Chapter 9

First Report of Mycotoxins in Second Peanuts Crop in Adana and Osmaniye at Harvest, Drying, Prestorage and Storage Periods

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.68210

Abstract

Aflatoxin (AF) and cyclopiazonic acid (CPA) contaminations are very important problems for peanuts and its products. The aim of the study was to detect aflatoxin (types B and G) and cyclopiazonic acid (CPA) occurrence and critical periods of toxin production in peanuts collected from different research areas of Osmaniye and Adana, Turkey, in 2015. Peanut kernels toxin analysis was performed in four different periods during the harvest, drying, prestorage, and storage. Total aflatoxin occurrence in peanut kernels was analyzed by immunoaffinity chromatography-reversed-phase high-performance liquid chromatography (IAC-HPLC) analysis and cyclopiazonic acid occurrence in peanut kernels was analyzed by thin layer chromatography (TLC). Aflatoxin levels in 76 out of 102 contaminated samples were from 0.3 to 1333.42 μg/kg. Cyclopiazonic acid levels in 18 out of 102 peanut samples were from 16.6 to 44.44 μg/kg. An unusual pattern of mycotoxin production (aflatoxin types B and G simultaneously with CPA) was seen in 11 of 102 peanuts samples. Six of nine samples were from the storage period. Aflatoxin contamination during harvesting (64%) and drying (75%) were higher than prestorage (53%). Aflatoxin (93%) and cyclopiazonic acid (30%) were the most produced during storage. The results showed that storage period was significantly important for the presence of two mycotoxins according to the statistical analysis.

Keywords: aflatoxin, cyclopiazonic acid, peanut, drying, harvest, prestorage, storage

1. Introduction

Peanut (*Arachis hypogaea* L., Family: *Fabaceae*) is a rich source of fat, proteins, and vitamins. Peanuts are grown on a large scale in almost all the tropical and subtropical countries, especially in India, China, the USA, and West Africa [1]. Also, peanut is a member of the legume family, an important food and oil crop. It is currently grown on approximately 42 million acres worldwide. It is the third major oilseed of the world after soybean and cotton [2]. Peanut is used for human consumption, oil production, food industries, and animal feeding [3]. It is grown in China (37%), India (20%), Nigeria (6.5%), and the USA (4.1%). Turkey supplies about 0.3% of the world production of peanut [4]. The total production of peanut was 147,537 tons harvested from 377,729 da, with an average yield of 391 kg/da in Turkey in 2015 [5].

Poor agricultural practices and postharvest treatments of peanuts can lead to an infection by mold fungus *Aspergillus flavus* and *Aspergillus parasiticus* releasing the toxic substance aflatoxins (AFs). Contamination may occur when either the grown crops [6] or more badly stored harvests are infested by molds [7, 8]. *A. flavus* also produces other mycotoxins such as cyclopiazonic acid (CPA) and indole-tetramic acid [9]. CPA occurs naturally in peanuts [10–12] and corn [12, 13].

The occurrence of aflatoxins in foods has been also recognized as a potential threat for human health. Aflatoxin, naturally occurring secondary metabolites, are potent hepatotoxic, mutagenic, and carcinogenic toxins, causing serious health hazards in humans and in animals. Aflatoxin B_1, B_2, G_1, and G_2 are found predominantly as the hydroxylated metabolic products of aflatoxins B_1 (AFB_1) and B_2 (AFB_2), respectively [14]. The most toxic aflatoxin known, AFB_1, is cited as a group I carcinogen by the International Agency for Research on Cancer [15, 16].

Another mycotoxin is cyclopiazonic acid (CPA), which causes necrotic foci in internal organs such as the liver and exerts neurotoxic effects [17]. Natural occurrence of CPA has been reported in peanuts, corn, cheese, tomato products, and also meat, eggs, and milk of animals that are fed by contaminated feeds [18]. Incidence of aflatoxigenic *A. flavus* strains was higher in peanuts (69%) than in wheat (13%) or soybeans (5%), while the ratio of CPA producers [12]. Risk of aflatoxin contamination hits top values in such commodities as nuts [19]. The maximum levels of AFB_1 and total aflatoxins allowed in peanut as determined by Commission of the European Communities are 2 and 4 μg/kg, respectively.

Fungal infection of seeds before and after harvest remains a major problem of food safety in most parts of Turkey. Problems associated with this infection include loss of germination, mustiness, moldy smell [20–22], and aflatoxin contamination [23–25]. These problems are, however, dealt with most developed world where a careful commodity screening and improved storage conditions are provided [7, 23, 26]. Though aflatoxins producing fungus are a natural contaminant of peanut and other agricultural commodities, it is aggravated due to poor agricultural practices, harvesting practice, postharvest handling, and storage methods. Some studies undertaken in Turkey in different foods show that aflatoxins levels are substantially higher. One important aspect is traditional harvesting and storage practices [22].

http://dx.doi.org/10.5772/intechopen.68210

In this study, aflatoxin and cyclopiazonic acid contamination were determined in the second peanut crops at harvest, drying, pre-storage, and the storage periods in Adana and Osmaniye provinces of Turkey in 2015. Aflatoxin is always the most important toxin for peanut because of its toxicity, and the CPA presence in peanuts is also important as it causes necrotic foci in internal organs such as the liver and exerts neurotoxic effects, therefore, in peanuts, aflatoxins and besides, CPA should be investigated.

2. Materials and methods

2.1. Location of the study

In the second crop of peanuts, 102 samples were collected randomly throughout the peanut fields, that is, about 5949.5 da from Adana and Osmaniye provinces during October and November in 2015. Peanut samples were collected from 72 representative fields of 16 different districts of Adana and Osmaniye. Sampling was done according to Bora and Karaca [27] and have been followed in 1% of the survey areas. Also, 30 peanut samples were collected from storage in Adana and Osmaniye provinces. The samples were collected during the following periods of production: preharvest, drying, and prestorage. Also, 30 unshelled peanut samples were collected from the storage.

2.2. Collection of peanut samples

Second crop peanut samples were collected during harvest, drying, prestorage of eliminated soil, and storage periods. Seventy-two crusted peanuts samples were collected at harvest, dried for 7 days, and eliminated soil to prestorage. Each sample has been obtained from different farmer fields. Samples of pods (about 5 kg each) were divided manually and homogeneously to obtain working samples (about 1 kg each) for mycotoxins analyzes. The shells were removed manually. Additionally, 30 unshelled peanut samples were collected from storage. The samples (about 1 kg each) were collected in paper bags for analyzing mycotoxins. All the samples were kept at +4°C [28].

2.3. Analysis of mycotoxins

2.3.1. Determination of AFs with immunoaffinity chromatography-reversed phase high-performance liquid chromatography (IAC-HPLC) analysis

Analysis of aflatoxins was performed using immunoaffinity columns, as described below. Identification and deperiodination of aflatoxins B_1, B_2, G_1, and G_2 in peanut product samples were carried out by high-performance liquid chromatography (HPLC) according to Arzandeh and Jinap [29]. To 50 g of tested sample, 5 g of NaCl and 125 ml of methanol and water (70:30)was mixed in a blender for 2–3 min at high speed. The mixture was filtered through Whatman no. 4 filter paper. Then 30 ml of water was added to a 15 ml of filtrate. About 10 ml of the second filtrate was quantitatively passed through the immunoaffinity

column at flow rate of 1 ml/min. The column was washed with 10 ml of water. Aflatoxins were eluted with 1 ml of methanol in an amber vial at flow rate of 1–2 ml/min. The elution step was repeated with 1 ml of water. Thus, Agilent 1100 HPLC was ready for injection. Fluorescence detector (excitation at 360 nm and emission above 440 nm). Mobile phase consisted of methanol/water/acetonitrile (300:600:200, v/v/v) with a flow rate of 1.0 ml/min followed by derivatization with bromine (132 mg/l KBr, 385 μL nitric acid) in C-18 (R-Biopharm Rhône). HPLC column was maintained at a constant temperature (T = 25°C). The results were expressed as a μg/kg.

2.3.2. Determination of CPA with thin-layer chromatography (TLC) analysis

The peanut samples were tested for cyclopiazonic acid (CPA) production following the method modified by Somuncuoglu [18]. On each samples, 45 g of the contents of each test plate was macerated in a waring blender with 150 ml of methanol and 2% sodium bicarbonate (7:3). The slurry was twice filtered through a Büchner funnel with Whatman no. 4 filter paper and then concentrated to dryness with a rotary evaporator. The residue was partitioned between 200 ml of dichloromethane-distilled H_2O (1:1), and the dichloromethane layer was extracted three times with a saturated $NaHCO_3$ solution (100 ml). The dichloromethane layer, containing AFs, was rotary evaporated and concentrated under a gentle stream of nitrogen. The aqueous layer, containing CPA, was acidified to pH 2.0 with 0.5 N HCl and extracted two times with 25 ml kloroform (500 ml). The extract was evaporated and concentrated as for CPA.

A total of 45 g of peanuts was used for extraction of CPA. After adding 150 ml methanol and sodium bicarbonate (2%) (7:3), it was stirred in a high-speed mixer for 5 min. The mixture was filtred using Whatman no. 4 filter paper, and then 80 ml was taken from the fitrate obtained. To the filtrate, 30 ml of 0.05 M solution of lead acetate was added and stirred and the precipitate was removed by filtration. 0.5 N HCl reduced the pH to 2 with 50 ml of the filtrate that was extracted twice with 25 ml of chloroform, and the bottom phase was collected. The water in the chloroform phase (lower phase) was removed with 10 g anhydrous sodium sulfate by filtrating through a paper filter. The extract was collected in the flask after extraction and dried at 40°C by rotary vacuum evaporator. The extract in the flask was taken to the tubes with 3-4 ml of chloroform and then the content is dried under nitrogen gas.

The qualitative presence of CPA was determined by thin-layer chromatography (TLC) separation on silica gel plates 60 EM-5721 (20 × 20 cm; Merck). The plates were first dipped in a 2% (wt/wt) solution of oxalic acid in methanol for 10 min, after being heated at 100°C for 1 h and cooled. The plates were spotted with 60–80 μl of the respective extract and developed in the solvent ethyl acetate/2 propanol/sodium hydroxide (50:15:10, v/v/v) for 35–40 min. After this, the plates were being heated at 35–40°C for 1 h and cooled. CPA was viewed after spraying with Ehrlich's reagent (1.0 g of 4-dimethylaminobenzaldehyde in 25 ml of HCl and 75 ml etanol) with subsequent development of a purple color in daylight [30].

The results were calculated applying the formula, and a concentration of CPA in μ/kg = (S × Y × V)/(X × W)

http://dx.doi.org/10.5772/intechopen.68210

where *S* is the μl aflatoxin CPA standard equal to unknown; *Y* is the concentration of CPA standard μg/ml; *V* is the μl of final dilution of sample extract; *X* is the μl of sample extract spotted to give fluorescent intensity equal to *S* (CPA standard); and *W* is the weight of sample in gram of original sample contained in the final extract [18].

2.4. Statistical analysis

To compare the aflatoxin and CPA, periods of harvest, drying, preharvest, and storage results were analyzed using the Kruskal-Wallis one-way analysis on Rank's test (H statistic) and then Mann-Whitney U nonparametric multiple comparison test. All statistical analysis were performed by using SPSS, version 21.0 (IBM Corp., Armonk, NY, USA). Statistical analysis also revealed significant differences among the storage periods.

3. Results and discussion

Of 102 peanut samples analyzed by HPLC, 76 (75%) peanut samples were contaminated with aflatoxins (**Table 1**). High levels of AFs (1333.42 μg/kg) were found in shelled peanuts and unshelled peanuts (1235.15 μg/kg), respectively (**Table 1**). Of the samples analyzed, 32 (31.37%) peanuts samples were above limit as recognized in Turkey (10 μg/kg for AFs) (FAO, 2004) and 27 (26.47%) peanuts samples were above limit as recognized in US Food and Drug Administration (FDA) (20 μg/kg for AFs) [58].

Aflatoxin contamination was determined in 16 (64%) peanut samples of 25 samples collected during harvest. Of these samples, three of them were determined over 10 μg/kg, two of them were over 100 μg/kg, and one of them over 1000 μg/kg (**Table 1**).

Aflatoxin contamination was determined in 24 (75%) peanut samples of 32 samples collected during drying period. Of these samples, four of them were determined over 10 μg/kg, four of them over 100 μg/kg, and one of them over 1000 μg/kg (**Table 1**). Aflatoxin contamination was determined in 8 (53%) peanut samples of the 15 samples collected during prestorage period. Of these samples, two of them were determined over 10 μg/kg, one of them over 100 μg/kg, and one of them over 1000 μg/kg (**Table 1**). A total of 30 peanut samples were taken from the various peanut storages from Adana and Osmaniye provinces. Peanut samples were determined to be infected with aflatoxin levels between 0.18 and 1235.15 μg/kg (**Table 1**). Aflatoxin contamination was determined in 28 (93%) storage samples. Of these samples taken from peanut storage, toxin contamination were determined in five of them over 10 μg/kg, seven of them over 100 μg/kg, and one of them over 1000 μg/kg.

From the 102 peanut samples analyzed by TLC, 18 (17%) peanut samples produced CPA (**Table 2**). Four peanut samples produced CPA in 22.22 μg/kg (16%) during the harvest period. Five peanut samples produced CPA in 22.22 μg/kg (16%) during the drying period (**Table 2**). Nine peanut samples produced CPA in 16.66–44.44 μg/kg (30%) during the storage period (**Table 2**).

Periods of peanuts	Samples (positive samples)	%[b]	Range of AFs (μg/kg)	Samples >10 (μg/kg) (%)[b]	Range of AFs (μg/kg)	Samples >100 (μg/kg) (%)[b]	Range of AFs (μg/kg)	Samples >1000 (μg/kg) (%)[b]	Range of AFs (μg/kg)
Harvest	25 (16)	64	0.03–1333.42	3 (12)	32.45–49.55	2 (8)	137.54–684.30	1 (4)	1333.42
Drying	32 (24)	75	0.06–1106.70	4 (13)	13.93–38.20	4 (13)	105.10–420.77	1 (3)	1106.70
Prestorage	15 (8)	53	0.19–1311.28	2 (13)	11.19–58.89	1 (7)	123.14	1 (7)	1311.28
Storage[a]	30 (28)	93	0.18–1235.15	5 (17)	13.28–37.10	7 (23)	243.25–663.08	1 (3)	1235.15
Total	102 (76)	75	0.03–1333.42	14 (14)	11.93–58.89	14 (14)	105.10–663.08	4 (4)	1106.70–1333.42

[a]Significant differences ($p < 0.05$).

[b]Percentage related to the total number of samples in each period.

Table 1. Occurrence of aflatoxins in peanuts samples ($n = 102$) collected from Adana and Osmaniye provinces in Turkey and analyzed by HPLC.

Periods of peanuts	CPA samples (positive samples)	% [b]	Range of CPA (μg/kg)	Positive CPA samples within AFB (μg/kg)	Positive CPA samples within AFB + AFG (μg/kg)
Harvest	25 (4)	16	22.22	2	2
Drying	32 (5)	16	22.22	2	3
Prestorage	15 (0)	–	–	–	–
Storage[a]	30 (9)	30	16.66–44.44	2	6
Total	102 (18)	17	16.66–44.44		

[a]Significant differences ($p < 0.05$).

[b]Percentage related to the total number of samples in each period.

Table 2. Occurrence of CPA in peanuts samples ($n = 102$) collected from Adana and Osmaniye provinces in Turkey and analyzed by TLC.

Aflatoxins type B and CPA were detected in 11 of the 102 samples of peanuts, suggesting the possibility of cooccurrence of these toxins. Based on the combination of mycotoxins (AFB+/AFG+/and CPA+/) that were existing in nine of the peanut samples, especially six of them from the storage period can be considered. Only one peanut sample was without contamination of aflatoxin, but it had produced CPA.

Molds may be divided into two main groups, namely the "field fungi" and the "storage fungi." The first contamination is considered to be in the field and during ineligible drying. The reduction occurred in quality due to the mistakes made during the growth period of the peanut plants, exposure to fungus and pest infestation of the fruit, and also when met with climatic conditions such as humidity and temperature; aflatoxin forms of fungi can lead to increased secondary contamination and development [31–33].

Peanuts are considered to be a high-risk product for contamination with aflatoxins since they are frequently contaminated with fungi, particularly *A. flavus* and *A. parasiticus*, and because of long drying times and occurrence of rainy periods after uprooting [34]. Fungi produce carcinogenic aflatoxins. Aflatoxins are highly regulated for both animal feed and food destined for human consumption. Of the naturally occurring aflatoxins, aflatoxin B_1 is the most toxic. *A. flavus* may also produce CPA, which is toxic in a variety of animals and has been implicated in human poisoning. CPA and aflatoxins commonly occur together in contaminated agricultural commodities [35].

CPA is a product of the ubiquitous genera of molds, *Aspergillus* and *Penicillium*. The molds are known to inhabit a number of food sources and may constitute parasitic infections of man and other animals. CPA effects may be masked by concurrent aflatoxicosis; for example, CPA and aflatoxins were isolated from peanut meal related to the Turkey "X" disease that caused the death of over 100,000 turkeys [35]. In this study, CPA was isolated from 18 peanut samples that include harvest, drying, and storage periods. Isolates of *A. flavus* that are able to produce simultaneously aflatoxins type B and CPA were detected in all substrates, suggesting the possibility of co-occurrence of these toxins. CPA occurs naturally in peanuts [10, 11] as a cocontaminant with AFs and may have contributed to the "Turkey X" syndrome in England in 1960 [36].

In general, CPA is produced by *A. flavus* alone or in combination with type B aflatoxins, but not in conjunction with type G aflatoxins. *A. flavus* and *A. parasiticus* are closely related species belonging to the *Aspergillus* section *Flavi*. Both species can produce aflatoxins, but not all isolates of either species do so [37]. Aflatoxins consist of a group of approximately 15–20 related secondary metabolites, although AFB_1 and AFB_2 are produced by *A. flavus*, AFB_1, AFB_2, AFG_1, and AFG_2 are produced by *A. parasiticus* strains. That means in our study, *A. parasiticus* could exist in peanuts during storage, and it would be able to produce CPA as well. Although Vaamonde et al. [12] found that isolates of *A. parasiticus* consistently produce both B and G aflatoxins, they do not produce CPA; according to Oktay and Dinh et al. [16, 37], they could find that *A. parasiticus* are able to produce CPA besides groups B and G.

In the survey areas, farmers do the drying by leaving piles of harvested peanuts on the ground and carry out the mixing with a shovel by shifting the piles. Because of damaged and broken peanuts that hold on to the soil surface and because of lack of ventilation, peanuts become vulnerable to pathogens contained in the surrounding air, so appropriate medium is provided for the development of fungi, such as *Aspergillus*, which infect, primarily, peanuts in the fields and harvest period [38, 39]. Meanwhile, peanuts especially raw, immature, or damaged for any reason with seed coat damage and peanut kernels separated from cotyledons have high potential for production of aflatoxin [40]. As the farmers' peanut drying process under the sun on the soil takes a long time, they can lead to the development of potential producers of aflatoxin fungus. Peanuts are considered to be at high risk of AFs because they are frequently contaminated with *Aspergillus*, especially, aflatoxigenic species. Recently, it has been reported that AFB_1 was detected in 25% of raw peanuts from China, ranging from 0.01 to 720 μg/kg [41]. On the other hand, Juan et al. [42] showed a weak contamination of the analyzed samples of peanuts with AFs (5%). Mphande et al. [43] reported that 78% of raw peanuts from Botswana contained AFs at concentrations ranging from 12 to 329 μg/kg.

The drying stage is very important to reduce attack and damage from insects and fungi. Traditional drying techniques in Turkey involve bare-ground drying and is a major source of fungal contamination. Some farmers do not dry peanuts immediately after harvest. They dry them as a cluster on the ground for a few days waiting for sunshine. They walk on the stacks of peanuts and mix by shovel. Cracks and breaks in peanut pods and testa are caused mainly during shelling by trampling. These practices, coupled with an inefficient and slow drying process under the humid conditions, enhance aflatoxin contamination greatly [38]. When the soil and other materials are removed from the harvested and dried peanuts before entering the storage the amount of aflatoxin possible on the peanut will be reduced. Kacmaz [46] reported that the content of peanut products from order processing contain 10–15% of impurities (stones, earth, garbage, fiber, hernia, etc.) in Osmaniye.

As peanuts come from the field, they are mixed with foreign materials such as rock sediments, moist soil particles, and outer shells of raw peanuts, and they must be removed from pods [45] to avoid forming optimum conditions for the aflatoxin development before entering storage [28, 44, 45].

http://dx.doi.org/10.5772/intechopen.68210

When determining the aflatoxin contamination compared with second-crop peanuts collected during different periods from the survey areas, it was found that contamination was significantly higher in the period of harvesting (64.00%) and drying (75.00%) than during the prestorage (53.33%) (**Figure 1**). The highest rate of aflatoxin contamination was detected during storage (93%), followed by drying and harvesting (**Figure 1**). Also, CPA was found higher in the period of storage (30.00%) than in harvesting (16.00%) and drying (16.00%) periods (**Figure 2**). The contamination of stored peanuts is the CPA content of peanut during harvesting and drying and the inability to maintain adequately during storage. As a result of statistical analysis it was found that only the storage period was important. Although the storage was statistically significant for samples containing differences in production of the two mycotoxins, and in some instances, a statistical analysis was not evident. Considering the samples taken during harvesting period, aflatoxin contamination continued in the storage conditions, and therefore, it is clear that aflatoxin contamination has increased [24, 28, 38, 39, 45, 47–51]. In 28% of the newly harvested peanuts aflatoxin contamination was found to be 0-5 ppb whereas in 48% of the stored samples it was found to be 0-22 ppb. [39]. According to Ding et al. [41] low AFs contamination is found in peanuts after harvest, but AF levels might be higher during storage and processing. It is therefore, necessary to monitor the AFs contamination status of peanuts during growth, storage, and processing [17].

CPA of kernel samples was detected. The frequency of detection was 60% for the Caiapó, with mean levels ranging from 304.1 to 2583.7 μg/kg, and 74.3% for the 886, with levels ranging from 288.0 to 4918.1 μg/kg [17]. Other studies investigating the production of CPA in peanuts also reported high rates of 89 [52], 93 [53], and 97% [54]. Aflatoxins and CPA were also detected simultaneously in kernel samples (11.4%). The co-occurrence of CPA and AFs has been reported by several investigators [11, 12, 55, 56]. In addition, Smith et al. [57] demonstrated possible synergistic and cumulative effects of the two mycotoxins [17].

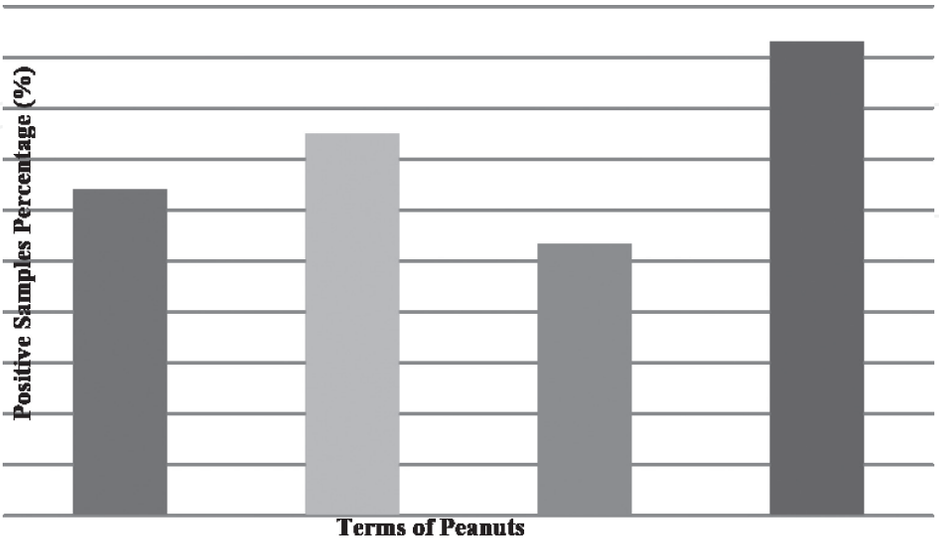

Figure 1. Distribution of the number of AFs found in all positive samples.

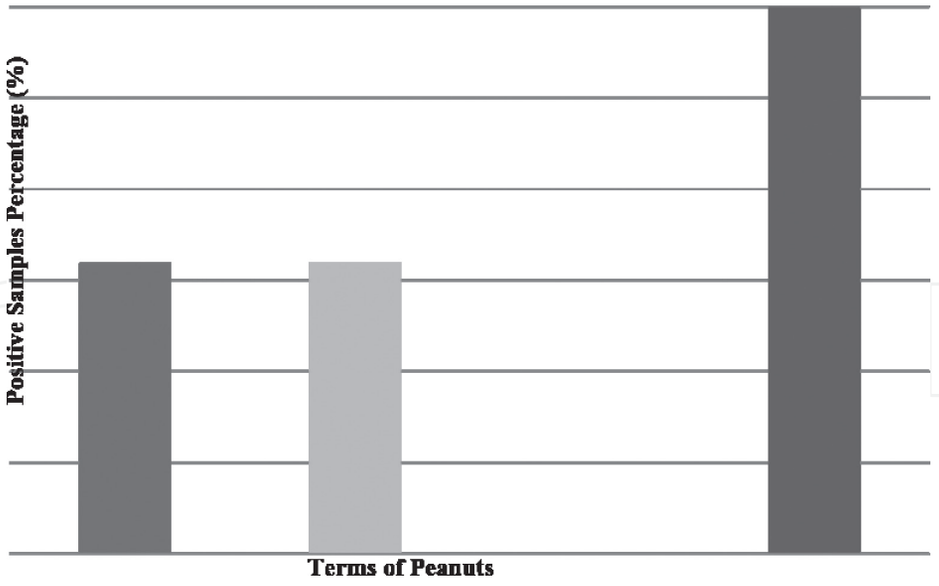

Figure 2. Distribution of the number of CPA found in all positive samples.

The levels of aflatoxin in 27 of the 102 samples analyzed were found to be over 20 μg/kg [58], determined by the FDA. In addition, aflatoxin levels in 32 of the 102 samples were found to be above the legal limit of 10 μg/kg [59] determined in Turkey. In the region where there are major problems in peanut harvest, drying, and storage periods, and as long as measures are not taken, these problems will continue to increase in the future is absolute.

All of these, besides difference in climate conditions, methods of harvesting, drying process, and transferring, leading to mechanical damages of peanuts and inadequate drying after rewetting for dehulling are deperiodinant for the final aflatoxins content. Our results showed that high aflatoxin contamination of 32 of the 102 samples were levels above "recognized" limits in Turkey. So far although aflatoxin is always the most important toxin because of its toxicity, the CPA presence in peanuts is also important by the end of this study.

4. Conclusions

In accordance with results of the study, it was concluded that when deperiodining the aflatoxin contamination compared during different periods, storage period is determined to be higher than the harvesting and drying. So it was concluded that aflatoxin began during the period of harvest, and increased during the drying period. In the period of the prestorage, it was found to decrease as a result of purifying of the soil or other foreign matter partially. Aflatoxin contaminations in peanut samples continue in the storage conditions, and aflatoxin contamination that was detected increased very much. Considering that if the samples were collected at harvest, it can be concluded that the creation of a suitable environment for the production of toxins is inappropriate during drying and storage conditions. In the region, where there are problems in peanut harvesting, drying, and storage periods and as long as

measures are not taken, these problems are expected to continue to increase in the future. Also, the sample contaminated with CPA and the simultaneous detection of AFs and CPA highlight the need to investigate factors related to the control and co-occurrence of these toxins in peanuts.

Acknowledgements

This work was financially supported by Tubitak of Turkey (Project no: 115O007). We thank Tubitak for their contributions to Tubitak.

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