RESEARCH ARTICLE

Identification and High-performance Liquid Chromatography Quantification of Aflatoxins in Red Chili (*Capsicum annuum L.*) in Guntur, Andhra Pradesh, India

Ommi Sailaja[1,2], G. Krishnaveni[2], M. Manoranjani[3]

[1]Department of Chemistry, Krishna University, Machilipatnam, Andhra Pradesh, India, [2]Department of Chemistry, KBN PG College, Vijayawada, Andhra Pradesh, India, [3]Department of Chemistry, PB Siddhartha College, Vijayawada, Andhra Pradesh, India

Abstract

Introduction: Aflatoxins are hepatogenic, teratogenic, immunosuppressive, and carcinogenic fungal metabolites found in feeds, nuts, wine-grapes, spices, and other grain crops. Humans are exposed to AFs through consumption of mycotoxin-contaminated foods. **Aim:** This study aimed to determine the prevalence of aflatoxin contamination in red chili samples collected at different locations in Guntur, Andhra Pradesh. **Materials and Methods:** Aflatoxins were extracted by liquid-liquid extraction method using chloroform solvent. The qualitative and quantitative analysis of aflatoxins present in the samples was analyzed using thin-layer chromatography and high-performance liquid chromatography techniques. **Results and Discussions:** Among the samples in the study, two samples S3 and S7 were not infected with aflatoxin producing fungi. In sample S5, aflatoxin G1, G2, and B2 were identified and G1 was found to be very high (21.32 ng/g). The high amount of aflatoxin B2 (34.02 ng/g) was observed in sample S2. Among the samples having aflatoxins, aflatoxin G2 was found in all the samples. Strict measures are necessary to produce high-quality red chilies. Red chili berry must be treated to reduce molds before the production of powders for consumption.

Key words: Aflatoxins, estimation, high-performance liquid chromatography, red chilies, thin-layer chromatography

INTRODUCTION

Aflatoxins are mycotoxins produced by certain molds (*Aspergillus flavus and Aspergillus parasiticus*) which can grow in soil, decaying vegetation, hay, and grains. These fungi can grow in wide range of food commodities, particularly cereals, oilseeds, spices, and tree nuts. Maize, groundnuts (peanuts), pistachios, brazils, chilies, black pepper, dried fruit, and figs.[1] Usually, these fungi are regularly found in improperly stored staple commodities. The aflatoxins are poisonous carcinogens consist of about 20 similar compounds belonging to a group called the difuranocoumarins, but only four (B_1, B_2, G_1, and G_2) are naturally found in foods. Aflatoxin B_1 is the most commonly found in food and also the most toxic.[2-5] High enough exposure levels, aflatoxins can cause acute toxicity and potentially death in mammals, birds, and fish as well as in humans. Children are particularly affected by aflatoxin exposure, which leads to stunted growth, delayed development,[3] liver damage, and liver cancer. Adults have a higher tolerance to exposure but are also at risk.[4] For most species, the LD_{50} (lethal dose) is between 0.5 and 10 mg/ kg body weight.[6] The liver is the principal target organ, although the site of the hepatic effect varies with species. A notable outbreak occurred in India in 1974 when almost 400 people became ill with fever and jaundice after eating maize contaminated with between 0.25 and 15 mg/ kg aflatoxin and more than 100 died. Major outbreaks have also occurred in

Address for correspondence:
Ommi Sailaja, Department of Chemistry, Krishna University, Machilipatnam, Andhra Pradesh, India.
Phone: +91-9985019882.
E-mail: sailajabotta@gmail.com

Received: 29-11-2017
Revised: 19-12-2017
Accepted: 27-12-2017`

Kenya, the largest in 2004 when 317 people were affected and 125 died, probably as a result of eating contaminated maize. Contaminated poultry feed is suspected in the findings of high percentages of samples of aflatoxin contaminated chicken, meat, and eggs in Pakistan.[7]

Chronic toxicity is probably more important from a food safety point of view, certainly in more developed regions of the world. It is important to recognize aflatoxins because these toxins are very stable and may pass through quite severe processes. For this reason, they can be a problem in processed foods. Aflatoxins are crystalline substances, freely soluble in moderately polar solvents such as chloroform, methanol, and dimethyl sulfoxide and dissolved in water to the extent of 10–20 mg/L. They fluoresce under ultraviolet (UV) radiation. Chilies are one of the most valuable crops grown all over India, which is used as vegetable, spice, condiment, sauce, and pickle. Guntur district is the main producer and exporter of most varieties of chilies and chili powder from India to countries such as Sri Lanka, Bangladesh, Middle East, South Korea, UK and USA, and Latin America.[8] The annual production of this type is approximately 280,000 tons. The present study is aimed to investigate the aflatoxin contamination on mold infected chilies collected from various storage area of Guntur, Andhra Pradesh, India. Few works are reported in literature for the estimation of aflatoxins in different red chili varieties.[9-18]

MATERIALS AND METHODS

Collection of samples

All the samples were randomly collected from different sources in Guntur, Andhra Pradesh.

The sample collection details and codes given for collected samples were given in Table 1.

Instrumentation

Chromatographic separation was performed on a PEAK chromatographic system equipped with LC-P7000 isocratic pump; Rheodyne injector with 20 µl fixed volume loop, separation was achieved on Inertsil ODS Column (250 mm × 4.6 mm, 5 um); variable wavelength programmable UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. 1.5 L ultrasonicator was used to sonicating the mobile phase and samples. Standard and sample drugs were weighed using Denver electronic analytical balance (SI-234) and pH of the mobile phase was adjusted using Systronics digital pH meter.

Table 1: Sample collection locations

Sample location	Sample code
Cold Storage, Near Mirchi yard, Guntur	S1
Mirchi Yard dump Area, Guntur	S2
Warehouses, Market Area, Guntur	S3
Supermarket, Market Area, Guntur	S4
Packing establishment warehouses, Chuttugunta, Guntur	S5
Household aged sample	S6
Smaller shops, Market Area, Guntur	S7

Materials

Analytical standard aflatoxin B1, G1 having concentration of 2 µg/mL in acetonitrile, aflatoxin B2, G2 with 0.5 µg/mL in acetonitrile were purchased from Sigma-Aldrich. Water, acetonitrile, and methanol used were of high-performance liquid chromatography (HPLC) grade and were purchased from Merck chemicals private limited, Mumbai. Samples and mobile phase were filtered using 0.2 µ nylon membrane filter paper purchased from Merck- Millipore private limited, Mumbai.

Preparation of standard solution

Accurately measured 0.1 ml from aflatoxin B1 and G1, 0.4 ml from aflatoxin B2 and G2 were made up to 20 ml separately using methanol. Standard concentration having 10 ng/ml aflatoxin was obtained. From this, required dilutions were prepared accurately. Equal volume of the prepared four aflatoxins B1, B2, G1, and G2 was mixed and the mixed solution was used for HPLC analysis.

Extraction of aflatoxins

For simultaneous extraction of aflatoxins modified method of Braic*u et al*., 2008[19] was used.

Thin-layer chromatography (TLC) separation of aflatoxins

The plate was first eluted with anhydrous diethyl ether, dried up in a fume hood for 5 min, and developed with chloroform and acetone in the ratio of 9:1 (v/v) at same direction.[21] The TLC plate was visually examined under ultraviolet light at 366 nm.

HPLC analysis of aflatoxins

Method conditions

HPLC analysis was carried for the quantification of aflatoxins present in the samples. For HPLC analysis, the method described by Herzallah *et al*., 2009[24] was adopted. Chromatographic separation was carried on Inertsil ODS C-18 (250 × 4.6 mm; 5 µ id) column using water, acetonitrile,

and methanol in the ratio of 60:20:20 (v/v) as mobile phase at a flow rate of 1.0 ml/min. UV detection was carried at a wavelength of 365 nm. Sample volume of 20 µL was injected into HPLC column maintained at 40°C.

Construction of calibration curve

The prepared aflatoxins calibration curve dilutions were analyzed in the HPLC method. The peak area response of each aflatoxin was used for the construction of calibration curve by considering prepared concentrations on x-axis and peak area response on y-axis. The obtained regression equation was used for the estimation of aflatoxin content in chili samples.

Sample analysis

The extracted aflatoxin samples were analyzed in the HPLC method and the peak area response of the resultant chromatogram was substituted in the standard regression equation and the aflatoxin content in the samples was calculated.

RESULTS AND DISCUSSIONS

TLC separation method was used for the qualitative determination of aflatoxins present in the samples. TLC results showed prominent spots for each aflatoxin. Samples S3 and S7 showed only one spot indicating one aflatoxin presents in those samples. S2 and S5 showed two and three spots indicated that two and three types of aflatoxins are present in those samples, respectively. No spots were observed in S1, S4, and S6 confirmed that the samples have no fungal infection and do not have aflatoxins. The results of aflatoxin TLC separation study were given in Figure 1.

HPLC analysis of collected naturally contaminated red chili samples showed the presence of aflatoxins. The amount of aflatoxins presents in the samples was estimated using standard aflatoxin calibration curve [Table 2]. Calibration curve was obtained in the concentration range of 0.5– 3.0 ng/ ml for all the four standard aflatoxins. Standard regression equation was found to be y = 17336x – 1044, y = 23420x - 270.7, y = 20670x – 1229, and y = 17641x - 96.76 for aflatoxin G1, G2, B1, and B2, respectively. The obtained standard regression equation was used for the estimation of aflatoxin content in samples. The standard chromatogram was given in Figure 2. Linearity results were given in Table 3 and calibration curves were shown in Figure 3.

The extracted samples were analyzed in the HPLC method. The chromatogram of samples S1, S4, and S6 do not show any detections confirms that the aflatoxins present in these samples were found to be below the detection limit or the samples do not infect with aflatoxin producing fungi. The chromatograms of S3 [Figure 4] and S7 [Figure 5] show single peak representing the presence of one aflatoxin and the retention time of the compound was found to be same as standard aflatoxin G2. This confirms that the samples S3 and S7 were found to be having aflatoxin G2 and the quantity was found to be 19.40 and 12.86 ng/g for S3 and S7, respectively. The chromatogram obtained for sample S2 [Figure 6] shows two peaks representing the presence of two aflatoxins, and by comparing the retention times, it was confirmed that the sample having aflatoxins G2 and B2. The high amount of aflatoxin B2 (34.02 ng/g) was observed in sample S2. Among the samples in the study, more number of aflatoxins (G1, G2, and B2) was identified in sample S5 [Figure 7] and the quantity of aflatoxin G1 was found to be very high (21.32 ng/g). It is well known that growth of molds and consequent mycotoxin production is dependent on a number of factors such as temperature, humidity, handling during the harvesting, and storage.[22,23] Aflatoxins continue to pose a health risk through human exposure to contaminated spices. Routine controls and survey studies have to be performed for the detection of aflatoxin contamination in spices. The data presented in this study showed one of the highest levels of aflatoxin accumulation was observed in red chili samples. The data also reflected that production of ground red chili on surfaces with soil contact could be a major reason of *A. flavus* and *A. parasiticus* contamination and further accumulation of aflatoxins. Strict regulations should be imposed for preventing the production of aflatoxins.

Table 2: Amount of aflatoxin content in the samples

Sample	Compound	Peak Area	Amount Present (ng/g)
S1	No peak	-	BDL
S2	G2	36281.6	15.607
	B2	59914.1	34.018
S3	G2	34120.8	19.397
S4	No peak	-	BDL
S5	G2	20138.4	8.714
	G1	35916.8	21.320
	B2	11693.5	6.683
S6	No peak	-	BDL
S7	G2	29846.2	12.859

Table 3: Standard aflatoxins calibration curve results

Concentration in µg/ml	Peak area obtained for			
	G2	G1	B2	B1
0.5	11391.4	8263.0	9124.2	9869.8
1.0	22957.5	15817.5	17289.1	19381.3
1.5	34587.1	24577.3	26040.3	28632.1
2.0	47570.6	33293.4	35158.2	39958.5
2.5	58094.4	42821.9	44059.5	50589.6
3.0	69681.7	50994.6	52983.5	61223.9

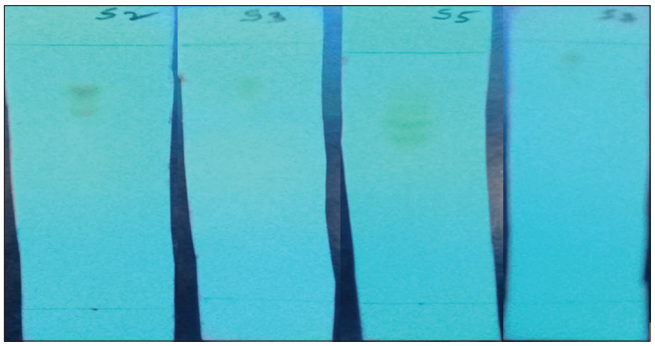

Figure 1: Thin-layer chromatography results

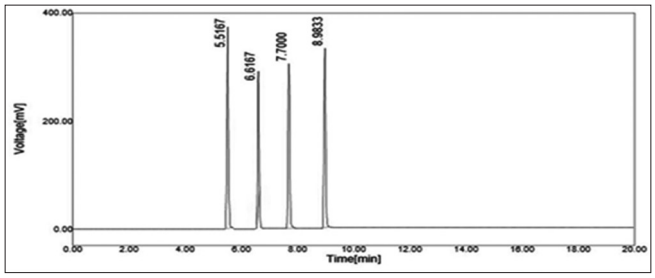

Figure 2: Standard chromatogram of aflatoxins at 4 ng/ml

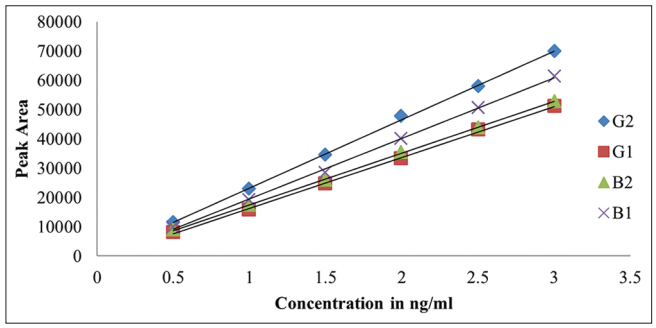

Figure 3: Standard calibration curves for aflatoxins

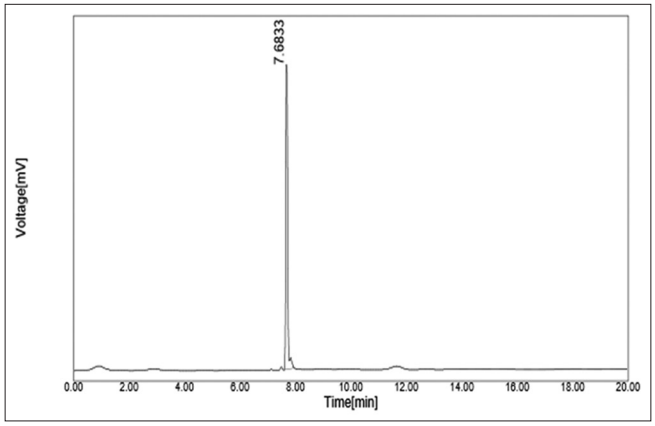

Figure 4: Chromatogram of S3

CONCLUSION

TLC separation and HPLC quantification methods were adopted for the estimation of aflatoxins content present in different red chili varieties collected at different locations

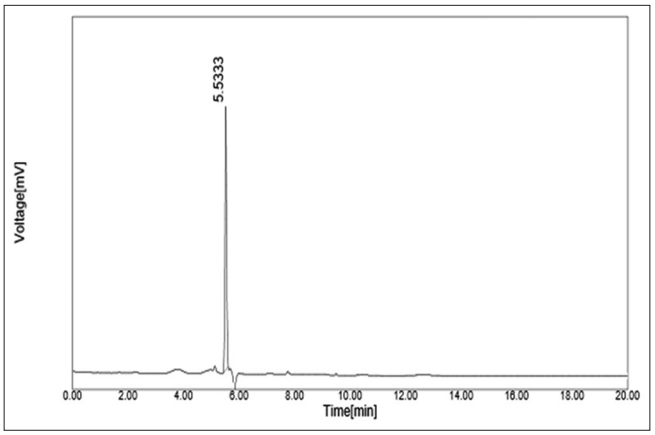

Figure 5: Chromatogram of S7

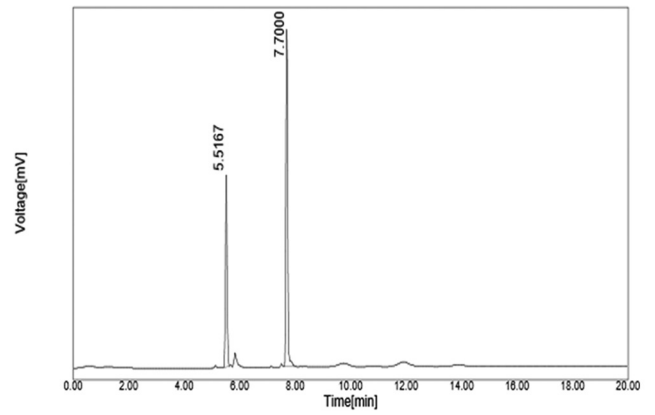

Figure 6: Chromatogram of S2

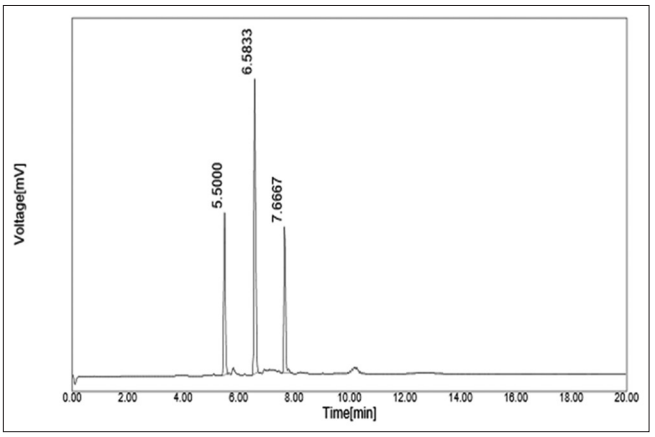

Figure 7: Chromatogram of S5

in Guntur, Andhra Pradesh, India. The results showed that four out of seven samples were found to having aflatoxins. On the basis of the achieved results, it is concluded that high levels of aflatoxins were detected in red chili samples, which may be controlled by adopting rather vigorous preventive measures, such as proper harvesting, drying, handling, packaging storage, as well as transportation. Finally, high standards are required to enhance exports and for the sake of the nation's health. Further, research on the prevention of

mold contamination growth and aflatoxins formation in red chilies from harvesting to sale is needed.

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Source of Support: None. **Conflict of Interest:** None declared.