SIMULTANEOUS DETERMINATIONS OF AFLATOXINS B_1, B_2, G_1, AND G_2 USING HPLC WITH PHOTODIODE-ARRAY (PDA) DETECTOR IN SOME FOODS OBTAINED FROM YOGYAKARTA, INDONESIA

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ABSTRACT

A method has been validated for simultaneous determinations of aflatoxins B_1, B_2, G_1 and G_2 using HPLC with photodiode-array (PDA) detector in some foods obtained from Yogyakarta. An optimum condition is reached using column ResolveTM C_{18} (150 mm x 3.9 mm i.d; 5 µm). The mobile phase used is a mixture of methanol-acetonitrile-water (17: 19: 63 v/v) delivered isocratically at 1.2 ml/min. All aflatoxins were detected at 365 nm. The samples of food (corn, rice, and pea nuts) were obtained from local markets and super markets in Yogyakarta. The sample was prepared by solid phase extraction using a Florisil cartridge.

The method revealed a good linearity for all aflatoxins evaluated by determination coefficients (r^2) > 0.99. The recoveries of aflatoxins B_1, B_2, G_1 and G_2 are 84.78; 82.14; 85.29; and 89.06 %, respectively. The detections limits are 13.20; 9.36; 7.54; and 6.75 µg/L. The RSD values of intra-day precisions are 0.38; 1.27; 3.09; and 1.76 %, respectively. The validated method has been used to quantify the level of flatoxins in foods.

Keywords: *Aflatoxin B_1, B_2, G_1 and G_2, HPLC, photodiode-array (PDA) detector, food*

INTRODUCTION

Aflatoxins (AFs) are a group of toxic, mutagenic, and carcinogenic secondary metabolites produced primarily by *Aspergillus flavus* and *A. parasiticus*. The most important members are aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 ($AFGB_1$), and G_2 (AFG_2) (Saleemullah *et al*., 2006). The chemical structures of aflatoxins are shown in Figure 1. Aflatoxins have been found to be potent naturally occurring carcinogens (Xiulan *et al.*, 2006) and it is classified by the International Agency of Research on Cancer (IARC) as Group 1 carcinogen (Zinedine *et al.*, 2006).

Several methods have been developed for determination of aflatoxins in some foods. Liu *et al.* (2006) has analyzed aflatoxins in food using HPLC with fluorescence detection. Kalcher *et al.* (2007) used post-column derivatization technique using bromine for determination of aflatoxin. The detection was carried out by fluorescence. Till now, the HPLC method using fluorescence detector is a method of choice for determination of aflatoxins. However, for purposes of method development and scientific reasons, it is necessary to propose an alternative method to quantify aflatoxins using photodiode-array detector.

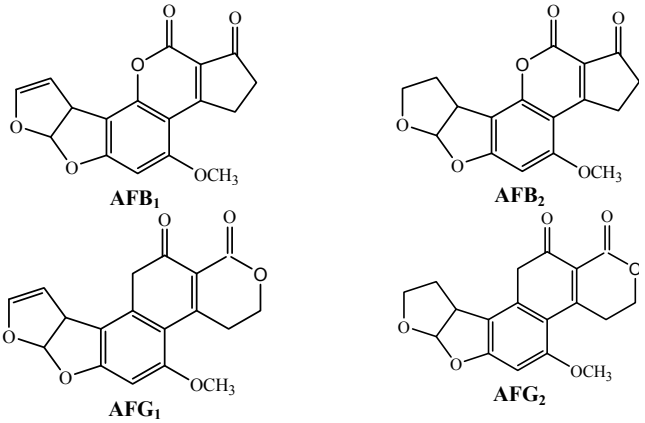

Figure 1. The chemical structures of Aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2).

MATERIALS AND METHODS

Materials

Samples of rice, corn, and bean are obtained from local markets and supermarkets in Yogyakarta, Indonesia in July 2007. Aflatoxins B_1, B_2, G_1 and G_2 were purchased from

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Sigma Co. Methanol and acetonitrile were purchased from E. Merck, Darmstat, Germany.

Extraction and Purification

Extraction and purification used in this study were performed according to Liu *et al.* (2006) with slight modifications. An amount of 25 g sample (corn, rice, and pea nut) was grounded to a fine powder. A-125 ml of chloroform was added with constant oscillation at 100 rpm for 30 min. The organic extract was further purified using a chromatography column primed with Florisil. The solutions of chloroform–hexane (1:1) and chloroform–methanol (9:1) were added sequentially to eliminate impurities from the samples, and chloroform–methanol (1:9) was used to elute the aflatoxins. The eluate was dried under a hood. Finally, the extracted toxins were dissolved in 200 ml of mobile phase in a glass vial for further HPLC analysis. The eluate was filtered through a 0.45 µm membrane filter before injection into chromatographic system.

Chromatographic Conditions

The HPLC instrument (Knauer) was equipped with pump (Smartline pump 1000), autosampler (Smart line 3800), and interface (Smartline Manager 5000). The detection was carried out by photodiode-array at 365 nm. The column used is ResolveTM C_{18} (150 mm x 3,9 mm i.d; 5 µm) using a mobile phase consisting of a mixture methanol-acetonitrile-water (17: 19: 63 v/v) with flow rate 1.2 ml/min.

Method Validation

Method validation was carried out by performing several tests according to ICH (international conference on harmonization) i.e. linearity, precision, sensitivity (limit of detection and limit of quantification), and accuracy.

For linearity determination, calibration graphs were established with five different concentrations of each aflatoxins.

System precision was performed by injecting six times of aflatoxin standars into HPLC. RSD value of retention times and chromatogram areas of each aflatoxins was used as parameters to evaluate its precision.

Sensitivity of analytical method was evaluated by determination a limit of detection (LOD) and limit of quantification (LOQ). LOD was defined as the concentration of analyte that gives a chromatogram with signal to noise (S/N) ratio of 3; whereas LOQ was the lowest amount of analyte with signal to noise (S/N) ratio of 10.

Accuracy was evaluated by analyzing blank matrices spiked with known quantities of aflatoxins in the linear concentration range and subjected to extraction and purification and injected in HPLC systems. The percentage of recovery was calculated for each aflatoxins evaluated.

RESULTS AND DISCUSSIONS

Method Optimization

Today, the development of analytical method is usually based on the existing literature using the same or quite similar instrumentation. It is rare today that an HPLC-based method is developed which does not relate or compare to the existing method or the existing literature (Swartz and Krull, 1997).

Figure 2 showed the 2D and 3D of the chromatographic separation of standard aflatoxin G_1, G_2, B_1, and B_2 using photodiode-array detector at 365 nm . The column used was ResolveTM C_{18} (150 mm x 3,9 mm i.d; 5 µm) with mobile phase consisting of a mixture of methanol-acetonitrile-water (17: 19: 63 v/v) with flow rate 1.2 ml/min. The Chromatogram revealed that all aflatoxins evaluated are well separated each other.

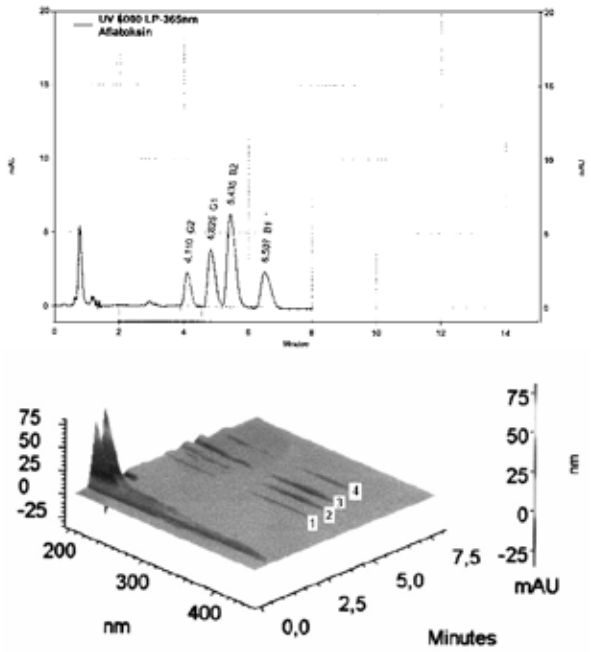

Figure 2. **A**. HPLC Chromatogram of two dimension (2D) and **B**, three dimension (3D) obtained from separation of aflatoxins. Column: ResolveTM C_{18} (150 mm x 3,9 mm i.d; 5 µm); mobile phase: Methanol-acetonitriel-water (17: 19: 63 v/v/v); flow rate: 1.2 ml/min; Vol. injection: 20 µl; Detector: photodiade-array at 365 nm. 1 = AFG_2 (0.30 µg/ml), tr = 4.11; 2 = AFG_1 (0.31 µg/ml), tr = 4.83; 3 = AFB_2 (0.16 µg/ml), t_r = 5.43; and 4 = AFB_1 (0.11 µg/ml), tr = 6.51.

Method Validation

Linearity test of the photodiode array detector was performed using external standard as shown in Table 1. The working standard solutions of aflatoxins were prepared three replicates at concentration ranges in Table 1. Linear calibration curves were obtained by plotting the peak area against the concentration of each aflatoxins.

Table 1. Linearity test of aflatoxins

Aflatoxin	Working concentration range (µg/ml)	Linier regression	r^2
B_1	0.11 – 0.33	y = 274.03x – 4899.7	0.9992
B_2	0.16 – 0.50	y = 451.43x – 15170.0	0.9962
G_1	0.31 – 0.94	y = 126.02x – 10006.0	0.9923
G_2	0.30 – 1.01	y = 70.09x – 8326.7	0.9907

All aflatoxins evaluated revealed a good linearity with coefficient correlation (r^2) > 0.99. Furthermore, the regression linier can be used to quantify the concentration of aflatoxins in food samples.

The precision of aflatoxins was evaluated by calculating a RSD value of retention times and chromatogram areas from six replications (Table 2). The RSD value of retention times and chromatogram areas is below 2.00 %; therefore it can be stated that the developed method is precise.

Table 2. The precision of aflatoxins (n = 6)

Aflatoxin	RSD value (%)	
	Retention times	Chromatogram area
B_1	0.38	1.23
B_2	1.05	0.96
G_1	0.86	1.35
G_2	0.45	0.75

The sensitivity of developed method was evaluated by its limit of detection (LOD) and limit of quantification values. LOD was assayed as the analyte concentration giving a chromatogram with signal to noise (S/N) ratio of 3; whereas LOQ was the lowest analyte concentration having signal to noise (S/N) ratio of 10. The LOD and LOQ values were shown in Table 3.

Recovery test was performed by adding an amount of aflatoxins standard in the blank samples. The spiked samples were further processed as in the sample preparation. All determination is performed in three replicates. The recovery results were shown in Table 3. From Table 3, it can be stated that the proposed method is sensitive enough. Also, the method revealed a good accuracy with average recovery ranges from 71.80 to 90.30 %.

Tabel 3. Limit of detection (LOD), limit of quantification (LOQ) and recovery of aflatoxin B_1, B_2, G_1, dan G_2

Aflatoxin	LOD (µg/ml)	LOQ (µg/ml)	Recovery (%)
B_1	0.013	0.043	90.30
B_2	0.008	0.263	85.25
G_1	0.030	0.094	77.87
G_2	0.051	0.169	71.80

Analysis of Aflatoxins in Some Foods

The validated method was further used to analyze the aflatoxin contents in some food obtained from local and super markets in Yogyakarta. An example of chromatogram obtained from sample of RT_1 (*see* abbreviation for Table 4 below) was shown in Figure 4. The level of aflatoxins was shown in Table 4.

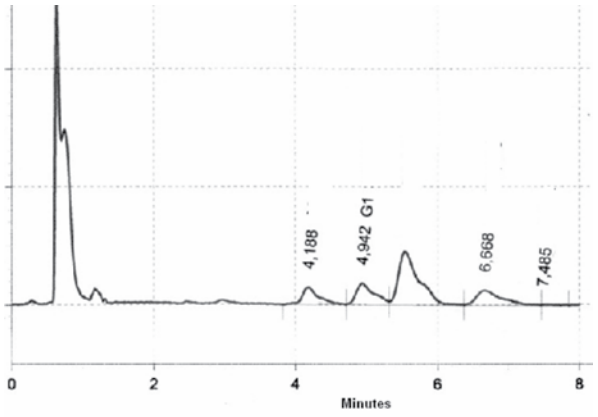

Figure 4. Chromatogram obtained from sample of RT1. For chromatographic conditios, *see* Figure 2.

Table 4. The aflatoxin content of some foods

Sample	Level of aflatoxins $\overline{X}$ ± SD (µg/kg) (n=3)			
	B_1	B_2	G_1	G_2
RT_1	n.d	n.d	2.72 ± 0.02	n.d
RT_2	1.12 ± 0.02	1.12 ± 0,02	1.12 ± 0,03	n.d
RT_3	n.d	3.45 ± 0.04	n.d	1.18 ± 0.03
RS_1	n.d	n.d	n.d	n.d
RS_2	n.d	n.d	n.d	n.d
CT_1	2.42 ± 0.03	n.d	2.42 ± 0.03	2.42 ± 0.03
CT_2	n.d	n.d	n.d	n.d
CT_3	n.d	n.d	n.d	n.d
CS_1	1.07 ± 0.03	n.d	n.d	1.07 ± 0.03
CS_2	n.d	2.05 ± 0.05	n.d	n.d
NT_1	n.d	n.d	n.d	n.d
NT_2	n.d	n.d	2.14 ± 0.06	n.d
NT_3	n.d	n.d	n.d	n.d
NS_1	n.d	1.85 ± 0.07	n.d	n.d
NS_2	n.d	n.d	n.d	n.d

RT = rice from traditional market; RS = rice from super market; CT = corn from traditional market; CS = corn from super market; NT = nut from traditional market; NS = nut from super market. For traditional market, 1 = Demangan; 2 = Kranggan; 3 = Beringharjo. For supermarket, 1= Mirota kampus; 2 = Alfa.
n.d = *not detected*

There is no report available describing the maximum amount of aflatoxins in Indonesia; however, some developing countries like Cina and Malaysia have set up the maximum allowed levels of aflatoxin in foods (Table 5).

Table 5. Maximum amount of aflatoxins allowed in foodstuffs in some countries in Asia (unit μg/kg) for human consumption and for trading (Liu *et al*., 2006).

Country	Aflatoxin (unit μg/kg)
China	20
India	30
Japan	10
Malaysia	35

An investigation on aflatoxin contamination in traditional and super markets in Yogyakarta showed that the average aflatoxin content (the highest concentration found is 3,45 ± 0,04 μg/kg) was much lower than the maximum aflatoxin contents allowed in some countries in Asia.

CONCLUSION

An HPLC method with photodiode-array detector (PDA) using reversed phase system has been developed and validated to determine the aflatoxin contents in some foods. The average level of aflatoxins found in some foods is lower than the allowed concentration in some developing countries like Malaysia and China.

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