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AFLATOXIN ANALYSIS IN DRIED RED PEPPER SAMPLES BY TLC AND HPLC

KURUTULMUŞ KIRMIZI BİBERLERDE İNCE TABAKA KROMATOGRAFİSİ VE YÜKSEK BASINÇLI SIVI KROMATOGRAFİSİ İLE AFLATOKSİN ANALİZİ

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In this study, aflatoxins (AFLs) B_1, B_2, G_1 and G_2 were determined in dried red pepper samples provided from markets, street bazaars and spice-sellers in İstanbul, using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The number of samples used in the analysis by TLC, HPLC and both methods were 27, 5 and 6 respectively. The results from TLC and HPLC studies were compared. In Turkey the recommended limits for $AFLB_1$ ise 5 ppb and total AFLs level is 20 ppb. In this research, aflatoxin B_1 levels were higher than 5 ppb in about 50% of the samples and total AFL levels were ≥ 20 ppb in about 40% of samples.

Bu çalışmada, İstanbul'daki marketler, baharatçılar ve semt pazarlarından sağlanan kurutulmuş kırmızı biber örneklerinde aflatoksin B_1, B_2, G_1 ve G_2 ince tabaka kromatografisi (İTK) ve yüksek basınçlı sıvı kromatografisi (YBSK) kullanılarak tayin edildi. Toplam 27 örnek İTK, 5 örnek YBSK, 6 örnek ise İTK ve YBSK yöntemi ile analiz edildi. İTK ve YBSK analizi sonuçları karşılaştırıldı. Türkiyede $AFLB_1$ için kabul edilen sınır değer 5 ppb ve toplam AFL için sınır değer 20 ppb dir. Bu çalışmada $AFLB_1$'i 5 ppb üzerinde içeren örneklerin miktarı yaklaşık %50, toplam AFL miktarını 20 ppb ve üzerinde içerenlerin miktarı yaklaşık %40'dır.

Keywords: *Aflatoxin; HPLC; TLC; Red pepper; Spices*

Anahtar kelimeler: *Aflatoksin; YBSK; İTK; Kırmızı biber; Baharat*

Introduction

Aflatoxins (AFLs) were first implicated in poultry diseases ('Turkey-X disease') in the UK in the 1960s, subsequently the fluorescent compounds in feed components, e.g. peanut meal as products of the genus *Aspergillus*, were discovered (1,2). The four naturally occuring AFLs B_1, B_2, G_1 and G_2 (Fig1) are acutely carcinogenic metabolites of the moulds *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare (2,3,4). The occurrence of aflatoxin-producing strains of *A.flavus* in herbs and spices has been reported (5). Spices are often produced in countries with tropical climates which have extreme ranges of rainfall, temperature and humidity and it is believed that the sun drying process presents the greatest potential for contamination. It is also possible for preharvest contamination to occur in tropical environments. Typically spices are laid out on the ground to dry in the open air where the climatic conditions of high temperature and humidity are ideal for growth of aspergilli and production of AFLs(5). A tolerance level of $AFLB_1$, in groundnut, cotton seed, maize and products derived from the processing thereof is 20 ppb in the European Communities Regulations 1991 (6). The FDA established enforcement levels of 0.5 ppb in milk and 20 ppb for other products. In the United States, concentrations of up to 20 ppb are permitted in animal feeds, although there may be a concern for secondary exposure to AFLs from the use of animal products (7). The maximal AFL levels accepted by the Ministry of Agriculture in Turkey(8) are as follows: Foods and agricultural products for $AFLB_1$ 5 ppb, milk and dairy products 0.5 ppb, total AFLs B_1, B_2, G_1 and G_2 20 ppb, feedstuff 50 ppb. The toxic limit for total AFLs given by WHO is above 30 ppb (9).

Epidemiological studies have shown a correlation between AFL exposure and primary hepatocellular carcinoma incidence in several Third World Countries (10). Very high levels of AFLs occur in foods in certain regions of Africa and Asia, and people in these regions suffer a high incedence of liver cancer. $AFLB_1$ requires metabolic activation by the cytochrome P-450-dependent mixed-function oxidase to be converted to the reactive 2,3-epoxide, the ultimate carcinogen. The AFLs, e.g., $AFLB_1$ are genotoxic carcinogens and the reactive

metabolites react with DNA. The major adduct formed with DNA in intracellular reactions is formed from the 2-position of $AFLB_1$ and the N-7 position of guanine in DNA(7). One possible mode of action of such adducts in cancer development has been indicated in contemporary studies of the *p*53 tumor-suppressor gene. Mutational hotspots have been detected in the gene from human livers obtained from South Africa where AFL exposure is known to be high (10). Toxicologically, AFL may be regarded as a quadruple threat: it can function as a potent toxin, a carcinogen, a teratogen, and a mutagen(1). $AFLB_1$ is acutely toxic in all species studied with an LD_{50} ranging from 0.5 mg/kg for the duckling to 60 mg/kg for the mouse (11). There is, of course, no established toxic dose for humans; but strong circumstantial evidence from Southeast Asia, India, and Africa, plus a suspect case in Germany, indicates that AFLs have been involved in human deaths, particularly among children.

The AFL problem was first encountered in Turkey in June 1967, when hazelnuts exported to Canada were returned to Turkey with a report from their Department of Health and Welfare asserting that they contained AFLs. Turkey was again confronted with the same problem in the summer of 1971 (12) and also with the red peppers in 1995 (13). In this study, AFLs were determined in dried red pepper samples using TLC and HPLC.

Materials and Methods

Analysis were carried out on the dried red pepper samples provided from markets, street bazaars and spice-sellers in Istanbul and on the samples from the known origin of Turkey (Urfa, Kahramanmaraş, Kayseri, Silifke) and India. Thin layer chromatographic (14) and high performance liquid chromatographic (15) methods were used for the determination of AFLs B_1, B_2, G_1 and G_2 in dried red pepper samples.

Chemicals: $AFLB_1$(Sigma A-6636), $AFLB_2$ (Sigma A-9887), $AFLG_1$ (Sigma A-0138), $AFLG_2$ (Sigma A-0263) were used as standards. AFLsB_1, B_2, G_1 and G_2 were dissolved in benzene/acetonitrile (98:2) so as to contain 20 ng of AFL/ml for HPLC and 10 µg of AFL/ml for TLC. SPE (Solid-phase extraction) column: J.T.Baker Part No 7086-07 and other chemicals were HPLC grade Merck products. Bidistilled water was used.

Apparatus: High Performance Liquid Chromatograph (HPLC, Waters Corp., U.S.A.). The attachments: Pump M 510 solvent delivery system, Detection: M 420-AC Fluorenscence Detector at 365 nm excitation, 425 nm emission, Rheodyne 7725 sample injector (100 µl accessory), Data Station: Unicam 4880 Chromatography Data Handling System, Column: µBondapak C_{18} 125 A^0 10 µ (3.9x300 mm) Waters.

Sample Preparation: 50 g dried red pepper samples were placed in a Waring blender and blended with 200 ml of methanol/water (85:15) for 30 min. After filtration 40 ml of 10% sodium chloride solution was added to 40 ml of filtrate and this solution was extracted with 2x25 ml hexane. Hexane extract was discarded and defatted solution was extracted with 2x25 ml chloroform and evaporated. SPE column was conditioned with 3 ml hexane followed by 3 ml methylene chloride. Sample residue was dissolved in 3 ml methylene chloride and transferred onto a SPE column and sample was aspirated. The column was washed with 3 ml hexane followed by 3 ml diethylether, followed by 3 ml methylene chloride. AFLs were eluted with 6 ml chloroform/acetone (9:1). The eluates were collected into a 10-15 ml screw cap vial and evaporated to dryness under the stream of nitrogen (Sample extract A).

Procedure for TLC: Sample extract (A) was dissolved with 0.2 ml chloroform/methanol (1:1) for TLC and 50 µl of sample extract were spotted 110 mm from the bottom of the aluminium silicagel foil (Merck 5553). The chromatogram was developed in the first direction with diethylether. The diethylether was evaporated and the plate was examined under an ultraviolet (UV) lamp. The top portion (about 55 mm) of the plate containing the interfering compounds was cut off. The plate was turned 180°, developed in chloroform/acetone (90:10), dried and examined under an UV lamp at 366 nm. To confirm the samples for AFLsB_1, B_2, G_1 and G_2 which give blue and green fluorescence colours, respectively, 25% H_2SO_4 was sprayed. Afterwards, from these samples that turned out to light yellow, the spots that contain AFLs emerged.

Procedure for HPLC : 0.2 ml of hexane was added to sample extract (A), vortexed for 10 seconds and after adding 0.2 ml of 80% trifluoroacetic acid (TFA) vortexed for 30 seconds. After 1 min. 2.3 ml of CH_3CN/2.5% acetic acid (10:90) was added and mixed well, the lower (aquaeous) layer was filtered through a Millex HV (0.45 µm). HPLC quantitation: The samples were injected into HPLC system under the same conditions used for preparing calibration graphs and analysed triplicate. Injection: 50 µl, flow rate: 1.7 ml/min., chart speed: 0.25 cm/min., mobile phase: CH_3OH/CH_3CN/5.0% acetic acid (14:14:72). The mobile phase was filtered through a Millipore HV

Table 1. Results of TLC and HPLC methods for aflatoxins in red pepper samples. (u.o., unknown-origin, n.d., not detected)

Red pepper	origin	Contamination level (ppb) by TLC				Contamination level (ppb) by HPLC			
		$AFLB_1$	$AFLB_2$	$AFLG_1$	$AFLG_2$	$AFLB_1$	$AFLB_2$	$AFLG_1$	$AFLG_2$
Markets (marked)	u.-o.	n.d.	n.d.	n.d.	n.d.				
	u.-o.	n.d.	n.d.	n.d.	n.d.				
	u.-o.	20	n.d.	n.d.	n.d.				
	u.-o.	n.d.	n.d.	n.d.	n.d.	1.4	n.d.	n.d.	n.d.
	u.-o.	40	n.d.	n.d.	n.d.				
	u.-o.	2.5	n.d.	n.d.	n.d.				
Spice-seller (unmarked)	u.-o.	n.d.	n.d.	n.d.	n.d.				
	u.-o.	100	20	20	n.d.	109.7	32	18.8	n.d.
	u.-o.	80	40	10	n.d.	71.9	33.1	10.4	n.d.
	Urfa	8	n.d.	n.d.	n.d.				
	Urfa	14	3.2	n.d.	n.d.				
	Urfa	16	n.d.	n.d.	n.d.				
	Urfa	1.7	n.d.	n.d.	n.d.	1.2	n.d.	n.d.	n.d.
	Urfa	n.d.	n.d.	n.d.	n.d.				
	Urfa					3.2	n.d.	n.d.	n.d.
	Urfa					n.d.	n.d.	n.d.	n.d.
	Urfa					n.d.	n.d.	n.d.	n.d.
	K.Maraş					n.d.	n.d.	n.d.	n.d.
	K.Maraş	40	n.d.	n.d.	n.d.				
	K.Maraş	80	4	4	n.d.				
	India	20	4	n.d.	n.d.				
Street bazaar (unmarked)	u.-o.	n.d.	n.d.	n.d.	n.d.				
	u.-o.	40	4	n.d.	2				
	u.-o.	n.d.	n.d.	n.d.	n.d.				
	u.-o.	20	n.d.	n.d.	n.d.	17.7	7.8	4.1	n.d.
	u.-o.	6	n.d.	n.d.	n.d.				
	u.-o.	60	3.4	0.9	n.d.				
	u.-o.	13	n.d.	n.d.	n.d.				
	u.-o.	13	n.d.	n.d.	n.d.				
	u.-o.					2.4	n.d.	n.d.	n.d.
	Kayseri	n.d.	n.d.	n.d.	n.d.				
	Silifke	n.d.	n.d.	n.d.	n.d.				

(0.45µm) membrane filter and degassed by immersion in an ultrasonic bath.

Results and Discussion

AFLsB$_1$, B$_2$, G$_1$ and G$_2$ can be readily separated and detected using either normal- or reversed-phase TLC or HPLC techniques. The samples were initially screened semi-quantitatively by TLC; this provides a rapid means of analysing a large number of samples. It was easy, fast and inexpensive. However, HPLC using fluorescence detection is now the method of choice for determining AFLs and is also growing in popularity for their identification(2). Table 1 shows results of TLC and HPLC methods for AFLs in samples.

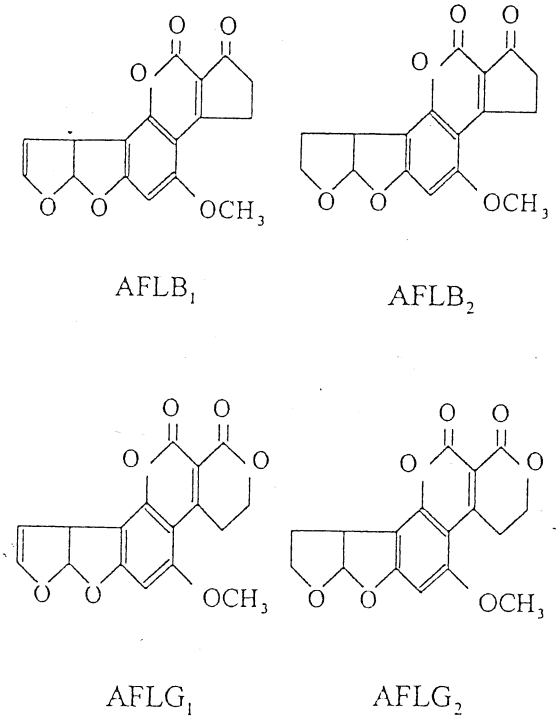
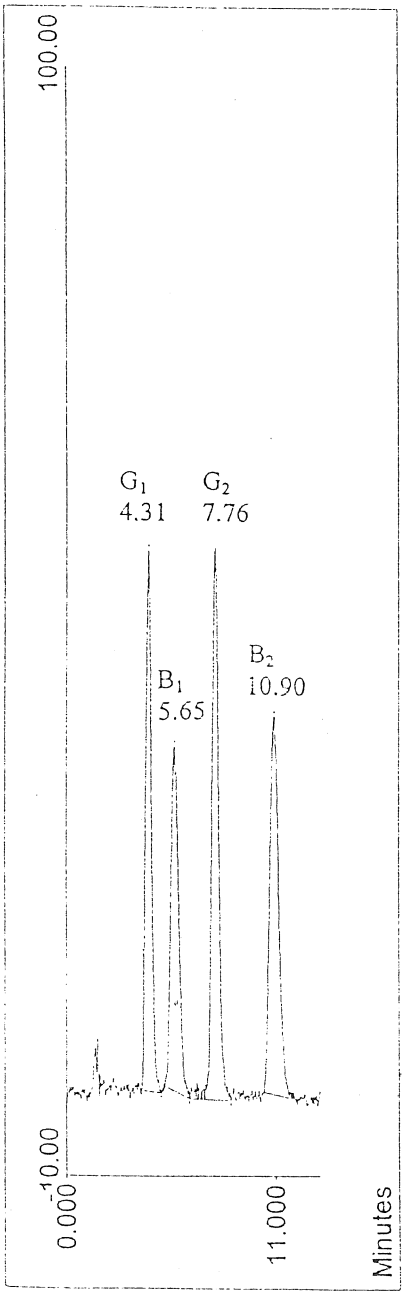


Fig.1. Structures of major aflatoxins

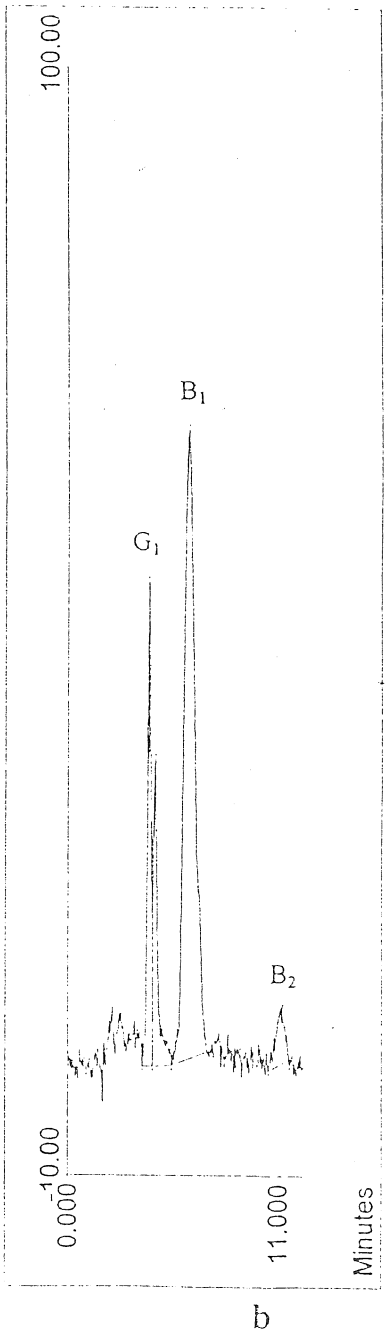
In this study, the detection limits of HPLC were 0.075 ng for AFLs B$_1$ and G$_1$ and 0.06 ng for B$_2$ and G$_2$. The detection limits of TLC were 1 ng for AFLs. Dried red pepper samples were spiked at 4 ppb level for each of the AFLs B$_1$, B$_2$, G$_1$ and G$_2$, and percentage recoveries of AFLs were found as 91, 71, 97 and 67 respectively. Fig. 2 shows HPLC chromatograms of AFLs standards and a sample. In a study, of the spices analysed, chili powder and ground ginger were the most likely to be contaminated; some samples contained over 20 ppb total AFLs. More than 50% of the spice samples were found to be contaminated at levels greater than 1 ppb (16). In another study, of 157 retail samples in UK which included curry powders,



a

Fig.2. a) HPLC chromatogram of aflatoxins standards

pepper, cayenne pepper, chilli, paprika, ginger, cinnamon and coriander, nearly 95% of samples contained below 10 ppb total AFLs and only nine samples had higher levels. The highest pepper, cayenne pepper, chili, paprika, ginger, cinnamon and coriander, nearly 95% of samples concentration in a retail sampl e was 48 ppb



b

Fig.2. b) HPLC chromatogram of a red pepper extract

in a chili powder (5). In a study on AFLs in various foods, spices and feedstuffs by HPLC in Turkey, the amounts of $AFLB_1$ of two red pepper samples have been found as 1.048, 1.273 ppb, respectively (4). In our study, the highest $AFLB_1$ level was found as 109.7 ppb in an unmarked sample collected from a spice-seller. For some of the unmarked pepper samples it was difficult to find an origin and most probably they were blended products.

The results of our TLC and HPLC studies were found as comparable. In Turkey the recommended limits for $AFLB_1$ is 5 ppb and total AFLs level is 20 ppb. In about 50% of samples $AFLB_1$ levels were higher than 5 ppb and total AFL levels were higher than 20 ppb in about 40% of samples. In our country, the use of dried red pepper in some special foods and the snack food business has increased. Hence, exposure to aflatoxins from these sources will also be increasing, causing additional health risk for the consumer. AFL contamination is not only of concern for public health, but also stands to be an economic problem. As a result of our study, we believe that AFL analysis has to be done and declared to the consumer for food products such as dried red pepper samples that can have a high possibility of AFL contamination in Turkey. It would be very helpful to the consumer if one can see a notice on the label about the result of the AFL analysis.

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