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Journal of Chromatography A, 932 (2001) 153–157

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Short communication

Determination of aflatoxins B1, B2, G1 and G2 in spices using a multifunctional column clean-up

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Received 8 March 2001; received in revised form 13 August 2001; accepted 13 August 2001

Abstract

A rapid and simple method using a multifunctional column, which contains lipophilic and charged active sites, was developed to analyse aflatoxins B1, B2, G1 and G2 in various spices, such as red pepper and nutmeg. After extraction by acetonitrile:water (9:1) and clean-up using MultiSep #228 column, the aflatoxins and aflatoxin–TFA derivatives are determined using LC with fluorescence detection. Recoveries of each aflatoxin B1, B2, G1 and G2 spiked to red pepper, white pepper, black pepper, nutmeg and tear grass at the level of 10 ng/g were over 80–85% in all instances. The minimum detectable concentration for aflatoxins in red pepper was 0.5 ng/g.

Keywords: Spices; Multifunctional column; Clean-up methods; Aflatoxins

1. Introduction

Aflatoxins are mycotoxins produced by certain fungi, especially, *Aspergillus flavus*, and they display strong carcinogenicity [1]. Therefore, they are dangerous food contaminants and many countries have set stringent regulatory demands on the level of aflatoxins permitted in imported and traded commodities [2].

In terms of aflatoxin analyses without using chloroform, highly successful procedures have been published [3–8]. Milk and biological fluids have been used as a sample in most of them. On the other hand, an analytical method of aflatoxins in foods using the multifunctional column (MFC) that allows the passage of aflatoxins and retains the interfering substances was reported and the usefulness of the method has been shown by a collaborative study [9,10]. This method has been adopted as an AOAC official method. We also developed a rapid and sensitive clean-up method with another commercial multifunctional cartridge column using LC with fluorescence detection for the analysis of aflatoxins in various cereals, nuts and corn [11].

These methods are used worldwide for foods except milk because they are simple and rapid while there is no need to use a toxic solvent, chloroform, for extraction and clean-up. Therefore, the methods using MFC are more useful and safer than the conventional method that consisted of extraction with chloroform and purification using silica or florisil [12–17]. Recently, we applied the MFC method to analyze aflatoxins in several spices using the combination of MFC clean-up and an affinity

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)01211-0

column clean-up [18]. However, the method involved time-consuming clean-up steps and was too costly for routine analysis because we had to use two clean-up columns. Obviously, it is still difficult to analyze aflatoxins using only a single MFC clean-up for various spices because of the many interferences.

In the present study, we describe a rapid and simple method for the analysis of aflatoxins B1, B2, G1 and G2 in various spices using only one MFC clean-up step followed by LC analysis with pre-column derivatization.

2. Experimental

2.1. Apparatus

The LC system consisted of Hitachi model L-7100 pumps (Tokyo, Japan), Tosoh Model AS-8020 auto-injector (Tokyo, Japan) and Shimadzu model RF-10AXL fluorescence detector (Tokyo, Japan). The guard column, Inertsil ODS-3 (1.0 cm×4.0 mm I.D., 3 μm, GL science, Tokyo, Japan) was placed between the autoinjector and the separative column, Inertsil ODS-3 (25 cm×4.6 mm I.D., 3 μm, GL science, Tokyo, Japan) column. The integrator used was a Hitachi model D-7500. MycoSep #226, #228 column (MycoSep type) and MultiSep #228 column (cartridge type) were purchased from Romer, Labs, Inc. (MO, USA). Isolute Multimode (cartridge type) was purchased from International Solvent Technology Ltd. (Hengoed, Mid Glamorgan, UK). A coffee mill SCM-40A (Sibata, Tokyo, Japan) was used for grinding samples.

2.2. Standard preparation

The aflatoxins B1, B2, G1 and G2 crystalline materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Each stock standard solution of aflatoxins with concentrations of 1 μg/ml was prepared in methanol. They were stored at 4°C in the dark. The stock solutions were evaporated to dryness under reduced pressure before using, then they were derivatized with TFA as the working standards. By means of a pre-column derivatization with TFA, the weakly fluorescent aflatoxins B1 and G1 are transformed into their highly fluorescent hemiacetals B2a and G2a. The highly fluorescent aflatoxins B2 and G2 are not affected by this conversion due to their saturated structure [19].

2.3. Samples

Red pepper was supplied by Center for Inspection of Imported Foods and Infectious Diseases in Yokohama Quarantine Station. The other spice samples were purchased commercially in Tokyo.

2.4. Extraction and purification of aflatoxins

Samples were ground to uniform consistency using the coffee mill, and 20 g test sample was extracted with 160 ml of acetonitrile–water (9:1) by shaking vigorously in a 300 ml glass flask fitted with a stopper for 30 min. The solution was filtered through Whatman No.5 filter paper. A 5-ml portion of the filtrate was transferred to a MultiSep #228 cartridge column and passed through at a flow-rate of 2 ml/min. The aflatoxins passed through the column. Then, a 0.5-ml portion of the first 1 ml eluate was evaporated to dryness at 40°C in a centrifuge glass tube and the residue was used for precolumn derivatization.

2.5. Precolumn derivatization

A 100 μl sample of the TFA solution was added to the residue from sample extracts or aflatoxin working standards to derivatize aflatoxins B1 and G1. The tube was allowed to stand at room temperature for 15 min in the dark. A 0.4 ml sample of the acetonitrile:water (1:9) solution was added to the tube. A 25-μl portion of the sample or standard solution in the tube was subjected to LC analysis.

2.6. LC conditions

The mobile phase was acetonitrile–methanol–water (8:27:65). The mobile phase was degassed by sonication. The Inertsil ODS-3 (4.6 mm I.D.×250 mm, 3 μm) was connected as the LC column. The column was maintained at 40°C with a flow-rate of 0.7 ml/min. The aflatoxins and derivatives were detected at the excitation and emission wavelengths

of 365 and 450 nm, respectively. The injection volume was 25 μl.

2.7. Determination of aflatoxins recoveries

Aflatoxins are not concentrated through extraction, clean-up using MFC and pre-column derivatization. Each aflatoxin in a sample solution was determined from respective standard curves.

We calculated the recoveries of aflatoxins from each aflatoxin concentrations in sample solutions and the expected value from extraction solvent spiked.

3. Results and discussion

3.1. Selection of extracting solvent

Previously, we reported that the extraction of aflatoxins using 90:10 acetonitrile/water was effective for their recovery from various nuts and corn [11]. Among some extracting solvents examined, we confirmed the solvent 90:10 acetonitrile/water resulted in a chromatogram with fewer interfering peaks than those using the other solvent combinations.

3.2. Clean-up of aflatoxins using multifunctional columns

Several commercial multifunctional columns have been studied for clean-up of aflatoxins from red pepper. The commercial columns we examined are MycoSep #226, #228 column (MycoSep type), MultiSep #228 column (cartridge type) and Isolute Multimode (cartridge type). Among the multifunctional columns that we examined, both the MultiSep #228 column (cartridge type) and MycoSep #228 column (MycoSep type) showed better results than the others in terms of the recoveries of aflatoxins from red pepper. But, it appeared to be still necessary to separate some aflatoxins, especially, aflatoxin G1 from some impurities.

Considering the chromatographic principle of MFC, we considered that the best purified solution would be obtained in the early eluate part if aflatoxins could pass through without retention while some impurities would be retained weakly in some degree on the MFC and elute after the aflatoxins. Therefore, each three fractions (1 ml) were eluated from MultiSep #228 cartridge column. The chromatograms of the first 1 ml eluate, second 1 ml eluate and third 1 ml eluate are shown in Fig. 1. The chromatogram of first eluate exhibited less impurity peaks than the chromatograms of the second or third eluate. The recoveries of aflatoxins from the first

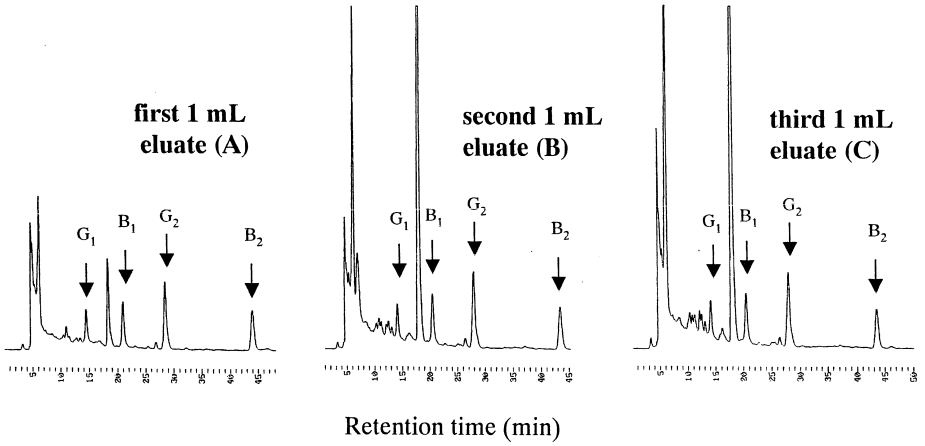

Fig. 1. Typical LC chromatograms of the first 1 ml eluate (A), second 1 ml eluate (B) and third 1 ml eluate (C) of the aflatoxin-spiked (each 10 ng/g) red pepper extract from MultiSep #228 cartridge column.

eluate were greater than 87.5% and almost the same as those from the second eluate and third eluate. This clean-up method using the first 1 ml eluted from MultiSep #228 cartridge column was more effective than that using MycoSep #228 column in terms of reducing impurity peaks. These findings suggested that the best chromatogram would be obtained in the analysis of the first eluate passed through the MultiSep #228 cartridge type column. Thereafter, we used only the first 1 ml eluate from the MultiSep #228 cartridge type column as the sample solution.

3.3. Examination of LC conditions

According to our previous papers [11,18], we modified several LC conditions to establish the optimum separation between aflatoxins and aflatoxin derivatives with TFA from impurities in spices. The mobile phase of acetonitrile–methanol–water (8:27:65) gave the more satisfactory separation of peaks of aflatoxins, derivatized aflatoxins and impurities than acetonitrile–water or methanol–water mixtures. Among several columns examined, the most suitable column was Inertsil ODS-3 (4.6 mm× 250 mm, 3 μm). The detection wavelengths used were according to the previous reported methods [11,18].

3.4. Recovery tests from various spices

The developed method was applied to the aflatoxins in red pepper, black pepper, white pepper, nutmeg and tear grass. Figs. 2 and 3 showed typical LC chromatograms of cleaned-up extracts from samples for blank and spiked samples in red pepper (Fig. 2) and black pepper (Fig. 3). The peaks corresponding to derivatized aflatoxin B1, G1 and aflatoxin B2, G2 were resolved from the background peaks of blank samples. The recoveries of aflatoxins B1, B2, G1 and G2 from samples spiked at 10 ng/g for all aflatoxins were quite good (Table 1). Recoveries of aflatoxins B1, B2, G1 and G2 in spiked red pepper, black pepper, white pepper, nutmeg and tear grass at the level of 10 ng/g were over 83% in all instances. Coefficients of variation were low, ranging from 2 to 6%. Repeatability for spiked samples was good. The detection limits for aflatoxins B1, B2, G1 and G2 were approximately 0.5 ng/g in

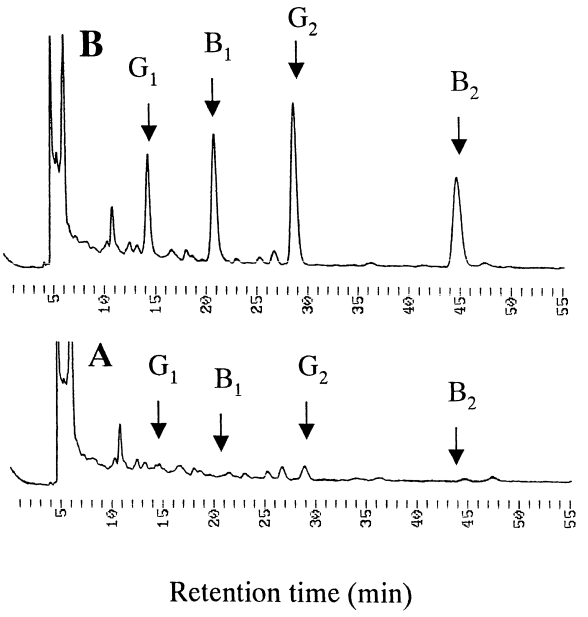

Fig. 2. Typical LC chromatograms of sample solutions from non-spiked red pepper (A) and red pepper spiked with aflatoxins B1, B2, G1 and G2 [10 ng/g].

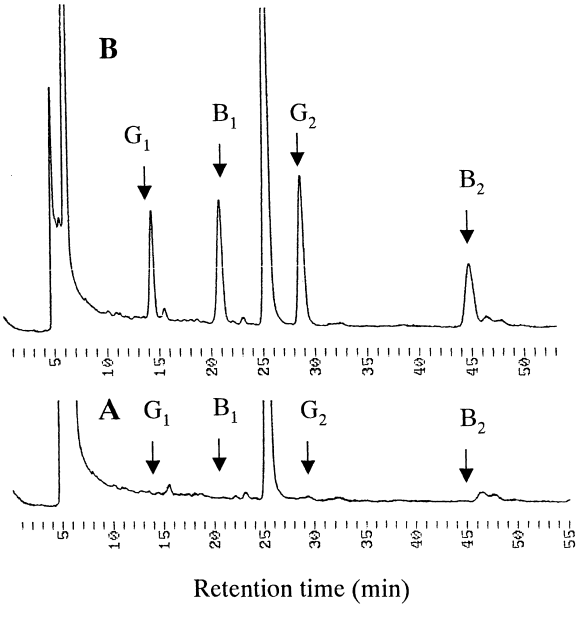

Fig. 3. Typical LC chromatograms of sample solutions from non-spiked black pepper (A) and black pepper spiked with aflatoxins B1, B2, G1 and G2 [10 ng/g].

Table 1
Mean recoveries from various spices spiked with 10 ng/g aflatoxins B1, B2, G1 and G2

Spices	Aflatoxins			
	B1	B2	G1	G2
Red pepper	94±4	103±3	102±3	105±5
Black pepper	98±4	97±4	97±4	85±3
White pepper	90±5	90±4	85±6	90±6
Nutmeg	82±5	87±4	95±2	85±5
Tear grass	96±5	96±4	98±5	99±2

Each value represents the mean±SD from triplicate analyses.

red pepper (signal-to-noise ratio, 3:1). These values were clearly similar to those reported by Wilson et al. [9] using MycoSep #224 in various nuts and cereals, showing high sensitivity of this proposed method.

4. Conclusions

This proposed method using MultiSep # 228 MFC is a rapid (within 2 h), simple and reproducible method for the analyses of aflatoxins in spices. Recoveries of aflatoxins B1, B2, G1 and G2 spiked to red pepper and nutmeg at the levels of 10 ng/g were greater than 83%, respectively. The detection limit in red pepper was 0.5 ng/g. This method can analyze aflatoxins more rapidly and safely than the methods using a chloroform extraction step.

Acknowledgements

We are grateful to Dr. J.L. Richard for reviewing the manuscript and to Mrs. Akiko Shinoda for supplying MFC columns. The study was supported by a grant for Scientific Research Expense for Health and Welfare Programs from the Japanese Government.

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