Applied Biological Research 25(1): 216-224; (2023)
DOI: 10.5958/0974-4517.2023.00025.3

DETECTION OF AFLATOXIN PRODUCING FUNGI IN RICE AND PEANUT, AND DETERMINATION OF AFLATOXIN BY HPLC-FLD METHOD

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(Received 8 February, 2023; accepted 5 May, 2023)

ABSTRACT

Peanut and rice are the widely consumed food material all over the globe. The contamination of these commodities with aflatoxin is a big challenge and a major health concern. In present study, two aflatoxin producing fungi were the isolated from rice and peanut and identified as *Penicillium charlesii* and *Aspergillus candidus* on the basis of morpho-molecular characteristics A sensitive, rapid and robust analytical method was developed and validated to determine the aflatoxin contamination level in aforementioned peanut and rice matrices. The method involved methanolic extraction of aflatoxin, immune affinity column clean-up followed by derivatisation with Kobra cell and fluorescence detection. The analysis of contaminated samples revealed only two peanut samples and one rice sample possessed aflatoxin B1 and traces of aflatoxin B2. The method showed 0.05 µg kg^{-1} as the limit of quantification which was below the maximum levels set by various global regulations. The method performance was in line with the performance criteria established by numerous regulatory bodies such as EU and SANTE.

Keywords: Aflatoxin, HPLC-FLD, identification, peanut, rice

INTRODUCTION

Mycotoxins are the major concern in human food safety, as they create many health issues to the consumers. Considerable losses occur in crop and livestock due to the contamination of toxigenic fungi and mycotoxin produced by them. Mycotoxins are produced by commonly occurring fungal species such as *Aspergillus* spp. (Moss, 1998). Aflatoxins (AFs) are mycotoxins mostly produced by *Aspergillus* and *Penicillium* species in various food materials such as cereals, nuts, and spices. Contamination of food material with AFs is globally a critical challenge especially in the developing countries where agricultural and food processing systems are not properly designed to minimise the food safety risks or issues (Mutegi *et al.*, 2013). These fungi grow on a diverse range of food products and produce aflatoxins once the environmental conditions favour their growth. The AF contamination occurs during agricultural processes such as harvesting, handling, shipping, and storage of the crops. AFs majorly include B_1, B_2, G_1, and G_2 which are commonly found in cereals, nuts, spices; and also, M_1 type which is commonly found in milk and milk based products (Zhang *et al.*, 2020; Chayya *et al.*, 2023). AFs are highly hazardous to the consumer that lead to severe toxic effects. AFB_1 is the most toxic in nature; therefore, the International Agency for Research on Cancer (IARC) has incorporated AFB_1 in group of type 1 carcinogen (IARC, 1993). There are also, study performed to reduce the level of AF in food matrices (Naumenko *et al.*, 2019).

Peanuts are rich in protein and vitamin E, and taste delicious. It is a very popular snack all over the world (Chen *et al.,* 2011). Peanuts are susceptible to various types of deterioration including aflatoxin contamination during storage which renders them unsuitable for consumption and so lead to the huge economic losses (Mutegi *et al.,* 2013). Rice is considered an extremely good substrate for the fungal growth and aflatoxin production (Allwood *et al.,* 2023). Pathogenic fungi are able to penetrate cuticle by producing the hydrolytic enzymes that degrade cell wall components (Sieber *et al.,* 2000; Roncero *et al.,* 2003). Due to the serious effects of AFs, European Union has set the regulatory limit in rice and peanut for AFB_1, and the total AF at 2 and 4 μg kg^{-1}, respectively (EC 1881/2006). However, Codex Alimentarius Commission (CAC) has set a maximum limit (ML) for total AF as 15 μg kg^{-1} (CODEX STAN 193-1995), while Food Safety Standards Authority of India (FSSAI) set it at 5 and 15 μg kg^{-1} for AFB_1 and total AFs, respectively (FSSAI, 2011). It is essential to explore the level of aflatoxin produced in commonly used food commodities such as peanut and rice; and determine the AF load in food samples as well as identify the fungal species involved. The present study was aimed to find the level of aflatoxin in stored peanut and rice, as well as identify the fungi responsible for aflatoxin production on morpho-molecular basis. In this study, we developed a validated analytical method which detects the traces of AFs, as per regulatory requirements (EC 401/2006, SANTE 12089) for aflatoxin analysis using high performance liquid chromatography, with fluorescence detection (HPLC-FLD).

MATERIALS AND METHODS

Test matrices

The peanut {*Arachis hypogaea* L.} (n = 7) and rice {*Oryza sativa* L.} (n = 8) samples were procured from different shops in a local market in Satara, Maharashtra (India). The samples were checked for toxin level by using HPLC-based method (Dhanshetty *et al*., 2019). As the toxin levels in different samples vary due to the non-homogeneous nature of the test matrices, so the samples from different shops were analysed separately. The samples with high aflatoxin load (> 8 ng g^{-1} for B_1 and total AF) were selected for the isolation of fungi. The samples with contamination < 0.1 ng g^{-1} (B_1 and total AF) were selected as control and used for method validation of aflatoxin analysis using high performance liquid chromatography with fluorescence detector (HPLC-FLD) method.

Isolation and identification of fungi

For fungal isolation and their screening, potato dextrose agar (PDA) was used as growth medium. In brief, PDA agar (39 g from Hi Media, Bengaluru, India) was added to 1 L distilled water and autoclaved for 15 min at 121°C. Following sterilisation, the medium was cooled to 50°C in a water bath and 8 mL of 1% chloramphenicol antibiotic solution added to it. The medium was then poured into the sterile petri-dishes and allowed to solidify. To prepare different sample dilutions, 1 g of each sample (rice and peanut) was transferred to sterile test-tubes containing 9 mL sterile saline water (0.85% NaCl) which was serially diluted to prepare 10^{-6} dilution of each sample. The diluted samples (0.1 mL) were poured on PDA medium in Petri-plates and incubated at 25°C for 20-25 days. The fungal growth was regularly monitored (Bills and Polishook, 1991). The standard manuals were used to tentatively identify the fungi based on their morpho-cultural characteristics *viz*., mycelial characteristics, fruiting bodies/spores, etc. (Ellis and Eds, 1971; Sutton, 1980; Barnett and Hunter, 1998). Morphological characteristics were observed under a compound microscope (Olympus CX41 microscope and Nikon Eclipse Ni-U, India). For fungal identification, the isolates were grown in 50 mL potato dextrose broth in 250 mL conical flasks at 28±2°C for 48-72 h, followed by filtration through Whatman filter paper (Hi media, India), washed with deionized water, and dried under liquid nitrogen (Caicedo *et al.,* 2019). The dried frozen mycelia were ground to fine powder and submitted for identification to Eurofins Laboratory, India. The identification of fungi was confirmed by

performing polymerase chain reaction (PCR) using specific ITS markers (5′-CTTGGTCATTTAG AGGAAGTAA-3′; 5′-TCCTCCGCTTATTGATATGC-3′). DNA was extracted by using DNA extraction kit (BioRad, India). The specific PCR were performed as per Gonzalez-Salgado *et al*. (2008).

Data analysis and phylogeny

Using BLASTN programme, the translated nucleotide sequences were evaluated for similarity in comparison with the fungal reference species present in the genomic database banks (Madden, 2013). The fungal 18S and ITS rDNA sequences were compared with NCBI (National Centre for Biotechnology Information; *http://www.ncbi.nlm.nih.gov*) database for identification. The molecular evolution genetic analysis (MEGA) software version 10.0 was used to perform phylogenetic analysis of the matched sequences from GenBank and aligned (Tamura *et al*., 2013). The phylogenetic tree was obtained (Marchler-Bauer *et al*., 2015) and confirmation done by assessing the similarities of the isolated fungi with the earlier reported fungal species, capable for aflatoxin production (Thathana *et al*., 2017). The nucleotide sequences were deposited in NCBI.

Determination of aflatoxins in peanut and rice

For the analysis of AFs, high performance liquid chromatography HPLC (Agilent Technologies) connected to fluorescence detector (G1321A) (HPLC-FLD*)* was used (Dhanshetty *et al.*, 2019). Separation of AFs was performed by using C18 column (150 mm × 4.6 mm, 5 µm) with column temperature 40°C and injection volume 20 µL. The separation was accomplished with a mixture of mobile phase comprising 72:28 of methanol: water; v/v. The flow rate was kept at 1.2 mL min^{-1} and run time 17 min. For estimation of AFs, the wavelength of fluorescence detector was set with excitation (λex) of 362 nm and emission (λem) 455 nm. Derivatisation unit of Kobra cell was used to achieve the increment in signals of less fluorescent AFs (AFB_1 and AFG_1). The column temperature was maintained at 40°C during the entire analysis. Identification of AFs was achieved by injecting the standard of AF and confirming the retention time of standards AFs with the AFs present in the analysed samples. The individual standards of aflatoxin were procured from Sigma Aldrich, India with purity > 98%. The response of analyte in reagent blank and control samples determines the specificity. Both control (blank and reagent blank) meet the requirement for specificity by not exhibiting any target peaks during the run. The limit of quantification (LOQ) recovery study of AFs was performed at three levels (0.05 ng g^{-1}, and 0.5 & 2 µg kg^{-1}) in rice and peanut matrix. Due to immuno-affinity column (IAC) clean-up, a clear peak of each targeted AF was observed without any interference (Fig. 1). The spiked samples for recovery studies were evaluated against the linearity of six-point solvent standard calibration.

Method validation

The single laboratory validation was performed by using HPLC-FLD method. Each test matrix was assessed in accordance with the requirements of global regulatory bodies (EC 401/2006, SANTE 12089). The LOQ recovery, precision and robustness were analysed at 0.05 µg kg^{-1} (LOQ). The solvent-based (methanol: water; 1:1) calibration standards were prepared in the range of 0.02 to 5 ng mL^{-1} (Dhanshetty and Banerjee, 2019). The chromatogram of blank (control sample) did not show any peak at the retention time (*tR*) of the targeted analyte. Recovery and precision studies were conducted at 0.05, 0.5, and 2 µg kg^{1} levels. To determine the repeatability, each study was performed in six replicates with three different analytes on three different days and precision relative standard deviation (RSD) [Oulkar *et al*., 2018].

Statistical analysis

In order to estimate the AF content in test samples, each analysis we performed in six replicates. Given that the data followed a normal distribution, analysis of variance (ANOVA) was performed using the Minitab18 software (Minitab Inc., State College, PA, USA).

RESULTS AND DISCUSSION

Isolation and identification of fungi from contaminated peanut and rice samples

Fifteen potential aflatoxin producing isolates were isolated from the tested matrices of peanut and rice (7 from peanut and 8 from rice samples). Two potential isolates (one each from matrix rice A and peanut A) were morphologically identified using the standard methods ((Thathana *et al*., 2017). These isolates, grown on PDA, were microscopically examined for their reproductive structures. The isolate from rice revealed milky white colony, septate mycelium, and produced conidia measuring 2.5 x 3.5 µm in size, which were globose, pale green, and smoothly thin-walled. The fungus was tentatively identified as *Aspergillus candidus* Link. The isolate from peanut showed typical branching septate hyphae which produced conidial heads at maturity. Typically, the conidia were dark green, oval to slightly elliptical, and measured 2.0 x 2.5 µm in size. The fungus was tentatively identified as *Penicillium charlesii* G. Smith. The enzymatic characteristics of these two have been discussed in our earlier communication (Dhanshetty and Mali, 2021). The confirmation of the identity of these isolates was done by PCR ITS markers. The isolates from rice and peanut showed similarity with *Aspergillus candidus* and *Penicillium charlesii*, respectively, after performing Blast analysis at the National Centre for Biotechnology Information (NCBI) and comparing the sequences with NCBI data bases (Tamura *et al.*, 2013). The nucleotide sequences of *A. candidus* and *P. chrlesii* were deposited under GenBank Accession No. OQ848043 and OQ848046, respectively. The nucleotide homology and comparative study of gene sequences displayed >90% similarity with the reference sequence of *A. cadidus* and *P. charlesii*. The phylogenetic tree of fungal isolates was developed (Fig. 1 and 2) and

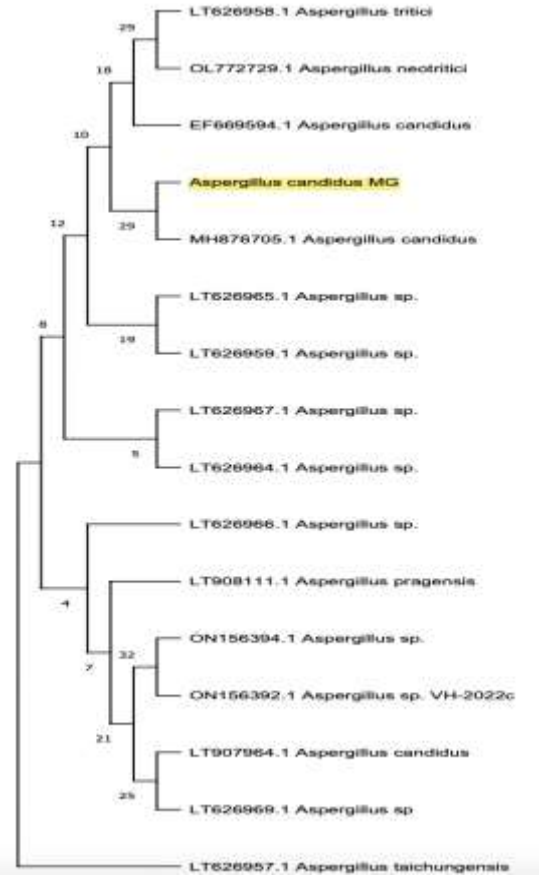

Fig. 1: Phylogenetic tree for *Aspergillus candidus* isolated from rice sample

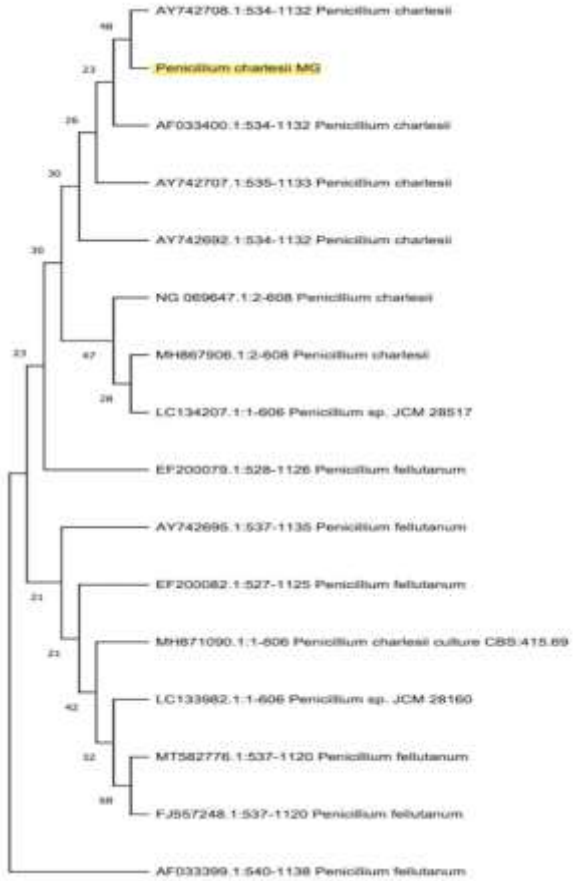

Fig. 2: Phylogenetic tree for *Penicillium charlessi* isolated from peanut sample

the values mentioned in these figures next to the branches define the percentage of replicate trees in which the allied taxa are clustered together. Thus, on the basis of morphological characterization, gene sequence and phylogenetic analysis, the isolates were confirmed. Earlier, Uka *et al*. (2019) studied the molecular phylogeny and genetic diversity for the emerging mycotoxin producing fungi by preforming using similar method. *A. candidus* is majorly responsible for onychomycosis disease majorly found in human nails after infection (Ahmadi *et al.,* 2012). The potential of *P. charlesii* to hydrolyse tea catechins and its impact on biotransformation of black tea polyphenols have earlier been reported (Raghuwanshi *et al.,* 2014). Earlier, Mislivec *et al.* (1968) and Gomez *et al*. (2022) reported aflatoxin production by *P. charlesii*. However, limited studies were performed for aflatoxin production by these fungi.

Optimisation of chromatographic conditions (HPLC-FLD)

The optimisation of chromatographic conditions were performed to achieve the proper separation of the analyte and to determine the traces of AFs in peanut and rice samples. The symmetric peak shape and proper retention time were determined by analysing the replicate of standards and samples. The retention time for AFs were noted at 7.3 min for AFG_2, 9.07 min for AFG_1, 11.05 min for AFB_2 and 14.85 min for AFB_1 with proper base to base separation of each AF (Fig. 2). The C_{18} column 150 mm with 5 µm diameter showed proper sensitivity of analyte by achieving the LOQ of 0.5 µg kg^{-1} in both rice and peanut matrices. Post-column derivatization is required to obtain the desired LOQs of AFs on HPLC-FLD analysis (Ozbey and Kabak, 2012). Ok *et al.* (2015) described an HPLC with FLD approach for simultaneous measurement of AFs in red pepper that featured a photochemical reaction using PHRED The approach produced LOQs of 1.5 ng g^{-1} for AFB_2 and AFG_2 and 5 ng g^{-1} for AFB_1 and AFG_1, but the cost of analysis increased by the use of a PHRED derivatization unit. So, we could achieve the LOQ of 0.5 µg kg^{-1} by using Kobra cell for derivatisation of B_1 and G_1.

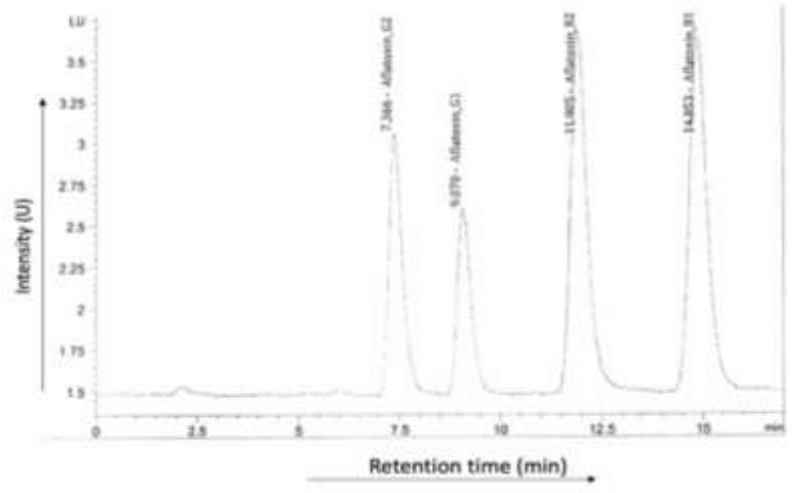

Fig. 3: Chromatogram of AFs in peanut sample at LOQ level

Sample preparation and method validation of AFs

The extraction solvent (72% methanol in water) showed the recovery of each targeted aflatoxin at 85-98% in rice and 88-97% in peanut matrix at common LOQ of 0.05 µg kg^{-1} level, which is in agreement with Oulkar *et al.* (2018) and Dhanshetty and Banerjee (2019). Further, the derivatisation step performed with Kobra cell, connected in between the column to detector, improved the signals of AFB_1 and AFG_1 which are necessary as these analytes have less fluorescent property than the remaining two AFs (AFB_2 and AFG_2). Post-column derivatisation is critical for achieving the desired AF LOQs on HPLC-FLD analysis (Ozbey and Kabak, 2012; Ainiza *et al.*, 2015; Ok *et al.*, 2015).

Method validation of aflatoxin analyses in peanut and rice

The study revealed that each AF showed the recoveries in-between 85-98% for rice and 88-97% in peanut matrix, with relative standard deviation (RSD) less than 10% for each level including LOQ (Table 1). All the compounds depicted precision *($RSDR_r$)* below 9% for rice and 10% for peanut matrix. This precision RSD_r was determined by analysing six replicates of each sample. In terms of

RSD, the overall precision was satisfactory (Table 1). The laboratory precision/ reproducibility *(RSD_R)* was determined by analysing the replicates of each spiked sample on three different days with three different analysts. Laboratory reproducibility showed recoveries in the range of 75-89% with RSDs ≤ 20%. All the method validation parameters successfully met the criteria of global regulatory requirements such as SANTE 12089 and EC 401/2006 showing that the method is fit for the purpose. In all the test matrices, the IAC clean-up effectively removed the matrix co-extractives and left no traces of co-eluting interfering signals at the retention periods of the target aflatoxins. The matrix effects were negligible (5%) when the signal intensities in solvent were compared with the standards that matched the matrix. Hence, for quantifications we used the solvent-based calibrations.

Table 1: Level of AFs (($\mu g\ kg^{-1}$) in contaminated sample analysed on HPLC-FLD

Matrix	AFB_1	AFB_2	AFG_2	AFG_1
Peanut sample 1	8.5	0.1	BLQ	BLQ
Peanut sample 2	8.4	0.2	BLQ	BLQ
Peanut sample 3	0.4	0.1	BLQ	BLQ
Peanut sample 4	0.5	0.2	BLQ	BLQ
Peanut sample 5	BLQ	BLQ	BLQ	BLQ
Peanut sample 6	0.6	0.3	BLQ	BLQ
Peanut sample 7	BLQ	BLQ	BLQ	BLQ
Rice sample 1	0.4	0.08	BLQ	BLQ
Rice sample 2	0.4	0.1	BLQ	BLQ
Rice sample 3	1.2	0.8	BLQ	BLQ
Rice sample 4	0.1	0.5	BLQ	BLQ
Rice sample 5	0.3	0.08	BLQ	BLQ
Rice sample 6	BLQ	BLQ	BLQ	BLQ
Rice sample 7	BLQ	BLQ	BLQ	BLQ
Rice sample 8	8.3	1.2	BLQ	BLQ

BLQ = Below limit of quantification; The results were below the detection limit for these samples

Table 2: Recovery of aflatoxins in peanut and rice samples at different level of spiking

Analyte	Spike level ($\mu g\ kg^{-1}$)	Recoveries (% RSD)	
		Peanut	Rice
G_2	0.05	85 (9)	89 (7)
	0.50	89 (8)	94 (8)
	2.00	97 (8)	96 (7)
G_1	0.05	84 (9)	88 (6)
	5.00	86 (5)	87 (6)
	2.00	95 (8)	88 (8)
B_2	0.05	88(8)	89 (7)
	0.50	89 (10)	92 (9)
	2.00	93 (8)	87 (9)
B_1	0.05	85 (6)	89 (5)
	0.50	88 (8)	96 (8)
	2.00	98 (8)	97 (7)

*Analysis done in 6 replicates for each level of spiking

Analysis of contaminated samples

The contaminated samples of rice and peanut were analysed for the load of AFs present in them. Among all the samples evaluated, two peanut samples and one rice sample exhibited the AF level of > 8 ng g^{-1} with contamination of B1. Each case, the precision RSD was found <5%. The AFG_1 and AFG_2 were below the limit of quantification for all the samples of peanut and rice (Table 2). AFB_1 levels in packaged rice sold in Lebanon were examined earlier by Hassan *et al*. (2022). Similarly, Dhanshetty and Banerjee (2019) performed the analysis of AF contaminated rice and peanut samples and determined the level of AF by using HPLC-FLD method.

Conclusion: Two aflatoxin producing fungi *Penicillium charlesii* (from peanut) and *Aspergillus candidus* (from rice samples) were identified. The level of aflatoxin varied in the contaminated samples, with AFB_1 as dominant contaminant, followed by AFB_2. The analytical approach followed is straightforward, quick, accurate, and repeatable. In peanut and rice matrices, the developed validated method offered satisfactory assessment of AFs. The approach can be appropriately adopted in regulatory and commercial food testing laboratories as the findings are in line with the performance standards established by numerous regulatory agencies, including the EU and SANTE.

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

Contribution of authors: Manisha Dhanshetty performed the studies and prepared manuscript under the supervision of Dr. G.V. Mali who conceptualized the research work and reviewed the manuscript.

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