# Ochratoxin A in raisins samples marketed in São Paulo, Brazil

Ocratoxina A em amostras de uva passa comercializadas em São Paulo, Brasil

RIALA6/1084

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**ABSTRACT**
The present study aimed to evaluate a methodology for analyzing ochratoxin A (OTA) in raisins samples marketed in São Paulo, Brazil. OTA was extracted with methanol:water (80:20, v/v) and diluted with phosphate buffer solution, and purified through an immunoaffinity column. OTA was separated and quantified using HPLC with fluorescence detection. The established detection and quantification limits were 0.24 and 0.80 ng/g, respectively. The recoveries values were 81.6, 80.4 and 81.9%, and the relative standard deviations (RSD) were 6.0, 4.3, and 6.1 % at 2.0, 5.0 and 10.0 ng/g levels, respectively. Of twenty black raisin samples analyzed 10 (50 %) contained OTA at levels ranging from 1.3 to 39.1 ng/g. All of twenty-two white raisin samples showed no contamination with OTA.
**Key words.** ochratoxin A, OTA, raisins, HPLC, immunoaffinity column.

**RESUMO**
O presente estudo teve como objetivo avaliar uma metodologia para análise de ocratoxina A (OTA) em amostras de uva passa comercializadas em São Paulo, Brasil. A OTA foi extraída com metanol:água (80:20, v/v) e diluída com solução tampão fosfato, utilizando-se coluna de imunoafinidade para purificação da amostra. A OTA foi separada e quantificada por meio de CLAE com detector de fluorescência. Os limites de detecção e de quantificação estabelecidos foram respectivamente de 0,24 e 0,80 ng/g. Os valores de recuperação foram 81,6; 80,4 e 81,9% e os coeficientes de variação foram 6,0; 4,3 e 6,1 % respectivamente para os níveis de 2,0; 5,0 e 10,0 ng/g. De um total de vinte amostras de uvas passas pretas, 10 (50 %) continham OTA na faixa de concentrações de 1,3 a 39,1 ng/g. Todas as vinte e duas amostras de uvas passas brancas não apresentaram contaminação por OTA.
**Palavras-chave.** ocratoxina A, OTA, uva passa, CLAE, coluna de imunoafinidade.

## INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced mainly by *Penicillium verrucosum* and some species of *Aspergillus* and it has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic to animals[1-3]. The carcinogenicity in rats and mice is well established. OTA has been associated with a high incidence of a kidney disease known as Balkan Endemic Nephropathy and related to the development of urinary tract tumours in humans[4]. The International Agency for Research on Cancer has classified OTA as a possible carcinogen to humans (Group 2B)[5].

OTA is a common contaminant of many foodstuffs[3,6,7] and consequently, is present in a large proportion of tested populations of humans and animals. Although reported levels are usually low in each commodity, it can be taken from a variety of food sources and beverages and the intake of different contamined foods and drinks might provide a total amount of OTA near the provisional tolerable levels. Recently, the Joint FAO/WHO Expert Committee on Food Addtives (JECFA) has established a provisional tolerable weekly intake of 100 ng/kg body mass[8].

In further years, the use of more accurate and sensitive analytical methods for this toxin, with lower limits of detection (LOD), led to its detection in different food commodities. The reported results of occurence studies have become more realistic and have been supported by a better evaluation of the human exposure and health risks. Nowadays, OTA has raised public health concerns because high toxic potency and the human population continuous exposure to it.

Several countries have established maximum levels for OTA in cereals and other products, however in Brazil, no regulatory limits for OTA have been established. There is limited data about this mycotoxin in food, cereals and beverages produced or commercialized in this country.

The occurrence of OTA in dried fruits, inclusive raisins, has been reported in several studies[9-10], showing a considerable contamination level with high toxin concentration and incidence. In the United Kingdom[11], the Ministry of Agricultural, Fisheries and Food (MAFF) has detected OTA in more than 80% of dried fruits analysed with the maximum concentration at 18 µg/kg. OTA has been detected in 80% of 61 samples of dried vine fruits in Australia[12]. Forty percent of these samples had OTA concentrations more than 1 µg/kg and in 10% more than 5 µg/kg. In Brazil[13], the OTA contamination was observed in dried vine fruits with 33% of the samples analysed containing more than 5µg/kg.

This study proposes an analytical method using high performance liquid chromatography with fluorescence detection (LC-FLD), which is sensitive to low concentrations of OTA in raisins and reports a survey to determine the levels of OTA in these products commercialized in the city of São Paulo, Brazil.

## MATERIAL AND METHODS

### Raisin samples

Fourty two raisin samples (22 white and 20 black without seeds from different brands and lots) were purchased from supermarkets in the city of São Paulo, Brazil from December 2005 to February 2006. A sample size of 250 g was ground, mixed and sub-sampled prior to analysis.

### Standard and reagents

OTA standard was purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade, excepting methanol and acetonitrile were HPLC grade. They were purchased from Merck (Darmstadt, Germany). All solutions were prepared with deionized water. OTA stock solution was prepared in toluene-acetic acid (99:1). The concentration was determined according to AOAC[14], checked with UV spectrophotometry in 333 nm, using $\varepsilon$= 5440 $cm^{-1}$ $mol^{-1}$. Working standards were prepared by evaporation of known volumes of the stock solution under $N_2$ stream, followed by dissolution in LC mobile phase. They were used to calibrate the LC detector response and for the recovery tests. The solutions were stored from -15°C to -20°C and protected from light. Phosphate-buffered saline (PBS) (NaCl 0.14 M, KCl 0.027 M, $Na_2HPO_4$ 0.077 M, $KH_2PO_4$ 0.0015 M) at pH 7.4 and sodium bicabonate aqueous solution 1% (w/v) were prepared in the laboratory.

### Sample preparation extraction and immunoaffinity clean-up

A OTA free-sample was spiked with known amount of OTA (2.0 ng/g) and the extraction procedure was tested using three extraction solvents, (i) acetonitrile:water (60:40, v/v), (ii) sodium bicarbonate aqueous solution 1%, and (iii) methanol:water (80:20, v/v) as follows: 25 g samples were extracted with 100 mL of extraction solvent by shaking vigorously for 3 minutes in a blender or for 1 hour in a rotational shaker. It was filtered through a paper filter. Then, 4 ml of the filtered solution diluted with 12 mL of PBS were passed through an immunoaffinity column (OchraStar™, Romer Labs Inc., EUA and Ochraprep®, R-Biopharm Rhone Ltd, Scotland). The column was washed with 2 portions of 10 ml of PBS and air-dried. Ochratoxin A was eluted under gravity with 2 ml of methanol:acetic acid mixture (49:1). The eluate was reduced to dryness under $N_2$ and the residue was solved in 300 µl mobile phase. The solution was filtered through 0.45 µm before LC analysis.

### LC-FLD determination

The detection of OTA was carried out using a GBC system (GBC, Dandenong, Victoria, Australia) equipped with a HPLC pump (LC 1110) and a fluorescence detector (model LC 1255). Liquid chromatography separation was performed on a LiChrosorb C18 (Merck, Darmstadt, Germany) 5 µm particle size 25 cm x 10 mm column. The mobile phase was

acetic acid aqueous solution 3.33%:acetonitrile:methanol (30:35:35, v/v) at a flow-rate of 0.8 ml/min. The excitation and emission wavelengths of the fluorescence detector were set at 332 nm and 476 nm, respectively. Sample volumes of 20 µl were injected in triplicate. The linearity was determined in the range of 2 to 256 ng/ml of OTA using 8 calibrators. The time retention of OTA was 6.5 ± 0.2 minutes and the confirmation of the OTA identity peak was performed by methyl ester formation according to AOAC[14].

**Recovery experiments**

Recovery experiments were performed on OTA-free raisin samples (OTA concentration < LOD) spiked with OTA levels of 2.0, 5.0, and 10.0 ng/g. Spiking was carried out in triplicate and a double analysis of the blank was also carried out. After leaving the spiked samples over night for the solvent to evaporate, extraction solvent was added and the OTA concentration was determined. In order to evaluate the experimental conditions of the recovery test, the clean-up has been done using two different immunoaffinity columns (OchraStar™, Romer Labs Inc., EUA and Ochraprep®, R-Biopharm Rhone Ltd, Scotland) with spiked sample at 2.0 ng/g level.

## RESULTS AND DISCUSSION

**Analytical**

Quantification of OTA in raisin samples by LC-FLD was performed using an external calibration curve obtained by diluting appropriate aliquots of OTA stock solution in the mobile phase. The experimental points fit well to a straight line in the range 2 - 256 ng/ml ($r^2$ = 0.99998). The detection (LOD) and quantification (LOQ) limits established were 0.24 and 0.80 ng/g, respectively.

Three different extraction solutions suggested by the manufactures of the immunoaffinity columns (IAC) and two shaking devices were tested. These tests were performed using spiked samples at 2.0 ng/g OTA level. The best results for recovery have been obtained when the mixture of methanol:water was utilized, since the recoveries for the two first procedures (i) acetonitrile:water (60:40, v/v) and (ii) sodium bicarbonate aqueous solution were 119 % and 55 % respectively. The recovery was similar for both blender and rotative shaker. The recoveries using the methanol mixture were 81.6, 80.4, and 81.9 % and the relative standard deviations (RSD) were 6.0, 4.3, and 6.1 % at levels of 2.0, 5.0 and 10.0 ng/g, respectively ( Table 1). These results were in agreement to the values recommended by The European Communities[15].

Typical procedures include extraction methanol- 1% sodium carbonate (70:30), followed by clean-up on IAC[16] and after column washing, OTA could finally be eluted with methanol.

Ianamata et al[12] extracted OTA from dried vine fruits with methanol- 1% sodium carbonate (70:30). The recovery was 81,0 % and the relative standard deviation (RSD) was 31,24%. However, when Magnoli et al[17] used the same extraction solution, the mean recoveries were 95 ± 2% and 90 ± 3% for black and white dried vine fruits, respectively. For both commodities, the mentioned authors utilized PBS containing 1% Tween 20 to wash the column and only methanol to elute OTA from the IAC. In the present study, the OTA extraction was done with a simple solution containing methanol-water (80:20) and only PBS solution was utilized to wash the IAC. The mean recoveries obtained in the present work were similar to those reported by Ianamata et al[12], however the relative standard deviation values were better than the results obtained by those authors.

**Table 1.** The recoveries of OTA in samples of raisins fortified.

| OTA added concentration (ng/g) | OTA obtained concentration (ng/g) | Mean (ng/g) | Recovery (%) | Relative standard deviations (RSD) (%) |
|---|---|---|---|---|
| | 1.74 | | 86.8 | |
| **2** | 1.62 | 1.63 | 80.8 | 6.0 |
| | 1.54 | | 77.2 | |
| | 4.05 | | 81.0 | |
| **5** | 3.83 | 4.02 | 76.6 | 4.3 |
| | 4.17 | | 83.4 | |
| | 8.58 | | 85.8 | |
| **10** | 8.35 | 8.19 | 83.5 | 6.1 |
| | 7.63 | | 76.3 | |

Two immunoaffinity columns were tested in this work: OchraStar™ (Romer Labs Inc) and Ochraprep® (R-Biopharm Rhone Ltd). The methanol:water (80:20) extraction solution suggested by Romer proved adequate for both columns. The columns efficiency was evaluated through OTA recoveries at 2.0 and 10.0 ng/g levels. The results obtained were 103.5 % and 85.7 % for OchraStar™ column and 81.6 % and 81.7 % for Ochraprep® column for 2.0 and 10.0 ng/g OTA levels, respectively. Both values did not show significant statistical difference (p>0.05), therefore both columns tested were adequate to clean-up step.

The experimental method proposed in the present work proved to be adequate to quantification of OTA in raisins. The extraction step was efficient and the IAC clean-up was suitable and no co-extractives were observed at OTA retention time during chromatographic analysis (Figure 1). At present, the clean-up with IAC is considered essential in obtaining optimal recovery of ochratoxin A in addition to the advantages of decreasing experimental time when comparing with other traditional techniques such as liquid-liquid partition.

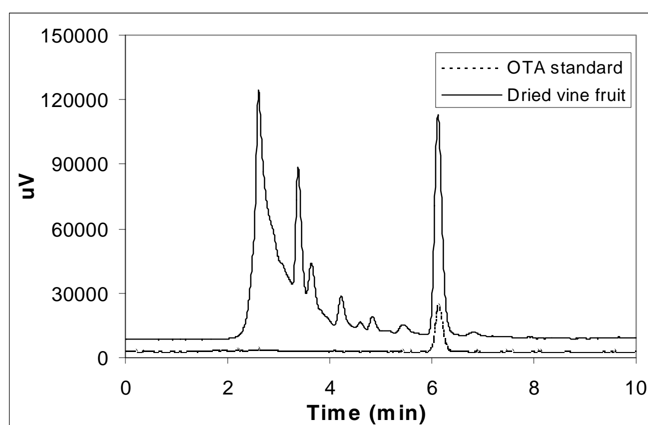


**Figure 1.** HPLC chromatograms – (a) a standard solution containing 10 ng/ml of OTA. (b) a raisin sample naturally contaminated with 18.7 ng/g.

### Incidence

Raisins in package and bulk are commercialized in Brazilian market, therefore the samples investigated included both types. OTA peak identity was confirmed by methyl ester derivative, which elutes at different time from OTA[14].

All white raisin samples showed no contamination with OTA, independent of the type of packaging. Of a total of twenty samples of black raisins 10 (50 %) contained OTA at levels ranging from 0.2 (LOD) to 39.1 ng/g. Considering the type of package, OTA was detected in 43 % of packaged samples and 54 % of bulk samples, a difference not statistically significant (p>0.05). The results of the OTA analysis of 42 different brands of raisins are presented in Table 2.

**Table 2.** Incidence of ochratoxin A in different brands of raisins.

| Sample | Kind of package | Positive/ Total | Percentage of positive (%) | Range (ng/g) |
|---|---|---|---|---|
| Black | Original package | 3/7 | 43 | LOD* – 39.1 |
| | Bulk | 7/13 | 54 | LOD – 4.3 |
| White | Original package | 0/7 | 0 | < LOD |
| | Bulk | 0/15 | 0 | < LOD |
| Total | Original package / Bulk | 10/42 | 24 | LOD – 39.1 |

*LOD (Limit of detection) = 0.24 ng/g.

The OTA levels observed in this work were similar to those obtained by Ianamata et al[13] in dried vine fruits commercialized in Brazil.

Ochratoxin A has been reported as naturally occurring in dried vine fruits in many countries. Magnoli et al[17] detected occurence of 67.7 and 84.2 % with mean levels of 6.3 and 4.4 ng/g in black and whitedried vine fruits, respectively. MacDonald et al[18] showed a high incidence of this toxin (88%) in sultanas, raisins and currants commercialized in the UK with a maximum level of 53.6 µg/g, however Stefanaki et al[10] observed low levels of OTA in Greek domestic dried fruits.

The European Union (EU) has established a maximum OTA limit of 10 ng/g for dried vine fruits (currants, sultanas and raisins)[19]. In the present work two samples of black raisins showed contamination above this limit (18.7 and 39.1 ng/g) and the OTA concentrations of six samples ranged from 1.32 to 8.59 ng/g. In Brazil raisins are consumed during all the year and specially at the end of the year during Christmas. The OTA determination in raisin is very important in order to protect consumers' health from the risk of exposure to this toxin. In addition, in Brazil there is not limit for OTA in this product. Considering the OTA high toxic potency and the continuous exposure of human population, the present work may give support to establish a OTA limits for raisins and others foodstuffs. Therefore, other surveys on the occurrence of OTA in dried black raisins are recommended in order to better measure the contribution of this foodstuff to OTA human exposure.

## CONCLUSIONS

The analytical method proposed in this work is easy to apply and allows the analysis of a large number of samples per day. All this due to the fact that the immunoaffinity column clean-up provides several advantages in comparison to traditional methods of purification, by reducing the use of hazardous solvents, by reducing analysis time, and by showing greater specificity.

Although a limited number of samples has been analysed, the high incidence of contamination in black raisin samples from which, a few presented high concentrations indicates a real risk of human exposure to OTA through the continuous consumption of these fruits.

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