Capillary Electrophoresis with Laser-Induced Fluorescence: Method for the Mycotoxin Ochratoxin A

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Ochratoxin A (OA) is a natural contaminant of a large variety of foods of plant and animal origin. Herein is reported the development of a capillary electrophoresis (CE) method for the quantification of OA in three very different commodities: roasted coffee, corn, and sorghum. The extraction and isolation procedures combine a silica column and an immunoaffinity cleanup column analogous to other chromatographic methods. After separation from interferences by CE, OA was exposed to light from an ultraviolet He/Cd laser, and the fluorescence of OA was measured [CE-laser-induced fluorescence (CE-LIF)]. When OA was added to several foods over the range 0.2−10 ng/g (ppb), the average recoveries were 86% for roasted coffee (SD = 12.2; $n = 8$), 99% for corn (SD = 10.1; $n = 8$), and 91% for sorghum (SD = 14.8; $n = 4$). Each instrumental analysis, after extraction and purification, required 13 min, equivalent to HPLC analysis. CE-LIF can be applied to the quantitation of OA in roasted coffee, corn, and sorghum, reducing organic solvent usage relative to HPLC.

Keywords: *Ochratoxin A; coffee; corn; sorghum; capillary electrophoresis*

INTRODUCTION

Ochratoxin A (OA) is a secondary fungal metabolite produced by ubiquitous molds within the genera *Aspergillus* (Varga et al., 1996) and *Penicillium* (El-Banna et al., 1987; Pitt, 1987). OA is well-known to be nephrotoxic, immunotoxic, and a potent teratogen (Hayes, 1981; Krogh, 1987; Kuiper-Goodman and Scott, 1989). Because of the well-established carcinogenicity to both male and female rats and mice, this toxin has been classified as a *possible* human carcinogen (IARC, 1993).

OA is found as a natural contaminant in a wide variety of foods of plant origin, farm animal feeds, and edible animal tissue (Pohland et al., 1992). OA is a fairly stable molecule and will survive most food processing (Scott, 1996). Furthermore, the large variety of different foods susceptible of OA contamination, such as cereals, meat products, and coffee, can greatly influence human OA intake (Jørgensen and Bilde, 1996; Gilbert, 1996). A Provisional Tolerable Weekly Intake for OA has been set at 100 ng/kg of body weight by the World Health Organization (1996). Due to the variety of matrixes contaminated with OA, various analytical methods exist to quantify it. Both biological and chemical procedures have been developed, and most instrumental methods are based upon high-performance liquid chromatography (HPLC) (Nesheim et al., 1992; Hald et al., 1993; Langseth et al., 1993; Valenta et al., 1993; Kuhn et al., 1995; Scott and Kanhere, 1995; Zimmerli and Dick, 1995).

In this study we analyzed three very different commodities, roasted coffee, corn, and sorghum, using a single purification procedure and capillary zone electrophoresis with laser-induced fluorescence (CZE-LIF) for separation and detection of OA. CZE-LIF offers the possibility of rapid and sensitive assay of small volumes of samples, which in turn reduces organic solvent usage relative to HPLC.

MATERIALS AND METHODS

Reagents. The OA was obtained from Sigma Chemical Co. (St. Louis, MO; lot 76H4084). The stock solution was prepared by dissolving 1.0 mg of OA in 2.0 mL of acetonitrile and was stored at 4 °C. The purity of the standard was checked spectrophotometrically in accordance with an AOAC (1990) procedure. An electrophoretic buffer was prepared from deionized (16.8 MΩ) water prepared with a Nanopure II purifier (Sybron/Barnstead, Boston, MA) and then passed through a 0.2 μm filter (Zapcap CR, Schleicher & Schuell, Keene, NH). All solvents (HPLC grade) and reagents (ACS grade) for cleanup and CZE analysis were purchased from Fischer Scientific (Fair Lawn, NJ) or EM Science (Gibbstown, NJ). Normal-phase cleanup columns (2 g Mega Bond Elut silica cartridges) were obtained from Varian (Harbor City, CA). Nylon membrane syringe filters, 0.45 μm, Spartan 25, were obtained from Schleicher & Schuell. Ochratest immunoaffinity columns were obtained from Vicam L.P. (Watertown, MA) and maintained at 4 °C until use.

Apparatus. The CE unit, a Beckman P/ACE System 5000 with LIF detector (Beckman Instruments, Inc., Fullerton, CA), was modified by replacing the emission filter with a 399 nm long-pass filter and a 465 nm band-pass filter (Oriel Corp., Stratford, CT). The long-pass filter excluded light below 400 nm while the band-pass filter transmitted light between 364 and 539 nm. The combination permitted the collection of light between 400 and 539 nm. Excitation light at 325 nm was provided by a helium− cadmium laser unit Omnichrome model 100 (Chino, CA). Data processing was performed with a System Gold data handling program (Beckman Instruments).

Sample Preparation. Corn was retained on a No. 5 sieve (4.00 mm opening; Fischer Scientific Co.) and then ground for 30 s in a model M-2 Stein mill (Seedburo Eg. Co., Chicago,

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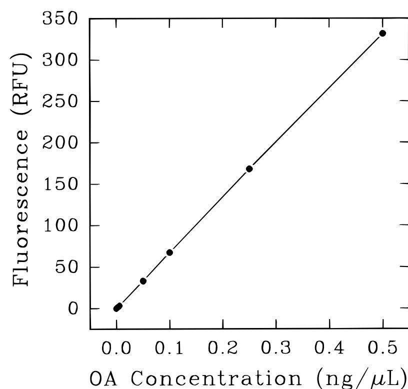

Figure 1. Calibration curve of OA standards. The curve is a fit of the average of four trials (r^2 = 0.999) (fluorescence = 0.6408 + 662.669[OA]).

IL) and blended in a Hobart mixer. Roasted ground coffee was obtained from a retail outlet in Peoria, IL. Ground sorghum samples were provided by Romer Laboratories (Union, MO).

A 20 g sample was weighed for the analysis and spiked with 8.0−400 μL of OA stock solution, 0.5 ng/μL in acetonitrile/deionized water solution (1:1). The sample was briefly shaken, and 100 mL of methylene chloride and 10 mL of 0.1 M phosphoric acid solution were added. Extraction was performed with a wrist-action shaker for 30 min, and then the extract was passed through a pleated paper filter, No. 588 (Schleicher & Schuell). Forty milliliters of filtered extract was collected for the first cleanup step. A 2 g silica column was preconditioned with 10 mL of methylene chloride, and the sample was applied to the column and allowed to drain through by gravity. The column was washed with 40 mL of a methylene chloride/ethyl acetate solution (98:2, v:v). The OA was eluted with 30 mL of a methylene chloride/acetic acid solution (98:2, v:v). The eluate was dried under a stream of nitrogen at 35−40 °C and dissolved in 10 mL of a water/acetonitrile solution (9:1, v:v) for the second cleanup step. The sample was filtered with a syringe filter (0.45 μm pore size) and then applied at the immunoaffinity column and allowed to drain through by gravity. The column was washed with 10 mL of a 20 mM phosphate buffer, pH 7.00, and 10 mL of deionized water. The OA was eluted with 1.5 mL of methanol. The sample was dried under a stream of nitrogen (35−40 °C) and reconstituted with 0.6 mL of an acetonitrile/water solution (1:1) before analysis by CE-LIF. Acetonitrile/water (1:1) was used rather than acetonitrile/phosphate buffer (1:1) because preliminary experiments (data not shown) indicated greater stability of OA in the acetonitrile/water solution.

CE Operative Conditions. A fused silica capillary (57 cm total length, 50 cm length to detector × 75 μm i.d.) was housed in a cartridge configured for LIF detection and was maintained at 27 °C. Before each sample was injected, the capillary was rinsed for 1 min with the electrophoretic buffer (20 mM sodium phosphate, pH 7.00) at 0.5 psi. Hydrodynamic injection of the sample was performed at 0.5 psi for 5 s (equivalent to a volume of 30 nL). After immersion of each end of the capillary into the electrophoretic buffer, the voltage was applied and maintained at 20 kV (approximate current = 60 μA). At the conclusion of the 10 min run, the capillary was rinsed once with 0.5 N NaOH for 1 min at 0.5 psi and once with deionized water for 1 min at 0.5 psi. OA standards were prepared from the stock solution at five different concentration levels within the range 0.005−0.5 ng/μL, in an acetonitrile/water solution (1:1). The standard solutions were injected, and OA recovery from spiked samples was determined by comparison to the fluorescence data of the standards fit with a calibration curve (fluorescence = 0.6408 + 662.669[OA]) (Figure 1).

RESULTS AND DISCUSSION

Ochratoxin A with CZE. There are only two reports on the use of CE for OA analysis (Holland and Sepaniak, 1993; Böhs et al., 1995). Both studies used UV detection for measuring OA standard solutions, with the concentrations used being relatively high (100 μg/mL). This level of sensitivity is not adequate for measuring the very low levels of OA found in naturally contaminated foods. Most HPLC methods for OA are fluorescence-based. CZE with LIF detection has been applied successfully to other mycotoxins such as fumonisins and aflatoxins (Maragos et al., 1996; Maragos and Greer, 1997; Maragos, 1997), reaching very low limits of detection—comparable to those of HPLC. The limit of detection of the procedure described in this paper for OA was 0.2 ng/g for all of the matrixes used (Figure 2). This amount gave an OA peak with a signal-to-noise ratio of approximately 4.

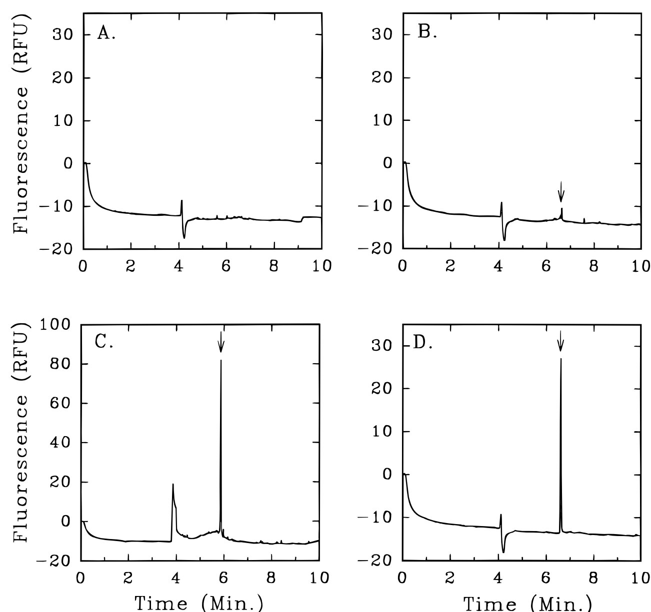

Figure 2. Detection of OA in coffee by CE-LIF: (A) control, roasted coffee with no detectable OA; (B) roasted coffee spiked with OA at the limit of detection of the method, 0.2 ppb; (C) 10 ppb spiked coffee; (D) OA standard, 0.05 ng/μL.

OA is a naturally fluorescent mycotoxin and does not require derivatization before CZE-LIF analysis, which permits a quick instrumental step. After extraction and cleanup, the analysis of each sample required only 13 min, 10 min for the electrophoresis and 3 min for the capillary rinsing. Although the samples required considerable cleanup (8 g sample equivalents in 0.6 mL of acetonitrile/deionized water, 1:1), clean electropherograms were obtained (Figures 2−4). The use of a 20 mM, pH 7.00, sodium phosphate buffer as electrolyte solution resulted in a good resolution of the OA with a relatively short migration time. The pH 7.00 phosphate buffer solution could be maintained for 10 days at room temperature without causing significant fluctuations in the migration time (Table 1).

Analytical Method. The procedure for extraction and cleanup described is similar to the method developed by Patel et al. (1997), and it is based on a tandem SPE cleanup using a silica column followed by an ochratoxin specific affinity column. The first step of cleanup by itself was not sufficient for a quantification of OA at levels below 5 ppb due to the great number of interfering peaks present in the electropherogram. Nevertheless, the silica column was efficient enough to purify the sample extract and allowed the passage of a larger amount of sample through the commercial affin-

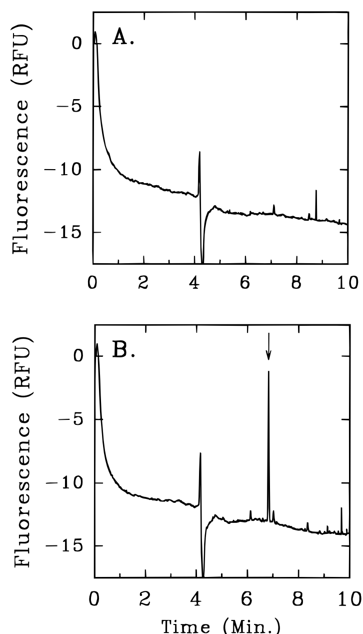

Figure 3. Detection of OA in corn: (A) control corn with no detectable OA; (B) corn spiked with 2 ppb of OA.

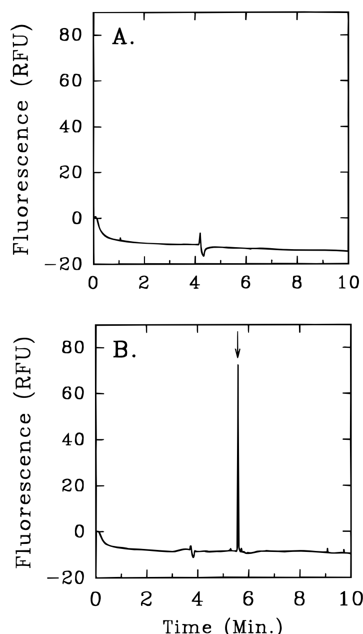

Figure 4. Detection of OA in sorghum: (A) control sorghum with no detectable OA; (B) sorghum spiked with 10 ppb of OA.

ity column (up to 9 g equivalent of sample), which resulted in an improved sensitivity for the method.

The repeatability and accuracy of the method was verified by recovery experiments using samples artificially contaminated with OA at levels from 0.2 to 10.0 ng/g (ppb). The recovery values for roasted coffee ranged from 76 to 107% with a mean of 86% (standard deviation = 12.2; coefficient of variation = 14.1%; n = 8). The recovery from spiked corn samples ranged from 79 to 110% with a mean value of 99% (standard deviation = 10.1; coefficient of variation = 10.2%; n = 8). The recovery from spiked sorghum samples ranged from 75 to 106% with a mean value of 91% (standard deviation = 14.8; coefficient of variation = 16.3%; n = 4).

Conclusions. CZE-LIF can be used for the separation and reliable quantification of OA in roasted coffee,

Table 1. OA Retention Time Variability

Day	Migration time* (min)			
New Buffer				
Day 2	5.67, 5.85, 5.75, 5.73	mean=5.75 SD=0.07 CV=1.21%	Mean=5.77 SD=0.12 CV=2.08%	Mean=6.07 SD=0.47 CV=7.86%
Day 8	5.77, 5.82, 5.94, 5.89	mean=5.85 SD=0.07 CV=1.20%		
Day 9	5.79, 5.70, 5.73, 5.59, 5.56	mean=5.67 SD=0.09 CV=1.59%		
Day15	5.70, 5.66, 5.93, 5.96	mean=5.81 SD=0.15 CV=2.58%		
New buffer				
Day 1	6.85, 6.83, 6.66, 6.64	mean=6.75 SD=0.11 CV=1.63%	Mean=6.74 SD=0.12 CV=1.78%	
Day 2	6.60, 6.63, 6.83, 6.88	mean=6.74 SD=0.14 CV=2.08%		

*The migration time referred to is for standard injections at the beginning, in between, and at the end of the sequence of sample injections

corn, and sorghum. The method described has sensitivity comparable to that of established HPLC methods. The excellent separation of OA from interferences with CE-LIF may allow for further improvements in analysis time and sensitivity. The substantial advantages of the CZE-LIF are as follows: the use of much smaller volumes of sample; the absence of organic solvent during the determinative step, consequently less hazardous waste generated; and the use of less expensive, versatile, capillaries. In conclusion, the worldwide interest in inexpensive, sensitive, and nonpolluting technologies suggests that CZE-LIF holds considerable promise for mycotoxin detection.

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