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The American University in Cairo  
School of Sciences and Engineering

# MYCOTOXINS DETECTION IN GRAINS AND NUTS USING LC – MS/MS

By

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**B.Sc. Agricultural Sciences, Biotechnology Major**

A Thesis Submitted in partial fulfillment of the requirements for  
the degree of Master of Science in Chemistry

Fall 2017

Under The Supervision of:

**Dr. Tamer Shoeib**

**Dr. Gihan El Moghazy**

## **Abstract**

Mycotoxins are natural toxins produced by fungus. These toxins are food and feed contaminants inducing diverse toxicological effects on human and animal health that may affect different organs. These adverse effects may be hepatotoxic, nephrotoxic and neurotoxic; these effects may be acute or chronic implying that even traces amount of mycotoxins, may accumulate over time and become problematic. The occurrence of mycotoxins in food and feed are thus an issue of critical importance and is of main concern worldwide.

Among the efforts that are being made to limits mycotoxins occurrence in food are the laws and regulations on their permissible limits. For instance, the permissible limits of Aflatoxins in peanuts are 4 ng/g for total aflatoxins and 2 ng/g for aflatoxin B1. These limits and regulations are based on human risk assessment studies, data about the occurrence of mycotoxins in various commodities, and the availability of sensitive and accurate analytical methods for their determination. In this work we developed a rapid, selective, sensitive, accurate and validated method for the simultaneous quantification of aflatoxins using LC-ESI-MS/MS. The developed method was applied on different sample matrices; peanuts (n = 45), lentils (n = 10), beans (n = 3), and wheat (n =2). The first ever reported data on the occurrence and levels of aflatoxins in peanuts in Greater Cairo are presented.

The results presented here show that the investigated peanuts samples from 5 different regions in Greater Cairo contain mean concentrations of total aflatoxin ranging from 16.69 to 101.93 ng/g which are 4 to 25.5 times higher than the permissible limits set by the European Union and FDA.

## Acknowledgement

First of all, all my gratitude to **Allah almighty**, the most merciful ever, who had never let me down, and who is always there for me, and who without him I would have been nothing.

Secondly, working under the supervision of **Dr. Tamer Shoeib** is a bless. Words cannot express my gratitude for him, he has always surprised me with his capabilities of dedication. He has taught a lot beyond the expected. During the thesis work, he was very supportive by every means. On day to day basis, he was following up all the details of the work, and all the aspects that might be affecting the work flow, and he has done everything he could to facilitate the working environment, despite his vast responsibilities. In my opinion he is a role model of how supervisors, mentors, and managers should be. Scientifically, technically, as well as personally, he has taught me a lot. Every time I face a problem during my practical work, he come up with simple and effective punch of solutions, and here when I learned from him to think calmly and widely. Besides, all the theoretical background and thinking skills, that I have acquired from him during the course work of the graduate studies, he had a magical way of linking the real world problems to science, and putting them in an extremely exciting syllabus, that taught us to see aspects from different point of views.

Thirdly, I would like to express my deepest gratitude for **Dr. Gihan El Moghazy** my advisor and manager, for her support, time and effort all over the past years, as well as for providing me with the required facilities and guidance for the practical work, and for helping me in being a stronger person, personally and academically, where she always encouraged me to push my limits upwards. Fourthly, all my appreciation for Dr. Amal Abdulaziz the head of the mycotoxins laboratory, in the Regional Centre for Food and Feed, for her kindness and generosity in helping me a lot during the practical work. Also, I want to thank the mycotoxin's laboratory team for their support.

I want to thank all my colleagues in the Regional Center for Food and Feed; and specially the LC-MS/MS laboratory team, Eng. Mohamed Sayed and Eng. Youmna Hassouna who have supported me in the research work. Also my friends and colleagues Dr. Sara Kamal, and Dr. Mohammed Hassan Abdula'al.

Moreover, I want to thank all the AUC academic staff for the learning environment they are providing and for their full support.

Last but not the least, the biggest gratitude goes to my parents Dr. Manal Ammar and Eng. Ali Abubakr who gave me life, who provided me with the most precious thing in the world; the unconditional love, care and support.

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## List of Abbreviations

ATP	Adenosine Triphosphate
AF	Aflatoxin
ALARA	As Low As Reasonably Achievable
CRM	Certified Reference Material
CXP	Collision cell Exit Potential
CE	Collision Energy
CFU	Colony Forming Unit
DP	Declustering Potential
DMDHST	Demethyldihydrosterigmatocystin
DMST	Demethylsterigmatocystin
DHOMST	Dihydro O- methylsterigmatocystin
DHST	Dihydrosterigmatocystin
EP	Entrance Potential
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
FDA	Food and Drug Administration
GC	Guanine Cytosine (DNA Bases)
HPLC-ESI-MS/MS	High Liquid Chromatography Electron Spray Ionization Mass spectrometry Mass spectrometry
HPLC	High Performance Liquid Chromatography
LOD	Limits Of Detection
LOQ	Limits of Quantification
m/z	Mass to charge
ML	Maximum Limit
M.W.	Molecular Weight
MRM	Multiple Reaction Monitoring
n	Nanogram
NOR	Norsolonic acid
N/A	Not Available
O-MST	O-methylsterigmatocystin
ppb	Part per billion
QuECHERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RH	Relative Humidity
RSD	Relative Standard Deviation
SRM	Selected Reaction Monitoring
SPE	Solid Phase Extraction
sp.	Species
TLC	Thin Layer Chromatography
TA	Thyamine Adenine (DNA Bases)
UHPLC-FLD	Ultra High Performance Liquid Chromatography Fluorescence Detector
UV	Ultraviolet
USDA	United States Department of Agriculture

# 1. Introduction

Mycotoxins are poisonous substances, which are secreted by fungi as secondary metabolites. The word mycotoxin originates from two words; myco comes from "mukes" a greek word that means fungus, and toxins come from "toxicum", latin word that means poison (DeVries, Trucksess and Jackson, 2002). However, not all toxins produced by fungus are called mycotoxins, for example, toxins that have a toxic effect on bacteria; are called antibiotics. Mycotoxins, on the other hand, are mainly compounds produced by fungi that have a toxic effect on vertebrates. Usually, fungus is not a common pathogen in vertebrates. There are, typically, low medical pathogenic fungi that affect vertebrates. However, problems arise when a fungus infects plants, where it produces mycotoxins as secondary metabolites on food and feed commodities, which are subsequently eaten by humans and animals and affect their health negatively. In addition when animals are fed on mycotoxin contaminated plants, these contaminants are stored in their tissues and milk, which are then eaten by humans and consequently, affect their health negatively. Several diseases are caused by mycotoxins, as they have varied toxicological effects. These diseases collectively are called mycotoxicosis (Bennett and Klich, 2003).

The hazardous effects of mycotoxins in general and aflatoxins specifically may be chronic or acute. Thus, exposure to small doses over time would cause many problems. It is very important to have data about the mean concentrations of mycotoxins quantities present in food and feed to be able to set regulations and determine the cause of the problem, in addition this allows for an estimation of the individual's exposure rate to mycotoxins. Also, a validated quick method that has low detection limit is essentially required for tracing small quantities of mycotoxins occurring in food and feed commodities.

## 1.1. Mycotoxicosis

When human and animals are exposed to a mycotoxins, it causes a poisoning effect. The diseases or symptoms caused are called mycotoxicosis. Several factors affect the mycotoxicosis; the type of mycotoxin ingested, age of the person, the ingested dose, the duration of the exposure, the sex and the health state of the person or the animal, besides the interactions between other mycotoxins, drugs, alcohols and diet. However, these are not the only factors; there are a lot of other factors that are being discovered every day.

There are two types of mycotoxicosis; acute mycotoxicosis, which occurs due to the instant exposure of an organism to mycotoxins, and chronic mycotoxicosis, which occurs due to the exposure of organism to small doses of mycotoxin over a long period of time. The exposure to mycotoxins occurs mainly through ingestion of contaminated plants, and sometimes may occur through dermal routes or inhalation (Bennett and Klich, 2003).

## 1.2. Discovery and History of Mycotoxins.

Ergotism, is thought to be the oldest known case of mycotoxicosis, it took place in central and northern Europe during the middle ages. For instance, in 1444 in France, 40,000 were killed due to the consumption of rye flour contaminated with *Claviceps purpurea*. The terrifying symptoms of that disease made people think at that time, that it was divine punishment and called it "Holy fire" (Steyn, 1995).

In 1961, near London, a disastrous disease outbreak among 100,000 turkey poults caused their death. This disease was called Turkey X disease. Later, the disease was linked to ingestion of mold contaminated peanut meals. Specifically, this was linked to a secondary metabolite produced by the mold, that was given the name Aflatoxins (Bennett and Klich, 2003 and Zain, 2011).

## 1.3. Types and Classifications of Mycotoxins

The only common property of all mycotoxins is their low molecular weight, they are considered natural products, since they are produced as a secondary metabolite by the fungus, and all have toxicogenic effect. However, each mycotoxin; displays different toxicogenic effect, has its own chemical properties, is produced by different fungus species, and has different biosynthetic pathway. This makes their classification very challenging. Mycotoxins now are categorized in many different ways according to the aspect being studied. For example, organic chemists classify them according to their chemical structures (e.g. coumarins, lactones,... etc.), mycologist categorize them with regards to the producer fungus (e.g. *Fusarium* toxins, *Aspergillus* toxins,...etc.), biochemists classify them according to their biosynthetic pathway (e.g. amino acid-derived, polyketides,... etc.), while clinicians group them by the organ that they effect (e.g. immunotoxins, hepatotoxins, nephrotoxins,... etc.), cell biologists use the effect of the toxin on the cell in their classification (e.g. mutagen, carcinogen, teratogen,...etc.). Therefore, a single mycotoxin will fall under different categories as described (Bennett and Klich, 2003).

## 1.4. Wide-spread Mycotoxins and Their Hazardous Effect

### 1.4.1. Aflatoxins

Aflatoxins are one of the most hazardous groups of mycotoxins (Goldblatt, 2012 and Yu, 2012). The median lethal dose of aflatoxins in some mammals may be as low as 0.34 mg/kg body weight (Hicks, Shimizu, & Keller, 2002). These toxins are produced by several *Aspergillus sp.* The exposure to aflatoxins may cause chronic or acute mycotoxicosis, depending on the state of the individual. The diseases caused due to exposure to aflatoxins are called aflatoxicosis. Aflatoxins are mutagenic, carcinogenic and toxicogenic; their consumption may lead to death or may lead to cancer (Vega, Young, & Todd, 2016).

Acute aflatoxicosis that leads to death has been reported several times in different countries, especially the developing countries. In 1974, for example, the consumption of aflatoxin contaminated maize lead to the death of 108 individuals in western India (Krishnamachari, Nagarajan, Bhat, and Tilak, 1975 and Yu, 2012). Other

affected individuals were shown to have consumed the toxin in the range of 2-6 mg/day for a period of a month, causing them to have hepatitis (Krishnamachari *et al.*, 1975).

Moreover, in 2004, deaths caused by aflatoxicosis were reported in Kenya, where 125 people died after the consumption of contaminated maize. The aflatoxin in this incident was produced by *Aspergillus parvisclerotigenus* not *Aspergillus flavus* (Lewis *et al.*, 2005 and Yu, 2012).

The deaths caused by acute aflatoxicosis are not less threatening than diseases caused by chronic aflatoxicosis. Exposure to low doses of aflatoxins over a long period of time may cause hepatocancer and thus, it is considered as hepatotoxin, as the most affected organ is the liver. Inside the liver, the cytochrome P450 enzyme metabolizes the aflatoxins to a reactive form of epoxides that bind to the guanine molecule in the DNA, forming Aflatoxin-DNA-adduct. This aflatoxin-DNA-adduct may cause mutation in a tumor suppressing gene in the liver which leads to cancer in the liver (Hamid, Tesfamariam, Zhang, and Zhang 2013).

Aflatoxins are found in many foods and feeds commodities, the highest plants with risk of aflatoxins contamination are corn, peanuts, cotton seed, copra and Brazilian nuts. Plants with moderate risk of contamination with aflatoxins are figs, almonds, pecans, walnuts and spices. Some plants such as soybeans, barley rice, oats, sorghum, millet and pulses are at low risk of aflatoxins contamination (MdQuadri *et al.*, 2017).

#### **1.4.2. Other Mycotoxins**

Fumonisin are one of the hazardous mycotoxins that have a special nature of being the only discovered hydrophilic mycotoxin, fumonisin molecule is illustrated in fig. (1). The discovery of fumonisin raised the probability that there might be other undiscovered mycotoxins because of their hydrophilic nature. Chemically, the fumonisin is an acetate derivative. A proposed mechanism was suggested that it is formed due to the condensation between 2 alanine molecules. Fumonisin B1 is the most abundant mycotoxins produced by the *Fusarium sp.*, moreover, most *Fusarium sp.* are fumonisin producer, specially *Fusarium verticillioides* (Bennett & Klich, 2003).

The toxicosis of fumonisin in animals occurs by its interaction with the sphingolipid metabolism, (Loiseau *et al.*, 2015), where a lot of disease may occur including: equine hydrothorax, leukoencephalomalacia in rabbits and pulmonary edema in pigs; liver toxicity, and carcinogenicity in rats liver. Fumonisin toxicosis is shown to have a positive correlation with esophageal cancer. Fumonisin are also suspected to be a cause of neural tube defects in humans as fumonisin causes it in experimental animals (Marasas, 2004).

Another group of mycotoxins produced by *Aspergillus sp.* and *Penicillium sp.* is Ochratoxin A, fig. (1), which causes toxicity in the kidney, thus is nephrotoxic. Ochratoxin A is thought to be a main risk factor of kidney diseases (nephropathy diseases). In addition, the occurrence of tumors in the urinary track is accompanied by Ochratoxin A. Moreover, ochratoxins A causes cancer as a consequence of its genotoxicity caused by its ability to form DNA adducts (Pfohl-Leschkowicz & Manderville, 2007). Disruption of cellular mechanisms caused by ochratoxin A occurs in several ways, including the suppression of the enzymatic activity involved in phenyl-alanine t-RNA, inhibition of ATP production in mitochondria, and peroxidation

of lipid enhancement (Darwish et al., 2014). In model animals, ochratoxins were found to be teratogenic, immunosuppressant and acute nephrotoxic (Darwish et al., 2014).

Citrinin is a nephrotoxic mycotoxin, which is produced by several species including *Monascus sp.*, *Aspergillus sp.*, and *Penicillium sp.* the chemical structure of citrinin is shown in fig. (1). Besides, its nephrotoxicity, it is embryocidal and fetotoxic (Flajs & Peraica, 2009). It usually occurs with ochratoxin A as they are produced from the same fungal species. There is a synergistic effect between both mycotoxins as in the kidney's murine, they cause the inhibition of the RNA synthesis (Bennett & Klich, 2003).

## 1.5. Factors Affecting Aflatoxins Occurrence.

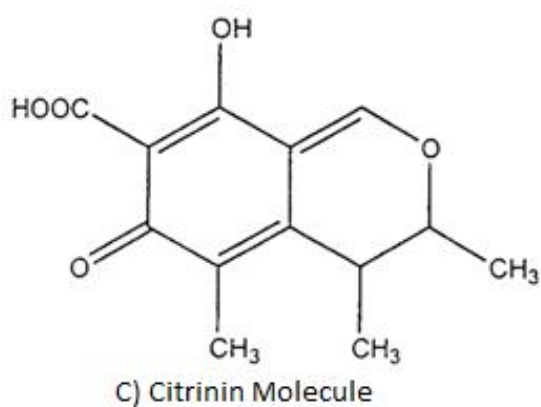
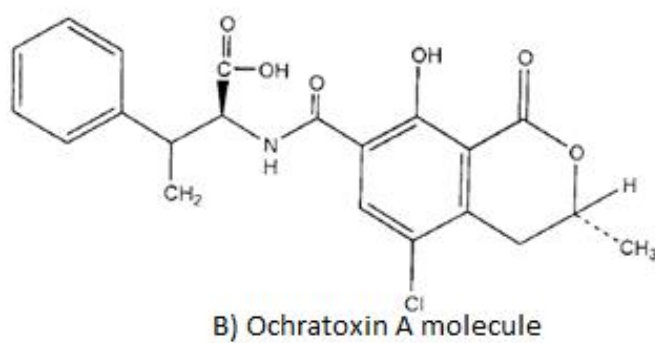
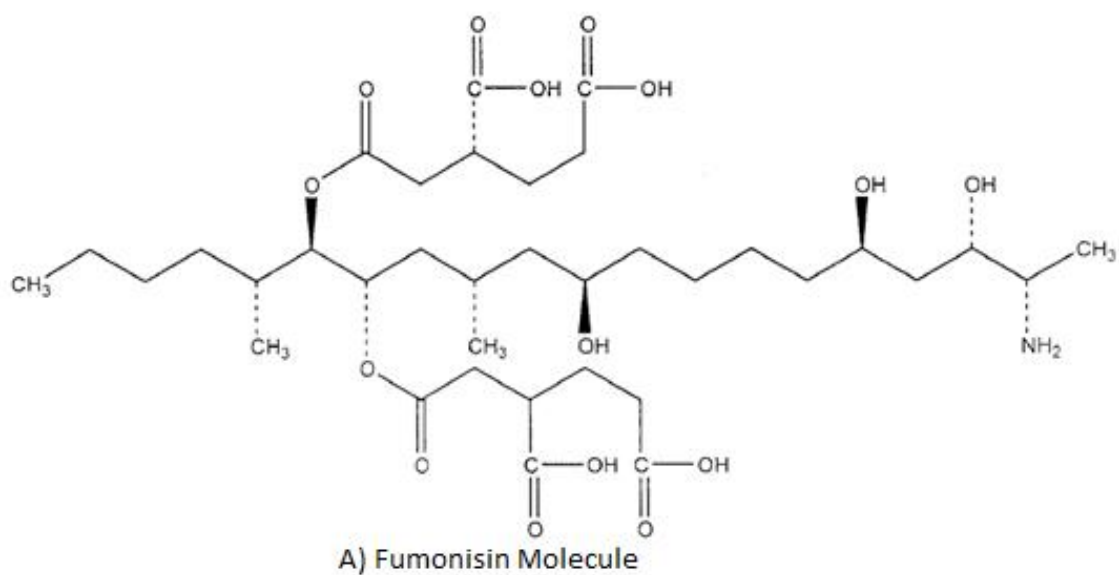
The production of aflatoxin in nature is influenced by several factors, the producing fungus strain, the matrix on which the aflatoxin is produced, the relative humidity and the moisture of the surroundings, time, temperature, time of harvesting, aeration conditions, and the microbiome of the environment. Usually, *Aspergillus flavus* and other storage fungus invade the crops only after harvesting (Goldblatt, 2012). However, sometimes a drought-stress associated invasion occurs in plants before harvest (Kebede, Abbas, Fisher and Bellaloui, 2012), a summarized scheme for the factors affecting aflatoxins production and how these factors can be used to reduce the occurrence of aflatoxins is represented in fig. (3).

### 1.5.1. Variation of Aflatoxins due to Producer Strains

Aflatoxins are produced by *Aspergillus sp.*, however not all species are producers, for instance, *Aspergillus flavus* and *Aspergillus parasiticus* produce aflatoxins, while *Aspergillus oryzae* and *Aspergillus tamaraii* are non-producers species. Not only does the ability to produce aflatoxins differs among species, but also differ among strains. Furthermore, different strains of the same species differ in their ability of aflatoxins production (Goldblatt, 2012).

A study done by Lai et al., showed that 37% of 127 *Aspergillus* strains isolated from rice produced aflatoxins B1 and B2 in the range from 175 to 124,101 µg/kg, the rest of the strains produced aflatoxins B1 and B2 in the range from below detection limit to 10,329 µg/kg (Lai, Zhang, Liu, and Liu, 2015).

In 2016, a study on the occurrence of aflatoxins in walnuts was carried out in Iran. In this study, 40 samples of walnut were analyzed for the presence of aflatoxins using an HPLC method. First, different species of fungus and bacteria were isolated, and the most predominant genus was reported to be *Aspergillus*, and the most predominant species was *Aspergillus flavus*. The highest levels of aflatoxins were found in samples contaminated with *Aspergillus flavus* and *Aspergillus parasiticus*. The aflatoxin G1 was found in the range of 1.7-18.2 ng/g, while aflatoxin B1 was found in the range of 0-8.2 ng/g with only 36% of *Aspergillus flavus* and *Aspergillus parasiticus* producing aflatoxins (Habibipour, Tamandegani, and Farmany, 2016).



**Fig (1): Chemical Structure of A) Fumonisin molecule, B) Ochratoxin A, and C) Citrinin. Adapted from Darwich et al., 2014.**

Researchers in Thailand studied aflatoxins producing species isolated from soil samples. All the investigated *Aspergillus flavus* and *Aspergillus parasiticus* were non-producer strains for aflatoxins B1 and G1. However, *Aspergillus nomius* was reported to be the most abundant aflatoxin producer strain (Ehrlich, Kobbeman, Montalbano, & Cotty, 2007). The variation of aflatoxin production by different strains and species occurs due to biotic and abiotic factors, in addition to, the genetic factors (Yu, 2012).

### **1.5.2. Impact of Substrate on Aflatoxin Production**

Some studies showed that aflatoxins production is enhanced when *Aspergillus flavus* is grown on natural substrates than when it is grown on a synthetic medium. The synthetic media produced relatively very low amount (Goldblatt, 2012). Thus for studying the matrix effect or the substrate effect on aflatoxin production, several natural substrates were sterilized and inoculated with the aflatoxin producing strain and incubated in the optimum temperature. Results showed that in laboratory conditions the aflatoxin production on wheat, corn and rice are more than in peanuts, sorghum and soybeans (Hesseltine, Shotwell, Ellis, and Stubblefield, 1966 and Goldblatt, 2012). Another study done by Mayne, Pons, Franz. and Goldblatt (1966) showed that aflatoxin production on glanded or glandless whole cotton seed and kernels were similar to that produced on wheat, while both produced double the yield of that produced on peanuts (Goldblatt, 2012). It was also shown that aflatoxin yield from fungus grown on wheat is greater than that grown on whole oats (Stubblefield, Shotwell, Hesseltine, Smith, and Hall, 1967 and Goldblatt, 2012).

Different varieties of peanuts showed similar susceptibility for aflatoxin production; however, some other observations contradicted these results, in which a variety of peanuts showed that aflatoxins were always not detected in a variety named U.S. 26 during inoculations studies (Goldblatt, 2012). Moreover, A study was done on two varieties of corn one was thought to be susceptible and the other was thought to be resistant, both varieties were incubated in several relative humidity conditions, levels of aflatoxins were significantly different between both varieties in relative humidity above 91%, while aflatoxins were not detected in both strains in relative humidity less than 80%, the resistant variety to aflatoxins were 98% lower in aflatoxin production than that of susceptible variety among different levels of relative humidity  $\geq 80\%$  (Guo, Russin, Brown, Cleveland, & Widstrom, 1996). Finally, under the same conditions *Aspergillus flavus* were shown to grow on brown rice producing higher populations than on white rice. (Choi *et al.*, 2015)

### **1.5.3. Impact of Relative Humidity and Moisture on Aflatoxin Production**

The relative humidity and moisture of the environment of a substrate plays a crucial role on fungus growth and fungus metabolic activity growing on that substrate. The optimum relative humidity for fungus growth is equal on all substrates. The safe storage humidity condition is 70%, where most fungus will not be able to grow. *Aflatoxin flavus* is considered a mesosphyte, thus the minimum relative humidity required for its growth and germination of spores is 85%, while the minimum relative humidity required for sporulation is 80%. However, the water requirement varies with the variation of temperature and nutrients availability (Goldblatt, 2012).

Usually, the fungus invades the kernels during the postharvest processes. When the drying process occurs rapidly and steadily without interruptions or retardations, safe storage is achieved and the crops are not contaminated with fungus, thus no aflatoxins will be expected to be produced. However, when high moisture content is allowed to exist during the curing and drying processes fungus growth is typically expected (Goldblatt, 2012).

Nakai et al., (2008), studied the occurrence of fungus and aflatoxins in different varieties of stored peanuts and reported a positive correlation between relative humidity, temperature and the occurrence of *Aspergillus flavus*.

Moreover, a study on two corn varieties was done to examine the resistance of aflatoxin contamination in corn using relative humidity. The varieties included two types, one variety that is susceptible to aflatoxins, and the other type is resistant to aflatoxins. Results showed that aflatoxins were not detected in kernels of corn incubated in relative humidity <80%, while kernels that were incubated in relative humidity ranging from 80-84.5% showed low amounts of aflatoxins B1. The amounts of aflatoxins in the susceptible variety ranged from 15-20 ppb, and in the resistant variety ranged from 4-7 ppb. On the other hand, aflatoxins B1 were very high in kernels grown on relative humidity > 91%. The amounts of aflatoxins B1 in susceptible variety ranged from 2216-3374 ppb, and the amounts of aflatoxins B1 in the resistant variety ranged from 45-79 ppb, the resistant mechanism in this variety was attributed to the increased layer of pericarp wax in the resistant variety (Guo *et al.*, 1996), which made it harder for fungus penetration.

Different samples of soybeans were tested in different temperature and different relative humidity showed that the highest yield of aflatoxin was in relative humidity of 90%, also this was the highest condition at which fungus grew. It was reported that at 70% relative humidity, the fungus could not grow and sequentially there was no aflatoxin production (Pratiwi *et al.*, 2015).

#### **1.5.4. Impact of Temperature on Aflatoxin Production**

The *Aspergillus flavus* is considered as a mesophilic organism. It grows in a wide range of temperature conditions, its optimum temperature preference is from 36 to 38 °C, with a minimum temperature required for growth from 6 to 8 °C while the maximum temperature it can tolerate is from 44 to 46 °C. However, the maximum and minimum temperatures required for growth are dependent on other factors like the availability of O<sub>2</sub>, CO<sub>2</sub> and nutrients, and relative humidity conditions (Goldblatt, 2012).

A study was done by Choi et al. (2015) to examine the effect of temperature on growth of *Aspergillus flavus* and aflatoxin production in rice. Results showed that at a temperature of 21°C and relative humidity of 85% the fungus did not grow and did not produce aflatoxin. However, at the same temperature and at higher relative humidity (97%) the fungus were able to grow and to produce aflatoxins.

In 2013, an interesting study was done to examine the growth of *Aspergillus flavus* and the production of aflatoxins on brown rice as a function of water activity and temperature, the study was conducted at temperatures ranging from 12 to 42 °C, and



water activity ranging from 0.82 to 0.92. The highest growth rate of *Aspergillus flavus* and the highest amount of aflatoxins were obtained with water activity ranging from 0.9 to 0.92 and a temperature of 21 °C with an incubation period of 21 days (Mousa, Ghazali, Jinap, Ghazali, & Radu, 2013).

#### **1.5.5. Impact of O<sub>2</sub> and CO<sub>2</sub> Availability on Aflatoxin Production**

Fungi are aerobic organisms, thus they require oxygen for respiration. However, their oxygen requirements and their tolerance to carbon dioxide vary (Goldblatt, 2012). Studying the suitable atmospheric condition for the *Aspergillus flavus* growth and aflatoxin production is important in providing information regarding storage conditions in order to control the fungus growth, and consequently control the aflatoxin production.

Taniwaki, Hocking, Pitt, and Fleet (2010) studied the effect of an atmosphere of 80% CO<sub>2</sub> and 20% O<sub>2</sub> on the growth of different fungus that included *Aspergillus flavus* and their mycotoxin production. Under these conditions, the colony diameters were reduced from 57.8 to 96.9 %, while the hyphal lengths were reduced from 73.7 to 99.6 %. More importantly, under these conditions no aflatoxin were detected.

Taniwaki, et al. (2009) investigated the impact of low oxygen and high carbon dioxide on the growth of some foodborne pathogenic fungus, including the *Aspergillus flavus* species. The experiment was done on three levels; all included < 0.5% O<sub>2</sub>, while CO<sub>2</sub> varied between 20, 40, and 60%. *Aspergillus flavus* was able to grow on < 0.5% O<sub>2</sub> and 20 % CO<sub>2</sub>.

The fact that low percentages of O<sub>2</sub> and high percentage of CO<sub>2</sub> control mold growth and consequently aflatoxins expression, was used to develop a safe storage technique called hermetic storage, which is being used in about 103 countries. The hermetic storage technique depends on isolating the atmosphere of the crop by using highly controlled airtight containers. Inside the hermetic stored containers, insects, crops, and natural microflora will breathe, where O<sub>2</sub> will be consumed and CO<sub>2</sub> will be produced, creating an environment of approximately 3% O<sub>2</sub> and 15% CO<sub>2</sub>, such condition will not allow the development of colony forming units from molds, and consequently aflatoxin production will not rise significantly (Villers, 2014).

Peanuts crops, however, reacted differently in hermetic storage, where it required a longer period, about 30 days, to reach the atmospheric condition of 3% O<sub>2</sub> and 15% CO<sub>2</sub>. Such a long period will allow the fungus to produced aflatoxins. Thus, hermetic storage is modified for peanuts, where CO<sub>2</sub> gas was injected or O<sub>2</sub> absorber sachets were used. The effect of the modified hermetic storage using CO<sub>2</sub> gas was compared to the effect of conventional hermetic storage on mold growth and aflatoxin production after 90 days of storage. The initial CFU mold count of the peanut sample before storage was  $3 \times 10^2$  whereas after 90 days samples stored in hermetic storage had a CFU mold count of  $1.7 \times 10^3 \pm 7 \times 10^2$ . Samples stored in the modified CO<sub>2</sub> hermetic storage on the other hand, had a CFU mold count of 970, while the control sample that was not stored in under hermetic storage conditions had a CFU mold count of  $1.3 \times 10^4 \pm 9 \times 10^3$ . Therefore, for peanuts storage specifically, the hermetic storage condition with CO<sub>2</sub> is better than the conventional hermetic storage condition (Villers, 2014). A summary of the factors impacting aflatoxins is presented in fig. (2).

### 1.6. *Aspergillus flavus*: The Most Abundant Aflatoxin Producer Strain

*Aspergillus flavus* is found naturally and abundantly in soil, where it is considered as "soil-borne mold". It is a durable saprophytic fungus that can survive on different organic nutrients, including, dead insects and animals, plant debris, compost, and grains including monocots and dicots. Sometimes, it invades immune-compromised individuals. Moreover, it is able to tolerate a wide range of temperatures from 12 to 48 °C with an optimum temperature from 28 to 37 °C (Yu, 2012). Due to its ability to survive relatively high temperatures; *Aspergillus flavus* can cause pathogenesis in humans.

Usually, *Aspergillus flavus* grows in the form of mycelium (a hair like fluffy structure formed of growing fungus) or conidia (asexual spores). In harsh conditions, mycelium transfer to sclerotia which is a hardened structure of mycelium is a very resistant structure to drought stress, and deficiency of nutrient. The occurrence of *Aspergillus flavus* is not specific to certain grains; it can infect monocots, dicots as well as crops growing above and beneath the ground. However, it grows abundantly in peanuts, pistachio, walnuts, corn and Brazilian nuts. The *Aspergillus flavus* may infect the crops before or after harvesting, in addition, it may infect crop during bad conditions of storage (Yu, 2012).

## Factors Affecting Aflatoxin Production

Aflatoxin Producer Strain

Substrate

Temperature

Humidity and moisture

CO<sub>2</sub> and O<sub>2</sub> availability

### Optimum and minimum Conditions for Fungal Growth

Some strains of the same sp. are producers while others are not.

Some varieties of the same plant are susceptible for growing fungus than others

### How this factors could solve the problem?

N/A

Bread, select and plant crop varieties that are resistant to aflatoxin production

Lots maybe stored at refrigeration conditions at 4 °C

Humidity should be kept below 80% during storage, probably 70%

Hermetic conditions can be used, where atmospheric conditions are starved for Oxygen

Optimum temperature for fungal growth [36-38 °C]  
Optimum T for aflatoxin production 21 °C  
Minimum T for fungal growth [6-8 °C]

Minimum Relative Humidity for:  
- Fungal germination, 85%,  
- Fungal sporulation, 80%

When ratios of O<sub>2</sub>:CO<sub>2</sub> is 20:80  
Fungi are not able to grow

Figure (2): Illustration of the impact of different factors on Aflatoxin production, and how it can be used to decrease the concentrations of aflatoxins in Peanuts.

## 1.7. Chemistry and Biosynthesis of Aflatoxins

### 1.7.1. Chemistry of Aflatoxins

Aflatoxins are difuranocoumarin derivatives, which are produced through a polyketide pathway. There are four major types of aflatoxins that were named according to their fluorescence activity. Aflatoxins B1 and B2 have blue fluorescence, while aflatoxins G1 and G2 have green fluorescence (Espinosa-Calderón, 2011), this fluorescence activity is due to the aflatoxins chemical structure that contain a conjugated system that enhance the electronic transitions under UV light (Valeur and Berberan-Santos, 2012). In 70:30 Mehtanol : Water the excitation wavelength of aflatoxins is about 365 nm, while the emission wavelength is about 455 nm (Huang and Elmashni 2007). Chemical structure is presented in table (1). There are other forms of aflatoxins that are not found naturally, but are formed as a result of metabolizing aflatoxins inside the body, like aflatoxins M1 and M2, that are found in cow's milk that have ingested contaminated feed (Goldblatt, 2012). Aflatoxin B1 is the most abundant and the most dangerous form (MdQuadri, Niranjan, Chaluvaraju, Shantaram, and Enamul, 2017; Goldblatt, 2012).

Moreover, aflatoxins are toxic small molecules that have low molecular weight. Chemical structure, chemical formulas, and molecular weights are illustrated in table (1). For these small molecules to be toxic, they must undergo epoxidation, which usually occurs in the liver. The groups that are labeled number 2 are the dihydro derivatives of the groups that are labeled number 1. Aflatoxin B2 is dihydroaflatoxin 1, and aflatoxin G2 is dihydroaflatoxin G1, table (1).

### 1.7.2. Biosynthesis of Aflatoxins

The biosynthetic pathways of aflatoxins involve 16 enzymes that start with acetate molecules and terminate with aflatoxin molecules. The aflatoxin biosynthetic pathway is illustrated in figure (3) First, a fatty acid molecule is formed from units of acetate molecules by the fatty acid synthase enzyme. The fatty acid molecule is then converted to an unstable polyketide precursor called noranthrone by the enzyme polyketide synthase. Noranthrone is immediately converted to an anthraquinone derivative called norsolonic acid (NOR) by the catalysis of anthrone oxidase (Hicks *et al.*, 2002; Baranyi, Kocsubé, Vágvölgyi, and Varga, 2013).

NOR produces an orange visible light, that can be seen by the naked eyes, thus some researchers search for mutant fungus that accumulate the orange color as this will increase the probability that this fungus is not a producer strain (Baranyi *et al.*, 2013). Then the NOR molecules is reduced to averantin by the catalysis of two enzyme which are the NOR- reductase and NOR-dehydrogenase (Baranyi *et al.*, 2013). The averantin is then converted to averufin which occurs in two steps. The averantin is first oxidized to hydroxyaverantin, a reaction that is catalyzed by Cytochrome P450 monooxygenase enzyme, followed by the action of the alcohol dehydrogenase enzyme, that catalyze the conversion of hydroxyaverantin to averufin.

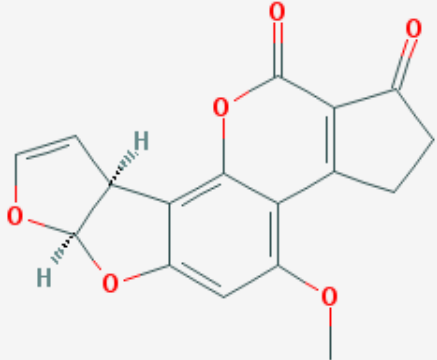
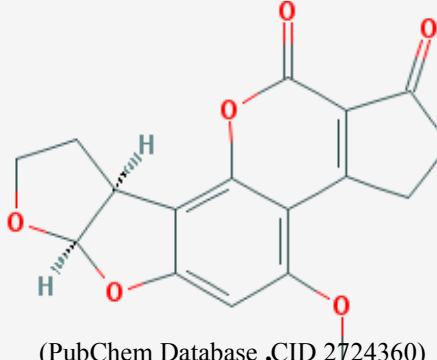
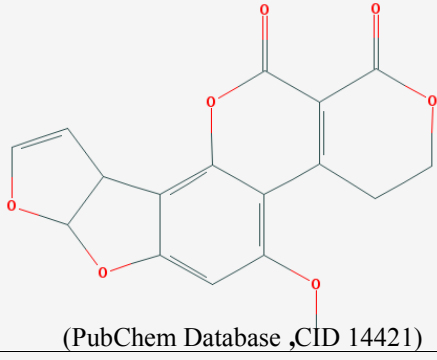
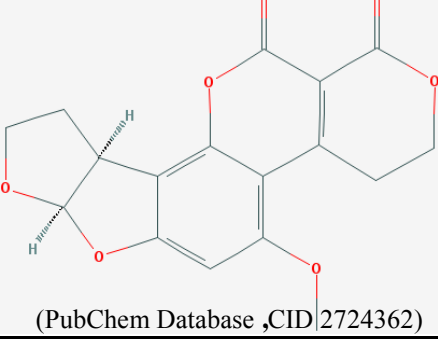
The averufin molecule is oxidized by the averufin monooxygenase enzyme to produce versiconal hemiacetal acetate that undergoes an esterification step under the calytic activity of cytosole esterase enzyme to give a versiconal compound. The versiconal compound is subsequently cyclized by the versicolorin B synthase enzyme to produce versicolorine B molecule (Hicks *et al.*, 2002;. Baranyi *et al.*, 2013).

The versicolorin B forms a branching point in the pathway, in which its final destination may be an aflatoxin B1 or G1 molecule, and produce sterigmatocystin as a byproduct, or it may be produce an aflatoxin B2 or G2 molecule as its final product. These processes occur through converting of versicolorin to demethyldihydrosterigmatocystin (DMDHST) or versicolorin A, both processes occurs under the enzymatic action of Cytochrome P450 monooxygenase/desaturase (Hicks *et al.*, 2002;. Baranyi *et al.*, 2013).

In the First branch, the demethyldihydrosterigmatocystin is converted to dihydrosterigmatocystin (DHST) by the enzyme O-methyl tansferase B, followed by the conversion of (DHST) to dihydro O- methylsterigmatocystin (DHOMST) by O-methyltransferase A. Finally DHOMST is converted to aflatoxin B2 or G2 molecule by monooxygenase enzyme (Hicks *et al.*, 2002;. Baranyi *et al.*, 2013).

In the second branch, the versicolorin A is converted to demethylsterigmatocystin (DMST) by the ketoreductase enzyme or cytochrome P450 monooxygenase. The DMST molecule is then converted to a sterigmatocystin molecule which is then converted to O-methysterigmatocystin (O-MST) by the O-methyltransferase II enzyme, OMST molecule is finally converted to aflatoxin B1 or G1 (Hicks *et al.*, 2002;. Baranyi *et al.*, 2013).

**Table (3): Chemical structure, formula, name and molecular wieght of different types of Aflatoxins.**

Aflatoxin Type	Chemical Formula/name	M.W. g/mol	Chemical Structure
B1	$C_{17}H_{12}O_6$ Aflatoxin B1	312.277	 <p>(PubChem Database, CID 186907)</p>
B2	$C_{17}H_{14}O_6$ Aflatoxin B2 or Dihydroaflatoxin B1	314.293	 <p>(PubChem Database ,CID 2724360)</p>
G1	$C_{17}H_{12}O_7$ Aflatoxin G1	328.276	 <p>(PubChem Database ,CID 14421)</p>
G2	$C_{17}H_{14}O_7$ Dihydroaflatoxin G1	330.292	 <p>(PubChem Database ,CID 2724362)</p>

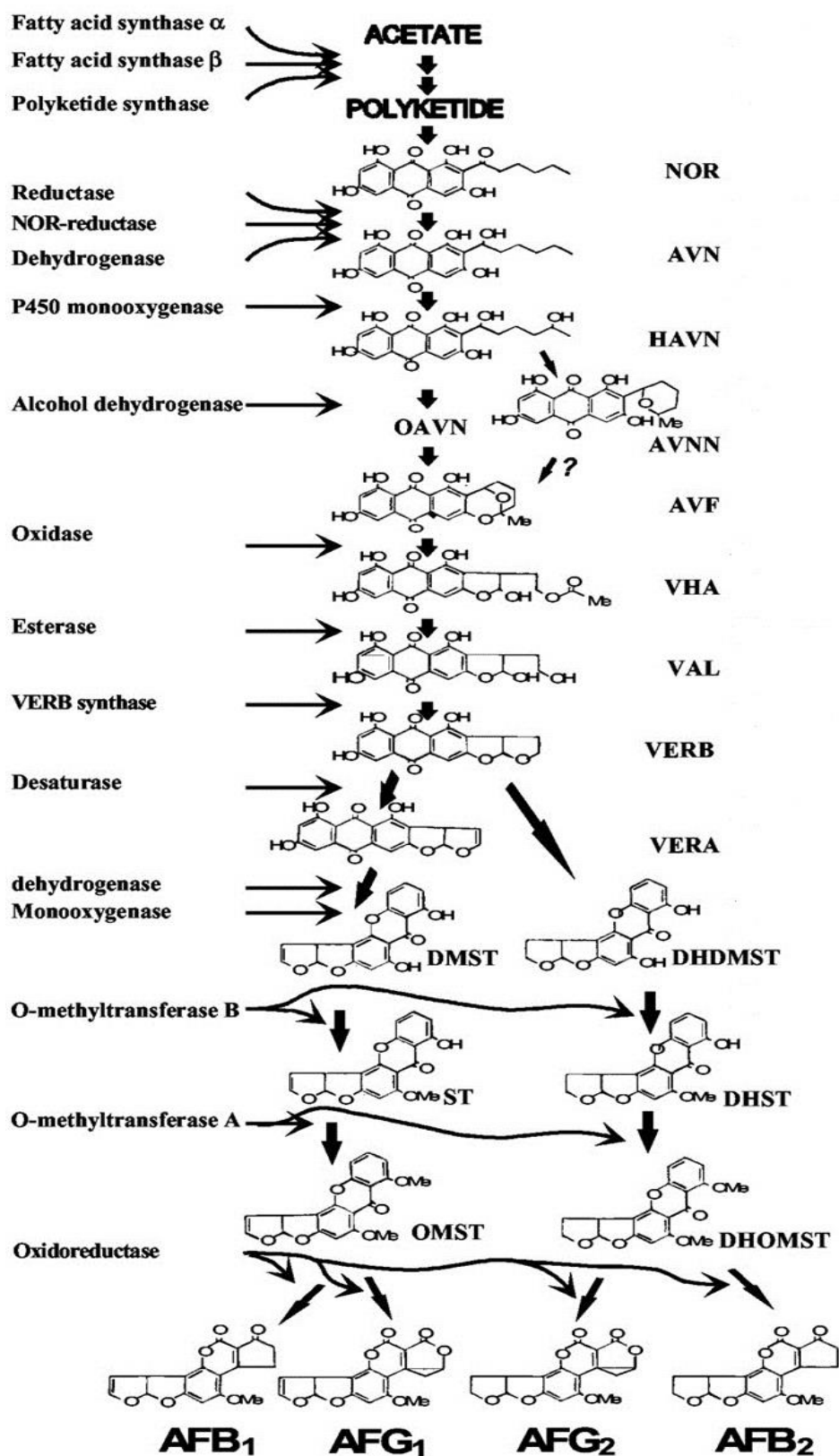


Figure (3): Aflatoxin Biosynthetic Pathway adapted from Yu, 2004

## 1.8. Occurrence of Aflatoxins in Food and Feed Commodities.

There are particular regions in the world that suffer from the contamination of their crops with aflatoxins, and that is because of their climatic conditions that is optimum for the *Aspergillus flavus* growth and aflatoxin production. Mainly these regions are the Southern United States Of America, Africa and Asia (Hicks, *et al.* 2002). Thus, the following review will be mainly focused on these regions that face aflatoxins challenges.

### 1.8.1. Aflatoxins incidences in Peanuts

Aflatoxin occurrence in Zimbabwe was studied in peanuts (n= 18) and peanut butter samples (n = 11) using high performance liquid chromatography (HPLC) with fluorescence detection. Out of the 11 samples of peanuts butter, 10 samples were contaminated with aflatoxins with a mean of 75.66 ng/g, and ranged from 6.1 to 247 ng/g. The aflatoxin B1 was the most abundant. It's occurrence mean was 51 ng/g, and ranged from 3.7-191 ng/g. However, only 3 samples out of the 18 peanut samples were contaminated with a mean of 51.0 ng/g, and a range of 3.7 to 191 ng/g (Mupunga, Lebelo, Mngqawa, Rheeder, and Katerere, 2014).

In Trinidad, in 2007, the occurrence of aflatoxin B1 in 186 peanuts samples was investigated using radioimmunoassay. All the samples were reported not to contain aflatoxin B1 (Offiah & Adesiyun, 2007).

Contamination of peanuts samples with aflatoxins in Brazil were analyzed using thin layer chromatography (TLC). A total of 60 samples were collected over a period of 12 months, from Tupa, State of Sao Paulo and analyzed on a monthly basis. The analysis of aflatoxins in hulls samples showed that 6.7 % of the samples were contaminated with aflatoxin B1 in range of 15 – 23.9 µg/kg, and contaminated with aflatoxin B2 in the range of 3.3 – 5.6 µg/kg. On the other hand, 33.3% of kernels samples were contaminated with aflatoxin B1 in the range of 7.0 – 116 µg/kg, while 28.3% of kernels samples were contaminated with aflatoxin B2 in the range of 3.3 – 45.5 µg/kg (Nakai *et al.*, 2008).

In State of Rio Grande do Sul, Brazil, 101 samples of peanuts and peanut products were collected from the market, to investigate the presence of aflatoxins. The analysis of aflatoxins was done using TLC. The results showed that 14% of the samples were contaminated with aflatoxin B1. In the peanut samples aflatoxins ranged from 24 to 87.5 µg/kg, while peanut products had a range of 22.0 to 84.6 µg/kg. The researchers stated that these findings showed that the aflatoxins concentrations exceed the Brazilian regulatory limits, which is 20 µg/kg total aflatoxins (Hoeltz, Einloft, Oldoni, Dottori, and Noll, 2012).

In Kenya, a very large sample of 1263 peanut and peanut products was investigated for the occurrence of aflatoxins. Samples were collected from market vendors from three provinces; Nairobi, Western (Busia District) and Nyanza. Analysis of the aflatoxins was done by indirect competitive immunosorbent assay (ELISA). The occurrence of aflatoxins was also examined in different packaging. The results of this large study showed that 37% of the samples contained mycotoxins in levels higher than



the regulatory levels set by the Kenyan bureau of standards, which is 10 µg/kg. In each province, different types of processed peanuts were analyzed. The effect of several factors on aflatoxins production were investigated in this work, including two important factors that may be common factors in different markets, which are the types of the peanut and the type of the packing material (Mutegi *et al.*, 2013).

In Nairobi, shelled raw peanuts, roasted peanuts, peanut butter, fried peanuts, spoilt peanuts, and others were studied with a total number of samples of 358. The average percentage of all samples types containing aflatoxin of  $\leq 4$  µg/kg was 20.3%,  $< 4 - 10$  µg/kg was 6.6%, and  $>10$  was 73.2 %. The types that were analyzed in Nyanza were podded raw peanuts, shelled raw peanuts, roasted peanuts, peanut butter, boiled peanuts, fried peanuts, spoilt peanuts and others with a total number of samples of 595. The average percent of the samples containing  $\leq 4$  µg/kg was 64.4%, while those containing  $< 4 - 10$  µg/kg was 2.2%, and samples containing  $>10$  was 31.4 %. From province of Western, a total of 203 of different types of peanuts and peanuts products samples were collected for analysis of aflatoxins, the mean percentage of samples containing  $\leq 4$  µg/kg was 52.0%, while those containing  $< 4 - 10$  µg/kg was 0%, and samples containing  $>10$  µg/kg was 48%. The detection limit of the method used in this study was 2 µg/kg (Mutegi *et al.*, 2013).

The packing materials studied in this work were jute bags, propylene bags, metal tins, PVC bags, paper, plastic jars, plastic basins, reeded baskets, and others. The jute bag seemed to be the best packaging material as the samples packed in it, showed the least concentrations of aflatoxins, where 100% of the samples had concentrations  $\leq 4$  µg/kg, and the maximum level reached inside such packing was 0.003 µg/g. The packing materials that showed lower aflatoxins contamination according to percentage of samples containing less than 4 µg/kg of aflatoxins are as follows in descending order: reeded basket 73.1%, plastic basin 69.5%, propylene bags 60.9%, PVC bags 55.7%, metal tins 55.6%, paper, 43.9% and plastic jars 29.7% (Mutegi *et al.*, 2013).

A survey was done in Taiwan on the occurrence of aflatoxins in peanuts and peanuts products (n = 1827), in which samples were collected from 1997 till 2011, and analyzed by HPLC equipped with a fluorescence detector. The study showed that 32.7% of all samples were contaminated with aflatoxins, the concentration of aflatoxins ranged from 0.2 µg/kg to 513.4 µg/kg. However, only 6.8% of the samples showed higher concentrations of aflatoxins than the allowed regulatory limits of Taiwan, which is 15 µg/kg. The most occurring type of aflatoxin reported is B1, followed by B2, G1 and G2 (Chen, Liao, Lin, Chiueh, & Shih, 2013).

### **1.8.2. Occurrence of Aflatoxins in other food and feed commodities**

In Sudan, 13 samples of lentils were analyzed for aflatoxins and pathogenic fungus. Aflatoxins were analyzed using thin layer chromatography. Only one sample was contaminated with aflatoxins with a concentration of 14.3 µg/kg (El-Nagerabi and Elshafie, 2001).

A survey of the contamination of rice with aflatoxins was conducted on 200 samples in the Canadian market. The rice samples rice originated from different countries including; Canada, Thailand, Pakistan, India, and the US. The analysis was done using two liquid chromatography methods. The limit of detection of both methods

was 0.002 ng/g. The samples analyzed in two groups over a period of two years. The first group showed 56% of the samples to be positive, while the second group this number was reduced to 43%. The mean concentrations of aflatoxins in both groups were 0.19 and 0.17 ng/g respectively. In the first group the 5 most contaminated samples contained aflatoxin B1 in the range of 1.44-7.14 ng/g, while those of the second group contained aflatoxin B1 in the range of 1.45-3.48 ng/g. The sources of the contaminated samples were mainly Pakistan, India and Thailand (Bansal *et al.*, 2011).

A survey of moulds and mycotoxins was performed on 99 rice samples taken from the Swedish retail market. The samples were analyzed for their content of aflatoxin B1, B2, G1, and G2 using HPLC and RIDAQUICK (a quick immunochromatographic qualitative kit). From the analyzed samples, 71% of the Basmati rice and 20% of jasmine rice contained concentrations of aflatoxin B1 above the limits of detection which was 0.1 µg/kg. However, 2 samples of jasmine rice and 10 samples of Basmati rice contained higher levels of aflatoxins than the European regulatory levels which are 2 µg/kg for aflatoxin B1 and 4 µg/kg for total aflatoxins (Fredlund, Thim, Gidlund Brostedt, Nyberg, Olsen, 2009).

A simultaneous detection of different types of aflatoxins in sorghum and pistachio were done in Tunisia. Pistachio samples, showed 55.5% of positive samples for aflatoxins, while sorghum samples showed 62%. The mean contamination levels was  $21.8 \pm 38.0$  ng/g for pistachio and  $9.9 \pm 11.5$  ng/g for sorghum (Ghali, Belouaer, Hdiri, Ghorbel, Maaroufi, and Hedilli, 2009).

## 1.9. Legislation and Regulations of Aflatoxins.

### 1.9.1. European Union Legislations

According to the EU commission in 1994, the scientific community of food expressed aflatoxins as genotoxic and carcinogen. Consequently, the total aflatoxin content of food should be limited, specifically, the aflatoxin B1 because it is the most toxic aflatoxin. To minimize the effect of limitations on trading, it is suitable to increase the allowed limits in such products that are not intended for direct human use. Besides, sorting and other physical treatments maybe used to reduce concentrations of aflatoxins in groundnuts and nuts. Thus, the EU regulatory limitations of aflatoxins vary according to the commodities intended use. Thus, it is mandatory to label the intended use of the packages, whether it is for human or animal use, and whether it is intended to be used as an end product or as part of a commercial recipe (OJ L 364, 20.12.2006, p.5). Limitations of aflatoxin concentration in different food commodities are shown in table (2).

**Table (2): The Limitations of Aflatoxins in Food and Feed Commodities, Set By EU Commission**

<b>Commodity</b>	<b>Aflatoxin B1 µg/kg</b>	<b>Total Aflatoxin (B1,B2, G1,G2) µg/kg</b>
<b>Peanuts And Other Oilseeds (To be subjected to sorting or physical treatment)</b>	8	15
<b>Peanuts, other oil seeds, peanuts products (Ready to use)</b>	2	4
<b>Hazelnuts and Brazil nuts (To be subjected to sorting or physical treatment)</b>	8	15
<b>Hazelnuts and Brazil nuts (Ready to use)</b>	5	10
<b>Almonds, Pistachios and Apricot kernels (To be subjected to sorting or physical treatment)</b>	12	15
<b>Almonds, Pistachios and Apricot kernels</b>	8	10
<b>All Cereals and Cereals product (with some exceptions)</b>	2	4
<b>Maize and Rice (to be subjected to sorting or other physical treatment)</b>	5	10

### **1.9.2. Regulations of FDA for aflatoxins in peanuts**

The FDA regulations are usually concerned with products ready for consumptions. Therefore raw peanuts regulation is the responsibility of the USDA, in which it will report to the FDA any samples that showed a concentration of more than 25 ppb. The FDA allows the shipments of peanuts containing a concentration of more than 25 ppb if the facility of the processor contains suitable equipment for sorting moldy and damaged peanuts prior to their consumption. However, peanuts that are ready to be consumed should not exceed 20 ppb of total aflatoxins. (FDA).

### 1.9.3. Proposed Maximum limits for ready-to-eat peanuts by the CODEX ALIMANTARIUS Commission

In 2017 in the 40<sup>th</sup> session in Geneva, Switzerland, two values (10 and 15 ppb) were proposed to be the maximum limits (ML) for ready-to-eat peanuts. There was a conflict between India (Chair of the EWG) and the delegations. India did not want to hold the ML of peanuts at 10 µg/kg, and it was concerned that this would lead to higher rejection rates, which would increase violations that are already at 9.7%. However, the delegations opposed the proposed ML as it was not following the GSCTFF criteria of (ALARA) in determining the MLs, where ALARA stands for "As Low As Reasonably Achievable", besides the idea that peanut MLs will not be consistent with other nuts. The delegation later, supported the point of view of the proposal because the violation rate was already more than the cut-off level of violation that should be less or equal to 5%, where the violation rate should be taken into consideration, since ALARA should be applied while taking into consideration to provide health protection with the minimum negative impact on trade. (PROGRAMME CODEX ALIMENTARIUS, Geneva, Switzerland 17 - 22 July 2017). Table (3) shows the permissible limits set by CODEX committee.

**Table (3): Maximum permissible limits of aflatoxins in different countries of some commodities according to CODEX ALIMANTARIUS (CODEX COMMITTEE, Brazil, 3-7 April 2017).**

Food Commodity	Country	Aflatoxin B1 µg/kg	Total Aflatoxins µg/kg
Nuts	Canada		15
All Food	Columbia		10
	Hong Kong		15
	India		30
	South Africa	5	10
	Jamaica		20
	Japan		10
	Morocco	10	
	Thailand		20
	Nigeria	20	
All food except for milk	United States		20
All Adult food	Singapore	5	5
Food for infants and young people	Singapore	0.1	NA

## 1.10. Quantitation Methods of Mycotoxins.

### 1.10.1. Thin Layer Chromatography

Thin layer chromatography (TLC) is one of the most cost effective, simple, and easy methods that are used for quantitation, characterization and detection of mycotoxins. This method employs a thin layer that is coated on a solid object for the adsorption of chemical compound through a solvent. Different chemical compounds run through the thin layer with different rates, thus compounds are separated from each other (Cheng, Huang, and Shiea, 2011). A linear relationship was reported to exist between the concentrations of aflatoxins per spot and the peak area of its fluorescence measure as observed by a densitometer. Thus, it was concluded that the peak area could be a measure of concentration when compared to a standard, this allowed for a precision of mycotoxin measurement to within 2-4% (Pons, Robertson, and Goldblatt 1966).

The advantages of TLC include it being a high sample through-put procedure, relatively non expensive and relatively easy to perform. A major disadvantage of the TLC method is the required laborious purification and cleanup of the samples. Conventional methods of TLC require a fluorescence detector for detection of separated compound, thus a pure analyte is required to avoid the interference of other compounds. Several studies were conducted for the detection and quantification of mycotoxins using TLC (Turner, Subrahmanyam, and Piletsky, 2009).

Var, Kabak, and Gök (2005), used the TLC method to quantify aflatoxin B1 in 102 helva samples. The detection limit of this method was reported as 1 µg/kg. Samples were cleaned and purified and run on TLC plate and then dried and exposed to UV light at 365 nm, the intensity and the retention factors of the samples were compared to the standard for quantification.

Sokolović and Šimpraga (2006), Conducted an experiment to test the occurrence of trichothecenes in grains and animal feed in Croatia. They used TLC as a semi quantitative method for the mycotoxin. The TLC method was used to analyze several types of trichothecenes including T-2, and deoxynivalenol and diacetoxyscirpenol. It was reported that TLC results are comparable to those of ELISA and HPLC among different concentrations in measuring deoxynivalenol and other *Fusarium* sp. Toxins. The limits of detection (LOD) of T-2 and DAS toxin were 0.1 mg/kg, while the LOD of DON was 0.01 mg/kg.

During the period from 1968 till 1994, several validation methods for mycotoxin quantitation using TLC were reported for the analysis of different matrices, including wheat, peanut and cocoa. These methods showed RSD values to be relatively high ranging from 12-64%. The applicability ranges for these methods were also relatively high where about 75% of these methods stated applicability ranges of 5 ng/g or above. All these factors make conventional TLC methods typically not suitable for the era requirements of high accuracy, precision and sensitivity (Gilbert and Anklaam, 2002).

### 1.10.2. HPLC Methods

High performance liquid chromatography is the most popular and most commonly used method in mycotoxins quantification. (Gilbert and Anklaam 2002) HPLC methods for measuring mycotoxins require aflatoxin extraction from the samples and the cleanup of analytes. The cleanup step is usually done by immune-affinity

columns, this is followed by injecting the samples on the HPLC where analytes are separated through a solid phase and detected typically by fluorescence detector.

Golge, Hepsag, and Kabak (2016), studied the incidence of aflatoxins in the confectionaries sold in turkey. They stated that the method showed high precision, linearity, recovery and selectivity. The limit of quantification ranged from 0.106 to 0.374 µg/ kg. Campos, et al. (2017) validated a method for simultaneous quantification of all four aflatoxins (B1, B2, G1, G2) in Brazilian nuts. Rahmani, Jinap, & Soleimany (2010) validated an HPLC-FLD method for quantitation of aflatoxins, ochratoxin and zearalenone in cereals. The limits of quantitation (LOQ) were as follows: aflatoxin B1 and G1 0.05 ng/g, aflatoxin B2 and G2 0.015 ng/g. The limits of detection (LOD) of aflatoxin B1 and G1 were 0.0125 ng/g, for aflatoxin B2 and G2 were 0.0037. The recovery rates of all mycotoxins ranged from 77-104% for different concentrations.

Brera et al. (2011) adopted a new idea for the validation of HPLC methods, where they used a simultaneous quantitation method for different matrices, they employed reversed phase HPLC for aflatoxins and ochratoxin in baby foods and paprika. Each investigated level were analyzed 10 times, the RSDr values ranged between 2-10% for aflatoxins B1. The LOD and LOQ for aflatoxin B1 in baby milk were 0.002 and 0.02 µg/kg while the recovery rate ranged from 86-96%. This makes the method valid for its analytical purpose according to EU regulations.

Tan and Wong (2011) optimized a HPLC method for aflatoxins quantitative analysis of aflatoxins in noodles using QuECHERS method (Quick, Easy, Cheap, Effective, Rugged, and Safe) for extraction of aflatoxins from samples instead of using solid phase extraction method to reduce time and cost of the procedure. The recovery rates 75% - 107%, the relative standard deviations were <13%, while the repeatability ranged from 2.0 – 12.3 % and the reproducibility ranged from 3.4 – 16.5%. The LOD ranged from 0.01 to 1.00 µg/kg, while the LOQ ranged from 0.05 – 1.80 µg/. These parameters were suitable for quantifying the legal limits set by the European Union.

Ibez-Vea et al. (2011) developed an UHPLC-FLD method for the simultaneous detection of aflatoxins B1, B2, G1 and G2, Ochratoxin and Zearalenone in barley samples. The extraction step of mycotoxins from the samples was done using acetonitrile:water. Immunoaffinity columns were used as a cleanup step. This method showed aflatoxin LOD ranging from 0.5 to 15 ng/kg, with the recovery ranging from 78.2% to 109.2%.

Zhu, Liu, Chen, and Cheng (2013), studied the incidence of aflatoxins B1, B2, G1 and G2 in rice samples. For the extraction procedure, methanol-water 60:40 (v/v) was employed. Immunoaffinity columns were used for the cleanup of the analytes. Extract were then diluted with tween 20 phosphate buffer saline in order to improve recoveries. The diluted extracts were subsequently injected onto HPLC with fluorescence detection. The recovery rates ranged from 75.2 to 94.7% and the mean of repeatability for total aflatoxin was 1.6%.

### 1.10.3. LC- MS-MS

Huang, et al., (2014), developed a simultaneous detection method for aflatoxin M1, zearalenone and  $\alpha$ -zearalenol, and ochratoxin A in milk samples. They employed UHPLC-ESI-MS/MS where Oasis HLB cartridges were used for sample purification.

The LOQ of mycotoxins ranged from 0.003 to 0.015 µg/kg with correlation coefficients  $\geq 0.996$  and recovery rates between 87 and 109 %. The repeatability ranged from 3.4 – 9.9% and reproducibility ranged from 4.0 - 9.9 %. In a similar study, Beltran *et al.*, (2011), reported on a method for the detection of aflatoxin (B1, B2, G1, and G2) and ochratoxin A in baby food and milk products using UHPLC-MS/MS. Extraction was done using acetonitrile : water (80:20 v/v). For high sensitivity a pre-concentration step using immune affinity columns was performed and the selectivity was enhanced by using the SRM mode. The method was tested on four matrices; raw milk, cereal milk, baby milk powder, and cereal infant formula. Recovery experiments were conducted on two levels, 0.025 and 0.1µg/kg. The recovery rates were satisfactory ranging from 80 - 110% in all food matrices with RSD values of less than 15%.

Liu *et al.* (2013), developed and validated an LC-ESI-MS/MS quantitation method for detection of the four types of aflatoxins: B1, B2, G1 and G2 in lotus seeds. Samples were extracted using methanol : water (80:20 v/v) followed by cleanup step using immunoaffinity columns. The limits of detection (LOD) of aflatoxin B1, B2, G1 and G2 were 0.007, 0.005, 0.003 and 0.005 µg/kg, respectively, the Limits of Quantitation (LOQ) of aflatoxin B1, B2, G1 and G2 were 0.02, 0.015, 0.01 and 0.015 µg/kg.

Liao, Lin, Chiueh, and Yang-Chih Shih (2011), reported an LC-ESI-MS/MS method for the analysis of aflatoxins types (B and G), Ochratoxin and Zearalenone in cereals samples. The extraction solvent used were methanol:water 80:20 (v/v), followed by immunoaffinity column clean up and the quantification was done using selected reaction monitoring (SRM). Average recovery rates of aflatoxins ranged from 5-95%. The LOQ of aflatoxin G1, G2, B1, B2 were 0.1, 0.3, 0.1, 0.2 ppb respectively.

Annunziata *et al.* (2017), developed a quantification method for aflatoxins (B1, B2, G1, G2), fumonisins (B1 and B2), T2 and HT2 toxins in products derived from cereals using tandem mass liquid chromatography. For validation spiked samples at 4 levels were employed with recovery rates ranging from 83.6 to 102.9%. The reported values for standard deviation of repeatability and reproducibility were 14.3 and 15.7 % respectively.

Spanjer, Rensen, and Scholten (2008), adopted a new approach aimed at determining all mycotoxins, in different matrices in one single extraction step. They developed a method that is able to simultaneously detect 33 mycotoxins in various products in a single experimental run. The extraction solvent used was acetonitrile/water, and the dilution was done with water. This was followed by reverse phase HPLC separation and detection by MS/MS. The LOQ of aflatoxin and ochratoxin were 1.0 µg/kg, and that of deoxynivalenol was 50 µg/ kg. This method has been used in several food matrices including freeze dried silage, baby food, maize, figs and nuts, including peanuts and pistachio.

Zheng, Xu, Wang, Zhan, and Chen (2014) described a cost-effective, rapid and simple LC-MS/MS method for the simultaneous analysis of several mycotoxins including, ochratoxin A, aflatoxins (B1, B2, G1 and G2) and Sterigmatocystin in 25 Chinese medicines that mainly have a herbal nature. They used a single extraction step using acetonitrile : water (84:16 v/v), the samples were extracted, diluted and injected directly. The SRM mode was used for quantification. The linearity was more than 0.995, and the sensitivity were determined by the LOQ that ranged from 1.6 – 25 ng/L.

The recovery rates ranged from 84.8 to 110.6 %. The precision was identified from the relative standard deviation that was less than or equal to 9.9% and the matrix effect was reported to range from 80.2 to 118.6%.

Škrbić, Koprivica, and Godula (2013), improved a previously developed simultaneous mycotoxin analysis method to a multi-matrix procedure. The method included the two types of spices matrices included in the EU regulations, the first type was red pepper and the second was black pepper. Samples were spiked with Aflatoxins (B1, B2, G1 and G2) and ochratoxin with the maximum allowed content for spices or less, according to the EU commission regulations No. 165/2010 and No. 105/2010. The obtained data shows the analysis of the crude extraction methods of spices to be feasible and sensitive at the same time. However, the validation parameters for the analysis of some mycotoxins did not match EU regulation.

#### 1.10.4. Other methods for Quantification of Mycotoxins

The fluorimetry assay employs the purification and cleanup of aflatoxins from the samples using immunochromatographic columns, followed by detection through the fluorimetry device, that detect the presence of the fluorescence of the analyte. Derivatization technique is sometimes used in mycotoxin detection for enhancement of the fluorescence signal, using a chemical label that will form a fluorescent derivative. The fluorescence emitted from the tested analyte is compared to standards of different concentrations for quantitation. The aflatoxins are first extracted from the sample typically by a methanol:water (70:30 (v/v)) solvent. The samples are then passed through an immune-chromatographic column that binds the aflatoxins, followed by several washing steps with water, and finally the aflatoxin bound to the immune-chromatographic column is eluted in methanol. The sample is then typically concentrated before analysis (Trucksess et al., 1990).

Methods that depend on antibody- antigen reactions are also used in the quantification of mycotoxins. These methods employ enzyme linked immunosorbant assays (ELISA). These methods have the advantages of not requiring laborious sample preparation and clean up steps, as well as they are rapid and sensitive. (Gilbert and Anklam, 2002 and Pei, Zhang, Eremin, Lee, 2009).

Pei, Zhang et al. (2009), fabricated an ELISA plate for quantifying aflatoxin M1 in milk using a monoclonal antibody. The recovery rates of their spiked samples were 98% and their reported limits of detection were 0.04 ng/ml.

Zheng, Humphrey, King and Richard (2005), validated AgraQuant® ELISA plates for the quantitation of mycotoxins. The method were validated for several matrices including corn and corn products, sorghum, peanuts, cotton seed, wheat, milled rice, popcorn and soybeans. In this study, no complicated sample cleanup was performed. The detection method was based on competitive ELISA, where the aflatoxins compete with a horse reddish peroxidase conjugate. The limits of detection for this method were 3.5 ppb, while the range of quantification was 4 - 40 ppb. In comparison to HPLC where the range of quantification is 0 – 320 ppb this method was shown to be precise, rugged, accurate, and sensitive.



The aim of this research project is to develop a reliable analytical method for simultaneous detection of aflatoxins in different food commodities using HPLC- ESI-MS/MS. Besides, employing a new, quick, relatively inexpensive and non-laborious extraction method for aflatoxins that require no clean up step.

Since regulations and legislations of mycotoxins are affected by several factors including the availability of toxicological data, the method will be applied on 45 samples of peanuts to report the occurrence of aflatoxins in the peanuts available in the market in Greater Cairo. Moreover, the variability of the occurrence of aflatoxins in peanuts samples at different stages will be investigated including: shelled raw, shelled roasted, unshelled raw, unshelled roasted. Also, the method will be tested on lentils samples and beans samples, which are two of the most consumed grains in the region.

A peanut is composed of three parts: the grain which is the inner edible part, the skin which is the red thin layer covering the grain, and the shell which is the brown hard crust that usually contains two grains.

## 2. Experimental Details

### 2.1 Instrumentation Principles and Background:

In this work, High Performance Liquid chromatography (HPLC) equipment 1200 series Agilent Technologies, coupled with mass spectrometry mass spectrometry (MS/MS) equipment AB Sciex 4000 Qtrap, with electrospray ionization (ESI) source were the analytical instrumentations used herein. To achieve our analytical goals in simultaneous quantification of aflatoxins in nuts and grains samples, the HPLC was employed for physical separation of analytes. On the other hand the MS/MS was used for its high capabilities of analyte analysis based on the analyte's mass/charge ( $m/z$ ).

The MS/MS instrument is mainly composed of four main parts, the ion source, MS1 (Q1), collision cell (Q2) and MS2 (Q3). The ion source, ionize the entering molecules to allow the influence of them through the instrument under electric currents. MS1 (Q1) determine the parent molecule, while the collision cell (Q2) is responsible for the fragmentation of the parent ion to the daughter ions under the influence of specific parameters, then come the role of MS2 (Q3) were the fragments are analyzed and then detected (**Gross & SpringerLink (Online service), 2011**).

### 2.2 Instrumentation and Materials

#### 2.2.1. Instruments

- a. Tandem Mass Spectrometry Applied Biosystems AB Sciex 4000 Q Trap.
- b. HPLC 1200 series Agilent Technologies
- c. HPLC Column C18 Eclipse XDB 5 $\mu$ m, 4.6 X 150 mm, Agilent technologies

#### 2.2.2. Tools, Equipment and Consumables

- a. Sensitive Balance (KERN) 4 decimals.
- b. Falcon Tubes (50 ml)
- c. Volumetric flask (100 ml)
- d. Measuring Cylinders ( 10 ml, 50 ml, 100 ml, 250 ml, 1000 ml)
- e. Automatic Micropipettes (10  $\mu$ l, 20  $\mu$ l, , 100  $\mu$ l, , 200  $\mu$ l,, 1000  $\mu$ l,)
- f. HPLC vials Agilent Technologies (1.5 ml)
- g. Synringe Filter 0.22  $\mu$ m
- h. Eppendorf tubes (1.5 ml) Blender 500w
- i. Whatsmen Filter Paper (12.5 cm) Double rings Funnels
- j. Solid Phase Extraction Column Aflatest (Vicam)

#### 2.2.3 Chemicals

- a. Milli-Q- Deionized Water (resistivity: 18.2 M $\Omega$ .cm)
- b. Methanol LC MS MS Grade Thermofisher
- c. Formic Acid 99-100% Chem. Lab.
- d. Ammonium Acetate Merck
- e. Sodium Chloride Salt MP, Biomedicals
- f. Aflatoxin Standard Mixture CRM dissolved in methanol from Sigma Aldrich, with concentrations shown in table (4).

**Table (4): The concentration of each mycotoxin in the Standard Vial**

Type	B1	B2	G1	G2
Concentration (µg/ml)	990	300	940	280

#### **2.2.4. Solutions and Reagents.**

- a. The Extraction Solvent: Methanol: Water (80:20)
  - Preparation:
    - Add 400 ml of water (2.3.1) and 1600 ml of methanol (2.3.2) into a volumetric flask, and mix thoroughly
- b. HPLC Isocratic mobile phase: Methanol: Water (70:30) + 5mM Ammonium acetate, 0.1% Formic acid.

The mobile phase gradient and components were chosen based on trial and error experiments with some guidance from literature that recommended using methanol: water as the mobile phase with ammonium acetate as buffering agent (Lattanzio, Solfrizzo, Powers, and Visconti. 2007)

- Preparation of 10 mM Stock solutions:
    - Add 600 ml of water (2.3.1) and 1400 ml of methanol (2.3.2) into a volumetric flask, and mix thoroughly (Mixture 1).
    - Put 950 ml of mixture 1 in a 1 L one-marked volumetric flask.
    - Weight 0.77 g of Ammonium acetate (2.3.4), add it and Mix thoroughly
    - Add 2000 µl of formic acid (2.3.3)
    - Add more of Mixture 1 until the mark of 1 L to form Mixture 2 (10 mM Ammonium acetate, Methanol : Water 70:30, 0.2% Formic acid) (Mixture 2).
  - Preparation of Working Buffer:
    - Dilute Mixture 2 with Mixture 1 with ratio of 1:1 to Obtain the working buffer of 5 mM ammonium acetate, methanol:water, 70:30, 0.1% formic acid.
- c. Standard solution for the Standard Curve:
    - Preparation of Standard Stock Solution:
      - Put each 50 µl of standard solution (2.3.6) on 950 µl methanol:water (Mixture 1), and store at -20 °c, Until use.
    - Preparation of Working Standards for Standard Curve:
      - Dilute the standard solution with Mixture 1 (methanol : water 80:20) in a serial dilution form starting with the stock solution. Details are shown in the table (5)

**Table (5): Preparation method of the points of the Calibration Curve**

Standard volume to be added (µl)	MeOH : Water 80:20 to be added (µl)	Name of Std	B1 conc. ng/ml	B2 conc. ng/ml	G1 conc. ng/ml	G2 conc. ng/ml
<b>500</b> (Stock vial)	500	S1	49.5	15	47	14
<b>500</b> <b>S1</b>	500	S2	24.75	7.5	23.5	7
<b>500</b> <b>S2</b>	500	S3	12.37	3.75	11.75	3.5
<b>500</b> <b>S3</b>	500	S4	6.18	1.875	5.87	1.75
<b>500</b> <b>S4</b>	500	S5	3.09	0.9375	2.93	0.87
<b>500</b> <b>S5</b>	500	S6	1.54	0.468	1.46	0.43
<b>0</b>	500	S0	0	0	0	0

**2.2.5. Samples:**

a. Certified reference material of wheat samples, obtained from the Regional Center for Food and Feed, the Agriculture Research Center.

b. Peanuts Samples:

Samples were collected from several regions of Greater Cairo. Considering that the Nile River is the center of Cairo, sample were collected from the following points: East Cairo away from the center by 15 km in Kirdasah point, North Cairo away from the center by 8 km in Shubra Point, and West Cairo away from the Center by 15 km in Heliopolis and Nasr City point, and finally from North Cairo away from the center by 6 km in Giza point. The areas that were covered in this work are shown in figure (4).

Sample codes and locations are shown in table (6). All samples were loosely packed samples in paper pack. Moreover, the storage conditions were not under controlled conditions, thus data about the storage conditions are not available.

Moreover, different types of peanuts samples were collected; shelled raw, shelled roasted, unshelled roasted and unshelled raw. The shells of all the shelled peanuts were removed before testing. As the edible part are the grains and sometimes the skin when the whole peanuts are salted.

To investigate the contribution of the skin in the total aflatoxins concentrations, samples no. 31,35,36,37, and 38 were peeled and their red skin were removed, and each of the skin and the grains were analyzed separately.

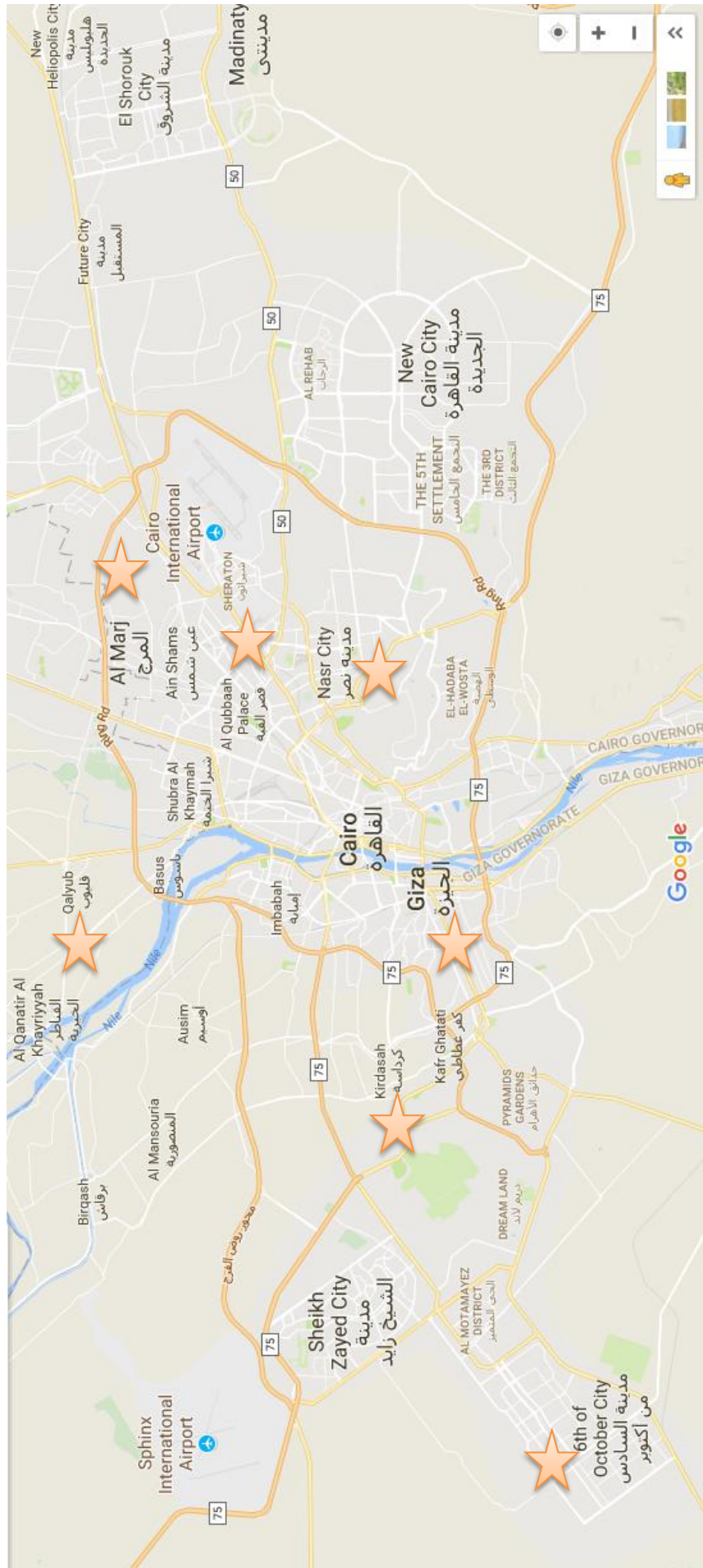


Figure (4): Orange Stars On Cairo Map, Shows the Covered areas and Region During Sampling

Peanuts samples showed variations in their morphological appearance. Where some samples were consistent, and their skin had pure red color, while other peanuts were wrinkled and their skin showed dark color figure (5). In the case of shelled peanuts, some samples had pure beige color while others had a black color covering the shell figure (6), finally, some shelled samples contained insects inside the shell. To investigate the relation between the morphological appearance of peanuts and it's contaminations with aflatoxins, three samples that were not included in the survey were sorted according to their morphological appearance as shown in fig. (5,6), where only healthy peanuts were picked and analyzed using the developed method.



Fig. (5): Shows the difference between healthy peanuts on the right side, and unhealthy peanuts on the left side.



Fig. (6): illustrates the difference in appearance between healthy and unhealthy shelled peanuts, the healthy peanuts on the right and the unhealthy is on the left.

**Table (6): The Peanuts samples code, type, and source.**

Code	Sample Type	Location
1.	Unshelled, Raw	Haroun street, Heliopolis
2.	Unshelled, Roasted	Haroun street, Heliopolis
3.	Unshelled, Roasted	Haroun street, Heliopolis
4.	Unshelled, Raw	Haroun street, Heliopolis
5.	Unshelled, Roasted	Dokki, Giza
6.	Unshelled, Roasted	Dokki, Giza
7.	Unshelled, Salted	Dokki, Giza
8.	Unshelled, Raw	Dokki, Giza
9.	Unshelled, Roasted	Qalyubia, Greater Cairo
10.	Unshelled, Roasted	Qalyubia, Greater Cairo

11.	Unshelled,Roasted	Qalyubia, Greater Cairo
12.	Unshelled,Roasted	Qalyubia, Greater Cairo
13.	Unshelled,Raw	Haroun Street, Heliopolis
14.	Unshelled,Roasted	Haroun Street, Heliopolis
15.	Unshelled,Roasted	Haroun Street, Heliopolis
16.	Unshelled,Roasted	Berket el Khayam
17.	Unshelled,Raw	Berket el Khayam
18.	Unshelled,Raw	El Meatamdia
19.	Unshelled,Roasted	El Meatamdia
20.	Unshelled,Raw	EL Haram street, Giza
21.	Unshelled,Roasted	EL Haram street, Giza
22.	Unshelled,Roasted	EL Haram street, Giza
23.	Unshelled,Roasted	El Talbia
24.	Unshelled,Roasted	El Talbia
25.	Unshelled,Raw	Ainshams
26.	Unshelled,Roasted	Ainshams
27.	Unshelled,Roasted	Ainshams
28.	Unshelled,Raw	El Maady
29.	Unshelled,Roasted	El Maady
30.	Shelled, Raw	Berket el Khayam
31.	Unshelled,Roasted	Shubra
32.	Shelled, Raw	El Meatamdia
33.	Shelled, Raw	Shubra
34.	Unshelled,Raw	Qalyubia
35.	Unshelled,Roasted	Marg
36.	Unshelled,Roasted	6 October
37.	Unshelled,Roasted	6 October
38.	Shelled, Roasted	Giza, Square
39.	Shelled, Roasted	Shubra
40.	Shelled, Roasted	Shubra
41.	Shelled, Raw	Shubra
42.	Shelled, Roasted	El Marg
43.	Unshelled, Roasted	Masr wel Sudan Street, Hadaayek el Kubba
44.	Shelled, Roasted	Masr wel Sudan Street, Hadaayek el Kubba
45.	Shelled, Raw	District 10, Nasr City
46.	Shelled, Roasted	District 10, Nasr City
47.	Shelled, Raw	District 7, Nasr City
48.	Shelled, Roasted	District 7, Nasr City

### c. Lentils Samples:

Ten samples of lentils were collected from the market, including brown and yellow lentil, as well as packed and unpacked samples. Locations and packing status are illustrated in table (6).

**Table (7): The lentil samples' code, type and source.**

Code	Type	Location
1.	Brown (local)	Haroun Street, Heliopolis
2.	Brown (Imported)	Haroun Street, Heliopolis
3.	Yellow	Haroun Street, Heliopolis

4.	Yellow	Shubra
5.	Brown	Shubra
6.	Yellow	Giza Unpacked
7.	Brown	Giza Unpacked
8.	Brown	Giza Packed
9.	Yellow	Giza Packed
10.	Yellow	Giza Packed

#### d. Beans Samples:

Three grounded beans samples were purchased from the markets and tested for the occurrence of aflatoxins in them, using the developed HPLC MS/MS method. The location and status of the packing is illustrated in table (7). All the samples were home grown in Egypt.

**Table (8): The beans, code, and source**

Serial No.	Description	Location
1.	No Skin, Ground, Unpacked	Haroun Street, Heliopolis
2.	No skin, Whole Bean, Unpacked	Haroun Street, Heliopolis
3.	No Skin Ground, Packed	Company A

### 2.3. Methods

#### 2.3.1. Building acquisition LC MS MS method for Aflatoxins (B1, B2, G1, G2) using Analyst® 1.6 software:

For quantitation analysis, MS MS is coupled with HPLC, where all the MS MS parameters optimized above are introduced to the instrument for each analyte, where it is applied first on known concentrations of the standard, to provide a standard curve, Followed by Injection of the samples.

- a. HPLC conditions are permanent for the 4 aflatoxins, conditions are as follows:
  - The solid phase used is immobilized in C18 Column, section (2.1.3)
  - The mobile phase was isocratic methanol : water, 70:30, 5mM ammonium acetate, and 0.1% formic acid, Section (2.4.2)
  - Run time is 8.0 minutes
  - Flow rate 500 µl/min
  - Injection volume 20 µl
  - Since it's isocratic mobile phase, thus there is no gradient, it is 100% isocratic solvent
- b. MS/MS conditions are as follows:
  - Ion Source parameters are shown in table (8).
  - The Scan mode: MRM
  - The Resolution: Unit
  - The Scan Rate: 300 ms
  - MS/MS parameters required are shown below in table (9).



Collision Gas used is Nitrogen Gas The gas used to collide with the Ion to fragment, however these are the factors that affect the fragmentation.

- 1- EP is the Entrance potential of the Ion, it focuses the ion path.
- 2- DP is the Declustering Potential, controls the potential in the orifice of the ion source, which controls the declustering of the ion.
- 3- CE is the Collision Energy, it controls the potential difference between Q0 and Q2, which controls the speed of the ion and thus the collision force with the gas at the collision cell.
- 4- CXP which is the Collision Cell Exit Potential which controls the potential difference between collision cell and Q2.

**Table (9): The Electro Spray Ion Source Parameters**

Parameter Name	Parameter Value or Type
<b>Polarity</b>	Positive
<b>Temperature</b>	500 °C
<b>Curtain Gas</b>	10 psi
<b>Gas 1</b>	35 psi
<b>Gas 2</b>	35 psi

**Table (10): MS/MS Conditions for the 4 Aflatoxins, and the Precursor Ions, and their Daughter Ions.**

Aflatoxin (AF)	Precursor Ion	Q1 m/z	Type of Daughter ion	Q3 m/z	EP	DP	CE	CXP
AF B1	[ M+H] <sup>+</sup>	313.2	Quantifier	285.0	10	90	37	46
			Qualifier	241.1	10	90	43	34
AF B2	[ M+H] <sup>+</sup>	315.2	Quantifier	287.0	10	121	37	54
			Qualifier	259.0	10	121	47	38
AF G1	[ M+H] <sup>+</sup>	329.3	Quantifier	243.0	10	116	43	34
			Qualifier	200	10	101	69	30
AF G2	[ M+H] <sup>+</sup>	331.2	Quantifier	313.2	10	80	39	50
			Qualifier	245.2	10	80	49	30

### 2.3.2. Quantitation Wizard Building the Calibration Curve for the 4 aflatoxins

6 points of known concentration was injected in the HPLC MS/MS, prepared as shown in table (5).

### 2.3.3. Building Quantitation:

Build a quantitation wizard, and use the standard curve points to plot the standard Curve.

### 2.3.4. Studying the Matrix Effect:

To study the effect of matrix on the signal and the reading of aflatoxins, dilute 5 standard points with a negative peanuts extract for aflatoxins and run at the same time with 5 standard points diluted with methanol: water 80:20.

Study the difference between the two regressions, the difference is the matrix effect as done by Lattanzio, et al. (2007)

### **2.3.5. Sample Preparation and Aflatoxin Extraction:**

Samples were purchased and analyzed directly, to ensure the investigation of the instant state, and to avoid the increase or the decrease of the aflatoxin content due to laboratory conditions.

Prior to starting the analysis, a peanut sample was physically sorted. The sample was subsequently spiked with the known concentrations of aflatoxins and extracted by solid phase extraction method and by the crude extraction method included in this work. Both extraction methods utilized a solvent to sample ratio of 4:1. In the solid phase extraction, 10 ml of extract was concentrated using the SPE column.

#### **1- Solid Phase Extraction Method:**

- a. Weight 10 g of samples
- b. Add 1 g of Sodium Chloride Salt
- c. Add 40 ml of Methanol : Water (80:20)
- d. Blend for 2 minutes
- e. Dilute the Extract with deionized water 4 times
- f. Filter Using Whatsman Paper
- g. Filter the Extract with Syringe Filter
- h. Run 20 ml from the Diluted Filtered Extract through the Column, with the rate of 1 to 2 drops per second
- i. Wash the Column with 20 ml Deionized Water
- j. Elute the Column with 1 ml of 100% Methanol.

#### **2- Crude extraction method:**

Two certified reference materials (CRM) of known concentrations of Aflatoxins were used to test the reliability of the extraction method.

No cleanup step required, similar to the method used by Elbert et al., (2008) and Zheng, et al., (2014).

- a. Weight 10 g of the samples
- b. Add 1 g of sodium chloride Salt, section (2.3.5)
- c. Put 40 ml of the extraction buffer, methanol : water 80:20, Section (2.4.1)
- d. Blend for 2 minutes in a blender, section (2.2.9)
- e. Filter with Whatsman filter paper, section (2.2.10)
- f. Dilute with milli-Q water with sample : water ratio of 5:1.
- g. Filter the extract through 0.22 µm filter, and transfer to HPLC vial.

### **2.4. Waste Management:**

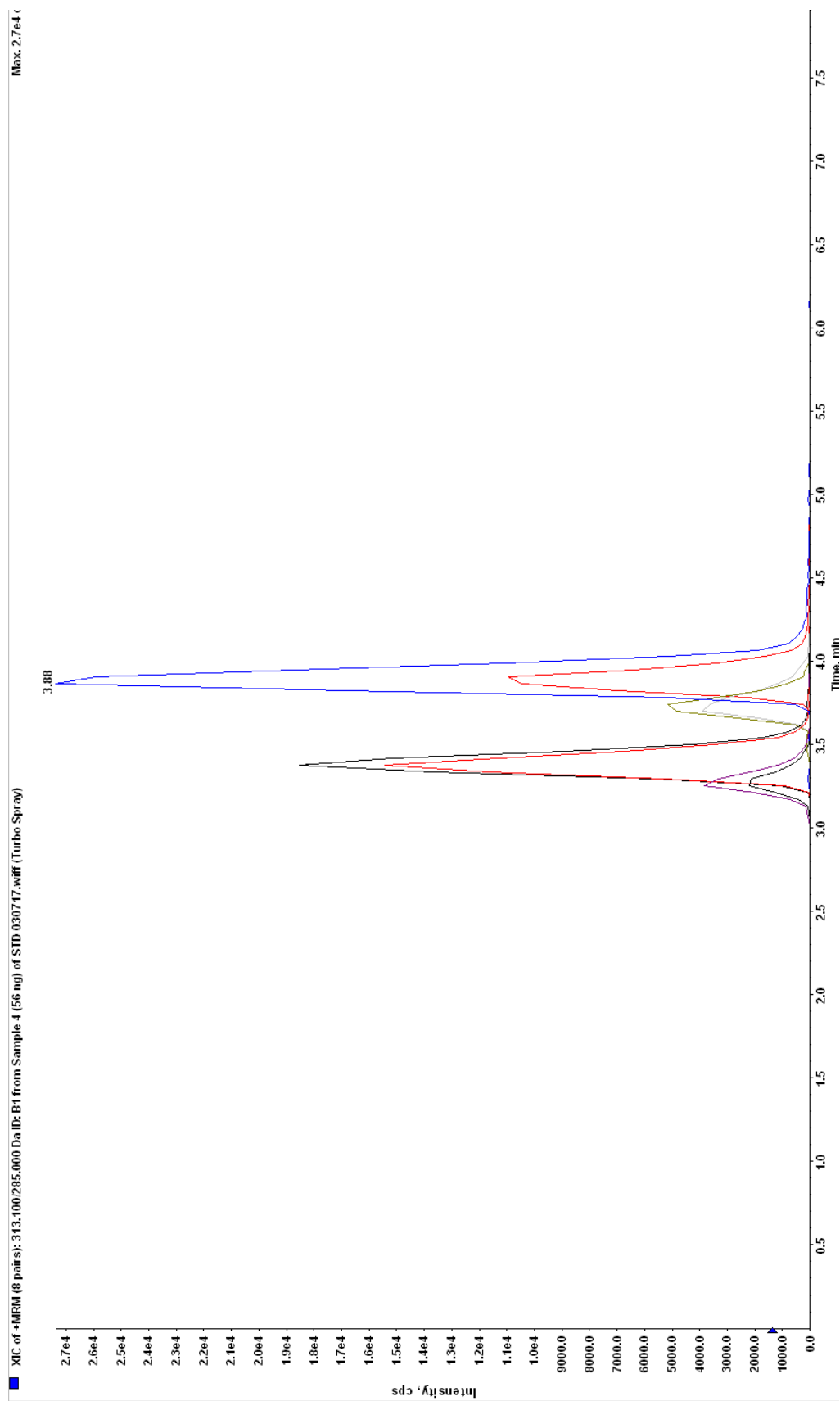
Wastes of the extraction steps and HPLC are disposed in a biohazard bag, and a special company is responsible for its treatment.

### 3. Results and Discussion

#### 3.1 Coupling the HPLC with the MS/MS Using the Optimized Method.

The use of the HPLC with the optimized MS/MS Conditions separated the 4 types of aflatoxins at different retention times, providing the distinct shape of the parent ion with high intensity and a smaller intensity peaks for the daughter ions, figure (7). The retention time of aflatoxin B1, B2, G1, and G2 were 3.88, 3.72, 3.38 and 3.27 min. respectively.

In this work, reversed phase HPLC was employed for the separation of aflatoxins using C18 Column which is packed with hydrophobic stationary phase. Thus, the more the polarities of the molecule the faster they elute from the stationary phase *i.e.* molecules with lower retention time are more polar than molecules with higher retention time (Scientific, 2009). From figure (7), it can be concluded that the polarity of aflatoxin G(1,2) is higher than the polarity of aflatoxin B(1,2) more over the aflatoxin B2 is higher than aflatoxin B1 and aflatoxin G2 is higher than aflatoxin G1, and that may be due to the more di-hydrogen atoms found in aflatoxin B2 and aflatoxin G2.



**Figure (7): The Four Analytes Peaks Separated on HPLC, RT 3.27 is the AF G2 peak, RT 3.38 is the AF G1 peak, RT 3.72 is the AF B2 peak and RT 3.88 is the AF B1 peak.**

By Injecting different standard concentrations (0 , 1.5, 3.09, 6.187, 12.3, 24.75, and 49.5 ng/ml) for AF B1, (0, 0.4687, 0.9375, 1.875, 3.75, 7.5, and 15 ng/ml) for AF B2, (0, 1.468, 2.9375, 5.875, 11.75, 23.5, and 47 ng/ml) for AF G1, and (0, 0.4375, 0.875, 1.75, 3.5, 7, and 14 ng/ml) for AF G2, 4 standard curves for each analyte was obtained. Each standard concentration was injected in triplicate.

The linearity was satisfactory with a correlation value of more than 0.999. This high linearity shows the accuracy and the sensitivity of the method and the instrument, where the intensity is clearly dependent on the concentration and no other factors are disrupting the correlation between the intensity and the concentration. The accuracy of AF B1, AF B2, AF G1, and AF G2 calibration curves were 94.6 to 101.06 %, 94.4 to 105%, 96.7 to 100.4%, and 87.2 to 101.6% respectively. Linearity and calibration curves are shown in fig (8-11). The obtained standard curves showed excellent linearity values of more than 0.999. Moreover, accuracy ranged from 95% to 113%, while the standard deviation between replicates did not exceed 2.1%.

The limits of detection (LOD) and the limits of quantitation (LOQ) were calculated according to the signal to noise ratio, where the LOD was estimated as the concentration of standards at which the signal to noise ratio is 3:1, while the LOQ is estimated as the concentration at which the signal to noise ratio is 10:1, this method was used as it a better method as compared to calculating the LOD and LOQ based on calibration curve (Şengül, 2016). The limits of detection (LOD) for AF B1, AF B2, AF G1 and AF G2 were 0.1, 0.1, 0.045, 0.096 ng/ml respectively, and the limits of quantitation (LOQ) for B1, B2. G1 and G2 were 0.3867, 0.2, 0.36 and 0.2 ng/ml respectively, which is a suitable parameter that is capable of measuring the EU legal limits. The RSD was  $\leq 3.1$  %.

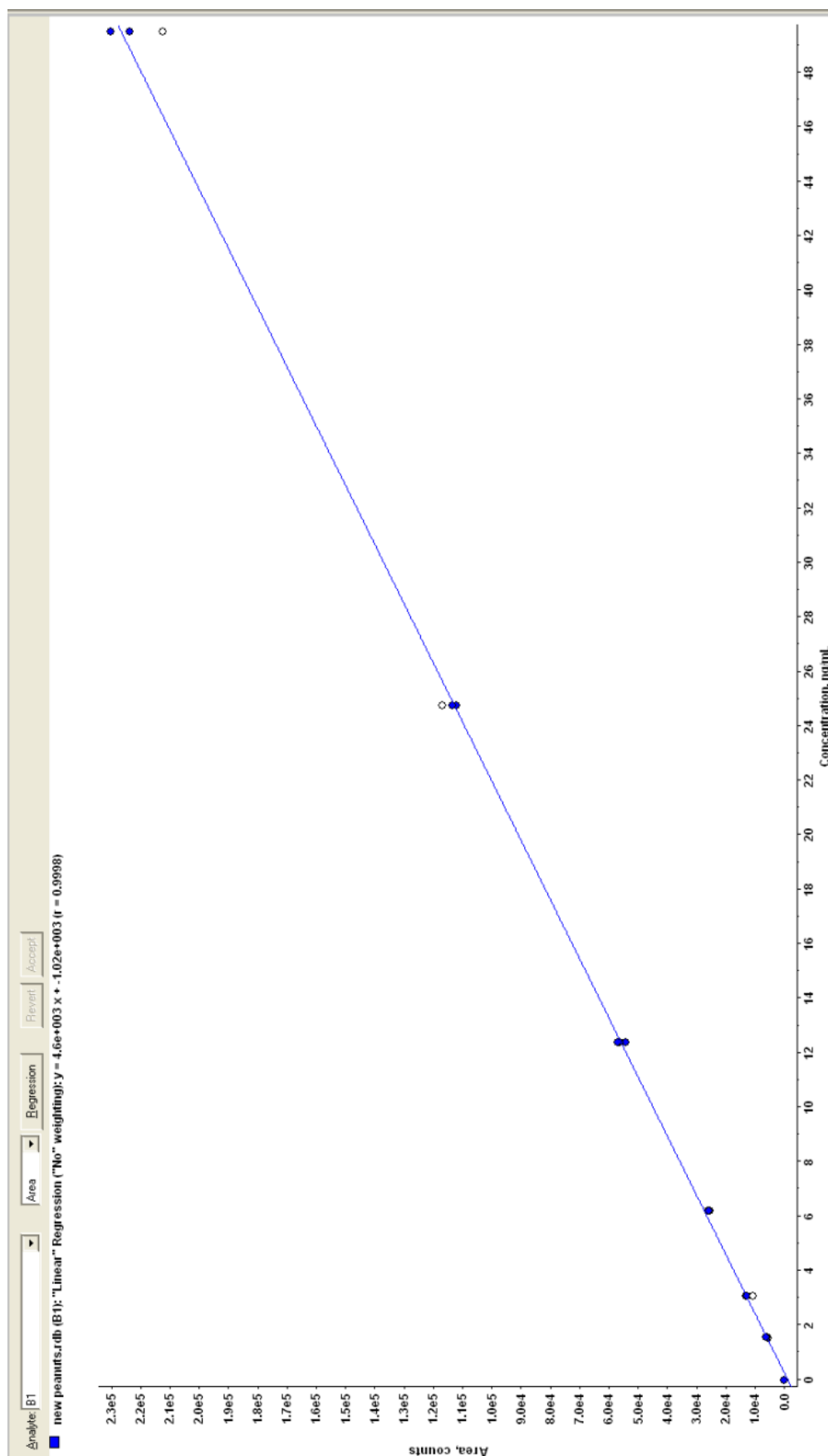


Figure (8): Calibration Curve of B1

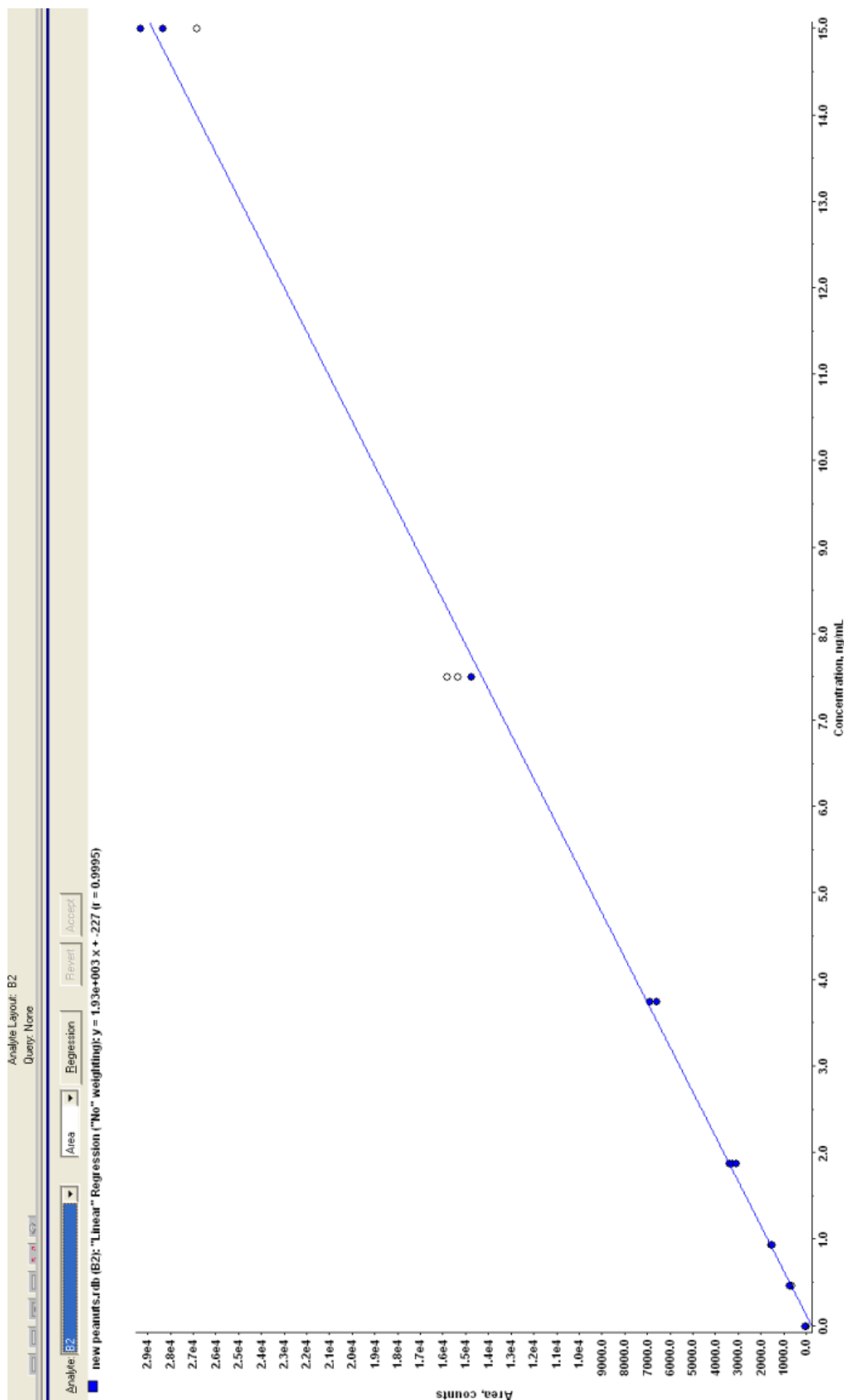


Figure (9): Calibration Curve of AF B2

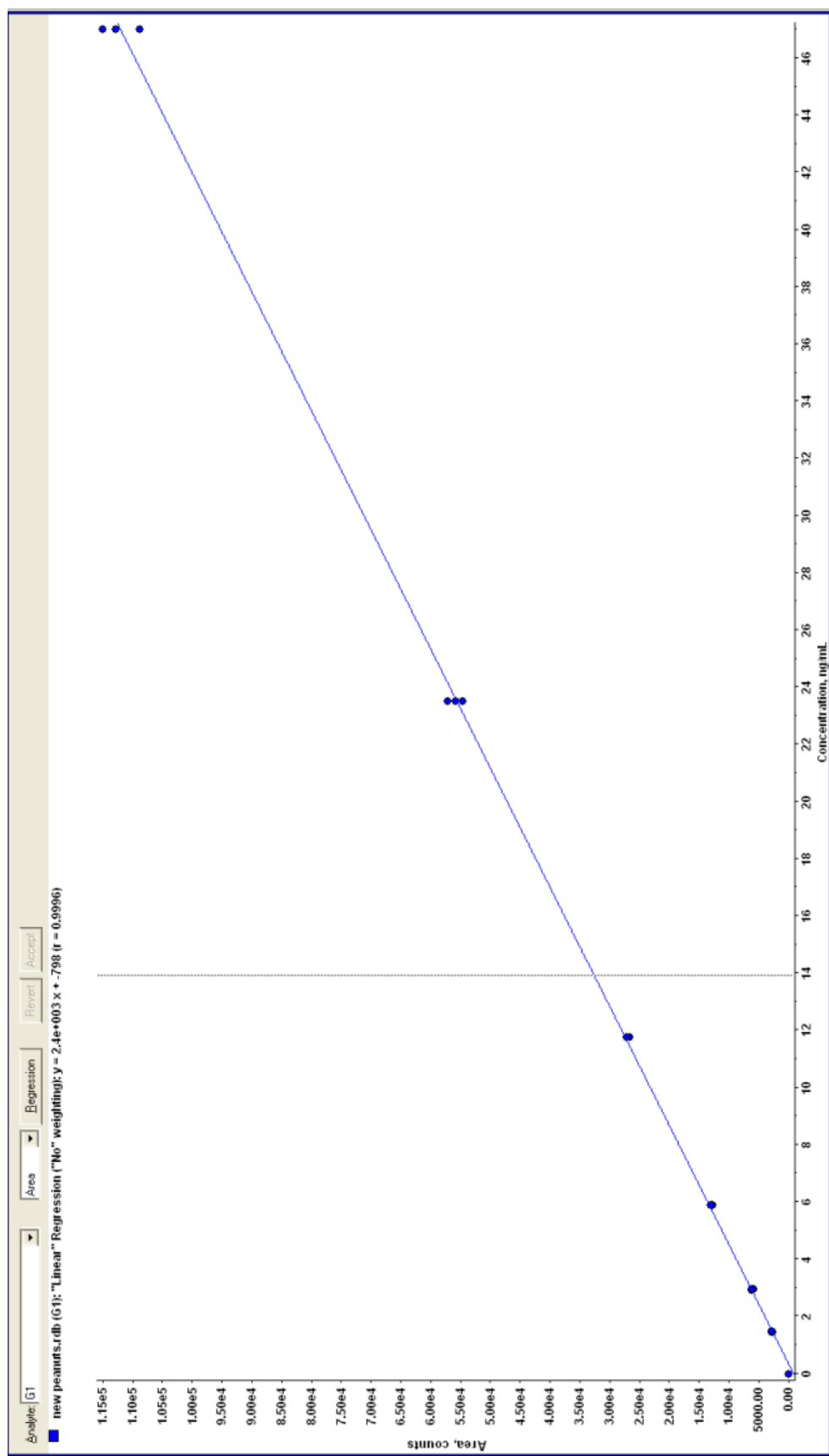


Figure (10): Calibration Curve of AF G1



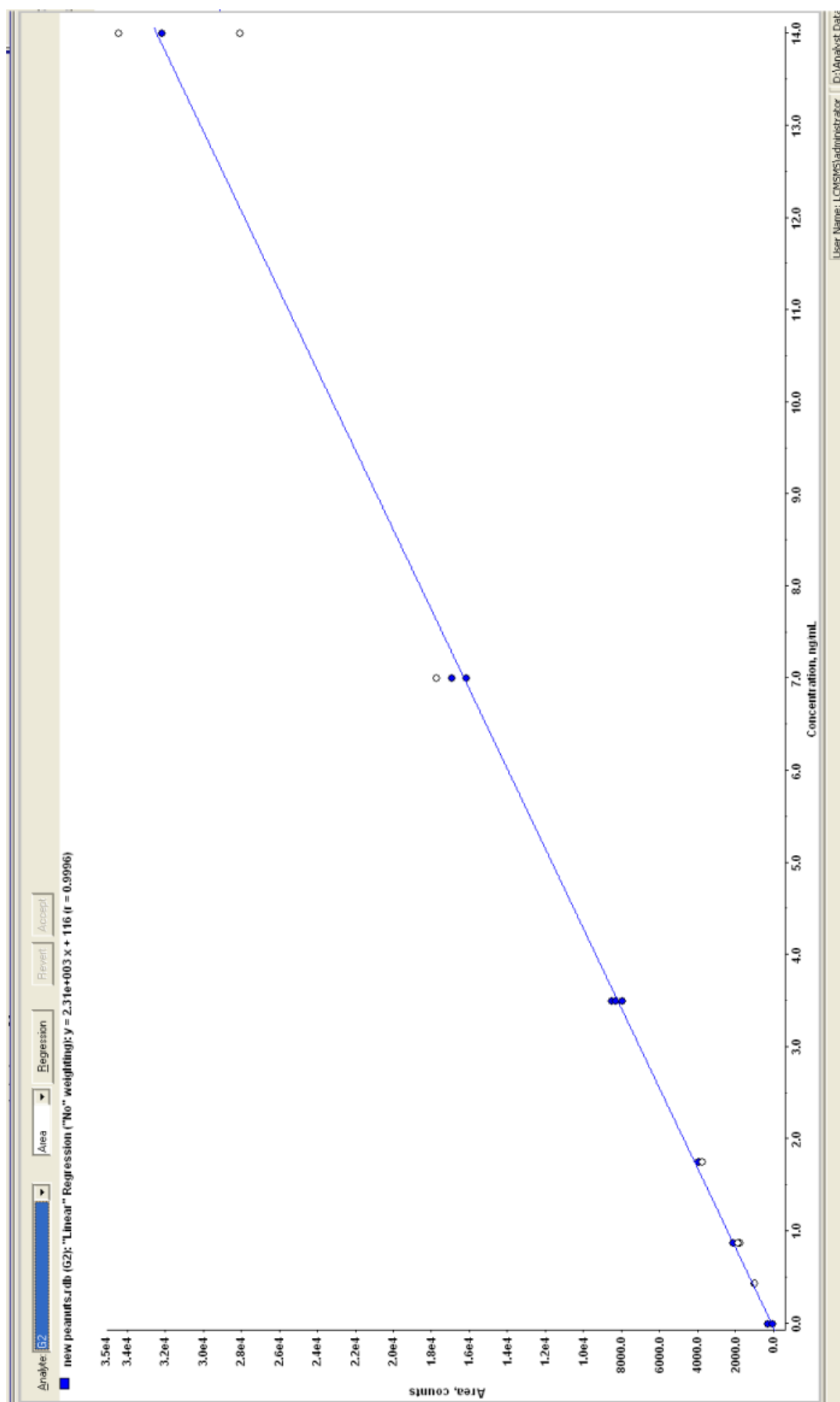


Figure (11): Calibration Curve of AF G2

### 3.2. Testing the reliability of the Crude Aflatoxin Extraction Method:

#### 3.2.1 Extraction of Peanuts Samples and Spiking:

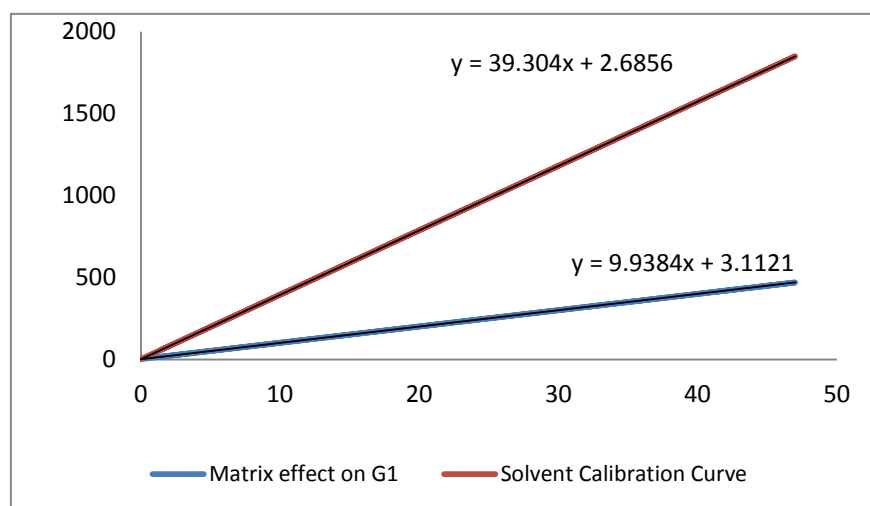
In the crude extract method was diluted and injected directly. The recovery rates for both methods were in the range of 40-50 %.

#### 3.2.2. Analysis of a known sample concentration using certified reference material (CRM):

The two certified reference materials of aflatoxins that were performed during the study provided accepted results. The first test showed recovery rate of 100%, while the second test showed a recovery rate of 99.92%.

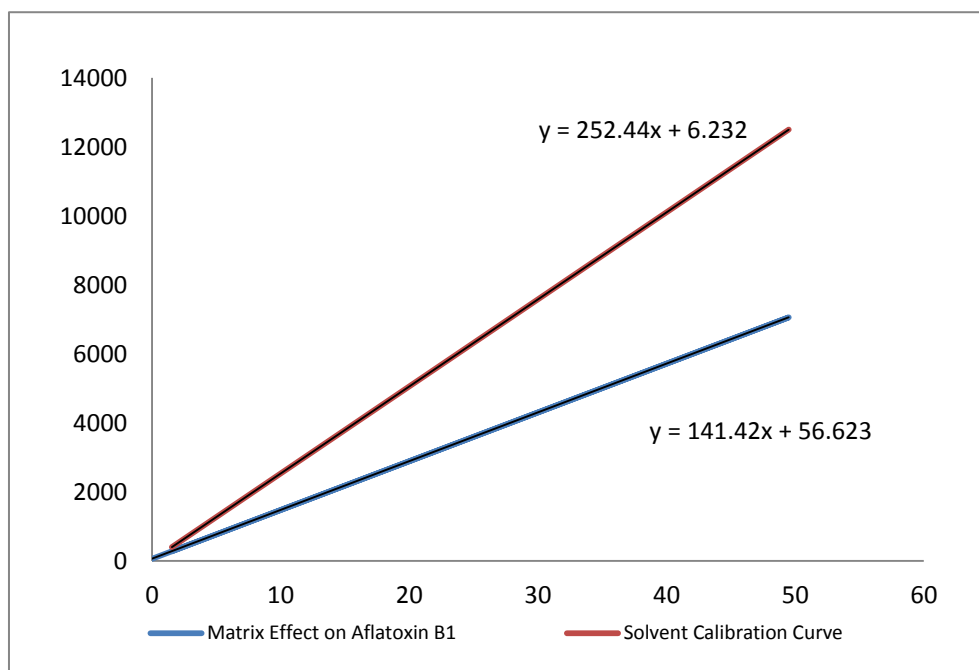
#### 3.2.3. The Matrix Effect:

The effect of the matrix on the reading was studied by comparing two calibration curves of the same points, the first calibration curve points were diluted in methanol : water 80:20, and the second calibration curve points were diluted in peanuts extract that does not contain aflatoxins, equations displayed by the instrument were used to plot the graphs below and investigate the effect. The linearity of both the matrix calibration curve and the solvent calibration curve was more than 0.999. Results showed that the matrix reduced the signal intensity of the analyte remarkably figure (12,13). Although the the matrix reduced the effect signal intensity, however the linearity of the curve shows that the effect of the matrix is consistent through different concentrations.



**Figure (12): The matrix effect on AF G1 Signal**

The magnitude of the matrix effect was investigated From the graph above, where it was calculated by dividing the slope of the matrix calibration curve by the slope of the solvent calibration curve, results showed that the matrix reduce the AF B and AF G signals of the samples about 76.5% and 42.3% respectively.



**Figure (13): The matrix effect on AF B1 Signal**

### 3.3. Simultaneous Quantification Analysis of Aflatoxins (B1, B2, G1, G2) in Peanuts Samples.

#### 3.3.1. The Incidence of Aflatoxins in Peanuts Samples.

Results are represented below in table (11). The reported values are blank corrected, while it's not matrix adjusted. Note that compensating the matrix effect would increase the values. Despite of the reduction effect of the matrix, the results are still tremendously high, compared to the European Union legislations and regulations, and the FDA as well. Only 4 samples out of the 45 samples were below the regulation limit (4µg/kg) (OJ L 364, 20.12.2006, p.5)

**Table (11): Occurrence of Aflatoxins in Peanuts Grains' Samples**

<b>Sample Code</b>	<b>AFB1 ng/g</b>	<b>AFB2 ng/g</b>	<b>AFG1 ng/g</b>	<b>AFG2 ng/g</b>	<b>Total AF ng/g</b>
1	0.48	1.18	1.43	2.70	5.79
2	1.16	3.48	1.66	1.66	7.96
3	4.73	2.96	1.76	1.76	11.22
4	10.36	3.88	9.98	14.54	38.75
5	66.13	12.76	1.38	22.24	80.27
6	8.96	3.45	1.49	1.12	15.03
7	0.20	1.38	1.49	0.00	3.08
8	59.75	10.70	0.75	1.18	72.39
9	0.19	1.66	1.07	24	2.92
10	3.44	1.35	0.72	0.00	5.51
11	3.42	2.51	1.02	0.00	6.95
12	16.73	5.32	1.10	1.10	24.24
13	4.50	1.33	1.78	1.78	9.39
14	15.62	9.97	2.16	2.16	29.91
15	10.58	1.01	2.07	0.36	14.01
16	119.33	32.47	1.45	0.00	153.25
17	28.87	5.90	1.34	0.00	36.10
18	14.73	6.04	1.89	0.19	22.84
19	1.86	3.80	1.89	0.19	7.73
20	0.46	0.86	1.41	0.00	2.73
21	0.21	1.49	1.29	0.00	2.99
22	0.00	1.11	0.00	0.94	12.05
23	4.83	2.29	0.00	19.64	7.12
24	188.49	33.65	0.00	25.61	247.75
25	92.25	23.41	1.79	23.73	141.18
26	0.49	0.25	0.00	15.83	16.57
27	152.60	28.93	2.07	21.18	204.78
28	37.35	8.27	1.77	27.27	74.65
29	22.46	8.39	0.26	21.11	52.21
30	0.40	4.31	1.69	0.00	6.39

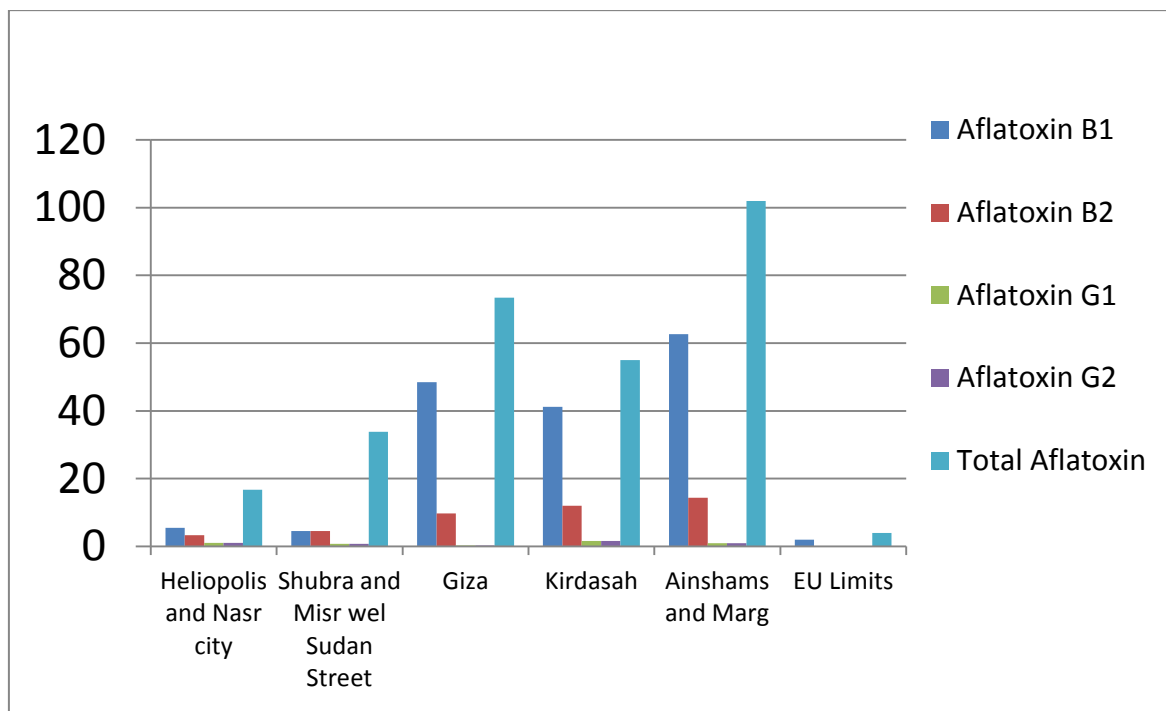
31	18.04	8.10	0.3	26.44	52.58
32	7.66	3.72	0.00	0.00	11.38
33	N/A	N/A	N/A	N/A	N/A
34	N/A	N/A	N/A	N/A	N/A
35	5.15	4.89	0.00	35.13	45.18
36	0.00	3.38	0.00	17.90	21.28
37	0.00	2.82	0.00	18.53	21.34
38	0.00	2.00	0.00	14.40	16.40
39	0.00	3.30	0.00	13.93	17.22
40	0.00	2.75	0.00	17.71	20.46
41	0.00	6.98	0.00	13.94	20.92
42	27.16	14.44	0.00	20.24	61.84
43	0.00	3.77	0.00	32.22	35.98
44	0.00	2.86	0.00	26.48	29.34
45	0.00	3.77	0.00	21.72	25.49
46	0.00	0.63	0.00	27.53	28.15
47	0.00	1.40	0.00	8.57	9.97

### 3.3.2. The Incidence of Aflatoxins in Peanuts Samples According to Different Regions

The means reported showed that Aflatoxins in peanuts are highest in the area of Ainshams and Marg, while the region with the lowest concentrations was in the region of Heliopolis and Nasr city table (12). Also for illustration the mean concentrations are represented in a bar chart in figure (14), including the regulatory limits of the EU commission. Results show that the mean concentrations are higher than the EU by 4-16 times higher. Moreover, the highest occurring type of aflatoxin differed from region to region. AF B1 was the most occurring aflatoxin in all regions except for Shubra, where Af B1 and B2 were present equally, and that is illustrated in figure (14).

**Table (12): The mean concentrations of the occurrence of each aflatoxin in different regions of Greater Cairo**

Location	AFB1 ng/g	AFB2 ng/g	AFG1 ng/g	AFG2 ng/g	Total ng/g
1. Heliopolis and Nasr city	5.49	3.308	1.0725	1.0725	16.69
2. Shubra and Misr wel Sudan Street	4.51	4.508	0.750	0.750	33.85
3. Giza	48.45	9.7	0.325	0.325	73.41
4. Kirdasah	41.2	12.053	1.6425	1.6425	54.98
5. Ainshams and Marg	62.62	14.370	0.9650	0.9650	101.93



**Fig (14): The aflatoxins mean concentrations of each region is represented in a bar chart, showing the different between each region.**

In Brazil, in 2017, 16 samples of ready to use unshelled peanuts were analyzed for the presence of aflatoxins by HPLC- FLD, their results showed that 4 samples out of the 16 samples were positive samples and their concentrations were 14.08, 0.82, 4.21 and 1.62 ng/g, 25% of the samples were contaminated with aflatoxins (Martins, et al., 2017). In Zimbabwe, out of 18 samples of peanuts, 3 samples were contaminated with aflatoxins with a mean of 51 ng/g. On the other hand, 10 out of the 11 peanuts butter were contaminated with aflatoxins with a mean concentration of 75.66.(Mupunga, Lebelo, Mngqawa, Rheeder, and Katerere, 2014). The finding of the Zimbabwe raises the probability of that peanuts used in the industry are of lower quality than ready to use peanuts, so the peanut butter and peanuts products might have higher concentrations of aflatoxins that results presented in this study, as several studies showed that peanuts butter contain high levels of aflatoxins as mentioned by Younis and Malik (2003).

The study done in Taiwan showed that 32.7% of all samples of peanuts were contaminated with aflatoxins, where the ranges of contamination were from 0.2  $\mu\text{g/kg}$  to 513.4  $\mu\text{g/kg}$ . However, only 6.8% of the samples showed higher concentrations of aflatoxins than the allowed regulatory limits of Taiwan, which is 15  $\mu\text{g/kg}$ . The most occurring type of aflatoxin reported is B1, which is similar to the results shown here.

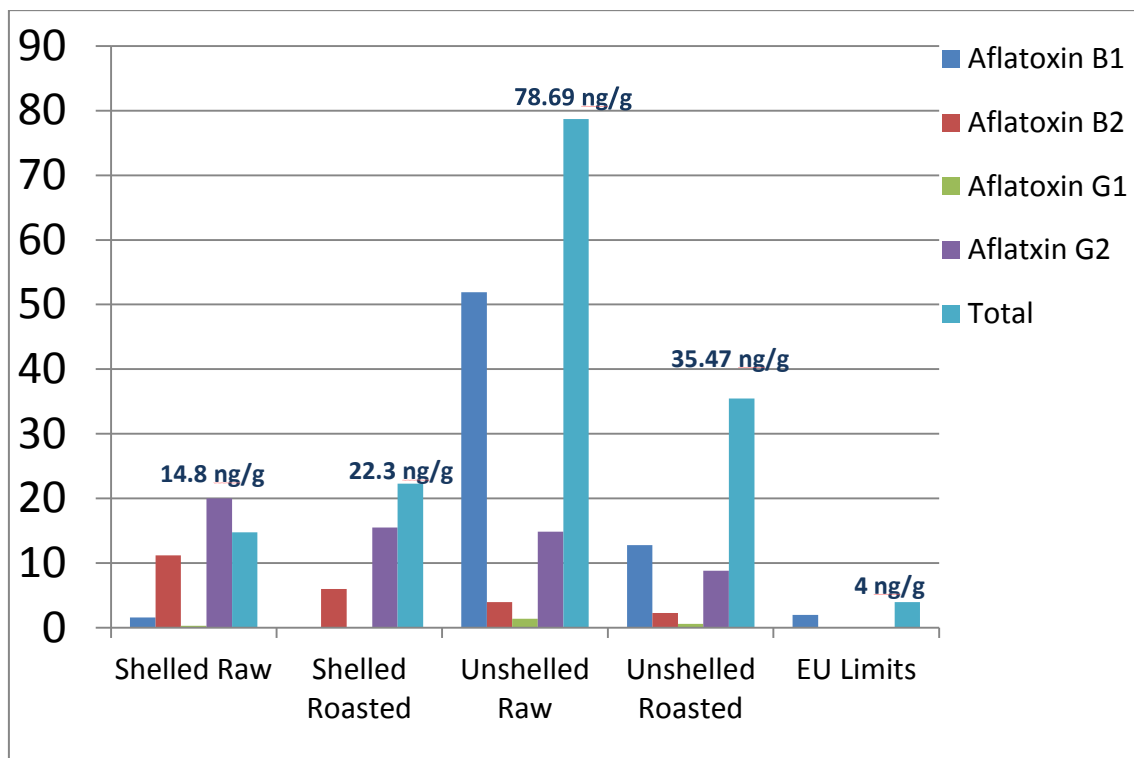
### 3.3.2. The Relationship Between Aflatoxins Occurrence and The Type of Peanuts Samples

The mean concentrations of each Aflatoxin in different types of peanuts are presented in table (13). The shelled raw samples were no. (30, 32, 41, 45 and 47), the shelled roasted samples were no. (38, 39, 40, 44, 46, 48), the unshelled raw samples were no. (5, 8, 20, 25, 28), the unshelled roasted samples were no. (6, 7, 29, 35 and 42), suggests that shelled peanuts seem to be protected slightly from aflatoxin production, as the shelled peanuts showed significantly lower (by about 60%) means than unshelled samples which was also supported by Mutegi, *et al.* (2017) and Kaaya and Warren, (2005).

More interestingly, all shelled samples examined did not contain AF B1 or AF B2. The data also suggests that the roasting process reduced the aflatoxin concentration; most likely through thermal decomposition as mentioned by Raters, M., and Matissek, R. (2008), aflatoxins are decomposed at 160 °c. Moreover, the shelled roasted peanut might be containing higher concentrations of aflatoxins than the shelled raw as aflatoxins might be migrated from the shell to the grain, the difference in concentrations of aflatoxins in the different types of peanuts is illustrated in a bar chart in figure (15).

**Table (13): Effect of different types of peanuts on the mean concentrations of the occurrence of aflatoxins:**

	<b>AFB1</b> ng/g	<b>AFB2</b> ng/g	<b>AFG1</b> ng/g	<b>AFG2</b> ng/g	<b>Total</b> ng/g
<b>Shelled Raw</b>	1.6	11.2	0.3	20.01	14.8
<b>Shelled Roasted</b>	0	6	0	15.524	22.31
<b>Unshelled Raw</b>	51.9	4	1.4	14.884	78.69
<b>Unshelled Roasted</b>	12.79	2.3	0.6	8.845	35.47



**Fig (15): The Effect of Different Types of Peanuts on the Occurrence of Aflatoxins**

During the research work, there was an assumption that aflatoxins in peanuts are found on the outer skin not the grain, so 7 samples of peanuts were peeled and aflatoxins were analyzed in each of peanuts' grains and peanuts' skin separately, results showed that most of the aflatoxins are accumulated in the grain not the skin. However, the weight of the peanut's skin contributes to the total weight of the grain by not more than 2.5%. For instance, the peanuts skin with the least aflatoxin concentration, sample no. 31, the skin weight contributed to the total weight by 2.1%, and the grain by 97.9%, and their total aflatoxins were 52.58 and 1.91 ng/g respectively table (14,15). Thus, the contribution of the skin in the total aflatoxin will be  $(1.91 \times 2.1)/100$  which is equals to 0.0411 ng/g while, the contribution of the grain will be  $(52.8 \times 97.9)/100$  which is equals to 51.69 ng/g, and for sample with the highest aflatoxin occurrence in the skin as sample no. 35 table (15)  $(38.88 \times 2.1)/100$  is equal to 0.816 ng/g, which is less than 0.2% of aflatoxin found in the whole peanut.

Sample No.	AF B1 ng/g	AF B2 ng/g	AF G1 ng/g	AF G2 ng/g	Total ng/g
31 skin	1.58	0.18	0.00	0.15	1.91
35 skin	0.00	0.00	0.00	38.88	38.88
36 Skin	0.00	11.99	0.00	15.37	27.35
37 Skin	0.00	9.19	0.00	10.99	20.18
38 Skin	0.00	0.00	0.00	0.00	0.00



<b>39 Skin</b>	0.00	0.00	0.00	0.00	0.00
<b>40 Skin</b>	0.00	0.00	0.00	0.00	0.00

**Table (14): Occurrence of Aflatoxins in Peanuts Skin Samples**

### 3.3.3. The Relation Between The Healthy Morphological Appearance Of The Peanuts And The Occurrence Of Aflatoxins:

Prior to analysis it was assumed that those peanuts which have accepted appearance have less mold and aflatoxins, while those which have lower quality appearance have higher content of mold and aflatoxins. This assumption was supported by experimental work done in this thesis, where three samples of peanuts that were not included in the survey were sorted out from the pack according to their appearance, and their results of aflatoxins were below the quantification limit, which is much lower than the regulatory limits of the EU and FDA. Therefore, physical sorting of peanuts would provide a reliable method for decreasing the aflatoxins incidences in the market. However, this is supported by the regulations set by EU where the peanuts to be sorted have much higher regulatory limits than ready to eat peanuts, table (2).

### 3.4. Simultaneous Quantification of Aflatoxins in Lentils and Beans Samples:

The extraction and LC MS/MS analysis were applied to 10 samples of lentils and 3 samples of beans, since they are of the most consumed grains in Egypt. The lentils samples showed the lowest concentrations of aflatoxins contamination, where the total aflatoxin ranged from 0.132 to 1.388 ng/g. The highest occurring aflatoxin, were aflatoxin G1 which ranged from 0.015 – 1.388 ng/g, table (17).

**Table (15): The Occurrence of Aflatoxins in Lentils Samples.**

<b>Lentils Samples</b>	<b>AF B1 ng/g</b>	<b>AF B2 ng/g</b>	<b>AF G1 ng/g</b>	<b>AF G2 ng/g</b>	<b>Total ng/g</b>
<b>1</b>	0	0	1.388	0	1.388
<b>2</b>	0	0.259	0.231	0.038667	0.528
<b>3</b>	0.112	0	0.02	0	0.132
<b>3</b>	0.024	0	0.015	0	0.039
<b>4</b>	0	0	0.893333	0	0.893
<b>5</b>	0.010667	0	0.9	0	0.910
<b>6</b>	0	0	0	0	0
<b>7</b>	0	0	0	0	0
<b>8</b>	0	0	0	0	0
<b>9</b>	0	0	0	0	0
<b>10</b>	0	0	0	0	0

A study done in Sudan, to investigate the occurrence of aflatoxins in lentils, show that only 1 sample of lentils seed out of 13 lentil seed was contaminated with aflatoxin with concentration of 14.3 µg/kg. (El-Nagerabi, and Elshafie 2001).

The beans samples analyzed in this work showed lower amounts of aflatoxins as compared to peanuts samples, and higher amounts of aflatoxins if compared to lentils samples as shown in table (12). Results of aflatoxins occurring in beans are shown below in table (16).

**Table (16): The occurrence of aflatoxins in bean samples**

<b>Bean Samples</b>	<b>AF B1 ng/g</b>	<b>AF B2 ng/g</b>	<b>G1 ng/g</b>	<b>G2 ng/g</b>	<b>Total</b>
<b>1</b>	0	0.828	1.493	0	2.321
<b>2</b>	0	1.961	1.56	0.5488	4.069
<b>3</b>	0	4.66	1.57	0.49	6.72

## **4- Conclusion**

This work addresses many analytical method challenges. A sensitive and accurate LC-ESI-MS/MS method for the simultaneous detection and quantification of aflatoxins types with a low LOD and LOQ was developed and reported. In addition to, a crude, quick, easy and cheap extraction method was investigated, and was employed with different matrices. The method developed was applied on 52 samples of peanuts and peanuts skin, 10 samples of Lentils, 3 samples of beans and 2 samples of wheat (Certified Reference Material).

The crude extraction emphasis a novel extraction process, that reduced the cost, time and effort consumed in the extraction process of aflatoxins. This may allow ease of analysis in order to obtain additional data about the occurrence of mycotoxins in food in Egypt as this data currently is scarce. This extraction method could be used with broader range of mycotoxins and matrices, without the need for specialized extraction columns. Thus, it may widen the research with respect to matrices types and target analytes as well.

Moreover, the data generated in this study provide a valuable information about aflatoxins levels in peanuts available in the Greater Cairo region; the results showed that level of aflatoxins in peanuts in Egypt is significantly higher (4-25.5 times) than the EU and FDA standards. This clearly suggest significant and serious human health hazard as these toxins are known to be cancer causing.

## **5. Future Researches**

Further research and development are required in this area, more feasible, quick, and accurate methods are required to facilitate the quality control procedure. Moreover, the interesting field of microfluidics would be applied in mycotoxin detection, as it would provide, easy, cheap, quick method for detection of mycotoxin, and the fascinating thing about it, that it will provide consumers and individual with a method to test their own exposure to hazards, such technology will raise the quality of the market, as stakeholders will know that they could be traced any time by authorities and consumers as well, thus there will be an innate quality control flow.

Moreover, risk assessments researchers are required in this area, to test the exposure rate of individuals, to be able to set regulations and legislations on ground base. The exposure rate could be studied by LC MS MS too by quantification of aflatoxins and biomarkers, that are found in urine, blood, and milk samples, as well as DNA adducts. An interesting area also, is the practical investigations of the claims of the beneficial detoxification effects of detox water and green smoothies, where research are needed to study wither certain food and drink may detoxify the body or not, if these claims were proven to be true it would enhance the public health and the functional food industries as well.

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