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Full Length Research Paper

Natural occurrence of heavy metal, fungi and mycotoxins in soybean meal samples used in animal feeding in Saudi Arabia

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20 samples of soybean meal used in animal feeding in Saudi Arabia were analyzed for their content of heavy metals and contamination with fungi and/or their mycotoxins. The analyzed heavy metals were lead, copper, cadmium, nickel, chromium, arsenic and mercury, which were detected using Atomic Absorption Spectrometer. The data shows that cadmium levels in soybean meal were higher in four samples when compared to the Current European Official Regulations. The amount and generic composition of microscopic fungi in feeding soybean have been monitored. Total counts of fungi were ranged from $3.9x10^4$ to 10^5 CFU/g. From the analyzed samples, 36 species belonging to 16 genera of microscopic fungi were isolated and identified. Among the isolated genera, *Aspergillus, Fusarium* and *Penicillium* belonged to the most dangerous fungi. The level of mycotoxins detected in feeding soybean was ranged from 1 to 14.8 ppb for aflatoxins, 1 to 14 ppb for ochratoxin A and 1 to 17 ppb for zearalenone. In conclusion, feeding soybean can be seriously contaminated by heavy metals, mycotoxigenic fungal genera as well as mycotoxins that cause serious health problems for man and animals.

Key words: Aflatoxins, ochratoxins A, zearalenone, mycotoxins, heavy metal, soybean.

INTRODUCTION

Soybean is extremely important as a relatively inexpensive source of protein and oil. Soybean meal is the product remaining after extraction of the oil from whole seeds followed by grinding of the solid residue (FAO, 2004). Soybean meal is among the most valuable protein feedstuff of vegetal origin. They have highly valued dietetic characteristics and are suitable for all kinds of feeding mixtures (Gatlin et al., 2007). Soybean flour and fat-free flour is used in feeding mixtures for poultry and young cattle. Nesheim and Wood (1995) reported that soybean meal is often contaminated with undesirable substances (heavy metals and mycotoxin residues) and forbidden substances (processed animal protein, hormones, some supplements and medications).

Heavy metals are a definite human health hazard because of their bio-cumulatively (Stwertka, 1996) and the most dangerous being lead, cadmium and mercury (Fergusson, 1990; Friberg, 1984; Nordberg, 2006; Stone, 1992). Heavy metals can be widely used in all fields of life (batteries, dyes, alloys, chemical compounds, pharmaceutical and cosmetic products but contamination of soil, food, feed or water with heavy metals is increasing problem (WHO, 2001; Reese 2003; Zarogianniss, 2005).

The raw feed ingredients (grains, seeds, meals) are an optimal media for fungal development due to their high level of nutrients. Certain environmental conditions, particularly the moisture, temperature, pH and light favor fungal growth and development. Infestation may occur in the fields, upon harvesting, during storage and even during processing leading to the loss of nutrients and to the development of toxic compounds known as mycotoxins (Pitt, 2000, Yaling et al., 2008), which can cause a variety of ill effects in humans, from allergic responses to immunosuppression and cancer (Schutze et al., 2010). The Food and Agriculture Organization estimates that mycotoxins contaminate 25% of agricultural crops world-

wide (Smith et al., 1994). The most important mycotoxins are aflatoxins (AFTs), ochratoxin A (OTA), fumonisins, trichothecenes and zearalenone (Bennett and Klich, 2003). AFTs are potent carcinogens and in association with hepatitis B virus, are responsible for many thousands of human deaths (Pitt, 2000). OTA A is a probable carcinogen, and may cause urinary tract cancer and kidney damage and zearalenone causes oestrogenic effects in animals and human (Pitt, 2000). Feeding soybeans, in general, are excellent substrates that enhance mould growth, which in turn cause direct losses and risks for human and animal health via production of allergen and toxin (Milicevic et al., 2010). Chelkowski (1991) established that the amount of microscopic fungi in fodder is an important indicator of its quality and should not be higher than $1x10^5$ CFU /kg.

Contamination of food supplies by these and other naturally occurring mycotoxins is of particular concern in rural communities of developing countries (Bhat et al. 1997). The limit of < 20 ppb of mycotoxin was allowed for fodder samples and more than this limit was not allowed to be used for animal feeding (FDA, 1997). The storage temperature, moisture content, presence of oxygen and gaseous composition are the most important factors influencing the development of fungi and mycotoxin production during storage. Physiological stage of grains or sensitivity by different hybrids to fungal growth is important as well (Diekman and Green, 1992; Pitt and Hocking, 1997). Accordingly, the aims of the present study were to measure the abundance of heavy metals, fungi and their toxins in soybean meal samples, collected from Jeddah, Saudi Arabia and used in animal feeding.

MATERIALS AND METHODS

Sample collection

A total of 20 samples of soybean meal were collected from fodder markets at Jeddah, Saudi Arabia during April to October 2009. The samples were collected in clean plastic bags, transferred directly to the microbiological laboratory and stored at 4°C until analyzed.

Soybean bean moisture content and chemical analysis

The moisture content of the samples was directly determined by dry weight method (Aziz, 1987). Chemical composition and nutritional quality of soybean meals were determined as described by Karr-Lilienthal et al. (2005, 2006).

Heavy metal analysis

Heavy metal concentrations of acid digested soybean meals were measured by atomic absorption (PERKIN ELMER 300 Spectroscopy), as described by AOAC (1995), and expressed in units of mg metal/kg sample. About 1 g of each feed samples was digested using 3 ml of a concentrated mixture of perchloric and nitric acid (1:1, v/v) on a hot plate until the solution become clear. Three replicates of each sample were included and the mean value was calculated.

Fungal isolation and identification

For determinations of fungal colony-forming units (CFU/g), 20 g samples of ground soybean were soaked in 180 ml sterile tap-water containing 0.02% Tween 80 and shaked on a rotary shaker at 50 rpm for 30 min. Serial dilutions (from 10^{-1} to 10^{-5}) in sterile tap water with 0.02% Tween 80 were prepared and 1 ml aliquots were inoculated on each of three plates of Czapek-Dox agar with 1% streptomycin, which was added to inhibit bacterial growth. Petri dishes were inoculated using spread-plate technique and incubated at 25°C. Total fungal counts (CFU/g) in each sample were determined after 10 days of incubation according to Pitt and Hocking (1997) and Samson et al. (1995).

The isolated fungi were identified according to macro- and microscopic characteristics as described in Raper and Fennell (1965), Moubasher (1993), and Samson et al. (1995). The total counts (TC) of each species of fungi were calculated for gram of the used sample (TC/g).

Quantification of mycotoxins using fluorometry method

The samples were analyzed for total aflatoxins; ochratoxin and zearalenone using immunoaffinity method based on Association of Official Analytic Chemists (AOAC) method (Trucksess et al., 1991 and Bennett et al., 1994). Each sample was ground and 100 g of the resultant powder was extracted with 100 ml of a methanol/water mixture (80:20 v/v). After shaking, the mixture was filtered through a filter paper (Whatman no. 1), the filtrate was diluted (1:4) with water, and 2 ml was used to determine total aflatoxins using Aflatest P immunoaffinity column (VICAM, Watertown, MA, USA). Ochratoxin A and zearalenone were determined using Ochraprep and ZearalaTest Immunoaffinity Column Procedures (VICAM, Watertown, MA, USA), respectively. The quantities of total aflatoxins, ochratoxin A and zearalenone were determined using recalibrated VICAM Series 4 fluorometer set at 360 nm (excitation) and 450 nm (emission) as described in Bokhari (2010).

RESULTS AND DISCUSSION

Soybean meal is the single most important animal feed in many countries including North America, Africa and Asia since it is a palatable feedstuff. It is widely used as a filler and source of protein in animal diets, including chicken, cattle, horse, sheep, and fish feed (Kacaniova, 2003). The benefits of using soybean meal prepared from clean, dry, and mold and mycotoxin free soybean seeds include a consistently improved animal growth rate and feed conversion efficiency, which ultimatey enhances the animal producer's profitability and reduces susceptibility of animal to diseases. Thus, the contamination of soybean meal, used for animal feeding, with heavy metals, fungi must be examined.

Heavy metals in the collected soybean meal samples

20 samples of soybean meals, commonly used as feed materials for sheep, cow, camel and poultry, were collected and chemical analysis of each sample was determined. The mean values of nutrient composition of the soybean meal samples are shown in Table 1. The quantities of heavy metals (lead, copper, cadmium, nickel, chromium,

Table 1. The chemical composition of soybean meal samples (mg/kg).

Tested material	Range	Mean*	Metal ion	Range	Mean
Carbohydrates	240 - 310	300	Calcium	3.00 - 3.80	2.60
Soluble sugars	34.0 - 75.0	50.0	Iron	0.10 - 0.18	0.16
Total fat	10.0 - 14.0	13.0	Magnesium	2.80 - 4.40	3.20
Saturated fat	2.00 - 6.00	3.0	Phosphorus	6.00 - 9.40	7.50
Unsaturated fat	9.0 - 13.0	11.0	Potassium	16.0 - 19.5	17.5
Protein	330 - 380	364	Sodium	0.04 - 0.06	0.03

*Mean of 20 samples.

Table 2. Concentration of heavy metals (µg/g) in soybean meal samples.

Sample number	Pb^{2+}	Cu^{2+}	Cd^{2+}	Ni^{2+}	Cr^{2+}	Ar^{2+}	Hg^{2+}
1	0.40	< 0.01	0.500	0.400	0.200	< 0.01	0.04
2	4.50	< 0.01	0.500	0.260	< 0.01	0.100	0.01
3	1.10	< 0.01	0.600	< 0.01	0.100	0.060	0.09
4	0.50	< 0.01	1.100	0.100	0.900	0.040	0.05
5	0.10	< 0.01	0.200	< 0.01	< 0.01	0.040	0.01
6	1.10	< 0.01	2.500	0.600	0.600	1.400	0.08
7	0.90	< 0.01	2.200	0.630	< 0.01	< 0.01	0.01
8	0.50	< 0.01	1.000	< 0.01	0.300	< 0.01	0.01
9	0.30	< 0.01	< 0.01	< 0.01	0.400	< 0.01	0.01
10	2.00	0.080	< 0.01	< 0.01	0.100	< 0.01	0.01
11	0.90	0.040	< 0.01	1.000	< 0.01	< 0.01	0.01
12	3.00	< 0.01	1.200	1.100	< 0.01	< 0.01	0.01
13	4.00	< 0.01	1.300	0.900	< 0.01	< 0.01	0.01
14	0.90	< 0.01	1.000	0.800	< 0.01	< 0.01	0.01
15	2.80	< 0.01	0.500	0.900	0.900	< 0.01	0.01
16	0.40	< 0.01	0.400	0.900	< 0.01	< 0.01	0.01
17	0.90	< 0.01	0.400	< 0.01	0.900	< 0.01	0.01
18	0.10	< 0.01	0.500	0.400	0.100	< 0.01	0.08
19	3.30	< 0.01	0.500	1.400	0.900	< 0.01	0.08
20	2.90	< 0.01	0.900	0.100	1.900	< 0.01	0.06
Mean ± SD	1.40 ± 0.11	0.006 ± 0.00	0.75 ± 0.22	0.44 ± 0.19	0.27 ± 0.12	0.075 ± 0.01	0.08 ± 0.04

arsenic and mercury) were measured (Table 2) using Atomic Absorption Spectroscopic method and compared with that recorded by Current European Official Regulations (1 mg/kg). The concentration of lead (Pb^{2+}) varied from 0.1 to 4.5 mg/kg with a mean value of 1.59 mg/kg. The data from the analysis of soybean meal used for animals revealed that only seven samples contained lead concentration higher than 1 mg/kg. The allowed lead content in feed ingredients according to the Current European Official Regulations is 1 mg/kg. The data showed that Cd^{2+} levels in soybean meal samples ranged from 0.0 to 2.5 mg/kg and that the levels were higher in four of the 20 collected samples when compared to Current European Official Regulations. The copper concentrations in the examined feed samples were very also low and ranged from 0 to 0.08 mg/kg. The concentration of nickel was ranged from 0.0 to 1.4 mg/kg. Moreover, the concentrations of Cr^{2+}, Ar^{2+} and Hg^{2+} were in the range of 0.0 to 1.9, 0.0 to 1.4 and 0.0 to 0.08 mg/kg with mean values of 0.27, 0.075 and 0.8 mg/kg, respectively.

In Bulgaria, analysis on 152 samples of animal feed revealed that 33.9 and 20.34% of the lead and cadmium samples were higher compared to Current European Official Regulations (1 mg/kg); however the detected levels of nickel, arsenic and mercury were lower (Alexieva *et al*., 2007). Contamination of soybean meals by heavy metals may be due to accumulation of these metals during plant growth or during soybean meals processing or shipping.

Mycotoxins and fungal contamination of the collected soybean meal samples

The amount and generic composition of microscopic

Table 3. % of moisture content, number of fungal isolates, % of toxigenic isolates and quantities of aflatoxins, ochratoxin A and zearalenone (ppb) of twenty collected natural feed soybean meal samples.

Sample number	%Moisture content	Total count of fungi x10^4	No. of fungal isolate	No. of toxigenic isolate	Quantity of the toxin detected (ppb)		
					AFT	OTA	ZON
1	14.0	6.9	13	2	<1	2.7	2.0
2	13.0	8.5	28	3	6.3	9.0	8.0
3	14.0	8.7	17	3	9.0	1.8	5.0
4	11.0	7.4	24	4	4.4	2.1	5.0
5	18.0	10.0	24	2	14.8	7.2	7.0
6	11.0	6.9	16	1	<1	<1	7.0
7	9.5	3.9	13	2	5.9	3.5	<1
8	12.0	6.8	11	2	6.1	2.4	7.0
9	10.3	6.9	10	0	<1	<1	<1
10	14.0	8.0	14	1	<1	<1	3.0
11	11.0	4.9	12	1	4.7	10.4	<1
12	14.0	5.5	11	3	<1	6	4.0
13	14.0	6.9	14	2	12.2	14	17.0
14	13.0	6.8	11	2	2.9	3.8	2.5
15	14.0	7.4	1	0	<1	3.0	2.0
16	11.9	5.9	12	3	4.9	6.2	9
17	12.4	6.7	22	3	5.5	5.3	<1
18	9.0	5.7	21	2	7.8	5.2	5
19	11.0	6.3	22	2	1	2.8	5
20	14.0	7.0	27	3	5.8	3.8	3
Mean±SD	12.4±1.3	6.8±2.2	16.2±4.4	2.1± 0.9	4.5±1.9	4.6±1.3	4.5±2.3

AFT, Total aflatoxin; OTA, ochratoxin A; ZON, zearalenone.

fungi in feeding soybean meal samples have been monitored. Total counts of fungi ranged from 3.9 to 10x10^4 CFU/g. The percentages of moisture content in the collected soybean meal samples ranged from 9.5 to 14%. The number of fungal isolates in each sample was counted and the percentage of toxigenic isolates was calculated. The most contaminated sample was sample no. 2 (collected from poultry farm), which carried 28 different fungal isolates, and sample no. 20 (collected from animal stable), which was contaminated with 27 fungal isolates. Additionally, three toxigenic fungal isolates were recorded in each of the two samples (no. 2 and no. 20).

The quantities of aflatoxins, ochratoxin A and zearalenone (ppb) of 20 collected natural feed soybean meal samples were detected using imminoaffinity methods. The quantities of total aflatoxins ranged from 0 to 14.8 ppb with mean of 4.5 ppb. Quantities of ochratoxins A were in the range 0 to 14 ppb with mean value of 4.6 ppb; and finally zearalenone ranged from 0 to 17 ppb with a mean value 4.5 ppb. The limit of <20 ppb of mycotoxin was allowed for fodder samples and more than this limit was not allowed to be used for animal feeding (http://www.sciencedirect.com/science/article/pii/S1319562X09000552FDA, 1997). It is clear that no samples of soybean meals were contaminated with higher level of mycotoxins which exceeded the regulatory limit. On contrast, Bokhari (2010) reported that some feed samples were contaminated with higher contamination levels of aflatoxins and ochratoxins than 20 ppb (regulatory limit). Aflatoxins are mainly produced in food staff by *Aspergillus flavus* and *Aspergillus parasiticus* and have carcinogenic and hepatotoxic actions, depending on the duration and level of exposure (Bennett and Klich, 2003). Whereas, ochratoxins A is a nephrotoxic and carcinogenic mycotoxin that produced by some strains of *Penicillium* and *Aspergillus* (Fung and Clark, 2004). Furthermore, zearalenone is produced by several field fungi such as *Fusarium* and is common in wheat, maize and maize products, but can be also found in soybeans (EFSA, 2004).

In Egypt, El-Kady and Youssef (1993) detected aflatoxins in 35% of soybean seed samples in the range of 5 to 35 µg/kg but the other mycotoxins were not detected. Mazen et al. (1990) found that cotton seed products were naturally contaminated by aflatoxin B1 and B2 and about 16% of samples were positive for aflatoxins contamination but no citrinin, ochratoxin A, patulin, sterigmatocystin, diacetoxyscirpenol, T-2 toxin or zearalenone were detected in the samples using thin layer chromatography.

From the analyzed samples, 36 species belonging to 16 genera of microscopic fungi were isolated using Czapek-Dox agar and identified (Table 4). Among the

Table 4. The detected fungal species in the collected soybean meal samples.

Isolated species of fungi	Number of occurrence/20 samples	Incidence remark	Frequency of incidence (%)	Total count/g dry matter x10^2
Absidia corymbifera	5	M	25	1.1
Alterneria alternate	8	M	40	3.1
Alterneria solani	11	M	55	1.8
Alterneria tentuis	5	M	25	1.9
Alterneria trtiticina	7	M	35	0.7
Aspergillus candidus	9	M	45	2.1
Aspergillus flavus	17	H	85	3.9
Aspergillus fumigatus	12	M	60	3.7
Aspergillus niger	11	M	55	2.6
Aspergillus ochraceus	14	H	70	2.0
Aspergillus sydowii	12	M	60	1.9
Aspergillus versicolor	11	M	55	1.4
Botrytis cinerea	4	R	20	1.2
Cladosporium sp	5	M	25	2.1
Curvularia lunata	3	R	15	0.4
Eurotium amstelodami	3	R	15	1.5
Eurotium chevalieri	4	R	20	0.5
Fusarium coeruleum	9	M	45	1.4
Fusarium graminearum	16	H	80	1.3
Fusarium. oxysporium	7	M	35	1.1
Fusarium solani	9	M	45	1.2
Geotrichum candidum	5	M	25	0.8
Humicola brevis	4	R	20	0.5
Mucor circinelloides	9	M	45	0.7
Mucor hiemalis	4	R	20	0.7
Mucor racemosus	3	R	15	1.1
Mycelia sterilia	2	R	10	0.1
Penicillium citrinum	7	M	35	2.6
Penicillium glabrum	8	M	40	1.8
Penicillium rubrum	15	H	75	2.3
Penicillium oxalicum	5	M	25	2.1
Penicillium sp.	2	R	10	0.4
Rhizopus oryzae	7	M	35	3.1
Rhizopus stolonifer	10	M	50	2.2
Scopulariopsis	3	R	15	0.5
Trichoderma sp.	11	M	55	3.9

H, More than 13, M, more than 5, R, less than 5.

isolated genera, *Alterneria solani A. flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus ochraceus, Aspergillus sydowii, Aspergillus versicolor, Fusarium graminearum, Penicillium rubrum, Rhizopus stolonifer* and *Trichoderma* sp. were the most commonest isolates. The genera *Aspergillus, Penicillium* and *Fusarium* were among the most dangerous fungi. *A. flavus, F. graminearum* and *P. rubrum* occurred in 85, 80 and 75% of the tested samples, respectively. Milicevic et al. (2010) reported that *Aspergillus, Alternaria, Claviceps, Fusarium, Penicillium* and *Stachybotrys* genera were found in food and feed commodities, both pre- and post-harvest, and caused adverse human health effects due to production of mycotoxins. El-Kady and Youssef (1993) isolated 73 species and eight varieties belonging to 32 genera and the commonest species were *A. flavus, A. fumigatus, A. niger* and *A. alutaceus*. Furthermore, Mazen et al. (1990) isolated 39 fungal species and 16 genera from Egyptian cotton seeds, cottonseed meal and cotton seed cake and *Aspergillus* was the most frequent genus, emerging in 87 to 100% of the samples. Similarly, the most common fungal species of *A. flavus, A. niger, A. tamarii* and *Penicillium*

chrysogenum were detected in broad bean (Saber, 2007) and in coffee bean seeds (Bokhari and Aly, 2009). Moreover, 16 fungal species were isolated from physic nut seeds during one year of storage including *Alternaria alternata, A. flavus, A. fumigatus, A. niger, A. terreus, Cephaliophora irregularis, Chaetomium globosum, Cladosporium cladosporioides, Curvularia lunata, Fusarium moniliforme, F. roseum, Penicillium citrinum, P. rubrum* and *Rhizopus stolonifer* (Srivastava et al, 2011).

The presence of *Alternaria* among the fungal flora isolated from the tested soybean meal samples perhaps means the high quality of the examined soybean samples. Christensen (1987) reported that *Alternaria* occurrence serves as an indicator of recently harvested soybean or good storage conditions. At harvest time, there is a high occurrence of *Alternaria* in soybean, which can persist during the whole storage if grain moisture is low. However when the grain moisture increased, the typical storage fungi (*Aspergillus, Penicillium*) begin to grow and antagonistically effect the vitality of *Alternaria* (Wilson et al., 2002).

It is reported that soybeans support the growth of many mold species, which can produce toxins such as aflatoxins, trichothecenes (such as T-2), and cytochalasins. The quality of soybeans can vary widely, depending on environmental, agronomic, and storage conditions. In Germany, aflatoxin B1 was detected in 32 of the 51 soybean meal samples but the maximal concentration was only 0.41 µg/kg. OTA could not be detected but zearalenone was detected in 23 of the 51 samples with a maximal concentration of 18 µg/kg. Ochratoxin A was found in four samples, with the greatest concentration detected being 1 µg/kg. Furthermore, zearalenone concentrations of up to 363 µg/kg were detected in four suspicious samples of high protein soybean meal (Valenta et al., 2002). Although, 80% of maize samples were highly contaminated with zearalenone and aflatoxins, only 50% of cotton-seed cake, 30% fish meal and 40% of wheat bran were contaminated with aflatoxin B1 and B2. Thus, soybean meal appeared to be a poor substrate for mycotoxins formation compared to other food materials (Mahmoud, 2007).

Conclusion

Animal feed, including soybean meal can be seriously contaminated with organic and inorganic compounds and damaged by mycotoxigenic fungi. Organic chemicals comprise the largest group and include mycotoxins whereas the inorganic compounds include heavy metals, which collectively cause series health problem for man and animals. Toxigenic and non-toxigenic fungi are common contaminants of feed. The effects of feed contaminants and toxins ranged from reduced intake to reproductive dysfunction and increased incidence of diseases. Residues transferred to edible animal products represent another reason for concern. Comprehensive legislation is in place for the control of several of these chemical compounds and pathogens in feed. However, in many developing countries, particularly in Africa and Asia statutory control of contaminants is at best rudimentary. The scope for decontamination of feeds is limited and generally uneconomic, and prevention is the most effective practical strategy.

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