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Rapid, Low Cost Thin-Layer Chromatographic Screening Method for the Detection of Ochratoxin A in Green Coffee at a Control Level of 10 μg/kg

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A thin-layer chromatographic (TLC) screening method was developed for the detection of ochratoxin A (OTA) in green coffee at a control level of 10 μg/kg. The method is based on extraction of OTA with a mixture of phosphoric acid and dichloromethane, purification by liquid−liquid partition into sodium hydrogen carbonate, separation by normal-phase TLC, and detection by visual estimation of fluorescence intensity under a UV lamp at 366 nm. The method was validated by performing replicate analyses of uncontaminated green coffee material spiked at 3 different levels of OTA (5, 10, and 20 μg/kg), and also by comparing results obtained on a series of test trial green coffees naturally contaminated with OTA (range 0.2 to 136.7 μg/kg) with those measured by a quantitative immunoaffinity/HPLC method. The agreement between the two methods was excellent, and neither false positive nor false negative results were recorded. This screening method is rapid, simple, robust, and very cheap, which makes it particularly well adapted for implementation in coffee-producing countries.

KEYWORDS: Ochratoxin A; green coffee; analysis; thin-layer chromatography; screening method

INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic and nephrocarcinogenic mycotoxin produced by several fungal species from the *Aspergillus* genus and by *Penicillium verrucosum*. It is most commonly found in cereals and cereal products, although a wide range of other commodities have been reported to contain the toxin. These include dried vine fruits (currants, raisins, and sultanas), green coffee and coffee products, wine, beer, grape juice, cocoa and cocoa products, spices, and meat products (*1*−*3*).

The natural occurrence of OTA in green coffee beans has been reported by several authors; it has been found in concentrations ranging between 0.1 and 360 μg/kg (*4*−*14*).

Although considerable inconsistencies are found in the literature regarding the influence of roasting and subsequent operations on the OTA content of coffee (*12*, *15*, *16*), experiments undertaken to assess the evolution of OTA along an industrial soluble coffee manufacturing line have recently shown that more than 80% of the OTA originally present in the green coffee is destroyed during the roasting process (*17*). This explains why OTA concentrations found in roast and ground coffee or soluble coffee are consistently low, with overall mean contamination levels of ca. 0.8 μg/kg ($n = 1337$) and 1.2 μg/kg ($n = 480$), respectively (*14*, *16*, *18*−*24*). According to a recent evaluation performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), this represents only ca. 4−7% of the total OTA weekly intake (*3*).

However, to minimize exposure and further decrease OTA concentrations to lowest achievable levels, an important prevention program has been set up by the International Coffee Organization (ICO, London) in collaboration with the Food and Agriculture Organization (FAO, Rome). This concerted effort is directed toward enhancement of green coffee quality through prevention of mold growth, and consists of establishing and disseminating guidelines for coffee production, harvesting, processing, storage, and transport. To support this program, and considering also the very inhomogeneous distribution of OTA in green coffee (*12*, *17*), rapid and inexpensive screening methods are needed to monitor OTA levels in green coffee at all stages of the production chain. This is particularly important for coffee-producing countries, where the cost of analysis is often the first factor considered before a method is adopted.

Modern analytical methods for the determination of OTA in green coffee and coffee products usually involve an immunoaffinity column cleanup and high-performance liquid chromatography (HPLC) with fluorescence detection (*11*, *16*, *19*, *21*, *22*, *25*−*27*), sometimes with chemical confirmation by liquid chromatography/tandem mass spectrometry (*25*, *26*). As immunoaffinity columns have been shown to give very clean extracts and well-defined chromatograms with no interference at OTA retention time, it should also be possible to perform the final quantitation by thin-layer chromatography (TLC) and scanning densitometry, as proposed recently for fumonisin B_1

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and the aflatoxins (*28*, *29*). However, such methods are not appropriate for implementation in certain coffee-producing countries, because they are too expensive and require sophisticated instrumentation. In addition to some rather old traditional TLC procedures that were shown to provide insufficient sensitivity (*4*, *30*−*32*) and/or inadequate cleanup of green coffee extracts (*31*, *32*), there are some rapid ELISA test kits commercially available, but none of these proved reliable for measuring OTA in green coffee because of a strong matrix interference which leads to 100% false positive results (A. Pittet, unpublished results).

The objective of the present work was to develop and validate a rapid, simple, and low cost TLC screening method allowing the detection of OTA in green coffee at a control level of 10 μg/kg without using expensive immunoaffinity columns for cleanup. This method is based on extraction of OTA with a mixture of phosphoric acid and dichloromethane, purification by liquid−liquid partition into sodium hydrogen carbonate, separation by thin-layer chromatography, and detection by visual estimation of fluorescence intensity under a UV lamp at 366 nm.

MATERIALS AND METHODS

Green Coffee Samples. Experiments were conducted with test trial samples of both Arabica and Robusta green coffee originating from Thailand, India, Kenya, Uganda, Salvador, and Guatemala. All these samples had been previously analyzed by HPLC according to a method described elsewhere (*16*) and identified as being either blank (OTA content < 0.2 μg/kg) or naturally contaminated with OTA concentrations ranging between 0.2 and 136.7 μg/kg. The green coffee material used for spiking experiments originated from Salvador and contained no detectable OTA.

Apparatus. Gastight microliter syringes were obtained from Hamilton Company (Reno, NV). The orbital shaker (IKA-electronic type KS-501 D) was purchased from IKA-Werke GmbH & Co. (Staufen, Germany). The stirrer for test tubes (Vortex Genie-2) was from Bender & Hobein (Zürich, Switzerland), and the Mini-Vap block heater was from Supelco (Bellefonte, PA). The Camag UV-Viewing Box 3, fitted with two 8-W longwave UV lamps (366 nm), as well as the twin trough chambers for chromatography, were obtained from Camag AG (Muttenz, Switzerland).

Chemicals and Reagents. A stock standard solution of OTA at 50 μg/mL in benzene/acetic acid (99:1) was purchased from Supelco (Bellefonte, PA). Acetic acid (glacial) 100%, acetonitrile, dichloromethane, diethyl ether, ethyl acetate, formic acid 98−100%, 1-octanol, *o*-phosphoric acid 85%, sodium hydrogen carbonate, anhydrous sodium sulfate powder, and toluene were all obtained from Merck (Darmstadt, Germany) and were of analytical grade. Silica gel 60 precoated TLC aluminum sheets (20 × 20 cm, layer thickness 0.2 mm, without fluorescent indicator) were also obtained from Merck (Darmstadt, Germany). Folded filters (medium fast S&S 597$^1/_2$, qualitative, diameter 240 mm) were purchased from Schleicher & Schuell GmbH (Dassel, Germany).

Safety. Ochratoxin A is a potent nephrotoxin and liver toxin that has been reported to have immunosuppressant properties. It is classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC). Gloves and safety glasses should be worn at all times, and all standard and sample preparation stages should be carried out in a fume cupboard.

Preparation of OTA Standard Solutions. A spiking solution at 10 μg/mL was prepared by diluting the stock standard solution (50 μg/mL) five times with toluene/acetic acid (99:1). This spiking solution was stored in the dark in a freezer at −18 °C. Then a working standard solution at 0.5 ng/μL was prepared by transferring 500 μL of spiking solution (10 μg/mL) to a 10-mL volumetric flask and diluting to volume with acetonitrile/toluene/acetic acid (50:49:1). This working standard solution was stored in the dark in a refrigerator at 4 °C.

Extraction. A 50-g portion of finely ground coffee beans was placed in a 500-mL Erlenmeyer flask, soaked with 25 mL of 0.1 M phosphoric acid (prepared by diluting 5.75 g of phosphoric acid 85% to 500 mL with water), and extracted with 125 mL of dichloromethane by shaking for 60 min on a mechanical shaker. The resulting suspension was filtered through a folded filter (S&S 597$^1/_2$).

Liquid−Liquid Partition. A 20-mL aliquot of filtrate was transferred to a 200 × 26 mm glass test tube with screw-cap, and 20 mL of 3% aqueous sodium hydrogen carbonate solution was added, followed by 3 drops of 1-octanol (to avoid formation of emulsions). The test tube was capped, the contents were mixed gently by hand, and the cap was slightly unscrewed to allow degassing. Then the test tube was tightly closed again and vigorously shaken for 30 s on a Vortex mixer. After phase separation, 18 mL of the upper aqueous layer was transferred to another 200 × 26 mm test tube and acidified to pH 2.0 with ca. 0.6 mL of phosphoric acid 85% (*Caution: as this reaction liberates carbon dioxide, the acid must be added in small portions of ca. 0.1 mL each, and the contents of the test tube mixed gently by hand after each addition to avoid excessive foaming*). Subsequently, 5 mL of dichloromethane was added into the test tube, which was then capped and vigorously shaken on a Vortex mixer for 30 s. After phase separation, the upper aqueous layer was removed by means of a Pasteur pipet, and the lower organic layer was dried by addition of ca. 1 g of anhydrous sodium sulfate powder so as to eliminate the last droplets of water. An aliquot of 3.5 mL of organic phase was transferred to a 5-mL conical vial and evaporated to dryness on a Mini-Vap block heater under a stream of nitrogen at 40 °C, and the residue was taken up in 100 μL of acetonitrile/toluene/acetic acid (50:49:1).

Thin-Layer Chromatography (TLC). Aliquots of 60 μL of sample extracts (representing 3.0 g of green coffee) and OTA working standard solution (0.5 ng/μL) were spotted onto a silicagel 60 TLC plate with a microliter syringe, along a virtual line situated at 100 mm from the bottom edge of the plate. Spots were applied at 13 mm intervals, according to the spotting scheme shown in Figure 1 (standards 1 and 2, samples 3−12, standards 13 and 14). During application, care was taken to dry spots with a stream of cold air so that they never exceeded a diameter of ca. 5 mm. The TLC plate was first developed in diethyl ether (saturated tank), air-dried, examined under UV light, and cut with scissors at ca. 1 cm above the spotting line so as to remove interfering substances that migrated with the solvent front. Then the plate was developed in the opposite direction (cut edge down) in toluene/ethyl acetate/formic acid (5:4:1), again in a saturated tank, until the solvent front reached ca. 5 mm from the upper edge of the plate. After this second development, the TLC plate was air-dried at room temperature and visually assessed under a longwave (366 nm) UV lamp. The OTA concentration in green coffee samples was estimated as greater than (>), equal to (≅), or less than (<) 10 μg/kg, by comparing the fluorescence intensity of OTA spots (at an R_f value of approximately 0.70) with that of pure OTA standards.

RESULTS AND DISCUSSION

The proposed method is a screening procedure for the qualitative determination of OTA in green coffee, i.e., determination of the presence or absence of OTA at a given concentration of 10 μg/kg. The extraction was adapted from the original conditions described by Stoloff and Scott (*32*) in AOAC Official Method 975.38, but chloroform was substituted by dichloromethane, and phosphoric acid was used instead of distilled water for wetting the test portion because this has been shown to improve OTA recoveries, for example for analysis of barley samples (*33*).

To optimize the extraction conditions, various solvent/sample ratios, as well as different shaking times, were tested for analysis of naturally contaminated green coffee. The extraction yield was compared with results obtained by immunoaffinity/HPLC according to the method described by Pittet et al. (*16*). For this purpose, aliquots of dichloromethane extracts were evaporated to dryness under a stream of nitrogen at 40 °C, and the residues were taken up in mobile phase before being subjected to HPLC quantitation. The results are summarized in Table 1. Increasing the amount of test portion from 25 to 50 g while keeping the

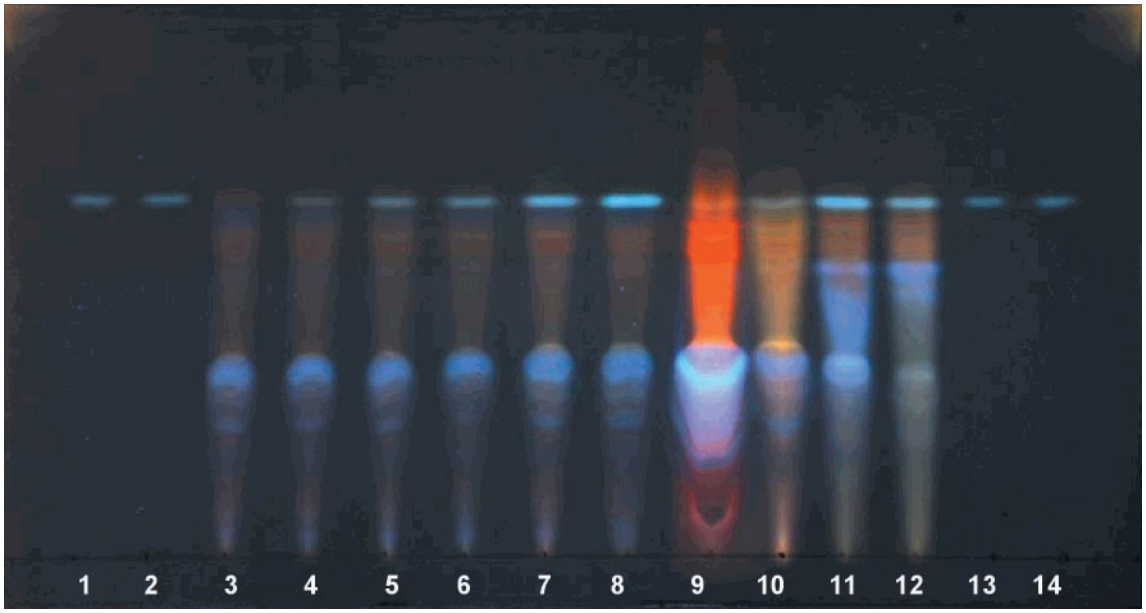

Figure 1. Example of a silica gel 60 TLC plate after development in toluene/ethyl acetate/formic acid (5:4:1), as visualized under 366 nm UV light. Tracks 1, 2, 13, 14 = OTA working standard solution, corresponding to the 10 μg/kg control level; track 3 = blank green coffee with no detectable OTA; tracks 4, 5, 6, 7, 8 = blank green coffee spiked with respectively 5, 10, 20, 30 and 50 μg/kg OTA; tracks 9, 10, 11, 12 = green coffee samples naturally contaminated with respectively 4.5, 12.7, 38.2, and 24.7 μg/kg OTA.

Table 1. Influence of Solvent/Sample Ratio and Shaking Time on the Extraction of OTA from Naturally Contaminated Green Coffee

amount of test portion (g)	volume of H_3PO_4 0.1 M (mL)	volume of CH_2Cl_2 (mL)	shaking time (min)	number of determinations (*n*)	mean recovery[a] (%)	RSD (%)
25[b]	12.5	125	30	2	53	12.1
50	12.5	125	30	2	49	21.9
50	25	125	30	4	72	10.9
50	25	125	60	2	98	5.1
50	25	125	120	2	105	2.0

[a] Recoveries are expressed as a percentage of the OTA contamination level measured by immunoaffinity/HPLC according to the method of Pittet et al. (*16*). [b] Original conditions proposed by Stoloff and Scott (*32*), but in their method distilled water was used instead of 0.1 M phosphoric acid, and chloroform was used instead of dichloromethane.

volume of dichloromethane unchanged (125 mL) was found necessary to achieve the desired sensitivity. However, a significant increase of the extraction yield (from 49 to 72%) was observed when the volume of phosphoric acid 0.1 M was increased from 12.5 to 25 mL, and a further improvement could be obtained by extending the shaking time from 30 to 60 min. These conditions led to an almost quantitative extraction of OTA (98%). Shaking for 120 min was shown to have practically no further influence on the amount of OTA extracted.

Regarding the cleanup procedure, attempts to use solid-phase extraction columns such as MultiSep no. 212 multifunctional columns (Romer Labs, Inc., Union, MO), diethyl-aminopropyl (DEA), and other anion exchange cartridges as proposed by Akiyama et al. (*34*), or silica cartridges as proposed by Langseth et al. (*35*) were all unsuccessful due to insufficient purification and/or poor recoveries. C_{18} cartridges used according to recommendations given by Nesheim et al. (*36*) gave satisfactory results, but the procedure was considered too tedious and therefore inappropriate for a rapid screening method. Clearly the best compromise was obtained with a liquid−liquid partition of OTA into a 3% aqueous sodium hydrogen carbonate solution, followed by acidification to pH 2.0 and partition into dichloromethane. This cleanup is an adaptation of the protocol described by Valenta et al. for the analysis of OTA in urine and feces of swine (*37*). However, our approach differs from that originally described by Valenta et al. in a number of ways, including the substitution of chloroform by dichloromethane, and the use of a more concentrated solution of aqueous sodium hydrogen carbonate (3% instead of 0.8%). The volume of sodium hydrogen carbonate solution was also increased from 10 to 20 mL in order to increase sensitivity and make the procedure more robust. Moreover, the subsequent purification of the extract by solid-phase extraction through a silica gel cartridge was found superfluous for analysis of green coffee and dropped. A critical parameter of this procedure is that it is very important to carefully dry the dichloromethane extract over anhydrous sodium sulfate powder before evaporation to dryness, otherwise the recovery and reproducibility of the method may be very poor (*37*).

Appropriate TLC conditions were selected by testing different types of commercially available TLC plates (both normal-phase and reversed-phase, with or without fluorescent indicator, and with or without concentrating zones), different spotting solvents, and different solvent systems for plate development. Among the many different solvent systems that were tested for the development of normal-phase TLC plates (which are cheaper

Table 2. Method Performance for Analysis of Green Coffee Material Spiked with Various Amounts of Ochratoxin A

OTA added (μg/kg)	number of analyses	assessment by visual TLC		
		< 10 μg/kg	≅ 10 μg/kg	> 10 μg/kg
0	2	2	0	0
5	5	5	0	0
10	5	0	5	0
20	5	0	0	5

Table 3. Agreement between Visual TLC and HPLC for Analysis of Ochratoxin A in Naturally Contaminated Green Coffee Samples

OTA concentration as determined by HPLC[a] (μg/kg)	no. of samples within range	assessment by visual TLC (μg/kg)				
		< 10	≅ 10	> 10	≫ 10	⋙ 10
< 0.2	9	9	0	0	0	0
0.2–5.0	9	9	0	0	0	0
5.1–10.0	6	5	1	0	0	0
10.1–20.0	4	0	2	2	0	0
20.1–50.0	3	0	0	1	2	0
> 50	5	0	0	0	3	2

[a] Analyses were performed according to method of Pittet et al. (*16*).

than reversed-phase plates), the best one was found to be toluene/ethyl acetate/formic acid (5:4:1) as proposed in AOAC Method 975.38 (*32*). However, with certain green coffee samples, e.g. those containing a relatively high proportion of coffee husks, a preliminary development of the plate in diethyl ether proved necessary to obtain adequate separation of OTA from background interferences (see track 9 in Figure 1), and therefore this approach was adopted in our protocol in order to improve the overall robustness of the method. Another important factor is the choice of the spotting solvent. Toluene/acetic acid (99:1) was found satisfactory in our preliminary experiments, but in a later stage considerable improvements could be obtained by using a mixture consisting of acetonitrile/toluene/acetic acid (50:49:1). With this solvent, manual spotting allowed us to get OTA spots appearing as thin bands of ca. 7−8 mm length after plate development, with minimal spreading and, consequently, a better sensitivity.

The accuracy of the method was assessed by performing replicate analyses of uncontaminated green coffee material spiked at 3 different levels of OTA, viz. 5, 10, and 20 μg/kg. The results are presented in Table 2. Blank green coffee extracts as well as the 5 samples spiked at 5 μg/kg were all clearly negative, while those spiked at 20 μg/kg were all clearly positive. Moreover, OTA spots corresponding to samples spiked at 10 μg/kg were very similar to the control spots with respect to their size, shape, and typical greenish fluorescence. As shown in Figure 1, a contamination level of 5 μg/kg is still visible on the TLC plate, but the fluorescence intensity is too low to consider this as the limit of detection of the method.

The method was further validated by screening a total of 36 test trial green coffee samples previously analyzed by HPLC according to the method of Pittet et al. (*16*) and identified as being either blank (OTA content < 0.2 μg/kg) or naturally contaminated with OTA concentrations ranging between 0.2 and 136.7 μg/kg. The results are presented in Table 3 and show that the agreement between the two methods was excellent. All samples containing less than 5.0 μg/kg OTA were recorded as negative, and no sample containing more than 20 μg/kg failed to give a definite positive result. Among the 10 samples falling within the ranges 5.1−10.0 μg/kg and 10.1−20.0 μg/kg, three were recorded as containing approximately 10 μg/kg OTA by visual TLC. A review of HPLC results confirmed that the OTA level in these samples was very close to the control level, with concentrations of 9.9, 10.5, and 10.6 μg/kg.

In conclusion, this TLC screening method is rapid, simple, and robust, and requires a much lower level of expertise than conventional analytical methods based on HPLC. With experience it is possible to analyze up to 20 samples in one working day. Moreover, the technique is well adapted for use in coffee-producing countries, particularly in those laboratories facing budget restrictions. Indeed, total investments necessary for implementation of this method have been estimated at ca. $6000 (U.S. $), and the cost per analysis (including all chemicals and reagents) is less than $4 (U.S. $).

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