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Determination of aflatoxin B_1 levels in deep-red ground pepper (isot) using immunoaffinity column combined with ELISA

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Abstract

Deep-red ground pepper, a variety of red ground pepper, is a special spices belonging to Sanliurfa and consumed both in Sanliurfa and other provinces of Turkey. The aim of this study was to determine the aflatoxin B_1 (AFB_1) levels of deep-red ground pepper. For this purpose, 75 samples of deep-red ground pepper (isot) marketed in Sanliurfa (Turkey) were purchased from bazaars and herbal shops. The occurrence and concentration range of AFB_1 in the samples were investigated by microtitre plate Enzyme Linked Immunosorbent Assay (ELISA) method using immunoaffinity columns. Seventy-two of the 75 ground deep-red pepper samples (96%) contained AFB_1 in the range of 0.11–24.7 μg/kg. Eleven (14.7%) samples were above the regulatory limits used in the European Union and in Turkey. More precaution should be taken on hygiene controls in order to prevent microbiological and chemical hazards.

Keywords: Aflatoxin B_1; Red ground pepper; Deep-red ground pepper; Spices

1. Introduction

The problem of food and feed contamination with toxigenic moulds especially *Aspergillus* species is of current concern and has received a great deal of attention during the last three decades (Rustom, 1997). These fungi are capable of growing on a great variety of food commodities and animal feed materials when the conditions of temperature, relative humidity and product moisture are favourable (Iamanaka et al., 2007; Rosi et al., 2007). Aflatoxins are a group of extremely toxic metabolites produced by some *Aspergillus* species namely *A. flavus*, *A. parasiticus* and the rare *A. nomius*, during the growth on foods and/or feeds. *A. flavus* produces only B aflatoxin, while the other two species produce both B and G aflatoxins (Sweeney and Dobson, 1998; Creppy, 2002). Aflatoxins are a significant threat to both human and animal health because they present toxigenic, carcinogenic, teratogenic and mutagenic potential. Among aflatoxins, aflatoxin B_1 (AFB_1) is considered to be the most potent naturally occurring hepatocarcinogen known, the risk assessment of which is very well established (Sweeney and Dobson, 1998). Therefore, International Agency for Research on Cancer (IARC) has classified as a Group 1 human carcinogen (International Agency for Research on Cancer, 1993).

Humans are exposed to aflatoxins via risky foods such as milk and dairy products, cereals, cacao, coffee, grapevine, dried fruits and various spices (Brera et al., 2002; Colak et al., 2006). Spices are often contaminated with aflatoxins. The climatic conditions prevailing in the tropics are especially favourable for mould contamination and aflatoxin production (Fazekas et al., 2005). Of the different mycotoxins, aflatoxin is the most common in spices. Contamination of spices with aflatoxins takes place in the field, during drying and during the storage and processing stages

Abbreviations: AFB_1, aflatoxin B_1; ELISA, enzyme linked immunosorbent assay; IARC, International Agency for Research on Cancer; EU, European Union; PBS, phosphate buffer solution.

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(Elshafie et al., 2002). Previous studies have demonstrated that spices are contaminated with various microorganisms including toxigenic moulds (especially *Aspergillus* spp.) which have aflatoxin producing potential (Martins et al., 2001; Fazekas et al., 2005; Reddy et al., 2001; Banerjee and Sarkar, 2003). Therefore, spices pose health problems because they are often added to foods without further processing or are eaten raw. Due to their frequent occurrence and toxicity, regulatory agencies are imposing uniformly rigorous standards on the level of acceptance in imported commodities. In the European Union (EU) an acceptable level of aflatoxins for spices has been set at 5 μg/kg for AFB_1 and 10 μg/kg for total aflatoxins ($B_1 + B_2 + G_1 + G_2$) (Commission Regulation, 2002). Turkey has been trying to become a member of the EU and has harmonized its aflatoxins limits accordingly (Turkish Food Codex, 2002).

Spices are largely produced in countries where tropical climates (high ranges of temperature, humidity and rainfall) are favorable to mycotoxin contamination. Furthermore they are usually dried on the ground in the open air in poor hygienic conditions that promote even more growth of moulds and production of mycotoxins (Martins et al., 2001).

Red ground pepper, the dried form of *Capsicum annuum* L., occupies a prominent place among the spices in Turkey consumed by the majority of people. It is commonly used for flavouring, seasoning and imparting aroma or colouring of foods. Deep-red ground pepper is a variety of red ground pepper. It is a special spices belonging to Sanliurfa and consumed both in Sanliurfa and other provinces of Turkey and also exported to other countries. It is known as Isot in the Sanliurfa and is produced both traditionally at home and commercially in the factory. Traditional deep-red ground pepper is generally produced at homesteads in order to sell or to consume at home. Traditionally, fresh peppers are cleaned and divided into four parts with knife. Then, they are spread and kept on concrete surface for a day to evaporate some of its water. In the next stage, they are transferred into plastic bags during daytime to perspire and are kept in plastic bags throughout the night. It is kept for 7–10 days in plastic bags until its colour turns into deep-red. Later, they are hammered and sieved. Finally, 250–300 ml of olive oil and 500 g salt are added into 100 kg peppers. In the industrial production of deep-red ground pepper, drying is achieved on pulsating trays in a hot air circulated tunnel (Hayaloglu et al., 2005).

Despite lots of studies on aflatoxin in agricultural products, only a few are concerned with spices that are more and more common in our daily diet and play an important role in the economy. The aim of this study was to therefore provide information on AFB_1 levels in deep-red ground peppers marketed in Sanliurfa, a southeastern province of Turkey. The result of this study can contribute to the evaluation of deep-red ground peppers (isot), consumed by a lot of people in Sanliurfa and other provinces of Turkey, from the point of view of food safety.

2. Materials and methods

2.1. Samples

In April, a total of 75 samples of deep-red ground pepper (isot) commercialized in Sanliurfa were randomly obtained from bazaars and herbal shops. These samples' origins are homestead production. Samples were stored at 4 °C in plastic bags until the analysis.

Sanliurfa has tropical climate that high range of temperature. Drying temperature conditions such as 60 and 70 °C have been used during production.

2.2. Analysis of AFB_1 by ELISA

AFB_1 concentrations of the samples were analysed by microtitre plate Enzyme linked immunosorbent assay (ELISA) method. AFB_1 test kit (Ridascreen Aflatoxin B_1 Art. No.: 1201, R-Biopharm, Darmstadt, Germany) (R-Biopharm GmbH, 2004) and immunoaffinity column (Rida Aflatoxin Column Art. No.: R5001/5002) were used to run ELISA analyses (R-Biopharm GmbH, 2005).

2.2.1. Samples preparation and separation with immunoaffinity column

Sample preparation and separation with aflatoxin column procedures were performed according to the instructions of the test kit manual (Rida Aflatoxin Column Art No.: R5001/5002) (R-Biopharm GmbH, 2005). 25 ml of methanol (70%) was added to 5 g of deep-red ground pepper and the solution was extracted by mixing gently for 10 min at room temperature. Afterwards, the extract was filtered through a paper filter and 15 ml of distilled water were added to 5 ml of filtered solution. Then, 0.25 ml Tween 20 were added and stirred for 2 min.

According to test kit manual, clean up procedure was performed as follows. The column was rinsed with 2 ml distilled water for equilibration. The column was filled with approximately 1 ml sample extract. A suitable adapter was attached on top of the column and a syringe was used as a sample reservoir. Syringe was filled with the rest of the sample extract. This was passed slowly and continuously through the column (flow rate: approximately 1 drop/s) and discarded. The column was rinsed with 10 ml distilled water and the passed solution was discarded. The column was dried by passing air through the column for approximately 10 s, in order to make sure that all the residual buffer would be removed from the column. The syringe was removed and a clean and closable vial directly placed below the column. 0.5 ml of methanol was passed slowly through the column (flow rate: approximately 1 drop/s). Toxin containing eluate was diluted 1:10 with the sample dilution buffer (Phosphate Buffer Solution (PBS), pH 7.2) and used 50 μl per well in the assay.

2.2.2. Test procedure of AFB_1

According to Ridascreen Aflatoxin B_1 (Art No.: 1201) test kit manual (R-Biopharm GmbH, 2004), 50 μl aflatoxin standard solutions and 50 μl prepared test samples were added into separate wells of micro-titer plate, in duplicate. Then, 50 μl of the diluted enzyme conjugate was added to each well, mixed gently and incubated for 2 h at room temperature (20–25 °C) in the dark. The liquid was then removed completely from the wells, the each well was washed with 250 μl washing buffer (PBS–Tween Buffer, pH 7.2). The washing procedure was repeated for three times in ELISA Washer (ELX 50, Bio-Tek Inst.). After the washing step, 50 μl enzyme substrate (urea peroxide) and 50 μl chromogen (tetramethyl-benzidine) were added to each well and incubated for 30 min at room temperature in the dark. Finally, 100 μl of the stop solution (1 M H_2SO_4) were added to each well and the absorbance was measured at 450 nm in ELISA plate reader (ELX 800, Bio-Tek Inst.).

2.2.3. Evaluation

The samples were evaluated according to the Rida Soft Windows program distributed by R-Biopharm GmbH. The detection limit of the AFB_1 test in the analytical procedure was 0.025 μg/kg, recovery rate was 50–70% and the average coefficient of variation was 8%.

3. Results

The AFB_1 content of the deep-red ground peppers is summarized in Table 1. Seventy-two of the 75 deep-red ground pepper samples (96%) contained AFB_1 in the range of 0.11–24.7 μg/kg. Sixty-one of them (81.3%) were in a concentration range of 0.11–5 μg/kg. Eleven samples (14.7%) were above the regulatory limit, which had been set at 5 μg/kg for AFB_1 in Turkey, in concentrations ranging from 5.1 to 24.7 μg/kg (Table 2). The average level of positive samples was 1.9 μg/kg.

Table 2
Aflatoxin B_1 levels exceeding legal limit[a] (>5 μg/kg) in deep-red ground peppers from Sanliurfa, Turkey

Sample no.	Level
1	5.1
2	5.3
3	6.1
4	6.7
5	7.2
6	9.4
7	8.7
8	10.9
9	13.5
10	15.2
11	24.7

[a] Turkish food codex.

4. Discussion

During the past decades a huge number of scientific papers have demonstrated that the list of raw materials and processed foods actually contaminated by aflatoxins is continuously increasing spanning from peanuts, known to be contaminated by aflatoxins since 60 s, to cereals, coffee, cocoa, dried fruits and spices (Zinedine et al., 2006). Among aflatoxins, AFB_1 is the most frequent toxin in spices with higher levels and chilli samples are the most frequent contaminated substrate (Romagnoli et al., 2007). In this study, AFB_1 in deep-red ground pepper samples was high percentiles (96%). About 15% of the samples contained AFB_1 in excess of the maximum permissible level, and the highest AFB_1 concentration found in these samples was high about five times the maximum level (24.7 μg/kg), posing a health risk to consumers. The reason for high levels of AFB_1 is a result of lying red peppers on soil and asphalt for drying, storage of the red peppers under high humidity levels, and insufficient control of transport and shop conditions. These results showed that AFB_1 occurrence in red pepper in Turkey could relatively be a critical point, regarding the quality of red peppers. It is well known that growth of moulds and consequent mycotoxin production is dependent upon a number of factors such as temperature, humidity, handling during the harvesting and storage. Therefore, deep-red ground pepper samples currently being marketed in Turkey pose serious health hazards to human beings.

There are few studies on aflatoxin levels in spices in Turkey. Hayaloglu et al. (2005) reported that in only one of 40 red-blackish ground pepper (isot) samples (2.5%) contained AFB_1 at level 3 μg/kg. In a similar study performed by Erdogan (2004), it was reported that total aflatoxin was found in only one isot pepper (5%) at level 13.8 μg/kg. Gurbuz et al. (1999) examined 75 red ground pepper samples and detected AFB_1 at levels between 0.25 and 10 μg/kg in 32% of the samples. Our results were higher than these results. On the other hand, Colak et al. (2006) found AFB_1 in 13 of 30 red-scaled pepper (red ground pepper) samples (43.3) in the range of 1.9–35.5 μg/kg. In another study performed by Bircan (2005), AFB_1 was found in 27 of 30 red ground pepper samples (90%) in the range of 0.5–116.4 μg/kg and in all chilli powder (100%) in the range of 1.6–80.4 μg/kg.

Furthermore, numerous studies have been performed in different countries for investigating of aflatoxin contamination in spices, particularly in red ground pepper and chilli powder. Martins et al. (2001) studied twelve different prepackaged spices marketed in Portugal and found 43% of the samples, including red ground pepper, were contaminated with AFB_1 in the range of 1–20 μg/kg. In Hungary, 70 ground red pepper samples were screened for aflatoxin contamination and 25.7% of the samples contained AFB_1. The aflatoxin concentration of ground red pepper was between 0.14 and 15.7 μg/kg (Fazekas et al., 2005). Fifty-nine percent of the 182 chilli samples collected in India were contaminated with AFB_1. Highest level of AFB_1 was detected in chilli pepper grade 3 samples at level 969 μg/kg (Reddy et al., 2001). In screening AFB_1 in ground red pepper in Ethiopia, Fufa and Urga (1996) found eight of 60 samples (13.3%) collected from markets, shops and storage facilities were contaminated with AFB_1 in concentrations of 250–525 μg/kg. These findings support the results of this study and red ground pepper must be

Table 1
Occurrence and distribution of aflatoxin B_1 in deep-red ground pepper samples from Sanliurfa, Turkey

Analysed (*n*)	Positive[a] *n* (%)	Distribution *n* (%)			Concentration[c] (μg/kg)	
		ND < 0.025 μg/kg	0.025–5 μg/kg	>5[b] μg/kg	Mean ± SD	Range
75	72 (96)	3 (4)	61 (81.3)	11 (14.7)	1.9 ± 4.2	0.11–24.7

[a] ⩾0.025 ng/l ND: not detected, <0.025 μg/kg.
[b] Exceed Turkish legal limit.
[c] Positive samples.

considered as a suitable substrate for aflatoxin biosynthesis.

5. Conflict of interest statement

This study confirmed that red ground pepper could be affected by mycotoxin contamination due to the climatic conditions, especially humidity and temperature of the region. More precaution should be taken on hygiene controls in order to prevent microbiological and chemical hazards.

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