



Occurrence of Aflatoxin B1 in Animal Feed Collected from the Northeastern Area of Morocco

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ABSTRACT

The carry-over of contaminants from feed to animal products is an important issue in the animal production chain, therefore, the quality control of those animal products should include the control of the animal feed. The current study was carried out to assess the contamination levels of three types of animal feed (dairy animal feed, poultry feed, and fish feed) by Aflatoxin B1. A total of 68 animal feed samples were collected from the Northeastern Moroccan area (Tangier-Tétouan-AL Hoceima). The samples were extracted with a mixture of acetone/water. The sample extractions were filtered, diluted with phosphate-buffered saline, and applied to an immunoaffinity column. Aflatoxin B1 was eluted with methanol then analyzed by high-performance liquid chromatography with fluorescence detection, after post-column photochemical derivatization. The analytical results for the level of Aflatoxin B1 in the animal feed samples revealed an average presence of 44.12% for all analyzed samples. The concentrations were between 1.02 and 13.59 µg/Kg, with a mean value of 4.08 ± 3.11 µg/Kg. The results indicated that there was a significant difference across the three types of animal feeds regarding the concentrations of Aflatoxin B1.

Keywords: Aflatoxin B1, Animal feed, HPLC, Morocco, Statistical data

INTRODUCTION

Aflatoxins are mycotoxins secreted by the species of *Aspergillus*, mainly *Aspergillus species*. Aflatoxin B1 (AFB1) is well known to be the most dangerous mycotoxin for humans. It is commonly found in a wide variety of food and feedstuffs (Oplatowska et al., 2016; Zinedine et al., 2016; Sasiprapa et al., 2018). This substance is classified in Group I of carcinogenic agents for humans (IARC, 2002; Ostry et al., 2017).

The consequences of food and animal feed contamination by aflatoxin B1 have been widely investigated. With acute or chronic intoxication, teratogenic, mutagenic, carcinogenic, immunotoxic, or hepatotoxic effects of aflatoxins were well documented and discussed, especially for dairy cattle and poultry (Mckean et al., 2006; Klingelhofer et al., 2018; Sirma et al., 2018). Recently, toxic effects of aflatoxins on human astrocytes and human gastric smooth muscle cells were investigated (Omotayo et al., 2019; Park et al., 2019 a).

Aflatoxins in animal feed exert detrimental effects at three levels of animal health, human health through the consumption of contaminated products of animal origin, and subsequent economic losses due to the seizure and destruction of the incriminated lots. For different animal species, many studies have looked at aflatoxin's effects on animal health. General health problems, such as diarrhea, mastitis, and respiratory disorders were reported in herds (Quezada et al., 2000; Yiannikouris and Jouany, 2002; Rawal et al., 2010; Alam et al., 2020). Other effects have been listed, including attenuation of cell viability and induction of endoplasmic reticulum-mediated cell death in a bovine mammary epithelial cell line (Park et al., 2019b). Additional effects on embryonic development and attenuation of sperm viability in cattle (Komsky et al., 2018; Jiang et al., 2019), toxico-pathological effects in broiler chickens (Kashif et al., 2020) as well as disruption of the blood-brain barrier and affection of fish behavior (Ayyat et al., 2018; Baldissera et al., 2018) were also reported.

The above-mentioned effects have two consequences. First, contamination of animal products including milk, eggs, and meat, leads to adverse effects on human health (Costamagna et al., 2019; Adegbeye et al., 2020; Elzupir and Abdulkhair, 2020). Second, a reduction of reproductivity (poultry, aquaculture, and dairy production) leads to important economic losses (Ayyat et al., 2018; Alam et al., 2020; Yang et al., 2020). Aware of all these consequences, several studies have been carried out by many countries with the aim of determining the natural presence of aflatoxins in animal feed (Khayoon et al., 2010; Arroyo-Manzanares et al., 2015; Xiong et al., 2018). However, a large number of studies in different countries have focused mainly on the occurrence of AFB1 eliminated in the form of Aflatoxin M1 in dairy

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animal feed due to the importance of milk contamination in human health, especially children. The aim of those studies was to investigate the carryover rate of AFB1 from feed to milk and to determine contamination levels of this important animal product (Battacone et al., 2003; Xiong et al., 2013; Alahlah et al., 2020).

To manage the risk associated with animal feed contamination, analysis methods were developed and regulatory limits for feed were set. At the analytical level, from TLC to LC-MS/MS, the determination of aflatoxins in food and feed has undergone a marked evolution (Shephard, 2009). At the regulatory level, several health authorities have established limits for AFB1 in feedstuffs. In European Legislation 574/2011/EC (Official Journal of the European Union, 2011), the maximum level of AFB1 in complementary and complete feed is 0.01 mg/kg (10 µg/kg), except for dairy and young animals for which maximum levels are 0.005 mg/kg (5 µg/kg). For other livestock, these levels are capped at 0.02 mg/kg (20 µg/kg). Those values are relative to a feed with a moisture value of 12%. The same maximum levels are established by Moroccan regulations (Bulletin Officiel N° 6184, 2016).

In Morocco, the first report on the contamination of animal feed dealt with poultry feed with a total number of 315 samples (Kichou and Wasler, 1993). Another subsequent study by Zinedine et al. (2007), including poultry feed (21 samples) revealed an incidence of 66.6%.

The present study aimed to investigate Aflatoxin B1 contamination levels, in three types of animal feed intended for dairy farms, poultry, and aquaculture fish.

MATERIALS AND METHODS

Sampling

A total of 68 samples of animal feed (44 dairy cattle feed, 12 poultry feed, and 12 fish feed) were collected in the northeastern area of Morocco, from October 2019 to July 2020. Samples, of 2 kg approximately each, were ground in a laboratory mill to ensure homogeneity and they were analyzed immediately.

Reagents

Liquid chromatography grade acetone, acetonitrile, and methanol, and phosphate buffered saline (PBS) were supplied by Sigma-Aldrich (Steinheim, Germany). The LC grade water was obtained by filtering deionized water with MilliQ system. Powders of Aflatoxin standards B1 were supplied by N^oTox (Novakits, France), and kept frozen at -20°C in darkness. Working solutions of 100 ng/mL AFB1 were prepared from the stock solution containing 1 µg/mL into acetonitrile-water (1V/9V) and were used to obtain a calibration curve ranging from 0.2 to 4 ng/mL. The dilution was made with methanol up to 4 mL and further diluted with water up to 10 mL (AOAC, 2005)

Apparatus

The cleanup was achieved with an immuno-affinity column (Afla Clean select, LCTech GmbH, Germany) using a vacuum extraction manifold system. The chromatography was performed on a Perkin Elmer series 200 system coupled with the fluorescence detector Perkin Elmer Flexar (HTDS, France). A Sepax Bio-C18 reverse-phase column (150 × 4.6 mm, 5 µm, 300 Å) with guard column C18 (30 × 4.6 mm, 5 µm) was used for the chromatographic separation. The post-column derivatization was realized with a Photochemical derivatization device- PHRED CE-12 with a knitted reactor coil KRC 20-25, provided by Aura Industries, USA (Joshua, 1993).

Methods

Extraction and immune-affinity clean-up were performed according to the AOAC Official Method 2003.02 (AOAC, 2005), and the manipulations were done in the dark.

Extraction

A test portion of 50 g (± 0.1 g) was weighed into a 500 ml flask, an extraction solvent (acetone/water, 85:15 v/v) was added. The mixture was shaken by hand for the first 15-30 s and then for 30 minutes with a flask shaker. The extract was filtered through a qualitative cellulose grade 4 Whatman paper filter and 5 ml of the filtered sample were taken into a 100 ml calibrated flask, diluted to volume with phosphate-buffered saline (pH 7.4), and then filtrated through a glass microfiber GF /A filter to obtain a clear solution (AOAC, 2005).

Immunoaffinity column clean up

The manufacturer's instructions concerning column conditioning were followed. The columns were brought to room temperature, the liquid contained was evacuated and a small portion was maintained on the top of the column until the sample solution was applied. Then, 50 mL of the filtered sample extract was passed through the column by gravity at a flow rate of approximately 3 mL/min (approximately 1-2 drops per second). The immunoaffinity column (IAC) was washed with 2 × 10 mL of water at a flow rate of a maximum of 5 mL/min and then allowed the column to run dry by

applying a little vacuum (AOAC, 2005). The elution was carried out in 2 steps. Firstly, 0.5 mL of methanol was applied into IAC and collected by gravity in a calibrated 5 mL volumetric flask. After 1 min, the second volume of 1.25 ml methanol was applied and collected completely by pressing air through and diluted to volume with water. The elute was filtered through a filter unit 0.45 before High-performance liquid chromatography (HPLC) injection (AOAC, 2005)

Derivatization

The post-column photochemical derivatization was performed according to Joshua (1993). It is done automatically, using PHRED system placed between the LC column and the detector. AFB1 was transformed to their corresponding hemiacetals under UV light.

High-performance liquid chromatography

A volume of 100 μ L (in full loop injection mode) was injected; the flow rate was 1 mL/min of the mobile phase containing water/acetonitrile/methanol solution (70/18/12, v/v/v). Detection was made with a fluorescence detector, excitation and emission wavelengths were set at 365 and 435 nm, respectively (AOAC, 2005). All samples were analyzed in duplicate.

Statistical analysis

Aflatoxin B1 concentrations (μ g/kg) were calculated based on a calibration curve. ANOVA test was conducted and then the means of the three feeds were compared by Tukey test using SPSS Software (IBM SPSS Statistics V21 x86, USA). The level of significance was $p \leq 0.05$. Results are presented as means \pm standard deviation.

RESULTS AND DISCUSSION

Results from AFB1 analytical determinations in the investigated feeds are summarized in Table 1. For the three animal feed types, the incidence of contamination was 44.12%. Out of the 68 samples analyzed, 30 were found to be positive for AFB1. Concentration levels varied between 1.02 μ g/kg (dairy animal feed) to 13.59 μ g/Kg (Poultry feed), with a mean value of 4.08 ± 3.11 μ g/Kg. Segregation of the results by the type of feed indicated an incidence of contamination of 40.91 % in dairy animal feed (18 samples out of 44). The concentrations varied between 1.02 and 4.13 μ g/Kg, with a mean value of 2.66 ± 0.9 μ g/Kg. All results were below 5 μ g/Kg.

In poultry feed, 7 samples were found to be contaminated (58.33%). The concentrations varied between 1.11 and 13.59 μ g/Kg, with a mean value of 5.96 ± 4.75 μ g/Kg. The Moroccan regulatory level is capped at 20 μ g/kg. A proportion of 41.67% of AFB1 levels in fish feed samples was above the limit of quantification. Concentration levels ranging between 2.08 and 9.47 μ g/Kg were found, with a mean value of 6.58 ± 3.08 μ g/Kg. None of the investigated samples exceeded the current regulatory maximum level of 10 μ g/kg.

Regardless of the number of samples, the analysis of the results showed that poultry feed is the most contaminated with the highest incidence, followed by fish feed and finally dairy animal feed. With respect to a level of significance $p \leq 0.05$, the ANOVA and Tukey test made it possible to conclude that there was no difference between poultry and fish feed ($p > 0.05$) (while there was a significant difference between the concentrations of AFB1 in dairy animal feed as well as and in fish and poultry ($p \leq 0.05$) feed on the other hand (Table 2).

Table 1. Occurrence of AFB1 in animal feed samples of the Northeastern area of Morocco

Type of animal feed	Distribution of samples by AFB1 (μ g/kg) concentration intervals						
	$\geq 1 - < 5$			$\geq 5 - < 10$		$\geq 10 - < 20$	
Dairy animal Feed (n = 44)	2.13 \pm 0.90	2.1 \pm 0.90	2.22 \pm 0.90	--	--	--	--
	2.51 \pm 0.90	3.34 \pm 0.9	2.01 \pm 0.90				
	3.35 \pm 0.90	2.14 \pm 0.9	2.55 \pm 0.90				
	4.13 \pm 0.90	3.46 \pm 0.9	1.33 \pm 0.90				
	4.09 \pm 0.90	2.80 \pm 0.90	1.02 \pm 0.90				
	3.56 \pm 0.90	1.75 \pm 0.90	3.35 \pm 0.90				
Poultry feed (n = 12)	1.11 \pm 4.75	1.23 \pm 4.75	2.86 \pm 4.75	5.33 \pm 4.75	7.12 \pm 4.75	10.45 \pm 4.75	13.59 \pm 4.75
Fish feed (n = 12)	2.08 \pm 3.08			9.47 \pm 3.08	6.94 \pm 3.08	--	
				5.14 \pm 3.08	9.25 \pm 3.08		

Table 2. Differences between the tree studied animal feed

(I) Feed	(J) Feed	Mean Difference (I-J)	Standard Error	Significance	95% Confidence interval		
					Lower Bound	Upper Bound	
Tukey Test	Dairy Animal	Poultry	-3.38794*	1.17	0.020	-6.2945	-0.4814
		Fish	-3.91822*	1.33	0.017	-7.2169	-0.6196
	Poultry	Dairy Animal	3.38794*	1.17	0.020	0.4814	6.2945
		Fish	0.53029	1.54	0.937	-4.3510	3.2905
	Fish	Dairy Animal	3.91822*	1.33	0.017	0.6196	7.2169
		Poultry	0.53029	1.54	0.937	-3.2905	4.3510

*The mean difference is significant at the level of 0.05 in a row.

In 2018, the production of poultry meat in Morocco reached 670000 metric tons and that of eggs was more than 5.8 billion units as reported by [Fisa Maroc \(2020\)](#). These values have been the highest since 2008. To reach these levels, feed production has also much evolved. In 2018, the production of poultry feed was 3.4 million metric tons. While the dairy feed is based on fodder production and agro-industry by-products, poultry feed is based only on industrialized feed (90% of the feed industry is focused on poultry feed). This may allow us to conclude that such a feed is the most likely to be contaminated by aflatoxins during production, transport, and/or storage and thus to have a tentative explanation for the high incidence found on a small number of samples.

For dairy animal feed, the obtained result of the current study indicated that the incidence was similar to that reported in other countries. Incidences of 42% (n = 200), 35.1% (n = 174), and 41.96% (n = 210) were reported from China during two studies ([Han et al., 2013](#); [Xiong et al., 2018](#)) and Iran ([Ehsani et al., 2016](#)) respectively. When comparing the results of the present study with those from some African countries, the incidence of AFB1 in the present study is lower than incidences reported in Nigeria (86.8%) by [Omeiza et al. \(2018\)](#), and Kenya (86%) by [Kang'ethe and Lang'a \(2009\)](#). In some countries of southern Europe, with a Mediterranean climate, the presence of aflatoxin B1 in dairy animal feed with low incidences has been reported as 14.9% in Portugal ([Martins et al., 2007](#)) and 8.1% in Italy ([Decastelli et al., 2007](#)). Differences in incidence between countries can be explained by the colder climatic conditions and by the reinforced feed control system in these countries.

The transfer of Aflatoxin B1 from feed to milk has been investigated by several authors ([Battacone et al., 2003](#); [Xiong et al., 2013](#); [Costamagna et al., 2019](#)). The carryover rate varied among the different studies. In dairy cattle, an average AFB1 carryover rate estimated at 0.84% was reported from Argentina ([Costamagna et al., 2019](#)) while a carryover estimated at 0.56% was reported from China ([Xiong et al., 2013](#)). In dairy ewes, a lower carryover rate of 0.112% was reported from Italy ([Battacone et al., 2003](#)). The current results may explain the occurrence of aflatoxin M1 in Ultra High Temperature (UHT) milk (35% incidence) and powdered milk (100% incidence) as evidenced from a previous study involving samples collected in the northern area of Morocco ([Alahlah et al., 2020](#)). Concentrations up to 44 ng/Kg were found in UHT milk, with an average concentration of 14.76±10.21 ng/Kg. Nevertheless, actual confirmation awaits future analysis of a larger number of dairy animals' feed samples.

With respect to AFB1 determinations in poultry feed in Morocco, the current levels are much lower as compared to the results of [Kichou and Wasler \(1993\)](#) with concentrations ranging from 20 to 5625 µg/kg. However, the current incidence is close to that reported by [Zinedine et al. \(2007\)](#) with a value of 66.6% based on 21 analyzed samples. The carryover rate of aflatoxin B1 from feed to eggs and laying hens (meat, liver, kidneys, and gizzards) has not been investigated as for milk, but AFB1 has been detected in such animal products ([Quezada et al., 2000](#); [Hussain et al., 2010](#); [Alam et al., 2020](#)).

Regarding the presence of AFB1 in fish feed, and to our knowledge, this is the first report of aflatoxin B1 in this type of feed in Morocco. The development of the aquaculture sector in Morocco has led the National competent authority of food safety (ONSSA) to set up a residue monitoring program for this type of feed. Although studies on the contamination of fish flesh with AFB1 are scarce, some have still looked at the effect of AFB1 on fish behavior and growth performances ([Ayyat et al., 2018](#); [Baldissera et al., 2018](#)).

Whether for poultry feed or fish feed, the limited number of investigated samples may not allow a true risk assessment. However, the results of the present study should drive an alert on underlying risks in animal feed safety with the necessary strengthening of official control over the entire production chain along with the implementation of good hygienic and manufacturing practices and quality assurance programs.

CONCLUSION

Findings of the current study revealed that no sample exceeded the limit of contamination set by the national authority and EU, however, a study on a large number of samples should be carried out to confirm these results, in particular for poultry feed. The incidence of 40.91% obtained for dairy animal feed should be taken into account. A study on aflatoxin M1 in raw milk becomes necessary to verify whether or not the level of contamination of cattle feed affects the quality of the milk. Poultry feed with an incidence of 58.33% should raise awareness of the national authorities with respect to strengthening the already used monitoring programs. Considering the fish feed, national studies on the quality of the products must be carried out to better exploit the results currently found in the current study.

DECLARATIONS

Authors' contributions

Naoual Alahlah conceived of the presented idea. Naoual Alahlah, Mohammed El Maadoudi, Nourredine Bouchriti, and Reda Triqui developed the theory and performed the computations. Meriem Stitou, Nour Houda Hafid and Oussama El Ouahabi carried out the experiment in animal feed samples. Naoual Alahlah, Mohammed El Maadoudi, and Nourredine Bouchriti verified the analytical methods and supervised the findings of this work. Naoual Alahlah wrote the manuscript with support from Mohammed El Maadoudi, Nourredine Bouchriti and Reda Triqui.

All authors discussed the results and contributed to the final manuscript.

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Competing interests

All authors declare no competing interests are to be reported.

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Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct data and/or falsification, double publication and/or submission, and redundancy) have been checked before submission.

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