VALIDATED HPTLC METHOD FOR AFLATOXIN B1 DETECTION IN FEED INGREDIENT AND FEED SAMPLES

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ABSTRACT

High performance thin layer chromatographic method was developed and validated according to the protocol on “Validation of Analytical Procedures: Methodology, Veterinary International Cooperation on Harmonization (VICH)” with respect to linearity, sensitivity, precision and accuracy for determination of aflatoxin B₁ in feed ingredients and feed. Chromatography was performed on thin layer chromatography (TLC) silica gel 60F₂₅₄ aluminium sheets by Camag Linomat-5 applicator, with mobile phase condition acetone : chloroform (1:9). Analysis of samples viz. feed ingredients and feed, for aflatoxin B₁ was carried by HPTLC method and compared with TLC method. Extraction of aflatoxin was done as per AOAC method with screening and quantification by TLC and further quantification by HPTLC using reference standards. Out of 38 samples of nine types of feed ingredients analysed, samples of Bengal gram and rice bran & wheat bran mixture were negative by both methods. The other ingredients like cumbu/bajra, de-oiled rice bran, groundnut oil cake, maize, soyabean meal and sunflower oil cake, by HPTLC method wherein the Aflatoxin B₁ was found to be ranging from 1.61 ppb to 630.73 ppb of 77.42% positive samples, whereas by TLC method it was from 05 ppb to 140 ppb in 70.97% positive samples. While 4 samples of wheat bran analysed were all negative for Aflatoxin B₁ by TLC method, whereas 50% (2 samples) found to be positive with HPTLC method with concentration ranging from 2.73 to 17.88. Similarly out of 59 feed samples analysed, 47 and 46 samples were positive for Aflatoxin B₁ representing 79.66% and 77.97% of the samples, with concentration ranging from 0.54 ppb to 204.72 ppb and from 05 ppb to 710 ppb by HPTLC and TLC respectively. In the present study, the Limit of detection by HPTLC was 0.5 ppb whereas it was 5 ppb with TLC method.

Key words: HPTLC, Aflatoxin, Feed, Feed ingredients.

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INTRODUCTION

Mycotoxins have received considerable attention due to their significance in agricultural loss and human health. Mycotoxins greatly resist decomposition or being broken down in digestion, so they remain in the food chain even after heat treatment, such as cooking and freezing (Hong et al., 2010). According to the FAO, more than 25% of the world’s agricultural production is contaminated with mycotoxins, resulting in economic losses. Most countries have adopted regulations to limit exposure to mycotoxins, which has strong impact on food and animal crop trade. Aflatoxin, the most potent of the mycotoxins was discovered some 30 years ago in England following a poisoning outbreak causing 100,000 turkey deaths. Aflatoxins are a group of polyketide-derived furanocoumarins which are carcinogenic among the known mycotoxins. At least 13 different types of aflatoxin are produced in nature (USDA, 2009). They are mainly of four major groups, AFB1, AFB2, AFG1 and AFG2 of which, AFB1 is a potent carcinogen (Prabakaran and Dhanapal, 2009). In addition, aflatoxin, M₁ and M₂ are hydroxylated metabolites of aflatoxin B₁ and B₂. Aflatoxin B₁ is metabolized into a variety of hydroxylated derivatives (aflatoxin P₁, M₁, B₂) which are less toxic than the parent compound, even though their presence in food is still a threat to human health (IARC – International Agency for Research on Cancer, 1993). Extrapolations for risk assessment studies have estimated that about 5 to 28% of liver cancers worldwide are due to aflatoxin contamination of food (Bessy, 2011). Aflatoxins are secondary metabolites of the fungi Apergillus flavus and Aspergillus parasiticus. These moulds are common contaminants of feed ingredients and feed, particularly in the tropical regions. While the presence of Aspergillus flavus does not always indicate harmful levels of aflatoxin, it does mean that the potential for aflatoxin production is present (USDA, 2009). Fungi produce aflatoxins in the presence of higher moisture, temperature and adequate substratum. Synthesis is highest when humidity is above 13% and temperature is between 24°C and 37°C. That is why warm and wet geographic regions are the most favorable environments for aflatoxins and usually are affected. Before harvest, the risk for the development of aflatoxin is greatest during major droughts. When soil moisture is below normal and temperatures are high, the number of aspergillus spores in the air increases. These spores infect crops through areas of damage caused by insects, and inclement weather. Once infected, plant stress occurs, the production of aflatoxin is favoured. During post-harvest stage, proliferation of aflatoxin can be exacerbated in susceptible commodities like feed ingredients and thereby feed under storage conditions such as hot and humid storage environment (Risk Assessment Studies, 2001). Aflatoxin contamination of food results in some toxic effects such as liver cancer and immunosuppression in various animals and humans. The presence of mycotoxins is unavoidable and, therefore, testing of raw materials and products is required to keep our food and feed safe (Romer labs guide to mycotoxins, 2012).

Thin layer chromatography (TLC) techniques were extensively used for aflatoxin
analysis, although recently an increase in the use of high performance thin layer chromatography (HPTLC) has been noted. The accuracy of TLC is less than that of high-performance liquid chromatography (HPLC) but the results obtained using HPTLC are similar to that of HPLC and more consistent than enzyme-linked immunosorbent assay (ELISA) data (Jaimez et al., 2000 and Ramesh et al., 2013). The present study was conducted to assess aflatoxin B<sub>1</sub> contamination in feed ingredients and feed samples using TLC for screening and quantification and further quantification by HPTLC using reference standards.

**MATERIALS AND METHODS**

The routine feed ingredient and feed samples received at the Pharmacovigilance Laboratory for Animal Feed and Food Safety (PLAFFS), Chennai were utilized for the study.

**Sampling** Moulds and aflatoxins occur in an extremely heterogeneous fashion in feed and food commodities. It is thus crucial that sampling is carried out in a way that ensures that the analytical sample be a true representative of the consignment. Failure to do this may invalidate the subsequent analysis. So care was taken to obtain a sample of grain which is as representative as possible of the whole bulk.

**Sample Preparation**: Aim to achieve maximum particle size reduction and thoroughness of mixing to achieve effective distribution of contaminated portions. Grind entire lot sample through hammer to pass number 14 sieve split sample sequentially in sample splitter. Regrind 1kg portions to completely pass number 20 sieve and mix thoroughly. Weigh 25 grams of sample for aflatoxin estimation.

**Method**: As per AOAC method by TLC and HPTLC and quantified with reference standards.

**Extraction of Aflatoxin**

Sample analysis was carried out by taking a known quantity (25g) of the powdered sample in a 250ml flask and treating with 19ml distilled water and 106ml acetone. This mixture was shaken for 50 minutes at 200 rpm on a shaker. It was then filtered through Whatman paper (No.1). To the 75ml of filtrate, 1.5g of cupric carbonate was added. Another solution of 85ml of 0.2N NaOH and 15ml of 0.4M FeCl<sub>3</sub> was prepared. This solution was mixed with filtrate containing cupric carbonate thoroughly and then filtered through Whatman No1 filter paper. Transfer the 100 ml filtrate into a 500ml separating funnel and add 100ml of 0.03% H<sub>2</sub>SO<sub>4</sub> and 25ml of chloroform. Shake the mixture vigorously releasing the fumes or gases and allow for separation. Then the lower layer was transferred to a 100ml seperating funnel. After 30 minutes the lower layer was seperated and treated with 1% KCl in 0.02M KOH solution in a 100ml seperating funnel, by gentle shaking and allowing for seperation. The lower layer was collected in a vial, by passing through anhydrus sodium sulphate bed. The extract was evaporated in a hot plate under fume hood. Finally the dried extract was re-dissolved in 0.2ml of chloroform and used for TLC and HPTLC analysis.
Sample Assay

Estimation of Aflatoxin B₁ by TLC: The dissolved residue was then spotted on to a silica gel plate of about 0.5mm thickness as 5 µl drops. The standard solution of aflatoxin B₁ was also spotted on to the same plate as drops of 1, 3, 5 µl. The plate was developed in chloroform- acetone (9:1) mobile phase upto 80mm from lower edge of plate. After each development, the plate was dried with an hair drier and observed under UV light. The fluorescence intensities of aflatoxin spots of sample were compared with those standard spots. The sample spot, which matches one of the standard spots, was selected. Standard was also used to compare the colour and rf value of unknown sample streak on the plate. The amount of aflatoxin B₁ was estimated.

Estimation of Aflatoxin B₁ by HPTLC:

Plate Material

Silica gel HPTLC plates in the format of 10x10 cm or 20x10 cm are used. For reproducibility studies and quantitative analyses, plates were prewashed as follows:

• In HPTLC plate was marked for the direction of development with pencil at the upper edge of the plate.
• The plate was developed with 20ml methanol per trough in a 20x10 cm twin-trough chamber (TTC) to the upper edge.
• After development the plates were dried by using hair drier.
• The plates were handled on the top edge.

Sample Application

The dried samples were applied as bands (spray-on technique) along with reference standard bands using Linomat-5 standard / sample applicator.

Preparation of Developing Solvents

Prepared 9:1 ratio of Chloroform and Acetone and poured 20ml per trough in TTC for development of plates.

Development

The spotted samples were developed first in di-ethyl ether upto 90mm from lower edge of plate and then in chloroform- acetone mobile phase in a pre-saturated TTC up to 80mm from lower edge of plate.

Derivatization

Transfer of reagent for derivatization of samples on a HPTLC plate may be accomplished by spraying or dipping. Dipping is the preferred method and should be used whenever possible. Spraying is done in a TLC spray cabinet or in the fume hood. If derivatization includes heating, a plate heater should be used.

Spraying

The developed plates were dried by using hair drier and sprayed with 20% H₂SO₄.

Heating

After spraying, the plates were dried using hair drier.

Scanning

Finally the plates were scanned in CAMAG HPTLC scanner-3 under 366nm wavelength to determine the levels of aflatoxin B₁ contamination in the samples.
Detection by TLC or HPTLC is based on their fluorescence under UV radiation, although aflatoxin B<sub>1</sub> needs derivatisation to enhance the fluorescence and thereby for confirming it in the samples.

**RESULTS AND DISCUSSION**

**Aflatoxin B<sub>1</sub> in feed ingredients and feed samples:**

The results of aflatoxin contaminated samples and % of contamination were presented in Table 1. Out of 97 samples of feed ingredients (38) and feed (59) analysed, 73 samples (75.26%) were found to be aflatoxin B<sub>1</sub> contaminated by HPTLC method whereas only 68 samples (70.1%) by TLC method.

The type and no. of samples of feed ingredients analysed, contaminated with aflatoxin B<sub>1</sub> and the range of contamination by TLC and HPTLC methods were shown in Table 2. Out of 38 samples of nine types of feed ingredients analysed, samples of Bengal gram and rice bran & wheat bran mixture were negative by both methods. The other ingredients like cumbu/bajra, de-oiled rice bran, groundnut oil cake, maize, soyabean meal and sunflower oil cake, by HPTLC method the aflatoxin B<sub>1</sub> was found to be ranging from 1.61 ppb to 630.73 ppb of 77.42% positive samples, whereas by TLC method it was from 05 ppb to 140 ppb in 70.97% positive samples. While 4 samples of wheat bran analysed were all negative for Aflatoxin B<sub>1</sub> by TLC method, whereas 50% (2 samples) found to be positive with HPTLC method with concentration ranging from 2.73 to 17.88. The groundnut oil cake and sunflower oil cake were 100 percent contaminated, followed by deoiled rice bran (DORB), maize, wheat bran, soyabean meal and others.

The results of aflatoxin B<sub>1</sub> contaminated samples of feed and the range of contamination is presented in Table 3. Similarly out of 59 feed samples analysed, 47 and 46 samples were positive for Aflatoxin B<sub>1</sub> representing 79.66% and 77.97% of the samples, with concentration ranging from 0.54 ppb to 204.72 ppb and from 05 ppb to 710 ppb by HPTLC and TLC respectively. In the present study, the Limit of detection by HPTLC was 0.5 ppb whereas it was 5 ppb with TLC method. Agreeing with the present results, Banu and Muthumary (2008) from their study revealed that of the 18 different commercial feed samples analysed, 14 showed contamination with aflatoxin B<sub>1</sub>. In a study by Prabakaran and Dhanapal (2009), analysis of aflatoxins in raw feed ingredients like bamboo rice, pani varagu, thiri varagu, cambu, saamai and koran thinai used for poultry feed production, it has been reported aflatoxins were detected in cumbu (220 ppb of aflatoxin B<sub>1</sub> and 45 ppb aflatoxin B<sub>2</sub>) and in saamai (15 ppb aflatoxin B<sub>1</sub>) only and absent in other samples.
Validated HPTLC method for aflatoxin B1 detection in feed ingredient

**Table 1**

Samples contaminated with aflatoxin B₁, % of contamination and concentration range by TLC and HPTLC

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>Number of Samples</th>
<th>Contaminated sample</th>
<th>% of Contamination</th>
<th>Concentration range (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>By HPTLC</td>
<td>By TLC</td>
<td>By HPTLC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Feed ingredients</td>
<td>38</td>
<td>26</td>
<td>22</td>
<td>68.42</td>
</tr>
<tr>
<td>Feed</td>
<td>59</td>
<td>47</td>
<td>46</td>
<td>79.66</td>
</tr>
<tr>
<td>Total</td>
<td>97</td>
<td>73</td>
<td>68</td>
<td>75.26</td>
</tr>
</tbody>
</table>

**Table 2**

Feed ingredients analysed, no. of samples contaminated with aflatoxin B₁ and the range of contamination by TLC and HPTLC

<table>
<thead>
<tr>
<th>Feed Ingredient</th>
<th>No. of samples analysed</th>
<th>Detection by HPTLC</th>
<th>Detection by TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive samples</td>
<td>Range (ppb)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Bengal gram</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cumbu</td>
<td>5</td>
<td>2</td>
<td>4.75</td>
</tr>
<tr>
<td>DORB</td>
<td>8</td>
<td>7</td>
<td>2.45</td>
</tr>
<tr>
<td>Groundnut oil cake</td>
<td>2</td>
<td>2</td>
<td>24.25</td>
</tr>
<tr>
<td>Maize</td>
<td>11</td>
<td>10</td>
<td>1.61</td>
</tr>
<tr>
<td>Rice bran &amp; wheat bran</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Soyabean meal</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Sunflower oil cake</td>
<td>2</td>
<td>2</td>
<td>1.64</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>4</td>
<td>2</td>
<td>2.73</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>26</td>
<td>1.61</td>
</tr>
</tbody>
</table>

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REFERENCES


