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Development of a Solid-Phase Cleanup and Portable Rapid Flow-Through Enzyme Immunoassay for the Detection of Ochratoxin A in Roasted Coffee

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A membrane-based flow-through enzyme immunoassay (patent application pending) for the detection of ochratoxin A (OA) in roasted coffee was developed. First, an extraction and solid-phase cleanup method was developed. A high partition coefficient for OA in the mobile phase was achieved by using methanol/5% aqueous $NaHCO_3$ as the sample extraction and cleanup solvent. The solid-phase (aminopropyl) cleanup was developed to chromatographically elute OA but retain cross-reacting compounds. Without using aminopropyl cleanup, cross-reacting compounds resulted in 100% false positives for both flow-through enzyme immunoassay and HPLC methods. However, after cleanup with aminopropyl, no false positives were observed. The flow-through results were visually evaluated. The sensitivity achieved for the flow-through was 4 μg kg^{-1} in spiked roasted coffee. The assay was used to screen roasted coffee samples. Results were confirmed with HPLC with a detection limit of 1 μg kg^{-1}.

KEYWORDS: Flow-through; enzyme immunoassay; ochratoxin A; coffee

INTRODUCTION

Ochratoxin A (OA) is mainly produced by *Aspergillus ochraceus* and *Penicillium verrucosum.* These OA-producing species are known contaminants of cereals and coffee. Martinez et al. (*1*) isolated mycotoxin-producing fungal species, including OA-producing *Penicillium* species and *A. ochraceus*, in a study on Venezuelan green coffee. OA levels were detected in 70% of the same samples and ranged from 0.9 to 15 μg kg^{-1}. Under normal conditions, OA is a relatively stable compound and may undergo slight decomposition when exposed to heat for a short period of time. A 77−87% reduction was reported by Levi et al. (*2*) when green coffee, spiked with OA, was roasted for 20 min at 200 °C. Gallaz and Stalder (*3*) reported 80−90% reduction after roasting of coffee beans which were spiked with OA or inoculated with *A. ochraceus*. However, Tsubouchi et al. (*4*) observed a 0−12% OA reduction in roasted coffee beans contaminated by inoculation with *A. ochraceus*. Their observations were supported by those of Studer-Rohr et al. (*5*). Pittet et al. (*6*) suggested that these inconsistencies between findings could be due to differences in the method of introducing the toxin into the coffee beans and that the analytical methods used were not sufficiently sensitive and selective to detect low levels of OA in roasted coffee. Recent studies confirmed the significant reduction of OA during roasting. Blanc et al. (*7*) found that the amount of OA naturally present in green coffee was reduced by 84% after roasting and grinding. They emphasized the importance of studying the distribution of toxins in green coffee beans when performing such research. Van der Stegen et al. (*8*) reported a 69% reduction of OA in naturally contaminated green coffee after roasting according to the normal commercial practice. Sampling of green coffee was done taking into account the inhomogeneity of mycotoxin contamination. Three explanations are given for this reduction: physical removal of OA with chaff, isomerization into another diastereomer, and thermal degradation with possible involvement of moisture. These data call for sensitive analytical methods at the onset of the coffee processing chain, usually on farms or in warehouses and roasting chambers, where contaminated lots should be segregated from high-quality coffee. Therefore, this article reports on the development of an easy and rapid flow-through enzyme immunoassay screening method for OA in roasted coffee.

MATERIALS AND METHODS

Immunoreagents. Anti-OA monoclonal antibodies and OA-horseradish peroxidase (OA-HRP) were produced by the Institute for Animal Sciences, Agricultural Biotechnology Center, Gödöllö, Hungary. Gyöngyösi-Horvath et al. (*9*) outlined the production of these monoclonal antibodies from hybridoma cell lines. The anti-OA monoclonal antibody was immunoglobulin IgG1 with κ light chains. The affinity

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Figure 1. Components of the flow-through device: (upper row, from left to right) plastic bottom member and absorbent material; (lower row, from left to right) plastic top member and membrane.

constant was 7.8×10^9 M^{-1}. The anti-OA immunoglobulin fraction (pure immunoglobulin fraction; protein content 2.5 mg mL^{-1}) was used for the flow-through assay. The cross-reactivity of this antibody was 100% for OA and 9.3% for ochratoxin B. There were no cross-reactivities with coumarin, 4-hydroxy-coumarin, and D,L-phenylalanine.

Flow-Through Assay: Membrane Preparation. The method used was a modification of that described by De Saeger and Van Peteghem (*10*, *11*) and Sibanda et al. (*12*−*14*). Briefly, a nylon Immunodyne ABC membrane (Pall France, Saint Germain-en-Laye, France; pore size, 0.45 μm) was cut into 4 cm^2 pieces with a central 1 cm^2 marked in pencil. Undiluted rabbit anti-mouse antibodies (2 μL) (Dako, Glostrup, Denmark; no. Z259; protein concentration, 3.5 g L^{-1}) were coated on one part of the inner 1 cm^2 of the membrane (sample spot). Goat anti-HRP antibodies (Sigma; 2 μL, 1:25000 in PBS−0.1% casein) were coated adjacent to the anti-mouse antibody spot (internal control spot). The membranes were dried at 37 °C for 30 min and blocked with PBS−2% casein for another 30 min. The blocked membrane was finally dried at 37 °C for 45 min and vacuum-packaged in a plastic bag containing silica as a moisture adsorbent.

Development of a Sample Extraction and Cleanup Method. Initial experiments with the flow-through enzyme immunoassay without a solid-phase cleanup step resulted in 100% false positives due to matrix interferences and brown-colored background on the membranes. At first, the extraction method used for roasted coffee was the same as that used for green coffee (*14*). Blank green coffee samples were roasted under industrial conditions and analyzed by HPLC with a detection limit of 1 μg kg^{-1} (*15*). All the blank roasted coffee samples showed a peak with a retention time identical to that of the OA standard. So, both the flow-through enzyme immunoassay and HPLC method resulted in false positives. It was suspected that the cross-reacting compound was the one with the same retention time as OA. To remove these matrix interferences of roasted coffee, an additional cleanup method had to be developed. Various solid-phase materials (aminopropyl, trimethylaminopropyl, *n*-propyl-ethylene-diamine, octadecyl, cyanopropyl, and diol) were evaluated for their ability to adsorb the brown color and other matrix interferences.

Determination of Solid- and Liquid-Phase OA Partitioning. The partition coefficient of OA between the solid phase and the mobile phase in the cleanup method using aminopropyl material (Bakerbond spe, J.T. Baker, Deventer, The Netherlands; 3-mL columns) was evaluated by analyzing the two fractions separately. Spiked roasted coffee samples (20, 40, 80, and 160 μg kg^{-1}; 20 g) were extracted with 60 mL of methanol/5% aqueous $NaHCO_3$ (1/1, vol/vol) by shaking for 15 min. After filtration through a Whatman no. 4 filter, 4 mL of filtrate was extracted over 400 mg of aminopropyl solid-phase material at a rate of 1 drop/s. The eluate that elutes directly from the column as the sample is applied is herein referred to as the frontal elution fraction. It was collected in a 100-mL flask and diluted to 60 mL with PBS (pH 7.4). The second fraction, herein referred to as the wash fraction, was obtained by washing the aminopropyl column with 4 mL of methanol/5% aqueous $NaHCO_3$ (1/1, vol/vol), followed by 2 mL of absolute methanol. This wash fraction was collected in another 100-mL flask and diluted to 100 mL with PBS (pH 7.4). The frontal elution fraction and the wash fraction were applied onto an Ochratest immunoaffinity column (Vicam, Watertown, MA). This column was washed with 10 mL of HPLC water and blown to dryness with one syringe volume of air (=20 mL). OA was eluted with 4 mL of absolute methanol, evaporated to dryness, and redissolved in 150 μL of methanol for HPLC analysis. The partition coefficient (K_d) was calculated using the formula K_d = [OA wash fraction]/[OA frontal elution fraction] or K_d = [OA solid phase]/[OA mobile phase].

HPLC Analysis of OA. Both frontal elution and wash fractions were analyzed by an HPLC method. Samples (50 μL) were injected through a manual Rheodyne injector and separated over a Supelco Discovery C18 (5 μm, 25 cm × 4.6 mm) column at room temperature. The mobile phase used was acetonitrile/water/acetic acid (99/99/2) at a flow rate of 1 mL/min. The HPLC system consisted of a Waters 600 Controller, a Waters 610 Fluid Unit, and a Waters 474 scanning fluorescence detector (330 nm excitation, 460 nm emission). Sample OA concentrations were quantified by comparing sample peak areas to those of standards.

Flow-Through Assay: Sample Preparation. The proposed cleanup method for roasted coffee was used for the development and optimization of the flow-through enzyme immunoassay. Therefore, spiked roasted coffee samples (0, 1, 2, 3, 4, 5, 10, 20, 40, and 80 μg kg^{-1}; 5 g) were extracted with 15 mL of methanol/5% aqueous $NaHCO_3$ (1/1, vol/vol) by shaking for 15 min. After filtration through a Whatman no. 4 filter, the filtrate (2 mL) was brought over 200 mg of aminopropyl solid-phase material at 1 drop/s. The eluate (1 mL) was diluted to 6.25 mL with PBS (pH 7.4) to reduce the methanol concentration to 8%. This solution was used in the flow-through enzyme immunoassay.

Flow-Through Assay Procedure. De Saeger and Van Peteghem (*10*, *11*) described a flow-through enzyme immunoassay carried out in a device format consisting of a plastic bottom and top member (**Figure 1**). In the device, cotton wool was acting as an adsorbent which actively drew liquid reagents through the membrane. The membrane was held above the cotton wool, directed at the center of the top member for reagent access. To analyze roasted coffee samples, anti-OA antibodies (100 μL; 1:75 in assay buffer) were first applied onto the membrane. The membrane was washed with 300 μL of PBS−Tween 0.05% (wash buffer), followed by 600 μL of sample extract. The membrane was washed with 300 μL of wash buffer, followed by 100 μL of OA-HRP (1:150 in assay buffer). The membrane was then washed with 900 μL of wash buffer. Finally, assay results were developed with 50 μL of ColorBurst Blue (Alercheck Inc., Portland, ME). Interpretation of the results was visual.

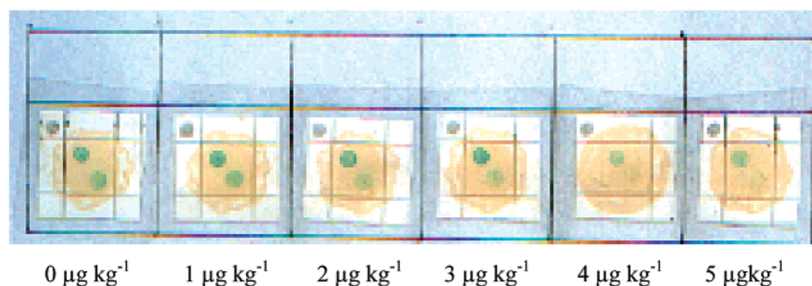

Figure 2. Dose response of the flow-through enzyme immunoassay to increasing ochratoxin A concentrations of spiked roasted coffee samples (left upper corner in the 1 cm^2 area of the membrane, internal control spot; right bottom corner in the 1 cm^2 area of the membrane, sample spot).

Table 1. Calculated Partition Coefficient Values for the Dispersion of OA between the Aminopropyl Solid Phase and the Methanol/5% Aqueous $NaHCO_3$ (1/1, vol/vol) Mobile Phase[a]

OA concn (μg kg^{-1})	OA concn, solid phase (μg kg^{-1})	OA concn, mobile phase (μg kg^{-1})	partition coeff (K_d = solid/mobile)
20	10	8	1.25
40	18	16	1.13
80	33	35	0.94
160	71	65	1.09

[a] $n = 3$.

Screening of Roasted Coffee Samples for OA. Roasted coffee samples (*9*) were received from Fort-E.P. Leidsche, Belgium, for OA screening and determination. The samples were screened by flow-through enzyme immunoassay and confirmed by HPLC.

RESULTS AND DISCUSSION

Analysis of OA in roasted coffee samples with flow-through immunoassay and HPLC without a solid-phase cleanup step resulted in false positives. HPLC chromatograms of blank roasted samples showed a peak with a retention time similar to that of the OA standard (*15*). This compound was isolated as an HPLC fraction and tested on the flow-through immunoassay but gave a negative result. The same fraction was again tested with the HPLC method to check whether the compound was still present. The fact that the compound could not be found anymore suggested that it was an unstable compound. Various solid-phase materials were tested, but only aminopropyl was capable of adsorbing the brown color and matrix interference while at the same time allowing OA to elute directly in the mobile phase. The use of an aminopropyl solid-phase cleanup step removed the cross-reacting compounds, and the interfering peak disappeared from the HPLC chromatograms. This interfering compound observed in the HPLC chromatograms must have been a result of the roasting process. In green coffee there are no such interfering compounds (*14*). The observed behavior of the compound suggests that anti-OA antibodies used in the flow-through and immunoaffinity columns for HPLC cleanup cross-react with this unknown compound. The compound might possess some similarities to ochratoxin A.

To elute OA rapidly over a polar solid phase, a strong eluotropic solvent has to be used. Methanol is considered the second strongest eluotropic organic solvent after acetic acid (*16*). Analyte solubility in the elution solvent also plays an important part in the chromatographic elution of an analyte. OA dissolves readily in methanol and in aqueous $NaHCO_3$. Therefore, to obtain a strong eluotropic mobile phase which was compatible with an enzyme immunoassay, the extraction solution [methanol/5% aqueous $NaHCO_3$ (1/1, vol/vol)] was considered to be sufficient to effect frontal chromatographic elution of OA.

Partition coefficient (K_d) values were calculated and are shown in **Table 1**. The distribution of OA between the aminopropyl solid phase and the methanol/5% aqueous $NaHCO_3$ (1/1, vol/vol) mobile phase was almost identical. OA was, on average ($n = 3$), partitioned almost equally between the two phases.

The flow-through test for spiked OA concentrations in roasted coffee showed a color extinction on the sample spot at 4, 5, 10, 20, 40, and 80 μg kg^{-1}. The sample spot appeared for 0, 1, 2, and 3 μg kg^{-1}, and the internal control spot appeared in all assays (**Figure 2**). The screening results were confirmed by HPLC, the method recovery of which ranged from 73 to 87%. It means that this flow-through enzyme immunoassay can be used to screen roasted coffee samples for OA levels using a cutoff point of 4 μg kg^{-1}. Commission Regulation (EC) No. 472/2002 of 12 March 2002 does not mention a maximum level for OA in roasted coffee, although there are limits for cereals (5 μg kg^{-1}), cereal products (3 μg kg^{-1}), and dried grapes (10 μg kg^{-1}). Although the blue spots were well resolved from the background color, instrumental colorimetric measurement of absorbances was not feasible. This was mainly due to the high absorbances caused by the brown background, resulting in no significant differences between background, internal control spot, and sample spot. Assay results, therefore, could only be achieved through visual evaluation.

Analysis of the nine roasted coffee samples using the flow-through enzyme immunoassay resulted in all nine samples being negative. The confirmation step with the HPLC analysis also resulted in nine negative samples. This means that no false negative or false positive results were obtained.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; Ig, immunoglobulin; K_d, partition coefficient; OA, ochratoxin A; PBS, phosphate-buffered saline.

SAFETY

OA has carcinogenic, genotoxic, immunosuppressive, nephrotoxic, and teratogenic properties. Gloves were worn when working with standards and samples. Standard and sample preparation was done in a fumehood. Glassware and OA waste were decontaminated by keeping them for 24 h in sodium hypochlorite solution (household bleach). Afterward, decontaminated glassware was washed with detergent and rinsed with water.

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