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Extraction and Purification of Aflatoxins in Bagasse for HPLC Fluorescence Determination

Yin Min Htun[1,2], **Hiroyuki Nakagawa**[2,3] **and Masayo Kushiro**[2*]

[1]*Department of Agriculture, Ministry of Agriculture, Livestock and Irrigation, Nay Pyi Taw, Myanmar*

[2]*Food Research Institute, National Agriculture and Food Research Organization (NARO), Tsukuba-shi, Ibaraki, Japan*

[3]*Advanced Analysis Center, Food Research Institute, NARO, Tsukuba-shi, Ibaraki, Japan*

[*]**Corresponding author:** Masayo Kushiro, Food Research Institute, National Agriculture and Food Research Organization (NARO), Tsukuba-shi, Ibaraki, Japan, Tel: +81298388037; E-mail: kushirom@affrc.go.jp

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Abstract

Using a multifunctional column which consists of lipophilic and charged active sites, a simple extraction and purification method with quick and accurate analysis of aflatoxins (B_1, B_2, G_1 and G_2) from sugarcane bagasse was developed by spike and recovery tests. After extraction with a mixture of acetonitrile and water (9+1, v/v) followed by clean-up method through MultiSep #226 multifunctional column (MFC), aflatoxins were detected via high-performance liquid chromatography with fluorescence (HPLC-FL) analysis. Recovery rates of each aflatoxin B_1, B_2, G_1 and G_2 spiked to the bagasse at different levels were 81.5 ± 3.3%, 87.5 ± 0.7%, 80.2 ± 4.3% and 86.4 ± 4.5% in samples spiked with 5 ng AF mix/g, and 83.1 ± 1.2%, 89.4 ± 2.0%, 81.0 ± 4.3% and 87.2 ± 2.1% in samples spiked with 10 ng/g, respectively. It is concluded that this proposed method is applicable for the practical determination of aflatoxins contamination in bagasse.

Keywords: Bagasse; Multifunctional column; Extraction method; HPLC-FL

Introduction

Aflatoxin (AF), a family of mycotoxin, is poisonous carcinogen that is produced by certain fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* which grow in soil, decaying vegetation, hay and grains [1]. AFs often contaminate food and agricultural products which lead to a health hazard to humans as well as animals. The most abundant AFs are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). Since they are dangerous food contaminants, various countries world-wide have established the limit and regulatory demands on the level of aflatoxins permits in traded commodities [2].

Sugar is an important nutritious food all over the world. Sugarcane was introduced to the Okinawa and Kagoshima, Japan in the 17th century from China, and it became an important crop in the regional economy of the Southwestern Islands of Japan [3]. Sugarcane is susceptible to microbial contamination due to its richness in carbohydrates with a higher water activity and it favors several fungi to appear the visible colonization and mycotoxin contamination [4]. Sugarcane bagasse can be converted to ethanol by enzymatic or acid catalyzed hydrolysis and fermentation of the released saccharides [5]. Recently, sugarcane bagasse has got an attention as a new food material rich in dietary fiber. Daily intake of total dietary fiber, non-digestible carbohydrates, and lignin that are intrinsic and intact in plants, was recommended as 20-25 g in Japan [6]. Tanaka et al. reported that 20 g/day of dietary fiber increases fecal weight, leading to better bowel movement [7,8]. The bagasse which can also mix with rice and drinks, have effect to adsorb the oil entering the body and discharge it out of the body. However, slight natural contamination of AFs in sugarcane has been occasionally found in Japan. Iwaya et al. also suggested that the contamination of AFs in brown sugar was derived from sugarcane [9]. Important fungi that produce aflatoxins such as *A. flavus* (18%) and *A. parasiticus* (65%) were discovered in sugarcane or soil of production plots in Japan [10,11]. Up to now, no study has attempted to examine the presence of aflatoxigenic fungi nor aflatoxins in the bagasse.

In terms of AFs analyses, the methods using multifunctional column (MFC) are more useful and safer than conventional method that consisted of extraction with chloroform and purification using silica or florisil [12-15], whereas a Vicam Aflatext immunoaffinity column was used for a clean-up method of AFs from creamy and crunchy peanut butter [16,17], cattle feed [18], corn [19] and baby food [20]. The MFC method was applied to analyze AFs in several spices [21], nuts, corn, cereals, spice and black teas [22] using combination of MFC clean-up and an affinity column clean-up. There is, however, no report yet about the extraction and purification of AFs from bagasse using MFC cartridge. In previous study, Zheng et al. reported the analytical method using MultiSep #226 for the determination of Fusarium mycotoxin zearalenone from edible oils [23]. In this study, a simple extraction and purification method with fast and reliable analysis of aflatoxins (B_1, B_2, G_1 and G_2) from sugarcane bagasse was described.

Methodology

Samples, materials and reagents

A product of dry fermented sugarcane fiber so called "bagasse" which was available on the market was purchased from food stores. The sample was kept in desiccator until use. Standard aflatoxins B_1, B_2, G_1 and G_2 crystalline materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The stock standard solution of aflatoxins with 50 ng/ml was prepared in acetonitrile and was stored in freezer at -20°C in the dark. A MultiSep #226 multifunctional column (MFC)

was purchased from Romer Labs®, Inc. Union, MO, USA. All reagents such as acetonitrile, methanol and distilled water were HPLC grade.

Extraction and purification

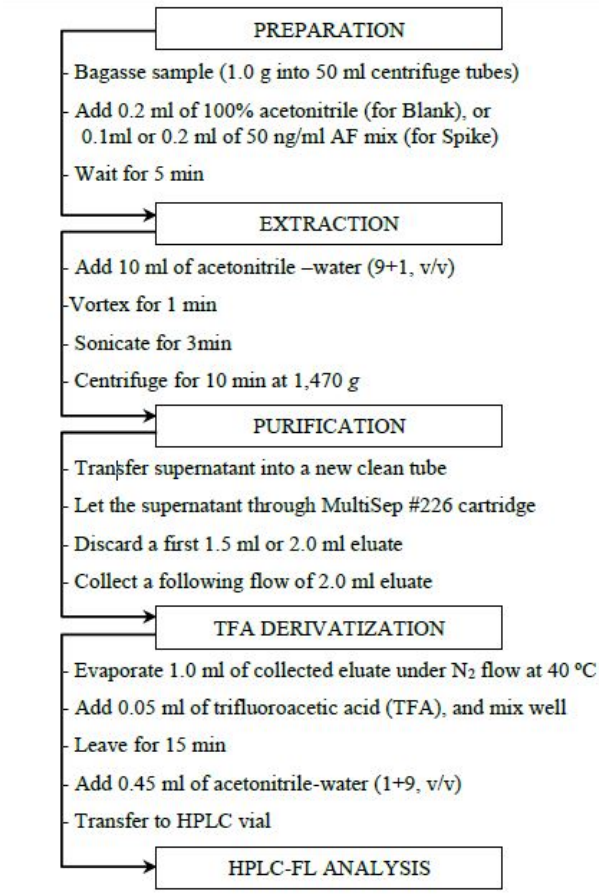
For extraction and purification of AFs (shown in Scheme 1), 1 g of bagasse was measured in a 50 ml centrifuge tube and extracted with 10 ml of acetonitrile and water (9+1, v/v). The sample was vortexed for 1 minute and sonicated for 3 minutes. Then the mixture was centrifuged at 1,470 g for 10 minutes. The supernatant was loaded onto a MultiSep #226 MFC for the purification of AFs and passed through at a gravity flow. The first 1.5-2.0 ml of eluate was removed, and the following 2.0 ml was collected in a new clean test tube. A 1.0 ml of aliquot of the collected solution was transferred into another test tube and was evaporated at 40°C under a gentle flow nitrogen gas. The dried residue was dissolved in 50 μl of trifluoroacetic acid (TFA) and was vortexed shortly. After keeping at room temperature for 15 minutes, 450 μl of acetonitrile and water (1+9, v/v) was added, and the sample was directly injected into HPLC-FL system (Shimadzu, Kyoto, Japan).

Determination on HPLC

HPLC-FL analysis was performed using a Capcell Pak C18 column of 3.0 × 250 mm with a 5-µm particle size (Osaka Soda, Osaka, Japan). The mobile phase was composed of acetonitrile, methanol, and water (10+30+60, v/v/v), and used with a flow rate of 0.3 ml/min. The column heater was set at 40°C, and the injection amount was 5 µl. The AF-TFA derivatives were detected at wavelength of 365 nm (excitation) and 450 nm (emission). Six standard concentrations of AFs working solution, covering a range from 1 to 20 ng/ml (1, 2, 5, 10, 15, and 20 ng/ml), were employed, and calibration curves were plotted with peak areas against the concentration of AFs (ng/ml). The linearity of the calibration plot was demonstrated by calculating the correlation coefficient. The limit of detection (LOD) was defined as the concentration that was three times higher than the standard deviation of the blank signal. The limit of quantification (LOQ) was set as three times higher than the value of LOD.

Spike and recovery tests of aflatoxins

For simple in-house validation of the analytical method for AFs, spike and recovery tests were performed for a blank (without matrix) in triplicate (n=3). The spike levels were set as 5 ng/g and 10 ng/g of AFs mixture (AFB_1, AFB_2, AFG_1 and AFG_2) standard, since EFSA's Panel on Contaminants in the Food Chain (CONTAM) announces a statement on the possible effects on human health in the maximum permitted levels of aflatoxins of 4 ng/g to 10 ng/g, and the regulation level in Japan is 10 ng/g for total AFs (B_1, B_2, G_1 and G_2). The procedure for sample extraction and cleanup of AFs is described in 2.2 (Scheme 1).



Scheme 1: Analytical method for aflatoxins in bagasse.

Results and Discussion

Analytical procedure for AFs in food and agricultural products is being normalized world-wide. However, the analytical method for extraction and purification of AFs in bagasse using MFC has never been established. In this study, the analytical method for AFs in bagasse sample was in-house validated using blank and spike analyses. As shown in Table 1, AFB_1 was observed in blank samples as 1.55 ± 0.06 ng/g whereas AFB_2, AFG_1 and AFG_2 showed less than LOD. Since the observed quantity of AFB_1 is higher than LOQ, the naturally contamination level was not negligible. Therefore, in this study, the recovery ratio of AFB_1 was calculated after subtraction of naturally contaminated level. Previously, Iwaya et al. reported that the contamination of AFB_1 in brown sugars made in Kagoshima was detected as 0.14~1.63 ng/g in 2013 [9] and 0.10~1.31 ng/g in 2014 [24], suggesting it derived from fungi during growth. A study from seven islands of Okinawa and Kagoshima Prefectures showed that aflatoxigenic fungi were detected in 74% of 53 soil samples from sugarcane fields [11]. The adherence of aflatoxigenic fungi in the bagasse sample used in the present study is also indicated.

Aflatoxins	Amounts of AFs in bagasse (ng/g)	LODa	LOQb
AFB_1	1.55 ± 0.06	0.49	1.47

AFB_2	<LOD	0.44	1.32
AFG_1	<LOD	0.52	1.56
AFG_2	<LOD	0.28	0.84

Table 1: Observed amount of AFs (mean ± standard error; n=3) of a bagasse sample used in this study.

Spiked level (ng/g)	AFB1	AFB2	AFG1	AFG2
5	81.5 ± 3.3	87.5 ± 0.7	80.2 ± 4.3	86.4 ± 4.5
10	83.1 ± 1.2	89.4 ± 2.0	81.0 ± 4.3	87.2 ± 2.1

Table 2: Recoveries of AFs (mean ± standard error; n=3).

For all samples, the recovery rates were ranging from 80.2% to 87.5% for spiked level of 5 ng/g, and from 81.0% to 89.4% for spiked level of 10 ng/g (Table 2). These values were similar to the results using MycoSep #224 in various nuts and cereals [25], to the results (greater than 83%) using MultiSep #228 in spices [21], and in line with the results (70% - 110%) adapted from Instituto Adolfo Lutz (IAL) extraction with chloroform in wheat grain [26]. The recovery ratios obtained in this study were from 80% to 90%, which is within the range of food GLP, Japan.

There were no substantial differences in the recovery rates of two spiked samples, indicating that almost all of AFs were extracted with a mixture of acetonitrile-water (9+1, v/v). Moreover, the relative standard deviations were between 0.7% and 4.5%, which were in line with the validation criteria (below 10%) [27]. On the other hand, the first eluate 1.5 ml or 2.0 ml should be discarded because the recovery rates of discarded eluate were always under 70% (data not shown). As a result, the extraction and purification procedure for AFs from the sugarcane bagasse used in this study is applicable for the practical determination of AFs in bagasse (Figure 1).

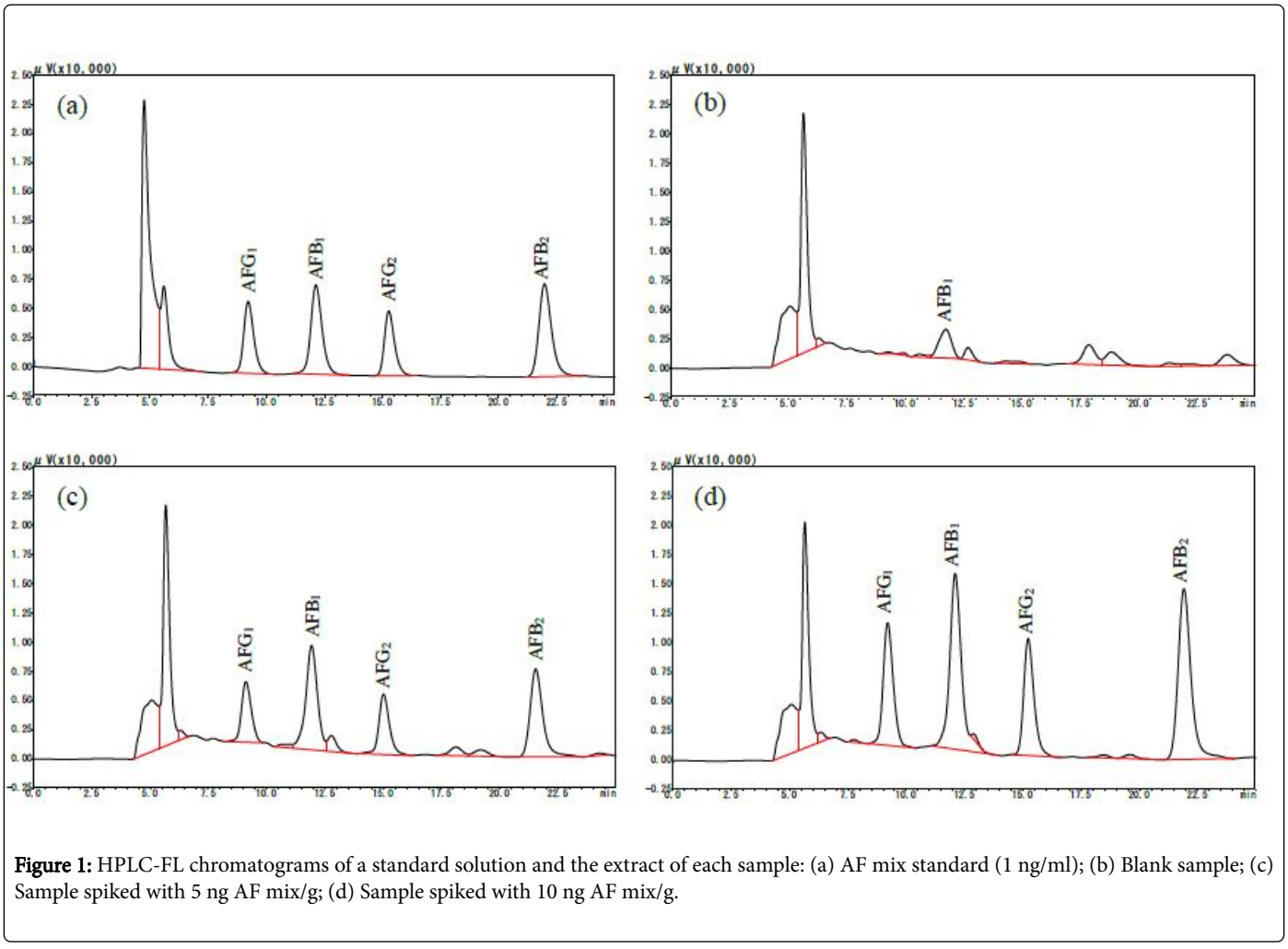


Figure 1: HPLC-FL chromatograms of a standard solution and the extract of each sample: (a) AF mix standard (1 ng/ml); (b) Blank sample; (c) Sample spiked with 5 ng AF mix/g; (d) Sample spiked with 10 ng AF mix/g.

Conclusion

This proposed method using MultiSep #226 MFC cartridge is a quick (within 2 hours), simple and accurate method for the analysis of aflatoxins in bagasse. The recoveries of AFB_1, AFB_2, AFG_1 and AFG_2 spiked to bagasse at both 5 and 10 ng/g levels were higher than 80%, respectively. Therefore, this method will be applicable for the practical determination of AFs in bagasse and will facilitate further risk management of undesired AFs contamination in bagasse. A slight natural contamination of AFB_1 was observed in a bagasse sample used in this study. Further studies to identify fungal species adherent to the bagasse would be conducted to clarify the source of AFs contamination.

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