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Analysis of Ochratoxin A in Coffee by Solid-Phase Cleanup and Narrow-Bore Liquid Chromatography−Fluorescence Detector−Mass Spectrometry

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A method for the analysis of ochratoxin A (OTA) in green and roasted coffee has been developed. OTA was extracted from coffee with 1% $NaHCO_3$, and the extract was filtered and purified by solid-phase cleanup using a polymeric column that exhibits reversed-phase and anion-exchange functionalities. OTA was analyzed on a narrow-bore reversed-phase C_{18} HPLC column with acetonitrile/water (0.1% formic acid) (40:60) as mobile phase and quantified with a fluorescence detector. The presence of OTA in coffee was confirmed by single-quadruple mass spectrometry using an electrospray ionization source. The method has been validated, obtaining a recovery of 82.5% and a detection limit of 0.1 ng/g. It has been applied to 20 coffee samples from various countries and different manufacturers with no detection of OTA.

KEYWORDS: Ochratoxin A; coffee; SPE; HPLC; MS

INTRODUCTION

Ochratoxin A (OTA) (**Figure 1**) is a mycotoxin produced by several fungal species from the genera *Aspergillus* and *Penicillium*. The International Agency for Research on Cancer has classified OTA in the group 2B as a renal carcinogenic to animals and possibly to humans (*1*). Ochratoxin A is widely found in cereals and their derivatives, wine, coffee, beer, nuts, dried fruits, and meat products (*2*−*5*). A recent evaluation performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) had established a Provisional Tolerable Weekly Intake of 112 ng/kg of body weight for OTA, in which 12% of ochratoxin A intake belongs to coffee (*6*).

Because the occurrence of ochratoxin A in coffee has been described in a wide range of literature (*7*−*11*) and a possible regulation for a maximum level of OTA in coffee will appear (*12*), coffee companies have taken a special interest in the mycotoxical quality of coffee in order to ensure food safety. It is therefore necessary to have available analytical methods for the analysis of this toxin. Most of the analytical methods recently published for the determination of OTA in coffee take a long time. Due to the low concentration levels of OTA usually found in coffee and the complexity of the sample, sensitive and selective techniques should be applied. The toxin is usually extracted with an organic solvent and a salt aqueous solution or a mixture of both. The extract is purified by solid-phase extraction (*13*, *14*), immunoaffinity columns (*15*−*18*) or a combination of both (*19*). Immunoaffinity columns have the disadvantage that they are matrix dependent and very expensive.

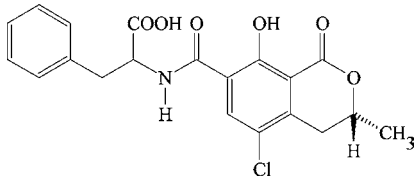

Figure 1. Ochratoxin A.

In this study a method for the analysis of ochratoxin A in green and roasted coffee was developed. Emphasis was put on the cleanup step, assaying a new polymeric solid-phase sorbent, not described previously, for the analysis of OTA in coffee. Positive OTA samples were also analyzed by coupling of the HPLC with electrospray ionization (ESI) mass spectrometry (*20*). The method has been applied to 20 samples of both Arabica and Robusta green and roasted coffees.

MATERIALS AND METHODS

Samples. Fifteen samples of green coffee and five samples of roasted coffee were purchased from a local roaster. Coffee samples were originally from different production regions (Zimbabwe, Brazil, India, Uganda, Colombia, and Indonesia) during 2001 and 2002. Details are shown in **Table 1**.

Solvents and Reagents. Crystalline ochratoxin A standard was supplied by Sigma-Aldrich (St. Louis, MO). The stock solution of 100 mg/L was prepared in HPLC grade acetonitrile (Merck, Darmstadt, Germany). All other standard solutions were prepared immediately before use by diluting the stock solution with mobile phase.

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Table 1. Green and Roasted Coffee Samples Analyzed

production region	no. of green coffee samples	no. of roasted coffee samples
Zimbabwe	3	
Brazil	2	1
India	2	1
Uganda	2	1
Colombia	3	1
Indonesia	3	1

Milli-Q quality water (Millipore, Bedford, MA) and other chemicals of analytical grade or HPLC quality needed for analysis were obtained from Merck.

Solid-Phase Extraction (SPE). Extraction was performed with Oasis MAX (3 cm^3/60 mg) cartridges (Waters, Milford, MA) on a vacuum manifold (IST, Hengoed, Mid Glamorgan, U.K.) connected to a Vacum-Sel 3001001 vacuum membrane pump (J. P. Selecta, Barcelona, Spain) to allow a flow rate of 1 mL/min.

HPLC—Fluorescence Detector. The HPLC system consisted of an Alliance 2690 and a 2475 fluorescence detector (Waters). Chromatographic separations were performed on a 100 × 2.1 mm i.d., 3.5 μm, XTerra MS C_{18} narrow-bore column (Waters) at 30 °C, with a mobile phase of acetonitrile/water (0.1% HCOOH) (40:60) at a flow rate of 300 μL/min. The injection volume was 10 μL. Excitation wavelength was 330 nm, and emission wavelength was 460 nm.

Extraction of Ochratoxin A from Coffee. The toxin was extracted essentially according to the method of Leoni et al. (*17*). Five hundred grams of coffee sample was ground. A portion of 10 g of coffee sample was weighed in an Erlenmeyer flask, and then OTA was extracted with 100 mL of a 1% aqueous solution of sodium bicarbonate with magnetic stirring for 30 min. The extract was filtered through Whatman filter paper (no. 3). An aliquot of 5 mL was adjusted to pH 6.5 with 1 M H_3PO_4 and sonicated for 2 min in an ultrasonic bath. It was then passed through the Oasis MAX cartridge at a flow rate of 1 mL/min, previously conditioned with 2 mL of methanol and equilibrated with 2 mL of Milli-Q water. The cartridge was washed with 2 mL of 50 mM sodium acetate/methanol (95:5) and then with 2 mL of methanol. OTA was eluted with 2 mL of methanol (2% formic acid) and collected in a clean vial. The eluted extract was evaporated to dryness under a nitrogen stream. The residue was dissolved in 100 μL of mobile phase and injected onto the HPLC.

Calibration Curves and Quantification. Standard solutions for the calibration curves were prepared daily (100, 400, 800, 1200, and 2000 ng of OTA). Calibration curves were constructed by plotting the observed peak area against the concentration. Quantification of coffee samples were performed by external standard by measuring peak area at OTA retention time and calculated with the calibration curve. Peak area values of calibration curves were proportional to the concentration over the range tested; we accepted ±10% response factor. Calibration curves were also fitted by linear least-squares regression and showed correlation coefficients >0.999.

Method Validation. The HPLC−fluorescence detector method was validated for the following parameters: linearity, recovery, repeatability, intermediate precision, specificity, and detection and quantification limits following the guidelines established by the International Conference on Harmonization (*21*).

Linearity was done by injecting duplicate OTA standards of 0.02, 0.1, 0.4, 0.8, 1.2, and 2 ng. Recovery was obtained by using the addition method. The minimum standard addition was less than the maximum level of OTA allowed in green coffee beans in the case of regulation (3 μg/kg). Coffee samples were spiked with 20, 40, 60, and 80 ng of OTA standard. The calibration curve of OTA was made at concentration levels that ranged from 0.1 to 2 ng.

Repeatability was estimated by assaying five replicates of coffee samples spiked with 20 ng of OTA standard on the same day by the same analyst.

Intermediate precision was estimated from the analysis of five samples, stored at 4 °C in darkness, by the same analyst on different days.

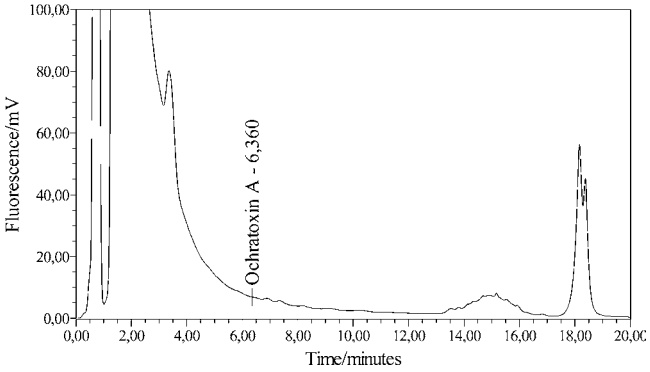

Figure 2. HPLC fluorescence chromatogram of green coffee beans. Ochratoxin A was not detected.

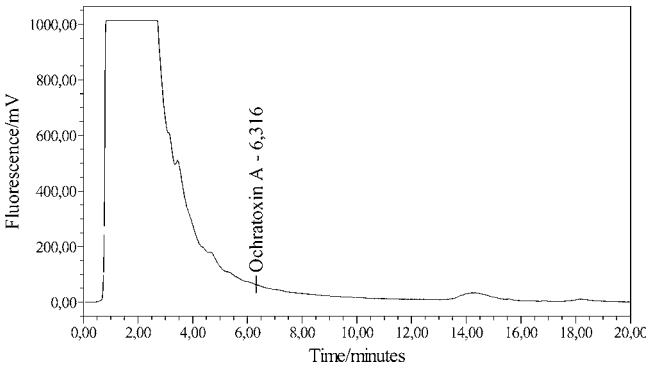

Figure 3. HPLC fluorescence chromatogram of roasted coffee beans. Ochratoxin A was not detected.

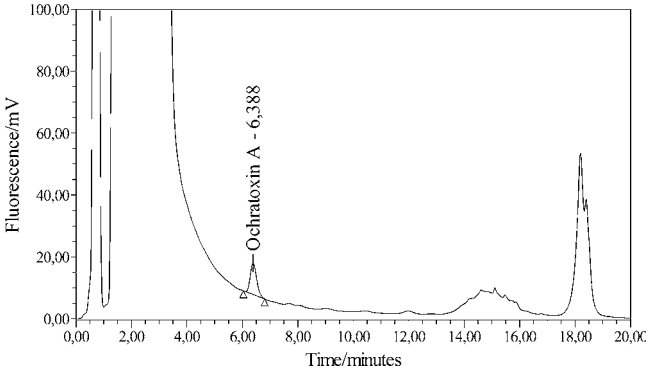

Figure 4. HPLC fluorescence chromatogram of green coffee beans spiked with ochratoxin A standard (3 ng/g).

Quantification and detection limits were determined by duplicate injections of spiked coffee samples based on signal-to-noise ratios of 10:1 for the quantification limit and 3:1 for the detection limit. For the quantification limit, samples were spiked with 5 ng of OTA, and 1 ng of OTA was used for the determination of the detection limit.

Specificity was tested by injecting the samples in the mass spectrometer with an ESI source, operating in the positive ion mode with recording at *m*/*z* 404 and 358.

Confirmation of Ochratoxin A by ESI-MS. Mass spectrometry was performed on a ZMD (Waters) single-quadrupole equipped with an ESI source and operating in the positive ion mode. The parameters used for the mass spectrometer in all experiments were as follows: capillary voltage, 3.0 kV; cone voltage, 20 V; source block temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas, 420 L/h; cone gas, 50 L/h; low mass resolution, 15; high mass resolution, 15; ion energy, 0.5; extractor, 5; R_f lens, 0.5; and electron multiplier voltage, 650.

The ions monitored were the protonated molecule $[M + H]^+$ at *m*/*z* 404 at a 20 V cone voltage and the loss of formic acid [MH −

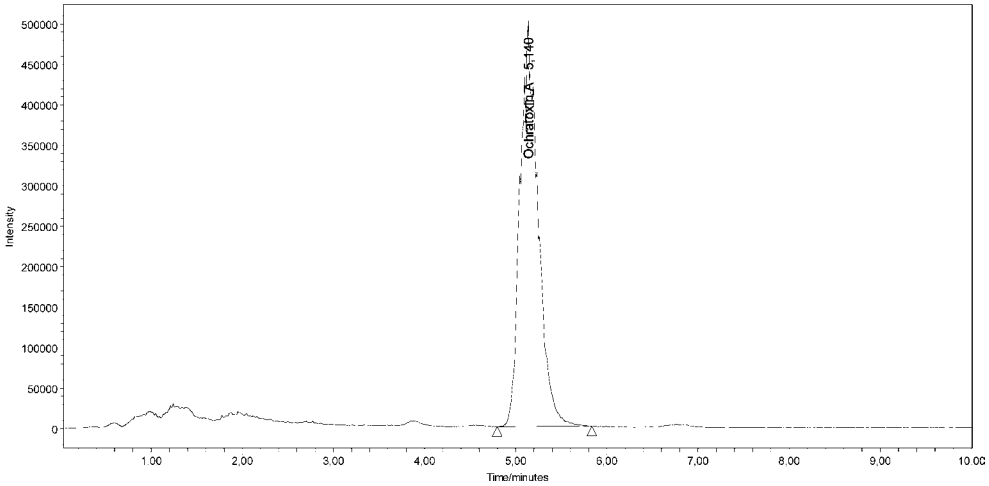

Figure 5. HPLC-MS chromatogram of green coffee sample spiked with ochratoxin A standard (3 ng/g).

$HCOOH]^+$ at *m*/*z* 358 at a 40 V cone voltage. A dwell time of 0.8 s was used throughout.

RESULTS AND DISCUSSION

Most recent publications for the analysis of ochratoxin A in coffee employ immunoaffinity columns in the sample purification step. We proposed a method not described previously, which uses Oasis MAX columns for the analysis of OTA in green and roasted coffees. These columns contain a polymeric reversed-phase sorbent that exhibits both reversed-phase and anion-exchange functionalities. The results described below show that it is a good alternative.

We have tested two types of columns: Oasis HLB and Oasis MAX. HLB columns contain a polymeric reversed-phase sorbent, and MAX columns contain quaternary amine groups on the surface of the polymeric reversed-phase sorbent that gives both reversed-phase and anion-exchange functionalities. The extraction of OTA from coffee was the same in both cases with 1% $NaHCO_3$. For the analysis using HLB columns, this was conditioned with 2 mL of acetonitrile and equilibrated with 2 mL of water. Different columns were loaded with 5 mL replicate extract spiked coffee samples adjusted to pH 1.1, 3.1, 4.0, and 6.5 with H_3PO_4 and then washed with 2 mL of H_2O containing 5% acetonitrile. The toxin was eluted with 3 mL of acetonitrile. The eluate was evaporated under a nitrogen stream, redissolved in 100 μL of mobile phase, and then injected into the HPLC. To test the MAX columns, replicates of 5 mL of extracted coffee samples adjusted to pH 2.1, 3.5, 5.0, and 6.7 were applied to the previously conditioned column with methanol and equilibrated with H_2O. The cartridge was washed with 2 mL of 50 mM sodium acetate/methanol (95:5) and then with 2 mL of methanol. The OTA was eluted with 2 mL of methanol containing 2% formic acid and collected in a clean vial. The eluted extract was evaporated to dryness under a nitrogen stream. The residue was redissolved in 100 μL of mobile phase and injected into the HPLC. The OTA peak in spiked coffee samples was detected at expected levels. Results of recovery experiments of the full analytical procedure proposed in the tested range were 82.4 $\pm$ 5.4% expressed as the percent coefficient of variation (CV). Repeatability defined as CV was 4.0%, and intermediate precision was 4.9% for a spiked sample at a level of 3 ng/g of OTA.

Typical HPLC fluorescence chromatograms of green and roasted coffee beans and green coffee beans spiked with 3 ng/g of OTA standard are shown in **Figures 2**, **3**, and **4**, respectively.

The quantification limit was 0.5 ng/g in coffee samples, based on a signal-to-noise ratio of 10:1, and the limit of detection was 0.1 ng/g in coffee samples, based on a signal-to-noise ratio of 3:1.

Mass spectrometric detection was optimized by injecting a standard of 100 mg/L of OTA in the positive and negative ionization modes at different cone voltages (20, 40, and 60 V). Full-scan mass spectra were recorded to select the most abundant *m*/*z* value. The relative intensity for the most abundant *m*/*z* was used to evaluate the performance of each ionization and the cone voltage value. In both ionization modes fragmentation is observed when the voltage is increased. The experimental fragments are at *m*/*z* 358, corresponding to [M $-$ COOH], and at *m*/*z* 257 when the molecule is cleaved at the amine group.

The HPLC-MS chromatogram of a coffee sample spiked with OTA standard at 3 ng/g is shown in **Figure 5**. The peak eluting at 6.7 min was identified as ochratoxin A from its retention time and from the characteristic ion at *m*/*z* 404. An average concentration of 2.7 ng/g $\pm$ 5.9% was found by MS detection. The results obtained show that results are equivalent so that all OTA positive samples obtained after HPLC fluorescence analysis could be confirmed by HPLC-ESI-MS. This proves that it is not necessary to use a triple-quadrupole detector, as described in the literature. Analysis of green and roasted coffee samples demonstrated that, in all cases, OTA was not detected.

We can conclude that the polymeric sorbent tested is a good alternative to use in the sample preparation step. It is easy to apply and time saving and does not require particular skills; it allows one operator to analyze many samples in a day. The use of a narrow-bore column provides more sensitivity, an important aspect for the analysis of ochratoxin A due to its being found in low concentrations. The selectivity and sensitivity of LC-MS will progressively replace conventional LC methods because of the low levels of regulatory exposure limits and the variety of complex samples matrices.

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