Chromatographic Analysis of Aflatoxigenic Aspergillus flavus Isolated from Malaysian Sweet Corn

Rahim Khan 1, Farinazleen Mohamad Ghazali 1,*, Nor Ainy Mahyudin 2,3 No and Nik Iskandar Putra Samsudin 1,4

1 Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang 43400, Selangor Darul Ehsan, Malaysia; sirifrahim1@yahoo.com (R.K.); nifikiskandar@upm.edu.my (N.I.P.S.)
2 Department of Food Service and Management, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang 43400, Selangor Darul Ehsan, Malaysia; norainy@upm.edu.my
3 Laboratory of Halal Science Research, Halal Products Research Institute, Universiti Putra Malaysia, Serdang 43400, Selangor Darul Ehsan, Malaysia
4 Laboratory of Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, Serdang 43400, Selangor Darul Ehsan, Malaysia
* Correspondence: farinazleen@upm.edu.my; Tel.: +60-122-198-912

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 AFB1 and AFB2, 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 AFB1 concentrations ranged from 0.09 to 50.68 ppb, while the AFB2 concentrations ranged between 0.33 and 9.23 ppb. The findings showed that six isolates produced more AFB1 and AFB2 than the acceptable limit of 5 ppb. The incidence of aflatoxigenic isolates of A. flavus in sweet corn and higher concentrations of AFB1 and AFB2 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

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, AFB1, AFB2, AFG1, and AFG2 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\textsubscript{2} and AFG\textsubscript{2} are the dihydroxy byproducts of AFB\textsubscript{1} and AFG\textsubscript{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\textsubscript{2} and AFG\textsubscript{2} provide a greater fluorescence quantum output than both AFB\textsubscript{1} and AFG\textsubscript{1} [11].

![Figure 1. Chemical structures of AFB\textsubscript{1}, AFB\textsubscript{2}, AFG\textsubscript{1}, and AFG\textsubscript{2}.](image)

The International Agency for Research on Cancer (IARC) classified AFB\textsubscript{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\textsubscript{1} is usually heat-resistant and can tolerate high temperatures during cooking or sterilization processes. In human food, the presence of AFB\textsubscript{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\textsubscript{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\textsubscript{1} and AFB\textsubscript{2}; recent studies have found that few strains of *A. flavus* produce AFG\textsubscript{1} and AFG\textsubscript{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 $1 \times 10^6$ 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.
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$_3$CN)/methanol (CH$_3$OH)/distilled water (H$_2$O) (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$_2$O) 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:

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

where $\sigma$ 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).

\[
\text{RSD} = \frac{S}{x} \times 100
\]

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).

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 AFB1 and AFB2 was used to construct a four-point calibration curve. The LOD for AFB1 was 0.072 ppb, while for AFB2 it was 0.062 ppb. In contrast, the LOQ for AFB1 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)  | LOQ (ppb) | Calibration Curve | \( R^2 \) |
|---------|------------|-----------|-------------------|----------|
| AFB1    | 0.072      | 0.220     | \( y = 55.012, 9.1 + 16 \) | 0.9960   |
| AFB2    | 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 (R²) ranged between 0.9952 and 0.9960 for the FLD detector, representing enhanced linearity for AFB₁ and AFB₂ (Table 1).

![Figure 4](image)

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

| AF    | LOD (ppb) | LOQ (ppb) | Calibration Curve | \( R^2 \) |
|-------|-----------|-----------|-------------------|----------|
| AFB₁  | 0.072     | 0.220     | \( y = 55,012,129.6632x \) | 0.9960   |
| AFB₂  | 0.062     | 0.180     | \( y = 66,012,133 + 07x \) | 0.9952   |

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 AFB₁ and AFB₂ 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 (%) |
|---------------------|----------------------------|
|                     | AFB₁ (ppb) | AFB₂ (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 AFB₁ and AFB₂

Regarding AFB₁ quantification, 24 strains of *A. flavus* produced AFB₁ ranging from 0.09 to 50.68 ppb, while the remaining 16 strains did not produce AFB₁ (Table 3). In these AFB₁-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 aflatoxicogenic, producing AFB$_1$ and AFB$_2$, while the remaining 18 isolates were non-aflatoxicogenic. This study also displayed that all aflatoxicogenic and non-aflatoxicogenic isolates produced olive-green colonies on PDA medium and rapidly grow at optimum temperature (25–32 °C). This observation suggests the presence of aflatoxicogenic and non-aflatoxicogenic 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 aflatoxicogenicity of *A. flavus* isolates. It has been identified that all S-type *A. flavus* strains producing small sclerotia (≤400 µM in diameter) are aflatoxicogenic, whereas the L-type strains producing larger sclerotia (>400 µM in diameter) include both aflatoxicogenic and non-aflatoxicogenic 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 aflatoxicogenic 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 aflatoxicogenic and non-aflatoxicogenic 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$_1$. 
AFB2 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 associated with sweet corn.

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 and AFB2 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 AFB1 and AFB2 greater than the permissible limit emphasizes the need for field trials to investigate their actual ability for AF production in corn crops.

Author Contributions: F.M.G., supervision, project administration, funding acquisition; R.K., method-ology, writing—original draft preparation; N.I.P.S., conceptualization, and methodology assistance; N.A.M. review, resources, and data curation. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Geran Inisiatif Putra Universiti Putra Malaysia, UPM/GP/2017/9568800.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors wish to acknowledge the financial contribution of the Ministry of Science, Technology, and Innovation (MOSTI), Malaysia, for funding this research under the Science Fund (Grant number: 05-01-04-SF0750).

Conflicts of Interest: The authors declare no conflict of interest regarding this paper’s publication.
References

1. Medina, A.; Schmidt-Heydt, M.; Rodriguez, A.; Parra, R.; Geisen, R.; Magan, N. Impacts of environmental stress on growth, secondary metabolite biosynthetic gene clusters and metabolite production of xerotolerant/xerophilic fungi. *Curr. Genet.* 2015, 61, 325–334. [CrossRef] [PubMed]

2. Abbas, H.K.; Accinelli, C.; Shier, W.T. Biological control of aflatoxin contamination in US crops and the use of bioplastic formulations of *Aspergillus flavus* biocontrol strains to optimize application strategies. *J. Agric. Food Chem.* 2017, 65, 7081–7087. [CrossRef] [PubMed]

3. Dias, C.; Aires, A.; Saavedra, M.J. Antimicrobial activity of isothiocyanates from cruciferous plants against methicillin-resistant *Staphylococcus aureus* (MRSA). *Int. J. Mol. Sci.* 2014, 15, 19552–19561. [CrossRef] [PubMed]

4. Assunção, R.; Martins, C.; Viegas, S.; Viegas, C.; Jakobsen, L.S.; Pires, S.; Alvito, P. Climate change and the health impact of aflatoxins exposure in Portugal—an overview. *Food Addit. Contam. Part A* 2018, 35, 1610–1621. [CrossRef] [PubMed]

5. Van der Fels-Klerx, H.; Vermeulen, L.; Gavai, A.; Liu, C. Climate change impacts aflatoxin B<sub>1</sub> in maize aflatoxin M<sub>1</sub> in milk: A case study of maize grown in Eastern Belgium and imported to the Netherlands. *PLoS ONE* 2019, 14, e0218956. [CrossRef]

6. Mohammed, A.; Chala, A.; Ojewo, C.; Dejene, M.; Fininsa, A.; Ayalew, A.; Arias, R.S. Integrated management of *Aspergillus* species and aflatoxin production in groundnut (*Arachis hypogaea* L.) through the application of farmyard manure and seed treatments with fungicides and Trichoderma species. *Afr. J. Plant Sci.* 2018, 12, 196–207.

7. Prakash, B.; Kedia, A.; Mishra, P.K.; Dubey, N. Plant essential oils as food preservatives to control molds, mycotoxin contamination, and oxidative deterioration of agri-food commodities—Potentials and challenges. *Food Control* 2015, 47, 381–391. [CrossRef]

8. Fente, C.; Ordaz, J.J.; Vazquez, B.; Franco, C.; Cepeda, A. New additive for culture media for rapid identification of aflatoxin-producing *Aspergillus* strains. *Appl. Environ. Microbiol.* 2001, 67, 4858–4862. [CrossRef]

9. Hruska, Z.; Yao, H.; Kincaid, R.; Brown, R.; Cleveland, T.; Bhatnagar, D. Fluorescence excitation-emission features of aflatoxin and related secondary metabolites and their application for rapid detection of mycotoxins. *Food Bioprocess Technol.* 2014, 7, 1195–1201. [CrossRef]

10. Maragos, C.; Appell, M.; Lippolis, V.; Visconti, A.; Catucci, L.; Pascale, M. Use of cyclodextrins as modifiers of fluorescence in the detection of mycotoxins. *Food Addit. Contam.* 2008, 25, 164–171. [CrossRef]

11. Chandra, H.; Bahuguna, J.; Singh, A. Detection of aflatoxin in *Zea mays* L. from Indian markets by competitive ELISA. *Octa J. Biosci.* 2013, 1, 62–68.

12. Ostry, V.; Malir, F.; Toman, J.; Grosse, Y. Mycotoxins as human carcinogens—the IARC Monographs classification. *Mycotoxin Res.* 2017, 33, 65–73. [CrossRef]

13. Hua, S.S.T.; Parfitt, D.E.; Sarreal, S.B.L.; Sidhu, G. Dual culture of atoxigenic and toxigenic strains of *Aspergillus* isolated from pre-harvest maize ears grown in two Argentine regions. *Crop Prot.* 2017, 92, 41–48. [CrossRef]

14. Waliyar, F.; Osiru, M.; Ntare, B.; Kumar, K.; Sudini, H.; Traore, A.; Diarra, B. Post-harvest management of aflatoxin contamination in groundnut. *World Mycotoxin J.* 2015, 8, 245–252. [CrossRef]

15. Xie, L.; Chen, M.; Ying, Y. Development of methods for determination of aflatoxins. *Crit. Rev. Food Sci. Nutr.* 2016, 56, 2642–2664. [CrossRef] [PubMed]

16. Camilletti, B.X.; Torrico, A.K.; Maurino, M.F.; Cristos, D.; Magnoli, C.; Lucini, E.I.; Pecci, M.I.P.G. Fungal screening and aflatoxin production by *Aspergillus* section *Flavi* isolated from pre-harvest maize ears grown in two Argentine regions. *Crop Prot.* 2017, 92, 41–48. [CrossRef]

17. Saldan, N.C.; Almeida, R.T.; Avíncola, A.; Porto, C.; Galuch, M.B.; Magon, T.F.; Oliveira, C.C. Development of an analytical method for identification of *Aspergillus flavus* based on chemical markers using HPLC-MS. *Food Chem.* 2018, 241, 113–121. [CrossRef] [PubMed]

18. Abbas, H.K.; Zablutowicz, R.; Weaver, M.; Horn, B.; Xie, W.; Shier, W. Comparison of cultural and analytical methods for determination of aflatoxin production by *Mississippi Aspergillus* isolates. *Can. J. Microbiol.* 2004, 50, 193–199. [CrossRef] [PubMed]

19. Sadhasivam, S.; Britzi, M.; Zakim, V.; Kostyukovsky, M.; Trostanetsky, A.; Quinn, E.; Sionov, E. Rapid detection, and identification of mycotoxigenic fungi and mycotoxins in stored wheat grain. *Toxins* 2017, 9, 302. [CrossRef]

20. Khan, R.; Ghazali, M.F.; Mahyudin, N.A.; Samsudin, N.I.P. Morphological characterization, and determination of aflatoxic and non-aflatoxic *Aspergillus* isolates from sweet corn kernels and soil in Malaysia. *Agriculture* 2020, 10, 450. [CrossRef]

21. AOAC. *Official Methods of Analysis of AOAC International*; AOAC International: Rockville, MD, USA, 2016; ISBN 978-0-935584-87-5.

22. Paez, V.; Barrett, W.B.; Deng, X.; Diaz-Amigo, C.; Fiedler, K.; Fuerer, C.; Hostetler, G.L.; Johnson, P.; Joseph, G.; Konings, E.J.; et al. AOAC SMPR®. 2016.002. J. AOAC Int. 2016, 99, 1122–1124. [CrossRef]

23. Alshannaaq, A.F.; Gibbons, J.G.; Lee, M.K.; Han, K.H.; Hong, S.B.; Yu, J.H. Controlling aflatoxin contamination and propagation of *Aspergillus flavus* by a soy-fermenting *Aspergillus oryzae* strain. *Sci. Rep.* 2018, 8, 16871. [CrossRef]

24. Dorner, J.W.; Lamb, M.C. Development and commercial use of Afla-guard®, an aflatoxin biocontrol agent. *Mycotaxon Res.* 2006, 22, 33–38. [CrossRef] [PubMed]

25. Khan, R.; Ghazali, F.M.; Mahyudin, N.A.; Samsudin, N.I.P. Co-Inoculation of aflatoxicogenic and non-aflatoxicogenic strains of *Aspergillus flavus* to assess the efficacy of non-aflatoxicogenic strains in growth inhibition and aflatoxin B<sub>1</sub> reduction. *Agriculture* 2021, 11, 198. [CrossRef]
26. Lahouar, A.; Crespo-Sempere, A.; Marín, S.; Saïd, S.; Sanchis, V. Toxigenic molds in Tunisian and Egyptian sorghum for human consumption. *J. Stored Prod. Res.* 2015, 63, 57–62.

27. Rank, C.; Klejnstrup, M.L.; Petersen, L.M.; Kildgaard, S.; Frisvad, J.C.; Gotfredsen, H.C.; Larsen, O.T. Comparative chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus* (NRRL 3357). *Metabolites* 2012, 2, 39–56.

28. Medina, A.; Mohale, S.; Samsudin, N.I.P.; Rodriguez-Sixtos, A.; Rodriguez, A.; Magan, N. Biocontrol of mycotoxins: Dynamics and mechanisms of action. *Curr. Opin. Food Sci.* 2017, 17, 41–48.

29. Khan, R.; Ghazali, F.M.; Mahyudin, N.A.; Samsudin, N.I.P. Biocontrol of aflatoxins using non-aflatoxigenic *Aspergillus flavus*: A literature review. *J. Fungi* 2021, 7, 381.

30. Dagnas, S.; Onno, B.; Membre, J.M. Modeling growth of three bakery product spoilage molds as a function of water activity, temperature, and pH. *Int. J. Food Microbiol.* 2014, 186, 95–104.

31. Probst, C.; Bandyopadhyay, R.; Cotty, P. Diversity of aflatoxin-producing fungi and their impact on food safety in sub-Saharan Africa. *Int. J. Food Microbiol.* 2014, 174, 113–122.

32. Bandyopadhyay, R.; Ortega-Beltran, A.; Akande, A.; Mutegi, C.; Atehnkeng, J.; Kaptoge, L.; Cotty, P. Biological control of aflatoxins in Africa: Current status and potential challenges in the face of climate change. *World Mycotoxin J.* 2016, 9, 771–789.

33. Sarma, U.P.; Bhetaria, P.J.; Devi, P.; Varma, A. Aflatoxins: Implications on health. *Indian J. Clin. Biochem.* 2017, 32, 124–133.

34. Mellon, J.E. Extracellular xylanolytic and pectinolytic hydrolase production by *Aspergillus flavus* isolates contributes to crop invasion. *Toxins* 2015, 7, 3257–3266.

35. Barrett, C.B.; Bevis, L.E. The self-reinforcing feedback between low soil fertility and chronic poverty. *Nat. Geosci.* 2015, 8, 907–912.

36. Ehrlich, K.; Moore, G.; Mellon, J.; Bhatnagar, D. Challenges facing the biological control strategy for eliminating aflatoxin contamination. *World Mycotoxin J.* 2015, 8, 225–233.

37. Rogers, H.A. How composition methods are developed and validated. *J. Agric. Food Chem.* 2013, 61, 8312–8316.

38. Currie, L.A. Detection and quantification limits: Origins and historical overview. *Anal. Chim. Acta* 1999, 391, 127–134.

39. Şengül, Ü. Comparing determination methods of detection and quantification limits for aflatoxin analysis in hazelnut. *J. Food Drug Anal.* 2016, 24, 56–62.

40. Trucksess, M.W.; Stack, M.E.; Nesheim, S.; Albert, R.H.; Romer, T.R. Multifunctional column coupled with liquid chromatography for determination of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> in corn, almonds, Brazil nuts, peanuts, and pistachio nuts: Collaborative study. *J. AOAC Int.* 1994, 77, 1512–1521.