Research Note

Two-Dimensional Thin-Layer Chromatographic Method for the Analysis of Ochratoxin A in Green Coffee

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ABSTRACT

A low-cost thin-layer chromatographic method has been developed for the presumptive measurement of ochratoxin A (OTA) at 5 μg/kg in green coffee beans. The analytical method consisted of extracting OTA by shaking the beans with a mixture of methanol and aqueous sodium bicarbonate solution, which was then purified by liquid-liquid partition into toluene. OTA was separated by normal-phase two-dimensional thin-layer chromatography and detected by visual estimation of fluorescence intensity under a UV lamp at 365 nm. The chromatography solvents were toluene–methanol–formic acid (8:2:0.03) for the first development and petroleum ether–ethyl acetate–formic acid (8:10:1) for the second dimension development. This method was tested with uncontaminated green coffee bean samples spiked with an OTA standard at four different concentrations (5, 10, 20, and 30 μg/kg). The method is rapid, simple, and very easy to implement in coffee-producing countries. It is highly selective and does not involve the use of chlorinated solvents in the sample extraction step. This inexpensive method has been applied to different types of green coffee samples from various countries (Zimbabwe, Brazil, India, Uganda, Colombia, and Indonesia) and different manufacturers, and no OTA below the detection limit of 5 μg/kg was detected in any samples analyzed.

Ochratoxin A (OTA) is a mycotoxin produced as a secondary metabolite by certain Aspergillus and Penicillium fungal species. OTA consists of a dihydroisocoumarin chlorate moiety coupled through its 7-carboxyl group by an amide bond to L-β-phenylalanine (Fig. 1). Natural OTA occurs in plant products such as cereals, coffee beans, cocoa beans, dried fruits, legumes, nuts, and spices (7, 12, 16, 17, 21) and has been detected in beverages such as wine, grape juice, and beer. It also has been found in products made from animals that have been exposed to large quantities of OTA (2, 20). The occurrence of this mycotoxin in foods and feeds is unavoidable and influenced by various environmental factors. Its presence varies with geographic location, agricultural and agronomic practices, and the susceptibility of commodities to fungal invasion during pre- and postharvest storage periods.

Several lines of evidence suggest that OTA can cause liver and kidney damage and has teratogenic, carcinogenic, and immunosuppressive properties. The International Agency for Research on Cancer classified OTA in group 2B as a renal carcinogen for animals and possibly humans (4). OTA is suspected as the cause of urinary tract tumors and of a chronic kidney disease found in southeastern Europe and known as Balkan endemic nephropathy (1, 10).

Since the 1960s, there has been much interest in evaluating mycotoxin contamination in many foodstuffs because the presence of these toxins decreases the quality of the product. This contamination results in economic losses and represents a risk to human health. In 2004, the European Union established a maximum concentration of 5 μg/kg for OTA in grain and ground coffee (3).

Most of the analytical methods currently used for the analysis of OTA in coffee employ reverse-phase high-performance liquid chromatography (HPLC) with a fluorescence detector. At the purification and concentration step, solvent extraction, immunoaffinity columns, or solid-phase extraction must be used because of the low concentration of OTA usually found in foods and the complexity of the samples (9, 15). Confirmation of OTA presence is done by derivatization or mass spectrometry coupled to HPLC (5, 19). Immunochemical methods based on enzyme-linked immunosorbent assays also have been used (13, 14). However, many coffee-producing countries cannot use these methods because they are very expensive and require professional skills and equipment. Other methods involve the use of solvent extraction and thin-layer chromatography (TLC). At the sample extraction step, most of these methods require the use of hazardous solvents such as chloroform and dichloromethane, which are chlorinated solvents with adverse environmental effects (6, 8, 11, 18).

The aim of this study was to develop an easy and inexpensive method for the analysis of OTA in green coffee that could be used in coffee-producing countries and would not require special equipment or laboratory training. The toxin is extracted by liquid extraction and purified by liquid-liquid partition into toluene. OTA is detected by nor-

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mal-phase TLC under a UV lamp at 365 nm. Confirmation of OTA is achieved with two-dimensional TLC.

MATERIALS AND METHODS

Reagents and chemicals. Crystalline OTA standard was supplied by Sigma-Aldrich (St. Louis, Mo.). Chemical products used were of analytical grade. Toluene was purchased from Romil (Barcelona, Spain); acetone, hexane, ethyl acetate, and bicarbonate sodium were supplied by Panreac (Barcelona, Spain); and formic acid and acetonitrile were obtained from Merck (Darmstadt, Germany).

Coffee samples. Green coffee beans were obtained from a local roaster. The beans came from different regions (Zimbabwe, Brazil, India, Uganda, Colombia, and Indonesia) and had been harvested during 2001 and 2002. The beans were stored at 4°C in the dark until used for analyses.

OTA standard solutions. A stock OTA solution of 100 mg/liter was prepared in acetonitrile and stored at 4°C in the dark. Standard OTA working solutions of 1, 0.5, and 0.1 mg/liter were prepared immediately before use by diluting the stock solution with acetonitrile.

Developing solvents. A TLC silica gel plate G60 (5 by 10 cm plastic, coated with 0.25 mm; Merck) was spotted with 5 ng of OTA. The plate was placed in an unsaturated Stahl-type tank (Desaga GmbH, Heidelberg, Germany). TLC solvents were toluene–methanol–formic acid (8:2:0.03) for the first dimension and petroleum ether–ethyl acetate–formic acid (8:10:1) for the second dimension. TLC plates were left to evaporate at room temperature and inspected under UV lamp at 365 nm.

Extraction and purification of OTA in green coffee samples. Approximately 500 g of green coffee beans was ground in a mill. A 10-g portion of ground coffee was spiked with 50 ng of OTA standard (5 μg/kg) and then shaken with 50 ml of a mixture of methanol–NaHCO₃ (50:50, 3% aqueous water) for 10 min with a magnetic stirrer. The sample was then filtered through filter paper to separate solid components, and the filtrate was adjusted to pH 3.0 ± 0.5 with H₃PO₄. Two extractions were conducted with 10 ml of toluene. The supernatant was collected with a Pasteur pipette in a conical flask and evaporated to dryness under vacuum, and the residue was dissolved in 500 μl of toluene.

TLC. For detecting OTA, 60 μl of sample extract was spotted on a TLC silica gel plate G60 (10 by 10 cm plastic, coated with 0.25 mm) 1 cm from the left bottom corner. At 1 cm from the bottom right corner, the plate was spotted with 6 μl of the OTA 1 mg/liter (6 ng) standard, and a second standard spot was applied 1 cm from the top left. The plate was placed in an unsaturated Stahl-type tank with toluene–methanol–formic acid (8:2:0.03) and then removed after 8 cm of development, and the solvent was allowed to evaporate at room temperature. The plate was then inspected under a UV lamp at 365 nm. OTA spots should be greenish blue. Samples with spots that matched both Rf and color of standard spots were considered presumptive positive samples and were submitted to a second TLC procedure for confirmation.

Confirmation procedure. For confirmation of OTA presence, the previously developed TLC plate was turned 90° and then placed in an unsaturated tank with petroleum ether–ethyl acetate–formic acid (8:10:1) as a second developing solvent. The plate was removed after 8 cm of orthogonal development and allowed to dry at room temperature. It was then inspected under a UV lamp at 365 nm. Samples with spots with the same Rf values and color as the standards, from the first and second development, were considered presumptive positive samples.

Solvent extraction optimization. Different solvents for extraction of OTA from green coffee were tested. Coffee samples were extracted with mixtures of methanol–NaHCO₃ (3% aqueous solution: 0:100, 25:75, 50:50, and 100:0) for 30 min with magnetic stirring. The 75:25 mixture was not miscible. Extracts were then filtered through filter paper, and the filtrate was adjusted to pH 3.0 ± 0.5 with H₃PO₄. Two extractions were conducted with 10 ml of toluene. The supernatant was collected with a Pasteur pipette in a conical flask and evaporated to dryness under vacuum, and the residue was dissolved in 500 μl of toluene. The extracts were then subjected to two-dimensional TLC.

Extraction time evaluation. To evaluate the extraction time, we used the same process described for extraction and purification of OTA but varied the amount of time allowed for mixing with the magnetic stirrer. Extraction times tested were 5, 10, 15, 20, and 30 min.

Detection limit. The lower limit of visual detection was tested by analyzing coffee samples spiked with the OTA standard at the legislated concentration (5 μg/kg) and three higher concentrations (10, 20, and 30 μg/kg). Four independent TLC plates were spotted with 6, 12, 24, and 36 μl of the 1 mg/liter OTA standard, respectively. Each of the TLC plates was spotted with 60 μl of the coffee sample spiked with 50, 100, 200, and 300 ng of toxin standard.

RESULTS AND DISCUSSION

TLC solvents tested. We used toluene–methanol–formic acid (80:20:0.3) as the first developing solvent (OTA Rf of 0.25) and petroleum ether–ethyl acetate–formic acid (8:10:1) as the second developing solvent (OTA Rf of 0.75) (Figs. 2 and 3). We avoided the use of chlorinated solvents.

To assess the performance of the method and to optimize the cleanup step and detection procedure, we assayed...
different extraction solvents and extraction times. These experiments were performed with coffee samples spiked with 100 ng of OTA standard (10 μg/kg).

**Solvent extraction.** The solvent extraction procedure was optimized to obtain the maximum recovery and to evaluate the TLC profile for the cleanest profile and the most intense OTA spot. Extraction solvents with ≥50% methanol have the same extracting ability. Extractions done with methanol give a dirty TLC profile, and the OTA spot is not very clear. We decided to work with methanol–NaHCO₃ (3%, 50:50) because the OTA spot was more intense.

**Extraction time.** The best extraction time was 10 min; shorter times yielded darker OTA spots in spiked coffee samples, which means that less OTA was extracted.

**Detection limit.** The detection limit for coffee samples spiked with ≥50 ng of OTA standard could be visually determined on the TLC plates by all evaluators when plates were examined under UV light at 365 nm. This limit is the same as that reported most often (8, 17, 18). This method of OTA evaluation was applied to green coffee beans originating from Zimbabwe, Brazil, India, Uganda, Colombia, and Indonesia, and no OTA was detected at the control concentration of 5 μg/kg.

The use of two-dimensional TLC instead of one-dimensional TLC allowed for more selectivity and did not require long sample-preparation steps. TLC plates are inexpensive, and the method does not require specialized equipment. This method is an effective laboratory tool for screening OTA at 5 μg/kg and should be very easy to implement in coffee-producing countries.

**REFERENCES**


