*Journal of Food and Drug Analysis, Vol. 14, No. 3, 2006, Pages 292-296* 藥物食品分析 第十四卷 第三期

# Determination of Aflatoxin Contamination in Red-Scaled, Red and Black Pepper by ELISA and HPLC

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(Received: March 21, 2006; Accepted: May 20, 2006)

## ABSTRACT

Mycotoxins are toxic metabolites produced by some species of mould genera such as *Aspergillus*, *Penicillium* and *Fusarium*. Mycotoxins are carcinogenic, mutagenic, teratogenic and immunosuppressive to most animal species and humans. Humans are exposed to aflatoxins *via* risky foods such as milk, cereals and various spices. This study aimed to determine the aflatoxin levels in red scaled, red and black pepper. For this purpose, 84 spice samples were randomly obtained from markets and bazaars in Istanbul. Thirty-six of 84 spice samples (42.9%) were found contaminated with aflatoxins in the range of 0.3-46.8 μg/kg. All positive samples were also analyzed and confirmed by HPLC. The results obtained by ELISA were closely related to those by HPLC. In conclusion, aflatoxins continue to pose a health concern *via* human exposure to contaminated spices.

Key words: Spices, Aflatoxin, Aflatoxin B1, ELISA, HPLC

## INTRODUCTION

Mycotoxins are toxic metabolites produced by some species of mould genera such as *Aspergillus*, *Penicillium* and *Fusarium*[1-2]. Among all mycotoxins, aflatoxins are a group of highly toxic secondary metabolic products named as aflatoxin B1(AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2)[3-4]. Aflatoxins are carcinogenic, mutagenic, teratogenic and immunosuppressive to most animal species and humans[5]. AFB1 has the highest potency as a toxin and is classified as group I carcinogen by International Agency for Research on Cancer (IARC)[6]. The order of toxicity, AFB1>AFG1>AFB2>AFG2, indicates that the terminal furan moiety of AFB1 is the critical point for determining the degree of biological activity of this group of mycotoxins[7].

Aflatoxins easily occur on feeds and foods during growth, harvest or storage[8]. Humans are exposed to aflatoxins *via* risky foods such as milk and dairy products, cereals, cacao, coffee, grapevine, dried fruits and various spices[9]. Spices are exposed to a wide range of microbial contamination due to poor collection conditions, unpretentious production process and extended drying times. In addition, spices can be contaminated through dust, waste water and animal/human excreta in unpackaged spices which are sold in markets and bazaars[10-11]. Several studies have demonstrated that spices are contaminated with various microorganisms including toxigenic moulds (especially *Aspergillus* spp.) which have aflatoxin producing potential[12]. Therefore, spices pose health problems because they are often added to foods without further processing or are eaten raw[13-14].

Due to their frequent occurrence and toxicity, guidelines and tolerance levels of aflatoxins have been set in several countries including Turkey. According to the Turkish Food Codex, the maximum residue limits for AFB1 and total aflatoxin in spices is 5 and 10 μg/kg, respectively[15].

Although several studies are available for aflatoxin levels in different food types which are consumed in Turkey, limited studies are done on aflatoxins in spices. Therefore, this study was planned to determine aflatoxin levels in red scaled pepper, red pepper and black pepper which are consumed to a great extend at Turkish kitchen and to compare the obtained results with maximum aflatoxin tolerance limits accepted by the Turkish Food Codex.

## MATERIALS AND METHODS

### I. *Samples*

A total of 84 samples of spices (30 red-scaled pepper, 30 red pepper and 24 black pepper) commercialized in Istanbul were randomly obtained from markets and bazaars. Samples were stored at 4°C in plastic bags until the analysis. All samples were analyzed in duplicate.

### II. *Analysis of aflatoxins by ELISA*

#### (I) *Sample Preparation*

Sample preparation procedures were performed according to the instructions of the test kit (Rida Aflatoxin Column Art No.: R5001/5002, R-Biopharm, Darmstadt, Germany) manual[16]. 25 mL of methanol (70%) was added to 5 g of spices. Afterwards, the solution was

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extracted by mixing gently for 10 minutes at room temperature. The extract was filtered through a paper filter and 15 mL of distilled water were added to 5 mL of filtered solution. 0.25 mL Tween 20 were added and stirred for 2 minutes, followed by entire amount of the sample solution (20 mL) passing over the column. Clean up procedure was performed according to the kit's manual. Toxin containing eluate was diluted 1:10 with the sample dilution buffer (supplied with the test kit) and used 50 µL per well in the assay.

(II) *Test Procedure of Total Aflatoxins*

According to Ridascreen Aflatoxin Total (Art No.: 4701) test kit manual[17], 50 µL of the standard solutions or prepared sample in duplicate were added to the wells of micro-titer plate. Then 50 µL of the diluted enzyme conjugate and 50 µL of the diluted antibody solution were added to each well. The solution was mixed gently and incubated for 30 min at room temperature (20-25°C) in the dark. The unbound conjugate was removed during washing for three times (ELISA Washer ELX 50, Bio-tek Inst.). Afterwards, 100 µL of substrate/chromogen solution was added to each well, mixed gently and incubated for 30 min at room temperature (20-25°C) in the dark. Then, 100 µL of the stop solution (1M $H_2SO_4$) was added to each well and the absorbance was measured at 450 nm in ELISA plate reader (ELX 800, Bio-tek Inst.). The mean lower detection limit is 0.25 µg/kg.

(III) *Test Procedure of AFB1*

According to Ridascreen Aflatoxin B1 30/15 (Art No.: 1211) test kit manual[18], 50 µL of the standard solutions or prepared sample in duplicate was added to the wells of micro-titer plate. Then 50 µL of the enzyme conjugate and 50 µL of the anti-aflatoxin antibody solution were added to each well, mixed gently and incubated for 30 min at room temperature (20-25°C). The washing procedure was applied for three times (ELISA Washer ELX 50, Bio-tek Inst.). After the washing step, 100 µL of substrate/chromogen solution were added to each well and mixed gently and incubated for 30 min at room temperature (20-25°C) in the dark. Finally, 100 µL of the stop solution (1M $H_2SO_4$) were added to each well and the absorbance was measured at 450 nm in ELISA plate reader (ELX 800, Bio-tek Inst.). The mean lower detection limit is 1.0 µg/kg.

III. *Analysis of aflatoxins by HPLC*

Aflatoxins were analyzed in spices according to the method described by Stroka *et al.*[19] and Zinedine *et al.*[1] First aflatoxin standart solutions (containing 1000 ng B1, 200 ng B2, 1000 ng G1 and 200 ng G2/mL) were prepared in toluene-acetonitrile (98+2). Working standard solutions were prepared daily from these standard solutions acoording to Stroka *et al.*[19] For the extraction procedure, 50 g of sample was added with 5.0 g of NaCl, extracted 300 mL methanol: water (80:20 v/v) in a blender at high speed for 3 min and filtered through a Whatman filter paper No.4. 10 mL were diluted with 60 mL of PBS, and 66 mL of the diluted filtrate was applied to the immunoaffinity column (Aflaprep, R- Biopharm) previously conditioned with 10 mL of PBS (flow rate of about 3 mL/min). The column was washed with 15 mL of water and air was drawn through the column until dry. Aflatoxins were eluted by applying 1.25 mL of methanol to the column. The eluate was diluted with 1.75 mL of water. A 100 µL aliquot was injected onto the HPLC system (Hewlett Packhard 110 HPLC Chromatograph, equipped with a Hewlett Packard 1100 fluorescence detector). Excitation and emission wavelengths were set at 333 and 460 nm, respectively. The eluate passed through a C18 column (5 µm particle size, 250 mm x 4.6 mm). The mobile phase was acetonitrile: water : methanol (17:54:29 v/v/v), and the flow rate was set at 1 mL/min. The limit of detection of the method was 0.02 µg/kg.

Quantification of each toxin was performed by measuring peak areas at their retention times, and by comparing them with their relevant standard calibration curve. The identity of each toxin was confirmed in all the analyzed samples by injecting sequentially sample extracts and comparing the peak area ratio with their corresponding standard.

IV. *Statistical Analysis*

Statistical analyses were performed by using the Statistical Package for Social Sciences (SPSS) (version10.0) for MS/Windows. Independent Sample T-test was used in comparison of sample mean values and Bivariate Correlation test was used in comparison of ELISA and HPLC method.

## RESULTS AND DISCUSSION

In this study, 36 out of 84 spice samples (42.9%) were found to be contaminated with aflatoxins in the range of 0.3-46.8 µg/kg. All positive samples were also analyzed and confirmed by HPLC. Statistically, comparison of the quantitative analysis of total aflatoxins and AFB1 by ELISA and HPLC revealed a good correlation for red scaled pepper (for total aflatoxins r = 0.983, for AFB1 r = 0.999), red pepper (for total aflatoxins r = 0.987, for AFB1 r = 0.985) and black pepper (for total aflatoxins r = 0.823, for AFB1 r = 0.995) between both methods. The results of the t-test for related samples revealed slightly higher results for ELISA ($p < 0.05$).

17 out of 30 red-scaled pepper samples (56.7%) contained total aflatoxins ranging from 0.7 to 46.8 µg/kg while 13 out of 30 samples contained AFB1 ranging from 1.9 to 35.5 µg/kg. In red pepper, 11 out of 30 samples

(36.7%) contained total aflatoxins ranging from 0.8-15.4 μg/kg. The concentrations of AFB1 were found to be ranging from 2.9-11.2 μg/kg. The levels of total aflatoxins were determined to be ranging from 0.3-16.7 μg/kg in 8 out of 24 black pepper samples. Only two samples contained AFB1 at the levels of 9.8 and 10.3 μg/kg (Table 1).

According to these results, 9 red-scaled, 3 red and 2 black pepper samples exceeded the maximum limits of AFB1 (5 μg/kg) and total aflatoxin (10 μg/kg) set in the Turkish Food Codex[15]. The samples obtained from bazaars had the highest contamination levels: red-scaled pepper (46.8 μg/kg), black pepper (16.7 μg/kg) and red pepper (15.4 μg/kg).

Few studies have been performed on aflatoxin levels in spices in Turkey. Yildirim *et al.*[20] found total aflatoxins in 8 out of 34 red pepper samples (23.5%) in the range

**Table 1.** Average amount of total aflatoxins and AFB1 in the contaminated red scaled, red and blck pepper samples

| *S | Sample no. | Source | Total aflatoxins (μg/kg) | | AFB1 (μg/kg) | |
|---|---|---|---|---|---|---|
| | | | ELISA | HPLC | ELISA | HPLC |
| RED SCALED PEPPER | No.1 | Bazaar | 29.7 ± 1.2 | 26.3 ± 1.2 | 9.9 ± 1.3 | 9.4 ± 0.6 |
| | No.2 | Market | 0.8 ± 0.3 | 0.4 ± 0.02 | **ND | 0.3 ± 0.1 |
| | No.7 | Market | 0.7 ± 0.2 | 0.3 ± 0.1 | ND | 0.2 ± 0.1 |
| | No.8 | Bazaar | 31.2 ± 2,7 | 28.4 ± 0.3 | 26.7 ± 2.1 | 24.2 ± 1.1 |
| | No.9 | Bazaar | 27.8 ± 3.0 | 26.1 ± 2.2 | 24.6 ± 1.3 | 22.8 ± 2.0 |
| | No.10 | Bazaar | 38.3 ± 5.8 | 35.0 ± 0.0 | 32.8 ± 2.2 | 31.4 ± 2.9 |
| | No.11 | Bazaar | 3.1 ± 1.9 | 2.4 ± 1.1 | 1.9 ± 0.9 | 1.7 ± 1.5 |
| | No.14 | Market | 26.4 ± 3.7 | 25.1 ± 1.4 | 10.8 ± 0.2 | 9.2 ± 0.7 |
| | No.15 | Bazaar | 42.4 ± 1.9 | 40.8 ± 2.8 | 34.5 ± 2.7 | 32.0 ± 0.0 |
| | No.17 | Bazaar | 5.6 ± 1.4 | 3.8 ± 1.0 | 2.3 ± 0.8 | 1.7 ± 0.9 |
| | No.18 | Bazaar | 1.6 ± 1.8 | 0.8 ± 0.3 | ND | ND |
| | No.19 | Bazaar | 22.3 ± 3.1 | 20.7 ± 2.5 | 15.2 ± 3.2 | 14.0 ± 2.3 |
| | No.22 | Market | 2.5 ± 2.0 | 2.0 ± 0.9 | 2.0 ± 0.6 | 1.6 ± 0.4 |
| | No.24 | Market | 8.4 ± 0.5 | 8.1 ± 0.4 | 4.2 ± 1.6 | 3.9 ± 0.9 |
| | No.27 | Bazaar | 25.6 ± 2.4 | 20.3 ± 1.7 | 16.3 ± 1.4 | 15.7 ± 2.4 |
| | No.28 | Bazaar | 1.2 ± 0.0 | 0.6 ± 0.0 | ND | ND |
| | No.29 | Bazaar | 46.8 ± 1.7 | 44.7 ± 1.1 | 35.5 ± 3.1 | 32.3 ± 1.0 |
| RED PEPPER | No.2 | Bazaar | 15.4 ± 0.0 | 14.2 ± 4.2 | 4.5 ± 0.2 | 3.8 ± 1.1 |
| | No.5 | Bazaar | 13.8 ± 1.4 | 12.1 ± 2.8 | 7.2 ± 2.3 | 7.0 ± 3.4 |
| | No.9 | Market | 3.1 ± 0.3 | 2.2 ± 0.4 | 2.9 ± 1.5 | 2.1 ± 0.6 |
| | No.12 | Market | 8.4 ± 2.7 | 8.0 ± 0.0 | 5.8 ± 2.1 | 4.2 ± 0.2 |
| | No.13 | Market | 1.3 ± 0.4 | 0.9 ± 0.1 | ND | 0.2 ± 0.1 |
| | No.15 | Bazaar | 13.8 ± 0.5 | 10.2 ± 2.3 | 11.2 ± 0.0 | 10.7 ± 3.4 |
| | No.17 | Market | 0.9 ± 0.2 | 0.3 ± 0.0 | ND | ND |
| | No.20 | Market | 5.5 ± 0.1 | 5.1 ± 2.3 | ND | ND |
| | No.21 | Bazaar | 7.9 ± 2.5 | 6.2 ± 1.8 | 5.4 ± 2.8 | 5.0 ± 1.9 |
| | No.28 | Bazaar | 0.8 ± 0.2 | 0.4 ± 0.0 | ND | ND |
| | No.30 | Market | 2.6 ± 1.4 | 1.7 ± 0.5 | ND | ND |
| BLACK PEPPER | No.3 | Market | 1.2 ± 0.1 | 0.7 ± 0.1 | ND | ND |
| | No.4 | Market | 0.9 ± 0.3 | 0.3 ± 0.2 | ND | ND |
| | No.7 | Market | 0.8 ± 0.0 | 0.1 ± 0.2 | ND | ND |
| | No.10 | Market | 0.3 ± 0.1 | 0.1 ± 0.0 | ND | ND |
| | No.12 | Bazaar | 11.6 ± 3.4 | 10.1 ± 4.6 | 9.8 ± 2.4 | 8.6 ± 3.4 |
| | No.15 | Bazaar | 2.3 ± 2.0 | 2.0 ± 0.5 | ND | ND |
| | No.19 | Market | 0.7 ± 0.5 | 0.3 ± 0.2 | ND | ND |
| | No.20 | Bazaar | 16.7 ± 4.9 | 15.3 ± 3.8 | 10.3 ± 03.9 | 9.5 ± 0.0 |

*S: Sample Type, **ND: Not Detected

*Journal of Food and Drug Analysis, Vol. 14, No. 3, 2006*

**Table 2.** The levels of total aflatoxins in spices in different countries

| Country | Sample number | Positive percentage (%) | Range (μg/kg) | Authors |
|---|---|---|---|---|
| Egypt | 120 | 16 | 8.0-35.0 | El-Kady *et al.*[24] |
| Ethiopia | 60 | 13 | 250-525 | Fufa and Urga[25] |
| England | 157 | 95 | 0.0-48.0 | McDonald and Castle2[6] |
| Egypt | 10 | 40 | 25.0* | Selim *et al.*[27] |
| Morocco | 55 | 84 | 0.0-9.68 | Zinedine *et al.*[1] |
| Qatar | 12 | 75 | 0.16-69.28 | Abdulkadar *et al.*[28] |
| Hungary | 70 | 18 | 6.1-15.7 | Fazekas *et al.*[29] |

*Average contamination value.

of 1.6-15.0 μg/kg. In another study performed by Erdogan[21], it was reported that total aflatoxins was found in 8 red-scaled pepper samples (18.2%) and in 3 red pepper samples (10.7%). Our results were found to be higher than these results. On the other hand, Hazir and Coksoyler[22] reported that 46 out of 141 red pepper samples (32.6%) contained aflatoxin. In a similar study in Van, Agaoglu[23] found the highest aflatoxin contamination level of 44.0 μg/kg in red-scaled pepper. These results are correlated well with our findings.

Furthermore, many surveys of aflatoxin contamination in spices are available in the literature. Results are summarized in Table 2.

Mostly, spices are grown in tropical and subtropical regions and harvested in poor sanitary conditions. These improper conditions are convenient for the biosynthesis of aflatoxins. Therefore, growing conditions, harvesting and processing methods, storage conditions and post-harvest treatments should be carefully controlled in order to prevent aflatoxin risks due to contaminated spices. In addition, training programs should be presented for producers.

In conclusion, aflatoxins continue to pose a health concern *via* human exposure to contaminated spices. Routine controls and survey researches have to be performed for the detection of aflatoxin contaminations in spices. The ELISA method showed a good correlation with HPLC, since ELISA (simplicity, rapidity, reliability, cost-effective etc.) can be used in routine screening of aflatoxin contamination in spices.

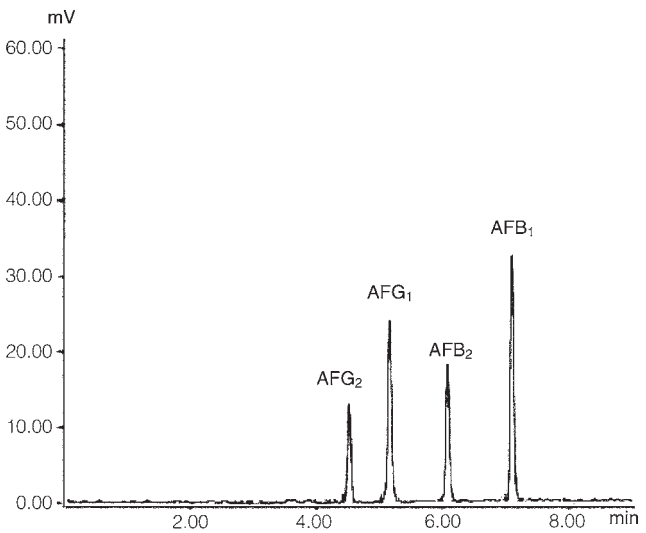


**Figure 1.** HPLC chromatogram of aflatoxin standard mixture: AFB1 and AFG1 at 0.4 ng/mL; AFB2 and AFG2 0.2 ng/mL; Chromatographic conditions: C18 column (5 μM; 250 × 4.6 mm); mobile phase, acetonitrile-water-methanole (17:54:29 v/v/v); flow rate; mL/min; fluorescence detector ($\lambda_{er}$ = 333 nm and $\lambda_{em}$ = 460 nm).

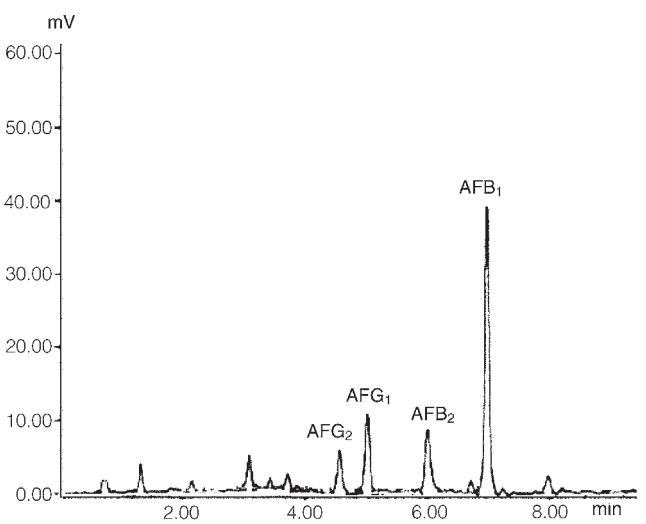


**Figure 2.** HPLC chromatogram of aflatoxins for red scaled pepper (Sample No. 1).

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