Short Communication

Analysis of United Kingdom purchased spices for aflatoxins using an immunoaffinity column clean-up procedure followed by high-performance liquid chromatographic analysis and post-column derivatisation with pyridinium bromide perbromide

R. Colin Garner*, Matthew M. Whattam, Patrick J.L. Taylor and Martin W. Stow

Biocode Limited, University Road, Heslington, York, YO1 5DE (UK)

ABSTRACT

An immunoaffinity column method has been developed to analyse aflatoxins with greater than 70% recoveries from a variety of spices purchased from local stores. Samples of chilli powder, ground ginger, black pepper, cayenne pepper, paprika and cumin were initially screened semi-quantitatively; this provides a rapid means of analysing a large number of spice samples. Those samples that had aflatoxin levels greater than 1 ppb (w/w) total aflatoxins were re-analysed quantitatively by high-performance liquid chromatography (HPLC) using a newly developed post-column derivatisation procedure with pyridinium bromide perbromide (PBPB). Some samples of spices, in particular chilli powder, were found to contain aflatoxins at 20 ppb (w/w) or higher.

INTRODUCTION

The aflatoxins are a group of mycotoxins produced by the food spoilage fungi *Aspergillus*, particularly *flavus* and *parasiticus*. There are four naturally occurring aflatoxins, *viz.* aflatoxin B_1, B_2, G_1 and G_2, and all have varying degrees of biological activity [1]. Aflatoxin B_1 is the most potent and has been shown to be a toxin, mutagen and animal carcinogen. The aflatoxins as a group on the basis of epidemiological evidence have been classified as human liver carcinogens by the World Health Organisation [2]. Most countries in the Western world have introduced regulations controlling the level of aflatoxins in human and animal food although contamination levels are much lower than in the Third World [3]. In the Third World as a result, particularly where hepatitis B infection is endemic, liver cancer is very common [4]. A wide variety of food matrices have been shown to be contaminated by the aflatoxins and it is therefore essential to have available simple and quantitative methods for aflatoxin analysis.

Traditionally aflatoxin analysis has been performed using solvent extraction, usually with chloroform, followed by sample clean-up by

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* Corresponding author.

silica gel chromatography, prior to either TLC or HPLC analysis. More recently immunological methods have been introduced which permit more rapid and reliable aflatoxin analyses to be conducted [5–8]. The immunoaffinity approach offers a simple means of specifically concentrating aflatoxin from blended food sample solvent extracts using columns containing an anti-aflatoxin antibody [9]. The technique enables a wide variety of food matrices to be analysed using a one-step extraction protocol without the need to use halogenated hydrocarbon solvents for extraction.

In this study we have used a commercially available immunoaffinity (aflatoxin EASI-EXTRACT) column to screen a large number of spices purchased from local stores using firstly a semi-quantitative procedure in which the immunoaffinity purified extract is assayed using silica–Florisil mini-columns [10] and fluorescence. The semi-quantitative procedure is very rapid and allows a large number of samples to be processed efficiently. Any samples containing aflatoxin levels higher than 1 ppb were re-analysed by HPLC. A new method of post-column derivatisation of the aflatoxins was developed using a solution of pyridinium bromide perbromide (PBPB). This mild brominating agent enhances the fluorescent signal produced by aflatoxin B_1 and G_1 and hence increases the sensitivity of the analytical method.

The following spices have been examined: chilli powder, ground ginger, black pepper, cayenne pepper, paprika and cumin.

EXPERIMENTAL

Materials

Total aflatoxin EASI-EXTRACT columns and analytical silica–Florisil columns were from Biocode Ltd (York, UK). The manufacturer's specifications for EASI-EXTRACT columns are that they will recover $>80\%$ total aflatoxins when 3.5 ng of each aflatoxin B_1, B_2, G_1 and G_2 is applied in 175 ml of diluted peanut butter extract (equivalent to 1 ppb contamination level), and that they have a total capacity of >5 μg when 10 μg aflatoxin B_1 is applied in 50 ml of

5% (v/v) methanol in phosphate buffered saline pH 7.4 (PBS). Analytical reagent grade acetonitrile, methanol and chloroform were purchased from FSA (Loughborough, UK). All water used was purified by reverse osmosis followed by ion exchange/activated carbon column clean-up. Aflatoxins B_1, B_2, G_1 and G_2 and PBPB were purchased from Sigma (Poole, UK). PBS was prepared using PBS tablets from Biocode Ltd. All other reagents were laboratory reagent grade.

Sample preparation

Powdered spice (10 g), purchased from local supermarkets and health food shops, was vigorously blended for two min using an Ultra Turrax homogeniser (Sartorius, Epsom, UK) with 40 ml methanol–water (80:20, v/v). The resulting suspension was centrifuged at 1600 g (av) for 15 min at room temperature and the supernatant collected. This was diluted to 10% (v/v) methanol with PBS and re-centrifuged at 1600 g (av) for a further 15 min. The supernatant was applied to immunoaffinity columns.

Immunoaffinity purification

Semi-quantitative analysis for initial screening of spice samples. The diluted spice extracts (112 ml) prepared as above were applied either manually via a syringe or using a peristaltic pump (Watson-Marlow, Falmouth, UK) to the aflatoxin immunoaffinity columns as per the manufacturer's instructions, *i.e.* at a flow rate of 5 ml/min. After sample application, the columns were washed with 20 ml purified water and any bound aflatoxins eluted slowly with 1.5 ml neat methanol. To this methanol eluate, 6 ml PBS was added followed by 3 ml chloroform. The whole mixture was vigorously shaken for one min, the chloroform layer removed and applied to an analytical column. These glass columns consist of a number of powders including alumina (to remove non-specific fluorescent material), silica and Florisil. Any aflatoxin is concentrated at the silica–Florisil interface and can be visualised under UV light. Once the chloroform extract had been allowed to drip through the analytical

column it was washed with 3 ml chloroform–acetone (9:1). The analytical column was then examined under long wave ultraviolet light (6 W at 360 nm) in a dark box. The intensity of the fluorescence at the silica–Florisil interface was compared with analytical columns to which known amounts of aflatoxin B_1 (0, 4, 10, 20 ng) had been applied in order to provide a measure of the level of aflatoxin contamination.

Quantitative analysis by HPLC. For spice samples that contained greater than 1 ppb total aflatoxin as determined by the above procedure, a quantitative analysis was performed using an automated HPLC system (Anachem, Luton, UK). The spice extracts were immunopurified as above and any bound aflatoxins slowly eluted with 1.5 ml methanol using an automated robotic sample preparation system (ASPEC, Anachem). The eluate was subsequently diluted by the system with 2 ml of purified water before 500 μl was injected onto the HPLC system. This automated system has been described previously for the analysis of mycotoxins by Sharman and Gilbert [11].

High-performance liquid chromatography and post-column derivatisation

For the analysis of aflatoxins, a reverse-phase Spherisorb ODS2 HPLC column (5 μm particle size, 25 cm × 4.6 mm I.D.) was used with an isocratic mobile phase of 40% (v/v) acetonitrile:methanol (5:4) in HPLC grade water at a flow rate of 0.75 ml/min. The post-column derivatisation reagent PBPB (at 0.05 mg/ml in HPLC grade water) was mixed at a flow rate of 0.3 ml/min with the eluate from the HPLC column at a T piece junction. The reagent was allowed to react for 30 cm length of tubing (0.25 mm I.D.) before monitoring for the presence of aflatoxins with an Applied Biosystems (Anachem) Model 980 fluorescence detector (excitation 362 nm, emission 418 nm).

When iodine was used as the post-column derivatisation reagent, a saturated solution in water was mixed with the eluate from the HPLC column at 0.6 ml/min. The reaction occurred over a 5-m length of coil heated to 50°C before entering the fluorescence detector.

RESULTS AND DISCUSSION

A semi-quantitative screening of a wide range of chilli powder, ground ginger, black pepper, cayenne pepper, cumin and paprika samples was conducted to detect aflatoxin contamination of > 1 ppb. The amount of the diluted extract applied to the column determines the detection limit. In this case, 112 ml of extract was applied, which is equivalent to 3.5 g of spice. As 4 ng of aflatoxin is required on the analytical column in order to see a fluorescent band, samples contaminated at a level of greater than 1 ppb gave a positive result on the analytical column. The results indicated that more than 50% of samples were contaminated at levels higher than 1 ppb. No positive samples were detected when either cumin or paprika were analysed. The black pepper samples gave large amounts of yellow-green fluorescence over areas of the analytical column and this interfered with the semi-quantitative analysis of aflatoxin. Those spices that were positive gave clear, sharp blue fluorescent bands at the silica–Florisil interface, indicative of the presence of aflatoxins.

The extraction efficiency of the 80% (v/v) methanol–water method was assessed by spiking spice samples (which had previously been shown to be non-contaminated using the semi-quantitative analysis procedure) at the 4 ppb level each with aflatoxins B_1, B_2, G_1, and G_2. These samples were then processed as described in the methods section and the eluates analysed by HPLC. Each spice sample was analysed using duplicate EASI-EXTRACT columns and the eluate from each column was injected in duplicate onto the HPLC. The percentage recoveries obtained are shown in Table I. The unspiked spice samples were also analysed at the same time and any natural aflatoxin contamination present taken into consideration during the recovery calculation.

The accuracy and reproducibility of the automated ASPEC–HPLC system was investigated by analysing multiple samples of naturally contaminated spice extracts through the EASI-EXTRACT columns. Five extracts of each spice were analysed and every eluate was injected in duplicate onto the HPLC system. The results

TABLE I

PERCENTAGE RECOVERIES OF AFLATOXINS FROM SPICE SAMPLES SPIKED AT 4 ppb LEVEL WITH EACH OF THE AFLATOXINS B_1, B_2, G_1 AND G_2

Spice	% Recovery			
	AFG_2	AFG_1	AFB_2	AFB_1
Black pepper	97	89	92	74
Chilli powder	78	69	78	73
Ginger powder	92.5	70	102.5	112.5

expressed as means and R.S.D.s are shown in Table II. The R.S.D.s of the cayenne pepper and chilli powder samples contaminated with aflatox-

TABLE II

THE REPRODUCIBILITY/ACCURACY OF THE AUTOMATED ASPEC/HPLC SYSTEM FOR THE ANALYSIS OF AFLATOXIN CONTAMINATION IN CHILLI POWDER AND CAYENNE PEPPER

R.S.D. values are shown in brackets.

Sample	Mean contamination level (ppb)	
	AFB_2	AFB_1
Chilli powder	0.9 (2.2%)	19.8 (4.7%)
Cayenne pepper	0	4.0 (4.6%)

in B_1 around the 4 ppb and 20 ppb levels were 4.6 and 4.7%, respectively.

With the described extraction procedure in conjunction with the HPLC system the limit of quantification of the method is 0.14 ppb of aflatoxin. The limit of detection will be less, around 0.06 ppb.

The spice samples which were found to contain > 1 ppb aflatoxin following an initial screening by semi-quantitative analysis were re-analysed using a HPLC based quantitative method. The results are detailed in Fig. 1. Typical HPLC chromatograms of a highly contaminated chilli powder sample and a contaminated ginger sample are shown in Fig. 2. The ground ginger samples typically show contamination by all four aflatoxins while the chilli powder contains predominantly aflatoxin B_1 and B_2.

The highest levels of total aflatoxin contamination were found in chilli powder and ground ginger samples.

A direct comparison between the use of iodine and PBPB as post-column derivatisation reagents was conducted. The results detailed in Table III are those obtained when the system for each reagent was optimised for sensitivity (as described in the method section). The results show that the fluorescence of all the aflatoxins is increased especially that of AFB_1. The day-to-day reproducibility of the system was found to be good.
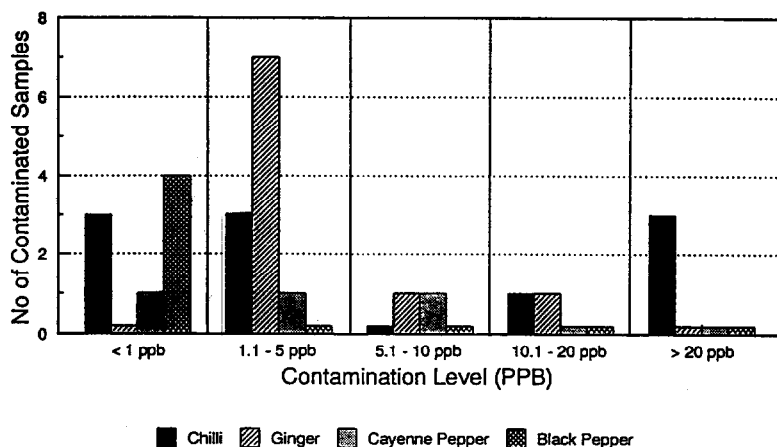

Fig. 1. Total aflatoxin levels in spices analysed by HPLC following initial semi-quantitative screening.
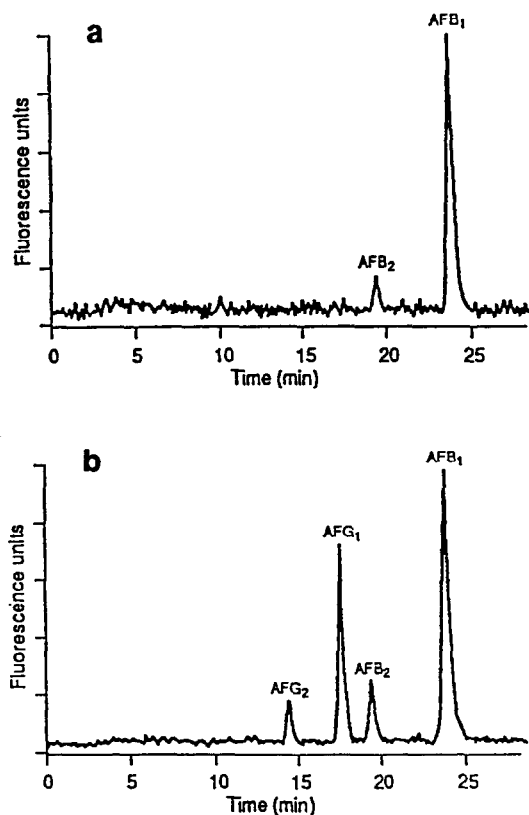
Fig. 2. HPLC chromatograms illustrating the analysis of aflatoxin peaks for aflatoxin B_1 normalised to full scale deflection in each chromatogram - (a) naturally contaminated chilli powder sample, (b) naturally contaminated ginger powder sample.

CONCLUSIONS

In the United Kingdom, regulations exist controlling the level of aflatoxin contamination in some human and animal food. Nevertheless the regulations, particularly for human food, apply to only certain food matrices (nuts, nut products, dried figs and dried fig products). In the study reported here we have developed simple quantitative methods for aflatoxin analysis and have applied this to a variety of spices. We chose these to study, since spices are difficult to analyse using conventional analytical methods and there were literature reports that aflatoxin contamination of spices does occur [12].

We have analysed a variety of powdered spices purchased in local supermarkets and health food stores. More than 50% of the spice samples were found to be contaminated at levels of greater than 1 ppb. Of these 17 out of 32 samples purchased from supermarkets and 3 out of 4 samples purchased from health food shops, were contaminated at these levels.

Immunoaffinity column methods for sample clean-up provide specific and sensitive procedures for food analysis. The chromatograms obtained from immunoaffinity purified extracts are relatively free from contaminating chemicals thus increasing the assay sensitivity. Spices are particularly difficult to analyse for aflatoxins, because of the highly coloured contaminating materials that are co-extracted with the aflatoxins. Immunoaffinity methods provide a quick, one-step solution to sample clean-up which should speed up aflatoxin analysis.

The post-column derivatisation method reported here is an improvement over existing procedures particularly the use of iodine [13]. PBPB provides a stable solution which needs no long reaction times or elevated temperatures to enhance aflatoxin fluorescence. Aflatoxin peaks are as a result narrower and in addition, peak

TABLE III

A COMPARISON OF AFLATOXIN PEAK AREAS AFTER POST-COLUMN DERIVATISATION WITH EITHER IODINE–WATER OR PBPB–WATER

Reagent system	Mean peak area				R.S.D.			
	AFG_2	AFG_1	AFB_2	AFB_1	AFG_2	AFG_1	AFB_2	AFB_1
Iodine–water	76073	43801	57033	32411	2.1	2.0	2.8	3.5
PBPB–water	112510	47683	86511	82063	2.0	3.8	2.8	4.5

height is increased over that produced by iodine–water again enhancing sensitivity. The system also has the advantage of not requiring expensive equipment such as the method of post-column derivatisation using electrochemically generated bromine [14].

Of the spices analysed, chilli powder and ground ginger were the most likely to be contaminated; some samples contained over 20 ppb total aflatoxins. Chilli powder is used in a wide variety of foods including ethnic dishes and snack foods. Ginger root powder is used as a flavouring agent in drinks, biscuits, cakes as well as ethnic foods. In the UK, the number of foods containing spices is increasing, particularly in the snack food business. Hence exposure to aflatoxins from these sources will also be increasing, putting some additional health risk to the consumer. It is important that all food with the potential for aflatoxin contamination is analysed and that regulations cover all sources of human exposure.

ACKNOWLEDGEMENTS

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