JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evaluation of Methods Used To Determine Ochratoxin A in Coffee Beans

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A comparative study was conducted to evaluate four previously reported methods that proved to have a recovery greater than 80% for the determination of different levels of ochratoxin A (OTA) in green and roasted coffee beans and to select an accurate, sensitive, and less-expensive technique between the existing methods. The results indicated that the Association of Official Analytical Chemists (AOAC) official method for the extraction of OTA in green coffee and determination by high-performance liquid chromatography (HPLC) is recommended as an efficient method for the routine analyses of OTA in green and ground roasted coffee beans. This method proved to be an accurate, sensitive, and less-expensive method that employs routine materials and available equipment. Although the immunoaffinity column/HPLC procedure tested showed a significantly higher percentage than the AOAC recommended method, it is recommended for use in processed coffee beans where low concentrations of OTA may be expected to be detected.

KEYWORDS: Methods; ochratoxin A; coffee (green and roasted)

INTRODUCTION

Ochratoxin A (OTA) is a toxic mold metabolite produced by *Asperigllus ochraceus* (*1*) and *Penicillium verrcosum* (*2*). It is mainly found as a natural contaminant in cereals, cereal products, and coffee beans (*3–5*). It causes a number of adverse effects in laboratory animals including nephrotoxcity, nephro- and heptocarcinogenicity, tetragenicity, neurodevelopmental toxicity, and immunotoxicity. The most notable and sensitive of these toxic effects are nephrotoxicity and nephrocarcinogenicity (*6*). Moreover, the overall evaluation of IARC (*7*) was "Ochratoxin A is possibly carcinogenic to humans (Group 2B)."

For the determination of OTA in foods, several methods have been reported including spectrofluorometry (*8*), thin-layer chromatography (TLC) (*9*), and liquid chromatography (LC) (*10–16*). Nevertheless, most of these methods could not be applied to coffee beans and its products because the interfering fluorescent products made the detection very difficult. Moreover, the official Association of Official Analytical Chemists (AOAC) method of analysis of OTA in green coffee beans (*17*) is based on TLC for separation, but the method is inapplicable to roasted coffee beans and coffee products since the detection in coffee products requires much higher selectively and sensitivity (*18*). Indeed, the analysis of coffee products presents a high degree of difficulty owing to the acidic substances extruded with OTA (*19*). However, it was only in 1986 when Terada et al. (*18*) established a new high-performance liquid chromatographic method (HPLC) for the determination of OTA in coffee beans and coffee products. Later, a major improvement was introduced by Nakajima et al. (*20*), who were the first to introduce the immunoaffinity column for cleanup for the analyses of OTA in coffee products. They developed their own monoclonal antibody (mAb) affinity columns and proposed ion-pair chromatography for the final OTA quantitation. The important analytical advantage of the cleanup method with the mAb affinity column is a one-step removal of interfering materials from sample extracts which should be carefully considered.

However, such a method involved complicated and time-consuming solvent cleanup steps. More recently, Pittet et al. (*19*) evaluated the applicability of commercially available immunoaffinity columns for cleanup and analyzed OTA elute by conventional reversed-phase HPLC with fluorescence detection, adopting the method of Nakajima et al. (*20*) but using a different extraction technique and a different solvent for the elution of OTA from the columns, as well as improved HPLC conditions. They observed that the extraction of OTA with a mixture of methanol and 3% aqueous sodium hydrogen carbonate (50:50) followed by high-speed homogenization as suggested respectively by Koch et al. (*21*) and Tsubouchi et al. (*22*) yielded higher OTA levels (by 15–25%) compared to those obtained with a 1% aqueous sodium hydrogen carbonate solution. Their initial results also showed that elution of OTA from immunoaffinity columns using 50% dimethyl sulfoxide as recommended by Nakajima et al. (*20*) was not effective in removing OTA from commercial Ochratest columns, which may be due

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10.1021/jf072558x CCC: $37.00
Published on Web 10/18/2007

to differences in the antibody or in the composition of the packing material (gel). They added that much better results were obtained by using pure methanol for the quantitative elution of bound OTA. Accordingly, the same authors reported that HPLC separations were carried out by using the mobile phase proposed by Baumann and Zimmerli (*23*) which consists of 45% acetonitiril and 55% water/acetic acid (41:2). In this study, Pittet et al. (*19*) found that significant changes occurred in the OTA retention time from day to day with a freshly prepared mobile phase, even when taking extra care to minimize batch-to-batch variations. The problem was solved by buffering the HPLC mobile phase, and the optimum composition eventually turned out to be 45% acetonitril and 55% 4 mM sodium acetate/acetic acid (19:1). They added that their method, which is adapted from that published by Nakajima et al. (*20*), proved to be extremely useful for the rapid cleanup of OTA not only in soluble coffee but also in green and roasted coffee beans.

In the current study, we aimed to evaluate four of the existing methods for OTA determination that cover different protocols: TLC, HPLC, and an immunoaffinity column cleanup procedure to select an accurate, sensitive, and less-expensive method for the detection of OTA in green and roasted coffee beans.

MATERIALS AND METHODS

Materials. *Chemicals and Reagents.* Ochratoxin A standard and diatomaceous earth were purchased from Sigma Chemical Company (St. Louis, MO). Analytical-grade methanol, benzene, acetic acid, chloroform, *n*-hexane, sodium hydrogen carbonate, phosphoric acid, sodium chloride, dihydrogen phosphate, dipotassium hydrogen phosphate, zinc sulfate, and phosphotungestic acid and HPLC-grade acetonitrile and acetic acid were obtained from Merck (Darmstadt, Germany).

Thin-Layer Chromatography Plates. Ready-made plates of thin-layer chromatography (20 × 20 cm) aluminum sheets with a 0.2 mm thickness of silica gel G 60 (without fluorescent indicator) were purchased from Merck (Darmstadt, Germany).

HPLC Equipment. The HPLC system consisted of a binary pump (LC Advp) controller, SCL 10AVP; florescence detector, Rf-10AxL; and degasser, DGV-14A, equipped with a reverse-phase analytical column packed with C18 material (Spherisorb 5 μm ODS2, 15 cm × 4.6 nm) with a 20 μL loop, working under software class VP (Shimadzu Co. Ltd., Kyoto, Japan).

Ochratest Immunoaffinity Column. The ochratest immunoaffinity columns were purchased from VICAM, Massachusetts.

Sep-Pak C18 Silica Cartridge. Sep-Pak C18 silica cartridges were purchased from Water Associates, Inc., Massachusetts.

Preparation of Ochratoxin A Stock Solution. A stock solution of OTA was prepared according to the AOAC official method (*24*) at a concentration of 10 μg/mL in benzene/acetic acid (99: 1 v/v). The stock solution was stored at −20 °C.

Methods. *Preparation of Spiked Green and Roasted Ground Coffee Bean Samples.* Samples of green and roasted ground coffee beans were spiked with the OTA stock solution at concentration levels of 0.250, 0.500, 0.750, 1.250, 1.500, 2.500, and 7.500 μg/mL.

Analysis of Ochratoxin A (OTA). The following four methods were adopted for the determination of OTA in green and roasted coffee beans:

I. AOAC Official Method (*17*), Ochratoxin A in green coffee using liquid chromatographic method (HPLC) for OTA separation instead of using the TLC
II. Immunoaffinity column/HPLC procedure of Pittet et al. (*19*)
III. AOAC Official Method (*24*), ochratoxin A in corn and barely; liquid chromatographic method (HPLC)
IV. A multimycotoxin detection method (*25*) (TLC)

The same conditions of HPLC given in method III were followed for methods I and II.

EXTRACTION AND CHROMATOGRAPHIC SEPARATION OF OTA FROM GREEN AND ROASTED GROUND COFFEE BEANS

Extraction Methods. *1. Chloroform Extraction Method (Method I).* A total of 25 g of ground coffee beans (green and/or roasted) were put into a 500 mL Erlenmeyer flask, followed by the addition of 12.5 mL of distilled water and 125 mL of chloroform. The flask was stoppered and shaken for 30 min with a shaking apparatus and filtered through 18.5 cm filter paper using a buchner funnel. Portions of 50 mL were collected and transferred to the prepared column chromatograph (*17*).

2. Methanol–Bicarbonate Extraction Method (Method II). A total of 25 g of ground coffee beans (green and/or roasted) was put into plastic bottles of a blender and mixed with 500 mL of methanol/aqueous sodium hydrogen carbonate 3% (50:50). The suspension was blended for 3 min. The homogenated sample was filtered through Whatman no. 4 filter paper. Then 4 mL of the filtrate was transferred to a graduated cylinder and diluted to 100 mL with a saline phosphate buffer consisting of 8.6 g of sodium chloride, 0.272 g of sodium dihydrogen phosphate, and 1.136 g of dipotassium hydrogen phosphate per litter of distilled water (*19*).

3. Phosphoric–Chloroform Extraction Method (Method III). A total of 50 g of coffee (green and/or roasted) was put into high-speed blender; 25 mL of phosphoric acid (0.1 M) and 250 mL of chloroform were added and blended for 3 min at medium speed. A total of 10 g diatomaceous earth was added just before the end blending time, then it was all filtered through Whatman no. 4 filter paper, and a 50 mL portion was collected and transferred to separatory funnel; 10 mL of sodium bicarbonate (3%) was added and shaken gently; then, the upper phase was collected for column separation (*24*).

4. Methanol–Water Extraction Method (Method IV). A total of 100 g of coffee (green and/or roasted) were put into a high-speed blender; 200 mL of methanol/water (8:2) was added and blended for 3 min and then filtered through Whatman no. 4 filter paper, and 50 mL of extract was collected and put into a 500 mL Erlenmeyer flask along with a 50 mL cleanup solution (150 g of zinc sulfate and 50 g of phosphotungestic acid per liter of distilled water), then it was all shaken for 10 min and filtered through Whatman no. 4 filter paper. A total of 75 mL of extract was collected in a separatory funnel, and 15 mL of chloroform was added, and it was all shaken gently. The chloroform phase was collected and filtered through filter paper covered with anhydrous sodium sulfate, and the chloroform extract was evaporated to dryness on a steam bath and transferred quantitatively with 2 mL of chloroform to a small vial and evaporated under nitrogen gas for subsequent TLC separation for OTA (*25*).

Chromatographic Separation. *1. Column Chromatography (Method I).* A pad of glass wool was inserted into the bottom of the chromatographic tube (22 × 300 nm). A total of 6 g of $NaHCO_3$–diatomaceous earth mixed with 25 mL of aqueous sodium bicarbonate solution (5%) was added to 50 g of acid-washed diatomaceous earth (900 g of acid washed celite 545 was soaked overnight in methanol, filtered through Whatman no. 1 filter paper, washed with 8 L of water, and dried for 12 h at 150 °C) and was transferred to the chromatographic tube; then, 50 mL of chloroform extract was added to the column, washed with 70 mL of hexane followed by 70 mL of chloroform. All washing reagents were discarded; OTA was eluted with 100 mL of acetic acid/benzene (2:98), and the elute was collected in a 125 mL Erlenmeyer flask and evaporated to

near dryness on a steam bath and transferred quantitatively with 2 mL of chloroform to a small vial and evaporated under nitrogen (*17*) for subsequent HPLC OTA separation.

2. Ochratest Immunoaffinity Column (Method II). The whole diluted extract was applied to an immunoaffinity column, at a slow steady flow rate of 2–3 mL/min. After washing of the column with 10 mL of distilled water, OTA was eluted with 4 mL of methanol. The elute was evaporated to dryness on steam bath under nitrogen for subsequent HPLC separation for OTA (*19*).

3. Sep-Pak C18 Column (Method III). A C18 column was placed on vacuum monifold ports, and the column was prewashed twice with 2 mL of methanol, 2 mL of water, and 2 mL of sodium bicarbonate (3%). A total of 5 mL of bicarbonate extract was added to the C18 column, followed by 2 mL of phosphoric acid (0.1 M) and 2 mL of water, and washings were discarded. OTA was eluted with 8 mL of ethyl acetate/methanol/acetic acid (95:5:0.5 v/v/v). The elute was collected in a vial containing 2 mL of water, and the elute was shaken with a tube-shaking machine (vortex genie) to mix the two phases. The OTA extract (upper phase) was pipetted to a 7 mL screw-cap vial. The remaining upper phase was rinsed from the tube with 2 × 1 mL of ethyl acetate, and this was added to the OTA. The extract was evaporated just to dryness on a steam bath under nitrogen for subsequent HPLC analyses (*24*).

Determination. *1. Determination of OTA by HPLC.* The aforementioned columns' elutes were dissolved in a 500 μL mobile phase consisting of acetonitrile/water/acetic acid (99:99:2) and filtered through 0.45 μm microfilter paper into a 5 mL screw-cap vial for subsequent HPLC analyses. The separation was performed at ambient temperature at a flow rate of 1.0 mL/ min, with an injection volume of 20 μL for both standard solutions and coffee sample extracts. The fluorescence detector was operated at an excitation wavelength of 330 nm and an emission wavelength of 460 nm (*24*).

2. Determination of OTA by TLC. The OTA residue was dissolved in 100 μL of acetic acid/benzene (1:99). The spots were carried out as 10 μL for the samples and the OTA standard. The plate was developed twice with benzene/methanol/acetic acid (18:1:1 v/v/v) and removed from the jar when the solvent reached 3 cm from the top of the plate. The solvent was evaporated from the plate at room temperature until dryness. The chromatoplate was examined by using a UV lamp at 340 nm, and the OTA spot showed a greenish blue fluorescence, and it was determined by densitometer at a wavelength of 340 nm. The OTA spot was confirmed by spraying the chromatoplate with an aluminum chloride reagent (20 g $AlCl_3 \cdot 6H_2O$ in 100 mL of alcohol), then air-dried and visualized under the same UV light (340 nm) as a bright blue spot (*25*). The concentration of OTA was calculated as micrograms per kilogram of sample according to the following equation:

$$\text{OTA } (\mu\text{g/kg}) = SYV/XW$$

where S = the microliters of OTA standard equal to an unknown value, Y = concentration of the OTA standard (μg/mL), V = microliters of the final dilution of the sample extract, X = microliters of the sample extract spotted giving a fluorescent intensity equal to S (OTA standard), and W = grams of the sample applied to the column.

Statistical Analysis. All data were subjected to a statistical analysis using the General Linear Medial Procedure of the Statistical Analysis System, SAS Institute Inc. (*26*). The significance of the differences among treatment groups was determined by Waller–Duncan K-ratio (*27*). All statements of significance were based on a probability of $P > 0.05$.

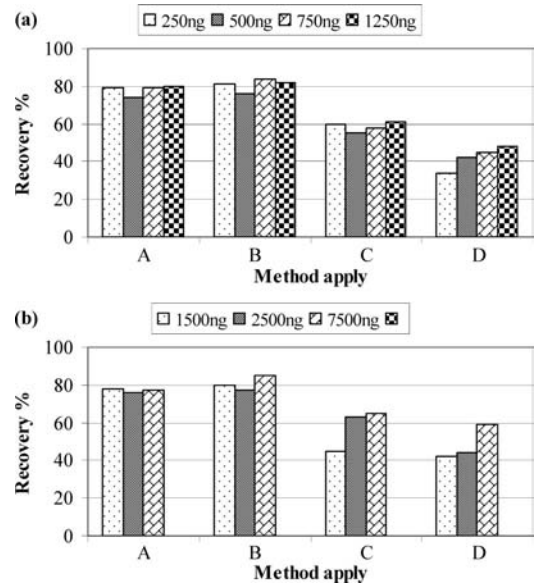

Figure 1. Recovery of OTA in green coffee beans (**a**) spiked with 250, 500, 750, and 1250 ng and (**b**) spiked with 1500, 2500, and 7500 ng using the four given methods (average of five determinations on separate extracts for each OTA concentration). (A) OTA in green coffee/HPLC, AOAC (*17*). (B) Immunoaffinity column/HPLC, Pittet et al. (*19*). (C) OTA in corn and barely/HPLC, AOAC (*24*). (D) Multimycotoxin detection method (TLC), Stoloff et al. (*25*).

RESULTS AND DISCUSSION

In the current study, different methods of extraction, chromatographic separation, and determination of OTA were performed to suggest a simple, validated method for the determination of OTA in green and roasted ground coffee beans. For this propose, four existing methods with higher than 80% recoveries were adopted to quantify different levels of OTA in green and roasted coffee beans to achieve an accurate, sensitive, time-consuming, and less-expensive technique.

Recovery Experiments. The results of recovery experiments on green and roasted coffee beans spiked with OTA are summarized in **Figures 1a**,**b** and **2a**,**b**. It is clear that the average percentage of recoveries was found to be descendingly fluctuated between 76–85, 74–80, 45–65, and 34–59 for methods II, I, III, and IV, respectively, for green coffee beans. The same trend was also true for the roasted coffee beans as the average percentage recoveries ranged from 77–90, 73–86, 41–57, and 38–53 in the same respective order for the four adopted mentioned methods (II, I, III, and IV, respectively). This indicated that the immunoaffinity column cleanup procedure followed by the AOAC method (*17*) for OTA in green coffee showed the best average recoveries compared to the AOAC method for OTA in corn and barely (*24*) and the multimycotoxin detection method (*25*) for both green and roasted coffee beans.

Although the recoveries of both the immunoaffinity column cleanup/HPLC procedure and the AOAC method for OTA in green coffee/HPLC for both green and ground roasted coffee beans were agreeable with the EC legislation (*28*) with regard to the suitability of the methods of detection, it is obvious that those of the immunoaffinity column cleanup/HPLC procedure (*19*) were relatively higher (85 and 90%) than those of the AOAC method for OTA in green coffee/HPLC (*17*), which recorded 80 and 86% recoveries.

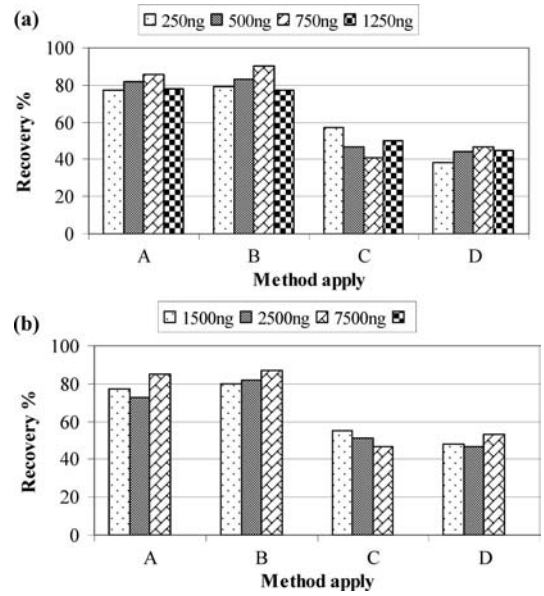

Figure 2. Recovery of OTA in roasted coffee beans (**a**) spiked with 250, 500, 750, and 1250 ng and (**b**) spiked with 1500, 2500, and 7500 ng using the four given methods (average of five determinations on separate extracts for each OTA concentration). (A) OTA in green coffee/HPLC, AOAC (*17*). (B) Immunoaffinity column/HPLC, Pittet et al. (*19*). (C) OTA in corn and barely/HPLC, AOAC (*24*). (D) Multimycotoxin detection method (TLC), Stoloff et al. (*25*).

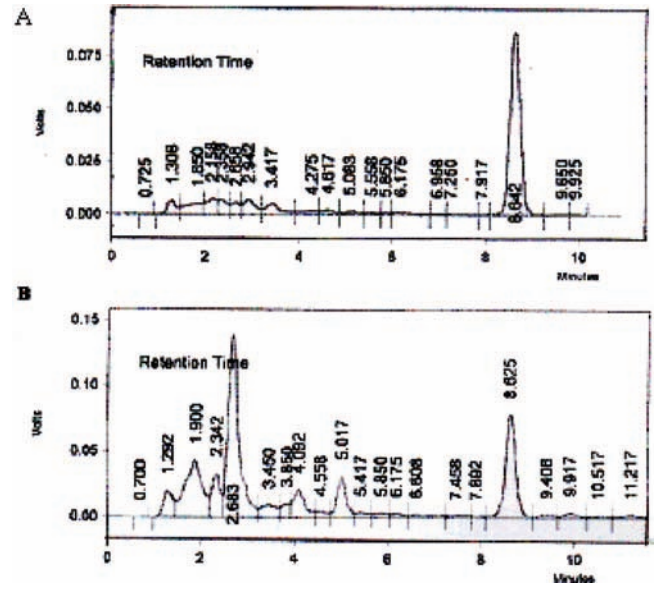

Figure 3. HPLC chromatograms illustrating the analysis of OTA added to coffee at a concentration of 250 ng/kg using (**A**) an immunoaffinity column procedure introduced by Pittet et al. and HPLC. (**B**) AOAC official method of analysis (2000) OTA in green coffee and HPLC. Conditions of the HPLC system are given in the methods section.

From the present results, it is obvious that the method of Pittet et al. (*19*) using the immunoaffinity column cleanup procedure and OTA elute analyzed by conventional reversed-phase HPLC lead to the highest recoveries. The relatively higher percentage recoveries achieved are mainly due to the extraction technique followed (*19*), which was effective in removing OTA from the ochratest column, and to the use of the immunoaffinity column cleanup procedure of Pittet et al. (*19*), which proved to be extremely useful for the rapid cleanup of OTA with the highest recovery and which gave cleaner HPLC chromatograms (**Figure 3**) than the AOAC method for OTA in green coffee (*17*) for both green and roasted coffee beans. This conclusion was also reached earlier by Pittet et al. (*19*) with pure and adulterated soluble coffee.

Moreover, it is important to shed light upon the statistical analysis data given in **Table 1**, which confirmed the presence of a significant difference ($P > 0.05$) between the transformed percentages of recovery of the immunoaffinity column cleanup/HPLC procedure of Pittet et al. (*19*) and those of the AOAC method for OTA in green coffee/HPLC (*17*) for both the green and roasted coffee beans. It ought to be considered that, although the immunoaffinity column/HPLC procedure of Pittet et al. (*19*) was apparently found to be more accurate than the AOAC method for the determination of OTA in green coffee/HPLC (*17*), the cost of the immunoaffinity column may be an obstacle against the use of such a method for routine analysis in green and roasted coffee bean survey studies. However, it is recommended for use in processed coffee bean studies where low concentrations of OTA may be expected to be detected and where interfering materials exist (*19*).

We have put in our consideration for the proposed European Community (EC) legislation, which specifies that recoveries of 70–110% should be achieved in order to ensure that a given method is suitable for use in the detection of OTA (*28*). Thus, it can be concluded that, although the immunoaffinity column cleanup/HPLC procedure (*19*) recorded a significantly higher percentage recovery than did the AOAC method for OTA in green coffee/HPLC (*17*), the AOAC method for OTA in green coffee/HPLC (*17*) is recommended as an efficient method for the routine analyses of OTA in green and ground roasted coffee beans. This method was found to be an accurate, sensitive, and less-expensive one which employs routine materials and readily available equipment.

Table 1. ANOVA for Differences between the Transformed Percentages of Recovery of the Different Methods Used for the Detection of OTA in Green and Roasted Coffee Beans

		MS[a]	
source of variation	df	green coffee beans	roasted coffee beans
between methods	3	8.195802[b]	10.36261[b]
error	24	0.14086	0.99026
total	27		

[a] MS: mean squares. df: degrees of freedom ($n - 1$). [b] Significant at $P \leq 0.05$.

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Received for review August 27, 2007. Accepted September 10, 2007.

JF072558X