



Original Research Article

A validated HPTLC method for detection of Ochratoxin A and Citrinin contamination in feed, fodder and ingredient samples

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A B S T R A C T

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A novel HPTLC method was validated for the detection of ochratoxin A and citrinin contamination in feed, fodder and ingredient samples. A HPTLC 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. Chromatography was performed on thin layer chromatography (TLC) silica gel 60F₂₅₄, aluminum sheets precoated with oxalic acid by Camag Linomat-5 applicator, with mobile phase condition toluene : ethyl acetate : formic acid (6:3:1). Extraction of OTA and CIT was done as per AOAC method with screening by TLC and quantification by HPTLC using reference standards. Out of 20 samples of feed and 4 ingredient samples analysed by HPTLC, 8 samples and 1 sample respectively found contaminated with OTA representing 40% and 25% with a concentration range of 2.05 – 31.34 ppb and 0.8 – 3.4 ppb respectively. Out of 25 samples of feed, 4 ingredient, 3 green fodder and 1 dry fodder sample analysed by HPTLC, 8, 3, 2 and 1 sample respectively found contaminated with CIT representing 32%, 75%, 66.67% and 100% with a concentration range of 0.42 – 15.31, 0.8 – 1.14, 0.43 – 1.01 and 2.15 ppb respectively. In the present study, the limit of detection by HPTLC was 0.8 ppb for OTA and 0.4 ppb for CIT. It is concluded that the present study validated the HPTLC method suitable for detection of OTA and CIT contamination in feed, fodder and ingredient samples. Further, presence of OTA and CIT in feed and feed ingredient sample warrants the necessity of implementation of HACCP at farm level.

Introduction

Mycotoxins are secondary metabolites of fungi which occur naturally in a variety of plant products. These compounds comprise several chemical structures

(Richard, 2007), which occur in mycelium of filamentous fungi, normally after a period of balanced growth followed by stress conditions (Rupollo *et al.*, 2006).

Mycotoxin residues may occur in livestock products like milk, egg and meat due to ingestion of mycotoxins from feed by food producing animals. 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. *Aspergillus*, *Penicillium* and *Fusarium* are among the fungal genera that occur in food and that have toxigenic species, such as *Aspergillus ochraceus* and *Penicillium citrinum*, the main producers of ochratoxin A and citrinin, respectively (Knasmuller *et al.*, 2004, Elmholt and Rasmussen, 2005).

Ochratoxin A (OTA) has been detected in several stored agricultural products (Chandelier *et al.* 2004). OTA, in combination either with sterigmatocystin or citrinin (CIT), has often been found in stored wheat, barley, oat and corn (Abramson *et al.* 1999). Cereals are frequently stored at the farm for economic reasons (low prices, for use as feed). Under such conditions, the mycotoxin risk increases because grains are not always harvested at a sufficiently low water activity to guarantee the absence of fungal growth during the storage period. In addition, the farmers' storage facilities (barns, lofts, attics, silos) are more difficult to clean up thoroughly in comparison with the more sophisticated commercial storage facilities. Moreover farmers' storages may have more important moisture sources like leaks in the roof, humidity from the walls, etc. increasing the risk of mycotoxin contamination at the farm (Tangni and Pussemier, 2005). So, Pussemier *et al.*, (2005) have reviewed data from different countries and concluded that atypical severe contaminations of cereals by OTA (up to 1000 ppb as observed in Poland, for

example) could be linked to bad storage conditions in individual farms from either the organic or the conventional side. Based on these findings, mycotoxins can be a serious contamination problem in poorly stored grains intended for human consumption. It can also be a problem for animal health because cereals can be used to prepare animal feed and also because grain dusts collected in large grain storage facilities can be used, as a source of fiber and/or cellulose, for incorporation into feed (Tangni and Pussemier, 2005).

Ochratoxin A (OTA) is classified by the International Agency for Research on Cancer (IARC from World Health Organization (WHO)) as a probably carcinogenic agent for humans (Group 2B, IARC) (WHO, 1993). It has been also correlated to the Balkan endemic nephropathy (Castegnaro *et al.*, 2006). Its presence was detected in many stored and dry foods (FAO, 1995; Dilkin, 2002) such as corn, wheat, oats, beans, nuts, peanuts, rice, barley, sorghum, cotton seed, coffee beans, cocoa and spices (Furlong *et al.*, 1999; Molinie *et al.*, 2005).

Citrinin (CIT) [(3*R*,4*S*)-8-hydroxy-3,4,5-trimethyl-6-oxo-4,6-dihydro-3*H*-isochromene-7-carboxylic acid, has been known since 1931, when it was isolated from *Penicillium citrinum* and, afterwards, from the Australian plant *Crotalaria crispate* (Chagas *et al.*, 1995). This mycotoxin is associated with cases of porcine nephropathy and has been found as a contaminant in corn, rice, wheat and other grains (Heber *et al.*, 2001; Meister, 2004). The adverse effects caused by CIT on the human body are chronic rather than acute. It has been demonstrated that its intake may cause hepatic and renal failure in the long run (Knasmuller *et al.*, 2004). According to European Food Safety

Authority (2012), no previous assessment of citrinin as a food contaminant could be identified. However, in various documents related to the assessment of ochratoxin A, reference is made to the concomitant occurrence of citrinin in food or feed material. To date, high performance liquid chromatography with fluorescence detection is the method of choice for routine citrinin analysis. One of the major challenges in citrinin analysis relates to its instability in various organic solvents and at higher temperatures. So far, none of the applied analytical methods has been validated by inter-laboratory studies. In addition, no certified reference materials or proficiency tests are available for the determination of citrinin in food or feed (European Food Safety Authority, 2012). Also literature on High performance thin layer chromatographic (HPTLC) method which is simpler, cost effective and with high throughput is not much available. In this context, present study was done to assess OTA and CIT contamination in feed, fodder and ingredient samples by a validated HPTLC method.

Materials and Methods

Sample collection

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

Sampling

Moulds and mycotoxins 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

To achieve maximum particle size reduction, thorough mixing and effective distribution of contaminated portions the samples were subjected to following steps. Grinded entire lot of sample through hammer and passed through number 14 sieve split sample sequentially in sample splitter. Re-grinded 1kg portions to completely pass number 20 sieve and mixed thoroughly. Finally, weighed 25 grams of sample for OTA and CIT estimation.

Analytical method

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

Matrix extraction

Sample analysis was carried out by taking a known quantity (25g) of the grinded sample in a 250ml flask and added 4 g of sodium chloride and treated with 100 ml of acidified acetonitrile (acetonitrile-88ml, 20% H₂SO₄-2ml and 5NHCl + 4%KCl-10 ml). This mixture was shaken for 30 minutes at 200 rpm in an orbital shaker. It was then filtered through whatman paper (No1). To 50 ml of filtrate in a 250 ml separating funnel, 50 ml distilled water and 50 ml hexane were added, and shaken gently for 3 minutes and the acetonitrile:water phase was collected, this operation was performed twice. To the final acetonitrile:water phase in another separating funnel 20 ml chloroform was added and shaken

vigourously. The organic phase (chloroform) was collected in a vial through anhydrous sodium sulphate bed. The extract was evaporated in a hot plate under fume hood. Finally the dried extract was reconstituted with benzene: acetonitrile (98:2) and used for TLC and HPTLC spotting.

Estimation of OTA and CIT by TLC

The dissolved residue was then spotted as 5 µl drops on to a silica gel plate of about 0.5mm thickness which was earlier precoated with oxalic acid and dried. The standard solution of OTA and CIT were also spotted on to the same plate as drops of 1, 3, 5 µl. The plate was developed in mobile phase condition toluene : ethyl acetate : formic acid (6:3:1). After development, the plate was air-dried and observed under UV light.

The fluorescence intensities (lemon yellow) of CIT spots of sample were compared with those of standard spots. The sample spot, which matches one of the standard spots, was selected. Standard was also used to compare the colour and *r_f* value of unknown sample streak on the plate. The amount of CIT was estimated. Later, the same plate was post derivatised by spraying with liquor ammonia and viewed under UV light for OTA spots (blue) if any in the samples by comparing with those of standard spots. The amount of OTA was estimated.

Estimation of OTA and CIT by HPTLC Plate Material

Silica gel HPTLC plates, precoated with oxalic acid and dried using air drier, in the format of 10×10 cm or 20×10 cm were used separately for OTA and CIT estimation.

Sample and standard application

The reconstituted dried samples were applied as 5 µl and 10 µl bands (spray-on technique) using Linomat-5 sample applicator. The standard solution of OTA was also applied as bands of 1, 3, 5 µl on to the same plate with samples. Similarly, for CIT estimation, standard solution of CIT was also applied as bands of 1, 3, 5 µl on to the same plate with samples.

Preparation of developing solvents

Prepared a mobile phase condition toluene:ethyl acetate: formic acid(6:3:1) and poured 20 mL per trough in TTC with 30 minutes pre-saturation time for development of plates.

Development

The spotted samples were developed in pre-saturated TTC up to 80mm from lower edge of TLC 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 was done in a TLC spray cabinet or in the fume hood. For OTA estimation, the developed plates were dried by using hair dryer and sprayed with liquor ammonia, near fume hood. After spraying the plates were dried using hair dryer for 5 minutes.

Scanning

Finally the plates were scanned in CAMAG HPTLC scanner-3 under 366nm wavelength to determine the levels of OTA or CIT contamination in the

corresponding 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.

Result and Discussion

Out of 20 samples of feed and 4 ingredient samples analysed by HPTLC, 8 samples and 1 sample respectively found contaminated with OTA representing 40% and 25% with a concentration range of 2.05 – 31.34 ppb and 0.8 – 3.4 ppb and mean concentration of 7.07 and 6.73 ppb respectively (Table 1).

Out of 25 samples of feed, 4 ingredient, 3 green fodder and 1 dry fodder sample analysed by HPTLC, 8, 3, 2 and 1 sample respectively found contaminated with CIT representing 32%, 75%, 66.67% and 100% with a concentration range of 0.42 – 15.31, 0.8 – 1.14, 0.43 – 1.01 and 2.15 ppb, and mean concentration of 27.42, 5.34, 1.44 and 2.15 ppb respectively (Table 2).

The results of the present study for OTA is in accordance with (Hackbart *et al.*, 2012), who have also reported that considering all 38 samples, OTA was found in the sample of rough rice which was stored in silos with 560 µg kg⁻¹ of contamination. In Belgium, analyses of different commodities including raw foodstuffs and processed food performed during 2001-2002 have shown that of the 184 samples investigated, OTA contamination was above the limit of quantification in 73 samples (40%) and that cereal grain samples were contaminated up to 25.4 ppb (Chandelier *et al.*, 2004). This amount is higher than the Commission of the European Communities recommendation (European Union regulatory agency), 40

which establishes a maximum limit of 5 ppb (EC, 2002). Hackbart *et al.*, (2012) reported that with Soares and Rodriguez-Amaya method and addition a partition with cyclohexane to find evidence of the presence of ochratoxin A and aflatoxins in 68 rice samples (in São Paulo State, Brazil), but ochratoxin A was not found in any sample.

Simas *et al.*, (2007) analyzed the occurrence of aflatoxins and ochratoxins in grains used for feeding dairy cattle (in Bahia State, Brazil). 80 samples were analyzed, but ochratoxins were not detected. However, the aflatoxins were detected in 33.75% of the samples with contamination levels ranging from 1 to 3 ppb.

Hackbart *et al.*, (2012) reported that OTA in rice samples (in Rio Grande do Sul State, Brazil), as well as the presence of *Penicillium* in non-positive samples due to the conditions of the analytical method. Dors *et al.*, (2009) evaluated the occurrence of aflatoxin B1, B2, deoxynivalenol, ochratoxin A and zearalenone in rice samples and only aflatoxin B1 was found.

Similarly, for CIT, the results of the present study are in accordance with Hackbart *et al.*, (2012), who have reported that the CIT occurred in one of the samples of parboiled rough rice with 120 ppb of contamination. The concentration that was determined for CIT can also be considered high in comparison with the maximum limits established by the same legislation for mycotoxins that have similar effect to OTA. The present validated HPTLC method is suitable for detection of OTA and CIT contamination in feed, fodder and ingredient samples.

Table.1 Ochratoxin A contamination in feed and ingredient samples

Type of Sample	Number of samples	Contaminated sample	% of Contamination	Concentration Range (ppb)	Concentration Mean±SE (ppb)
Feed	20	8	40	2.05-31.34	7.07±3.5
Ingredients	4	1	25	0.8-3.4	6.73±0.00
Total	24	9	37.5	0.8-31.34	63.3±3.08

Table.2 Citrinin contamination in feed, ingredients and fodder samples

Type of Sample	Number of Samples	Contaminated sample	% of Contamination	Concentration Range (ppb)	Concentration Mean±SE (ppb)
Feed	25	8	32	0.42-15.31	27.42±1.77
Ingredients	4	3	75	0.8-1.14	5.34±0.82
Green Fodder	3	2	66.67	0.43-1.01	1.44±0.29
Dry fodder	1	1	100	2.15	2.15±0.00
Total	33	14	42.42	0.42-15.31	36.35±1.04

Further, presence of OTA and CIT in feed and feed ingredient sample warrants the necessity of implementation of HACCP at farm level.

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