Natural Occurrence of Aflatoxins Contamination in Commercial Spices in Iran

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ABSTRACT
A total of 80 sample of spices (red pepper, black pepper, turmeric and cinnamon), commercialized in Iran, was analyzed for aflatoxins B1, B2, G1 and G2 content using high-performance liquid chromatography (HPLC) with a fluorescence detector (FD). A mixture of acetonitrile–methanol–water (17:29:54; v/v) was used as the mobile phase and an immunoaffinity column (IAC) applied as a cleanup method. All kinds of spice samples were spiked with aflatoxins B1, B2, G1 and G2 at levels of 1, 10, and 30 ng/g and recovery values were determined. Results showed recoveries ranged from 76.4±5.6 to 98.3±3.2 for AFG1 in cinnamon (spiked at 1ng/g) and AFB2 in turmeric (spiked at 10ng/g) respectively. Thirty-two out of 80 (40%) samples were contaminated with aflatoxins ranged from 0.85±0.10 to 24.60±0.12. Aflatoxin B1 was detected in all of the contaminated samples at the highest concentration as compared with other aflatoxins. Red pepper was significantly ($p \leq 0.05$) more contaminated than other spices.

Keywords: Aflatoxins; Spices; Red pepper; Black pepper; Turmeric; Cinnamon

INTRODUCTION
Fungus contamination, one of the serious problems involving agricultural products, may occur at all stages of food production and storage. Every year, 10-20% of agricultural commodities are spoiled because of mold growth to the extent that they could not be consumed by human and animal [1]. Moreover, molds may produce toxic compounds that are harmful to human. Therefore, fungus contamination is very critical for the economic, food safety and human health perspectives [2].

Aflatoxins, the secondary fungi metabolites, may grow on different agricultural commodities, including cereal and cereal products, coffee, nuts, and spices. They are produced by species of molds such as, Aspergillus particularly A. flavus and A. parasiticus. Among the approximately 20 different types of aflatoxins, only the aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) have got more attention since they are associated with acute liver damage and cirrhosis [3]. Due to the teratogenic, mutagenic and carcinogenic effects of AFB1, it has been classified as group 1, as a human carcinogen, by the International Agency for Research on Cancer [4].

Spices are common food additives, used for their properties as flavoring, seasoning, imparting aroma, colorant or pungent products. Spices are likewise the most common additives used to cook various foods In Iran. However, with the exception of the red pepper, other spices (black pepper, turmeric and cinnamon) are not cultured in Iran. They are imported in bulk from other countries and packed in Iranian factories. Red pepper is cultivated in Iran and imported also from other countries. Iran after Japan and USA is one of the main importers of spices in the world. India, United Arabic Emirates, China and Vietnam are the main countries export spices (pepper, turmeric and cinnamon) to Iran. However, small portions of pepper are imported from Turkey, Germany and France. About 41% of turmeric consumed in Iran is imported from India [5].

In terms of food safety, spices usually suffer from a wide range of microbial contamination because of poor collection conditions, non-sanitary production process and extended drying time, especially when the sun drying is applied. In addition, spices can be contaminated through dust, waste water and animal/human excreta, when they are unpacked and sold in wholesale and retail [6].

Elshafie and coworkers analyzed mold contamination of 105 samples of seven spices (cumin, cinnamon, clove, black pepper, cardamom, ginger, and coriander) and reported that toxigenic Aspergillus spp. was able to grow on spices and produce aflatoxins [7]. Martins reported that 43% of the spice samples marketed in Portugal was contaminated with AFB1 [8]. In a related study, total aflatoxins were detected in 12 spices (13.6% of incidence) at the level of 0.08–4.66ng/g [9]. In Turkey, 5.0–18.2% of samples of different pepper types contained 1.1–97.5mg kg^{-1} aflatoxins (B plus G) [10]. Colak reported thirty-six out of 84 spice samples (42.9%) were contaminated with AFs in the range of 0.3-46.8ng/g [11]. The AFB1 also was detected in 58 out of 93 analyzed organic spices in Turkey. The author reported that organically produced spices were

heavily contaminated with AFB1, especially cinnamon and red pepper samples [12].

A related study was carried out in Iran, to detect the presence of AFB1 in 36 samples of chilli powder and black pepper. AFB1 was found in all the spices samples, ranged from 0.312 to 0.627ng/g [13]. Salari reported that Aflatoxin B1 and B2 were found in nine out of 36 red pepper samples (25%) from Khorasan Razavi [14]. However, to the best of the researcher's knowledge, there is no study on the determination of aflatoxins in turmeric and cinnamon in Iran. The current study was conducted to determine and compare the level of aflatoxins contamination in the most commonly used spices (red pepper, black pepper, turmeric and cinnamon) marketed through diffrent outlets in Iran.

MATERIALS ANDMETHODS

Sampling

A total of 80 spice samples in commercially available sizes (50–100g) were collected during the period from May to September 2014. Five to 10 samples were purchased to obtain about 500g of each sample. The collected samples were made up of 4 different kinds of spices including red pepper (Capsicum annuum) (20 samples), black pepper (Piper nigrum) (20 samples), Turmeric (Curcuma longa L.) (20 samples), and cinnamon (Cinnamomum verum) (20 samples). These samples were randomly purchased from different markets in Tehran the capital of Iran. A wide range of different brands was collected in order to ensure that the survey was as comprehensive as possible and a representative of the commercial spice products available to consumers.

Chemicals

A stock solution of AFB1, AFB2, AFG1 and AFG2 (3000ng/ml), dissolved in benzene–acetonitrile (98:2, v/v), was purchased from Supelco (Bellefonte, PA, USA). The working standard solution (500ng/ml) of each mycotoxins was prepared, kept in an amber vial and stored at -20$^{\circ C}$ until used. The aflatoxins standard calibration curves for HPLC determination were prepared by solving an appropriate amount of working standard solution in the same solvent to obtain the final concentration. The glass microfiber filter Whatman (11cm, 934-AH), fluted filter paper (24cm) and immunoaffinity columns for aflatoxins were supplied by Vicam (Watertown, MA, USA). The Methanol, acetonitrile (HPLC grade) and sodium chloride were purchased from Merck (Darmstadt, Germany). Decontamination of the glassware was performed using a sodium hypochlorite solution and washing to a neutral pH with distilled water.

Extraction and purification of aflatoxins from spices

The samples (red pepper, black pepper, Turmeric, and cinnamon) were analyzed for AFB1, B2, G1 and G2 with a HPLC-fluorescence detector (FD), as described by Zinedine [15], with some modifications. A 25g of sample and 5g sodium chloride was placed in a 200 ml conical flasks. A volume of 100ml of methanol–water (80: 20, v/v) was added and the mixture was homogenized using a Waring blender (Middleton, MA, USA) for 3 min. The mixture was passed through a Whatman fluted filter paper, then 5ml of the filtrate was transferred to a glass beaker to which 20ml of a (90:10, v/v) solution of Tween 20–water was added. The solution was centrifuged at 10,000 rpm for 10 min, then filtered through a Whatman glass microfiber filter 934-AH (Vicam); a volume of 10 ml of the filtrate was passed through IAC aflatest (Vicam) at a flow rate of 1.0 ml/ min. Ten ml of distilled, de-ionized water was passed through IAC at a flow rate of 1ml/ min. Aflatoxins were subsequently eluted from the column with 1.5 ml of methanol and collected in a glass vial and 1.5 ml of de-ionized water was also added to the vial. The volume of 20 µl of elute was injected into the HPLC.

Apparatus

The detection of AFs was carried out using HPLC apparatus (Waters 600, Milford Massachusetts, USA) consisted of Water 600 controller high performance liquid chromatography system equipped with a Waters 600E pump, Waters 717 auto sampler and Waters in line degasser AF. The system was controlled by the Empower PDA software (Waters, Milford Massachusetts, USA). Liquid chromatography separation was performed on a reversed phase C18 (5mm, 25cm, 0.46cm) Purospher star column from Merck (Darmstadt, Germany). The fluorescence detector was set to an excitation wavelength of 365nm and an, emission wavelength of 435nm. The mobile phase consisted of a mixture of acetonitrile–methanol–water (17: 29: 54, v/v). The mobile phase was filtered using a Millipore filtration apparatus and maintained at a flow rate of 1 ml/ min. A photochemical reactor (model PHRED, New York, NY, USA) was used as a post-column derivatization. Aflatoxins were identified by constant retention times. Calibration curves of peak areas, versus aflatoxin concentration was then plotted and used for the determination of AFs in samples. A Waring blender (Middleton, MA, USA) and a water purifier (Elga, Marlow, UK) was applied.

Method validation

For linearity, a seven-point calibration curve was constructed with concentrations of 0.1, 0.5, 1, 2, 5, 10 and 20ng g^{-1} for AFB1, AFB2, AFG1 and AFG2. The calibration curves were obtained using the linear least squares regression procedure of the peak area

versus the concentration. The limits of detection (LOD) and limits of quantification (LOQ) were determined by using the signal-to-noise approach, defined as that level, resulting in a signal-to-noise ratio of approximately 3:1 and 10:1, respectively. With regard to the accuracy of the method applied and because there was no certified reference material (CRM) available, 25 g of aflatoxin-free of each spices sample was spiked with AFB1, AFB2, AFG1 and AFG2 at levels of 1, 10, and 30ng g^{-1}. The spiked samples were analysis by HPLC, as described above and then the recovery and standard deviation (SD) were calculated. All tests were carried out in three replicates.

Statistical analysis

The descriptive statistics (mean, standard deviation, range), and one-way analysis of variance (ANOVA) were conducted using Minitab (Version 17, State College, PA., USA). Statistical differences of AFs in four types of spices (black pepper, red pepper, turmeric and cinnamon) were determined using one-way ANOVA. A probability value of 0.05 was used to determine the statistical significance.

RESULTS

Method validation

All the aflatoxins (B1, B2, G1 and G2) were well separated from each other in standard and sample chromatogram. AFG2 was elected first, followed by AFG1, AFB2 and AFB1. The linearity in the working standard solutions at three determinations of five concentration levels was reliable, between 0.9992 for AFB1 and 0.9995 for AFG2 (Table 1).

Table 1: Recovery values obtained for red pepper, black pepper, turmeric and cinnamon at three different spiking levels (n=3).

Spiking level	Aflatoxins	Recovery % (mean±SD)			
		Red pepper	Black pepper	Turmeric	Cinnamon
1 ng/g	B1	81.5±5.6	77.7±5.1	84.4±6.5	82.1±4.8
	B2	89.0±4.1	83.0±5.6	79.6±4.7	80.6±5.6
	G1	79.9±5.6	78.2±3.1	81.0±4.6	76.4±5.6
	G2	81.0±2.3	76.9±3.1	79.3±5.5	77.5±3.7
10 ng/g	B1	97.3±4.8	96.8±5.8	91.5±3.9	91.3±4.6
	B2	91.1±3.5	89.2±5.9	98.3±3.2	90.1±4.6
	G1	86.1±5.9	92.1±4.2	92.3±4.9	87.9±5.6
	G2	83.1±6.0	80.0±5.3	85.7±4.2	88.7±6.0
25 ng/g	B1	87.8±7.1	94.1±4.2	87.8±4.0	85.5±3.2
	B2	92.9±4.8	86.5±3.1	96.7±4.7	87.4±6.5
	G1	84.9±5.8	89.2±4.8	85.0±5.5	85.0±6.0
	G2	86.0±4.4	84.3±3.7	85.1±6.4	82.1±3.5

SD: Standard deviation

The LODs of each of the aflatoxins B1, B2, G1 and G2 were found to be 0.02, 0.02, 0.08 and 0.06, respectively as has been demonstrated in Table 1. The values obtained for LOD and LOQ were comparable with the previous results [9]. To assess the method specificity, three clean matrixes of each sample (black pepper, red pepper, turmeric and cinnamon) were prepared and injected onto the HPLC. No appreciable signal at the retention time of aflatoxins was observed. Recovery values ranged from 76.4±5.6 to 98.3±3.2 for AFG1 in cinnamon (spiked at 1ng/g) and AFB2 in turmeric (spiked at 10ng/g) respectively, which is within the legislated levels described by the European Commission [16]. The recovery values also were in agreement with previous studies. Fazekas reported that recovery values for AFB1, AFB2, AFG1 and AFG2 were 81.25%, 85.3%, 83.1% and 75%, respectively [17].

Occurrence

The AFs (B_1, B_2, G_1 and G_2) were analyzed in 80 spices including red pepper, black pepper, cinnamon and turmeric samples. Results showed 32 out of 80 (40%) samples were contaminated with total aflatoxins ranged from 0.85±0.10 to 24.60±0.12. Eleven out of 20 red pepper (55%), 8 out of 20 black pepper (40%), 6 out of 20 cinnamon (30%) and 7 of 20 turmeric samples (35%) contained aflatoxins. The highest concentration of total aflatoxins (24.60±0.12) was found in a red pepper sample (Table 2).

AFB_1 was detected in all of the contaminated samples at the highest concentration as compared with other aflatoxins. While nine out of 80 (11.1%) and 11 out of 80 (13.7%) of the samples contained AFB_2 and AFG_1 respectively. Only in two samples AFG_2 was detected.

The results of one way ANOVA (Table 3) showed that the occurrence of aflatoxins contamination in red pepper samples was significantly more than other spice samples ($p \leq 0.05$). However, there was no significant difference between black pepper, cinnamon and turmeric samples.

Table 2. Total and individual aflatoxins in four types of pepper samples analysed with HPLC-FD.

Type of sample	Number of samples	Number of positive samples (%)	Aflatoxins concentration in positive samples (ng/g)				
			Total AFs	AFB_1	AFB_2	AFG_1	AFG_2
Red pepper	20	11 (55)	1.73-24.60	1.73- 17.99	0.66-4.16	1.47-2.45	nd-0.52
Black pepper	20	8 (40)	2.11-7.01	2.11-6.64	0.32-1.15	1.12-1.34	Nd
Cinnamon	20	6 (30)	0.85-5.04	0.85-4.53	0.50-0.52	nd-0.19	nd-0.41
Turmeric	20	7 (35)	1.48-5.68	1.48-4.33	0.56-0.57	1.27-2.07	Nd
Total	80	32 (40)	0.85-24.06	0.85-17.99	0.32-4.16	0.19-2.45	0.41-0.52

AFs: Total aflatoxins, nd: Not detected

Table 3. Analysis of variance for total aflatoxins (B_1, B_2, G_1 and G_2) concentration in 80 analyzed spices sample (red pepper, black pepper, turmeric and cinnamon).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	4	316.1	105.35	8.79	0.00
Error	236	2827.9	11.98		

DF: Degree of freedom, Adj: SS adjusted sums of squares, Adj: MS adjusted mean squares

The maximum acceptable level of aflatoxins in spices is not established in Iran, While Commission Regulation [18] sets limits of 5ng/g for AFB1 and 10 ng/g for total AFs in Capsicum spp. (chillies, chilli powder, cayenne and paprika), Piper spp. (White and black pepper) Myristica fragrans (nutmeg), Zingiber officinale (ginger), Curcuma longa (turmeric) as well as mixtures containing one or more of any of these individual spices.

In the current study only two samples (red pepper) were contaminated with total AFs higher than the maximum permitted by the European Union. Five red pepper (25%) and 1 black pepper (5%) samples showed AFB1 at the level of higher than the permitted value (5ng/g). While AFB1 in all turmeric and cinnamon samples was lower than 5ng/g.

Concentration levels of the AFs in the current study were much lower than those reported by Jeswal and Kumar [19], who analyzed different types of spices produced in India and reported that aflatoxins were detected in 32 out of 42 black pepper (mean: 185.0±22.0ng/g), 24 out of 35 turmeric samples (mean: 163.8±25.7ng/g) and 47 out of 55 samples (mean: 219.6±21.3ng/g). In Turkey also, 5.0–18.2% of samples of different pepper types contained 1.1–97.5ng g^{-1} total AFs [10]. In Turkey, high levels of AFB1 contamination (up to 40.9lg/kg) were reported in red pepper powder [20]. In Kenya, 34 out of 46 samples (73.9%) were positive for AFs ranged from 2 to 99.6 ng/g (mean=15.1ng/g) [21]. In a related study, four samples out of six chilli powders prepared in Qatar, were contaminated with AFs in the range of 5.60–69.28 ng/g [22].

In contrast with these results, some researchers reported lower levels of aflatoxins in spices. Al-juraifani showed 31 out of 50 cinnamon samples were found to be contaminated with AFB1; however none exceed 4.67µg/kg. The author believes that the presence of essential oils with anti-mycotic effects, may inhibit the production of AFs or reduce fungal infestation and/or subsequent AFs production [23]. In a related study conducted in Turkey [24], aflatoxin was detected in 6 (75%) of 8 cinnamon and in 6 (75%) of 8 black pepper ranged from 0.5 to 1 ng/g. In Malaysia, 70 out of 126 (55.5%) samples were contaminated with total aflatoxins, although only low levels of aflatoxins were found ranging from 0.1 to 4.9ng g_1 [25].

The results of the current study showed that spices may be contaminated by aflatoxins. Therefore, more study is required to determine the Afs content of different type of spices.

CONCLUSION

A survey of the four different types of spices from Iran was performed for determining the aflatoxins (B1, B2, G1 and G2) contents. The results demonstrated that imported spices to Iran may be contaminated with aflatoxins. The highest concentration of total aflatoxins was detected in a red pepper. Red pepper samples were significantly more contaminated than other spices. AFs contamination in spices could be a serious problem even at low levels. Therefore, imported shipments must be controlled seriously by regulating bodies to ensure that the consignments are free of aflatoxins.

ETHICAL ISSUES

Ethical issues have been completely observed by the author.

CONFLICT OF INTEREST

The author declares that there are no conflicts of interest.

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