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HPLC Assay for Aflatoxins in Dried Red Peppers and Feedstuffs in Turkey

Türkiye'de Kurutulmuş Kırmızı Biberlerde ve Yemlerde HPLC ile Aflatoksin Aranması

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Abstract

The purpose of this study was to investigate aflatoxins (AFs) B_1, B_2, G_1 and G_2 in contaminated dried red peppers and feedstuffs consumed in Turkey. The AFs were detected using high performance liquid chromatography (HPLC) and the results higher than 1 ppb were further confirmed by thin layer chromatography (TLC). In HPLC, the detection limit of B_1 and G_1 was 0.075 ng and for B_2 and G_2 it was 0.060 ng. In TLC, the detection limit of AFs was 1 ng. A total of 26 commercially available dried red pepper and 53 feedstuff specimens were analyzed. In Turkey for spices the allowed upper B_1 level is 5 ppb and for all foods total AF level is 10 ppb. In dried red peppers, the B_1 level higher than 5 ppb

was 42.3% and the total AF level greater than 10 ppb was 38.4%. In Turkey for mixed feedstuffs the allowed upper total AF level is 50 ppb and in 7.5% of the feedstuffs the total level was higher than 50 ppb.

Key Words: Aflatoxin; feedstuff; dried red pepper; HPLC.

Introduction

Aflatoxins (AFs) are highly toxic, carcinogenic compounds that are of major concern as a toxic contaminant in foods, feedstuffs, herbs and spices. It is believed that the sun-drying process of spices presents the greatest potential for AF contamination. The AFs are produced on agricultural commodities in the field under stress condition or when high moisture and warm temperature exist in storage (Holcomb *et al.*, 1992). "Turkey-X disease" was the outbreak first in the UK and these compounds were discovered in the 1960s (Macdonald and Castle, 1996). AF B_1, B_2, G_1 and G_2 are acutely carcinogenic metabolites of the *Aspergillus flavus* and *Aspergillus parasiticus* (Scott, 1978). The International Agency for Research on Cancer has placed B_1 on their list of probable human carcinogen (WHO, 1987). Toxicologically, AF may be regarded as a quadruple threat: it can function as a potent toxin, a carcinogen, a teratogen, and a mutagen (Ciegler, 1975). Epidemiological studies have shown a correlation between AF exposure and primary hepatocellular carcinoma incidence in several Third World Countries and people in these regions suffer a high incidence of liver cancer. After metabolic activation of B_1, it is converted to the B_1 8,9-epoxide, the ultimate carcinogen (Garner *et al.*, 1993; Harrison *et al.*, 1993). B_1 single dose (per os) is acutely toxic in all species studied, for example with an LD_{50} ranging from 0.3 $mg.kg^{-1}$ for the duckling to 9.0 $mg.kg^{-1}$ for the mouse (Ciegler, 1975).

It was reported that in some samples of spices, especially chilli powder, over 20 ppb total AFs were found and more than 50% of the spice samples were contaminated at levels greater than 1 ppb (Garner *et al.*, 1993). In 20% of the 10 red pepper samples collected in Tokyo, the average B_1, B_2, G_1 amounts detected were 5.9, 0.2 and 0.2 ppb, respectively (Tabata *et al.*, 1993). In a study of 157 retail samples in the UK which included curry powders, pepper,

cayenne pepper, chilli, paprika, ginger, cinnamon and coriander it was found that nearly 95% of samples contained below 10 ppb total AFs and only nine samples had higher levels with a maximum concentration of 48 ppb in one chilli powder sample (Macdonald and Castle, 1996). About 8700 ppb AF was found in mouldy corn in the USA (Cavalheiro, 1983). The Moroccan poultry feeds were surveyed from 1989 to 1991 and the levels of AFB_1 contamination ranged from 20 to 200 ppb except for four samples which contained higher levels of B_1 as 2000-5625 ppb (Kichou and Walser, 1993). In Cyprus, AF contamination in peanut butter was found as 56.7% and the highest level of AFB_1 was 700 ppb (Ioannou-Kakouri *et al.*, 1999). Various methods have been used for the determination of AFs based on HPLC (Dunne *et al.*, 1993; Garner *et al.*, 1993; Holcomb *et al.*, 1992; Howell and Taylor, 1981; Paulsch *et al.*, 1988), gas chromatography-mass spectrophotometry (GC/MS) (Holcomb *et al.*, 1992), TLC (Ehrlich and Lee, 1984; Majerus and Zakaria, 1992; Nesheim *et al.*, 1999), HPTLC (Ioannou-Kakouri *et al.*, 1999), enzyme-linked immunosorbant assay (ELISA) (Adachi *et al.*, 1991; Kichou and Walser, 1993), immunoaffinity (Trucksess *et al.*, 1990), mini column chromatography (Arim *et al.*, 1999).

In many countries the upper limits for the AF levels as ppb in foods is as; 20 ppb in the USA, 15 ppb in Canada, 10 ppb in Japan, France and UK, 5 ppb in Australia (Tabata *et al.*, 1993). In the USA, concentrations of up to 20 ppb are permitted in feedstuffs (Cavalheiro, 1983). Italy established maximum tolerated levels in spices as 20 ppb (FAO, 1997). The AF, T-2 toxin and fumonisin B_1, B_2, are all mycotoxins, yet in Turkey the guideline appear only for AF (Omurtag *et al.*, 1998; Omurtag, 2001; Omurtag and Yazıcıoğlu, 2000; Omurtag and Yazıcıoğlu, 2001). The upper limits are as 5 ppb AFB_1 in spices/all foods and 10 ppb total AF in all foods (T.C. Resmi Gazete, 1997). The upper limit in mixed feedstuffs is 50 ppb for total AF (T.C. Resmi Gazete, 1990). The AF problem in Turkey was encountered first in 1967 with hazelnut and later in 1972 and 1974 with pistachio and dried fig and in 1994 with red peppers (Çamlıbel, 1995). The purpose of this study was to determine by HPLC the extend of AF in dried red peppers and feedstuffs consumed in Turkey.

Materials and Methods

Materials: Total number of samples was 79 of which 26 were dried red peppers and 53 were feedstuffs. All samples were collected from various cities of Turkey. The collected dried red pepper samples were powdered. Each feedstuff sample was blended and finely ground using an Erweka mill (AR-400 TG2S-57222) with Retsch Test Sieve (Serial number 703429, mesh 20). Using this sieve mixture a 250 g subsample was obtained (stored – 20° C) and finally a 50 g was taken from it. The sample preparation, HPLC and TLC procedures were reported elsewhere (Omurtag *et al.*, 1998).

Chemicals: AF B_1, B_2, G_1 and G_2 (Sigma A-6636, 9887, 0138 and 0263 respectively) were used as standards. B_1, B_2, G_1 and G_2 were dissolved in benzene-acetonitrile (98:2 v/v) so as to contain 20 ng of AF.mL^{-1} for HPLC and 10 µg of AF.mL^{-1} for TLC. The SPE (Solid-phase extraction) column was a silica cartridge (JT Baker, 7086-07) and other chemicals were of HPLC-grade Merck products. Bidistilled water was used.

Sample preparation: 50 g of sample was mixed in a Waring Blender with 200 mL of methanol-water (85:15, v/v) for 3 min. After filtration, 40 mL of 10% sodium chloride solution was added to 40 mL of filtrate and this solution was extracted with 2×25 mL of hexane. The hexane extracts were discarded and the defatted solution was extracted with 2×25 mL of chloroform and organic phase evaporated. The SPE column was conditioned with 3-mL hexane followed by 3-mL diethylether, and 3 mL methylene chloride. AFs were eluted with 6-mL chloroform-acetone (9:1 v/v). The eluates were collected into 10mL screw cap vials and evaporated to dryness under a stream of nitrogen.

Procedure for the HPLC: A volume of 0.2 mL of hexane was added to the residue, vortexed and 0.2 mL of 80 % trifluoroacetic acid (TFA) was added and revortexed. 2.3 mL of CH_3CN- 2.5% acetic acid (10:90, v/v) was added and mixed thoroughly; then the lower (aqueous) layer was filtered through a Millex HV filter (0.45 µm). 25 µL and/or 50µL of this sample was injected into the HPLC column. Samples were prepared and analysed in triplicate. The areas of the peaks were used for quantification.

Apparatus: The HPLC (Waters Corp., U.S.A.) was used with a Model 510 Pump and a M 420-AC Fluorescence Detector (Waters). Fluorescence was recorded at excitation (λ_{Ex}) and emission (λ_{Em}) wavelengths of 365 and 425 nm, respectively. The injector was a Rheodyne 7725 sample injector (100 µL accessory). A reverse phase, µBondapak C_{18} (10µm)(Waters), 3.9×300 mm I.D. analytical column was protected by a C_{18} guard column. The data station was an Unicam 4880 Chromatography Data Handling System.

Operating conditions: The mobile phase was methanol-acetonitrile-5.0 % acetic acid (14:14:72, v/v/v); flow rate was 1.7 $mL.min^{-1}$; chart speed was 0.25 $cm.min^{-1}$ and column temperature was ambient. The mobile phase was filtered through a Millipore HV (0.45 µm) membrane filter and sonicated. An AF (B_1+B_2+G_1+G_2) mixed standard (0.02 $ng.\mu L^{-1}$) was derivatisated with TFA. An aliquot (25 and/or 50 µL) injected to the HPLC column and linear regression lines for B_1, B_2, G_1 and G_2 were found to be y=2.6325x-33.3 (r=0.9984) y=3.3825x+21 (r=0.9998) y=3.0375x-24 (r=0.9984) and y=3.57x-18.7 (r=0.9998), respectively. The retention times of B_1, B_2, G_1 and G_2 were 5.65 min, 10.90 min, 4.31 min, and 7.76 min, respectively.

Results and Discussion

All the specimens were analyzed by HPLC and TLC was used to verify the positive results higher than 1 ppb. The B_1 detected in dried red peppers and feedstuffs were 65% and 17%, respectively and the complete list of the specimen results with the mean values of AFs are given in the Table 1. HPLC chromatograms of AFs standards and one dried red pepper sample is given in Figure 1. The highest level of B_1 in dried red peppers and feedstuffs were 56 ppb and 59.9 ppb, respectively. In dried red peppers the B_1 level higher than 5 ppb was 42.3% and the total AFs level higher than 10 ppb was 38.4% of the total specimens. In Turkey for mixed feedstuffs the allowed upper total AF level is 50 ppb and in 7.5% of the feedstuffs this value was exceeded. In 1997, 32 dried red pepper samples were collected in Turkey, the detected highest B_1 level was 109.7 ppb and about 50% of the samples B_1 level

was higher than 5 ppb (Omurtag *et al.*, 1998). Multi-mycotoxin residue analysis by TLC with fluorodensitometric procedures was made in totally 302 feeds and feedstuffs obtained from 25 feed factories in Turkey and incidences of AFB_1, G_1, G_2 were 11.2%, 1.98% and 0.3%, respectively (Özkazanç *et al.*, 1992).

In this study, the detection limits of HPLC were twice the signal-to-noise ratio, 0.075 ng for B_1 and G_1 and 0.06 ng for B_2 and G_2. The detection limits of TLC were 1 ng for AFs. The HPLC recoveries of B_1, B_2, G_1 and G_2 from 4 ppb spiked AF-free dried red peppers and feedstuffs were 91±1.79%, 71±3.16%, 97±2.28%, 67±2.37% and 82±6.24%, 74±4.09%, 82±4.42% and 65±5.03%, respectively. The recovery results were expressed as the mean of five different manipulations including the extraction from the same AF-free specimen.

For protection of food supplies from mycotoxin contamination, special care must be given from the earliest point of production until consumption. As a consequence, the exposure to AFs will not only put some additional health risks to the consumers, but also arises to be an economic problem for the producers.

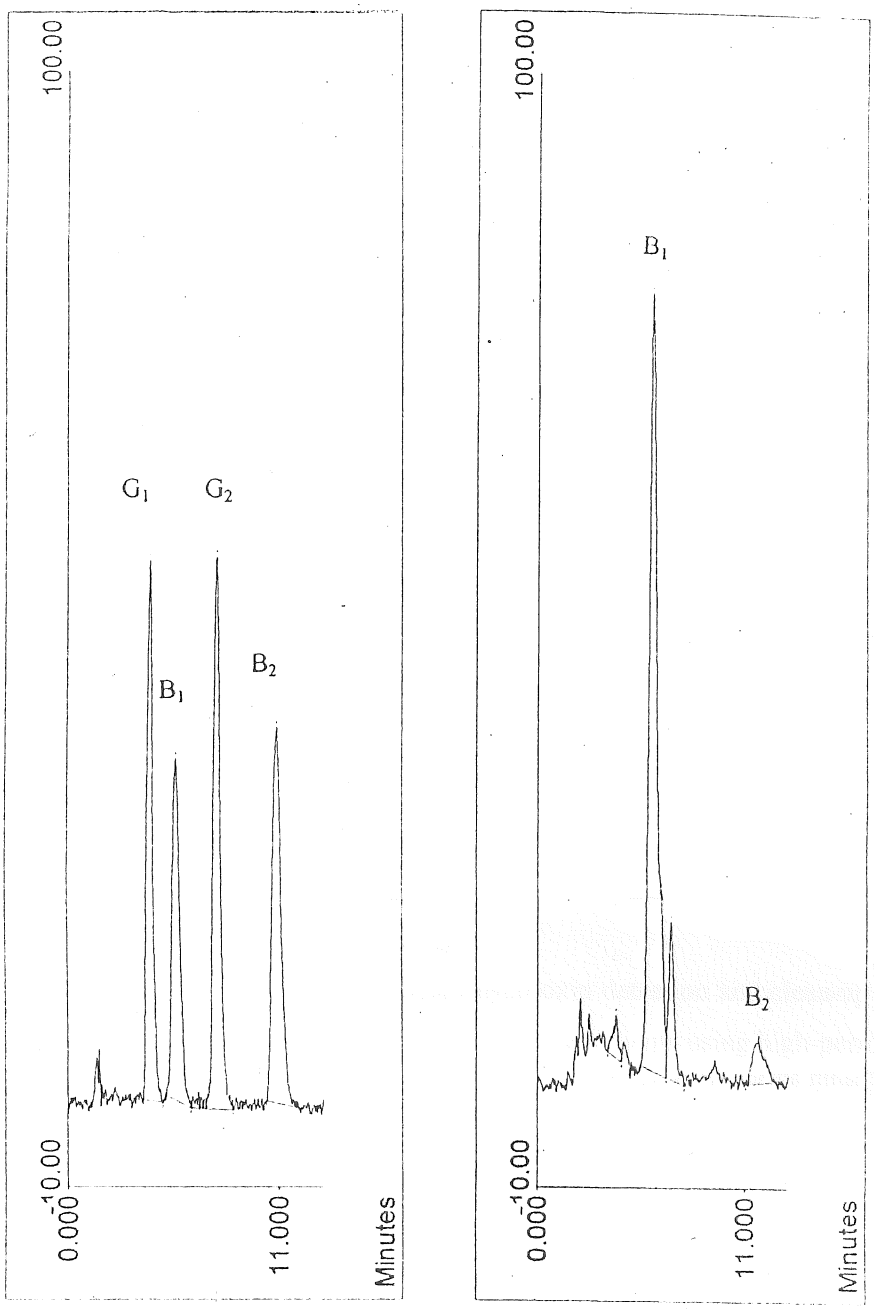

Figure 1. HPLC chromatograms: a) Aflatoxin standards (AF G_1 , B_1 , G_2 , B_2 ; 400 pg), b) Naturally contaminated dried red pepper extract (AF B_1 ;38.2 $\mu g.kg^{-1}$, B_2 ;2.3 $\mu g.kg^{-1}$)

Table 1. HPLC results of aflatoxins in red pepper and feedstuff specimens. Origin of specimens in Turkey are; UO: Unknown Origin, SE: Southeast, NW: Northwest, S: South, ND: Not Detected. (Results are in ppb).

	AFs	Red pepper			Feedstuff
		SE	S	UO	NW
AFs Positive / Total	B_1	5/8	2/5	10/13	9/53
	B_2	2/8	0/5	3/13	3/53
	G_1	0/8	0/5	1/13	1/53
	G_2	0/8	0/5	0/13	0/53
Range	B_1	3.1-56	2.7-10.3	0.6-38.2	5.1-59.9
	B_2	1.3-1.6	ND	0.4-2.3	15.3-48.7
	G_1	ND	ND	0.6	29.3
	G_2	ND	ND	ND	ND
Means of AFs positive	B_1	22.94	6.50	16.94	28.81
	B_2	1.45	-	1.47	30.66
	G_1	-	-	0.6	29.30
	G_2	-	-	-	-

Özet

Bu çalışmanın amacı, Türkiye'de tüketilen kurutulmuş kırmızı biber ve yemlerde aflatoksin (AF) B_1, B_2, G_1 and G_2 'yi araştırmaktır. AF analizinde yüksek basınçlı sıvı kromatografisi (HPLC) kullanıldı ve 1 ppb den yüksek sonuçlar ince tabaka kromatografisi (İTK) ile doğrulandı. HPLC'de B_1 ve G_1'in deteksiyon sınırı 0.075 ng ve B_2 ve G_2'nin deteksiyon sınırı 0.060 ng'dır. İTK da, AF lerin deteksiyon sınırı 1 ng dır. Piyasadan temin edilen toplam 26 kurutulmuş kırmızı biber ve 53 yem örneği analiz edildi. Türkiye'de baharatlarda AFB_1 için kabul edilen sınır değer 5 ppb ve tüm gıdalar için toplam AF düzeyi 10 ppb'dir. Bu çalışmada, AFB_1 'i 5 ppb üzerinde içeren kurutulmuş kırmızı biber örneklerinin miktarı %42.3 ve toplam AF miktarını 10 ppb üzerinde içerenlerin miktarı %38.4 olarak

saptandı. Türkiye'de karma yemlerde toplam AF için kabul edilen sınır değer 50 ppb olup, örneklerin %7.5' u bu sınır değeri aşmıştır.

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