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Assessment of aflatoxins in wheat flour from different areas of Punjab in Pakistan

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Abstract

Pakistan is an agricultural country and agriculture sector contributes approximately 35-40% to the national income. The Punjab is leading in the production of wheat. Wheat is more susceptible to infestation of different kinds of Fungi. Mycotoxins are perilous toxic metabolites fabricated abundantly by such Fungi. For a prolonged period of time, mycotoxins are familiar to attribute grievous diseases in mankind and animals. For this purpose, wheat flour was purchased from local markets and flour mills of different areas in the Punjab. A total of 108 whole wheat flour samples were collected and analyzed (73 samples from markets and 35 samples from flour mills) for aflatoxin, ash and moisture contents. The overall moisture of wheat flour sampled collected from flour mills of Punjab were in the range of 8.315% to 13.937% and from markets the values were in the range 10.302% to 13.803%.The overall ash content of wheat flour samples from flour mills were in the range of 0.618% to 0.983% and for market samples the values were 0.547% to 1.100%. All of the samples were analyzed and found negative for aflatoxin level in whole wheat flour. Results indicated that moisture content (%) in entire the flour samples were below 14%. Investigation of aflatoxin revealed that AFs (AFB_1, AFG_2, AFB_2 and AFG_1) were not established within detectable limits of Thin Layer Chromatography i.e. for B_1 and G_1 detectable limit is ≥0.72ppb while for B2, G2 is ≥0.20 ppb. However, aflatoxin was not be detected by thin layer chromatography, so for more reliable results, sophisticated techniques such as HPLC need to be used to determine aflatoxin in wheat flour samples.

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Introduction

Wheat is the most important cereal crop and a preferable staple food in Pakistan. The Punjab province is leading in the production of wheat among all provinces of Pakistan and it is one of the vital crops to meet the food security challenges in Pakistan. Wheat has been grown on 8,740,000 hectares showing as 0.6% decrease the last year area with the production of 25.195 million tons during the year 2018-2019 (Economic Survey of Pakistan, 2018-2019). Igrejas *et al.* (2020) report that the wheat grown all over the world has now become the excellent source of food. Pakistan stands on 6th position for wheat production in the world with 25,600,000 metric tons production. The country has been exporting whole wheat grains and wheat flour since previous years. Major wheat exporters in the international market are advanced countries having scientific and rapid techniques for wheat production and harvesting and its quality assurance as stated by the international organization such as FDA, WTO and WHO standards. So, Pakistan has to face many challenges to compete in the export market (Iqbal *et al.*, 2013). Nowadays wheat is the most important crop in the world as it is an important source of essential nutrients for animals and humans (Shurtleff and Aoyagi, 2020).

Wheat flour release energy, which is an important source of starch (carbohydrates, protein (gluten), vitamin, bran and minerals. Many kinds of items are produced from whole wheat flour that is beneficial in the bakery industry for different baked food items (Maliha *et al.*, 2010). Wheat is a high glycemic food that has a potential effect on prediabetics patients (Davis, 2019). Wheat is an especially delicate to warm strain. Presentation to temperatures over 30°C can hurt leaf photosynthetic process and accelerate the senescence, bringing about diminished grain making. (Atwell and Finnie, 2016).

Aflatoxins (AFs) are toxic metabolites created by Aspergillus fungi. There are four main types of AFs (B1, B2, G1, and G2) all are toxic to humans and animals (Hanioka *et al.*, 2012). AFB1 is the largest toxic form in which there are fewer tolerance levels in the food product. Dietary sullying with AFs may prompt a major measure of wellbeing impacts (Nakai *et al.*, 2008), making pollution of foodstuff by AF a medical problem of fundamental concern. AF B1 is mutagenic carcinogenic, and teratogenic, causing damage the liver (Calderari *et al.*, 2013), but also to other systems and organs (Bakırdere *et al.*, 2012), Natural incidence and inadequate long-term storage are the major routes leading to the existence of Aspergillus in food. The mold finds explicitly good situations for its spread in the Amazon rainforest, with temperatures above 25°C and relative humidity of more than 80 % (Atayde *et al.*, 2012).

More than four hundred mycotoxins have previously been discovered. However, aflatoxins (AFs) have become the most broadly known mycotoxins created by flavus and parasiticus under favorable conditions (Khan *et al.*, 2014), among them just four aflatoxins (AFB1, AFB2, AFG1 and AFG2), found naturally on a different variety of agricultural commodities insignificant levels (Asghar *et al.*, 2016). AFs have been described as immunosuppressive, mutagenic, hepatotoxic, neoplastic, carcinogenic and classified by the International Agency for Research on Cancer (IARC) as Class 1 human carcinogens (Varga *et al.*, 2015). AFs are related with numerous chronic diseases including reproductive problems, immune suppression, blood and nerve defects, digestive problem, jaundice and anemia (Ajani and Pasha, 2017).

The well organized and comprehensive check and balance is a significant issue in Pakistan to ensure the food security and safety as well as for export objectives. Numerous techniques and approaches for the qualitative and quantitative determination of aflatoxins have been established. Techniques based on TLC (Thin layer chromatography), ELISA (enzyme linked immune sorbent assay) and HPLC (high-performance liquid chromatography) are major techniques for aflatoxins determination. The present research was planned to determine the aflatoxins contamination in wheat flours through the Thin Layer

Chromatography. Further, the level of moisture content was also analyzed in the wheat flour samples to analyze its relationship, if any, with the contamination of aflatoxins.

Materials and methods

Study Design and Sampling

This research was conducted to determine ash and moisture content in wheat flour samples and thereafter, to evaluate the quantitative level of aflatoxin in whole wheat flour samples collected from the markets and flour mills in different districts of Punjab in Pakistan. A total of 108 whole wheat flour samples were collected and analyzed (73 samples from markets and 35 samples from flour mills).

Sample Collection

Random samples of whole wheat flours were collected from different markets and flour mills of Punjab Province. For the purpose 1-kilogram sample was collected from each point via random sampling from selected areas. Samples was used to collect homogeneous & uniform sample from randomly selected flour bags from markets and flour mills. Each sample was collected from 3 parts of bags (Upper, Middle and Lower). Samples were collected in clean polyethylene bags, transported safely to the labs and stored at ambient room temperature following the standard protocols to prevent the contamination. All collected samples were free from chemical preservation during the storage and there was no insect infestation in the samples.

Moisture and Ash analysis was performed in Food Technology and Biochemistry labs of Pakistan Council for Scientific and Industrial Research (PCSIR), Lahore. Aflatoxin detection was performed in the Organic lab of Chemistry Department at Government College University Faisalabad (GCUF).

Chemical Composition of Wheat Flour

Moisture content was estimated by drying 5g of the sample in a hot air oven at 105± 5 °C for 24 hours according to AACC Method No. 44-01 (AACC, 2000) (Model: B115 No. 86490), PCSIR, Pakistan) till the weight of the sample became constant. The cry matter was measured as per following relationship.

$$\text{Dry matter (\%)} = \frac{\text{Wt. of dried sample (g)}}{\text{wt. of fresh sample (g)}} \times 100$$

The ash contents were estimated from 3g flour sample taken in crucible according to AACC Method No. 08-01 (AACC, 2000). Afterwards, charring of the samples was done on the low flame until sample became free of smoke. The crucible was placed in the muffle furnace (Thermolyne 1400 Furnace, PCSIR) at 550±5 oC for 5-6 hours. After that the samples were placed in the desiccator until room temperature was attained. The ash was calculated according to the following formula.

$$\text{Ash contents} = \frac{\text{Wt. of ash (g)}}{\text{Wt. of sample (g)}} \times 100$$

Thin Layer Chromatography (TLC)

Thin layer chromatography was determined using the method described in Sherma and Fried (2003). Chemicals used were of analytical grade and acquired from different distinguished companies in current research. The chemicals were obtained from E. Merck (Darmstadt, Germany), Sigma-Aldrich (St.Loius-MO, USA) and Distill water purchased from BDH (Poole, England). Aflatoxins Standard AFB1 (2.03 µg/ml), AFG1 (2.02 µg/ml), AFB2 (0.503 µg/ml), and AFG2 (0.504µg/ml) were purchased from Romer Labs Inc. and stored at -4°C in a chiller after wrapping in an aluminum foil. Whatman no.4 filter paper, wrist shaker, electrical hot plate, TLC tank, TLC plates (having silica gel coating on aluminum sheets with a layer thickness of 0.25 mm), 20x20 cm (E.Merck, Darmstadt, Germany) were used. Sample grinder with sieve size 20 (Cyclone 1093 mill, Sweden), beakers 250 ml, graduated cylinder 100 ml, separating funnel 500 ml, automatic pipette 300 µl, funnel 100 mm diameter and UV detector (Germany) were also used during various analyses.

Extraction and Purification of Aflatoxins

It is marked that aflatoxins are generally found in most food commodities. That is the reason; for precise determination of aflatoxins, AOAC official

method number 972.26 was followed (Helrich, 1990). Each representative sample of wheat flour (powder form) weighed up to 50g accurately and precisely through calibrated weighing scale and added in 25 ml distilled water to moist the sample for easy extraction. Then 250ml analytical chloroform were added to the sample and suspension of the matrix was mixed at 30 rpm (rotation per minute) for 30 minutes at wrist action shaker. The integrated matrix was then filtered via Whatman filter paper No.4. After that 50ml filtrate was transferred into a 250ml beaker for chloroform evaporation on a hot plate at 70 °C temperatures for 15 minutes and then placed at room temperature for further dryness at 25 °C for further examination through thin layer chromatography.

TLC Plates Development

The dried matrix of the sample was liquefied in 0.5ml chloroform (analytical grade) and shaken accurately. To check the level of aflatoxins, spots of different sizes of wheat flour samples were spotted on TLC plate. Then plates are placed into first mobile phase having 50 ml diethyl ether to remove impurities from the sample. After that plate placed on a hot plate for dryness for 1 minute and transferred into 2nd mobile phase having extra pure acetone (45mL) and chloroform (5ml). The tank was covered with lid and left the plates inside the tank till the complete development of spots to stop line. After TLC plates development, these plates were removed from 2nd mobile phase and dried for further examination. After that interpretation of TLC plates were conducted under the UV light detector at 366 nm and 254nm wavelengths. Aflatoxins standard (AFB1, AFB2, AFG1, and AFG2) was developed with Acetonitrile in TLC tank to check the precision and accuracy of the method. Retention factor was calculated by following equation:

$$\text{Retention factor} = \frac{\text{Distance travled by solute}}{\text{Distance traveld by solvent}}$$

The Concentration of Aflatoxins was individually calculated by using following equation.

$$\text{AFB1}(\mu g/kg) = \frac{S \times Y \times V}{X \times W}$$

In equation,

S = Volume in μl of AFB1 standard equal to unknown.

Y = Concentration in $\mu g/ml$ of AFB1 standard.

V = Volume in μl of final dilution of sample extract.

X=Volume

in μl of sample extract spotted to given flourescent intensity equal to S.

W = Weight of sample in grams present in the final extract.

Same procedure to calculate concentrations were followed for the AFB_2, AFG_1 and AFG_2 spots. The concentrations of TAFs (B_1, B_2, G_1, and G_2) were calculated by using underlying equation.

Total AFs (TAFs) = Concentration of AFB_1 + AFB_2 + AFG_1 + AFG_2

Statistical Analysis

Data was statistically analyzed by using SPSS. Standard deviation (SD). Significance level of samples were determined following the respective method described by Steel *et al.* (1997).

Results and discussion

Chemical composition

Flour Mills and Grain Markets

There was significant different of moisture content among all of the collected flour samples. In case of samples from flour mills, the overall moisture of wheat flour sampled from selected districts of Punjab, Pakistan was in the range of 8.315 ± 0.037% to 13.937 ± 0.024%. Least moisture was recorded in Rawalpindi (8.315 ± 0.037%) and maximum moisture contents were found in Mianwali (13.937 ± 0.024%) as shown in Table 1. In case of samples obtained from markets, significant difference was found in moisture content and the level was found in the range of 10.302% to 13.803% as shown in the Table 2. Least percentage moisture contents were recorded in the samples collected from Sahiwal (10.302 ± 0.066%) district, while maximum percentage of moisture contents (13.803 ± 0.052%) were found in the samples collected from markets. The results for moisture analysis showed that moisture content (%) in all the samples was less than 14%.

Table 1. Comparison of means for different district of Punjab regarding moisture (%) and ash content from flour mills sample.

North Punjab	No. of Samples	Moisture (%)	Ash (%)
Islamabad	6	11.025±1.816^{A}	10.980±0.341^{H}
Rawalpindi	6	8.315±0.037^{B}	11.110±0.053^{GH}
South Punjab			
Bahawalnagar	3	13.103±0.048^{A}	0.683±0.012^{B}
Bahawalpur	3	12.657±0.018^{B}	0.733±0.015^{B}
Layyah	3	10.557±0.032^{G}	0.860±0.017^{A}
Lodhran	3	10.923±0.030^{E}	0.707±0.027^{B}
Multan	3	11.260±0.035^{D}	0.883±0.055^{A}
Muzaffargarh	3	13.080±0.012^{A}	0.857±0.024 ^{A}
Rahim Yar Khan	3	12.340±0.042^{C}	0.733±0.015^{B}
Rajanpur	3	10.773±0.035^{F}	0.657±0.024^{B}
Vehari	3	10.257±0.029^{H}	0.667±0.024^{B}
Central Punjab			
Bhakkar	3	10.980±0.341^{H}	0.863±0.027^{CD}
Chiniot	3	11.110±0.053G^{H}	0.830±0.012^{DE}
Faisalabad	6	11.902±0.247^{E}	0.722±0.017^{G}
Gujranwala	3	13.360±0.044^{BC}	0.810±0.021^{DEF}
Gujrat	3	9.820±0.021^{I}	0.787±0.050^{EF}
Hafizabad	3	12.470±0.082^{D}	0.767±0.028^{FG}
Jhang	3	11.550±0.012^{EF}	0.787±0.009^{EF}
Kasur	3	13.153±0.037^{C}	0.643±0.015^{H}
Lahore	6	13.248±0.011^{C}	0.618±0.016^{H}
Mianwali	3	13.937±0.024^{A}	0.917±0.015^{BC}
Nankana Sahib	3	13.680±0.059^{AB}	0.653±0.027^{H}
Narowal	3	11.413±0.167^{FG}	0.820±0.017^{DEF}
Okara	3	11.847±0.06^{4E}	0.983±0.007^{A}
Pakpattan	3	10.950±0.031^{H}	0.943±0.027^{AB}
Sahiwal	3	11.153±0.015^{FGH}	0.933±0.015^{AB}
Sargodha	3	12.647±0.041^{D}	0.830±0.017^{DE}
Sheikhupura	3	13.437±0.024^{BC}	0.637±0.009^{H}
Sialkot	3	9.583±0.018^{I}	0.817±0.022^{DEF}
Toba Tek Singh	6	11.272±0.080^{FGH}	0.915±0.008^{BC}

Means sharing similar letters in a column are statistically non-significant (P>0.05).

It means that wheat was stored with safe moisture levels as fungi require 14% to cause food spoilage by the production of secondary metabolites i.e. aflatoxins. Moisture content analysis of present survey was critical, as this is well reported by different researchers that moisture content (%) is strongly associated with the level of aflatoxin contamination (Iqbal *et al.*, 2010).

Several researchers have reported a higher level of aflatoxins at higher moisture contents. Aflatoxin production by A. flavus and A. parasiticus at a given temperature has been described in relation to moisture content who detected aflatoxin in almonds stored at 100 % relative humidity whereas no detection was reported at lower relative humidity (Rogel-Castillo *et al.*, 2015).

Ash Content Analysis

Flour Mills and Grain Markets

Ash percentage was measured in all of the samples collected from flour mills, of the Punjab Province.

Table 2. Comparison of means for different district of Punjab regarding moisture (%) and ash content from market sample.

North Punjab	No. of Samples	Moisture (%)	Ash (%)
Islamabad	9	12.702±0.362^{A}	0.664±0.029 ^{B}
Rawalpindi	9	12.918±0.322^{A}	0.880±0.019^{A}
South Punjab			
Bahawalnagar	6	12.702±0.362^{A}	0.910±0.022^{C}
Bahawalpur	6	12.918±0.322^{A}	0.913±0.033^{C}
Layyah	6	12.702±0.362^{A}	0.895±0.018^{C}
Lodhran	6	12.918±0.322^{A}	0.987±0.015^{AB}
Multan	6	12.702±0.362^{A}	1.043±0.014^{A}
Muzaffargarh	6	12.918±0.322^{A}	0.950±0.015^{BC}
Rahim Yar Khan	6	12.702±0.362^{A}	0.803±0.019^{D}
Rajanpur	6	12.918±0.322^{A}	0.923±0.015^{C}
Vehari	6	12.702±0.362^{A}	0.950±0.036^{BC}
Central Punjab			
Bhakkar	6	11.470±0.087^{EF}	1.027±0.033^{ABC}
Chiniot	6	13.160±0.296^{AD}	0.643±0.013^{HI}
Faisalabad	12	13.188±0.137^{ABC}	0.793±0.017^{EF}
Gujranwala	9	13.388±0.106AB	0.626±0.010^{HI}
Gujrat	6	13.280±0.108^{ABC}	0.598±0.017^{IJ}
Hafizabad	6	13.295±0.100^{ABC}	0.607±0.030^{IJ}
Jhang	6	13.542±0.090^{AB}	0.882±0.024^{D}
Kasur	6	13.637±0.100^{A}	0.650±0.013^{HI}
Lahore	18	12.649±0.163^{CD}	0.812±0.020^{E}
Mianwali	6	12.320±0.034^{DE}	0.955±0.053^{CD}
Nankana Sahib	6	13.578±0.117^{A}	0.660±0.012^{GHI}
Narowal	6	13.803±0.052^{A}	0.547±0.01^{4J}
Okara	6	10.438±0.094^{G}	1.018±0.039^{BC}
Pakpattan	6	10.838±0.051F^{G}	1.100±0.044^{A}
Sahiwal	6	10.302±0.066^{G}	1.073±0.015^{AB}
Sargodha	6	13.472±0.104^{AB}	0.697±0.026^{GH}
Sheikhupura	12	12.813±0.097^{BCD}	0.731±0.025^{G}
Sialkot	6	12.313±0.136^{DE}	0.740±0.012^{FG}
Toba Tek Singh	12	11.357±0.180^{F}	1.061±0.023^{AB}

Means sharing similar letters in a column are statistically non-significant (P>0.05).

The overall ash content of wheat flour sampled from selected districts of Punjab; Pakistan was in the range of 0.618 ± 0.016% to 0.983 ± 0.007%. Least ash was recorded in Lahore (0.618 ± 0.016%) and maximum ash contents were found in Okara (0.983 ± 0.007%) as shown in the Table 1. The samples acquired from markets; ash level was found in the range of 0.547 ± 0.019% to 1.100± 0.044%. Least percentage ash contents were recorded in the samples collected from Narowal (0.547 ± 0.019%) district samples, while maximum percentage ash contents (1.100 ± 0.044%) were found in the samples from Pakpattan district

(Table 2). The ash contents in the different types of wheat flour was calculated using spectral and chemometric methods and the ratio ranged from 1.679 to 1.688% (Moroi *et al*., 2011). A similar result was found in the study of Ferrão and Davanzo (2005) in which the ash content was fixed from 0.330 to 1.287%. According to Czaja *et al*. (2020) the Ash and moisture were calculated using the Raman spectroscopy and the ratio ranged from 0.4 to 5%.

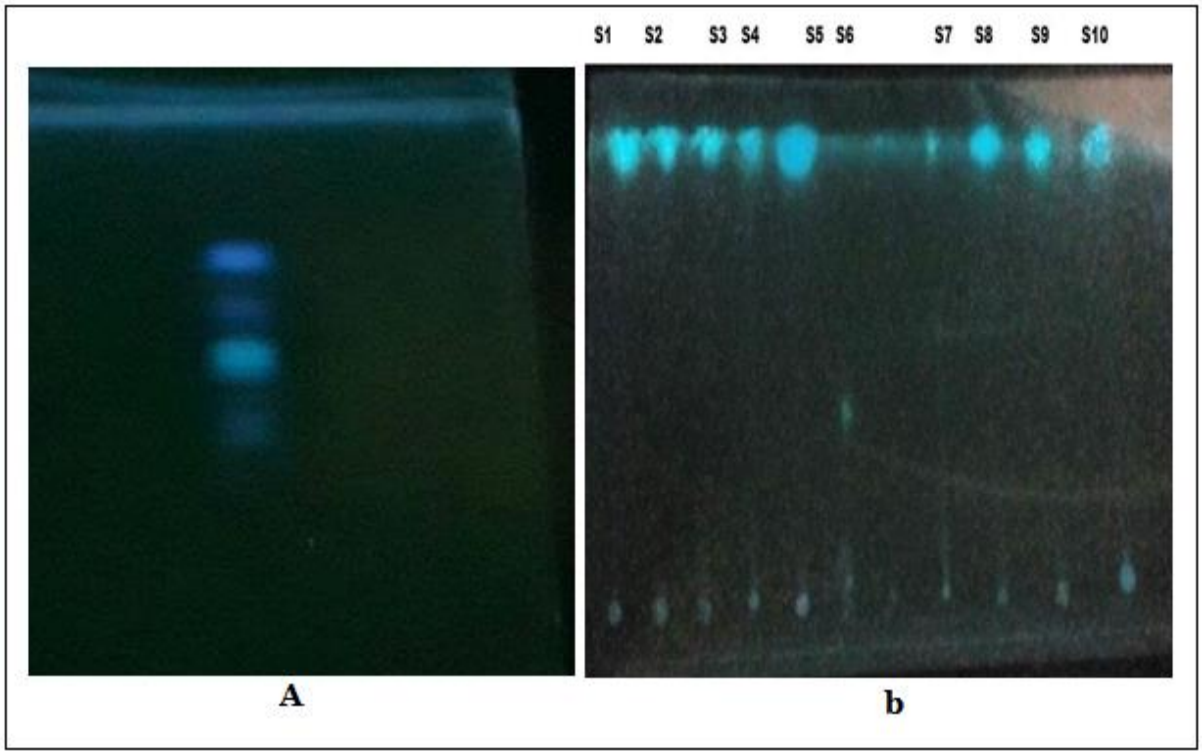

Fig. 1. AFB1, AFB2, AFG1, AFG2 standard under UV (366nm) light (a) TLC plate's visualization under UV Light of wheat flour samples collected from flour mills (b).

S1= 1 F-FM-ISB

S2= 3 F-FM-RWP

S3= 5 F-FM-GUJ

S4= 9 F-FM-NRW

S5= 10 F-FM-LHR

S6= 14 F-FM-SKP

S7= 15 F-FM-SRG

S8= 18 F-FM-FSD

S9= 24 F-FM-SHW

S10= 30 F-FM-BWP

Aflatoxin optimization

The aflatoxin could not be detected in the wheat flour samples collected from the flour mills in the imaging of the TVC plate under UV light, as shown in Fig 1. Moreover, the aflatoxin also could not be detected in the wheat flour samples collected from the market flour samples in the imaging of the TVC plate under UV light, as shown in Fig 2. After investigation of all samples, it was found that aflatoxins were not present in all 108 (100%) wheat flour samples within detectable limits of Thin Layer Chromatography i.e. for B1 and G1 detectable limit is ≥0.72ppb while for B2 and G2 is ≥0.20 ppb. The absence of aflatoxins in the wheat flour samples might be because of low moisture level, due to well-processing storage conditions.

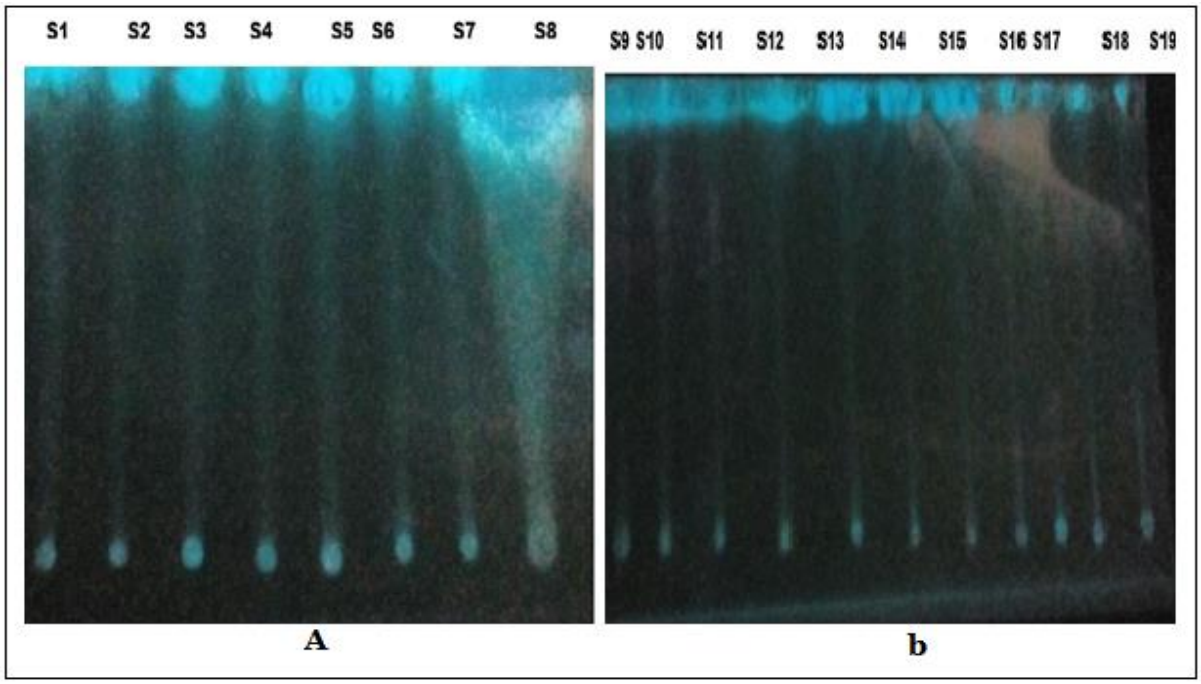

Fig. 2. TLC plate's visualization under UV Light of wheat flour samples collected from market.

S1= 36 F-MKT-ISB
S2= 39 F-MKT-RWP
S3= 44 F-MKT-GRW
S4= 47 F-MKT-SKT
S5= 58 F-MKT-LHR
S6= 59 F-MKT-KSR
S7= 62 F-MKT-NKS
S8= 63 F-MKT-SHK
S9= 69 F-MKT-BKR
S10= 71 F-MKT-MWL
S11= 73 F-MKT-FSD
S12= 81 F-MKT-TTS
S13= 88 F-MKT-OKR
S14= 91 F-MKT-MLT
S15= 99 F-MKT-BWN
S16= 101 F-MKT-RYK
S17= 104 F-MKT-MZG
S18= 108 F-MKT-LAH

The results indicated that, in present study no sample was found contaminated with aflatoxins. Similarly, Maliha *et al.* (2010) and Sahar *et al.* (2009) reported no aflatoxin detection in stored wheat grain samples taken from different regions of Punjab, and Sindh. This has also been verified by the reading showed in China by Houng *et al.* (2016) examined 42 composite samples of Rice and Maize for three mycotoxins i.e. Aflatoxins, Fumonisins, and Ochratoxin A. Approximately similar study was conducted by Iqbal *et al.* (2014) in which out of 185 samples 137 samples were not contaminated with aflatoxins within detectable limits and among forty-six samples of different cereals including corn, wheat, and rice; it contained no aflatoxins but detected with ZEA and OTA, though far below the EU legal permissible limits when examined for aflatoxins and other mycotoxins. This may be due to firstly improved management

conditions have been improved, secondly, moisture percentage not exceeding the threshold limits. The moisture contents in all the samples used in the current study have lesser than 14%. Furthermore, we can also assume that there may be the contamination of aflatoxins in wheat flour, but their levels may be lower than the detectable limits of thin layer chromatography (TLC), which are far below than Pakistan Standards and Quality Control Authority (PSQCA) limits i.e. 20ppb and as limits set by Codex Alimentarius which are 15ppb for cereals (Nisa *et al*., 2015). Same kind of results were observed by Kumar *et al*., (2017) during analysis of aflatoxins in water damaged and moldy wheat in Queensland. International market requirements vary from country to country as Pakistan Standard and Quality Control Authority (PSQCA) MTL is 20µg/Kg while EU has a different maximum residual limit for aflatoxins B1 and for total aflatoxins i.e. 2µg/Kg and 4 µg/Kg, respectively. Hence the samples having aflatoxin in the range of 0 to 20µg/Kg can be used in international trade. The result of current study suggested and depicted the wheat flour with moisture content less than 14 %, which is a true demonstration of good processing condition applied during the milling process. Similarly, the non-detection of aflatoxins in our study depict the clear food safety status of wheat flour being consumed by common man in Pakistan.

Conclusion

For moisture content highest moisture was found in the samples from Central Punjab regions. Whereas lowest moisture content was calculated in the North Punjab regions. Overall, 12.60 % moisture was found in all samples. For ash contents more ash was observed in samples from Central Punjab regions. While lowest ash was calculated in samples from Central Punjab regions. As for as aflatoxins are concerned, no sample was found to contain aflatoxins in the 108 samples that shows that wheat flour being consumed by the consumers in all these regions is quite safe for consumption. However, there is need to determine the aflatoxins in wheat flour sample through sophisticated technique like HPLC for more reliable findings.

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