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Determination of Aflatoxin B_1 Contamination of Commercial Mixed Feeds (For Dairy Cow) by Immunoaffinity Column Using High Performance Liquid Chromatography

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In this study, aflatoxin B_1 levels of commercial mixed feeds (for dairy cow) produced in Thrace region, Turkey were determined by immunoaffinity column and HPLC and the results were compared with the values accepted by Turkish Feed Legislation. Aflatoxin B_1 levels of 104 feed samples collected from 8 factories in 2 different seasons (June-July-August; December-January-February) ranged between 0 to 7.83 µg kg. Two samples (5.19 and 7.83 µg kg) had exceeded the tolerance limit accepted by Turkish Feed Legislation (5 µg kg). It was also recorded for samples collected in the second season (December-January-February) from the point of seasonal variation. It was noted that aflatoxin B_1 content of the samples collected in the winter. Seasonal variations with regard to aflatoxin B_1 were statistically significant ($p < 0.01$). As a result, aflatoxin B_1 levels in 98.07 % of the samples provided throughout the year did not exceed the maximum tolerance limit established by Turkish Feed Legislation.

Key Words: Aflatoxin B_1, Commercial mixed feed (for dairy cow), HPLC.

INTRODUCTION

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* Link and *A. parasiticus* Speare[1-4]. These fungi survive in a wide range of enviroments and can be found in soil, in plant and animal remains and in grains and seeds such as maize, peanuts and tree nuts[5]. These two fungi are responsible for spoilage of stored grains around the world[6]. *Aspegillus flavus* is the main fungus that causes pre-harvest aflatoxin contamination in field crops. The Food and Agriculture Organization of the United Nations (FAO) estimated that at least 25 % of the world's cereal grains are contaminated by mycotoxins, including aflatoxins[7]. As a consequence humans as well as animals, may be exposed to mycotoxins through the consumption of contaminated feed. Mycotoxins cause acute, sub-chronic or chronic toxicity[8].

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The mycotoxins of greatest significance in feed are aflatoxins, the major aflatoxins being B_1, B_2, G_1, G_2[9]. Aflatoxin contamination is common in Latin America, Africa, Asia and Australia[10]. Aflatoxin B_1 (AFB_1) is the most toxic and is hepatotoxic, hepatocarcinogenic and mutagenic to humans and animal species[9,11]. Aytug[12] reported that the toxic level of aflatoxin in feed was to 100-300 µg/kg for chickens and 100 µg/kg for ruminants. Mabbett[10] reported that the toxic level of aflatoxin in feed was 200-500 µg/kg for all animal species.

The European Union (EU) and Turkey have established maximum limits of AFB_1 in all feed materials. In EU, the maximum limit of AFB_1 was set as 20 µg/kg in all feed materials, complete feedingstuffs for cattle, sheep, goats, pig and poultry (except for young animals)[13]. The legal limits[14] (5 µg/kg) for AFB_1 for dairy cow.

The objective of the present study was to investigate the contamination level of AFB_1 commercial mixed feed (for dairy cow) produced in Thrace region, Turkey. The findings of this study are important in advising policy makers where put more emphasis when designing mould and aflatoxin management strategies in the region.

EXPERIMENTAL

Source of samples: A total of 104 feed samples obtained from 10 kg each, were collected during the period of June 2006 to February 2007 in 2 different seasons (June-July-August; December-January- February) from 8 factories in the Thrace region, Turkey. They were collected directly from the production line and sent to the laboratory. The samples were homogenized, quartered to obtain 1 kg laboratory sample and tested for fungal analysis. They were then stored at 4 ºC for mycotoxin analysis.

Mycotoxin analyses: Aflatoxin B_1 analysis was performed by HPLC according to the methodology proposed by AOAC[15]. A 50 g portion of feed was extracted with 100 mL acetonitrile:water (90:10, v/v) by blending for 2 min into a blend jar. The mixture was filtered through filter paper Whatmann No. 4 (Whatmann, Inc., Clifton, New Jersey, USA) and a 3 mL aliquot was taken and placed into 10 mL culture tube. A multifunctional column (Mycosep 224 MFC, Romer Labs® USA) was pushed into the culture tube. The extract was forced through frit, through 1-way valve and through packing material. The purified extract (0.5 mL) was collected in a column reservoir. An aliquot (200 µL) was derivatized with 700 µL trifluoroacetic acid-acetic acid-water (20:10:70, v/v). The derivatized aflatoxin was analyzed by using a HPLC system. Chromatographic separations were performed on a reversed phase column (VARIAN, 150 mm × 4.6 mm id., 5 µ particle size). Water-methanol-acetonitrile (4:1:1, v/v) was used as mobile phase at a low rate of 1 mL min^{-1}. Fluorescence of aflatoxin derivatives was recorded at excitation and emission wave lengths of λ 360 nm and λ 460 nm, respectively. Standard curves were constructed with different levels of AFB_1. This toxin was quantified by correlating peak heights of sample extracts with those of standard curves (Fig. 1). The detection limit of the analytical method was 1 ng g^{-1}.

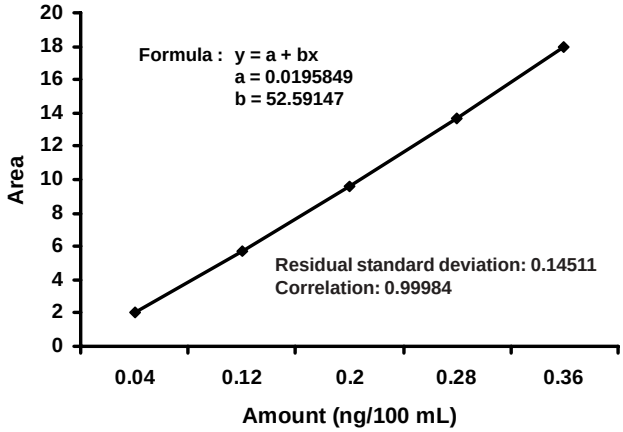

Fig. 1. Calibration curve of AFB_1

Isolation and identification of moulds: 104 Commercial mixed feeds (for dairy cow) each sample lot was assayed by the direct plating technique for internal mould infection[16,17]. The commercial mixed feed samples were surface disinfected for 1 min with sodium hypochlorite (10 % commercial bleach, Jik, Rickitt Benkiser, East Africa Ltd), washed 3 times sterile distilled water and placed directly on the surface of malt salt agar prepared by mixing 68 g of sodium chloride, 10 g of malt extract, 20 g of agar and 1 L of distilled water[18]. This media is used for growing mould species requiring a high osmotic concentration. The non-osmophilic moulds were identified on malt extract agar (Becton Dickinson Microbiological Systems, Becton Dickinson and Company, Sparks, MD 21152, USA) prepared by mixing 33. 6 g in 1 L of distilled water as recommended by the manufactures. Feed each samples were placed directly on each agar plate. The plates were incubated upright at 30 ºC for 42-72 h. After sufficient growth, some of the cultures that could not be identified were transferred onto Potato Dextrose Agar (PDA) for purification and were identified using the keys recommended by Tuite[19] and Singh *et al.*[20].

Data analysis: Statistical analysis was carried out to determine differences of AFB_1 contents commercial mixed feed (for dairy cow) samples collected different seasons. All data were subjected to analysis of variance (ANOVA), whereas differences between means were significance by Duncan's multiple range test in the general linear model of SPSS statistical programme (SPSS ver 10.0, SPSS Ltd. Working, UK). Differences between means were considered significant at $p < 0.01$.

RESULTS AND DISCUSSION

Mycological survey: The frequency of fungal genera is shown (Table-1). The most frequent genera found were *Penicillium*, *Aspergillus* followed by *Rhizopus* and *Mucor*. This study reveals the commercial mixed feed samples and the natural occurrence of *Penicillum* spp. and *Aspergillus* spp. mycotoxins.

TABLE-1
COMMERCIAL MIXED FEEDS FUNGAL GENERA FREQUENCY

Fungal genera	Frequency (%)	Fungal genera	Frequency (%)
Penicillium spp.	32.5	*Rhizopus* spp.	2.5
Aspergillus spp.	4.5	*Mucor* spp.	2.5

Moderate levels of colony counts were determined in present study. They did not exceed the feed hygienic quality limits (1×10^5 CFU g^{-1})[21,22]. Present results showed that *Penicillium* species had the highest isolation frequencies followed by *Aspergillus, Mucor, Rhizopus* spp. Many studies have shown that most feeds have species from *Aspergillus* and *Penicillium* genera as predominant flora Bragulat *et al.*[23] whereas Magnoli *et al.*[24] found *Fusarium* and *Penicillium* species as prevalent in poultry feeds.

Aspergillus and *Fusarium* toxins are the greatest concern for animal health. However, the high frequency of *Penicillum* spp. as well as the presence of *F. subglutinans*, must be considered a potential risk factor[24].

Most studies indicate that there is no correlation between the presence of a toxin and the producing fungus in the same substrate, but the presence of toxicogenic fungi in feeds may be an indicative of their potentiality to produce mycotoxins. When the storage conditions are not appropriate and the toxigenic fungus is present, this may produce a mycotoxin. Further integrate studies of the mycotoxin natural occurrence and efficient prevention methods during feed processing should be encouraged and conducted simultaneously.

Mycotoxin analyses: The occurrence of mycotoxins was defined as the percentage of feed samples in which AFB_1 (68.26 %) was detected (Table-2, Fig. 2 and 3). It was also recorded there to samples collected in the second season (December-January-February), from the point of seasonal variation. It was realized that the AFB_1 content of the samples collected in the winter. Seasonal variations with regard

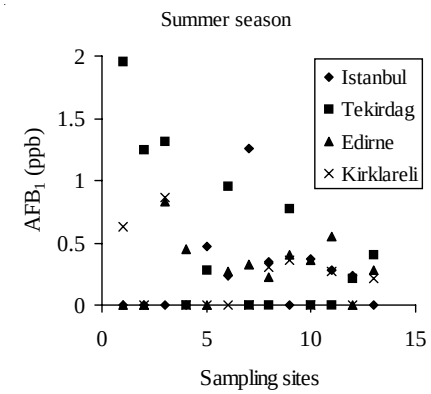

Fig. 2. Distribution of AFB_1 levels of commercial mixed samples according to summer season

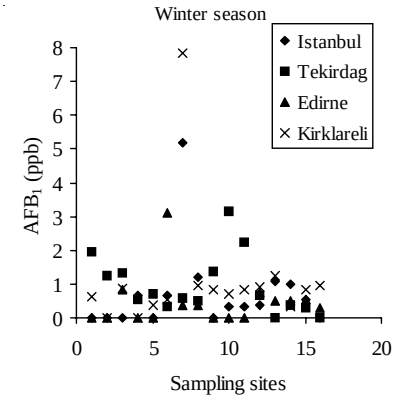

Fig. 3. Distribution of AFB_1 levels of commercial mixed samples according to winter season

to AFB_1 were statistic-ally significant ($p < 0.01$). As a result, AFB_1 levels in 98.07 % of the samples provided throughout the year did not exceed the maximum tolerance limit established by Turkish Feed Legislation. Dalcero *et al.*[25] found AFB_1 occurring in 48 % of feedstuff samples. Magnoli *et al.*[24] observed that fumonisins and AFB_1 co-occurred in 60 % of the samples. These studies coincide with present studies.

Although the amounts of the toxins detected on feedstuff, in Turkish region, were lower than the regulation limits established, present results showed that many samples had AFB_1 levels close to the permissible maximum and which could affect young animals. Moreover, a synergistic toxic response is possible in animals on simultaneous exposure. The recent association of AFB_1 with carcinogenesis in human beings has increased concern over the possibility that *Fusarium* mycotoxins may be transferrred into milk, eggs and meat[26]. In general, the highest AFB_1 levels were found during the winter season of sampling. It is suggested unsuitable storage conditions of raw materials in the sampling area. Mycotoxins in feed require effective surveillance. For that reason, quality control procedures may facilitate a good fungal identification and toxins detection.

TABLE- 2
AFB_1 CONTENTS OF THE COMMERCIAL MIXED FEEDS
COLLECTED IN THE SUMMER AND WINTER

	Summer season (June-July-August)				Winter season (December-January-February)			
	Istanbul	Tekirdag	Edirne	Kirklareli	Istanbul	Tekirdag	Edirne	Kirklareli
1	BD	1.95	BD	0.63	0.65	0.52	BD	BD
2	BD	1.25	BD	BD	BD	0.69	BD	0.37
3	BD	1.32	0.83	0.87	0.66	0.35	3.09	0.52
4	BD	BD	0.45	BD	0.87	0.60	0.36	7.83
5	0.47	0.28	BD	BD	5.19	0.48	0.39	0.94
6	0.24	0.95	0.27	BD	BD	1.37	BD	0.82
7	1.26	BD	0.33	BD	0.34	3.14	BD	0.69
8	0.35	BD	0.23	0.30	0.32	2.25	BD	0.81
9	BD	0.78	0.41	0.36	0.39	0.67	0.75	0.90
10	0.37	BD	0.36	BD	1.08	BD	0.49	1.25
11	0.28	BD	0.55	0.27	0.98	0.36	0.49	0.34
12	0.24	0.21	BD	BD	0.52	0.28	0.55	0.83
13	BD	0.41	0.28	0.21	BD	BD	0.31	0.94
Min.	0	0	0	0	0	0	0	0
Max.	1.26	1.95	0.83	0.63	5.19	3.14	3.09	7.83
Average	0.15	0.55	0.29	0.20	0.85	0.82	0.49	1.25

BD = Below detection limit.

Conclusion

In conclusion, the results of the study showed that AFB_1 contents of commercial mixed feed (for dairy cow) collected from 8 factories in 2 different seasons did not exceed the tolerance limit suggested by Turkish Feed Legislation (0.5 µ/kg). Mycotoxicosis is very important for animal health and there is not enough data in Turkey. For this reason, more emphasis should be given to routine aflatoxin and also to

other mycotoxins in feed materials. To protect the animal health against mycotoxins, factors influencing mycotoxin production in feedstuffs have to be clearly defined and preventive measures should be taken to decrease the risk of mycotoxicosis.

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