INCIDENCE OF AFLATOXIN CONTAMINATION IN SELECTED SPICE SAMPLES IN ANDHRA PRADESH

By

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CERTIFICATE

Ms D. KIRAN MAYI has satisfactorily prosecuted the course of research and the thesis entitled **"INCIDENCE OF AFLATOXIN CONTAMINATION IN SELECTED SPICES IN ANDHRA PRADESH"**. submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by her for a degree of any University.

Date: 19-12-17
Place : Hyderabad

(Dr. M. UMA REDDY)
Major Advisor

CERTIFICATE

This is to certify that the thesis entitled **"INCIDENCE OF AFLATOXIN CONTAMINATION IN SELECTED SPICES IN ANDHRA PRADESH"** submitted in partial fulfilment of the requirements for the degree of **'MASTER OF SCIENCE IN HOME SCIENCE'** of the Acharya N.G.Ranga Agricultural University, Hyderabad, is a record of the bonafied research work carried out by **Ms D. KIRAN MAYI** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

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(KIRANMAYI D)

DECLARATION

I, **Ms D. KIRAN MAYI** hereby declare that the thesis entitled **"INCIDENCE OF AFLATOXIN CONTAMINATION IN SELECTED SPICES IN ANDHRA PRADESH"** submitted to Acharya N.G. Ranga Agricultural University for the degree of **'MASTER OF SCIENCE IN HOME SCIENCE'** is the result of the original work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

Date:
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ABSTRACT

Aflatoxin B_1 is a potential carcinogen, teratogen, mutagen and immunosuppressive agent produced as a secondary metabolite by the storage fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Various agricultural commodities were found to be contaminated with aflatoxins like rice, wheat, maize, peanuts, coconut, milk, spices etc.

The present survey was designed to estimate the extent of aflatoxin contamination in selected spices in Andhra Pradesh. A total of 188 samples of five kinds of spices namely red chillies (n = 59), black pepper (n = 28), coriander (n = 50), turmeric (n = 26) and dry ginger (n = 25) were collected from different market yards, wholesale and retail shops. Amount of aflatoxin in these collected spice samples was estimated using Indirect Competitive ELISA method of aflatoxin analysis. Cooking experiment was also done with red chilli sauce prepared from naturally contaminated red chillies to know the effect of cooking on the stability of aflatoxins.

The results of the aflatoxin analysis of spices showed that red chillies was the most often contaminated commodity, having high aflatoxin levels upto 400 μg/kg. Incidence of aflatoxins in black pepper was also high but the maximum levels remained below 100 μg/kg. In the remaining three types of spices i.e., coriander, turmeric and dry ginger, all the spices recorded an aflatoxin level of below 10μg/kg. Among all the spices coriander was found to contain lowest levels of contamination and the maximum level of aflatoxin recorded was only 3.2 μg/kg.

The results of the cooking experiment showed that aflatoxin was stable to heat as the aflatoxin levels in chilli sauce remained unchanged even after subjecting to microwave cooking at high temperature. Thus microwave heating was proved to be ineffective in destroying the aflatoxins in redchillies.

From the results of the present study it can be concluded that besides other major commodities, spices are also contaminated with aflatoxins beyond permissible limits. Although the consumption of these spices is limited when compared to essential commodities like cereals and oilseeds, their long-term adverse effects on the health of people cannot be ruled out. Hence, there is a need for regular and frequent checking of these commodities also.

LIST OF ABBREVIATIONS

%	=	Percentage
µg	=	microgram
µl	=	micro litre
>	=	More than
A.P	=	Andhra pradesh
AFB_1	=	Aflatoxin B_1
ALP	=	Alkaline Phosphatase
BSA	=	Bovine Serum Albumin
ELISA	=	Enzyme linked immuno sorbant assay
FDA	=	Food and Drug Administration
gm/g	=	gram
HPLC	=	High Pressure Liquid Chromatography
IgG	=	Immunoglobulin G
kg	=	Kilogram
lt	=	litre
mg	=	milligram
min	=	minutes
ml	=	millilitre
ng	=	nanogram
nm	=	nanometer
$^{\circ}C$	=	degree centigrade
OD	=	Optical Density
PBS	=	Phosphate-Buffered Saline
Ppb	=	Parts per billion
ppm	=	Parts per million
rpm	=	Rotations per minute
wt	=	weight

Introduction

CHAPTER –1

INTRODUCTION

In the year 1960 more than 1.00.000 young turkeys, poultry birds and ducks in England died in the course of a few months from an apparently new disease that was termed Turkey "X" disease. Critical review of the early outbreaks showed that the mortality of the young turkeys was associated with feeds namely Brazilian peanut meal. Speculations made regarding the nature of the toxin suggested that it might be of fungal origin. In 1961, a toxic compound was isolated from the moldy Brazilian groundnut meal used for turkey's feed. Since the compound was produced by *Aspergillus flavus Linkex Fries*, it was named as "Aflatoxin".

The aflatoxin is not one compound but a group of more than fifteen toxins, which are closely related to difuranocoumarins, some of them occur naturally on different types of food as a result of growth of the yellowish green moulds *A. flavus* and *A. parasiticus*. Some members of the group represent products of metabolism by cells of organisms (mostly animals) exposed to toxic effects of the parent fungus metabolites. Aflatoxin B_1 a prototype of the aflatoxins, is widely recognized as the most potent hepato carcinogenic compound and along with certain other members of the group, possess additional toxic properties including mutagenicity (cause mutations), teratogenicity (cause malformations), acute cellular toxicity and it suppresses the immune system.

Aflatoxin contamination of foods and feeds has gained global significance as a result of its deleterious effects on human as well as animal health .The marketability of food products is adversly affected by aflatoxin contamination .The harmful effects of consuming aflatoxin contaminated food products is well documented. The reported outbreaks of aflatoxicosis in man were due to the consumption of contaminated food. Circumstantial evidence has implicated groundnut meal containing aflatoxin as causing Indian childhood cirrhosis. Studies carried out in Kenya, Swaziland, Mozambique and Thailand have found a positive correlation between hepatocellular carcinoma and aflatoxin ingestion by man. The harmful effects of consuming contaminated groundnut meal cake have mainly been observed in poultry and milch cattle. The effects in poultry include mortality, feed refusal, slow growth, fertility and reproduction problems. A 3 % live weight loss of poultry in USA representing a total weight loss of over 100 million kg of meat, worth 143 million US dollars has been estimated to be due to consumption of feed contaminated with mycotoxins.

Aflatoxins have been detected as natural contaminants of many different foods such as corn, peanuts, cottonseed, nuts, almonds, figs, spices and a variety of other foods and feeds. Milk, eggs and meat products are sometimes contaminated because of consumption of aflatoxin contaminated feed by the animals. However, the commodities with the highest risk of aflatoxin contamination are corn, peanuts and cotton seed.

Toxin contamination of foods such as peanuts and cereal grains occur both at pre harvest and post harvest stages and also during storage as a result of improper drying or because of subsequent wetting of products not adequately protected from weather. This contamination is affected by an interrelationship of several factors including climate, geographical location, type of storage container and the mode of commodity handling and transport. Insect and rodent infestations also facilitate mold invasion of some stored commodities.

Information with regard to aflatoxin contamination in cereals and oil seeds has been, related in thousands of scientific articles and news reports but comparitively lesser information is available about aflatoxin contamination in spices, which also form an important component of human diet. These spices were found to contain active principles, which are inhibitory to fungal growth and aflatoxin production, but they are quite vulnerable to mould contamination, depending on the climatic conditions under which they are stored (Madhyastha and Bhat 1985). Among the moulds, the species most frequently isolated are *A. glaucus, A. restrictus, A. ochraceus* and *A. flavus*, the latter capable of synthesising aflatoxins (Guarino 1973).

India has been famous for her many and varied spices for many centuries. The word "India" conjures up a vision, in the mind of foreigners of a land of spices, among other things. In India, spices besides forming an essential ingredient in its cuisine play an important part in its national economy as India is the largest exporter of spices to the rest of the world including

Europe and USA. Spices export is one of the major foreign exchange earner to India. Aflatoxin contamination also affects the international trade of the food products. To minimise the health risk, legislation has been passed in several countries restricting the level of aflatoxins in food products. Recently, several consignments having huge quantities of chillies, from Guntur in Andhra Pradesh were turned down by the importing countries because these chillies were contaminated with aflatoxins. So, analysis of spices for aflatoxins has become mandatory.

Looking into the gravity of the aflatoxin problem, considerable efforts have been made to develop simple, specific and sensitive methods for analysis of aflatoxin in different commodities. Conventional chemical methods for aflatoxin analysis such as Thin Layer Chromatography, Mini column Method, High Pressure Liquid Chromatography, Gas and Mass Spectroscopy are time consuming, laborious, expensive and require extensive sample clean-up. Recently efforts have been made to develop and use Enzyme- Linked Immunosorbant Assay (ELISA) for the determination of aflatoxin in food and feeds. The commercial kits for aflatoxin analysis based on enzyme immuno assays are very expensive and many times import of kits becomes a problem. Aflatoxin estimation by ELISA method is very simple, specific, fast and cost effective also. In addition to this large number of samples can be analysed with in a short period.

As aflatoxins are considered as unavoidable contaminants of our food supply, efforts have been directed towards detoxification of aflatoxin

contaminated food. The application of physical methods, gamma irradiation, microwave treatment (Straton 1980 and Pluyer *et al* .,1987) and chemical methods(treating with chlorinating agents, oxidising agents and hydrolytic agents) alone or in combination provide varying effective means of aflatoxin degradation. Such treatment procedures are expected to be cost effective but their applicability is restricted by safety problems that may arise from chemical residues. (Samarajeewa *et al.*, 1990). So physical detoxification by homescale cooking methods like roasting, boiling, fermentation, frying, baking, steaming etc was extensively investigated. *(Farah et al., 1983, Reddy 1984, Pluyer et al., 1987, Mahjoab 1988 and Maria et al., 1988). These normal food processing and* preparation methods appear to cause an average of 60% degradation under laboratory conditions.

However all aflatoxins do not react equally to heating, for example aflatoxin B_1 is heat stable, while aflatoxin G_1 can be destroyed by heat (Reegner 1967). Also the sensitivity of aflatoxins to heat is governed by environmental conditions. On one hand, the presence of moisture in foods may enhance degradation by hydrolising lactone ring at critical moisture and temperature levels. On the other hand aflatoxins may be protected in foods in part by their binding or association with proteins and other constituents.

As a result of above investigations it was decided to ascertain the occurrence of aflatoxins in spices and the present study was undertaken with the following objectives:

General objective:

To assess the levels of aflatoxin B_1 in selected spice samples.

Specific objectives:

1. To identify the factors associated with aflatoxin contamination
2. To identify the stages of contamination in market samples
3. To assess the effects of cooking on the stability of aflatoxin in selected samples.

Review of Literature

CHAPTER - 2

REVIEW OF LITERATURE

The Aflatoxin problem came into light in 1960's when there was a severe outbreak of Turkey 'X' disease in England killing about 100,000 turkeys and other farm animals. Affected birds lost appetite, became lethargic and died within 7 days after the onset of symptoms. The cause of this disease was traced to a feed component, peanut meal, which was infested heavily with *Aspergillus flavus*. On analysis of the feed, it was discovered that a series of fluorescent compounds later termed, as 'aflatoxins' were responsible for the outbreak. This very singular event initiated the international interest which now exists in mycotoxins. Later on many studies were conducted which extensively investigated the occurrence, chemical structure, biosynthesis, factors affecting the biosynthesis and health hazards of these aflatoxins.

The work done in the area of aflatoxin research in the past is reviewed under following categories and presented in this chapter.

2.1. Studies on chemistry of Aflatoxins

2.2. Studies on health hazards of aflatoxins

2.2.1. Effects of aflatoxins in animals.

2.2.2. Effects of aflatoxins in humans.

2.3. Studies on methods of estimation of aflatoxins

2.4. Studies on aflatoxin contamination in agricultural commodities.

2.5. Studies on aflatoxin contamination in spices

2.5.1. Aflatoxin contamination in red chillies.

2.5.2. Aflatoxin contamination in black pepper.

2.5.3. Aflatoxin contamination in coriander.

2.5.4. Aflatoxin contamination in turmeric.

2.5.5. Aflatoxin contamination in dry ginger.

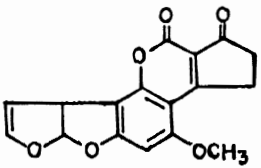
2.6. Studies on effect of cooking on the stability of aflatoxins.

2.1. CHEMISTRY OF AFLATOXINS

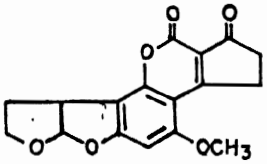
Chemically aflatoxins are a group of difuranocoumarins produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*. (Diener and Davis 1969). Presently 18 different types of aflatoxins have been identified with aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 being the most common (Beuchat 1987). Of these, the four naturally occurring major aflatoxins are aflatoxin B_1, B_2, G_1 and G_2 (Heathcote and Hibbert 1978). The letters B and G refer to the flourescent colours observed under long wave ultraviolet light and the subscripts 1 and 2, to the separation patterns of these compounds on thin layer chromatographic plates. (Fig.1) (Bullerman 1979).

Aflatoxins have high melting points of 260° with thermal degradation temperature of 269° C and hence very stable to heat (Table 1). (Ciegler and Vesonder 1983). However they are destroyed by strong oxidising agents and alkalis and are decomposed on exposure to air and ultra violet light and visible light. (Heathcote and Hibbert 1978).

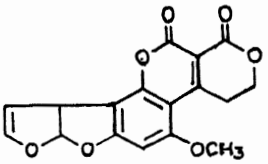
Aflatoxins are produced on a variety of substrates at an optimum temperature of 30-35° C with a relative humidity of 80-85%. Among the substrates



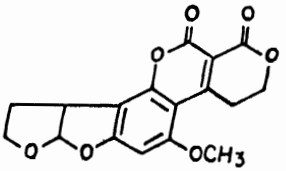
AFLATOXIN B_1



AFLATOXIN B_2



AFLATOXIN G_1



AFLATOXIN G_2

Fig. 1. Structures of aflatoxins.

rice, groundnut and maize have been shown to yield substantial amounts of aflatoxins under laboratory conditions. (Hesseltine *et al.*,1966)

Table 1: Chemical and Physical properties of aflatoxins

Aflatoxin	Molecular formula	Molecular weight	Melting point	Molar absorption in methanol (362-363 nm)	Fluorescence emission (nm)
B1	C17H12O6	312	268-269	21,800	425
B2	C17H14O6	314	268-269	23,400	425
G1	C17H12O7	325	244-246	16,100	450
G2	C17H14O7	330	237-240	21,100	450

Source: Wyllie T D and Morehouse L G 1977

2.2 HEALTH HAZARDS OF AFLATOXINS

In general, aflatoxins produce a number of adverse effects in a range of biological systems including plants, animals and humans. Aflatoxin B_1, the most toxic compound in the series of aflatoxins, has been found to be one of the most potent carcinogens occurring naturally. Because of frequent contamination of aflatoxin B_1 in agricultural commodities such as peanuts, corn and animal feed stuffs, aflatoxin problems become a potential hazard to human and animal health. (Busby and Wogan 1979).

2.2.1 Effects of aflatoxins in animals

The history of aflatoxin investigations began with the discovery of their harmful effects in turkey poults. Ducklings have been found to be highly susceptible to aflatoxin toxicity. Besides these, a variety of animals such as quail, chickens, rainbow trout, rats, mice, guinea pigs, hamsters, rabbits, dogs, cattle, sheep, swine, horses, monkeys and mink are known to be affected by aflatoxins.

Aflatoxins have been shown to be lethal to many domestic and experimental animals. (Goldblatt 1969 and Wogan 1977). Animal susceptibility to carcinogenesis by aflatoxins varies with the sex, age, species, strain within the species, hormonal and nutritional status of the animal. The duckling is the most susceptible species and the mouse the most resistant one to the lethal toxicity of aflatoxin (Patterson and Allcroft 1970). Protein deficiency was observed to sensitise the animal to acute toxic effects of aflatoxin but was protective towards its carcinogenic effects (Madhavan and Gopalan 1968).

Consumption of mycotoxin contaminated feed has resulted in a number of disease outbreaks in farm and domestic animals. Outbreaks mostly attributable to aflatoxins have been reported in turkeys (Austwick 1978), pigs (Cook 1989), horses (Vesonder 1991), dairy cattle, poultry, rabbits, dogs and camels (Bhat and Nageswara Rao 1989)

2.2.2 Effects of aflatoxins in humans

Several attempts have been made in various parts of the world to correlate the consumption of various foods contaminated with aflatoxins with acute or chronic diseases in humans such as primary liver cancer, Indian childhood cirrhosis, chronic gastritis, kwashiorkar, reyes syndrome and some respiratory diseases.

Cases in which acute poisoning occurred due to the consumption of foods heavily contaminated with aflatoxins were reported mainly from tropical countries such as Taiwan (Tung and Ling 1968), Uganda (Serck-Hanssen 1970) and Thailand (Shank *et al* .,1971). In India an acute aflatoxicosis outbreak occurred, affecting man and dogs due to consumption of aflatoxin contaminated maize. (Krishnamachari *et al.*, 1975a)

Data on aflatoxins and human cancer have shown a positive correlation between aflatoxin ingestion and liver cancer in population studies in which aflatoxin intake and the incidence of primary liver cancer were estimated concurrently. (Shank *et al.*, 1972, Peers and Linsell 1973, Van Rensburg *et al.*, 1974 and Campbell and Stoloff 1974).

The presence of aflatoxins in human breast milk and cord sera, the teratogenic, carcinogenic and immunotoxic effects of aflatoxins in humans and the possible relationships between aflatoxin exposure and kwashiorkar and Indian childhood cirrhosis were discussed by Raisuddin (1993). In 1994 Adhikari *et al.*, investigated the possible effects of consumption of aflatoxin contaminated food on

Kwashiorkar and reported that exposure of dietary aflatoxins compounded the effects of Kwashiorkar.

The possibility that some cases of Reye's syndrome (Encephalopathy with fatty degeneration of the viscera) might be due to aflatoxin ingestion has been reported by Dvorackova *et al* .. (1977) in Czechoslovakia, where they detected aflatoxins in the livers of patients with Reye's syndrome.

Aflatoxin B_1 has also been found to be a potent mutagen. Mutagen studies on bacteria suggest that the possible mechanism of mutagenesis may be initiated by the ability of the aflatoxin to bind the nucleic acids such as DNA. Aflatoxins are being implicated to cause genetic mutations in people suffering from primary liver cancer particularly in areas of sub-Saharan Africa and southeast Asia(Bressac *et al.*, 1991, Harris 1991 and Hsu *et al.*, 1991).

2.3 STUDIES ON METHODS OF ESTIMATION OF AFLATOXINS

The occurrence of aflatoxins in a wide variety of agricultural commodities led to the development of various analytical methods. These analytical approaches for the detection of aflatoxin in foods and feeds mainly involved chemical or physico-chemical methods as they are more specific, rapid, reproducible and possess lower limits of detection than biological methods. (Van Egmond and Paulsch 1986). Aflatoxins fluoresce strongly under UV light. This property formed the basis for practically all the analytical methods for their detection and quantification.

Separation, detection and quantification of aflatoxins in foods have been achieved mostly by chromatographic techniques such as Thin layer chromatography (TLC). High pressure liquid chromatography (HPLC) and Mini column chromatography (Romer 1984). These techniques have been used for screening, presumptive or semi quantitative and quantitative purposes depending upon the limits of detection, precision and accuracy required for the analysis.

In India, a pressure mini column technique that is simple, rapid, economical, reasonably accurate and practical in field situations to detect aflatoxin has been developed and collaboratively tested. (Sashidhar *et al* .,1988 and 1989)

Immunoassay methods such as ELISA are gaining more popularity in recent years. (Pesska 1988) as they have been found to be rapid, highly sensitive, specific and require little sample clean up. They have been developed and collaboratively tested (Cher *et al* .,1987 and Trucksess *et al*., 1989) for detecting aflatoxin in human serological fluids. (Groopman and Donahue 1988)

A number of biological methods like chick embryo test, physical methods like mass spectroscopy and chemical tests like reaction with triflouroacetic acid or hydrochloric acid and have been developed for confirmation of aflatoxin. (Nesheim and Brumley 1981).

All the above discussed methods or techniques have been well tested and developed for detecting aflatoxin in a number of commodities like groundnut, maize, rice, wheat etc. But a review of available literature on aflatoxin research in spices indicates that detection of aflatoxins in spices was done mostly by

chromatographic techniques namely HPLC (Awe and Schranz 1981, Finoli and Ferrari 1994, Putzka 1994 and Mc Donald and Castle 1996), LC (Taguchi *et al.*, 1995 and Selim *et al.*, 1996) and TLC (Shank *et al* .,1972, Beljaars *et al* .,1975, Trucksess and Stoloff 1980, Singh 1983, Misra 1987 and El Kady *et al* .,1995) and no other method like physical, biological or immuno-assay method has been till now employed for this purpose.

2.4. AFLATOXIN CONTAMINATION IN AGRICULTURAL COMMODITIES

Aflatoxin contamination has been reported in various agricultural commodities which are listed in table 2. (Bhat 1992). Among these the two commodities which gained much importance in aflatoxin research are groundnut and maize. Occurrence of aflatoxin in groundnut has been most extensively studied because it was in groundnut meal that aflatoxin was first discovered. In the cereal crops, by far the most important contamination occurs in maize, which forms a staple diet in many regions of the world. In addition to groundnut and maize, a number of other commodities currently known to be contaminated with aflatoxins are barley, copra (dried coconut), cotton seed, parboiled rice, wheat, tree nuts, legume and spice crops.

Aflatoxin contamination in groundnuts has been reported from sixty countries in the African, American, Asian and European continents (Mehan *et al.*, 1991). Many surveys have been initiated in the African and Asian regions where it is mostly grown. From the results of these surveys in such regions it was felt that the

problem of aflatoxin contamination was more during transit and storage of the harvested produce (Bhat 1988). Higher levels of contamination were encountered during storage in the retail or wholesale markets than during harvest or storage in the farm (Quitco *et al.*, 1989). Aflatoxin contamination in groundnut was also reported from regions of South America, where the frequency of contamination ranged from 38-50% with maximum levels of 5000 µg/kg in Brazilian markets (Sabino 1989).

Aflatoxin contamination in maize has been reported from the African, American and Asian continents. Maize tends to have the highest incidence of aflatoxin contamination because of normal practice of harvesting this crop with moisture levels suitable for mold growth (Stoloff 1976). In India, maize heavily contaminated with aflatoxins to a level of 15,600 µg/kg (Krishnamachari *et al.*, 1975) resulted in an outbreak of aflatoxicosis in human and animals. In parts of Africa (Peers and Linsell 1973 and Van Rensburg *et al.*, 1974) and China (Tu et al., 1985 and Yeh *et al.*,1985), high incidence of aflatoxin contamination in maize has been associated with primary liver cancer in many parts of these regions.

In a study conducted in Thailand, in which more than 2000 samples of market foods and food stuffs representing some 170 different human foods were analysed for aflatoxins showed that peanuts and corn were the most frequently and most highly contaminated with aflatoxins (Shank *et al* .,1972).

A study conducted by ICMR involving rural and urban regions of 11 states in India revealed that 21 percent of the 2062 samples of groundnut analysed

exceeded the Indian tolerance limit of 30 µg/kg (Bhat *et al* .,1996). A similar analysis of maize samples showed that 26 percent of 2074 samples analysed exceeded the tolerance levels (Bhat *et al* .,1997).

In cereal commodities such as rice and parboiled rice, aflatoxin contamination occurred at low levels in normal conditions. However, under conditions of stress such as heavy rains or floods, high aflatoxin contamination was observed to occur (WHO 1988). In India, aflatoxin levels ranging from 30-1130 µg/kg were observed in cyclone affected rice.(Vasanthi and Bhat 1990)

Table 2: Occurrence of aflatoxins in foods and feeds

Cereal grains	Maize, rice, wheat, sorghum
Oilseeds	Groundnut, cottonseed, coconut, soy bean, sunflower
Vegetable oils	Groundnut oil, coconut oil, cottonseed oil & olive oil.
Pulse	various beans (Africa) Peas (Asia/Africa)
Root crops	Cassava, sweet potato
Tree nuts	Pistachio nuts, almonds, walnuts, and areca nuts
Commercial crops	Coffee, cocoa, figs, peaches, spices
Animal feeds	Extractions of cottonseed, groundnut, and coconut
Animal products	milk, cheese, fish, shrimp
Fermented products	Alcohol, beer, sauces, wines.

Source: Bhat 1992

In a recent survey in Mysore (India) various foods collected from selected big shops and petty shops were analysed for aflatoxin B_1 content. The results of the study indicated that, the contamination levels ranged from 6-1000 ppb. The highly susceptible commodities were parboiled rice, maize, groundnut and spice powders. Noticeably high quantities were present in maize and groundnut ranging between 10-1000 and 6-111 ppb respectively (Girija *et al.*, 1998).

2.5 AFLATOXIN CONTAMINATION IN SPICES

Extensive surveys have been undertaken with regard to aflatoxin contamination in cereals and oilseeds and number of reviews have also appeared on these aspects. Comparatively lesser information is available about aflatoxin problem in spices which constitute an important fraction of human diet. These substrates are also known to favour the growth of toxigenic fungi and help in aflatoxin production. (Hanssen 1971 and Pal and Kundu 1972).

Results of a study on aflatoxin contamination in spices reported upto 120 ppb aflatoxin levels in 18 of 125 samples of black pepper, ginger and turmeric collected from drying yards of Kerala, ware houses of Karnataka (India) and some industrial belts of Canada (Seenappa and Kempton 1980)

Samples of common Egyptian foods (17 nuts and seeds, 10 spices, 31 herbs and medicinal plants, 12 dried vegetables and 28 cereal grains) were collected from markets in Cairo and Giza for aflatoxin analysis. The results indicated that highest prevalence of aflatoxin B1 was in nuts and seeds (82%) followed by spices (40%)

and the mean concentration of aflatoxin B_1 in spices was 25 ppb. (Selim *et al.*, 1996).

2.5.1 Aflatoxin contamination in Red Chillies

Aflatoxin contamination was found in various kinds of spices like chillies, ginger, nutmeg, coriander and turmeric. Of all these spices, red chillies/red pepper/chilli peppers were reported to be showing highest aflatoxin levels and frequency of occurrence. (Scott and Kennedy 1973, Flannigan and Hui 1976, Michael and Schranz 1981, Singh 1983, Prakash *et al* .,1988, Garrido *et al.*, 1992, Tabata *et al.*, 1993, Putzka 1994, Taguchi *et al* .,1995 and Patel *et al.*, 1996).

High aflatoxin levels in dried chilli peppers were reported from Thailand. With 11% incidence in contamination, aflatoxin levels reached a maximum of 966 µg/kg (Shank *et al* .,1972). In India, an aflatoxin level of 10-60 µg/kg was observed in red chillies (Madhyastha 1985). In another study in Germany, where a total of 185 samples of spices were analysed for mycotoxins, aflatoxins were detected in 4 samples of red chillies, ranging from 8.4-24 µg/kg. (Majerus and Woller 1985)

When a total of 15 samples of chilli powder were analysed for aflatoxins, 7 samples were positive, containing aflatoxins B_1 and B_2 upto 15.3 µg/kg. (El-Dessouki 1992). In a study of aflatoxins in various spices like ginger, turmeric, coriander, pepper, nutmeg and red chillies, highest contamination of aflatoxins upto 120 µg/kg was found in red chillies (Llewellyn *et al* .,1992).

Samples of black pepper, cayenne pepper, chilli powder, cumin, ground ginger and paprika from retail outlets in UK have been analysed for aflatoxin contamination. More than 50% of the samples were contaminated with >1 ppb aflatoxin. Among these chilli powder was the most often contaminated with some samples containing >20 ppb total aflatoxins. (Garner *et al.*, 1993).

A total of 22 pepper (*capsicum annum*) samples from local markets and farms of 6 districts in Punjab, NWFP (North West Frontier Province) and Sindh, Pakistan were screened for aflatoxin B_1 and B_2 contamination in pepper ranged from 32.2-48.1 µg/kg (Jaffar *et al* .,1994)

Samples of sun dried, matured red pepper were analysed for aflatoxin content and it was reported that AFB_1 values varied from non-detectable to 2.2 µg/kg (Adegoke *et al.*,1996). Aflatoxin contamination of Shiro and ground red pepper samples collected from government owned food stores, retail shops and open markets of Addis Ababa, Ethiopia was investigated. Out of 60 samples of ground red pepper. 8 (13.3%) were positive for aflatoxins, the contamination levels ranging from 250-515 ppb (Fufa and Urga 1996).

In UK a survey of aflatoxins in 157 retail samples of herbs and spices which included curry powders, pepper, cayenne pepper, chilli, ginger, cinnamon and coriander nearly 95% of samples contained below 10 µg/kg total aflatoxins and only 9 samples had higher levels. The highest concentration in a retail sample of chilli powder was 48 µg/kg (Mac Donald and Castle 1996).

2.5.2 Aflatoxin contamination in Black Pepper

With respect to incidence of aflatoxin contamination in black pepper, contradictory reports have been documented. The earlier studies by Scott and Kennedy (1973) Beljaars *et al.*, (1974) and Awe and Schranz (1981) reported lack of aflatoxin contamination in black pepper. However fungal invasion by *Aspergillus flavus* has been documented for black pepper. (Christensen *et al.*, 1967 and Moreno-Martinez and Christensen 1973).

Later on quite a number of studies reported fungal contamination and aflatoxin production upto different levels in black pepper. (Suzuki *et al.*, 1973, Mat-Isa-Awang and Nazarifah Ibrahim 1987, Prakash *et al.*, 1988, Abidin *et al.*, 1995 and Guglielminetti *et al.*, 1996)

In India a study conducted on aflatoxin contamination in spices reported an aflatoxin level of 1.5-57.5 µg/kg in black pepper samples. (Madhyastha 1985)

In a survey carried out to detect aflatoxins and isolate aflatoxigenic moulds contaminating fresh and processed meat products, out of 100 samples of spices analysed, four black pepper samples were found to contain about 35 µg/kg of AFB_1 (Aziz and Youssef 1991)

A total of 120 different samples belonging to 24 kinds of spices collected from different places in Assiut, Egypt were examined for natural occurrence of mycotoxins. The analysis of these spice extracts revealed aflatoxins (8-35 µg/kg) in 16 samples of black pepper (El Kady *et al.*, 1995)

2.5.3 Aflatoxin contamination in Coriander

In addition to the other spices like the chillies, pepper, turmeric, nutmeg and ginger, coriander was also found to be contaminated with fungus *Aspergillus flavus* and aflatoxin production was also observed. (Prakash *et al* .,1988, Saxena and Mehrotra 1989 and El-Kady *et al*., 1995).

The first ever study on aflatoxin contamination in coriander, recorded low to high incidence of aflatoxins upto 45.5 ppb aflatoxin B_1 (Scott and Kennedy 1975). In a survey in which 185 samples of spices were analysed for mycotoxins, aflatoxins were detected in two samples of coriander upto 5.2 µg/kg (Majerus and Woller 1985)

An investigation on the natural occurrence of aflatoxins in coriander reported that maximum level of contamination for AFB_1 was found to be 75 µg/kg. (Misra 1987). A study in which 100 spice samples were analysed for aflatoxins, 2 samples of coriander were found to be contaminated with aflatoxin upto 8 µg/kg.(Aziz and Youssef 1991). Another analysis of spice samples for aflatoxins showed that the incidence of aflatoxins in coriander samples ranged between 2-75 µg/kg. (Llewellyn *et al*., 1992).

Contrary to all the above studies Belijaars *et al*., (1974) in a survey on aflatoxin contamination in spices reported that no aflatoxin was detected in coriander samples.

2.5.4. Aflatoxin contamination in Turmeric

When compared with aflatoxin research in all other spices, very few studies investigated the aflatoxin contamination in turmeric. In a study on growth and aflatoxin production by *Aspergillus parasiticus* on various spices, Madhyastha and Bhat (1985) found that turmeric supported comparatively less fungal growth and aflatoxin production.

An investigation on aflatoxin incidence in spices showed that turmeric samples were contaminated with aflatoxins upto 3.8 ppb AFB_1 (Scott and Kennedy 1975).

A survey carried out to detect aflatoxins and isolate aflatoxigenic moulds contaminating fresh and processed meat products reported that two samples of turmeric contained AFB_1 upto 12 µg/kg. (Aziz and Youssef 1991). Llewellyn *et al.*, (1992) showed that turmeric samples were contaminated with aflatoxins upto 2-75 µg/kg.

2.5.5 Aflatoxin contamination in dry ginger

Ginger samples were also found to be contaminated with the aflatoxin producing strains of *Aspergillus flavus*. (Flannigan and Hui 1976 and Madhyashta and Bhat 1985). And the results of many surveys indicate the presence of aflatoxins in ginger samples. (Awe and Shranz 1981, Tabata *et al.*, 1987, Garrido *et al.*, 1992 and Patel *et al.*, 1996).

Scott and Kennedy (1975) reported low to high incidence of aflatoxins upto 25 ppb aflatoxin B_1 in ginger samples. In a TLC(Thin Layer Chromatography) determination of aflatoxins in dry ginger roots and ginger oleoresin, aflatoxins were found in the majority of the lots examined. Most of the reported results were below the FDA administrative guidelines of 20 ppb total aflatoxins. (Trucksess and Stoloff 1980).

In India, an aflatoxin level of 12.5-25 µg/kg was observed in ginger (Madhyashta 1985). A study in U.K on aflatoxin contamination in various spices like black pepper, cayenne pepper, chilli powder, cumin and ground ginger showed that ginger was the most often contaminated with some samples containing >20 ppb total aflatoxins. (Garner *et al.*, 1993).

2.6 STUDIES ON THE EFFECT OF COOKING ON THE STABILITY OF AFLATOXINS

While there have been a considerable number of publications examining the fate of aflatoxins during food processing which have been thoroughly reviewed by Scott (1984), less work has been reported on domestic cooking. With respect to domestic cooking also, number of studies were published on the stability of aflatoxins in commodities like peanuts, rice, corn, wheat, Bengal gram dhal etc. But till date only one survey reported the effect of cooking on the stability of aflatoxins in spices.

Rehana *et al* (1979) studied the effect of different cooking methods on the destruction of aflatoxins in rice. Rice naturally infested (0.02-0.08 ppm) or rice to which mouldy rice or aflatoxin B_1 has been added (0.2- 4ppm) when subjected to normal cooking; aflatoxin destruction was 49%, 82% and 73% respectively. The levels of aflatoxins being low in rice, pressure cooking can be safely used as it was found to reduce about 70% of the aflatoxin.

Stoloff and Trucksess (1981) made boiled corn grits with naturally contaminated corn. After boiling, only 72% of the aflatoxin was recovered, indicating 28% of the destruction of the aflatoxin. In the same study nearly 50-70% loss of aflatoxins has been observed during high temperature roasting of peanuts but during milder processes such as boiling and baking, losses have only amounted to 20-30% of aflatoxins.

Microwave roasting was reported to completely destroy aflatoxins in contaminated peanuts, although it is not clear whether the microwave process itself or the high temperatures were responsible. (Luter *et al.*, 1982).

In the baking of the bread with aflatoxin-contaminated flour, losses were observed during fermentation and the baking itself. (El-Banna and Scott 1983). While during cooking of tortillas losses were observed, associated primarily with the alkaline conditions employed. (Arriola *et al.*, 1988).

Sarita (1991) experimented on the effect of boiling on aflatoxin reduction in Bengal gram dhal and in upma made with wheat semolina. She reported a 75%

destruction after boiling bengal gram and 50% decrease after roasting semolina and 17% after upma preparation.

The one study published on the effect of cooking on the stability of aflatoxins in spices was by Mac Donald. S and Castle L (1996). The results of the cooking experiments showed that aflatoxin levels in spice sauces are not reduced by domestic cooking with either microwave or conventional gas oven cooking.

Materials and Methods

CHAPTER-3

MATERIALS AND METHODS

The present study "Estimation of aflatoxins in spices" was conducted in two phases. The first phase included the collection of various spice samples from different areas of A.P. In the second phase, estimation of AFB_1 in the collected spice samples by ELISA method was carried out.

3.1 COLLECTION OF SAMPLES FOR AFLATOXIN ANALYSIS

To obtain a representative sample, all the spice samples [Red chillies (*capsicum annum);* Black pepper *(piper nigrum*); Coriander (*coriandrum sativum*); Turmeric *(curcuma longa*) and ginger (*Zinger officinale*)] were collected by random sampling method. The collection areas comprised of regulated market yards, retail shops and wholesale shops. Red chilli samples were collected from regulated market yards situated in major chilli growing regions of Andhra Pradesh namely Guntur and Khammam. The rest of the spice samples were collected taking the quality as the criterion. A total of 188 samples of selected spices were collected for aflatoxin analysis. Table- 3 shows the collection area and the number of spice samples collected. The quantity of sample collected ranged from 500-1000 gm per sample. In the collection of red chillies from market yards, about 500-1000gm sample was drawn from different bags (100kg) and made into three sub samples before the analysis was done.

Table 3 Collection area and number of spice samples analysed

Sample	Total no. of samples	Collection area		
		Market yard	Whole sale shop	Retail shop
Red chillies	59	59	-	-
Black pepper	28	-	11	17
Coriander	50	-	22	28
Turmeric	26	-	16	10
Dry ginger	25	-	14	11

3.2 PREPARATION OF SAMPLES FOR AFLATOXIN ANALYSIS

All the samples were first kept at 45°C for overnight drying in a hot air oven. These dried samples were then ground to fine powder in waring blender.20gm portion of each sample was used for aflatoxin analysis.

3.3 ANALYTICAL METHODOLOGY FOR AFLATOXINS

3.3.1 Indirect competitive ELISA procedure for aflatoxin analysis

Apparatus

- ELISA reader
- Hot air oven
- Incubator

- Shaker
- Refrigerator
- MicroPipettes
- HPLC equipment
- Vortex Mixer
- Waring blender

Materials

- Microtitre plates(Nunc Maxisorp)
- Aflatoxin B_1 (Sigma A 6636)
- Aflatoxin B_1 – BSA conjugate (Sigma A 6655)
- Carbonate buffer (coating buffer)

  Na_2Co_3 : 1.59 gm
  $NaHCo_3$: 2.93 gm
  Distilled Water : 1.0 l

  pH of the buffer should be 9.6. No need to adjust the pH.

- Preparation of AFB_1 standard solution: (AOAC 1995)

  Weigh 1mg aflatoxin standard to nearest 0.001mg and transfer quantitatively to 100ml volumetric flask. Dissolve in and dilute to volume with methanol. Measure the absorption of the solution at maximum absorption. Calculate concentration of AFB_1 in solution using the following formula.

- AFB_1 concentration($\mu g/ml$) = $\frac{\text{Absorption(OD) at 360nm x312 x1000}}{21500}$

- Phosphate buffer (PBS)

  Na_2HPo_4 : 2.38 gm
  KH_2Po_4 : 0.4 gm
  KCl : 0.4 gm
  NaCl : 16.0 gm
  Distilled Water : 2.0 l

- Phosphate –buffered Saline with Tween (PBS-Tween): PBS:1l; Tween – 20: 0.5ml.

- Polyclonal antibodies to AFB_1: Prior to this study, polyclonal antibodies to AFB_1 were produced at ICRISAT centre. For production of polyclonal antibodies, commercially available(Sigma) AFB_1-BSA(0.25ml) was mixed with equal volume of Freund's complete adjuvant and injected to Newzealand White inbred rabbit into hind leg at multiple sites.After four immunisations at weekly intervals the rabbit was bled and the titer was determined by indirect competitive ELISA. Serum was lyophilised and stored at –20°C till used.

- Goat anti rabbit Ig G – Alkaline Phosphotase Conjugate.

- Albumin bovine serum (Sigma A 6793):
  Dissolve 200 mg BSA in 100ml PBS - Tween

- **Substrate buffer for alkaline phosphatase system:** p-nitrophenyl phosphate (PNPP) should be stored at –20°C. It is preferable to buy the chemical in tablet form (5, 15 or 20mg tablets available). Prepare 10% diethanolamine (v/v) in distilled water. Adjust pH to 9.8 with conc. HCl. This solution can be stored but pH should be adjusted to 9.80 prior to use. Prepare 0.5mg /ml PNPP in 10% diethanolamine, pH 9.8 (for each 15 mg tablet 30 ml solution is required). Ensure that the PNPP solution does not turn yellow. This may sometimes happen because of ALP contamination from skin.

3.3.1.1 Preparation of sample extracts

In the method (AOAC; 1995 modified by ICRISAT) followed for the present study, the aflatoxins were extracted from food matrix with methanol-water and then used for aflatoxin analysis. Following is the schematic representation of the preparation of spice extracts.

Titrate the spice powder in 70% methanol containing 0.5% KCL in a blender, until the powder is thoroughly ground (Proportion used is 100 ml for 20 g seed)

Transfer the extract to a conical flask and shake it forl hour at 250 rpm.

Filter the extract through whatman no.41 filter paper

Dilute the extract 1:10 in B.S.A (Bovine Serum Albumin) and use for analysis by ELISA

3.3.1.2 Detection and Determination of aflatoxins

In all the extracted samples, aflatoxin was detected by using Indirect Competitive ELISA method.

Procedure

i. Prepare AFB_1-BSA conjugate in carbonate coating buffer at 150-ng/ml concentration. Dispense 150 µl of the diluted toxin-BSA to each well of ELISA plate. Incubate the plate overnight in a refrigerator or at 37°C for atleast 1 hour.

ii. Collect the toxin and store in a large glass bottle for disposal.

iii. Wash the plates in three changes of PBS-tween, allowing 3 min for each wash.

iv. Add 0.2% BSA prepared in PBS-tween at 150 µl per each well of ELISA plate. Incubate at 37°C for 1 hour.

v. Wash the plates in three changes of PBS-tween allowing 3 min for each wash.

vi. Prepare the sample extracts as explained earlier.

vii. Preparation of aflatoxin B_1 standards.

Prepare healthy extracts as mentioned above. Dilute aflatoxin B_1 standards (using 1:10 healthy extract) at concentrations ranging from 10ng to 10 picogram in 100 µl.

viii. Addition of polyclonal antisera prepared for aflatoxin B_1-BSA conjugate.

Prepare a 1:25,000 dilution of antiserum (for each polyclonal anti-serum this should be predetermined) in PBS-tween containing 0.2% BSA. Incubate this for 1 hour at 37°C. Add 50 µl of anti-serum to each of the dilution of aflatoxin standards (100 µl) and sample extracts (100-µl) intended for analysis. (Anti-sera produced at ICRISAT labs)

ix. Incubate the plate for 1 hour at 37°C.

x. Wash the plate in three changes of PBS-tween allowing 3 min for each wash.

xi. Prepare 1:1000 dilution of goat anti-rabbit Ig labelled with alkaline phosphatase in PBS-tween containing 0.2% BSA. Add 150 µl to each well and incubate for 1 hour at 37°C.

xii. Wash the plate in three changes of PBS-tween allowing 3 min for each wash.

xiii. Add substrate solution (p-nitrophenyl phosphate) prepared in10% diethanolamine buffer. (pH 9.8) and incubate for 1 hour at room temperature.

xiv. After satisfactory development of yellow colour in each ELISA plate (in well where low aflatoxin concentrations were used for competition). Measure absorbance at 405 nm in an ELISA reader (preferably automatic). It usually takes 1 hour.

xv. Using the values obtained for aflatoxin B1 standards draw a curve, with the help of a computer, taking aflatoxin concentrations on the X-axis and optical density values on the Y-axis.(Fig-2)

xvi. Calculations

$$AFB_1\ (\mu g/kg) : \quad \frac{A \times D \times E}{G} \quad \text{or} \quad \frac{A \times E}{C \times G}$$

A = AFB_1 concentration in diluted or concentrated sample extract (ng/ml)

D = Times dilution with buffer.

C = Times concentration after clean up.

E = Extraction solvent volume used (ml)

G = sample weight (gm)

3.3.2 HPLC determination of Aflatoxins

The procedure for aflatoxin estimation in spices by ELISA method was standardised by comparing the results obtained by ELISA method with that of HPLC. For this purpose, a sample analysis of red chillies was done by HPLC method (AOAC 1990). The procedure followed is given in Appendix –1 in detail. The amount of aflatoxin in spices was determined as follows.

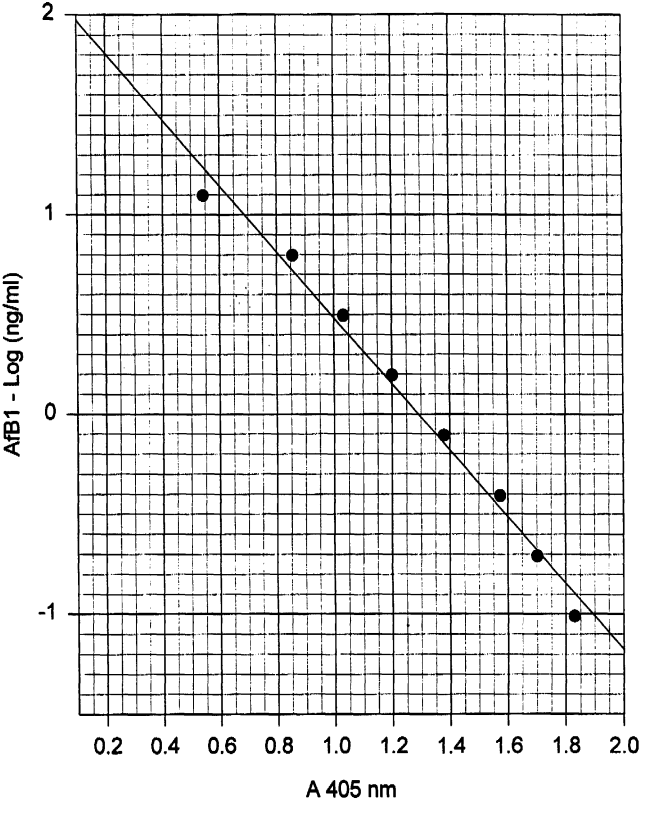


Fig. 2 Linear regression line for estimation of aflatoxin content by ELISA method (correlation coefficient 0.99)

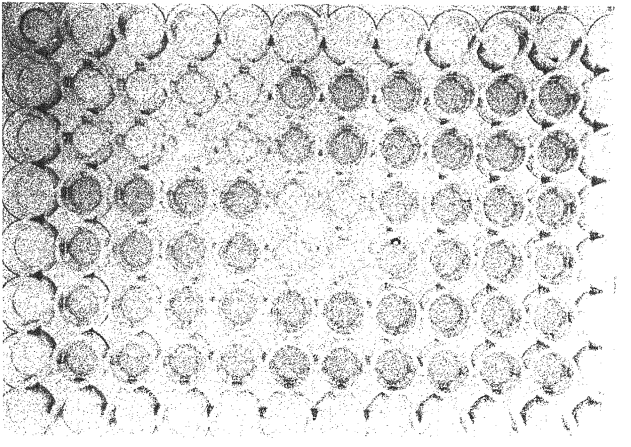


PLATE- 1 Aflatoxin analysis in ELISA plate

AFB_1(ng/100 gm) = PA x ARF x 100/20 x 100/wt. of the sample

Where,

P A - Peak Area

A R F - Area Resolution Factor of AFB_1 standard as obtained from standard graph.

3.4 COOKING EXPERIMENT WITH CHILLI SAUCE

Stability of aflatoxin during cooking was tested by preparing red chilli sauce with naturally contaminated red chillies. For this, first 25-g of red chillies and 10 g each of ginger and garlic were ground together in an electric mixer. 5 g of corn flour mixed in water was then added. This mixture was kept for cooking and other ingredients i.e. 35 ml of vinegar. 5-ml Soya sauce, 5-g salt and 10 g of sugar were added. Towards the end ajinomoto was added and kept for cooking for 3-5 min. The chilli sauce thus prepared was mixed thoroughly and portions of about 50 g each were taken in 6 different bowls. The 6 bowls of chilli sauce were then heated one after another in a microwave oven for 1,2,3,4,5 and 6 minutes successively. This treated chilli sauce was then analyzed for aflatoxins as described above.

3.5 STATISTICAL ANALYSIS

The results obtained after estimation of aflatoxins in different spices were subjected to analysis by one-way ANOVA.

Results and Discussion

CHAPTER -4

RESULTS AND DISCUSSION

Aflatoxins are toxic, secondary metabolites produced by common mold, *Aspergillus flavus* and *Aspergillus parasiticus* in a variety of food products. A global significance survey carried out in different parts of the world revealed that maize, groundnut, cotton, soybean, wheat, rice etc are commodities most affected by aflatoxins. The search for these aflatoxins in agricultural products has expanded to even spices during the last decade. Various spices, which were found to be contaminated with aflatoxins, are red chillies, ginger, nutmeg, coriander and turmeric. Among these spices, contamination of chilli or red pepper appeared to be the most prevalent. Studies on incidence of aflatoxin contamination in various spices in India is quite limited more so in Andhra Pradesh. Hence the present study is focussed on this aspect.

The present study was designed to provide an insight into the magnitude of aflatoxin contamination in spices in Andhra Pradesh, India. For this purpose, a total of 188 samples of 5 kinds of spices were collected from different areas of Andhra Pradesh and subjected to aflatoxin analysis by ELISA method. The spices selected were red chillies, black pepper, coriander, turmeric and dry ginger. The results of this study were presented and discussed in this chapter under the following heads:

4.1 Standardisation of Analytical Method

4.1 STANDARDISATION OF ANALYTICAL METHOD

Methods of detecting aflatoxins are broad and complex This complexity is due to the diversity of commodities contaminated with aflatoxins Several assay methods specific for commodities such as peanuts, cottonseed, corn, green coffee and mixed feeds have been developed based on the inherent nature of the commodity

In the present study the samples of spices were analysed using ELISA method for determination of aflatoxins Initially sample analysis was done in a few red chilli samples following the usual procedure in which the aflatoxin standard was prepared in buffer (B S A –9 3 ml + methanol – 0 7 ml) and used for aflatoxin estimation in the samples The AFB_1 values obtained by this method were very high with no consistency between the replicates of the samples (Table -4) To overcome this problem, the method was slightly modified and the standard was prepared in a healthy chilli extract (B S A – 9 ml + 1 ml healthy extract) instead of a buffer The standard curve is

presented in Fig- 3 for both the methods and there are significant differences between the values obtained by the two methods. The results obtained in the modified

Table 4: Aflatoxin estimation in red chillies with different method Variations

Sample No.	HPLC (μg/kg)	ELISA	
		I (μg/kg)	II (μg/kg)
1.	2.98	4.5	2.32
2.	242.36	572.6	283.00
3.	19.88	22.4	20.20
4.	129.06	388.9	165.30
5.	1.54	23.3	1.80

I - Standard prepared in buffer

II – Standard prepared in healthy extract

method were considerably low and consistent. To substantiate these results, another method of analysis: HPLC, which is considered to be more sensitive, was used. As is shown in the above table, a good correlation was found between the results of HPLC and modified ELISA methods. Hence, aflatoxin estimation of all the spice samples was done using this modified version of the ELISA method.

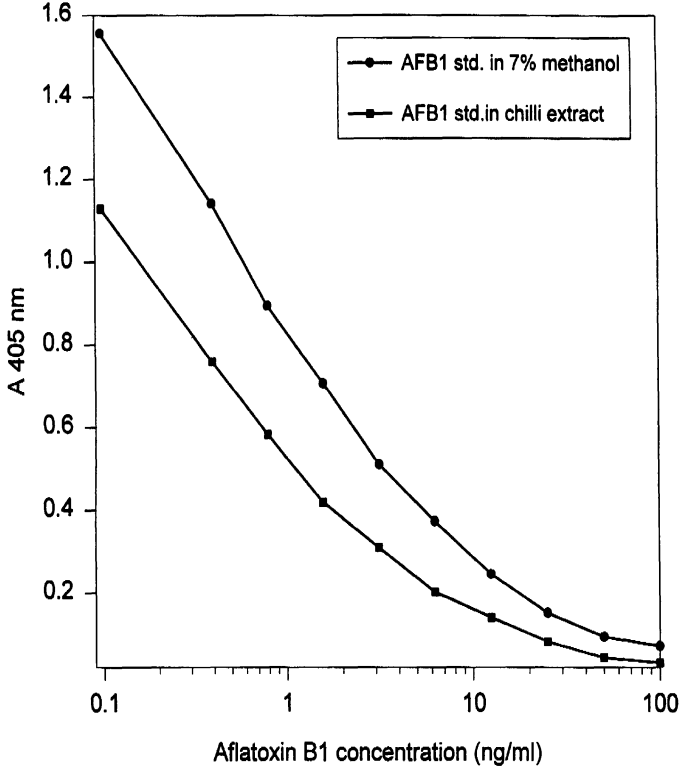


Fig. 3 Comparision of AFB1 standard curves prepared in chilli extract and 7% methanol

The very high and inconsistent values obtained in the normal ELISA method might have been due to presence of some interfering components in the spices. This particular problem of interfering co-extractives has been encountered by others also (Madhyastha and Bhat 1985, Hansen *et al.*, 1994 and Mac Donald and Castle 1996).

4.2 AFLATOXIN CONTAMINATION IN RED CHILLIES

Table 5: Aflatoxin levels in chilli samples (n = 59)

Range of Aflatoxins (µg/kg)	Number of samples	Percentage
0	26	44.06
1-10	18	30.50
10-50	7	12.00
50-100	2	3.40
> 100	6	10.00

Analysis was done in triplicates and mean values were taken

Level of significance between replicates – NS

A total of 59 red chilli samples were collected from major market yards of Andhra Pradesh for aflatoxin analysis. Results shown in Table 5 indicate the aflatoxin levels in red chilli samples. Out of these 59 samples analysed, 26 samples had no aflatoxin. While 18 samples had less than 10 µg/kg. In seven

samples aflatoxin levels ranged between 10-50 μg/kg and 2 samples had above 50 μg/kg aflatoxin. Highest levels i.e., above 100 μg/kg were detected in 6 chilli samples.

The data on aflatoxin contamination in red chillies shows that nearly half of the samples (44%) were not contaminated with aflatoxins. And 30.50 per cent of samples with less than 1 μg/kg aflatoxin. All the seven samples (12%) which had aflatoxin in the range of 10-50 μg/kg, recorded an average of 25 μg/kg aflatoxin. Thus more than 75 per cent of the red chilli samples had aflatoxin level below the permissible level of 30μg/kg.

While only 2 samples (3.40%) had aflatoxin levels above 50 μg/kg, 10 per cent of the samples were detected to be having higher levels of aflatoxin i.e., above 100μg/kg. The maximum level of aflatoxin recorded was 401 μg/kg. Such high toxin level (above 500 μg/kg) in red chillies, were reported in earlier studies (Shank *et al.*, 1977 and Fufa and Urga 1996).

Normally, matured chilli fruit, which is red in colour, is harvested during Jan-Mar every year in Andhra Pradesh. The harvested chilly fruits are spread on the drying floor for natural drying under the bright sunshine. After drying, the farmers separate the red chillies into different grades based on size, colour of the chilli pod and extent of damage (insect/physical damage) to the chillies. Thus the graded chilli samples were collected from the different market yards and the physical observations were recorded. The chilli samples

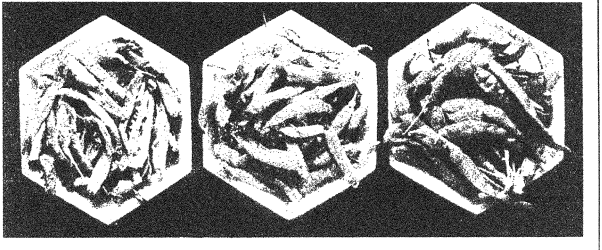


Plate 2 Three grades of chilli samples

which are not fully grown, dicoloured and severely damaged were categorised as low grade and those which were slightly damaged with medium size and colour were considered as medium quality samples. Superior grade samples were those which were brightly coloured and fully-grown with no damage.

After drying and grading of the chillies, before packing into gunny bags, farmers add little water to the fully dried chillies. The practice of addition of water to the dried chillies, is to break the brittleness of the dried chillies. Due to addition of water the chilli pod becomes soft, flexible and becomes convenient for packing in the gunny bags without any further pod damage. Moreover farmers add water to chillies with an intention to increase the weight of the produce also. Then these chillies with moisture packed in the bag goes to storage before they are used. At storage, the chillies with this moisture are prone to *A. flavus* infection and aflatoxin contamination. To avoid aflatoxin contamination during storage, there is a need to develop suitable packing and storage practice for redchillies in Andhra Pradesh.

The comparison of extent of aflatoxin contamination among these three grades of samples shows that the frequency of contamination is almost similar in all the samples but only differing only in the level of aflatoxins. (Fig-4)

The poor quality samples when compared with the other two grades of samples had highest percentage (46%) of samples having aflatoxin levels more than 30 μg/kg. Majority of the samples having high aflatoxin level (even above 100 μg/kg) were those belonging to the poor quality samples. In the

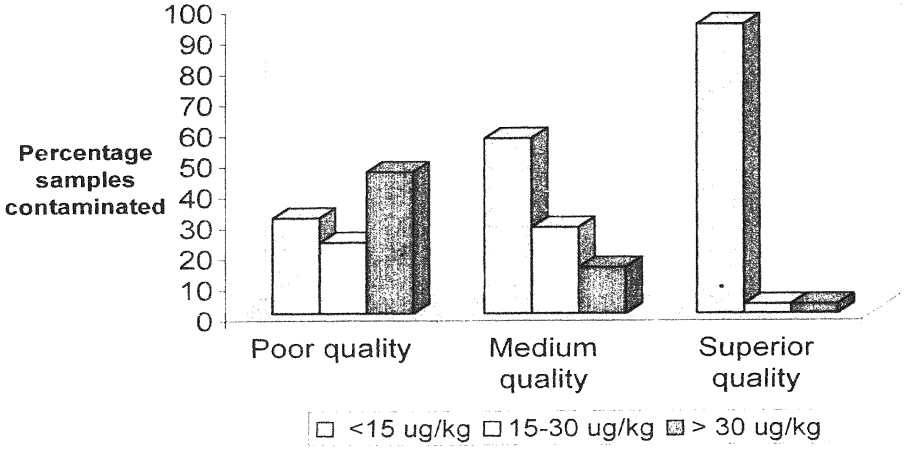


FIG.4 Percentage of chilli samples contaminated according to aflatoxin levels in different grades of chillies

medium quality samples, only 15 per cent of the samples were having above 30 µg/kg toxin, while majority of these samples (57%) had aflatoxin below 15 µg/kg. The superior quality samples had maximum number of samples (94%) below 15µg/kg aflatoxin range and only negligible percentage of samples (3%) had above 15 µg/kg aflatoxin.

The high aflatoxin levels as well as frequency of contamination in the low and medium quality samples might have been due to the high insect/physical damage and discolouration seen in these samples.

Similar observations were made by a study on aflatoxin contamination in spices (Singh 1983). It was reported that spices in general and red chillies in particular were more prone to aflatoxin contamination because of faulty agricultural as well as storage practices.

4.3 AFLATOXIN CONTAMINATION IN BLACK PEPPER

From the Table- 6, which shows the aflatoxin levels in pepper samples, it is evident that out of 28 samples, 10 had aflatoxins in the range of 1-10 µg/kg and 16 samples had between 10-50 µg/kg. And in only 2 samples aflatoxin levels of above 50 µg/kg were detected.

Table 6: Aflatoxin levels in pepper samples (n = 28)

Range of aflatoxins (µg/kg)	Number of samples	Percentage
0	0	0.0
1-10	10	35.7
10-50	16	57.1
50-100	2	7.2
> 100	0	0.0

Analysis was done in triplicates and mean values were taken

Level of significance between replicates – NS

In more than half of the samples (60%) the aflatoxin levels ranged between 10-15 µg/kg with an average value of 20 µg/kg, while 36.00 per cent of the samples were detected to be having less than 10 µg/kg aflatoxin. Thus the results of the aflatoxin contamination in pepper showed that in maximum number of samples (93%), the aflatoxin level was below the permissible level of 30 µg/kg. A maximum level of 64 µg/kg was detected in one pepper sample.

The above results conform with those of other studies which indicate aflatoxin contamination upto 35 µg/kg in black pepper (Aziz & Youssef 1991

and El Kady *et al.*, 1995), and also the results of a study in India which reported an aflatoxin level of 1.5-57.5 μg/kg (Madhyastha 1985).

Fig. 5 shows the level of aflatoxin contamination in wholesale and retail samples. In the wholesale samples, all the samples had aflatoxin level below 30μg/kg with 55.00 per cent of the samples having below 15 μg/kg and 45.00 per cent ranging between 15-30 μg/kg. While in retail samples, 41.00 per cent of samples were detected to be having below 5 μg/kg and an equal percentage of samples had between 15-30 μg/kg. The remaining 18.00 per cent of the samples had above 30 μg/kg. This shows that though the frequency of contamination is similar between the two varieties of pepper samples, high aflatoxin levels were detected in the samples bought from retail shops. Similar results were observed in earlier studies conducted by Bhat(1988) and Quitco *et al.*,(1989) indicates that aflatoxin contamination may occur during transit and during storage in retail shops.

4.4 AFLATOXIN CONTAMINATION IN CORIANDER

The results of the aflatoxin analysis of the coriander samples indicate that all the 50 samples were having less than 10μg/kg aflatoxin (Table- 7). Out of these 50, 44 samples were without any contamination. Only in 6 samples (12%) the aflatoxin levels ranged between 1-10 μg/kg. The maximum level detected was only 3.2 μg/kg.

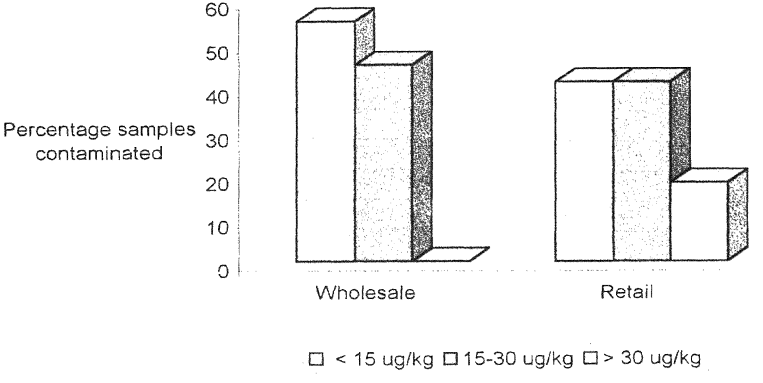


FIG. 5 Percentage of pepper samples contaminated according to aflatoxin levels in wholesale and retail shops

Table 7 : Aflatoxin levels in coriander samples (n=50)

Range of aflatoxins (μg/kg)	Number of samples	Percentage
0	44	88
1-10	6	12
10-50	0	0
50-100	0	0
> 100	0	0

Analysis was done in triplicates and mean values were taken

Level of significance between replicates – NS

The results of the present study are lower when compared to some studies that reported a toxin level of 75 μg/kg in coriander (Misra 1987 and Llewellyn *et al.*, 1992). But Majerus and Woller (1985) and Aziz and Youssef (1991) made similar observations. They reported a maximum aflatoxin level of about 8 μg/kg in coriander. Toxin levels can vary depending on several factors such as level and stage of contamination, method of storage and other favourable factors.

4.5 AFLATOXIN CONTAMINATION IN TURMERIC

Table 8 : Aflatoxin levels in turmeric samples (n=26)

Range of aflatoxins (μg/kg)	Number of samples	Percentage
0	12	46
1-10	14	54
10-50	0	0
50-100	0	0
> 100	0	0

Analysis was done in triplicates and mean values were taken

Level of significance between replicates – NS

All the 26 turmeric samples analysed for aflatoxins showed toxin level below 10μg/kg (Table 8). Out of these 26 samples, 12 had no aflatoxin and 14 samples had aflatoxin level between 1-10 μg/kg, i.e., nearly half of the samples (46.00%) had below 1 μg/kg. The maximum level recorded in the turmeric samples was 9.3 μg/kg.

Similar results were reported in studies by Scott and Kennedy (1975) and Aziz and Youssef (1991) which indicated maximum values of 3.8 μg/kg and 12μg/kg respectively in turmeric samples.

The lower levels of aflatoxin observed in turmeric may be due to its composition and other protective factors such as curcumin present in turmeric.

4.6 AFLATOXIN CONTAMINATION IN DRY GINGER

In the analysis of ginger samples for aflatoxins, out of 25 samples only 9 samples were detected to be having aflatoxin levels in the range of 1-10 μg/kg and the rest of the samples (16) had no aflatoxin (Table 9).

Studies on aflatoxin contamination in ginger reported aflatoxin levels upto 25 μg/kg (Scott and Kennedy 1975, Madhyastha 1985 and Garner *et al.*, 1993). However, the results of the present study showed a toxin level of below 10 μg/kg in all the ginger samples and majority of the samples (64%) were without any toxin (Table 9). Such variations in toxin levels are possible depending on varietal differences, place of cultivation and other agro-climatic variations.

Table 9 : Aflatoxin levels in ginger samples (n=25)

Range of aflatoxins (μg/kg)	Number of samples	Percentage
0	16	64
1-10	9	36
10-50	-	-
50-100	-	-
> 100	-	-

Analysis was done in triplicates and mean values were taken

Level of significance between replicates – NS

4.7 SUMMARY OF SPICES ANALYSED FOR AFLATOXINS

Table- 10 shows the levels of aflatoxin contamination in all the five kinds of spices. A perusal of the data shows that of all the spices analysed for aflatoxins, pepper showed the highest percentage of contamination (100%) followed by red chillies, ginger and turmeric with 56, 54 and 36 percentages respectively. The lowest percentage of contamination was seen in coriander with 12.00 per cent. (Fig-6)

Table 10: Aflatoxin contamination in 5 kinds of spices

Sample	Total number	Samples contaminated		Samples with aflatoxin B_1 levels		Range of aflatoxin B_1 levels (µg/kg)
		Number	%	>30µg/kg	<30µg/kg	
1. Red chillies	59	33	56	9	24	2-401
2. Black pepper	28	28	100	3	25	3.3-64
3. Coriander	50	6	12	ND*	6	0.2-3.2
4. Turmeric	26	14	54	ND	14	2.0-9.3
5. Dry ginger	25	9	36	ND	9	2.2-9.4

* ND = Not detected

However, a real picture of extent of aflatoxin contamination is given by the data on the number of samples with AFB_1 levels below or above the

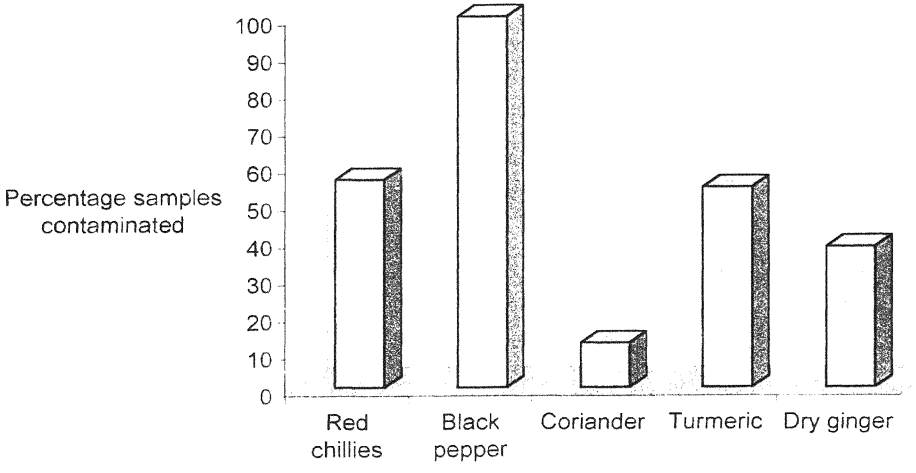


FIG.6 Percentage aflatoxin contamination in spices

prescribed limit for human consumption given by W.H.O (30 μg/kg) and the range of aflatoxin levels in these commodities.Of all the commodities analysed, 9 red chilli samples and 3 pepper samples had aflatoxin levels above 30 μg/kg. The highest toxin level was recorded in a red chilli sample with 401 μg/kg. The maximum level in pepper sample was 64 μg/kg. In the rest of the commodities, all samples had aflatoxin levels below 10μg/kg.

Similar observations of high aflatoxin levels as well as high frequency of occurrence in red chillies were reported in earlier studies also (Shank *et al.*, 1972, Flannigan and Hui 1976, Garrido *et al.*, 1992, Llewellyn *et al.*, 1992 and Mac Donald and Castle 1996).

4.8 EFFECT OF COOKING ON THE STABILITY OF AFLATOXIN

Table 11: Aflatoxins in chilli sauce after microwave cooking

Time (min.)	Aflatoxin B_1 (μg/kg)
0	130.3
1	134.6
2	135.5
3	176.5
4	134.3
5	165.4

Analysis was done in duplicates and mean values were taken

Red chilli sauce prepared with naturally contaminated red chillies was subjected to microwave heating at high temperature for different lengths of time (in min) to test the stability of aflatoxins during cooking From Table- 11 which shows the aflatoxin levels in chilli sauce after microwave cooking, it can be seen that no change occurred in the aflatoxin levels of chilli sauce even after subjecting it to microwave heating The initial and final AFB_1 values remained almost equal with 130 3 and 165 4 µg/kg respectively

The samples when heated at high temperature for more than 5 min got completely burnt So, the experiment was terminated at 5 min The present results indicate the stability of aflatoxins to heat as no loss has occurred even after microwave heating

Aflatoxins were found to have high melting points of 260°C with thermal degradation temperature of 269 °C and hence are very stable to heat (Ciegler and Vesonder 1983) The results also conform with those reported by Mac Donald and Castle (1996) which showed that aflatoxin levels in spice sauces are not reduced by domestic cooking with either microwave or conventional gas oven cooking The present experiment also proves that microwave heating is not effective in destroying the aflatoxins in foods

Results of the present study indicate that the frequency and level of aflatoxin contamination in spices studied is quite high In India, these spices are used in various food preparations almost daily Among these spices red

chillies are consumed in significant amounts, especially among low-income population in preparation of chutneys which is consumed along with rice/rotis.

Under these circumstances, consumption of contaminated chillies leads to high levels of aflatoxin intake and the effects of toxicity is high in a malnourished host. Hence, it is essential to take steps to prevent the aflatoxin contamination in not only major agricultural commodities like cereals, pulse and oil seeds but also in spices which are consumed almost everyday by Indians.

Summary and Conclusions

CHAPTER –5

SUMMARY AND CONCLUSION

A variety of contaminants are found naturally occurring in foods. Of these mycotoxins, the secondary metabolites of various species of fungi have gained much significance both in the health and economic sector. Among the mycotoxins, much attention has been focussed on aflatoxins produced by *A. flavus* and *A.parasiticus* in stored grains. AFB_1 the most toxic and abundantly found among the series of aflatoxins, continues to be a major problem in risk commodities like groundnut, maize and chillies. While aflatoxin contamination in commodities like cereals assumes significance from the health point of view, its problem in cash crops like groundnut and chillies affects trade and economy.

The present study was conducted to determine the extent of aflatoxin contamination in spices in Andhra Pradesh. The samples of spices were collected from market yards, wholesale and retail shops in different areas of Andhra Pradesh. A total of 59 red chillies samples, 28 black pepper, 50 coriander, 26 turmeric and 25 dry ginger samples were collected for the estimation of aflatoxins. The collected samples were subjected to aflatoxin estimation by ELISA method of analysis.

Of the 59 red chilli samples analysed for aflatoxins, nearly half of the samples (44 %) had no aflatoxin. Higher levels of aflatoxin i.e., above 100 µg/kg were recorded in 10 per cent of the samples. The maximum level of

AFB_1 seen in red chillies was 401 μg/kg. On the whole, 75per cent of red chilli samples, recorded AFB_1 levels below the permissible level of 30 μg/kg. Among the three grades of chillies analysed, superior grade samples recorded low aflatoxin levels with 90 per cent of them having less than 15 μg/kg while low-grade samples recorded high aflatoxin levels and high frequency of contamination.

AFB_1 estimation in 28 black pepper samples showed that all the samples ranged between 1μg/kg and 100 μg/kg. Majority of these samples (93%) had AFB_1 below the 30 μg/kg and only 2 samples had above 50 μg/kg. The maximum level recorded was 60.4μg/kg. The results showed little variation in the incidence of contamination between the wholesale and retail samples. But two pepper samples which recorded high values of above 50 μg/kg were from retail shops.

In the remaining three kinds of spices i.e., coriander, turmeric and dry ginger all the samples recorded less than 10 μg/kg toxin. Among these, coriander had lowest level of contamination with only 12 per cent, while in turmeric and dry ginger 54 and 36 percentage contamination was seen respectively.

Heat stability of aflatoxins in spices was tested by performing cooking experiments with red chilli sauce prepared with contaminated red chillies. The results showed that microwave heating was not effective in destroying the

aflatoxin in foods as no change was observed in AFB_1 levels even after subjecting to microwave heating at high temperature for different lengths of time (upto 5 min.).

In conclusion it can be said that among all the spices, red chillies is the most often contaminated spice. Red chillies form a major ingredient in many of the Indian food preparations like chutneys and pickles, in addition to this, red chillies are one of the important export commodities in India. So, contamination of red chillies has both health and economic implications. As the results of the present study indicate that homescale cooking methods like microwave cooking prove to be ineffective in reducing the amount of aflatoxins in spices. Extensive research need to be done to identify the exact factors responsible for aflatoxin contamination in spices and take necessary preventive measures like following proper drying methods and safe storage practices to minimise the incidence of contamination.

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Appendices

APPENDIX-1

Aflatoxin estimation by HPLC (AOAC, 1990):

Sample preparation

For 1 gm sample

50 gm of powdered sample

↓ 250 ml of acetone-H_20 (85:15)

Add 5 ml of Acetone-H_20 (85:15)

↓

Take in a conical flask and shake for half an hour

↓

Filter and add 10ml of 20%lead acetate 3 H_20

(Prepared in 0.3% acetic acid in H_20) to the filtrate

↓

Let the precipitate settle completely (allow for 10min.)

↓

Add 40 ml H_20, shake and filter into a separating funnel – Extract the filtrate with 50 ml of chloroform

↓

Collect lower chloroform layer and dry over anhydrous sodium sulphate

↓

Filter and evaporate $CHCl_3$

Cleaning of sample on column

Pack the column with silica gel (column grade 60-120 Qualigens SQ)

↓

Prepare a slurry by adding 25 g of silica gel in 3:1 of ether-hexane mixture

↓

Transfer the slurry completely to the column. Add 1-2 gm of anhydrous Na_2S0_4 to the top of the column and let the solvent layer be 1 inch on top of the Na_2S0_4 layer

↓

Dissolve the dried residue in 1 ml of chloroform and slowly add to the column. Rinse 3-4 times with 1 ml of chloroform each time and slowly add to the column

↓

Open stopper and drain it out

↓

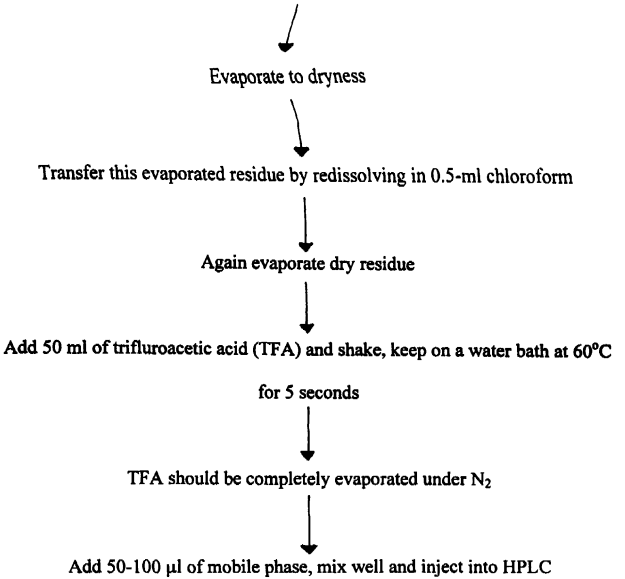
Wash the column with benzene acetic acid (25 ml of 9:1 and open the stop cock to maximum flow rate)

↓

Next wash with 50 ml of (3:1) ether: hexane

↓

Finally dilute the toxin into a fresh beaker with 60 ml of (9:1) chloroform:acetone


Evaporate to dryness
Transfer this evaporated residue by redissolving in 0.5-ml chloroform
Again evaporate dry residue
Add 50 ml of trifluroacetic acid (TFA) and shake, keep on a water bath at 60°C
for 5 seconds
TFA should be completely evaporated under N_2
Add 50-100 µl of mobile phase, mix well and inject into HPLC

APPENDIX-2

ANOVA tables for 5 types of spices analysed:

RED CHILLIES

Source	Degrees of freedom	Sum of squares	Mean square	F value
Model	60	990405.67830508	16506.76130508	24.96
Error	116	76705.66011299	661.25569063	
Corrected total	176	1067111.33841808		

BLACK PEPPER

Source	Degrees of freedom	Sum of squares	Mean square	F value
Model	29	16553.41440476	570.80739327	13.21
Error	54	2333.12880952	43.20608907	
Corrected total	83	18886.54321429		

CORIANDER

Source	Degrees of freedom	Sum of squares	Mean square	F value
Model	51	130.78553333	2.56442222	4.66
Error	98	53.90040000	0.55000408	
Corrected total	149	184.68593333		

TURMERIC

Source	Degrees of freedom	Sum of squares	Mean square	F value
Model	27	434.68243590	16.09934948	6.05
Error	50	132.98794872	2.65975897	
Corrected total	77	567.67038462		

DRY GINGER

Source	Degrees of freedom	Sum of squares	Mean square	F value
Model	26	474.66720000	18.25643077	11.83
Error	48	74.06426667	1.54342222	
Corrected total	74	548.75146667		

APPENDIX-3

Estimated aflatoxin levels (μg/kg) in five kinds of spices:

I.	RED CHILLIES			II.	CORIANDER		
1.	3.53	40.	33.9	1.	0	40.	0
2.	21.9	41.	2.8	2.	0	41.	0
3.	23.3	42.	3.7	3.	0	42.	3.2
4.	5.7	43.	19.03	4.	0	43.	2.4
5.	11.2	44.	0	5.	0	44.	0
6.	18.7	45.	8.1	6.	0	45.	0
7.	21.03	46.	4.4	7.	0	46.	0
8.	0	47.	109.06	8.	0	47.	2.3
9.	0	48.	2.4	9.	0	48.	0
10.	0	49.	6.06	10.	0	49.	0
11.	0	50.	0	11.	0	50.	3.4
12.	401.1	51.	73.3	12.	0		
13.	0	52.	0	13.	0		
14.	0	53.	6.5	14.	0		
15.	0	54.	0	15.	0		
16.	0	55.	0	16.	0		
17.	7.5	56.	0	17.	2.1		
18.	5.8	57.	0	18.	0		
19.	10.1	58.	0	19.	0		
20.	53.1	59.	0	20.	0		
21.	0			21.	0		
22.	285.8			22.	0		
23.	4.2			23.	0		
24.	4.2			24.	0		
25.	0			25.	0		
26.	196.3			26.	0		
27.	0			27.	0		
28.	0			28.	0		
29.	234.9			29.	0		
30.	0			30.	0		
31.	7.8			31.	0		
32.	150.1			32.	0		
33.	5.4			33.	0		
34.	0			34.	0		
35.	3.5			35.	0		
36.	2.1			36.	0		
37.	0			37.	2.4		
38.	0			38.	0		
39.	10			39.	0		

III. BLACK PEPPER	IV. TURMERIC	V. DRY GINGER
1. 63.9	1. 3.1	1. 0
2. 9.3	2. 2.3	2. 0
3. 26.7	3. 0	3. 6.06
4. 60.03	4. 2.7	4. 0
5. 19.3	5. 2.8	5. 0
6. 12.2	6. 5.1	6. 0
7. 20.8	7. 4.5	7. 0
8. 15.03	8. 9.3	8. 5.06
9. 18.06	9. 0	9. 0
10. 21.2	10. 0	10. 2.2
11. 22.8	11. 0	11. 3.8
12. 10.4	12. 4.03	12. 0
13. 3.3	13. 0	13. 0
14. 5.3	14. 3.1	14. 0
15. 10.2	15. 3.4	15. 0
16. 9.96	16. 2.8	16. 0
17. 19.8	17. 0	17. 0
18. 18.7	18. 2.1	18. 0
19. 21.8	19. 0	19. 9.6
20. 20.5	20. 0	20. 8.3
21. 9.6	21. 0	21. 3.7
22. 20.4	22. 0	22. 3.4
23. 4.3	23. 3	23. 2.8
24. 8.1	24. 2.06	24. 0
25. 45.06	25. 0	25. 0
26. 5.2	26. 0	
27. 14		
28. 15.8		