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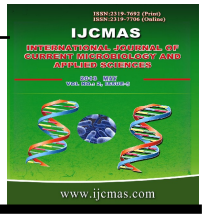

Original Research Article

Analysis of feed samples for aflatoxin B_1 contamination by HPTLC - a validated method

J. Ramesh, Ghadevaru Sarathchandra* and V. Sureshkumar

Pharmacovigilance Laboratory for Animal Feed and Food Safety, Directorate of Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, India- 600 051.
**Corresponding author e-mail: gsarathchandra@rediffmail.com*

Keywords

Feed, aflaoxinB_1; analysis; HPTLC; VICH;

A B S T R A C T

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 B_1 in feed. Chromatography was performed on thin layer chromatography (TLC) silica gel $60F_{254}$, aluminum sheets by Camag Linomat-5 applicator, with mobile phase condition acetone : chloroform (1 : 9). Analysis of feed samples, for aflatoxin B_1 was carried by HPTLC method. Extraction of aflatoxin was done as per AOAC method with screening by TLC and quantification by HPTLC using reference standards. Out of 59 samples of feed analysed by HPTLC, 47 samples were positive for Aflatoxin B_1 representing 79.66% with a concentration of 25.53±5.31ppb (mean±S.E.). In the present study, the Limit of detection by HPTLC was 0.5 ppb.

Introduction

Mycotoxins are by-product of fungal growth which occur naturally in a range of plant products. Due to ingestion of mycotoxins from feed by food producing animals, mycotoxin residues may occur in their products like milk, egg and meat. Mycotoxins have received considerable attention due to their significance in agricultural loss and human health. Amongst the mycotoxins that are known to cause human diseases, aflatoxins have been studied most. Aflatoxin was discovered some 30 years ago in England following a poisoning outbreak causing 100,000 turkey deaths. Aflatoxins are secondary metabolites of the fungi *Apergillus flavus* and *Aspergillus parasiticus*. These moulds are common contaminants of feed ingredients, particularly in the tropical regions. 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 24ºC and 37ºC. 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 feed under storage conditions such as hot and humid storage environment (Risk Assessment Studies, 2001). Aflatoxin is classified into a number of subtypes. However, the most important ones are B_1, B_2, G_1 and G_2, distinguished by their fluorescenct colour under ultraviolet light. Aflatoxin B_1, the most potent one, is metabolized into a variety of hydroxylated derivatives (aflatoxin P_1, M_1, B_2) which are less toxic than the parent compound, although their presence in food is still a threat to human health. Among the aflatoxins, aflatoxin B_1 is the most toxic form for mammals and presents hepatotoxic, teratogenic and mutagenic properties, causing damage such as toxic hepatitis, hemorrhage, edema, immunosuppression and hepatic carcinoma (Speijers and Speijers, 2004).

Taking into account the developments in Codex Alimentarius, recently EC has introduced the maximum accepted / residue levels (MRL) for aflatoxins in animal feeds as 0.02 mg/kg i.e., 20 ppb in all feed materials and in most complete and complementary feedstuffs for cattle, sheep, goats, pigs and poultry, while it is 0.005 mg/kg in complete feeding stuffs for dairy animals and 0.01 mg/kg for complete feeding stuffs for calves and lambs (Commission European Communities, 2003). Because of the toxicity of these molecules and considering the MRL set in food and in feedstuffs, analytical identification and quantification of such contaminants at these low levels has to be carried out with reliable methods: they must be able to provide accurate and reproducible results to allow an effective control of the possible contamination of food and feed commodities (Manetta, 2011). The combination of TLC methods with much-improved modern clean-up stage offers the possibility to be a simple, robust and relatively inexpensive technique (Vargas *et al.*, 2001), that after validation can be used as viable screening method. Moreover, given the significant advantages of the low cost of operation, the potential to test many samples simultaneously and the advances in instrumentation that allow quantification by image analysis or densitometry, TLC can be used also in laboratories of developing countries in alternative to other chromatographic methods that are more expensive and require skilled and experienced staff to operate. Improvements in TLC techniques have led to the development of high-performance thin-layer chromatography (HPTLC), successfully applied to aflatoxin analysis (Nawaz *et al*., 1992). The study was done to assess aflatoxin B_1 contamination in feed samples by High performance thin layer chromatographic (HPTLC) method.

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 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 is truly 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 106 ml acetone. This mixture was shaken for 50 minutes at 200 rpm on a shaker. It was then filtered through whatman paper (No1).To the filterate1.5g of cupric carbonate was added. Another solution of 85 ml of 0.2N NaOH and 15 ml of 0.4M FeCl3 was prepared. This solution was mixed with filterate containing cupric carbonate thoroughly and then filtered through Whatman No1 filter paper. To the filtrate, 100ml of 0.03% H_2SO_4 and 25ml of chloroform were added and the mixture was transferred to a separator funnel. After 30 minutes the lower layer was separated and treated with 1% KCl in 0.02M KOH solution, again in a separating funnel. The lower layer was collected in a vial. The extract was evaporated in a hot plate under fume hood. Finally the dried extract was redissolved in 0.2ml of chloroform and used for TLC and HPTLC spotting.

Sample Assay By Estimation of Aflatoxin B_1 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 B_1 was also spotted on to the same plate as drops of 1, 3, 5 µl. The plate was developed in chloroform-acetone (1:9). After development, the plate was air-dried 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 B_1 was estimated.

Estimation of Aflatoxin B_1 by HPTLC

Plate Material

Silica gel HPTLC plates in the format of 10×10 cm or 20×10 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 20 ml methanol per trough in a 20×10 cm twin-trough chamber (TTC) to the upper edge. After development the plates were dried by using hair dryer. The plates were handled on the top edge.

Sample Application

The dried samples were applied as bands (spray-on technique) using Linomat-5 sample applicator.

Preparation of Developing Solvents

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

Development

The spotted samples were developed in 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. If derivatization includes heating, a plate heater should be used.

Spraying

The developed plates were dried by using hair dryer and sprayed with 20% H_2SO_4.

Heating

After spraying the plates were dried using hair dryer.

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 B1 needs derivatisation to enhance the fluorescence and thereby for confirming it in the samples.

Results and Discussion

Out of 59 samples of feed analysed, 47 samples (79.66%) were found to be aflatoxin B1 contaminated by HPTLC method (Table 1) and the concentration (mean±S.E.) of aflatoxin B1 in the samples of feed analysed was found to be 25.53±5.31.

Table.1 Aflatoxin B_1 contamination in feed samples

No. of samples analysed	No. of samples positive	% of samples contaminated	Range (ppb)		
			Low	High	Mean ± S.E.
59	47	79.66	0.54	204.72	25.53±5.31

Substantiating the present result, Dhand *et al.*, (1998) have reported 75% of feed samples (21 of 28) contaminated with aflatoxin B_1. It can be concluded that for analysis of aflatoxin B_1 levels in feed samples, High Performance Thin Layer Chromatography (HPTLC) is the simplest and validated method with high throughput.

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