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Single Corn Kernel Aflatoxin B_1 Extraction and Analysis Method

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Abstract

Aflatoxins are highly carcinogenic compounds produced by the fungus *Aspergillus flavus*. *Aspergillus flavus* is a phytopathogenic fungus that commonly infects crops such as cotton, peanuts, and maize. The goal of this research was to design an effective method for sample preparation and analysis of aflatoxin B_1 from *Aspergillus flavus* infected maize kernels. The method involves liquid extraction with a methanol/water solution. The samples were analyzed by liquid chromatography coupled to an electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) operating in positive ion multiple reaction monitoring (MRM) mode. The spiked levels for the recovery experiment were 4 ppb and 20 ppb, respectively. Recoveries ranged between 72% and 113% (90.8% average), with Relative Standard Deviation below 15% (10.6% on average).

Keywords

***Aspergillus flavus*, Aflatoxin, Maize, HPLC, MS/MS, Extraction**

1. Introduction

Aspergillus flavus (*A. flavus*) is a fungus that commonly grows in the soil and is known to infect a variety of crops including cotton, peanuts, and corn. *A. flavus* can cause ear rot in maize, aflaroot in peanuts, and yellow spot disease in cotton [1]-[3]. Environmental conditions such as high temperatures, high humidity, drought stress, and poor crop storage can cause *A. flavus* to start producing aflatoxins [4]. Aflatoxins are secondary metabolites

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of the fungus and are extremely carcinogenic. The four major types of aflatoxins are aflatoxin B_1, B_2, G_1, and G_2. Aflatoxin B_1 and B_2 fluoresce blue under UV light while aflatoxin G_1 and G_2 fluoresce green in the presence of UV light. Aflatoxin B_1 can also be metabolized to aflatoxin M1 which would be found in the milk of lactating mammals. Aflatoxin B_1 is the most common and carcinogenic of these compounds as seen in **Figure 1**. Aflatoxin B_1 is converted *in vivo* to an aflatoxin B_1-exo-8, 9-epoxide by the liver enzyme cytochrome p450 oxidase [5]. The epoxide that is formed is a highly reactive electrophile and has an extremely high regiospecificity for the N7 position of the guanine residue in DNA [6]. It specifically binds to the p53 encoding region to form a DNA adduct [7]. This results in nonfunctioning p53 proteins which are important tumor suppressors in humans.

In 1988 the International Agency for Research on Cancer classified aflatoxin B_1 as a Class 1 human carcinogen [8]. Therefore, the U.S. Food and Drug Administration (FDA) has restricted the amount of aflatoxin in food for human consumption to 20 parts per billion (ppb) in the United States. The European Commission (EC) limits aflatoxins in food for human consumption to 4 ppb in the European Union [9] [10]. The Council of Agricultural Science and Technology has estimated that the US has an annual loss of almost a billion dollars due to crop damage from mycotoxins. An estimated $225 million of that are due to aflatoxin contamination in maize crops [11]. Study of the maize genome has increased in recent years in hopes to discover genes that are involved with aflatoxin resistance in corn. The DNA from a single corn kernel can be used to analyze the gene expression that occurs in *Aspergillus flavus* inoculated maize. In order to compliment this procedure, aflatoxin extraction should be equally versatile. Aflatoxin extraction methods normally require several (10 - 250) grams of ground maize which may not be feasible in determining aflatoxin accumulation for a smaller region of the *A. flavus* infected ear. A single kernel extraction method is needed in order to accurately track aflatoxin concentration and maize gene expression at specific *A. flavus* infected sites on the cob.

2. Materials and Methods

2.1. Reagents and Chemicals

All solvents and reagents were Optima LC/MS grade. Methanol, water, formic acid, and ammonium acetate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Purified aflatoxin B_1 and M_1 standards were acquired from Sigma Aldrich (St. Louis, MO, USA).

2.2. Sample Preparation

Maize kernels were flash frozen in liquid nitrogen. The kernels were ground into a fine powder with a mortar and pestle. 200 mg of each ground sample was placed into a 1.5 mL micro-centrifuge tube. Aflatoxin free ground maize was spiked with aflatoxin B_1 to yield two levels of spiked samples (4 ppb and 20 ppb) of aflatoxin B_1 for recovery. These concentrations were chosen because they are the limit for aflatoxin in food for human consumption in the European Union and the United States, respectively.

A solution of 1 mL (70/30, v/v) methanol/water was added to each 1.5 mL microcentrifuge tube. The samples were mixed for 1 minute and then centrifuged for 5 minutes at 14,000 rpm. Polytetrafluoroethylene (PTFE) syringe filters (0.45 µm) were used to remove any large particles from the samples after centrifugation as shown in **Figure 2**. The liquid extracts were transferred to auto-sampler vials and analyzed using an Agilent 6460 LC/MS/MS Triple Quadrupole with electrospray ionization. Aflatoxin M_1 was added as an internal standard at a concentration of 10 ppb. The calibration curve was matrix-matched with extracted aflatoxin-free maize solution.

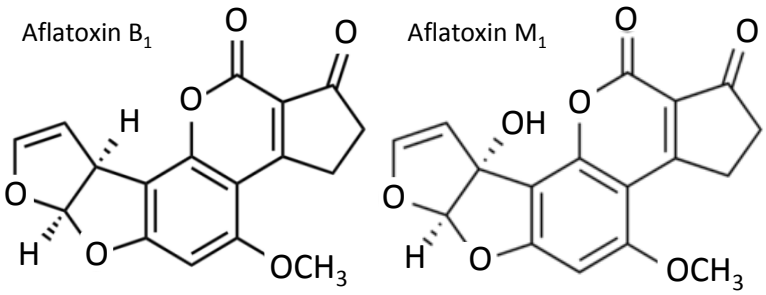

Figure 1. Structures of aflatoxin B1 and aflatoxin M1 (ISTD).

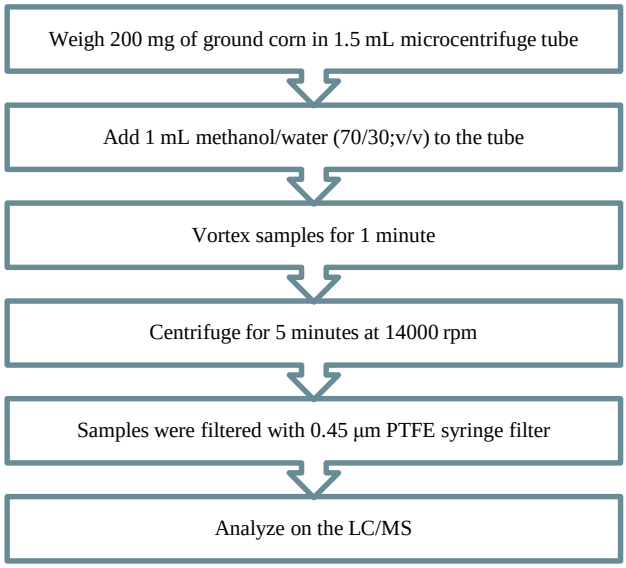

Figure 2. Sample Preparation workflow detailing single kernel aflatoxin extraction.

3. Experimental

The samples were analyzed using an Agilent 6460 LC/MS/MS Triple Quadrupole Mass Spectrometer with ESI, using an Agilent 1200 Series High Performance Liquid Chromatography (HPLC). The HPLC system consists of a binary pump (G1312B), infinity high performance degasser (G1379B), high performance autosampler (G1367E), thermostatted column compartment (G1316B), sampler thermostat (G1330B), and Mass Hunter data software. Aflatoxin B_1 and M_1 were optimized using the Agilent Optimization software. Aflatoxin M_1 was used as an internal standard (ISTD) at 10 ppb. The Agilent Optimization software produces the ideal fragmentor voltage and collision energy for each MRM transition of aflatoxin B_1 and M_1 as listed in **Table 1**.

4. Instrumentation

4.1. HPLC Method Conditions

An Agilent 6460 LC/MS Triple Quadrupole Mass Spectrometer equipped with electrospray ionization (ESI) was used to analyze the aflatoxin concentration of the samples. The autosampler temperature was set to 4˚C. The HPLC used a Zorbax Eclipse Plus-C18 Narrow Bore 2.1 × 50 mm, 5 µm column with a temperature of 50˚C. The mobile phase consisted of 5 mM ammonium acetate with 0.1% formic acid in HPLC-grade water and 5 mM ammonium acetate with 0.1% formic acid in methanol. The flow rate of the mobile phase during the analysis was consistently 0.6 mL/min. The mobile phase gradient was transitioned from 95% water to 100% methanol during the six minutes of the analysis time. Then for the final three minutes of the run, the mobile phase reversed from 100% methanol back to 95% water. The total run time of the method was nine minutes which includes a six minute analysis time and an additional three minutes for the system to get back to equilibrium. The calibration curve was matrix-matched in order to reduce matrix effects. The six point calibration curve consisted of the concentrations 1 ppb, 5 ppb, 10 ppb, 25 ppb, 50 ppb, and 100 ppb as shown in **Figure 3**.

4.2. MS Method Conditions

The parameters for the mass spectrometer were the following. The Agilent 6460 Triple Quadrupole Mass Spectrometer (MS/MS) system coupled to an electrospray analyzed the samples while in positive mode. The drying gas temperature was 325˚C while the gas flow was set to 10 liters per minute. The nebulizer gas pressure was set to 50 psi and the capillary voltage was 4000V. The Sheath Gas Flow had an output of 11 liters per minute and the sheath gas temperature reached temperatures of 350˚C. The delta electron multiplier voltage (EMV) was

800V and the dwell time lasted for 200 msec. The precursor ion for aflatoxin B_1 was 313.1 m/z and 329.1 m/z for aflatoxin M_1. While in multiple reaction monitoring (MRM) mode, the mass spectrometer was set to look for the daughter ions after the precursor ion entered the collision cell. The transitions for aflatoxin B_1 included 313.1 > 285.1 m/z with a collision energy of 20kEV, 313.1 > 269.1 m/z with a collision energy of 25 kEV, and 313.1 > 241.1 m/z with a collision energy of 35 kEV. The fragmentor value was 166 for aflatoxin B_1 and for aflatoxin M_1 was 131. The cell accelerator (7) values were the same for both aflatoxin transitions. The two transitions used for identifying aflatoxin M_1 are 329.1 > 273.1 m/z and 329.1 > 229.1 m/z. The retention time of aflatoxin M_1 was 3.0 minutes and 3.4 minutes for aflatoxin B_1 as shown in **Figure 4**. Agilent MassHunter Quantitative Analysis Workstation Software v. B.04.0.225.19 was used to analyze the quantitative data obtained from the samples and the calibration curve.

Table 1. MRM transitions for aflatoxin B1 and the internal standard, aflatoxin M1.

Compound (Aflatoxin)	Retention Time (min)	Fragmentor Voltage (V)	Collision Energy (eV)	Precursor Ion (m/z)	Product Ion (m/z)
B_1	3.4	166	21	313.1	285.1
B_1	3.4	166	33	313.1	269.1
B_1	3.4	166	41	313.1	241.1
M_1 (ISTD)	3.0	131	25	329.1	273.1
M_1 (ISTD)	3.0	131	41	329.1	229.1

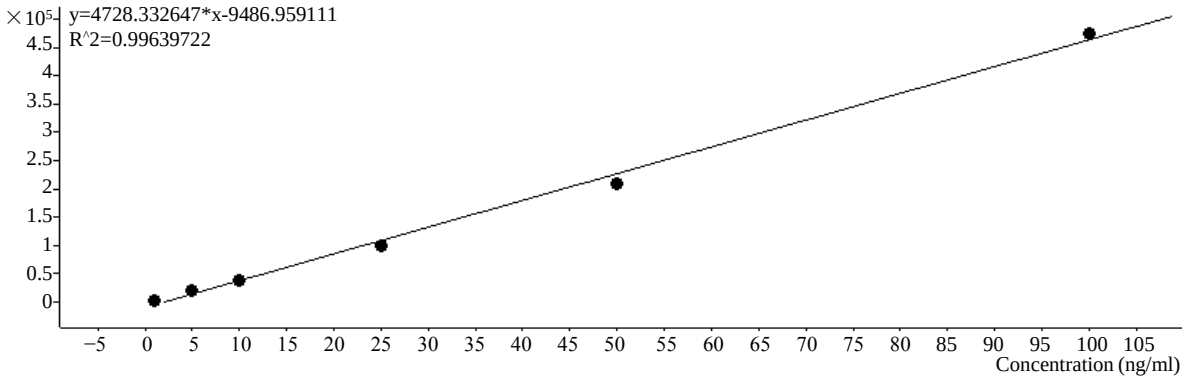

Figure 3. Matrix matched calibration curve for aflatoxin B1 from concentration of 1.0 ppb to 100 ppb.

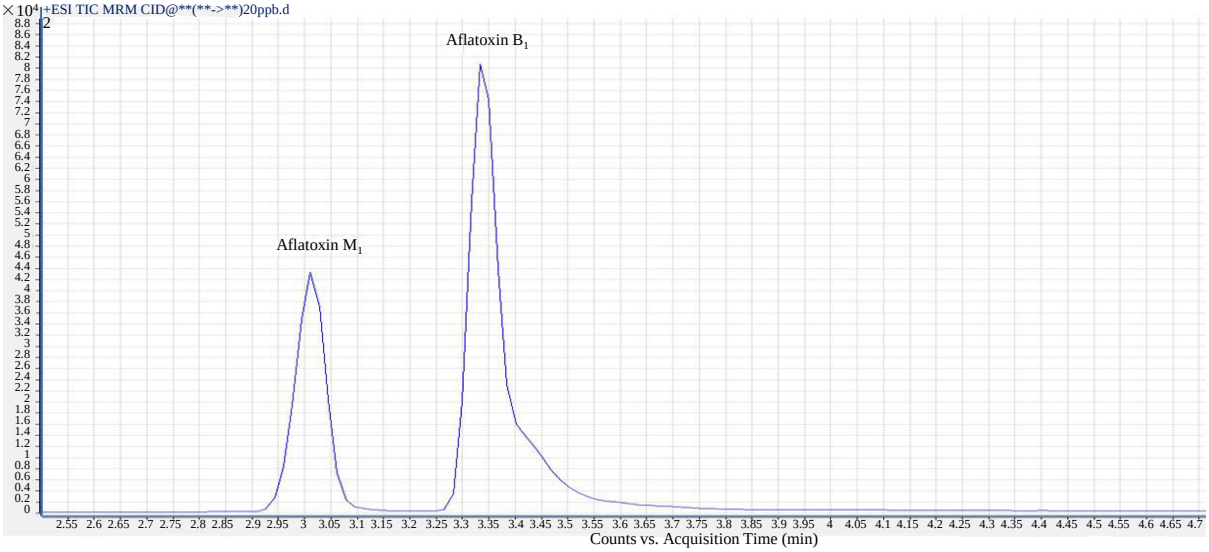

Figure 4. Chromatogram of aflatoxin B_1 standard at 20 ppb with aflatoxin M_1 as the internal standard at 10 ppb.

Table 2. Average percent recoveries and RSD values obtained from spiked corn samples.

	4 ng/mL spiked maize	20 ng/mL spiked maize
Aflatoxin B_1	**% Recovery**	**% Recovery**
Replicate 1	91.65	93.23
Replicate 2	86.7	113
Replicate 3	97.38	72.384
Replicate 4	87.82	87.087
Replicate 5	90.59	87.88
Avg % Recovery	**90.828**	**90.716**
RSD (n = 5)	**4.11**	**14.45**
LOD (ppb)	**0.344**	**0.344**
LOQ (ppb)	**1.042**	**1.042**

5. Results and Discussion

Table 2 demonstrates the average percent recoveries and relative standard deviation (RSD) values obtained from the spiked corn samples after performing the single maize kernel aflatoxin extraction. The analysis was performed in replicates of five at each of the two levels. The average percent recoveries for the 4 ppb and the 20 ppb aflatoxin B_1 spiked maize were 90.83% with a RSD of 4.11% and 90.72% with a RSD value of 14.45% respectively. Calibration standards were matrix matched with a range from 1 ppb to 100 ppb with a linear correlation (R^2) of 0.996 as shown in **Figure 3**. In The limit of detection (LOD) and limit of quantification (LOQ) were estimated from the concentration of aflatoxin B1 required to give a signal to noise ratio of 3:1 and 10:1 respectively. The LOD was determined to be 0.344 ppb and the LOQ was calculated to be 1.042 ppb. The internal standard, aflatoxin M_1, injected at 10 ppb accounts for possible instrument variation. Aflatoxin M_1 was chosen to be the internal standard because it has a structure similar to aflatoxin B_1 but is not produced by *A. flavus*. Aflatoxin M_1 is also less expensive and more readily available commercially than the radiolabeled form of aflatoxin B_1. Methanol was the preferred solvent for aflatoxin extraction due to its compatibility with the ELISA testing and the cost effectiveness over other solvents like acetone and acetonitrile [12] [13]. This simplified aflatoxin extraction method is analogous to other extractions methods in terms of aflatoxin recovery without the need for solid phase extraction or clean up columns [14]. The single maize kernel extraction method was needed in order to more accurately determine the changes in aflatoxin production, gene transcription, and protein production between inoculated and control maize kernels.

6. Conclusion

This process demonstrates a fast, simple, and effective analytical method for determining aflatoxin concentrations in a single maize kernel using an Agilent 6460 Triple Quadruple Mass Spectrometer. The detection levels for aflatoxin B_1 were below both the limit set by the FDA in the United States and the limit set by the EC in the European Union. The recovery percentages for aflatoxin B_1 were 90.83% for 4 ppb and 90.72% for 20 ppb with a satisfactory average RSD less than 15%. The single kernel extraction method will be a useful technique in determining how aflatoxin producing *Aspergillus flavus* affects infected maize.

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References

[1] Smart, M.G., Shotwell, O.L. and Caldwell, R.W. (1990) Pathogenesis in Aspergillus Ear Rot of Maize: Aflatoxin B1 Levels in Grains Around Wound Inoculation Sites. *Phytopathology*, **80**, 1283-1286. http://dx.doi.org/10.1094/Phyto-80-1283

[2] Guchi, E. (2015) Implication of Aflatoxin Contamination in Agricultural Products. *American Journal of Food and Nutrition*, **3**, 12-20.

[3] Zeringue, H.J., Shih, B.Y., Maskos, K. and Grimm, D. (1999) Identification of the Bright-Greenish-Yellow-Fluorescence (BGY-F) Compound on Cotton Lint Associated with Aflatoxin Contamination in Cottonseed. *Phytochemistry*, **52**, 1391-1397. http://dx.doi.org/10.1016/S0031-9422(99)00432-X

[4] Trenk, H.L. and Hartman, P.A. (1970) Effects of Moisture Content and Temperature on Aflatoxin Production in Corn. *Applied Microbiology*, **19**, 781-784.

[5] Smela, M.E., Currier, S.S., Bailey, E.A. and Essigmann, J.M. (2001) The Chemistry and Biology of Aflatoxin B1: From Mutational Spectrometry to Carcinogenesis. *Carcinogensis*, **22**, 535-545. http://dx.doi.org/10.1093/carcin/22.4.535

[6] Greenblatt, M.S., Bennett, W.P., Hollstein, M. and Harris, C.C. (1994) Mutations in the p53 Tumor Suppressor Gene: Clues to Cancer Etiology and Molecular Pathogenesis. *Cancer Research*, **54**, 4855-4878.

[7] Clewell, R.A., Sun, B., Adeleye, Y., Carmichael, P., Efremenko, A., McMullen, P.D., Pendse, S., Trask, O.J., White, A. and Andersen, M.E. (2014) Profiling Dose-Dependent Activation of p53-Mediated Signaling Pathways by Chemicals with Distinct Mechanisms of DNA Damage. *Toxicological Sciences*, **142**, 56-73. http://dx.doi.org/10.1093/toxsci/kfu153

[8] Vainio, H. and Wilbourn, J. (1992) Identification of Carcinogens within the IARC Monograph Program. *Scandinavian Journal of Work, Environment & Health*, **18**, 64-73.

[9] Otsuki, T., Wilson, J.S. and Sewadeh, M. (2001) Saving Two in a Billion: Quantifying the Trade Effect of European Food Safety Standards on African Exports. *Food Policy*, **26**, 495-514. http://dx.doi.org/10.1016/S0306-9192(01)00018-5

[10] Wu, F. and Guclu, H. (2012) Aflatoxin Regulations in a Network of Global Maize Trade. *PLoS ONE*, **7**, e45151. http://dx.doi.org/10.1371/journal.pone.0045151

[11] Robens, J. and Cardwell, K. (2003) The Costs of Mycotoxin Management to the USA: Management of Aflatoxins in the United States. *Journal of Toxicology*: *Toxin Reviews*, **22**, 139-152. http://dx.doi.org/10.1081/txr-120024089

[12] Bertuzzi, T., Rastelli, S., Mulazzi, A. and Pietri, A. (2011) Evaluation and Improvement of Extraction Methods for the Analysis of Aflatoxins B1, B2, G1 and G2 from Naturally Contaminated Maize. *Food Analytical Methods*, **5**, 512-519. http://dx.doi.org/10.1007/s12161-011-9274-5

[13] Spanjer, M.C., Rensen, P.M. and Scholten, J.M. (2008) LC–MS/MS Multi-Method for Mycotoxins after Single Extraction, with Validation Data for Peanut, Pistachio, Wheat, Maize, Cornflakes, Raisins and Figs. *Food Additives & Contaminants*: *Part A*, **25**, 472-489. http://dx.doi.org/10.1080/02652030701552964

[14] Khayoon, W.S., Sadd, B., Yan, C.B., Hashim, N.H., Ali, A.S.M., Salleh, M.I. and Salleh, B. (2010) Determination of Aflatoxins in Animal Feeds by HPLC with Multifunctional Column Clean-Up. *Food Chemistry*, **118**, 882-836. http://dx.doi.org/10.1016/j.foodchem.2009.05.082


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