Incidence of ochratoxin A in rice and dried fruits from Rabat and Salé area, Morocco.

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Incidence of ochratoxin A in rice and dried fruits from Rabat and Salé area, Morocco.

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Running title: Ochratoxin A in rice and dried fruits from Morocco

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Abstract

One hundred samples of dried fruits (20 dried raisins, 20 walnuts, 20 peanuts, 20 dried figs and 20 pistachios) and twenty samples of rice purchased from retail shops in the Rabat and Salé area in Morocco were analyzed for ochratoxin A (OTA) by immunoaffinity (IAC) clean-up and LC with fluorescence detection. The limit of quantification (LOQ) (S/N, 10:1) of OTA was 0.02 ng/g in rice, 0.03 ng/g in pistachio, peanut and walnut and 0.03 ng/g in dried raisins and dried figs. The incidence of occurrence of OTA in dried raisins, walnuts, peanuts, dried figs and rice was 30, 35, 25, 65 and 90 % respectively. Analytical results showed that pistachio samples contained no detectable OTA, but concentrations ranged from 0.02±0.01 to 32.4±2.10 ng/g in rice, from 0.10±0.05 to 2.36±0.75 in peanut, from 0.03±0.01 to 1.42±0.45 in dried figs, from 0.05±0.02 to 4.95±0.02 in dried raisins and from 0.04±0.01 to 0.23±0.05 in walnuts. Results showed also that 15% of total number of rice samples which were analyzed of rice exceeded the regulatory limit set by European Union regulations for cereals (European Commission 2002). This is the first report on the occurrence of OTA in dried fruits and rice available in Morocco.

Keywords: *Ochratoxin A, rice, dried fruits, Morocco*

Introduction

Ochratoxin A (OTA), chemically known as N-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl]-carbonyl}-3-phenyl-L-alanine, is a mycotoxin described the first time by Van der Merve et al. (1965). OTA is a ubiquitous secondary fungal metabolite primarily produced by the genera of *Aspergillus* (e.g. *A. ochraceus*) and *Penicillium* (e.g. *P. verrucosum*). Several other *Aspergillus* species have been described as producers of this toxin, including strains of *A. alliaceus*, *A. ostianus*, *A. sclerotiorum*, *A. sulphureus*, *A. melleus*, *A. petrakii*, *A. glaucus, A. niger*, *A. awamori*, *A. foetidus*, *A. carbonarius*, *A. albertensis*, *A. auricomus* and *A. wentii* (Varga et al., 1996). In colder climates, *Penicillium* species were found to be responsible for OTA contamination of several agricultural products. OTA has been widely detected in cereals (barley, wheat, maize, oat) (Speijers & Van Egmond, 1993; Trucksess et al., 1999), green coffee (Leoni et al., 2000), grape juice (Zimmerli & Dick, 1995), and wine (Blesa et al., 2004a; Miraglia & Brera, 2002). OTA contamination of dried fruits was found to be due to the action of black aspergilli in Europe including Spain (Abarca et al., 2003), France (Sage et al., 2004), the Czech Republic (Ostry et al., 2002) and in other parts of the world such as Argentina (Romero et al., 2005) and Australia (Leong et al., 2004).

OTA is receiving increasing attention for its toxic effects and high incidence in a wide range of food commodities. OTA has been shown to be nephrotoxic, carcinogenic, immunotoxic, genotoxic and teratogenic to all animal species tested. The genotoxicity of OTA has been postulated *in vivo* and *in vitro* (Creppy 2002). Genotoxic effects such as DNA strand breaks, sister chromatid exchanges, chromosomal aberrations and induction of micronuclei have been observed in some mammalian cell systems in

response to OTA exposure (Mally & Dekant, 2005). The presence of OTA in blood from healthy humans confirms a continuous and widespread exposure. A positive correlation among human nephropathies and dietary OTA exposure or plasma concentrations arises from several epidemiological studies. OTA has been implicated in a human disease of kidney referred to as Balkan endemic nephropathy, characterized by tubule interstitial nephritis and associated with high incidence of kidney, pelvis, ureter and urinary bladder tumors in some Eastern European countries in particular Bulgaria, Romania, Serbia, Croatia, Bosnia and Hertzegovinia, Slovenia and Macedonia (Pfohl-Leszkowicz et al., 2002). Consumption of food contaminated with OTA during pregnancy and/or childhood is suspected to induce lesions in testicular DNA that puberty could promote testicular cancer (Monaci & Palmisano, 2004). Sufficient experimental evidence for carcinogenicity in animal studies has led to the classification of OTA as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC 1993). The Joint Committee FAO/WHO of Experts on Food Additives (JECFA) has established the provisional tolerable weekly intake (PTWI) of OTA at 100 ng/kg of body weight corresponding to approx. 14 ng/kg b.w./day (JECFA 2001).

In North African countries, the most suspected food susceptible to be contaminated by OTA are domestic and imported cereals like wheat and sorghum, olives, poultry products and spices (Grosso et al., 2003). Data published suggested the evidence association of elevated exposure to OTA with cases of human nephropathies in Tunisia (Abid et al., 2003). In Morocco, a North African country surrounded by the Atlantic and Mediterranean seas, there is a lack of investigations related to OTA occurrence in food and feed, even though climatic conditions, crop production and handling practices are

favourable to mycotoxin contamination. In a recent report, the natural occurrence of OTA in barley, corn and wheat from Morocco was reported and the co-occurrence of OTA with *Fusarium* toxins (Zearalenone and Fumonisin B_1) has been also confirmed in corn samples by Zinedine et al. (2006). Although cereals (corn, wheat, barley) were studied for mycotoxin occurrence, rice has never been investigated. The Moroccan population consumes huge amounts of dried fruits directly or as ingredient included in popular foods such as "Chebbakia", "Sellou/Sefouf", "Zammita" and "Mkharka" especially during the "Ramadan" fasting month and festival days. Almost all nuts such as pistachio, walnuts and peanuts consumed in Morocco are imported and little is known about their quality. Consequently, there are no legal parameters concerning the presence of mycotoxins, especially OTA, that control their entry to the country and, up to now, there is a lack of information in the literature about the occurrence of OTA in these products. The purpose of this survey is to investigate the presence of OTA in rice and dried fruits available in the area of Rabat and Salé in Morocco.

Material and methods

Sampling

One hundred samples of dried fruits (20 raisins, 20 walnuts, 20 peanuts, 20 dried figs and 20 pistachios) and twenty samples of rice were purchased in July – August (2005) from retail shops of Rabat and Salé in Morocco, stored in plastic bags at 4ºC, taken to the Laboratory of Food Chemistry and Toxicology of the University of Valencia of Spain and immediately analyzed for OTA.

Chemical and reagents

Acetonitrile, cyclohexane, formic acid, acetic acid and LC grade methanol was supplied by Merck (Darmstadt, Germany). LC-grade water was obtained by filtering deionised water through a 0.45-µm filter with a Waters-Millipore (Milford, MA, USA) system. Solvents and water were degassed for 20 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA). Phosphate-buffered saline (PBS) was bought from Sigma (St. Louis, MO, USA).

OTA crystalline material was purchased from Sigma (St. Louis, MO, USA). Stock standard solution with concentration of 500 µg/ml was prepared in methanol, kept in a closed container at -20 °C, wrapped in aluminium foil, and held for less than 3 months. Standard working solutions were prepared by appropriate dilution in the same solvent and stored in glass-stopped tubes at -20 °C.

Extraction procedure

- OTA in rice

The method used for OTA in rice was developed in the laboratory and reported by Blesa et al. (2004b). Briefly, samples of rice (200 g) were prepared using a food processor and mixed thoroughly. An aliquot (2.5 g) of the sample was placed into a mortar (50 ml capacity) and was gently blended with 1.5 g of the solid phase C_8 (50 µm, Análisis Vínicos, Tomelloso, Spain) for 5 min using a pestle, to obtain a homogeneous mixture. This mixture was introduced into a 100 mm x 9 mm i.d. glass chromatographic column with a coarse frit (no. 2) and covered with a plug of silanized glass wool at the top of the column. OTA was eluted with 20 ml methanol–formic acid (99:1, v/v) with a

vacuum manifold. The eluate was evaporated to 3 ml with a gentle stream of N_2 at 45 °C, and then, it was filtered through a nylon acrodisk (0.45 µm) and centrifuged at 5000 rpm for 10 min (Heraeus, Germany). The extract was filtered again and evaporated to dryness with N_2 at 55 °C and reconstituted in 500 µl of the mobile phase for LC analysis.

- OTA in dried fruits

The method of Stroka et al. (2000) slightly modified was used. Briefly, 10 g of samples of pistachio, peanut and walnut added with 1g of NaCl were blended with 40 ml of methanol-water (80:20) and 20 ml of cyclohexane for 3 minutes. After separation of the two phases, cyclohexane was eliminated. For figs and raisins, samples were extracted only with 40 ml of methanol-water (80:20). The extracts were filtrated on Whatman filter paper No. 4. An aliquot of 10 ml was diluted with 60 ml of PBS buffer (pH 7.4). An immunoaffinity column (IAC OchraTest™, Vicam, USA) for ochratoxin analysis was conditioned with 10 ml of PBS buffer by gentle syringe pressure at a flow rate of 5 ml/min. Then, the mixture of the filtrate diluted extract (70 ml) was applied to the IAC column (1–2 drops per second), followed by a washing with 20 ml of LC grade water and then dried with air. OTA was then slowly eluted from the IAC with 2 ml methanol into a glass vial; the eluate was evaporated to dryness with a gentle stream of N_2 at 55 ºC and reconstituted in 500 µl of the mobile phase for LC analysis.

LC analysis

The chromatographic system consisted of a Shimadzu (Kyoto, Japan) SCL-6A system LC equipped with two LC-6A pumps, a Rheodyne Model 7125 injector and an SRF-535

fluorescence detector. Separation was carried out on a C18 column Phenomenex (150×4.6 mm I.D, 5 µm., Scharlau, Barcelona, Spain). A 20-µl aliquot of the sample was injected onto the column. The mobile phase consisted of acetonitrile/water/acetic acid (49.5/49.5/1, v/v/v) pumped at a flow rate of 0.8 ml/min. Determination of OTA was performed at 333 and 464 nm as excitation and emission wavelengths, respectively.

Confirmation

The identity of OTA in positive samples was confirmed by methyl ester formation according to the method of Zimmerli and Dick (1995). Briefly 200 µl of the extract was diluted to 2.5 ml methanol and 0.1 ml concentrated HCl was added. The solution was left standing overnight at room temperature. Thereafter, the methanol was evaporated and the residue was taken up in 200 µl of mobile phase. Then 20 µl were injected to the LC system. 90% of the OTA was methylated by this method. The resulting methyl ester has a quite different retention time than OTA, this allows the confirmation of OTA identity by an almost complete disappearance of the first OTA peak and the presence of a new one.

Results and discussion

Recoveries for OTA on samples spiked at a level of 10 ng/g for dried raisins and dried figs and at a level of 5 ng/g for pistachio, peanut, walnut and rice are reported in Table I. The limit of quantification (LOQ) (S/N, 10:1) of OTA was 0.02 ng/g in rice, 0.03 ng/g in pistachio, peanut and walnut and 0.03 ng/g in dried raisins and dried figs. As shown, the limit of quantification (LOQ) allows OTA determination at the maximum levels indicated in the European legislation. Results of the study reflected that the analysis

gave clean chromatograms and recoveries were considered as valid for analyzing residues of OTA in cereals and dried fruits according with European specification (European commission 2002)

[Insert Table I about here]

OTA in rice

Results of occurrence of OTA in rice samples showed that OTA was present in 18 out of 20 analyzed samples, the incidence is 90%. Figure 1 shows a chromatogram of a rice positive sample. Levels of contamination in positive samples ranged between 0.02±0.01 and 32.4±2.10 ng/g, where the average of OTA in positive rice samples is 4.15±1.45 ng/g. Our findings showed high incidence of OTA in Moroccan rice comparing with data described for non-organic and organic rice from Spain where the incidence of occurrence of OTA were 7.8 and 30 % respectively (González et al., 2006). Worldwide contamination of rice with OTA was reported in UK (Scudamore et al., 1999) in Vietnam (Trung et al., 2001), in rice germs and rice germ cake from Egypt (Abdelhamid 1990), in Portugal (Pena et al., 2005), in France (Leblanc et al., 2005) and in polished rice from Korea (Park et al., 2005).

[Insert Figure 1 about here]

In Morocco, rice cultivation fluctuates vastly depending especially on climatic conditions. On a potential of 25 000 ha in the Gharb plain, the harvested area varies from 500 ha to 13000 ha. One average, the Moroccan population consumes 60 000 tons each year (2 kg/person/year). Due to drought the country has endured during the last two decades, rice yield production decreased dramatically from 44 000 tons in 1993 to 2500 tons in 1995 leading to extensive importation from other countries. Rice (*Oryza*

sativa L.) is an important food crop worldwide along with wheat and corn, and has been major food in several countries. Park et al. (2005) has been reported that rice is naturally contaminated with *A. ochraceus*. Rice is an aquatic plant and is usually harvested at very high moisture levels (35-50 %). Therefore, mycotoxin-producing moulds could contaminate the grain and produce important quantities of OTA during storage. Furthermore, rice is a better substrate for the characterization of OTA-producing *A. ochraceus* strains.

Up to now, no Moroccan government regulations for the maximum permitted levels of mycotoxins in foods and feeds are still in force. However, a project prepared by the Inter-ministerial Committee for Food Control and Frauds Repression (Comité Interministériel pour le Contrôle Alimentaire et la Répression des Fraudes, CIPCARF) and the Moroccan Service of Industrial Normalization (Service de Normalisation Industrielle Marocaine, SNIMA) to protect both human and animal health is under discussion. The maximum limit for OTA in cereals set by the Moroccan project is 30 ng/g (FAO 2004). This value is higher than maximum residue level (MRL) fixed by European regulations for OTA in cereals and cereal products at levels that cannot be greater than 5 and 3 ng/g, respectively (European Commission 2005). The maximum limit of OTA established by the codex in cereals and cereal products is about 5 ng/g (JECFA 2001). Our findings showed that 15% of analyzed samples exceeded the maximum permitted level of OTA set by the European Union in cereals.

OTA in dried fruits

The incidence of OTA in dried raisins, dried figs, walnuts, and peanuts were 30, 65, 35, and 25% respectively (Figure 2). In pistachio samples, OTA was below the

quantification limit. Analytical results showed that OTA levels ranged from 0.10±0.05 to 2.36±0.75 in peanut, from 0.03±0.01 to 1.42±0.45 in dried figs, from 0.05±0.02 to 4.95±0.02 in dried raisins and from 0.04±0.01 to 0.23±0.05 in walnut. The average of OTA in positive samples of peanut, dried figs, dried raisins and walnut are 0.68±0.15, 0.33±0.12, 0.96±0.25 and 0.11±0.06 respectively. All positive samples of dried fruits were below the maximum tolerable level of OTA (10 ng/g) set by European regulations in dried vine fruits (FAO 2004). However, no maximum limit is set by the Moroccan project of mycotoxin regulations for OTA in dried fruits.

In comparison, previous UK surveys reported an incidence of OTA in raisins of 85% (MacDonald et al., 1999) and 97% (MAFF 1999), while a German survey (Engel 2000) reported a 95% overall incidence of OTA in raisins and currants. Data from Finland and France have indicated incidences of 71 and 46%, respectively (Miraglia & Brera, 2002). Recent data from Canada reported that OTA was present in 79% of samples of raisins (Lombaert et al., 2004). OTA was present in 95% of dried fig samples from Brazil and 26% contained levels higher than 5 ng/g, and samples were infected particularly with *A. niger* (Imanaka et al., 2005). In Turkey, Senyuva et al. (2005) reported the contamination of fig samples from the 2003 and 2004 crops with both OTA and aflatoxins. Bayman et al. (2002) reported high levels of OTA (up to 1850 ng/g) in figs grown in California. In the European Commission's 2002 Scientific Co-operation (SCOOP) project report, the analysis of 20 samples of fig imported to The Netherlands reported an incidence of 10% with a maximum concentration of 0.8 ng/g (Miraglia & Brera, 2002).

[Insert Figure 2 about here]

In Morocco, traditional techniques for the transformation and conservation of fruits are still used. Theses practices are very optimal conditions (especially temperature, humidity and fruits damages) for mould growth and mycotoxin production. The natural drying, which may consists in direct exposition of the fruit to the sun, is widely used especially in rural area. Fruits (raisins, figs etc.) having reached a sufficient degree of maturity are gathered and transported to drying places such as the terrace of house or a piece of ground fenced-off to prevent access of animals. These surfaces of drying are in general exposed to a maximum sunning and are papered with herbs to avoid the contact with the ground. Fruit are spread out over these surfaces without preliminary treatment. After drying, fruit are collected and stored. During the process of fruit drying, the sugar is concentrated as the moisture content decreases resulting in an almost selective medium for xerotolerant moulds such as *A. niger* section nigri species. Among black aspergilli, *A. carbonarius* is the most important as OTA producing isolate observed more frequently (Abarca et al., 2003; Sage et al., 2004). Other black aspergilli including the *A. niger* aggregate and A. *aculeatus* have also been found to produce OTA on grapes (Battilani et al., 2003). Recently, OTA production in *A. tubingensis* isolates originating from grapes was observed by Medina et al. (2005).

Conclusion

As far as we know, this is the first report that has shown the contamination of rice and dried fruits available in Morocco by OTA. The acceptable level of OTA which are fixed by European regulations was exceeded in 15% of analyzed samples of rice. It can be concluded that the occurrence of OTA in some dried fruits and rice samples is due to the fact that may be some food safety and quality standards (good agricultural practices

(GAPs), good manufacturing practices (GMPs), and the hazard analysis and critical control point (HACCP) system) need to be applied and performed in most of Moroccan food units to control growth of moulds and mycotoxin production during harvesting, distribution and storage periods. A survey of a large number of prepared food samples during theses periods needs to be investigated.

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Figure caption

Figure 1. LC fluorescence chromatograms of (A): standard solution of OTA at 0.05 ng/ml. (B): positive sample of rice. (C): a not contaminated sample of rice (< LOQ).

Figure 2: Percent distribution of OTA in different analyzed matrixes.

Table I. Recoveries and limit of quantification (LOQ) for OTA in different analyzed matrixes

Food commodity	Recovery (%)	LOQ (ng/g)
Rice	83.0	0.021
Peanut	93.6	0.027
Pistachio	86.8	0.027
Walnut	72.1	0.027
Dried figs	82.4	0.030
Dried raisins	75.5	0.030

Figure 1

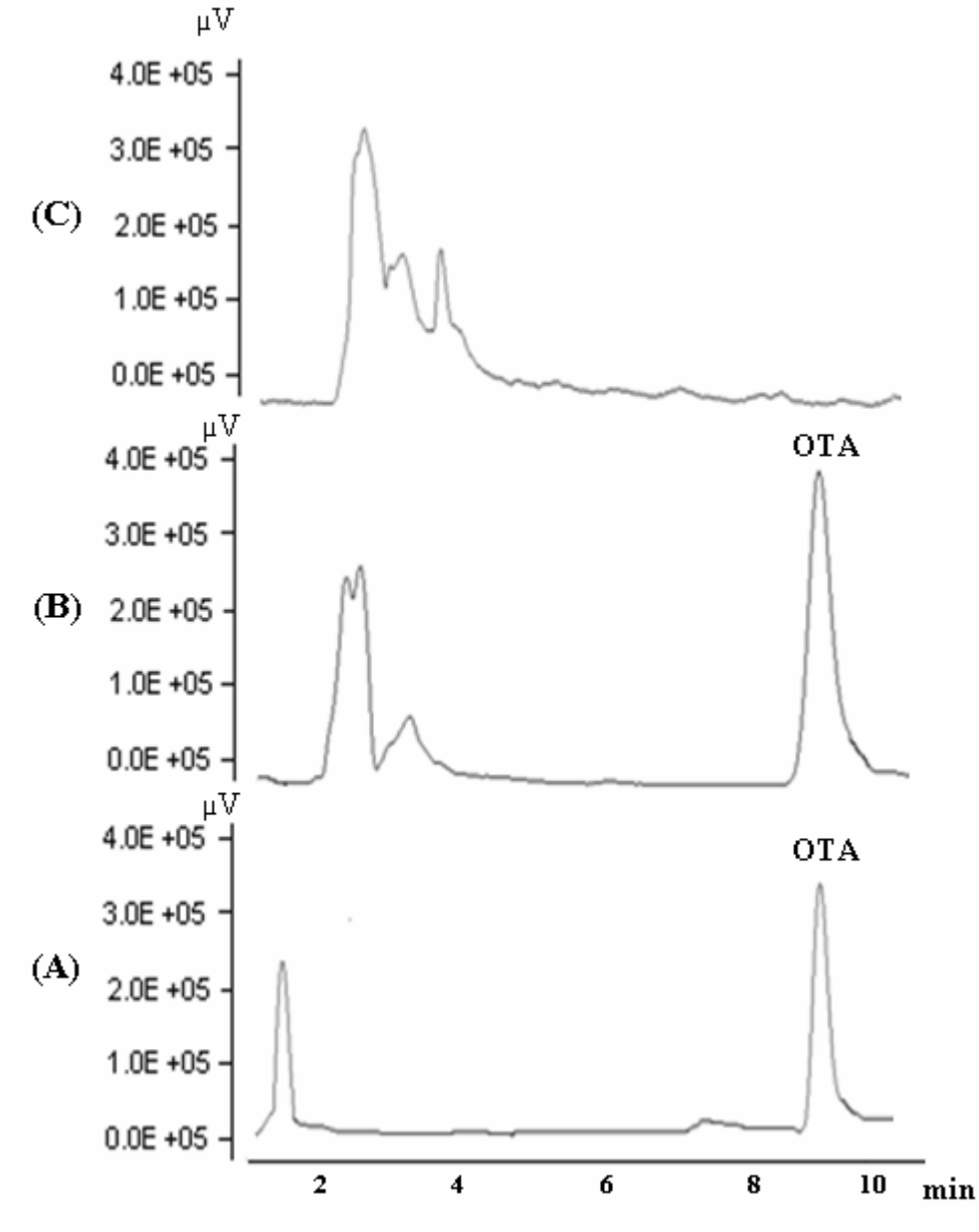

Figure 2