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Monitoring of Aflatoxin B_1 in Livestock Feeds Using ELISA and HPLC

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Abstract Because of potential health hazards of aflatoxins for humans, the present study was conducted to monitor aflatoxin B_1 (AFB_1) in livestock feeds. A total of 249 samples of feeds collected in Korea were analyzed by DC-ELISA for qualitative analysis of AFB_1. Then, 27 samples that were verified to contain AFB_1 by DC-ELISA were quantitated by HPLC/FLD. HPLC/FLD analysis revealed that only one sample collected from a farm contained 11 ppb of AFB_1, whereas the other samples collected from feed companies did not contain AFB_1. The presence of AFB_1 was further confirmed by LC/MS analysis. TLC analysis indicated that the result of the DC-ELISA was most likely due to possible contamination of other mycotoxins rather than AFB_1. In conclusion, HPLC/FLD analysis following DC-ELISA is necessary for rapid and accurate detection of AFB_1.

Key words: Aflatoxin B_1, feed, ELISA, HPLC/FLD, LC/MS

Aflatoxins, which are potent carcinogenic, mutagenic, and teratogenic metabolites, can contaminate human foods and animal feeds [2, 5, 6, 18, 19]. Such contamination is the result of invasion by the molds before and during harvesting, or improper storage of agricultural commodities [5, 15, 18]. Humans can be exposed to aflatoxins by direct ingestion of contaminated foods or indirect consumption of foods prepared from animals previously exposed to aflatoxins in feeds [7, 18]. Aflatoxins are secondary metabolites produced by the molds, such as *Aspergillus flavus*, *A. parasiticus*, and *A. nonimus*, which are found worldwide in air, and dead plants and animals [15, 19]. Four main aflatoxins [aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2)] are difuranocoumarin derivatives, and they are potent liver carcinogens in a wide variety of animal species, including humans [13, 21]. Among this group of toxins, AFB_1 was found to be one of the most potent environmental carcinogens [4, 9]. Their toxicity has caused severe health and economic problems [4]. The recent rapid development of knowledge concerning the aflatoxins has led to an increasing awareness of these potential chemical environmental contaminants [9]. The regulation in Korean limits AFB_1 and total aflatoxins including AFB_1, B_2, G_1, and G_2 to 10 ppb for young animals and 20 ppb for mature animals, respectively, in the feeds. The United States and Europe also regulated that feeds must contain total aflatoxins less than 20 ppb. In Korea, most agricultural products used as the feeds have come from other countries, and therefore, they have a great possibility to be contaminated by aflatoxins. However, the feeds newly produced by the feed companies are not likely to be seriously contaminated, because many feed companies mix the contaminated feeds with the fresh ones to dilute AFB_1 to lower than 10 ppb. Severe contamination is likely to occur during transportation and storage in farms. Therefore, continuous monitoring of aflatoxins in livestock feeds is necessary to ensure the health of livestock and humans. The present study was undertaken to effectively monitor AFB_1 in livestock feeds using ELISA and HPLC/FLD.

A total of 171 samples was collected from the feed companies located in Gyeongin (99 samples), Chungcheong (19 samples), Jeolla (21 samples), Gyeongsang (25 samples), and Jeju (7 samples) regions. Seventy-eight samples were directly collected from the animal farms located in the Gyeongin (24 samples), Chungcheong (20 samples), Jeolla (20 samples), and Gyeongsang (14 samples) regions. A total

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Table 1. Numbers of AFB_1-positive feed samples detected by DC-ELISA, HPLC/FLD, LCMS, and TLC analyses.

Sampling region	Sampling place	Number of samples	Number of AFB_1-positive samples detected by			
			ELISA	HPLC/FLD	LC/MS	TLC
Gyeongin	Feed company	99	16	-	-	-
	Farm	24	1	1	1	-
Chungcheong	Feed company	19	2	-	-	-
	Farm	20	-	-	-	-
Jeolla	Feed company	21	7	-	-	-
	Farm	20	-	-	-	-
Gyeongsang	Feed company	25	1	-	-	-
	Farm	14	-	-	-	-
Jeju	Feed company	7	-	-	-	-
	Farm	-	-	-	-	-
Sub total	Feed company	171	26	-	-	-
	Farm	78	1	1	1	-
Total		249	27	1	1	-

of 249 samples of feed was analyzed for the contamination of AFB_1 by DC-ELISA (direct competitive enzyme-linked immunosorbent assay) [1]. Thus, 60% methanol (100 ml) and NaCl (5 g) were added to 20 g of feed sample, shaken for 20 min, and centrifuged at 3,000 rpm for 5 min at 4°C for extraction. The supernatant was filtered with filter paper No. 1 (Whatman, Maidstone, England) and glass microfiber paper pretreated with 1 ml of 100% methanol. An aliquot was taken and diluted 10 times with PBST (PBST [1 l] contains Tween 20 [0.05%], NaCl [8 g], KCl [0.2 g], Na_2HPO_4 [1.44 g], KH_2PO_4 [0.24 g], and distilled water) for ELISA application [10, 11, 20]. The samples shown to contain AFB_1 by DC-ELISA were further analyzed by HPLC/FLD for quantification. The HPLC/FLD analysis of aflatoxins followed the determination of mycotoxin method in the 2002-06 official feed regulation, announced by the Ministry of Agriculture and Forestry, Korea. The standard solution for HPLC/FLD analysis was prepared by dissolving AFB_1 (Sigma, St. Louis, U.S.A.) in acetonitrile-benzene (v:v, 2:98) solution [1, 10, 12]. The analytical procedures consisted of extraction, purification (clean-up), and quantitative determination [5]. Feed sample was extracted with methanol, diluted with water and n-hexane, and the extracts were purified using a chromatograph column (22×330 mm) with chloroform, sodium sulfate anhydrous (Na_2SO_4), and silica gel (Merck A.G., Darmstadt, Germany). HPLC (Agilent 1,100, Agilent Technology, U.S.A.) was used for separation and determination of AFB_1 in the extracts. The LC conditions were as follows: flow rate, 0.7 ml/min; column, Reverse-phase C_{18} column (4.6 m×150 mm); mobile phase, 30% acetonitrile (CH_3CN); detector, fluorescence detector; excitation filter, 365 nm; emission filter, 418 nm [17]. Mass spectrometry (LC/MS) was carried out to confirm the AFB_1 under the conditions as follows: column, SCX (2.0 m×150 mm); mobile phase, 40% methanol, 10 mM ammonium acetate; flow rate, 200 μl/min; AFB_1 standard solution, 0.0313 ng/ml. The thin layer chromatography (TLC) analysis was also performed according to the determination of mycotoxin method in the 2002-06 official feed regulation, announced by the Ministry of Agriculture and Forestry. Ten μl of the filtrates were spotted on a TLC plate (20 m×20 m, Merck, Art.5554), and the plate was developed by chloroform-acetone (v:v, 90:10) solution. AFB_1 on the plate was detected under a UV lamp at 365 nm [14, 22].

The numbers of AFB_1-positive samples determined by DC-ELISA, HPLC/FLD, LC/MS, and TLC are described in Table 1. Among the 249 feed samples examined by DC-ELISA, 27 samples were found to be AFB_1-positive. Most of them (26 samples) were collected from feed companies, which was quite unexpected: We expected that the samples collected from farms should be more contaminated. The 27 AFB_1-positive samples revealed by DC-ELISA were subjected further to quantification of AFB_1 through HPLC/FLD. HPLC/FLD analysis revealed that only one sample collected from a farm contained 11 ppb of AFB_1, whereas the remaining 26 samples that were collected from feed companies did not contain AFB_1: The 26 samples collected from feed companies had peaks similar to that of AFB_1. This was most probably due to the presence of compounds chemically closely related to AFB_1, such as AFB_2, AFG_1, and AFG_2 [14]. The result of LC/MS analysis for AFB_1 was in accordance with HPLC/FLD analysis, confirming that only one sample, which was confirmed to be AFB_1-positive by HPLC/FLD analysis, contained AFB_1 (Table 1). Although the ELISA procedure described in this study was a relatively simple and rapid test to perform [16], it still needs to be improved for more accurate analysis. Therefore, HPLC/FLD analysis following DC-ELISA performed in this study appears to be recommendable for both rapid and accurate detection of AFB_1. The TLC analysis could not

Table 2. R_f values on silica gel TLC plate.

Sample	R_f values
AFB_1	0.53
Gyeongin farm	0.17
Jeolla farm	0.31
Gyeongsang farm	0.51

Mobile phase: chloroform/acetone (90/10) 100 ml.

detect AFB_1 in all the samples, indicating its low sensitivity (Table 1) [12, 14, 22]. The R_f values of some samples obtained from the TLC analysis are given in Table 2. Although the same R_f value with AFB_1 was not detected, each sample showed a distinctive developed phase, indicating the possibility of the presence of mycotoxins other than AFB_1.

Among the 249 samples examined in this study, only 1 sample was found to contain 11 ppb of AFB_1, which is lower than the Korean regulation (20 ppb for blended feed). However, many different commodities are prone to aflatoxin contamination [2, 3, 9, 12, 15, 18, 19, 23] and aflatoxin-contaminated diet has been associated with the elevated incidence of liver cancer, decreased immunity, kwashiorkor, and growth stunting in the subregion [3]. Naturally occurring mixtures of aflatoxins and AFB_1 have been classified as group 1 human carcinogens [3, 8]. Because of these widespread and high-toxicity aflatoxins, many organizations worldwide continually monitor aflatoxin-susceptible commodities for the incidence and levels of aflatoxin contamination [18]. The International Agency for Research on Cancer (IARC) has classified aflatoxin B_1 as a probable human carcinogen [22]. However, in its evaluation of acceptable aflatoxin intake levels, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) did not identify an acceptable daily intake value. They did recommend, however, that chronic exposure to aflatoxin contamination be reduced to the lowest practical level. Therefore, in view of the importance of aflatoxins to the economy and public health, systematic control of both local and imported cereals, food, and feed products, and continuous monitoring should be established in Korea to obtain scientific data for the national mycotoxin control regulation system.

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