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DETECTION OF OCHRATOXIN A IN INSTANT COFFEE BY UPLC-MS/MS

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ABSTRACT. A rapid, accurate and economic procedure has been applied to extract and analyze trace level of ochratoxin A (OTA) in instant coffee. The OTA was extracted and cleaned-up by methanol extraction followed by retention on IRA-400 anion exchanger and quantified by Ultra Performance Liquid Chromatography (UPLC) with MS/MS detection. Under optimum conditions, the detection limit (S/N = 3) was 0.05 ng/g and recoveries of OTA at spiking levels of 5, 10, and 20 ng/mL, were 82.67 ± 8.05%, 96.87 ± 4.63%, and 98.25 ± 4.46% respectively.

Keywords: *ochratoxin A; mycotoxin; instant coffee; contamination; UPLC-MS.*

INTRODUCTION

Ochratoxin A (OTA) is one of the most dangerous secondary metabolite from out of more than 300 mycotoxins that have been isolated and described. OTA is a substituted isocumarin derivative of phenylalanine and is produced in temperate climates especially by species of *Penicillium* and in tropical and subtropical regions mostly be *Aspergillus* species [1,2]. This mycotoxin contaminate a large variety of food and feed products being a major concern for the international trade. Studies made in different countries from all over the world have pointed out the presence of OTA in numerous products with vegetal origin: peanuts, cereals, coffee, beer, wine and fruit juice [3-10]. OTA was reported to be present in dangerous levels in animal products like milk [11], pork and poultry meat [12-13].

The international legal bodies have established very low limits of permitted levels of OTA in products designated to human consumption due to the fact that a lot of studies have shown OTA to be a teratogenic, carcinogen and a foetotoxic

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agent [14-15]. Also, OTA was advocated to be nephrotoxic, being associated with Balkan endemic nephropaty [14-16]. The International Agency of Research on Cancer (IARC) classified OTA as a possible carcinogen for humans - 2B group [17], based on sufficient evidence in animals and for kidney carcinogenicity of OTA but inadequate evidence in humans. The presence of OTA was signaled in human milk [18] and in human blood [19].

Many countries have established maximum levels for OTA in cereals and other products but only a few have rules for coffee and coffee derived products despite the extensive studies regarding the presence of OTA in green, roasted and instant coffee [6, 20-25].

The European Union has established the highest permitted level of OTA in roasted coffee beans at 5 ng/g and in instant coffee at 10 ng/g [26]. The European Commission had established a Tolerable Weekly Intake (TWI) for OTA at 120 ng/kg b.w./week [27].

RESULTS AND DISCUSSION

Sample extraction and purification

The first step in optimization was to select an appropriate extraction solvent. The suitability of methanol, chloroform, acetone, cold and hot water was tested. The results (see Figure 1) showed that the highest extraction efficiency was obtained with methanol.

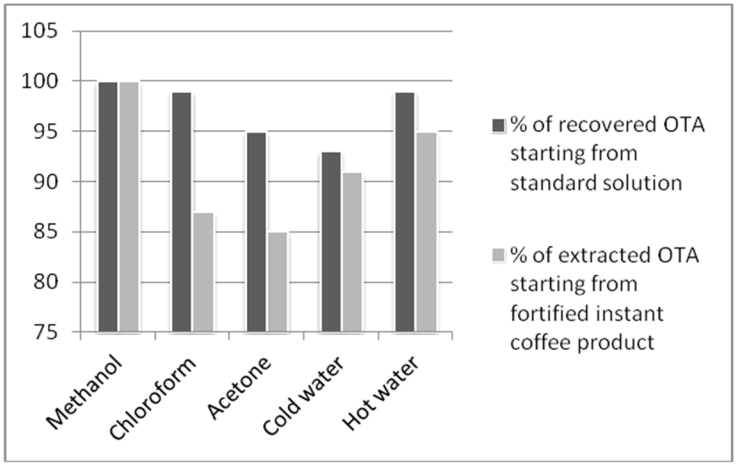

Figure 1. The influence upon the yield of extraction of OTA following the same extraction protocol except for the nature of the extraction solvent (the recovery values for each solvent are normalized, considering the best yield 100%).

The influence of the matrix upon the yield of the extraction of OTA is also presented in Figure 1. There were compared the results obtained when a solution of 20 ng/mL OTA prepared in the extraction solvent was subjected to the extraction procedure with those obtained when 2.5 g of instant coffee product free of OTA was subjected to the extraction procedure starting with 20 mL extraction solvent containing 20 ng/mL OTA.

Qualitative analysis

The analytical characteristics of the UPLC-MS/MS method including the calibration curve, repeatability, limits of detection and quantitation were investigated under the optimized conditions.

Calibration curve was prepared for the target analyte after the extraction of a standard series of spiked fresh instant coffee samples with the regression equation being y = 402.773x + 975.965 and the determination coefficient of 0.999. The precision of the proposed method was calculated by 3 replicated extractions and analysis using 3 levels of fortification of spiked sample (5, 10 and 20 ng OTA/g coffee), and the relative standard deviations (RDSs) of OTA were between 4 and 8 % (see Table 1). The limits of detection (LOD, S/N = 3) and quantitation (LOQ, S/N = 10) for the processed spiked fresh instant coffee were 0.05 and 0.2 ng/g, respectively.

Validity of the method

To assess the applicability of the proposed method, commercial instant coffee samples were obtained and analysed; the extraction procedure followed by separation with UPLC-electrospray tandem MS being applied.

Table 1. Recovery of OTA from spiked instant coffee samples.

Concentration Added (ng/g)	Concentration Detected (ng/g)	Mean (ng/g)	Recovery (%)	Relative Standard Deviation (%)
5.00	4.51	4.17	82.67	8.05
	4.12			
	3.88			
10.00	10.8	9.69	96.87	4.63
	9.21			
	9.75			
20.00	19.01	19.65	98.25	4.46
	20.65			
	19.29			

Confirmation

The results showed that the presence of OTA was confirmed in over 90% of the analyzed samples of instant coffee, always being below the European legal limit. The identity of OTA in samples was confirmed by the fragmentation patterns obtained in MS2 spectra of the key ion at *m/z* 239 Da. These patterns were similar to those found in the MS2 spectra of the standard of OTA (see Figure 2).

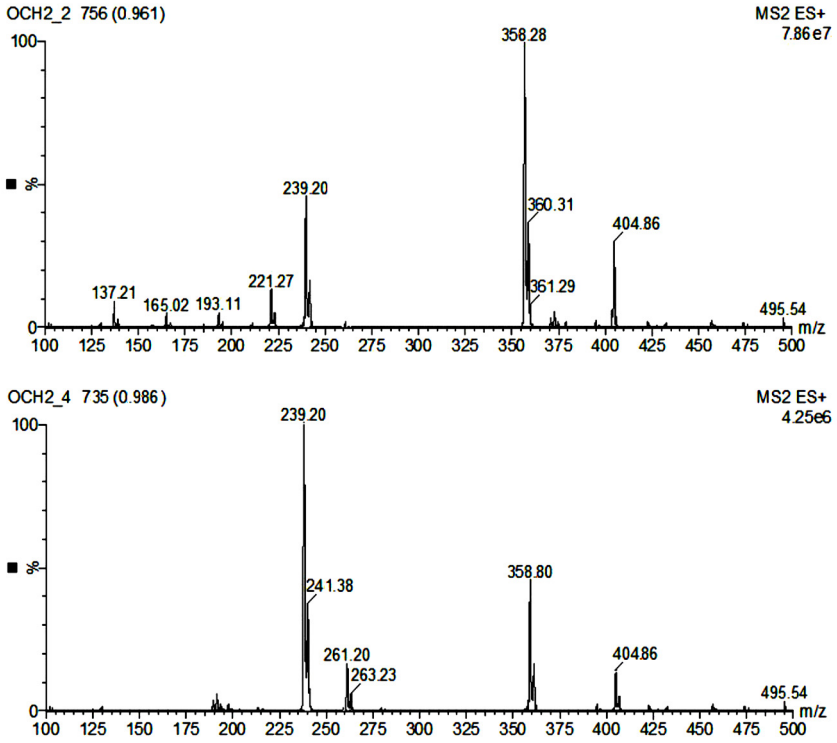

Figure 2. The MS2 spectra of an OTA standard solution obtained at 15V collision energy (upper panel) and used for quantification (404.8> 358.7 m/z) and at 23 V (lower panel) used for confirmation (404.8> 239.6 m/z) of the presence of the analyte in the sample

In Figure 3 there are presented the two chromatograms of the same instant coffee product, subjected to the sample preparation procedure, but the sample corresponding to the chromatogram B was fortified with 10 ng OTA/g instant coffee product before the sample preparation. For comparison, in the lower panel there is presented the chromatogram of a standard solution of OTA (100 ng/mL).

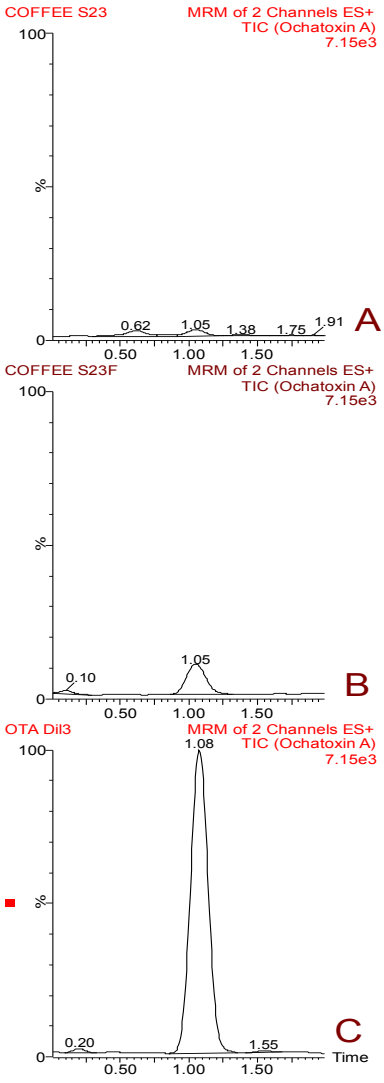

Figure 3. UPLC chromatograms (A) instant coffee sample naturally contaminated with 2.5 ng OTA/g product, injection 10 µL, (B) the same instant coffee sample, fortified with 10 ng OTA/g product before starting the sample preparation procedure, injection 10 µL, (C) a standard solution containing 100 ng/mL of OTA

CONCLUSIONS

This study reports the successful analysis of OTA in instant coffee samples based on UPLC-MS/MS technique. The presented method offers suitable features of merit such as low detection limit (0.05 ng OTA/g product), good recovery (over 82%), and relative standard deviation lower than 8%, together with other advantages such as rapidity (less than 15 min), low cost of analysis and minimal use of organic solvents.

EXPERIMENTAL SECTION

Sample collection

Instant coffee products were processed in 24 h after purchase. Quantities of 10 g of samples were grounded, mixed and sub-sampled prior to analysis.

Reagents

The OTA standard was purchased from Sigma (Redox Lab Supplies Com S.R.L. Bucharest, Romania). A stock solution of 0.5 mg/mL OTA was prepared in methanol and stored at -20 ºC and protected from light. Methanol, formic acid and Amberlite® IRA-400 chloride form were also purchased from Sigma-Aldrich.

Working solutions (in the range 0.2 – 200 ng/mL) for the recovery tests and calibration curve were prepared by appropriate dilution of the OTA stock solution in 0.1% formic acid in 30% methanol.

Sample preparation

In 20 mL methanol was added 2.5 g instant coffee product and vortexed for 3 min at 2.500 rpm. For OTA extraction other solvents, like chloroform, acetone, cold and hot (boiling) water were also tested. The samples were centrifuged for 5 min at 5.500 rpm (Rotofix 32A, Hettich). From the supernatant were collected 16 mL (equivalent to 2 g of initial coffee product) and mixed with an equal quantity of 1 mM $NaHCO_3$. This solution was percolated on a small column filled with 0.5 mL Amberlite IRA-400 (previously washed with 5 mL methanol and further with 10 mL of 1 mM $NaHCO_3$). The column was washed with 5 mL of 1 mM $NaHCO_3$. The analyte was eluted with 0.5 mL methanol (containing 0.1% HCOOH), followed by 0.5 mL of 0.1% HCOOH. From the eluate, 10 µL were injected in UPLC system after filtration on 0.2 µm membrane filter.

UPLC analysis

The chromatographic analysis was carried out on a Waters Acquity UPLC-MS system (Binary Solvent Manager, Xevo TQD MS-detector equipped with an electrospray ionization interface) with a UPLC BEH C18, 1.7 µm (2.1×100 mm) column, using a gradient elution procedure. Mobile phase A consisted in 0.02% formic acid in 5% methanol and mobile phase B

was 0.02% formic acid in methanol. The gradient profile was: 0 – 0.2 min, 30% A and 70% B; 0.2 – 1 min, linearly increase until 100% B; 1 – 1.5 min, hold 100% B; 1.5 – 1.6 min, linearly decrease until 70% B (initial condition). The column temperature was set at 30°C. The analyses were run at a flow rate of 0.3 mL/min, and the sample volume injected was 10 µL.

Calibration

To prepare the calibration curve, appropriate volumes of OTA stock solutions were diluted with 0.1% formic acid in 30% methanol to prepare a series of working solutions containing 0.02–500 ng/mL. A calibration curve was constructed by plotting the peak area for each standard against the mass of OTA injected. Slope and intercept data of the calibration curve were used to compute the quantity of the analyte in coffee extracts.

For quantitative determination of OTA peak areas of the sample, chromatograms were correlated with the concentrations according the calibration curve. Standard solutions and sample volumes of 10 µL were injected in triplicate. The linearity was determined in the range of 0.2-200 ng/mL of OTA using 7 calibrators. The retention time was 1.08 min. An OTA standard solution at 10 ng/mL was daily injected at the beginning of the analysis.

Validation

The coffee samples, spiked with various concentrations of standard solutions, were analyzed. Limits of detection were based on a signal to noise (S/N) ratio with 3:1 as the minimum. Recovery tests were performed in triplicate by spiking standards at 3 different levels into OTA-free samples: 5, 10, and 20 ng/mL in coffee. The spiked samples and blank samples without standard were analyzed by UPLC.

Confirmation and quantification

The ESI parameters for Xevo TQD MS detector were fixed as follows: capillary voltage at 3 kV, source temperature at 150°C, desolvation temperature at 300°C, and desolvation gas at 500 L/h. Nitrogen was used as the desolvation gas, and argon was employed as the collision gas. The detailed MS/MS detection parameters for the analyte are presented in Figure 4 and Table 2 and were optimized by direct injection of a 1 µg/mL OTA standard solution into the detector at a flow rate of 10 µL/min.

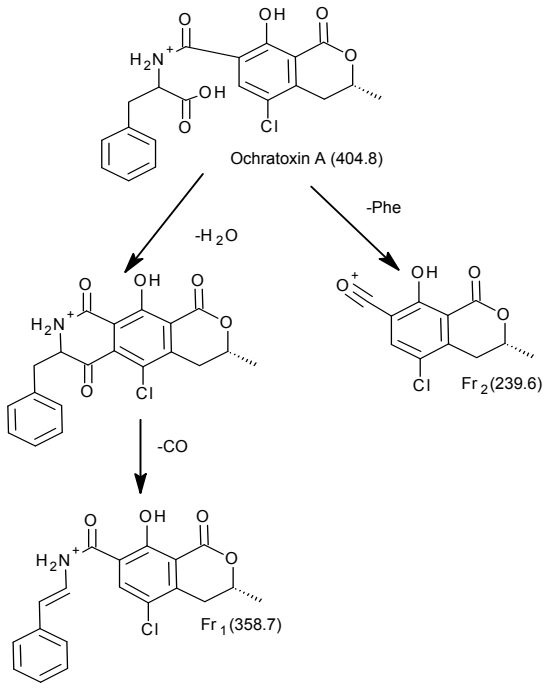

Figure 4.
Fragmentation pattern of ochratoxin A, obtained at cone voltage 30 V and collision energy 15 V, for daughter fragment with m/z 358 and 23 V for the fragment with m/z 239

Table 2. Mass spectrometer parameters for OTA detection.

Transition monitored	Precursor ion (*m/z*)	Fragment ion (*m/z*)	Cone voltage (V)	Collision voltage (V)
Quantification	404.8	358.7	30	15
Confirmation	404.8	239.6	30	23

Analyses were carried out in multiple reactions monitoring mode (MRM), using two specific transitions for the analyte and the detector was fixed at maximum Extended Dynamic Range with peak mass widths of 2 and 1.5 Da for the first and third quadrupole, respectively. The dwell time for all transitions was 0.01 seconds.

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