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Full Length Article

Analysis of Aflatoxins in the Cereals from Hepatic Affected Areas in Faisalabad, Pakistan

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ABSTRACT

The worldwide increasing prevalence of liver diseases in humans over the last two decades is a strong indication that environmental factors are of great significance in the development of these diseases. Exposure to toxic metabolites such as aflatoxins may be one of the causes. An area-based study was carried out to determine the effect of aflatoxins for the prevalence of liver diseases. Two areas in Faisalabad division were selected showing most and least prevalence of liver diseases. Samples of wheat, rice and corn taken from effected and control area of Faisalabad Division were analyzed by HPLC using ODS column in isocratic mode at 365 nm wavelength. Food contamination analysis showed that a significant proportion of them are contaminated to a degree for above the Codex Alimentarius. Maize samples were found to be highly contaminated with Aflatoxin B1 (AFB_1). The results indicated that aflatoxins might have played a role for liver damage of inhabitants of the area showing prevalence of the diseases.

Key Words: Aflatoxins;, *Aspergillus flavus; A. parasiticus;* Codex alimentarius; Epidemic

INTRODUCTION

Naturally occurring toxins such as mycotoxins pose profound challenges to food safety. The mycotoxins of public health importance within the region are aflatoxins. Mycotoxins are substances produced by moulds that contaminate various agricultural commodities either before harvest or under post-harvest conditions (FAO, 1991). Aflatoxins are toxic metabolites produced predominantly by two species of mould, *Aspergillus flavus* and A. *parasiticus* that are widely distributed in the nature and form the toxins at temperatures ranging from 12 to 42°C and at relative humidity higher than 80%. All aflatoxins are acutely and chronically toxic. There is compelling evidence of the association between exposure to aflatoxins and primary liver cancer. Aflatoxin B_1 is considered to be one of the most potent hepatotoxins and is also a human carcinogen. It is linked with the development of human hepatocellular carcinoma as regarded by the International Agency for Research on Cancer. Toxicity for aflatoxins occurs at very low levels and aflatoxin B_1, for example, at concentrations of parts per billion (ppb) can cause very damaging effects (Bhat & Vasanthi, 2003).

Human exposure directly to AFB_1 consuming contaminated food, meat, egg, milk and other edible products from animals that consume aflatoxin-contaminated food are the main source of its exposure. Exposure of AFB_1 is a serious problem in countries, where hygiene and food safety are not vigorously enforced (Jackson & Groopman, 1999). Aflatoxin B_1 (AFB_1) has been demonstrated to be carcinogenic in many animal species including rodents, non-human primates and fish (Busby & Wogan, 1984; Groopman & Kensler, 1999). Another published study identifies a form of cytochrome P450 (abbreviated as "P450") from human liver, which is likely to be at least partially responsible for generation of the active AFB_1 metabolite (Shimada & Guengerich, 1989).

It has also been reported that AFB1 exerts liver-specific carcinogenicity by inducing a guanine (a purine) to thymine (a pyrimidine) substitution at codon 249 on the P53 gene (Bressac *et al.*, 1991; Hsu *et al.*, 1991). In Africa and Southern China, the high level of dietary AFB1 may represent a special environmental hazard for chronic HBV (Eaton & Gallagher, 1994; Bailey *et al.*, 1996; Chen *et al.*, 1996). Maqbool *et al.*, (2004) determined aflatoxins in poultry feed. A survey on cancer prevalence was carried out in Karachi by Yasmin *et al.* (2000). In this study, an attempt was made to estimate aflatoxins ingestion in subjects by means of a food frequency with a determination of aflatoxin levels in selected locally grown or stored cereals.

MATERIALS AND METHODS

Epidemiological studies. A Performa for epidemiological analysis was constructed by surveying hospitals and joining vaccination camps managed by various NGOs. It was used as a tool for epidemiological analysis to decide the area at high and low risk and to decide the factors responsible for

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epidemic of hepatic diseases. Most of the liver diseased patients were searched by visiting hospitals in Faisalabad division, which was divided in to three zones as under:

Zone 1: Faisalabad city and allied areas.

Zone 2: Samundary, Gojra and Toba Tek Singh (TTS).

Zone 3 Jhang and Chiniot.

Vaccination camps with the collaboration of different NGO's for epidemiological studies were also attended. Persons showing increased levels of liver enzymes, HBsAg positive and HCV positive were interviewed for study by using a questionnaire.

Food sampling. Samples of cereals (wheat, maize & rice) were collected randomly from the areas prevalent to hepatic diseases (Skerritt, 1998). Quality and quantity of food used by subjects was evaluated in terms of aflatoxins.

Determination of aflatoxins. Determination of aflatoxins in food is usually carried out with thin layer chromatography (TLC), enzyme Linked Immunosorbent Assay (ELISA) and HPLC with fluorescent after TFA derivatization. Many methods have been used for the determination of aflatoxins in corn, wheat and other food materials in the past (Garner, 1975; AOAC, 1984). In the present study, for the analysis of aflatoxin residues in food (rice, maize & wheat) taken from hepatic diseases prevalent and control area was performed following AOAC Official Methods of Analysis (AOAC, 1984) with little modifications.

Aflatoxins were extracted with acetonitrile. High performance liquid chromatography (HPLC) was used for the determination of aflatoxins in cereals (wheat, rice & maize). The conditions used for the analysis of aflatoxins were as:

Shimadzu HPLC System	LC-10 A
System Controller unit	SCL-10 A
Liquid pumps	LC-10 AS
Column oven	CTO-10 A
UV-Vis detector	SPD-10A (190-600 nm)
Column	250 x 4.6 mm (i.d.), 5 μm
Column temperature	30°C
Injection volume	20 μL
Acetonitrile:methanol:H_2O	(22.5:22.5:55)
Flow rate	1.5 mL min^{-1}
Wavelength	365 nm
Pressure	140 kg cm^{-2}.

Aflatoxins standard (B_1, B_2, G_1 & G_2) was injected and the chromatogram of standard peaks was obtained.

Samples of wheat, rice and corn taken from effected and control area of Faisalabad Division were analyzed by HPLC using ODS column in isocratic mode at 365 nm wavelength. Before analyzing the un-known samples of the present dissertation, the instrument was validated to see its precision, accuracy and limit of detection (LOD). The separation (resolution) pattern of aflatoxins assayed by reverse phase HPLC column is shown in Figs. 1-5.

Fig. 1. Resolution of aflatoxins in reverse phase HPLC system; Peaks 1: AFG2, 2: AFG1, 3: AFB2 , 4: AFG1, wavelength (λ): 365 nm

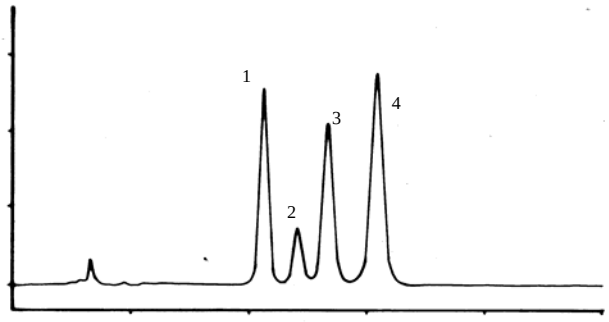


Fig. 2. Calibration of AFG_2

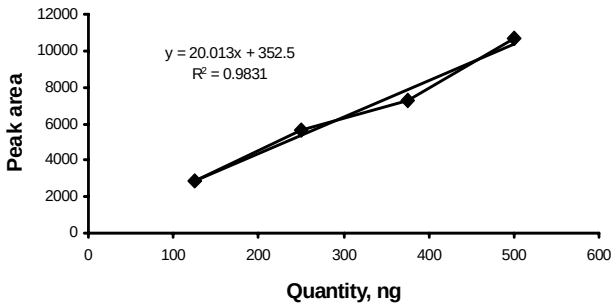


Fig. 3. Calibration of AFG_1

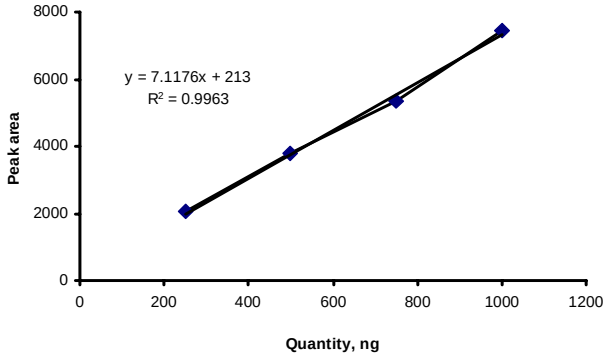
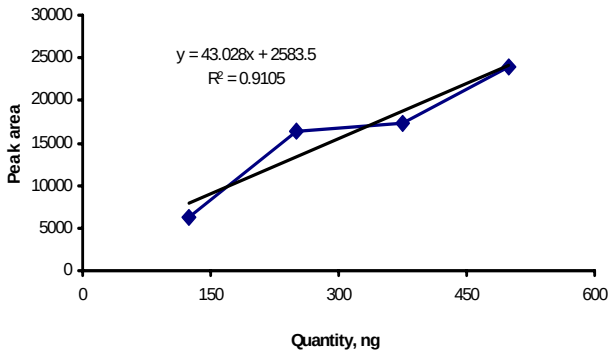


Fig. 4. Calibration of AFB_2



The graphs presented in Figures showed that the behavior of aflatoxin working solution was linear. The data was analyzed for regression coefficient and found in the range of 0.9105-0.9963. Some problem was observed in the behavior of AFB_2. It may be due to the impurity of the solvent or may be other hidden artifacts, which could not be rectified during the analysis. The overall response of HPLC parameters was good enough to analyze the un-known samples. The limit of detection was also noted at high sensitivity and found that for AFB_1 and AFG_1, the sensitivity of the instrument was 0.1 μg kg^{-1} and for AFB_2 and AFG_2 0.2 μg kg^{-1} (response to noise ration was 3:1). The precision and reproducibility was noted and found 5.2% ± 1.2 and 6.5% ± 2.2, which seems to be good for the analysis of food samples.

RESULTS AND DISCUSSION

The comprehensive survey performed to find the areas most prevalent and least prevalent to liver diseases showed Zone 2 (Samundary, Gojra & TTS) and Zone 3 (Jhang & Chiniot) in former and latter areas. So, analytical study was focused on these zones. Food contamination data showed that wide ranges of commodities were contaminated and that a significant proportion of them are contaminated to a degree for above the Codex Alimentarius. From the data it became evident that maize collected from TTS contained higher aflatoxins as compared to rice and wheat of the same area. From twelve (12) samples of maize, samples 3, 4, 5, 6, 7, 8 and 10 contained significant residues of aflatoxins. Aflatoxin B1 was dominant among others in maize samples. The intensity of aflatoxins found in maize samples is displayed in Fig. 6-8. Significantly high residue of total aflatoxins in wheat samples taken from TTS is illustrated in Fig. 7.

Rice samples were also collected from bins of residents in TTS and wholesale markets and found reasonable residue of aflatoxins. The highest residues of rice were calculated in samples No. 2, 5, 6, 7, 8 and 9. Least residue of aflatoxin B_1 was detected in sample 10 (2.39 μg kg^{-1}). Substantially high residues of total aflatoxins were observed in rice samples (Fig. 8).

The samples of wheat, maize and rice collected from Jhang area were analyzed and found that some samples were contaminated with compounds excreted by fungus. Most of the analyzed samples had lower level of aflatoxins than the recommended MRL and no aflatoxins were detected in sample 6 and 7. The data revealed that the aflatoxins are below the MRL set by WHO, USA.

Food quality assessment has gained more prominence in the wake of globalization of trade and attending sanitary, Phytosanitary as well as CODEX Alimentarius stipulations. Consequently, monitoring of toxic chemicals presence in food has gained priority over other aspects particularly pesticide residues, metal residues and aflatoxins (Paroda, 2004).

Fig. 5. Calibration of AFB_1

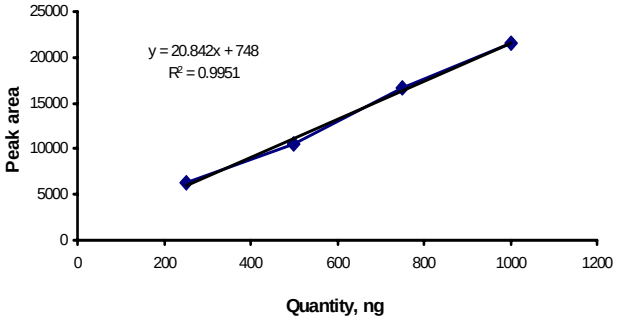


Fig. 6. Intensity of aflatoxins in maize sample from TTS

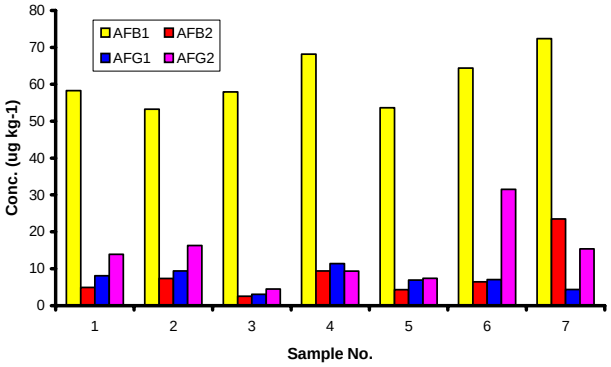
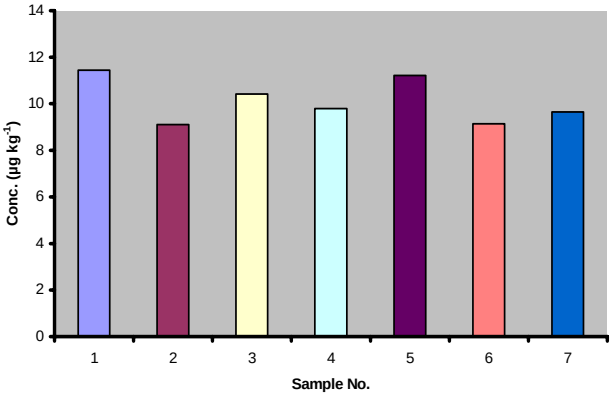


Fig. 7. Total aflatoxins found in wheat samples from TTS



The presence of aflatoxins or any other toxic residues beyond limits has a serious bearing on the safety and ultimate marketing of food and food products. Fungi are a major cause of deterioration and spoilage in stored crops. They render unfit for human and animal consumption perhaps as much as 1% of the world's supply of grain and oilseeds (Johnson, 1948). Human foods are allowed 4-30 ppb aflatoxin, depending upon the country involved (Food & drug Administration, 1995; Henry *et al*., 1999).

The concentration of AFB_1 and total aflatoxins (ΣB_1, B_2, G_1 & G_2) in samples of TT Singh was significantly

Table I. Comparison of aflatoxins in cereals (wheat, maize & rice) between control and effected area

Aflatoxin	Area	Wheat				Maize				Rice			
		Mean ± SE		CV%	t-value	Mean ± SE		CV%	t-value	Mean ± SE		CV%	t-value
AFB1	T.T.Singh	3.88 ± 0.370		32.97	4.08**	49.37 ± 5.224		36.65	6.96**	5.84 ± 0.516		30.63	5.44**
	Jhang	2.11 ± 0.227		37.17		11.67 ± 1.418		42.10		2.23 ± 0.416		64.53	
AFB2	T.T.Singh	1.72 ± 0.300		60.24	3.24**	7.26 ± 1.585		75.65	3.03**	3.68 ± 0.350		32.93	6.05**
	Jhang	0.55 ± 0.203		127.72		2.28 ± 0.444		67.57		0.98 ± 0.278		98.64	
AFG1	T.T.Singh	0.48 ± 0.108		78.78	4.40**	6.12 ± 0.809		45.79	3.74**	1.57 ± 0.183		40.35	4.63**
	Jhang	0.00 ± 0.000		0.00		2.46 ± 0.551		0.00		0.35 ± 0.189		0.00	
AFG2	T.T.Singh	1.94 ± 0.294		52.43	3.43**	12.00 ± 2.018		58.29	3.65**	1.79 ± 0.196		37.92	2.14*
	Jhang	0.64 ± 0.240		129.98		4.04 ± 0.828		70.97		0.97 ± 0.329		117.40	
Overall	T.T.Singh	2.01 ± 0.225		77.71	4.39**	18.69 ± 2.97		109.99	4.45**	3.22 ± 0.300		64.57	5.95**
	Jhang	0.83 ± 0.148		123.94		5.11 ± 0.71		96.05		1.13 ± 0.182		111.22	

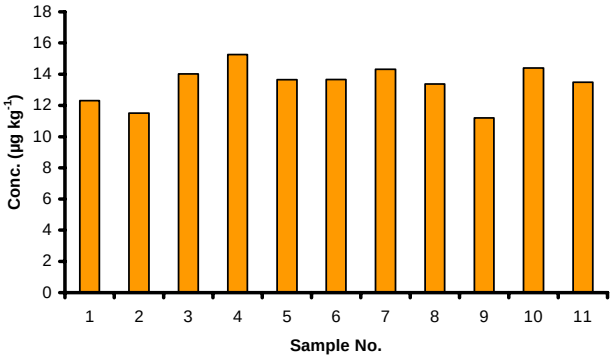
* = significant (P<0.01), ** = highly significant (P<0.01) and ns = non-significant (P>0.05) difference between control and affected area (t-test) (n=24).

higher as compared to samples of Jhang. The concentration of individual AFB_1 in wheat samples was high as compared to MRL (2 μg kg^{-1}) and concentration of total aflatoxins in wheat samples was high in comparison of EU MRL (4 μg kg^{-1}) but less than FDA, USA (20 μg kg^{-1}), which is in agreement with the reported results of Toteja *et al*. (2006).

The data indicated the presence of aflatoxins in maize samples. All samples were found highly contaminated with aflatoxins. This may be due to the invasion of fungus during harvesting season or it may be the high moisture level of the storage place. It is normal practice in Pakistan that people of village store their maize in mud made bins, which absorb high water content during rain time. The highest concentration of AFB_1 (>68 μg kg^{-1}) was detected in maize samples (TTS) comparable with Jhang (>19 μg kg^{-1}). The residue levels found in subtropical area of India was higher than that of our results (666 μg kg^{-1}). The median level of reported study was < 5 μg kg^{-1} in states of Gujarat, Haryana, West Bengal and Andhra Pradesh (Bhat *et al*., 1997). The total residue of aflatoxins was significantly high in all samples of maize collected from TT Singh, which is a highly prevalent area with liver diseases, except sample number 11 and 12. Many factors could be accounted for the proliferation or increase in aflatoxins concentration in maize seed like soil, humidity during pre-and post-harvest, weather condition and above all the storage place or containers. Our results are in agreement with the published data of Vargas *et al*. (2001). They found aflatoxin B1 in the range of 0.2 to 129 μg kg^{-1} in Brazilian corn through in-house validated methods. The occurrence of aflatoxins in corn samples of Egypt was high as compared to the results of present dissertation. Aly (2002) found the distribution of aflatoxins in corn and corn products during wet-milling process fractions. The order of the aflatoxins was $G_1 > G_2 > B_1 > B_2$. After milling the aflatoxins were found in gluten, fibre and germ. Gluten, fibre and germ were the most highly contaminated fractions. The presence of aflatoxins in processed corn product is increasing cancer of liver in Egyptian population. The reported data in the under taken study also agreed earlier reports (Tutelyan *et al*., 1989).

The level of AFB_1 in rice sample from TTS was in the range of 2.39-8.20 μg kg^{-1}, AFB_2 in the range of 1.25-5.25 μg kg^{-1}, AFG_1 in the range of 0.56-2.45 μg kg^{-1} and AFG_2 in

Fig. 8. Total aflatoxins measured in rice samples from TTS



the range of 0.90-3.28 μg kg^{-1}, whereas in Jhang samples the concentration was in the range of 0-4.50 μg kg^{-1} for AFB_1, 0-2.67 μg kg^{-1} for AFB_2, 0-1.83 μg kg^{-1} for AFG_1 and 0-3.20 μg kg^{-1} for AFG_2, respectively. Total aflatoxins in both areas were in the range of 7.27-15.26 μg kg^{-1} and 0-10.36 μg kg^{-1}, respectively. The rice samples were least contaminated with fungi secondary metabolites among cereals collected from TTS and Jhang areas. Our results are contradictory to earlier reported data by Sugita-Konishi *et al*. (2006). They reported nil aflatoxins in corn and corn products, rice but other contamination was detected (fumonisins & ochratoxin A) in retail foods in Japan.

Samples were also taken from control area and analyzed for aflatoxin residues. The data was further compared statistically (Table I). Foods of control and effected area of Faisalabad Division were found contaminated with aflatoxins. The concentration of AFB1 is higher in maize as compared to other staple foods. The least food (rice) was found contaminated with fungi producing compounds. The samples collected from TTS contained substantially high aflatoxins as compared to District Jhang. The residue of aflatoxins in most samples of wheat, rice and maize exceeds the established MRL of EU but found below than the FDA, USA MRL in cereals commodities.

In conclusion, aflatoxins found in cereals play a significant role in the hepatic diseases though virus aided a lot in the proliferation of disease.

REFERENCES

Aly, S.E., 2002. Distribution of aflatoxins in product and by-product during glucose production from contaminated corn. *Nahrung*., 46: 341–4

AOAC, 1984. *Official methods of Analysis of the Association of Official Analytical Chemists*, 14th edition. Arlington, VA 22209, USA

Bailey, E.A., R.S. Lyer, M.P. Stone, T.M. Harris and J.M. Essigmann, 1996. Mutational properties of the primary a.atoxin B1-DNA adduct. *Proc. Natl. Acad. Sci. USA*, 93: 1525–39

Bhat, R.V. and S. Vasanthi, 2003. *Food Safety in food Security and Food Trade: Mycotoxin Food Safety Risk in Developing Countries*. Vision 2020 for Food, Agriculture and the Environment, National Institute of Nutrition, Indian Council of Medical research, India

Bhat, R.V., S. Vasanthi, B.S. Rao, R.N. Rao, V.S. Rao, K.V. Nagaraja, R.G. Bai, C.A. Prasad, S. Vanchinathan, R. Roy, S. Saha, A. Mukherjee, P.K. Ghosh, G.S. Toteja and B.N. Saxena, 1997. Aflatoxin B1 contamination in maize samples collected from different geographical regions of India-a multicentre study. *J. Food Addit. Contamin*., 14: 151–6

Bressac, B., M. Kew, J. Wands and M. Ozturk, 1991. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature*, 350: 429–31

Busby, W.F. and G.N. Wogan, 1984. Aflatoxins. *In*: Searle, C.E. (ed.), *Chemical Carcinogens*, pp: 945–1136. American Chemical Society, Washington, DC

Chen, C.J., L.Y. Wang, S.N. Lu, M.H. Wu, S.L. Ypu, Y.J. Zhang and L.W. Wang, 1996. Elevated a atoxin exposure and increased risk of hepatocellular carcinoma. *Hepatology*, 24: 38–42

Eaton, D.L. and E.P. Gallagher, 1994. Mechanism of aflatoxin carcinogenesis. *Annul. Rev. Pharmacol. Toxicol.*, 34: 135–72

Food and Drug Administration, 1995. *Section 555.400 Foods-adulteration and Aflatoxin Aflatoxin (CPG 7120.26)*. Internet http:/www.fda. gov/oral/ compliance- ref/cpg/cpgfod /cpg 555-400,html (accessed 10 March 2004)

FAO, 1991. *Food, Nutrition and Agriculture*. *In*: *Food for the Future*. Food and Agriculture Organization (FAO) letter No. 1, 1991

Garner, R.C., 1975. Aflatoxin separation by high pressure liquid chromatography. *J. Chromatograph*, 103: 186–8

Groopman, J.D. and T.W. Kensler, 1999. The light at the end of the tunnel for chemical-specific biomarkers: day-light or headlight. *Carcinogenesis*, 20: 1–11

Henry, S.H., F.X. Bosch, T.C. Troxell and P.M. Bolger, 1999. Reducing liver cancer-global ontrol of aflatoxin. *Sci.*, 286: 2453–4

Hsu, I.C., R.A. Metcalf, T. Sun, J.N. Welsh, N.J. Wang and C.C. Harris, 1991. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature*, 350: 427–8

Jackson, P.E. and J.D. Groopman, 1999. Aflatoxin and liver cancer. *Baillieres Best Pract. Res. Clin. Gastroenterol*., 13: 545–55

Johnson, A.G., 1948. The role of fungi in the deterioration of grains in storage. *In*: "*Preservation of Grains in Storage*", FAO Agricultural Studies No. 2: 88–91

Maqbool, U., A. Maqbool, H. Anwar and M.I. Mohsin, 2004. Determination of aflatoxin-B_1 in poultry feed and its components employing enzyme-linked immunosorbent assay (ELISA). *Toxicol. Environ. Chem.*, 86: 213–8

Paroda, R.S., 2004. Foreword. *In*: Mahindru, S.N.(ed.), *Food Contaminants-Origin, Propagation and Analysis.* Published by A.P.H. Publishing Corporation, 5-Ansari Rd., Darya Ganj, New Delhi, India

Shimada, T. and F.P. Guengerich, 1989. Evidence for Cytochrome P450-NF, the Nifedipine Oxidasc, Bcing the Principal Enzyme Involved in the Bioactivation of Aflatoxins in Human Liver. *Proceed. Natl. Acad. Sci. USA*, 86: 462–5

Skerritt, J.H., 1998. Appropriate Analytical technologies for monitoring agrochemical residues. *In*: Kennedy, I.R., J.H. Skerritt, G.I. Johnson and E. Highley (eds.), *Seeking Agricultural Produce Free of Pesticide Residues.* ACIAR Proceeding No. 85e published in 1998

Sugita-Konishi, Y., M. Nakajima, S. Tabata, E. Ishikuro, T. Tanaka, H. Norizuki, Y. Itoh, K. Aoyama, K. Fujita, Kai and S. Kumagai, 2006. Occurrence of aflatoxins, ochratoxin A and fumonisins in retail foods in Japan. *J. Food Protec*., 69: 1365–70

Toteja, G.S., A. Mukherjee, S. Diwakar, P. Singh, B.N. Saxena, K.K. Sinha, A.K. Sinha, N. Kumar, K.V. Nagaraja, G. Bai, C.A. Prasad, S. Vanchinathan, R. Roy and S. Parkar, 2006. Aflatoxin B1 contamination in wheat grain samples collected from different geographical regions of India: A multicenter study. *J. Food Protect.*, 69: 1463–7

Tutelyan, V.A., K.I. Eller and V.S. Sobolev, 1989. A survey using normal-phase high-performance liquid chromatography of aflatoxins in domestic and imported foods in the USSR. *Food Addit. Contamin*., 6: 459–65

Vargas, E.A., R.A. Preis, L. Castro and C.M. Silva, 2001. Co-occurance of aflatoxins B1, B2, G1, G2, zearalenone and fumonisins B1 in Brazillian corn. *J. Food Addit. Contamin*., 18: 981–6

Yasmin, B., A. Bhurgri, H. Sheema, Hassan, S.H.M. Zaidi and A. Rahim, 2000. Cancer incidence in Karachi, Pakistan: First results from Karachi Cancer Registry. *Int. J. Cancer*, 85: 325–9

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