

## Occurrence of Aflatoxin B in Some Feedstuffs in Isfahan

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### ABSTRACT

Certain food and feed products are threatened by mycotoxins. Aflatoxins are a group of mycotoxins produced mainly by fungi *Aspergillus flavus* and *Aspergillus parasiticus*. There are four major aflatoxins: B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> that are usually formed together in various foods and feeds. Aflatoxin B<sub>1</sub> is known as the most commonly occurring type of aflatoxin and a potent cancer-causing agent. Because aflatoxigenic fungi can grow on a variety of feedstuff at any stage of production, this study was undertaken to determine the presence of aflatoxin B<sub>1</sub> in certain foodstuff in Isfahan. From July 2007 to May 2008, ninety seven samples of animal feeds, such as corn, wheat, barley, rice bran, cottonseed bran, dried roughage feed, and recycled bread were collected from Eastern municipal districts of Isfahan. The samples were treated with methanol and aflatoxin was extracted from the aqueous phase with chloroform, and analyzed using the thin layer chromatography (TLC). Nineteen samples out of the 97 analyzed samples were contaminated with aflatoxin B<sub>1</sub>. Highest number of contamination was seen in recycled bread samples (64%). Bread is probably the food of greatest concern in Iran, because of its predominant consumption. However, the toxin was detectable neither in wheat nor in wheat flour. Contamination of corn, cottonseed bran and rice bran were 38, 25 and 17% respectively. Results indicated that high contamination of recycled bread can be associated with a very poor storage habit. However contamination of corn, cottonseed bran and rice bran may occur during processing and storage.

**Keywords:** Mycotoxins, Aflatoxins, Aflatoxicosis, *Aspergillus*, Feedstuff

### INTRODUCTION

Globally, certain food and feed products are threatened by mycotoxins. The Food and Agriculture Organization estimates that mycotoxins contaminate 25% of agricultural crops worldwide (Gourama and Bullerman, 1995; Vasanthi and Bhat, 1998). Aflatoxins are a group of mycotoxins produced mainly by fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins have received greater attention than any other mycotoxins because of their demonstrated potent

carcinogenic effects in susceptible laboratory animals and acute toxicological effects in human (Eaton and Groopman 1994; Gourama and Bullerman, 1995; Dogliotti, *et al.*, 1998; Omer *et al.*, 1998). There are four major aflatoxins: B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> plus two additional metabolic products, M1 and M2 that are of significance as direct contaminants of foods and feeds that have toxicological and carcinogenic effects in animals. Aflatoxin B<sub>1</sub>, which is generally present together with B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> in many agricultural commodities and have been

detected in cereals, corn cottonseed and some nuts (Heathcote and Hibbert, 1976; Pitet, 1998). These toxins are usually formed together in various foods and feeds in various proportions; however, aflatoxin B<sub>1</sub> is usually the most abundant naturally occurring member of the family (Pitet, 1998). Because aflatoxigenic fungi can grow on a variety of feeds at any stage of production, processing, transportation and/or storage (Gourama and Bullerman, 1995; Pitet, 1998), this study was undertaken to determine the presence of aflatoxin B<sub>1</sub> in certain feedstuff in Isfahan.

## MATERIALS AND METHODS

### *Samples*

From July 2007 to may 2008, ninety seven samples of animal feeds, such as corn, wheat, barley, rice bran, cottonseed bran, dried roughage feed, and recycled bread were collected from Eastern municipal districts of Isfahan. Each sample was collected from three different points in the feed storage: one sample in the upper third of the storage, one sample in the middle third and the last one in the lower third (Whitaker *et al.*, 1999). The collected materials from the three points were homogenized and 200 g of the mixed sample was weighed out and powdered in a mortar.

### *Extraction*

Extraction was carried out by the method described by Whitaker *et al.* (1999) with a minor modification. Fifty gram of each powdered sample was added into a 1 liter blender containing 300 ml methanol/water (80%+2%) and blended at high speed for 3 min. The mixture was filtered through folded filter paper into a 400 ml beaker. Into 180 ml of the filtrate 20 ml of lead acetate (20%) and 100 ml of

water were added and mixed vigorously. The mixture was allowed to stand for 5 min to coagulate precipitate and filtered again. 200 ml (equal to 20 g of sample) of the resulted filtrate was transferred into a separatory funnel and defatted with 50 ml of hexane. The hexane was discarded and 50 ml of sodium chloride solution (5%) was added to the remaining aqueous phase and aflatoxin was extracted twice with 50 ml portions of chloroform. The chloroform extract was washed with 100 ml of sodium chloride solution dried by filtering into a 300 ml beaker through 50 g of granular anhydrous sodium sulfate contained in a fluted filter paper. The chloroform fraction was the evaporated to dryness under nitrogen. For analyzed of aflatoxins the residue was redissolved in 100 µl of chloroform.

### *Detection*

The thin layer chromatography (TLC) methodology described by Whitaker *et al.* (1999) was used to detect and estimate aflatoxins in the samples. The thin layer chromatographic procedure requires a 20 x 20 cm glass plate coated with 0.2 mm microcrystalline silica gel (Merck Inc. Germany). The standard used for the TLC technique was aflatoxin B<sub>1</sub> (Sigma Inc. USA), prepared at the concentration of 1 µg/ml. Five µl of each redissolved sample extracts or aflatoxin B<sub>1</sub> standard was spotted at a position 3 cm from the bottom of the plate and was developed in a solvent mixture containing chloroform / methanol (97+3). After the development was complete, the plate was examined under UV light at 365 nm in the dark room. The blue fluorescent spots with R<sub>f</sub> of about 0.68 for both the sample and standard were taken as a positive indication of the presence of aflatoxin B<sub>1</sub>.

The presence of aflatoxin B<sub>1</sub> was then confirmed by preparation of a derivative as described by Trucksess (1976) using the

remaining 100  $\mu$ l of the extract solution. As illustrated in Fig 1, after development of the TLC plate in the first direction with chloroform /methanol (97+3) by preparation of a characteristic derivative formed by treatment with trifluoroacetic acid, the plate was allowed to be seated in the dark for 30 min and was developed in the second direction with chloroform/methanol (97+7). The plate was then examined under long wavelength UV light for the blue fluorescent spot of aflatoxin B<sub>1</sub> derivative.

### RESULTS

Thin layer chromatography (TLC), also known as flat bed chromatography or planer chromatography is one of the most widely used separation techniques in aflatoxin analysis. Since 1990, it has been considered the AOAC official method and the method of choice to identify and estimate aflatoxins at levels as low as 1 ng/g (Whitaker *et al.*, 1999). The examination of the TLC plate under long wavelength UV light (Figure 2) showed the blue fluorescent spots with R<sub>f</sub> about 0.68 for both the likely positive samples (lines 2, 3 and 9) and standard (line 1). Figure 2 shows a chromatogram taken from 12 extracts of cottonseed bran samples. As can be seen the blue fluorescent spots from the sample extracts numbered 2, 3, and 9 are identical to the standard fluorescent spot numbered 1. The presence of the blue fluorescent spot of those extracts at the R<sub>f</sub> of about 0.68 was taken as positive indication of presence of aflatoxin B<sub>1</sub> in the corresponding samples. The confirmation of aflatoxin B<sub>1</sub> in a sample extract with the positive blue fluorescent spot was performed by formation of a derivative aflatoxin B<sub>1</sub> known as aflatoxin B<sub>2a</sub> (Heathcote and Hibbert, 1976; Truckess, 1976) using trifluoroacetic acid. A typical

chromatogram of the two dimensional TLC is demonstrated in Figure 3.

The size and the brightness of the blue fluorescent spots of the sample extracts under the UV light as compare to that of the aflatoxin B<sub>1</sub> standard (5 ng / spot) was taken as a measure to estimate aflatoxin contamination levels. Considering the percentage recovery of aflatoxin B<sub>1</sub> (65%) in the extraction procedure (Whitaker *et al.*, 1999), when 5  $\mu$ l of spotted sample extracts is visible under the UV light, it is reasonable to assume that the contamination levels in the positive samples could be higher than 20 ppb. Table 1 summarized the results obtained from the analyzed samples were confirmed by to be contaminated with aflatoxin B<sub>1</sub>. The highest contamination of aflatoxin B<sub>1</sub> was seen in recycled bread (64%). Contamination of corn, Cottonseed bran and rice bran were 38.25 and 17% respectively.

### DISCUSSION

The results of the present study indicate that the contaminations of corn, cottonseed bran and rice bran are significantly higher than the maximum limit suggested by FAO and WHO (Whitaker *et al.*, 1999). The action level for human food is 20 ppb total aflatoxins, with the exception of milk which has an action level of 0.5 ppb for aflatoxin M<sub>1</sub>. The action level for most feeds is also 20 ppb. However, the toxin was undetectable in the certain feedstuffs, such as wheat, wheat flour, barley and dried roughage feed (Table1). Wheat is probably the commodity of greatest worldwide concern, particularly in Iran, because of its predominant consumption. Although, wheat is grown in climates that are likely to have persistent contamination with aflatoxins, it seems that the quality of the procedures used in the storage and transport of this commodity have been adequate to prevent getting infected with

*Aspergillus*. Contrary to wheat and wheat flour, recycled bread samples were found to be highly contaminated with the aflatoxin B<sub>1</sub>. It appears that contamination has occurred mainly after the bread processing, most probably because of the poor storage facilities. It is very well known that food items stored in lack of proper storage facility for a long time before its consumption are vulnerable to get contaminated with aflatoxin. It has been reported that recycled bread may be consumed occasionally as the append food for dairy animals in Isfahan suburbs (Gheisari and Messripour, 2003). The ingestion of aflatoxin B<sub>1</sub> contaminated feeds by farm animals may be affected as a

result of the conversion of aflatoxin B<sub>1</sub> to the metabolite aflatoxin M<sub>1</sub> excreted in milk of dairy animals (Galvano *et al.*, 1998 Kim *et al.* 2000 and Yarsan *et al.* 2002 ). Therefore, a safety program should be established to meet the requirements for processing and storage of animals feed products. Furthermore, in order to achieve an acceptable level of safety of feedstuffs, it is necessary to have a regular and standard aflatoxin survey of the agricultural products for use as food and feed.

Table 1. Aflatoxin contamination of feedstuff

Kind feeds (NO)	Number contaminated	%	Aflatoxin levels
Corn (13)	4	31	> 20 ppb
Wheat (12)	0	0	ND
Barley (12)	0	0	ND
Rice bran (12)	2	17	> 20 ppb
Cottonseed bran (12)	3	25	> 20 ppb
Dried roughage feed (10)	0	0	ND
Recycled bread (14)	9	64	> 20 ppb
Wheat flour (11)	0	0	ND
Total (96)	18	19.6	> 20 ppb

ND=Not Detected

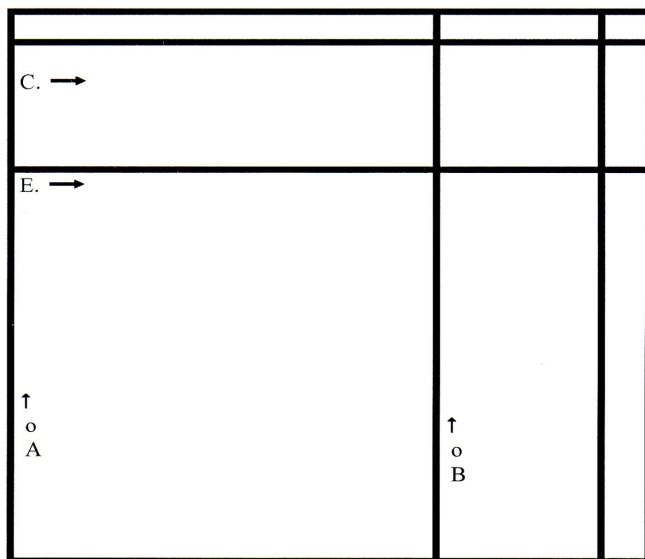


Figure 1. Schematic drawing of two dimensional TLC for derivatization procedure for confirmation of aflatoxin B<sub>1</sub>; A, represents the sample extract; B, standard of aflatoxin B<sub>1</sub>; E, aflatoxin B<sub>1</sub> from sample after development in first direction, and C, standard of aflatoxin B<sub>1</sub>, trifluoacetic acid superimposed on both C, and E, and the TLC was developed for the second direction

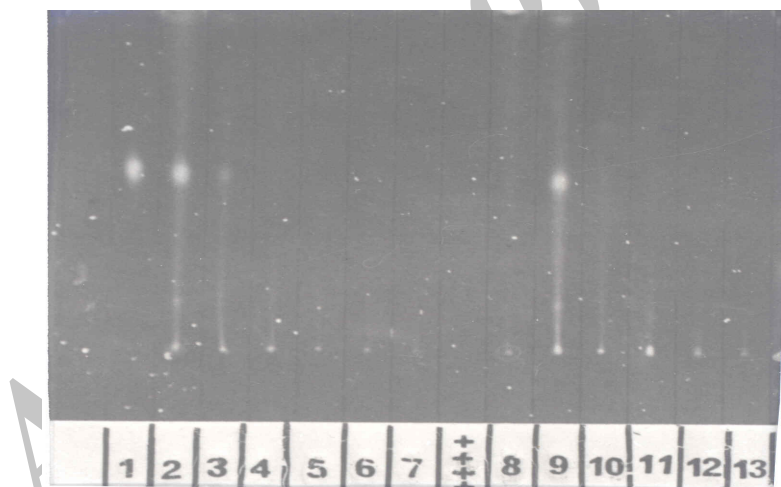


Figure 2. Chromatogram taken under UV light from of a silica gel TLC plate. Aflatoxin B<sub>1</sub> standard (line1), and 12 cottonseed bran sample extracts (lines 2-13) were developed and examined under UV light at 365 nm in the dark room. Fluorescent spots (lines 2, 3 and 9) indicated as possible contamination of the samples

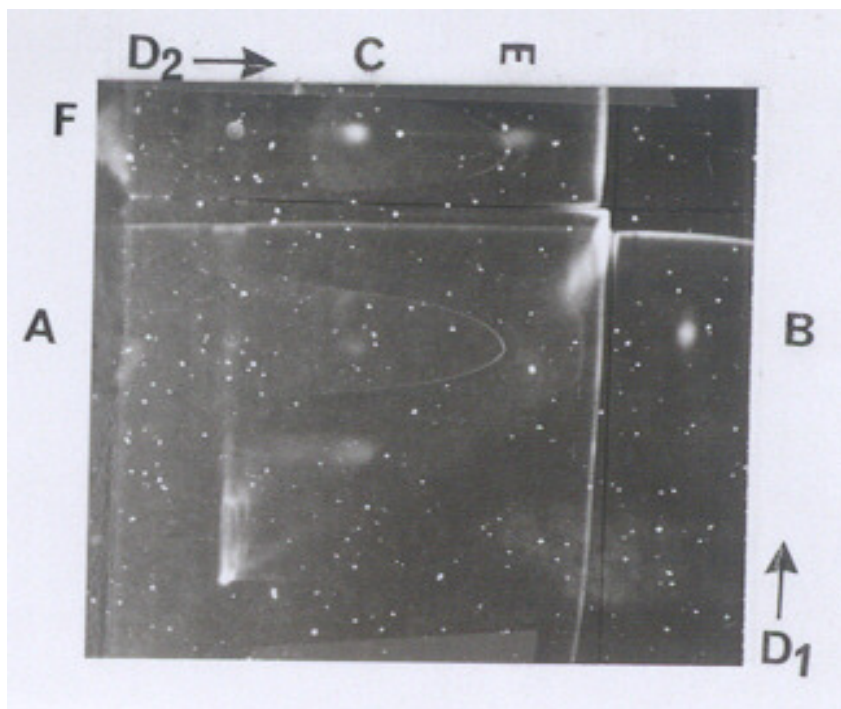


Figure 3. Two dimensional TLC for confirmation of aflatoxin B<sub>1</sub>. In the first direction (D1), aflatoxin B<sub>1</sub> from a sample extract and aflatoxin B<sub>1</sub> standard moved to A and B spots respectively. Trifluoacetic acid superimposed to the fluorescent spots at A and F spots under UV light and developed for the second direction. C, represents aflatoxin B<sub>2a</sub> and E, unreacted aflatoxin B<sub>1</sub>

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