Artigo

# DETERMINATION OF OCHRATOXIN A IN CORIANDER (*Coriandrum sativum* L.) BY HPCL/FLUORESCENCE DETECTION

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An analytical study based on extraction with acetonitrile-water, immunoaffinity column cleanup, and HPLC/fluorescence detection for separation and identification of ochratoxin A in coriander was carried out. Validation of the applied methodology was done through accuracy and precision studies. Homogenized samples of coriander were spiked in triplicate with ochratoxin A at 0.5, 1.0, 2.0, and 5.0 μg/kg levels. Recovery values were in the range of 98% for a fortification level at 0.5 μg/kg to 109.1% at 1.0 μg/kg. Application to coriander samples available in Portuguese markes showed no contamination with ochratoxin A.

Keywords: ochratoxin A; coriander; HPLC.

## INTRODUCTION

Coriander (*Coriandrum sativum* L.) is originated from Middle East and Southern Europe and reached other areas centuries ago[1,2]. It has a long traditional use in Portuguese culinary, initially in Alentejo zone, but nowadays its utilization was expanded at all country in different cooked dishes or minced or puréed in sauces, with aromatic function. It is sold in fresh or in dry state. In last case was frequently found in all markets and has a general use between Portuguese populations.

Ochratoxin A (OTA) is a secondary fungal metabolite produced by several molds, mainly by *Aspergillus alutaceus* var. *alutaceus* Berkely and Curtis (formerly *A. ochraceus* K. Wilh.), in warmer and tropical parts of the world, and *Penicillium verrucosum*, in temperate climates[3,4]. Its production and the growth of the fungi responsible are dependent upon factors such as temperature, humidity, handling during harvesting and conditions of storage. *Penicillium verrucosum* is especially associated with stored cereals, although it has also been isolated from meat and fish[4-7]. *Aspergillus ochraceus* is common on coffee beans and spices[8]. The occurrence of OTA in food has been recognized as a possible threat to human health[9]. In humans, the consumption of OTA contaminated food exhibits nephrotoxic, teratogenic and immunosupressive effects[10]. The OTA is also suspected to cause the Balkan Endemic Nephropaty, a kidney disease observed in countries such as Romania, Bulgaria, and Bosnia. The IARC (International Agency for Research on Cancer) has classified OTA as a possible carcinogen to humans (Group 2B)[11], based on sufficient evidence for carcinogenicity in experimental animal studies and inadequate evidence in humans. Human exposure to OTA is well documented in some studied Portuguese populations[12-14]. Despite extensive data in the literature concerning the occurrence of OTA in different type of food, only few related its presence in coriander, what is important in Mediterranean diet.

The monitoring of OTA requires simple, quick, accurate, precise, and sensitive methods, but unfortunately few reports on the analytical methods are disposable for its determination in coriander, nevertheless there are more reports for its determination on spices or herbs, in general. All those procedures require a suitable sample extraction step using chloroform mixed with orthophosphoric acid[15], or methanol[16]. For other foods, such as cereals, acetonitrile-water was tried for barley[17], for various cereals[18,19], or for wheat grains[20], at different proportions.

Also for cleanup, different procedures have been tried for different extracts, but utilization of immunoaffinity columns (IAC) has been well documented for different types of foods, beverages, human blood or serum[12,21], milk[22], and urine[23], due to its specificity.

The aim of this work was to monitor the degree of OTA contamination of coriander samples available in Portuguese market. At first, a method based in sample extraction with acetonitrile-water and immunoaffinity cleanup to purify and concentrate OTA in coriander extracts, for further detection and quantification by HPLC /spectrofluorimetric detection was validated.

## EXPERIMENTAL PART

### Reagents

HPLC grade acetonitrile, toluene, and methanol were purchased from Carlo Erba (Milan, Italy). Analytical grade acetic acid, hydrochloride acid, sodium hydroxide, potassium chloride, potassium dihydrogen-phosphate, anhydrous di-sodium hydrogen-phosphate, and sodium chloride were obtained from Merck (Darmstadt, Germany). Water was purified by distillation and passage through Milli Q system (Millipore, Bedford, MA).

Ochratoxin A (OTA) was obtained from Sigma Chemicals Co, St. Louis, USA.

Ochratest™ immunoaffinity columns were from VICAM, Watertown, USA.

The phosphate buffer solution (PBS solution) was prepared from potassium chloride (0.2 g), potassium dihydrogen-phosphate (0.2 g), anhydrous disodium hydrogen-phosphate (1.2 g), and sodium chloride (8 g) added to distilled water (900 mL). After dissolution the pH was adjusted to 7.4, with 0.1 mole $L^{-1}$ HCl, or 0.1 mole $L^{-1}$ NaOH as appropriate. The solution was made to 1 L.

### Ochratoxin A standards

OTA is toxic and probably carcinogenic for humans. A fume

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hood was used; gloves, protective clothing, and eyewear should be worn.

Standard solution of ochratoxin A was prepared from the ochratoxin A vial purchased from Sigma Chemicals Co. The concentration and purity of standard solutions were evaluated by AOAC Official Methods[24]. One stock solution was made in 4 mL toluene-acetic acid (99:1) at 250 µg/mL. Intermediate solution was prepared at 10 µg/mL, diluting 1 mL of stock solution with 25 mL toluene-acetic acid (99:1). For fortification assays, work solution was prepared in toluene: acetic acid (99:1) at 1000 ng/mL.

For calibration curve, one work solution for calibration was prepared evaporating 10 µL of stock solution at dryness, and diluting to 10 mL with mobile phase (250 ng/mL). The others were prepared diluting this solution at the following concentrations: 25.0, 20.0, 15.0, 10.0, 5.0, and 2.5 ng/mL. All solutions were keep in amber flasks to protect from light.

### Sampling

Random ten samples of coriander were collected in supermarkets in Coimbra city, in January 2002.

### Linearity studies

Linearity was evaluated in the range of 0.05 ng to 0.5 ng, using six working standard solutions at concentration levels between 2.5-25 ng/mL.

### Recovery studies

The recovery studies were made spiking each of three replicates of 25 g of coriander with OTA working solution prepared in toluene: acetic acid (99:1) at different levels (Table 1 – Graphic 1).

### Extraction and cleanup

The sample (25 g) was homogenized with a 100 mL acetonitrile-water mixture (60:40) for 3 min, and filtered. The filtrate (4 mL) was placed in a 100 mL flask, and added of PBS solution (44 mL). After mixing, the totality of the mixture was passed through the immunoaffinity column for cleanup, without drying. After, the column was washed by 10 mL of water and dried by air with a syringe, and ochratoxin A was eluted with 2 mL of methanol. The methanol was dried at ±50 ºC, under nitrogen stream, and the residue was redissolved in 250 µL of mobile phase and injected in the HPLC system.

### Chromatographic conditions

The detection was made using an HPLC system consisted by a pump, model 307 (Gilson, France), one 20 µL injection valve, model 7125 (Rheodyne, Cotati, California, USA), a guard column, $C_{18}$ – 5 µm Nucleosil 120 KS (30 x 4 mm i. d.), and a column, $C_{18}$ – 5 µm Nucleosil 100 (250 x 4.6 mm i. d.). A spectrofluorimeter, model LS-3B (Perkin-Elmer, USA) was connected to the HPLC. The analyses were made using 333 nm for excitation and 460 nm for emission. An integrator, model 3390A (Hewlett-Packard - USA) was used to measure peak areas. The mobile phase consisted of water: acetonitrile: glacial acetic acid (49.5:49.5:1.0 v/v/v) at a flow rate of 1mL/min.

### Accuracy and precision

The validation studies of the methodology were verified by evaluation of accuracy and precision. Accuracy was evaluated by recovery studies, using four different fortification levels, 0.5, 1.0, 2.0, and 5.0 µg/kg. For each level, one homogenized coriander sample was spiked with OTA in triplicate. Precision was evaluated on the basis of intra-day repeatability (three replicates for each one of the fortification levels, within one day by the same analyst) (standard deviation (RSD%). Intermediate precision was calculated on the basis of inter-day repeatability for three days.

## RESULTS AND DISCUSSION

Figures 1 and 2 show chromatograms of a standard and of a coriander sample fortified at 2 µg/kg, respectively.

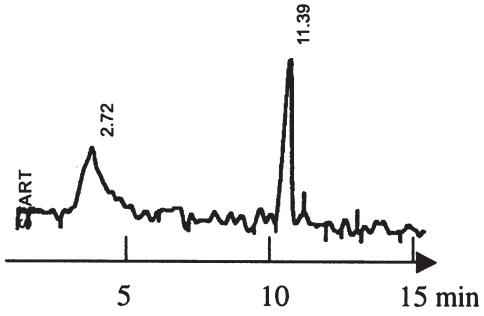



***Figure 1.*** *Chromatogram of ochratoxin A standard: retention time = 11.39 (0.1 ng). Column: $C_{18}$ – 5 µm Nucleosil 100 (250 x 4.6 mm i. d.); guard column: $C_{18}$ – .5 µm Nucleosil 120 KS (30 x 4 mm i. d.); injection volume: 20 µL; mobile phase: water: acetonitrile: glacial acetic acid (49.5:49.5:1.0 v/v/v); flow rate: 1 mL/min; detection: HPLC-spectrofluorimeter at 333 nm for excitation and 460 nm for emission*

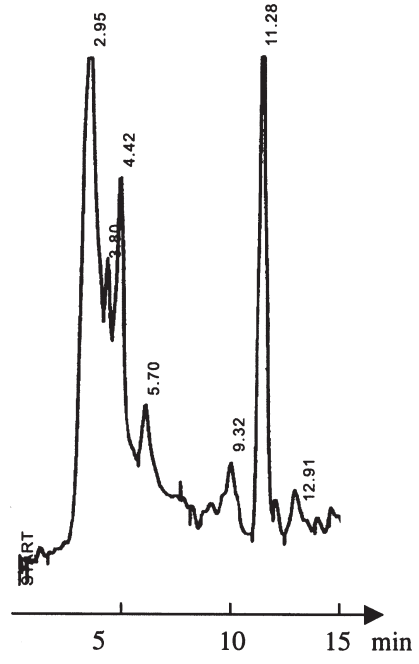



***Figure 2.*** *Chromatogram of one coriander sample fortified at 2.0 µg/kg. Column: $C_{18}$ – 5 µm Nucleosil 100 (250 x 4.6 mm i. d.); guard column: $C_{18}$ – .5 µm Nucleosil 120 KS (30 x 4 mm i. d.); injection volume: 20 µL; mobile phase: water: acetonitrile: glacial acetic acid (49.5:49.5:1.0 v/v/v); flow rate: 1 mL/min; detection: HPLC-spectrofluorimeter at 333 nm for excitation and 460 nm for emission*

### Linearity

The calibration curve was obtained using the linear least squares regression procedure of the peak area versus the concentration. The linearity for OTA, in the working standard solutions at three determinations of six concentration levels, between 2.5-25 ng/mL (0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 ng), was good as shown by the fact that the determination of the correlation coefficients ($r^2$) are above 0.9914.

### Limit of detection

The limit of detection (LOD), based on a signal-to-noise ratio of three, was 0.05 µg/kg. Good results were obtained, with standard deviation of 3.3%.

### Limit of quantification

The limit of quantification for OTA was determined by the signal-to-noise approach, defined as that level resulting in a signal-to-noise ratio of approximately 10:1. The LOQ of the method was 0.2 µg $kg^{-1}$.

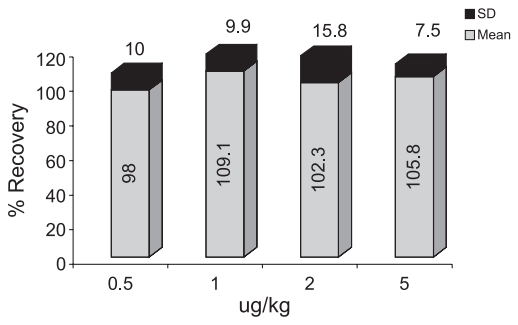
### Accuracy and precision

Accuracy was determined calculating the mean recovery values for each fortification level (Table 1 - Graphic 1). Both values, for studied levels, indicated adequate methodology for quantification of OTA in coriander samples. Intermediate precision on the basis of analysis at 5 µg $kg^{-1}$ per day for three days was 11.6% (Table 2).

**Table 1.** Results of coriander samples spiked with four different levels of ochratoxin A with recovery and precision values

| Fortification level* (µg/kg) | Recovery % | Mean Recovery % | Repeatability RSD% |
|---|---|---|---|
| 0.5 | 88.0 | 98 | 10.0 |
| | 98.0 | | |
| | 108.0 | | |
| 1.0 | 119.6 | 109.1 | 9.9 |
| | 100 | | |
| | 107.5 | | |
| 2.0 | 102.3 | 102.3 | 15.8 |
| | 86.5 | | |
| | 118.0 | | |
| 5.0 | 101.0 | 105.8 | 7.5 |
| | 114.35 | | |
| | 101.9 | | |

*n=3




***Graphic 1.*** *Accuracy and precision of analytical methodology for determination of OTA in coriander*

**Table 2.** Results of repeatability and intermediate precision of ochratoxin A from coriander

| Level µg/kg | Repeatability[a] RSD% | Intermediate precision[b] RSD% |
|---|---|---|
| 5.0 | 7.5 | 11.6 |

[a] Repeatability on the basis of three replicates at 5 µg/kg within the same day; [b] Intermediate precision on the basis of analysis at 5 µg/kg per day for three days.

The maximum limits for OTA set by Standing Committee on the Food Chain and Animal Welfare in 2002[25] range between 3 µg/kg for derivatives of cereals to 10 µg/kg for dried fruits, but no maximum limits were established for spices, condiments, and aromatic herbs. Furthermore, there are also national regulations in member states. Romania, Greece, and France set up OTA maximum limits, in all foods, of 5, 20, and 5 µg $kg^{-1}$, respectively[26]. The detection limit of the established methodology for determination of OTA in coriander is in compliance with the maximum limits purposed.

None of ten analysed samples was contaminated with OTA. In a German survey, OTA was detected in 21 of the 31 coriander analysed samples, showing a mean OTA level of 1.48 µg/kg, ranging from lower than LOD to 3.88 µg/kg[27]. In India, levels of OTA ranged from 10 to 51 µg $kg^{-1}$ in 20 of fifty coriander samples[15].

## CONCLUSIONS

The analytical methodology purposed provides good results in terms of accuracy, intermediate precision, repeatability, and sensitivity for determination of OTA in coriander.

The established methodology fulfils the Standing Committee on the Food Chain and Animal Welfare requirements, attending to maximum levels of OTA in foods.

Owing the importance of coriander in Portuguese diet, its contribution to the dietary OTA exposure of consumers must assumes relevance from a public health point of view. How its presence was not detected in any analysed sample, the coriander consumption for Portuguese consumers seems to be safe.

## ACKNOWLEDGMENTS

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