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ORIGINAL RESEARCH PAPER

DETERMINATION OF AFLATOXINS IN RICE USING QUECHERS AND FLUORESCENCE HPLC

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Abstract: This study presents the determination of aflatoxins B1, B2, G1, and G2 in rice, applying a QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction followed by high-performance liquid chromatography-fluorescence detection (HPLC-FLD). The objective of the proposed method is a fast, inexpensive, and easy analysis of aflatoxins. The aflatoxins were separated on a C18 column by isocratic elution with water: methanol: acetonitrile (65 : 25 : 10, v/v/v), and detection was achieved at excitation/emission wavelengths of 360/450 nm, without derivatization. The calibration curves showed good linearity ($R^2 > 0.99$), and the limits of detection and quantification were ≤ 6 and ≤ 8 $\mu g \cdot kg^{-1}$, respectively. Average intraday and interday recoveries were in the range 104 - 119 % and 104 - 113 % with RSD % ≤ 12 % for concentrations between 6 - 20 $\mu g \cdot kg^{-1}$. The proposed method was tested on rice samples sold on markets, of which none exhibited presence of aflatoxins.

Keywords: *HPLC-FLD, mycotoxins, Oryza sativa, xenobiotics*

INTRODUCTION

Aflatoxins are highly toxic and carcinogenic secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus* [1, 2]. Contamination of food products with aflatoxins is common, particularly in developing countries located in hot and humid regions, and it is estimated to affect up to 25 % of the food supply [3]. Aflatoxins B1, B2, G1, and G2 (Figure 1) occur naturally when these fungi infest food products including rice, peanuts, corn, and spices [1]. B type aflatoxins are produced by *A. flavus*, whereas *A. parasiticus* produces both B and G types. The B and G denominations refer to the blue and green fluorescence emitted when the compounds are exposed to UV light [4]. Aflatoxins have been classified by the International Agency for Research on Cancer (IARC) as Group 1 carcinogens [5] and aflatoxin B1, the most common one, is considered one of the most potent carcinogens [2]. Aflatoxin exposure increases the risk of liver cancer, particularly hepatocellular carcinoma, and if the exposure is high, it can result in acute aflatoxicosis and death [3].

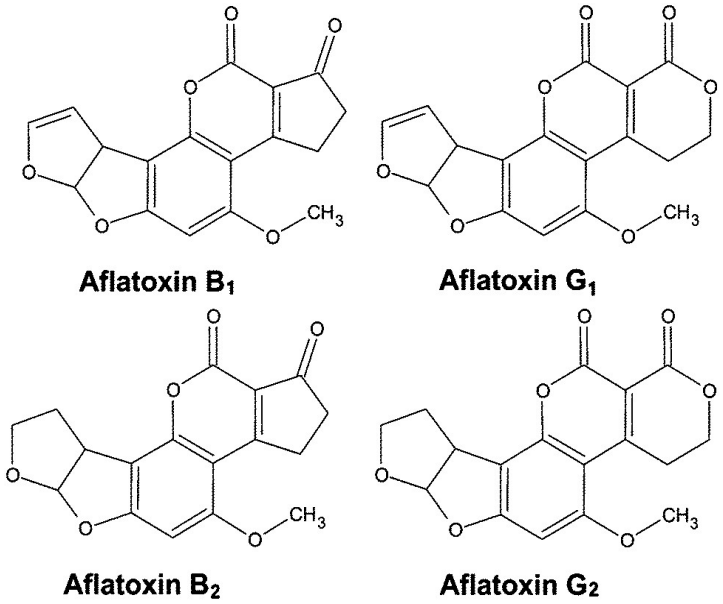

Figure 1. *Chemical structure of aflatoxins B1, B2, G1, and G2*

Aflatoxin analysis is challenging because of the generally low concentrations at which they occur and the complexity of the food matrices, and is generally achieved by solvent extraction followed by the use of immunoaffinity columns [6, 7] and high performance liquid chromatography-fluorescence (HPLC-FLD) [4] or liquid chromatography-mass spectrometry (LC-MS) [1]. Enzyme-linked immunosorbent assay (ELISA) can also be used for detecting aflatoxins [8, 9]. Nevertheless, these methods are costly and the former is also time consuming and requires the use of hazardous solvents.

The Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method was first reported in 2003 [10]. QuEChERS is a microscale extraction in which pesticides are isolated using a minimal amount of acetonitrile and the extract is cleaned-up by dispersive solid phase extraction (d-SPE). Thus, this method allows for fast sample preparation using only a small amount of reagents, while providing high and reproducible recoveries for a wide range of pesticides in various food products [11].

Even though QuEChERS was originally developed for pesticide analysis, it has been applied successfully to the analysis of veterinary drugs [12] and aflatoxins in noodles [4], eggs [13], and honey [14].
Rice is a staple food for half of the world's population and even though it is considered a low risk food for aflatoxin exposure, contamination can still occur particularly in tropical regions. A survey of contamination of rice and rice by-products with aflatoxins reported that more than a third of rice samples exhibited detectable levels of aflatoxins [2]. Therefore, monitoring of aflatoxins in this important staple food cannot be neglected.
The goal of this study was to develop a modified QuEChERS method for the analysis of aflatoxins in rice, considering all naturally occurring aflatoxins (B1, B2, G1, G2) and using HPLC-FLD for detection, without derivatization. The method aims for a fast, environmentally friendly, and low cost detection of these mycotoxins in rice.

MATERIALS AND METHODS

Samples

Pesticide-free (organic) white rice (*Oryza sativa* L.) was purchased from a local supermarket in Daegu, Korea, and used for the validation and recovery experiments. For the analysis of market samples, six rice samples belonging to different varieties were analyzed; the samples comprised four basmati rice of Indian and Pakistani origins (three purchased in Korea and one purchased in Saudi Arabia), one Korean short-grain rice, and one Korean brown rice.

Chemicals

All solvents used were HPLC-grade. Methanol was obtained from Fisher Scientific (Seoul, Korea) and acetonitrile was obtained from Daejung Chemicals (Gyeonggi-do, Korea). The water used was from a Milli-Q water purification system (Millipore, Bedford, MA, USA).
Primary secondary amine (PSA) was purchased from Agilent (Little Falls, DE, USA), NaCl and anhydrous $MgSO_4$ were obtained from Samchun Pure Chemicals (Gyeonggi-do, Korea).
Analytical standards of aflatoxins B1, B2, G1, and G2 (purity > 98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA).
Stock solutions containing all analytes (1 $mg \cdot mL^{-1}$ of each) were prepared in acetonitrile, stored in the dark at -21 °C and used over a period of 3 months. Serial dilutions in acetonitrile were prepared to make working standard solutions at concentrations of 0.001 – 1 $mg \cdot mL^{-1}$.

Calibration solutions

Aflatoxin standards were prepared in acetonitrile at concentrations of 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 $\mu g \cdot kg^{-1}$. Measurements were conducted in triplicate.

QuEChERS sample preparation

Organic rice was ground in a blender (7011HS, Waring, USA) until a fine and homogeneous powder was obtained. A QuEChERS method [4, 10], with modifications, was applied. Ground rice (3.3 g) was weighed in a 50 mL Teflon centrifuge tube, and aflatoxins were spiked at concentrations of 6, 12, and 20 $\mu g \cdot kg^{-1}$. The spiked homogenates were stored in the dark at room temperature for 6 h to allow aflatoxin absorption prior to QuEChERS extraction. Water (6.6 mL) was added to the spiked rice, the tube was shaken for 3 min to obtain a homogeneous mixture, 10 mL of acetonitrile was added, and the tube was shaken again for 1 min. Subsequently, 4 g of anhydrous $MgSO_4$ and 1 g of NaCl were added, the tubes were shaken for 1 min and centrifuged at 4000 rpm and 4 °C for 5 min. An aliquot of 6 mL of the supernatant was transferred into a 15 mL Teflon centrifuge tube containing 150 mg PSA and 600 mg $MgSO_4$, subsequently shaken for 1 min and centrifuged at 4000 rpm and 4 °C for 5 min. Then, an aliquot of 1 mL of the supernatant was transferred into an Eppendorf tube, and 20 µL of this sample was injected into the HPLC. Five replicates were used to determine recoveries.

Safety considerations

Because aflatoxins are toxic and carcinogenic compounds, handling was performed carefully and nitrile gloves, disposable face mask, and safety glasses were worn for protection. Aflatoxin standards and working solutions were stored in the dark. Glassware was decontaminated by submerging in bleach (sodium hypochlorite) for 72 h prior to washing.

HPLC analysis

A Shimadzu LC-20A HPLC (Kyoto, Japan) equipped with a fluorescence detector and a Prominence autosampler was used for the analysis. Data were recorded using the LCsolution software (Shimadzu). The column was a Waters C18 (3.9 × 300 mm, 10 µm; Milford, MA, USA).
The mobile phase was water: methanol: acetonitrile (65 : 25 : 10, v/v/v), pumped in isocratic mode at a rate of 1 $mL \cdot min^{-1}$ for 30 min. The injection volume was 20 µL. The column was maintained at 30 °C and detection was performed at 360 nm excitation/ 450 nm emission wavelengths.

Method validation

The parameters determined were linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and recovery, applying published equations [15, 16].
To determine linearity, the concentrations of the aflatoxins were determined and the peak areas of the standards were recorded. Calibration equations were obtained by linear regression of the response versus concentration:

$$y = ax + b \quad (1)$$

where: y is the response, a is the slope, x is the concentration, and b is the intercept.
In this study, the LOD and LOQ were defined as 3 times the baseline noise and 6 times the baseline noise, respectively, in a part of the chromatogram close to the retention time of each analyte [4, 15].
The accuracy of the method was evaluated in terms of recovery, which was calculated using eqn. (2):

$$\%RE = \frac{Area\ of\ \ preextraction\ spike}{Area\ of\ \ postextraction\ spike} \times 100 \quad (2)$$

The intraday and interday precision of the method was evaluated in terms of repeatability (%RSD), and was calculated using eqn. (3):

$$\%RSD = \frac{STDEV\ \ Area\ of\ \ postextraction\ spike}{AVERAGE\ \ Area\ of\ \ postextraction\ spike} \times 100 \quad (3)$$

RESULTS AND DISCUSSION

Optimization of the method

QuEChERS extraction was initially performed in three ways: (i) omitting the dSPE clean-up step, (ii) including the dSPE clean-up step as described in the materials and methods section, (iii) using 9: 51: 40 (v/v/v) methanol: acetonitrile: water as described in a previous report [4]. Of these extraction methods, (ii) provided cleaner samples and was thus used in further experiments.
Because QuEChERS was originally developed to analyze pesticides in products with high water content such as fruits and vegetables, dry samples require the addition of water [17]; thus, here, we added water to 3.3 g of rice to obtain about 10 mL of slurry for extraction.
Two mobile phases were tested: 65 : 25 : 10 (v/v/v) and 70 : 20 : 10 (v/v/v) of water: methanol: acetonitrile; the former provided better separation in a shorter time, and was thus chosen for the study.
Regarding FLD detection, various excitation/emission wavelengths were evaluated for optimizing the detection of the aflatoxins: 365/440, 360/440, 365/425, and 360/450 nm. Among these, the 365/425 nm excitation/emission wavelengths provided the strongest fluorescence signal for aflatoxin B2, while yielding the weakest fluorescence signal for aflatoxins G2 and G1. The 360/440 nm wavelengths yielded the weakest fluorescence signal for aflatoxin B1. By contrast, the 360/450 nm excitation/emission wavelengths provided the strongest fluorescence signals for all aflatoxins, except B2 although the peak obtained was clear, and was thus selected as the most suitable combination for the simultaneous detection of all aflatoxins.
Therefore, complete separation of all aflatoxins was achieved using an isocratic elution with water: methanol: acetonitrile (65 : 25 : 10, v/v/v) as the mobile phase on a C18 column. The separation provided clear resolution in an analysis time of 30 min. Maximum absorption was attained at 360 nm excitation/450 nm emission wavelengths. The chromatogram of a rice sample spiked with 20 $\mu g \cdot kg^{-1}$ of aflatoxins compared with the chromatogram of a blank rice sample is shown in Figure 2. As can be observed, the

blank sample was mostly free from interfering peaks in the region of interest, although a small peak or baseline noise was observed at approximately 12 min.

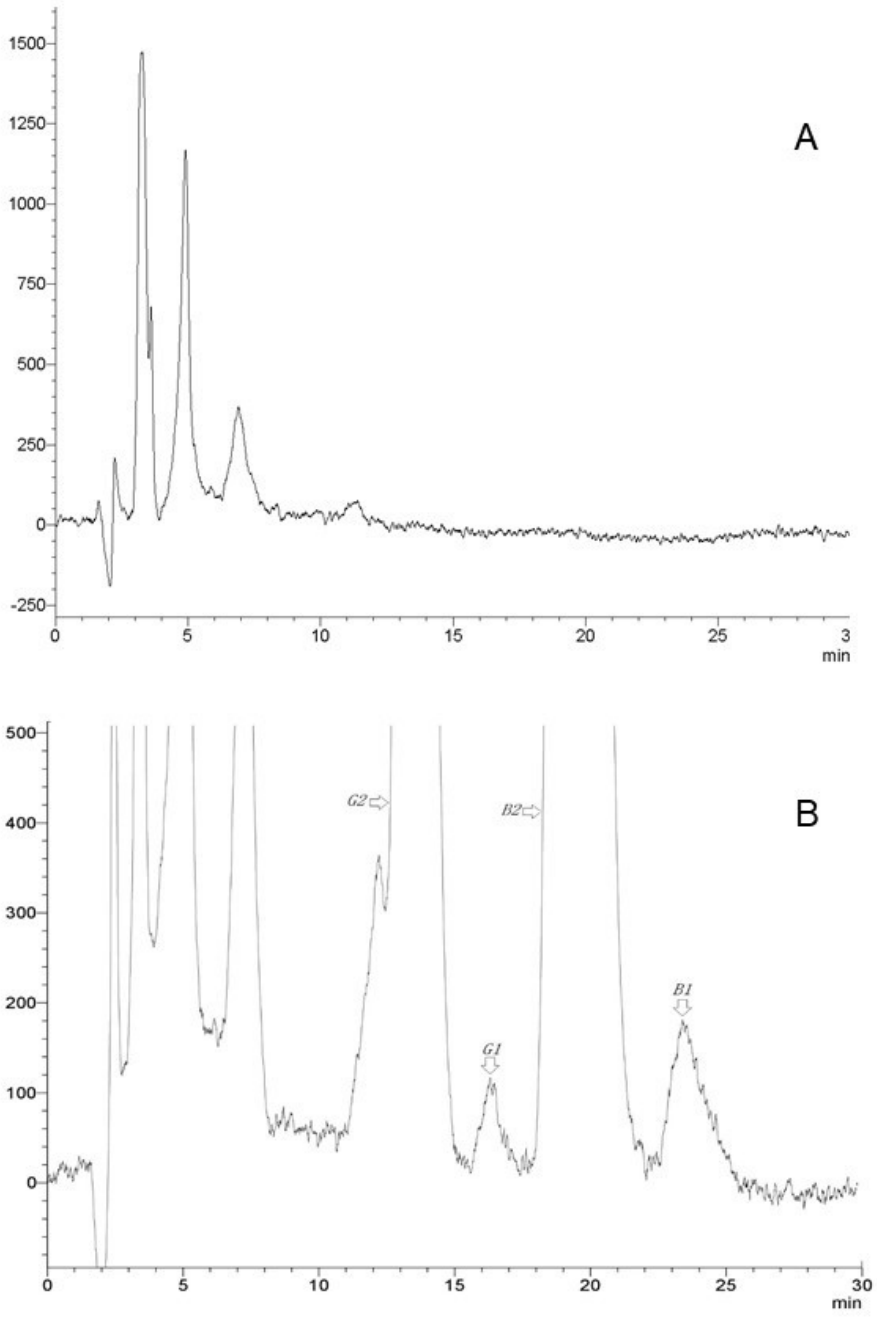

Figure 2. *HPLC-FLD chromatograms of (A) unspiked organic rice and (B) organic rice sample spiked with 20 µg·kg^{-1} of aflatoxins B1, B2, G1, and G2*

Method validation

Linearity

For constructing the calibration curves, aflatoxin concentrations equivalent to 4 – 40 µg·kg^{-1} in rice, spaced evenly, were prepared in acetonitrile and peak areas were

integrated. As a result, the calibration curves obtained were linear and showed good regressions (correlation coefficients (R^2) > 0.99) (Table 1).

Table 1. *Calibration data (4 – 40 µg·kg^{-1}), LODs, and LOQs of aflatoxins*

Aflatoxin	Calibration equation	R^2	LOD [µg·kg^{-1}]	LOQ [µg·kg^{-1}]
G2	y = 25138x - 5550.8	0.9984	0.30	0.80
G1	y = 299.44x - 299.07	0.9906	6.0	8.0
B2	y = 894.41x + 62.193	0.9982	0.05	0.15
B1	y = 1073.3x + 1434.4	0.9918	4.0	6.0

Limits of detection and quantification

The LODs and LOQs were estimated by spiking blank rice samples with a mixture of the four aflatoxins, and are summarized in Table 1. The LODs were in the range 0.05 -.0 µg·kg^{-1}, and the LOQs were in the range 0.15 - 8.0 µg·kg^{-1}. These values are higher than those reported in some other studies, probably because no derivatization step was included in our method. For example, Hassan *et al*. [18] reported LODs of 0.01 0.02 ng·g^{-1}, but the sample was derivatized prior to HPLC analysis. Another method that omitted the derivatization step attained LODs of 0.07 - 0.79 µg·kg^{-1} [4]. Using UHPLC-MS/MS, Koesukwiwat *et al.* [19] obtained LODs of 0.5 - 1 µg·kg^{-1}.

The maximum tolerated level (MTL) of aflatoxins in rice and other foods vary according to the country, ranging from 0 - 50 µg·kg^{-1} [20]. The European Union has established the lowest limits for aflatoxins, allowing 2 and 4 µg·kg^{-1} of aflatoxin B1 and total aflatoxins, respectively, in cereals for direct human consumption [21]. Elsewhere, MTLs for aflatoxin B1 vary from 5 µg·kg^{-1} in Russia to 10 µg·kg^{-1} in China, Japan, and Korea; limits for total aflatoxins are 20 µg·kg^{-1} in the United States and 30 µg·kg^{-1} in Brazil and India [6, 8, 22].

Therefore, the LODs and LOQs reported here are low enough to allow the application of the proposed method in several major countries including the US, China and India, although further increase in sensitivity would be necessary to allow its application in Europe. This could be achieved by including a concentration step before HPLC analysis.

Recovery

The accuracy and precision of the method were determined in terms of recovery and repeatability (% RSD), respectively. Samples were spiked with the mixture of aflatoxins at three different concentrations, and left in the dark for several hours to allow aflatoxin absorption into the rice matrix.

The intra and interday recoveries obtained for rice samples spiked with 6, 12, and 20 µg·kg^{-1} of aflatoxins (n = 5) are shown in Table 2. G2 exhibited intraday recoveries of 104 - 109 %, G1 of 107 - 128 %, B2 of 102 - 106 %, and B1 of 100 - 116 %. The interday recoveries for these four aflatoxins were in the range of 102 - 118 %. The intra and interday RSDs in all cases were 12 % or lower.

The acceptable recoveries for trace analysis are 70 - 120 % with % RSD ± 20 % [23]; thus, the values obtained for the aflatoxins are adequate.

Analysis of market samples

No samples were found to be contaminated with the investigated aflatoxins, suggesting that the rice available in the Korean market is safe for consumption. In 1991, approximately 3 % of the rice in Korea exhibited aflatoxin contamination, thus it is plausible that improvements in farming and storage and more stringent controls have further reduced the incidence of these mycotoxins in the rice sold in Korea [2].

Table 2. *Mean recoveries, intra and interday precision (% RSD) of the aflatoxins*

Aflatoxin	Concentration [µg·kg^{-1}]	Recovery (RSD) intraday [%]	Recovery (RSD) interday [%]
G2	6	109 (2.0)	108 (4.0)
	12	104 (3.0)	103 (3.0)
	20	104 (1.0)	102 (3.3)
G1	6	128 (12.0)	117 (8.9)
	12	123 (5.5)	102 (6.5)
	20	107 (1.1)	112 (8.9)
B2	6	106 (1.4)	107 (4.5)
	12	102 (1.9)	103 (4.7)
	20	105 (1.2)	103 (1.7)
B1	6	116 (1.8)	117 (2.5)
	12	116 (1.5)	118 (3.7)
	20	100 (3.3)	103 (0.4)

CONCLUSIONS

The proposed method allows simple and rapid determination of aflatoxins in rice, using QuEChERS and HPLC-FLD. It also allows fast, easy and inexpensive aflatoxin analysis without the need for derivatization. The LODs obtained are below the MTLs set by various major countries including the US, China and India, and thus the method could be used to monitor the occurrence of aflatoxins in this important food staple. Concentrating the sample and re-dissolving in a small amount of solvent is recommended to increase the sensitivity of the method.

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