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Investigation of Groundnut Cake Contamination by Aflatoxins, Ochratoxins and Zearlenone from Three States in Sudan.

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Abstract

This survey examined 75 samples of groundnut cake from three state (Khartoum, Kordofan and Gadarif) of Sudan for aflatoxin B1,B2,G1,G2, (AFB1, AFB2, AFG1, AFG2) ochratoxin (OTA, OTB) and zearalenone (ZON) using High Performance Liquid Chromatography (HPLC) method with fluorescence detection. The limit of detections (LODs) and limit of quantifications (LOQs) were found to be in range between 0.01–0.6 ng g^{-1} and 0.03–2.0 ng g^{-1}, respectively. The frequency of contaminated samples with AFB1 from Khartoum, Gadarif and Kordofan state was 46.0%, 34.0%, and 32.0%, respectively. Only one samples of groundnut cake from Khartoum state was found to be contaminated with AFG2 (2.0%). Concentrations of OTA, and OTB were low and may not cause problems. The highest contaminated samples with mycotoxins were found in Khartoum state.

Keywords: Groundnut cake; Mycotoxins; Aflatoxins; Ochratoxin A; Zearalenone; HPLC; Sudan

Background

Mycotoxins are produced by several fungi, particularly by many species of *Aspergillus, Fusarium, Penicillium, Claiceps*, and *Alternaria*. They comprise a group of several hundreds of chemically different toxic compounds. They are low molecular weight compounds with great structural variation resulting in diverse physicochemical properties[1]. The most common mycotoxins are aflatoxins, ochratoxin A, trichothecenes, zearalenone, and fumonisins.

Mycotoxins pose a hazard to animal health due to the harmful biological effects they possess. These effects vary from general symptoms to more severe, the consumption of mycotoxin contaminated diet may induce acute and long-term chronic effects resulting in a teratogenic, carcinogenic (mainly for liver and kidney), oestrogenic, or immunosuppressive impact not only on animals but also on man whereas animals usually suffer more due to grain of lower quality[2]. In addition to the toxic effects, a mycotoxin contaminated diet may lead to other consequences like feed refusal, poor feed conversion, diminished body weight gain, increased disease incidence due to immune suppression, and interference with reproductive capacities which are responsible for great economical losses.

Among the numerous mycotoxins that have been identified, aflatoxins (AFs) ochratoxin A (OTA) and andzearalenone (ZON) are of importance for control because of their frequent and worldwide contamination of agricultural products. The European Union (EU) has established regulatory limits for mycotoxins in crops and foodstuffs[3].

Although there are a lot of improvements on analytical methods for the analysis of mycotoxins over the years, there is a shortage on the reports of the occurrence of mycotoxins in feedstuffs and feed. In 2007, Binder[4] reported the occurrence of mycotoxins in 30% of samples from Asian-Pacific countries and 50% in European and Mediterranean samples, and concluded that the incidence of mycotoxins relevant for animal production is quite high in animal feed.

Aflatoxins (AF), mainly produced by *Aspergillus flavus* and *A. parasiticus*, are a group of naturally occurring fungal metabolites that have long been recognized as significant environmental contaminants. Aflatoxins are classified as carcinogen-

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ic by the International Agency for Research on Cancer (IARC 1993) and within that group, aflatoxin B1 (AFB1) is the strongest naturally occurring carcinogen known[5].

AFB 1 is a potent hepatocarcinogen that can induce tumors in many species of animals, including rodents, nonhuman primates, and fish[6]. While the liver is the major target organ, under certain circumstances, significant numbers of tumors have been found in lung, kidney, and colon[7].

Aflatoxins contamination of foods and feeds can occur prior or during harvesting, transport or storage. The favorable conditions for the growth of these moulds are high humidity and temperature. The conditions can be best represented by water activity range 0.84 – 0.86 and a temperature range, 25 – 40°C[8].

Ochratoxin A (OTA) is one of the several fungal mycotoxins that have aroused significant public concern worldwide. The disease caused by OTA exposure is known as *ochratoxicosis*, and the primary target is the kidney. Epidemiological studies show that OTA may be involved in the pathogenesis of different forms of human nephropathies, including kidney cancer[9,10].

Tumor incidence data from long-term animal studies also provides reasons for concern about the effect of OTA exposure on the human population. Thus, OTA was classified as a possible carcinogen (Group 2B) to humans by The International Agency for Research on Cancer[11]. OTA may be encountered in a host of common foodstuff and beverages. The highest reported occurrence of OTA was found in cereal grains, and to a lower extent in other foodstuff of plant origin, OTA can also be found in tissues and products of animal origin, pork and poultry, and dairy products, among others[12,13].

Zearalenone (ZON) is a mycotoxin that contains a non-steroidal resorcyclic acid lactone and it is produced mainly by several species of Fusarium fungi which grown in cereal grains (*eg. F. culmorum and F. graminearum*). If grains contaminated with ZON are used for animal feed, it is considered to be potentially hazardous to animals[14]. The aim of this study is to investigate the presence of AFB1, AFB2, AFG1, AFG2, OTA, OTB and ZON in groundnut cake samples from three states in Sudan.

Material and Methods

Samples: All the samples analyzed have been collected randomly from shops of animal feed in markets of three states in Sudan (Khartoum, Kordofan and Gadarif). The samples size was 0.5-1 kg. The samples were kept at freezer till tested. At the time of analysis samples were brought up to room temperature[15]. Extraction, clean up and determination of aflatoxins, ochratoxins and zearalenone was done using AOAC official method 990.33, 991.44 and 994.01 respectively with some modifications[16].

Chemicals: Methanol (CL chem. Lab) HPLC grade, n-hexane (CARLO ERBA) RPE, hydrochloric acid, 37 % dichloromethane were obtained from Scharlau, (Barcelona, Spain), sodium chloride (CARLO ERBA, MARSEILLE, France), acetone, benzene (AnalaR), acetonitrile HPLC grade, trifluroacetic acid (TFA), chloroform were purchased from (ROMIL, UK), phosphoric acid (AnalaR), and acetic acid were obtained from (Riedel-de Haën, Hannover, Germany), sodium sulphate anhydrous was purchased from Prabhat Chemicals, (Mumbai, India), citric acid, diethyl ether (Labtech Chemicals) were all profiled by El Walidien Trade and Investment Co. (Khartoum , Sudan). AFB1, AFB2, AFG1 and AFG2 were purchased from Immunolab GmbH (Kassel, Germany), OTA and OTB was purchased from Sigma–Aldrich Chemical GmbH (Germany), ZON was purchased from Supelco (USA).

Apparatus: HPLC system consisted of an LC 20AB pump, DGU 20A3 degasser unit, auto sampler (SIL-20A) and fluorescence detector (RF 10AXL) (Shimadzu, Kyoto, Japan). To measure the peak area software LC Solution Version 1.22 was used.

Analysis of Aflatoxins (AFB1; AFB2, AFG1, and AFG2)
Extraction and Clean up: Fifty gram of groundnut cake samples was transferred to one liter blender jar, containing 200 ml methanol and 50 ml 0.1N hydrochloric acid and blended for 3 min at high speed. The solution was filtered through 24 cm Whatman No 1. 50 ml of the filtrate was transferred into 250 ml separation funnel; 50 ml of 10% sodium chloride solution was added and the solution was swirled. 50 ml hexane was added and the solution was shaken gently for 30s. The two phases were separated and lower layer was drained into 250 ml separation funnel and was extracted three times with 25 ml dichloromethane. The dichloromethane extracts was combined and concentrated to 2 ml, approximately. The concentrated extract was then carefully transferred into silica gel chromatography column and then 30 ml ether: hexane (3: 1) v (volume) / v (volume) were added to wash the column. 2.0 ml of the chloroform extract was poured into the column and the beaker was washed with 0.5 ml chloroform, and thereafter 25 ml benzene: acetic acid (9: 1) v/ v was added and received into 250 ml beaker. Then 30 ml ether and, 100 ml of dichloromethane: acetone (90: 10) v/ v were added to the column to elute the aflatoxins and to evaporate the solvents. To derivatize the aflatoxins 200 µl hexane and 50 µl trifluroacetic acid (TFA) were added. The extract the mixture was shaken vigorously using vortex – Genie2 for 30 seconds and was left to stand for 5 minutes. Thereafter 1.95 ml acetonitrile: water 1: 9 (v / v) was added and the mixture was shaken for 30 seconds and was left for 10 minutes to separate. The lower aqueous layer was collected by automatic pipette and used for HPLC analysis. Similarly derivation of a working standard mixture was done, for separation and detection the HPLC operating conditions were as follows

- Column type and size: Supelcosil LC18; 150 x 4.6mm (id); 5 micron particle size.
- Oven temperature: 40°C.
- Fluorescent Detector: excitation 360 nm and emission 440 nm
- Mobile phase: water: acetonitrile: methanol (700:170:170).
- Flow rate: 1.0 ml min^{-1}.
- Injection volume (20 µL).

Analysis of Ochratoxins (OTA and OTB)
Extraction and cleanup: Briefly 50 g groundnut cake powder was transferred into 500 ml conical flask and 250 ml chloroform 25 ml 0.1 M phosphoric acid was added. The flask was securely stoppered and was shaken on a wrist action shaker for 30 min. The solution was filtered through filter paper cover with diatomaceous earth. About 50 ml of the filtrate was mixed with 40

ml hexane and loaded into chromatography column containing mixture of 2.0 g diatomaceous earth and 1ml 1.25% sodium bicarbonate. After that the OTA and OTB were removed with 75 ml acetic acid: chloroform (1:99) and was collected and evaporated to dryness in steam bath. The dry extract was dissolved in 500 µL mobile phase and was injected into HPLC, for separation and detection the HPLC operating conditions were as follows

- Column type and size: C18; 250 X 4.6 mm I.D.; 5 micron particle size
- Temperature: ambient temperature 25°C
- Fluorescence detector: excitation 333 and emission 460 nm
- Mobile phase: acetonitrile :water :acetic acid (99: 99: 2)
- Flow rate: 1 ml min^{-1}
- Injection Volume: 20 µL

Analysis of ZEN

Groundnut cake powder (50 g) was transferred into 500 ml conical flask with stopper. 25 g of diatomaceous earth and 20 ml water were added and the flask was rotated. 250 ml chloroform was added, and the flask was securely stoppered and was shaken on a wrist action shaker for 30 min and the solution was filtered through filter paper. 50 ml of the extract was transferred to 250 ml separation funnel then 10 ml saturated sodium chloride and 50 ml of 2 % sodium hydroxide were added and shaked vigorously for 1.0 min. The layers were separated and the lower layer was discarded. 50 ml chloroform was added and the solution was shaken vigorously for 1 min, the layers were separated and the lower layer was discarded. 50 ml of citric acid solution (106g in 1 L water) was added then the ZON was extracted with 50 ml dichloromethane. The extract was dried with 40 g of sodium sulphate anhydrous and was re-extracted with 50 ml of dichloromethane and the organic layer was drained through anhydrous sodium sulphate. The dry extract was dissolved with 500 µL mobile phase and was injected into HPLC, for separation and detection the HPLC operating conditions were as follows

- Column type and size: C18; 250 X 4.6 mm I.D.; 5 micron particle size
- Temperature: ambient temperature 25°C
- Fluorescence detector: excitation 236 nm and emission 418 nm
- Mobile phase: methanol :acetonitrile: water (1.0: 1.6: 2.0)
- Flow rate: 2 mL min^{-1}
- Injection Volume: 20 µL

Result and discussion

Method performance: The calibration curves, using different concentrations, was generated by plotting mycotoxins peak areas against the corresponding concentrations of calibration samples All objectives for linearity validation have been matched: coefficient of correlation r = in range from 0.993 to 0.997 was obtained for all of the analyte, indicating good calibration curves. The limit of detection (LOD) (signal-to-noise ratio = 3) was calculated to be 0.017, 0.01, 0.017, 0.01, 0.13, 0.3 and 0.6 ng / g for AFB1, AFB2, AFG1, AFG2, OTA, OTB and ZEN; respectively. The limit of quantification (LOQ) (signal-to-noise ratio = 10) was calculated to be 0.05, 0.03, 0.05, 0.03, 0.4, 0.9 and 2.0 ng / g of AFB1, AFB2, AFG1, AFG2, OTA, OTB and ZEN; respectively (Table 1).

Table1: Linearity range, limit of detection (LOD) and limit of quantification (LOQ) for different mycotoxins.

Analyte	Linear range (ng/ml)	Slope	Intercept	R^2	LOD (ng/g)	LOQ (ng/g)
AFB_1	2-18	3434627.02	+52628.24	0.991	0.017	0.05
AFB_2	0.4- 3.6	5911589.33	+12887.47	0.995	0.01	0.03
AFG_1	2-18	200000	+12733.01	0.995	0.017	0.05
AFG_2	0.4- 3.6	2695521.41	+2974.84	0.996	0.01	0.03
OTA	12.5 - 250	72.93	+2.63	0.994	0.13	0.4
OTB	25- 500	10225	+5216	0.998	0.3	0.9
ZEN	16- 128	94690	+7937	1	0.6	2

LOD: Limit of Detection, LOQ: Limit of Quantification, R^2: Correlation coefficients.

Recovery experiments were determined by spiking AFB1, AFB2, AFG1, AFG2, OTA, OTB and ZEN at different level of concentrations. The average recoveries percent and relative standard deviation percent for the analyzed mycotoxins were in range of 90.1 to 98.7 % and 0.46 to 4.88 % respectively (Table 2).

Table 2: Recovery and relative standard deviation (RSD) of different mycotoxins in groundnut cake spiked samples

Analyte	Sp.C	Groundnut cake	
		R %	RSD %
AFB_1	2.5	93.3	3.98
	5	91.7	3.11
AFB_2	1.25	90.1	4.55
	2.5	94.5	1.91
AFG_1	2.5	93.4	4.88
	5	94.9	1.5
AFG_2	1.25	95.2	1.68
	2.5	94.3	2.14
OTA	5	97.3	0.93
	10	98.7	0.46
OTB	5	97.5	0.83
	10	98.4	1.04
ZEN	25	96.1	1.91
	50	95.5	2.41

Sp.C : Spiked Concentration . R %: Average recovery Percent, RSD %: Relative Standard Deviation Percent.

Occurrence of AFB1, AFB2, AFG1, AFG2, OTA, OTB and ZON: The results for the concentration of various mycotoxins AFB1, AFB2, AFG1, AFG2, OTA, OTB and ZON in groundnut cake samples from three different states were summarized in Tables 3 and 4.

Table 3: The mean and standard deviation of different mycotoxins in groundnut cake at the three states

Mycotoxins	State	N	Mean	Std. D
AFB_1	Khartoum	15	133.84	141.7
	Gadarif	7	19.02	21.93
	Kordofan	9	140.53	106.12
AFB_2	Khartoum	11	43.23	58.17
	Gadarif	5	6.95	6.83
	Kordofan	9	26.2	17.64
AFG_1	Khartoum	2	10.76	10.61
	Gadarif	1	1.51	-
	Kordofan	1	4.32	-
OTA	Khartoum	7	7.27	3.23
	Gadarif	4	1.68	0.59
	Kordofan	3	7.29	1.99
OTB	Khartoum	3	1.25	0.26
	Gadarif	0	-	-
	Kordofan	1	2.41	
ZEN	Khartoum	3	4.67	2.87
	Gadarif	2	3.62	0.37
	Kordofan	5	4.33	1.68

N: Number of positive samples, Std. D: standard deviation.

Table 4: Mycotoxins assayed and detected in the groundnut cake at the three states

Mycotoxins	State		
	Khartoum (n=33)	Gadarif (n=21)	Kordofan (n=21)
AFB_1 (+) Rang (µg/kg) Positive (%)	15 439.31-2.93 45.45	7 64.53-3.23 33.3	7 339.51-6.12 33.3
AFB_2(+) Range (µg/kg) Positive (%)	11 188.51-3.22 33.3	4 17.99-0.21 19.1	7 50.45-1.31 33.3
AFG_1(+) Range (µg/kg) Positive (%)	2 18.26-3.25 6.1	1 1.51 4.8	2 4.32-1.34 9.5
AFG_2(+) Range (µg/kg) Positive (%)	1 11.11 3.03	0 - 0	0 - 0
OTA(+) Range (µg/kg) Positive (%)	7 12.34-2.76 21.2	4 2.54-1.21 19.1	3 9.53-5.75 14.3
OTB(+) Range (µg/kg) Positive (%)	3 1.54-1.02 9.1	0 - 0	1 2.41 4.8
ZEN(+) Range (µg/kg) Positive (%)	3 7.76-2.08 9.1	2 3.35-3.88 9.5	5 6.7-2.98 23.8

The most frequently found mycotoxin was AFB1 followed by AFB2 and OTA then ZEN. This order of the AFB1and AFB2 is consistent with the results obtained by Younis[17] for peanut and peanut products from Sudan. An amount of 15 out of 33 (45.45%) of animal feed from Khartoum state were naturally contaminated by AFB1, and 7 out of 21 (33.3%) from Gadarif and Kordofan states. AFB2 was detected in 11 (33.3%), 4 (19.1%) and 7 (33.3%) samples from Khartoum, Gadarif and Kordofan states respectively. AFG1was detected in 2 (6.1%), 1 (4.8%) and 2 (9.5%) samples from Khartoum, Gadarif and Kordofan states respectively. AFG2 was detected in one sample (3.03%) from Khartoum state.

An earlier study carried out in Sudan by Elamin[18] showed that aflatoxin was present in 10-100% of groundnut samples with an average concentration of 8.37 ± 0.61 µg kg^{-1}.

Elzupir[19] have studied the contamination of animal feed in Khartoum state by aflatoxins and ochratoxin A and he found that 66.67% of the samples analyzed were contaminated by aflatoxins at concentration ranged between 2.79 to 147.13 ng / g while the ochratoxin A has detected in 77.78% of the sample analyzed at the range of 0.22-1.59 ng / g. The different values obtained in different studies may be due to differences in the techniques used and types of samples analyzed. Recently Rodrigues[20] have investigated the occurrence of mycotoxins in feeds and feed ingredients from Middle East and Africa and have found that OTA was present in 67% of samples from Sudan and from 100% of Nigerian samples.

Salah[21] have studied the contamination of animal feed with aflatoxins and he found that 60% of the sample analyzed contaminated with aflatoxins and the types of aflatoxins found in the samples were AFB1 and AFB2.

Conclusion

The results obtained in the present investigation have shown that mycotoxins contamination in the animal feed samples is alarmingly high. This is evidently posing a dangerous problem to the poultry and livestock industry as well as to human health; a wide range of harmful effects and chronic toxicity in Sudanese animals can be easily overlooked.

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