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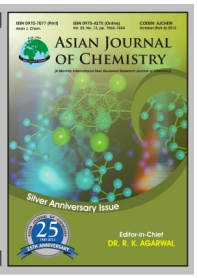

HPLC Determination of Aflatoxins in Wheat Grains Collected from Central Areas of the Punjab, Pakistan

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Occurrence of aflatoxins (B1, B2, G1 and G2) in samples of wheat from different localities in Faisalabad division, Punjab, Pakistan was determined with latest technique by using HPLC-UV and HPLC-FLD in isocratic mode using MycoSep # 226 columns. Total 40 samples were analyzed each taken from urban, semi-urban and rural areas. Contaminated samples, their percentage, mean value of aflatoxins, highest aflatoxin B1 and total B1 and B2, G1 and G2 was determined. Samples collected from semi-urban areas were found more contaminated 36 μg kg^{-1} (limit 2-25 μg kg^{-1} FDA, USA).

Key Words: Aflatoxins, HPLC-FLD, Cereals, Validation, Food, Wheat.

INTRODUCTION

Food is one of the most intimate and important components of our chemical environments. Good food is not only expected to look fresh and tasty but it must be free from all contaminants. Mycotoxins are natural food and feed contaminants mainly produced by moulds of genera *Aspergillus flavus, Aspergillus paraciticus, Aspergillus nomius*[1]. Mycotoxin contaminated food consumption is associated with several cases of human poisoning or mycotoxicosis and some times resulting in death[2]. Fungus invades, most commonly, cereal foods in hotter countries due to which shortage of food was faced[3].

Wheat (*Triticum aestivum* L.) is the leading food grain of Pakistan and being the staple diet of the people. Wheat provides energy, which is a rich source of carbohydrates, magnesium, manganese, proteins, vitamin, fiber and minerals. Different by-products are formed from raw wheat that are used in bakeries for different food products. It occupies a central position on agricultural policies and economics of the country. It contributes 12.5 % to the value added in agriculture and 2.9 % to GDP. Wheat is cultivated on an area of 8 million hectares and its annual production was about 18.47 million tons in 2008-2009. At farm level, the storage consists of mud bins, metallic bins, concrete rooms, jute bags and wooden boxes. Whole sellers also practice open storage by putting bagged grain on plinth, which is covered with tarpaulin for protection. Storage owned by flour millers is for operational stock only[4]. Due to poor drying and storage facilities the post-harvest losses are quite substantial. It has been estimated total aggregate losses during various post-harvest operation of wheat in Pakistan is about 15.3 %[5].

The occurrence of aflatoxins, in food has been recognized as potential threat to human health, either caused by direct contamination *via* grains and grain products or by carry over of mycotoxins and their metabolites in animal tissues, milk and meat after intake of contaminated feedstuffs[6]. There exist a great number of reports that suggest intoxication of humans by the consumption of aflatoxins contaminated agricultural products[7-11].

Aspergillus flavus, parasiticus and nomius are main fungal species which infect the wheat. These species grow well in range of 19-35 ºC produce maximum aflatoxins at 28 ºC. Humidity also results in maximum production of aflatoxins[12]. Pakistan is also considered as an agricultural country and major part of its economy depends upon agriculture growth particularly on wheat, rice and cotton crops. Basic focus of this research project was to quantify the aflatoxins concentration in wheat grains samples obtained from different parts of Punjab

TABLE-1
QUANTITATIVE ANALYSIS OF AFLATOXIN (AFB_1, AFB_2, AFG_1, AFG_2) IN WHEAT SAMPLES COLLECTED FROM DIFFERENT AREAS OF FAISALABAD DIVISION, PAKISTAN

Area of sample	Total sample	Wheat Aflatoxin = 20 ppb (%)	Wheat aflatoxin > 20 ppb (%)		
			21-25 ppb	26-30 ppb	> 30 ppb
Urban	15	1 (7)	2 (13)	3 (20)	6 (40)
Semi-Urban	15	1 (7)	0 (-)	2 (13)	10 (67)
Rural	10	1 (10)	1 (10)	2 (20)	4 (40)
Total	40	3 (8)	3 (8)	7 (17)	20 (50)

province. The sample was collected from different wheat grain markets during harvesting seasons when temperature, humidity and other plant stresses conditions were most favourable for the attack of *Aspergillus fungi*.

EXPERIMENTAL

Acetonitrile, methanol (HPLC grade) purchased from Merck (Germany). The MycoSep® columns-226 (AflaZon+ Multifunctional columns; Recorder # COCMY2226; Romer Lab., USA) were purchased locally. Double distilled water was prepared with Bibby distillation system, UK. Standards of aflatoxins *i.e.* AFB1, AFB2, AFG1 and AFG2 (50 μg mL^{-1} in acetonitrile) was obtained from Punjab Feeds Ltd., Lahore, Pakistan and stored in freezer of Pesticide Chemistry Lab., NIAB, Faisalabad. A series of working standard solutions (0.1 to 0.5 μg mL^{-1}) were prepared from stock solution and were stored in a borosilicate glass vials below 4 ºC.

Wheat samples: Samples of wheat grains were procured directly from whole sale market (grain market), vendors (grocery shops) and super markets during April 2008 to April 2009 covering the two harvesting seasons of wheat production. It was pre-planned to get random sample from urban, semi-urban and rural areas and stored at 4 ºC in polythene bags with proper identification codes until these were analyzed for aflatoxins.

Method: The method used for analysis of aflatoxins in wheat samples was made following the procedure described by Fu *et al.*[13] with little modifications.

Extraction procedure: The samples of cereal were thawed for two hours and grinded 250 g samples of wheat collected from different areas of Faisalabad, Pakistan using Centrifugal Grinding Mill (Retsch, ZM 200, Germany). A portion of 25 g of ground wheat sample was taken in an Erlenmeyer flask (250 mL) and added 20 % sodium chloride. Aflatoxins were extracted in 100 mL of a mixture of acetonitrile-water (84:16) by shaking for 45 min. The extract was filtered through Whatman (Maidstone, UK) filter paper (whatman No. 1). The filtrate (9 mL) was transferred to a glass tube, acidified with acetic acid (70 μL) and then passed through a Mycosep-226 AflaZon+ columns (Romer Labs.) with a flow rate of 2 mL/min. A portion of 2 mL of solution was evaporated to dryness with gentle stream of N_2 at 60 ºC in a centrifuge glass tube and the residue was dissolved with 0.3 mL of mobile phase solution.

HPLC analysis using UV-VIS and fluorescence detectors: The analyses of cleansed extracts were carried out using Shimadzu HPLC system equipped with UV-VIS (SPD-10A) and fluorescent detector (RF-530) without and with derivitization using triflouroacetic acid. The system validated/standardized with known standards individually and in mixture form. All analyses were performed in triplicate using discovery C_{18} column (250 × 4.6 mm, 5 μm), Supelco, USA in isocratic mode. Calibration curve was determined using a series of calibration solution of aflatoxin in acetonitrile. Each experiment was repeated triplicate and data obtained was presented as mean ± S.D. values.

RESULTS AND DISCUSSION

The quality of analytical data was determined by evaluating limit of detection, percentage recovery, reproducibility, linearity of instrument and checking of sample artifacts. The instrument was checked daily with mixture solution of aflatoxins. Instrument detection limits for all aflatoxins (B_1, B_2, G_1 and G_2) were determined according to published guidelines at a signal-to-noise ratio (S/N) of three[14]. Blank and pre-cleaned samples were prepared, treated and analyzed in the same manner as the real samples. The validation experiment followed the Guidance for Industry-Bioanalytical Method Validation recommended by the Food and Drug Administration of the United States[15].

The data obtained from our studies highlights that maximum samples of wheat were contaminated with > 30 ppb aflatoxins and least number of samples (13 %) collected from Faisalabad Division fall in the range of 21-25 ppb aflatoxins as shown in Table-1. It is also threatening that wheat grains of the Faisalabad vicinity were exhibited high contamination of aflatoxins. Anyhow Only 3 samples showed 20 ppb aflatoxin contaminations, which is below maximum limit set by FDA, USA.

For the sake of awareness and public demand, it is very much subtle that such solvents are used that extract the whole contaminants (aflatoxins) from commodity under observation. Different solvents such as methanol, acetonitrile in combination with water along with/without sodium chloride were used for the extraction of aflatoxins from cereal samples. The samples were spiked at 10 μg kg^{-1} equivalent concentrations for AFB_1 and AFG_1 and 5 μg kg^{-1} for AFB_2 and AFG_2 respectively.

Spike samples of wheat were mixed well, extracted with acetonitrile: water (84:16), filtered, cleaned up using multifunctional column 226 and analyzed by Shimadzu HPLC in reverse phase system. The recovery percentage from each commodity is listed in Table-2. Each sample was treated and analyzed in five times. The data mentioned revealed that recovery percentage of aflatoxins in cereal samples is ≥ 85 %. The co-extraction in the sample did not effect on recovery of aflatoxins. The solvent used (acetonitrile and water) showed good behaviour to trap the hydrophobic aflatoxins in all type of cereals.

TABLE-3
ANALYTICAL PARAMETERS EXECUTED WITH THE HPLC METHOD FOR THE ANALYSIS OF AFLATOXINS (AFB_1, AFB_2, AFG_1 AND AFG_2)

Aflatoxin	Retention time (min)	Linearity (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	Precision (% RSD)	
					Repeatability	Reproducibility
AFB_1	7.58 ± 1.16	1-10	0.1	0.35	4	8
AFB_2	6.28 ± 0.78	0.1-5	0.2	0.60	2	12
AFG_1	5.76 ± 1.05	1.10	0.1	0.35	2	12
AFG_2	4.85 ± 0.56	0.1-5	0.2	0.60	5	10

TABLE-2
YIELD OF AFLATOXIN FROM WHEAT SAMPLES COLLECTED FROM FAISALABAD DIVISION

Aflatoxin	Spiking level	Recovery of aflatoxins from wheat (%)
AFB_1	10	92 ± 0.08
AFB_2	5	88 ± 0.16
AFG_1	10	89 ± 0.09
AFG_2	5	85 ± 0.12

Values are mean ± SD for triplicate determinations

TABLE-4
AFLATOXINS LEVEL (μg kg^{-1}) IN WHEAT SAMPLES COLLECTED FROM URBAN, SEMI-URBAN AND RURAL AREAS

Area	Total samples	Contaminated samples (% age)	(μg kg^{-1})
Urban	15	12 (80 %)	52 ± 5.24
Semi-urban	15	13 (87 %)	64 ± 6.05
Rural	10	8 (80 %)	85 ± 3.05

*Aflatoxins = AFB_1, AFB_2, AFG_1 and AFG_2

The analytical method was evaluated to prove its applicability to the analysis of quality parameters of the chromatographic method such as limit of detection, limit of quantification, precision and sensitivity following the method as described earlier[16]. Validation results are presented in the Table-3. In order to evaluate the precision of the proposed method and reproducibility were estimated. Both were expressed as percent relative standard deviation and compared with the established values by Codex Alimentarius Commission of the World Health Organization and European Union (EU Directive 110/2001) (2001/110/EC, 20 December 2001). All these data was in good agreement with standard values.

The retention time of each peak was compared with HPLC data of standards aflatoxins and calculate the concentration and sample contamination. Table-4 showed that more than 80 % of the wheat samples were contaminated with fungi producing mycotoxins. Wheat grains of semi-urban were highly contaminated (87 %) with mean residues of total aflatoxins (64 ± 6.05 μg kg^{-1}). The mean aflatoxins in collected samples of urban areas were calculated 52 μg kg^{-1}. The samples belong to rural areas contain mean residue of aflatoxins 85 ± 3.05 μg kg^{-1}.

High moisture levels in the samples and high temperature are favourable for the growth of aflatoxins producing fungi. Optimum conditions are 16-24 % moisture at 20-38 ºC. However, it is reported that aflatoxins production can also take place at temperatures as low as 7-12 ºC[17]. After harvest, if grains are not dried quickly or during storage remains at moisture high enough, mycotoxins such as aflatoxins may produce. The wheat contamination level was compared with the values given in Table-5 in particular with USA (Food and Drug Administration; FDA) allowable limits *i.e.* 20 μg kg^{-1} for sum of aflatoxins in food commodities. The distribution of aflatoxins level in wheat consumed by residents of Faisalabad Division is summarized in Table-5.

Aflatoxins contamination in foods, which causes the economic loss and threats to public health, is a world wide problem encountered from time to time in most countries of the world. This issue was not studied on required level in Pakistan. Although appreciable data was repoted world-wide[18,19].

The collected samples from urban areas (38 %) analyzed and the percentage of samples with detectable aflatoxins levels in this area were found to be 42, 15, 10 and 55 % for AFB_1, AFB_2, AFG_1 and total aflatoxin respectively. Samples collected from semi-urban areas (38 %) of Faisalabad Division for determination of total aflatoxins were found to have detectable levels. This ratio is low as compared to urban area although having the same metrology conditions. The samples from rural areas are less as compared to other areas due to less access to the residents. The percentage of positive samples with detectable aflatoxins levels and permissible limits of different types of aflatoxins are shown in the Table-5. The concentration of AFG_2 was low as compared to other aflatoxins found in wheat samples of Faisalabad Division. The climatic conditions prevailing in the tropics are especially promoted the fungal proliferation and consequently production of mycotoxins.

TABLE-5
PERCENTAGE OF POSITIVE SAMPLES WITH DETECTABLE AFLATOXINS LEVELS AND PERMISSIBLE LIMITS OF DIFFERENT TYPES OF AFLATOXINS

Area	No. of samples	AFB1		AFB2		AFG1		AFG2		TOTAL	
		% of positive samples	Range (μg/kg)	% of positive samples	Range (μg/kg)	% of positive samples	Range (μg/kg)	% of positive samples	Range (μg/kg)	% of positive samples	Range (μg/kg)
Urban	12	42	4-25	15	0.5-12	10	0.5-8	0	-	55	12-65
Semi-Urban	13	36	2-25	10	0.5-14	5	0.8-12	4	0.6-4	40	4-16
Rural	8	50	6-34	25	0.810	40	4-10	0	-	65	6-85
Total	33	45	6-45	30	5-20	42	2-16	8	0.8-6	58	5-80

The hot and humid weather conditions in Pakistan promote the attack of fungi with maximum production of aflatoxins. Wheat is sown in October-December and collected in April-May (a rabi crop). Wheat crop covered thousand hectares area of Pakistan with production of 20956.8 thousand tones. Wheat is usually grown during hot weather and harvested during humid summer in Pakistan. These conditions promote the attack of fungi with maximum production of aflatoxins which is a continuous threat for hepatitis B infection in the areas. The wheat was stored through out the year in different types of bins but in remote areas in clay bins. Clay is a good absorbent for water during rainy season which may increase the moisture level of grains in bins.

Ratio of contaminated sample was found high in semi-urban areas as compared to urban and rural areas of the Division. From the data collected, it is clear that most of the residents stored wheat through out the year in steel bins and put in open air and sun light directly that affect the aflatoxins value of wheat. The increase in temperature inside the bins may be the cause of aflatoxins production because the toxigenic fungi proliferate greatly in high temperature[20].

Mould growth and mycotoxin production are related to the presence of fungal inoculum on susceptible crops. Plant stress caused by extreme weather, faulty water and fertilization imbalancing, insect damaging and inadequate storage conditions. In general, biotic and abiotic stresses (heat, water and insect damage) cause plant stress and predispose plants in the field to mycotoxin contamination[21].

Conclusion

HPLC determination of aflatoxins was found simple, cost effective and time effective. Distribution of aflatoxins, determined by HPLC technique in wheat samples showed that 36-50 % samples contaminated with AFB1, 10-25 % with AFB2, 5-40 % with AFG1 and 0-4 % with AFG2. Overall 45 % samples were contaminated with AFB1, the major carcinogen and the least with AFG2 (8 %). The presence of several mycotoxins, even at such low levels, could pose chronic effects on human health and livestock fed on the contaminated wheat products. Periodical monitoring of stored grains through HPLC will facilitate the authorities to reduce the risk factors associated with aflatoxin contaminations.

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