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Measurement of Aflatoxins Using Capillary Electrophoresis

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1. Introduction

1.1. The Aflatoxins

The aflatoxins are a group of mycotoxins produced by certain *Aspergillus* species, in particular *Aspergillus flavus* and *Aspergillus parasiticus*. The aflatoxins are extremely potent mutagens, are suspected human carcinogens, and can adversely affect animal health and agricultural productivity. Many countries routinely screen agricultural commodities for the presence of the aflatoxin B_1 (AFB_1), the most potent member of the group. In the United States, the Food and Drug Administration has established a guideline level of 20 ng/g (ppb) total aflatoxins in food destined for human consumption ***(1)***. For breeding cattle, breeding swine, and mature poultry the limit is 100 ppb. For finishing swine and finishing beef cattle the limits are 200 and 300 ppb, respectively. Because of the importance of this group of mycotoxins to human and animal health, all of the common tools of analytical chemistry, including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry, immunoassay, biosensors, and capillary electrophoresis, have been used for their detection. The literature dealing with chromatographic methods for the mycotoxins is extensive and several excellent reviews have been published ***(2–6)***.

The major aflatoxins have a strong UV absorbance with a maximum in the range of 360-365 nm when measured in methanol, with reported extinction coefficients in the range of 21,700 to 27,300 for AFB_1 ***(7)***. The characteristic UV/Vis spectra of the aflatoxins is helpful in confirming the presence of the toxins when they are isolated from complex matrices. Many analytical methods for the aflatoxins, including TLC and HPLC methods rely upon the

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fluorescence of this group of mycotoxins ***(8,9)***. The aflatoxins were given their trivial names in part from their fluorescence characteristics: the AFB toxins exhibit a blue fluorescence whereas the AFG toxins exhibit a blue-green fluorescence on TLC plates when exposed to longwave UV light.

1.2. Capillary Electrophoresis

Although chromatographic separations are based upon the interactions between an analyte and a stationary phase, electrophoretic separations are based upon the behavior of an analyte in the presence of an electrical field. Electrophoretic separations are performed in capillaries, principally because of the high electrical field strengths used and the need to rapidly remove the heat generated. The permutations of capillary electrophoresis (CE) are numerous and range from free zone CE, where separations are performed in simple buffers, to micellar electrokinetic capillary chromatography (MECC), where a pseudostationary phase is used. Capillary chromatography is a hybrid of CE and HPLC where the electrical field is used to draw the buffer through a stationary phase, rather than pumping it through as with HPLC. The application of CE to mycotoxin analysis was recently reviewed ***(10)***.

The simplest and most widely used form of CE is free zone capillary electrophoresis. In free zone CE the composition of the buffers placed at the anode and cathode are identical. With the application of an electrical potential across the capillary the buffer components begin to separate, with the anions moving toward the anode and the cations toward the cathode. In the case where bare fused-silica capillaries are used the application of an electrical potential causes a flow of buffer from the anode toward the cathode. This flow, termed electroosmotic flow (EOF), results from the movement of cations with an accompanying shell of hydration toward the cathode. If the EOF is sufficiently vigorous even compounds having a substantial negative charge can be made to flow toward the cathode. In this manner the components carrying positive charges will elute first, followed by neutral compounds and then negatively charged compounds. The magnitude of the EOF is influenced by a host of factors including the pH, ionic strength, viscosity, and temperature of the solution as well as the magnitude of the applied voltage and the surface characteristics of the capillary wall. Where it is desirable, the surface of the silica capillaries can be treated to reduce, eliminate, or even reverse the EOF.

The major difference between free zone CE and MECC is the use of a buffer containing a pseudostationary phase, usually composed of micelles formed from surfactants such as sodium dodecyl sulfate (SDS) or bile salts such as sodium deoxycholate (NaDC). The components of the sample interact with the micelles and the buffer as they migrate through the capillary. The micelles therefore serve a function analogous to the stationary phase in HPLC. The

major aflatoxins differ from each other in polarity and can therefore be separated by either reversed-phase HPLC or MECC ***(11)***.

1.3. Separation of the Aflatoxins by CE

The aflatoxins G_2, G_1, and B_2 were first separated using CE by Balchunas et al. ***(12)***. The separation was performed using MECC, with fluorescence detection (MECC-LIF). Several papers refining the separation have appeared from the same group ***(11,13,14)***. Cole et al. ***(11)*** formed the micelles for MECC with the bile salt surfactant NaDC rather than SDS. With the use of NaDC buffer the addition of organic modifiers was not necessary to achieve baseline separation of these three aflatoxins. A full examination of the factors affecting separation of aflatoxins, including AFB_1, by MECC-LIF was provided by Cole et al. ***(13)***. AFG_2, AFG_1, AFB_2, and AFB_1 were separated from one another within 30 s. This very rapid separation was achieved by applying 36 kV across a 40 cm capillary (35 cm length to detector, 25 μm id). By using a buffer composed of 50 m*M* SDS, 10 m*M* dibasic sodium phosphate, 6 m*M* sodium borate, and 10% (v/v) acetonitrile the aflatoxins were separated within 5 min when 20 kV was applied. Several samples of spiked corn meal or *Aspergillus flavus* cultures were examined. The limit of detection (LOD) was estimated to be 1 ppm for spiked maize. Whereas the LOD was rather high for spiked maize, the LOD for aflatoxins standards was quite good: ranging from 2.64×10^{-8} *M* (8 ng/mL) for AFB_2 to 4.36×10^{-7} *M* (143 ng/mL) for AFG_1. In part, the difference may be due to the optimization of the system for standards in solution, rather than in purified corn matrix, and the clean-up and concentration steps used.

Recently, MECC-LIF has been applied to the quantitation of aflatoxin B_1 in maize (***15***; **Fig. 1**). AFB_1 was isolated from maize using one of two extraction methods: either a chloroform extraction followed by cleanup on a silica column or a methanol/water extraction followed by cleanup on an affinity column. The electrophoresis buffer contained 50 m*M* NaDC, 10 m*M* dibasic sodium phosphate and 6 m*M* sodium borate (pH 9.1). Baseline separation of AFG_1, AFG_2, AFB_2, and AFB_1 was achieved within 10 min. The order of the four aflatoxins was the same as that reported with reversed-phase HPLC ***(16–18)***. With the silica column cleanup the limit of detection (LOD), defined as the level of AFB_1 required to give a signal to noise ratio of 4, was 0.5 ppb in maize. With the affinity column cleanup the LOD was 1 ppb. This limit of detection compares favorably to the desired range for quantitation of aflatoxins in foods.

2. Materials

1. Sample Grinder: a Stein Mill or equivalent that can be used to attain a particle size able to pass through a number 20 sieve (roughly the consistency of ground coffee).

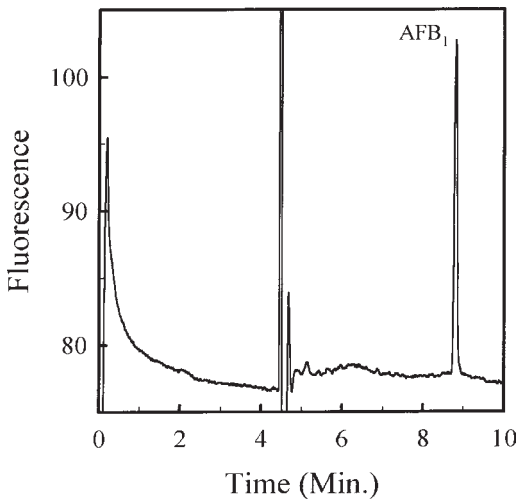

Fig. 1. Electropherogram of a 50 ng/mL solution of AFB_1 in NaDC buffer. The migration time for AFB_1 was 8.8 min. The peak shown at less than 1 min is an artifact from the NaDC buffer. The positive and negative peaks shown at 4.5–5 min arise from the 1% acetonitrile present in the standard.

2. Extraction Solution: 80% methanol/water (v/v), 100 mL per sample.
3. Blender: explosion proof blender made of methanol-resistant materials.
4. Sample Filtering and Dilution: Filter paper, Schleicher & Schuell number 588 (fast), or equivalent. Funnel, vial to collect the filtered extract (volume less than 100 mL), and vial to collect the diluted extract.
5. Aflatoxin Immunoaffinity Columns: AflaTest P columns, Vicam LP (Watertown, MA) (*see* **Note 1**).
6. Nitrogen gas for sample dry-down.
7. CE system:
   a. A capillary electrophoresis unit capable of delivering a voltage of 20 kV or a current of 105 µA, equipped with a fluorescence detector. Preferably a unit with automated sample injection. A Beckman P/ACE 5000 unit has been used previously.
   b. Filters for the fluorescence detector. A 400 nm long-pass filter (Oriel Corp., Stratford, CT) to exclude light from the excitation source and a 400 nm band-pass filter capable of transmission of wavelengths up to 462 nm. Alternatively, a tunable detector with an appropriate monochromator. The emission maximum occurs at 427–440 nm under the described conditions (*see* **Note 2**).
   c. A 19 mW Helium/Cadmium laser with 325 nm output connected as the excitation source (Model 3056, Melles Griot, Carlsbad, CA). For the Beckman P/ACE 5000 unit this involves disconnecting the Argon-ion laser and connecting the He/Cd laser to the fluorescence detector housing. The excitation

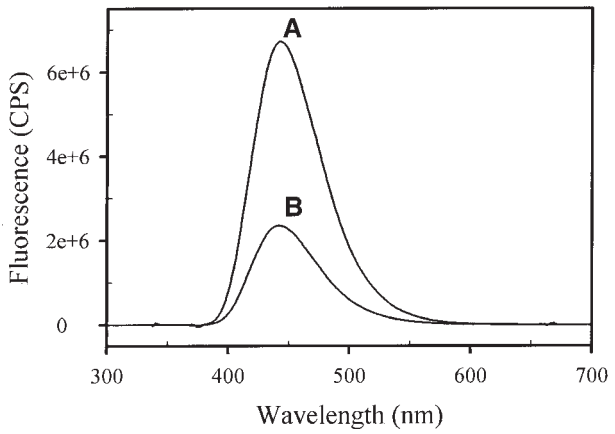

Fig. 2. Fluorescence emission spectra of 1 µg AFB_1/mL in methanol when excited with either light of wavelength 360 nm **(A)** or 325 nm **(B)**. Light from the He/Cd laser excitation source is 325 nm.

maximum for AFB_1 occurs at 360 nm under the described conditions; however the 325 nm source is sufficient to excite the aflatoxins, even though this wavelength does not result in maximum fluorescence (**Fig. 2**).

d. Fused silica capillary: 57 cm total length, 50 cm length to detector, 75 µm inner diameter.
e. Minivials: vials with a capacity of 0.6 mL and which can fit into the CE autosampler (*see* **Note 3**).

8. Electrophoresis buffers:

To avoid blockage of the capillary, all buffers should be filtered (0.45 µm or smaller) before using them with the CE.

a. Electrophoresis buffer: 50 m*M* sodium deoxycholate, 6 m*M* sodium borate, and 10 m*M* sodium phosphate, in deionized water, final pH 9.1 (*see* **Note 4**).
b. Sodium hydroxide, 0.1 *N* in deionized water.
c. Deionized water: 16 *M*Ω or better.

3. Methods

3.1. Extraction and Isolation of Aflatoxins from Maize

For accurate results it is important that the sample which is ground reflect the composition of the original sample, and that the original sample be obtained using a validated sampling plan. Add 100 mL of extraction solution to 50 g of ground maize and blend for 3 min. Filter the extract, allowing gravity to determine the flow rate. Dilute 10 mL of filtrate with 40 mL of deionized water and filter once more. Quantitatively transfer 10 mL of diluted filtrate, equivalent to 1 g of maize, onto a Vicam Aflatest P column. Wash with 20 mL deionized water

and elute the aflatoxins with 1 mL methanol. Dry the purified extract under a gentle stream of nitrogen and store refrigerated until the day of analysis.

3.2. Separation and Detection by CE-LIF

1. Aflatoxin Standards: Prepare AFB_1 stock solutions in acetonitrile over the range of 0.5 to 100 µg/mL and store at –20°C. Working standards should be prepared fresh daily to minimize the effects from degradation of AFB_1 in the buffer. Prepare the working standards over the range of 5 to 1,000 ng AFB_1/mL by diluting the stock solutions 1:100 (v/v) with electrophoresis buffer.
2. Samples: Prepare the maize samples by dissolving the extract from the immunoaffinity column in 0.8 mL electrophoresis buffer (a level equivalent to 1.25 g maize/mL). Transfer to a minivial for injection.
3. Capillary Electrophoresis: Rinse the capillary with the electrophoresis buffer by applying a pressure of 20 psi for 2 min. The volume of this rinse is equivalent to roughly 10 times the capillary volume. Inject the sample for 5 s at 0.5 psi, approx 30 nL. Transfer the capillary ends to the electrophoresis buffer and apply 20 kV. This should result in a current of approximately 104 µA. The aflatoxins G_2, G_1, B_2, and B_1 will elute within 10 min. After 10 min stop the voltage and rinse the capillary with 0.1 *N* sodium hydroxide for 1.5 min at 20 psi. Rinse the capillary an additional 1.5 min with deionized water. For best results intersperse samples with standards.
4. Data Analysis: The aflatoxins will elute as sharp symmetrical peaks in the order AFG_2, AFG_1, AFB_2, and AFB_1. Determine the peak height of the appropriate aflatoxin in the sample. The peak area, which can be calculated by many software programs designed for CE, can be used in place of the peak height. However, in our hands we found better reproducibility using the height because it is not corrected for mobility, which influences peak area. Calculate the concentration of aflatoxin in the injected sample by comparing the sample to the aflatoxin standard curve (5–1,000 ng/mL). It is important that the aflatoxin concentration be calculated using the appropriate standard curve (i.e., AFG_1 from an AFG_1 standard curve, and so on), due to the differences in fluorescence intensity among the aflatoxins. Calculate the concentration of individual aflatoxins in the maize as follows in **Eq. 1**:

$$[AFB_1]_{maize} \text{ in ng/g} = ([AFB_1]_{sample} \text{ in ng/mL}) \div (1.25 \text{ g equivalents maize/mL}) \quad (1)$$

The total aflatoxin content is obtained by summing the values for the individual aflatoxins.

4. Notes

1. The immunoaffinity cleanup used here is simple and rapid, but is not the only cleanup compatible with the method. Previously we have used the Contaminants Branch (CB) cleanup as well ***(15)***. The CB method, while more laborious, was also slightly more sensitive and gave a slightly cleaner extract for injection. Other

solid phase extraction columns, such as the popular MycoSep columns (Romer Labs, Union, MO) may also be used, provided sufficient sample is purified (i.e., if the equivalent of 1 g maize or greater is purified).

2. The intensity of the fluorescence signal can be increased substantially by removing the bandpass filter in the fluorescence detector. However, more interferences can be expected as the components having emission at wavelengths greater than 462 nm are no longer excluded.
3. For injection of samples the minivials can be purchased from several suppliers. It is just as effective, and considerably less expensive, to remove the caps from 0.6 mL "Eppendorf" style centrifuge tubes and fit these into the standard autosampler vials using adapter springs such as those sold by Beckman-Coulter (Fullerton, CA). Vials with even smaller capacities (so-called microvials, with a capacity of 30 µL) can also be purchased. In our experience the use of microvials has led to a dramatic increase in variability relative to when minivials are used.
4. The buffer composition will influence the fluorescence intensity observed from the aflatoxins and should be rigidly controlled.

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