

AN INVESTIGATION ON AFLATOXIN QUANTITIES IN THE PREPARED AND PROCESSED AUTUMN AND SPRING WHEAT FLOUR SAMPLES COLLECTED FROM 5 SUPERIOR PROVINCES OF IRAN

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ABSTRACT

By increasing the health cultures around the world and advancement in science technology and also development of food hygiene, infections and food poisoning due to bacteria and their toxins has been decreasing. Unfamiliarity of most farmers, especially during maintenance of wheat and wheat flour which is an important component of the food chain in most countries around the world; is destructive to the conditions of growth and development of microorganisms and fungi which resulted in a significant economic losses and irreparable physical damages to people and governments annually. Hence, in this study, flour samples from different areas of five providences of Iran (Mazandaran, Gilan, Zanzan, Kermanshah and Khuzestan) are collected and the level of the aflatoxins production was measured by using ELISA tests. The mean 9.25 and standard deviation of 1.70 in fourteen samples from fourteen city areas that are sampled indicates the presence of aflatoxin in domestically produced wheat flour. After analyses of the Pearson's Correlation significant at $p < 0.05$ it is determined that the amount of aflatoxin in wheat and also in mixed flour was reversed (PC; -0.135), and this association was not statistically significant (sig: 0.65). At the end, results of this experiment and similar previous studies which used different methods for evaluating amount of aflatoxin, indicate that ELISA test is a renewable, simple, selective, sensitive, quick and affordable method which is proper for determination of mycotoxins in food products.

KEY WORDS: *Aflatoxin, Wheat flour, ELISA, Fungal infections*

Introduction

By increasing the health cultures around the world and advancement in science technology and also development of food hygiene, particularly food hygiene infections and food poisoning due to bacteria and their toxins has been decreasing. Since fungal contamination of food and effects of using such food products is increasing day by day (FAO., 2004). Unawareness of most farmers and livestock owner about growth and development of microorganism and fungi leads to a considerable economic losses and irrecoverable physical damages to people yearly. Food and Agriculture organization of the United Nations (FAO) estimates the contamination of microorganism's financial loss to the national wealth around the world, which is about 10% of total food production. Environmental factors such as temperature, humidity, air composition and also general conditional grains play a significant role in deterioration of grains. In general, more than 13% of seed grains

humidity and more than 65% of relative humidity barn and also the temperature between 5-10% provide the conditions for microbial activity (FAO., 2004). Fungi during their growth on different food products, causes reduction in the quantity of food due to elimination of the fungal contaminated portion and also reduce the nutritional value of the food products, because the adverse effect on the nutrients of the food components, produce secondary metabolites which is called mycotoxins or a toxin products by fungi. If these toxins are taken by living organisms, severe deleterious effects such as carcinogenicity, malformations, growth retardation, immune suppression and mutagenesis are caused. Mycotoxins are a group of toxic metabolites that are relatively resistant. Mycotoxins are produced by fungi in secondary metabolic pathways in fungal cells, results in contamination of food production and maybe in the surrounding environment. In addition, to the production of mycotoxins and other toxic metabolites. The significant changes that occur in an activity of microorganisms in the cereals including: Reduction of the products nutritional value due to the decomposition of proteins. Lipids and sugars also reduced Aroma production, reduction of gluten, resulting in devaluation of the processed flour and effects on the rheological properties of dough. Most known mycotoxins in products derived from acetate or amino acids that are produced by species belonging to the genus *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* (Gareis, et al., 2003). Many fungi which produced mycotoxins grow well in hot and humid weather conditions and produce and secrete significant amounts of toxin. Dispersion and distribution of mycotoxins and fungi in nature are largely dependent on geographical conditions (Gareis, et al., 2003). In Iran due to varied climatic conditions, the possibility of exciting a wide range of toxigenic fungi and toxins in the environment is high. Diversification of agriculture has an important role in the development of more types of mycotoxin-producing fungi growing on food (Rajab Zadeh., 1375). For example, products such as wheat, corn, rice and peanuts that grow in slightly different terms are suitable for growing fungi such as *Aspergillus* and *Fusarium* on them. The fungi which are producing toxins by contamination of grain directly, cause reduction in the quality and quantity of food grains and indirectly have bad effects on value of meat and dairy products (Rajab Zadeh., 1375). According to the above description, on a fungal infection of wheat flour, several studies have been conducted that in some of them the most isolated fungal species belonging to the genus *Aspergillus* and *Fusarium*. According to important role of wheat flour in food chain in this research the mycotoxins contamination is investigated.

Materials and Methods

Sampling and preparation of cell extracts

Sampling freshly wheat (harvesting time: late of April till at the end of August) from 7 wheat-producing provinces including southern province (Khuzestan) West (Kermanshah and Hamedan) and North (Zanjan, Ardabil, Mazandaran and Golestan). For each hundred tonnes, one kilogram sample were provided which after preparation wheat sample for consumption (separation of debris and winnowing sieve special flour factory, drying and adjust the humidity and mixing and re-mixing) was done for each sample. Afterwards, four samples (100 gr) randomly selected for measurement sample, the control sample, stoke sample and respectively. Then wheat sample was taken, and trituration done by laboratory mills. At the end wheat, sample is ready to start the extraction of toxin.

Releasing of toxin in separation solution

Releasing of the toxin in the separation solution is done by solvent extraction, which contain of 40 ml methanol, 40 ml ethanol and 20 ml acetone. First 10 gr of triturated wheat sample was taken and transferred to a proper plate. Then 20 ml normal saline and 20 ml solvent extraction was added and was shaken for about 30 minutes. In the next step, the sample transfers to water bath, and it was kept at the volume of the extract in Falcon tubes reaches less than 10 ml. Then filtrated the extract through a Whatman no.1 filter paper that does not have activated charcoal. The simultaneous transfer operations with 10 ml of deionized distilled water to wet the filter and dilute the extract and speeding the movement occurs.

Results

Provinces where the samples were obtained.

In this study, wheat flour samples are collected from 7 provinces of Iran (Khuzestan, Golestan, Zanzan, Ardabil, Mazandaran, Kermanshah and Hamedan). The total number of wheat flour samples collected from North, West and South, 14 samples out of 14 cities. Area selected for sampling were chosen according to their recent 5-year production volume. The selected areas are located in the northern region: Golestan, Zanzan, Ardabil, Mazandaran. Kermanshah province in the west and Khuzestan in south. Unfortunately, collected samples from the province of Fars and Bushehr were excluded from the extraction and measurement due to the water damage and disorder in microbial health especially in fungal biomass. We have chosen 50 tons of wheat from Aliabad, Gonbad, Kordkuey the cities of Golestan province; Ardabil province, Garmi and Maghan; Mazandaran province, Nek, Sari, Ijrood and khudabande from Zanzan province.

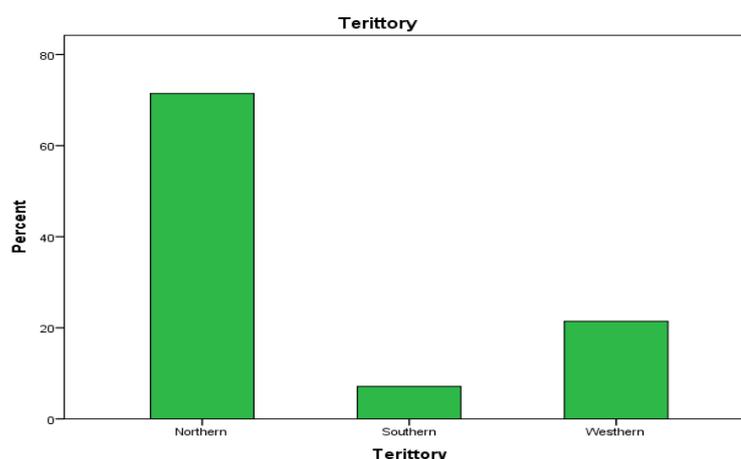


Chart 1: Percent distribution of samples obtained from three regions of North, West and South of Iran.

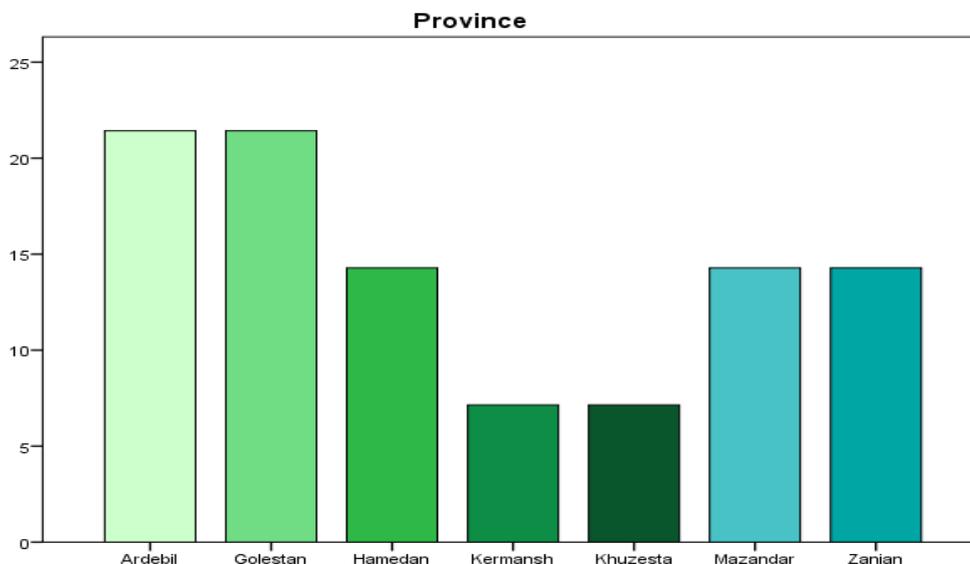


Chart 2: Percent distribution of samples from seven provinces

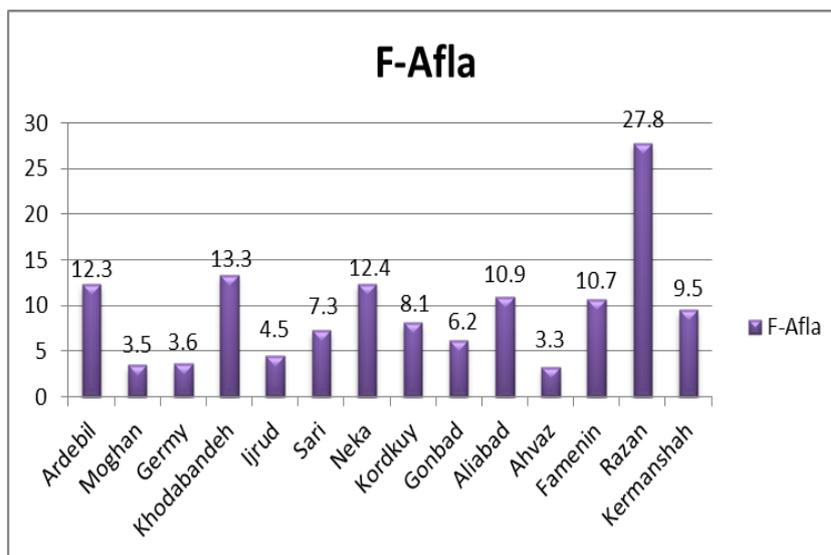


Chart 3: The level of aflatoxin in flour samples obtained from the every city.

Sampling method

In this part, samples, the sampling distribution of site evaluation and preparation of the samples was obtained and then, measurement and analysis of numerical values in Table 4-1 were obtained. According to Table -1, the Skn and Kut number for flour aflatoxin.

Table 1: Statistical values obtained for samples of wheat flour.

N	FAfla
Valid	14
Missing	0
Mean	9.52857
Std. Error of Mean	1.69178
	5
Median	8.80000
Std. Deviation	6.33007
	8
Variance	40.070
Skewness	1.895
Std. Error of Skewness	.597
Kurtosis	5.049
Std. Error of Kurtosis	1.154
Range	24.500
Minimum	3.300
Maximum	27.800

The mean value and standard deviation for 14 cities were 52.9 and 14 respectively that represent the existence of aflatoxins in wheat flour. The amounts of aflatoxins which were measured in stored wheat flours in contrast in comparison with bakeries samples that in order to protect the legitimate means of poison mixed different kinds of flour, the mean value and standard food with approved practices of the country. After reviewing statistic results and Pearson Correlation with significant at $p < 0.05$, it was found that in mixed wheat flour the amounts of aflatoxins is significantly lower than stored wheat flour.

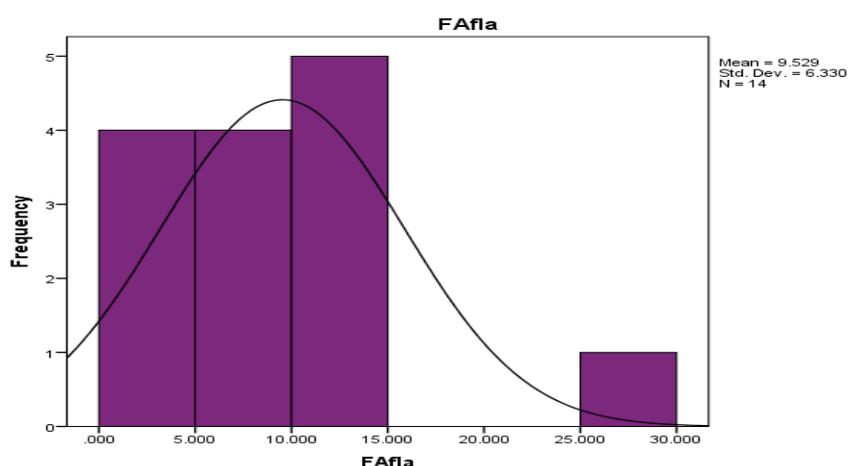


Chart 4. Frequency distribution of Data.

As seen in Chart 4, the skewness is to the right and it means that mixing the Gilan wheat flour (Wet region) with Khozestan wheat flour (Hot and dry region) can cause reduction in the amount of aflatoxins. Wilcoxon curve was also examined in this study. By determination of statistically significant correlation, the numerical difference between the values obtained. According to the amount of aflatoxin in processed wheat, it was found that the amount of aflatoxin in wheat and wheat flour was countercurrent and the disalignment is meaningful and what is the difference between the numerical results obtained by the degree of -0.16 (Sig: 0.88).

Discussion

Environmental factors such as temperature, humidity, air components and general status of grains, also play important roles in grain spoilage. In general, humidity of more than 13%, relative humidity of more than 65% and temperature of more than 5-10% °C, provide the situation for microbial activities. During the growth on different nutrients, fungi in addition to reducing the food's quantity due to removing the affected parts and also lowering the value of nutrients due to affecting on nutrients which are available in foods, can produce secondary metabolites called mycotoxins or fungus toxins that if the living organism receive these toxins, will generate devastating and severe effects like carcinogenesis, malformations, lowering the growth, inhibiting the immune system and mutagenesis in them. Mycotoxins are groups of relative resistant toxic metabolites that produced by producing fungi in the pathway of secondary metabolism of fungus cells and cause contamination in foods and possibly in surroundings. In addition to producing mycotoxins and other toxic metabolites, main changes that are results of microorganism's activity in grains are: reducing the value of nutrients in products due to protein, lipid and carbohydrate degradation, producing the metabolites that can reduce Aroma, lowering the extent of gluten, affecting on rheologic properties of dough in result of decreased value of dough processing. Most of known mycotoxins are actually products of acetate and/or amino acids that produced by species belong to aspergillus, penicillium, fusarium, claviceps, alternaria, stachybotrys, myrothecium fuma and diplodia genera. Many of producing fungi related to mycotoxins can grow well in warm and humid weather and produce and secrete significant amounts of toxins. Dispersal and distribution of mycotoxins and their producing fungi in nature greatly depend on geographical conditions. In Iran, due to diverse weather conditions, presence of wide spectrum of producing fungi as well as related toxins in milieu is possible. Diversity of agricultural products in Iran, also help spread more types of producing fungi related to mycotoxins and their growth on foods in the country. For instance, products like wheat, maize, rice and peanut which can grow in partly different conditions, are appropriate sites for growth of fungi such as aspergillus and fusarium. Mycotoxin's producing fungi directly can reduce quality and quantity of grains or indirectly lower the value of meat and dairy products. Results of this study showed that amounts of aflatoxin in wheat flour produced in northern cities was higher than central and southern regions and if we mix these two doughs together, the extent of toxins will decrease significantly and reach to allowed level. This study apart from investigating the level of aflatoxin in dough samples, also discuss about ELIZA method and in following we will consider other studies in this field. Study of Ramesh et al (2013) showed that all of flour factories were contaminated with aflatoxin and ochratoxin. In one factory, survey of mycotoxins contamination, showed the standard limit. The suggested standard by Iran was according to standard institute. Maximum permissible consumed amounts of dough samples was 5ppb and total aflatoxin was 15 ppb. In the present study, it was indicated that 24.28% of samples

which are contaminated with aflatoxin B1, have been exceeded. The total Aflatoxin and Ochratoxin was 11.42% and 14.28% respectively. However, the average of 10 samples contamination, was only unit out of 7. Different levels of contamination were observed in the samples. One of the reasons for this phenomenon is a grain storage. According to Mahmoudi, et al., (2012), 7 out of 14 units randomly in the province of Mazandaran was chosen. Production rate and the number of bags of flour for each group based on a random table, 0.5 kilogram out of 70 samples studied. 17 samples (24.28%) indicates the presence of large amounts of contamination. The maximum contamination of AFB1 in flour according to Standards Institute should be 5ppb. The average contamination of samples were unit 1 and unit 6, the minimum and maximum contamination respectively. Minimize contamination of sample 3 from unit 1 (with contamination of 0.728) and maximum contamination, samples 5 and 6 (with contamination of 6.71) were obtained. Wheat flour samples were analyzed by using ELIZA method which the average level of aflatoxin B1 93/2 ng / g and 6.5% of samples set more than the maximum limit they had by the international laws of about aflatoxin B1 (Azizi et al., 2007). In another study, 2.54% of the samples, were contaminated with aflatoxins and aflatoxin B1, G1 2.54 and 3.39% in samples was observed respectively. Levels of total aflatoxins and aflatoxin B1 in the sample, respectively in the range of (1.3-7.1 ng /g) and (1.36-1.78 ng /g) is (Hedayati., 2005). Zinedine and colleagues, reported 0.07 ng / grand 0.07 ng / g which are the average total aflatoxins and aflatoxin B1 in wheat respectively and aflatoxin B2, G1 and G2 were not observed in any sample (Zinedine et al. 2007). In Malaysia, 21.7% of samples were contaminated with aflatoxins and aflatoxin G2, G1, B2 and B1, respectively in 1.2%, 4.8%, 4.8%, 13.3% have been diagnosed samples (Abdullah et al., 1998). In other studies, 41 samples analyzed and 59% of wheat samples with aflatoxin were contaminated the outbreak of aflatoxin G2, G1, B2 and B1, respectively, 42%, 12%, 37% and 12% respectively (Giray et al., 2007). Baliukoniene and colleagues indicate that the conditions of storage has a profound impact on aflatoxin levels (Baliukoniene et al., 2003). In summer, the temperature and the humidity are highly desirable for producing mycotoxin, but in their research, was found that aflatoxin levels were higher in winter than in summer. Period storage of wheat is an important factor in the degree of aflatoxin contamination in winter. As a harvest of wheat is in the first half of the year and had been stored for few months, this period can have a significant result in high contamination of aflatoxin in the winter time. However, present results shown that there is a relationship between humidity and the total level of aflatoxin. Basilico has shown that fungal growth and toxin production is the environment that can affected the type and quantity of toxins (Basilico et al., 1995). It has been determined that the product variety, weather patterns, temperature, humidity, water activity, oxygen levels, low maintenance requirements, not dry enough, insect or rodent activity and other problems have an effect on *Aspergillus* growth and aflatoxin production in stored products (OBrain et al., 2007; Cotty et al., 2007). Pamela (2001), stated that the mold contamination in stored grain and animal food is a criterion for determining the quality of the product. Evaluation of wheat grains in terms of fungal infection indicated that all samples had a fungal contamination, but the amount is little. *A.flavus* isolates in this study had a higher number of isolates of *A. parasiticus* which Barros and colleagues (2005), were obtained more *A.flavus* strains of peanuts (73%), compared to wheat grains (13%) were obtained. Shape and size of the isolated structural features of this study were according to the results of Raper and Fennel (1965) and Domsch and colleagues (1980).In addition, in areas of the province of Punjab, Sindh, and NWFP, *A. parasiticus* was isolated in the AFPA environment. For this reason, this environment is suitable for the detection of fungi. AFPA

environment that is used in this research was not industrial product. Frandberg (2003) also showed that none of the samples in this study were not contaminated with aflatoxin and this observation was similar to Carlos and colleagues (2000) study in the analysis of aflatoxin contamination of rice. Halt (1994), found the aflatoxin in wheat samples but in the range 20 µg / kg (standard of FAO and WTO), respectively. Escobar and Reguerio (2002) studied aflatoxin contamination, it is found in wheat, but much less than other analysis such as peanuts and corn. Polley and colleagues (1991) reported that the levels of mycotoxins in England wheat, with variable values, is little. In Giray B. and et al (2007) study, it has been undertaken to determine the AFB₁, AFB₂, AFG₁, AFG₂ levels by HPLC in forty-one wheat samples grown and consumed in some regions of Turkey. The concentrations of total AFs in the wheat samples were determined to be ranging from 10.4 to 643.5 ng/kg. Fifty-nine percent of the samples were found to be positive for total AFs. The percentage of positive samples for AFB₁, AFB₂, AFG₁, and AFG₂ were 42, 12, 37, and 12%, respectively. Although the detected levels are under the permitted levels for AFs in cereals, these amounts should be considered in regard to overall daily exposure to mycotoxins. In Anand et al (2009) study, thirty wheat samples procured from storage units in different area of Coimbatore, Tamilnadu, India were processed to isolate the predominant fungal contaminants. Heterogeneous group of fungi were enumerated by standard plate count, among which four predominant organisms namely *Aspergillus flavus*, *Aspergillus tamarii*, *Rhizopus* spp. and *Fusarium* spp. are identified by macroscopic and microscopic observations. Since reputed journal reports, continuously highlight the impact of mycotoxin production in wheat by *Aspergillus flavus*, the isolate was chosen and processed to examine the production of aflatoxin and further analysis and confirmation was done using Albino rats and analytical techniques such as Thin Layer Chromatography, Immuno diffusion and Immuno electrophoreses. Coconut extract broth was used for the production of aflatoxin. Thin Layer Chromatography revealed the presence of G2 type of mycotoxin at a concentration of 15 ppb. For the future confirmation the extract was injected to Albino rat, antisera was raised, which when subjected to Immuno diffusion and Immuno electrophoreses, showed the presence of specific antibody against mycotoxin. There are several methods for measuring the amounts of aflatoxins like thin layer chromatography, high performance liquid chromatography and immunological methods. In the present study, the immunological tests (ELISA) was used for aflatoxin and ochratoxin measurement. ELISA is a renewable, easily, selectivity, sensitivity, speed and cost-effective method and can be used for the determination of mycotoxins in food products.

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