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Chromatographic Analysis of Aflatoxigenic *Aspergillus flavus* Isolated from Malaysian Sweet Corn

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Abstract: High-performance liquid chromatography (HPLC) provides a quick and efficient tool for accurately characterizing aflatoxigenic and non-aflatoxigenic isolates of *Aspergillus flavus*. This method also provides a quantitative analysis of AFs in *Aspergillus flavus.* The method's recovery was assessed by spiking a mixture of AF at different concentrations to the testing medium. The validity of the method was confirmed using aflatoxigenic and non-aflatoxigenic strains of *A. flavus*. The HPLC system, coupled with a fluorescence detector and post-column photochemical reactor, showed high sensitivity in detecting spiked AFs or AFs produced by *A. flavus* isolates. Recovery from medium spiked with 10, 20, 60, and 80 ppb of AFs was found to be 73–86% using this approach. For AFB_1 and AFB_2, the limit of detection was 0.072 and 0.062 ppb, while the limit of quantification was 0.220 and 0.189 ppb, respectively. The AFB_1 concentrations ranged from 0.09 to 50.68 ppb, while the AFB_2 concentrations ranged between 0.33 and 9.23 ppb. The findings showed that six isolates produced more AFB_1 and AFB_2 than the acceptable limit of 5 ppb. The incidence of aflatoxigenic isolates of *A. flavus* in sweet corn and higher concentrations of AFB_1 and AFB_2 emphasize the need for field trials to explore their real potential for AF production in corn.

Keywords: *Aspergillus flavus*; extraction; HPLC; limit of detection; limit of quantitation

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1. Introduction

Aspergillus flavus is a ubiquitous saprophytic or parasitic fungus commonly found in decaying vegetation, crops, and seeds. Contamination of cereal crops by *A. flavus* is one of the worst food security problems due to its acute and chronic effects on humans and animals [1]. Together with *A. parasiticus*, *A. flavus* is the largest agricultural fungal species, producing aflatoxins (AFs) in corn, peanuts, and nuts [2]. Tropical conditions, including high temperatures, high humidity, heavy rains, and floods, cause mycological dissemination and AF production [3]. Due to Malaysia's tropical environment, the temperature remains between 28 and 31 °C and humidity between 60 and 80%, providing suitable conditions for *A. flavus* to contaminate cereal crops. The colonization of *A. flavus* on ripening corn results in contamination with AFs. As climate change progresses, *A. flavus* is predicted to extend its growing area, contributing to an increasing threat of AF infection throughout the globe [4,5]. AFs are secondary metabolites that cause severe disease in humans and animals [6,7]. While approximately 13 types of AFs are currently identified, AFB_1, AFB_2, AFG_1, and AFG_2 are the most harmful to humans and animals, predominantly found in foods and feeds. The letters "B" and "G" represent their blue and green

fluorescence released under ultraviolet (UV) radiation, while the digits show their major and minor compounds. Furthermore, AFB_2 and AFG_2 are the dihydroxy byproducts of AFB_1 and AFG_1 [8,9]. AFs possess specific fluorescence activities due to their oxygenated pentaheterocyclic framework known as the coumarin nucleus (Figure 1). The tendency to fluoresce has motivated several analytical approaches to detect and quantify such toxins [10]. Owing to the lack of double bonds within the furan rings, AFB_2 and AFG_2 provide a greater fluorescence quantum output than both AFB_1 and AFG_1 [11].

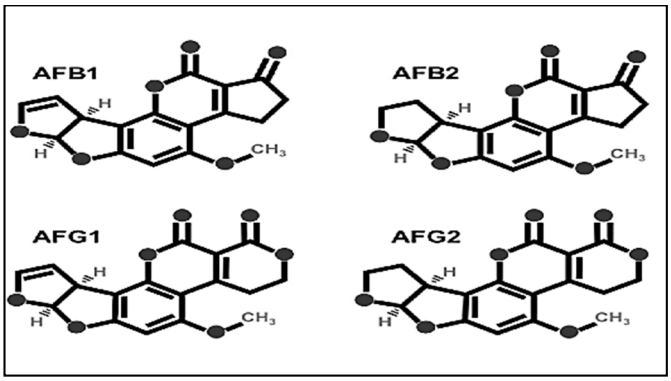

Figure 1. Chemical structures of AFB_1, AFB_2, AFG_1, and AFG_2.

The International Agency for Research on Cancer (IARC) classified AFB_1 as a Group 1 human carcinogen, causing liver cancer in humans [12]. This active site might be involved in a reduction reaction, leading to a significant shift in its functions [13,14]. AFB_1 is usually heat-resistant and can tolerate high temperatures during cooking or sterilization processes. In human food, the presence of AFB_1 can cause acute and chronic health effects, including immunosuppressive diseases, stunting in children, hepatic carcinoma (HCC), and even death. Inhaling AF-contaminated dust may induce tumors in human and animal respiratory tracts [15]. Moreover, AFB_1 is directly linked to jaundice, diarrhea, depression, low-grade fever, and liver cancer. The communities of *A. flavus* existing in diverse agroecosystems are composite sets of different populations.

Therefore, understanding the ability of *A. flavus* to produce AFs is an essential factor in the forecast of the prevalence and intensity of AF contamination. Previous studies presumed that *A. flavus* produces only AFB_1 and AFB_2; recent studies have found that few strains of *A. flavus* produce AFG_1 and AFG_2 [16,17]. Several strategies, including fungal cultures and molecular marker-based methods, have been developed to identify and distinguish aflatoxigenic and non-aflatoxigenic *A. flavus* strains [18,19]. Culture-based methods are rapid, cost-effective, and involve limited research expertise, however, the development of new analytical methods could be considered a promising alternative to culture-based methods, as they may have a broad range of applications, a shorter total analysis time, and high efficiency, sensitivity, specificity, and reproducibility. High-performance liquid chromatography (HPLC) is an advanced analytical separation method since it perfectly complements other known chromatographic techniques (conventional column chromatography, thin-layer chromatography, and gas chromatography). Furthermore, HPLC, equipped with a fluorescence detector (FLD) and post-column photochemical reactor, ensures a relatively fast, efficient, sensitive, specific, and global method for the detection of AF. Therefore, the HPLC-FLD system is a very versatile separation/detection system that allows the identification of chemical compounds. Given the advantages of HPLC-FLD, this study aimed to develop an analytical method for the identification of AF-producing isolates of *A. flavus*, isolated from Cameron Highlands' sweet corn [20], and the quantification of these different AFs.

2. Materials and Methods

2.1. Chemicals and Reagents

Standards including AFB_1, AFB_2, AFG_1, and AFG_2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile were acquired from Merck KGaA (Darmstadt, Germany) and used to prepare the mobile phase. HPLC-grade chloroform was obtained from Sigma-Aldrich (St. Louis, MO, USA) and used for the extraction of the AFs. Ultra-pure water was acquired from Elga LabWater (High Wycombe, UK) and used to prepare the mobile phase and culture media. Whatman filters with 0.2 µm pore size and a polytetrafluoroethylene (PTFE) syringe filter (0.22 µm with 13 mm diameter) were obtained from HmbG Chemicals (Hamburg, Germany). Potato dextrose agar (PDA) was bought from Oxoid Ltd. (Basingstoke, UK).

2.2. Aflatoxin Standards Preparation

Standard solutions of AFB_1 and AFB_2 were prepared in acetonitrile at a fixed volume of 10 ppb (parts per billion) using a slightly modified method of the Association of Official Analytical Chemists (AOAC) [21,22]. In preparing 10 ppb of each AF stock standard, 10 mg of individual AF was dropped in 100 mL volumetric flasks. In each volumetric flask, 50 mL acetonitrile was poured and stirred for 30 s. Next, a 10 mL solution was emptied into a volumetric flask and mixed with the acetonitrile. The working solutions (individual and mixture solutions) were prepared with acetonitrile and retained in HPLC vials (Thermo Scientific, Milford, MA, USA) at −4 °C. Standard solutions of AFs were developed by dissolving the mobile phase's working solutions in the calibration curve.

2.3. Strains of Aspergillus Flavus

Forty isolates of *A. flavus* were used in this study. For the recovery experiments, *A. flavus* NRRL 21,882 was used as a non-aflatoxigenic strain as it cannot produce AFs due to the deletion or mutation of gene clusters responsible for AF biosynthesis [23,24]. Alternatively, ATCC 200026 (synonym: NRRL 3357) was employed as a positive control since it produces AFB_1 and AFB_2 in laboratories and fields [25]. The *A. flavus* isolates were grown for 7 d at 30 ± 2 °C. PDA was preferred for this study, as it is rich in carbohydrate content and possesses an acidic pH (5.1), providing favorable conditions for *A. flavus* to grow and produce AFs [23]. Following seven days of incubation, conidia were harvested, counted, and adjusted to 1×10^6 using a hemocytometer and sterile distilled water. Spore suspensions of *A. flavus* were preserved at −4 °C until further analysis.

2.4. Inoculation of Aspergillus Flavus

Spore suspensions were used as an inoculum source throughout the inoculation process. Forty isolates of *A. flavus* with negative control (NRRL 21,882) and positive control (NRRL 3357) were inoculated in petri dishes (100 mm × 20 mm), comprising roughly 25 mL PDA. After inoculation, the dishes were incubated at 30 ± 2 °C for seven days.

2.5. Extraction of Aflatoxins

A flowchart for the extraction of AFs from *A. flavus* culture is presented in Figure 2. AFs were extracted from *A. flavus* cultures using a solid–liquid extraction method. We transferred 10 mL of ultrapure water into each culture, and spores were harvested by gently scratching the mycelial surface and transferred into 50 mL conical centrifuge tubes. Spore suspensions were then vortexed (LMS Co., Ltd., Tokyo, Japan) for 30 s, before 1 mL was emptied into new 15 mL centrifuge tubes, mixed with 1.5 mL chloroform, and vortexed for 30 s. Next, the mixture was subjected to centrifugation (Sartorius, Germany) for 5 min at 13,000× *g*. The residual from the bottom phase was shifted into new HPLC vials. An additional 1.5 mL chloroform was used to extract the sample residue and recover traces of AFs following the first extraction. The chloroform extracts were mixed and vaporized to achieve adequate aridity. The extract was then diluted with a mobile phase of 1 mL and filtered into an HPLC vial using a PTFE syringe filter.

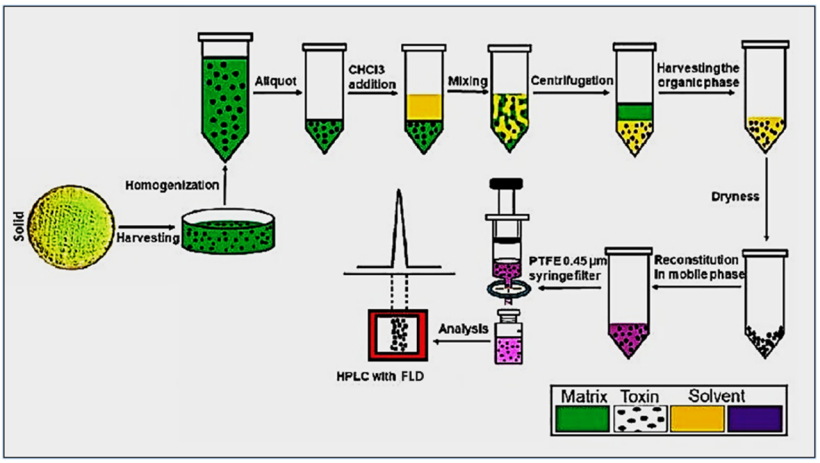

Figure 2. Flowchart showing the extraction of AFs from an *A. flavus* culture.

2.6. High-Performance Liquid Chromatography Procedure

In this study, samples were tested for AFs by a reversed-phase HPLC system (Waters 600, Milford, MA, USA) equipped with a fluorescence detector (FLD) (Waters 2475, Milford, MA, USA) and post-column photochemical reactor. The separation was accomplished through a C_{18} column (Ymc Triart, 5 µM, 12 nm, 150 mm × 4.6 mm; YMC, Tokyo, Japan) at 40 °C. The excitation and emission wavelengths were 360 nm and 440 nm, respectively. A mobile phase of acetonitrile (CH_3CN)/methanol (CH_3OH)/distilled water (H_2O) (10:35:55 $v/v/v$) with a flow rate of 1 mL/min was employed to elute the samples. The mobile phase (acetonitrile, methanol, and dH_2O) was filtered using a Whatman filter (0.2 µM × 47 mm diameter; Merck, Darmstadt, Germany) and degassed for 30 min in an ultrasonic bath (Power sonic 420, Seoul, Korea). The volume of injection was 20 µL. The data software Empower-2 Chromatography (Waters, Milford, MA, USA) was used for data acquisition and data processing.

2.7. Validation of HPLC

The HPLC process was validated by assessing recovery, accuracy, linearity, and sensitivity under the AOAC guidelines [21], with minor changes. A mixture of known concentrations of AFB_1 and AFB_2 (10, 20, 60, and 80 ppb) was spiked into the blank samples to validate recovery. The spike of each concentration was achieved in triplicate, and the tests were conducted in triplicate each day for three consecutive days. Accuracy was observed through reliability. Reliability was measured by the relative standard deviation (RSD) of spiked toxins recurrent on the first day. Blank samples were developed by inoculating the negative control (NRRL 21,881) on PDA, harvested, and analyzed by HPLC coupled with FLD and a post-column photochemical reactor. The selectivity of the method was confirmed, as the chromatographic peaks did not conflict with the retention time of the AFs. The linearity for AFs was observed in triplicate, ranging between 10 and 80 ppb. The calibration curve for each concentration (10, 20, 60, and 80 ppb) was constructed employing the peak area of the AF against the analyte concentration. The linearity was assessed through the correlation coefficient (R^2), interception (y), and slope (s) of the regression line. The sensitivity of the HPLC method was assessed by evaluating the LOD and LOQ through the following equation:

$$\text{LOD} = 3.3\ \sigma/\text{s and LOQ} = 10\ \sigma/s. \quad (1)$$

where σ is the standard deviation of blank samples, and s is the calibration curve slope.

2.8. Statistical Analysis

The HPLC system has been enhanced and verified using statistical analysis to improve the recovery of the AFs and avoid chemical loss. The quantities were averaged and shown as a mean ± standard error. The peak areas of the AFs were separated without any interruption. The significance ($p < 0.05$) of the data was analyzed through the ANOVA test (analysis of variance) with a confidence interval of 95% using the SPSS® version 25 software (IBM SPSS® Inc., Chicago, IL, USA). The following equation determines the relative standard deviation (RSD).

$$RSD = Si \times 100/\times \quad (2)$$

where S represents the standard deviation, while x represents the mean of the data.

3. Results

3.1. Aspergillus Flavus on PDA

On PDA, *A. flavus* isolates produced olive-green conidia, which dominated the appearance of the colony. After three days of incubation, sporulation began from the center and progressed radially, covering the colony's surface. The conidia produced had a yellowish to olive color. As the sporulation spread outwards, it gave a characteristic white border encircling the sporulating mycelia. The white border eventually covered as the entire mycelia continued to sporulate and produce more conidia by day seven. The colonies produced brown or colorless exudates (droplets). Some isolates produced a compact mass of dark brown fungal mycelia (sclerotia). The reverse of the *A. flavus* colonies was pale in color. As the colony grew, it slightly raised as the mycelia piled, and the center became floccose and rough (Figure 3).

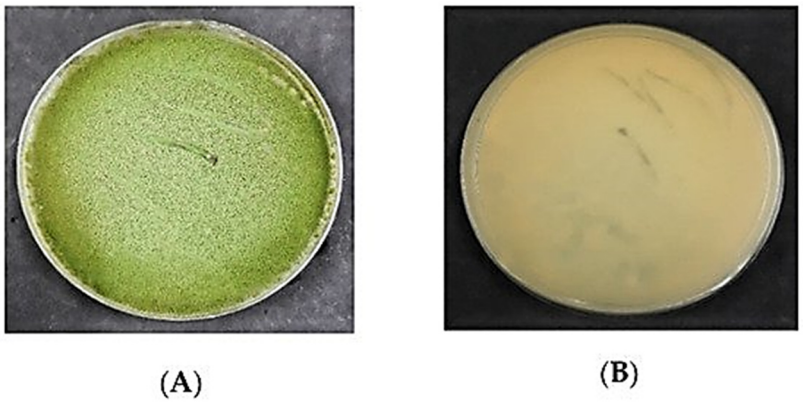

Figure 3. Colony morphology of *A. flavus* on the potato dextrose agar (PDA); (**A**) = obverse, (**B**) = reverse.

3.2. LOD and LOQ for Aflatoxin

The LOD and LOQ for AFs were used to validate the HPLC system's performance. A standard solution of 20–80 ppb/mL of AFB_1 and AFB_2 was used to construct a four-point calibration curve. The LOD for AFB_1 was 0.072 ppb, while for AFB_2 it was 0.062 ppb. In contrast, the LOQ for AFB_1 was 0.220 ppb, while for AFB2 it was 0.180 ppb (Table 1).

Table 1. Validation of the quantification of AFs by HPLC.

AF	LOD (ppb) [a]	LOQ (ppb) [b]	Calibration Curve [c]	R^2
AFB_1	0.072	0.220	y = 55,012, 9.1 + 16	0.9960
AFB_2	0.062	0.180	y = 1.92317 +16	0.9952

[a]. LOD, [b]. LOQ, [c]. x = concentration of AF (ppb); y = intensity.

3.3. Linearity

The linearity was determined by four-point calibration curves over the range of 10 to 80 ppb for individual AFs to determine a relative association between response and AF concentration. Calibration curves were constructed by plotting the peak area (y) against the AF concentration (x) (Figure 4). Linear regression (R^2) ranged between 0.9952 and 0.9960 for the FLD detector, representing enhanced linearity for AFB_1 and AFB_2 (Table 1).

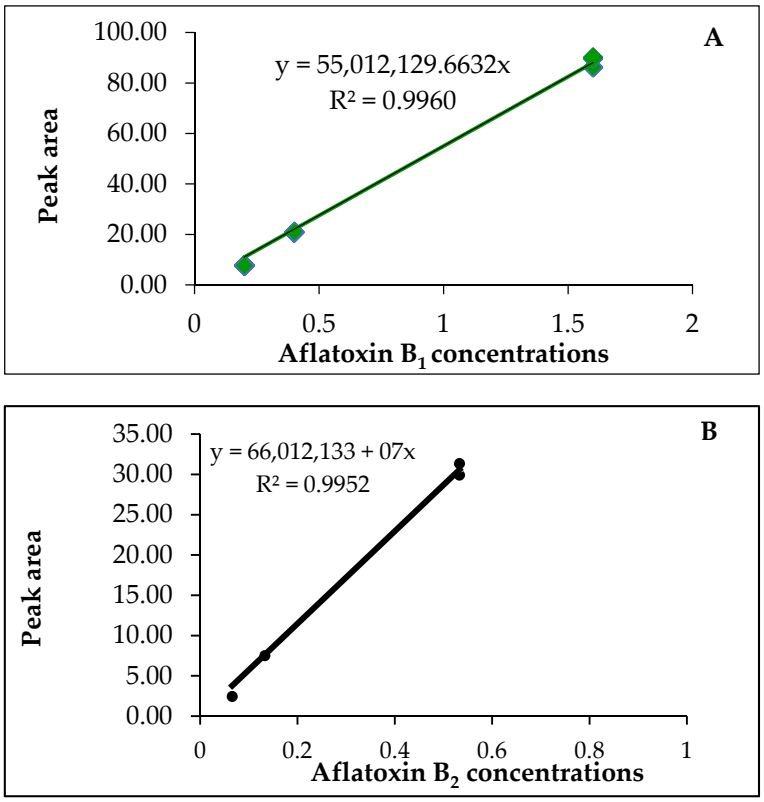

Figure 4. Calibration curves of standard solutions of AFB_1 (**A**) and AFB_2 (**B**) concentrations of 10, 20, 60, and 80 ppb as detected by FLD.

3.4. Recovery of Aflatoxins

The percentage recovered of the analytes when the test sample is assessed with the complete method is known as the recovery of the method [26]. Table 2 represents the recovery percentage of AFB_1 and AFB_2 at different concentrations of a spike in culture conditions. Recovery of AFs exhibited the same retention time with a total recovery of 73–86%.

Table 2. The recovery percentage of spiked aflatoxins from *A. flavus* culture.

Spiked Levels (ppb)	Recovery of Aflatoxins (%)	
	AFB_1 (ppb)	**AFB_2 (ppb)**
80	81.3	86.0
60	77.5	82.5
20	77.6	76.1
10	73.0	79.4

3.5. Quantification of AFB_1 and AFB_2

Regarding AFB_1 quantification, 24 strains of *A. flavus* produced AFB_1 ranging from 0.09 to 50.68 ppb, while the remaining 16 strains did not produce AFB_1 (Table 3). In these AFB_1-producing strains, two strains surpassed the maximum acceptable limit of 5 ppb.

Alternatively, 22 strains were found to produce AFB_2 with levels of 0.33 to 9.23 ppb. The results indicated that 6 of the 40 isolates produced AFB_1 and AFB_2 in quantities higher than the permissible limit of 5 ppb in food. Moreover, the positive control (NRRL 3357) produced both AFB_1 and AFB_2 with concentrations of 3.96 to 1.14 ppb. In contrast, the negative control (NRRL 21,882) did not produce any type of AFs (AFB_1, AFB_2) when cultured on the PDA medium, as seen in previous studies [23,27].

Table 3. The concentrations of AFB_1 and AFB_2 produced by *A. flavus*.

Strain No.	Sclerotial Type	Concentrations	
		AFB_1 (ppb)	**AFB_2 (ppb)**
AKR1	-	-	-
AKR2	L	0.278 ± 0.12	-
AKR3	S	0.221 ± 0.05	-
AKR4	S	0.315 ± 0.11	-
AKR5	L	-	-
AKR6	S	0.636 ± 0.51	-
AKR7	S	0.428 ± 1.04	-
AKR8	S	2.290 ± 0.68	2.481 ± 1.04
AKR9	S	-	2.113 ± 0.64
AKR10	L	0.462 ± 0.39	0.548 ± 0.62
AKR11	L	0.508 ± 0.26	-
AKR12	L	0.609 ± 0.11	-
ARV13	L	0.284 ± 0.09	-
ARV14	S	0.423 ± 0.12	-
ARV15	-	0.265 ± 0.59	-
ARV16	-	0.488 ± 0.94	5.715 ± 0.94
ARV17	S	3.848 ± 0.31	5.198 ± 0.05
ARV18	S	1.550 ± 0.53	2.165 ± 0.35
ARV19	L	0.309 ± 0.48	-
ARV20	L	1.163 ± 0.16	-
ARV21	L	3.538 ± 0.53	0.640 ± 0.01
ARV22	L	2.512 ± 0.89	-
AK23	S	1.575 ± 0.13	0.332 ± 0.29
AK24	L	-	-
AK25	S	0.659 ± 0.34	0.751 ± 0.35
AK26	S	-	1.191 ± 0.39
AK27	S	-	0.536 ± 0.39
AK28	S	-	0.339 ± 0.26
AK29	S	-	0.362 ± 0.21
AK30	S	-	2.142 ± 0.11
AKL31	S	-	1.213 ± 0.14
AKL32	S	0.429 ± 0.02	0.330 ± 0.13
AKL33	S	0.267 ± 0.21	-
AKL34	L	-	-
AKL35	L	-	-
AKL36	L	-	-
AKL37	L	-	-
AKL38	S	-	8.665 ± 0.19
AKL39	L	-	-
AKL40	S	0.659 ± 0.12	4.928 ± 0.30
NRRL 21,882	-	-	-
NRRL 3357	S	1.142 ± 0.11	4.928 ± 0.12

Note: (-) stands for nil.

4. Discussion

The contamination of sweet corn with AFs represents one of the worst global food security problems, due to their acute and chronic adverse effects on humans and animals [28]. *A. flavus* is the leading food contaminant since it can produce AFs and persists as a pathogen in both pre-and post-harvest food supply [26]. The growth of *A. flavus*

and AF production are linked to various environmental factors, including water activity, temperature, pH, and content of CO_2 [1,29,30]. In Malaysia, the environmental conditions are characterized by high temperature, high humidity, and inadequate storage practices that contribute to the potential for a substantial exposure of the Malaysian people to AFs. Recent studies have found that species of *A. flavus* and *A. niger* are the most common fungi isolated from contaminated cereal crops [31]. The results of the current study demonstrate that among the 40 isolates of *A. flavus*, 24 isolates were aflatoxigenic, producing AFB_1 and AFB_2, while the remaining 18 isolates were non-aflatoxigenic. This study also displayed that all aflatoxigenic and non-aflatoxigenic isolates produced olive-green colonies on PDA medium and rapidly grow at optimum temperature (25–32 °C). This observation suggests the presence of aflatoxigenic and non-aflatoxigenic isolates in A. *flavus* species. The results also revealed that the *A. flavus* isolates producing small sclerotia could produce both AFB_1 and AFB_2, while the *A. flavus* isolates producing large sclerotia did not synthesize AFB_1 and AFB_2.

The results obtained from the current study demonstrated significant variability in the AF-producing potential of *A. flavus*. According to Bandyopadhyay et al. [32] and Sarma et al. [33], the size and formation of sclerotia are strongly related to the aflatoxigenicity of *A. flavus* isolates. It has been identified that all S-type *A. flavus* strains producing small sclerotia ($\leq$400 μM in diameter) are aflatoxigenic, whereas the L-type strains producing larger sclerotia ($\geq$400 μM in diameter) include both aflatoxigenic and non-aflatoxigenic strains. Similarly, Mellon [34] has reported a close relationship between small sclerotia and AF production. On the contrary, Barrett and Bevis [35] and Ehrlich et al. [36] found the highest level of AFs in isolates having large sclerotia. Hence, the relationship between AF production and sclerotial size and was observed in this study. This method has shown efficient separation capacity and selectivity, allowing the simultaneous quantification of the AFB_1 and AFB_2 produced by aflatoxigenic strains. The FLD detection method proficiently distinguishes the peaks of AFB_1 and AFB_2 in the same HPLC run without interruption. Besides that, method validation is a vital criterion for conducting the HPLC analysis [37]. LOD and LOQ were applied to validate the performance of the HPLC method. LOD is the lowest analyte concentration detected under specified laboratory conditions but not simply quantitated [38]. In contrast, LOQ is the smallest analyte concentration that can be quantified [39]. In this study, multiple concentrations of 3.0 ppb were injected to evaluate the sensitivity of FLD for detecting AFB_2. It was noticed that FLD easily detected AFB_2 at parts per trillion (ppt) as predicted, since it lacks a double bond in furan rings. To the best of our knowledge, an LOD of 1.0–5.0 ppb is adequate for a researcher to distinguish between aflatoxigenic and non-aflatoxigenic strains of *A. flavus* and to quantify the concentration of AFs. In this study, we used reversed-phase chromatography in which AFs were eluted in a sequence of AFB_2 and AFB_1 (Figure 5). The sequence has been confirmed by matching the retention time attained in the AF mixture with the retention time of the individual AFs. The extracted AFs were then identified using the FLD detector (Figure 5). Note that AFB_2 can also be observed in low quantities while using the FLD detector as it fluoresces 40 times as higher as AFB_1.

The percentage recovered of the analytes when the test sample was assessed with the complete method is known as method recovery [40]. FLD has detected all spiked samples in the sequence, and their mean was calculated. The recovery spectrum agreed with the criteria of AOAC and Codex Alimentarius' acceptable recovery limits. The acceptable recovery limit of the AOAC at 10 ppb is from 70 to 125%, while for Codex Alimentarius, it is 60–120% at 1−10 ppb. The results indicated that 6 of the 40 isolates had produced AFB_1 and AFB_2 in amounts higher than the maximum acceptable limit of 5 ppb in food. Therefore, further research on AFs contamination of sweet corn in the field and storage is needed to provide data on the Malaysian population's exposure towards AFs, particularly AFB_1.

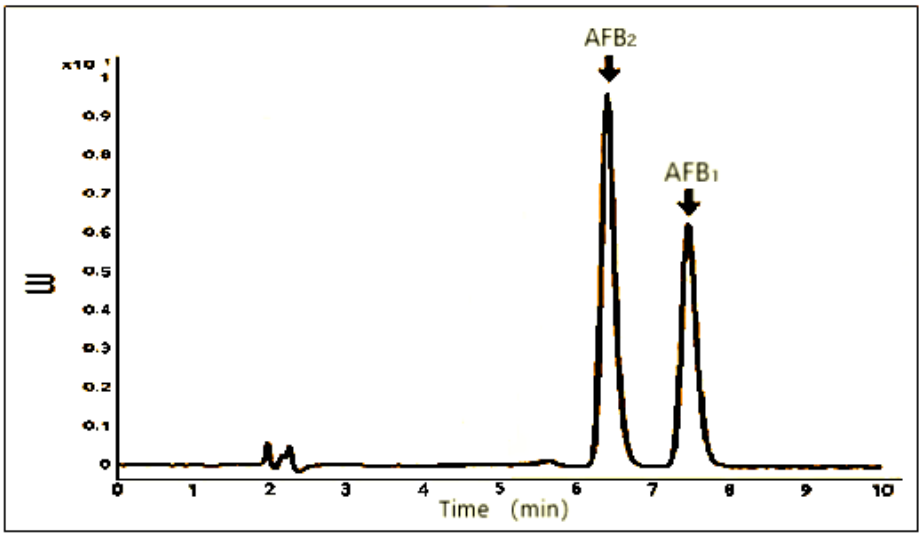

Figure 5. Representative HPLC chromatograms of AFB_1 and AFB_2 in the culture of *A. flavus* detected by FLD.

5. Conclusions

In conclusion, this is the first detailed study on *A. flavus* associated with sweet corn collected from the Cameron Highlands, Malaysia. This study demonstrated that an HPLC instrument (Waters 600, USA) coupled with a fluorescence detector (Waters 2475, USA) assured precision and linearity in the quantitative determination of AFs produced by aflatoxigenic *A. flavus* isolates. Chloroform was used for the extraction of AFs to prevent emulsion production—a two-layer mixture with AF existing in the chloroform layer, minimizing toxin loss and leaving other substances in the aqueous layer. Peaks of AFB_1 and AFB_2 were differentiated within 15 min with excellent selectivity, linearity, and recovery. AFB_1 was detected in 24 of 40 *A. flavus* isolates ranging from 0.09 to 50.68 ppb, and 22 isolates were found to be producing AFB_2 ranging between 0.33 and 9.23 ppb. The results indicated that 6 of the 40 strains had produced AFB_1 and AFB_2 in quantities greater than the permissible limit of 5 ppb. The occurrence of AF-producing *A. flavus* isolates in sweet corn and the quantities of AFB_1 and AFB_2 greater than the permissible limit emphasizes the need for field trials to investigate their actual ability for AF production in corn crops.

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