

## DETERMINATION AND DETOXIFICATION OF AFLATOXIN IN CEREAL SAMPLES COLLECTED FROM DIFFERENT AREAS OF LAHORE

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### ABSTRACT

**Objectives:** Aflatoxin contamination of cereals and poultry feed causes significant financial loss and poses a risk with serious health implications for both humans and animals. Present research was conducted to check the occurrence of aflatoxins B1 in cereals including pulses and poultry feed and to evaluate the potential of different techniques in removing aflatoxin contamination from cereals.

**Methodology:** 112 cereal samples which comprised of varieties of pulses and cereals used as poultry feed (yellow split chickpeas, Barley, sorghum, wheat bran, corn seeds and oat) were collected from various locations of Lahore. Quantitative analyses were performed through Thin Layer Chromatography and High-Performance Liquid Chromatography techniques. Detoxification of mycotoxin (Aflatoxin) contaminated samples was carried out through both chemical and physical methods.

**Results and Conclusion:** Aflatoxins were found in 36% (n=40) of the samples, with concentrations ranging from 2.073 to 23.03µg/kg-1. Selective 7 samples belonging to seven food types i.e., sorghum, yellow split chickpea, barley, wheat bran, corn and poultry feed mixture rendering negative aflatoxin results, were subjected to HPLC to confirm and validate the results of TLC. HPLC having declared as more sensitive technique gave positive results for three out of seven samples that previously were found negative for AFB1 in samples subjected to TLC analysis. The concentration detected ranges between 0.02-0.42µg/kg-1. Detoxification studies conducted through implementation of both physical and chemical methods proved the efficacy of both methods under variable conditions. Study hence suggested that a comprehensive and regular national level intensive monitoring and surveillance plan is required to improve the quality and storage of pulses and poultry feed in Pakistan.

**Keywords:** Aflatoxins, Cereals, poultry feed, UV light, TLC, HPLC, Lahore, detoxification.

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### INTRODUCTION

As influential teratogenic, mutagenic and hepatotoxic properties aflatoxins cause many serious damages that includes hemorrhage, edema, hepatitis, immuno-suppression and liver cancer. Aflatoxins are produced mostly by *Aspergillus flavus* and *Aspergillus parasiticus* before or after fruiting (Paterson *et al.*, 2010).

Contamination with aflatoxin is a severe food safety issue for ground crops grown in tropical and subtropical climates, where high temperatures and humidity enhance *Aspergillus* spp. growth and dispersion. Beans, dried fruit oil seeds, spices, almonds, and rice are the most common foods affected by aflatoxin. Aflatoxin is exceedingly difficult to remove since it is persistent and heat resistant in dried items<sup>3</sup> (Lee *et al.*, 2015).

Soybean meal, cereal grains, animal by-product eals, lipids, and vitamin and mineral premixes, among other ingredients, constitute the majority of the poultry

feed. Proteins and amino acids, carbohydrates, lipids, minerals, and vitamins are all necessary nutrients for a fowl's development, health, and basic growth and reproduction processes. As a result, these foodstuffs, as well as water, are given to the birds (Anjum *et al.*, 2014). As the growing demand for poultry meat and poultry by-products, the poultry industry has grown to become one of the country's largest industries. Despite the industry's widespread commercialization, poultry industry faces numerous challenges. Mycotoxicosis is one such issue, which is regarded as the industry's second most concerning issue behind rising chicken feed prices (Abidin *et al.*, 2011). According to one of the global feed surveys, 20% of complete diets were found to be contaminated with Aflatoxin, out of which 5% were above risk threshold (Anco, 2016).

TLC and HPLC have been declared as the effective techniques involving separation, detection, and quantification, as a result of which two techniques, TLC and HPLC, and are in frequent use nowadays (Sobolev *et*

*al.*, 2007). These methods are exceedingly effective, they can detect very low levels of aflatoxins as few picograms (AOAC). Aflatoxins in food can now be identified and measured in less than 10 minutes using highly specific antibody-based testing (Sobolev *et al.*, 2007).

Botanical extracts are another method for biologically breaking down AFB1. Liquid extracts of Malabar-nut leaf and lemon-scented gum have been found to be highly effective, against AFB1 with degradation rates of >95 percent in both cases (Velazhahan *et al.*, 2010; Vijayanandraj *et al.*, 2014).

Mycotox is a medicinal premix that is used to treat mycotoxicosis in chickens. Oxyquinol, dichlorothymol, and micronized yeast are among the ingredients. Antifungal and anti-mycotoxin effects are known to exist in all of these substances. The potential for AFB1 breakdown has been examined using isolated enzymes from various biological sources. Many aflatoxin-degrading enzymes have recently been discovered like, laccases, bacillus and manganese peroxidase etc. These technologies have a high efficacy, however their effectiveness on food substrates has not been studied, therefore their efficacy on food items is not known. The therapy takes several days to complete, as it does with all biological control techniques, this may be impractical in industrial applications (Yehia *et al.*, 2014; Loi *et al.*, 2016).

Chemical additives have also proven to be a popular solution for contaminated foods. Succinic acid, acetic acid, ascorbic acid, and formic acid have been found only marginally effective at oxidizing AFB1-contaminated foods. Infected samples soaked in acidic solutions for a specific time period. Even at atmospheric pressure, high AFB1 deterioration could be evident in as less as 24 hours. (Safara *et al.*, 2010; Lee *et al.*, 2015; Rushing *et al.*, 2016).

Current study was conducted to detect the levels of mycotoxin contaminations in the selected cereal samples, through TLC and HPLC analyses. Furthermore decontamination strategies involving both traditional physical as well as chemical methods were applied to find the aflatoxin degrading efficacy of these methods.

## MATERIALS AND METHODS

**Collection of samples:** The study was conducted at Food and Biotechnology Research Centre of PCSIR Laboratories Complex, Lahore. Total 112 samples of cereals were collected from different areas of Lahore, during the duration of 3 months from March-May 2021. Samples were collected from godown, fields and market. Aflatoxin identification and assessment was done by comparing it with aflatoxin standard varying concentrations according to Association of Official Analytical Chemist (AOAC) 2005.

**Thin Layer Chromatography:** For aflatoxins determination 50 g of ground poultry feeds were placed in a 500 mL conical flask, along with 150 mL chloroform and 25 mL water. After proper shaking the mixture was filtered. Spotting of 5, 10, 15 and 25  $\mu$ L of samples was done on TLC plate after filtration. 5 or 10  $\mu$ L spots were also run on the same TLC plate as standards. TLC plate was placed in chromatographic tank - 1 having diethyl ether and allowed it to move to half. After plate development in tank-1, plate was removed and dried, followed by immersion in chloroform-acetone in 9:1 by volume. Results were recorded for the presence or absence of aflatoxin under UV light at 365 nm wavelength Nisa *et al.* (2014).

**High Performance Liquid Chromatography (HPLC):** For HPLC analysis, 25 g sample was mixed in water and acetonitrile solution ( $H_2O$ : ACN) in the ratio 1:4. After filtration the total volume of filtrate was noted down. 9 mL filtrate was transferred to the Mycosep glass tube and 70  $\mu$ L Acetic acid was added followed by vortexing. 2 mL of the solution was taken and, allowed to evaporate under moderate nitrogen stream at normal room temperature. The process was repeated twice. 200  $\mu$ L hexane was added to re-dissolve aflatoxins followed by the addition of 50  $\mu$ L Tri-Fluoro Acetic Acid (TFA). After subjecting to darkness for few minutes 1.95 mL ACN:  $H_2O$  (1:9 v/v) was added for the separation of layers. 20  $\mu$ L of the filtrate was injected in HPLC for analysis (AOAC, 1995).

**Detoxification Methods:** All contaminated samples were detoxified by applying two different methods i.e., physical, and chemical methods.

**A) Physical methods:** Out of 40 aflatoxin contaminated samples, three highly contaminated sample (>10  $\mu$ g/Kg) from each sample category were selected for detoxification by physical methods i.e., washing and heating. Washing was performed in two batches. First batch of contaminated samples was washed with sterilized distilled water at room temperature, whereas the second batch was treated with hot distilled. For second physical treatment method, contaminated samples were subjected to high heat treatment through boiling of the samples. Amount of detoxified aflatoxin was determined by using the TLC method mentioned earlier.

**B) Chemical methods:** From aflatoxin positive samples, three of the most contaminated samples (>10  $\mu$ g/Kg) were studied for reduction of aflatoxins by chemical treatment. Hydrochloric acid and citric acid were used for detoxification of contaminated samples following the procedure of Zahra *et al.*, (2012).

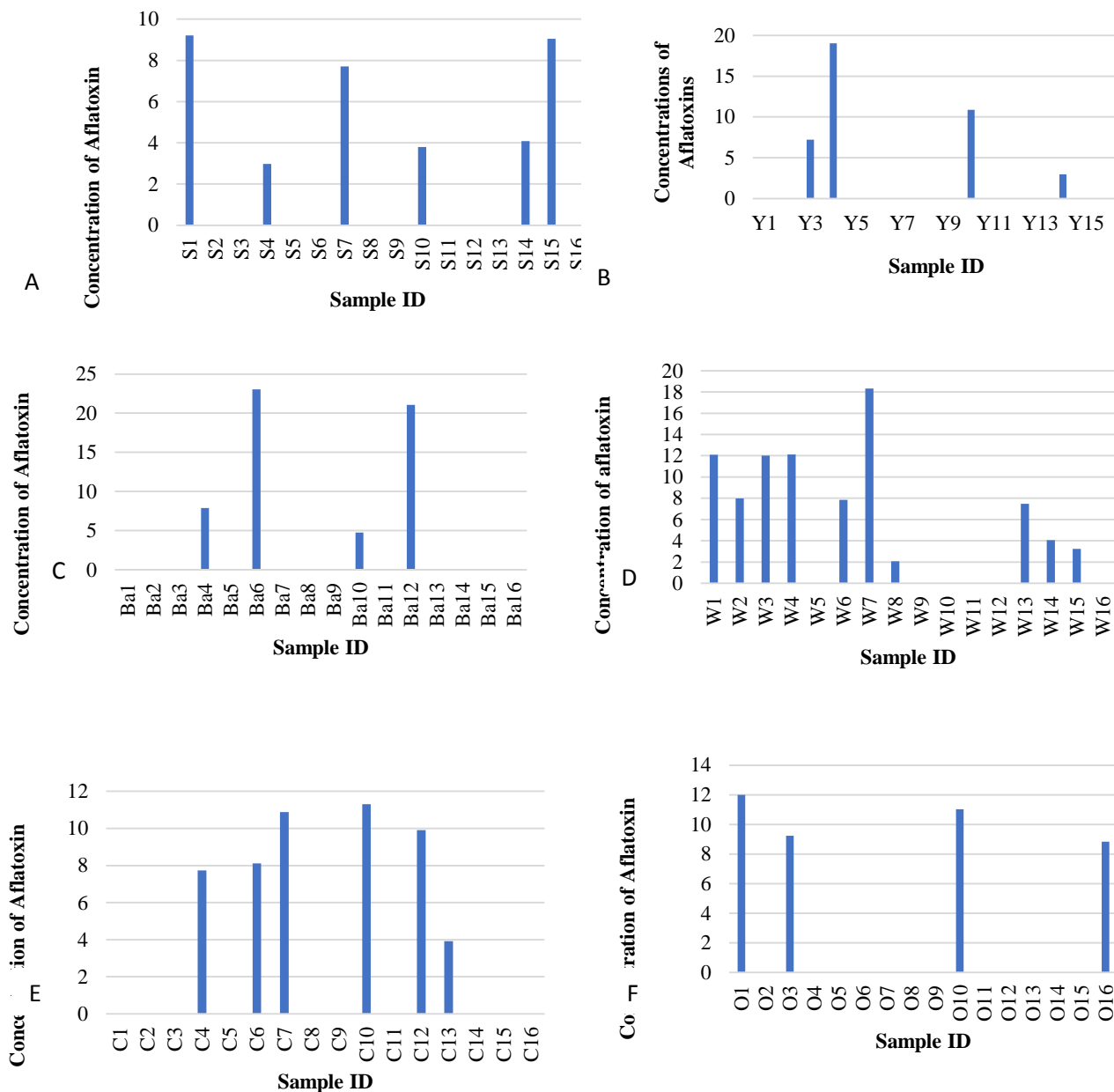
For chemical detoxification, each pre weighted ground sample was treated with HCl at pH 2.0 and 10% Citric Acid. After filtration the filtrate was heat dried. Chemical detoxification of aflatoxin was quantified by

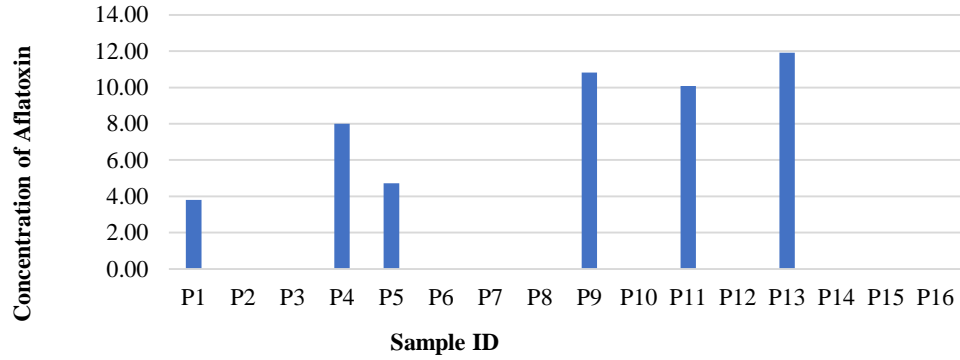
thin layer chromatography using TLC procedure mentioned earlier.

## RESULTS

**Thin Layer Chromatography:** All 112 collected samples were screened by using TLC method for the estimation of aflatoxins. Out of 40 contaminated samples 25 were found contaminated within the permissible limit and 15 beyond the permissible limit (Fig. 1).

**High Performance Liquid Chromatography:** Samples for HPLC testing were selected at random from the batch of samples that were aflatoxin contaminated at levels negligible enough not to be detected by TLC technique. 7 such samples were finally detected. Samples Ye01, Ba09, W05, C03, O08, and P02 (6 in number) were positive for AFG1, Ye01, C03 and P02 samples (3 in number) were positive for AFB1, Ye01, P02 were positive for AFG2 and sample P02 (1 in number) was positive for AFB2 (Table 1; Figs. 2)



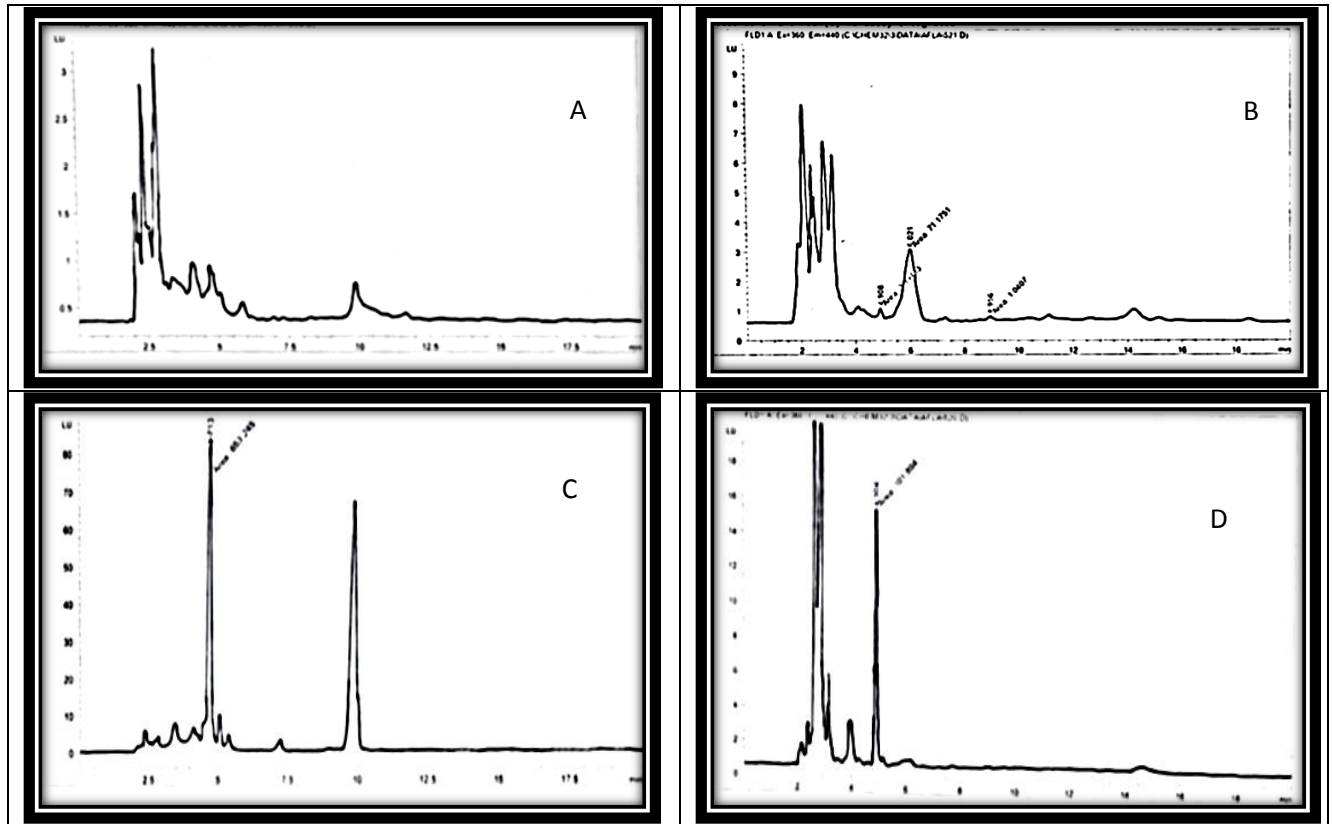


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Figure 2: A: Concentration(µg/Kg) of Aflatoxin in sorghum samples B: Concentration(µg/Kg) of Aflatoxin in Yellow Split chickpeas samples C: Concentration(µg/Kg) of Aflatoxin in Barley samples D: Concentration(µg/Kg) of Aflatoxin in Wheat bran samples E: Concentration(µg/Kg) of Aflatoxin in Corn seeds samples F: Concentration(µg/Kg) of Aflatoxin in Oat samples G: Concentration(µg/Kg) of Aflatoxin in poultry feed samples

Table 2: Levels of Aflatoxin detected by HPLC in cereals samples, not detected by TLC (µg/Kg)

Sample name	sample ID	AFG1	AFB1	AFG2	AFB2
Sorghum	S3	ND	ND	ND	ND
Yellow Split chickpeas	Ye1	0.05 ± 0.02	0.42± 0.03	0.02± 0.01	ND
Barley	Ba9	11.01± 0.45	ND	ND	ND
Wheat bran	W5	2.86± 0.16	ND	ND	ND
Corn seeds	C3	0.37± 0.06	0.11± 0.02	ND	ND
Oat	O8	0.18± 0.03	ND	ND	ND
Poultry Feed	P2	0.19± 0.03	0.07± 0.05	0.02± 0.01	0.02± 0.01



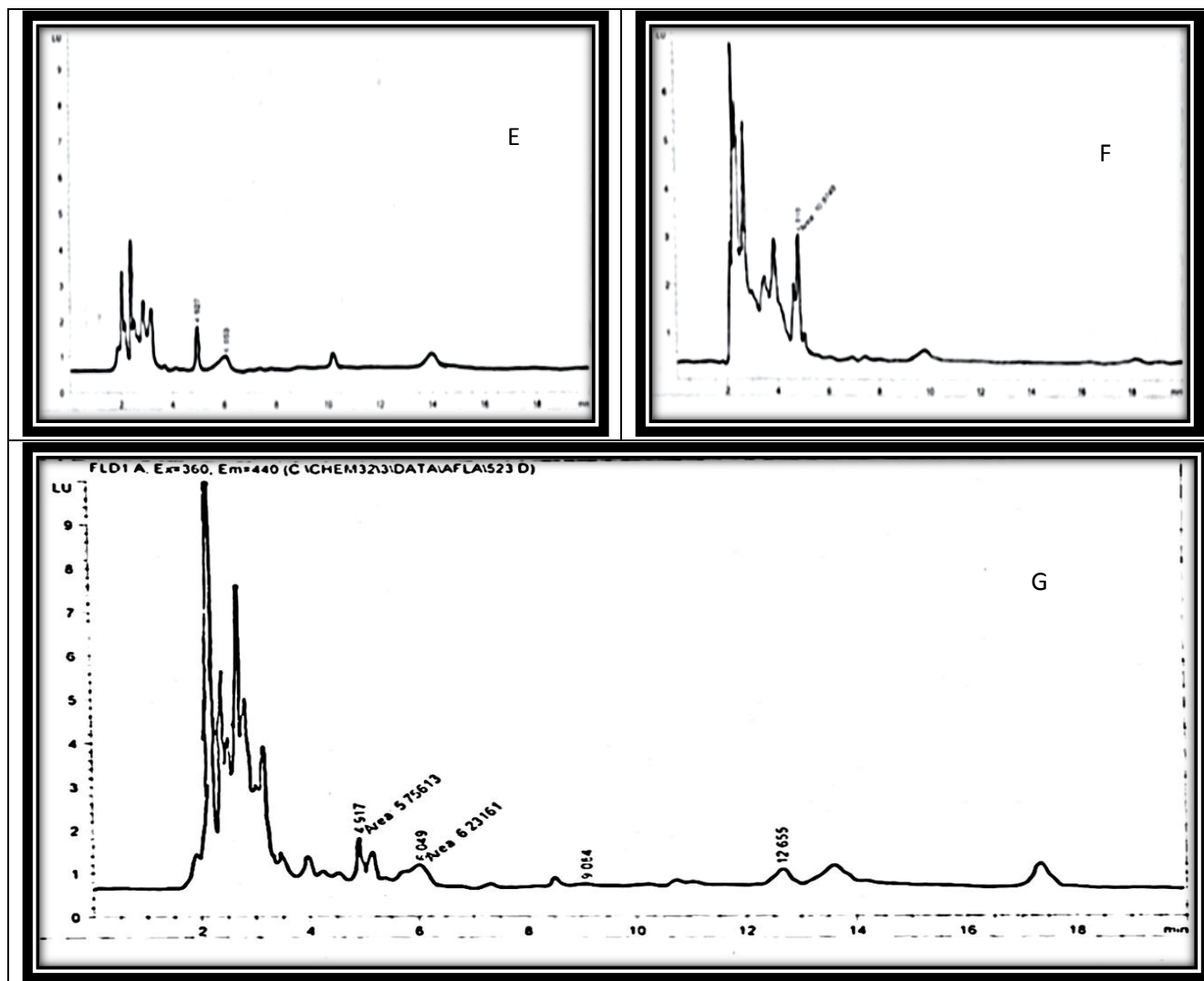


Figure 2. A: Concentration of Aflatoxin in Sorghum B: Concentration of Aflatoxin in Yellow Split Chickpea C: Concentration of Aflatoxin in Barley D: Concentration of Aflatoxin in wheat bran E: Concentration of Aflatoxin in Corn F: Concentration of Aflatoxin in Oat G: Concentration of Aflatoxin in Poultry Feed

#### Detoxification:

**A) Detoxification by Physical methods:** Two physical methods were employed for detoxification of highly contaminated samples i.e., Y4, W7 and B6. Samples with higher contamination value were subjected to washing and boiling. Simple washing resulted in 29.19%, 21.24% and 29.57% reduction respectively which was lower than washing with hot water. When Y4, W7 and B6 samples were washed with hot water, AFB1 reduced from initial concentration of 19.04 $\mu\text{g}/\text{Kg}$ , 18.31 $\mu\text{g}/\text{Kg}$  and 23.03 $\mu\text{g}/\text{Kg}$  to final concentration of 11.9 $\mu\text{g}/\text{Kg}$ , 11.33 $\mu\text{g}/\text{Kg}$  and 13.11 $\mu\text{g}/\text{Kg}$  respectively. The highest reduction percentage was shown when Y4, W7 and B6 were subjected to boiling for 10min at 120

$^{\circ}\text{C}$ . Trend of detoxification by physical methods is shown in Fig 3.

**B) Detoxification by Chemical methods:** Highly contaminated samples i.e., S1, Y4 and W7 samples were treated with chemical solutions of varying concentration of citric acid and hydrochloric acid. Hydrochloric acid reduced the AFB1 levels to 45.37%, 44.84% and 48.42% respectively at its pH2. Citric acid was most effective in reducing AFB1 and showed remarkable reduction percentage of 49.05%, 45.93% and 50.28% respectively at 10% concentration. Overall effectiveness of chemical reagents is shown in Fig 4.

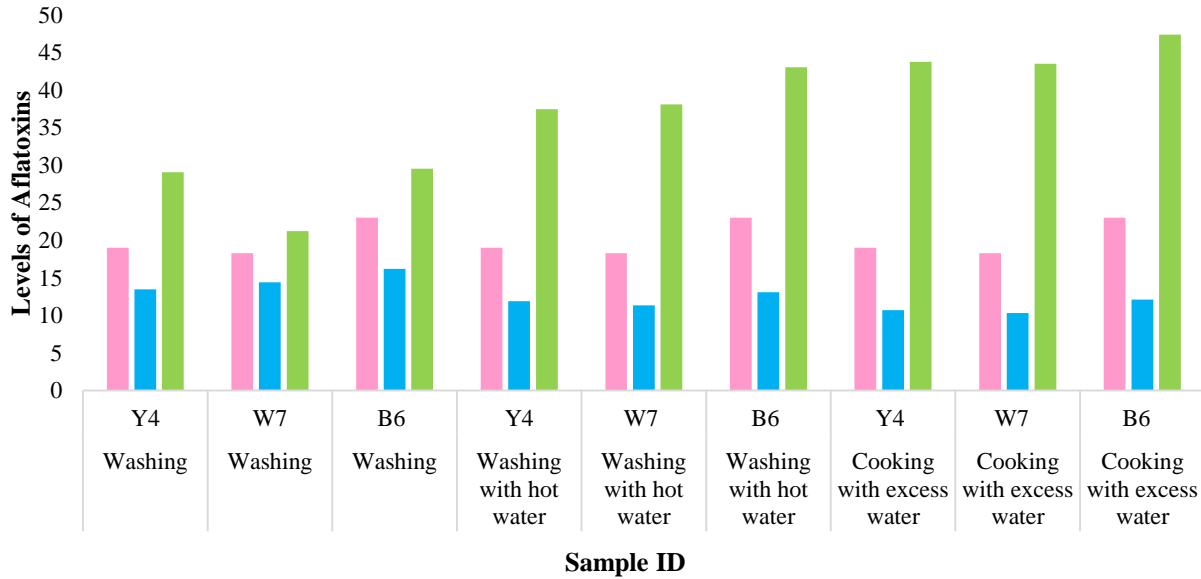


Figure 3. Detoxification by Physical methods

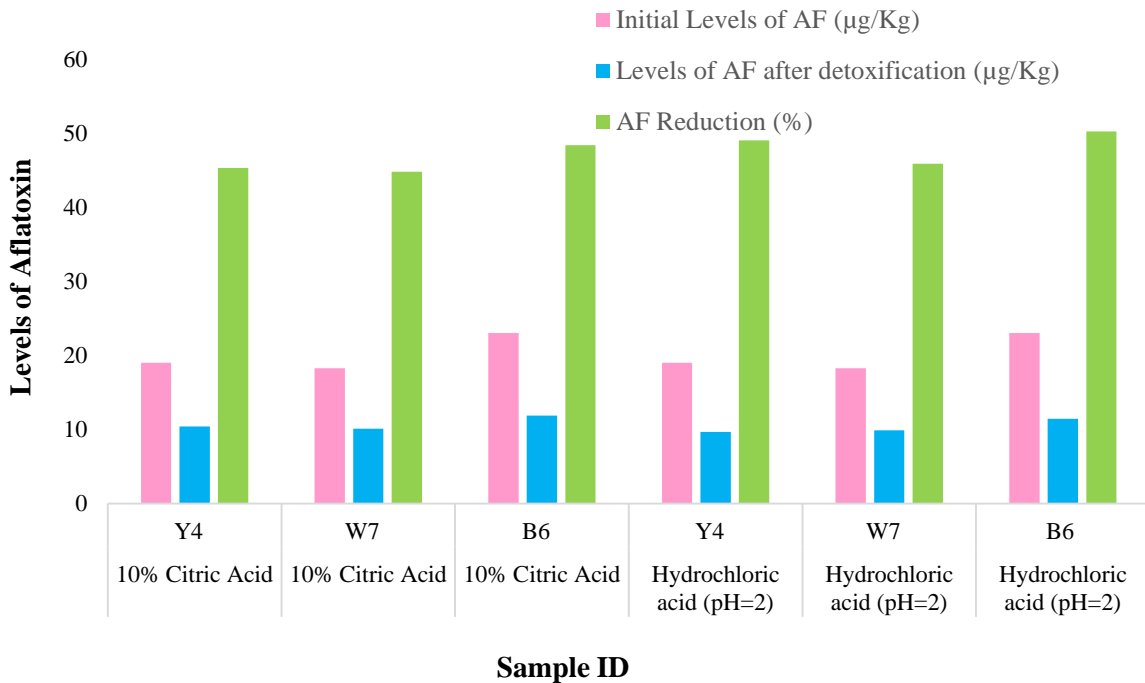


Figure 4. Detoxification by Chemical methods.

## DISCUSSION

Current study was conducted with the main objective to assess the potential presence of mycotoxin aflatoxin in poultry feed. The presence of Aflatoxin at any step of food chain presents a potential risk for all the levels of food chain up to tertiary consumer's level.

Literature reports that aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1), and M2 (AFM2) are the most common types, there are more than 20 varieties of aflatoxin compounds (AFM2). Aflatoxins are found in dry foods (cereals, spices, and dried fruits), while aflatoxins' metabolic metabolites, such as AFM1 and AFM2, are common in milk (Akhtar *et al.*, 2017;

Udomkun *et al.*, 2017). Our focus was AFB1 type aflatoxin due to its abundance and frequent contamination in cereals.

Un Nisa *et al.*, (2021) studied fifty samples for the estimation of aflatoxin. It was observed that 62% of the tested samples were contaminated with aflatoxin, as detected by TLC method. Among them, 22% were infected with aflatoxin B1 and B2 and the rest of samples were found contaminated with aflatoxin B1 type. Samples under study were subjected to TLC analysis, revealing the presence of aflatoxin B1 type. Furthermore, out of 37% samples detected positive for AFB1 with TLC, 13% samples were found contaminated beyond permissible limit. The study somehow corresponds to the study conducted in Croatia where out of 38% total contaminated samples, and 29% of the samples contained aflatoxin at levels higher than the upper permissible limits (Pleadin *et al.*, 2012).

A study conducted by Wacoo *et al.* (2014) for the evaluation of different methods of identification for Aflatoxin detection *i.e.*, TLC, HPLC, MS, ELISA and EIS. Methods were reported to have limitations. During the present study TLC has initially been chosen as an inexpensive method for Aflatoxin detection in cereals. Bringing into account the sensitivity and accuracy offered by a more advanced technique samples were subjected to HPLC analysis for the detection of contamination level in the targeted samples

Detoxification of aflatoxins by physical, chemicals, microbes and enzymes are normal practices that have been in use for many years (Lalah *et al.*, 2019; Guan *et al.*, 2021). Aflatoxin contaminated rice containing 20000-30000 ppb Aflatoxin B1 was exposed to direct sunshine heating (temperature ranging from 37-42 °C). This treatment caused a negligible decrease in AFB1 concentration, ranging from 18000 to 25000ppb corresponding to the reduction of aflatoxin levels up to 10% to 17%. Results from two physical methods applied *i.e.*, washing and boiling with water, clearly supported the temperature dependent detoxification because boiling with water proved more effective than washing with plain water.

The outcomes of this study support the prevalence of risks that the poultry sector faces when it comes to Aflatoxin contaminated poultry feed and additives. The present study must be appreciated for its significance as an indicator of Aflatoxin contamination in poultry feed that should be addressed on priority to ensure healthy nutrition for the livestock.

**Conclusions:** It is concluded that cereal samples from different areas of Lahore showed variable contamination ratio. The samples of Barley collected from the fields of Chung showed highest aflatoxin incidence as compared to other areas. Cereal samples collected from fields and warehouses were more infected by *Aspergillus* as

compared to cereals from utility stores. Utility stores have better storage conditions like suitable temperature and controlled humidity. Field samples were more contaminated due to improper handling and storage, varying temperature and high humidity that favored growth of aflatoxigenic fungus. This study provides recent information regarding aflatoxin contamination of cereals in the samples from different areas of Lahore. The research also provides suggestions for the management of food commodities to prevent aflatoxin contamination during harvesting, transport and storage phase. For contamination removal different detoxification strategies can be used to degrade affected cereals. Most common methods used for detoxification were physical and chemical methods. Further studies with novel approaches to remediate mycotoxins are required to address this potential threat of food contamination.

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