Food Control 20 (2009) 716–720

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

HPLC determination of ochratoxin A in high consumption Tunisian foods

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ARTICLE INFO

Article history:
Received 14 February 2008
Received in revised form 8 September 2008
Accepted 13 September 2008

Keywords:
Foods
Ochratoxin A
HPLC–FLU
Immunoaffinity

ABSTRACT

Samples (180) of high consumption food commodities from various regions of Tunisia were analysed to determine ochratoxin A contamination levels. A high performance liquid chromatography method for ochratoxin A determination was optimized. Samples were extracted with acetonitrile/water (80:20, v/v) solution and purified by immunoaffinity column. Average recoveries at 0.5 and 2 ng/g levels ranged from 84 ± 3.1 to 94 ± 1.2% with a between-day coefficient of variation (RSDR) of 3.8%. The method detection limit was 0.1 ng/g and ochratoxin identity was confirmed by methyl ester formation. The whole procedure was simple and fast if compared with other existing procedures. Performed analysis indicates that 45% of monitored samples were contaminated with levels ranging from 0.11 to 33.9 ng/g. The most contaminated commodities were barley, sorghum and wheat.

1. Introduction

Ochratoxin A (OTA) is a toxic secondary metabolite of several *Aspergillus* and *Penicillium* species frequently present in food and feeds (Atkins & Norman, 1998; Meri, Marika, & Aldo, 2005). OTA is a well-characterized nephrotoxin (Coulomb, 1993) and has been found in food and blood samples in areas with endemic porcine and human nephropathy (Petkova-Bocharova, Chernozemsky, & Castegnaro, 1988; Khalef et al., 1993; Wafa et al., 1998). In addition, immunosuppressive, teratogenic and carcinogenic effects for OTA have been reported (Roger, 1993). It was suspected as a possible cause of urinary tract tumors (Dorrenhaus et al., 2000) and was classified by the international agency for research on cancer (IARC) as a possible human carcinogen (group 2B) (International Agency for Research on Cancer (IARC), 1993). OTA occurrence in human foods is widespread, contamination of cereals (barley, wheat, maize) and derived products have been well-documented (Ruprich & Ostry, 1993). In Mediterranean area, OTA has been found in other products such as green coffees, dried fruits, spices coffee, beer, and, in wine (Gauchi & Leblanc, 2002). Those commodities are widely consumed in Tunisia and are a possible source of OTA human exposure. Preliminary mycotoxins surveys in Tunisia showed relatively high levels of OTA in frequently consumed food, especially in cereals products, species and dried fruits (Ghali, Hmaissia-khlifa, Ghorbel, Maaroufi, & Hedili, 2008; Maaroufi et al., 1995). Many countries and international organisations have regulated the OTA content in several commodities. The European commission has enforced the limits of OTA in cereals and cereal products with the following levels: 5.0 μg/kg for raw cereal grains, 3.0 μg/kg for cereals and cereal products intended for human consumption, 0.5 μg/kg for baby food and cereal-based food intended for young children (2005; European Commission Regulation, 2002). There are currently no legal limits for OTA in spices, however, the European Commission has been discussing a limit of 10 μg/kg for OTA in spices. (Goryacheva et al., 2006). For the dried vine fruits, soluble coffee and some dried fruits, the European commission has set a maximal permissible limit for ochratoxin A at 10.0 μg/kg. Numerous methods for OTA determination in food have been described, including Enzyme-linked immunosorbent assay (ELISA) and thin layer chromatography (TLC) (El-Kady, El-Maraghy, & Eman, 1995). Liquid chromatography linked to fluorescence detection (HPLC/FD) was extensively used for OTA confirmatory Analysis (Fazekas, Tar, & Kovács, 2005; Gonzalez, Juan, Soriano, Molto, & Manes, 2006). Current analytical methods for ochratoxin A have achieved low limits of detection, and require one or more clean-up steps involving liquid–liquid partition, solid-phase extraction or immunoaffinity clean-up. Present work describes a sensitive analytical method for OTA analysis in some food matrix using a HPLC technique preceded by an immunoaffinity clean-up step. A total of 180 of various Tunisian food samples were checked for OTA contamination.

2. Materials and methods

2.1. Chemicals

Ochratoxin A solid standard and boron trifluoride (BF_3, methanol solution 14%) were obtained from Sigma (St. Louis, MO, USA). OTA stock solution (1 mg/ml) was prepared in toluene–acetic acid (99:1) and kept at −20 °C. The stability of the stock solution was checked spectrophotometerically and all working standard solu-

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doi:10.1016/j.foodcont.2008.09.004

tions were prepared immediately before use by diluting the stock solution with acetonitrile. Acetic acid, acetonitrile, and methanol (HPLC grade) were purchased from Merck (Merck, Darmstadt, Germany). Water HPLC grade, was obtained using a Simplicity system (Millipore Corporation, Bedford, MA, USA). Ochraprep® immunoaffinity columns (IAC) were obtained from R-Biopharm. (Rhone Ltd, Glasgow, UK). Whatman® filter paper and a 47 mm membrane filters were used with a MILLIPORE® filtration system (Millipore Corporation, Bedford, MA, USA) for extract and solvent filtration, respectively. The OTA Extraction solution (pH 1.6) was composed of 33.7 ml 85% orthophosphoric acid and 118.0 g sodium chloride per litre. Phosphate buffered saline (pH 7.4) was prepared by adding potassium chloride (0.20 g), potassium dihydrogen phosphate (0.2 g), anhydrous disodium hydrogen phosphate (1.16 g) and sodium chloride (8.00 g) to 900 mL of water. The pH was adjusted with 0.1 M NaOH as appropriate and the volume made to 1 L with water. OTA methyl ester (OTA-Me) was prepared using a modified Hunt et al. method (Hunt et al., 1980). About 300 μl of OTA extract was mixed with 300 μl of BF3 solution. The mixture was heated at 70 °C for 20 min and used directly for HPLC analysis.

2.2. Samples

A total of 180 food samples for human consumption were collected during the years 2005 and 2006 from local markets and traditional family reserves in Tunisia. The samples were stored in plastic bags at +4 °C until grinding and analysis. The selected commodity groups were: cereals as wheat, barley, maize, sorghum, rice and their derived products, spices including cumin, red pepper and black pepper, dried fruits as peanuts and pistachio.

2.3. Apparatus

The HPLC apparatus consisted of a WATERS HPLC system (Waters, Milford, MA, USA) including a gradient pump (Waters 600) connected to an automatic sampler (waters 717) and a waters model 477 fluorescence detector. This system was piloted by a Millennium 4.0 data system. The analytical column was a MACHERY-NAGEL (Duren, Germany) C18 (4.6 mm × 150 mm; 5 μm) column kept at 25 °C. A centrifuge (Heraeus CHRIST, LABOFUGEL, Germany) and a coffee mill SCM-40A (Sibata, Tokyo, Japan) were used during the extraction steps.

2.4. Sample preparation and immunoaffinity clean-up

Samples were extracted and cleaned using Ochraprep® immunoaffinity columns according to the manufacturer's instructions with some modifications. The food samples was finely ground and homogenized. About 10.00 g were mixed with 40 ml of acetonitrile/water (80:20, v/v) mixture and 20 ml of hexane and then horizontally stirred for 20 min. The extract was filtered through filter paper and all filtrate was collected and centrifuged for 15 min at 4000 g at ambient temperature and the upper hexane phase was discarded while 8 ml of the lower layer was transferred to a 20 ml tube and evaporated to dryness at 60 °C under a lower stream of nitrogen. After addition of 0.5 ml acetonitrile and 2 ml of phosphate buffer saline (PBS), the tube was mixed-vortex for 2 min before adding 18 ml of PBS. The diluted extract (equivalent to 2 g sample) was loaded into the IAC column at 2 ml/min flow followed by 20 ml of PBS at 4 ml/min flow rate for washing. OTA was then eluted with 2 ml of methanol/acetic acid 2% solution. The eluate was evaporated to dryness under nitrogen stream at 60 °C and reconstituted with 1 ml of acetonitrile. 50 μl were injected into the chromatograph. For recovery calculation, OTA free samples spiked at 0.5 and 2 ng/g were extracted in quadruplicate as it was described previously during three consecutive days.

2.5. HPLC ochratoxin A determination

OTA determination was carried out under isocratic conditions, with a mobile phase constituted of acetonitrile/water/acetic acid (50:48:2, v/v/v) at flow rate of 1 ml/min. Respective fluorescence excitation and emission wavelengths of 334 and 460 nm were 108 used for OTA and OTA-Me detection. The calibration curve linearity was verified by linear regression analysis in the 0.1–8 ng/ml range. Ochratoxin A quantification was performed by measuring peak areas at OTA retention time, and comparing them with the relevant calibration curve. The OTA identity was confirmed in all positive samples. For samples with OTA concentration lower than 1 ng/g, IAC eluate was spiked with OTA and injected into the chromatograph then spiked and unspiked chromatograms were compared. For samples containing more than 1 ng/g, OTA derivatization to OTA-Me was applied.

2.6. Validation and statistical analysis

Analytical method validation was carried out on the basis of the Harmonized Guidelines for Single Laboratory Validation of Methods of Analysis. (Thompson, Ellison, & Wood, 2002). This method was initially validated by analysis of replicate of standard solutions and spiked samples ($n = 4$) at 5.0 ng/g for a variety of wheat, red pepper, barley, rice and sorghum. Spiked samples were allowed to equilibrate for 1 h prior to extraction. In addition, a matrix blank was also analysed to determine any residual mycotoxin levels. Validation data were analysed by SPSS software program (SPSS Institute, Inc., 2000, Version 10.0). The calibration curve used for quantification was calculated by the least-124 squares method and means comparison was made by ANOVA test ($p < 0.05$).

3. Results and discussions

3.1. Analytical method characterisation

The proposed HPLC method enabled the OTA quantification in analysed food commodities with higher selectivity and sensibility. The use of acetonitrile and acidified water with (2% acetic acid) as mobile phase allowed a good separation of OTA and OTA-Me from the matrix compound with respective retention times of 5.3 and 12.5 min. The reproducibility of the retention time was good (CV = 2.1%). Figs. 1 and 2, respectively, show the chromatographic profiles of OTA standard solution and the correspondent OTA-Me. Many emission/excitation wavelengths couples were tested in order to assess the optimum conditions for OTA detection. The best fluorescence signals in terms of signal-to-noise ratio and sensibility were obtained with the 310 nm excitation/465 nm emission wavelength combination. The detection and quantification limit (signal-to-noise ratio of 3) obtained with a direct standard solution injection was, respectively, less than 0.l and 0.2 ng/ml. Quantification was performed using a linear calibration curve established in the range of 0.1–8 ng/ml with a correlation coefficient (r) of 0.996. The treatment of extracts that contained OTA or the OTA standard solution by BF3 resulted in the disappearance of OTA peak and the appearance of new one at 12.5 min corresponding to OTA-Me. The OTA confirmation step allowed to overcome some analytical problems such as co-elution of interfering compounds, resulting in erroneous positives results. Typical elution profiles for OTA naturally non-contaminated and contaminated food samples are shown, respectively, in Figs. 3 and 4. The mean extraction recoveries are presented in Table 1. For cereal samples, recoveries at 2 ng/g spiking level were, respectively, 87 ± 2.2, 84 ± 6.8 and 89 ± 2.3 for wheat, barley and rice. Recoveries for 0.5 spiking concentrations are slightly more significant than those of 2 ng spiking concentra-