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Original article

The occurrence and seasonal variation of aflatoxin B1 and zearalenone concentrations in poultry feeds

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Abstract

The present study aimed to investigate the contamination of poultry feed with aflatoxin B1 and zearalenone at laying hen farms in Tehran suburbs. The poultry feed was selected from five laying hen farms. A total of 60 poultry feed samples were collected from each farm during four consecutive seasons, from spring to winter of 2021. High-performance liquid chromatography was used to determine the amount of aflatoxin B1 and zearalenone. The mean aflatoxin B1 and zearalenone concentrations in various seasons showed significant differences ($p<0.01$). The highest reported aflatoxin concentration was in winter, with a mean concentration of 1366.53±77.85 ng/kg. The lowest concentrations were reported in autumn and summer, indicating a significant difference ($p<0.01$). The highest concentration of zearalenone was reported in summer, with a mean concentration of 150.72±10.35 µg/kg. The lowest concentration was reported in winter, with a mean concentration of 22.87±10.35 µg/kg, indicating a statistically significant difference ($p<0.01$). Overall, the concentrations of aflatoxin B1 and zearalenone toxins significantly differed in various poultry farms. The poultry farm D had the highest aflatoxin contamination with a mean concentration of 648.08±59.89 ng/kg. Poultry farms A, B, and C had the highest zearalenone concentrations with mean concentrations of 125.17±20.61, 96.04±20.61, and 99.49±20.61 µg/kg, respectively. Autumn was the only season showing significant differences regarding zearalenone toxin concentration in poultry farms.

Keywords: aflatoxin B1, high-performance liquid chromatography, poultry diet, zearalenone

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Introduction

Mycotoxins (fungal toxins) are secondary toxic mold metabolites produced in a wide range of nutrition products under different conditions (Omotayo et al. 2019). Consumption of mycotoxin-contaminated food can harm human and animal health and cause serious diseases (Negash 2018). More than 4.5 billion individuals in developing countries are exposed to food contamination caused by mycotoxins (Hassan et al. 2016).

Various factors, including the toxin concentration and the animal's exposure duration to the toxin, play roles in mycotoxin poisoning incidence and severity (Mohsen et al. 2022). Poultry are among the most sensitive animals to mycotoxins, and the toxic effects of mycotoxins depend on their age, sex, and physiological and nutritional condition at the time of exposure (Xu et al. 2022, Furian et al. 2022).

Mycotoxin-contaminated feeds for poultry are among the top nutrition safety challenges that can harm the economy (Haque et al. 2020). Mold growth can increase the toxin concentration during various stages of poultry feed production and distribution, making investigating the influential factors in poultry farm contamination more difficult (Filazi et al. 2017, Xu et al. 2022,). *Aspergillus*, *Fusarium*, and *Penicillium* fungi can produce mycotoxins under different conditions before, during, or after harvest, drying, and crop storage (Magan and Aldred 2007). The produced mycotoxins have high physicochemical stability. Considering their high toxicity and concentration level in cereal and food products, mycotoxins are considered one of the most dangerous toxins for human and animal (especially livestock and poultry) health (Zain 2011). Aflatoxins and zearalenone have been suggested as the most critical poultry feed mycotoxin contaminants. Various studies have reported nutrition contamination with such toxins (Mayahi et al. 2007, Gruber-Dorninger et al. 2019, Mohammadi et al. 2021).

Aspergillus species (including *Aspergillus flavus*, *Aspergillus parasiticus, Aspergillus nomius, and Aspergillus pseudotamari*) produce aflatoxins (Choudhary and Kumari 2010). Different types of aflatoxin include B1, B2, G1, and G2, among which aflatoxin B1 has the highest toxicity and is considered among the carcinogenic, mutagenic, and malformation-causing agents (Tahir et al. 2018). Aflatoxins (especially aflatoxin B1) primarily affect cereals and legumes in hot and humid regions (Lalah et al. 2019). Aflatoxin consumption through contaminated diets affects animal health and reproduction. Since the toxic metabolites will be present in meat, milk, and eggs, they would also endanger humans (Negash 2018). Aflatoxicosis in poultry and animals cause biochemical parameter alterations, hepatic and renal abnormalities, and immune system disorders, which can increase the sensitivity to infectious diseases. Various countries have imposed different legal restrictions on animal feed diets (Yiannikouris and Jouany 2002). Generally, the permissible limit of aflatoxins for animal diets is higher than human limits. The United States Food and Drug Administration (FDA) has recommended a maximum permissible aflatoxin B1 and other aflatoxins in cereals for human consumption as 2 and 4 µg/kg, respectively. The suggested permissible aflatoxin B1 for poultry feed diets is 10 µg/kg (Hussain et al. 2016, Shi et al. 2016). Accordingly, poultry feed contaminations beyond the mentioned limits are considered a threat to human health.

Zearalenone is a secondary metabolite produced by *Fusarium graminearum* and other related species (Assumaidaee et al. 2020). *Fusarium sp.* grows in various agricultural products, such as corn, rice, wheat, barley, sesame, oats, and soy. It is present in different types of poultry feed diets (Cinar and Onbaşı 2019). Zearalenone is among cereals and legumes' most important fungal pathogens on the global level, imposing critical damage to the agriculture industry (Ayofemi Olalekan Adeyeye 2020). Zearalenone toxin shows intense estrogen activity and causes animal complications, including reduced feed consumption, decreased milk production, increased milk somatic cells, abdominal pain, breast swelling, ovary atrophy, and abortion (Ropejko and Twarużek 2021). Various countries have determined different permissible limits for this toxin in cereals and their products, ranging from 50 to 1000 µg/kg (FAO 2004).

Generally, the damages of these toxins are not limited to the reduction of animal and agricultural products quality. On one hand, the increasing rate of meat consumption (especially poultry) caused preventive measures and toxin-neutralizing actions for poultry feed diets to be highly significant to the poultry industry. Hence, investigating poultry feed diets concerning contamination with mycotoxins has become critical. Accordingly, the present study aimed to investigate aflatoxin B1 and zearalenone concentration in poultry feed of the laying poultry farms in Tehran suburbs using high-performance liquid chromatography method during different seasons. Considering the conducted search in available data banks, the current study was the first research in this field in Tehran suburbs poultry farms.

Table 1. The primary components ratio in poultry farms.

Poultry farm name	Capacity	Daily ration (g)	The Ratio of Ration Components						
			Corn	Wheat	Soybean	Powdered meat	Calcium carbonate	Bran	Concentrate
Unit A	141000	120	56.5%	_	28%	_	12%	1%	2.50%
Unit B	181500	108.75	45.5%	11%	25%	5%	11%	_	2.50%
Unit C	191000	120	55%	_	28%	_	12%	-	5%
Unit D	47500	115.25	54.5%	_	26%	5%	11%	1%	2.50%
Unit E	24500	113.25	48.5%	5.5%	29%	_	12%	_	5%

Materials and Methods

Sample collection

Tehran is the capital and largest city of Iran and the largest city in Tehran Province. Out of 25 actives laying poultry farms in Tehran suburbs, five units were selected using the simple random method. The samples were collected from the feed diets consumed by each unit during four consecutive seasons (from spring to winter 2021) following the protocols based on the National Standard of Iran (no. 7570) (Iran Standard and Industrial Research Institute, 2019). The primary components of the samples included corn, soybean, wheat bran, sorghum, animal proteins, and powdered grain products (Table 1). Three samples were collected from different locations on each farm. After homogenization, a one-kilogram sample was prepared for analysis. Overall, 15 samples in each season were collected (three repetitions) from each farm. Over the four seasons, 60 samples were collected for mycotoxin analysis. The samples were transferred in cold conditions (attached to ice) to the laboratory to stop the activity of microorganisms. After grinding, the samples were kept at -20°C until the experiment. From each poultry farm, a research questionnaire regarding the farm's capacity, flock age and type, feed storage method, temperature, and moisture was obtained at each season.

Mycotoxin's analysis

The high-performance liquid chromatography (HPLC) method was used to determine aflatoxin B1 and zearalenone concentrations.

Measuring aflatoxin B1 concentration

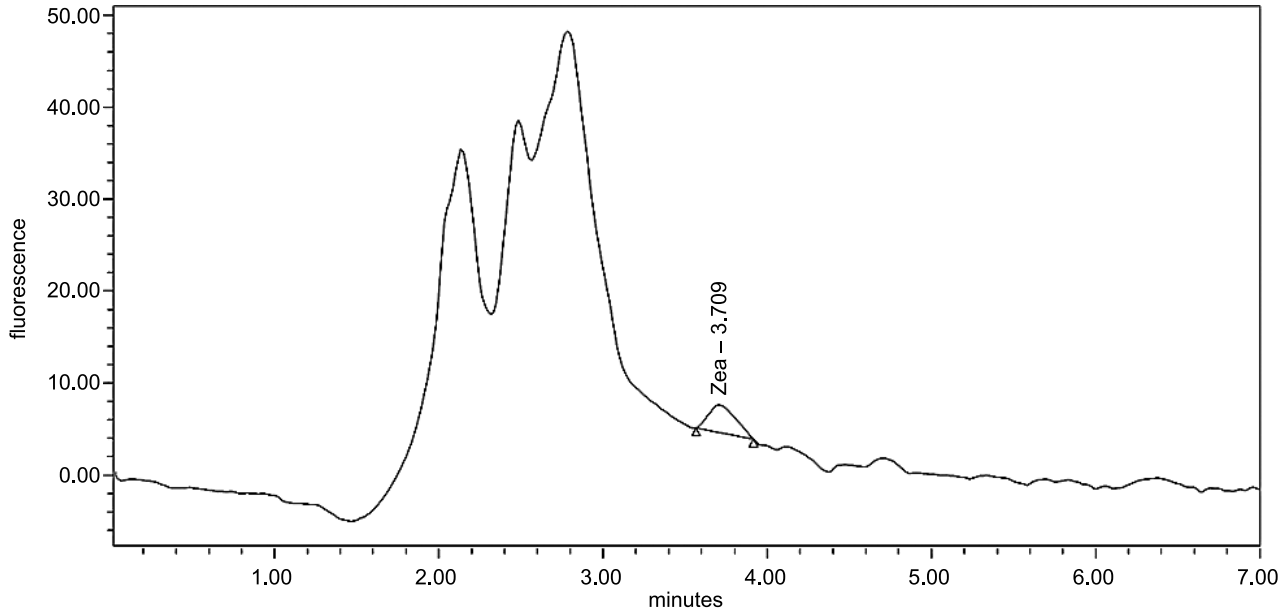
From each sample, 10 g were collected and mixed with 50 mL methanol (33%). The obtained solution was shaken with a rotational movement for 2 minutes. After 15 minutes, the solution was filtered using a Whatman Grade 42 filter paper. Eventually, the samples were diluted with methanol in a 1:10 ratio. In this method, the toxin present inside the sample solution was extracted by the solvent (methanol). The extract was passed from the immunoaffinity column (The flow rate = 2 to 3 ml/min).

When passing from the immunoaffinity column, the toxin inside the extract was attached to the specific antibody of the column as an antigen. If needed, the column was washed using a buffer solution. Eventually, the pure toxin molecules remained inside the column as antigen to the antibodies. The immunoaffinity column was washed with 10 ml of water and dried with air pressure (National Standard of Iran, 2003).

From the vial content, 20 µL was injected into the HPLC device (Waters 2695, Alliance, USA) equipped with a fluorescent detector. The detection was conducted at 365 nm wavelength. Every analysis was repeated three times. Eventually, the obtained chromatograms were compared with the standard chromatograms regarding retention time. The contamination type and concentration were determined using the standard curve (National Standard of Iran, 2003).

Measuring zearalenone concentration

A volume of 100 mL acetonitrile-water (90:10) extraction solvent was added to 10 g of each ground feed sample. After shaking the solution for one hour, the extracted solution was filtered using a paper filter. A volume of 15 mL of the filtered extract was diluted with 85 mL of phosphate-buffered saline (PBS) and purified using an immunoaffinity column. The present zearalenone in the obtained extract was detected and measured by an HPLC device using the reverse-phase chromatography method and a fluorescent detector. The detection was conducted at 275 nm wavelength (National Standard of Iran 2009).



	Peak name	RT	Area	% Area	Height	Amount
1	Zea	3.709	34310	100.00	2983	185.221

Fig. 1. Chromatogram of poultry feed sample containing zearalenone.

Statistical analysis

Central tendency (such as mean) and dispersion indicators (such as variance and standard deviation) were determined. Analysis of variance (F-test) and Duncan's mean comparison were used to investigate the differences between the seasons and poultry farm conditions. The statistical analysis was conducted using SPSS (version 23). The p values lower than 0.05 were considered statistically significant.

Results

The high-performance liquid chromatography (HPLC) method was used to determine the aflatoxin B1 and zearalenone toxin concentrations. The retention time of the resulting chromatograms was compared with standard chromatograms, the contamination type was determined, and the contamination level was calculated using the standard curve. Figures 1 and 2 present poultry farm samples of zearalenone and aflatoxin chromatograms.

Aflatoxin and Zearalenone concentrations in poultry feed samples

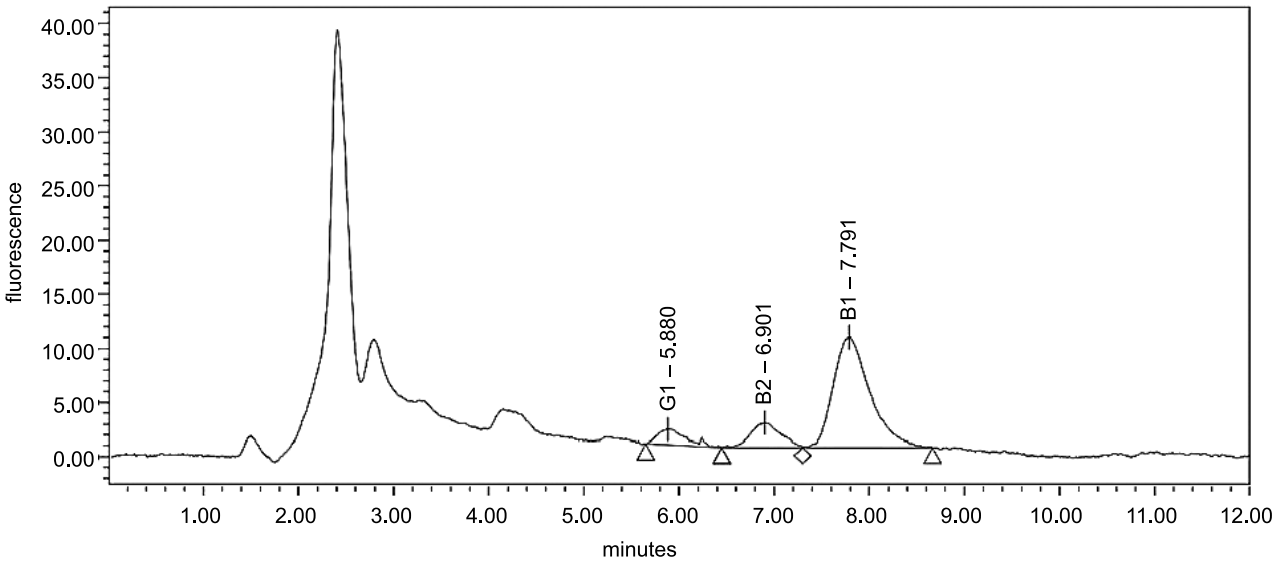
Table 2 presents the aflatoxin B1 and zearalenone concentrations. Table 3 compares the mean aflatoxin B1, and zearalenone concentrations in poultry feed diets of Tehran suburbs poultry farms during four seasons.

In all samples, aflatoxin B1 concentrations were lower than the permissible limit (10000 ng/kg). As the table indicates, the zearalenone toxin concentrations were also lower than the permissible limit in all samples (2000 μg/kg).

Table 4 compares the mean aflatoxin and zearalenone concentrations in poultry feed diets. According to the results, the feed sample from farm D indicated the highest mean aflatoxin concentration. The feed sample of farm A also had the lowest concentration. Farms A, B, and C showed the highest mean zearalenone concentrations, and farm E had the lowest mean ($p<0.01$). There was a significant difference between the zearalenone concentrations of the different farms during the autumn. The feed sample of farm E indicated the lowest zearalenone toxin concentration. There was no significant difference between aflatoxin B1 and zearalenone concentrations in different farms during other seasons.

Discussion

Mycotoxins-fungal toxins are secondary metabolites produced by some genera, *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps, Alternaria,* and *Stachybotrys* in cereals and fodders under unfavourable environmental conditions. Since mycotoxins play critical roles in public health, food safety, and the national economy of developing countries, this study aimed to investigate the aflatoxin B1 and zearalenone concentrations in poultry feed diets of the laying poultry farms.



Peak results

	Name	RT	Area	Height	Conc Ng/Ml	Amount	Final_Amount	Units
1	G2	5.424						
2	G1	5.880	30565	1437	0.28	0.827	1.181	ppb
3	B2	6.901	49976	2275	0.06	0.194	0.277	ppb
4	B1	7.791	273874	10179	0.83	2.481	3.544	ppb

Fig. 2. Chromatogram of poultry feed sample containing aflatoxin B1.

Table 2. The mean aflatoxin B1 and zearalenone concentrations in poultry diets of Tehran suburbs poultry farms in different seasons of the year.

Season	Mean		Standard deviation		Minimum contamination		Maximum contamination	
	AFB1 (ng/kg)	ZEN (µg/kg)	AFB1 (ng/kg)	ZEN (µg/kg)	AFB1 (ng/kg)	ZEN (µg/kg)	AFB1 (ng/kg)	ZEN (µg/kg)
Spring	401.8	61.54	489.77	46.68	ND	8	1544	185.22
Summer	ND	150.72	ND	140.23	ND	3.71	ND	467.01
Fall	ND	105.95	ND	43.98	ND	27.48	ND	184.62
Winter	1366.53	22.87	351.84	11.97	823	5.77	1841	45.81

The results of the present study indicated significant differences between mean variables (aflatoxin and zearalenone concentrations) in various seasons ($p<0.01$). The highest reported aflatoxin B1 and zearalenone concentrations were in winter and summer, respectively. The highest aflatoxin concentration was in winter and the lowest in autumn and summer; the differences were significant. The highest reported zearalenone concentrations were also in summer and autumn, while the lowest was in winter ($p<0.01$). In a study by Ersali et al. (2008) on 91 animal feed diet samples, the samples indicating higher contaminations than the permissible limit in spring, summer, autumn, and winter were 15.78, 56.25, 75, and 33.33%, respectively. In another study, Rahimi et al. (2008) reported that 17.6% of the investigated animal feed had contamination levels higher than the permissible limit. In another study conducted in Austria on 21 poultry feed samples, the aflatoxin contamination had a 6.66% incidence rate with a mean 1.26 µg/kg concentration (Zinedine et al. 2007). Considering the obtained information from farms through the questionnaires in this study and the temperature increase during the summer, we expected aflatoxin production to increase. However, despite the expectations, the aflatoxin concentration in summer was lower than the detectable limit due to the steady and uniform humidity fluctuation in all seasons at every farm unit. Nemati et al. (2014), in a study (Tabriz, Iran) on aflatoxin B1 concentration in poultry feed, reported aflatoxin and fungal contamination of the diets and their components. They reported the highest contaminations of feed components in soybean meal, wheat bran, corn,

Table 3. Comparison of the mean aflatoxin B1 and zearalenone concentrations in diets of Tehran suburbs poultry farms.

Season	Aflatoxin B1 (ng/kg)	Zearalenone (µg/kg)
Spring	401.8[b]	61.54[bc]
Summer	ND[c]	150.72[a]
Fall	ND[c]	105.95[ab]
Winter	1366.53[a]	22.87[c]
SEM	77.85	10.35
p value	0.0001	0.0001

[a,b,c] – values within a column with different superscripts differ significantly at $p<0.01$
ND – not determined

Table 4. Comparison of the aflatoxin and zearalenone concentrations in diets of poultry farms in different sampling seasons.

Season farm	Spring		Summer		Fall		Winter		All season	
	AFB1 (ng/kg)	ZEN (µg/kg)	AFB1 (ng/kg)	ZEN (µg/kg)	AFB1 (ng/kg)	ZEN (µg/kg)	AFB1 (ng/kg)	ZEN (µg/kg)	AFB1 ng/kg	ZEN µg/kg
A	62	88.72	ND	284.08	ND	100.55[a]	1964.32	27.31	339.92[c]	125.17[a]
B	ND	76.37	ND	147.10	ND	138.23[a]	619.33	22.03	404.83[bc]	96.04[ab]
C	394.33	66.72	ND	183.67	ND	117.46[a]	2203.27	30.57	524.50[ab]	99.49[a]
D	1552.67	67.91	ND	75.51	ND	138.23[a]	1206.33	8	648.08[a]	72.42[c]
E	ND	8	ND	63.45	ND	34.85[b]	839	26.69	293.08[c]	33.24[d]
p value	0.182	0.261	-	0.319	-	0.001	0.149	0.144	0.001	0.038

[a,b] – values within a column with different superscripts differ significantly at $p<0.01$
ND – not determined

and wheat grain. In the present study, the same components were the base compound of the poultry feed in every farm. In Tanzania, Kajuna et al. (2013) reported that 68% of the poultry feed samples had aflatoxin B1 contamination. They also reported the lowest toxin concentrations and highest contamination in corn bran and broiler feed. In a study by Monge et al. (2012) in Argentina on the relationship between raw material contamination and final poultry feed diet contamination with *Aspergillus* and *Fusarium* and their mycotoxins, results indicated that 19% of the raw material samples and 79% of the final poultry feed diets had contamination levels higher than the permissible limit. They isolated *Aspergillus flavus* from the samples; however, *Fusarium sp.* was the most prevalent. The result analysis indicated that the primary contamination source of *Fusarium* was corn. A study between 2009 and 2016 in the Republic of Korea indicated that 98% of the 58 poultry feed samples were contaminated with zearalenone (maximum contamination concentration of 262 µg/kg). The values were higher than the permissible limit by the European Union and South Korean regulations (250 µg/kg for the poultry) (Chang et al. 2017). The highest production level of zearalenone is at approximately 16% humidity and temperatures lower than 25°C (Waśkiewicz and Goliński 2015). The peak zearalenone concentration in the present study was during the summer. According to the questionnaires, the recorded temperature at every poultry farm (except farm B) was higher than 25°C. The humidity was in the 30 to 45% range (the humidity of the production hall and feed storage). According to the study by Waśkiewicz et al. (2015) the present study lacked the necessary conditions in terms of humidity and temperature for Fusarium species during the summer. The high concentration of this fungi during summer was inconsistent with Waśkiewicz et al. (2015) which indicates that other factors play critical roles in feed diet contamination. The preparation date of the feed may be among the possibilities.

Generally, climate changes are the primary cause of increased diet contamination with mycotoxins (Battilani et al. 2016). Comparing the poultry farms at different seasons indicated a significant difference between the zearalenone concentration in Farm E and other farms during the autumn. The zearalenone con-

centration in Farm E was lower than in others. Considering the results of the questionnaires in the autumn, the lower concentration of zearalenone in this farm compared with others was probably the lower capacity of the farm (24000 birds), lower storage capacity, lower production hall humidity, lower crushed grains percentage (1%), and eventually, use of toxin binder in the mentioned farm. Also, farm E had no reports of aflatoxin except for winter. The zearalenone concentrations in spring and summer were also lower than in other poultry farms. Overstocking of poultry feed and higher relative humidity of winter compared with other seasons may be the causes of higher aflatoxin and zearalenone contamination in winter. Feed prone to contamination with various fungi can produce toxic metabolites, such as aflatoxin and zearalenone.

In spring and winter, the aflatoxin B1 concentrations of poultry farm A were 62 and 1964.35 ng/kg, respectively. The aflatoxin B1 concentration in winter was lower than the detection limit ($p<0.01$). Comparing the questionnaires and analysing the obtained data reveals that the humidity of feed storage did not change significantly during the winter (the humidity of poultry farms in four seasons was between 26% and 31%). Only the temperature decreases of the feed storage to 15°C could provide a suitable condition for aflatoxin B1 contamination in this poultry farm. However, the crushed grains percentage in autumn and winter (5%) increased from the mean percentage in spring and summer (1%). The humidity level of the feed, which was lower during the winter compared with other seasons (measured as 7%), can be another influential factor, as the humidity levels in spring, summer, and autumn were 10, 9, and 8.5 percent, respectively.

Unlike the mentioned factors for poultry farm A, based on the findings of the questionnaires, the high aflatoxin B1 concentration of the poultry farm B during the winter depended on factors including humidity (48% in the winter) and the temperature of the production hall (decreased to 18°C). The decreased humidity of the feed storage (from 31% to 28%) and the change of the toxin binder (zeolite and toxin trap in winter, and zeolite in other seasons) can also be the causes of higher aflatoxin concentration in this farm. The temperature of the feed storage decreased to 15°C (similar to the poultry farm A). Factors including the crushed grain percentage and the basic composition of the feed were among the causes of the stability in this farm.

The highest reported aflatoxin concentration in poultry farm C was during the winter. The temperature of the production hall in spring and winter was 23°C, and during the summer and autumn was 26°C. Decreased temperature of the feed storage (from 27°C to 20°C) can also be another factor contributing to the increase in aflatoxin B1 concentration during the winter. Increased humidity of the production hall during the winter (44%) was another factor causing the increase in aflatoxin B1 concentration in poultry farm C.

The highest observed aflatoxin B1 concentrations in poultry farm D were in spring and winter. The humidity of the production hall increased from 38.5% to 49% during the winter, and the temperature fluctuation in the production hall was slight (23°C during the spring and 21°C during the winter). According to the information obtained from the questionnaires, the only changed factor of the farm was toxin binders (zeolite during the summer and autumn and zeolite in addition to clay in spring and winter).

Considering the fixed capacity of each poultry farm and the obtained results of the study, there was no relationship between the farm capacity and toxin production. However, considering that the daily feed per bird in farms A and B were higher than in other farms, results demonstrated that the zearalenone toxin load of these farms was higher, indicating the relationship between feed consumption and the contamination level.

Findings indicated no significant difference in mean zearalenone concentration in each poultry farm during different sampling seasons. However, there were statistically significant differences between the farms, possibly due to the different poultry feed storage conditions and Fusarium species. Hence, considering the temperature and humidity levels, the zearalenone concentrations of the farms were different.

Overall, the aflatoxin and zearalenone concentrations in the 60 studied samples were lower than the permissible limits (10000 ng/kg and 2000 µg/kg, respectively). Nevertheless, constant monitoring of poultry feed is the primary method to prevent mycotoxins from entering the human food chain. Accordingly, continuous investigation of poultry feed with laboratory methods is necessary.

The present study's findings revealed the incidence rate and the concentration of mycotoxin contamination of poultry feed of five poultry farms in the Tehran suburbs. Despite the toxin concentrations being lower than the permissible limit, preventing such contaminations requires taking necessary controlling measures, including dehumidifying, storing a sufficient amount of feed after harvest, utilizing suitable toxin binders, and constant monitoring of poultry feed.

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