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Natural Incidence of Aflatoxins and Ochratoxin A Nuts Collected from Local Market in Tripoli

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A B S T R A C T

Aflatoxins and ochratoxin A are mycotoxins produced by fungi belonging to the *Aspergillus* and *Penicillium* genera. The present study was carried out to study the natural occurrence of aflatoxins (AFS) and ochratoxinA (OA) in 90 nut samples collected from Tripoli, Libya during 2013 were determined by using a high-performance liquid chromatography technique. Aflatoxins B1, B2, G1 and G2, in the local market in Tripoli Libya during summer, 2013. Natural occurrence of aflatoxin B1, B2, G1 and G2 in almonds, Br. almonds, hazelnuts, cashews, walnuts and peanuts samples collected from local market in Tripoli. The percentages of positive samples with aflatoxins were 33.3, 40.0, 20.0, 13.3, 26.6 and 53.3% for almonds, Brazilian almonds, hazelunts, cashews, walnuts and peanuts, respectively. The concentrations of aflatoxin B1 were ranged between (0.9-5.3, 1.4- 7.8, 1.2 -5.4, 2.1- 3.4, 1.6-7.8 and 2.4-10.9 µg/kg) for almonds, Brazilian almonds, hazelunts, cashews, walnuts and peanuts, respectively. Aflatoxin B2 were found in Brazilian almonds ranged from (2.2-3.5 µg/kg) and in peanut (7.6-8.4 µg/kg). These results indicate the contamination of nuts with aflatoxins B1 and B2 were in various concentrations, this difference depends on type of nuts and environmental conditions, as well as the availability of nutrients to the fungus. The percentages of nut samples (almonds, Brazilian almonds , hazelunts, cashews, walnuts and peanuts) were contaminated with ochratoxin A 26.6, 33.3, 13.3, 20.0, 13.3 and 33.3%, respectively, also the concentrations were ranged between (3.5-5.0,1.5- 2.2, 1.2 -3.7, 1.3- 2.5, and 4.0-6.5 µg/kg) for Brazilian almonds, hazelunts, cashews, walnuts and peanuts, respectively. However almonds free of ochratoxin A. The highest concentration found in peanut samples (6.5 µg/kg). The difference concentration of AFS or OA may be depends on type of nuts and environmental conditions, as well as the availability of nutrients to the fungus.

Introduction

Mycotoxin contamination is a worldwide food safety problem that has attracted global attention due to significant losses associated with its impact on human and animal health and consequently, national and economic implications (Makun *et al.*, 2009).

Dry fruits including tree nuts are highly susceptible to fungal attacks both while in the field and during storage which may result in production of mycotoxins in them. Most of these toxins are thermostable at high temperatures (Kabak, 2009) and can,

therefore, contaminate processed foods and enter the human food chain throµgh derived foods. The mycotoxin of prime concern, due to their toxicity and prevalence, are aflatoxins (AFs) and ochratoxin A (OTA).

AFs are found as contaminants in various agricultural commodities.

The commodities with the highest risk of AFs contamination include corn, peanut, cottonseed, Brazilian nuts, pistachio nut, fig, spice and copra (Pittet, 1998).

Aflatoxins are difuranocoumarin derivatives synthesized by polyketide pathway by various strains of *Aspergillus*, mainly *A. flavus* and *A. parasiticus*. Besides this, several other species of *Aspergillus* belonging to section *Flavi*, *Ochraceorosei* and *Nidulantes* have been claimed to produce aflatoxins. Aflatoxins have immunotoxic, mutagenic and carcinogenic effects (Moss, 1998) and have been classified as group 1 carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 1993). Occurrence of AFs contamination on several agricultural products such as maize, wheat, rice, spices, dried fruits and nuts has been reported worldwide (Grajewski *et al.*, 2012; Nguyen and Ryu, 2014).

Ochratoxin A (OTA) is a ubiquitous mycotoxin produced by several fungal species belonging to the genera *Aspergillus* (e.g. *Aspergillus ochraceus*) and *Penicillium* (e.g. *Penicillium verrucosum*) (Alvarez *et al.*, 2004). OTA is a potential nephrotoxin with carcinogenic potential and hepatotoxic and teratogenic mycotoxin in animal species and it is classified by the IARC as a possible human carcinogen of Group 2B (IARC, 1993). The toxin has also been found in human sera from people. Living in areas where Balkan endemic nephropathy occurs, and it is sµggested to be a possible determinant of this fatal human disease (Hult *et al.*, 1982). It has also been extensively documented as a contaminant of a wide variety of foods including cereals, green coffee, spices, nuts, dried fruits, beer, wine, grapes, and grape juice (Ghali *et al.*, 2009). Many countries and international organizations have regulated the OTA content in several commodities.

Materials and Methods

Sample collected

Total of 90 nut samples (almonds, Br. Almonds, hazelnuts, cashews, walnuts and peanut) were collected from Tripoli, Libya during 2013.The sample was stored in polyethylene bags at -18 ^{O}C for determination of aflatoxins.

Extraction of aflatoxins (AFs) nuts according to CB method of AOAC (2007) as follow

Fifty grams of each ground samples were weighed into 500ml Erlenmeyer flask containing 25 g diatomaceous earth, 250 ml chloroform and 25 ml distilled water. The mixture was shacked with a horizontal shaker for 30 min. The extract was filtered throµgh Whatman No.4 filter paper. The first 50 ml of extract was collected and transferred for clean up using silica gel column.

Clean up: Chromatographic columns were prepared by initially packing anhydrous sodium sulphate (5 g) into glass tube (22 x300 mm) with plµg of glass wool. Chloroform was added to 10 g silica for column to create slurry, which was added to the chromatographic column. The stopcock was opened to allow the silica gel packing to settle, while the excess chloroform was drained. During draining, another 10 g anhydrous sodium sulphate was added to the

top of silica gel to prevent column from drying. A portion of filtrate (50ml) was loaded to the column and allowed to flow at a rate of one drop/second. Then, 150 ml n-hexane followed by 150 ml diethyl ether was passed throμgh and discarded. Subsequently, aflatoxins were eluted with 150 ml chloroform: methanol (97: 3, v/v) into 250 Erlenmeyer flask and then was evaporated to dryness on steam bath. The residue was quantitatively transferred to a small vial with chloroform and evaporated to dryness on steam bath under nitrogen and reserved for TLC and HPLC analysis.

Determination of aflatoxins by HPLC

Derivatization

The derivatives of tested samples and standards were done as follow: Tow hundred μl hexane were added to the clean up dry film of standard and tested samples followed by 50 μl Trifluroacetic acid (TFA), cap vial, and their mixed by vortex vigorously for 30 s. The mixture was left to stand for 5 min. To the mixture 450 ml water- acetonitril (9 +1 v/v) by pipet were added and mixed well by vortex for 30 s, and the mixture was left to stand for 10 min. to form two separate layers. The lower aqueous layer was used for HPLC analysis (AOAC, 2007).

Apparatus

High performance liquid chromatography (HPLC) was used to aflatoxins determination. A mobile phase consists of water: acetonitril: Methanol (240:120:40). The system equipped with (Waters 600) delivery system. HPLC column a reverse phase analytical column packed with C18 material (Spherisorb 5 μm ODS2, 15cm×4.6nm). The detection was performed using the fluorescence detector was operated at an excitation wave length of 360 nm and an emission wave length of 440 nm. The separation was performed at ambient temperature at a flow rate of 1.0 ml/ min,. Data were integrated and recorded using a Millennium Chromatography. Manger Software 2010 (Waters, Milford MA 01757)

Quantization

The mixed solutions of standard as well as sample extract after derivatisation were filtered throμgh a 0.22 μm membrane filter and loaded (50 μl) into a 200 μl injection loop. The elution order of the four aflatoxins was G_2, B_2, G_{2a} (G_1 derivative), B_{2a} (B_1 derivative).AFs contents in samples were calculated from chromatographic peak areas using the standard curve.

Detection and determination of ochratoxinA

Extraction

Fifty grams of nuts were put into high speed blender; 25 ml phosphoric acid (0.1M) and 250 ml chloroform were added and blended for 3 min. at medium speed. Ten gram diatomaceous earth were added just before the end blending time then filtered throμgh Whatman No.4 filter paper and 50 ml portion were collected, transferred to separator funnel, 10 ml sodium bicarbonate (3%) were added and shacked gently, then the upper phase was collected for column separation.

Clean up

A Sep-Pak C18 Column was placed on vacuum monifold ports, column prewashed twice with 2 ml methanol, 2 ml water, and 2 ml sodium bicarbonate (3%). Five ml bicarbonate extract were added to the C18 column, followed by 2 ml phosphoric acid (0.1M) and 2 ml water, and washings were discarded. OTA was eluted with 8 ml ethyl acetate: methanol: acetic acid (95: 5: 0.5

v/v/v). The elute was collected in vial containing 2 ml water and the elute was shacked with tube shaking machine (vortex genie) to mix the two phases. Pipette OTA extract (upper phase) to 7 ml screw- cap vial. Rinse remaining upper phase from tube with 2 x l ml ethyl acetate and add to OTA. Evaporate extract just to dryness on steam bath under nitrogen for subsequent HPLC analyses (AOAC, 2007).

Determination

Determination of OTA by HPLC

The fore-mentioned columns elutes were dissolve in 500 µl mobile phase consists of acetonitrile: water: acetic acid (99:99:2) and filter throµgh 0.45 µm microfilter into 5 ml screw cap vial for subsequent HPLC analyses. High performance liquid chromatography (HPLC) was used to ochratoxins A Determination. The system equipped with (Waters 600) delivery system. HPLC column a reverse phase analytical column packed with C18 material (Spherisorb 5 µm ODS2, 15cm×4.6nm). The detection was performed using the fluorescence detector was operated at an excitation wave length of 330 nm and an emission wave length of 460 nm. The separation was performed at ambient temperature at a flow rate of 1.0 ml/ min. Data were integrated and recorded using a Millennium Chromatography [Manger Software 2010 (Waters, Milford MA 01757)].

Quantization

Calculated from chromatographic peak areas using the standard curve.

Results and Discussion

Natural occurrence of aflatoxin

The obtained results of table 1 and figures 1,2 and 3 showed the natural occurrence of aflatoxin B1, B2, G1 and G2 in almonds, Brazilian almonds, Hazelnuts, cashews, walunts and peanuts samples collected from local market in Tripoli. The percentages of positive nuts sample (Almonds, Brazilian Almonds, Hazelunts, Cashews, Walnunts and Peanuts) were contaminants with aflatoxins 33.3, 40.0, 20.0, 13.3, 26.6 and 53.3%, also the concentrations of aflatoxin B1 were ranged between (0.9-5.3,1.4- 7.8, 1.2 -5.4, 2.1- 3.4, 1.6-7.8 and 2.4-10.9 µg/kg, respectively. Aflatoxin B2 were found in Br. Almonds ranged from (2.2-3.5 µg/kg) and in peanut (7.6-8.4 µg/kg).

Higher values of contamination with aflatoxin B1 and B2 were observed in peanut. These results may be attributed to type of cultivation or poor transportation, handling and storage condition of nuts. Storage, whether in the farm, on in manufacturing premises or in the grocery store, is considered one of the most critical post-harvest phases in food handling. Inappropriate environmental conditions, improper packaging or spoiled foodstuffs can cause mycotoxin contamination during this stage (FAO/WHO/UNEP, 1999). Aflatoxins were detected in 90% of hazelnut samples (25–175 µg/kg) and 75% of walnut samples (15–25 µg/kg). Jimenez *et al*., (1991) reported moulds and mycotoxins in almonds, peanuts, hazelnut and pistachio nuts and detected aflatoxins at up to 95 µg /kg in the samples.

According to many reports, peanuts are the main susceptible products for aflatoxin contamination. Tree nuts such as almonds, walnuts, and pistachios may be contaminated with aflatoxin, though at lower levels than for cottonseed and corn; however, the problem is very significant to producers because: (1) the crop has a high unit value, and (2) much of the crop is sold to European markets that enforce limits significantly lower than those in some countries (Shephard, 2003). For over

all sanitary precaution, the European Union has enacted in 1998, very severe aflatoxin tolerance standards of 2 µg / kg AFB1 and 4 µg / kg total aflatoxins for nuts and cereals for human consumption and this has come into effect from January, 2001. Consumers in the developed world are well aware of the carcinogenic effect of aflatoxins and will thus stay away from a product that has aflatoxin beyond the acceptance level. Exports of agricultural products particularly groundnuts and other oilseeds from developing countries have dropped considerately in recent years resulting in major economic losses to producing countries as a result of this restriction. According to the World Bank estimate, the policy change by the European Union will reduce by 64%, imports of cereals, dried fruits, oil seeds and nuts from nine African countries namely Chad, Egypt, Gambia, Mali, Nigeria, Senegal, South Africa, Sudan and Zimbabwe and this will cost African countries about US $670 million in trade per year. However, the new rule of the EU has been criticized as being too stringent. There is the need for mycotoxin surveillance because of its wide occurrence in contaminated commodities (Negedu *et al*., 2011).

Table.1 Survey of aflatoxins in nuts collected from Tripoli, Libya during (2013)

Kind of nuts	No. of samples	No. of positive samples	Percentage of positive samples	*Concentration of Aflatoxins µg/kg			
				AFB1		AFB2	
				Min	Max	Min	Max
Almonds	**15**	**5**	**33.3**	**0.9**	**5.3**	**ND**	**ND**
Br.Almonds	**15**	**6**	**40.0**	**1.4**	**7.8**	**2.2**	**3.5**
Hazelnuts	**15**	**3**	**20.0**	**1.2**	**5.4**	**1.5**	**ND**
Cashews	**15**	**2**	**13.3**	**2.1**	**3.4**	**ND**	**ND**
Walnuts	**15**	**4**	**26.6**	**1.6**	**7.8**	**ND**	**ND**
Peanuts	**15**	**8**	**53.3**	**2.4**	**10.9**	**7.6**	**8.4**

ND: Not Detectable.
* aflatoxins G1 and G2 not detectable

Figure.1 The percentages of positive nuts sample

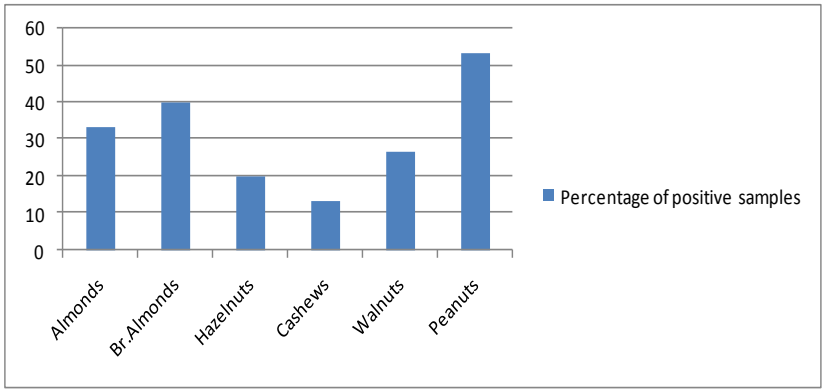


Figure.2 The minimum and maximum concentrations of aflatoxin B1 (µg/kg)

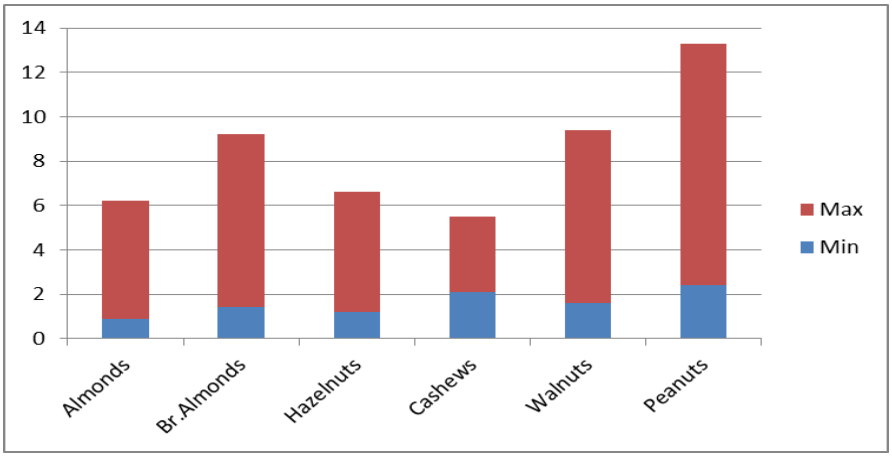


Figure.3 The minimum and maximum concentrations of aflatoxin B2 (µg/kg)

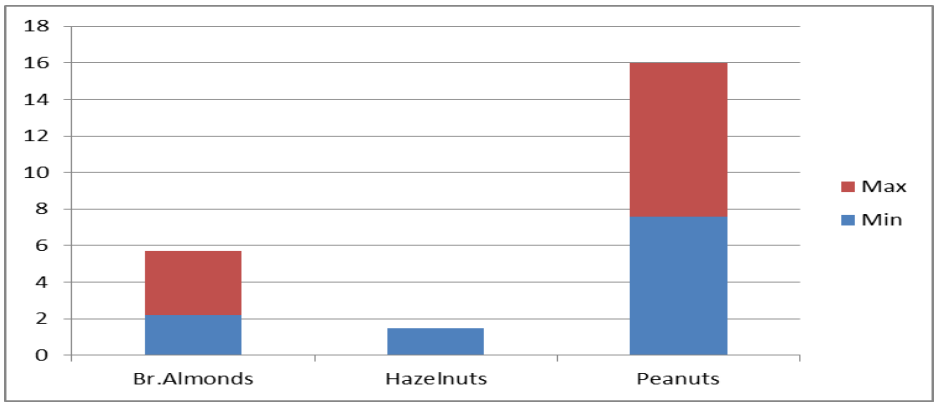


Table.2 Survey of ochratoxin A in nuts collected from Tripoli, Libya during, 2013

Kind of nut	No. of samples	No.of positive samples	Percentage of positive samples	Ochratoxin A concentration µg/kg	
				Min	Max
Almonds	15	ND	26.6	ND	ND
Brazilian Almonds	15	5	33.3	3.5	5.0
Hazelnuts	15	2	13.3	1.5	2.2
Cashews	15	3	20.0	1.2	3.7
Walnuts	15	2	13.3	1.3	2.5
Peanuts	15	5	33.3	4.0	6.5

Figure.4 The percentages of positive nuts sample

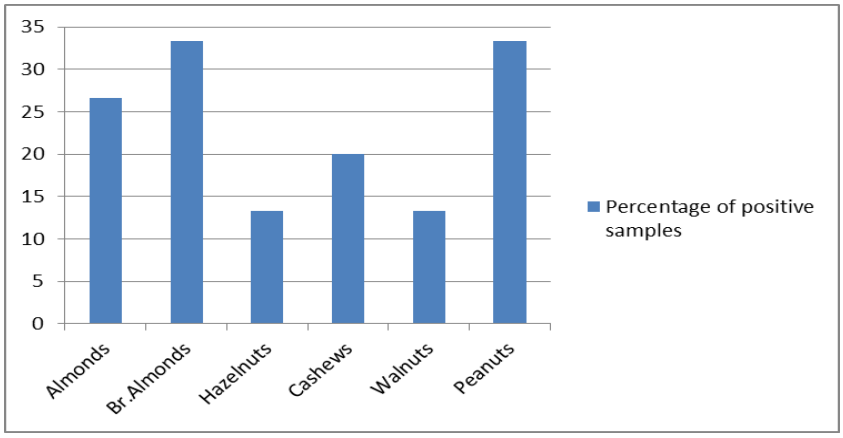
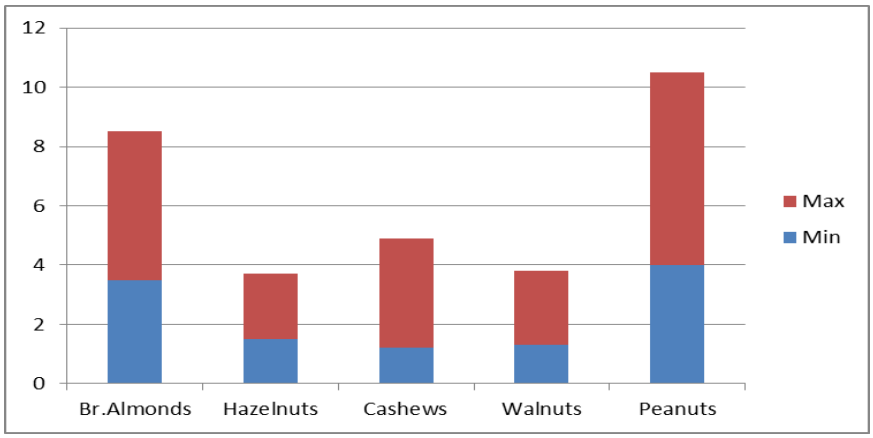


Figure.5 The minimum and maximum concentrations of ochratoxin A (µg/kg)



Natural occurrence of ochratoxin A

The obtained results of table 2 and figures 4 and 5 showed the natural occurrence of ochratoxin A in nuts samples collected from local market in Tripoli. The percentages of positive nuts sample (Almonds, Brazilian Almonds, Hazelnuts, Cashews, walnuts and peanuts) were contaminants with ochratoxin A 26.6,33.3, 13.3,20.0, 13.3and 33.3%, respectively, also the concentrations were ranged between 3.5-5.0, 1.5- 2.2, 1.2 -3.7, 1.3-2.5, and 4.0-6.5 µg/kg for Brazilian Almonds, Hazelnuts, Cashews, walnuts and peanuts. However almonds free of ochratoxin A.

Several studies have reported the incidence of black aspergilla in pistachios and other tree nuts. *A. niger* was found in 42% of nuts samples including pistachios, almonds, walnuts and Brazilian nuts (Bayman *et al*., 2002). They have been recently associated with the presence of ochratoxin A (OTA) in grapes and their derivatives (Battilani *et al.*, 2003). The main species involved in OTA contamination is *Aspergillus carbonarius* and a low percentage of isolates of the closely related species in *A. niger* aggregate (Belli *et al.*, 2004).

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