Aflatoxin M₁ Contamination in Fluid Milk Products, Khartoum State 2015

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بسم الله الرحمن الرحيم

﴿وَإِنَّ لَكُمْ فِي الْأَنْعَامِ لَعِبْرَةٌ نِسْقِيكُمْ مَمَّا فِي بُطُونِهِ مِنْ بَيْنِ فَرَثٍ وَدَمٍ لَبَنًا خَالِصًا سَائِعًا لِلشَّارِعَيْنَ﴾

صدق الله العظيم

سورة الأنعام

 الآية (66)
Dedication

I dedicate this work to everyone who encouraged me to complete this study.

The soul of my father

My mother

My dear friends and family

With love and respect
Acknowledgement

Firstly Praise for Allah the lord of the world who gave me the ability to perform this research

I would like to send great thankful to my supervisor;

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List of Abbreviations

AFB1: aflatoxin B1
AFM1: aflatoxin M1
AOAC: Association of Official Analytic Chemists
CAST: Council for Agricultural Science and Technology
CEC: Commission of European Communities
EC: European Commission
EFSA: European Food Safety Authority
FAO: Food and Agriculture Organization
IARC: International Agency for Research on Cancer

ICMSF: International Commission on Microbiological Specifications of Food
JECFA: The joint FAO/WHO Expert Committee on Food Additives
LOQ: Limit of Quantification
PPb: part per billion
PPT: part per trillion
SCF: Scientific Committee on Food
UHT: Ultra - High – Temperature
UKFSA: United Kingdom Food Standards Agency
WHO: World Health Organization
ABSTRACT

Background: Aflatoxins are toxic fungal metabolites found in foods and feeds. When ruminants eat Aflatoxin B1 (AFB1)– contaminated feedstuffs, they metabolize the toxin and excrete AFM1 in milk.

The aim of this study was to determine contamination of aflatoxin M1 (AFM1) in fluid milks in Khartoum State, Sudan

Methods: The samples were analyzed by aflasensor kits, using screening method; eighty seven samples of fluid milks were collected from different sources randomly. About 25 samples from dairy farms (raw) and 62 samples of processed milk from 3 factories; 40 UHT, 12 flavored UHT and 10 pasteurized milk. Rapid test was used for detection of aflatoxin M1 (Aflasensor kit).

Result: The presence of AFM1 contamination was detected in most of the milk samples (85.06%). High prevalence (88.7%) of aflatoxin M1 was detected in the processed milk (flavored, pasteurized and UHT revealed 100%, 100% and 82%, respectively) and the raw milk showed 92%. Almost 85.06% of the contaminated milk samples exceeded the European Commission level (0.05 µg/kg) and Codex Alimentarius recommended limit (0.5 µg/kg).

Conclusion: The presence of aflatoxin M1 in raw milk and processed sample might suggested high occurrence of AFM1 in milk samples which might constituted a possible hazard for human health. Therefore, there is a need to limit exposure to aflatoxins by imposing regulatory limits. Further studies are also needed on large scale basis to investigate the AFM1 and other mycotoxins in milk and dairy products.
المقدمة: الافلاتوكسين هي عبارة عن نواتج إيضية سامة تنتجها الفطريات وهي توجد في الأغذية وفي اعلاف الحيوانات. عندما تتناول المجترات أعلاف احتوت بها الافلاتوكسين من النوع B1 يتحول هذا النوع بعد تفككه إلى الافلاتوكسين M1 الذي يتم إفرازه مع اللبن.

تهدف هذه الدراسة التي أجريت بولاية الخرطوم بالسودان إلى تحديد التلوث بالافلاتوكسين M1 في الالبان السائمة.

طريقة البحث: جمعت سبع وثمانون عينة عشوائية من مصادر لبن مختمق. حممت العينات بواسطة اختبار الافلاسينسر السريع. اخذت 25 عينة لبن خام من مزارع الألبان و 62 عينة من مصانع تجهيز UHT منكة و 10 عينة لبن مبستر، استخدمت الدراسة الاختبار السريع (الافلاسينسر) للكشف عن وجود الافلاتوكسين M1.

النتيجة: أظهرت الدراسة وجود تلوث بالافلاتوكسين M1 في معظم عينات اللبن (85.06%). ووجد ارتفاع في معدل حدوث الافلاتوكسين M1 في الألبان المصنعة (88.7%) كمشكلة مستمرة والممتنع (100% و 100% و 82% على التوالي)، وفي اللبن الخام 92%. تجاوزت تقلبات 85.06% من عينات اللبن الملوث الحد الموصى به من قبل الاتحاد الأوروبي (0.05 ميكروغرام/كغ).

وهيئه الدستور الغذائي الكونكس (0.5 ميكروغرام/كغ).

الخلاصه: أن وجود الافلاتوكسين M1 في عينات اللبن الخام والمصنعة قد يشكل خطاً على صحة الإنسان. لذلك هناك حاجة للحد من التعرض بالافلاتوكسين من خلال فرض قيود تنظيمية. وكذلك يجب إجراء المزيد من الدراسات على نطاق أوسع للتحقيق من السموم الفطرية الأخرى في الألبان ومنتجاتها.
Chapter one

1.1 Introduction

Milk is one of the most nutritious foods. It is rich in high quality protein by providing all ten essential amino acids. It contributes to total daily energy intake as well as essential fatty acids, immunoglobulins, and other micronutrients (FAO, 1997). Cow’s milk is predominant type in several countries, although goat, buffalo, sheep, and camel milks are also consumed. Milk is also consumed in fermented forms such as cheese, yoghurt, kefir, and buttermilk, and as butter. Commercially available milk can be classified into two major groups: liquid milk and dried or powdered milk (FAO, 1997).

Milk is a good source of many nutrients, and it is used extensively even as a main food in many countries, it could also be a source of toxic substances such as aflatoxin M₁ (AFM₁) (Stoloff, 1980). Aflatoxins are a group of naturally occurring toxins produced by mould such as *Aspergillus flavus* (Kamkar, 2005). Aflatoxins are found in food and feed, when ruminants eat aflatoxin B₁ (AFB₁) –feedstuffs, it is metabolized and excreted in milk as AFM1 (Prandini *et al*., 2008). Aflatoxin is associated with both toxicity and carcinogenicity in human and animal populations (Verma, 2004). Although the potency of AFM1 is less than that of its parent compound, it is also known to be hepatotoxic and carcinogenic (Bullerman, 1979). AFM1 remain stable when milk is heat-treated; there is no evidence that cold storage, concentrating or drying changes the level of AFM1 (Park, 2002).

It has been stated that the contamination of milk and milk products with AFM1 displays variations according to geography, countries and seasons. The contamination level of AFM1 is differentiated further by hot and cold seasons, due to the fact that grass, pasture, weed, and rough feeds are found more commonly in spring and summer than winter (Battilani, 2004).
1.2 Objectives

1.2.1 General Objective:
To evaluate of the occurrence and to detect the aflatoxin M₁ in fluid milks in Khartoum State.

1.2.2 Specific objectives:
1. To investigate the occurrence of aflatoxin M₁ contamination in raw milk from some dairy farms.
2. To investigate the occurrence of aflatoxin M₁ in processed fluid milk products and to compare that contamination level with international standards.
1.3 Literature Review

1.3.1 Definition and significant of aflatoxins

Aflatoxins are toxic metabolites produced by several species of fungus of the Aspergillus genus, which grow on plants and food of vegetable origin. The main aflatoxin-producing species are Aspergillus flavus and Aspergillus parasiticus, although other species that produce aflatoxins in food have also been identified such as A. nomius (Kruzman et al., 1987), A. tamarii (Goto et al., 1997), A. pseudotamarii (Ito et al., 2001) or A. australis (IARC, 2002). These compounds were discovered at the end of the 1950s and beginning of the 1960s as a result of an investigation conducted to evaluate the high mortality rate in poultry and other food-producing animals as a consequence of ingesting feed containing peanuts originating from South America (Blount, 1961 and Sargeant et al., 1961). Aflatoxins are fluorescent compounds with a condensed coumarinic structure with one bifurane and one pentanone (aflatoxins B) or one lactone (aflatoxins G) group. The letters B and G refer to the colour of the aflatoxins under ultraviolet light (Blue and Green), and the numbers 1 and 2 refer to their position in chromatographic separation. The more relevant aflatoxins in terms of food safety are B1 and B2 (produced by A. flavus and A. parasiticus), G1 and G2 (produced by A. parasiticus) and M1 and M2 (metabolites of aflatoxins B1 and B2 which are excreted in milk). Among them, aflatoxin B1 stands out from a health safety point of view as it is the most common in food as well as the most toxic for humans (Deng et al., 2010).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has evaluated aflatoxins B and G on several occasions since 1987 (JECFA, 1999 and 2007) and has recommended that, due to their carcinogenic potential, dietary exposure to aflatoxins should be minimised as much as possible. In this way, the 2007 report by the Panel on Contaminants of the European Food Safety Authority...
(EFSA) indicated that exposure to aflatoxins from any food source should be kept as low as reasonably as possible, due to their genotoxic and carcinogenic properties (EFSA, 2007). Recently, Codex Alimentarius has established maximum limits for total aflatoxins (the sum of aflatoxins B1, B2, G1 and G2) in some nuts (almonds, peanuts, hazelnuts, pistachios and Brazil nuts) intended for further processing, at 15 μg/kg in comparison to the 10 μg/kg allowed for the same ready to eat products, based on the information provided by JECFA (Codex Alimentarius, 2008).

1.3.2 Factor influencing the development of aflatoxin production

The factors implicated in the growth of the fungus belonging to the Aspergillus genus in foods are those relating to the environment in which they develop (pH, composition of the food or wateractivity) or extrinsic factors: ambient humidity, storage temperature and microbial competition (Zinedine and Mañes, 2009). *A. flavus* and *A. parasiticus*, the principal aflatoxin-producing fungi, have similar growth and toxinogenesis patterns. Although the conditions described vary slightly depending on the bibliographical source, some authors report that *A. flavus* or *A. parasiticus* grow in a temperature range between 10-12 and 42-43 ºC, and optimally between 32 and 33 ºC. They can grow in a wide pH range (2.1 to 11.2), with optimal growth between 3.5 and 8 (ICMSF, 1996). In terms of water activity (aw), the minimum values for growth are between 0.80 and 0.83, and the optimum is 0.99 (Sweeney and Dobson, 1998).

With regard to toxinogenesis, the temperature range in which the toxins are produced is from 12 to 40 ºC, with the optimum between 25 and 30 ºC. In *A. parasiticus*, the proportion of production of aflatoxins B compared to G is greater at high temperatures (ICMSF, 1996). They are produced in a pH range between 3.5 and 8, with an optimum pH of 6. In terms of water activity (aw), the minimum for production is 0.82 for *A. flavus* (Sweeney and Dobson, 1998) with an optimum of 0.99 (Cousin *et al.*, 2005). The production of aflatoxins also
depends on the sources of carbohydrates and nitrogen, phosphates, lipoperoxides and trace elements (Luchese and Harrigan, 1993). As a result, this production is favoured by an environment rich in carbohydrates, although some substrates rich in fat and proteinlike peanuts also allow aflatoxin production (Marth, 1990). In some cases, the presence of other fungi may reduce aflatoxin synthesis (Wicklow et al., 1980) and some components of various foods also reduce aflatoxin production. Thus, for example, oleuropein, a phenolic iridoid compound contained in olives, reduces aflatoxin production by A. flavus and A. parasiticus, caffeine inhibits Aspergillus growth and aflatoxin production (CAST, 1998). It has also been reported that some vitamins, such as vitamin K5, which present antimicrobial activity, slow down the growth of aflatoxin-producing Aspergillus species, as well as aflatoxin production by those fungi (Miranda et al., 2011).

It is a well-known fact that some lactic acid bacteria produce compounds with antimicrobial and antifungal properties, and can be used to control the growth of pathogenic bacteria, spoilage bacteria and fungi. Lactic acid bacteria with the greatest capacity to inhibit or reduce the growth of toxin-producing fungi belong to the genus Lactococcus, Lactobacillus, and, to a lesser degree, Pediococcus and Leuconostoc (Dalié et al., 2010). It has also been demonstrated that, as well as inhibiting fungal growth, certain lactic acid bacteria have the capacity to capture aflatoxins, thus reducing their bioavailability. This process is rapid and reversible (Bueno et al., 2006), and is dependent as much on the dose as on the strain studied (Kankaanpää et al., 2000). This capacity to capture aflatoxins (particularly aflatoxin B1) is due to the peptidoglycans in the bacterial wall of some lactic acid bacteria (Lahtinen et al., 2004). This mechanism has also been observed in other microorganisms, such as Saccharomyces cerevisiae (Bueno et al., 2006). Moreover, it is known that certain substances have an inhibitory effect on the toxicity caused by aflatoxins, whether through metabolising them or inhibiting their oxidising effects. One example of this is
saffron (*Crocus sativus*), the aqueous extract of which is capable of preventing lipid peroxidation induced by various substances such as aflatoxins, as well as of promoting detoxifying enzymatic systems, and whose tests on mice have verified that it contains protective substances against diverse hepatotoxic agents, among them aflatoxin B1 (Premkumar et al., 2003). Furthermore, it has been verified that the administration of green tea capsules, due to their polyphenolic content, protects against aflatoxin-induced cellular damage, not only because of their antioxidant properties but also because they activate metabolism (Kensler et al., 2011). Aspergillus is an aerobic microorganism, the vacuum packaging or modified atmospheres packaging could also reduce oxygen availability and inhibit aflatoxin production (Ellis et al., 1993).

### 1.3.3 Condition for growth

Humidity and low temperatures influence development of *Aspergillus* spp. and production of toxins (Ghorbanian et al., 2008). *Aspergillus parasiticus* is able to produce sclerotia from 25 to 35°C, with relative humidity between 65 and 80% (Giorni et al., 2007).

The season affects the level of toxins which produced from fungi. Moreover the levels of aflatoxins M1 were higher in winter and spring than in summer and autumn (Tajkarimi et al., 2007).

Elsayed (1990) indicated that aflatoxin production was increased with advanced period of storage. Damaged pods were highly contaminated with *Aspergillus flavus* and accumulated large amount of aflatoxin (Ozay et al., 2008). However, sound intact pods recorded lower fungal contamination and were almost free of aflatoxins. Hag Elamin et al. (1988) and Pazzi et al. (2005) reported that aflatoxins content of stored groundnut seeds increased with increasing period of storage. Omer et al. (2004) indicated that moisture is the major factor affecting the rate of deterioration in sound seeds of haricot bean, faba bean and maize as compared to significantly higher levels in damaged and mouldy seeds.
of the same crops. Moreover, Mixon and Roger (1973) reported that when the seed coat of the resistant seeds was picked with a needle, \textit{A. flavus} developed in the area of injury.

1.3.4 Physical and chemical properties of aflatoxin

Aflatoxin are potent toxic, carcinogenic, mutagenic, immunosuppressive agents, produced as secondary metabolites by the fungus \textit{Aspergillus flavus} and \textit{Aspergillus parasiticus} on variety of food products (Ozay \textit{et al.}, 2008). Among eighteen different types of aflatoxins identified, major members are aflatoxins B\textsubscript{1}, B\textsubscript{2}, G\textsubscript{1} and G\textsubscript{2}. Aflatoxins B\textsubscript{1} (AFB\textsubscript{1}) is normally predominant in amount in cultures as welll as in food products. Pure AFB\textsubscript{1} is pale-white to yellow crystalline, odorless and solid (Guzman de Pena, 2007). Aflatoxins are soluble in methanol, chloroform, acetone, acetonitrile. \textit{A. flavus} typically produces AFB\textsubscript{1} and AFB\textsubscript{2}, whereas \textit{A. parasiticus} produce AFG\textsubscript{1} and AFG\textsubscript{2} as well as AFB\textsubscript{1} and AFB\textsubscript{2}. Four other aflatoxins M\textsubscript{1}, M\textsubscript{2}, B\textsubscript{2}A, GA which may be produced in minor amount were subsequently isolated from cultures of \textit{A. flavus} and \textit{A. parasiticus} (Ghorbanian \textit{et al.}, 2008). A number of closely related compounds namely aflatoxins GM1, parasiticol and aflatoxicol are also produced by \textit{A. flavus}. Aflatoxins M\textsubscript{1} and M\textsubscript{2} are major metabolites of aflatoxins B\textsubscript{1} and B\textsubscript{2} respectively, and are found in milk of animals that have consumed feed contaminated with aflatoxins (Reddy \textit{et al.}, 2008).

1.3.5 Aflatoxins occurrence in raw agricultural products

Aflatoxins often occur in crops in the field prior to harvest, post harvests contamination can occur if crops drying is delayed and during storage of the crop (Martins \textit{et al.}, 2007). Aflatoxins are detected occasionally in milk, cheese, corn, peanuts, cotton seed, nuts, almonds, figs species and a variety of other foods and feeds (Ghorbanian \textit{et al.}, 2008). Milk, eggs and meat products are sometimes contaminated because of the animal consumption of aflatoxins contaminated feed (Martins \textit{et al.}, 2007).
(Dickens and Pattee, 1966) reported that under unfavorable climatic and storage conditions, oil seeds and many other agricultural products are subjected to invasion by toxigenic strains of *A. flavus* and *A. paracticus*. They added that although aflatoxins, has been found in variety of food--stuffs, the most pronounced contamination has been encountered in groundnut seeds, cakes and in the meals produced from these oil seeds. Maize samples in Sudan Savanna were associated with higher aflatoxins levels (Hell *et al.*, 2000).

**1.3.6 Aflatoxins occurrence in processed foods**
Corn is probably the commodity of world wide concern because it is grown in climates that are likely to have potential contaminated corn and cotton seed meals in dairy rations have resulted in aflatoxins. Moreover, M1 contaminated milk and milk products, including skimmed milk, cheese and yoghurt (Van Eijkeren *et al.*, 2006) and pasteurized milk (Zinedine *et al.*, 2007).

Aflatoxins were detected in some cereals and leguminous seed in Sudan (Wad Madani and Khartoum), the levels of aflatoxins in the seeds were below the reported hazard threshold. Moreover, sound intact seeds contained low or no aflatoxins compared to significantly higher levels detectable in damaged and mothly seeds of the tested crop plants (Abdel-Rahim *et al.*, 1989).

**1.3.7 Aflatoxin in dairy products**
The aflatoxins B$_1$ occur in peanuts, milk and animal feed (Offiah and Adesiyun, 2007). Transfer of aflatoxins B$_1$ from feed to milk and from milk to curd and whey in dairy sheep fed artificially contaminated concentrates was reported (Manetta *et al.*, 2005).

Aflatoxins M$_1$ in lactating cow, B$_1$ in feed of dairy cattle were detected (Van-eijkeren *et al*.  2006). Moreover they assuming a linear relationship between the cow’s lactation status and feed intake, the model relates daily milk production and B1 concentration in total feed to M$_1$ levels in milk.
A survey was undertaken to determine the aflatoxin M$_1$ contamination of milk and some locally produced dairy products in Nigeria. Samples of human and cow milk, yoghurt and "Wara "ice cream were collected randomly within the local governments and analyzed for aflatoxins M$_1$ and the result showed a high contamination level of aflatoxins M$_1$ (Atanda et al., 2007). Aflatoxins occur in UHT milk (Unusan, 2006) and aflatoxins M$_1$ was reproduced in randomly selected north African milk and cheese samples (Atanda et al., 2007). Ali et al.,(2014) detected aflatoxin M$_1$ in fresh cow milk samples and imported milk powered in Sudan.

1.3.8 Aflatoxins in milk

Aflatoxins are toxic secondary metabolites of moulds Aspergillus flavus and A. parasiticus and are the main mycotoxin which could be detected in milk (Boudra et al., 2007). Eighteen aflatoxins have been identified up to now, and B$_1$, B$_2$, G$_1$, G$_2$ and M$_1$ are the aflatoxins of most concern (Decastelli et al., 2007). Moreover, aflatoxin M$_1$ (AFM$_1$) is the only mycotoxin that has been set a legal limit in milk by many national governments and international organisations, such as the Codex Alimentarius Commission (Food and Agriculture Organisation, 2004 and Sugiyama et al., 2008).

Aflatoxin B$_1$ (AFB$_1$) is the most toxic aflatoxin and has been designated as a primary carcinogenic compound by the International Agency for Research on Cancer (IARC) of the World Health Organisation (IARC, 1993). Dairy cows consuming feeds contaminated with AFB$_1$ can excrete a hydroxylated metabolite of AFB$_1$ as AFM$_1$ in milk (Frobish et al., 1986 and Chopra et al., 1999). AFM$_1$ has been classified as a second-class possible human carcinogen by the IARC (1993). Moreover its carcinogenicity was estimated to be 1/10th of that of AFB$_1$ by the Joint Expert Committee on Contamination and Food Additives in 2001 (Sugiyama et al., 2008). Furthermore, AFM$_1$ is very stable, and it is assumed that neither storage nor processing, such as pasteurisation, autoclaving, fermentation
or other methods commonly used to process milk products, destroys its toxin (Tajkarimi et al., 2008).

A legal limit for AFM$_1$ in milk existed in 60 countries at the end of 2003. Among them, 50 ng L$^{-1}$ set by the EU and 500 ng L$^{-1}$ by the United States are two peak limits (Food and Agriculture Organisation, 2004). Further, many countries such as New Zealand, United Kingdom, Brazil and Iran have conducted national surveys to assess AFM$_1$ contamination in raw milk in order to ensure safety of milk products (UKFSA, 2001; Sassahara et al., 2005 and Tajkarimi et al., 2008). The Chinese government has established 500 ng L$^{-1}$ as the national legal limit of AFM$_1$ in milk. Pei et al. (2009) measured the AFM$_1$ content in 12 raw milk samples collected from the market in northeastern China in 2008. AFM$_1$ was detected in all samples with contents in the range of 160–500 μg L$^{-1}$, and 75% of samples had AFM$_1$ concentrations in the range of 320–500 μg L$^{-1}$, which were much closer to the Chinese national legal limit. The findings suggested that the contamination of AFM$_1$ in raw milk in the northeast of China is serious and this stressed the need for continued assessment of AFM$_1$. AFM$_1$ contamination in 360 raw milk samples collected from five provinces, covering the north and south of China was assessed using ELISA (Zheng et al., 2013).

The occurrence of AFM$_1$ in milk is a public health concern, as it was classified as category 2B by the International Agency of Research on Cancer, so it is possibly carcinogenic to humans (Boudra et al., 2007). Contamination of milk and milk products with AFM$_1$ displays variations according to geography, countries and seasons (Battilani, 2004). The European Union set a limit of 0.050 μg/kg AFM$_1$ in liquid milk and dried or processed milk products, 10 times lower than the Codex Alimentarius limit (Codex Alimentarius Commission, 1995). In Egypt, the Ministry of Health stated that fluid milk and dairy products should be free from AFM$_1$ (Amer and Ibrahim, 2010).
1.3.9 Effect of milk processing on AFM$_1$ content

1.3.9.1 Heat treatments

The results of studies on heat processing of dairy products on the amount of AFM$_1$ are ambiguous but most of the studies indicate that treatments like pasteurization and sterilization do not cause an appreciable change in the amount of AFM$_1$ in these products (Park, 2002).

1.3.9.2 Storage at low temperature

Studies on the stability of AFM$_1$ in milk during cold and frozen storage have given variable results. Because of their variability, a final conclusion cannot be made from results of these studies; however storage of frozen contaminated milk and other dairy products for a few months does not appear to affect their content of AFM$_1$ (JECFA, 2001).

1.3.9.3 Manufacture of cultured dairy products

Cultured dairy products are manufactured by heating milk and adding a starter culture to initiate the fermentation. Studies have shown that there was no significant decrease in the AFM$_1$ content of cultured dairy products, such as kefir and yoghurt (Park, 2002).

1.3.9.4 Concentration and drying of milk

Evaporated, concentrated or dried milks are results of partial or complete removal of water from milk, without heating, that leads to concentration of milk solids and contaminants such as AFM$_1$ (JECFA, 2001).

1.3.10 Human aflatoxicosis

Human exposure to levels of aflatoxins from nanograms to micrograms per day occurs through consumption of contaminated maize, peanuts and other contaminated foodstuffs. Maize is the staple food in Kenya. It is milled into flour for various delicacies such as Ugali(Lunyasunyaet al., 2005). The human gastrointestinal tract rapidly absorbs aflatoxin after consumption of contaminated food and the circulatory system transports aflatoxin to the liver (Fung and Clark,
From 1-3% of ingested aflatoxin irreversibly bind to proteins and DNA bases to form adduct such as aflatoxin B1 lysine in albumin. Disruption of protein and DNA bases in hepatocytes causes liver toxicity (Tandon et al., 1978). Early symptoms of hepatotoxicity from aflatoxicosis can manifest as anorexia, malaise, and low grade fever. Aflatoxicosis can progress to potentially lethal acute hepatitis with vomiting, abdominal pain, hepatitis and death (Etzel, 2002). Symptoms of B1 also include yellow eyes, swollen legs, vomiting, abdominal pain and bleeding. The health impact of aflatoxin exposure in animals mainly depends on dosage and response. Low dosages produce nutritional interference and immunological suppression, while high doses lead to acute illness and death (Lunyasunya et al., 2005). Aflatoxins have been detected in the blood of pregnant women, in neonatal umbilical cord blood, and in breast milk in African countries, with significant seasonal variations (Maxwell et al., 1989). Levels of aflatoxins detected in some umbilical cord blood at birth are among the highest levels ever recorded in human tissues and fluids (Coulter, 1984). Aflatoxins have been suggested as etiological factor in encephalopathy and fatty tissue degeneration of viscera, similar to Reye syndrome, which is common in countries with a hot and humid climate (Turner et al., 2002). The clinical picture includes enlarged pale liver and kidneys and severe cerebral oedema. Aflatoxins have been found in blood during the acute phase of the disease, and in the liver of affected children (Turner et al., 2002). The evaluation of epidemiological and laboratory results carried out in 1987 by the International Agency for Research on Cancer (IARC) found that there was sufficient evidence in human for the carcinogenicity of naturally occurring mixtures of aflatoxins, which are therefore classified as Group 1 carcinogens, except for aflatoxins M1, which is possibly carcinogenic to human (IARC, 1987). In recent studies, aflatoxins have been found in the brains and lungs of
children who have died from kwashiorkor and those who had died from various other diseases (Oyelami et al., 1995; Oyelami et al., 1997).

1.3.11 Biological safety
Spores and other viable propagules of Aspergillus flavus, A. parasiticus, and other fungi can cause three types of disease in humans: allergy, poisoning, and infection (Hill et al., 1984). A. flavus infection in humans is uncommon but possible. Airborne spores and dust containing propagules of the A. flavus group may cause allergies in some people and the inhaled particles may contain aflatoxins (Shotwell et al., 1981).

1.3.12 Analytical methods for aflatoxin detection
1.3.12.1 Thin layer chromatography (TLC)
Thin-Layer Chromatography (TLC), also known as flat bed chromatography or planar chromatography is one of the most widely used separation techniques in aflatoxin analysis (Suzana et al., 1993). Since 1990, it has been considered the AOAC official method and the method of choice to identify and quantitate aflatoxine at levels as low as 1 ng/g (Stroke and Anklam, 2000). The TLC method is also used to verify finding by newer, more rapid techniques (Grosso et al., 2004).

1.3.12.2 High performance liquid chromatography (HPLC)
High performance liquid chromatography is a method of choice for separating potentially carcinogenic substances. Detection normally is performed by fluorescence spectroscopic method on trifluoroacetic acid derivative. For analyzing aflatoxins, reverse phase liquid chromatography (RPLC) and normal phase liquid chromatography (NPLC) are used (Park, 1995). The RPLC for analysis of aflatoxins employs silica based HPLC columns bonded with C8 and C18 groups which are used with mobile phase consisting of binary and ternary mixture of polar solvents. The commonly used solvents mixtures include deionized water, methanol and acetonitrile. In the reverse phase mode, the
elusion order of the common aflatoxins is G2, G1, B2 and B1. Aflatoxins may be separated and detected by UV detection. However the sensitivity is not sufficient to detect these compounds at parts per billion (ppb) concentrations required for food analysis, with fluorescence detector (Nollet, 1992).

1.3.12.3 Immunochemical methods

Thin layer chromatography and liquid chromatography (LC) methods of determining aflatoxin in food are laborious and time consuming (Cordova – Izquierdo et al., 2007). Often, these techniques to solve separation and interference problems (Pal et al., 2005). Through advances in biotechnology, highly specific antibody-based tests are now commercially available that can identify and measure aflatoxins in food in less than 10 minutes (Soblev, 2007). These tests are based on the affinities of the monoclonal or polyclonal antibodies for aflatoxins (Salter et al., 2006). The three types of immunochemical methods are radioimmunoassay (RIA), enzyme-linked immunosobent assay (ELISA), and immunoaffinity column assay (ICA) (Grosso et al., 2004).

1.3.12.4 Confirmation and identities of the aflatoxins

Although analytical methods might consist of different extraction, clean-up and quantitation steps, the results of analysis by such methods should be similar when the methods are applied properly. Since the reliability of quantitative data is not in question, the problem still to be solved is confirmation of identity of aflatoxins. The confirmation techniques used involve either chemical derivatization or mass spectrometry (MS) (Cordova-Izquierdo et al., 2007).

1.3.12.5 Presumptive and screening methods

Some applications require only presumptive or screening tests, while others require the quantification of only B or other several of the aflatoxins. Groundnuts at the buying point are visually inspected in the United States for evidence of A. flavus conidial heads and if present the suspect lots are not allowed into commerce for human consumption. This visual examination is not a chemical
test and may result in family acceptances or rejections. The other commonly used screening technique is the application of one of several minicolumn procedures to detect aflatoxin contamination above a predetermined level (Romer et al., 1979). Shannon and Shotwell (1979) conducted a collaborative study of two minicolumn methods and found that a combination method using the Holaday extraction and the Velasco minicolumn was the most satisfactory method. Minicolumn techniques should not be used for quantitative purposes where accurate quantitative data are required. Madhyastha and Bhat (1984) developed a minicolumn confirmation method for aflatoxins. These workers confirmed the identity of aflatoxins on the developed minicolumn by applying 20% HSO₄, 20% HCl, or trifluoroacetic acid (TFA) in 20% HNO₃. All acids changed the fluorescence from blue to yellow, with the TFA in 20% HNO₃ having the lowest detection limit.

1.3.12.6 Biosensors and other methods
Biosensors, an alternative to improve the disadvantages of the previous methods, are multidisciplinary tools with an enormous potential in detection and quantification of aflatoxin. There are all kinds of biosensors that base their performance on different physical or biochemical principles, such as optical, optoelectronic, electrochemical, piezoelectric, DNA and combined. Thus, such devices have a huge impact in healthcare, food management, agro-economical economy and bio-defense (Nayak et al., 2009). Many kinds of biosensors are applied to detect aflatoxin. However, they mainly work in conjunction with immunochemical methods. Such junctions are based on the high affinity of antigen-antibody interaction and have the aim of increasing the sensitivity and shortening the detection time of the toxic element (Dinçkaya, et al., 2011).

Further methods exist which are less common than the previously described methods but have a wide utility as well. The most important are those ones that base their principle on electrochemistry, spectroscopy and fluorescence.
Compared with traditional methods for aflatoxin determination, electrochemical techniques offer some advantages such as reliability, low cost, in-situ measurements, fast processes, and easier methodology than common chromatography techniques through a similar performance. Especially for measurement of AFM1, the disposable immunosensors have been applied directly in milk following a simple centrifugation step without dilution or other pretreatment steps. Exhibition of a good working range with linearity between 30 and 240 ng/ml makes this method useful for AFM1 monitoring in milk (maximum acceptable level of AFM1 in milk is 50 ppt) (Micheli et al., 2005). Spectroscopy techniques have been popularized due to the characteristics that fast, low-cost and non-destructive analytical methods suitable to work with solid and liquid samples. Among them, near infrared spectroscopy (NIRS) is an excellent method for a rapid and low cost detection of aflatoxin in cereals (Fernández-Ibáñez et al., 2009). When incorporated with a bundle reflectance fiber-optic probe, NIRS was successfully applied to quantify aflatoxin B1, ocharatoxin A and total aflatoxins in paprika (Hernández-Hierro et al., 2008). Aflatoxins have a native fluorescence due to their oxygenated pentaheterocyclic structure, which is the basis of most analytical and microbiological methods for detection and quantification of aflatoxins (Rojas-Durán et al., 2007).

1.3.12.7 Lateral flow immunoassay for aflatoxins
Lateral flow immunoassay for aflatoxins applies the same principle and reagents as in the micro-well type immunoassay, except for the fact that in lateral flow immunoassay (LFIA), the separation of bound and unbound antibody sites is obtained by means of lateral flow on suitable support (nitrocellulose membrane). The liquid flow transports immune reagents along the membrane where they encounter their partners in spatially confined zones of the membrane itself where immunoreactions take place. Lines (test and control lines) are traced on the nitrocellulose membrane by means of dedicated dispenser which enables
dispensing of small volumes with high productivity. Interpretation of assay result depends on the presence and intensity of both test and control line. The indirect competitive format in which the antigen (protein conjugate of the target toxin) is coated on the membrane and the antibody labeled is preferred for aflatoxins (Anfossiet al., 2011).

1.3.12.8 Competitive enzyme immunoassay

The bases of this test are the antigen- antibody reaction. The well in the microtiter strips are coated with capture antibodies directed against anti-aflatoxin antibodies. The standard or the sample solution, aflatoxin- enzyme conjugate and anti-aflatoxin antibodies are added. Free and enzyme conjugated aflatoxin compete for the aflatoxin antibody binding sites. At the same time the anti-aflatoxin antibodies are bound by the immobilized capture antibodies. Any unbound enzyme conjugate is then removed in a washing step. Enzyme substrate and chromogen are added to the wells and incubated. Bound enzyme conjugate converts the colourless chromogen into a blue product. The addition of the stop solution leads to colour change from blue to yellow. The measurement is made photometrically at 450nm; the absorption is inversely proportional to the aflatoxin concentration in the sample (R-Biopharm, 2003).

Competitive enzyme immunoassay is a preferred choice of testing aflatoxins because it is simple, sensitive, and cost effective and can be used to test over 100 samples unlike HPLC and lateral flow immunoassays which although sensitive are not cost effective and cannot be used on over 100. They are normally used as confirmatory tests ( Waliyar ad Reddy, 2009). immunochemical methods are quite specific and can be used to screen flatoxins in grains and grain products (Trucksessset al., 2007).
P2. Materials and Methods:

2.1 Study area
This study was carried out in Khartoum State that located in central Sudan, comprising the semi-arid zone between the latitude 15.08°N to 16.39°N and longitude 31.36°E to 34.25°E. The president livestock in Khartoum is about 236,909 head of cattle.

2.2 Source of samples
Eighty seven (87) samples of milk were collected randomly from Khartoum, 62 samples from different dairy factories (n=3) and 25 raw milk from dairy farms (n=8). The sample were collected directly from bulk tank milk in sterile tubes from different farms, while the processed milk were obtained from different sales centers distributing the dairy products that supplied to them by the selected dairy factories. The objective is to determine the aflatoxin M1 residues using Aflasensor kit (Unisensor, Belgium). Heatsensor was used as incubator according to heatsensor accompanied manual.

2.3 Test Procedure of Aflasensor
It is rapid test for Aflatoxin M1 quantification in milk that required incubation temperature of 40°C.

2.3.1 Background
Aflasensor is a rapid quantitative test that allows the quantification of the presence of aflatoxin M1 (AFM1) molecules in raw milk samples. The dipstick assay does not require any samples processing, cleaning or extraction. The test take 10 minutes at 40°C (using Heatsensor). The results are either directly interpreted by visual observation with a threshold level at 100 ppt of AFM1. The LOQ of the Aflasensor is 20 ppt with a quantification range going from 20 to 150 ppt.
2.3.2 Reaction mechanisms

Alflasensor is a competitive test involving specific antibody with high affinity for aflatoxin M₁ molecules. The test requires the use of two components. The first component is a microwell containing predetermined amount of antibody linked to gold particles. The second is a dipstick made up of a set of membranes with specific capture lines. For a valid test, the upper red control line has to be visible after the second incubation. When the reagent from microwell is re-suspended with a milk sample, specific antibodies will bind the analytes if present during the first incubation. When the dipstick is dipped into the sample, the liquid starts running vertically on the dipstick and passes through capture zones. When the sample is free of aflatoxin M₁, a color development occurs at the test line indicate the absence of aflatoxin M₁ in the milk sample. On the opposite, the presence of aflatoxin M₁ in the sample will not cause the appearance of the coloured signal at the test capture line. Based on this, intensity accuracy will determined the aflatoxin M₁ concentration present in the milk sample (UNISENSOR, 2013).

2.3.3 Composition of Aflasensor kits

Aflasensor milk kits contain everything needed to perform 96 measurements. 12 pots with 1 strip of 8 reagent microwell and 8 dipsticks, one micropipette of 200µl, one positive standard containing powder to reconstitute raw milk of aflatoxin and negative standard containing powder to reconstitute raw milk (Appendix 1).

2.3.4 Additional material needed

2.3.4.1 Heatsensor

2.3.4.1.1 Installing the device
Heat sensor was used at incubation of 40°C according to the reference supplied with the heat sensor manual. The Heatsensor-DUO was placed onto a level, horizontal surface. The device was connected to a power source via the adapter provided in the delivery package. The incubator was switched on by pressing the main power button (main switch is on the rear of the device).”I” should be pushed to power on the device, and “O” should be pushed to power off the device. The incubator is ready to operate when the display becomes visible. The top was opened and a reagent of amicrowell was placed into the block (Appendix 2).

2.3.4.1.2 Creating a new program
The key ”prog” was pressed, while holding it up “▲” or dawn to select the right program, if P08 was selected (to create anew program on P08),”>” key was pressed while holding it, “prog.” key was pressed. The cursor displayed on the screen (P08).Then continue “>” to remove the cursor from left to right and pressed up” ▲” or dawn”▼” key to set a new value. The first temperature (corresponding to the first incubation), the first timing, the second temperature (corresponding to the second incubation) and second timing. When the sing “■” appeared, the first temperature and first time were set, and then the sing”■■”indicated setting of the second temperature and second time.

2.3.4.1.3 Bell mode at the end
In the main interface dawn “▼”key was pressed for 2 seconds continuously. The coming in the Bell mode interface, the alarm times could be modify by pressing down or upkey.

2.3.4.1.4 Maintenance and cleaning
The well in the block should be cleaned by cloth stained with alcohol to assure good heat translation between the block and the test tube. If there are smutches on the instrument they should be cleaned with a cloth;
power should be off when cleaning the instrument. During cleaning, the cleaning liquid should not be dropped into the well. Corrosive cleaning liquid should be avoided.

2.4 General remarks
The kits should be stored in dry place at low temperature between 2°C and 8°C before opening the plastic pots, they should be brought to room temperature in order to avoid exposure of the product to moisture and light. The best temperature to perform the test should be 40°C±1°C, the heatsensor or alternatively waterbath appropriate to perform the aflasensor assay.

The result must be read within 2 minutes time after the second incubation. The content of a pot should be used before opening the next one. The clotted milk should not be used with Aflasensor (UNISENSOR, 2013).

2.5 Direction for use
This procedure is described to easily run one single sample or a set of many samples. In that case, try to perform the test in cascade and avoid any delays mixing reagent and milk but also when adding and removing dipsticks. The same incubation time and temperature must be used for each sample. Moreover, more than 8 samples must not be used at one time and multi-pipette should be used if there are more than 3 samples. The test should be performed in clean and dry place and the hands are to be washed before start connecting the heat sensor.

To open a pot of dipsticks, the safety ring should be stopped by pressing it down the pot, the ring must be taken off to get the stopper of the pot with the thumb. The pot with dipsticks should always be well closed after the reagents been out. Individual caps should not be separated and should be put immediately
back into the white pot without damaging the dipsticks; they should be closed and tightly sealed.
The microwell(s) should be placed in the heating block which shows 40°C. New tip should be placed on the micropipette and 200µl of the milk sample is transfer into each of the microwell, the reagent and the milk are in contact, the reaction beings. Quickly and immediately the start after mixing, the three minutes countdown starts, during that first incubation, both receptors and monoclonal antibodies would detect whether or not there is aflatoxin in the milk sample. It takes three minutes for the reaction to be completed and during that time the same pot has to be opened as before, by take out as many dipsticks as there is analysis in progress then the pot has to being closed the pot.
The dipsticks should be placed on a clean sheet when the three minutes are over; the dipsticks should automatically dipped into each of the microwells laid in the incubator. After the second incubation time is over, the stop sound signal of the running tone has to be pressed and the dipsticks should be taken out from the microwells and laid down on a sheet of paper.

2.6 Negative and positive standards reconstitution
The cap and rubber stopper from the vial were removed and 1 ml of the distilled or deionised water was added. The sample was then vigorously shaken during 10 seconds. The sample was checked for properly, dissolved and left to stand for 1 minute and a half (Appendix 3).

2.7 Visual interpretation of the result
After checking the control (CTRL) line, the analysis was considered as invalid if the control line was not visible. When the top control line could be seen, the test lines were examined; the intensity of the line colour of the test line was compared with the intensity of the line colour of the test line. For interpretation of the result, it should be started from bottom line of aflatoxin M1 for example (Appendix 4). When the test line was darker in colour like the control line, the
result was negative, which means that at the given sensitivity of the test, the milk samples contain no aflatoxin M1 or aflatoxin M1 at a lower level than the value stated in the enclosed aflatoxin M1 limit of detection. When the line was as the same intensity or lighter in colour than the control line (CTRL), the result is considered positive(+) and the sample considered to contain Aflatoxin M1 at equal or higher concentration than 100ppt. When there was no test line at all, the milk sample should contain higher concentrations of aflatoxin M1 residues and considered as full positive (++) as shown in (Appendix4).

2.8 Summary of the protocol
About 200 µl of the milk sample was added into reagent microwell and was mixed 10 times to be homogenized. The sample was incubated for 3 minutes at 40°C. The dipstick was dipped automatically into the reagent microwell. The second incubation time was continued for 7 minutes at 40°C. Finally the test lines colour intensities was read and compared with the control line for interpretation of the result.

2.9 statistical analyses
The data of the present study were illustrated on a percentage basis
3. Results

A total of 87 milk samples were collected from dairy farms (25) and different batches of dairy factories (62) in Khartoum State. The result of milk samples tested by aflasensor kit for the residues of aflatoxin. Table 1 illustrated that approximately 78% of milk samples were contaminated with aflatoxin M1: About 55% of the processed samples from different factories and about 23% from dairy farms (raw) were positive for the presence of aflatoxin.

Table 2 showed that the fluid milk samples (both pasteurized and UHT) revealed high occurrence of aflatoxin M1. The contamination was in 38 (100%), 14 (100%) and 3 (30%) of the samples collected from factory A, B and C, respectively. Moreover Table 3 showed that aflatoxin M1 was detected in 12 (100%), 10 (100%) 33 (82%) of the flavored UHT, pasteurized and plain UHT milk samples, respectively. Also all samples of flavored milk samples (12) were positive for aflatoxin M1 (100-150ppt), the cacao UHT milk, strawberry UHT milk and vanilla UHT milk that were 6 (100%), 4 (100%), 2 (100%) respectively.
Table (1): Presence of aflatoxin M$_1$ (AFM$_1$) in the milk samples

<table>
<thead>
<tr>
<th>Type of milk</th>
<th>Number of samples</th>
<th>Positive (++) (100-150 ppt)</th>
<th>Positive (+) (20-100 ppt)</th>
<th>Negative (-) (&gt;20 ppt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>25</td>
<td>18 (72%)</td>
<td>5 (20%)</td>
<td>2 (8%)</td>
</tr>
<tr>
<td>Processed milk</td>
<td>62</td>
<td>45 (72.6%)</td>
<td>10 (16.1%)</td>
<td>7 (11.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>87</td>
<td>63 (72.4%)</td>
<td>15 (17.2%)</td>
<td>9 (10.4%)</td>
</tr>
</tbody>
</table>

(++) ≡ Strong positive (100 – 150 ppt).
(+) ≡ Positive (20-100 ppt).
(-) ≡ Negative (>20 ppt).
Table (2): Occurrence of aflatoxin (AFM₁) in fluid milk samples from different dairy factories

<table>
<thead>
<tr>
<th>Source of milk samples</th>
<th>Number of samples</th>
<th>Positive (++)</th>
<th>Positive (+)</th>
<th>Negative (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factory (A)</td>
<td>38</td>
<td>38 (100%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Factory (B)</td>
<td>14</td>
<td>4 (28.6%)</td>
<td>10 (71.4%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Factory (C)</td>
<td>10</td>
<td>3 (30%)</td>
<td>0 (0.0%)</td>
<td>7 (70%)</td>
</tr>
<tr>
<td>Total</td>
<td>62 (100%)</td>
<td>45 (72.6%)</td>
<td>10 (16.1%)</td>
<td>7 (11.3%)</td>
</tr>
</tbody>
</table>
Table (3): Aflatoxin M<sub>1</sub> contamination in different types of fluid milk samples

<table>
<thead>
<tr>
<th>Type of milk samples</th>
<th>Positive (++)</th>
<th>Positive (+)</th>
<th>Negative (-)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain UHT milk</td>
<td>23 (57.5%)</td>
<td>10 (25%)</td>
<td>7 (17.5%)</td>
<td>40 (100%)</td>
</tr>
<tr>
<td>Flavored UHT</td>
<td>12 (100%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>12 (100%)</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>10 (100%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>45 (72.6%)</td>
<td>10 (16.1%)</td>
<td>7 (11.3%)</td>
<td>62 (100%)</td>
</tr>
</tbody>
</table>
Table (4): Aflatoxin M$_1$ detection in flavored UHT milk samples

<table>
<thead>
<tr>
<th>Type of milk</th>
<th>Positive (++)</th>
<th>Positive (+)</th>
<th>Negative sample</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cacao UHT milk</td>
<td>6 (100%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>Vanilla UHT milk</td>
<td>2 (100%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>Strawberry UHT milk</td>
<td>4 (100%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>4 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>12 (100%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>12 (100%)</td>
</tr>
</tbody>
</table>
4.1 Discussion

The presence of AFM1 was detected in a concentration that ranged between 20 - 150 part per billion (ppt) as shown in table 1. The fluid milk samples were found to be contaminated with aflatoxin M₁ in 55(88.7%) of the processed milk compared to the raw milk samples (23;92%). The results obtained in this study were lower than the result obtained by Ali et al., (2014) in Khartoum State, Sudan that showed the average concentration for AFM₁ was between 0.1 and 2.52 μg/kg. Also the concentration reported during this study was lower than that obtained by Elzupir and Elhussein (2010) in Khartoum State. Almost in this study 85.06% of contaminated milk samples exceeded the European communities/Codex Alimentarius recommended limits (0.05 μg/kg and 1.5 μg/kg respectively). Many countries have established a limit for aflatoxin M₁ (AFM₁) such as European countries (05 μg/kg) (CEC, 2001). In a previous study it was also found that an average concentration for AFM₁ was 2.04 μg/kg in Abeokuta and Odeda in Nigeria (Atanda et al., 2007). Similarly in India, the contamination of AFM₁ ranged between 28 and 164 μg/l with 99% of the contaminated samples exceeded the European Communities recommended limit (Shipra et al., 2004). In some countries the level concentration of AFM₁ obtained from milk samples exceed the limit adopted by the Europe countries (05 μg/kg = 0.05 ppb = 50 ppt). Similarly Elzupir and Elhussein (2010) found that all contaminated samples (95.45%) had a level of AFM₁ above EU standard with contamination levels ranging between 0.22 and 6.90 μg/kg and an average concentration of 2.07 μg/kg. AFM₁ was detected in 92% of raw cow milk samples examined and the contaminated levels were higher than the EU limit. Zheng et al., (2013) found that 78.1% of the raw milk samples (360) collected from China was contaminated at concentrations of 5–123 ng L⁻¹, which was far below the Chinese and Codex limits and that only 10% of the raw milk samples exceeded the EU limit. Iqbal et al. (2013) found 42% of milk samples from urban and 27%
from rural farmhouses from Punjab, Pakistan, were above the EU limit. However, only 15% and 8% of the milk samples from urban and rural farmhouses, respectively, exceeded the Codex limit. Iqbal et al. (2011) found only 16.3% of raw buffalo’s and cow’s milk samples collected in Pakistan exceeding the EU limit and the data revealed significant differences (P<0.05) between mean AFM1 concentrations in milk during morning (0.043 μg/kg) and evening (0.028 μg/kg) lactation. Also Rohani et al. (2011) found that in Iran, 50% of the milk samples had higher levels than the EU limit and all concentrations were above the maximum Iranian level of 0.05 μg l−1 in liquid milk. Moreover, Kamkare et al. (2013) reported 18 (28%) and 32 (52%) of cow and buffalo raw milk samples were above the 50 ng/l limit, respectively. Abbes et al. (2012) found that 84.4% of the feed samples (mean 18.7 ± 1.4 mg/kg) were contaminated with AFB1 at levels higher than the Tunisian and the EU limit for dairy animals, which are 20 and 5 mg/kg, respectively.

The results of this study showed that AFM1 concentration in 23 of raw milk samples (92%) ranged between 0.05 and 1.5 μg/kg, which exceeding the EU limit. This result is higher than that obtained in Iran (Kamkar, 2005; Gholamreza et al., 2007). Their results for AFM1 concentration ranged between 0.02–0.09 μg/kg and 0.00–0.89 μg/kg with 79.92% and 5.4%, respectively, exceeding the EU limit. Moreover Rohani et al. (2011) reported that AFM1 in cow milk samples in Iran measured with HPLC to range from <0.01 to 0.41 μg/L. The contamination detected in this study could probably be linked with the type of feed used for these dairy cows as explained previously by Elzupir and Elhussein (2010); Elteib et al., (2012); Ali et al. (2014). The Commission of the European Communities established a limit for aflatoxin B1 (AFB1) of 15 μg/kg for supplementary feedstuffs for lactating dairy cattle in order to reduce the contamination of AFM1 in milk and milk products (EC, 2006).
The fluid milk samples (both pasteurized and UHT) showed occurrence of aflatoxin M₁ in 38 (100%), 14 (100%) and 3 (30%) in the samples collected from factory A, B and C, respectively. (Table 2). Similarly, Santini et al. (2013) found AFM₁ contamination in 42% of the investigated dairy products (ultra-high temperature treated milk, milk cream and cheese), of which 83% contained less than 5.0 ng L⁻¹ and 17% contained 10.0–20.0 ng L⁻¹ AFM₁. They concluded that these findings justify continuous monitoring for public health reasons in order to reduce consumer exposure.

Aflatoxin M₁ was detected in 12 (100%), 10 (100%) 33 (82%) in flavored, pasturized and UHT milk samples, respectively. (Table 3) The study also showed the presence of aflatoxin in 12 samples according to the flavored UHT milk. All samples were contaminated with aflatoxin (cacao, strawberry and vanilla). Overall, the AFM₁ ranged from concentrations of 20 to 150 ppt. These results are comparable with the results recently reported in a Turkish survey in 129 UHT milk samples in Central Anatolia, which showed an average AFM₁ level of 108.2 ng L⁻¹ was reported and the incidence of positive samples of 58% were found (Unusan, 2006). Similarly in an Iranian survey, UHT milk samples from popular markets in central Iran were positive in 62.3% of the analyzed samples, with an average value for AFM₁ of 46.4 ng L⁻¹ (Fallah, 2010). This might be because most of UHT milk prepared from powder milk in some factories. The processed milk samples were contaminated by AFM₁ and 55 (88.7%) of the samples exceeded the EU standard (table 1). In Sudan, most of dairy products (yoghurt, pasteurized milk and UHT milk were processed using imported milk powder. This supported JECFA reports which stated high AFM₁ level in milk powder could be due to complete removal of water, which will concentrate milk solids and contaminants such as AFM₁ (JECFA, 2001). On the other hand, pasteurised milksamples from five commercial trademarks produced indifferent areas in Thailand showed that only one trademark brand revealed
significant differences of AFM1 levels between seasons. It was suggested that farm management factors, rather than environmental factors, were likely to be the main cause of AFM1 contamination in dairy products (Suriyasathaporn and Nakprasert, 2012).

4.2 Conclusion
According to the results obtained in this study, high occurrences and levels of contamination of AFM$_1$ in fluid milk samples were found using Aflasensor, which is a highly specific test for the detection of aflatoxin. The higher occurrence of aflatoxin (AFM1) in raw milk samples and processed milk might have a negative impact on public health, especially infants and children who consumed large amounts of fluid milk.

4.3 Recommendations
1. Monitoring programs should be implemented and limits of aflatoxin $M_1$ should be stated for milk and milk products.

2. Milk and dairy products have to be inspected and analyzed continuously for AFM$_1$ contamination.

3. Reduction of the contamination of raw material and supplementary feedstuffs for dairy cattle with aflatoxin $B_1$.

4. Education and awareness should be conducted especially among farmers and livestock producers on the health hazards of aflatoxins.

5. Strict legislations should be implemented for the imported milk products in order to minimize the occurrence of aflatoxins and to ensure the quality of milk and dairy products in country.

4.4 References


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4-5 Appendixes:

Appendix (1): Aflasensor Kit used to detect aflatoxin
Appendix (2) Heatsensor used as incubator refer to heatsensor manual
Appendix (3) negative and positive control standard
Appendix (4) A valid test for Aflasensor