Journal of Microbiological Methods 158 (2019) 14–17

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Note

HPLC quantitation of aflatoxin B_1 from fungal mycelium culture

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ARTICLE INFO

Keywords:
Aspergillus flavus
Aflatoxin B_1
HPLC
Mycotoxins

ABSTRACT

Aflatoxins are mycotoxins that contaminate agricultural products when infected by toxigenic *Aspergillus flavus*. Methods for quantifying aflatoxin from culture using chromatography are available but are not optimized for population studies. We provide details of a method for preparation and quantitation of aflatoxin B_1 from fungal cultures that satisfy those needs.

1. Introduction

Aflatoxins are carcinogenic secondary metabolites produced by *Aspergillus flavus* and allied species that contaminate agricultural commodities (Horn, 2005; Horn, 2007). Aflatoxins are known to accumulate in fungal mycelia, spores and sclerotia, and are also excreted into their environment (Carbone et al., 2007a; Wicklow & Shotwell, 1983). Of the four aflatoxin metabolites (B_1, B_2, G_1, G_2) that can potentially accumulate in these fungi, aflatoxin B_1 is the most carcinogenic and most abundantly produced. Due to its toxicity to animals and humans the United States Food and Drug Administration (FDA) tightly regulates acceptable levels of aflatoxin B_1 in food products for human consumption at less than 20 ppb and animal feed at less than 20 ppb to less than 300 ppb depending on crop, livestock, and animal's maturity (Action Levels for Aflatoxins in Animal Feeds; Compliance Policy Guides 683, 1994; Adulteration with Aflatoxin; Compliance Policy Guides 555, 2005). These analyses are based on aflatoxin found in the grain or feedstuff, which could be derived from several aflatoxigenic fungi. Commercially available methods for detection and determination of aflatoxin levels based on antibodies or enzyme-linked immunosorbent assay (ELISA) are designed to extract and measure aflatoxins from plant derived substrates, at levels as low as 0.5 ppb to levels higher than 100 ppb (Khalil et al., 2013; Wacoo et al., 2014). However, these approaches do not indicate if one or multiple strains or species are producing the toxins nor do they distinguish between the different aflatoxin metabolites of those strains.

While this information may not be critical for regulatory purposes, understanding the species and lineage composition of strains that are aflatoxigenic and non-aflatoxigenic as well as the relative amounts of B_1 aflatoxin produced between aflatoxigenic strains is important to population genetic studies with the ability to improve agricultural management strategies (Moore et al., 2009). Increasing evidence exists that balancing selection is maintaining fungal lineages that are aflatoxigenic and non-aflatoxigenic in natural populations (Horn & Dorner, 1999; Horn, 2003; Carbone et al., 2007b; Drott et al., 2017). A better understanding of the aflatoxin producing potential of strains in these lineages is important when evaluating the population genetic consequences of management strategies that deploy non-aflatoxigenic strains as biological control agents (Faustinelli et al., 2017; Olarte et al., 2012).

Previous approaches to aflatoxin quantification for research explored extractions directly from fungal mycelium grown in culture and then with aflatoxins found in agricultural products (e.g. oil-seeds and kernels) using simple chemical cleanup columns (Sobolev & Dorner, 2002; Horn et al., 1996). To further facilitate quantitation of aflatoxin production across isolates in a fast but increasingly reliable approach and provide a detailed methodology for aflatoxin quantitation needed by the community, we adapted these methodologies using a HPLC method that consistently quantifies aflatoxin B_1 in the media of fungal mycelium cultures after extraction with chloroform and purification by chemical cleanup columns. Obtaining aflatoxin concentrations only from culture media reduces the problematic non-aflatoxin signals during HPLC increasing our ability to replicate previous approaches and permits relevant quantitation from isolates that are non-aflatoxigenic and those that produce aflatoxin at low levels; this is important because even very low doses (20 ng/mL) of aflatoxin B_1 can be toxic to humans and animals (Mehrzad et al., 2017).

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https://doi.org/10.1016/j.mimet.2019.01.008
Received 22 October 2018; Received in revised form 20 January 2019; Accepted 21 January 2019
Available online 22 January 2019
0167-7012/

2. Materials and methods

2.1. Solid and liquid fungal cultures for aflatoxin analysis

Cultures for aflatoxin testing were grown on Potato Dextrose Agar (PDA) plates for 7 days (5-dark, 2-light) at 30 °C, which is sufficient for yielding abundant sporulation. Following sporulation, liquid cultures were grown in 8 mL of YES Media, a media containing 2% yeast extract and 20% sucrose known to facilitate the production of aflatoxins (Koehler et al., 1975), with 75 μg/mL ampicillin within a 16 mL vial (ThermoSci Screw Vial Convenience Kit, Solid-top Cap from Thermo Scientific, B7800-4). A 1 μL inoculating loop (VWR, 12000-808) was used to inoculate by touching the surface of the strain's plate in a region with conidia, obtaining a ball of conidia approximately the size of the hole in the inoculating loop, and then inserting it into a YES vial by touching the loop to the media and gently agitating. Caps were screwed onto each vial by closing completely and then half turn backward to allow for airflow. Vials were sealed by a single wrap of parafilm to secure and prevent loss of volume due to dehydration without impacting oxygen permeability and incubated under constant light at 30 °C for 7-days. Three liquid cultures were produced as replicates for each strain tested.

2.2. Aflatoxin extractions

In a biosafety cabinet, 1 mL of each fully incubated culture was taken and transferred to a 4 mL vial (ThermoSci Screw Vial Convenience Kit, Solid-top Cap from Thermo Scientific, B7800-2). The culture samples were obtained from just below the mat of mycelium while minimizing the amount of detritus transferred.

In fume hood, 1 mL of Chloroform HPLC Grade, Alfa Aesar (VWR, AA43685-K2) was added to each culture being extracted, and vortexed vigorously for 10 s. Samples were left at rest for 30 min to allow for separation of layers; if any emulsive cultures remained, they were centrifuged for 2 min at 3000*g*. A 1 mL pipetman was used to transfer 0.5 mL of the chloroform layer to a new 4 mL vial minimizing any media or tissue debris contaminating the sample. Each extraction was placed in a heated bead bath under a low-pressure stream of UHP GR 5.0 Nitrogen until chloroform completely evaporated. Caps were re-sealed rapidly after removing the nitrogen stream to limit oxygen exposure. Extractions were then stored sealed and dry at 4 °C in the dark.

2.3. Extract purification

One milliliter of methanol (HiPerSolv CHROMANORM® gradient for HPLC; VWR, BDH20864.100E) was added to each dried sample and suspended by agitating. The suspended samples were passed through 1 mL polypropylene SPE tubes (Sigma-Aldrich, 57023), containing 200 μL of alumina basic, 60–235 mesh (Fisher, A941-500) between PE frits, 20 μm porosity (Sigma-Aldrich, 57023) into new 4 mL vials as the purified sample. The bottom tips of clean-up columns each containing trace amounts of its purified sample were placed under long-wave UV, which causes aflatoxin to fluoresce. The fluorescence of samples' clean-up columns was compared to previous results to determine dilution amounts needed to allow for only one injection. Purified samples were stored stably at 4 °C in the dark for 0–2 days before HPLC injection which allows for multiple sets of extractions to be analyzed at once.

2.4. HPLC

Aflatoxin quantifications were performed by the Biomanufacturing Training and Education Center's Bioprocess and Analytical Services at North Carolina State University following previously published specifications (Huang & Elmashni, 2007). In summary, samples were analyzed by reversed phase HPLC on a Shimadzu Prominence system interfaced with Shimadzu Labsolutions Version 5.54 using a Phenomenex Kinetex, 2.6 μm, C18, 100 Å, 150 × 4.6 mm column and fluorescence detection. Mobile phases consisted of deionized water (A) and methanol (B) filtered through a 0.22 μm membrane filter prior to use. A gradient program was utilized, with a starting condition of A:B (50:50, v/v) held for 11 min. The proportion of A:B was changed (5:95, v/v) over the course of 1 min, and held for 5 min. The proportion of A:B was returned to initial conditions (50:50, v/v) over the course of 0.5 min and held for 7.5 min for a total run time of 25 min. Sample injection volume was 10 μL, with a flow rate of 0.5 mL/min, an excitation wavelength of 365 nm, an emission wavelength of 455 nm and a column temperature of 40 °C. The working aflatoxin B_1 (Sigma-Aldrich, A6636-5MG) standard curve was based on a five-point calibration curve for the range of 0.0625–1.0 μg/mL. Serial dilutions were used to determine the limit of aflatoxin B_1 detection at 0.0020 μg/mL and quantification at 0.0039 μg/mL.

2.5. Aflatoxin recovery from spiking

Aflatoxin B_1 (Sigma-Aldrich, A6636-5MG) dissolved in methanol to a concentration of 1000 μg/mL was added to each sample at the appropriate volume; 1, 6, 20, and 50 μL respectively. Each IC201 and IC1179 spiking sample was processed as above until the subsampling 1 mL of liquid culture step. Each spiked media sample contained only the 7 mL of YES media with no introduced strain of *Aspergillus flavus*. For non-chloroform spiking samples, aflatoxins were added to the 1 mL of culture after it was transferred to its new vial and vortexed, before adding chloroform and being processed as other samples. Each chloroform spiking sample contained 1 mL chloroform amended with the appropriate aflatoxin spiking amount followed by vortexing and processing of the top 0.5 mL of chloroform as the sample for drying under nitrogen and handling, as the other dried samples.

2.6. Known aflatoxigenic strains and population sample isolates

Five strains of *A. flavus* previously shown to produce aflatoxins were retrieved and tested for aflatoxin concentration. These five strains native to Georgia were originally harvested from corn kernels (IC218, IC225), soil samples (IC229, IC278), and peanut seeds (IC308) (Horn & Dorner, 1999).

Forty-two isolates obtained as part of an ongoing exploration of genetic structure in *A. flavus* field populations (Molo, 2018) were also tested for aflatoxin concentration with this methodology. These strains came from soil and kernel samples collected between 2013 and 2014, in North Carolina and Texas.

3. Results and discussion

3.1. Aflatoxin recovery from spiking

The percent recovery of aflatoxins with this method was determined by extraction and quantification of non-aflatoxigenic samples spiked with aflatoxin B_1 to achieve four different concentrations: 1, 6, 20, and 50 μg/mL of culture. To understand the source of any variation a variety of non-aflatoxigenic sample types were tested including media inoculated with *A. flavus* biocontrol strains Afla-Guard (=NRRL 21882, IC201) and AF36 (=NRRL 18543, IC1179) to examine the impact of any cellular debris and other secreted metabolites, media-only samples that were not inoculated with any strain to examine how well aflatoxin is transferred between the media and chloroform, and samples where aflatoxin was spiked directly into 1 mL of chloroform to explore the amount of aflatoxin lost due to the resuspension and purification with the chemical cleanup column.

Recovery of aflatoxins under all experimental conditions and concentrations tested showed fairly consistent retention with an overall average percent recovery of 77.68% ± 9.52% (Table 1). There was no substantial variation between results observed under experimental

Table 1
Aflatoxin concentrations and recovery in experimental and spiked conditions.

Spiked solution	Strain	Spiked aflatoxin B_1 Conc. (μg/mL)	Recovered aflatoxin B_1 Conc.[a] (μg/mL)	% Recovery	Avg. % recovery by media	Std. Dev % recovery by media
YES Media	IC201	1	0.7	70.00	73.27	2.70
YES Media	IC201	6	4.28	71.33		
YES Media	IC201	20	15.02	75.10		
YES Media	IC201	50	38.32	76.64		
YES Media	IC1179	1	0.74	74.00	76.92	9.49
YES Media	IC1179	6	4.1	68.33		
YES Media	IC1179	20	14.48	72.40		
YES Media	IC1179	50	46.48	92.96		
YES Media	Not Inoculated	1	1.02	102.00	84.53	12.89
YES Media	Not Inoculated	6	4.12	68.67		
YES Media	Not Inoculated	20	18.2	91.00		
YES Media	Not Inoculated	50	38.22	76.44		
Chloroform	Not Inoculated	1	0.72	72.00	76.00	5.40
Chloroform	Not Inoculated	6	4.28	71.33		
Chloroform	Not Inoculated	20	15.16	75.80		
Chloroform	Not Inoculated	50	42.44	84.88		

[a] The aflatoxin concentrations obtained from HPLC correspond to the 1 mL of methanol in which dried aflatoxin samples are resuspended. This only contains the amount of aflatoxins within 0.5 mL of the chloroform, which is half of the initial 1.0 mL of chloroform introduced to each sample. To represent the aflatoxin concentration within 1.0 mL of culture, the concentrations reported were obtained by doubling each concentration initially conveyed by HPLC results to factor in the previous halving of that concentration.

conditions where the aflatoxins were removed from media, with or without non-aflatoxigenic cultures present, and results where aflatoxin B_1 was applied directly to the chloroform supporting the extraction method. The consistent loss, which was observed in all samples, appears to be during either resuspension or purification. Additional variations of these steps were not tested due to the risks of aflatoxin degradation with prolonged handling and excessive dilution of samples with low concentrations. Variations from this expected loss occurred across experimental conditions and concentrations providing no clear indications of what causes this phenomenon but in no case did this variance alter the magnitude or relative concentration of the sample. None of the experimental conditions reported any aflatoxin when no aflatoxin was introduced.

3.2. Aflatoxin recovery from known aflatoxigenic strains

To initially assess the protocol's ability to quantitate aflatoxin from fungal mycelium culture, extractions were performed on five strains of *Aspergillus flavus* known to produce aflatoxins across a spectrum of concentrations (Horn & Dorner, 1999). Three replicate cultures were grown and aflatoxins extracted in parallel for each strain to reduce sample variance that could occur due to environmental variability while examining what variation remains due to growth differences and extraction variability.

Aflatoxin B_1 concentrations ranged from 0.08 μg/mL to 29.58 μg/mL across the control samples with the highest Relative Standard Deviation (RSD) calculated as 58.08 for a set of three replicates (Table 2). Several strains were low producers with aflatoxin concentrations less than 0.50 μg/mL. For most of the strains, the results showed proportionally larger deviations from their mean than was produced with the spiking experiment. This variation in aflatoxin B_1 production by strains has been reported previously and explained by genotype by environment effects (Olarte et al., 2012; Olarte et al., 2015; Fountain et al., 2014). Mean aflatoxin concentrations between samples were comparable and show noticeably distinct levels across the selected strains supporting this protocol's use as a simple method for aflatoxin quantitation across an *A. flavus* population's diverse phenotype range.

3.3. Aflatoxin recovery from population sample isolates

Aspergillus flavus isolates can be separated genetically into two lineages: lineage IC which are known to produce higher levels of aflatoxin B_1 and lineage IB which have more nontoxigenic isolates and toxigenic isolates that produce less aflatoxins (Moore et al., 2009; Geiser et al., 2000; Moore et al., 2017). Aflatoxin B_1 concentrations of the toxigenic isolates from the high producing lineage had a broader range from averages of 0.06 μg/mL to 39.12 μg/mL and RSD averages 40.93% compared to those from the low aflatoxin producing lineage with averages of 0.02 μg/mL to 1.99 μg/mL and RSD averages 60.81% (Table S1). Ten of the isolates produced no aflatoxin from any of the three extractions performed for each, suggesting that these isolates are likely non-aflatoxin producers. Of these ten nontoxigenic, only two were from the high toxin producing lineage while eight were from the lineage expected to contain fewer toxigenic isolates. Only three isolates (IC6510, IC14611, and IC14677) of thirty-two aflatoxigenic isolates had an extraction where aflatoxin was not detected, supporting the consistency of this methodology for the detection of aflatoxin from aflatoxigenic isolates.

Table 2
Aflatoxin B_1 concentrations from liquid culture of known aflatoxigenic strains.

NRRL number	IC number	B_1 Conc.[a] (μg/mL)	Avg. B_1 Conc. (μg/mL)	Relative standard deviation (%)
20025	IC218	0.4 0.2 0.28	0.29	34.32
20027	IC225	0.16 0.08 0.28	0.17	58.08
29459	IC229	1.06 1.1 1.16	1.11	4.55
29507	IC278	29.58 27.36 29.36	28.76	4.25
29537	IC308	0.36 0.2 0.2	0.25	36.46

[a] For an explanation of aflatoxin concentrations see footnote of Table 1.

4. Conclusions

We describe an HPLC method for quantitation of aflatoxin B_1 in the media of fungal mycelium cultures. The method is scalable and can

determine relative aflatoxin producing potential across the spectrum of possible yields as shown by quantitation of aflatoxin B_1 from over forty isolates as part of a larger population genetics study.

This study focused on the results for aflatoxin B_1, the most carcinogenic and most abundantly produced of the aflatoxins. However, fluorescent peaks corresponding to aflatoxin B_2, G_1, and G_2 were visualized during development of this methodology (Fig. S1) suggesting it has the potential to quantify all aflatoxin metabolites produced by *A. flavus* and allied species, but development of the expanded method will require additional validation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.01.008.

Funding

This work was supported by the Agriculture and Food Research Initiative Competitive Grants Program grant no. 2013-68004-20359 from the USDA National Institute of Food and Agriculture (NIFA).

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

We thank Victor Sobolev for helpful insights, Vicki Cornish for assistance with fungal strain management and Brian Mosley (Golden LEAF Biomanufacturing Training and Education Center) for processing the samples through HPLC.

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