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Investigation of aflatoxin and metal concentrations in animal feeds and feed ingredients from Turkey

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Abstract: The contents of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) aflatoxin G_2 (AFG_2), aluminium (Al), arsenic (As), barium (Ba), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), selenium (Se), tin (Sn) and zinc (Zn) in animal feed samples in Turkey were screened. Eighty animal feeds and feed ingredients were collected from different cities in Turkey. Aflatoxins were determined using the HPLC instrument after pre-separation using immuno affinity column, and also the instrument of ICP-MS was used for metal determinations. All types of animal feed samples have led concentrations lower than the maximum EU and Turkey regulation limit, while 1.25% and 11.8 of mixed and feed additive samples had AFB_1 and Hg concentrations higher than the maximum limits, respectively. A single correlation analysis was used to determine the relationship between AFB_1 and total AFs and metal contents in mixed animal feed samples ($p<0.05$). A strong positive correlation was found between As and AFB_1 and total AFs contents; whereas Cr was correlated negatively to AFB_1 and total AFs, using single correlation analyses.

Keywords: Animal feed; aflatoxins; metals; HPLC, ICP-MS; correlation analyses.

1. Introduction

Mycotoxins possessing low molecular weights (MW ≤ 700) are chemical compounds produced by certain molds (*Aspergillus*, *Fusarium*, *Penicillium*, *Aternaria*, *Claviceps*, *etc.*). Today, more than 500 species of mycotoxin are known while aflatoxin, ochratoxin, deoxynivalenol, zearalenone, fumonisin, and patulin are among the well-known mycotoxins. Mycotoxins can be produced in plant products by means of fungal contamination during either pre-harvest (field level) or post-harvest conditions (storage, transport, and processing) [1,2]. Aflatoxins are secondary metabolites of *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius*, *Aspergillus bombycis* species [3,4]. There are four types of aflatoxins: aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin B_1 (AFB_1), and aflatoxin B_2 (AFB_2). *Aspergillus flavus* is responsible for the production of aflatoxin B_1 and B_2 while aflatoxin G_1, G_2, B_1, and B_2 can be produced by *Aspergillu sparasiticus* [5]. Aflatoxins are difuran-based coumarin compounds. They are classified in two sub-groups as difurocoumarocyclopentenone (AFB_1 and AFB_2) and difurocoumarolactone (AFG_1 and AFG_2). They have fluorescence property and absorb strongly UV light

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at 362 nm; and blue (from AFB_1 and AFB_2), green (AFG_1), and blue-green (AFG_2) colors are exhibited by emitting light at 425-450 nm [6].

The suitable factors for the production of aflatoxins are of biological, chemical, and environmental origins. Biological and chemical factors are the source elements of plant products or foodstuffs involving water activity, pH, chemical composition, and level of maturity at harvest. Environmental factors are the temperature conditions, relative humidity conditions, oxygen level, amount of light, climate of the place where the product is grown, the geographic region, and the processing of the product (storage, drying techniques) [1].

Aflatoxins are commonly present in herbal products. Nuts (hazelnuts, pistachios, peanuts), grains (wheat, corn, barley, rye, rice, oats), cereal products (bran, flour), oil seeds (sesame, sunflower, rapeseed, cottonseed), coffee beans, cocoa, spices (black pepper, red pepper), and dried fruits (raisins, dried figs) are the most encountered plant-derived products [7]. Animal feeds are contaminated with aflatoxins due to the raw materials used in feeds like millet, corn, sorghum, and bagasse of peanut, sunflower, soy, and cotton seed. Additionally, aflatoxins can be found in dairy milk and milk products as aflatoxin M_1 metabolite [8[. The effects of aflatoxins show varieties in animals (monkeys, trout, rats etc.) depending on exposure dose, duration, type, genus, dietary or nutritional status, and general health status. Additionally, they can cause serious health complications such as cancer, liver damage, decrease in milk production, immune suppression, and anemia [6]. People can be directly or indirectly exposed to aflatoxins [5]. According to a report published in 1993 by the International Agency for Research on Cancer, aflatoxin B_1 is classified as a carcinogenic substance (group 1) for humans [9].

Determination of aflatoxins in food and feed samples is generally based on the extraction procedure of the toxin from the matrix and quantitation with different analytical techniques. Immunosensors-immunochemical methods [10-12], Thin-layer chromatography (TLC) [8], Gas chromatography [13], High-performance liquid chromatography (HPLC) [8,14] and Fourier transform infrared spectrometry (FTIR) [15] have been performed for qualitative and quantitative analyses of aflatoxins. In the extraction of the aflatoxins from matrices, some organic solvents such as chloroform, dichloromethane, methanol, acetonitrile, and acetone [16,17] were used.

Aflatoxins and elements are potential environmental contaminants that cause serious problems for human health, and it is important to monitor these pollutants in animal feed [18]. Metal-based fertilizer application, pesticides, post-harvest contamination, industrial-based activities, and anthropogenic activities can cause soil contamination [18,19]. Plant materials grown in contaminated soils can be eaten by grazing animals and then transferred to human food through milk, meat, or other animal products [20]. Animal foods containing healthy food proteins are important for the human diet. For this reason, eaten by animal are the main determinants of human health and quality [21,22]. However, all elements of a high level in different animal feed species can cause some negative health problems in animals. Some elements such as Cd, Pb, Zn, and Cu have harmful effects on poultry feeds. In poultry, these elements accumulate in the kidneys and liver and cause toxic effects [23]. For these reasons, the determination of the elements in animal feeds is necessary for human health [24]. Limited information is available on the presence and determination of elements in feed materials, premixes, and finished feed. In recent years, many atomic spectroscopic techniques (flame atomic absorption spectrometry, graphite-furnace atomic absorption spectrometry, inductively coupled plasma optical emission or mass spectrometry) have been widely used in quantitative determinations of elements in different matrixes [25-28]. Inductively coupled plasma mass spectrometry (ICP-MS) is preferred to other spectroscopic methods because of its advantages such as precision, high selectivity, wide linear range, and multiple element determinations.

In this work, the determination of elements and AFB_1, AFB_2, AFG_1, and AFG_2 have been undertaken in different types of animal feed samples. The interrelationships between the elements and the content of aflatoxins in animal feed samples have not been given in the literature. In addition, the relationship between the concentrations of elements and aflatoxins in animal feeds have been undertaken statistically using correlation analysis for the first time.

2. Experimental

2.1. Reagents and Solutions

KBr (99.9 %), HNO_3(65 %) (Suprapur®), H_2O_2 30%, NaCl (99.9 %), H_2SO_4 (99.9 %), KOH (99.9 %), and HPLC grade acetonitrile, acetone, and methanol were purchased from Merck (Darmstadt, Germany). The mix standard stock solution containing AFB_1 (1000 ng/mL), AFB_2 (300 ng/mL), AFG_1 (1000 ng/mL), and AFG_2 (300 ng/mL) were obtained from Supelco Chemical Company (USA). The mix standard stock solution was diluted with methanol to 10 mL in order to prepare second level standard stock solution. Working standard solutions were prepared from second level standard solution with methanol-water mixture. The certificated reference material (ERM-BE 376 Compound Feeding stuff) was taken from European Reference Material. Argon and helium purity was higher than 99.999 %.

2.2. Insturuments

Agilent 7700 ICP-MS instrument (Santa Monica, CA, USA) was used for elemental analysis and the auto-sampler (ASX-500) was also used. Operating conditions for ICP-MS measurements were: forward power 1.6 kW, sampling depth 10 nm, sample uptake flow rate 0.3 mL/min, carrier gas flow rate 0.35 L/min, dilution gas flow rate 0.6 L/min, cell gas flow rate He 5 mL/min and spray chamber temperature 2 ºC. A microwave device (MARSXpress, CEM Corporation, Matthews, North Carolina) was used for element digestion from the feed samples. In order to homogenize of samples *blender* (Waring 1200) was used.

The chromatographic analyses of the aflatoxins were carried out by utilizing an Agilent 1100 Series HPLC system (Palo Alto, CA, USA). The chromatographic separations of aflatoxins were achieved on the C18 analytical column (250 mm × 4.6 mm, 5 µm) obtained from ACE (Aberdeen, Scotland). The aflatoxins were detected by performing an Agilent Fluorescence Detector (FLD). Immunoaffinity columns (AflaTest) were purchased from VICAM (USA). A stainless steel blender possessing 1 L volume was taken from Waring Products, Inc. (USA).

2.3. Sample Collection and Preparation for Aflatoxin and Metal Determinations

Eighty different types of animal feed (three sample of each) were supplied from various feed factories or companies in Kocaeli, Sakarya, and Yalova areas in Turkey. From the 80 feed samples, 36 of them were compound feed, 18 of them were cattle feed (feeding, milk and young cattle feed), three of them were lamb feed, six of them were poultry feed (chicken growth feed and chick feed), and 17 of them were feed materials (7 types of soybean meal, 8 types of sunflower meal, one type of corn, and one type of feedstuff).

All samples were ground to fine powder with a stainless-steel blender. Each 500 g of sample was sieved, and then the powder containing particle of one mm in size was collected. After the samples were placed into polyethylene storage containers, they were labeled, numbered, and kept at +4 ºC until analysis. The concentrations of total aflatoxin, AFB_1, AFB_2, AFG_1, AFG_2 and studied elements were screened using the procedures given below.

2.4. Metal Determination

The total concentrations of studied elements were screened by using after microwave digestion. One gram of compound feed, lamb feed, cattle feed, poultry feed, and feed additive samples were transferred into microwave digestion vessels containing six mL of 65% HNO_3, and then two mL of 30% H_2O_2 was added to each vessel. Digestion conditions for the microwave system were applied as follows: 250 W for two min, 0 W for two min, 250 W for six min, 400 W for five min, and 550 W eight min, vent for 15 min. After cooling to room temperature, ultra-pure water was added to a volume of 50 mL, and then the element concentrations were determined by ICP-MS [29]. All experiments were performed in triplicate. The calibration graphs were prepared using multi-element standard solutions contained 0.5, 1.0, 5.0, 10.0, 20.0, 30.0, 40.0, and 50.0 µg/L for As, Ba, Cd, Co, Cr, Hg, Ni, Pb, and Sn 25.0, 50.0, 100.0, 250.0, and 500.0 µg/L for Al, Ca, Cu, Fe, Mg, Mn, Na, Se, and Zn. The limits of detection calculating to the 3*s* criterion were 16.4, 7.6, 4.8, 200, 10.3, 4.6, 8.5, 12.3, 3.8, 36.7, 78.0, 12.3, 578, 0.97, 7.8, 9.6, 46.4,

and 0.40 µg/L for Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, Mg, Mn, Na, Ni, Pb, Se, Sn, and Zn, respectively.

2.5. Afltoxin Determination

The AOAC official standard method was used for the analysis of the animal feeds, feed ingredients, and certified materials [30]. Ten gram of NaCl and 250 mL of MeOH:H_2O (70:30) solution was added to 50 g of the sample, respectively for HPLC analysis. The sample was mixed by using the blender at medium speed for 2 min. The extracts were filtered by Whatman (No-4) filter paper. 20 mL of filtrates were diluted with 40 mL of double distilled water. 15 mL of mixture was passed through immune affinity column at a flow rate of about 1-2 drop/s using a syringe. Then, the immune affinity column was washed with 10 mL of water. Aflatoxins were eluted by using one mL of methanol. The eluate was diluted with water and then transferred into amber HPLC vials for injection.

Aflatoxin B_2 and G_2 can produce analytically useful fluorescence at adequate intensity in aqueous mobile phases. Yet, fluorescent intensities of aflatoxin B_1 and G_1 were very weak. Therefore, a post-column derivatization (PCD) treatment was conducted using an electrochemical cell (Coring-Cell, Coring System Diagnostics GmbH, Germany, 100 µA) in order to increase fluorescence intensities of aflatoxin B_1 and aflatoxin G_1. The derivatizing agent was bromine formed in the cell using bromide present in the mobile phase. Because free bromine absorbs some light in the cell [30], baseline drops down. Thus, 120 mg KBr and 350 µL of 4 M HNO_3 were added into each one L mobile phase. This system is capable of derivatizing in a short period of four seconds, so the analysis completed in a short time. On this count, four main types of aflatoxins were simultaneously observed in the same chromatogram. The experiments were performed in triplicate.

The mobile phase (isocratic) composition was H_2O:MeOH:ACN (56:26:18, v/v/v) with 120 mg KBr and 350 µL of 4 M HNO_3. The mobile phase was delivered at a flow rate of 1.0 mL/min with an isocratic mode. Fluorescence detector was used. The excitation wavelength was 360 nm while emission wavelength was 430 nm, the injection volume was 100 µL; and the column temperature was 25 ºC.

2.6. Validation of the HPLC Method

Prior to measurement of aflatoxins in animal feeds and feed ingredients, this HPLC method was validated for its linearity, selectivity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and recovery. Linearity was evaluated by injecting aflatoxins standard solutions in the following ranges: 0.40-7.20 ng/mL for AFB_1 and AFG_1; 0.12-2.16 ng/mL for AFB_2, and AFG_2 with five calibration levels, each injected in triplicate. To test the recoveries of proposed HPLC method used for aflatoxins, non-infected animal feeds and feed ingredients samples were spiked with 0.50 µg/kg AFB_1, AFG_1, and 0.15 µg/kg of AFB_2, AFG_2. Ten experiments were repeated on spiked samples for each matrix. LOD and LOQ were determined by the spiked samples based on signal-to-noise (S/N) ratio of 3:1 for LOD and 10:1 for LOQ. The accuracy of the method was tested with certified reference material ERM-BE 376 Compound Feeding stuff. Precision was obtained by performing eleven analysis of the reference material and given by relative standard deviation (RSD %).

2.7. Statistical Analysis

The correlation between aflatoxins and metals were made using Minitab 18.1, and *t* values were calculated using Microsoft Excel 2013 programme. A probability value of 0.05 was used to determinate the statistical significance.

3. Results and discussion

3.1. Optimization of HPLC Extraction Process

Acetone, acetonitrile, methanol, and chloroform have been used at varying rates for extracting the aflatoxins from different types of samples since aflatoxins are soluble in polar solvents [16,17]. In this study, acetone, methanol, and acetonitrile mixtures was examined for optimizing the proposed extraction procedure. Due to its environmentally polluting nature, chloroform was not used. Extraction yield studies

of AFB_1, AFB_2, AFG_1, and AFG_2 in different concentration of spiked (1.3, 2.6, 5.2, 10.4 and 15.6 mg/mL) animal feed and feed ingredients samples were performed by using different proportions of MeOH:H_2O (60:40 %, 70:30 %, 80:20 %), ACN:H_2O (55:45 %, 70:30 %, 85:15 %), and Acetone:H_2O (55:45 %, 70:30 %, 85:15 %) solvent mixture. When acetone:H_2O mixtures were used, the recovery values were found in the ranges of 0-167 %, 6-175 %, 0-37 %, and 11-154 % for AFG_2, AFG_1, AFB_2, and AFB_1, respectively. In addition, the extraction yields obtained by using ACN:H_2O mixtures were found to be 0-145%, 0-20%, 0-175%, and 0-146% for AFG_2, AFG_1, AFB_2, and AFB_1, respectively. In the MeOH:H_2O solvent mixtures, the most stable and highest recovery rates of the aflatoxins studied were obtained. When using different MeOH:H_2O solvent mixtures for different concentrations of AFG_2, the highest recovery was found at 60:40, and the lowest recovery was 80:20. However, since the standard deviation values of the 60:40 MeOH:H_2O mixture were greater than 70:30, the mixture of 70:30 MeOH:H_2O was decided with AFG_2 analysis for animal feed and feed ingredients. Similar results were obtained in AFB_1 and AFB_2 for studied MeOH:H_2O solvent mixtures. For AFG_1, it was observed that the highest recovery values were in 70:30 and 80:20 MeOH:H_2O solvent mixtures, and the standard deviation values were in low these two solvent mixtures. Therefore, for all aflatoxin species studied, 70:30 MeOH:H_2O was decided as an extract of solution in the following studies.

3.2. Aflatoxin Methods Validation

When samples with non-infected animal feeds and feed ingredients were analyzed, no interferences were observed with the aflatoxin peaks at the retention of each compounds. However, toxin peaks were observed without the appearance of shoulders and interferences when added to actual samples containing aflatoxin. Chromatograms obtained from a standard solution, standard reference material, and mixed feed sample (No 12) are shown in Figure 1, 2, and 3.

Selectivity, linearity, detection limit (LOD), quantitative limit (LOQ), recovery, accuracy, and precision were determined to test the validity of the procedures used in detecting AFs. The linearity, LOD, LOQ, and recovery percentages for each aflatoxin in animal feeds, and ingredients samples are given in Table 1.

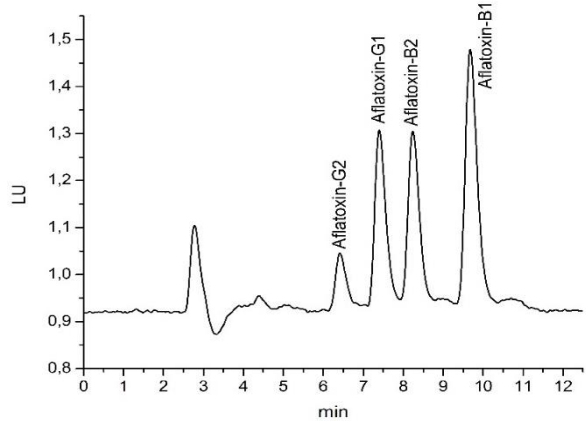

Figure 1. Chromatogram of the mixed standard solution of AFB_1 and AFG_1= 6.0 ng/g, AFB_2 and AFG_2 = 1.80 ng/g and totally = 15.60 ng/g
(Ex = 360 nm; Em = 430 nm)

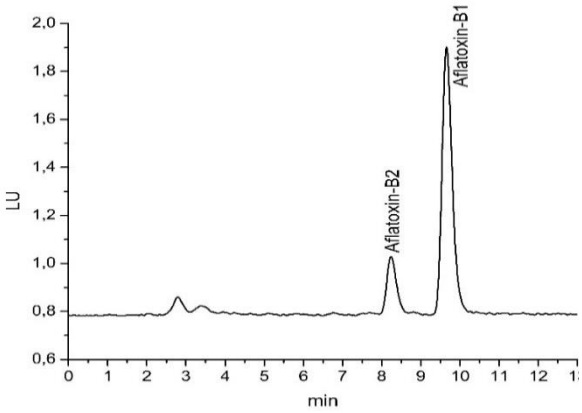

Figure 2. Chromatogram of the ERM-BE 376 Compound Feedingstuff solution
(Ex = 360 nm; Em = 430 nm)

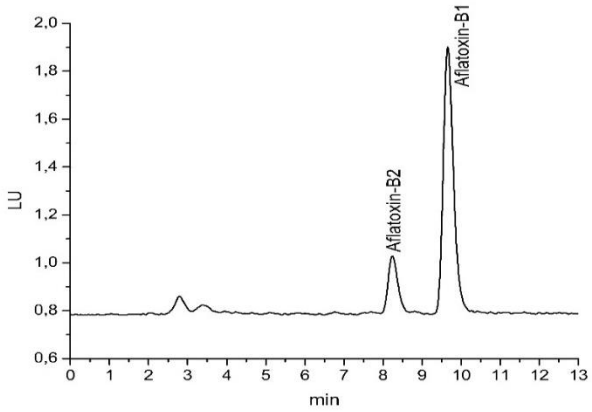

Figure 3. Chromatogram of the mixed feed sample (No 12) containing AFB_1= 122.51 ng/g and AFB_2 = 10.76 ng/g (Ex = 360 nm; Em = 430 nm)

In general, good linearity was obtained for all aflatoxins studied. The correlation coefficients R^2 were in the range from 0.9935 to 0.9996. The mean recoveries of aflatoxins from non-infected animal feeds samples were spiked with AFB_1 and AFG_1 at concentrations of 0.50 µg/kg and were between 96% and 104%. The recoveries values obtained for AFB_2 in spiked samples at a concentration of 0.15 of µg/kg were found to be 80% while it was 40% for AFG_2. The LODs (S/N = 3) were 27, 11, 16, and 10 ng/kg for AFB_1, AFB_2, AFG_1, and AFG_2, repectively, and the LOQs (S/N=10) 90, 4, 54, and 34 ng/kg for AFB_1, AFB_2, AFG_1, and AFG_2, respectively. Accuracy was examined by the determination of the certified reference material ERM-BE 376 Compound Feedingstuff of the AFs. The results obtained showed that proposed method is selective and accurate (Table 2). They are in good agreement considering one sample *t* test at a 95% confidence limit. The precision of the methods was evaluated calculating the relative standard deviation (RSD) of eleven analyses performed on each reference material ERM-BE 376. The average RSD for repeatability of reference material ERM-BE 376 measurements were in the range of 6.21-10.6%. A typical chromatogram obtained for AFs in certified reference material is shown in Figure 3.

3.3 Aflatoxin Determination

The results of the analyses of AFB_1, AFB_2, AFG_1, AFG_2, and total AFs in the 80 animal feed samples are given in Table 3. In the samples of various animal feeds analyzed, AFB_1 in 50 samples, AFB_2 in 32 samples, AFG_1 in 9 samples, and AFG_2 in eight samples were determined. In only three of the samples, four types of aflatoxins were present together. There were three types of aflatoxins present at the same time in seven samples. The number of samples with two aflatoxins determined at the same time was found to be 24, and 19 samples were found to contain only one species of aflatoxin. When the results of AFB_1 were analyzed; AFB_1 was not detected in 37.5% of the samples. However, in 35% of the samples analyzed, aflatoxin amount was in the range of 0.1-1.0 µg/kg. The sample containing AFB_1 in the range of 1.0 to 10.0 µg/kg was 20% of all samples analyzed and 6.25% in the range of 10.0-20.0 µg/kg. In addition, the number of samples containing AFB_1 determined at a concentration greater than the 20.0 µg/kg was determined as 1.25% of all samples. Aflatoxin B_1 was detected in most samples analyzed, and the levels ranged from 0.12 to 122.51 µg/kg.

According to the regulation in the European Union countries, AFB_1 levels for feed materials and feed compounds (cattle, sheep and poultry) are expressed as 20 µg/kg, while the AFB_1 level feed compounds for dairy animals (cattle, sheep, lamb, and young poultry) and complementary and complete feeds is 5 µg/kg and 10 µg/kg, respectively[31]. For feed materials and compound feed for dairy animals (cattle, sheep, lamb) given by Republic of Turkey Ministry of Agriculture and Rural Affairs AFB_1 limit values are given as 20 µg/kg and 5 µg/kg, respectively [32]. When the animal feed samples analyzed in this study, only one sample was found exceeding the limit of 20 µg/kg AFB_1 limit value for compound feeds according to Turkish regulation (Table 3).

Table 1. Method performance parameters determined in feed samples

Analyte	Linear Range (ng/mL)	Regression Equation[a]	R^2	Retention time (min)	AFs spike concentration (µg/kg) [b]	Recovery %	RSD %	LOD (ng/kg)	LOQ (ng/kg)
AFB_1	0.4-7.2	y=5.77x-0.19	0.9996	9.71	0.50	96	10.8	27	90
AFB_2	0.12-2.16	y=11.46x-0.25	0.9972	8.26	0.15	80	3.3	11	4
AFG_1	0.4-7.2	y=4.00x-1.51	0.9948	7.42	0.50	104	4.0	16	54
AFG_2	0.12-2.16	y=4.25x-0.43	0.9935	6.43	0.15	40	14.4	10	34

[a] n=3
[b] n=10

Table 2. Determination of aflatoxins in the Certified Reference Material (ERM-BE376) (µg/kg, n=11)

Analyte	Certified value	Found	RSD %	Recovery %	$t_{observed}$
AFB_1	12.9±1.76	10.97±1.16	10.6	87.4	-3.56
AFB_2	0.68±0.1	0.45±0.07	15.6	64.8	-7.63
AFG_1	5.2±0.8	4.51±0.28	6.21	85.3	-2.86

$t_{ciritical}$=2.23

Table 3. Presence of aflatoxins in different animal feed samples by HPLC analysis

Sample category	Aflatoxins (µg/kg)									
	ΣAFs[a]		AFB_1		AFB_2		AFG_1		AFG_2	
	No.[b]	Range	No.[b]	Range	No.[b]	Range	No.[b]	Range	No.[b]	Range
Mixed Feed	36/24	0.24-19.70; 133.28	36/24	0.14-7.3; 122.51	36/14	0.04-0.63; 10.76	36/1	0.41	36/1	1.20
Lamb Feed	3/3	0.63-1.23; 19.70	3/3	0.38-18.12	3/2	0.079-1.57	3/0	ND[c]	3/1	0.85
Cattle Feed	18/14	0.12-8.79; 12.43	18/14	0.12-11.62	18/9	0.03-0.84	18/3	0.11-0.84	18/1	0.10
Poultry Feed	6/4	0.20-4.54; 12.4	6/4	0.20-3.2; 11.72	6/3	0.04-0.67	6/2	0.2-0.88	6/0	ND
Feed Additives	17/7	0.21-1.15; 10.78	17/5	0.21-0.75;10.16	17/4	0.037-0.62	17/3	0.11-0.26	17/5	0.065-0.30

[a] ΣAFs= Σ (AFB_1+ AFB_2+AFG_1+AFG_2).
[b] Incidence no was represented by total/positive sample of particular category.
[c] Not detected

Additionally, the limit of 5 µg/kg of AFB_1 determined for dairy animals was exceeded in only two samples (6.89 and 11.62 µg/kg). The limit value of AFB_1 in complementary and complete feeds is 10 µg/kg in the regulation of the European Union Countries [31]. When the results obtained according to this regulation were evaluated, five samples exceeding the limit value were determined. The European Community has established that maximum levels of AFM_1 in raw milk, heat-treated milk and milk for the manufacture of milk-based products should not exceed 50 ng/kg [33,34]. In addition, the daily amount of AFB_1 passing through the milk as AFM_1 is 0.17 to 3.3% [35]. As a result, AFB_1 values in the feed samples analyzed for this study exceeded the AFM_1 limit values in only one sample (122.51 µg/kg).

The main reason for observing such low AFB_1 values is the strict follow-up of aflatoxins from field to storage and from feed production to the animal. In particular, it is thought that the avoiding the use of moldy and low-quality raw materials with is effective in keeping aflatoxin production at low rates. Aflatoxin levels and aflatoxin contamination in animal feedstuffs depend on many factors such as feed composition, feed raw material, storage conditions, geographic area, and seasonal conditions [36].

3.4. Metal Determination

The results of the analyses elements concentrations in the compound feed, lamb feed, cattle feed, poultry feed, and feed additive samples are given in Table 4. The European Union and the Republic of Turkey Ministry of Agriculture and Rural Affairs in accordance with the regulations concerning undesirable substances in animal feed, As (in feed materials and complete feed), Cd (in feed materials and complete feed for cattle, sheep, goat), Hg (in feed materials and complete feed) and Pb (in feed materials and complementary feed) limits for 2 mg/kg, 1 mg/kg, 0.1 mg/kg and 10 mg/kg was given [31,32].

Table 4. Element concentrations in animal feed samples (mg/kg)

Element	Mixed Feed (*n*=36)	Lamb Feed (*n*=3)	Cattle Feed (*n*=18)	Poultry Feed (*n*=6)	Feed Additives (*n*=17)
Al	64±14	48±2	55±10	47±4	61±16
As	0.11±0.05	0.092±0.002	0.12±0.05	0.063±0.002	0.11±0.04
Ba	4.6±1.9	6.9±0.1	3.7±1.1	3.2±0.7	2.8±0.9
Ca[a]	0.11±0.04	7.2±0.2	0.070±0.014	0.070±0.007	0.028±0.006
Cd	0.063±0.047	0.039±0.005	0.042±0.005	0.067±0.004	0.044±0.017
Co	0.22±0.08	0.18±0.05	0.15±0.05	0.38±0.09	0.16±0.05
Cr	0.48±0.16	0.35±0.02	0.35±0.08	1.3±0.4	0.37±0.12
Cu	17±6	14±3	15±4	22±4	21±6
Fe[a]	0.34±0.06	0.33±0.02	0.35±0.05	0.35±0.04	0.36±0.08
Hg	nd~0.061±0.001[c]	nd	nd	nd	nd~0.19±0.09[b]
K[a]	27±5	23±1	28±5	25±7	48±9
Mg[a]	3.1±0.9	3.7±0.5	3.3±0.3	1.8±0.2	4.6±1.1
Mn	49±16	85±2	75±14	43±10	40±7
Na[a]	2.4±1.2	4.4±0.3	3.3±0.8	1.4±0.1	nd
Ni	2.7±0.8	2.6±0.1	2.8±0.5	1.9±0.3	4.1±1.8
P[a]	0.29±0.06	0.37±0.02	0.31±0.04	0.27±0.05	0.43±0.12
Pb	0.047±0.013	0.18±0.03	0.085±0.005	0.060±0.009	0.050±0.013
Se	225±42	252±11	357±29	281±22	224±29
Sn	nd	nd	nd	nd	nd
Zn	61±15	45±4	42±7	92±6	49±12

nd: below the limit of detection
[a] g/100g
[b] mean of two sample
[c] mean of six sample

The mercury concentrations in two of the samples exceeded the legal limit value of Republic of Turkey Ministry and EU regulations, exceeding 0.1 mg/kg, in this study on feed materials. However, the

mean mercury concentration determined in six compound feed samples was lower than the legal limits of Turkey Ministry and EU regulations [31,32]. The maximum content of As, Cd, and Pb in determined feed samples were found below the legal limits in this study. The contents of elements in different types of animal feed samples has been reported by several authors from different countries. Zhou et al. found the mean concentrations of Mn, Fe, Co, Ni, Cu, Zn, Se, Ba, Al, Cr, As, Cd, Hg, and Pb 117.96, 664.16, 0.84, 1.18, 19.52, 98.42, 1.50, 10.99, 492.00, 5.23, 0.34, 0.06, 0.01, 0.99 mg/kg, respectively [28]. The mean Mn, Fe, Co, Zn, Ba, Al, Cr, and Pb levels detected in the current study in cattle feed samples are lower than those reported in China by Zhou et al. In this study, the average Mn, Co, Zn, Ba, Al, Cr, and Pb levels in cattle feed samples were found to be lower than those of Zhou et al. In addition, the Cu, Se, As, Cd, and Ni levels were similar to those reported in the studies above [28]. In comparison to the other published work, the results of present study for Cu, Fe, and Mg levels showed higher values than Kerr et al [37]. However, other analyzed elements (Al, Ca, Cd, Mn, Ni, and Zn) were found to be compatible with Kerr et al [37]. When this study was compared with another study in animal feeds, it was found that calcium, copper, iron, manganese, and zinc concentrations were lower in this study [38]. In another study by Pereira et al. on cattle feed, Co, Cu, Mn, and Ni contents were found to be compatible with this study [39].

The relationship between both aflatoxin and element contents in different animal feeds is given in the Table 5. As can be seen from Table 5, the amounts of aflatoxin B_1 and Cd obtained in this study were found to be lower than those obtained from other studies, while the amounts of nickel and selenium were found to be slightly large.

3.5. Correlation Analysis

The relationships between the element and aflatoxin toxins (AFB_1 and total AFs) concentrations in compound feed samples were evaluated using single correlation analysis. Significant r values ($>|\pm 0.404|$, $n=24$) at the 95% confidence level are represented bold in Table 6. Table 6 shows that arsenic and selenium elements have a positive correlation with total Aflatoxin and AFB_1 in feed samples, and these correlations are also significant ($p<0.05$). However, the correlation of arsenic with AFB_1 and total AFs was found to be very strong compared to that of selenium. Although there is a weak negative correlation between chromium and nickel elements and AFB_1 and total AFs, these correlations are not statistically significant.

Although there is a positive correlation between arsenic and selenium contents and AFB_1 and total aflatoxin, many factors such as feed composition and type of raw materials used, storage type and conditions, geographical areas and climatic conditions, sampling area, and preparation of samples might be critical factors for findings of toxin content in animal feed samples [36]. In conclusion, these results indicate that samples of animal feed contaminated with total aflatoxin and AFB_1 are also contaminated with arsenic and selenium.

4. Conclusions

In conclusion, the elements and AFB_1 and total AFs contents were compared using a correlation analysis. A positive correlation was found between arsenic and selenium and AFB_1 and total AFs contents in compound feed samples. Arsenic correlation was significant; but selenium was not significant ($p< 0.05$). Also, this study underlines the importance of regular monitoring of aflatoxins and element contents in different types of animal feeds in Turkey. To evaluate the main sources of element and aflatoxin contamination, it is necessary to analyze the animal feed materials separately according to their types. Although there is a strong correlation between some elements and aflatoxin contents, some other factors, such as humidity, temperature, and material type that may influence the toxin content in feed samples. Fifty of the total eighty samples analyzed were contaminated with AFB_1, and thirty samples were contaminated with AFB_2, nine with AFG_1, and eight withAFG_2.

Table 5. Comparison of element and aflatoxin concentration in different types of animal feeds

AFB_1 µg/kg	**AFB_2 µg/kg**	**AFG_1 µg/kg**	**AFG_2 µg/kg**	**ΣAFs µg/kg**	**As mg/kg**	**Cd mg/kg**	**Co mg/kg**	**Cr mg/kg**	**Cu mg/kg**	**Hg mg/kg**	**Ni mg/kg**	**Pb mg/kg**	**Se mg/kg**	**Sn mg/kg**	**Zn mg/kg**	**Ref.**
15-35	5-25	<0.05-30	<0.03-16	28-90	<LOD-3	<LOD-2.5				<LOD-0.444		<LOD-4.9				[36]
<LOD-18.4				<LOD		0.05-0.27		0.01-16.92				0.39-3.69		3.26-14.26		[40]
				<0.0005	0.20-0.22	0.11-0.13	0.1-0.6		13.3-34.1	0.03-0.03	0.1-0.6	0.10-0.41	1.4-3.5		22.4-45.8	[41]
0.12-18.12	0.03-1.57	<LOD-0.88	<LOD-1.20	0.12-19.70	0.063-0.12	0.039-0.067	0.15-0.38	0.35-1.3	14-22	<LOD-0.19	1.9-4.1	0.047-0.18	224-357	<LOD	42-92	In this study

Table 6. Correlation coefficients (*r*) between elements and aflatoxin B_1 and total aflatoxins

Element	AFB_1	AFs
Al	-0.001	-0.001
As	**0.768**[a]	**0.733**[a]
Ba	0.304	0.297
Ca	0.052	0.055
Cd	-0.067	-0.080
Co	-0.091	-0.063
Cr	**-0.466**	**-0.407**
Cu	-0.029	-0.036
Fe	0.119	0,075
K	0.114	0.111
Mg	-0.042	-0.048
Mn	-0.083	-0.070
Na	-0.243	-0.268
Ni	**-0.417**	**-0.503**
P	0.074	0.053
Pb	0.131	0.090
Se	**0.535**[a]	**0.520**[a]
Zn	0.180	0.193

[a]Correlation is significant at 95% confidence level ($r_{critical}$ =**0.404**, n=24

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