Brazilian Journal of Microbiology (2007) 38:300-303
ISSN 1517-8382

OCHRATOXIN A IN BRAZILIAN INSTANT COFFEE

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Submitted: May 15, 2006; Returned to authors for corrections: August 03, 2006; Approved: September 18, 2006

ABSTRACT

The aim of this study was to determine the ochratoxin A (OTA) contamination of instant coffee samples collected in the market of the city of São Paulo, Brazil from August to December, 2004. The EN 14133/2003 method, originally developed to quantify OTA in wine, grape juice and beer samples, was evaluated and approved for analyzing OTA in instant coffee samples. OTA was isolated in an immunoaffinity column and quantified by HPLC with fluorescence detection. The established detection and quantification limits were 0.16 and 0.52 ng/g, respectively. The recoveries from spiked samples were 92.6 ± 1.7, 83.7 ± 0.8, and 91.0 ± 1.2 % at levels of 3.0, 5.0, and 8.0 ng/g, respectively. Of a total of 82 samples analised, 81 (98.8%) contained OTA at levels ranging from 0.17 to 6.29 ng/g. The high frequency of OTA occurrence in the instant coffee samples demonstrates the importance of an effective control of this product by governmental authorities and industries. The rapid methodology for OTA analysis in instant coffee used in this study was defined and validated, permitting it´s use for quality control of this product.

Key-words: ochratoxin A, instant coffee, immunoaffinity column, HPLC determination

INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic and nephrocarcinogenic mycotoxin produced by *Penicillium verrucosum* in temperate climates of Europe and North America and by species of *Aspergillus* in warmer climates such as South America and Africa (12).

OTA has received considerable attention since 1993, when the International Agency for Research on Cancer (5) classified it as a possible carcinogen for humans - 2B group. Several studies have shown OTA to be a potent nephrotoxin, immune suppressant, teratogen, carcinogen, as well as a potent foetotoxic agent (5). It may be also associated with Balkan Endemic Nephropathy, a chronic kidney disease, and the development of urinary tract tumors in humans (18).

OTA has been reported to occur in a wide variety of foods and beverages, e.g. pork and poultry meat (6), wheat, barley, oats, rye (20), spices (10), beer (15), maize products (16), wine and grape juice (21) and green, roasted and instant coffee (7,8,10,12-14,20).

The presence of OTA in human blood has been suggested as a contamination risk indicator. The results of analyses of human serum samples have demonstrated wide and continued OTA exposure through the ingestion of contaminated foods (9).

Several countries have established maximum limits for OTA in cereals and other products; however, only a few have legislation for coffee grains. Indonesia does not permit any quantity of OTA in cereals and grains (3). EC have established regulations for OTA in roasted coffee beans (5 µg/kg) and instant coffee (10 µg/kg) (European proposals). In Brazil, there is no specific legislation for this mycotoxin.

This paper reports a survey on the levels of OTA in instant coffee marketed in the city of São Paulo, Brazil, using immunoaffinity column (IAC) and high performance liquid chromatography (HPLC) methods.

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MATERIALS AND METHODS

Instant coffee samples

Eighty-two instant coffee samples (22 different brands, constituted by 3 or 4 lots per brand) representing a range of products were purchased from supermarkets in the city of São Paulo, Brazil from September to December 2004. A sample size of 100 g was ground, mixed and sub-sampled prior to analysis.

Standard and Reagents

OTA standard was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were of HPLC grade from Merck (Merck, Darmstadt, Germany). Glacial acetic acid, sodium chloride and sodium hydrogen carbonate were purchased from Merck and polyethylene glycol (PEG 8000) from Oxiteno (Oxiteno, São Paulo, Brasil). OTA stock solution was prepared in toluene-acetic acid (99:1). The concentration was determined according to AOAC (1), checked with UV spectrophotometry in 333 nm, using ε = 5440 $m^2 mol^{-1}$. Working solution for the recovery tests and calibration curve was prepared by appropriate dilution of the OTA stock solution in toluene-acetic acid (99:1). The solutions were stored at -15ºC to -20ºC and protected from light.

HPLC Method

1. Extraction

The European Standard EN 14133 (19) method, originally applied to OTA quantification in wine, grape juice and beer samples, was tested for instant coffee samples. An aliquot of 2 g was added to 40 mL of water and 40 mL of a diluting solution (10 g polyethylene glycol and 50 g sodium hydrogen carbonate dissolved in approximately 950 mL water and volume adjusted to 1000 mL with water) and mixed vigorously for 5 minutes. The solution was filtered through a glass microfibre filter and 40 mL of this filtrate was passed through a Ochraprep column (Rhône Diagnostics Tecnologies Ltd., Glasgow UK). The column was washed with 10 mL of the washing solution (25 g sodium chloride and 5 g sodium hydrogen carbonate dissolved in approximately 950 mL water and volume adjusted to 1000 mL with water) and with 10 mL of water and dried by passing air through it. OTA was slowly eluted from the column with 2 mL of methanol. The eluate was brought to dryness under a gentle stream of N_2.

The residue was dissolved in 300 µL HPLC mobile phase and filtered through a 0.45 µm Millex-HV filter units (Millipore Corporation, Bedford, MA, USA) before chromatographic analysis. Despite of the high specificity of the immunoaffinity column, the identity of OTA was confirmed by the preparation of the methyl ester derivative according to AOAC (1).

2. Chromatographic conditions

The determination of OTA was carried out in a GBC LC 1110 high performance liquid chromatography (HPLC) system equiped with a pumping system (GBC Pump LC 1110, Dandenong, Victoria, Australia) and a fluorescence detector (GBC LC 1255, Dandenong, Victoria, Australia). Liquid chromatography was performed on a LiChrosorb C_{18} (Merck, Darmstadt, Germany) 5 µm particle size 25 cm x 10 mm column, operated at 0.5 mL/min with 3.33% acetic acid-acetonitrile-methanol (30:35:35) as mobile phase. The excitation wavelength of the fluorescence detector was set at 332 nm and the emission wavelength was 476 nm.

3. Quantification

To prepare a calibration curve, appropriate volumes of the OTA stock solution were diluted with 3.33% acetic acid-acetonitrile-methanol (30:35:35). For quantitative determination of OTA peak areas of the sample, chromatograms were correlated with the concentrations according the calibration curve. Standard solution and sample volumes of 20 µL were injected in triplicate. The linearity was determined in the range of 2- 256 ng/mL of OTA using 8 calibrators. The retention time was 6.5 ± 0.2 min. A OTA standard solution at 10 ng/mL was daily injected since the beginning of the analysis.

4. Validation

Recovery studies were performed by adding known concentration of OTA standards (3.0, 5.0 and 8.0 ng/g) to a noncontaminated sample before the extraction and purification steps. The detection and quantification limits were established as three and ten times, respectively, the standard deviation obtained from the analysis of the blank instant coffee extract (n = 10) at the retention time of OTA.

RESULTS AND DISCUSSION

The mean recovery percentages of the method established for determination of OTA were 92.6, 83.7 and 91.7%, with coefficients of variation of 1.7, 0.8 and 1.2%, for concentrations of 3.0, 5.0 and 8.0 ng/g respectively (Table 1). Therefore the limit of detection was 0.16 ng/g. The limit of quantification was 0.52 ng/g, which is the minimum level at which the analyte can be quantified with acceptable accuracy and precision (4).

The method, originally developed for quantification of OTA in wine, grape juice and beer samples (19), has proven to be adequate for instant coffee as well, presenting. high selectivity and good precision (Fig. 1).

As shown in Fig. 1 the extraction step was effective and the clean-up with IAC was suitable, without co-extractive interferences on OTA retention time during chromatographic analysis. The clean-up with IAC is an essential step for optimal recovery of ochratoxin A (Table 1), besides decreasing the experimental time when compared to traditional liquid-liquid partition.

Eighty one (98.8%) out of 82 samples were contaminated with OTA, at concentrations ranging from 0.17 to 6.29 ng/g,

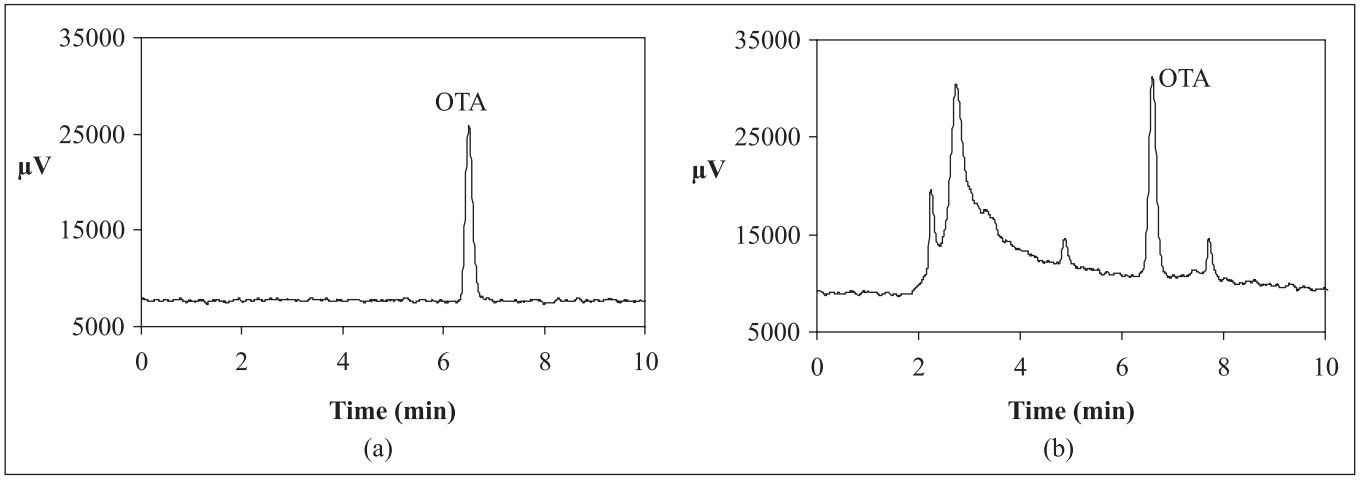

Figure 1. HPLC chromatograms – (a) a standard solution containing 2.4 ng/g of OTA. (b) a instant coffee sample naturally contaminated with 3 ng/g of OTA.

with an average of 1.24 ng/g (Table 2). These results are similar to those reported in countries where there are legal limits for this mycotoxin (3). However, in two samples of instant coffee from Parana state, the levels of OTA were above or equal to the legal limits in Italy, Cuba, Singapore and Indonesia (3).

The results did not indicate differences, in the levels of OTA contamination, in the 22 brands of instant coffee that were produced in the states of São Paulo and Minas Gerais (Southeast region), Paraná (South region) and Rio Grande do Norte (Northeast region). The highest levels of OTA contamination were found in samples from the state of Paraná (4.07 and 6.29 ng/g).

Pittet *et al.* (13) analysed 101 instant coffee samples from several countries and found OTA in 75 (74.3 %) samples, at concentrations ranging from 0.2 to 6.5 ng/g, with an average of 1.0 ng/g. Patel *et al.* (11) detected OTA in 64 of 80 samples, at concentrations ranging from 0.1 to 8.0 ng/g. In another study, carried out with 149 samples from several European countries, almost all were contaminated with OTA with concentrations ranging from non-detectable levels to 27.2 ng/g, with an average of 1.0 ng/g (17).

In Brazil, few studies regarding OTA occurrence in instant coffee are available. Prado *et al.* (14) analysed 37 samples (10 brands) collected in the city of Belo Horizonte, Minas Gerais, from October, 1998 to May, 1999 and found the presence of OTA in 31 (83.8%) samples, at concentrations in the range of 0.31 to 1.78 ng/g. Leoni et al. (7) analysed 16 samples marketed in Campinas, SP, and reported the presence of OTA in 100% of the samples, at levels of 0.5-5.1 ng/g.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has set a Provisional Tolerable Weekly Intake (PTWI)

Table 1. Recovery of Ochratoxin A from spiked instant coffee samples.

Concentration Added (ng/g)	Concentration Detected (ng/g)	Mean (ng/g)	Recovery (%)	Relative Standard Deviation (%)
	2.75			
3.00	2.75	2.78	92.6	1.7
	2.83			
	4.19			
5.00	4.22	4.19	83.7	0.8
	4.15			
	7.18			
8.00	7.29	7.28	91.0	1.2
	7.36			

Table 2. Positivity of OTA in 81 instant coffee samples.

Concentration range of OTA (ng/g)	Absolute frequency (number of positive samples)	Relative frequency (%)
< LOD*	1	1.2
0.16 – 0.50	13	16.0
0.51 – 1.00	20	24.7
1.01 – 1.50	22	27.2
1.51 – 2.00	18	22.2
2.01 – 2.50	3	3.7
2.51 – 5.00	3	3.7
5.01 – 10.00	1	1.2

*LOD = Limit of detection (0.16 ng/g).

for OTA of 14.3 ng/kg bw/day. Camargo et al. (2), analysing coffee consumption in the city of Campinas, SP, in 1993, found that 75% of the adults and children consume about five cups of coffee per day (10 g of instant coffee). Based on this information and considering that the mean value found in this study was 1.24 ng/g, coffee consumption contributes with 1.24% of the PTWI of OTA. When the maximum value (6.29 ng/g) is considered, coffee consumption may be responsible for 6.29% of the PTWI of OTA.

The occurrence of OTA in almost all instant coffee samples analysed in the present study demonstrates the importance of effective control by governmental authorities and industries, due to both the economic importance of this product in Brazil and the effect of over-consumption on human health. In Brazil, there are no legal limits established for ochratoxin contamination and there are few studies on the occurrence of OTA in foods and beverages. More research is necessary to evaluate the real exposure of the population to this mycotoxin through the ingestion of contaminated food.

RESUMO

Ocratoxina A em café solúvel brasileiro

O objetivo do presente estudo foi determinar a contaminação por OTA em amostras de café solúvel comercializadas na cidade de São Paulo, Brasil no período de agosto a dezembro de 2004. O método EN 14133/2003, originalmente desenvolvido para quantificar OTA em amostras de vinho, suco de uva e cerveja, foi avaliado e aprovado para análise de OTA em amostras de café solúvel. OTA foi isolada em coluna de imunoafinidade e quantificada por CLAE com detecção em fluorescência. Os limites de detecção e quantificação do método foram 0,16 e 0,52 ng/g, respectivamente. Os percentuais médios de recuperação foram de 92,6% (3 ng/g), 83,7% (5 ng/g) e 91,0% (8 ng/g), com coeficientes de variação de 1,7 (3 ng/g), 0,8 (5 ng/g) e 1,2 (8 ng/g). A análise das 82 amostras de café solúvel revelou a presença de ocratoxina A em 81 amostras (98,8%), com concentrações variando de 0,17 a 6,29 ng/g. A elevada ocorrência de OTA nas amostras analisadas indica a importância de um controle efetivo desse produto por parte das autoridades governamentais e das indústrias alimentícias. A metodologia rápida utilizada nesse estudo para análise de OTA em amostras de café solúvel foi definida e validada, podendo ser utilizada no controle de qualidade deste produto.

Palavras-chave: ocratoxina A, café solúvel, coluna de imunoafinidade, determinação por cromatografia líquida de alta eficiência

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