Method validation for the determination of ochratoxin A in green and soluble coffee by immunoaffinity column cleanup and liquid chromatography.

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Abstract

A method was validated for the determination of ochratoxin A (OTA) in soluble and green coffee. Performance parameters evaluated included selectivity, accuracy, intermediate precision, linearity, limit of detection, limit of quantitation, and ruggedness. The method was found to be selective for OTA in both matrices tested. Recovery rates from soluble coffee samples ranged from 73.5 to 91.2%, and from green coffee samples from 68.7 to 84.5%. The intermediate precision (RSDr) was between 9.1 and 9.4% for soluble coffee and between 14.3 and 15.5% for green coffee analysis. The linearity of the standard calibration curve (r^2) was >0.999 for OTA levels of 1.0-20.0 µg/kg in coffee samples. The limit of detection was determined to be 0.01 ng of OTA on column, while the limit of quantitation was found to be 0.03 ng on column. The limit of quantitation is equivalent to 0.6 µg/kg in soluble coffee samples and 0.3 µg/kg in green coffee samples. The results of the ruggedness trial showed two factors are critical for soluble coffee analysis: the extraction method, and the flow rate of the mobile phase. For green coffee analysis two critical factors detected were the extraction method and the storage temperature of the immunoaffinity column.

Five samples of soluble coffee and 42 of green coffee were analysed using the validated method. All soluble coffee samples contained OTA at levels that ranged from 8.4 to 13.9 µg/kg. Six of the 42 green coffee samples analysed

(14.3%) contained OTA at levels ranging from 0.9 to 19.4 µg/kg. The validated method can be used to monitor OTA levels in Colombian coffee for export or for local consumption.

Keywords: ochratoxin A, coffee, green coffee, soluble coffee, immunoaffinity, HPLC

Introduction

Ochratoxin A (OTA) is a nephrotoxic and carcinogenic mycotoxin capable of causing adverse effects on animal health and productivity (1). OTA also appears to play a role in certain human diseases and it has been included in group 2B by the International Agency for Research on Cancer (2). The general toxicology of ochratoxin A was recently reviewed by the World Health Organization (3). Ochratoxin A is produced by a single Penicillium species (P. verrucosum), by Aspergillus alutaceus (formerly A. ochraceus), and by A. carbonarius. P. verrucosum is the main source of OTA contamination of foods and feeds in temperate climates while Aspergillus spp. predominate in warmer climates (3).

The natural occurrence of OTA in plant products was first reported in 1969 in a corn sample that contained approximately 150 µg/kg OTA (4). Subsequent surveys established that OTA occurs in cereal grains, coffee beans, and other products in several areas of the world (5). OTA was first reported in green coffee beans by Levy et al. (6) and subsequent studies have also revealed the presence of OTA in roasted (7) and soluble coffee (8).

Six million tonnes of coffee are produced annually in about 70 countries, being Colombia the second largest producer after Brazil. Around 80% of coffee is exported to industrialized countries, which normally require that OTA levels are tested and garanteed to be below certain level; the standard for OTA in coffee is normally around a maximum content of 3-5 µg/kg, depending upon the country (9). The analysis of OTA in green and soluble coffee (the two forms in which Colombian coffee is exported) is a required quality control analysis for coffee and therefore appropriate validated analytical methods are necessary.

Several chromatographic methods for the determination of ochratoxin A in green and/or soluble coffee have been published (8, 10, 11, 12). However, the more recent trend is towards the use of methods based on immunoaffinity cleanup of the coffee extracts combined with high-performance liquid chromatography (HPLC) for the determination and quantification of the analyte. The aim of the present study was to validate a method for the determination of ochratoxin A in green and soluble coffee, using an immunoaffinity column for cleanup and HPLC.

Materials and methods

Standard solutions

Stock standard solutions of OTA (approximately 10 µg ml^{-1}) were prepared by dissolving a crystalline standard (Sigma Chemical Co., St. Louis, MO) in toluene-acetic acid (99:1 v/v). The exact concentration was determined spectrophotometrically according to AOAC International (13). A standard solution of

OTA (1.0 μg ml^{-1}) in methanol-acetic acid (99:1 v/v) was prepared for spiking purposes. Standard solutions ranging from 1.0 to 20.0 ng ml^{-1} were prepared in mobile phase: acetonitrile-methanol-water-glacial acetic acid (35:35:29:1 v/v/v/v) for calibration of the HPLC (12).

Immunoaffinity column

Contains antibodies against OTA with a total binding capacity of ≥100 ng OTA and a recovery of ≥85% when a diluted solution containing 100 ng OTA is applied onto the column (Ochraprep®, R-Biopharm Rhone Ltd., Glasgow, Scotland).

Samples and sample preparation

Before conducting the validation study, two different extraction solvent systems were evaluated using spiked green and soluble coffee samples at a level of 10 μg/kg. The two solvent systems evaluated were 1% aqueous sodium bicarbonate (w/v) (R-Biopharm Rhone protocol for green coffee) and methanol-3% aqueous sodium bicarbonate (50:50 v/v) (8). No significant differences ($P<0.05$) in recovery rates were found between the two solvents, and the simpler solvent (1% aqueous sodium bicarbonate) was selected for the validation study.

Blank (<0.2 μg/kg OTA) and spiked samples of ground green coffee beans (10 g) or soluble coffee (5 g) were extracted with 100 ml 1% aqueous sodium bicarbonate (w/v). Extraction was carried out during 2 minutes in a 200 ml glass blender jar using a blender at high speed. Extracts of soluble coffee samples were then filtered using high speed qualitative paper. Extracts of green coffee samples were first centrifuged at 5,000 rpm for 10 minutes, prior to filtration through high speed qualitative paper. This centrifugation step helps clarify the sample and makes it easier for the green coffee extract to flow through the immunoaffinity column. After filtration, 20 ml of each of the processed extracts were diluted with another 20 ml of phosphate buffer saline (PBS) solution.

In addition to the samples processed for the validation study, 42 samples of green coffee and five of soluble coffee, collected from different local coffee traders, were analysed using the validated method. Also, an interlaboratory green coffee sample from FAPAS® (Food Analysis Performance Assesment Scheme) was analyzed using the validated method (green coffee test material number 1733 127).

Immunoaffinnity clean-up

The diluted extract was transferred to the immunoaffinity column and allowed to pass through the column at a flow rate of 2-3 ml min^{-1}. For this step, a plastic 50 ml syringe barrel was attached to the top of the column, which in turn was attached to a vacuum manifold (12-port Alltech vacuum manifold, Alltech Associates, Inc., Deerfield, IL, USA). After washing the column with 20 ml HPLC water, the column was dried using vacuum. OTA was then eluted into a silanized conical amber vial with 4 ml HPLC grade methanol. Silanization was conducted by submersing the vials overnight in a 5% solution of dichlorodimethyl silane in isooctane; after silanization the vials were rinsed twice with methanol and allow to air dry. During the elution, backflushing (or reversing the direction of the flow) was done 3 times in order to ensure the complete elution of the OTA. The dried residue was then dissolved with 1 ml mobile phase, filtered through a 0.45 μm membrane filter, and 50 μl injected into the LC.

Liquid chromatography

Chromatographic determinations were performed with a Perkin-Elmer LC-410 pump equipped with a Rheodyne manual injector (50 µl loop) and a Perkin-Elmer 650-15 fluorescence detector (λ_{ex} = 330 nm; λ_{em} = 475 nm). Data was analysed with a Shimadzu C-R4A chromatopack system. Separation was carried out using a reversed-phase Prodigy ODS, 5 µm, 250 x 4.6 mm column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of an isocratic mixture of acetonitrile-methanol-water-glacial acetic acid (35:35:29:1 v/v/v/v) (12).

Determination of the single laboratory validation parameters

The characteristics of the method, including selectivity, accuracy, precision, linearity, limit of detection, limit of quantitation, and ruggedness were evaluated as follows.
Selectivity (the degree to which the method can quantify the target analyte in the presence of potentially interfering materials, other analytes, or matrices) was determined by measuring the resolution (Rs) of the analyte peak from interfering peaks. A resolution of at least 1.5 minutes is usually sought and one of 1.0 is the minimum usable separation (14). Accuracy (trueness) was assessed by means of recovery tests with spiked samples of green and soluble coffee, at two levels of contamination (1 and 20 µg/kg) in triplicate. Precision was determined as "intermediate precision", known as such because its value lies between within-laboratory and among-laboratory precision (15). This parameter was determined by performing two measurements (1 and 20 µg/kg) by two different analysts at two different days each, in duplicate. The time period between each analysis was 7 days.
Linearity was determined for standard solutions and for spiked extracts of soluble and green coffee which did not contain detectable levels of ochratoxin A. The linearity of the standard calibration curve was determined by injecting five different levels of OTA in triplicate (0.05, 0.25, 0.5, 0.75 and 1.0 ng on column). The linearity of the method for green and soluble coffee was carried out by triplicate analysis of blank extracts spiked at five different levels: 1, 5, 10, 15 and 20 µg/kg. The calibration line was fit with the help of an statistical program and the residuals (the difference of the experimental points from the fitted line) were plotted as a function of concentration. An acceptable fit produces a random pattern of residuals with a 0 mean. Limit of detection and limit of quantitation were determined as the mean blank response plus 3 and 10 standard deviations, respectively (16).
Ruggedness was investigated by means of the Youden Ruggedness Trial (17), in which 7 factors are explored in a experiment requiring 8 determinations. The factors evaluated for soluble coffee were extraction (blender for 2 minutes vs. orbital shaker for 60 minutes), storage of column (refrigerator vs. 7 days at room temperature), methanol elution volume (4 ml vs. 2 ml), silanization of the vial (yes vs. no), mobile phase flow rate (0.8 ml min^{-1} vs. 1.0 ml min^{-1}), analytical column temperature (room temperature vs. 30°C), and percent acetic acid in mobile phase (1.0% vs. 0.5%). The factors evaluated for green coffee were the same as for soluble coffee, except that the parameter mobile phase flow rate was replaced by centrifugation of the sample prior to dilution (yes vs. no).

Statistical analysis

All variables were subjected to one-way analysis of variance using SAS. When only two means were compared the t-test procedure was used. Statements of significance are based on a probability of $P<0.05$.

Results and discussion

The preliminary evaluation conducted to determine the best extraction solvent system for both green and soluble coffee samples indicated no significant differences ($P<0.05$) in the recovery rates of the two solvent systems tested. The mean recovery rate from spiked soluble coffee samples was 82.9% with 3% bicarbonate-metanol (50:50 v/v) solution, and 82.2%, using aqueous 1% bicarbonate alone. The mean recovery rate from spiked green coffee samples with 3% bicarbonate-metanol (50:50 v/v) was 81.5%, while with 1% bicarbonate was 72.9%. The method was validated using as extraction solvent 1% bicarbonate.

The method was found to be specific for ochratoxin A for both green and soluble coffee samples. No interfering peaks appeared within 1.5 minutes of the retention time of the analyte (Figure 1).

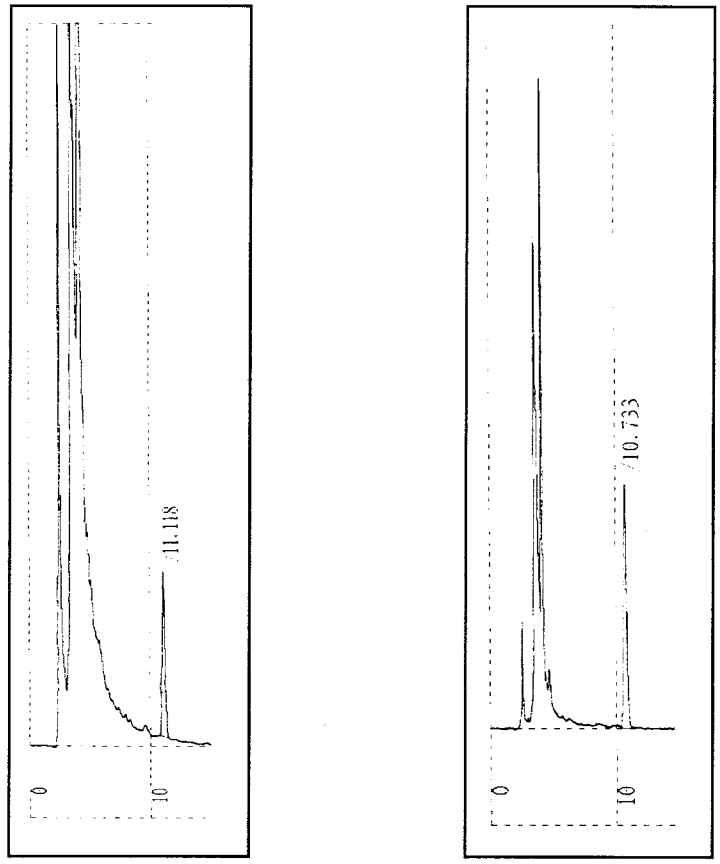

Figure 1. Chromatograms of spiked soluble and green coffee samples (10 µg/kg) showing no infering peaks at ±1.5 min from the analyte peak.

These results were expected since high selectivity is usually achieved when monoclonal antibodies raised against the analyte are used for purification of the analyte from its matrix.

The linearity of the standard calibration curve (r^2) when using neat standards was

>0.999 (y = 7642.7x + 610) for OTA amounts of 0.05, 0.25, 0.5, 0.75 and 1.0 ng on column. Residuals of the plot correspondend to a random pattern with a mean <0.00. The calibration curve was also shown to be linear when spiked soluble coffee extracts were used as standards. The linearity (r^2) was >0.998 (y = 7640.3x + 1922) for OTA concentrations equivalent to 1 to 20 µg/kg. Residuals of this calibration curve again corresponded to a random pattern with a mean <0.00. The linearity (r^2) of the calibration curve for spiked soluble coffee extracts was >0.996 (y = 7394.0x + 1695) for OTA concentrations equivalent to 1 to 20 µg/kg. Residuals of this calibration curve also had a random pattern with a mean <0.00. The results of these measurements show that the method is linear for a range of analyte concentrations of 1 to 20 µg/kg. No significant differences (P<0.05) were found among the calibration curves which indicates that there is no effect of the matrix on the linearity of the response.
The results of the accuracy tests are shown in Table 1.

Table 1. Recoveries from soluble and green coffee extracts spiked at two levels.

Spiking level	Recovery ± SD[a] (%)	RSD (%)
Soluble coffee		
1.0 µg/kg	91.2 ± 5.4	5.8
20.0 µg/kg	73.5 ± 4.8	6.6
Green coffee		
1.0 µg/kg	84.5 ± 7.8	9.4
20.0 µg/kg	68.7 ± 2.6	3.8

[a]Standard deviation (n = 4).
[b]RSD, relative SD.

Higher recovery rates were found for the low level of contamination tested compared with the high level, both for soluble and green coffee. Also, greater recovery rates were obtained for soluble than for green coffee. The recovery limits commonly accepted for a level of contamination around 10 µg/kg are 70-125% (14). Three of the recovery rates obtained in the present study were between these two limits; however, the recovery rate for 20 µg/kg OTA from green coffee was slightly lower than 70% (68.7%). Horwitz (14) recommends that recoveries less than 60-70% be subject to investigations leading to improvement. Therefore, the method needs to be improved in order to obtain greater recovery rates for OTA from green coffee. Recovery rates for both soluble and green coffee samples were found to differ significantly from 100% (P<0.05); therefore, any analytical result obtained using the present methodology needs to be corrected for recovery.
The intermediate precision data of the method is shown in Table 2.

Table 2. Intermediate precision for the analysis of soluble and green coffee extracts spiked at two levels (two analysts, two different days, each)

Spiking level	Mean ± SD[a] (µg/kg)	RSDr[b] (%)	Calculated[c] RSDr (%)	HORRAT ratio
Soluble coffee				
1.0 µg/kg	0.88 ± 0.08	9.4	22.6	0.42
20.0 µg/kg	14.85 ± 1.35	9.1	14.4	0.63
Green coffee				
1.0 µg/kg	0.72 ± 0.10	14.3	22.6	0.63
20.0 µg/kg	12.67 ± 1.97	15.6	14.4	1.08

[a]Standard deviation (n = 2).
[b]RSD, relative SD for repeatability.
[c]Calculated RSDr = $C^{-0.1505}$ (C = concentration).

Repeatability values were found to be lower for soluble coffee than for green coffee. The repeatability standard deviation varies with concentration and its value can be calculated using the formula RSDr = $C^{-0.1505}$ (18). Calculated RSDr values for the two concentrations tested (1 and 20 µg/kg) are 22.6% and 14.4% , respectively. Acceptable values for repeatability are between 0.5 and 2.0 times the calculated values. Alternatively a ratio can be calculated between the RSDr found and the RSDr calculated. This parameter is known as the HORRAT ratio (HORRATr) and acceptable values are typically 0.5 to 2.0. The HORRATr values determined ranged from 0.42 to 1.08 and are therefore approximately within acceptable levels.

The limit of detection was determined to be 0.01 ng of OTA on column, while the limit of quantitation was found to be 0.03 ng on column. The limit of quantitation is equivalent to 0.6 µg/kg in soluble coffee samples and 0.3 µg/kg in green coffee samples.

The results of the ruggedness trial showed that two factors are of critical importance in soluble coffee analysis: the extraction method (blender/shaker), and the flow rate of the mobile phase. For green coffee analysis also two critical factors were found: the extraction method and the storage of the immunoaffinity column prior to analysis. These results indicate that the extraction method affects the outcome of the analysis, being extraction with blender superior to extraction using a shaker. Also, the immunoaffinity columns need to be stored according to the manufacturer's recommendation (at around 4°C). This factor can be particularly critical during shipping of the columns from the manufacturer or dealer to its final destination (laboratories). Finally care must be taken in order to avoid fluctuations in the mobile phase rate of the chromatographic pump.

The results of the OTA determination performed to the 42 green coffee and 5 soluble coffee samples are summarized in Table 3.

Table 3. Natural occurrence of ochratoxin A in soluble and green coffee samples analysed using the method validated in the present study.

Type of coffee	Number of samples analysed	Number of positive samples[a]	Mean (µg/kg)	Minimum-maximum (µg/kg)
Soluble coffee	5	5	6.8	0.9 - 19.4
Green coffee	42	6	10.0	8.4 - 13.9

[a]Limits of quantitation: soluble coffee, 0.6 µg/kg; green coffee, 0.3 µg/kg.

All soluble coffee samples contained detectable levels of OTA, which ranged from 8.4 to 13.9 µg/kg, with a mean of 6.8 µg/kg. Six of the 42 green coffee samples analysed had detectable levels of OTA ranging from 0.9 to 19.4, with a mean of 10.0 µg/kg. The level of OTA detected in all soluble coffee samples and in 5 of the 6 positive green coffee samples is above the 5 µg/kg limit accepted for human foods in most countries (19). This finding indicates that surveillance on the OTA levels of Colombian coffee for local consumption is required. Further studies are required in order to evaluate the occurrence of ochratoxin A in Colombia coffee.
The interlaboratory sample from FAPAS® (green coffee test material number 1733 127) was found to contain 9.82 ng/g OTA when it was analyzed using the validated method. The assigned value for this material was 9.6 ng/g OTA, and the z–score obtained was 0.1. These figures indicate the goodness of fit of the validated method.

In conclusion, the method validated in the present study is applicable to soluble and green coffee samples. Linearity was demonstrated for contamination levels between 1.0 and 20.0 µg/kg. The validation parameters measured are within acceptable limits. The method has the advantage of using only an aqueous bicarbonate solution for extraction, in contrast to the methods that use a mixture of methanol and water. The detection limit of the method (<1.0 µg/kg for both soluble and green coffee samples) is sufficiently low for surveillance purposes. The method can be used to determine OTA content in soluble and green coffee samples.

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