5 Chemical and Molecular Methods for Detection of Toxigenic Fungi and Their Mycotoxins from Major Food Crops

S. Chandra Nayaka, M. Venkata Ramana, A.C. Udayashankar, S.R. Niranjana, C.N. Mortensen, and H.S. Prakash

Abstract

Mycotoxins are the secondary metabolites produced by certain molds on a wide range of agricultural commodities and are closely related to human and animal food chains. Mycotoxins are capable of causing disease in humans and other animals, and their detection is largely dependent on the sample matrix and the type of fungus causing their contamination. The strict regulations on trade of contaminated grains and seeds and other produce in industrial countries lead to economic burdens on farmers. In developing countries, the situation is aggravated where regulations may be nonexistent or not enforced and where consumption of home-grown cereals leads to a wide exposure to toxins. Important mycotoxins that occur quite often in food are deoxynivalenol/nivalenol, trichothecenes, zearalenone, ochratoxin A fumonisins, and aflatoxins. High concentrations of mycotoxins such as aflatoxins are consumed by humans in areas of the world with higher-than-average levels of liver cancer, childhood malnutrition, and disease. This chapter introduces rapid, robust, and user-friendly protocols currently applied in the identification of toxigenic fungi and important mycotoxins.

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Introduction

The term "mycotoxin" combines the Greek word for fungus "mykes" and the Latin word "toxicum" meaning poison. Mycotoxins have received considerable attention, especially over the last few decades. The problem related to mold damage and the hazard of consuming damaged grains have been recognized since historical times. The term "mycotoxin" is usually reserved for the toxic chemical products formed by a few fungal species that readily colonize crops in the field or after harvest and thus pose a potential threat to human and animal health through the ingestion of food products prepared from these commodities [1].

The possibility of human diseases occurring as a result of the consumption of mold-damaged rice and wheat was raised in Japan and other Asian countries during the first half of this century. Awareness of risks from eating overwintered millet was reported in the USSR [2]. However, the serious worldwide concern about mycotoxins began in the early 1960s after it was discovered in the United Kingdom that Turkey "X" disease is caused by aflatoxins. More than 300 mycotoxins have been identified, although only around thirty with toxic properties that are genuinely of concern for human beings or animals were reported by the French Agency of Food, Environmental and Occupational Health and Safety [3]. Some mycotoxins are rather rare in occurrence; others—such as aflatoxin, ochratoxin, fumonisins, and trichothecenes—are quite common in some years. The molds primarily responsible for producing mycotoxins are *Aspergillus*, *Fusarium,* and *Penicillium* spp. Occurrence of mycotoxins in food and animal feed often exhibits a geographical pattern; for example, *Aspergillus* species meet optimal conditions in tropical and subtropical regions, whereas *Fusarium* and *Penicillium* species are adapted to the moderate climate of North America and Europe [4]. The toxins can be produced in major food crops like, maize, wheat, sorghum, rice, soybeans, peanuts, and other food and feed crops in the field, during transportation, or improper storage. Moreover, in animals consuming contaminated feed, mycotoxins can deposit in different organs and also subsequently affect food of animal origin (e.g., meat, eggs, milk, and milk products). Worldwide trade with food and feed commodities has resulted in a wide distribution of contaminated material [5]. One of the characteristics of mycotoxins is that they can exude toxic properties in minute quantities; thus, sensitive and reliable methods are required for their detection and quantification, which generally involves sophisticated sampling, sample processing, extraction, and assay techniques.

Different methods have been applied in the detection and quantification of mycotoxins from food and feed samples, including ELISA (enzyme-linked immunosorbent assay), immunoaffinity cartridge, solid-phase ELISA, and selective adsorbent mini-column procedures [6]. TLC (thin-layer chromatography) and HPLC (high-performance liquid chromatography) [7] are more accurate for quantification of mycotoxins in food and feedstuffs' produce. Under practical storage conditions monitoring for the occurrence of fungi is often conducted; however, in practice, it is difficult to distinguish several toxigenic fungal species from their close relatives, and accurate identification based on traditional methods is very difficult owing to their genetic variation and high morphological similarity.

The conventional scheme of isolation and identification of toxigenic fungi from food samples is cumbersome and requires skilled personnel to achieve proper identification. Even

with taxonomical expertise, identification is commonly difficult regarding some fungi genus that contains a large number of closely related species [8]. Robust DNA-based tools often offer accurate, rapid, and sensitive identification and characterization of species (e.g., *Fusarium*) that belong to a complex genus [9]. The application of molecular biology techniques is an alternative to cumbersome and time-consuming conventional culture methods for precise identification of toxigenic fungal species before they can enter the food chain. The polymerase chain reaction (PCR) assay has allowed rapid, specific, and sensitive detection of toxigenic species without the need for prior growth of the organisms. The traditional molecular markers are mainly based on ribosomal DNA, β-tubulin, and calmodulin genes or have been based on anonymous DNA sequences. These DNA sequences are obtained from an unbiased sampling of genomic DNA, and these may or may not contain functional genes involved in toxin production [10].

Developing markers from anonymous sequences requires comparative analyses among related species of DNA profiles generated from randomly amplified fragments by using RAPD (random amplified polymorphic DNA) or AFLP (amplified fragment length polymorphism). In the last decade, numerous PCR assays have been developed for rapid detection and differentiation of toxigenic and nontoxigenic fungi from major commodities by using specific genes associated with mycotoxin biosynthesis [11].

Some Important Mycotoxins

Aflatoxins (*Aspergillus* spp.)

Aflatoxins are chemical derivatives of difurancoumarin, mainly produced by *Aspergillus flavus*/*A. parasiticus*. Aflatoxins have been implicated in subacute and chronic effects in humans. These effects include primary liver cancer, chronic hepatitis, jaundice, hepatomegaly, and cirrhosis through repeated ingestion of low levels of aflatoxin. It is also considered that aflatoxins may play a role in a number of diseases, including

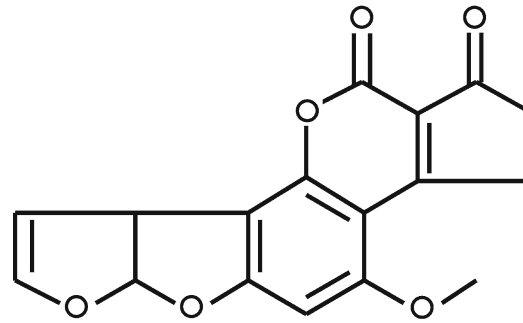

Fig. 5.1 Structure of aflatoxin B_1

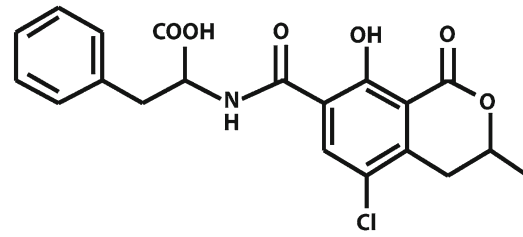

Fig. 5.2 Structure of ochratoxin A

Reye's syndrome, kwashiorkor, and hepatitis [12]. Aflatoxins can also affect the immune system. *A. flavus* infects many of our food crops, such as nuts, grains, and culinary herbs. Primary economic concerns are infestations that occur in corn and peanuts. The major aflatoxins consist of aflatoxins B1 (Fig. 5.1), B2, G1, and G2.

Ochratoxin

Ochratoxin A is the most important and most commonly occurring structurally related group of compounds; it is often abbreviated to OTA or OA (Fig. 5.2). Ochratoxin A is the major mycotoxin of this group, and it is an innately fluorescent compound produced primarily by *Aspergillus ochraceus* and *Penicillium verrucosum* [13]. Ochratoxin A is a potent toxin that affects mainly the kidneys, in which it can cause both acute and chronic lesions. Ochratoxin A is a potent teratogen in mice, rats, hamsters, and chickens, and a nephrotoxic effect has been demonstrated in all mammalian species.

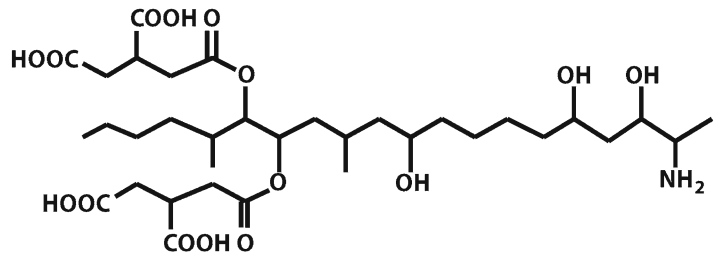

Fig. 5.3 Structure of fumonisin B_1

Fumonisins

The fumonisins are a group of nonfluorescent mycotoxins—FB_1 (Fig. 5.3), FB_2, and FB_3 being the major entities—produced primarily by *Fusarium verticillioides* and *F. proliferatum* [13], *Fusarium nygamai,* as well as *Alternaria alternata* f. sp. *lycopersici*. Fumonisins are thought to be synthesized by condensation of the amino acid alanine into an acetate-derived precursor. Numerous species-specific diseases have been attributed to fumonisin-contaminated feed, including leukoencephalomalacia in horses and pulmonary edema and hydrothorax in swine [14]. These compounds have been shown to have carcinogenic potential in animal models and are the only known inhibitors of ceramide kinase, a key enzyme involved in inflammatory cascades.

Deoxynivalenol

Deoxynivalenol, also known as DON or vomitoxin, is one of about 150 related compounds known as the trichothecenes that are mainly produced by *Fusarium graminearum* and, in some geographical areas, by *F. culmorum* (Fig. 5.4) [15]. These two species are important plant pathogens and cause Fusarium head blight in wheat and Gibberella ear rot in maize. Toxicity of deoxynivalenol is characterized by vomiting, particularly in pigs, feed refusal, weight loss, and diarrhea. A study reporting human food poisoning by infected wheat containing deoxynivalenol in India showed a range of symptoms, including abdominal pains, dizziness, headache, throat irritation, nausea, vomiting, diarrhea, and blood in the stool [16].

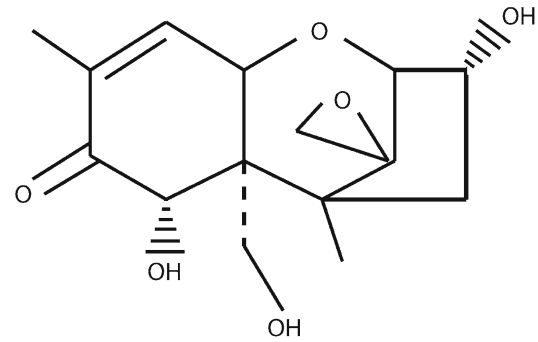

Fig. 5.4 Structure of deoxynivalenol

The potential presence of toxins in the food supply means that expensive testing and remedial actions are necessary to assure that they do not reach dangerous levels in our food. This testing and losses in crop quality and yield associated with these fungal diseases are estimated to cost agriculture billions of dollars annually, and the presence of fungal toxins in our crops places the competitiveness of our agricultural exports at risk. The presence of mycotoxins is unavoidable; therefore, testing of raw materials and products is required to keep our food and feed safe. The presence of mycotoxins in food crops is a serious and common quality problem that has become more obvious as a result of the research of recent years. Several chemical and biological detection systems exist for the determination of mycotoxins. Biological assays were used when analytical and methods were not available for routine analysis because biological assays are qualitative and often are nonspecific and time-consuming. Various analytical methods for mycotoxin analysis have been developed, such as TLC, HPLC, and HPTLC [7]. Many research laboratories have also adopted molecular detection methods for rapid and accurate

detection of toxigenic molds from major food crops.

This chapter presents information on the general protocols adopted for detection of important toxigenic *Aspergillus*, *Penicillium*, and *Fusarium* species by PCR and their mycotoxins mainly by TLC and HPLC.

Materials

(See Note 1)

DNA Extraction

1. Sterile distilled water.
2. 1× lysis buffer (made freshly approximately every 2 weeks): 100 mM Tris–HCl pH 7.4 (37°C), 100 mM EDTA, 6% SDS, 2% β-mercaptoethanol.
3. Vortex mixer.
4. Water bath.
5. Phenol: chloroform (1:1).
6. Microcentrifuge.
7. Disposable polypropylene microcentrifuge tubes: 1.5 mL conical; 2 mL screw-capped.
8. 3 M ammonium acetate.
9. Isopropanol.
10. Tris EDTA: 10 mM Tris–HCl pH 7.5 (25°C), 0.1 mM EDTA.
11. Phenol solution equilibrated with 10 mM Tris–HCl pH 8, 1 mM EDTA (Sigma-Aldrich, Gillingham, UK).
12. Ethanol (70%).
13. Agarose (e.g., molecular biology grade agarose from Sigma-Aldrich, USA).
14. TBE buffer: 50 mM Tris, 50 mM boric acid, 1 mM EDTA. Dilute when needed from a 10× stock.
15. Ethidium bromide: 0.5 mg/mL stock.
16. Gel loading mixture—40% (w/v) sucrose, 0.1 M EDTA, 0.15 mg/mL bromophenol blue.
17. Horizontal electrophoresis equipment (e.g., Biorad Wide Mini Sub Cell).
18. UV transilluminator and camera suitable for photographing agarose gels (e.g., Syngene Gene Genius Bioimaging System).

Basic Equipment Required for Polymerase Chain Reaction

1. Thermal cycler.
2. Micropipettes.
3. Agarose gel electrophoresis unit.
4. Centrifuge.
5. UV-gel documentation chamber.
6. Eppendorf tubes.

PCR Reagents

1. Agarose.
2. dNTP mix.
3. PCR buffer.
4. $MgCl_2$.
5. Template DNA.
6. Taq DNA polymerase.
7. Oligonucleotide primers.
8. Ethidium bromide.
9. TBE buffer (Tris–EDTA–boric acid buffer).

PCR Assay Set-up

Prepare a master mix (for 50 μL reaction) containing the following:

1. 5 μL 10× PCR buffer.
2. μL MgCl2 (25 mM)×(number of reactions+1).
3. μL dNTP mix (1.25 mM).
4. 30 μL sterile distilled H_2O.
5. Dispense the master mix at 43 μL per PCR tube.
6. Dispense primers in pairs at 2.5 μL per tube.
7. Dispense template at 1 μL per tube.
8. Prepare the Taq solution by diluting the appropriate amount of stock Taq DNA polymerase to 1 unit/μL with sterile distilled H_2O.
9. Dispense diluted Taq polymerase at 1 μL per tube.

General PCR Conditions

After setting up the reaction, the following conditions can be used for proper amplification of target genes:

1. Initial denaturation: 94°C/5 min.
2. Denaturation: 94°C/1 min.
3. Annealing temperature: 52–60°C/1 to 1.30 min.
4. Extension temperature: 72°C/1 to 2 min.
5. Repeated for 30 to 35 cycles.
6. Final extension temperature: 72°C/5 to 10 min.

After successful amplification load 5 μL of amplified PCR products to agarose gel (1%) in containing 1% ethidium bromide and visualize under UV.

Materials Required for Chromatography-Based Method

1. Silica gel-coated plates (Merck chemicals).
2. Solvents, analytical grade and HPLC grade (Sigma-Aldrich, USA).
3. Purified mycotoxin standards (Sigma-Aldrich, USA).
4. Micropipettes (Eppendorf 1–1,000 μL).
5. Chromatography chamber (CMAG).
6. Spray reagents.
7. UV scanner (CMAG).
8. Mechanical cup shaker (CMAG).
9. Conical flasks (250 mL).
10. Filter papers.
11. Separating funnels.
12. HPLC system with UV and fluorescent detectors (Hitachi F-4500).
13. C18/C8 cartridges.
14. Immunoaffinity/Solid phase extraction columns for clean up.

Methods

DNA Fingerprinting Methods

The methods detailed as follows describe the general protocol for obtaining pure DNA from fungal culture, food, and grain samples [17]. The volumes and number of tubes used per sample may need to be varied, depending on the type of sample and the quantity of mycelia being processed.

Extraction of DNA from Pure Cultures of Fungi [18]

1. Take small pinch of mycelium into microcentrifuge tubes.
2. Add 500 μL of lysis buffer, and macerate the mycelium with the help of a sterile glass rod.
3. Add 50 μL of 10% SDS, vortex, and incubate at 65°C for 10 min.
4. Add 500 μL phenol: chloroform (1:1) to each tube and vortex briefly.
5. Centrifuge 5 min at 10,000 rpm in the microfuge, then carefully transfer as much as possible of the top aqueous layer to a clean tube. Do not disturb the debris at the interface.
6. Add 40 μL 6 M ammonium acetate, 700 μL isopropanol, invert gently to mix, and spin for 2 min, and incubate the mixture at −20°C for 1–2 h.
7. Centrifuge at 10,000 rpm for 10 min and discard the supernatant.
8. Add 300 μL cold 70% ethanol, centrifuge for 2 min, and discard the supernatant.
9. Centrifuge for 10 s and remove the remaining liquid with a micropipette.
10. Allow the pellet to dry for 20 min in a fume hood and then resuspend it in 50 μL TE or sterile distilled water.

Extraction of DNA from Contaminated Food Samples (e.g., Maize)

This method has been used to prepare DNA from contaminated food grains at several laboratories. The steps are as follows:

1. Ground the contaminated food grains.
2. Add 400 μL of lysis buffer, vortex, and incubate at 65°C for 10 min.
3. Add 500 μL phenol:chloroform (1:1) to each tube and vortex briefly.

4. Centrifuge 15 min at 12,000 rpm in the microfuge, then carefully transfer as much as possible of the top aqueous layer to a clean tube. Do not disturb the debris at the interface.
5. 40 μL 6 M ammonium acetate, 600 μL isopropanol, invert gently to mix and spin for 2 min. Incubation of the mixture at −20°C for 10–60 min before centrifugation may improve recovery of DNA but can result in reduced purity of the sample. Remove the supernatant.
6. Add 50 μL RNase in TE to the pellet and incubate at 37°C for 15 min.
7. Pool into 200 μL samples or add 150 μL TE, and then add 200 μL phenol, vortex, and centrifuge at 10,000 rpm for 6 min.
8. Transfer carefully the top aqueous layer to a fresh tube.
9. Add 10 μL 6 M ammonium acetate, 600 μL isopropanol, and incubate at −20°C for 10 min.
10. Centrifuge at 12,000 rpm for 10 min and discard the supernatant.
11. Add 800 μL cold 70% ethanol, centrifuge at 10,000 rpm for 2 min, and discard the supernatant.
12. Centrifuge at 10,000 rpm for 15 s and decant the remaining liquid with a micropipette.
13. Allow the pellet to dry for 20 min in a fume hood and then resuspend it in 50–200 μL TE buffer.

Polymerase Chain Reaction

During the last few decades great advances have been made in molecular diagnostic technology, especially in the development of rapid and sensitive methods for the detection of plant pathogenic fungi [19]. A number of DNA-based techniques that have been developed include restriction fragment length polymorphism, pulse field gel electrophoresis, and PCR. PCR has been gaining popularity mainly because of its ease of application compared to other DNA-based techniques.

There are already many examples of PCR-based assays developed for the detection of fungi in plant pathology, but the reports on their use in specific detection of toxigenic fungi are limited. Many mycotoxin biosynthetic pathway genes are present within gene clusters, and some of these appear to have undergone horizontal transfer from one species to another and are now present in several species [7]. Regions of homology within mycotoxin biosynthetic gene from the different species can be used to develop primers to detect the presence of the relevant mycotoxigenic species. This strategy was successfully applied for aflatoxin producers [20], trichothecene-producing fungi [18], fumonisin-producing *Fusarium* species [18], and also for producers of ochratoxin [21]. PCR-based detection has been applied as an alternative assay, replacing cumbersome and time-consuming microbiological and chemical methods for detection and identification of the most serious pathogenic and mycotoxin producers in the fungal genera *Fusarium*, *Aspergillus*, and *Penicillium* spp. (Table 5.1).

Polymerase Chain Reaction-Based Detection of Aflatoxigenic Fungal Species

Target gene and primers: *Nor1*—5′CGCTACGCCGGCACTCTCGGCA3′ (forward)and5′TGGCCGCCAGCTTCGACACTC3′ (reverse). Amplicon size 400 bp.

Reaction conditions:

1. Initial denaturation: 94°C/5 min.
2. Denaturation: 94°C/1 min.
3. Annealing temperature: 58°C/1 min.
4. Extension temperature: 72°C/1 min. Repeated for 30 cycles.
5. Final extension temperature: 72°C/5 min.
6. After successful amplification, load 5 μL of amplified PCR products to agarose gel (1%) containing 1% ethidium bromide and visualize under UV (Fig. 5.5).

Polymerase Chain Reaction-Based Detection of Ochratoxigenic Fungi

Target gene and primers: *pks*1—5′AGT CTTCGCTGGGTGCTTCC3′ (forward) and 5′ AGCACTTTTCCCTCCATCTATCC3′ (reverse). Amplicon size 630 bp.

Table 5.1 Primer sequences developed for metabolic pathway genes for the detection of toxigenic fungi

S. No.	Toxin	Target gene	Primer sequence 5′–3′	Tm (°C)	Amplicon size (bp)	Reference
1	Trichothecenes	*Tri5*	GAGAACTTTCCCACCGAATAT	58	450	[18]
			GATAAGGTTCAATGAGCAGAG			
		Tri6	GATCTAAACGACTATGAATCACC	58	541	
			GCCTATAGTGATCTCGCATGT			
			AGA GCC CTG CGA AAG(C/T) ACT GGT GC			
2	Fumonisins	*Fum5*	GTC GAG TTG TTG ACC ACT GCG	62	845	[22]
			CGT ATC GTC AGC ATG ATG TAG C			
		Fum13	AGTCGGGGTCAAGAGCTTGT	58	998	
			TGCTGAGCCGACATCATAATC			
3	Aflatoxins	*Aflr1*	CGC GCT CCC AGT CCC CTT CAT T	65	1,032	[20]
			CTT GTT CCC CGA GAT GAC CA			
		Ver1	GCC GCA GGC CGC GGA GAA AGT GGT	65	537	
			GGG GAT ATA CTC CCG CGA CAC AGCC			
		Nor1	ACCGCTACGCCGGCACTCTCGGCAC	65	400	
			GTTGGCCGCCAGCTTCGACACTCCG			
		Omt1	GTG GAC GAA CCT AGT CCG ACA TCAC	65	797	
			GTC GGC GCC ACG CAC TGG GTT GGGG			
4	Ochratoxin	*Pks1*	AGTCTTCGCTGGGTGCTTCC	56	550	[21]
			AGCACTTTTCCCTCCATCTATCC			

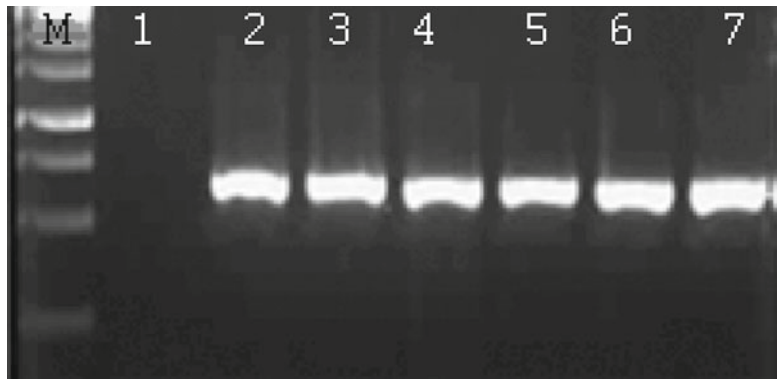

Fig. 5.5 Detection of aflatoxin-producing *Aspergillus* species targeting *aflR* gene (400 bp). Lane M,1-kb DNA ladder; lane 2, negative control; lanes 3–8 aflatoxigenic *Aspergillus* spp

Reaction conditions:

1. Initial denaturation: 94°C/5 min.
2. Denaturation: 94°C/1 min.
3. Annealing temperature: 56°C/1 min.
4. Extension temperature: 72°C/1 min. Repeated for 30 cycles.
5. Final extension temperature: 72°C/5 min.
6. After successful amplification, load 5 μL of amplified PCR products to agarose gel (1%) containing 1% ethidium bromide and visualize under UV (Fig. 5.6).

Polymerase Chain Reaction-Based Detection of Tricothecene-Producing *Fusarium* Species

Target gene and primers: *tri6*—5′GATCTAAACGACTATGAATCACC3′ (forward) and 5′GCCTATAGTGATCTCGCATGT3′ (reverse). Amplicon size 446 bp.

Reaction conditions:

1. Initial denaturation: 94°C/5 min.
2. Denaturation: 94°C/1 min.

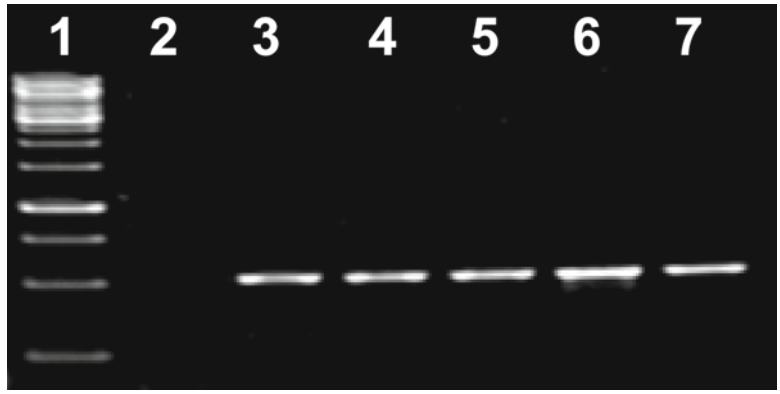

Fig. 5.6 Detection of ochratoxin-producing fungi targeting PKS gene (630 bp). Lane 1, 1-kb DNA ladder; lane 2, negative control; lanes 3–5, OTA-producing *Aspergillus*; lanes 6 and 7, OTA-positive strains of *Penicillin* spp

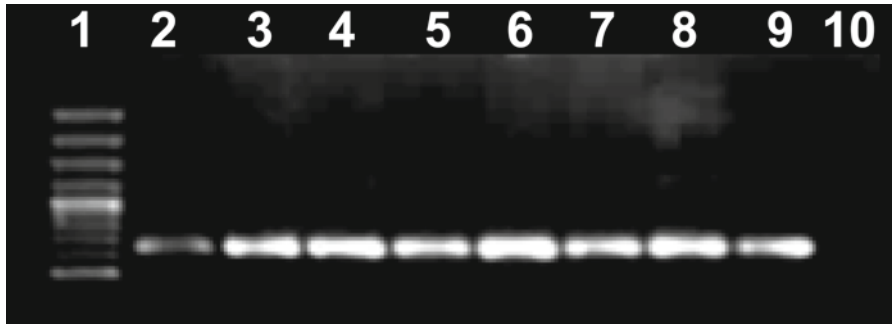

Fig. 5.7 Detection of trichothecene-producing *Fusarium* spp. by targeting *tri6* gene (440 bp). Lane1, 1-kb DNA ladder; lanes 2–9, positive strains of *Fusarium* spp.; lane 10, negative control

3. Annealing temperature: 56°C/1 min.
4. Extension temperature: 72°C/1 min. Repeated for 30–35 cycles.
5. Final extension temperature: 72°C/8 min.
6. After successful amplification, load the PCR amplicons into ethidium bromide- containing agarose gel and visualize bands under UV (Fig. 5.7).

Polymerase Chain Reaction-Based Detection of Fumonisins Producing *Fusarium* Species

Target gene and primers: *Fum13*—5′AGTCGGGGTCAAGAGCTTGT3′ (forward) and 5′TGCTGAGCCGACATCATAATC3′ (reverse). Amplicon size 998 bp.

Reaction conditions:

1. Initial denaturation: 94°C/5 min.
2. Denaturation: 94°C/1 min.
3. Annealing temperature: 58°C/1 min.
4. Extension temperature: 72°C/1.30 min. Repeated for 30 to 35 cycles.
5. Final extension temperature: 72°C/8 min.
6. After successful amplification, load 5 μL of amplified PCR products to agarose gel (1%) containing 1% ethidium bromide and visualize under UV (Fig. 5.8).

Chromatography Methods

Sample Extraction and Clean-up for Mycotoxins Analysis

During the chromatographic methods the determination step is usually preceded by a number of

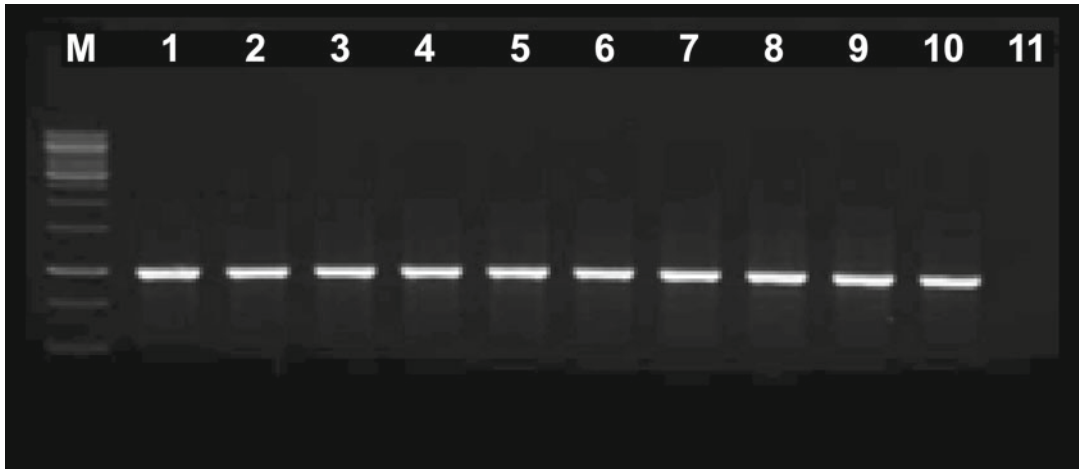

Fig. 5.8 PCR amplification *fum 13* gene (998 bp) of toxigenic *F. verticillioides* and *F. proliferatum.* Lane M, 1-kb DNA marker; lanes 1–5, *F. verticillioides* standard strains; lanes 5–10, *F. proliferatum* isolates; lane 11, non-toxigenic *F. verticillioides* isolate

operations such as sampling, sample preparation, extraction, and clean-up. The reliability of the results obtained by these procedures is highly dependent on the efficiency of these steps. A large number of components that are originally present in the sample must be reduced, and interfering compounds that show the same behavior in the chromatographic column must be removed as much as possible [7].

Conventional techniques such as column chromatography and liquid–liquid extraction usually require high amounts of solvent, are time-consuming, tedious to apply, and expertise is needed. Therefore, new approaches have been investigated to simplify the extraction and clean-up procedures. A number of clean-up columns have been developed that are used after the conventional extraction step. These procedures make use of different principles (immunoaffinity columns, solid phase extraction, ion exchangers, and others), but all have in common that they are commercially available and are easy to use. They have the additional advantages that less solvent is required and sample preparation can be speeded up considerably. The immunoaffinity columns (IACs) reveal high selectivity, as only the analyte is retained on the column and can then be eluted easily after a rinsing step in order to remove interfering components. Clean-up procedures are used for the removal of interfering compounds such as lipids, carbohydrates, and proteins [23].

IACs for clean-up purposes have become increasingly popular in recent years because they offer high selectivity. IACs are easy to use, and their application for purification of samples that are contaminated with several mycotoxins has already been well investigated. Because mycotoxins are low weight molecules, they are only immunogenic if they are bound to a protein carrier. If this problem is overcome, specific antibodies can be produced and bound to an agarose, sepharose, or dextran carrier. The mycotoxin molecules bind selectively to the antibodies after a preconditioning step, and subsequent to a washing step the toxin can be eluted with a solvent, causing antibody denaturation. Interfering substances do not interact and the column is therefore washed to remove the matrix [24].

Thin-Layer Chromatography

TLC was a very popular technique to separate and detect mycotoxins. TLC is the most commonly utilized test because more than one mycotoxin can be detected from each test sample. TLC

is based on the separation of compounds by their migration on a specific matrix with a specific solvent. The distance that a compound will travel is a unique identifier for specific compounds and a retention factor (R_f) has been determined for most mycotoxins. As with any detection system, a positive control containing purified mycotoxins must be run in parallel to ensure accuracy. For mycotoxin assays, silica gel TLC, with both precoated and self-coated plates, can be used. Detection and identification procedures have been specifically developed for each single mycotoxin, making use of molecular properties or reactions with spray reagents [25].

High-Performance Liquid Chromatography

HPLC or high-pressure liquid chromatography became available for the analysis of foodstuffs in the early seventies and gained importance in the determination of mycotoxins, particularly when several types of column packings and detectors became available. HPLC is the method of choice because it offers the advantages of good resolution, high degree of precision, reproducibility, and sensitivity. HPLC methods are mainly used for the final separation of matrix components and detection of the analyte of interest. Nowadays, HPLC methods are widespread, because of their superior performance and reliability compared with TLC. HPLC methods have been developed for almost all major mycotoxins in cereals and other agricultural commodities. Reversed phase (RP) chromatography is most commonly used for the determination of mycotoxins in agricultural samples—for example, a C_8 or C_{18} hydrocarbon phase with mixtures of polar solvents (e.g., water: methanol or water:acetonitrile combinations). Detection is mainly performed using diode array detection; alternatively, fluorescence detection (FLD), which utilizes the emission of light from molecules that have been excited to higher energy levels by absorption of electromagnetic radiation, is employed. FLD features superior sensitivity, although frequently derivatization of the analyte has to be performed in order to make the detection possible at all or enhance the sensitivity even further [26]. A short summary for the determination of the common toxins of *Aspergillus* and *Fusarium* spp. by chromatographic method is presented in the following section.

Detection of Aflatoxin by Thin-Layer Chromatography

A number of methods have been developed for the determination of aflatoxins by TLC. Silica plates are most commonly in use, with a number of solvent systems based on chloroform and small amounts of methanol or acetone. However, a shift can be observed to less toxic and more environmentally friendly mixtures (e.g., toluene/ethylacetate or acetone/isopropanol).

Extraction and Clean-up

Place 50 g of finely ground sample in a wide-mouth polypropylene screw-cap bottle with 100 mL of chloroform and water mixture (1:1) and place on a wrist-action shaker for 30 min. Allow the contents to settle, and a 10- to 25-mL aliquot of the solvent extract is filtered through four layers of Whatman filter paper. Dry the organic layer by rotary evaporator and reconstitute the compound in 3–4 mL of methanol. Load onto a preconditioned C18 clean-up column. Wash the column with 5 mL of phosphate buffered saline and then elute with chloroform–methanol (97:3) mixture. Eluants are allowed to dry for a few minutes and re-dissolved in suitable solvents for TLC analysis, as recommended by the supplier.

Thin-Layer Chromatography

Spot the sample (0.5–2 mL) by using a capillary tube on TLC plate. The spot should be as small and compact as possible, with a distance of 1–2 cm from the edges of the plate and between the spots.

1. Place TLC plate in chromatography chamber and run until solvent front is 2–3 cm from top of the plate (approximately 30–45 min).
2. Developing solvent: 80% benzene or toluene, 15% methanol, 5% acetic acid.

Table 5.2 R_f values and visible color of aflatoxin

	R_f value	Color			Color after spray treatment
			UV light		
Mycotoxin	Solvent system(B:M:A)	Visible light	Long wave	Short wave	Long-wave ultraviolet light
Aflatoxin B1	0.14	Yellow	Green	Faint green	Blue
Aflatoxin B2	0.20		Blue	Faint blue	Pink
Aflatoxin G1	0.23		Blue	Faint blue	Pink

Table 5.3 R_f value and visible color of Ochratoxin A

	R_f value	Color			Color after spray treatment
			UV light		
Mycotoxin	Solvent system (TEF)	Visible light	Long wave	Short wave	Long-wave ultraviolet light
Ochratoxin A	0.55	Yellow	Green	Green	Faint blue

3. Observe the TLC plate under UV scanner at 256 nm compared with standard toxin. Toxins were visualized in visible or ultraviolet light, before and after the plate was sprayed with freshly prepared mixture of 0.5 mL of *p*-anisaldehyde in 85 mL of methanol containing 10 mL of glacial acetic acid and 5 mL of concentrated sulfuric acid and then heated at 130°C for 10 min. The 5- to 10-min heating time was better for fluorescence development (Table 5.2).

Detection of Ochratoxin by Thin-Layer Chromatography

OTA detection by TLC can be performed by spotting samples and spikes onto a SG-60 plate and development with a mixture of toluene/methanol/acetic acid or toluene/ethyl acetate/formic acid. Under long-wavelength UV light OTA will appear blue–green at a retention value of 0.55.

1. Extraction and clean-up: As mentioned above.
2. Spot the sample (0.5–2 mL) by using a capillary tube on TLC plate. The spot should be as small and compact as possible with a distance of 1–2 cm from the edges of the plate and between the spots.
3. Developing solvent: 90% toluene, ethyl acetate, formic acid (5:4:1, v/v/v)
4. Observe the TLC plate under UV scanner at 256 nm compared with standard toxin.
5. Observation: Toxins were visualized in visible or ultraviolet light, before and after the plate was sprayed with a freshly prepared mixture of 0.5 mL of *p*-anisaldehyde/silver chloride in 85 mL of methanol containing 10 mL of glacial acetic acid and 5 mL of concentrated sulfuric acid and then heated at 130°C for 10 min. The 5- to 10-min heating time was better for fluorescence development (Table 5.3).

Detection of DON by Thin-Layer Chromatography

TLC is still common, and with the introduction of high-performance TLC (HPTLC) and scanning instruments, separation efficiency and precision have increased. Reagents (e.g., sulfuric acid or para-anisaldehyde) are necessary to visualize the only short wavelength absorbing DON. Other spray reagents include 4-para-nitrobenzyl-pyridine or nicotinamide in combination with 2-acetyl-pyridine) or $AlCl_3$, which is the most useful visualization reagent for DON. Typical detection limits by TLC are in the range of 20–300 ng/g.

1. Extraction and clean-up: 50 g of finely ground sample were placed in a wide-mouth polypropylene screw-cap bottle with 100 mL of a methanol–water mixture (1:1) and placed on a wrist-action shaker for 30 min.
2. The contents were allowed to settle, and a 10- to 25-mL aliquot of the solvent extract was

Table 5.4 R_f value and visible color of DON

	R_f value	Color			Color after spray treatment	
			UV light			
Mycotoxin	Solvent system (CMW)	Visible light	Long wave	Short wave	Visible light	Long-wave ultraviolet light
Deoxynivalenol	0.45	Yellow	Brown	Brown	Brown	Brick red

filtered through Whatman 4 filter paper and extracted with 25 mL of ethyl acetate.
3. Ethyl acetate was completely evaporated by rotary evaporation and the pellet resuspended in 3 to 4 mL of acetone–water (1:1).
4. Extracts were passed through preconditioned C18 column, and elutes were dried and reconstituted with 1 mL of a methanol–water mixture (1:1).
5. These extracts were used for TLC analysis.
6. Thin-layer chromatography: Spot the sample (0.5–2 mL) by using a capillary tube on TLC plate. The spot should be as small and compact as possible, with a distance of 1–2 cm from the edges of the plate and between the spots.
7. Place TLC plate in chromatography chamber and run until solvent front is 2–3 cm from top of plate (approximately 30–45 min).
8. Developing solvent: chloroform: methanol: water (9:1:0.2).
9. Observe the TLC plate under UV scanner at 256 nm compare with standard toxin. Toxins were visualized in ultraviolet, before and after the plate was sprayed with a freshly prepared mixture of 0.5 mL of *p*-anisaldehyde/silver chloride in 85 mL of methanol and then heated at 130°C for 20 min (Table 5.4).

Detection of Fumonisins by Thin-Layer Chromatography [27]

TLC is the simplest and most frequent screening method used for detection of fumonisins, but like all other methods, extraction and clean-up make a major contribution to accuracy and precision of obtained data. Derivatization is necessary before fluorescent detection can be performed, because fumonisins do not contain a chromophore to exhibit radiation. Reversed phase TLC (on C_{18} modified silica plates) has also been employed with acidic vanillin or fluorescamine/sodium borate buffer as a spray reagent.

1. Extraction and clean-up: 50 g of finely ground sample were placed in a wide-mouth polypropylene screw-cap bottle with 100 mL acetonitrile: water (1:1) and placed on a wrist-action shaker for 30 min.
2. The contents were allowed to settle, and a 10- to 25-mL aliquot of the solvent extract was decanted and filtered using Whatman 4 paper.
3. A C18 clean-up column was preconditioned with 5 mL of methanol followed by 5 mL of 1% aqueous potassium chloride (KCl). Two milliliters of the filtrate was combined with 5 mL 1% aqueous KCl and applied to the column.
4. The column was washed with 5 mL 1% aqueous KCl followed by 2 mL acetonitrile: 1% aqueous KCl (1:9), and the eluants were discarded.
5. The fumonisins were eluted with 4 mL acetonitrile: water (7:3), and the column eluant was evaporated to dryness under a stream of air on a heating module for TLC analysis.
6. Thin-layer chromatography: The sample residue was dissolved in 100 μL acetonitrile: water (1:1).
7. 10 μL was spotted on a C_{18} TLC plate along with 10-μL fumonisins standards (5, 10, and 100 ppm) dissolved in acetonitrile: water (1:1).
8. Observation: The TLC plate was developed in methanol: 1% aqueous KCl (3:2), air dried, and sprayed with 0.1 M sodium borate buffer (pH 8–9) followed by fluorescamine (0.4 mg/mL in acetonitrile). After 1 min, the plate was sprayed with 0.01 M boric acid: acetonitrile (40:60). The plate was then air dried at room temperature and examined under long-wave UV light. Fumonisin levels were estimated by visual comparison with standards (Table 5.5).

Table 5.5 *R*f value and visible color of fumonisins

Mycotoxin	R_f value Solvent system (M:Kcl)	Color after spray treatment Long-wave ultraviolet light
Fumonisins	0.5 (FB1) 0.1 (FB2)	Bright yellowish-green fluorescent bands

Detection of Aflatoxins by High-Performance Liquid Chromatography

1. Instrument: Liquid chromatography methods for the determination of aflatoxins in foods by reversed-phase HPLC (Hitachi F-4500). The emitted light is detected at 435 nm after excitation at 365 nm. Stationary phases for HPLC usually include C_{18} material, with mobile phases being mixtures of water, methanol, or acetonitrile. A fluorescence detector and a suitable data system are required to provide sensitive and specific detection and quantification of aflatoxins.
2. Solvents: All solvents shall be of HPLC grade, and all reagents should be analytical grade.
3. Extraction: A ground sample (20 g) is extracted with a methanol–water (7:3) mixture (80 mL). Corn and wheat samples will be kept in a vibrating shaker for 15–30 min. Extracts are filtered immediately after extraction through filter paper. After filtration the sample is evaporated to dryness at 40°C in a rotary evaporator.
4. Clean-up by IAC: The use of IACs is now well established in aflatoxin determination. MycoSep® (Romer Labs, Union, MO) columns, which remove matrix components efficiently and can produce a purified extract within a very short time, are also available. Conventional clean-up with silica columns has also been reported [16].
5. Standard preparation: Aflatoxin B1, B2, G1, and G2 standard can be purchased from private companies (Sigma-Aldrich, USA). Each of aflatoxins was diluted in methanol to 1 mg/mL solution of G2, 1 mg/mL of B2 10 mg/mL of G1, and 10 mg/mL of B1. 100 μL aliquot of each aflatoxin solution was then combined in a 2-mL glass vial and mixed well. This mixture was further diluted in series to 100,000 folds in water: methanol (7:3 v/v) and used as the standard solution.
6. Chromatography conditions: Column: Hypersil GOLD®, 3 μm, 100 × 2.1 mm; Flow Rate: 800 μL/min λex: 365 nm λem: 455 nm; Mobile Phase: Water: Methanol (7:3 v/v) (isocratic elution); Column Temperature: 40°C; Injection Volume: 10 μL of the prepared standard solution; Analytes: aflatoxin B1 and aflatoxin B2. The instruments will be controlled and the data analyzed using the suitable data system. No step changes of the excitation and emission wavelengths will be used during the run.
7. Observation: Aflatoxins fluoresce strongly on illumination with 365-nm ultraviolet light. Figure shows the fluorescence chromatogram of the two common aflatoxins with an excitation wavelength of 365 nm and an emission wavelength of 435 nm.

Detection of Ochratoxins by High-Performance Liquid Chromatography

1. Instruments: The liquid chromatograph equipped with quaternary pump, autoinjector with a stainless steel reverse phase 150 × 4.6 mm, 3-mm particle size C18 Supelco HPLC column (Supelco, USA). A fluorescence detector and a suitable data system are required to provide sensitive and specific detection and quantification of ochratoxins derivatized with OPA/mercaptoethanol.
2. Solvents: All solvents shall be of HPLC grade, and all reagents should be analytical grade.
3. Extraction: Sample extraction is generally performed with a mixture of water and organic solvents depending on the type of matrix. An IUPAC/AOAC method for the determination of OTA in barley uses a mixture of $CHCl_3$ and H_3PO_4 [28]; for green coffee, $CHCl_3$ is only employed [29]. For determination in wheat, a number of extraction solvents are used, including mixtures of toluene/HCl/$MgCl_2$, CHCl3/ethanol/acetic acid, and dichloromethane/H_3PO_4.

4. Clean-up by IAC: The use of IACs is now well established in ochratoxin determination. The extract is forced through the column, and ochratoxins are bound to the antibody. Five milliliters of the final extract, corresponding to 5% (v/v) of the original material, was placed into the IAC. The sample was allowed to pass though the column at a flow rate of 2–3 mL/min. Slowly elute the bound ochratoxin from the column using 1.5 mL of desorption solution; allow this to pass through the column by gravity and collect in a sample vial.
5. Standard preparation: Ochratoxin will be purchased from private companies (Sigma-Aldrich, USA). Ochratoxin was diluted 50 μg/mL in benzene: acetic acid (99:1). 50 μL aliquot of each solution was then combined in a 2-mL glass vial and mixed well. This mixture was further diluted in series to 100,000 folds in acetonitrile: water (7:3 v/v) used as the standard solution.
6. Chromatography conditions: Reversed phase HPLC approach with a C_{18} column [21]. Flow Rate: 800 μL/min λex: 365 nm λem: 455 nm; and an acidic buffer (acetic acid) in an acetonitrile/water mixture as a mobile phase. Column Temperature: 40°C; Injection Volume: 10 μL of the prepared standard solution; Analytes: Ochratoxin A.
7. The instruments will be controlled and the data analyzed using the suitable data system. No step changes of the excitation and emission wavelengths will be used during the run.
8. Observation: Quantify the ochratoxin A concentration by comparing the sample peak area to that of a standard.

Detection of Deoxynivalenol by High-Performance Liquid Chromatography

1. Instruments: The liquid chromatograph equipped with quaternary pump, autoinjector, and UV detector was used with a stainless steel reverse phase 150 × 4.6 mm, 3 mm particle size C18 Supelco HPLC column.
2. Solvents: All solvents shall be of HPLC grade, and all reagents should be analytical grade.
3. Extraction: Place 10 g of the ground sample into the ultraturax and then add 40 mL of distilled water and 2 g of polyethylene glycol. The mixture is stirred for 1 min. The extract is filtered through a fluted filter and then through a microfiber filter.
4. Clean-up by immunoaffinity chromatography: Place 1 mL of the final extract into the IAC. Use 10 mL of redistilled water for column washing. The elution of DON is conducted with 1 mL of methanol. The elution solvent is removed by a gentle stream of nitrogen and re-dissolved in 300 μL mobile phase.
5. Standard preparation: DON purchased from private companies (Sigma-Aldrich) is diluted to 200 μg/mL in ethyl acetate: methanol (95:5). 50 μL. Aliquot of the solution is then combined in a 2 mL glass vial and mixed well. Serially dilute this mixture to 1,000 folds in methanol: water (7:3 v/v) used as the standard for HPLC.
6. Chromatography condition: Samples of 50 μL are injected into the HPLC column and heated to 30°C. The used mobile phase consisted of a methanol: water solution (8:2 v/v). The flow rate is of 0.6 mL/min. Deoxynivalenol is determined at a wavelength of 218 nm by using UV detector.
7. Observation: Quantify the deoxynivalenol concentration by comparing the sample peak area to that of a standard.

Detection of Fumonisins by High-Performance Liquid Chromatography

1. Instrument: HPLC system consisting of an isocratic pump capable of a flow rate of 1 mL/min and a suitable injector capable of 10 μL injections. Columns containing C_{18}- or C_8-modified silica packing material of 3- to 5-mm particle size. A fluorescence detector and a suitable data system are required to provide sensitive and specific detection and quantification of fumonisins derivatized with OPA/mercaptoethanol.
2. Solvents: All solvents will be used of HPLC grade and all regents should be analytical grade.

3. Extraction: Place finely ground sample (25 g) into a container suitable for centrifuging (250-mL polypropylene centrifuge bottle). Add 100 mL extraction solvent (methanol–water, 3:1) and homogenize the contents for 3 min. Centrifuge the container at 10,000 rpm for 10 min at 4°C. Filter the supernatant through a Whatman 4 filter paper.
4. Solid phase extraction (SPE) cartridges: Sample extracts are generally cleaned up on SPE columns containing strong anion exchange material. For optimal simultaneous handling of cartridges, the use of a commercial SPE manifold is recommended.
5. Standard preparation: Fumonisin standards are prepared in acetonitrile: water (1:1) and stored at 4°C. Stock solutions of individual fumonisins standards of concentration 250 μg/mL are used, from which a working standard is prepared containing 50 μg/mL of each analog. Derivatize standards by mixing 25 μL working standard with 225 μL OPA reagent at the base of a small test tube. Inject 10 μL into the HPLC using a standardized time of 1–2 min between the addition of OPA reagent and injection.
6. Chromatography conditions: The HPLC mobile phase is a mixture of methanol and 0.1 M sodium dihydrogen phosphate in water. For most reversed-phase columns, a solvent composition of 75% to 80% methanol will be required. The pH of the mixture is adjusted to 3.35 with *o*-phosphoric acid and filtered through a 0.45-mm membrane filter.
7. OPA reagent: OPA reagent for derivatizing the fumonisins is prepared by dissolving 40 mg OPA in 1 mL of methanol and diluting with 5 mL of 0.1 *M* disodium tetraborate.
8. Observation: Quantify the fumonisins' concentration by comparing the sample peak area to that of a standard.

Summary

Many agricultural commodities are vulnerable to attack by fungi that produce mycotoxins. Detection of mycotoxins and toxin-producing fungi from food and feeds are very essential. In the present chapter we discussed available techniques for detection and quantification of major mycotoxigenic fungi and their toxins from agricultural produce. The standard methods varied from lab to lab and toxin to toxin and also from commodity to commodity. International agencies such as International Union of Pure and Applied Chemistry (IUPAC), Association of Official Analytical Chemists (AOAC), and The European Mycotoxin Awareness Network have developed their own methodologies for detection of mycotoxins from different food matrices. In conclusion, a broad range of techniques for practical analysis and detection of a wide spectrum of mycotoxins are available. This chapter presented some recent developments in scientific and technological basis analytical methods that offer flexible and broad-based methods for analysis of toxins and toxigenic fungi.

Notes

DNA Extraction and Polymerase Chain Reaction Conditions

1. Make use of suitable microbiological aseptic technique when working with DNA. Wear gloves to prevent nuclease contamination from the surface of the skin. Use sterile, disposable plasticware and automatic, aerosol-resistant pipettes reserved for DNA work.
2. Wipe pipettes with Dnase-removal solutions when transitioning between handling crude extracts to handling more purified material.
3. Equilibrated phenol can typically be purchased from commercial sources. Alternatively, you can equilibrate it yourself. There are also commercial sources of phenol and chloroform mixed together and equilibrated. The pH is important because chromosomal DNA will end up in the phenol phase if the pH is acid (around pH 5).
4. Phenol and chloroform should be used in a hood. Phenol is a dangerous substance that will burn you if it gets on your skin. Always wear gloves and be careful. A solution of

PEG 400 is recommended for first aid. Phenol is both a systemic and local toxic agent.

5. DNA should be kept frozen in a non-frost-free freezer. DNA should not be allowed to defrost between uses, as this will break long molecules.
6. Make a PCR master mix for 50 μL reaction containing DNA and PCR ingredients. After setting up the reaction, specific reaction conditions can be used for proper amplification of target genes. After successful amplification, the PCR amplicons can be stained with ethidium bromide-containing agarose gel and bands can be visualized under UV.

Mycotoxin Analysis

7. A laboratory or part of a laboratory should be reserved for mycotoxin analysis only and the work confined to that area. The bench top should be of a nonabsorbent material, such as formica, for example (Whatman Benchkote can also be used, but it must be removed and destroyed after use), and should be screened from direct sunlight.
8. Analyses should be performed in a well-ventilated laboratory, preferably under an efficient extraction hood, and fume cupboard facilities should be available.
9. Many of the solvents used are highly flammable and have low flash points. Bunsen burners, electric fires, and sparking apparatus such as centrifuges should not be used in the same laboratory. The amount of flammable solvents in the laboratory should be kept to a minimum and stored in a fire-resistant cupboard or bin.
10. Swab accidental spills of toxin with 1% NaOCl bleach, leave 10 min, and then add 5% aqueous acetone. Rinse all glassware exposed to aflatoxins with methanol, add 1% NaOCl solution, and after 2 h add acetone to 5% of the total volume. Allow a 30-min reaction and wash thoroughly.
11. Weighing and transferring mycotoxins in dry form should be avoided; they should be dissolved in a solvent. The electrostatic nature of a number of the mycotoxins in dry form results in a tendency for them to be easily dispersed in the working area and to be attracted to exposed skin and clothes.
12. Containers of mycotoxin standard solutions should be tightly capped, and their weights may be recorded for future reference before wrapping them in foil and storing them in a freezer.
13. During the grinding and weighing of samples, there is a risk of absorbing toxin either through the skin or by the inhalation of dust. There is also the risk of developing allergic reactions due to spores and organic material. These risks should be minimized by working under an extraction hood, by good hygiene, and by wearing protective clothing and masks.
14. Glassware and TLC plates should be decontaminated by soaking for 2 h in a 1% sodium hypochlorite solution. After this time an amount of acetone equal to 5% of the total volume of the bleach bath should be added, and the glassware soaked for an additional 30 min. Spraying of TLC plates must be carried out in an efficient fume cupboard or spray cabinet. Always ensure that this equipment is working before commencing use. When viewing chromatograms under UV light the eyes should be protected by UV filter or by wearing protective spectacles.

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