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DETERMINATION OF AFLATOXIN CONTENTS OF SEBINKARAHISAR WALNUT VARIETY BY ELISA METHOD

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ABSTRACT
Aflatoxins are carcinogenic toxic metabolites for human foods and animal feeds in terms of pharmaceutical produced by *Aspergillus flavus* and *Aspergillus parasiticus*. This study was carried out to determine the aflatoxin changes in Şebinkarahisar walnut under different seasonal changes between the years by enzyme-linked immunosorbent assay (ELISA) method. The presence of aflatoxin was found in all of the samples within measurable limits. But in none of the total 20 different walnut samples examined, aflatoxin was not observed above the limits determined by the Turkish Food Codex Contaminants Regulation. Each treatment included 3 wells were carried out triplicates to determination of AFT and AFB1 and correlation analysis showed a significant relationship between AFB1 and AFT for 2 different years. In addition, although there was an increase in aflatoxin content due to seasonal changes between the years, there was no statistically significant difference and it was supported by correlation analysis.
Keywords: Aflatoxin, *Aspergillus flavus*, seasonal changes, walnut, ELISA

ŞEBİNKARAHİSAR CEVİZ ÇEŞİDİNDE AFLATOKSİN İÇERİĞİNİN ELISA YÖNTEMİYLE BELİRLENMESİ

ÖZ
Aflatoksinler, özellikle *Aspergillus flavus* ve *Aspergillus parasiticus* tarafından üretilen farmasötik olarak insan yiyecekleri ve hayvan yemleri için kanserojen toksik metabolitlerdir. Bu çalışma, yıllar arasındaki mevsimsel değişimlere bağlı olarak Şebinkarahisar'da yetişen ceviz çeşidindeki aflatoksin miktarındaki değişimi enzim bağlı immünosorbent testi (ELISA) yöntemi ile belirlemek amacıyla yapılmıştır. Tüm örneklerde aflatoksin varlığı ölçülebilir limitler dahilinde bulunmuştur. Ancak incelenen toplam 20 farklı ceviz numunesinin hiçbirinde, aflatoksin, Türk Gıda Kodeksi Bulaşanlar Yönetmeliği tarafından belirlenen sınırların üstünde gözlenmemiştir. Her bir deneme örnek başına 3 kuyucuk içerecek şekilde 3 tekrarlı yapılmıştır ve korelasyon analizi 2 farklı yıl boyunca AFB1 ve AFT arasında anlamlı bir ilişki olduğunu göstermiştir. Ek olarak, her ne kadar yıllar arasındaki mevsimsel değişimlere bağlı olarak aflatoksin içeriğinde artışlar görülse de istatistiksel anlamda bir fark oluşmamıştır ve bu durum korelasyon analizi ile desteklenmiştir.
Anahtar kelimeler: Aflatoksin, *Aspergillus flavus*, mevsimsel değişiklikler, ceviz, ELISA

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INTRODUCTION

Mycotoxins, mostly produced by *Aspergillus* species, are considered to be very important natural toxins because moulds can be found almost everywhere and can develop their toxins in many foodstuffs and animal feeds from the field until consumed (Pohland, 1993; Shukla, 2016). These microorganisms generate various types of toxic metabolites and the major ones known as aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin (AFG2) (Chun et al., 2007; Shukla, 2016). These four aflatoxin groups were classified as group 1 carcinogens by the international agency for research on cancer (IARC, 1993). Among these metabolites, AFB1 is considered to be the most hazardous group and is often detected in combination with total aflatoxin (AFT) (Moss, 1998). These natural metabolites have carcinogenic, mutagenic, teratogenic and immuno-suppressive effects on certain dozes and can be found in walnut, nut, peanut, spices, cereals, dried fruit varieties and foodstuff made from these products and other many commodities associated with human nutrition and animal feed (Leszczyńska et al., 2001; Park et al., 2004; Castells et al., 2008; Erkmen and Bozoglu, 2008a). According to the 2013 FAO data, Turkey ranks fourth in walnut production. (Anonymous, 2013a; Anonymous, 2015b). In this production, Şebinkarahisar has an important place due to its climate characteristics. Variety of Şebinkarahisar walnut, named after the region and registered in 1993, is preferred by many producers and consumers in our country due to the late foliage tolerance to late spring frosts, enough fruit in the side branches, at least 2-4 fruit in the bunch, thin crust, easy and complete removal of the inner, light coloured, low inner shrinkage and high total fatty acids ratio (Karadeniz and Şişman, 2015). Due to their high biological content, the Şebinkarahisar walnut variety is preferred primarily as dry food as well as being used in many areas of the food industry, especially by baklava producers in Gaziantep.

In recent years, enzyme-linked immunosorbent assays (ELISA) are frequently preferred by researchers to determine toxic metabolites formed in foods due to high temperature, humidity and other storage conditions. Climate change influences the growth of the fungi producing the mycotoxins in commodities (IPPC, 2007). Several studies have been reported on mycotoxigenic fungal infection and contamination with mycotoxins in foods due to climate changes (Paterson and Lima, 2011; Magan et al., 2011; Wu et al., 2011) Again in 2003 and 2004, it was reported that a high percentage of *A. flavus* occurred due to climatic changes in maize used as animal feed in Italy (EU ETS, 2003).

ELISA method have advantages over other methods of aflatoxin detection due to its properties such as low cost, simplicity, the use of safe reagents, determination of contaminant in a large number of samples in a shorter time, and high precision results (Chun et al., 2007; Reza et al., 2012). In Turkey, the upper limit of aflatoxin acceptable for walnut according to the Turkish Food Codex is 8 μg/kg for AFB1 and 15 μg/kg for AFT (TGK, 2011). However, this ratio is considered as 5 μg /kg for AFB1 and 10 μg/kg for AFT according to European Union criteria (EC, 2006).

Nuts and their products have an important role in our daily diet as well as part of the ingredient. So it is important to control their level of aflatoxin occurrence in food. As a result of increasing food safety demand, it is essential to determine the aflatoxin contents of walnut species produced in Şebinkarahisar according to the seasonal changes. This report was designed to detect the AFT and AFB1 ratios of walnut variety produced in the seasonal changes of Şebinkarahisar in 2016 and 2017.

MATERIALS AND METHODS

Samples Collection

The walnut samples of the years 2016 and 2017 were collected from producers from different points of Şebinkarahisar in September-October which is the walnut harvest time for Şebinkarahisar (Table 1). Samples, taken from different points, were placed in plastic containers and the point of receipt was labelled. Samples were transferred to the laboratory and stored in

laboratory conditions (room temperature) as shelled until analysis. Samples were analyzed one month later.

Table 1. Location of walnut variety collected from different points of Şebinkarahisar, Giresun

Variety Name	Locality Information
Şebin T1	Tamzara District 20.x.2016-217, N 40 19'35.24" E 038 26'26.45",1254 m
Şebin K1	Kırkgöz District 21.x.2016-217, N 40 17'14.26" E 38 27'09.26", 1093 m
Şebin K2	Kırkgöz District 21.x.2016-217, N 40 17'14.37" E 38 27'10.35", 1092 m
Şebin K3	Kırkgöz District 21.x.2016-217, N 40 17'15.10" E 38 27'07.11", 1095 m
Şebin Y1	Yıltarıç Village 21.x.2016-217, N 40 18'28.31" E 38 29'40.31", 1218 m
Şebin Y2	Yedikardeş Village 12.x.2016-217, N 40 16'47.57" E 38 15'22.71", 1169 m
Şebin Y3	Yedikardeş Village 12.x.2016-217, N 40 16'47.05" E 38 15'22.95", 1166 m
Şebin E1	Yedikardeş Village 12.x.2016-217, N 40 20'28.32" E 38 19'54.63", 1565 m
Şebin B1	Biroğul District 10.x.2016-217, N 40 17' 11.40" E 38 27'47.74", 1028 m
Şebin A1	Avutmuş District 11.x.2016-217, N 40 18'44.35" E 38 25'17.05", 1034 m
Şebin O1	Ovacık Village 11.x.2016-217, N 40 19' 20.04" E 38 21'55.78", 1671 m
Şebin C1	Ovacık Village 10.x.2016-217, N 40 18' 29.02" E 38 20' 04.74", 1404 m
Şebin K4	Kütküt District 16.x.2016-217, N 40 18' 14.28" E 38 27' 01.87", 1105 m
Şebin A2	Kütküt District 24.x.2016-217, N 40 21' 57.42" E 38 35' 25.42", 1603m
Şebin T1	Toplukonak Village 20.x.2016-2017, N 40 21' 48.44" E 38 35' 02.44", 1606 m
Şebin G1	Gürpınar Village 23.x.2016-2017, N 40 22'30.54" E 38 33'02.48", 1693 m
Şebin T2	Turpçu Village 24.x.2016-2017, N 40 19' 43.59" E 38 34' 51.74", 1360 m
Şebin K5	Kavaklar District 24.x.2016-2017, N 40 17'27.27" E 38 27'24.12", 1215 m
Şebin K6	Konak Village 14.x.2016-2017, N 40 21'30.13" E 38 32'13.39", 1447 m
Şebin C2	Konak Village 14.x.2016-2017, N 40 20'21.36" E 38 30'39.39", 1141 m

x = October, N = North, E = East, m = meter

Extraction Procedure

5 grams of each walnut sample taken from different points were individually weighed and taken to separate filter bags both for AFT and aflatoxin B1. 25 mL of 80:20 methanol:water (v:v) mixture was added via macropipette onto the each sample. All samples were then disintegrated with the fully automatic CLS-400D Stomacher mixer for 5 min. The liquid fraction was filtered through whatman cellulose filter paper. 1 mL aliquot of the extract was taken with the help of the micropipette and diluted 9 mL of 80:20 methanol:water (v:v) mixture and stored at +4 °C until further analysis. In the latter case, the ratio of sample to extraction solvent is 1:50 (w/v). For the determination of both AFT and AFB1 three different extract was prepared and each experiment was repeated as triplicates

Reagents and Tests

Total Aflatoxin

The AFT test kit contains the following components: 96 wells (12 x 8 well strips) in a microwell holder coated with mouse anti-aflatoxin monoclonal antibodies; 96 non-coated wells in a microwell holder for the mixing wells. Standard solutions of AFT are at the following concentrations: 0.0, 0.02, 0.05, 0.1, 0.2 and 0.4 ng/mL in 50:50 methanol:water (v:v) mixture. In addition to the standards a 12 ml HRP conjugate of peroxidase in a buffer; 2 x 12 mL proprietary sample diluent; a unit of stabilized

tetramethylbenzidine substrate reagent solution; an acidic reagent for termination process; a phosphate buffered saline for washing process (PBS 0.1 mol/dm^3 , pH 7.4, 0.05%, Tween 20).

Aflatoxin B1

A set of AFB1 kit consists of 96 wells (12 x 8 well strips) in a microwell holder coated with antibodies to aflatoxin; 1 set of 12 x 8 non-coated mixing wells consisting of 96 pieces. Standard solutions of AFB1 are at the following concentrations: 0.0, 0.02, 0.05, 0.1, 0.2 and 0.4 ng/mL in 50:50 methanol:water (v:v) mixture. In addition to the standards a 12 ml HRP conjugate of peroxidase in a buffer; 2 x 12 mL proprietary sample diluent; a unit of stabilized tetramethylbenzidine substrate reagent solution; an acidic reagent for termination process; a buffer for washing (PBS 0.1 mol/dm^3 , pH 7.4 0.05%, Tween 20). ELISA method was used in this study. AFT and AFB1 values were determined by Helica test kits (Helica Biosystems Inc. USA).

Apparatus and Equipment

Samples of walnut were weighed with DENVER SI-234 analytical balance (Germany). The samples were homogenously smashed in methanol with a fully automated CLS-400D Stomacher mixer (USA). Multi-purpose sterile Pasteur pipettes and micropipettes with various volumes were also used for the insertion of standards and extracts into the wells (Germany). The plates were washed using a special washing machine Biotech Elx50 (Biotek Instruments INC, USA). Finally, absorbances of the examined and standard solutions were measured by Biotech ELx808 reading apparatus for ELISA tests (Biotek Instruments INC, USA).

Experiment

First of all, mixing wells were prepared according to the standard and sample number from the mixing wells in the kit. 200 µL of dilution solution were added to each of these mixing wells prepared. Then 100 µL of the standards and prepared extracts in the kit are added separately to each well. The mixture in the well is thoroughly homogenized by means of micropipette. 100 µL of this diluted mixture was transferred to the antibody-coated well and allowed to incubate for 30 min at room temperature in the darkness. The wells were washed 3 times with the fully automatic Biotech Elx50 strip washer with PBS. Washed wells were inverted in case of a drop of water. 100 µL of HRP conjugate was added to each well and allowed to incubate again in the dark for 30 min.

After the conjugate application, the above-mentioned washing was repeated and 100 µL of substrate was added and allowed to incubate for 10 min. After the incubation period, color changes in the wells were evaluated according to standards. Finally, 100 µL of stop solution was added to each well and readings were performed with Biotech ELx808 reading apparatus for ELISA tests within 15 min at the latest. This procedure was performed three times for both AFT and AFB1, separately. The content of aflatoxins was evaluated using the previously prepared standard curve.

Collection of Climate Data

All climate data of Şebinkarahisar in 2016 and 2017 were recorded in T.C. the General Directorate of Meteorology of the Ministry of Agriculture and Forestry was obtained from Giresun Meteorology Station Directorate.

Statistical Analysis

Each treatment included 3 wells were carried out triplicates to determination of AFT and AFB1. All data were conducted with SPSS Statistics program (SPSS 21.0) by using analysis of variance (ANOVA) with Pearson's correlation. Pearson correlation was preferred because all of the data did not conform to normal distribution. Correlations analyses were carried according to Spearman method (Spearman, 1904).

RESULTS

Determination of Aflatoxin Content

In the ELISA method, it is easier to process than the other methods because there is no need for the processes such as isolation and purification of the component to be tested. In addition, the preparation process consists of the only extraction. In the present report, AFT and AFB1 contents of walnut samples grown in

Şebinkarahisar in 2016-2017 years were determined. The effects of annual climatic changes on the aflatoxin content in walnut growing in the Şebinkarahisar region were investigated. The mean temperature, humidity and precipitation rates were analyzed for the changes in aflatoxin content over the years. Humidity of the most important parameters affecting aflatoxin content in foods was calculated to be higher in 2016 (Table 2). Therefore, AFT and AFB1 ratios were higher in all samples examined in 2016. The highest AFT ratio was determined in the Kırkgöz III sample which is accepted as the origin of Şebinkarahisar walnut variety with 0.845 ± 0.02 µg/kg. Surprisingly, the highest total aflatoxin ratio was obtained in the sample taken from the same location in 2017 samples with 0.828 ± 0.02 µg/kg. However, these calculated rates are not above the standards determined by the Turkish food codex. It is estimated that a decrease of 6.15% in humidity in 2017 resulted in a 2.01% decrease in the total aflatoxin ratio compared to 2016. Similar to AFT, the highest AFB1 ratios were obtained from 2016 data. Walnut samples taken from the Çiftlik region gave the highest AFB1 ratio with 0.630 ± 0.01 (Table 2). Although it was determined that all values obtained from the samples of 2016 increased significantly compared to the values obtained from the samples of 2017, it was determined that these values were acceptable according to the regulations of the Turkish Food Codex.

Table 2. Total aflatoxin (B1, B2, G1 and G2) and aflatoxin B1 contents of Şebinkarahisar walnut variety collecting different years

Product	2016		2017	
	AFT (µg/kg)	AFB1 (µg/kg)	AFT (µg/kg)	AFB1 (µg/kg)
Tamzara	0.464 ± 0.01	0.394 ± 0.01	0.422 ± 0.01	0.387 ± 0.01
Kırkgöz-I	0.477 ± 0.01	0.396 ± 0.01	0.459 ± 0.01	0.382 ± 0.01
Kırkgöz-II	0.461 ± 0.01	0.401 ± 0.01	0.438 ± 0.01	0.373 ± 0.01
Kırkgöz-III	0.845 ± 0.02	0.628 ± 0.02	0.828 ± 0.02	0.415 ± 0.01
Yıltarıç	0.427 ± 0.01	0.383 ± 0.01	0.410 ± 0.01	0.372 ± 0.01
Yedikardeş-I	0.466 ± 0.01	0.377 ± 0.01	0.439 ± 0.01	0.356 ± 0.01
Yedikardeş-II	0.561 ± 0.01	0.436 ± 0.01	0.515 ± 0.01	0.418 ± 0.01
Evcili	0.415 ± 0.01	0.406 ± 0.01	0.407 ± 0.01	0.392 ± 0.01
Biroğul	0.488 ± 0.01	0.458 ± 0.01	0.464 ± 0.01	0.433 ± 0.01
Avutmuş	0.592 ± 0.02	0.511 ± 0.01	0.583 ± 0.01	0.499 ± 0.01
Ovacık	0.502 ± 0.01	0.424 ± 0.01	0.489 ± 0.01	0.400 ± 0.01
Çakır	0.575 ± 0.01	0.457 ± 0.01	0.556 ± 0.01	0.434 ± 0.01
Kütküt	0.481 ± 0.01	0.411 ± 0.01	0.434 ± 0.01	0.398 ± 0.01
Altınçevre	0.500 ± 0.01	0.466 ± 0.01	0.429 ± 0.01	0.440 ± 0.01
Toplukonak	0.530 ± 0.01	0.416 ± 0.01	0.488 ± 0.01	0.373 ± 0.01
Gürpınar	0.538 ± 0.01	0.438 ± 0.01	0.512 ± 0.01	0.394 ± 0.01
Turpçu	0.575 ± 0.01	0.407 ± 0.01	0.519 ± 0.01	0.399 ± 0.01
Kavaklar	0.564 ± 0.01	0.424 ± 0.01	0.474 ± 0.01	0.374 ± 0.01
Konak	0.447 ± 0.01	0.403 ± 0.01	0.445 ± 0.01	0.401 ± 0.01
Çiftlik	0.672 ± 0.02	0.630 ± 0.02	0.640 ± 0.01	0.308 ± 0.01

AFT: Total Aflatoxin, AFB1: Aflatoxin B1

Climatic Conditions

In recent years, climatic characteristics have started to change in Turkey as in all over the world. Therefore, determining the effects of climatic characteristics in healthy product production or storage is important in terms of reliable food. In the present study, climatic data for the sampling months such as temperature,

humidity, and precipitation were analyzed according to months and years. It has been determined that the humidity rate of 2016 is 6.15% wider than in 2017. During the year 2016, the highest humidity was recorded in month of January with 72.5%. These rates were determined as 56% and 53.3%, respectively in September and October which is the walnut harvest and drying time. Also, the lowest humidity content was obtained from April with 45.4% (Figure 1). The highest average precipitation rate was found as 106.10 kg/m² in May 2016 as in the humidity rate. This rate was 5.09% higher than in May 2017. When the total average precipitation rate was examined throughout the year, it is determined that 2017 has received 4.23% more precipitation than in 2016 (Figure 2). When the temperature changes were compared, it was calculated that 2017 was 6.7% warmer than in 2016. During the year 2017, the highest temperature of 22.4 °C was recorded in the month of August with the lowest temperature (3.0 °C) recorded in February and the average for was determined to be 10.4 °C. In July, August and September, which is the walnut growing, harvesting and drying seasons, the average temperature is determined as 21.37 °C. 2016 was a colder year with 18.77 °C. The average annual temperature in this year is 9.7 °C (Figure 3).

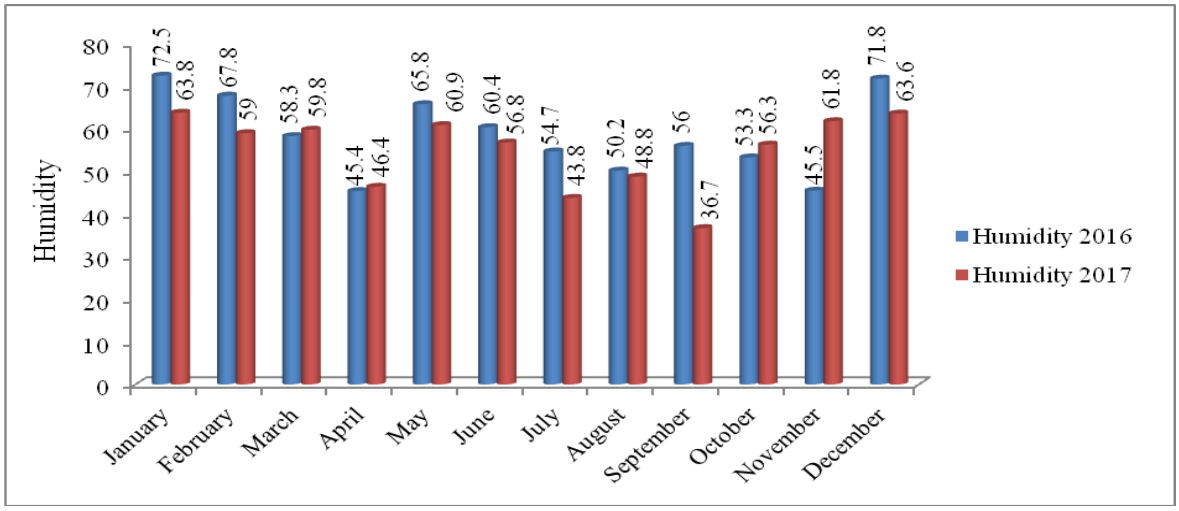

Figure 1. Mean values of monthly humidity distributions for different years

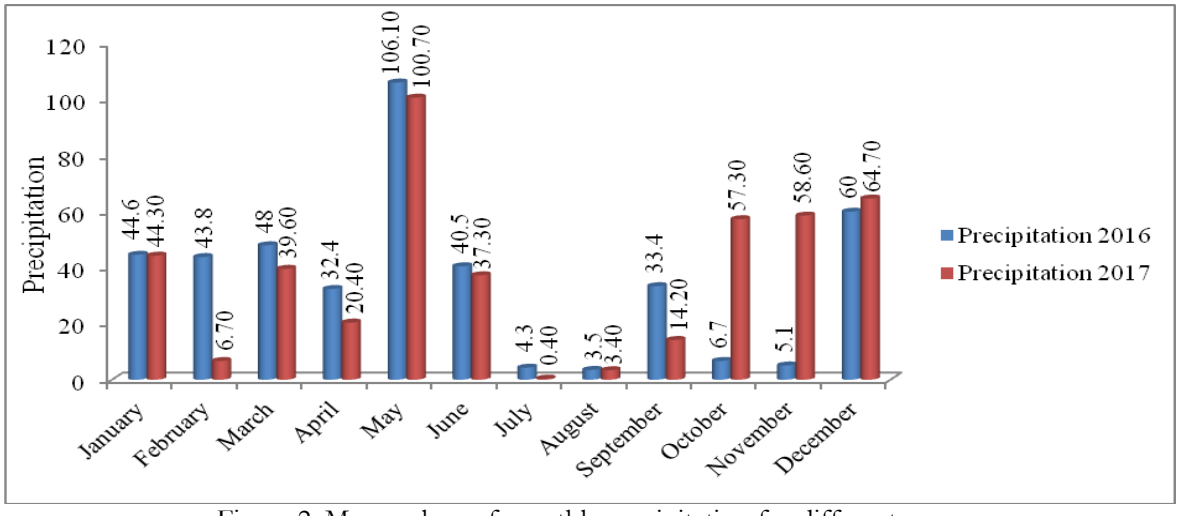

Figure 2. Mean values of monthly precipitation for different years

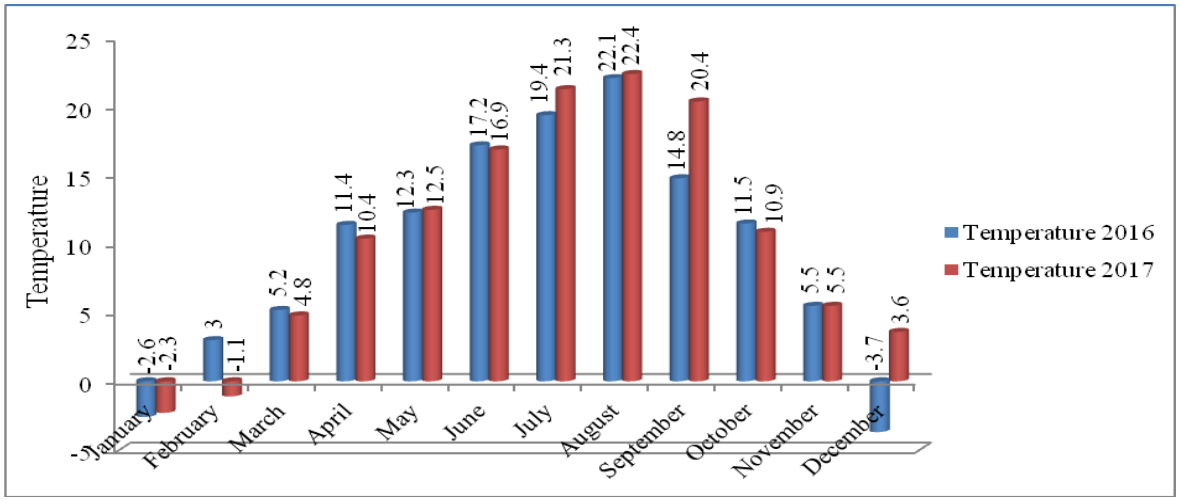

Figure 3. Mean values of monthly average temperature distributions for different years

The Relationship between Climate Data and Aflatoxin

The results of the statistical analysis revealed a correlation between AFT and AFB1 in both 2016 and 2017. In 2016, a strong correlation was found between AFT and AFB1 with r = 0.920. In 2017, this ratio was calculated as r = 0.543 due to the decrease in the average humidity content on an annual basis (Table 3, $P < 0.01$). In contrast, statistical analyzes showed that the seasonal changes (humidity, temperature, and precipitation) in 2016 and 2017 were not effective on AFT and AFB1 (Table 4). In 2017, the annual average precipitation and temperature values increased by 4.48% and 7.2%, respectively, compared to the previous year and the humidity rate decreased by 6.33% compared to 2016 year. This situation did not cause increase in AFT and AFB1 ratios in Şebinkarahisar which has continental seasonal changes.

Table 3. Spearman correlation coefficient between climatic factors and AFT and AFB1 of Şebinkarahisar walnut in 2016

	Humidity	Temperature	Precipitation	AFT	AFB1
Humidity	1.000		- 0.648*		
Temperature		1.000	- 0.608*		
Precipitation	- 0.648*	- 0.608*	1.000		
AFT				1.000	0.920**
AFB1				0.920**	1.000

** Correlation is significant at the 0.01 level (2- tailed), * Correlation is significant at the 0.05 level (2- tailed), AFT: Total Aflatoxin, AFB1: Aflatoxin B1.

Table 4. Spearman correlation coefficient between climatic factors and AFT and AFB1 of Şebinkarahisar walnut in 2017

	Humidity	Temperature	Precipitation	AFT	AFB1
Humidity	1.000	0.734**	- 0.748**		
Temperature	0.734**	1.000			
Precipitation	- 0.748**		1.000		
AFT				1.000	0.543*
AFB1				0.543*	1.000

** Correlation is significant at the 0.01 level (2- tailed), * Correlation is significant at the 0.05 level (2- tailed), AFT: Total Aflatoxin, AFB1: Aflatoxin B1

DISCUSSION

The world population tends to rise rapidly. This increase in the need for reliable clean food is increasing day by day. Turkey has a rich variety in terms of nutrient diversity. Reliable food consumption in this variety is important in our country as well as the whole world. Turkey is a pioneer in the production of tea, hazelnuts, peanuts, walnuts, dried fruits, spices and corn products. The fact that the aflatoxin content of these products are, at the determined standards, is important for health and for the country's economies. In this variety, Şebinkarahisar walnut variety holds an important place. Although there are many studies in the literature about the determination of aflatoxin content of dried eggplant and green bell pepper (Çağındı and Gürhayta, 2016), flour (Özturan et al., 2007), dark red ground pepper, hazelnut and roasted chickpeas (Gürses, 2006) in Turkey, there is no study to determine the aflatoxin contents of walnut variety growing in Şebinkarahisar by evaluating the seasonal changes. Besides, aflatoxin contents in foods produced under different conditions have tried to be determined by many researchers in the world. A detailed literature survey has revealed the importance of founding these toxic harmful metabolites in foods. (Leong et al., 2010; El tawila et al., 2012; Adaya-González et al., 2015). Set and Erkmen (2010) have reported that AFT and AFB1 content of packaged and unpackaged ground red paper and pistachio nut samples was determined on the allowed legal limits. Gürses (2006) was studied on different groups of commodities as hazelnut, walnut, peanut, almond and roasted chickpea collected from retailers, markets, and dried fruit retail shops in Erzurum. The researcher found that in 27.66% of 94 analysed samples had an AFB1 ratio above the allowed legal limits. In a similar report, 28 of 126 different food products sold in Qatar markets were found to have AFB1 ratios ranging from 0.14 to 81.64 µg/kg (Abdulkadar et al., 2004). These researchers have found that the highest ratio of aflatoxin was in pistachio samples with 81.64 µg/kg. In a study conducted by Luttfullah and Hussain (2011) on the products offered for sale in Pakistan, 40% and 70% aflatoxin was found in shelled walnuts and unshelled walnuts, respectively.

CONCLUSION

Aflatoxin contamination in foodstuffs is an undesirable situation in all processes (from raw material to field development, harvesting, storage, transportation, product processing and product production) from the field to table. As well as all over the world, this situation should be followed up in all processes of the products produced up to consumption in Turkey, and good agricultural practices should be achieved. None of the 20 different walnut samples examined within the scope of the study was found to have AFT and AFB1 ratios above the allowed legal levels under Şebinkarahisar seasonal changes.

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