Chapter 1

INTRODUCTION

Mycotoxins, which literally mean “fungus poison”, are secondary metabolites produced mainly by three genera of fungi namely Aspergillus, Penicillium and Fusarium. They consist of a large and diverse group of fungal toxins causing toxic effect in humans and animals. The outbreak of ‘Turkey X’ disease in 1960, which caused the death of 100,000 turkeys due to consumption of aflatoxin contaminated peanut, brought the problem of mycotoxins into the spotlight. Since then a number of mycotoxins have been identified and the period between 1960 and 1975 has been known as the mycotoxin gold rush (Bennett and Klich 2003). According to De Koe (1993), about 100,000 moulds have been identified; from which over 400 can be considered potentially toxic and about 5% of them are known to produce toxic compounds or classes of compounds causing problems in various parts of the world. An estimate of potentially 20,000 to 300,000 mycotoxins has been estimated to exist in the environment (CAST 2003).

The important mycotoxins of significant health hazards are aflatoxins, ochratoxin, trichothecenes, zearalenone, fumonisins, patulin, citrinin and ergot alkaloids (Hussein and Brasel 2001). Ergotism or St. Anthony’s fire refers to the human disease associated with gangrene which affects the blood supply and convulsive ergotism affects the central nervous system (Bennett and Bently 1999). Deoxynevalenol, also known as vomitoxin causes nausea, vomiting, diarrhea and suppress the immune system in animals (Pestka and Bondy 1994). Citrinin is a hepatotoxin and nephrotoxin and is known to co-occur with ochratoxin (Bilgrami et al. 1988; Vrabcheva et al. 2000). Ochratoxin primarily affects the kidney of all animal species and at high concentration can affect the liver. It is also an immune suppressor, teratogen and a carcinogen (Kuiper-Goodman and Scott 1989). The role of ochratoxin A in the human kidney disease, Balkan Endemic Nephropathy has been implicated (Hult et al. 1982; Pfohl-Leszkowicz et al. 2002). Zearalenone, also known as a mycoestrogen or phytoestrogen, mainly affects swine causing hyperestrogenism (Kurtz and Mirocha 1978). Fumonisin B₁ and B₂ are known to cause leukoencephalomalacia in horse (Marasas et al. 1988); pulmonary edema and hydrothorax in swine (Colvin and Harrison 1992). The high incidence of esophageal cancer in South Africa, China and Italy has been correlated with fumonisin B₁ (Peraica et al. 1999). Aflatoxins are
acutely and chronically toxic to humans and animals. They cause liver and kidney damage, and induce mutagenic, carcinogenic and immunosuppressive effects. Among the mycotoxins, AFB₁ is considered the most toxic and is classified as a group I carcinogen by the International Agency for Research on Cancer (IARC 2002). Most mycotoxins are fluorescent compounds and this property is often used for their detection. The commonly used detection methods include chromatographic techniques such as thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Other techniques include enzyme-linked immunosorbent assay, fluorometry and mass spectrometry. Detection by TLC is one of the oldest methods and it enables screening of more than one mycotoxin in a sample. Mass spectrometry is often used for detecting non-fluorescent mycotoxins especially trichothecenes (Croteau et al. 1994).

Mycotoxins occur as natural and unavoidable contaminants on a variety of food commodities. The Food and Agricultural Organisation has reported that 25% of the world’s food crops are contaminated with mycotoxins. The ubiquitous nature of fungi makes food crops vulnerable to fungal contamination during pre-harvest and post-harvest conditions. From field to plate, food undergo several stages of processing such as harvest, storage, transportation etc., and at every stage there is a risk of contamination by toxigenic fungi and their mycotoxins (Hill et al. 1983; Ellis et al. 1991). Mycotoxin contamination in cereals such as rice, wheat, maize, sorghum and barley has been reported from various countries (Giray et al. 2007; Janardhana et al. 1999; Mateo et al. 2011; Nguyen et al. 2007; Ratnavathi et al. 2012). Fruits, nuts, spices and medicinal plants are also prone to fungal and mycotoxin contamination (Bokhari 2007; Goncalez et al. 2008; Sanchez-Hervas et al. 2008; Santos et al. 2009; Senyuva et al. 2008). The extent of contamination by fungi depends on various factors like geographic location, processing and storing periods of the crops. Environmental factors like temperature, water activity or pH, damage of crop by insects, crop densities etc. influences the growth of fungi and mycotoxin production. All these factors leads to increased susceptibility of foods to fungal and mycotoxin contamination.

Consumption of food contaminated with mycotoxins leads to a number of adverse health effects. They are toxic to animals as well as humans and affect the immune system, nervous system, kidneys, liver etc. Fungal and mycotoxin contamination in food and feed are unavoidable, and prevention is not always possible. Hence the best way to deal with mycotoxin
contamination is to remove or detoxify the toxins. Some common methods include physical removal of the contaminated grains, density segregation, thermal inactivation (Levi 1980; Kitabatake et al. 1991) and UV irradiation (Liu et al. 2011). Microorganisms like lactic acid bacteria (Maing et al. 1973), Flavobacterium aurantiacum (Ciegler et al. 1966), Bacillus subtilis (Ono and Kimura 1991), Streptomyces (Sakuda et al. 1996) and fungi such as Trichoderma viride and Aspergillus niger (Aziz and Shahin 1997) have been studied for the removal of mycotoxin. Chemicals such as sodium hydroxide (Trivedi et al. 1992), chlorine (Samarajeewa et al. 1991), ammonia (Park et al. 1988), sodium bisulfite, citric acid (Mendez-Albores et al. 2005), etc. are being used for degrading mycotoxin. The level of degradation often depends on treatment temperature and exposure time. Though several detoxification methods have been developed, only few have been accepted for practical use.

The occurrence of mycotoxins in foods and medicinal plants is a persistent problem worldwide. In this study, medicinal herbs and spices were screened for the presence of fungal and mycotoxin contamination. To abate the problem of mycotoxins, spices were used to control the growth of Aspergillus flavus and Penicillium citrinum and toxin production in the present study. Degradation of AFB$_1$ was carried out using natural products and organic acids.
OBJECTIVES OF THE STUDY

1. Survey of medicinal herbs and spices for fungal and mycotoxin contamination
2. Study of fungal load
3. Extraction and identification of mycotoxins
4. Control of mycotoxin production using spices
5. Development of detoxification method
6. Biological assay
REVIEW OF LITERATURE

MYCOTOXINS

The word mycotoxin is derived from the Greek word “mykes” meaning “fungus” and Latin “toxicum” meaning “poison”. They are low molecular weight molecules produced as secondary metabolites by saprophytic fungi especially Aspergillus, Penicillium and Fusarium. Mycotoxins were known to man since the ancient times as ‘St. Anthony’s Fire’ caused by ergot alkaloids in the 1800s AD and ‘Alimentary Toxic Aleukia’ caused by T2 toxins during World War II in Russia (Richard 2003). It was not until 1960s, with the outbreak of ‘Turkey X’ disease in England, that mycotoxins were identified as important toxins. Turkey X disease refers to the death of 100,000 Turkey poults due to consumption of peanut meal contaminated with fungi (Blount 1961). The responsible fungus was identified as *Aspergillus flavus* and the toxin as aflatoxin.

As the name implies, mycotoxins are toxic to a wide range of animal species and humans. The important mycotoxins of significant health hazards are listed in Table 1.1 and their structures in Figure 1.1. The disease caused by the exposure to mycotoxins is known as mycotoxicosis. The symptoms of mycotoxicosis can be acute or chronic and depends on the type and concentration of toxin, age, health and sex of an individual (Bennett and Klich 2003). Mycotoxins may cause birth defects, exhibit hormonal activity, and affect the reproductive system, immune system and specific target organs. They may also affect the gastrointestinal system, cause skin irritation and reduce growth (Kuiper-Goodman 2004). There have been several outbreaks of mycotoxicosis in human population. In 1974, an outbreak of hepatitis in India resulted in the death of 100 people due to consumption of contaminated maize (Krishnamachari *et al.* 1975). In another case in India, an outbreak of gastrointestinal disorder associated with consumption of bread made of contaminated wheat was reported in 1987. The contaminating moulds consisted of Fusarium sp. and Aspergillus sp., and the toxins were identified as deoxynivalenol, nivalenol, acetyl-deoxynivalenol and T2 toxin (Bhat *et al.* 1989). The Kenyan outbreak in 2004 was one of the largest outbreaks, where 125 people died due to liver failure caused by acute aflatoxicosis after consuming contaminated maize (Muture and Oqana 2005).
<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Producer fungi</th>
<th>Commodities</th>
<th>Toxic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxins (B₁, B₂, G₁, G₂, M₁, M₂)</td>
<td>A. flavus, A. parasiticus, A. nominus, A. tamari</td>
<td>Peanuts, corn, wheat, rice, milk, cheese, figs, herbs</td>
<td>Mutagenic, carcinogenic, hepatotoxic, immunosuppressive</td>
</tr>
<tr>
<td>Citrinin</td>
<td>P. citrinum, P. viridicatum</td>
<td>Wheat, barley, corn, rice</td>
<td>Nephrotoxic, carcinogenic.</td>
</tr>
<tr>
<td>Deoxynivalenol (Trichothecenes)</td>
<td>F. graminearum, F. culmorum</td>
<td>Corn, wheat, barley, oats</td>
<td>Vomiting, immunosuppressive</td>
</tr>
<tr>
<td>Fumonisin</td>
<td>F. verticillioides, F. proliferatum</td>
<td>Corn, wheat</td>
<td>Tumors of the kidney and liver</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>A. ochraceus, A. flavus, P. viridicatum</td>
<td>Cereals, beans, peanuts, cheese, coffee, dried fruits, grapes, wine</td>
<td>Nephrotoxic, hepatotoxic, carcinogenic,</td>
</tr>
<tr>
<td>Patulin</td>
<td>P. patulum, P. expansum</td>
<td>Apples, apple juice</td>
<td>Subcutaneous sarcomas, hemorrhage, carcinogenic</td>
</tr>
<tr>
<td>T-2 toxin (Trichothecenes)</td>
<td>F. sporotrichoides, F. poae, F. roseum</td>
<td>Corn, wheat, barley, oats</td>
<td>Emetic, cytotoxic, teratogenic</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>F. graminearum, F. tricinctum, F. Culmorum</td>
<td>Corn, hay</td>
<td>Hyper-estrogenic, abortion</td>
</tr>
</tbody>
</table>
Figure 1.1 Structure of the major mycotoxins
AFLATOXINS

Aflatoxins are a group of structurally related metabolites produced by Aspergillus flavus, A. parasiticus, A. nomius and A. tamarii (Nagarajan and Bhat 1973; Kurtzman et al. 1987; Goto et al. 1996). They are found in various food materials such as groundnut, tree nuts, dried fruits, spices, herbs and a number of food grains (Romagnoli et al. 2007; Trucksess and Scott 2008). In order of their toxicity, the four naturally occurring aflatoxins (AFs) are AFB$_1$ > AFG$_1$ > AFB$_2$ > AFG$_2$. The letters B and G denotes their fluorescence colour under ultra violet (UV) light (B= blue and G= green). A. flavus produces only AFB$_1$ and B$_2$, whereas A. parasiticus produces AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$. AFM$_1$ and M$_2$ are hydroxylated metabolites of AFB$_1$ and B$_2$ respectively, found mainly in milk and dairy products (Sugiyama et al. 2008). Though AFM$_1$ and M$_2$ are products of detoxification, they too possess some level of toxicity which is lesser than AFB$_1$ and B$_2$. AFB$_1$ is the most toxic among them and is genotoxic, hepatotoxic, mutagenic, carcinogenic and immunosuppressive in animals and humans (Hussein and Brasel 2001). AFB$_1$ has been classified as group I human carcinogen by the IARC (IARC 2002).

Aflatoxins are difuranocoumarin derivatives, synthesized via the polyketide pathway involving one acetyl CoA and 9 malonyl CoA (Figure 1.2) (Bennett and Christensen 1983). The basic structure of AFB$_1$ contains a coumarin nucleus fixed to a bifuran and a pentanone structure attached to the common nucleus. It has been reported that the double bond in the terminal furan ring and the lactone ring in the coumarin moiety are the main site responsible for the toxicity of AFB$_1$ (Wogan et al. 1971; Nicolas-Vazquez et al. 2010). There are at least 28 metabolites and derivatives of aflatoxins reported. The liver microsomal enzymes (m) transform AFB$_1$ into AFM$_1$, AFP$_1$ and AFQ$_1$, whereas a cytoplasmic NADP-dependent dehydrogenase (c) is involved in the transformation into aflatoxicol (AFL) and AFLH$_1$ via AFQ$_1$ as given in Figure 1.3 (WHO 1979). AFB$_1$ is activated by cytochrome P450 to form AFB$_1$-8,9-epoxide, which is responsible for the mutagenic activity of AFB$_1$ (McLean and Dutton 1995). AFB$_1$-8,9-epoxide specifically bind to the N$^7$ position of guanine of DNA and RNA to form AFB$_1$-N$^7$-guanine adduct (Croy et al. 1978). AFB$_1$ inhibits DNA, RNA and protein synthesis resulting in immuno-suppressive, hormonal and teratogenic effects (McLean and Dutton 1995). The mutagenic and carcinogenic potential of AFB$_1$ and its metabolites has been demonstrated using Ame’s assay by Wong and Hsieh (1976). All the metabolites were reported to be less mutagenic than AFB$_1$. Among the
metabolites, AFL possessed the highest activity followed by AFM₁, AFH₁, AFQ₁, AFB₂, AFP₁; whereas AFB₂ₐ was found to be non-mutagenic. AFB₁ primarily affects the liver and development of Hepatocellular carcinoma due to chronic exposure to AFB₁ has been strongly implicated (Wild and Gong 2010).

Figure 1.2 Biosynthetic pathway of aflatoxin B₁ in *Aspergillus flavus* (Shier *et al.* 2005)
Figure 1.3 Aflatoxin B₁ metabolisms in liver (WHO 1979)
CITRININ

Citrinin (C$_{13}$H$_{14}$O$_{5}$) is a yellow coloured compound with a molecular weight of 250.25 g/mol. It was first isolated from *Penicillium citrinum* and later isolated from several species of *Penicillium* (*P. camemberti, P. expansum*) and *Aspergillus* (*A. terreus, A. niveus, A. oryzae*) (Hetherington and Raistrick 1931; Kurata 1990). Few citrinin producing fungi also produces ochratoxin A and patulin, and co-occurrence of citrinin with these toxins has been reported (Martins *et al.* 2002; Vrabcheva *et al.* 2000). Citrinin has also been isolated from *Monascus ruber* and *M. purpureus* which are used to produce red pigment (Blanc *et al.* 1995). The biosynthetic pathway of citrinin in *M. ruber* is illustrated in Figure 1.4 as reported by Hajjaj *et al.* (1999). They suggested that a tetraketide is the precursor for citrinin synthesis which aroused from condensing of one acetyl-CoA molecule with 3 malonyl-CoA molecules. Citrinin decomposes at 175 °C by dry heating and in moist condition decomposes at 140 °C. However heating at 140 °C resulted in the formation of a degraded product, citrinin H1, which was more toxic than citrinin (Trivedi *et al.* 1993). Citrinin is known to have antibiotic activity against gram positive bacteria, but due to its toxicity its use was discouraged.

Citrinin is found as natural contaminants in food and feed grains, fermented and processed food (Li *et al.* 2012; Zaied *et al.* 2012). Red yeast rice, which is used as dietary supplement, food preservative and colourant in many Asian foods, is reported to be often contaminated with citrinin (Gordon *et al.* 2010, Li *et al.* 2012). Consumption of such contaminated food is hazardous to both humans and animals. Citrinin has been associated with yellow rice disease in Japan (Saito *et al.* 1971). Citrinin is hepatotoxic and nephrotoxic to a number of animal species (Bilgrami *et al.* 1988). It primarily affects the kidney and the possible role of citrinin and ochratoxin A in the Balkan endemic nephropathy was implicated (Vrabcheva *et al.* 2000). Although there are no prescribed regulations for citrinin contamination, the European Food Safety Authority has determined a level of no concern for nephrotoxicity of 0.2 µg/kg body weight per day for citrinin (EFSA 2012).
Figure 1.4 Biosynthesis pathway of citrinin in *Monascus ruber* (Hajjaj et al. 1999)
OCCURRENCE OF MYCOTOXINS

Mycotoxins are found as natural contaminant of foods. Foods in the field or under stored conditions are susceptible to fungal contamination. In the field, the contaminating fungi are airborne or transmitted by insects, and damaged kernels often become infected. Stress conditions like drought, floods, insect infestation and delayed harvest increases the level of contamination. Post harvest conditions such as inadequate drying, warm humid environment during storage leads to mould formation (Hussein and Brasel 2001; Magan and Olsen 2004). Contamination of feeds and food grains during harvest, storage and processing with toxigenic fungi and their mycotoxins is a very common problem world over. Food and Drug Administration (FDA) has reported AFB$_1$ as an unavoidable contaminant of foods and that not only affects human health but causes great economic loss by decreasing the yield and quality of crops.

MYCOTOXINS IN FOOD AND FEED

A survey of mycotoxin contamination in animal feeds in European and Mediterranean markets and Asia-Pacific regions was carried out by Binder et al. (2007). The results showed that the mycotoxins deoxyxovalenol, zearalenone and T2 toxin were the major contaminants in the European samples. Aflatoxins, fumonisins, deoxyxovalenol and zearalenone were mainly found in the samples from Asia and Pacific regions. Herzallah (2009) has reported the presence of Aflatoxin M$_1$ and M$_2$ in milk samples and AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$ in meat samples collected from the local markets in Jordan. Rice is the staple food of India, consumed almost daily in every household, and if contaminated with aflatoxins will have high impact on human health. A survey by Reddy et al. (2009a) revealed the presence of several species of Aspergillus and AFB$_1$ in rice samples consisting of paddy and milled rice. Occurrence of Aspergillus and aflatoxins in rice grains were also reported from other countries like China, Nigeria and United Arab Emirates (Hussaini et al. 2007; Osman et al. 1999; Zouxin et al. 2006). Maize samples in Vietnam intended for human and animal consumption were reported to be contaminated with high fungal load and AFB$_1$ and fumonisin B$_1$ (Trung et al. 2008). Alborch et al. (2012) isolated several species of Aspergillus, Fusarium, Penicillium and Mucorales from maize flour and popcorn kernels in Spain, and reported the natural contamination of AFB$_1$ and ochratoxin A in the samples. Most mycotoxins are chemically stable they are not easily destroyed during
domestic cooking or food processing (Kitabatake et al. 1991; Trivedi et al. 1992; Bullerman and Bianchini 2007), hence contamination still occurs. Mycotoxins such as aflatoxins, ochratoxin and fumonisins have been detected in processed foods which were sold in the market (Sugita-Konishi et al. 2006; Romagnoli et al. 2007; Mushtaq et al. 2012). Aflatoxins and fumonisins were detected in maize-based products intended for human consumption in Glasgow, UK (Candlish et al. 2000).

MEDICINAL HERBS

Plants have been used for their medicinal properties since ancient times. Herbal medicines are used worldwide to alleviate symptoms, treat common ailments or to promote an overall wellness. The World Health Organisation (WHO) has estimated that 80% of the population of some Asian and African countries and many developed countries use herbal medicines as primary health care or as alternate medicine (WHO 2008a). *Phyllanthus emblica* (Amla) is rich in vitamin C and is used for treating cough, cold and diabetes. *Withania somnifera* (Ashwagandha) is known to relieve stress, nerve disorders, used as restorative tonic and aphrodisiacs. *Asparagus racemosus* (Shatawer) is used to enhance lactation and treating general weakness and fatigue. *Terminalia chebula* is used for treating ulcers, leprosy, anemia, etc. *Acorus calamus* (Sweet flag) is well known for its aroma and is often used as sedative, analgesic, laxative and diuretic agent. The therapeutic properties of these plants can be attributed to the secondary metabolites such as alkaloids, polyphenols, glycosides and terpenes.

MYCOTOXINS IN MEDICINAL HERBS

Herbs and the raw materials for herbal drugs are generally dried naturally in sun or shade drying and stored without any processing or decontamination, thereby increasing the chances of fungal growth and mycotoxin production. The presence of fungi and their mycotoxins in medicinal herbs have been reported from around the world. Studies in India showed the natural occurrence of AFB1 and citrinin in medicinal plants and herbal drugs such as *Piper nigrum*, *Mucuna prurita* (Roy et al. 1988; Roy and Kumari 1991). AFB1, citrinin, ochratoxin A and zearalenone were detected in several medicinal plants such as *Asparagus racemosus*, *Carum ajmoda*, *Cinnamomum zeylanicum*, *Cuminum cyminum*, *Elettaria cardamomum*, *Emblica officinalis*, *Piperlogum*, *P. nigrum*, *Saraca indica* and *Zingiber officinale* (Chourasia 1995).
Thirumala-Devi et al. (2001) reported the contamination of Coriandrum sativum, Piper nigrum, Zingiber officinale, Curcuma longa with ochratoxin A in India. The incidence of toxigenic fungi producing aflatoxins, ochratoxin A and fumonisins on medicinal herbs was reported from Argentina (Rizzo et al. 2004). The medicinal herb ginseng was reported to be contaminated with AFB₁, ochratoxin A and zearalenone (Gray et al. 2004; Trucksess et al. 2006). An investigation from South Africa showed the presence of fumonisin B₁ in dietary (Rumex lanceolatus, Zantedeschia aethiopica, Raphanus raphanistrum, Solanum nigrum) and medicinal (Catha edulis, Dalbergia obovata, Brunsvigia sp., Datura stramonium) wild plants (Sewram et al. 2006). Aflatoxins, ochratoxin A and citrinin producing Aspergillus and Penicillium were isolated from medicinal herbs in Brazil (Bugno et al. 2006). The presence of AFB₁ in Pimpinella anisum, Piper nigrum, Mentha piperita and Origanum majorana was reported by Bokhari (2007) in Saudi Arabia. In Korea, a survey was conducted on spices and processed spice products for aflatoxin contamination, where AFB₁ was detected in 13.6% of the spices (Cho et al. 2008). Multicontamination of mycotoxins with T-2 toxin, zearalenone, aflatoxins, ochratoxin A, deoxynivalenol, citrinin and fumonisin were detected in 84 medicinal herbs surveyed in Spain (Santos et al. 2009). There is always a risk of fungal and mycotoxin contamination during processing and packaging of herbal drugs. The lack of effective surveillance of the efficacy and quality of herbs subjects humans to potent health threats. The consumption of medicinal herbs contaminated with mycotoxins may cause ill effects rather than improving the well-being of an individual.

DETECTION OF MYCOTOXINS

Mycotoxins occur naturally and frequently in food and feeds as mentioned above. The toxic nature of mycotoxins makes their detection an absolute necessity. Several detection methods have been developed, among them chromatographic techniques are widely used. The procedure for detecting mycotoxins involves extraction from sample material, purification, and qualitative and quantitative analysis. The most common methods currently used are described here.

1. THIN LAYER CHROMATOGRAPHY (TLC)
TLC is one of the traditional methods of detecting mycotoxins. This technique enables screening large number of samples, easy identification and is cost effective. Silica gel layer is most commonly used, however phenyl non-polar bonded, silanized and polyamide are also been used (Lin et al. 1998). Mycotoxins are visualized on TLC plate by observing under UV light or by spraying chemicals which react with mycotoxins and enhances the fluorescence or produces colour products (Betina 1985). Aflatoxins, citrinin and ochratoxin are naturally fluorescent compounds hence they are identified based on their fluorescent properties. For example, the B and G aflatoxins are differentiated by blue and green fluorescence respectively, while citrinin is identified by yellow fluorescence (Betina 1985). Aflatoxins have been identified by chemical confirmation by spraying trifluoroacetic acid and sulphuric acid on the TLC plate (Stack and Pohland 1975). Serralheiro and Quinta (1985) have reported that spraying sulphuric acid improves the limit of detection of AFM\textsubscript{1} from 0.5 µg/kg to 0.3 µg/kg. Semi-quantitative analysis has been carried out for mycotoxins by TLC however the method has low sensitivity.

2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC provides more accuracy and precision of mycotoxin determination. Normal and reversed-phase HPLC are used with a variety of detection systems. UV and fluorescence detector are most commonly used. Pons and Franz (1978) reported accurate and sensitive detection of all aflatoxins at levels of 0.3-1 µg/kg, where aflatoxins B\textsubscript{1} and B\textsubscript{2} were detected by UV detector at 360-365 nm and G\textsubscript{1} and G\textsubscript{2} by fluorescence. Akiyama et al. (1998) detected non-fluorescent mycotoxins, fumonisins, by using o-pthalaldehyde post-column derivatisation and then detected by fluorescence detector. The detection limit of fumonisin by this method was reported to be 10 µg/kg of corn. Ochratoxin A in wine has been accurately detected by HPLC following immunoaffinity clean-up with a detection limit of 0.01 ng/ml (Visconti et al. 1999). HPLC with fluorescence detector has been used for detecting AFB\textsubscript{1}, citrinin and ochratoxin in rice and a detection limit of 0.07, 0.11 and 0.08 µg/kg, respectively, for these mycotoxins was reported by Nguyen et al. (2007).

3. GAS CHROMATOGRAPHY (GC)

This method is also used for detecting mycotoxins especially trichothecenes in food samples. Most mycotoxins are non-volatile and hence are derivatised for detection (Scott 1995).
Electron capture detection (ECD), mass spectrometry (MS) and flame ionization are the common detectors used with GC. Croteau et al. (1994) analysed trichothecenes in corn using GC. The trichothecene mycotoxins were derivatised using heptafluorobutyric anhydride and detected by ECD. The limit of quantification was reported in the range of 50-200 µg/kg of corn. In another report, trichothecenes were determined in corn by MS detector with a detection limit of 10-40 µg/kg and limit of quantification of 70-200 µg/kg (Milanez and Valente-Soares 2006). GC method has also been used for analysing multi-mycotoxins such as patulin, zearalenone and trichothecenes in wheat (Rodriquez-Carrasco et al. 2012). There are few disadvantages with this method such as the need for derivatisation and thermal stability of mycotoxins, where heating degrades the samples.

4. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA technique has been used for determining aflatoxin in a large number of foods. This method is based on direct and indirect competitive assay. ELISA has been used for detecting deoxynevalenol and zearalenone in maize (Cavaliere et al. 2005). Reddy et al. (2009a) have used indirect competitive ELISA for detecting AFB₁ in rice with a detection limit of 0.02 ng/kg. Other mycotoxin like fumonisin has also been detected using this method with a detection limit of 3 ng/ml of beer (Torres et al. 1998). This method has the advantage of screening bulk samples and is highly specific.

The performance of various detection methods differs for different food materials. Dell et al. (1990) has reported the determination of aflatoxin in peanut butter using high performance-TLC which gave precise and consistent data than HPLC and ELISA.

DETOXIFICATION OF MYCOTOXINS

1. PHYSICAL TREATMENT

Physical treatment includes cooking, boiling, roasting, microwave heating, extrusion, irradiation, etc. Food undergo heat treatment during the processing stage, hence thermal inactivation of mycotoxins is practical. Mycotoxins are relatively heat stable and so are not easily destroyed (Bullemen and Bianchini 2007). The level of mycotoxin degradation by
thermal process depends on factors like temperature, moisture content and time period. In heat treatment, temperature and time period are important in determining the level of degradation. Higher level of aflatoxin degradation was achieved when heated at 200 °C for longer exposure time (Levi 1980). The moisture content of a product also plays an important part in degrading aflatoxins. At high moisture content, degradation was found more efficient (Mann et al. 1967). Under dry conditions, citrinin was decomposed at 170 °C, where under moist condition it was detoxified at 140 °C (Kitabatake et al. 1991). Heating ochratoxin A in the presence of sodium hydroxide (NaOH) resulted in the detoxification of the toxin (Trivedi et al. 1992). Roasting fumonisin B₁ contaminated cornmeal at 218 °C for 15 min resulted in almost complete degradation of the toxin (Castelo et al. 1998). The presence of ammonia during extrusion of AFB₁ led to higher amount of degradation (Hameed 1993).

Aflatoxins are photosensitive in nature; hence various radiations such as sunlight, UV light and gamma rays were employed for degradation studies. Sunlight was efficiently used for degrading AFB₁ in olive oil, groundnut oil, etc. (Mahjoub and Bullerman 1988; Shantha and Sreenivasa 1977). AFB₁ was found to be more susceptible to irradiation when present in liquid medium than in solid media. The cytotoxicity and mutagenecity of AFB₁ has been shown to reduce after treatment with UV in aqueous medium (Liu et al. 2011). On the other hand, it was reported that irradiated fungal inocula may produce increased levels of mycotoxins especially aflatoxins (Applegate and Chipley 1974) and ochratoxin (Applegate and Chipley 1976; Paster et al. 1985).

2. CHEMICAL TREATMENT

Treatment with chemicals efficiently degrades AFB₁, however formation of degradation products was observed. Acids convert AFB₁ into several products such as AFB₂, B₂a, D₁, etc. rather than complete degradation. Shukla et al. (2002) reported the conversion of AFB₁ into AFB₂ and AFG₁ into AFG₂ by lactic acid. Citric acid causes the hydration of AFB₁ at the 8,9-olefinic bond of the terminal furan ring to form AFB₂a (Ciegler and Peterson 1968). Treatment of AFB₁ with hydrochloric acid at elevated temperature completely destroyed the toxin without the formation of toxic residues (Williams and Dutton 1988; Wattanapat et al. 1995). Other acids like salicylic, sulphamic, sulposalicylic, anthranilic, benzoic, boric, oxalic and propionic acids were
efficiently used for degrading AFB$_1$ by more than 90% in sorghum (Hasan 1996). Alkalis cause the hydrolysis of the lactone ring in AFB$_1$ however it can revert back under acidic conditions (Price and Jorgensen 1985; Camou-Arriola and Price 1989). Boiling AFB$_1$ contaminated corn with NaOH decreased the level of AFB$_1$ by 93%, with 18% reversion level after treatment with acid (Camou-Arriola and Price 1989). Nixtamalization (alkaline cooking of grains) was reported to be an efficient method in degrading fumonisin. Fumonisin-contaminated kernel corn on nixtamalization resulted in reduced toxicity of fumonisin (Voss et al. 2012). Sodium bisulfate and hydrogen peroxide were also used in degrading AFB$_1$ efficiently (Hagler et al. 1982; Altug et al. 1990).

Among the many chemicals used for detoxification of mycotoxins, ammonia is the most efficient and it has been accepted for use by the corn production industry. Ammonia degrades AFB$_1$ into AFD$_1$ which has reduced toxicity and mutagenic potential (Lee and Cucullu 1978). Ozone is well known for possessing anti-microbial property (Kim et al. 1999). Ozone degraded AFB$_1$ by more than 90% without any effect on the animal (McKenzie et al. 1998; Prudente and King 2002). Maeba et al. (1988) showed that ozone-treated aflatoxins were not toxic and mutagenic. AFB$_2$ and G$_2$, fumonisin, ochratoxin, patulin and zearalenone were also efficiently degraded by ozone.

3. BIOLOGICAL TREATMENT

MICROORGANISM

Few strains of lactic acid bacteria have been reported to remove AFB$_1$ and M$_1$ by binding non-covalently (El-Nezami et al. 1998; Haskard et al. 2000). Heat-treated and acid-treated _Lactobacillus rhomosus_ GG and _L. rhamosus_ were able to remove zearalenone, indicating that binding and not metabolism is the mechanism by which the toxins are removed (El-Nezami et al. 2002). Another bacteria _Flavobacterium aurantiacum_ B-184 was found to remove AFB$_1$ irreversibly (Ciegler et al. 1966; Lillehoj et al. 1967). Line et al. (1994) studied the mechanism and suggested that the degradation of AFB$_1$ by _F. aurantiacum_ is probably a mineralization phenomenon. A number of fungal species, especially _Phoma_ sp., were reported to prevent the synthesis of AFB$_1$ and degrade the toxin as well (Shantha 1999). Degradation of mycotoxins occurs during fermentation of various foods such as milk (Megalla and Mohran 1984), dough
fermentation in making bread (El-Banna and Scott 1983) or during beer brewing (Chu et al. 1975). Some toxigenic fungi (*Aspergillus parasiticus, A. flavus*) are able to degrade their own toxin under certain conditions (Shih and Marth 1975; Doyle and Marth 1978; Hamid and Smith, 1987). Degradation of AFB₁ depends on the type of substrate and the fungal strains used.

ENZYMES

Several fungal enzymes have been reported to degrade AFB₁. *Armellaria tabescens* produced a multienzyme system which detoxified AFB₁ by opening the difuran ring (Liu et al. 1998). The enzyme peroxidase from *A. flavus* and *A. parasiticus* has been shown to degrade AFB₁ and AFG₁ (Singh 1998; Doyle and Marth 1979). A horseradish peroxidase enzyme from the plant *Raphinus sativa* has also been reported to degrade AFB₁ (Das and Mishra 2000).

PLANT EXTRACTS

Use of botanicals as anti-fungal and anti-mycotoxin agent is considered safe to humans and environmental friendly. Various extracts from plants such as piperine from black and long peppers (Singh et al. 1994); lutein and xanthophylls from Aztec marigold (Meija et al. 1997); carotenoids from fruits and vegetables (Rauscher et al. 1998) were reported to suppress the toxicity and mutagenicity of AFB₁. The essential oils of several plants have been documented to possess strong antimicrobial property. The oil of *Illicium verum, Cymbopogon martini, Eucalyptus globulus, Cinnamon zylenium*, etc. are reported to be anti-fungals (Bansod and Rai 2008; Huang et al. 2010). The powder and essential oil of *Cymbopogon citratus* have been successfully used for inhibiting AFB₁ contamination and preserving the quality of melon seed under storage (Bankole and Joda 2004; Paranagama et al. 2003). The essential oils of *Cinnamomum jensenianum* (Tian et al. 2011), *Ocimum sanctum* (Kumar et al. 2010) and *Zataria multiflora* (Gandomi et al. 2009) were efficiently against toxigenic fungi and AFB₁ and their safe use as natural preservative of food has been implicated. Among the number of plants, *Syzygium aromaticum* (clove) has been extensively studied for its anti-microbial property (Pinto et al. 2009). The oil of clove and its main component, eugenol, has been reported to inhibit Aspergillus growth and AFB₁ production by various investigators (Bullerman et al. 1977; Jayashree and Subramanyam 1999). Whole clove has also been shown to exhibit anti-fungal and anti-aflatoxicogenic activity in culture media and rice grains as reported (Aiko and Mehta 2013).
Removal of toxigenic fungi and mycotoxins by botanicals are usually preferred over chemical treatments.

MANAGEMENT AND REGULATION OF MYCOTOXINS

Mycotoxigenic fungi are ubiquitous in nature and their occurrence in foods is unavoidable. 25% of the world’s food crops are contaminated with mycotoxins as reported by the Food and Agricultural Organisation. This not only causes economic loss but also reduces the world’s food supply. The contaminating fungi and mycotoxins are found in food crops as well as in a number of processed foods intended for human consumption. Mycotoxins pose higher risk of causing cancer than contaminants in food such as anthropogenic contaminants, pesticides, phycotoxins and food additives (Kuiper-Goodman 1998).

Several national and international organizations and agencies have set regulations and safety limits of various mycotoxins. The maximum levels for mycotoxins in foods and feeds have been set to ensure the safety of the consumers. The US Food and Drug Administration (FDA) and European Union (EU) have set the maximum limit of the major mycotoxins in human food as given in Table 1.2. With the recognition of AFB1 as a human carcinogen, several countries have set the regulation of AFB1 in foods (Table 1.3). Control of mycotoxins largely depends on taking proper care during pre-harvest and post-harvest conditions. Use of fertilizers, pest control and fungal resistant crops, and maintaining low moisture content and temperature during storage conditions can prevent fungal and mycotoxin contamination. Prevention of mycotoxin contamination is not always possible; hence many reduction or detoxification methods have been developed as mentioned above. These methods either degrade mycotoxins completely or reduce the toxin concentration to a safe level. Ammonia is currently used for degrading AFB1 in feedstuffs; however it also forms a degradation product AFD1 which is not completely non-toxic (Lee and Cucullu 1978). There is still a need for an efficient and safe method for mycotoxin detoxification.
### Table 1.2 The regulation of mycotoxins in human food (µg/kg)

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>European Union</th>
<th>US FDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B₁</td>
<td>2-8</td>
<td>20</td>
</tr>
<tr>
<td>Aflatoxin M₁</td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>200-700</td>
<td>1000</td>
</tr>
<tr>
<td>Fumonisins (FB₁, FB₂, FB₃)</td>
<td>200-1000</td>
<td>2000-4000</td>
</tr>
<tr>
<td>Ochratoxin</td>
<td>3-10</td>
<td>-</td>
</tr>
<tr>
<td>Patulin</td>
<td>10-50</td>
<td>-</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>20-200</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 1.3 Permissible limits of Aflatoxin B₁ in food set by various countries (Moss 2002)

<table>
<thead>
<tr>
<th>Country</th>
<th>AFB₁ (µg/kg)</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>0</td>
<td>Groundnuts, maize and products</td>
</tr>
<tr>
<td>Brazil</td>
<td>15</td>
<td>All foodstuffs</td>
</tr>
<tr>
<td>China</td>
<td>10</td>
<td>Rice and edible oils</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>5</td>
<td>All foods</td>
</tr>
<tr>
<td>Hungary</td>
<td>5</td>
<td>All foods</td>
</tr>
<tr>
<td>India</td>
<td>30</td>
<td>All foods</td>
</tr>
<tr>
<td>Japan</td>
<td>10</td>
<td>All foods</td>
</tr>
<tr>
<td>Nigeria</td>
<td>20</td>
<td>All foods</td>
</tr>
<tr>
<td>Poland</td>
<td>0</td>
<td>All foods</td>
</tr>
<tr>
<td>South Africa</td>
<td>5</td>
<td>All foods</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>5</td>
<td>Foods</td>
</tr>
</tbody>
</table>