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# Analysis of aflatoxins in herbal medicine and health functional foods

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ABSTRACT

Analytical methods of aflatoxins (AFs: $B_1$, $B_2$, $G_1$ and $G_2$) in herbal medicines (HMs) and health functional foods (HFFs) were optimized, which were used to analyze the representative samples that were directly collected from the users (total 2348 examinees) of HMs and HFFs. Two analytical methods, trifluoroacetic acid and Kobra cell derivatization methods, were compared; The latter was selected based on high linearity and sensitivity. The limits of detection of AFs using the Kobra cell method were 0.07–0.32 ng $g^{-1}$. Recoveries of AFs using various matrixes such as solid, semi-solid, liquid samples and CRM were 81.81–119.87%. The *Z*-score and linearities of calibration curves were 0.53 and 0.9996–0.9999, respectively. Among 241 samples, only *Angelica gigas NAKAI* extract products (2 products) were detected to have 7.93 and 5.70 ng $g^{-1}$ of aflatoxin $G_2$.



## 1. Introduction

Aflatoxins (AFs) are secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* (Romagnoli, Menna, Gruppioni, & Bergamini, 2007). Both fungal growth and AF production depend on a variety of physical, chemical, and biological factors (Bacaloni et al., 2008). These metabolites have high toxicity including carcinogenic, teratogenic and mutagenic properties (Ali et al., 2005; Cho et al., 2007). Among 20 types of aflatoxins, 4 types of AFs including $B_1$, $B_2$, $G_1$, and $G_2$ commonly occur in the natural environment (Bacaloni et al., 2008). Aflatoxins are converted into aflatoxin adducts, aflatoxin $M_1$ and $Q_1$, by a human metabolizing system (Bacaloni et al., 2008). Aflatoxin $B_1$, $M_1$, $G_1$, $M_2$, $B_2$, and $G_2$ have high toxicity and aflatoxin $B_1$ and $G_1$ have higher biological toxicity. International Agency for Research on Cancer (IARC) classified aflatoxin $B_1$ as Group 1 carcinogen (carcinogenic to humans) (IARC, 1993).

Aflatoxins occur in grains and herb medicines in humid conditions (Cho et al., 2007). Many studies on AFs have been conducted in foods, feeds and herb medicines (Reiter, Zentek, & Razzazi, 2009). In particular, herb medicines were reported as the main source of AFs ingestion (Bahk & Marth, 1983). According to previous studies, AFs-producing-microorganisms were detected in 42.9% of botanical herbs in Brazil (Bugno, Almodovar, Pereira, Pinto, & Sabino, 2006). Weaver and Trucksess (2010) reported 0.5 ng $g^{-1}$ of aflatoxins in the medicine from *Piper methysticum G. Forst*. In Malaysia and Indonesia, the levels of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ were reported to be in the range of 0.03–1.57 ng $g^{-1}$ in commercial traditional herb medicines (Ali et al., 2005). Previous studies reported that 1–7% of medicinal herbs and herbal medicines (HMs) in Korea contain various aflatoxins (Kim et al., 2010; Park et al., 2009). Botanical dietary supplements also have a possibility of containing hazardous residues such as AFs (Patwardhan & Mashelkar, 2009). Tassaneeyakul, Razzazi-Fazeli, Porasuphatana, and Bohm (2004) analyzed 28 herbal medicinal products in Thailand and detected AFs ranging from 1.1 to 14.3 ng $g^{-1}$ in 5 samples (Tassaneeyakul et al., 2004). Trucksess et al. (2009) analyzed 194 ginger dietary supplement capsules and reported the total AFs contamination mean and median levels of 7.34 and 7.19 ng $g^{-1}$, respectively. In Korea, dietary supplements are classified into Over The Counter (OTC) and Health Functional Food (HFF) by law. To date, there have been a few studies regarding hazardous residues such as AFs in HFFs. In addition, the analytical methods have not been compared for the analysis of AFs in HFFs and HMs.

Analytical methods of AFs using HPLC–FLD with derivatization by trifluoroacetic acid (TFA) and Kobra cell (bromination) were reported in previous studies (Reiter et al., 2009). In the present study, two analytical methods (TFA and Kobra cell derivatization methods) of aflatoxins (AFs: $B_1$, $B_2$, $G_1$ and $G_2$) in HMs and HFFs were optimized, which were used to analyze the representative samples that were directly collected from the users (total 2348 examinees) of HMs and HFFs. Various validation factors such as recovery rate, coefficients of variation (CV) values, *Z*-score, and limit of detection (LOD) were conducted for the accurate analysis of

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AFs in this study. Recoveries were conducted by solid, semi-solid, and liquid phases and certified reference material (CRM) for accurate validation of botanical HFFs in various forms such as pills, oils etc.

## 2. Experimental

### 2.1. Regents and materials

Aflatoxins standard mixture ($B_1$, $B_2$, $G_1$ and $G_2$, R-Bio pharm, Glasgow, Scotland) with methanol (J.T. Baker, Philipsburg, NJ, USA) was prepared as a standard solution. An immuno-affinity column (Alfarhone wide, R-bio pharm, Glasgow, Scotland) was used for the clean-up sequence. Water, acetonitrile and methanol were of HPLC grade (J.T. Baker, Philipsburg, NJ, USA). Trifluoroacetic acid (TFA, Sigma–Aldrich, MO, USA) and Kobra cell (R-Bio pharm, Glasgow, Scotland) were prepared for derivatization. All experimental supplies were disposable products for the safety of the experimenter. Certified response material (CRM, corn, 19 ± 2.3 ng $g^{-1}$, Aflatoxin $B_1 + B_2$, Trilogy A-C 720, USA) was used for validation.

### 2.2. Sampling

Samples were collected nationwide except Jeju Island in South Korea. Subjects were comprised of 1015 adults (over 19 ages), 557 children and youths (7–18 ages), 488 infant and toddlers (0–6 ages) with 288 their parents. Sampling was conducted through dietary surveys. Samples were classified as HFF, herbal medicine, generic medicine and so on by MFDS's HFFs and drugs database. Among the HFF and herbal medicine samples, 241 plants source samples were selected by MFDS database (MFDS, Foodnara). A total of 241 samples (185 HFF and 56 herbal medicines) were collected from HFFs and herbal medicines users. The amber colored sampling bottles were cleaned in $H_2SO_4$ (Dong Woo Fine-Chem, Iksan, Korea) for 24 h, upon they were rewashed in ultrapure deionized water and air dried. After collecting the samples, they were stored in freezer (−20 °C) until the analysis.

### 2.3. Validation of the analytical method for aflatoxins

Recoveries, CV (coefficient of variation) values, *Z*-score, limit of detection (LOD), limit of quantitation (LOQ), linearity and *R*-squared ($R^2$) were conducted for the validation of the analytical method. Recoveries and CV were performed by solid, semi-solid, liquid sample matrixes, and CRM. *Z*-score was calculated by CRM (corn, 19 ± 2.3 ng $g^{-1}$, Aflatoxin $B_1 + B_2$) by dividing the disparity between certified concentrations and mean values of measured AFs by the standard deviation of measured AFs. LOD of aflatoxins was carried out by 3.3 × sigma ($\sigma$)/slope factor of the calibration curve. Sigma was obtained by the standard deviation of the *y*-intercept of 7 specific calibration curves. Each specific calibration curve was constituted by 3 point concentrations. Linearity and $R^2$ were calculated by each standard curve of AFs.

### 2.4. Extraction of aflatoxins and clean-up procedure

Two gram samples with 20 ml of 70% methanol (v/v) were put into a 50 ml conical tube. The tube was shaken at 250 rpm for an hour. It was then centrifuged at 160 g (10 min) and filtrated by a filter paper (110 mm × 100 circles, Whatman, UK). Ten-milliliter filtered solutions were diluted by 20 ml of water. The 10-ml diluted extracted solution was ran in the immuno-affinity column. Ten milliliters of water was added for cleaning, and then 1 ml of methanol was eluted in columns for AFs elution.

### 2.5. Derivatization of aflatoxins by trifluoroacetic acid

The eluted AF extracts were dried up by nitrogen gas at 40 °C. For derivatization, 0.2 ml of TFA with 1 ml of hexane was added and stayed for 20 min in the dark. After that, the solution was messed up to 1 ml with 50% (v/v) methanol, mixed by vortex, and the bottom layer was injected for analysis.

### 2.6. Analytical condition of HPLC–FLD analysis

Waters HPLC 1525 system (Milford, MA, USA) and 474 fluorescence detector (Milford, MA, USA) were used. For the TFA method, the mobile phase was acetonitrile:water (7:3, v/v) and the column was Agilent XDB-C18 (250 mm*4.6 mm and 5 μm: Palo Alto, CA, USA). The wavelengths for excitation and emission were 360 nm and 450 nm, respectively. For the Kobra cell method, the mobile phase was a mixture of water, methanol and acetonitrile (6:2:2, v/v) with 119 mg KBr (0.001 M KBr) and 350 μl of 4 M nitric acid (1 L mobile phase). The column was Agilents Sb-Aq (250 mm*4.6 mm and 5 μm: Palo Alto, CA, USA). The wavelengths for excitation and emission were 365 nm and 435 nm, respectively. In both conditions, the column temperature was 40 °C, and the flow rate was 1 ml/min. The injection volume was 20 μl.

## 3. Results and discussion

### 3.1. Method validation for the analysis of aflatoxins

Method validation factors of AFs are shown in Table 1. The Kobra cell method had lower LOD than the TFA method. In particular, LOD of aflatoxin $G_2$ in TFA was higher than that in the Kobra cell method by 32 times. Limit of detection of aflatoxin $G_2$ in TFA and Kobra cell method were 2.28 and 0.07 ng $g^{-1}$, respectively. Coefficient of correlation was closer to 1.00 in the Kobra cell method. Chromatograms of aflatoxins are shown in Fig. 1. The baseline of the chromatogram of the Kobra cell method was more stable than that of the TFA method. In addition, the resolution and sensitivity (AF $B_1$ and $G_1$) of the Kobra cell method was better than those of the TFA method.

Based on the method validation results, the Kobra cell method was selected for further analysis in this study. Recovery rates of each matrix and CRM are listed in Table 2. Recoveries and coefficient of variation (CV) of solid, semi-solid and liquid were 81.81–119.46% and 1.18–9.52%, respectively. Recovery rate, CV and *Z*-score of CRM were 93.39%, 2.39% and 0.53, respectively. In previous studies, the recovery rates of AFs using Kobra cell method were 50.0–100% (Reif & Metzger, 1995; Romagnoli et al., 2007; Zhang, Liu, & Chen, 2005). Also, CV was 1.5–9.7% (Zhang et al., 2005), respectively. In the LC–MS/MS methods, LOD, LOQ, the recoveries

**Table 1**
Limit of detection (LOD), limit of quantification (LOQ), linearity and *R*-squared ($R^2$) of the analysis of aflatoxins using TFA and kobra cell methods.

| | Aflatoxin types | LOD (ng $g^{-1}$) | LOQ (ng $g^{-1}$) | Linearity | $R^2$ |
|---|---|---|---|---|---|
| Kobra cell | $B_1$ | 0.32 | 0.96 | $y = 9284.3x - 86{,}00.4$ | 0.9998 |
| | $B_2$ | 0.22 | 0.66 | $y = 16{,}511x - 12{,}745$ | 0.9999 |
| | $G_1$ | 0.25 | 0.75 | $y = 7238x - 7520.7$ | 0.9999 |
| | $G_2$ | 0.07 | 0.21 | $y = 7872.5x - 5020$ | 0.9999 |
| TFA | $B_1$ | 0.32 | 0.95 | $y = 2429.8x + 2617$ | 0.9969 |
| | $B_2$ | 0.59 | 1.76 | $y = 29{,}566x + 82{,}367$ | 0.9880 |
| | $G_1$ | 0.49 | 1.48 | $y = 1108.3x + 2220.5$ | 0.9993 |
| | $G_2$ | 2.28 | 6.83 | $y = 18{,}522x + 19{,}539$ | 0.9878 |

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