Evaluation of Aflatoxins contamination in barley feeds distributed in Mashhad city, Iran

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Abstract

Background and objective: Aflatoxins (AFs) are toxic metabolites that produced predominantly by three species of Aspergillus. Current study was conducted to determine quantity of AFs in barley feed.

Materials and methods: A total of 97 samples were analyzed with high-performance liquid chromatography (HPLC). The method was based on extraction of finely ground samples by methanol as solvent. An aliquot of 20 µl of extract was injected to HPLC after filtration. Aflatoxins were detected by ZORBAX Eclipse XDB C18 column (150×4.6×5 µm). Mobile phase of water:methanol:acetonitrile (60:20:20 v/v/v) at linear gradient mode and flow rate of 1 ml min\(^{-1}\) was used. Experiments were done at excitation and emission wavelengths of 365 and 445 nm, respectively, after post-column UV derivatisation.

Results and conclusion: The obtained results showed that AFB\(_1\), AFB\(_2\), AFG\(_1\) and AFG\(_2\) were detected in 19, 9, 4 and 1 samples, respectively. Relatively low LOD and LOQ were achieved in our analytical approach that approved a desirable goodness of fit for the analysis. Trueness was examined by determination of the relative recoveries of AFs that were quite good. This survey provides valuable information on aflatoxin contamination in barely feed products marketed in Mashhad city.

Keywords: Aflatoxin, Barley feed, High Performance Liquid Chromatography, Mashhad

1. Introduction

Barley is a valuable cereal grain with nutlike flavor. Barley is an ancient crop dating back to 7000 B.C. and is considered as one of the most widely adapted grain crops in the world. Egyptians and Greeks in ancient times consumed grain for a nourishing food source as well as medicinal purposes. Pearl barley is a human food made of grain by using abrasive disks to grind the hulls and bran of kernels. Half or more of the grown grain is used for livestock feed. Nutritive value of corn kernel for feed is nearly equal to barley. It is especially valuable as hog feed [1]. Fungi are widespread microorganisms in the environment. When food preservation is not suitable, inappropriate level of moisture and heat will result in growth and propagation of fungi. In addition, inadequate harvesting practices, drying,
handling, packaging and transport increase the risk of contamination with mycotoxins [2]. Of the most essential contaminant fungi which play critical role in toxin production are Aspergillus flavus and A. parasiticus and to lesser extent A. niger which contaminate plants and products [3]. Studies have shown that different seasons can also effect on contamination level [4]. High moisture and heat in autumn and summer compared to other seasons, if are not controlled in storage time, are the reason of higher AF contamination [5]. Contamination of animal feed to fungus, especially those of Aspergillus, can produce AF and transfer it to dairies [6]. AFB1 is the most important and toxic metabolite in all animal species and liver is the main target organ of toxicity [7]. When animals eat AFB1 contaminated feed, the toxin metabolizes to AFM1 in their body [3]. AFM1 and AFM3, are heterocyclic metabolites of AFB1 and AFB2, respectively, and may be found in dairy products. AFM1 resists against thermal process such as pasteurization and sterilization [8]. The main harm of mycotoxicosis is their adverse effects on liver and kidney. However, some mycotoxins interfere initially in synthesis of amino acid in body and consequently causing skin sensation, necrosis or immune system impairment [9]. International agency for research on cancer (IARC) reported AFB1 and AFM1 as primary group of carcinogenic compounds [10]. Since AFs accumulate in body, it is necessary to pay particular attention to reducing their entrance and formation in foodstuff [11]. Following to documentation of approved health concern to human, regulatory levels have been determined by international organizations including World Health Organization (WHO), U.S. Food and Drug Administration (US FDA) and Food and Agriculture Organization (FAO). In accordance, limit of AFB1 and total aflatoxins set on 0-5 ng g⁻¹ and 0-20 ng g⁻¹, respectively [3]. In 2002, Iranian National Standards Organization (INSO) set a maximum tolerated limit (MTL) for mycotoxins in foods and feeds. The regulatory limit for AFB1 were 5.0 ng g⁻¹ in animal feeds [12]. Today, some measures have been taken by ministry of health and other regulatory authorities; however, the results show that AFs contamination in feeds and foods is still substantial and a matter of concern for consumers. Some of the most common and sensitive techniques like thin layer chromatography (TLC) and liquid chromatography (LC) were used for AFs analysis in food and feed samples [1,13,14]. Currently, sensitive methods such as HPLC with fluorescence detection (HPLC–FLD) coupled with pre- or post-column derivatization, and immune-affinity clean-up is the most widely used method for AFs determination in complex matrices. To best of our knowledge, current data on AFs in barely feed is rare and more information is required to get a better estimation of potential problem. Therefore, the objective of present study was to study the occurrence and distribution of AFs in barely feed.

2. Materials and methods

2.1. Sampling

All of the feeding barely samples were purchased from wholesalers in Mashhad city, Iran. Average weight of 2-3 kg of samples was mixed and homogenized. Then, they were transferred to laboratory at ambient temperature and stored at 4°C until initial sample preparation. After which, the prepared analytes were stored at -20°C until analysis. In day of analysis, samples were defrosted at ambient temperature.

2.2. Chemicals

All chemicals used for analysis were HPLC grade and supplied by Merck Company (Darmstadt, Germany). AF standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard solutions including B1, B2, G1 and G2 were prepared and used for calibration curves and trueness experiments. Neogene immune-affinity column (Neogene Europe, Ltd, Scotland, UK) was used for analysis.
2.3. Apparatus
Analysis was performed by HPLC, Agilent Technologies SL 1200 Series (Waldbrohn, Germany) composed of fluorescence detector, a ZORBAX Eclipse XDB C18 column (150 × 4.6 mm × 5 μm, Agilent Technologies), thermostated auto-sampler and binary pump equipped with 152 micro vacuum degasser. On-line photochemical derivatization with a commercially available system UVE™ LCTech GmbH (Dorfen, Germany) was used. UV spectrophotometer was used for determination of AF stock solution concentration (10 mg 1⁻¹) [15]. A Waring blender (PB25E, USA) was used for mixing the samples.

2.4. HPLC condition
The experiments were performed with a mixture of water:methanol:acetonitrile (60:20:20, v/v/v) as mobile phase at flow rate of 1.0 ml min⁻¹ in isocratic elution mode. The column temperature and injection volume were 40°C and 20 μl, respectively. The fluorescence detection was carried out at 365 and 445 nm as the excitation and emission wavelengths, respectively [16].

2.5. Sample preparation
Five g of sodium chloride and 50 g of ground barley sample were weighted, blended for 5 min at high speed with 200 ml methanol 80% and then filtered. Twenty ml of filtrate was mixed with 130 ml of phosphate buffer solution. This solution was filtered with a glass microfiber filter (Whatman, Inc., Clifton, NJ). One hundred ml of final solution was transferred to a reservoir and passed through immune-affinity column at a flow rate of 1.0 ml min⁻¹. For further purification and clean-up, 10 ml of distilled deionized water was passed through immune-affinity column, at a flow rate of 5.0 ml min⁻¹. AF was eluted with methanol and water according to the following procedure. Firstly, 2 ml of HPLC grade methanol was passed through the column and then 2 ml of HPLC grade water was given and collected to a glass vial [16]. For AFs determination in the samples, standard curve was plotted by using the absorbance against concentration of external standards of AFs.

2.6. Statistical analysis
All experiments were performed three times. The statistical analysis was done using Minitab 16 and Excel 2010 software. Differences were significant at p≤0.05.

3. Results and discussion
In this study, 97 barley samples were analyzed to evaluate their concentration of AFs B₁, B₂, G₁ and G₂. As it is shown in Table 1, AFs B₁, B₂, G₁ and G₂ were detected in 19.59%, 9.28%, 4.12% and 1.03% of the samples with a mean value of 3.53, 1.73, 1.46 and 0.29 ng g⁻¹, respectively. In similar research carried out by Beheshti and Asadi in 2014 in Iran, 60 barley samples were examined. In their study, AFB₁ was detected in only five samples with a mean value of 0.48 ng g⁻¹ and AFs B₂, G₁ and G₂ were not detected [17]. Another research was done by Eskandari and Pakfetrat in same year in Iran. They studied occurrence of AFs and heavy metal in forty brands of animal feed that produced from samples of southwest of Iran. The results of this study showed that all samples were contaminated with aflatoxins. Importantly, AFB₁ and total aflatoxins were higher than maximum permitted levels in the all [18]. In comparison, lower contamination in our samples may be due to the fact that our sampling was done in summer and autumn of 2018 that weather was not humid.

Table 1. Concentration of mycotoxins (ng g⁻¹) in barley feed samples

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Average</th>
<th>Number of negative samples (&lt;LOD)</th>
<th>Numbers of samples in the range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0-5.0</td>
</tr>
<tr>
<td>AFB₁</td>
<td>3.53</td>
<td>78</td>
<td>14</td>
</tr>
<tr>
<td>AFB₂</td>
<td>1.73</td>
<td>88</td>
<td>7</td>
</tr>
<tr>
<td>AFG₁</td>
<td>1.46</td>
<td>93</td>
<td>3</td>
</tr>
<tr>
<td>AFG₂</td>
<td>0.29</td>
<td>96</td>
<td>1</td>
</tr>
</tbody>
</table>
Wu et al. determined AFB$_1$ in corn and corn germ meal. AFB$_1$ was detected in 176 out of 220 (80%) corn and 25 out of 34 (74%) corn germ meal samples. The mean concentration of AFB$_1$ in corn and corn germ meal was 3.9 and 7.4 ng g$^{-1}$, respectively. In their study, AFB$_1$ was also analyzed in cattle feed and it was detected in all samples with mean of 4.5 ng g$^{-1}$ [7]. A total of 97 samples containing 48 livestock feeds and 49 feed ingredients from different livestock farms and farmers were analyzed by high-performance thin layer chromatography (HPTLC). In total, 16 contaminated feeds and 13 contaminated feed ingredients were found. The results of that research showed that HPTLC had good recovery, precision and linearity in quantitative determination of aflatoxin B$_1$ [19]. Mateo et al., determined AFs and ochratoxin A in 105 barely grain in Spain from 2008 to 2010. Twenty-nine out of 105 samples were found to be contaminated with at least one of the mycotoxins. Species-specific PCR assays were used for detection of A. flavus, A. parasiticus, A. ochraceus, A. steynii, A. westerdijkiae, A. carbonarius and A. niger in mycotoxin-positive samples. The obtained results indicated the presence of all fungi species, except A. westerdijkiae [20]. Analytical data are summarized in Table 2. For repeatability measurement, standard solutions containing 3.6 ng ml$^{-1}$ of AFB$_1$ and AFG$_1$ and 0.72 ng ml$^{-1}$ of AFB$_2$ and AFG$_2$ were used. The calibration curve was drawn by seven concentrations of aflatoxins resulted in correlation coefficient ($r^2$) of AFB$_1$=$0.9970$, AFB$_2$=$0.9973$, AFG$_1$=$0.9967$, and AFG$_2$=$0.9942$. The calibration curves exhibited good linear regression. Limit of detections (LODs) for AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$ were 0.008, 0.017, 0.017 and 0.025 and limit of quantifications (LOQs) were 0.025, 0.05, 0.05 and 0.075 ng g$^{-1}$, respectively. Accuracy was examined by determination of relative recoveries of AFs. The relative recovery was calculated by comparing the AF concentration in spiked samples (containing a known amount of added analyte) to the concentration in native samples. Recovery of spiked samples at 10 ng g$^{-1}$ for AFB$_1$ and AFG$_1$ and 2 ng g$^{-1}$ for AFB$_2$ and AFG$_2$ are shown in Table 2. In mix standard solution of AFs, concentration of B$_1$ and G$_1$ was five times of B$_2$ and G$_2$. The recovery range was within the guideline of acceptable recovery limits of AOAC (Association of Official Analytical Chemists) and the Codex Alimentarius. AOAC acceptable recovery at 10 ng g$^{-1}$ level is 70-125% and Codex acceptable recovery range is 70-110% for a level of 10-100 ng g$^{-1}$ and 60-120% for a level of 1-10 ng g$^{-1}$ [21].

Table 2. Method validation of aflatoxin analysis by high performance chromatography in barley samples

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Retention time (min)</th>
<th>Repeatability (%RSD, n=6)</th>
<th>Limit of detection (ng g$^{-1}$)</th>
<th>Limit of quantification (ng g$^{-1}$)</th>
<th>Relative recovery (%)</th>
<th>Linear dynamic range (ng ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB$_1$</td>
<td>9.98</td>
<td>1.3</td>
<td>0.008</td>
<td>0.024</td>
<td>96.08</td>
<td>0.40-10</td>
</tr>
<tr>
<td>AFB$_2$</td>
<td>8.25</td>
<td>2.4</td>
<td>0.017</td>
<td>0.051</td>
<td>99.20</td>
<td>0.08-2</td>
</tr>
<tr>
<td>AFG$_1$</td>
<td>7.48</td>
<td>1.5</td>
<td>0.017</td>
<td>0.051</td>
<td>93.90</td>
<td>0.40-10</td>
</tr>
<tr>
<td>AFG$_2$</td>
<td>6.32</td>
<td>3.2</td>
<td>0.025</td>
<td>0.075</td>
<td>98.70</td>
<td>0.08-2</td>
</tr>
</tbody>
</table>

4. Conclusion
Our results showed that the current analytical method was able to determine aflatoxin amount in feeding samples with high recovery percent. In addition, 19 out of 97 barley samples contained AFB$_1$. Among them, AFB$_1$ in five contaminated samples were higher than the level of Iranian National Standard and European regulation (5 ng
g⁻¹). However, total aflatoxins were within the acceptable range of 15 ng g⁻¹ in all samples. Due to lack of fresh forage, elimination of AFs in animal feed is not applicable but their concentration can be reduced by proper monitoring and storage practices. In this regard, barley feed should be checked regularly and storage condition must be under strict control. At the same time, surveillance programs must be continuous and widespread in the market. What is important is that now animal being is at risk of daily exposure to fungal toxins mainly AFs. Feedings whose levels of toxin are higher than acceptable limit should not be consumed by animals because there is a risk of their transfer to human body through consumption of animal foods. Therefore, consideration of standards and regulations that highlight livestock-based food products to assess toxins and other contaminants is recommended.

5. Conflict of interest
The authors declare that they have no conflict of interest.

References


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