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Immunoaffinity Column Cleanup with Liquid Chromatography Using Post-Column Bromination for Aflatoxins in Medicinal Herbs and Plant Extracts

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Abstract

A new and accurate method to quantitate aflatoxins in medicinal herbs is developed. This method consists of extraction of the sample with MeOH–H_2O (70:30) followed by clean-up of the extracts with immunoaffinity columns and, finally, high-performance liquid chromatographic determination with fluorescence detection. Aflatoxins B_1 and G_1 are determined as their bromine derivatives, produced in an online post-column derivatization system. The overall average recoveries for three different medicinal herbs spiked at levels of 1.3 and 2.6 ng/g of total aflatoxins range from 93% to 97%. The detection limit is 0.15 ng/g for both G_2 and B_2 and 0.20 ng/g for both G_1 and B_1, based on a signal-to-noise ratio of 3:1 and a precision (within-laboratory relative standard deviation) ranging from 0.8% to 1.4%. The use of immunoaffinity columns provides excellent clean-up of these particular extracts, which are generally difficult to analyze. The method is applied successfully to 96 samples of natural drugs.

Introduction

Aflatoxins B_1, B_2, G_1, and G_2 are four major toxic metabolites of fungal strains *Aspergillus flavus* and *Aspergillus parasiticus*. The B_1 component is usually predominant and is also the most toxic on a mass basis. These mycotoxins are known to be highly toxic and carcinogenic. Therefore, the contamination of foods and animal feed with these mycotoxins is controlled by legal limits worldwide. The European Commission Regulation (1) sets limits for aflatoxin B_1 and total aflatoxins of 2 and 4 ng/g, respectively, in groundnuts, nuts, dried fruits, cereals (including buckwheat), and processed products for human consumption. The same limits have been set in Germany (2). For human dietary products, such as infant nutrition, there are stricter legal limits, such as 0.05 ppb for aflatoxin B_1 and the sum of all aflatoxins (3).

Previously, most determinations of aflatoxins have involved two-dimensional high-performance thin-layer chromatography with fluorescence detection (4). Enzyme-linked immunosorbent assay tests and, high-performance liquid chromatography (HPLC) methods have most often been described (5). The official method in China using two-dimensional thin-layer chromatography is time consuming and cannot be used for every matrix. The method using trifluoroacetic acid as the derivatizing agent has poor reproducibility and is difficult to automate.

In order to meet the tolerance limits for monitoring and research work, better methods for aflatoxins are needed. Many reports searching for the best methods to detect mycotoxins in

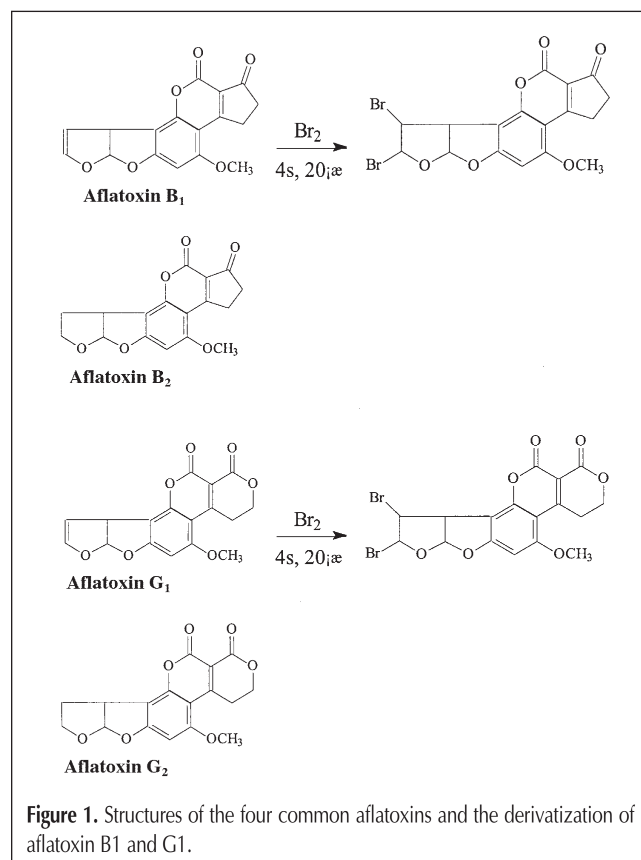

Figure 1. Structures of the four common aflatoxins and the derivatization of aflatoxin B1 and G1.

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different substrates, mainly foods and feeds, have been published. Conversely, only a few reports are available on medicinal plants used as active ingredients in phytotheraputic drugs.

Because the native fluorescences of aflatoxin B_1 and G_1 are not high enough to reach the required detection limits, a derivatization step is often applied (Figure 1). Taking into account these demands, the aim of this research was to improve or develop a method to detect aflatoxins in medicinal herbs using an HPLC technique.

Experimental

Materials and reagents

Codonopsis pilosula (Franch.) Nannf., *Prunus persica* (L.) Batsch. and Massa Medicata Fermentata were obtained from Tong Ren Tang crude drugs store (Beijing, China). The aflatoxins reference standards were available from Sigma (St. Louis, MO). Methanol and acetonitrile were analytical-reagent grade and purchased from Fisher Scientific (Fair Lawn, NJ).

The aflatoxin working standard solutions were prepared by dilution of a stock standard solution [0.96 µg of B_1, 0.29 µg of B_2, 1.0 µg of G_1, and 0.30 µg of G_2 per 1 mL in benzene–acetonitrile (98:2)] with methanol. The solution should be stored at 4°C in the dark.

Table I. Recoveries from Blank Samples Spiked with Aflatoxins at Different Levels

Samples spiked level	Recovery (%)				RSD (%)				Mean of means	
	G2		G1		B2		B1		Rec (%)	RSD (%)
***Codonopsis pilosula* (Franch.) Nannf.**										
1*	99	7.3	100	6.9	97	6.5	90	5.2	97	3.1
2†	99	1.5	99	2.6	97	4.6	96	3.5		
***Prunus persica* (L.) Batsch.**										
1	94	5.4	95	6.0	95	9.4	93	9.7	96	2.3
2	98	4.1	99	2.9	98	3.5	96	5.3		
Massa Medicata Fermentata										
1	92	6.4	87	4.9	96	5.5	90	8.8	93	3.8
2	95	4.7	94	9.5	98	5.1	96	4.6		

* Spiked at levels of 0.15 µg/kg aflatoxin G_2 and B_2 , 0.50 µg/kg aflatoxin G_1,and 0.48 µg/kg aflatoxin B1.
† Spiked at levels of 0.30 µg/kg aflatoxin G_2 and B_2, 1.00 µg/kg aflatoxin G_1, and 0.96 µg/kg aflatoxin B_1.

Table II. Linear Regression Results

Compound	Regression analysis equation	Correlation coefficient (*r*)	Linear range (ppb)
Aflatoxin G_2	$Y = 4.79 \times 10^5 X - 67.3$	0.9996	0.15–0.75
Aflatoxin G_1	$Y = 2.00 \times 10^4 X - 3.97 \times 10^2$	0.9991	0.50–2.5
Aflatoxin B_2	$Y = 7.66 \times 10^5 X - 2.28 \times 10^2$	0.9997	0.144–0.72
Aflatoxin B_1	$Y = 3.20 \times 10^4 X + 6.55$	0.9998	0.48–2.4

All work was carried out in the absence of daylight. Glassware was decontaminated using sodium hypochlorite solution (5.2% active chlorine) (Xi-Zhong chemical plant, Beijing, China). All glassware was cleaned with dilute hydrochloric acid and water. Pyridinium bromide perbromide (25 mg) was dissolved in 1 mL methanol and then diluted with 500 mL of water to prepare the derivatization solution.

Apparatus

A sonication bath was used for the extraction of the aflatoxins. As a clean-up step, monoclonal antibodies column (Aflatest P–Vicam, Watertown, MA) was used. The HPLC system (Waters, Milford, MA) consisted of a 2475 multi λ fluorescence detector, online degasser, and autosampler, and the analytical column was a Waters Symmetry C_{18} (150- × 3.9-mm i.d., 5-µm particle size). The post-column derivatization system was equipped with a second LC pump model 501 and the post-column reactor. The detector output was interfaced using SATIN box to the Waters Millennium 32 chromatographic manager system loaded on a digital computer for data handling and chromatogram generation.

Preparation of sample solution

A 10-g, finely pulverized (180 ± 7.6 µm particle size) sample was weighed into blender jar. An amount of 100 mL of methanol–water (70:30) was added, extracted by sonification for 30 min, and then filtered through Whatman N4. A 10-mL filtrate (equivalent to 1-g sample) was transferred by pipette into evaporating dish where

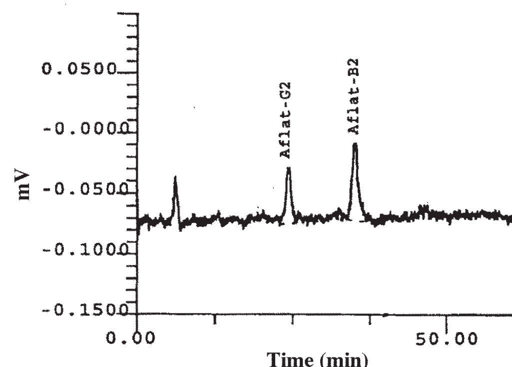

Figure 2. Chromatograms of 1.92 ng/mL B_1, 2 ng/mL G_1, 0.58 ng/mL B_2, and 0.6 ng/mL G_2 standard solution without post-column derivatization.

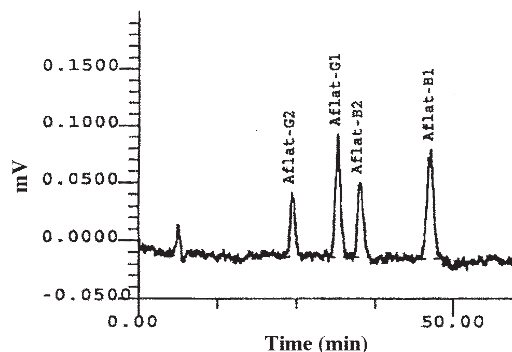

Figure 3. Chromatograms of 1.92 ng/mL B_1, 2 ng/mL G_1, 0.58 ng/mL B_2, and 0.6 ng/mL G_2 standard without post-column derivatization.

Table III. The Determination Results of Aflatoxins B_1, B_2, G_1, and G_2 in 33 Species of Medicinal Herbs and 11 Kinds of Patent Medicines (Total 96 Samples)

Sample name	Results	Sample name	Results
Cordyceps sinensis (Berk. Sacc.)	–	Niuhuang Qingxin pills (lot: 2010213)	×
Rheum palmatum L.	–	Niuhuang Qingxin pills (lot: 2010200)	×
Cassia obtusifolia L.	–	Niuhuang Qingxin pills (Lot: 2010100)	×
Salvia miltiorrhiza Bge.	–	Niuhuang Qingxin pills (lot: 2010102)	×
Lonicera japonica Thunb.	–	Niuhuang Qingxin pills (lot: 2010202)	×
Fagopyrum tatarium Gaertn	–	Da huoluo pills (lot: 2010190)	×
Scutellaria baicalensis Georgi	–	Da huoluo pills (lot: 2010224)	×
Glycine max (L.) Merr.	–	Da huoluo pills (lot: 2010153)	×
Coix lacryma-jobi L. var. ma-yuen (Roman.) Stapf	–	Da huoluo pills (lot: 2010314)	×
Massa medicata fermentata	–	Da huoluo pills (lot: 2010228)	×
Setaria italica (L.) Beauv.	–	Da huoluo pills (lot: 2010182)	×
Dashanzha Pills (wet)	–	Da huoluo pills (lot: 2010223)	×
Platycodon grandiflorum (Jacq.) A. DC.	–	Da huoluo pills (lot: 2010204)	×
Asparagus cochinchinensis (Lour.) Merr.	–	Da huoluo pills (lot: 2010152)	×
Glehnia littoralis Fr. Schmidt ex Miq.	–	Da huoluo pills (lot: 2010226)	×
Atractylodes macrocephala Koidz.	–	Da huoluo pills (lot: 2010203)	×
Prunus persica (L.) Batsch	–	Da huoluo pills (lot: 2010150)	×
Amomum kravanh Pierre ex Gagnep	–	Da huoluo pills (lot: 2010151)	×
Aplinia oxyphylla Miq.	–	Baifeng pills (lot: 2030016)	×
Sterculia lychnophora Hance	–	Baifeng pills (lot: 2030041)	×
Raphanus sativus L.	–	Baifeng pills (lot: 2030019)	×
Poria cocos (Schw.) Wolf	–	Baifeng pills (lot: 2030032)	×
Gastrodia elata Bl.	–	Baifeng pills (lot: 2030040)	×
Trichosanthes kirilowii Maxim.	–	Baifeng pills (lot: 2030066)	×
Sophora flavescens Ait.	×	Baifeng pills (lot: 2030069)	×
Citrus reticulata Blanco (*Exocarpium citri rubrum*)	×	Baifeng pills (lot: 2030047)	×
Ephedra sinica Stapf	×	Baifeng pills (lot: 2030020)	×
Citrus reticulata Blanco (*Pericarpium citri reticulatae*)	×	Baifeng pills (lot: 2030021)	×
Massa Pinelliae Fermentata	×	Baifeng pills (lot: 2030022)	×
Chenxiang Shuqi Pills	×	Jingzhi kesou tanchuan pills (lot: 2080016)	×
Angelica dahurica (Fisch. Ex Hoffm.) Benth. et Hook.f.	×	Jingzhi kesou tanchuan pills (lot: 2080017)	×
Glycyrrhiza uralensis Fisch	×	Jingzhi kesou tanchuan pills (lot: 2080018)	×
Cuscuta chinensis Lam.	×	Jingzhi kesou tanchuan pills (lot: 2080019)	×
Dashanzha pills? Dry?	×	Jingzhi kesou tanchuan pills (lot: 2080021)	×
Gingko Tablet (lot: 030314-1)	×	Jingzhi kesou tanchuan pills (lot: 2080022)	×
Gingko Tablet (lot: 030314-2)	×	Jingzhi kesou tanchuan pills (lot: 2080023)	×
Gingko Tablet (lot: 030313)	×	Jingzhi kesou tanchuan pills (lot: 2080024)	×
Weisu spread (lot: 030326)	×	Jingzhi kesou tanchuan pills (lot: 2080027)	×
Weisu spread (lot: 030327)	×	Jingzhi kesou tanchuan pills (lot: 2080029)	×
Weisu spread (lot: 030328)	×	Jingzhi kesou tanchuan pills (lot: 2080028)	×
Niuhuang Qingxin pills (lot: 2010386)	×	Jingzhi kesou tanchuan pills (lot: 2080030)	×
Niuhuang Qingxin pills (lot: 2010201)	×	Jingzhi kesou tanchuan pills (lot: 2080031)	×
Niuhuang Qingxin pills (lot: 2010101)	×	Jingzhi kesou tanchuan pills (lot: 2080032)	×
Niuhuang Qingxin pills (lot: 2010214)	×	*Gentiana macrophylla* Pall.	B_2 0.6757 ppb
Niuhuang Qingxin pills (lot: 2010198)	×	Fujian Massa Medicata Fermentata	B_1 0.9312 ppb; B_2 0.1094 ppb
Niuhuang Qingxin pills (lot: 2010194)	×	*Hordeum vulgare* L.	B_1 0.6345 ppb
Niuhuang Qingxin pills (lot: 2010211)	×	*Peucedanum praeruptorum* Dunn.	B_1 4.2755 ppb; B_2 1.0462 ppb; G_1 1.1536 ppb; G_2 1.1536 ppb
Niuhuang Qingxin pills (lot: 2010212)	×	*Prunus armeniaca* L. var. ansu Maxim.	B_1 2.6198 ppb; B_2 0.1921 ppb

* All samples were obtained from the market in Beijing (China) randomly. The names of patent medicines are not written in italics. The mark "–" means that no peaks after immunoaffinity column cleanup. The mark "×" means that samples also have some peaks which don't interfere with the detection of aflatoxins after immunoaffinity column cleanup.

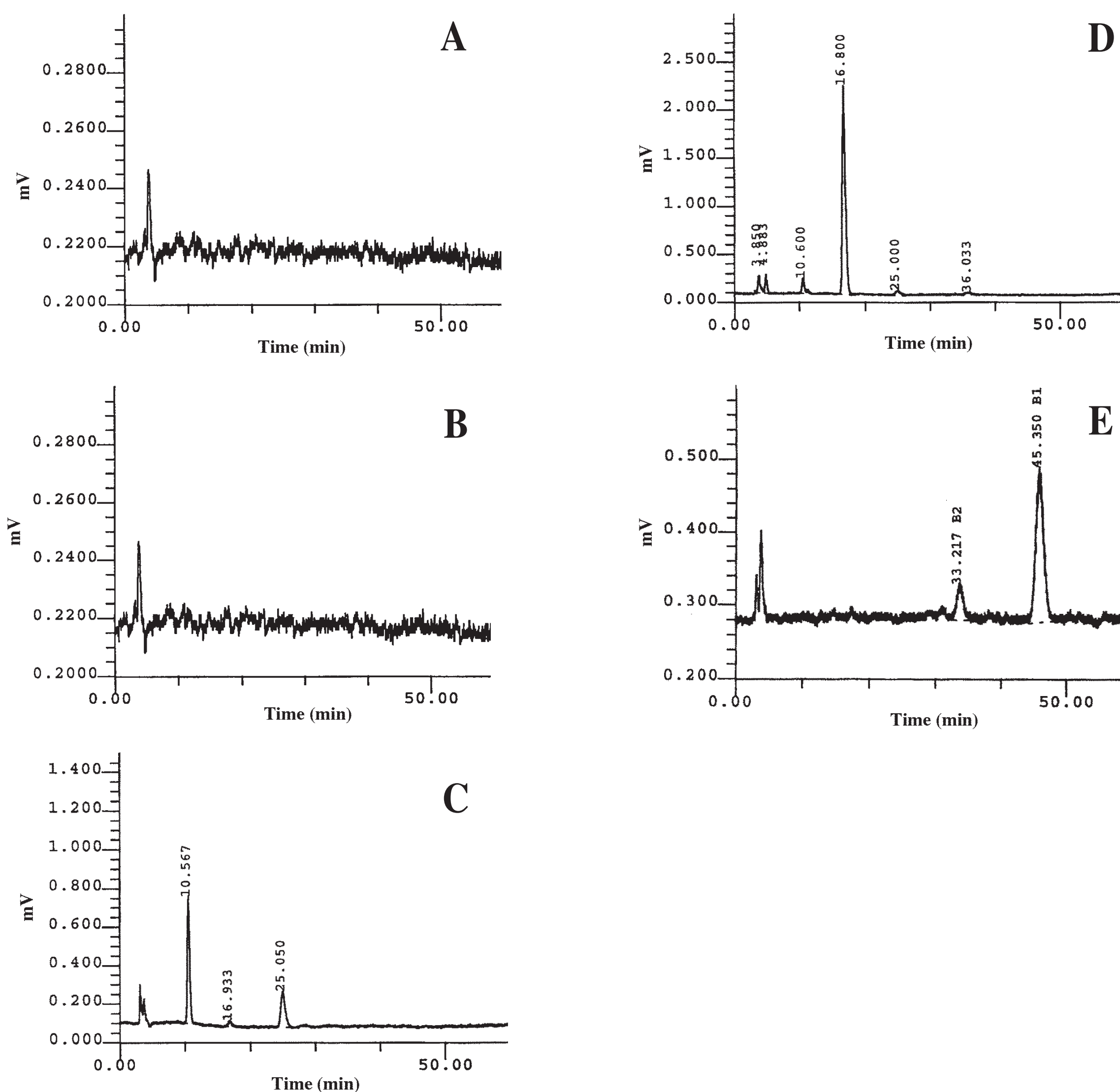

Figure 4. Typical chromatograms of samples: (A) *Codonopsis pilosula* (Franch.) Nannf. (< 0.15 ng/g for both G_2 and B_2 and < 0.20 ng/g for both G_1 and B_1); (B) *Codonopsis pilosula* (Franch.) Nannf. (sample spiked at levels of 4.8 ppb aflatoxin B_1, 5.0 ppb aflatoxin G_1, and 1.5 ppb aflatoxin B_2 and G_2); (C) *Ephedra sinica* Stapf; (D) *Citrus reticulata* Blanco; and (E) naturally contaminated *Prunus armeniaca* L.

most of the solvent was evaporated under a nitrogen stream at room temperature. Five milliliters of H_2O was added and mixed with 5 mL of 15% (v/v) tween-20 aqueous solution. The diluted extract was cleaned up through an Aflatest (Vicam) immunoaffinity column at a flow rate of approximately 1 drop/s. The column was washed with 10 mL H_2O at flow rate of 6 mL/min. The water bath was discarded and 2–3 mL air was passed through the column. The aflatoxins were eluted with 1 mL methanol and collected in a clean vial. The filtrate should then have been cleared; if not, it was refiltered. Column chromatography then proceeded immediately.

Chromatographic conditions

The mobile phase used for the separation consisted of a mixture of acetonitrile–methanol–water (14:17:69) at a flow rate of 1.0 mL/min. The column temperature was 25°C, and the injection volume was 10 µL.

The post column reactor temperature was maintained at 40°C. The flow rate of the post-column reagent (the derivatization solution) was 0.3 mL/min, and the total rate through reaction coil was 1.3 mL/min.

The aflatoxins were detected with λ_{ex} = 360 nm and λ_{em} = 450 nm. Prior to each run, the HPLC system was allowed to warm up for 30 min, and the pumps were primed using the protocol suggested by the manufacturer. Using a freshly prepared mobile phase, the baseline was monitored until stable before the samples were run.

Results and Discussion

Clean-up procedures were essential for these dirty extracts, particularly when HPLC was used for quantitation. The procedures

usually employed for clean-up—including liquid–liquid partition, addition of metal salts, and column chromatography—were assayed. All of them were slow or had poor analyte recovery (< 70%) and were nonspecific. To remove any interference from extracts of medicinal herbs, Aflatest P monoclonal antibody column was the most rapid and efficient clean-up system.

The dilution procedure recommended by Vicam for the analyses of aflatoxins in different matrices (such as corn grains, sorghum, soybeans, and feeds) was applied to medicinal herbs, but poor recoveries of 50–60% were obtained. The use of a surfactant improved aflatoxins recovery.

Extraction of the analytical sample was another important step. The combination of extraction with MeOH–H_2O (70:30) and elution with MeOH was selected. The volume of extraction solvent was greater than other substrates because dried medicinal herbs are more hygroscopic.

Results of the recovery experiments (by six replicates) carried out in medicinal herbs with aflatoxins at different levels are reported in Table I. Within the spiking range of total aflatoxins studied (1.3–2.6 ng/g) the overall average recoveries (mean of the means) were: 97 ± 3.0 for *Codonopsis pilosula*, 96 ± 2.2 for *Prunus persica*, and 93 ± 3.5 for *Massa Medicata Fermentata*. The detection limit of the method was 0.2 ng/g for both B_1 and G_1 and 0.15 ng/g for both B_2 and G_2, based on a signal-to-noise ratio of 3:1.

Linearity was tested by injecting a group of six standard solutions. Linearity relationships were set up between the elution peak areas and the concentrations for each of the compounds. Correlation coefficients were then calculated by linear regression analysis (listed in Table II).

Figure 2 shows a chromatogram of a standard solution of the four aflatoxins without the the post-column reagent (the derivatization solution) and Figure 3 shows it with the post-column reagent at the level of 1.92 ng/g B_1, 0.58 ng/g B_2, 2.0 ng/g G_1, and 0.60 ng/g G_2.

The reproducibility was studied by repeated injection of a standard mixture of the four aflatoxins. Twenty consecutive injections were made, containing 0.25 ng of each aflatoxin, with a delay of 1 h between injections. The relative standard deviation of analysis was ± 1.3% for aflatoxin B_1 and G_1 and ± 1.2% for aflatoxin B_2 and G_2.

The results of aflatoxin B_1, B_2, G_1, and G_2 determinations in 96 species of natural drugs are listed in Table III. Typical chromatograms of some samples are shown in Figure 4. Chromatograms of many samples, such as *Codonopsis pilosula* (Franch.) Nannf. (Figure 4A and 4B) and *Prunus persica* (L.) Batsch. (Table III), have no peaks after immunoaffinity column cleanup, although the chromatograms of many samples such as *Ephedra sinica* Stapf. (Figure 4C), *Citrus reticulata* Blanco, (Figure 4D) and others in Table III have some peaks that do not interfere with the detection of the aflatoxins. Several samples (Table III and Figure 4E) had detectable amounts of the aflatoxins.

Conclusion

The proposed method offers several advantages over other methods: high reproducibility, good recoveries for a variety of plant materials, easy automation, and inexpensive equipment.

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