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Investigation of Aflatoxin B1, Ochratoxin A, and Fumonisin B1 in Poultry Feeds in Nineveh Province

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ABSTRACT

Aflatoxin B1 (AFB1), ochratoxin A (OTA), and fumonisin B1 (FB1), the most commonly encountered mycotoxins, constitute serious human and animal health threats as a result of their toxigenic, carcinogenic, and mutagenic influences. The study aimed to investigate the occurrence of these mycotoxins in poultry feeds and determine the percentage of the samples that exceeded the legal limits approved by the European Commission (EC). Sixty poultry feed samples were collected from poultry feed plants and poultry farms in Nineveh Province and analyzed for detection mycotoxins using competitive Enzyme-Linked Immunosorbent Assay (ELISA). Results reported co-occurrence of AFB1 and FB1 in all samples examined (100%), while AFB1, OTA, and FB1 co-occurred in 53 samples (88.33%) at values ranging between 3.15–43.96, 0–168.24, and 220.6–6935.12 ppb, respectively. Also, results showed that FB1 existed at a mean value (2164.01 ppb) significantly higher ($P<0.05$) than those reported for AFB1 and OTA (16.48 and 32.09 ppb, respectively). Results revealed that 38.33% and 10% of feed samples exceeded the maximum permissible limits for AFB1 and OTA established by EC, whereas all feed samples were within the EC limit for FB1. As a result, strict procedures should be implemented to achieve legal limits concerning AFB1 and OTA in poultry feeds to preserve public health.

Keywords: aflatoxin B1, ELISA, fumonisin B1, ochratoxin A, poultry feeds, Nineveh

INTRODUCTION

Mycotoxins, the toxic secondary metabolites produced by mycotoxigenic molds, constitute a serious threat to human and animal health. Mycotoxigenic molds can produce mycotoxins through a variety of biosynthetic pathways, resulting in various pathological mechanisms ranging from acute toxicity to immunosuppressive or carcinogenicity (1). Although more than 400 mycotoxins have been discovered, only a few have special importance in public health, agriculture, and economics and their synthesis may be limited to specific strains within a mold species. Aflatoxins, ochratoxins, and fumonisins are assorted within the most predominant mycotoxins responsible for inducing many toxicological effects in humans and animals (2,3). Aflatoxin B1 (AFB1), the most toxic and widely spread aflatoxin, is produced primarily by *Aspergillus flavus* and *A. parasiticus* in several food and feed commodities (4). AFB1 is of great concern as it is the most vigorous naturally occurring carcinogen, mutagen, teratogen, and immune system suppressor. It is also classified as a Group I human carcinogen by the International Agency for Research on Cancer (IARC) (5). Ochratoxin A (OTA), the most occurring and most toxic

member of ochratoxins, is produced mainly by *Aspergillus ochraceus*, *Penicillium verrucosum*, and *A. carbonarium*. It is well documented for its involvement in many pathological effects, particularly nephrotoxic, immunotoxic, hepatotoxic and carcinogenic effects (6, 7). Fumonisin B1 (FB1), the most predominant member of fumonisins, is produced by *Fusarium verticillioides* and *F. proliferatum*. FB1 can be implicated in neurotoxicity, nephrotoxicity, hepatotoxicity, and mammalian cytotoxicity (8).

The majority of mycotoxigenic molds are phytopathogens, which can infect cereal crops both before and after harvest. Cereal crops are entered as main components in human foods and animal feeds (3, 9). Entering mycotoxins into the food chain, results in human exposure to these mycotoxins either directly through consuming contaminated food or indirectly through consuming foods of animal origin gained from animals fed contaminated feed ingredients (1, 10).

Although several studies investigated the presence of mycotoxins in poultry feeds and feed ingredients worldwide including Iraq (11-19), only three studies touched on the presence of AFB1 and OTA in poultry feeds in Iraq. The first study was presented by AL-Warshan et al. (20) for the determination of AFB1 levels in poultry rations collected from feedstuff production factories and local markets in Baghdad province. The second study was conducted in Sulaymaniyah to detect the occurrence of five mycotoxins including OTA in poultry feeds and feed ingredients (17). The third one was performed in Duhok to investigate the existence of three mycotoxins including AFB1 and OTA in sheep, cattle, and poultry feeds and feedstuff ingredients (21). Whereas no studies related to the occurrence of FB1 in poultry feeds.

In Nineveh province, there are no studies indicating the presence of AFB1, OTA, and FB1 in poultry feeds. Thus, the current study aimed to investigate the existence of these mycotoxins in poultry feeds in Nineveh province and to compare the results with the regulatory limits established by the European Commission (EC).

MATERIALS AND METHODS

Ethical Approval

The procedures of the study were reviewed and approved by the local Research Ethics Committee, College of Veterinary Medicine, University of Baghdad (Approval Number 1132 dated 20th May 2022).

Sample Collection

A total of 60 pelleted poultry feed samples were collected from poultry feed plants and poultry farms in Nineveh Province from April to September 2022. The samples were homogenized and quartered according to (22) to obtain 1 kg of representative samples. The samples were ground using a laboratory mill and sieved through a No. 18 mesh sieve.

Sample Pretreatment

Feed sample pretreatment was achieved according to the instructions of the ELISA kit manufacturer (Elabscience Biotechnology Inc., USA) for the detection of AFB1 (E-TO-E016), OTA (E-TO-E001), and FB1 (E-TO-E020).

For AFB1, sample pretreatment included taking 2±0.05 g of crushed homogenized sample into a 50 mL centrifuge tube, oscillating for 5 min. with 10 mL of 70% methanol and centrifuging for 10 min. at 4000 rpm at room temperature. Then, 2 mL of supernatant was taken, oscillated for 5 min. with 4 mL of chloroform and centrifuged for 10 min. at 4000 rpm at room temperature. The lower liquid was kept (lower liquid A), while the upper liquid was oscillated sufficiently for 5 min. again with 4 mL of chloroform and centrifuged for 10 min. at 4000 rpm at room temperature. The upper liquid was discarded, and the lower liquid was kept (lower liquid B). The lower liquids A and B were thoroughly mixed and 2 mL of mixture was dried in a water bath at 50-60℃. Thereafter, 0.5 mL of 70% methanol was added to dried materials to dissolve thoroughly, then 0.5 mL of deionized water was added and mixed well. Fifty microliters were taken for analysis.

Pretreatment of feed sample for OTA detection was achieved by putting 2±0.05 g of homogenized sample into a 50 mL centrifuge tube and 10 mL of 70% methanol was added to it, vortexed for 5 min., then centrifuged for 10 min. at 4000 rpm at room temperature. One milliliter of supernatant was taken to another centrifuge tube and mixed fully with 1 mL of 0.1 M $NaHCO_3$ solution (solution prepared by dissolving 4.2 g of $NaHCO_3$ in 500 mL of deionized water). Fifty microliters were taken for analysis.

For FB1, the sample pretreatment procedure was carried out by placing 1±0.05 g of crushed homogenized sample into a 50 mL centrifuge tube and 5 mL of deionized water was added and vortexed for 5 min., then centrifuged for 10 min. at 4000 rpm at room temperature. Subsequently, 0.1 mL of supernatant was taken and 0.9 mL of reconstitution buffer was added to it, then vortexed for 2 min. Fifty microliters were taken for analysis.

ELISA Procedure

Competitive ELISA procedures for the qualitative and quantitative determination of AFB1, OTA, and FB1 in naturally contaminated poultry feeds were implemented according to the kit manufacturer's instructions.

ELISA procedures for AFB1 and FB1 detection were the same. The standards and samples were examined in duplicate. Fifty microliters of standards or samples were put in the specific microwells, then 50 µL of horseradish peroxidase (HRP) conjugate and 50 µL of antibody working solution were added for each well. The plate was covered with the sealer, oscillated, and incubated for 30 min. at 25 °C. After liquid removal, each well was washed five times with 300 µL of washing buffer. After that, 50 µL of substrate reagent A and 50 µL of substrate reagent B were added to

each well, oscilated, and incubated for 15 min. at 25 °C for color development. To stop the reaction, 50 µL of stop solution was added to each well and oscillated. The optical density (OD) values were determined using a microplate reader (HumaReader HS, Germany) at 450 nm within 10 min. of stopping the reaction.

For OTA analysis, the ELISA procedure involved placing 50 µL of standards or samples (in duplicate) in the specific microwells, then 50 µL of antibody working solution was added for each well. The plate was covered, oscillated, and incubated for 30 min. at 37 °C. Each microwell was washed with 300 µL of washing buffer five times. HRP conjugate was added to each well (100 µL) and incubated for 30 min. at 37 °C. The washing step was repeated. For color development, 50 µL of substrate reagent A and 50 µL of substrate reagent B were added to wells, oscillated, and incubated for 15 min. at 37 °C. The reaction was stopped with 50 µL of stop solution for each well. The OD values were estimated at 450 nm with a microplate reader within 10 min. after the addition of the stop solution.

Statistical Analysis

Statistical analysis of the data was implemented using the One-way analysis of variance (ANOVA) procedure of the Sigma Stat for Windows Version 3.10 (23). Duncan's Multiple Range Test was achieved for comparison of the means at $P \leq 0.05$ (24).

RESULTS

Results of the investigation of AFB1, OTA, and FB1 in poultry feed samples showed that all samples (100%) were contaminated with AFB1 and FB1 and 53 samples (88.33%) with OTA, at levels ranging between 3.15–43.96, 0–168.24 and 220.6–6935.12 ppb, respectively. Results clarified that FB1 present in feed samples at a mean value (2164.01 ppb) significantly higher ($P<0.05$) than the mean values reported for AFB1 and OTA (16.48 and 32.09 ppb, respectively). Co-occurrence of AFB1 and FB1 was noticed in 100% of samples and the three targeted mycotoxins in 88.33% of samples (Figure 1 and Table 1).

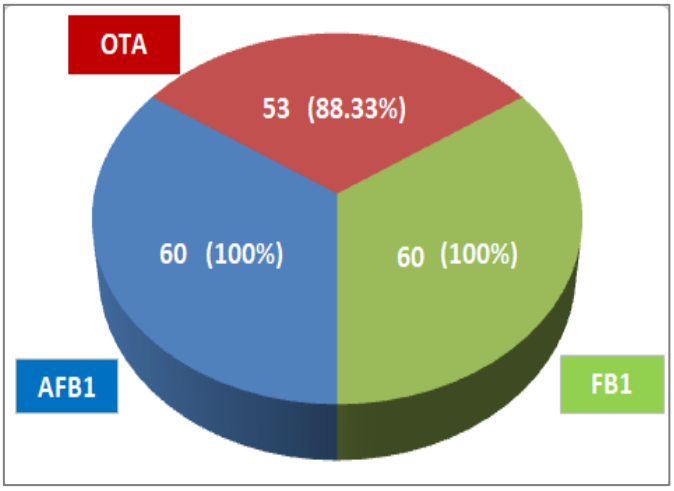

Figure 1. Number and percentage of the poultry feed samples naturally contaminated with aflatoxin B1 (AFB1), ochratoxin A (OTA), and fumonisin B1 (FB1)

Table 1. The range and mean concentrations (ppb) of aflatoxin B1 (AFB1), ochratoxin A (OTA), and fumonisin B1 (FB1) in poultry feed samples

		Concentration (ppb)		
		Range		
Mycotoxin	No. of Examined Feed Samples	Minimum	Maximum	Mean ± SD
AFB1	60	3.150	43.96	16.48±9.345 [b]
OTA	60	0.000	168.24	32.09±40.42 [b]
FB1	60	220.6	6935	2164±1646 [a]

Vertically different small letters are significantly different at $P \leq 0.05$

Our results exhibited that 31.67% of poultry feed samples were contaminated with AFB1 at levels ranging between 3.150–9.280 ppb, 30% at levels ranging between 10.12–19.84 ppb, 31.67% at levels ranging between 20.38–27.40 ppb, 3.330% at levels ranging between 32.94–36.76 ppb, and 3.330% at levels ranging between 41.09–43.96 ppb. European Commission (EC), No. 574/2011 established AFB1 regulatory limit of 20 ppb in poultry feeds (25). According to this limit, results related to the occurrence of AFB1 in poultry feed samples revealed that 37 samples

(61.67%) out of 60 samples were within the EC limit. Whereas 23 samples (38.33%) were higher than the EC legal limit (Table 2).

Table 2. Occurrence of aflatoxin B1 (AFB1) in poultry feed samples in Nineveh Province

AFB1 levels (ppb)	Sample No.	%	Range (ppb)
3.000–10	19	31.67	3.150–9.280
10.01–20	18	30.00	10.12–19.84
20.01–30	19	31.67	20.38–27.40
30.01–40	2	3.330	32.94–36.76
40.01–50	2	3.330	41.09–43.96
Total samples	60	100	3.150–43.96

Concerning OTA, results demonstrated that 11.67% of samples were negative for OTA presence, whereas, 50% of samples were contaminated with OTA at concentrations ranging between 10.21–19.92 ppb, 16.67% at concentrations ranging between 20.19–28.5 ppb, 6.67% at concentrations ranging between 32.61–36.14 ppb, 1.67% at a concentration of 49.3 ppb, 1.67% at a concentration of 73.81 ppb, 1.67% at a concentration of 94.23 ppb, and 10% at concentrations ranging between 113.13–168.24 ppb. The majority of the feed samples (54 samples, 90%) were within the desired limit for OTA recommended by the EC (100 ppb) (26). Only 6 samples (10%) exceeded the maximum permissible limit of the EC for OTA in the feedstuffs of poultry (Table 3).

Table 3. Occurrence of ochratoxin A (OTA) in poultry feed samples in Nineveh Province

OTA levels (ppb)	Sample No.	%	Range (ppb)
0	7	11.67	0
10.0–20	30	50	10.21–19.92
20.01–30	10	16.67	20.19–28.5
30.01–40	4	6.67	32.61–36.14
40.01–50	1	1.67	49.3
70.0–80	1	1.67	73.81
90.0–100	1	1.67	94.23
100.01–200	6	10	113.13–168.24
Total samples	60	100	·–168.24

Regarding the presence of FB1 in poultry feed samples, results pointed out that 23.33% of samples were contaminated with FB1 at values ranging between 220.6–972.05 ppb, 33.33% at values ranging between 1052.25–1814.5 ppb, 21.67% at values ranging between 2361.16–2990.2 ppb, 11.67% at values ranging between 3231.15–3912.4 ppb, 1.67% at a value of 4468.47 ppb, 3.33% at values ranging between 5523.12–5712.8 ppb, and 5% at values ranging between 6150.25–6935.12 ppb. Results showed that all feed samples were lower than the maximum permissible limit for fumonisin B1+fumonisin B2 in the complementary and complete feeding stuffs of poultry (20000 ppb), as there is no maximum level set by the EC related to FB1 alone in poultry feeds (26) (Table 4).

Table 4. Occurrence of fumonisin B1 (FB1) in poultry feed samples in Nineveh Province

FB1 levels (ppb)	Sample No.	%	Range (ppb)
200–1000	14	23.33	220.6–972.05
1000.01–2000	20	33.33	1052.25–1814.5
2000.01–3000	13	21.67	2361.16–2990.2
3000.01–4000	7	11.67	3231.15–3912.4
4000.01–5000	1	1.67	4468.47
5000.01–6000	2	3.33	5523.12–5712.8
6000.01–7000	3	5	6150.25–6935.12
Total samples	60	100	220.6 – 6935.12

DISCUSSION

Poultry fed on mycotoxin-contaminated feeds could pose a risk to human health by consuming poultry products contaminated with the residues of these mycotoxins, in addition to their hazardous effects on poultry health and performance (27,28). Monitoring of mycotoxin levels in feeds and foods of animal origin and establishing the maximum permissible limits to these mycotoxins in animal feed and human food become inevitable and applied strictly by many countries. However, in Iraq, strict regulations related to setting the acceptable levels for these mycotoxins in foods of animal origin and feed were not established yet.

Results presented the presence of AFB1 in 100% of the analyzed samples at values ranging between 3.15–43.96 ppb with a mean value of 16.48 ppb. AFB1 levels were higher than the maximum allowable limit set by EC in 38.33% of samples. Results were higher than those presented by Alshawabkeh et al. (29) which referred to the occurrence of AFB1 in 40% of feed samples collected from various regions of Jordan with values varied between 3.23-39.41 ppb. In addition to results obtained by Alahlah et al. (30) which reported the presence of AFB1 in 58.33% of poultry feed samples collected from the Northeastern Moroccan area with levels ranging between 1.11 - 13.59 ppb, with a mean concentration of 5.96 ppb. Per contra, results were lower than the results found by Al-Warshan et al. (20) regarding AFB1 mean levels in poultry feeds collected from feedstuff production factories and local markets in Baghdad Governorate which determined between 20-150 ppb, with AFB1 occurrence between 25-100%. Furthermore, Oruc et al. (31) recorded the presence of AFB1 in broiler feed in the Bursa region, Turkey at a range of 8.40-49.80 ppb and a mean value of 27.6 ppb. Abdou et al. (32) assessed AFB1 presence in poultry feeds in Egypt and also reported the occurrence of this mycotoxin in all five examined farms at mean values higher than our results which reached 70, 47, 42, 41, and 60 ppb, which were significantly higher than the EC regulation.

In our study, OTA was detected in 88.33% of the examined samples at levels ranging between 0–168.24 ppb

with a mean level of 32.09 ppb and 10% of samples exceeding the maximum allowable limit set by EC. OTA was surveyed in poultry feeds in three different cities in Morocco and recorded its presence in 30.6% with values varied between 0.24-26.8 ppb and mean values of 11.3, 0.8, and 9.4 ppb in samples collected from the three cities, without exceeding any sample the EC limit (33). These results were lower than our results. Also, our results were higher than those reported by Alkhalaileh (34) in Jordan, relating to the ranges (1.72-3.70 and 2.10-27.11 ppb) and mean concentrations (2.90 and 10.30 ppb) of OTA in feed samples not present under sunlight and those present under sunlight, respectively, where all the tested samples were within the acceptable EC limits. As well, the results were higher than the results found in laying hen feeds in Turkey, regarding the mean and maximum concentration of OTA, as they reached 27.28 and 96.30 ppb, respectively (35). Rahim et al. (17) recorded the presence of OTA in poultry feeds in Sulaymaniyah at levels varied between 1–6 ppb and a mean value of 3.4 ppb, which was also lower than our results. Conversely, the results were lower than those found by Fraga et al. (36) in Brazil, which indicated the presence of OTA in 100% of the tested poultry feed samples at concentrations varied between 17 to 197 ppb and a mean concentration of 98.2 ppb. Krnjaja et al. (37) reported OTA occurrence in 100% of feed samples of chickens and laying hens in Serbia with mean values of 34.40 and 43.89 ppb, respectively, which is higher than our results, although there are no samples exceeded the EC limits. Furthermore, the results were lower than the results obtained by Sherazi et al. (38) in Pakistan, concerning the mean level and number of samples exceeding the EC maximum regulatory limit which reached 75 ppb and 12.5%, respectively. Likewise, the results were lower than the results detected by Abidin et al. (39) in Pakistan, who recorded OTA presence in poultry feeds at levels ranging between 2.88-178.78 ppb.

FB1 was found in 100% of the tested samples at concentrations ranging between 220.6–6935.12 ppb with a mean value of 2164.01 ppb. FB1 levels were lower than the EC desired limit in all the examined samples. Results were in agreement to a certain extent with the results obtained by Kumi et al. (40) in Ghana which reported the presence of FB1 in 100% of poultry feed samples in a range of 500-4600 ppb with no samples exceeding the EC limit.

Results clarified a higher presence for AFB1 and FB1 (100%) in poultry feed samples comparable with OTA (88.33%) with FB1 mean value (2164.01 ppb) significantly higher ($P<0.05$) than those reported for AFB1 and OTA (16.48 and 32.09 ppb, respectively). Results were in coincidence with the results perceived by Elalfy and Abdein (41) which also showed a higher incidence of AFB1 in poultry feed samples collected from Dakhalia Governorate, Egypt than OTA with mean values of AFB1 in the starter and finishing rations (17.22 and 9 ppb, respectively) lower than those recorded for OTA (22.9 and 13.8 ppb, respectively). In Sulaymaniyah, OTA was detected in 100% of poultry feed samples, which was higher than our results, while aflatoxins and fumonisins were detected in 90.7 and 68.8% of samples, which was lower than our results, however, fumonisin was found at a mean value (1159 ppb) higher than the mean values of aflatoxins and OTA (4.5 and 3.4 ppb, respectively), which was in accordance with our results (17). Our results were higher than those stated by Akinmusire et al. (42) regarding FB1 and AFB1 incidence, which reported the presence of these mycotoxins in 97% and 83%, respectively of poultry feed samples in Nigeria. Also, results were higher than the results detected by Sadeeq (21) in poultry feeds in Duhok which showed the presence of AFB1 and OTA in 10% and 40% of samples analyzed with values ranged between 0-0.49 and 0.02-0.2 ppb with mean values of 0.113 and 0.111 ppb, respectively.

Co-occurrence of AFB1, OTA, and FB1 was also indicated by other researches. Akinmusire et al. (42) examined poultry feeds for multiple mycotoxins in Nigeria and referred to the contamination of examined feed samples with at least four mycotoxins, among them, aflatoxins and fumonisins co-occurred in 80% of the samples, which was lower than our results. Also, the results were higher than the results observed by Al-Said and El-Tedawy (43) who reported co-occurrence of total aflatoxin and OTA in 63% of poultry feedstuff samples in El Behaira, Egypt. In contrast, the results were lower than those noticed by Kumi et al. (40) who reported co-occurrence of total aflatoxin and OTA in 100% of poultry feed samples in Ghana.

These variations in the levels and occurrence of mycotoxins recorded in our study comparable with the other studies could be attributed mainly to the considerable diversity in the climatic conditions between different regions. Furthermore, the continuous climate changes witnessed by the world in recent years, as the rise in the environmental temperature, the increment in the levels of carbon dioxide, and extremes in water availability, possess a significant impact on mycotoxigenic mold development and mycotoxins production between various geographical regions (44, 45).

In brief, the present study revealed substantial information about AFB1, OTA, and FB1 levels in poultry feeds in Nineveh Province. Although all feed samples were within the EC desired limit for FB1, strict procedures should be imposed towards AFB1 and OTA levels, which exceeded the EC allowable limits in 38.33% and 10% of samples, respectively. This can be implemented by adopting strict legislation to limit exposure to mycotoxins in poultry feeds and feed ingredients, to prevent their negative impacts on poultry health and minimize their levels in poultry products for consumer health protection. Also, several strategies should be applied to prevent or minimize the presence of mycotoxins in food and feed commodities. Furthermore, regular monitoring of

mycotoxins in foods and feeds in our country may be essential due to the availability of suitable environmental conditions for the production of several types of mycotoxins by certain mold species, in addition to the continuous changes in climatic conditions annually.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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التحري عن سم الأفلا B1 وسم الأوكرا A وسم الفومونيزين B1 في أعلاف الدواجن في محافظة نينوى

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الخلاصة

يشكل كل من سم الأفلا B1 وسم الاوكرا A وسم الفومونيزين B1 والذين يعدون من بين السموم الفطرية الأكثر شيوعًا تهديدا كبيرا على صحة الإنسان والحيوان نتيجة لتأثيراتهم السمية والمسرطنة والمطفرة. هدفت الدراسة التحري عن تواجد هذه السموم الفطرية في أعلاف الدواجن وتحديد النسبة المئوية للعينات المتجاوزة للحدود القانونية المقرة من قبل المفوضية الأوروبية (EC). تم جمع ٦٠ عينة من أعلاف الدواجن من مصانع أعلاف ومزارع الدواجن في محافظة نينوى وتم تحليلها بحثًا عن السموم الفطرية باستخدام مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA). سجلت النتائج تواجدا مشتركا لكل من سم الأفلا B1 وسم الفومونيزين B1 في جميع العينات المفحوصة (١٠٠٪)، في حين سجل كل من سم الأفلا B1 وسم الاوكرا A وسم الفومونيزين B1 تواجدا مشتركا في ٥٣ عينة (٨٨,٣٣٪) وبمدى تراوح بين 3.15-43.96، ٠-168.24 و ٢٢٠,٦-٦٩٣٥,١٢ جزء بالبليون على التوالي. أظهرت النتائج ارتفاعا معنويا (P<0.05) في معدل تركيز سم الفومونيزين B1 (٢١٦٤,٠١ جزء بالبليون) مقارنة بمعدل تركيز سم الأفلا B1 وسم الاوكرا A (16.48 و 32.09 جزء بالبليون، على التوالي). كشفت النتائج عن تجاوز ٣٨,٣٣٪ و ١٠٪ من عينات الأعلاف للحدود القصوى المسموح بها من قبل المفوضية الأوربية لكل من سم الأفلا B1 وسم الاوكرا A، على التوالي، بينما كانت جميع عينات الأعلاف تقع ضمن الحدود المسموح بها لسم الفومونيزين B1. لذلك، لابد من اتباع إجراءات صارمة لتحقيق الحدود القانونية المتعلقة بسم الأفلا B1 وسم الاوكرا A في أعلاف الدواجن للحفاظ على الصحة العامة.

الكلمات المفتاحية: : أعلاف الدواجن، سم الأفلا B1، سم الاوكرا A، سم الفومونيزين B1، مقايسة الممتز المناعي المرتبط بالإنزيم، نينوى