). This mechanism induces dysfunction of the cell's normal process (Wild and Hall, 2000a) and consequently results in reduced mitochondrial function and damages critical cellular components such as DNA, lipids and proteins (Essigmann *et al*., 1982; Wild and Turner, 2002). Bond formation between AfB1 and DNA changes DNA's structure and biological activity, leading to the mutagenic and carcinogenic mechanism of AfB1 (Essigmann *et al*., 1982; Hsieh and Atkinson, 1991). In addition to forming AfB_1-8, 9-epoxide, AfB_1 is also metabolized in the liver to form hydroxylated metabolites called aflatoxin M1 (AfM_1), aflatoxin Q1 (AfQ_1), aflatoxin P_1 (AfP_1), and aflatoxicol (Wild and Turner, 2002; Egner *et al*., 2003; Kensler *et al*., 2011). The AfM_1 metabolite can be activated further to form a new metabolite called AfM_1-8, 9-epoxide, which binds to AfM_1-N7-guanine (Egner *et al*., 2003; Verma, 2004). In the liver, AfB_1 metabolites can also be excreted by bile into the small intestine (Bbosa *et al*., 2013b), and can be reabsorbed and returned to the liver via portal circulation (enterohepatic circulation). Both the ingested AfB_1 and its metabolites are excreted in urine, faecal matter and breast milk. Unabsorbed gastrointestinal aflatoxin and absorbed AfB_1 metabolites from biliary excretion into the intestine are both excreted through faeces (Hsieh *et al*. 1993). The urinary route forms a significant pathway for the excretion of absorbed metabolites of aflatoxin and aflatoxin (Groopman *et al*., 1985; Egner *et al*., 2003).

Mothers who are lactating who eat food contaminated with aflatoxin can excrete aflatoxin metabolites in her milk (IARC, 2002; Polychronaki *et al*., 2007b).

2.2.3 Impact of Aflatoxin on human health

Poisoning that result from aflatoxin ingestion is called aflatoxicosis (Çelik *et al*., 2005). There are two forms of aflatoxicosis identified; the first is extreme acute toxicity that results in damage to the liver and subsequent illness or death. The second type is called the sensitivity to chronic sub-symptoms. Dose and length of exposure to aflatoxins can have major effects on toxicology, and can have a range of health effects. Chronic sublethal doses have nutritional and immunological effects, whereas all doses cumulatively affect cancer risk (Williams *et al*., 2004).

2.2.4 Liver carcinogen

Aflatoxins have been extensively studied and cause adverse health effects in both human and animal body systems and organs (Bbosa *et al*., 2013a). Aflatoxins are potent hepatocarcinogens in animals and sufficient evidence of carcinogenicity has been provided by human epidemiological studies, which has led to the classification of AfB_1 as "group 1 human carcinogen" (IARC, 2002). High incidence of hepatocellular carcinoma (HCC) occurs most often in communities where chronic hepatitis B virus (HBV) infection and chronic aflatoxin dietary exposure are also prevalent. The Chinese population has shown synergistic association between AfB_1 and HBV in the cause of liver cancer (IARC, 1993; Groopman *et al*., 2005; Wild and Montesano, 2009; Kensler *et al*., 2011). Cancer effects due to aflatoxin are attributed to its ability to form adducts through the binding of DNA with AfB_1-8, 9-epoxide metabolite, which is generated during AfB_1 biotransformation. Formation of adducts causes alteration of DNA, a gene mutation, which is believed to be responsible for liver cancer (IARC, 1993; Wild and Turner, 2002a, Bbosa *et al*., 2013a). From the point of view of public health, these findings suggest that measures to reduce exposures to aflatoxin along with HBV vaccination may potentially have an effect on reducing the burden of liver cancer (Kensler *et al*., 2003).

2.2.5 Immune suppression

Upon ingestion of aflatoxins by infected foods, the intestine is the primary target for the damage caused by toxins (Turner *et al*., 2012). Effects on the gastrointestinal tract as a result of aflatoxin exposure have been reported in several animal studies (IARC, 1993; Applegate *et al*., 2009). Intestine impairment disturbs nutrient absorption in the body, which

contributes to nutritional deficiencies. In experimental studies in cell models and in animal studies the immunomodulatory effects of aflatoxins were considered (IARC, 2002; Williams *et al*., 2004). Higher exposure to aflatoxin has been associated with lower salivary immunoglobin A in Gambian children (IgA), (Turner *et al*., 2003). In Ghana, a study reported positive human immunodeficiency virus (HIV) individuals with higher levels of CD4 + T regulatory cells compared to HIV-positive people with lower levels of AfB1 biomarkers (Jiang *et al*., 2008). Although the results available do not allow a conclusion to be drawn about the effect of aflatoxin exposure on human immunity, the evidence still indicate that immune parameters may be impaired in populations chronically exposed to aflatoxins (Pitt *et al*., 2012). Impaired immune functions contribute to increased vulnerability to infectious diseases in the exposed population, and particularly the vulnerable.

2.2.6: Impairment of childhood growth

Studies in animal species indicate that chronic exposure to aflatoxin can have serious consequences for growth and development (Pitt *et al*., 2012). In the veterinary sector, animal exposure was related to reduced weight gain, reduced feed intake and nutrient uptake, impaired feed conversion capacity, impaired immunity, and growth failure (Han *et al*., 2008; Andretta *et al*., 2012; Da Rocha *et al*., 2014; Rezaei *et al*., 2014).

In human studies, exposure to aflatoxin in utero, suggested with loss in infant development during breastfeeding and complementary feeding. One of the earliest studies in Sudanese children documented the correlation between aflatoxin exposure and kwashiorkor (Hendrickse *et al*., 1982). High prevalence of waste was associated with intake of cereals infected with high aflatoxin levels in Kenya (Okoth and Ohingo, 2005). A cross-sectional analysis in Togo and Benin found an inverse correlation between the AF-alb concentration and the Z-scores growth indicator in children (Gong *et al*., 2002). In Benin, a longitudinal study showed a strong negative correlation between AF-alb levels and height increases over eight months of follow-up with children in the highest exposure group associated with a mean height decrease of 1.7 cm compared to the lowest exposure group (Gong *et al*., 2004). There was also correlation between exposure to aflatoxin in utero and lack of growth in Gambian children during the first year of life (Turner *et al*., 2007). A study in Ghana found that mothers with higher levels of AfB1-lysine during pregnancy were more likely to have babies with a low birth weight compared to mothers with lower levels (Shuaib *et al*., 2010).

Given the associations recorded on exposure to aflatoxin and growth impairment, however, the biological mechanisms of action by which aflatoxin can have an effect on

growth are currently not clear and therefore need further investigation (Pitt *et al*., 2012). Possible Hypotheses include impaired bowel integrity and nutrient absorption through altered barrier activity due to endothelial cell toxicity or immune suppression (Gong *et al*., 2008a; Smith *et al*., 2012). Other possible reasons include inhibition of protein synthesis by binding AfB1 with DNA, RNA and proteins and thus causing interference with the enzymes and substrates required for protein synthesis processes (Bbosa *et al*., 2013a). Further study of the health effects of aflatoxin, however, may be complicated by exposure to other forms of mycotoxins co-occurring in the same food (Strosnider *et al*., 2006) as well as other confounding factors.

2.2.7: Acute toxicity and death

Acute exposure to aflatoxins can lead to aflatoxicosis, which is shown to be serious, acute hepatotoxic. Anorexia, malaise, and low-grade fever may be the early symptoms of serious aflatoxicosis. Acute exposure to high levels can lead to vomiting, abdominal pain, jaundice, hepatic failure and death (Strosnider *et al*., 2006). Recurrent outbreaks of acute toxicity and deaths have caused acute dietary exposure to high levels of aflatoxin. Consecutive outbreaks of acute aflatoxicosis triggered by contamination of aflatoxin in homegrown maize occurred in Kenya between 2004 and 2005, resulting in 125 confirmed deaths of 317 cases of acute liver failure (Gieseker and CDC, 2004; Probst *et al*., 2007), thus expressing the serious public health issue due to exposure to aflatoxins.

2.3 Occurrences of Aflatoxins in Breast Milk

AfB_1, AfB_2, AfG_1 and AfG_2 are a group of contaminants produced naturally by *Aspergillus* species *(A. flavus and A. parasiticus),* and these mycotoxins often occur in foodstuffs such as nuts and maize derived products (CAST, 2003). Quite toxic is the most critical compound AfB, and a prime example of potent human carcinogen (Kensler *et al*., 2011). It is biotransformed to a DNA-reactive and mutagenic epoxide and other metabolites in humans and animals, including the hydroxylated metabolite AfM_1 excreted with milk and urine (Figure 2.l). The first studies on the occurrence of mycotoxin in human milk date back to the mid and late ' 80s. Early surveys were carried out in Germany of the occurrence of aflatoxins in human breast milk (DFG, 1984) and Sudan (Coulter *et al.,* 1984). Although sensitive analytical techniques have been applied to 120 Kiev and 65 Munich samples (LOD 3 and 6 ng / kg), a total of 185 breast milk samples (DFG, 1984) have not found the milk metabolite AFM1. Conversely, all 75 Sudanese samples tested positive (Coulter *et al.,* 1984). Then Wild *et al*. (1987) confirmed the presence of aflatoxins from mothers in Zimbabwe in

11 percent of milk samples (6/54), with rates of up to 51 ng / l. Nevertheless, none of the breast milk samples collected in France from mothers (0/42) contained detectable levels of aflatoxins. Analysis of Italian breast milk samples found a very low detection frequency (1-5 per cent) and a maximum of less than 200 ng/1 (Galvano *et al.*, 2008; Turconi *et al.,* 2004).

A number of studies in Africa have revealed the presence of aflatoxins (mainly AFB1 and AFM1) in breast milk from Ghana (32% positive), Kenya (28%), Nigeria (12%) and Sudan (37%) at levels up to several μg/1 (Lamplugh *et al*., 1988; Maxwell *et al*., 1989). Similar results for Sierra Leone were reported on later (Jonsyn *et al*., 1995). Such data show that babies are regularly exposed to aflatoxins in several African countries, and sometimes at very high levels of exposure. More recent data show that aflatoxins (AfM_1) also occur frequently in Egyptian breast milk (El-Sayed *et al*, 2002; Hassan *et al*., 2006a; Polychronaki *et al*., 2006; Tomerak *et al*., 201l), with a wide range of concentrations. As reported in several studies, the levels of AfM_1 breast milk show seasonal fluctuations most likely associated with variable levels of AfB_1 contaminants in the diet of mothers due to seasonal climatic conditions favoring mould growth and toxin production (example; El-Sayed *et al*., 2002; Maxwell *et al*., 1989; Polychronaki *et al.*, 2007).

The amount of AfM_1 detected in Egyptian mothers ' breast milk (average 13.5 ng/1; Polychronaki *et al*., 2006) is lower than that recorded in some other countries. In a large survey of 445 mothers ' aflatoxin in Abu-Dhabi breast milk, 99.5 percent of samples tested positive for AfM_1 at concentrations ranging from 2 ng/1 to 3,000 ng / l and a geometric mean of approximately 68 ng / l (Saad *et al.*, 1995). A more recent United Arab Emirates breast milk analysis found positive median levels (92 per cent of 140 samples) of 560 ng / ml and 5 to 3,400 ng / l (Abdulrazzaq *et al*., 2003). A small number of breast milk collected in Thailand was also reported to have high maximum AfM_1 levels (El-Nezami *et al*., 1995). In addition to the geographic patterns, differences also exist in the occurrence of AfM_1 in breast milk samples collected in different regions of a given country. Such differences are indicated by recent studies in Iran (Mahdavi *et al*., 2010; Sadeghi *et al*., 2009) and in Turkey (Gürbay *et al*., 2010a; Keskin *et al*., 2009), for example.

Aflatoxins are harmful compounds in breast milk, for sure. Reported detection rates and mean concentrations of these pollutants suggest that infant exposure in Europe is small or negligible, but may reach critical levels in Tropical Africa and some Middle East countries.

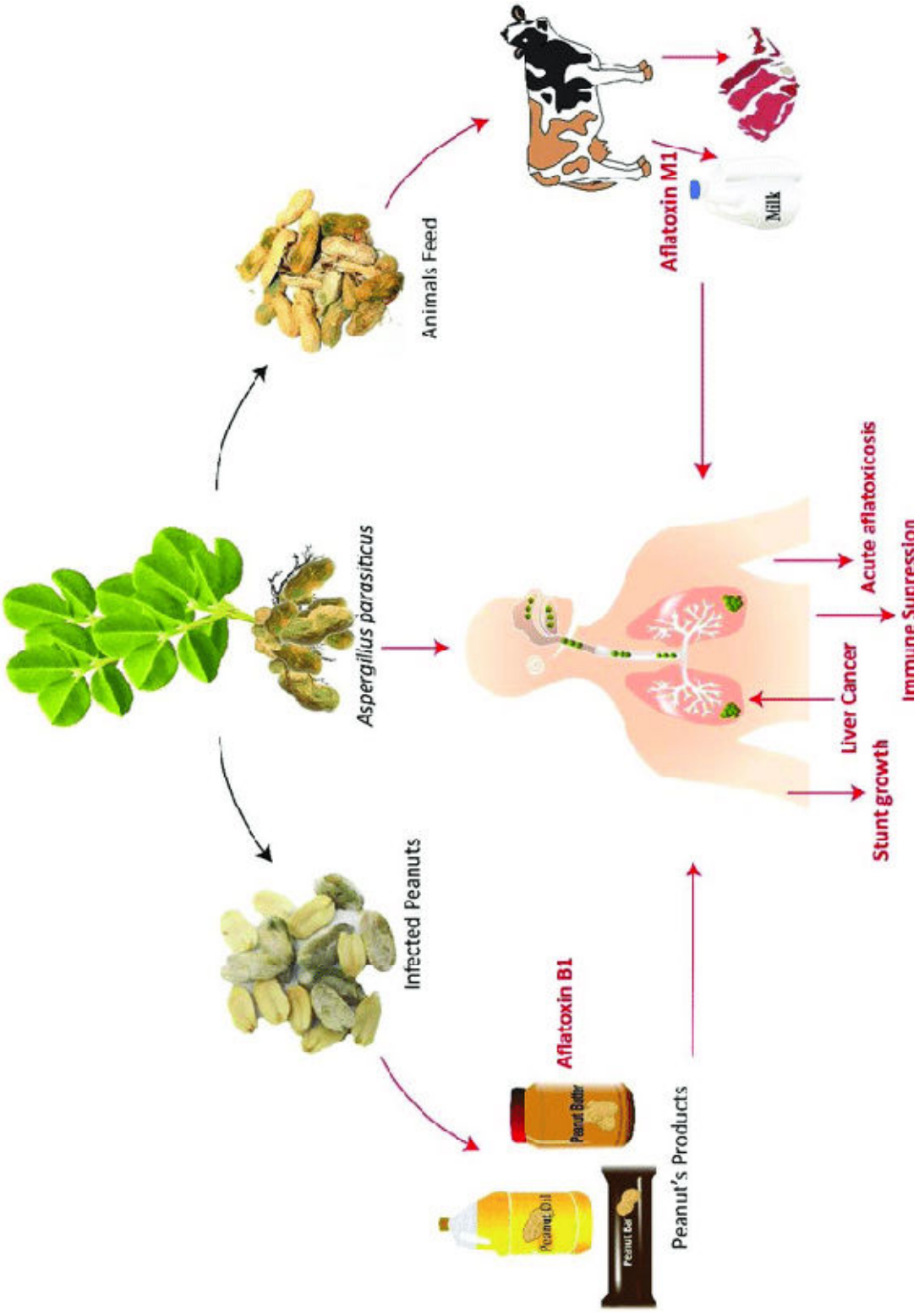


Fig. 2.3 Sources of Aflatoxin exposure and its flow in the food chain.

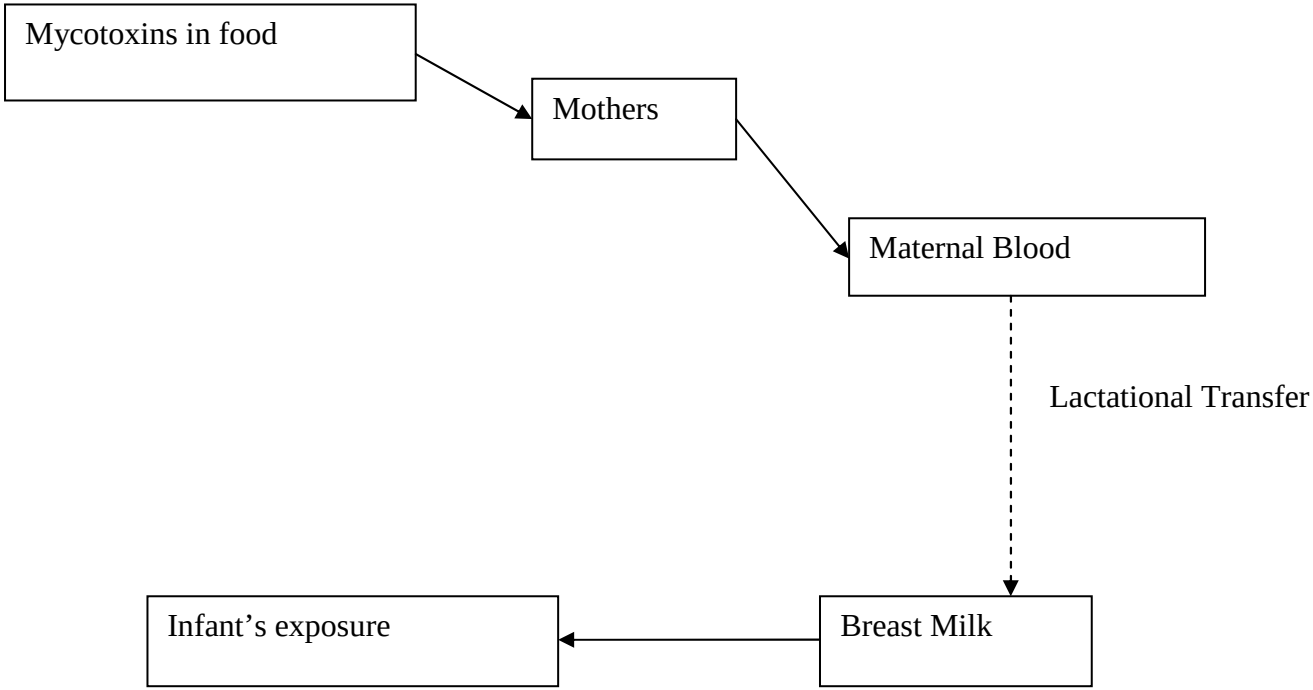


Figure 2.4: Factors influencing the exposure of infants to mycotoxins in early life.

2.4 Lactational transfer for Aflatoxins B_1 and M_1

The relationship between dietary intake of aflatoxins and metabolite excretion in breast milk of 5 mothers was explored in an early biomonitoring / molecular dosimetry study conducted in Gambia, West Africa. Estimates of the percentage of aflatoxins excreted in milk as AfM_1 ranged from 0.09 to 0.43 percent in the diet (Zarba *et al*., 1992). Since breastfeeding is not given, one can only speculate whether the range of variables reflects early and/or late nursing stages. The only study correlating the duration and occurrence of AfM_1 in breastfeeding is that of Polychronaki *et al*., (2006). They observed the frequency of detectable AfM_1 in breast milk varied greatly depending on the duration of the lactation. Thus, in breast milk, AfM_1 was observed most frequently in the first month of lactation than at the later stages of breastfeeding. The same authors recruited 50 Egyptian mothers (pre-selected based on AfM_1, breast milk levels) where the lactation duration was fitted into a statistical analysis as a linear term, a one-month increase in the lactation stage contributed to an 8 percent increase in breast milk risk of AfM_1 (Polychronaki *et al*., 2007). Yet the months (season) during which breast milk samples were collected appeared to be the dominant factor deciding AfM_1, presence, and levels. Another study reported mean levels of contamination in Egyptian mothers ' milk (1.9 ng / ml) and serum (8.9 ng / ml) (Hassan *et al*., 2006a); values estimating lactational transfer for aflatoxins with an M / P ratio of approximately 0.21.

2.5 Exposure of infants to Aflatoxins in early life

It has been shown that AfM_1 is a biomarker of nursing mothers ' dietary exposure to AFB1 in breast milk and, consequently, an exposure to aflatoxin in breastfed infants (Earba *et al*., 1992). Data available on AfM_1, breast milk levels from different countries indicate a wide range of maternal and infant exposures (Degen *et al*., 2013). There is no or little concern about average levels of human milk from Europe and some other countries, although peak concentrations can translate into undesirable exposures of breastfed children. The high concentrations found in samples of breast milk from several tropical West African countries, but also in the United Arab Emirates and Egypt, exceed the maximum tolerance limit for AfM_1 in milk and infant foods (25 mg / kg) set out in the European and US regulations (EC, 2006; Van Egmond *et al*., 2007). The high concentrations found in samples of breast milk from several tropical West African countries, but also in the United Arab Emirates and Egypt, surpass the maximum tolerance limit for AfM_1 in milk and infant foods (25 mg / kg) set out in the European and US regulations (EC, 2006; Van Egmond *et al*. 2007). Yet, what

breastfeeding choices are there in developed countries with poor quality staple foods where weaning foods are as likely to be contaminated as parental foods. Studies in high-risk African countries that compared aflatoxin exposure in fully sewed infants with those still partially sewed show that contaminant exposure significantly increases the following sewage and that early life exposure is associated with reduced growth (Gong *et al.*, 2003). This reinforces the need for intervention strategies in countries at high risk to reduce the exposure to aflatoxin (Kensler *et al.*, 2011; Wild and Gong, 2010).

2.6 Metal Toxicity

Metals are substances with high electrical conductivity, malevolence and luster, which lose their electrons willingly to form cations. Metals are naturally found in the earth's crust, and their compositions differ from one locality to another, resulting in spatial differences in regional concentrations. The distribution of metals in the atmosphere is monitored by the properties of the metal and by different environmental factors (Khlifi and Hamza-Chaffai, 2010).

Heavy metals are generally referred to as metals with a particular density exceeding 5 g / cm^3 and which adversely affect the environment and living organisms (Jarup, 2003). These metals are quintessential in maintaining various biochemical and physiological functions in living organisms at very low concentrations; however when they exceed certain threshold concentrations they become noxious.

Though these heavy metals are known to have many adverse health effects and last for a long time, heavy metal exposure persists and is growing in many parts of the world. Heavy metals are important threats to the atmosphere and their toxicity is a concern of increasing importance for economic, biological, nutritional and environmental reasons (Jaishankar *et al*., 2014; Nagajyoti *et al*., 2010). The most commonly found heavy metals in wastewater include arsenic, cadmium, chromium, and copper, lead, nickel, and zinc, all of which pose risks for human health and the environment (Lambert, 2000). Heavy metals enter the atmosphere by natural means and by human activity. Various heavy metal sources include soil erosion, natural weathering of the Earth's crust, mining, industrial effluents, urban runoff, sewage discharge, insect or disease control agents used in crops, and many others (Morais *et al*., 2012).

Although these metals have essential biological functions in plants and animals, they have sometimes been given an additional advantage by their chemical coordination and oxidation-reduction properties so they can avoid control mechanisms such as homeostasis,

transportation, compartmentalization and binding to the necessary cell constituents. Such metals bind to protein sites that are not intended for them by removing the original metals from their normal binding sites that cause cell failure and eventually toxicity. Previous research found that the oxidative deterioration of biological macromolecules mainly results from the binding of heavy metals to DNA and nuclear proteins (Flora *et al*., 2008).

2.7 Heavy Metals and Their Toxicity Mechanisms

2.7.1 Arsenic

Arsenic is one of the most important heavy metals causing disquiet from both ecological and individual health standpoints (Hughes *et al.*, 1988). It has a semi-metallic property, is prominently toxic and carcinogenic, and is extensively available in the form of oxides or sulphides or as a salt of iron, sodium, calcium, copper, *etc.* (Singh *et al*., 2007). Arsenic is the twentieth most abundant element on earth and its inorganic forms such as arsenite and arsenate compounds are lethal to the environment and living creatures. Humans may encounter arsenic by natural means, industrial source, or from unintended sources. Drinking water may get contaminated by the use of arsenical pesticides, natural mineral deposits or inappropriate disposal of arsenical chemicals. Deliberate consumption of arsenic in the case of suicidal attempts or accidental consumption by children may also result in cases of acute poisoning (Mazumder, 2008; Saha, 1999). Arsenic is a protoplastic poison since it affects primarily the sulphydryl group of cells causing malfunctioning of cell respiration, cell enzymes and mitosis (Gordon and Quastel, 1948).

***Mechanism of Arsenic Toxicity*:** In arsenic biotransformation, harmful inorganic arsenic compounds get methylated by bacteria, algae, fungi and humans to give monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). In this biotransformation process, these inorganic arsenic species (iAs) are converted enzymatically to methylated arsenicals which are the end metabolites and the biomarker of chronic arsenic exposure.

iAs (V) →iAs (lll) →MMA (V) →MMA (lll) →DMA (V)

Biomethylation is a detoxification process and end products are methylated inorganic arsenic such as MMA (V) and DMA (V), which excreted through urine, are bioindication of chronic arsenic exposure. However, MMA (III) is not excreted and remains inside the cell as an intermediate product. Monomethylarsonic acid (MMA III), an intermediate product, is found to be highly toxic compared to other arsenicals, potentially accountable for arsenic-induced carcinogenesis (Singh *et al*., 2007).

2.7.2 Lead

Lead is a highly toxic metal whose extensive use in many parts of the world has caused considerable environmental contamination and health problems. Lead is a bright, slightly bluish silvery metal in a dry atmosphere. It starts to tarnish on air contact, thus forming a complex mixture of compounds depending on the conditions given. Lead exposure sources include mainly industrial processes, food and smoking, drinking water, and domestic sources. The lead sources include petrol and house paint, which has been generalized to lead bullets, plumbing pipes, pewter pitchers, storage tubes, toys, and faucets (Thurmer *et al*., 2002). In the US, vehicle exhausts release more than 100 to 200,000 tons of lead per annum. Some are taken up by plants, fixed to the soil and drained into water bodies, and human exposure to lead is either due to food or drinking water in the general population (Goyer, 1990).

Lead is an extremely toxic heavy metal that disturbs physiological processes of various plants and, unlike other metals such as zinc, copper and manganese, it does not play any biological functions. A plant with a high concentration of lead fastens the development of reactive oxygen species (ROS), causing damage to the lipid membrane that ultimately results in damage to chlorophyll and photosynthetic processes and suppresses overall plant growth (Najeeb *et al*., 2014). Some research has revealed that lead can inhibit tea plant growth by reducing biomass and degrading the tea quality by changing the quality of its components (Yongsheng *et al*., 2011). Even at low concentrations, lead treatment has been found to cause considerable instability in plant ion uptake, which in turn leads to major metabolic changes in photosynthetic efficiency, and ultimately to strong plant growth inhibition (Mostafa, 2012).

***Mechanisms of Lead Toxicity*:** Lead metal causes toxicity in living cells by following the ionic mechanism and that of oxidative stress. Many researchers have shown that oxidative stress in living cells is caused by the imbalance between the production of free radicals and the generation of antioxidants to detoxify the reactive intermediates or to repair the resulting damage. Antioxidants, such as glutathione, present in the cell protect it from free radicals such as H_2O_2. Under the influence of lead, however, the level of the ROS increases and the level of antioxidants decrease. Since glutathione exists both in reduced (GSH) and oxidized (GSSG) state, the reduced form of glutathione gives its reducing equivalents ($H^+ + e^-$) from its thiol groups of cysteine to ROS to make them stable. In the presence of the enzyme glutathione peroxidase, reduced glutathione readily binds with another molecule of glutathione after donating the electron and forms glutathione disulfide (GSSG). The reduced form (GSH) of glutathione accounts for 90% of the total glutathione content and the oxidized

form (GSSG) accounts for 10% under normal conditions. Yet under the condition of oxidative stress, the concentration of GSSG exceeds the concentration of GSH. Another biomarker for oxidative stress is lipid peroxidation, since the free radical collects electron from lipid molecules present inside the cell membrane, which eventually causes lipid peroxidation (Wadhwa, 2012; Flora, 2012). At very high concentrations, ROS may cause structural damage to cells, proteins, nucleic acid, membranes and lipids, resulting in a stressed situation at the cellular level (Mathew, 2011).

The ionic mechanism of lead toxicity occurs mainly due to the ability of lead metal ions to replace other divalent cations like Ca^{2+}, Mg^{2+}, Fe^{2+} and monovalent cations like Na^{+}, which ultimately disturbs the biological metabolism of the cell. The ionic mechanism of lead toxicity causes significant changes in various biological processes such as cell adhesion, intra- and inter-cellular signalling, protein folding, maturation, apoptosis, ionic transportation, enzyme regulation, and release of neurotransmitters. Lead can substitute calcium even in picomolar concentration affecting protein kinase C, which regulates neural excitation and memory storage (Flora, 2012).

2.7.3 Mercury

The metallic mercury is a naturally occurring metal which is a shiny silver-white, odourless liquid and becomes colourless and odourless gas when heated. Mercury is very toxic and exceedingly bioaccumulative. Its presence adversely affects the marine environment and hence many studies are directed towards the distribution of mercury in water environment. Major sources of mercury pollution include anthropogenic activities such as agriculture, municipal wastewater discharges, mining, incineration, and discharges of industrial wastewater (Chen *et al*., 2012).

Mercury exists mainly in three forms: metallic elements, inorganic salts and organic compounds, each of which possesses different toxicity and bioavailability. These forms of mercury are present widely in water resources such as lakes, rivers and oceans where they are taken up by the microorganisms and get transformed into methyl mercury within the microorganism, eventually undergoing biomagnification causing significant disturbance to aquatic lives. Consumption of this contaminated aquatic animal is the major route of human exposure to methyl mercury (Trasande *et al*., 2005). Mercury is extensively used in thermometers, barometers, pyrometers, hydrometers, mercury arc lamps, fluorescent lamps and as a catalyst. It is also being used in pulp and paper industries, as a component of batteries and in dental preparations such as amalgams.

***Mechanism of Mercury Toxicity*:** Mercury is well known as a hazardous metal and its toxicity is a common cause of acute heavy metal poisoning with cases of 3,596 in 1997 by the American Association of Poison Control Centres. Methylmercury is a neurotoxic compound which is responsible for microtubule destruction, mitochondrial damage, lipid peroxidation and accumulation of neurotoxic molecules such as serotonin, aspartate, and glutamate (Patrick, 2002). The total amount of mercury emission into the environment has been assessed at 2,200 metric tons annually (Ferrara *et al.*, 2000). It is estimated that 8 to 10% of American women have mercury levels that would induce neurological disorders in any child they gave birth to, according to both the Environmental Protection Agency and National Academy of Science (Haley, 2005). Animals which are exposed to toxic mercury have shown adverse neurological and behavioural changes. Rabbits, when exposed to 28.8 mg/m^3 mercury vapour for 1 to 13 weeks, have shown vague pathological changes, marked cellular degeneration and brain necrosis (Ashe *et al*., 1953). The brain remains the target organ for mercury, yet it can impair any organ and lead to malfunctioning of nerves, kidneys and muscles. It can disrupt the membrane potential and interrupt with intracellular calcium homeostasis.

Mercury binds to freely available thiols as the stability constants are high (Patrick, 2002). Mercury vapours can cause bronchitis, asthma and temporary respiratory problems. Mercury plays a key role in damaging the tertiary and quaternary protein structure and alters the cellular function by attaching to the selenohydryl and sulphydryl groups which undergo reaction with methyl mercury and hamper the cellular structure. It also intervenes with the process of transcription and translation resulting in the disappearance of ribosomes and eradication of endoplasmic reticulum and the activity of natural killer cells. The cellular integrity is also affected causing free radical formation. The basis for heavy metal chelation is that even though the mercury sulphydryl bond is stable and divided to surrounding sulphydryl consisting ligands, it also contributes free sulphydryl groups to promote metal mobility within the ligands (Bernhoft, 2012).

2.7.4 Cadmium

Cadmium is the seventh most toxic heavy metal as per ATSDR ranking. It is a by-product of zinc production which humans or animals may get exposed to at work or in the environment. Once this metal gets absorbed by humans, it will accumulate inside the body throughout life. This metal was first used in World War I as a substitute for tin and paint industries as a pigment. In today's scenario, it is also being used in rechargeable batteries, for

special alloys production and also present in tobacco smoke. About three-fourths of cadmium is used in alkaline batteries as an electrode component, the remaining part is used in coatings, pigments and platings and as a plastic stabilizer. Humans may get exposed to this metal primarily by inhalation and ingestion and can suffer from acute and chronic intoxications.

Cadmium distributed in the environment will remain in soils and sediments for several decades. Plants gradually take up these metals which get accumulated in them and concentrate along the food chain, reaching ultimately the human body. In the US, more than 500,000 workers get exposed to toxic cadmium each year as per The Agency for Toxic Substances and Disease Registry (Bernard, 2008; Mutlu *et al*., 2012). Researchers have shown that in China the total area polluted by cadmium is more than 11,000 hectares and its annual amount of industrial waste of cadmium discharged into the environment are assessed to be more than 680 tons. In Japan and China, environmental cadmium exposure is comparatively higher than in any other country (Han *et al*., 2009). Cadmium is predominantly found in fruits and vegetables due to its high rate of soil-to plant transfer (Satarug *et al*., 2011). Cadmium is a highly toxic nonessential heavy metal that is well recognized for its adverse influence on the enzymatic systems of cells, oxidative stress and for inducing nutritional deficiency in plants (Irfan *et al*., 2013).

***Mechanism of Cadmium Toxicity*:** The mechanism of cadmium toxicity is not understood clearly but its effects on cells are known (Patrick, 2003). Cadmium concentration increases 3,000 fold when it binds to cysteine-rich protein such as metallothionein. In the liver, the cysteine-metallothionein complex cause hepatotoxicity and then it circulates to the kidney and gets accumulated in the renal tissue causing nephrotoxicity. Cadmium can bind with cysteine, glutamate, histidine and aspartate ligands and can lead to the deficiency of iron (Castagnett *et al*., 2002). Cadmium and zinc have the same oxidation states and hence cadmium can replace zinc present in metallothionein, thereby inhibiting it from acting as a free radical scavenger within the cell.

2.7.5 Chromium

Chromium (Cr) is the 21st most abundant mineral in the crust of the earth and may occur in all oxidation states from -2 to +6. It is most often found in 0, +2, +3 and +6. Elemental chromium (0) is not naturally present in the earth crust and is biologically inert. Almost all naturally found Cr is trivalent while hexavalent Cr is mostly of industrial origin. Most chromium compounds are halides, oxides or sulphides.

Divalent chromium (Cr^{2+}) is a strong reductant; the form is readily oxidised when in contact with air, producing Cr^{3+}. This explains why divalent Cr is not available in biological systems. Hexavalent chromium (Cr^{6+}) is the second most stable form and a strong oxidising agent, especially in acidic media. Hexavalent chromium is bound to oxygen as chromate ($Cr0_4^{2-}$) and dichromate ($Cr_20_7^{2-}$) with strong oxidative capacity. This form of Cr crosses biological membranes easily, reacting with protein components and nucleic acids inside the cell while being deoxygenated to Cr^{3+}. The reaction with genetic matter provides for the carcinogenic properties of Cr^{6+}.

Trivalent chromium (Cr^{3+}) is the most stable oxidation state in which chromium is found in living organisms. It cannot cross the cell membranes easily (Mertz, 1992) and has low reactivity, which is the most significant biological feature distinguishing it from Cr^{6+}. Trivalent Cr forms several coordination complexes, hexadentate ligands being the basic form. Some forms of Cr^{3+} (e.g. Cr_2O_3) are, thanks to their low reactivity and absorption from the gastrointestinal system, used as markers in the study of digestion processes (Furnival *et al*., 1990).

***Mechanism of* Chromium *Toxicity*:** Chromium toxicity is associated mainly with hexavalent chromium, whole trivalent chromium is believed to be a highly safe mineral. Hexavalent chromium is more soluble than trivalent Cr and at least five times as toxic (Barceloux, 1999). The safety limit for Cr^{3+} is approximately 1:10,000. Cr^{3+} toxicity is, in fact, lower than the toxicity of all other essential elements such as Cu, Fe, Zn, Mn and especially Se (Lindemann, 1996).

The toxicity of Cr^{6+} compounds is most probably based on oxidative DNA impairment (Cohen *et al*., 1993). The details of Cr6+ toxic activity are however not known. It is assumed that genotoxicity may be due to a transient form (Cr^{5+}) of intracellular origin formed by the reduction of Cr^{6+} to Cr^{3+} (Stearns *et al*., 1995). Extracellular reduction of Cr^{6+} to Cr^{3+} is regarded as a protective reaction (De Flora *et al*., 1989). The main protection mechanism against Cr^{6+} activity in the lungs and the stomach is the reduction of Cr^{6+} to Cr^{3+} by an NADPH-dependent mechanism involving ascorbate. Animal trials show that glutathione plays an important role in Cr^{6+} reduction in erythrocytes, also showing certain reduction activity in the lungs (Suzuki and Fukuda, 1990). Cr intoxication is characterised by pathological anatomical changes in the lungs, kidneys and liver. The lungs are affected with hyperaemia, erosion and an inflammatory change in the respiratory system mucosa developing after Cr inhalation. With Cr^{6+} compounds sensitising the lungs, a bronchial spasm or even an anaphylactic reaction may develop. Chronic exposure to Cr has been observed to

cause nose septum perforation (Lee *et al*., 2002) and small cell cancer of the lung tissue has been reported. Acute intoxication with Cr^{6+} leads to acute renal tubular necrosis characterised by significant interstitial change and subsequent renal failure (Ellis *et al*., 1982; Saryan and Reedy, 1988). Renal glomeruli usually remain intact. The hepatic parenchyma develops necrosis only at very high Cr^{6+} doses.

2.7.6 Copper

Various cells and tissues are known to contain copper with the liver and brain containing the highest concentrations (Turnlund, 1998). Cu mainly exists in biological systems as cupric form (Cu^{++}), although several separate types of the bound Cation can be found in Cu containing enzymes, often in combination within a single protein (Su *et al* 1982; Divertie *et al*. 1982). Essentially all of the body's Cu in normal healthy humans is linked to enzyme prosthetic groups or tightly bound to Cu transport or chaperone proteins (Rosenzweg, 2001, Prohaska, 2008, Boal and Rosenzweig, 2009). Cu chaperones help to minimize the probability of unbound (free) Cu from participating in redox reactions (Burkitt, 2001; Evans and Halliwell, 1994) and ensure delivery of Cu ions to specific target proteins (Boal and Rosenzweig, 2009, Fields *et al*., 2001, Prohaska, 2008). Cu absorbed more than metabolic requirements are normally excreted through bile. The amount of Cu ingested in food and water is relatively low, and the body can control excess amounts of Cu in the body by either decreased absorption or increased excretion under normal conditions. As tight control of Cu homeostasis prevents the excess accumulation of Cu in the body, acute and chronic Cu toxicity is relatively rare. However, Cu toxicity may result from exposure to excess Cu caused by accident, occupational hazard, environmental contamination, as well as adrenal gland insufficiency, inborn errors of Cu metabolism and other factors. A recommended dietary allowance (RDA) of 0.9 mg/day (0.013 mg/kg/day) has recently been established.

Mechanism of Copper toxicity: Most organisms possess a combination of regulated import, sequestration, and enhanced export mechanisms to protect against metal-induced toxicity. These mechanisms regulate metal status through metal-binding proteins at transcriptional, translational, and enzymatic levels. As stated above, the presence of a complex system of metal ion transporters and chaperones to regulate Cu homeostasis ensures Cu is provided to essential proteins without causing cellular damage. Disruptions in the homeostasis of Cu is associated with tissue damage and many diseases (Bleackley and Macgillivray, 2011; de Romana *et al*., 2011). In addition to the direct interact with essential macromolecules and minerals, several mechanisms, notably free radical-induced oxidative

damage have been proposed to explain Cu-induced cellular toxicity. In addition to the free radical-induced oxidative damage, information available suggests that the cellular response to Cu overload, particularly at the early stages of Cu accumulation, involves more specific mechanisms and pathways. This includes regulation of lipid metabolism; antimicrobial defence; neuronal activity; the resistance of tumour cells to chemotherapeutic drugs; kinase-mediated signal transduction; and other essential cellular processes (Hasan and Lutsenko 2012).

2.7.7 Zinc

Zinc, a divalent cation, is an essential micronutrient for human and its importance can be gauged from the fact that it is an essential component of more than 300 metalloenzymes and over 2000 transcription factors that are needed for the regulation of lipid, protein and nucleic acid metabolism and gene transcription. It is involved in gene transcription at various levels, via participation in histone deacetylation reactions and via factors possessing the zinc finger motifs (Bibi Nitzan and Cohen, 2006). An important family of zinc finger proteins is the steroid or thyroid hormone receptors that bind hormones and facilitate their wide range of effects. Zinc also plays an important role in maintaining the proper reproductive function, immune status and wound repair through regulation of DNA and RNA polymerases, thymidine kinase and ribonucleases. It maintains macrophage and neutrophil functions, natural killer cell activity and complement activity. It activates natural killer cells and phagocytic functions of granulocytes and stabilizes the plasma subcellular membranes especially the lysosomes. It inhibits the expression of integrins by keratinocytes and modulates the production of TNF-α and IL-6 and reduces the production of inflammatory mediators like nitric oxide. It is also proposed that it is toll-like receptors mediated regulation of zinc homeostasis which influences dendritic cell function and immune processes (Kitamura *et al*., 2006). Zinc also possess the antioxidant property and has been found useful in preventing UV-induced damage and reducing the incidence of malignancies. It has also been demonstrated to possess antiandrogenic properties as it causes modulation of 5α-reductase type 1 and 2 activity (Bibi Nitzan and Cohen, 2006; Brocard *et al*., 2007; Sharma, 1985).

Mechanism of Zinc Toxicity: The main causes of zinc toxicity include food poisoning, inhalation of zinc chloride, high dose zinc supplements and parenteral zinc poisoning (Pluhator *et al*., 1996). Intakes of 100to 300 mg/day zinc may be prescribed by physicians as a treatment for various medical problems, such as sickle cell anaemia and celiac

disease. However, prolonged therapy with such high doses causes severe copper deficiency (Fosmire, 1990; Prasad *et al*., 1978). This level of supplementation is thought to affect copper metabolism by blocking copper absorption from the intestine (Fosmire, 1990; Guthrie, 1989). Zinc toxicity may also induce a loss of copper (Guthrie, 1989). Both these losses may result in anaemia.

Other reported consequences of zinc intakes of 100 to 300 mg/day include changes in the immune responses and blood lipids. In one study, subjects took 150mg zinc twice a day for six weeks which resulted in a demonstrated reduction in lymphocyte stimulation responses, chemotaxis and phagocytosis of bacteria by polymorphonuclear leukocytes (Chandra, 1984). In addition, an increase in low-density lipoprotein and a decrease in high-density lipoprotein (HDL) was observed, although triglyceride and total cholesterol concentrations did not vary significantly from baseline.

2.7.8 Iron

Iron is the most abundant trace mineral in the body and is an essential element in most biological systems (Goyer, 1996; Greentree and Hall, 1995). About 70% of the iron in mammals is found in haemoglobin and about 5% to 10% is found in myoglobin, iron is in the ferrous (Fe^{2+}) form (Goyer, 1996; Greentree and Hall, 1995; Hillman, 1995). Ferric iron is used in iron-containing enzymes such as peroxidase, catalase and cytochrome C.

Mechanism of Iron Toxicity: the toxicity of iron depends on the amount of iron already in the body. Consequently, some animals develop clinical signs of toxicosis even when they receive doses that cause no problems in other animals. Iron is most toxic when given intravenously. Intramuscular injections are less toxic, and iron given orally is the least toxic probably because the amount of iron absorbed orally is not 100% of the dose ingested (Hillman, 1995). When assessing the potential toxicity of an iron overdose the amount of elemental iron in the products must be determined (Hillman, 1995). No clinical signs of toxicosis are expected in dogs ingesting less than 20 mg/kg. Dogs ingesting between 20 and 60mg/kg of elemental iron can develop mild clinical signs. When the amount of elemental iron ingested is greater than 60 mg/kg, serious clinical signs can develop (Greentree and Hall, 1995). In all animals, oral doses between 100 and 200mg/kg are potentially lethal (Greentree and Hall, 1995).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

The chemicals and reagents used in this experiment were of analytical grade. They include the following; Sodium Chloride from Fisher Scientific Company (New Jersey, USA), Chloroform from BDH (Poole, England), Acetonitrile from LOBA Chemie PVT Limited (Mumbai, India), Nitric acid, Hydrochloric acid from BDH (Poole, England), and Hydrogen Peroxide from Hydrox Laboratories (Illinois, U.S.A).

3.1.2 Equipment

The equipment used in this research were those available in the Department of Biochemistry Laboratory, Federal University of Technology Owerri laboratory, Technology Partners International, Rivers State and International Institute of Tropical Agriculture, Ibadan, Oyo State and they includes the following; Centrifuge from Techmel and Techmel (USA), UNISCOPE 801A Laboratory Water Bath, Surgifriend Medicals (England), Weighing balance from Ohaus (China), Oven from (Accumax, New Delhi, India), High-Performance Liquid Chromatography (Waters 2695 Alliance HPLC System, Milford, USA), Atomic Absorption Spectrophotometer (Model AA-6300) from Shimadzu, Japan. Manual Breast Milk Pump, Weighing Scale, and Measurement tape was procured locally.

3.2 Methods

3.2.1 Study Area: The study was conducted in Imo State, which is located in the Southeastern part of Nigeria. The state geographically lies within latitudes 4°45'N and 7°15'N, and longitude 6°50'E and 7°25'E with a total landmass of around 5,100 square kilometres. Imo State shares boundary with Abia State on the East, River Niger and Delta State to the West, Anambra State on the North and Rivers State to the South. The State has an estimated population of about 5,408,756 people as of 2016 with about 230 - 1,400 persons per square kilometre, and 3.25% growth rate annually (National Bureau of Statistics, 2018). Occupation of city inhabitants is mostly farming, trading, commerce, with a small percentage of the population working in private and public sectors.

3.2.2 Ethical Approval and consent: Ethical approval (Appendix III) for the study was obtained from the ethics committee of the Federal Medical Centre Owerri, Imo State, Nigeria (Protocol No. 7932). The participants were informed about the objectives, assured of the confidentiality of the study and if they agree to participate in the study, were asked to sign written informed consent agreement (Appendix I). Primary data and breast milk were subsequently collected.

3.2.3 *Sample Size Determination:* the sample size was calculated by a single cluster using the WHO formula (MSF, 1995). Since aflatoxicosis is estimated at 17.9% for (South Western) Nigeria (Atanda *et al*., 2007) and the precision required was 5% of the sample size, the sample size was calculated as below.

$N = Z^2PQ \times D/d^2$

Where

N = sample size

Q = 1- P

P = expected prevalence of aflatoxicosis reported elsewhere in Nigeria which is 17.9%

Z = Standard normal deviation for risk error a 95% confidence interval which is 1.96.

d = degree of accuracy which is 0.05

D = Design effect (1).

A sample size of 225 lactating mothers attending Federal Medical Centre Owerri was taken as the study subjects. Only subjects who were within the inclusion criteria and those who agreed and signed the consent form participated in the study.

3.2.4 Sampling: About 30 ml of breast milk samples was required in this study. The breast milk samples were collected from 40 volunteering lactating females in Owerri, Imo state July and August 2019. The samples were collected using manual breast pumps during the normal feeding of the infants into sterile plastic containers and kept in a refrigerator (4^0C) until further analysis.

3.2.5 Inclusion Criteria: The inclusion criteria established for this study shall include:

a. Evidence of breastfeeding
b. Record of breastfeeding
c. Duly completed food and sociodemographic questionnaires.

3.2.6 Exclusion Criteria: The exclusion criteria shall include volunteers under the age of 18 years and mothers with infants less than three months of age (Only matured milk was required and not colostrums or transition milk).

3.2.7 Collection of participant's personal information and food consumption data: A sociodemographic questionnaire was issued to the participants to supply information concerning their age, height, weight, number of children, date of birth of their baby, highest educational qualification, career, profession and place of residence. Additional details were collected on the type of breastfeeding (supplemented with baby commercial formulas or not and at the breast or not), and the weight of the baby at birth and the point of breast milk collection. The participant mothers also completed a semi-quantitative food questionnaire (Appendix II) about their consumption habits in the previous week (over 7 days). This document shall include the following food categories: maize, yam chips, plantain chips, potato chips, groundnuts, groundnut oil, melon, cassava-based foods, beans, rice, bread, yoghurt, cakes and dry fruits.

3.2.8 Determination of AfM_1

3.2.8.1 *Determination of AfM_1 in Breast Milk Sample*: The modified liquid-liquid extraction method as described by Campone *et al*. (2013) for simultaneous protein precipitation and extraction of aflatoxin M_1 followed by dispersive liquid-liquid microextraction (DLLME) and ultrahigh-pressure liquid chromatography-tandem mass spectrophotometer was used for determination of aflatoxin M_1 in breast milk. Briefly, 5 ml of each of the breast milk samples were placed in 15ml falcon tubes and centrifuged at 3000rpm and 4^0C for 10 minutes to eliminate the cream in the upper phase. The defatted samples were then added 3.8ml acetonitrile and 1.0g NaCl, shaking for 10 seconds, heated to 37^0C in a water bath, then mixed and centrifuged at 3000 rpm for 10 minutes. The organic phase (acetonitrile upper phase) containing the AfM_1 was quantitatively transferred to another conical flask and used as a disperser in the dispersive liquid-liquid microextraction step. The dispersive liquid-liquid microextraction was performed by adding 1.5 ml chloroform (DLLME extractant) and 5ml water in turn to the acetonitrile extract obtained by protein precipitation procedure. The three-component system was vigorously shaken by hand for 10 seconds until a cloudy solution is formed. The mixture was centrifuged for 15 minutes at 3000 rpm to separate the two phases. The settled phase was quantitatively transferred to a 2ml Eppendorf vial using a syringe and

blown to dryness. The residue was reconstituted with 1ml of acetonitrile/water (1:1 v/v), centrifuged and then injected into the chromatographic system.

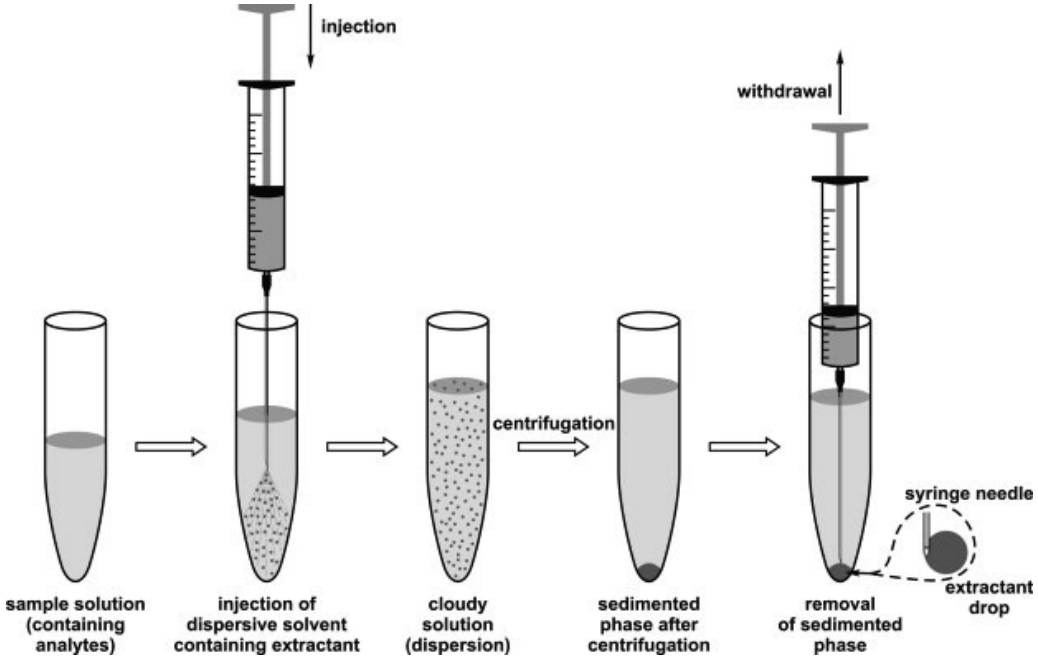


Figure 3.1: The dispersive liquid-liquid microextraction procedure (Zgoła-Grześkowiak and Grześkowiak, 2011).

The dried samples were taken to the National Research Institute for Chemical Technology Zaria, Kaduna State, Nigeria for HPLC analysis.

3.2.8.2 High-Performance Liquid Chromatography (HPLC) Analysis

A high performance liquid chromatographic system (Waters 2695 Alliance HPLC System, Milford, USA) with a quaternary, low-pressure mixing pump and inline vacuum degassing, complete with Waters 474 Fluorescence detector, Waters TC2 temperature control module, Waters column heater module and Masslynx 4.1 PC workstation was used for the determination of concentration of AfM_1 in breast milk extracts. An isocratic system (mobile phase) comprising Acetonitrile:Methanol: Water (17:17:66) was also used for separation, including a flow rate of 1ml/minute, an oven temperature of 40^0C and an injection volume of 30µl. The Detector (Waters 474 Fluorescence Detector) was set at 360nm and 440nm for excitation and emission. The concentrations of AfM_1 in milk were estimated from a standard curve (0.02 – 1.0ng/ml methanol) prepared from AfM_1 in chloroform (Appendix IV).

3.2.8.3 Trueness and Repeatability of Measurement: Trueness shall be determined by percentage recovery obtained from experiments conducted with 50 millilitre of skimmed milk

samples measured into three 100ml volumetric flasks and spiked at five different fortification levels (10 ng/ml AfM_1, 15ng/ml AfM_1, 20ng/ml AfM_1 and 25ng/ml AfM_1, 30 ng/ml AfM_1) with two other samples acting as control. AfM_1 shall be extracted as previously described and percentage recovery rate calculated. Repeatability was expressed as the relative standard deviations obtained in the recovery study. The average percentage of aflatoxin recovered was 92±8 from skimmed milk. Repeatability was taken as 8.

3.2.9 Determination of Heavy Metals:

3.2.9.1: *Digestion of breast milk sample:* Heavy metals in breast milk were determined according to the method described by Kunter *et al*. (2017) with slight modifications. Briefly, about 10±0.01g of breast milk samples was digested with 20 ml 1:1 concentrated HNO_3: HCl in an acid digestion unit and incubated for 10 minutes at 155^0C for further 7 mins at 200^0C. The digested sample was filtered with Whatman filter paper which was pre-rinsed with the acid solution to avoid contamination. The digested sample was rinsed to make up the sample to 50±0.05ml with distilled water. The levels of Pb, Cd, Cr, Cu, Zn, Fe, As, and Hg in the digested samples were then determined with an Atomic Absorption Spectrophotometer. The AAS equipment was calibrated with a primary standard from a certified reference materials producer (ISO 17025:2017 and ISO Guide 34 certified) from inorganic ventures, USA.

3.2.9.2 AAS Analysis: The levels of Pb, Cd, Cr, Cu, Fe, Zn, As, and Hg in the digested samples as determined with Atomic absorption spectrophotometer (AAS) under instrument parameters (Table 1). The results obtained were calculated using AAS wizard software. The concentration of each metal was calculated using the formula;

$$\text{Final concentration (mg/l)} = \frac{\text{Concentration of Metal (mg/kg)}}{\text{Dilution Factor} \times \frac{Nominal\ volume\ (ml)}{Sample\ weight\ (g)}}$$

Table 3.1: Recommended instrument parameters for Atomic Absorption Spectrometer

Element	Wavelength (nm)	Slit width (nm)	Lamp current (mA)	Fuel	Support
Arsenic	193.7	0.5	10	Acetylene	Air
Cadmium	228.8	0.5	4	Acetylene	Air
Chromium	357.9	0.2	7	Acetylene	Air
Copper	324.7	0.5	4	Acetylene	Air
Iron	248.3	0.2	10	Acetylene	Air
Lead	217.0	1.0	10	Acetylene	Air
Mercury	253.7	0.5	4	No flame	Air
Zinc	213.9	1.0	5	Acetylene	Air

3.2.10 *Determination of estimated daily intake of AFM_1*: The combination of the average body weight of the babies (kg), the babies' daily consumption of milk (ml/kg) and the average concentration of AFM_1 in the samples was used in a deterministic method to calculate the estimated daily intake of AFM_1 (Bogalho *et al*., 2018). The equation is as follows;

$$\text{EDI (ng/kg b.w/day)} = [\text{concentration of } AFM_1] \times \frac{(Daily\ consumption\ of\ milk)}{Babies'weight}$$

3.2.11 *Estimated daily intake (EDI) of heavy metals*: The estimated daily intakes for the analysed metals in the breast milk sample was calculated by multiplying the respective mean concentration of the metals (mg/kg) determined in the breast milk by the babies daily consumption of milk divided by the average body weight of the babies such that

$$\text{EDI (mg/kg b.w/day)} = [\text{metal concentration}] \times \frac{(Daily\ consumption\ of\ milk)}{Babies'weight}$$

Note: The daily breast milk consumption estimates of 150 ml/kg (< 7kg baby weight) and 1 L/kg (≥7kg baby weight) was used (Bogalho *et al*., 2018). The average body weight and AFM_1 concentration were calculated for the groups.

3.2.12 Statistical analysis: Statistical analysis was performed using statistical software (SPSS version 20.0). The results of AFM_1 concentrations and heavy metals concentration in breast milk were presented as mean±SEM and analyzed using multiple linear regressions to evaluate the association between AFM_1 concentrations and heavy metals concentration in breast milk and maternal diet as well as with sociodemographic factors. The p values of <0.05 and <0.01 were considered statistically significant. Simple percentages and Bar charts were also used to

present data on the sociodemographic factors and estimated daily intakes of AFM_1 concentrations and heavy metals concentration.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Sociodemographic information of the infants and breastfeeding mothers

Table 4.1 shows the personal information of infants and breastfeeding mothers. The average age of the lactating mothers was 29.13±3.69, range 24 – 39. All the mothers weighed between 55kg – 125kg with an average weight of 79.20±14.14kg. The average height of the lactating mothers was 1.66±0.09 meters which were within the range of 1.5 – 1.9. Only 47.5% of the lactating mothers have a Body Mass Index (BMI) of 25 – 29.9 (Overweight) while 27.5% of the lactating mothers had BMI above 30 (Obese). The average infant weight at birth and at the time of sampling was 3.50±0.58kg and 7.20±1.68kg respectively. Exactly 50% of the infants in our study were between the ages of 3 – 5 months, those between 6 – 8 months constitute 20% of the infants and 30% were above 8 months of age.

Table 4.1: Participants Personal Information

Variables	Range	No.	Mean±SD
Mothers age (years)	24 – 39	40	29.13±3.69
Mothers weight (Kg)	55 – 125	40	79.20±14.14
Mothers height (M)	1.5 – 1.9	40	1.66±0.09
Mothers BMI			
✓ <20	19.48 – 19.48	1 (2.5%)	19.48±0.00
✓ 20 – 24.9	20.20 – 24.74	9 (22.5%)	23.41±1.53
✓ 25 – 29.9	25.15 – 29.66	19 (47.5)	27.86±1.64
✓ >30	30.47 – 40.81	11 (27.5%)	34.96±3.33
Infants age (Months)			
✓ 3 – 5	3 – 5	20 (50.0%)	3.40±0.75
✓ 6 – 8	6 - 8	8 (20.0%)	7.25±0.70
✓ >8	9 – 12	12 (30.0%)	9.50±1.00
Infants weight at birth (kg)	2.2 – 4.9	40	3.50±0.58
Infants weight at the time of sampling (kg)	4.7 – 10.0	40	7.20±1.68

No. = number, SD=Standard Deviation

Table 4.2: Profile of the Analysed Population of Lactating Mothers

The profile of the analysed population of lactating mothers was presented in Table 4.2. A total of 40 lactating mothers were selected for the study and completed the questionnaires. The majority of the lactating mothers lived in the urban area (65%), but only 35 % lived in the suburban area. Only 75% of the lactating mothers were employed as public servants or in business. All the lactating mothers were well-educated and the highest academic qualification obtained by the lactating mothers was a bachelor's degree, few of them (27.5%) were at the highest school graduates. About 40% of the study population in our study may have in one way or the other been exposed to heavy metals arising from industrial/occupational exposure, air/water pollution, foods, medicines, improperly coated food containers or ingestion of lead-based paints/cosmetics.

	Mothers age, years				
Variables	18 – 25	26 – 30	31 – 35	36 - 40	Total
Number of Mothers	06	21	08	05	40
Place of Residence					
✓ Urban	04	14	04	04	28 (65%)
✓ Suburban	05	05	03	01	14 (35%)
Number of Children					
✓ 1	05	11	02	04	22 (55%)
✓ 2 - 4	05	06	06	-	17 (42.5%)
✓ >4	-	-	-	01	01 (2.5%)
Breastfeeding Stage					
✓ 3 – 5 months	04	09	03	03	19 (47.5%)
✓ 6 – 8 months	02	01	02	01	06 (15.0%)
✓ 9 – 12 months	03	07	02	01	13 (32.5%)
✓ 12 - above	-	01	-	-	01 (2.5%)
Time spent on breastfeeding	296	278	300	400	318
Employment status					
✓ Employed	07	12	07	04	30 (75%)
✓ Unemployed	02	06	01	01	10 (24%)
Educational level					
✓ College	06	15	07	01	29 (72.5%)
✓ High School	04	03	-	04	11 (27.5%)
*Exposure to heavy metals	10%	15%	5%	10%	40%

*None of the participants in the study were smokers, so exposure was mostly from industrial/occupational exposure, air/water pollution, foods, medicines, improperly coated food containers or ingestion of lead-based paints/cosmetics (NORD, 2006).

4.2 Food Consumption Determinants Data of the Study

As illustrated in figure 4.1, bread, cassava-based diet, groundnut, groundnut oil, maize (corn), rice and tomatoes were more commonly consumed at least once daily, while beans, cakes, maize, melon, plantain chips and potatoes chips were more commonly consumed at least once weekly.

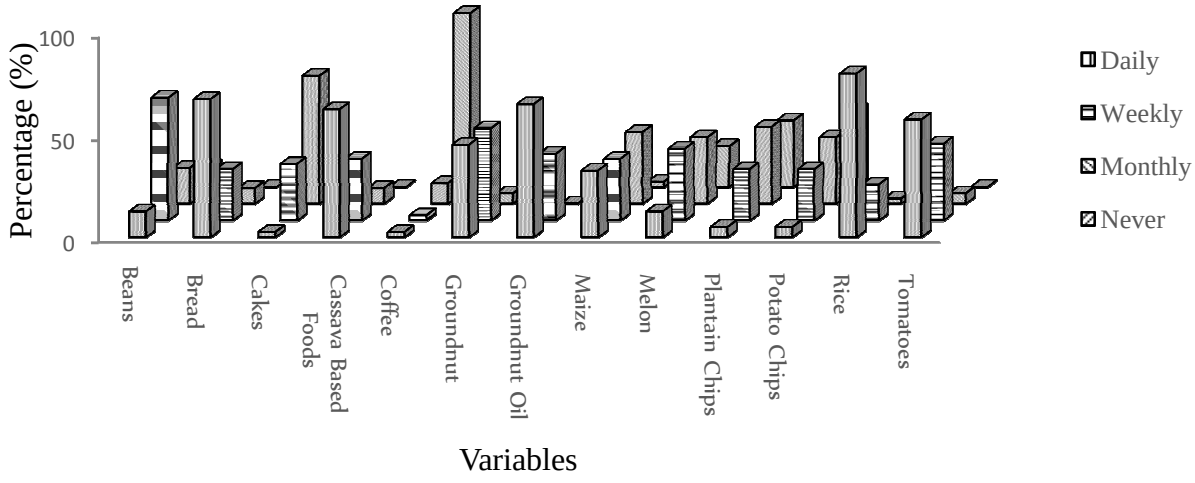


Figure 4.1: Food Consumption Data.

4.3 Levels of AfM_1 in Human Breast Milk

Table 4.4 shows the results of the occurrence of Aflatoxin M_1 in Breast Milk of Mothers in our study area. The result showed that the mean (±standard deviation) aflatoxin concentration was 4.02±1.12 ng/L. Lactating mothers between the age of 31 – 35 years had a higher aflatoxin M_1 concentration that the other age groups.

Table 4.3: Occurrence of Aflatoxin M_1 in Breast Milk of Mothers

		AfM_1 Concentration (ng/L)	
Sample	No.	Range	Mean±SD
18 – 25	6	2.51 – 5.06	3.78±0.80
26 – 30	21	2.35 – 7.08	4.02±1.23
31 – 35	8	3.28 – 6.14	4.68±1.18
36 – 40	5	2.33 – 5.01	3.52±1.15
Total Aflatoxin	40	2.33 – 7.08	4.02±1.12

AfM_1 – Aflatoxin; No. = number; SD=Standard Deviation

4.4 Estimated Daily intake of Aflatoxin M_1 by the age of infants.

Figure 4.1 shows the mean concentration of AfM_1 in all the breast milk samples consumed by the infants as well as by the infants between the ages of 3- 5 months, 6 – 8 months and above 8 months which was 0.34±0.04 ng/kg of body weight/day for all the infants but 0.34±0.06 ng/kg of body weight/day for infants between 3 – 5 months, 0.29±0.07 ng/kg of body weight/day for infants between 6 – 8 months and 0.38±0.04 ng/kg of body weight/day for infants above 8 months.

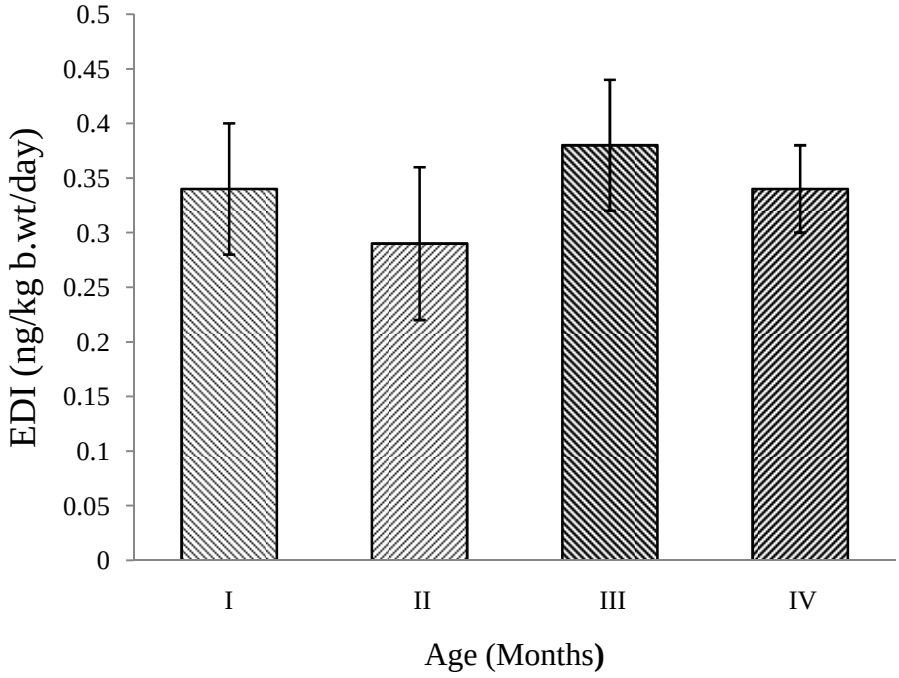


Figure 4.2: Estimated Daily intake of Aflatoxin M_1 in Breast Milk of Mothers by age of infants. I = 3 – 5 months, II = 6 – 8 months, III = >8 months, IV=Total number.

4.5 Association between Maternal Diet, Employment Status, Educational Level and Aflatoxin M_1 in Breast Milk.

Table 4.6 shows the result of the correlations between maternal diet, employment status, educational level and aflatoxin m_1 level in breast milk. The result indicated that the daily consumption of cassava-based foods, groundnut oil, maize, Tomatoes and Dry fruits was positively correlated with AfM_1 contamination ($p<0.01$, 0.05) although the association was found to be non-significant ($p>0.01$, $p>0.05$). The result also showed a non-significant weak positive correlation between education level, employment status and AfM_1 concentration

Table 4.4 Correlations between Maternal Diet, Employment Status, Educational Level and Aflatoxin M_1 Level in Breast Milk.

Parameters			Aflatoxin M_1 Level In Breast Milk
Beans	Daily	r	0.123
		p	0.450
	Weekly	r	-0.301
		p	0.059
Bread	Daily	r	0.183
		p	0.259
	Weekly	r	-0.038
		p	0.817
Cakes	Daily	r	0.040
		p	0.806
	Weekly	r	0.040
		p	0.805
Cassava based food	Daily	r	0.510**
		p	0.001
	Weekly	r	-0.407**
		p	0.002
Groundnut	Daily	r	0.243
		p	0.131
	Weekly	r	-0.205
		p	0.203
Groundnut oil	Daily	r	0.427**
		p	0.006
	Weekly	r	-0.328**
		p	0.039
Maize	Daily	r	0.479**
		p	0.002
	Weekly	r	-0.049
		p	0.770
Rice	Daily	r	0.279
		p	0.081
	Weekly	r	-0.211
		p	0.191
Tomatoes	Daily	r	0.579**
		p	0.000
	Weekly	r	-0.422**
		p	0.007
Dry fruits	Daily	r	0.347*
		p	0.028
	Weekly	r	0.032
		p	0.843
Employment Status		r	0.243
		p	0.130
Educational level		r	0.204
		p	0.206

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

Table 4.5: Recently Reported Prevalence and Level of Aflatoxin M_1 in Breast Milk of Mothers in Nigeria and other countries

Country (year)	Incidence rate (%)	Mean level ± SD (ng/L)	Range (ng/L)	References
Brazil (2016)	5/94 (5.35%)	18±5	13 – 25	Ishikawa *et al*., 2016
Colombia (2015)	45/50 (90%)	5.2	0.9 – 18.5	Diaz and Sanchez, 2015
Cyprus (2017)	40/50 (80%)	7.84±1.72	5.36 – 28.44	Kunter *et al*., 2017
Egypt (2011)	87/125 (69.6%)	74.413±7.070	7.3 – 328.6	El-Tras *et al*., 2011
Iran (2012)	8/132 (6.06%)	9.45±1.50	7.1 – 10.8	Ghiasain and Maghsood, 2012
Iran (2015)	85/100 (85%)	5.91±2.03	2.0 – 10.0	Maleki *et al*., 2015
Iran (2017)	39/250 (15.6%)	4.54±0.47	11.1 – 39.3	Jafari *et al*., 2017
Jordan (2012)	80/80 (100%)	67.78±4.6	9.71 – 137.18	Omar (2012)
Lebanon (2016)	104/111 (93.8%)	4.32±1.8	0.22 – 7.89	Elaridi *et al*., 2017
Nigeria (2019)	9/40 (22.5%)	4.02±1.12	2.33 – 7.08	This study
Portugal (2015 - 2016)	22/67 (32.8%)	7.4±1.9	5.1 – 10.6	Bogalho *et al*., 2018
Turkey (2014)	18/73 (24.6%)	3.01±1.42	1.3 – 6.0	Atasever *et al*., 2014
Turkey (2016)	66/74 (89.2%)	19.0±13.0	9.6 – 80	Kiliç *et al*., 2016

4.6 Heavy Metals in Human Breast Milk

Table 4.6 presents the levels of some heavy metals (Cd, Cr, Cu, Fe, Pb and Zn) as toxic contaminants. The concentrations of the selected heavy metals were in the order Fe>Pb>Cu>Cd>Cr>Zn. Iron (Fe), Lead (Pb) and Copper (Cu) were the selected heavy metals detected in high concentrations in the breast milk. Mercury (Hg) and Arsenic (As) were not detected in the breast milk samples. The mean (±standard deviation) values of these heavy metals were Cd: 0.029±0.013mg/L, Cr: 0.019±0.011mg/L, Cu: 0.035±0.013mg/L, Fe: 0.049±0.039mg/L, Pb: 0.038±0.013mg/L and Zn: 0.009±0.008mg/L.

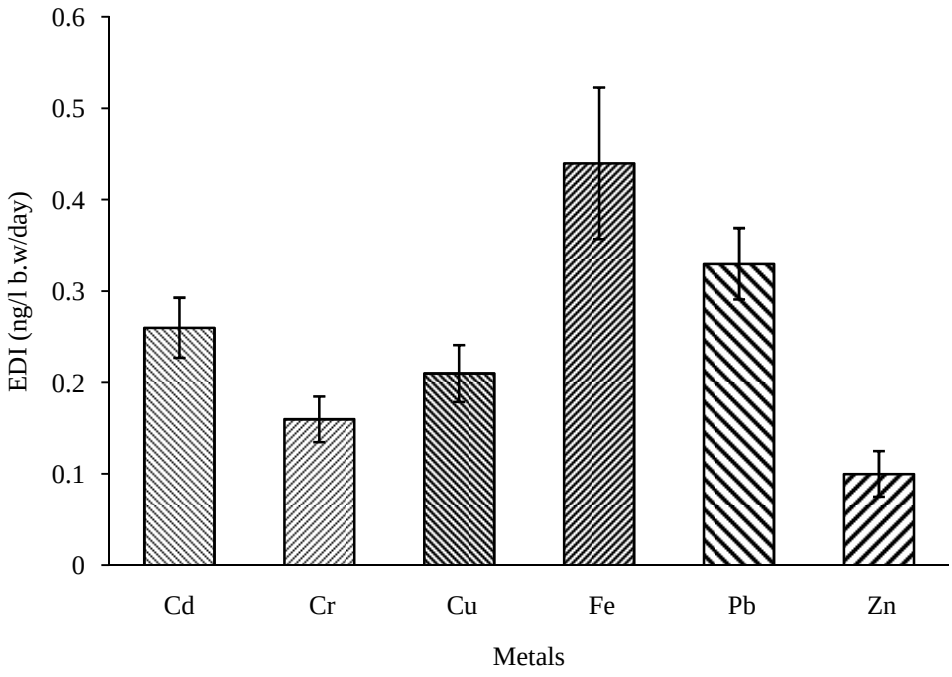
Table 4.6: Concentrations of Pb, Cd, Cr, Cu, Zn, As, Fe and Hg in Breast Milk of Mothers and the effect of mothers age, mg/L

Age of mothers, years	No.	Cd	Cr	Cu	Fe	Pb	Zn
18 – 25	6	0.025 ± 0.013^{a}	0.020±0.013	0.034 ± 0.011^{a}	0.051±0.059	0.044 ± 0.013^{a}	0.007 ± 0.051^{a}
26 – 30	21	$0.031\pm0.014^{a,b}$	$0.018\pm0.012^{a,b}$	$0.037\pm0.014^{a,b}$	0.047±0.024	0.039 ± 0.012^{b}	0.008 ± 0.006^{b}
31 – 35	8	0.028 ± 0.013^{b}	$0.026\pm0.011^{a,c}$	0.045 ± 0.006^{b}	0.067±0.057	0.039 ± 0.015^{c}	$0.018\pm0.013^{a,b,c}$
36 – 40	5	0.027±0.016	$0.014\pm0.042^{b,c}$	0.029±0.014	0.031±0.016	$0.031\pm0.012^{a,b,c}$	0.006 ± 0.021^{c}
Mean	40	0.029	0.019	0.035	0.049	0.038	0.009
Standard Deviation	40	0.013	0.011	0.013	0.039	0.013	0.008
Minimum Value	40	0.011	0.006	0.013	0.015	0.012	0.016
Maximum Value	40	0.051	0.048	0.059	0.017	0.059	0.038
Median	40	0.037	0.016	0.036	0.036	0.038	0.064
25^{th} percentile	40	0.016	0.011	0.022	0.026	0.024	0.048
75^{th} percentile	40	0.041	0.027	0.044	0.059	0.052	0.014
Effect of Mother's Age	40	p>0.05	p>0.05	p>0.05	p>0.05	p<0.05	p>0.05

Note: The Concentrations As and Hg in the breast milk samples were found to be below detection limits by the AAS machine and therefore were not presented in the table.

4.7 Mean Daily Intake of Heavy Metals in Breast Milk

Figure 4.3: Estimated daily intake of Pb, Cd, Cr, Cu, Zn, and Fe from breast milk per kg of infant's weight. Note: Values for Arsenic (As) and Mercury (Hg) were not represented in the chart because their mean values were below the detection limit. The result of the estimated daily intake of breast milk by the breastfed infants shows that heavy metals such as Pb, Fe, and Cd are ingested more daily than other metals analysed.



4.8 Association between Daily and Weekly Maternal Diet, Heavy Metal Exposure and Heavy Metals in Breast Milk.

There was a weak positive but non-significant correlation between heavy metal content and daily intake of maternal diet except for beans where a significant correlation ($p<0.01$) was found with Cr, Cu and Zn. A weak positive but non-significant correlation was also observed between exposure to heavy metals such as Cu, Fe and Pb and maternal diets.

Table 4.7; Correlations between Daily and Weekly Maternal Diet, Heavy Metal Exposure and Heavy Metals in Breast Milk

Diet			Heavy Metals In Breast Milk					
			Cd	Cr	Cu	Fe	Pb	Zn
Beans	Daily	r	0.204	0.403**	0.423**	0.271	0.258	0.587**
		p	0.207	0.010	0.007	0.091	0.109	0.000
	Weekly	r	-0.149	-0.417**	-0.160	-0.149	-0.416**	-0.174
		p	0.358	0.008	0.324	0.359	0.007	0.282
Bread	Daily	r	0.003	0.037	0.064	-0.117	-0.083	0.114
		p	0.986	0.822	0.696	0.473	0.612	0.484
	Weekly	r	0.188	0.022	-0.037	0.206	0.118	-0.055
		p	0.245	0.895	0.822	0.202	0.469	0.735
Cakes	Daily	r	0.233	-0.159	-0.189	0.071	-0.325*	-0.074
		p	0.166	0.329	0.242	0.662	0.041	0.650
	Weekly	r	0.303	0.173	-0.035	0.420**	0.287	0.201
		p	0.057	0.284	0.830	0.007	0.073	0.214
Cassava based food	Daily	r	0.037	0.120	0.135	0.158	0.130	0.278
		p	0.821	0.461	0.407	0.332	0.425	0.082
	Weekly	r	-0.177	0.005	0.074	-0.278	-0.276	-0.184
		p	0.276	0.975	0.651	0.082	0.085	0.255
Dry fruits	Daily	r	0.089	-0.071	0.013	-0.124	0.154	0.155
		p	0.585	0.661	0.937	0.446	0.341	0.341
	Weekly	r	0.122	0.201	0.024	0.266	0.086	0.095
		p	0.452	0.213	0.881	0.097	0.596	0.561
Groundnut	Daily	r	0.100	-0.021	0.013	-0.136	-0.034	0.118
		p	0.538	0.899	0.939	0.404	0.836	0.467
	Weekly	r	-0.019	0.232	0.032	0.163	0.068	0.068
		p	0.908	0.151	0.847	0.316	0.676	0.675
Groundnut oil	Daily	r	0.021	-0.036	0.095	0.032	0.073	-0.041
		p	0.897	0.826	0.561	0.843	0.655	0.799
	Weekly	r	-0.161	0.000	0.093	-0.055	0.015	0.019
		p	0.322	0.999	0.569	0.734	0.927	0.909
Maize	Daily	r	0.222	-0.042	-0.080	0.228	0.087	0.193
		p	0.169	0.797	0.625	0.157	0.592	0.234
	Weekly	r	0.235	0.047	-0.112	0.208	0.028	-0.019
		p	0.144	0.772	0.491	0.197	0.862	0.905
Rice	Daily	r	0.067	0.232	0.208	0.291	0.184	0.286
		p	0.683	0.150	0.197	0.068	0.257	0.074
	Weekly	r	-0.146	-0.310	-0.067	-0.210	-0.256	-0.232
		p	0.369	0.051	0.679	0.194	0.111	0.149
Yoghurt	Daily	r	-0.046	0.005	0.176	0.002	0.126	0.145
		p	0.778	0.974	0.278	0.992	0.439	0.371
	Weekly	r	0.033	0.110	-0.068	-0.001	-0.043	0.056
		p	0.838	0.498	0.677	0.996	0.791	0.730
Tomatoes	Daily	r	0.189	0.209	0.138	0.091	0.283	0.230
		p	0.243	0.195	0.397	0.577	0.077	0.153
	Weekly	r	-0.240	-0.024	0.092	-0.072	-0.294	0.035
		p	0.137	0.884	0.570	0.661	0.066	0.830
Exposure to heavy metals		r	-0.068	-0.090	0.108	0.079	0.086	-0.078
		p	0.675	0.581	0.507	0.627	0.597	0.634

Note: As and Hg in the breast milk samples were found to be below detection limits by the AAS machine and therefore were not presented in the table. **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).

Infancy is considered a unique period in human life, due mainly to the dependence of infants on breast milk from mother as the only nutritional source (Emmett and Rogers, 1997). The most bio-available source of nutrients for infants is human breast milk, which also provides the hormones and immunological factors that protect infants from potential disease-causing agents (Turck, 2005). Several factors have been reported to affect the quality of human breast milk, among which the primary cause of breast milk contamination is considered to be maternal consumption of contaminated foods. Aflatoxins as a highly carcinogenic, mutagenic, and teratogenic category of mycotoxins (McLean and Dutton, 1995) are known to be one of the most active toxins that endanger human health. Because the rate of biotransformation in children is lower than in adults and because of a higher rate of growth along with increased consumption of food and water / kg weight (WHO, 1986), children are considered highly susceptible to the detrimental effects of environmental toxins, especially aflatoxins. Early childhood exposure to aflatoxins has been shown to cause immunosuppression, impairment of growth and underweight. Several studies in different countries have confirmed the presence of AFM_1 both in infant formula and in human breast milk. Hence, AFM_1 in lactating mothers ' breast milk can be viewed as a possible health-threatening concern for children.

The data presented here give a sign for mother to child transmission of Aflatoxin M_1 and selected heavy metals through breast milk. Therefore, there are few issues for concern about child exposure to aflatoxin M_1 and heavy metals through breast milk. In developing countries like Nigeria, infants are breastfed for as long as 2 years of age (Adewuyi and Adefemi, 2016). Thus long term introduction to aflatoxin M_1 and heavy metals may add to long term health problems.

In Eastern Nigeria, most people predominantly consume their traditional foods such as maize (corn), cassava-based diet, rice, yam, groundnut and plantain. These traditional foods are always available all through the year, due to high production yield and availability of traditional storage methods. It is important here to state that storage factors can potentially add up to the risk factors for AfB_1 contamination of food and feed. In Nigeria, a major factor to aflatoxin exposure is poor harvest management of the harvested crops. In small farm communities, the crops harvested are usually left to dry over plastic or synthetic sheets, which may promote Aspergillus growth and toxin production (Galvano *et al*., 1996). Most of the crops are consumed locally by either the household or close communities. Worldwide,

two of the major sources of aflatoxin exposure are groundnuts and Maize (corn). These two crops are mainly consumed in Nigeria in several forms as boiled corn, roasted corn with groundnut, and groundnut oil.

During the lactation phase, mothers are exposed to numerous naturally occurring and/or synthetic contaminants and nearly all nutrients are also polluted to different degrees with these kinds of pollutants. For example, in different systems, determining their levels in biological systems is important for breast milk when considering infants ' vulnerability. AfM_1 contamination was detected in 100% of all the breast milk samples. This high prevalence in Nigeria matches the recent reports within other regions in Nigeria; Northwest region (77.5%, Makun *et al*., 2016), Southwest region (17.9%, Atanda *et al*., 2007) and from other countries such as Cyprus (80% by Kunter *et al*., 2017), Iran (85% by Maleki *et al*., 2015), Turkey (89.2% by Kilic *et al*., 2016), Colombia (90% by Diaz and Sanchez, 2015) and Jordan (100% by Omar, 2012). However, the prevalence in Nigeria was lower than those reported in Brazil (5.35% by Ishikawa *et al*., 2016) and Portugal (32.8% by Bogalho *et al*., 2018). The concentration range of Aflatoxin M_1 in breast milk in our study was 2.33 – 7.08 ng/L, which is lower than that reported for Turkey (9.5 – 8.0 ng/L by Kilic *et al*., 2016), Portugal (5.1 – 10.6 by Bogalho *et al*., 2018), Jordan (9.71 – 137.18 ng/L by Omar, 2012), Iran (11.1 – 39.3 ng/L by Jafari *et al*., 2017), Egypt (7.3 – 328.5 ng/L by El-trans *et al*., 2011), Cyprus (5.36 – 28.44 ng/L by Kunter *et al*., 2017), Brazil (13 – 25 ng/L by Ishikawa *et al*., 2016) but higher than that reported for Turkey (1.3 – 6.0 ng/L by Atasever *et al*., 2014).

The mean concentration of Aflatoxin M_1 in our study was 4.02±1.12 ng/L which was lower than the recent reports from other countries presented in table 4.3 except for the report from turkey in 2014 (3.01±1.42 ng/L) by Atasever *et al*., (2014). The differences in prevalence, range and concentration of AfM1 reported for breast milk obtained from lactating mothers in different countries are due to differences in analytical methods used to quantify AfM1 and factors such as climate and environment for food storage, especially humidity and temperature (Elaridi *et al*., 2017). Under these favourable conditions AfB1 is produced from these products in agricultural products and after consumption, AfB1 is metalized into AfM1 and secreted into breast milk. The differences in dietary habits between populations in different countries are another relevant factor (Elaridi *et al*., 2017). In our study, none of the contaminated samples exceeded the legal limit (0.025 ng/mL) for AfM_1 in breast milk as set by the Nigerian National Food Administration and Control (NAFDAC) and European Commission as reported in various literatures (Galvano *et al*., 1996; Makun *et al*., 2016).

The mean concentration of AfM_1 in all the breast milk samples consumed by the infants as well as by the infants between the ages of 3- 5 months, 6 – 8 months and above 8 months were used to determine the estimated daily intake of AfM_1 which was 0.34±0.04 ng/kg of body weight/day for all the infants but 0.34±0.06 ng/kg of body weight/day for infants between 3 – 5 months, 0.29±0.07 ng/kg of body weight/day for infants between 6 – 8 months and 0.38±0.04 ng/kg of body weight/day for infants above 8 months. This estimated daily intake value was lower than that reported in Lebanon (0.69 ng/kg of body weight/day, Elaridi *et al*., 2017), Mexico (2.35 ng/kg of body weight/day, Cantú-Cornelio *et al*., 2016) and Egypt (6.7 ng/kg of body weight/day, Polychronaki *et al*., 2007). Aflatoxins are carcinogenic and genotoxic substances and currently, there is no existing threshold value below which the risk of human health is equal to zero. Although the National Food and Drug Administration and Control (NAFDAC) in Nigeria as well as the Joint FAO/WHO Expert Committee on food additives (JEFCA) has not established a tolerable daily intake for aflatoxin yet, however, they strongly recommend that the concentration of aflatoxins should be as low as reasonably achievable. Therefore, the toxicological significance of aflatoxin in maternal milk should not be overlooked. Accordingly, it is beneficial to reduce the mother's exposure to aflatoxins and thus minimize infant exposure by breast milk. Where the source of contamination is clearly defined, post-harvest management of risk products, efficient labelling and food processing and restricting the exposure of lactating mothers to contaminants in the environment should be encouraged.

In this study, food consumption by lactating mothers was in agreement with the traditional Nigerian diet (Okeke *et al*., 2009). Analysis of AfM_1 concentrations in breast milk and consumption of various food groups indicated that the daily consumption of cassava-based foods, groundnut oil, maize, Tomatoes and Dry fruits was positively correlated with AfM_1 contamination ($p<0.01$, 0.05) (Table 4.5) although the association was found to be non-significant ($p>0.01$, $p>0.05$). In Africa, a correlation between ingested food and AfM_1 in body fluids has been observed (Adejumo *et al*., 2013; El-tras *et al*., 2011). Reports by authors elsewhere have also reported a relationship between AfM_1 concentration in breast milk and consumption of certain foods (Elaridi *et al*., 2017; Bogalho *et al*., 2018; Polychronaki *et al*., 2007; Sadeghi *et al*., 2009). A strong association has been established between employment statuses, education level and AfM_1 concentration in lactating mothers (Elaridi *et al*., 2017). Unemployed mothers with a lower educational level are assumed to have lower socioeconomic status and thus are more likely to consume food contaminated with AfB_1 and AfM_1 because higher quality products tend to be more expensive (Polychronaki *et al*., 2007).

This assumption was not completely supported in our study where a correlation between AfM_1 and education and employment status showed a non-significant weak positive correlation between education level, employment status and AfM_1 content.

The mean (±standard deviation) values of heavy metals in breast milk were Cd: 0.029±0.013mg/L, Cr: 0.019±0.011mg/L, Cu: 0.035±0.013mg/L, Fe: 0.049±0.039mg/L, Pb: 0.038±0.013mg/L and Zn: 0.009±0.008mg/L. The result of the estimated daily intake of breast milk by the breastfed infant show that heavy metals such as Pb, Fe, and Cd are ingested more daily than other metals analysed. There was a weak positive but non-significant correlation between heavy metal content and daily intake of maternal diet except for beans where a significant correlation ($p<0.01$) was found with Cr, Cu and Zn. A weak positive but non-significant correlation was also observed between exposure to heavy metals such as Cu, Fe and Pb and maternal diets.

The presence of the selected heavy metals in the lactating mothers ' breast milk points to the fact that these mothers were put at risk from these toxic contaminants. Those heavy metals are known to affect the health of the infant. Exposure of breastfed infants by lactating mothers to these heavy metals induces risk in breastfed infants after birth (Yurdakök, 2015). Lead, which is one of those toxic heavy metals have no known biological function in humans. The agency for toxic substances and disease registry in their report has outlined the main sources of exposure to lead (ATSDR, 1993). However, air pollution has remained the major route of lead exposure. Leaded gasoline is a major source of air pollution in urban areas and is the major factor responsible for elevated lead in urban lactating mothers (Markowitz 2000). It is transported to selected tissues in the body once the lead is absorbed and concentrated more in the bones (Poponikolaou *et al*., 2005). Much of the lead in breast milk does not come from exposure to diet or mother during lactation but from the bones of the mother. No reports of toxicity due to lead from breast milk have been published. The concentration of lead in the breast milk of lactating mothers was below the permissible limit (0.01 – 0.02 mg/ml) for lead in breast milk by FAO/WHO Codex Alimentarius Commission (Codex, 2011). The results were also found to be higher than those reported for other countries but similar to that reported elsewhere in Nigeria (Table 4.6).

Cadmium is another toxic heavy metal which has no known function in the human body (Yurdakök, 2015). The major source of cadmium exposure is smoking tobacco (Yurdakök, 2015). Higher levels of cadmium have also been detected in certain seafoods and in the liver and kidneys of mammals fed a diet rich in cadmium. Cadmium is gradually excreted through the renal path, when ingested (ATSDR, 2011). Over time, cadmium

accumulates gradually mainly in the liver and kidney, and to a lesser extent in the muscles and bones (HSDB, 2006). The placenta and breast milk also excrete cadmium (ASTDR, 2009). A significant association has been established between breast milk cadmium concentration and calcium secretion in breast milk (ATSDR, 2011). This indicates that maternal exposure to cadmium may result in insufficient calcium in breast milk (Honda *et al*., 2003). Results of the present investigation showed that the mean (±standard deviation) cadmium concentration (0.029±0.0013mg/L) in the breast milk of lactating mothers was below the International Food Standards level (0.01 – 0.05mg/ml) set by FAO/WHO (Codex, 2011) but was higher than those reported for breast milk of lactating mothers from other countries (Table 4.6).

Chromium is another heavy metal that plays essential roles in the body. It is reported to improve insulin sensitivity enhance carbohydrate, lipid and protein metabolism. There is a paucity of information on the amount of chromium required by the body. Available reports in literature so far contain conflicting information. The FAO/WHO joint committee on food standard provided a permissible limit of 0.05µg/L for breast milk chromium (Codex, 2011). The result of breast milk chromium from our study showed that chromium concentration was above the permissible limit and very much higher than the results reported from Sweden (Björklund *et al*., 2012). Postnatal administration of infants to hexavalent chromium through breast milk exposes them to excessive oxidative stress (Stanley *et al*., 2013). No significant positive correlation was observed between breast milk chromium in our study. This is in agreement with the previous study by Anderson *et al*. (1993).

Iron, copper and zinc are trace elements that have known metabolic functions in the human body. Their deficiency symptoms are associated with certain metabolic dysfunctions including anaemia, neutropenia, growth retardation and impaired immune function. Previous studies have shown no significant correlation between maternal iron, copper and zinc status and breast milk iron, copper and zinc concentrations (Domellöf *et al*., 2004; Örun *et al*., 2012; Friel *et al*., 2018). The association between copper, zinc and iron concentrations and certain sociodemographic factors like maternal and infant age, weight, height etc are still contentious (Örun *et al*., 2012; Friel *et al*., 2018) with some authors reporting a positive association between the metals and these factors. The result of the present investigation showed a positive correlation between daily maternal diet and concentration of Cu, Zn and Fe in the breast milk.

4.9 Some Reports on the Concentrations of Toxic Heavy metals (µg/L) by Country

Table 4.8; Cd, Cr, Cu, Fe, Pb, and Zn concentrations in the breast milk of mothers from Nigeria and other countries

Country	Cd (µg/l)	Cr (µg/l)	Cu (mg/l)	Fe (mg/l)	Pb (µg/l)	Zn (mg/l)	References
Greece	0.19	-	0.38	-	0.48	4.90	Leotsinidis *et al*., 2005
Iran	0.62 – 6.35	-	-	-	3.18 – 24.67	-	Rahimi *et al*., 2009
Italy	<LOD	-	0.35 – 0.42	-	0.85 – 1.07	0.70 - 0.90	Aballe *et al*., 2008
Nigeria	9.7	-	0.83	-	8.7	0.7	Adesiyan *et al*., 2011
Poland	0.21 – 7.35	-	0.03 – 0.46	-	0.49 – 12.0	0.04 – 8.16	Winiarska-Mieczan, 2014
Portugal	-	-	0.33 – 0.97	-	0.07 – 4.03	0.39 – 5.09	Almeida *et al*., 2008
Saudi Arabia	1.73	-	-	-	31.67	-	Al-Saleh *et al*., 2003
Spain	0.6 – 11.3	-	-	-	0.1 – 32.3	-	Rodriguez *et al*., 1999
Sweden	0.028 – 0.27	0.026 – 1.6	0.33 – 0.67	0.14 – 0.80	0.74 – 6.4	1.24 – 5.71	Björklund *et al*., 2012
Tanzania	24.1 – 35.9	-	-	-	32.4 – 1630	0.23 – 1.46	Khamis *et al*. 2017
Nigeria	11.02 – 51.00	5.90 – 48.20	0.01 – 0.06	0.02 – 0.17	12.07 – 59.50	0.01 – 0.03	This study

LOD – limit of Detection.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The present report on the occurrence and levels of some heavy metals and AfM_1 concentration in the breast milk of nursing mothers is the first of its kind in Eastern Nigeria. A high prevalence (100%) of AfM_1 was detected in the breast milk of all the study subjects. AfM_1 concentration was also positively and significantly associated with the daily consumption of foods such as cassava, groundnut oil, maize, tomatoes and dry fruit. Although none of the samples exceeded the National and International legal limit for AfM_1 in breast milk, the high prevalence of AfM_1 in the breast milk of lactating mothers in Nigeria suggests that infants may be exposed to this toxin through breast milk.

Given the results obtained, it is clear that the control and analysis of mycotoxins and heavy metals in the breast milk samples are necessary to know if preventive measures and good agricultural handling practices are implemented. These are the first tools to avoid the appearance of these mycotoxins and heavy metals and reduce exposure to the population.

5.2 Recommendation

Attempts should be directed to finding ways to reduce the presence aflatoxins and heavy metals in breast milk and infants' exposure, people, especially mothers should be educated about ways of conveyance of aflatoxins and heavy metals into foods, and associated hazards following unsuitable food storage and ingestion of contaminated foods. This warrants the need for monitoring the AfM_1 levels and heavy metal concentration in human breast milk samples over the breastfeeding period. Considering the benefits of breast milk to the health and nutrition of infants, this study should be repeated periodically with other areas within Nigeria included and findings from the study should be interpreted thoughtfully and made public. Adequate prophylactic and control measures should be devised to decrease AfM_1 presence in human breast milk and the resulting exposure of lactating newborns.

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APPENDIX I

Informed Consent Form

SIMPLE BREAST MILK EXTRACTION (JULY - SEPTEMBER)

CONSENT FORM TO PARTICIPATE IN RESEARCH

Study title: Levels of Aflatoxin M_1 and Heavy Metals in the Breast Milk of Lactating Mothers in Owerri, Nigeria.

Preamble: This is a biomedical research/study sponsored by the principal researcher for the partial fulfilment of a degree of Masters of Science in the Department of Biochemistry at the Federal University of Technology Owerri, Imo state. In this study, you will be required to give about 50.0 ml of your breast milk once for the purpose of the our research exercise. The laboratory personnel involved in collection of your breast milk or any other member of the research team available at the time of collection of your breast milk sample will explain the aim and objectives of this study if you wish to know. Please, feel free to ask questions about the research/study in the course of this study.

What you are required to do as a breast milk donor? If you agree to participate in this study as a breast milk donor, breast milk will be collected from you using a manual breast pump. This will take about 5 minutes. You will also be required to complete a semi-quantitative food questionnaire about your food consumption habit in the previous week (over 7 days). This include the following food categories: maize, yam chips, plantain chips, potato chips, ground nuts, ground nut oil, melon, cassava based foods, beans, rice, bread, yoghurt, cakes and dry fruits.

What are the risks of my participation? There are no life threatening risks; but the breast milk pump may hurt a little during breast milk collection.

Are there benefits? There is no benefit to you. The breast milk sample will be used solely for research.

Will my medical information be kept confidential? We will do our best to protect the information. Information that identifies you will be kept secure and restricted. However, your personal information may be given out if required by law. If information from this research is published or presented at scientific meetings, your name and other identifiers will not be used. Information that identifies you will be destroyed when this research is complete.

Are there any costs or payments? No. Your participation in this study is solely voluntary. Likewise, you will not be charged for donating breast milk sample.

What if I get injured? The research team shall provide the necessary first aid and medical treatment at no cost to you.

Can I say "No"? Yes, you do not have to donate breast milk sample for this study. If you decide not to be in this study, you will not be sanctioned or lose any benefits.

Who can answer my questions about the study? You may ask any questions, concerns, or complaints to laboratory personnel or the principal investigator/researcher

Name: Mrs Lynda C. Ekeanyanwu.
Institution: Federal University of Technology Owerri, Imo State, Nigeria.
Department: Biochemistry
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CONSENT

- ✓ I conform that I have read, or it has been read to me, the foregoing information and I understand the contents of the **INFORMED CONSENT FORM**.
- ✓ I have had the opportunity to ask questions about the study and understand what is involved.
- ✓ I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or other legal rights being affected, and I understand that any remaining sample will be destroyed at my request, but data collected up to the point of my withdrawal may still be used.
- ✓ I agree to provide 50.0ml of breast milk sample once during the study visit (equivalent to approximately one-third of a standard manual massage breast pump).
- ✓ I also agree to complete a semi-quantitative food questionnaire about my food consumption habit in the previous week (over 7 days).
- ✓ I agree to allow information about me to be collected, analyzed, reported and transferred to other approved collaborators and I understand that my identity will remain anonymous.

IF YOU WISH TO PARTICIPATE IN THIS STUDY, PLEASE SIGN BELOW

----------	----------
Date	Participant's Name and Signature for Consent
----------	----------
Date	Person obtaining Consent
----------	----------
Date	Witness Name and Signature – only required if the participant is a non English speaker

Cc;

Participant

Principal Researcher/Investigator

Laboratory/Hospital Notes (if required)

APPENDIX II

Questionnaire

Declaration

I, ……..declare that I gave consent for the collection of breast milk for subsequent determination of aflatoxin M_1 and heavy metals.

Date…….

Signature……

The questionnaire aims to evaluate a potential correlation between the consumption of certain foods, normally associated with the presence of aflatoxin M_1 and heavy metals in breast milk.

Individual characteristics:

1. Career/profession:
2. Mother's Date of birth:
3. Date of birth of your baby:
4. Mother's Height (cm):
5. Height of your baby at birth (cm):
6. Highest education qualification:
7. Number of children:
8. Place of residence:
9. Mother's Weight (kg):
10. Weight of your baby at birth (kg):

Characteristics of breast feeding:

1. Age of the baby (month):
2. Time spent on breast feeding (minutes) each day:
3. Type of breast feeding (supplemented with baby commercial formula or not and at the breast or not):
4. Weight of the baby at the time of milk collection (kg):

Note: Try to answer these questions in a sincere way, indicating the frequency of consumption of the foods mentioned in the table. The questionnaire aims to identify the consumption of foods associated with the presence of aflatoxin M_1 and heavy metals prior to

the collection of breast milk. So for each food, you must indicate (by filling in a "×" in the respective option) how many times per day or per week you ate on average each of the foods referred to in this list over the last month.

Instruction: Indicate the frequency of consumption (check with a "×") of the food consumed at the last month.

Food/amount	Daily frequency			Weekly frequency				Month
	1 daily	2 daily	>3 daily	1*- 2*	3*- 4*	5*- 6*	Never	1*- 3*
Beans								
Bread								
Cakes								
Cassava based foods								
Coffee								
Dry fruits								
Groundnut								
Groundnut oil								
Maize								
Melon								
Plantain chips								
Potato chips								
Rice								
Tomatoes								
Yam chips								
Yoghurt								

*Number of times each food is consumed.

APPENDIX III

Ethical Approval

FEDERAL MEDICAL CENTRE

P. M. B. 1010, Orlu Road Owerri, Imo State, Nigeria

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7932

June 2, 2019

Mrs. Lynda C. Ekeanyawu,
Department of Biochemistry,
School of Biological Sciences,
Federal University of Technology,
Owerri.

RE: APPLICATION FOR ETHICAL APPROVAL

TOPIC: "OCCURRENCE OF AFLATOXIN M1 AND HEAVY METALS IN THE BREAST MILK OF LACTATING MOTHERS IN OWERRI, NIGERIA.

The Ethical Committee has considered further corrections you made on your research proposal.

Sequel to this, ethical approval is hereby given for you to carry out the above study.

Note that you are to abide strictly by your methodology as stated in the proposal.

On completion of your study you are to submit a copy of your dissertation to this Committee.

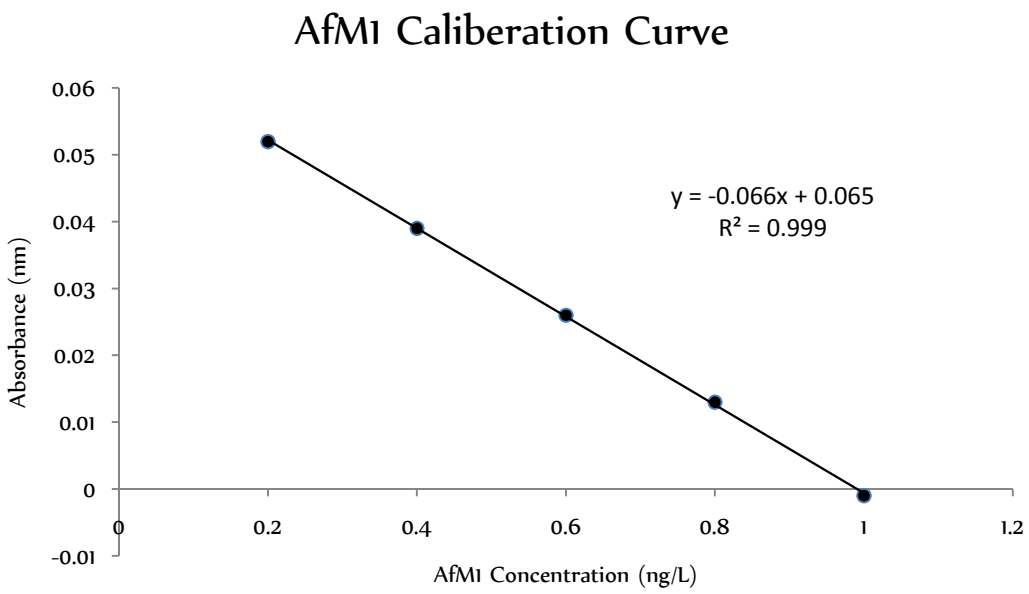
This approval is valid for one year.

Yours sincerely,

DR. I.I. IKE (MBBS, FMCPAED)
Ag. *CHAIRMAN ETHICAL COMMITTEE*

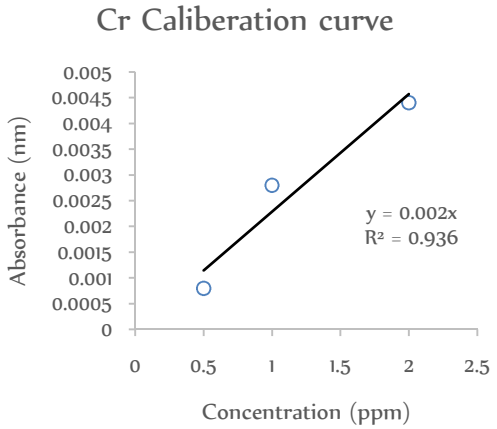
APPENDIX IV

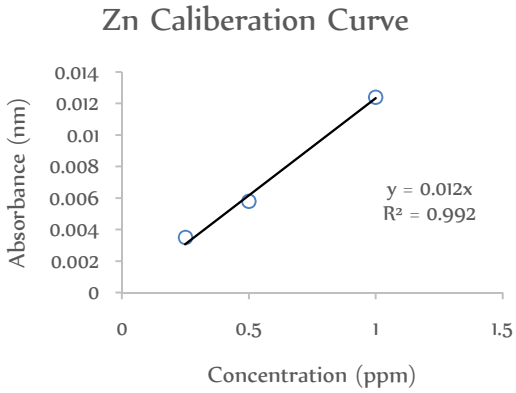
Aflatoxin standard calibration curve

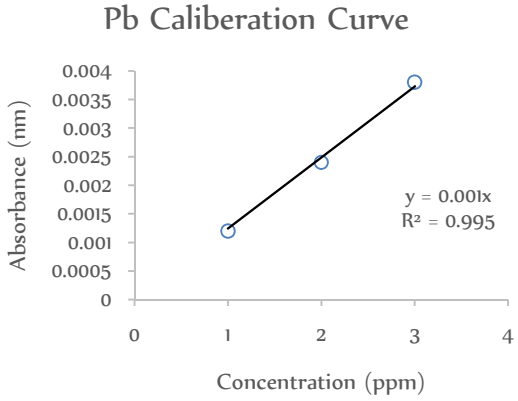


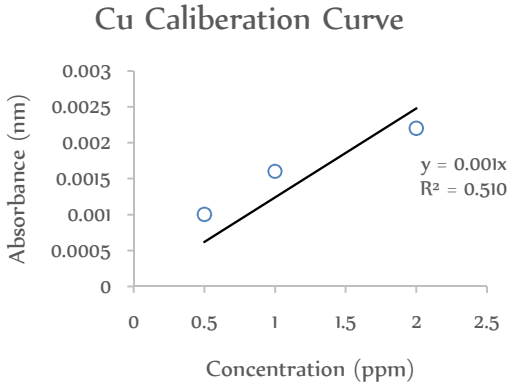
APPENDIX V

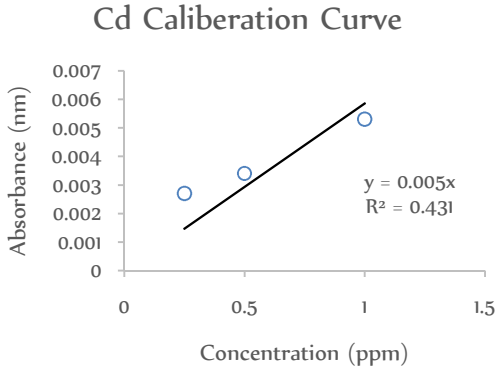
Heavy metals Standards Calibration curves for AAS

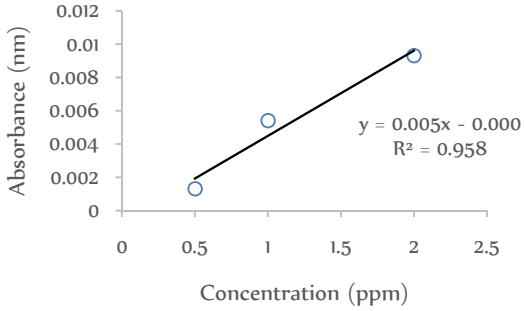


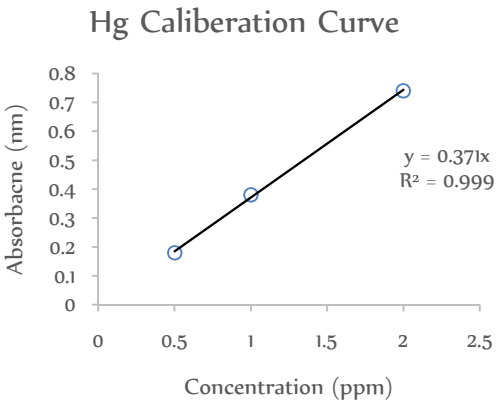


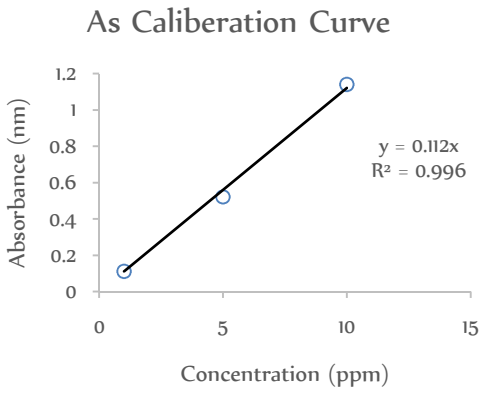









Hg Caliberation Curve
Absorbacne (nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2
2.5
Concentration (ppm)
y = 0.371x
R² = 0.999


As Caliberation Curve
Absorbance (nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
Concentration (ppm)
y = 0.112x
R² = 0.996

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Levels of Aflatoxin M_1 and selected heavy metals (Pb, Cd, Cr, Cu, Zn, Fe, As, and Hg) in the breast milk of lactating mothers in South Eastern, Nigeria

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ABSTRACT

In this study, the level and frequency of breast milk AfM_1 and selected heavy metals as biomarkers of maternal exposure was assessed. Selected heavy metals (Pb, Cd, Cr, Cu, Zn, Fe, As, and Hg) were analyzed using Atomic Absorption Spectrophotometry while AfM_1 levels were assessed by HPLC with fluorescence detection after aflatoxin extraction. The mean (± standard deviation) concentration of AfM_1 in the breast milk samples was 4.02 ± 1.12 ng/L and 100% of all the samples contained AfM_1 at 2.33–7.08 ng/L. AfM_1 concentration was positively and significantly ($p < 0.01$) associated with the daily consumption of cassava-based foods, groundnut oil, maize, tomatoes and dry fruit ($p < 0.05$). The mean (± standard deviation) values of these heavy metals were Cd: 0.029 ± 0.013 mg/L, Cr: 0.019 ± 0.011 mg/L, Cu: 0.035 ± 0.013 mg/L, Fe: 0.049 ± 0.039 mg/L, Pb: 0.038 ± 0.013 mg/L and Zn: 0.009 ± 0.008 mg/L. The result of the estimated daily intake of breast milk by the breastfed infant show that heavy metals such as Pb, Fe, and Cd are ingested more daily than other metals analyzed. There was a weak positive but non-significant correlation between heavy metal content and daily intake of maternal diet except for beans where a significant correlation ($p < 0.01$) was found with Cr, Cu, and Zn exposure. A weak positive but non-significant correlation was also observed between exposure to heavy metals such as Cu, Fe and Pb and maternal diets. None of the samples exceeded the national and international legal regulatory limits for AfM_1 and the selected heavy metals in breast milk except chromium. Nevertheless, the presence of these contaminants still presents a health risk.

1. Introduction

Several documented reports in the literature have proven that breast milk is the best source of nutrition/diet for newborns/infants, probably because of its reported nutritional characteristics and health beneficial properties (Kunter et al., 2017). Important dietary components and balanced nutrients such as carbohydrates, fats, proteins found in breast milk contribute to infant growth, ability to fight infection/diseases and maintenance (Ballard & Morrow, 2013). These assets make the breast milk a distinctive source of food for infants, and nutritionists and medical practitioners have always encouraged and recommended breastfeeding (Kulkarni et al., 2013). In spite of breast milk's nutritionally and immunologically valuable constituents, it may sometimes contain substantial quantities of toxic chemicals because of exposure of the mother to certain food and environmental contaminants (Cherkani-Hassani, Mejemmi, & Mouane, 2016). These contaminants which include heavy metals, pesticides, and fungal toxins may affect the health of the infant negatively.

Mycotoxins include a very diverse group of chemicals with a wide spectrum of toxic effects. Mycotoxins are known to occur naturally and they are usually highly toxic fungal metabolic products of secondary metabolism of moulds such as *Aspergillus flavus* and *Aspergillus parasiticus* (Kunter et al., 2017). The US Food and Drug administration considers mycotoxins as unavoidable food contaminants (Kim et al., 2000; Williams et al., 2004). The unavoidable problem is not just peculiar to the western world but heavily predominant in sub-Saharan Africa. In addition to diverse organ-specific actions (liver, kidney, central nervous system, and lungs), mycotoxins are known to adversely affect the digestive tract, cause skin irradiation, have haematological effects and reduce growth (Etzel, 2006; Sherif, Salama, & Abdel-Wahhab, 2009). A common type of mycotoxin is Aflatoxin (Kim et al., 2000; Williams et al., 2004). Humans may be exposed to aflatoxins directly from the ingestion of contaminated foods. When breastfeeding mothers consume contaminated foods containing aflatoxins, the substance, and its metabolites may accumulate in breast milk (Leong, Latiff, Ahmad, & Rosma, 2012; Prandini et al., 2009). Other known

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