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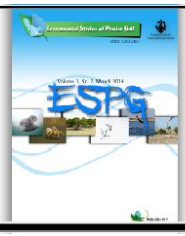

Aflatoxins in Tissues and Diets of Farmed White Shrimp (*Litopenaeus vannamei*)

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ABSTRACT

Aflatoxins (AFs) are one of the most important mycotoxins due to their common occurrence in feedstuffs and feeds that pose a serious threat to humans and animals. Although many outbreaks of acute and chronic diseases have been attributed to consumption of aflatoxin-contaminated foods, the most significant effect of aflatoxin is hepatotoxicity in farm animals, especially aflatoxicosis in shrimps. In this study, Aflatoxins content in tissues and diets of white shrimp collected from farms of Helleh, Delvar, Mond, Bandar Rig sites, located in Bushehr province were determined by isocratic reverse-phase liquid chromatography (HPLC). Results showed that the highest content of aflatoxin (4.12±0.14 μg kg^{-1}) was obtained from the food that had been used in Mond shrimp farm among all the examined farms. Although there were negligible differences between all the groups, significant difference were found between AFG1 group and the other groups ($P<0.05$).Overall, the means of aflatoxins B1, B2, G1 and G2 for all shrimp samples were 0.05, ND (< LOD), 0.01 and 0.03, respectively, while they were 0.057, 0.112, 0.278, and 0.745 ppb for their corresponding diets. In conclusion, continuous aflatoxin measurement of foods is suggested to prevent the contamination of shrimp farms in Bushehr province.

1 INTRODUCTION

The Pacific white shrimps, *Litopenaeus vannamei*, the most important penaeid shrimp species farmed worldwide (Armando et al., 2011), are the species of choice for the shrimp farming industry because of their high tolerance to low salinity and the year-round availability of healthy post-larvae (Allen et al., 2000; Marcelo et al., 2008). Production and export of shrimp play an important role in the fisheries economy in southern Iran (Salehi, 2010; Yousefi et al., 2009). The productions were 136, 163, 523 and 870 tonne in 1996 and 1998, respectively (Mojahedi, 2001).The trend of shrimp culture increased from 0.6 tonne in 1993 to 5,600 tonne in 2004 in Bushehr, located in south of Iran (Salehi, 2010). According to the report of Department of Fisheries in Bushehr,

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the production was 5,150 tonne of farmed shrimp in 2011 forming 70% of the country's shrimp production. This production is mostly exported to Spain, Portugal, United Arabic Emirates, Egypt and Lebanon (Irna, 2012).

A wide variety of animals can be affected by aflatoxins (Mohammadi et al., 2012). They may cause loss or illness of farm animals, as well as shrimps. According to FDA, the maximum safe level of aflatoxin in human and animals feeds is 20 ppb (FDA Regulatory Guidance).

Available literature on the toxicity of AFB1 indicates that aflatoxicosis is a potentially serious problem and different pathology and pathohistology changes occur in shrimps (Bautista et al., 1994; Tuan et al., 2002). They were sensitive to feed-borne aflatoxin (Boonyaratpalin et al., 2001; Burgos-Hernadez et al., 2005).

Aflatoxins (B1, B2, G1, and G2) are a group of both acutely and chronically toxic, mutagenic, carcinogenic and teratogenic polypeptide secondary metabolites that are naturally produced by certain Aspergillus (*A.*) species, in particular *A. parasiticus, A. flavus, A. nomius, and A. pseudotamarii* (IARC, 2002; Bennett and Klich, 2003;Yousefi et al., 2009; Mohammadi et al., 2012).They can affect animals and humans (Pitt, 2000**)**. The pathological states arising from the consumption of feeds contaminated with aflatoxins (AFs) are termed Aflatoxicosis. According to International Agency for Research on Cancer (IARC), because of sufficient evidence in humans for the carcinogenicity of naturally occurring mixtures of aflatoxins, aflatoxin B1 is especially confirmed as a potential carcinogen and is classified as Group 1 (IARC, 1987; IARC, 1993; Castegnaro and MC Gregor, 1998). The toxicity mechanism of AFB1 is understood**.** The main target organ for the toxicity and carcinogenicity is the liver (Peraica et al., 1999). After consumption, aflatoxin B1 is biotransformed in the liver by monoxygenases and then metabolized by CYP450 into aflatoxin 8,9 epoxide as a highly toxic metabolite (Emerole et al., 1979). Other metabolic products such as aflatoxicol, aflatoxin Q1, aflatoxin P1, and aflatoxin M1 depend on the genetic predisposition of the species (Smela et al., 2001).

In addition to the occurrence of different diseases like parasitic, bacterial and viral infections, hepatopancreatic damage with biochemical changes of the hemolymph, currently most of the problems facing the shrimp farming are related to several toxicities like presence of mycotoxins in shrimp feed. They can greatly influence the success of growth and health status of shrimps and result in lowering the production of shrimp (Pedro, 2008; Bintvihok et al., 2003). According to WHO report in 1979, quite small amounts of these substances can harm health (Jakić-Dimić et al., 2005).

Shrimp farms located in Bushehr, north part of Persian Gulf, face the potential risk of aflatoxin contamination most probably due to the hot and humid climate as reported by Butkeraitis (2011). There is no research on the contamination levels of AFT in cultured shrimp and their diets indifferent areas of shrimp farming in Bushehr coasts. Therefore, this study aimed to measure different species of aflatoxin (total AFT= B1+B2+ G1+ G2) and also individual aflatoxins of B1, B2, G1 and G2 (EFSA, 2013). This paper emphasized on the quantitative evaluation of the levels of AFT in tissues and their diets of farmed white shrimp (*Litopenaeus vannamei*).

2 MATERIALS AND METHODS

2.1 Materials

All chemicals (KCl, HNO3 and HCl), all HPLC grade solvents (methanol, acetonitrile, 2-propanol, chloroform and n-hexane), and AF standards were obtained from Sigma Chemical

Fig. 1. Map of the location of study area, Bushehr, an Iranian southern province located at north of Persian Gulf.

company in USA. The afla test immunoaffinity columns (IAC) were purchased from VICAM company in Watertown, Massachusetts, US. Data analyses were performed by the Kruskal–Wallis one-way analysis of variance (ANOVA) and Mann–Whitney–Wilcoxon test.

2.2 Sample preparation

In this study, 40 samples were analyzed. Overall, 20 samples of shrimp farms, lacking any diseases from Helleh, Delvar, Mond, Bandar Rig sites in Bushehr province (5 samples from each area) in addition to five samples of feed used for farmed shrimp from each mentioned region were randomly collected for analysis (Fig. 1).

The required amount of shrimp for each sampling was approximately 1000 g from each shrimp ponds. The samples were transported to the laboratory by a specific car for transferring the shrimp and fish, and immediately the extraction process started. In cases of delay in work, the samples were keptat-18°C in the laboratory. Adult shrimps were 2.5 months in age and ranged in weight from 10 to 15 g. Further, 50-60%of their body weight was muscle. Therefore, from each sample, 500 g muscle was obtained. After grinding and homogenization of the samples, 50 g of each sample was taken for the analysis. Next, after shaking for 30 minutes, the test portion was extracted with a solvent mixture of 300 ml methanol-water (8 + 2) plus 100 ml n-hexane. Following the filtration through a Whatman filter-paper (No.1, 185mm, F2042), the extract was diluted with water and filtered through glass micro fiber filter. IAC afla-test was used for samples clean-up and previously conditioned with 10 ml of phosphate buffer saline (PBS). Then, it was followed by 75 ml of the filtrate at a flow rate of 1 ml/min. The column was washed with water and dried using the vacuum. Finally, AF was washed with methanol using the following procedure. First, 0.5 ml methanol was used on the column that passed through by gravity. After 1 min, the second portion of 0.75 ml methanol was applied and collected. The afla-test was diluted with water and analyzed using HPLC. All samples were run in triplicate.

2.3 Analysis of AFs using HPLC

The determination of AFs levels in samples extracts was carried out by isocratic reverse-phase liquid chromatography (HPLC)Waters 1525 binary pump with post column

derivatization (PCD) and with 2475 Multy λ fluorescence detector. HPLC column (C18, 250 x 4.6 mm: 4 µm) was purchased from Waters in the US. Also, Breeze Software was used to control the system.

Detection of AFT was performed using 365 nm and 435 nm as fluorescence excitation and emission wavelengths, respectively. 100 µl was injected into HPLC.The mobile phase was a water-acetonitrile-methanol solution (6 +2+3, v/v/v) and 350 µl of nitric acid 4 M and 120 mg of KBr, and the flow rates were 1.00 ml/min. Standard AFB_1, AFB_2, AFG_1 and AFG_2 (Sigma-Aldrich) working solutions were prepared according to AOAC official method No. 971.22 using toluene/acetonitrile solvents (9 + 1) (10 µg/ml). Their purities were checked by UV spectrometry and TLC, according to AOAC official method No. 968.22. These standards were used to prepare mixed working standards for HPLC analysis according to AOAC official method No. 990.33 in area separated from analytical laboratory. The LOD were 0.07, 0.08, 0.1, 0.07 and 0.32 ng/g for AFB1, AFB2, AFG1, AFG2 and total AF, respectively. Recovery averages were 90.0%, 90.0%, 87.5% and 85.0% for AFB1, AFB2, AFG1 and AFG2, respectively.

3 RESULTS AND DISCUSSION

The results of aflatoxin contamination in shrimp tissue (T) and their diets (F) from farms located in Helleh, Delvar, Mond, Bandar Rig sites in Bushehr province are presented in Table 1. Among 40 samples analyzed, 25% of them did not show any AFT (sum of B1, B2, G1, and G2) contamination (<LOD: ND (not detectable)), and a high proportion of samples (75%) showed positive contamination. The highest level of AFB1 (0.5 ppb) was isolated from shrimp's tissues in Helleh area. In addition, the maximum levels of B2, and G1 were 1.3 and 1.63 ppb in Mond and Helleh food samples, respectively. This study showed that the highest level of measured aflatoxin, among all groups was 4.12 ppb that was related to Mond food sample. Overall, the means of aflatoxins B1, B2, G1 and G2 for all shrimp samples were 0.05, ND (< LOD), 0.01 and 0.03, respectively, while their corresponding diets were 0.057, 0.112, 0.278, and 0.745 ppb. However, differences between groups and their levels were lower than MTL of AFT established by FDA (20ppb). FDA has established the following action levels for aflatoxins present in human food, animal feed and animal feed ingredients (Table 2). The maximum allowable level of aflatoxin in human and animals feed is 20ppb (FDA Regulatory Guidance). As the results showed, despite the differences between all groups of individual aflatoxines, only AFG1 had a significant difference (P-value= 0.031) and there was no significant difference between the other mentioned groups (Table 3).

Table1

The means (±SD) of aflatoxin groups (B1, B2, G1, and G2) in shrimp tissue (T) and their feeds (F) from different shrimp farm sites of Helleh, Delvar, Mond, and Bandar Rig in Bushehr province.

AF	B1				B2				G1				G2			
Location	Mean		Standard Deviation		Mean		Standard Deviation		Mean		Standard Deviation		Mean		Standard Deviation	
	[a]T	[b]F	T	F	T	F	T	F	T	F	T	F	T	F	T	F
Rig	‹LOD	0.019	‹LOD	0.043	‹LOD	0.100	‹LOD	0.141	‹LOD	‹LOD	‹LOD	‹LOD	0.023	0.233	0.045	0.468
Mond	0.044	0.103	0.098	0.144	‹LOD	0.260	‹LOD	0.581	‹LOD	0.018	‹LOD	0.040	0.057	0.862	0.085	1.823
Helleh	0.157	0.106	0.228	0.147	‹LOD	0.087	‹LOD	0.195	0.040	0.981	0.089	0.778	‹LOD	‹LOD	‹LOD	‹LOD
Delvar	‹LOD[c]	‹LOD	‹LOD	‹LOD	‹LOD	‹LOD	‹LOD	‹LOD	‹LOD	0.114	‹LOD	0.255	0.039	0.432	0.088	0.689

[a]T: shrimp tissue [b]F: shrimp feed [c]LOD: limit of detection

Table 2
The maximum allowable levels for aflatoxins present in human food, animal feed and animal feed ingredients according to FDA.

Aflatoxin level (in parts per billion)	Commodities and species
20 ppb	For corn, peanut products, cottonseed meal and other animal feeds and feed ingredients intended for dairy animals; for animal species or uses not specified below, or when the intended use is not known.
20 ppb	For corn, peanut products and other animal feeds and feed ingredients, but excluding cottonseed meal, intended for immature animals.
100 ppb	For corn and peanut products intended for breeding beef cattle, breeding swine or mature poultry (e.g., laying hens).
200 ppb	For corn and peanut products intended for finishing swine (100 pounds or more).
300 ppb	For cottonseed meal intended for beef cattle, swine or poultry (Regardless of age or breeding status).
300 ppb	For corn and peanut products intended for finishing beef cattle (e.g., feedlot cattle).

In a similar study, aflatoxin B1 in 150 samples of shrimp feed from the eastern and southern regions of Thailand were analyzed by Bintvihok et al. (2003). The study concluded that the AFB1 contamination ranged from a non-detectable level (< LOD) to 0.651 ppb. In another research, Bernardo et al. (2009) conducted a study with 27 samples of feed for fish analyzed for aflatoxins and aflatoxin; these two (i.e. aflatoxins and aflatoxin) were found in two samples with levels of 5 and 6 μg.kg (Almeida et al., 2011). Altug and Beklevik (2003) detected aflatoxins in many of the 153 samples of fish feed during their research (1998-2000). In 56% of positive samples, the levels of contaminations were over 21 $\mu g.kg^{-1}$.

Bautista et al. (1994) surveyed commercial shrimp feeds in the Phillippines and reported aflatoxin B1 contaminations in various range of values from not detected to 120 $\mu g\ kg^{-1}$ using high-performance thin-layer chromatography. Their results also showed a significant reduction in performance of pre-adult shrimp (*Penaeus monodon*) at AFB1 concentrations of 75 ppb in a 60-day study period.

Table 3
One-way analysis of variance (ANOVA) between different groups of aflatoxin (B1, B2, G1, and G2) in shrimp tissue (T) and their feeds (F) from shrimp farm sites of Helleh, Delvar, Mond, and Bandar Rig in Bushehr province.

Null Hypothesis, (The distribution of AF is the same Across categories of location).	B1t	B1f	B2t	B2f	G1t	G1f	G2t	G2f
Sig.	0.222	0.364	1.000	0.584	0.392	0.031	0.520	0.447
Decision	Retain	Retain	Retain	Retain	Retain	Reject	Retain	Retain

Asymptotic significances are displayed. The significance level is 0.05.

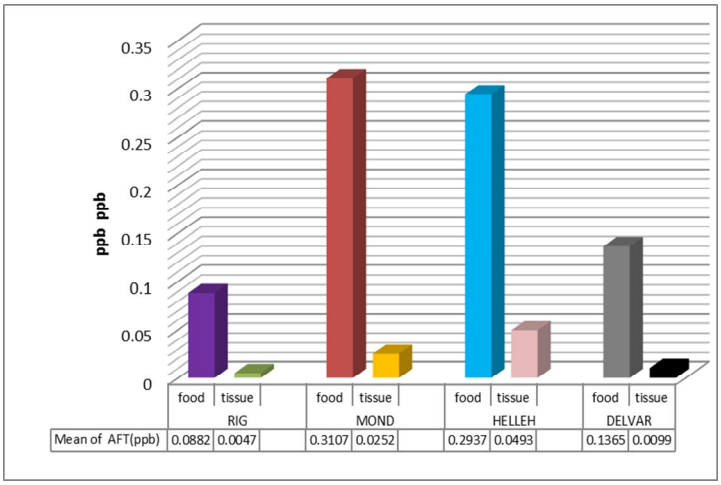

Fig. 2. The means of AFT (sum of B1, B2, G1, and G2) in shrimp tissues and their feeds samples from four areas of Helleh, Delvar, Mond, and Bandar Rig in Bushehr, a southern province of Iran.

Results of Tuan et al. (2002) on evaluating the effects of aflatoxin B1 on Nile tilapia (*Oreochromis niloticus*) showed that their diets containing 100 mg AFB1/kg reduced body weight gain, caused significant damages to liver and also 60% mortality in population. In the current investigation, both levels of aflatoxins that occur as expected in shrimp tissue, in addition to their natural values in feeds, not adding toxin to the samples by hand, were measured together. Our data can be considered as the inspiration for further surveys.

The average of total aflatoxins (AFT) in shrimp tissue and food samples in areas of Bandar Rig, Delvar, Helleh, and Mond were 0.0047, 0.0881; 0.0098, 0.1365; 0.0492 , 0.2937; and 0.0251, 0.3107 $\mu g \cdot kg^{-1}$, respectively. The distribution of AFT (sum of B1, B2, G1, and G2) in shrimp tissues and their feeds from four areas of interest are presented in Fig. 2. The mean of total aflatoxin in food and tissue samples were significantly different ($p=0.008$). As shown in Fig. 3, the total levels of aflatoxin in food samples have a direct correlation with their levels in tissue.

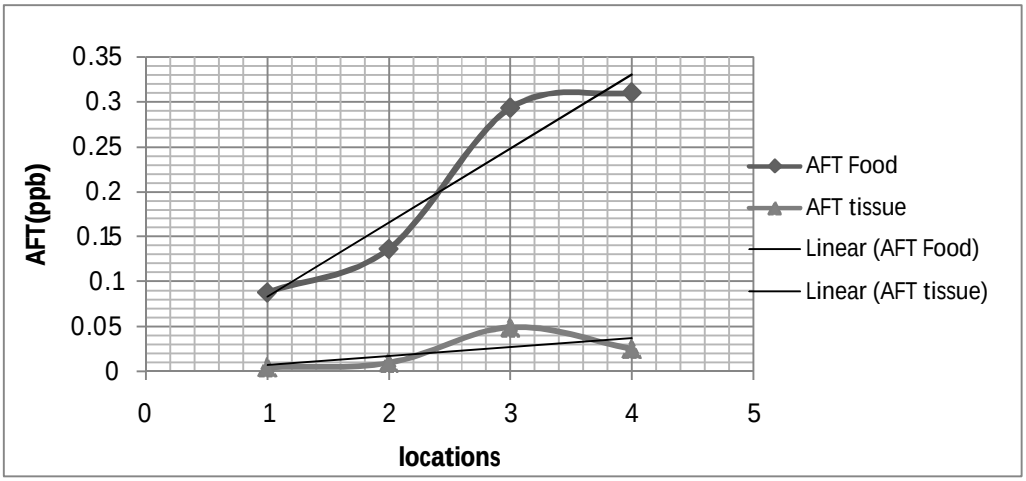

Fig. 3. Correlation between total levels of aflatoxin (AFT) in food samples (■) and their levels in shrimp tissue (▲) samples from four areas of Helleh (1), Delvar (2), Mond (3) and Bandar Rig (4).

Namely, with increasing the food samples, their levels in tissue samples have also increased. Moreover, an early linear relationship was obtained in the present study (Fig. 3). Overall, results of the research comparing mycotoxins mainly aflatoxins B1 (AFB1) in different aquatic species have demonstrated that they pose a risk to their performance and health such as effects on growth performance, feed conversion, apparent digestibility coefficients, physiological disorders and histological changes, in particular on hepatopancreatic tissue were studied (Wiseman et al., 1982; Bautista et al., 1994; Boonyaratpalin et al., 2001; Bintvihok et al., 2003; Burgos-Hernadez et al., 2005). In these studies, aflatoxins have been found in organs and tissues of animals that have ingested aflatoxin-contaminated feeds (Stubblefield and Shotwell, 1981).

4 CONCLUSION

Results found in the present study may be an expression of an adequate control system to implement in feed plants that produce this kind of feed. Foods that have been recently prepared and stored correctly must be purchased for shrimp farms. The shrimp food, as possible, should be stored in garners with the ability of temperature and humidity control. It should not be kept outside the warehouse for more than 2 weeks. Regular testing of aflatoxins in foods seems a good idea in this regard. In primary step, the shrimp foods should be sampled and controlled their moisture, durability, taste, and smell. Their use should be avoided, if inappropriate.

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