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Short Communication

Determination of Aflatoxin Levels in Maize Grain by High Performance Liquid Chromatography Using an Immunoaffinity Column Cleanup [1]

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[1] *The results of this study were presented in part as an abstract at the 3rd National Veterinary Pharmacology and Toxicology Congress from 29th September to 2nd October, 2010 in Kusadasi/Aydin, Turkey*

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Abstract

The aim of this study was to determine aflatoxin contamination levels in maize grain produced in Samsun Province, Turkey. Forty samples of maize grain intended for animal feed in Samsun Province were analyzed. The amounts of the aflatoxins, B_1, B_2, G_1 and G_2, were quantified with a liquid chromatography (HPLC-FLD) and post-column derivatization. Recoveries ranged between 89 and 98%. The detection limits were 0.007 and 0.02 ng/g and the corresponding quantification limits were 0.022 and 0.063 ng/g. As a result, it has been shown that the accurate and sensitive HPLC-FLD method was confirmed as being appropriate for the detection of AFs in maize grain, besides, the incidence of aflatoxins in maize was very low (2.5%).

Keywords: *Aflatoxin, Immunoaffinity column, High performance liquid chromatography, Maize*

Tane Mısır Örneklerinde Aflatoksin Düzeylerinin Yüksek Performanslı Likit Kromatografi-İmmunoafinite Kolon Yöntemi İle Belirlenmesi

Özet

Bu çalışmada Samsun ilinde üretilen tane mısırlarda aflatoksin kontaminasyonunun araştırılması amaçlandı. Bu amaçla, Samsun ilinde hayvan yemi olarak kullanıma sunulan 40 adet tane mısır örneği analiz edildi. Aflatoksin B_1, B_2, G_1 ve G_2 düzeyleri kolon sonrası türevlendirmenin yapıldığı likit kromatografi (HPLC-FLD) sistemi ile ölçüldü. Aflatoksinlerin geri kazanım oranları %89-98, belirleme alt limitleri (LODs) 0.007-0.02 ng/g ve hesaplama alt limitleri (LOQs) 0.022-0.063 ng/g aralığında belirlendi. Sonuç olarak, tane mısırlarda aflatoksinlerin tespitine yönelik olarak uygulanan immunoafinite kolon temizleme ve kolon sonrası brom türevlendirmesini içeren doğruluk ve hassasiyeti yüksek HPLC-FLD metodunun, aflatoksinlerin tespitinde uygun bir yöntem olarak kullanılabileceği, bunun yanında, Samsun ilinden elde edilen tane mısır örneklerinde aflatoksinle kontaminasyon oranının düşük düzeyde (%2.5) olduğu ortaya konulmuştur.

Anahtar sözcükler: *Aflatoksin, İmmunoafinite kolon, Yüksek performanslı likit kromatografi, Mısır*

INTRODUCTION

Aflatoxins (AFs) are toxic metabolites produced by a large number of *Aspergillus* species that include *Aspergillus flavus*, *A. parasiticus*, *A. nomius, A. pseudotamarii, A. parvisclerotigenus, A.bombycis, A. ochraceoroseus and A. rambellii.* Although 18 types of AFs have been identified, the well-known ones are AFB_1, AFB_2, AFG_1 and AFG_2 [1]. *Aspergillus* spp. grow in hot and humid environments, producing AFs during harvesting and storage that affect the quality and safety of food and feedstuffs [2]. The ingestion of AFB_1 and AFB_2-contaminated food or feed by female mammals leads to the excretion in milk of the partially detoxified hydroxylated analogues, aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2). In dairy cows, these contaminants can occur in commercial milk and be processed in cheese, and in many developing countries, the presence of AFM_1 in human milk can lead to ingestion by infants [3].

Maize is a major cereal crop for both livestock and human nutrition worldwide. In Turkey, maize is produced in large quantities in the Black Sea Region; in 2013, 271.808 tonnes of maize were produced in the Black Sea Region

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and Samsun Province was the top producer with 81.209 tonnes [4].

A reliable risk assessment of mycotoxin contamination for humans and animals relies on their identification and accurate quantification in food and feedstuffs [5]. Aflatoxins are low molecular mass polar compounds which possess UV absorption and fluorescence properties. For this reason, liquid separation techniques have predominated in their analysis, initially thin layer chromatography (TLC), but subsequently LC [3]. However, immunoaffinity column clean-up, a modern sample clean-up technique, has been applied to increase method specificity and sensitivity by the selective enrichment and isolation of the target aflatoxins [5,6].

In the present study, aflatoxin contamination levels in maize grain in Samsun Province, were determined to test whether the method has the appropriate level of sensitivity for the task and to survey the level of contamination of samples from various sources.

MATERIAL and METHODS

Collection of Maize Samples

Maize grain samples were obtained from feed sellers in Bafra, Carsamba, Terme and Samsun districts of Samsun Province. A total of 40 samples, with 10 from each district, were collected.

Extraction of Aflatoxins and Immunoaffinity Column Clean-up

The extraction and clean-up procedures were performed according to the AOAC method [7], with some modifications. Each sample (500 g) was ground to a fine powder with a laboratory mill (IKA®, Staufen, Germany). A 50 g sub-sample was taken and 4 g sodium chloride (Merck, Darmstadt, Germany) and 100 ml of ultrapure water (Millipore Simplicity®, Molsheim, France) were added. They were mixed vigorously for 1 min with a magnetic stirrer (WiseStir®, Daihan Scientific, Seul, South Korea). One hundred and fifty millilitres of methanol (Merck, Darmstadt, Germany) were added and the mixture was stirred vigorously for 2 min. The extract was filtered through a filter paper (Whatman® No.4) and 5 ml of clear filtrate (equivalent to 1 g of product) was transferred to a beaker and 15 ml of phosphate buffered saline (PBS) (Merck, Darmstadt, Germany) solution was added.

The Aflaprep® immunoaffinity column (R-Biopharm, Glasgow, Scotland) were at room temperature prior to conditioning. The column was loaded with 10 ml of PBS which was passed through the column at a speed of 2-3 ml/min under gravity. Twenty millilitres of diluted filtrate was then passed through the column at a flow rate of 3 ml/min under gravity. The column was then washed with two, 10 ml volumes of water at a flow rate of 5 ml/min and dried by applying a light vacuum for 10 s.

One millilitre of methanol was then passed through the column under gravity. Following that, 1 ml of ultrapure water was passed through the column under gravity. The remainder of the elution solvent in the column was collected by forcing pressurised air through the column after most of the eluent had passed through under gravity. The eluent was filtered through a disk filter unit (0.45 µm) prior to HPLC injection.

HPLC-FLD Analysis

The mobile phase consisted of methanol-water (45+55, v/v). One hundred and twenty milligrams of potassium bromide and 350 µl of 4 M nitric acid (Merck, Darmstadt, Germany) were added to each liter of the mobile phase. The flow rate was 1 ml/min isocratically. Post-column derivatization was carried out with bromine in the KobraCell® (R-Biopharm, Glasgow, Scotland) with an electrochemical reaction current of 100 µA. Fluorescence detection (RF-10AXL, Prominence LC 20A, Shimadzu, Kyoto, Japan) was performed at an excitation wavelength of 360 nm and emission wavelength of 430 nm. A reversed-phase C18 column (Inertsil® ODS-3V, 5 µm, 4.6 x 250 mm, GL Science, Tokyo, Japan) was used for separation. The column's oven temperature was set at 40°C and the run time was 30 min per analysis [7].

Calibrant solutions were prepared by diluting the total aflatoxin standard stock solution (Aflastandard®, 1.000 ng/ml, R-Biopharm, Glasgow, Scotland) with the mobile phase at concentrations of 0.0625, 0.125, 0.25, 0.625, 1.25, 1.875 and 2.5 ng/ml. They were injected into the HPLC at a volume of 100 µl. Recovery studies were carried out by spiking uncontaminated samples with three different levels (0.5, 1 and 2 ng/ml) of each aflatoxin.

RESULTS

The values of R^2, LOD, LOQ and RSD% (Relative Standard Deviation) are shown in *Table 1*.

Average recoveries were 89, 89, 98 and 93% and retention times were 11.8, 15.0, 18.3 and 23.6 min for AFG_2, AFG_1, AFB_2 and AFB_1, respectively *(Fig. 1)*.

Table 1. *R^2, LOD, LOQ and RSD% values of aflatoxins*
Tablo 1. *Aflatoksinlerin R^2, belirleme, hesaplama alt limitleri ve %RSD değerleri*

Aflatoxin	R^2	LOD (ng/g)	LOQ (ng/g)	RSD%
B_1	0.999	0.013	0.038	8.0
B_2	0.999	0.007	0.022	4.4
G_1	0.999	0.021	0.063	8.1
G_2	0.999	0.016	0.049	2.8

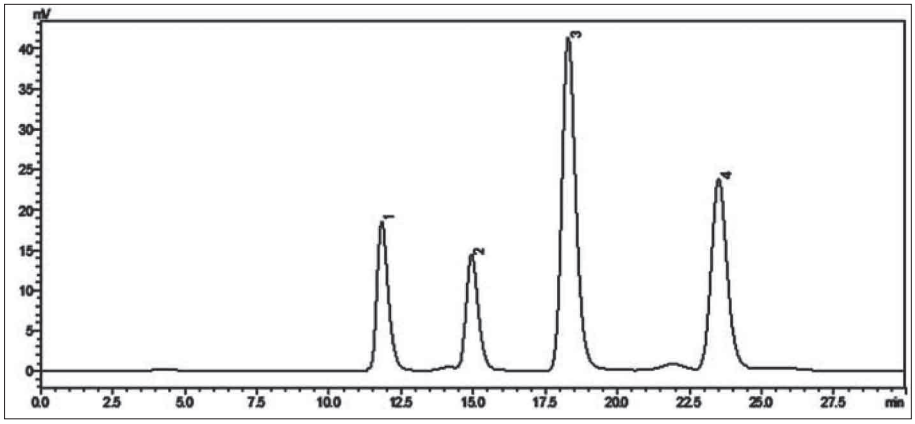
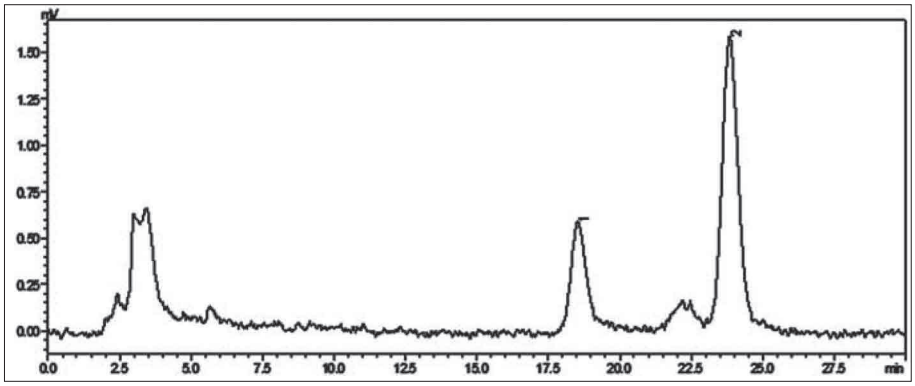


Fig 1. HPLC-FLD chromatogram of total aflatoxin standards at a concentration of 2.5 ng/ml. **1-** AFG_2, **2-** AFG_1, **3-** AFB_2, **4-** AFB_1

Şekil 1. 2.5 ng/ml konsantrasyonunda total aflatoksin standartına ait HPLC-FLD kromatogramı, **1-** AFG_2, **2-** AFG_1, **3-** AFB_2, **4-** AFB_1

Fig 2. HPLC-FLD chromatogram of the aflatoxin positive sample, **1-** AFB_2, **2-** AFB_1

Şekil 2. Aflatoksin pozitif numuneye ait HPLC-FLD kromatogramı, **1-** AFB_2, **2-** AFB_1



Only one maize sample collected from Samsun Province was contaminated and the contaminants were AFB_1 (0.193 ng/g) and AFB_2 (0.055 ng/g) *(Fig. 2)*.

DISCUSSION

In the present study, only one sample of a total of 40 maize grain samples from Samsun Province was contaminated with aflatoxins (AFB_1 and AFB_2); the AFB_1 concentration was lower than the limit set by the EU and Turkey (20 ng/g), but an AFB_2 limit has not been set [8,9]. Various mycotoxin screening studies of food and feedstuffs have been carried out in Turkey but there have been few undertaken with HPLC-FLD. Our results for aflatoxin levels were lower than in several previous studies performed in the same region or in other regions of Turkey. Specifically, Giray et al.[10] used ELISA to determine AFs and ochratoxin A (OTA) levels in 47 corn samples collected from various street bazaars and market outlets in different regions of Turkey. The AFs contamination range was between 0.625 and 8.57 ng/g for the Black Sea region, with 30% of samples contaminated. Oruc et al.[11] analyzed for AFB_1, T-2 toxin, fumonisin, deoxynivalenol and zearalenone with ELISA in various feedstuffs collected from a feed manufacturer that had obtained the samples from different regions of Turkey. They found seven samples of maize grains contaminated with AFB_1 in the range of 1.86 to 58 ng/g.

Previous reports indicate that certain volatile compounds produced by plants inhibit AF formation [12]. Plant lipoxygenase in maize and peanuts and its product, the 13(S)-hydroperoxide derivate, were also shown to interfere with AF formation [13]. Genetic modification of mold susceptible plants also plays a role in food safety. This involves increasing production of compounds such as anti-fungal proteins, hydroxamic acids and phenolics that reduce fungal contamination [14]. In the present study, the low AF contamination levels in maize may be related to genetic modification and/or proper harvesting, handling procedures and storage by agricultural producers.

The monitoring of AFs depends on their detection with precise analytical methods. While common analytical methods employ different detection techniques (ELISA, LC or TLC), all procedures require a suitable sample extraction step [15]. The extracts of most matrices are unsuitable for direct chromatographic analysis due to the large number of co-extracted impurities [5]. An immunoaffinity method is highly advantageous, as it is rapid and inexpensive [16]. In the present study, the KobraCell® was also used. It is an electrochemical cell that generates a reactive form of bromine for derivatization of aflatoxins, resulting in more sensitive detection. We achieved more precision in recovery of LODs and LOQs than several studies that used ultraviolet detection and/or TFA derivatization. Fu et al.[17] used an ultra-high pressure LC method with ultraviolet detection for the determination of AF levels in corn and peanuts but did not include a derivatization process. Their LODs for AFB_1, AFB_2, AFG_1 and AFG_2 were 0.32, 0.19, 0.32 and 0.19 ng/g and quantification limits were 1.07, 0.63,

1.07 and 0.63 ng/g, respectively. We achieved detections at 11 to 26 fold lower levels than their results and also better recoveries. Majeed et al.[18] determined AF and OTA levels in rice, corn and corn products in Pakistan with HPLC-FLD by using an AflaTest® immunoaffinity column and TFA derivatization. Their LODs and LOQs for AFB_1, AFB_2, AFG_1 and AFG_2 were 0.05/0.15, 0.10/0.30, 0.05/0.15 and 0.10/0.30, respectively, which were higher than in the present study.

The HPLC-FLD method with immunoaffiniy column clean-up involving post-column bromination that we applied to maize for detection of AFs is sensitive and accurate. AF contamination levels in the maize sampled in Samsun Province were very low but should be checked regularly because AFs threaten both human and animal health. For the future, the systematic use of new analytical techniques to measure AF levels with greater precision can help ensure that public health standards are improved.

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