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Prevalence of aflatoxin contamination in pulses and spices in different regions of Punjab

Arif Nazir*[,1], Imran Kalim[2], Muhammad Sajjad[1], Muhammad Usman[1] and Munawar Iqbal[1]

[1]Department of Chemistry, The University of Lahore, Lahore, Pakistan
[2]Food & Biotechnology Research Centre, PCSIR Laboratories Complex, Lahore, Pakistan
*Corresponding author's E. mail: anmalik77@gmail.com

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ABSTRACT

The aflatoxin contamination of food products is a prime parameter to analyze the quality of food. The present work specifically involves the determination of aflatoxin content of food through chromatographic technique. Pakistan Food Authority and European Union Regulations were followed for quantitative analysis of AFTs in pulses and spices. The samples were collected from different regions of Punjab including Sialkot, Narowal, Gujranwala and Gujrat. The AFTs detected in 120 samples, as per detection limits defined by regulatory authorities i.e. 50 ppb for spices and 4 ppb for pulses through Thin Layer Chromatography (TLC). Maximum AFTs was observed in (spices) red chili samples from Gujranwala city (55.5 ppb), black pepper samples from Narowal city (65.9 ppb), coriander samples from Shakar Garh (67.9 ppb), cumin samples from Daska (63.9) and aniseed samples from Sambrial (52.5 ppb). On the other hand, maximum AFTs for pulses was observed in split chick pea sample from Lahore (11.2 ppb), lentils samples from Kingra (8.6 ppb) and black gram beans from Gujrat Fuwara Chowk (15.4 ppb). This study provides awareness to the public for AFTs contamination in different food commodities.

Capsule Summary: Analysis of AFTs in different food samples including pulses (split chick pea, lentils, black gram beans) and spices (red chili, black pepper, cumin, coriander) was performed using chromatographic approach as a detection tool and most of the samples revealed AFTs contamination.

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INTRODUCTION

Food is one of the fundamental needs of life. The rapid growth in population raised the food intake requirement. The extremely safe handling, storage and processing conditions are required as the number of commodities increased day to day. Microorganisms are present everywhere in our environment. Depending upon the state of occurrence, the microorganisms can either be poisonous or nonpoisonous (Hassan et al., 2017; Iqbal et al., 2019; Mouhamd et al., 2017). Many malignant metabolites are being produced by plant pathogens like Fungi. Mycotoxins, the vesicant secondary metabolites, are being produced by threadlike fungus during chemical or enzymatic reactions. Human beings, animals and plants are largely affected by these microorganisms (Abbas et al., 2018a; Abbas et al., 2018b; Binder et al., 2007; Iqbal et al., 2017; Noreen et al., 2017). Mycotoxins contamination spoil

Table 1: Mycotoxins limits fixed by some countries for spices and other pulses products (FAO 2016)

S. No.	Country/Organization	Product	Aflatoxin B1 (µg/kg)	Total Aflatoxin (µg/kg)
1	Bulgaria "**	Spices	2	5
2	Croatia	Spices	30	NA
3	Cuba	All pulses	NA	5
4	Czech Republic "**	Spices	20	NA
5	European Union	Spices*	5	10
6	Finland" **	All Spices	NA	10
7	Hong Kong	All pulses stuffs	15	15
8	Iceland	Spices	5	10
9	India	All Pulses	NA	30
10	Indonesia	Spices powder	15	20
11	Iran	Spices	5	10
12	Jamaica	Pulses and Grains	NA	20
13	Japan	All pulses	10	NA
14	Liechtenstein	Spices	5	10
15	Malaysia	All pulses	NA	35
16	Mauritius	All pulses	5	10
17	Morocco	All pulses	10	NA
18	Nigeria	All pulses	20	
19	Norway	Spices	5	10
20	Oman	Complete pulses stuffs	10	10
21	Pakistan	Chili		30
22	Singapore	All pulses	5	5
		Pulses for infants	0.1	NA
23	Pakistan	All pulses stuffs	4	4
	Sri Lanka	All pulses		30
24	Switzerland	Spices	5	10
	Thailand	All pulses		20
25	USA	All pulses except milk***	NA	20
	Uruguay	All pulses and spices	5	20
26	Vietnam	All Pulses	NA	10
	Zimbabwe	All Pulses	5	NA

huge amount of crop per year (Sabahat et al., 2010). The most common mycotoxin found in staple food is aflatoxins (AFTs). The temperate regions provide most favorable conditions for the growth of AFTs producing fungi. Unfortunately, Pakistan is present in this region. IARC ranked AFTs as 1A class human carcinogen (IARC, 2002). Liver cancer, reduction in immunity system function and impaired growth are the most effects of AFTs.

AFTs are chemically similar toxic fungal moulds that are formed by a specific genus of moulds named *Aspergillus*; these are *Aspergillus nomicus, Aspergillus flavus*, and *Aspergillus parasiticus*(Eaton and Groopman, 2013; Gerbaldo et al., 2012; Varga et al., 2011). During inadequate storage and pre-harvest condition, their spores can spread and contaminate crops (Pitt, 2000). More than 18 types of AFTs are discovered so for. *Aspergillus parasiticus and Aspergillus nomius* produces Aflatoxin G_1 and G_2 (Ibisi and Asoluka, 2018; Lerda, 2010). The chances of getting AFTs contamination by various kind of food commodities which may include spices e.g., red chili and black pepper, oilseeds, corn, pulses, cereals, earthnuts, milk, cheese and other dairy products. Many countries including Pakistan are being affected by aflatoxin contamination of staple food.

Aflatoxin contamination can occur during harvesting season (pre and post), drying, packing, transportation and reposition. All these conditions make crops more prone to attacked by wide range microbes. The places where humid conditions, high temperature and heavy rainfall occur provide most favorable condition for the growth of toxigenic molds which result in aflatoxin production (Asghar et al., 2016). The AFTs are reported to express toxicity in a periodical manner and order of toxicity for AFTs is AFM2 < AFM1 < AFG2 < AFB2 < AFG1 < AFB1 (Kabak and Dobson, 2017).In tropical regions, like Pakistan, the pre-harvest conditions i.e. moisture and high temperature are mainly responsible for aflatoxin development (Zahir et al., 2007). The risk of fungal growth may be enhanced by increasing storage time (N'Dede, 2009).

The mycotoxins permissible limit in food is greatly controlled by European Union. The EU food law proposed the limit for all aflatoxin in food is 5-10μg/kg (Commission et al., 2010). According to FDA & FAO (Table 1) the aflatoxin limit in food commodities is 20 μg/kg (Food and Administration, 2010). For the quantitative determination of AFTs in food commodities, a number of techniques have been developed including TLC, HPLC, spectrometry, florescence, biosensors and enzyme linked immunosorbent assay (ELISA) has been reported to be used for this purpose (Espinosa-Calderón et al., 2011). Normally, all types of AFTs showed maximum absorption at 360 nm (Akbas and Ozdemir, 2006). Based on the fluorescent colours property of AFTs, they have been classified as "B" for blue (425 nm) and "G" for green- blue (450 nm). G toxins are more fluorescent than B toxin (Alcaide-Molina et al., 2009). It is significant to control the production of AFTs by suppressing mycotoxins growth (Banerjee and Sarkar, 2003). The inculcation of AFTs into the blood through inhaling, adsorption through skin or digestion causes teratogenic, Carcinogenic, mutagenic and hepatotoxic effects occur on humans and animals (Qazi and Fayyaz, 2006). The main objective of this study was qualitative and quantitative analysis of AFTs found in different food samples including pulses and spices like red chili, black pepper, cumin, coriander and cumin using chromatographic technique.

MATERIAL AND METHODS

Chemical and reagents

Standards for AFG1, G2, B1, B2 has been purchased from Trilogy Analytical Laboratory. All the analytical grade solvents and reagents were used to carry out experiments and were purchased from Merck.

Samples collection

Around 70 samples of spices (red chili, black pepper, coriander, cumin and aniseed) and 50 samples of pulses (split chick pea, lentils and black gram beans) were collected from different areas of Punjab. 50 g sample of each type was milled and used for aflatoxin detection. Each sample of 50 mg of each spice was taken into 500 ml conical flask. After that 150 ml chloroform ($CHCl_3$) and 25 ml of water (H_2O) were added. Then this flask was fixed on a wrist arm shaker for 30 min for thorough mixing. The sample was filtered and filtrate was evaporated on hotplate at 60 °C.

Thin layer chromatography (TLC)

About 1.5 cm above from the base, the spotting was done on TLC plate (size 8 cm×5 cm). In order to avoid dissolving of spot in solvent, its level must be below the spot. Spots of sample were applied by using micro syringe. One spot of standard was also applied on TLC plate to make comparison. Two tanks for the developing purpose were used i.e. 1st comprising anhydrous ether and 2nd with acetone-chloroform. After applying spots on the plate, this was placed in a 1st tank containing anhydrous ether. After homogenizing the internal environment of tank, the plate after about half an hour was taken out of tank and dried on hot plate. When the TLC plate was fully dried, it was put into 2nd tank comprising chloroform-acetone v/v ratio 9:1. Ratio was adjusted according to R_f values. AFTs presence and absence can be determined using 365 nm UV Light. The intensity of fluorescence is used to estimate aflatoxin content.

Quantitative determination of AFTs

Test solution was dried before determination of AFTs quantitatively. Acetonitrile-benzene in ratio 1:49 was added in this test solution. In this way, the spots of size 6.5, 5.0 and 3.5 μl of test solution were applied onto the plate uniformly. Subsequently, same size spots of standard AFTs have been applied to same TLC plate. TLC plates were interpreted on the basis of comparison between spots of samples and of standard AFTs applied to the plate. Plate was observed under UV-Vis light. These glowing spots indicate the presence of AFTs present in sample. Intensity of fluorescence indicates the quantity and presence of AFT in sample. In this experiment, the sample spot fluorescence intensity was compared with the standard. The spots of sample and standard were marked quantitatively. To get more precise and accurate results, spots were handled with much care so it does hinder in getting the accurate reading. In case of very small spots, solution was diluted and repeated the TLC procedure for better result. The absorption of the Aflatoxin in sample was calculated using relation shown in Eq. 1.

$$\text{AFTs contents}\left(\frac{\mu g}{kg}\right) = \frac{S \times Y \times V}{W \times Z} \quad (1)$$

Where, S: volume in mL of aflatoxin standard of equivalent intensity to Z = mL of sample, Y: concentration of aflatoxin standard in mg/mL, Z: volume in mL of sample extract required to give fluorescence intensity comparable to that of S = mL of the Aflatoxin standard, V: volume in mL solvents required to dilute final extract and W: Weight, in grams of original sample contained in final extract.

Table 2: Summary of contaminated samples of Split Chick Pea (SCP), Lentils (L) and Black gram Beans (BGB) from Punjab, Pakistan

Sample code	Sampling Area	AFT Conc. (ppb)
SCP1	Pasrur katchery	ND
SCP2	Sialkot Cannt	
SCP3	Sialkot Lalazar colony	10.2
SCP4	Sialkot Pakagarah	ND
SCP5	Lahore Lari adda	8.3
SCP6	Sialkot Shahab pura	ND
SCP7	Lahore cantonment	11.2
SCP8	Sialkot Gandum mandi	3.6
SCP9	Gulshan Iqbal park Sialkot	ND
SCP10	Bajwat Sialkot	
SCP11	Adda Sialkot	
SCP12	Sialkot Saddar	3.7
SCP13	Ugoki Sialkot	ND
SCP14	Sialkot Sublime Chok	
SCP15	Lahore Defence	6.2
SCP16	Zafarwal Sialkot Bipass	ND
SCP17	Lahore Gulberg	10.2
SCP18	Sialkot Duburji	ND
SCP19	Nandi Pur Kharana	
SCP20	Shahdara Lahore	8.7
L1	Chawinda	ND
L2	Allama Iqbal Town Sialkot	
L3	Pasrur Saddar	3.8
L4	Zafarwal Main bazar	ND
L5	Lahore Mall road	
L6	Shakargarh	
L7	Narowal Jassar Bipass	6.8
L8	Rang Pura Sialkot	ND
L9	Narowal Main bazar	
L10	Kingra	8.6
L11	Mirajkay	ND
L12	Mandi throo	3.5
L13	Lahore Saddar	ND
L14	Urra Chok Sialkot	7.9
L15	Narowal Sahara Hospital	ND
BGB1	Gujranwala wan chok	
BGB2	Gujrat near University of Gujrat	
BGB3	Gujranwala Wapda town	
BGB4	Gujranwala Saddar	3.9
BGB5	Gujrat Kachehri road	ND
BGB6	Gujrat Jail chowk	
BGB7	Gujrat Bhimber road	
BGB8	Gujranwala Bypass	11.2
BGB9	Multan road Lahore	ND
BGB10	Gujranwala Alam chowk	
BGB11	Gujranwala Khiali	
BGB12	Gujrat Fuwara chowk	15.4
BGB13	Gujranwala model town	ND
BGB14	Gujrat Kharyinwala	
BGB15	Gujranwala People's colony	10.2

RESULTS AND DISCUSSION

Samples of pulses and spices (red chili, black pepper, cumin, and aniseed) collected were analyzed using TLC for quantitative and qualitative analysis. Samples eluted on TLC were observed under UV spectrophotometer. Experimental work was carried out to detect the presence or absence of aflatoxin in pulses. TLC plate viewed under UV light gives blue fluorescence which matches with the standard solution spot. Sulphuric acid is sprayed on spot for qualitative analysis of fungal spore. The aflatoxin was detected in 17 samples of pulses (Fig. 1) and 21 samples of spices while remaining 33 samples of pulses and 49 of spices did not contain AFTs. Tables 2 and 3 summarize the results. The aflatoxin B1 usually found in each sample. The AFB2, AFG1 and AFG2 were not detected in any samples.

Aflatoxin in pulses

There are 15 samples of lentil out of which 5 samples are contaminated but 10 are uncontaminated overall contamination in lentil sample is 33.33%. Maximum detection AFT in contaminated sample of lentil is 8.6ppb. Twenty samples of split chick pea (SCP) were analyzed, out of which 8 samples are contaminated but 12 are uncontaminated and AFT percentage in SCP is 40%. In SCP contaminated samples maximum detection is 11.2ppb. In 15 samples of BGB, 4 samples are contaminated but 11 are uncontaminated and overall percentage of AFT in BGB is 26.67%. Maximum detection of AFT in BGB is 15.4ppb.

Different limits of AFTs tolerance are set by EU is 4 ppb in dried pulses. This study showed contamination range between 3.8-8.6 ppb. Five samples of lentils are contaminated in which two samples are within the FDA limit but 3 samples are not within the limit given by FDA standard. Detection range of aflatoxin in split chick pea was between 3.6-11.2 ppb. About 8 SCP samples were also exceeding FDA approval and 12 were within the permissible limit. The highest amount of aflatoxin detected in one SCP sample 11.2 ppb which was beyond the regulations set by FDA and WHO.

Four samples of black gram beans (BGB) are contaminated, out of which BGB4 is within the permissible limits of AFTs but three samples including BGB8, BGB12 and BGB15 are those samples which are beyond the permissible limit given by FDA. Detection range of aflatoxin in BGB was between 15.4-3.9 ppb. The highest amount of aflatoxin detected in one BGB sample 15.4 ppb which was beyond the regulations set by FDA and WHO. Occurrence of toxic fungus in pulses is not a healthy sign. Therefore, its reduction is necessary to minimize toxic effects on human health.

Aflatoxin in spices

The samples of red chili were analyzed through TLC. Table 3 shows the area from where samples have been collected and results after analysis. Figs. 2 and 3 show the pictorial elaboration of results obtained during experimental work. The results about this experimental work reveals that 9 out of 14 samples of aniseed were found uncontaminated. The contamination level in 5 out of 14 samples ranges from 29.5ppb-55.5ppb (Table 4). In these samples, one sample exceeds the limit of contamination set by EU but remaining four did not exceed the maximum permissible limit.

The Thin Layer Chromatographic analysis of black pepper was performed that were collected from the different area of Northern Punjab. It is evident from Fig. 2 that aflatoxin B1 exist in samples collected from vicinity of Punjab. The results about this experimental work reveals that 10 out of 14 samples of black pepper were found uncontaminated. The contamination level in 4 out of 14 samples ranges from 39.7-65.9ppb. In these samples, one sample exceeds the limit of contamination set by EU but remaining three did not exceed the maximum permissible limit. The Thin Layer Chromatographic analysis of coriander was performed that were collected from the different area of Northern Punjab. Fig. 3 shows the pictorial evidence of results obtained during experimental work. The results about this experimental work reveals that 8 out of 14 samples of aniseed were found uncontaminated. The contamination level in 6 out of 14 samples ranges from 33.4 ppb-67.9 ppb. In these samples, two samples exceed the limit of contamination set by EU but remaining four did not exceed the maximum permissible limit.

The Chromatographic analysis using TLC was performed for samples of cumin collected from the vicinity of Northern Punjab. The graphical explanation of experimental work performed to detect AFTs in Cumin is shown in Fig. 3. The results about this experimental work reveals that 10 out of 14 samples of cumin were found uncontaminated. The contamination level in 4 out of 14 samples ranges from 24.9 ppb-63.9 ppb. In these samples, three samples exceed the limit of contamination set by EU but remaining one did not exceed the maximum permissible limit. Aniseed samples collected from the different area of Northern Punjab were analyzed through

Table 3: Amount of aflatoxins determined in spices from different regions of Punjab, Pakistan

Sr. No.	Sampling Area	District	Aflatoxin Concentration (ppb)				
			Red chili	Black pepper	Coriander	Cumin	Aniseed
1	Saddar Bazar Cantt		ND	ND	ND	24.9	ND
2	Tehsil Bazar		42.5			ND	
3	Sambrial	Sialkot	ND	45.8	45.8		52.5
4	Daska			ND	47.2	63.9	
5	Pasrur		29.5		39.7		ND
6	Main Bazar		ND	39.7		ND	
7	Zafarwal		38.6	ND	ND		42.6
8	Main Bazar	Narowal				57.8	
9	Shakar Garh		ND	65.9	67.9		ND
10	Zafarwal					ND	
11	Main Bazar		55.5	ND	ND		35.5
12	Kamonke	Gujranwala	39.6			59.5	
13	Saddar Bazar		ND	49.6	59.6	ND	ND
14	Wazirabad			ND	33.4		

ND: Not Detected

Table 4: Screening analysis of spices and pulses for aflatoxins by TLC

AFTs Samples	Red Chilies	Black pepper	Coriander	Cumin	Aniseed	Lentils	Split chick pea	Black gram beans
AFT Detected					B1			
Samples	14	14	14	14	14	15	20	15
Contaminated	5	4	6	4	3	5	8	4
Uncontaminated	9	10	8	10	11	10	12	11
Contamination (%)	35.7	28.5	42.8	28.5	14.3	33.3	40	26.7
Max AFB1 (ppb)	55.5	65.9	67.9	63.9	52.5	8.6	11.2	15.4
Min AFB1 (ppb)	29.5	39.7	33.4	24.9	35.3	3.8	4.5	4.8
EU Limits (ppb)	50	50	50	50	50	4	4	4

TLC. The results about this experimental work reveals that 11 out of 14 samples of aniseed were found uncontaminated. The contamination level in 3 out of 14 samples ranges from 35.3 ppb-52.5 ppb. In these samples, one sample exceeds the limit of contamination set by EU, but remaining two did not exceed the maximum permissible limit. Present findings and previous studies (Al-Zoreky and Saleh, 2019; Asare Bediako et al., 2019a; Asare Bediako et al., 2019b; Fountain et al., 2019; Iamanaka et al., 2019; Karunarathna et al., 2019; Mahuku et al., 2019; Mwakinyali et al., 2019; Singh and Cotty, 2019) revealed that the AFTs contamination in food items is a serious issue, which needs to be tackled accordingly.

CONCLUSIONS

This study was intended to find out mycotoxins that cause different health issues to human and animals, their detection and analysis in pulses and spices from Punjab. The samples of pulses and spices including red chili, black pepper, coriander, cumin, and aniseed were analyzed. The results showed that very small number of contaminated samples was detected with minor amount of AFTs that can be harmful for both humans and animals. In this work, out of 50 samples of pulses, 17 (34%) samples depicted the AFTs presence, in the range of 3.5-15.4 ppb while out of 70 samples of spices, 21 (30%) samples depicted the AFTs presence, in the range of 24.9-67.9 ppb. There should be a strict check and balance on food controlling authorities working in Pakistan. The analysis of food commodities including staple food must be ensured to avoid public health risk. The most important thing is to promote an awareness campaign especially in farmers should be started that will also help to root out this problem. The awareness about farming and handling the samples to avoid contamination in samples which ultimately ends in deemed contamination of AFTs in food commodities.

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