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Monitoring of ochratoxin A exposure of the Portuguese population through a nationwide urine survey — Winter 2007

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by a variety of fungi, such as *Penicillium verrucosum* and *Aspergillium* spp., which has been found to have a wide number of potentially deadly toxic effects, and can enter the human organism through a variety of means. It then finds its way into the bloodstream and, after a lengthy process, is eventually excreted through the urine. It can thus be detected in its original form not only in blood samples but also in this biological medium. As such, and in an attempt to evaluate the exposure of the Portuguese population to this mycotoxin, morning urine samples were collected during the Winter of 2007, from each of five geographically distinct Portuguese locations — Bragança, Porto, Coimbra, Alentejo, and Algarve — and subjected to extraction by immunoaffinity columns and to OTA quantification through liquid chromatography coupled with fluorescence detection. Prevalent incidence was higher than 95% with Coimbra being the exception (incidence of 73.3%). In nearly all locations, the OTA content of most samples was found to be above the limit of quantification (LOQ) of 0.008 ng/ml. Indeed, excluding Coimbra, with an OTA content level of 0.014 ng/ml, all regions featured content values over 0.021 ng/ml.

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1. Introduction

OTA (structure in Fig. 1) is an UV-decomposable mycotoxin which has been proven to have a variety of toxic effects, including nephrotoxic — having been linked to the Balkan Endemic Nephropathy (Vrabcheva et al., 2004) — immunotoxic (Harvey et al., 1992), carcinogenic (IARC, 1993), teratogenic (Wangikar et al., 2005), while also being a possible genotoxic. It is produced, especially when in less than optimal growing conditions, by the fungi *Penicillium verrucosum* and *Aspergillium* spp. mainly in temperate and tropical climates, respectively (Eskola, 2002).

These fungi are often found growing in such items as cereals, grains, beans, and fruits, and thus the mycotoxin finds its way easily into the human organism through a variety of media, ranging from direct consumption to inhalation, including indirect consumption by ingestion of animal products tainted by virtue of contaminated feed (Clark and Snedeker, 2006; Duarte et al., 2009a). As both the fungi and the consumption of the foodstuffs they so often grow on are widespread on a global scale, OTA can be found practically anywhere and merits worldwide concern. As such, monitoring OTA exposure in populations is of crucial importance.

Two major ways of determining OTA exposure have been described: testing the aforementioned foodstuffs for occurrence, and direct analysis of biological samples. The former, however, tends to be less accurate due to the fact that only an estimation can be made regarding the eating habits of a population, an estimation that, moreover, results in an average value, failing to account for those who would fall in the high end of the contamination scale — exactly those who are in greater risk (Turner et al., 2008).

As far as biological samples are concerned, OTA detection has been performed both by testing for the mycotoxin or one of its subunits directly and by detection of its toxicological effects, such as β2-microglobulinuria (Hassen et al., 2004). The latter, however, requires already unhealthy levels of OTA contamination and has little to no use in prevention procedures. Furthermore, it is also highly non-specific — for instance, most subjects suffering from nephropathy, regardless of cause, tend to show increased β2-microglobulin levels.

Direct testing has been performed mainly in muscle tissue, plasma/serum, and urine. Muscle tissue analysis, for obvious reasons, has been applied only to animal subjects, post-slaughter. Between the remaining two sample types, urine presents several advantages as opposed to plasma/serum testing. These advantages begin to appear as soon as the collection process, with urine collection being a non-invasive procedure. OTA levels in urine have also been shown to correlate better to ingested amounts than those in plasma/serum,

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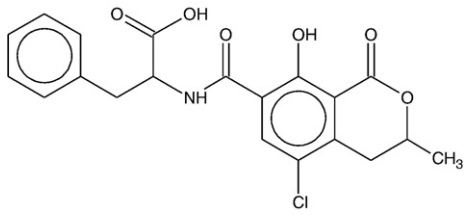

Fig. 1. Chemical structure of ochratoxin A.

which are also more prone to possess endogenous contaminants (Gilbert et al., 2001; MAFF UK, 1999; Costa, 2008). Also, though urine levels are lower than those of plasma/serum, continuous advances in analytical methodologies have proven capable of offsetting the difficulties thus arisen. Furthermore, a lack of knowledge concerning the toxicokinetics of OTA renders the plasma monitoring approach more adequate for assessing relative amounts of contamination rather than providing any quantitative information (MAFF UK, 1999).

In the specific case of Portugal, previous studies have found a worrying degree of OTA incidence in bread in several regions (Bento et al., 2009; Duarte et al., 2009b; Juan et al., 2007, 2008), a foodstuff that is both an integral part of the staple diet and has been indicated as one of the most important contributors to OTA intake for humans (Miraglia and Brera, 2002). As such, further data was deemed necessary to glean information concerning the contamination levels of the Portuguese general population.

The goal of this study was to monitor the OTA exposure of the Portuguese population of different regions through the evaluation of its detection frequency and levels in urine during the winter of 2007, and, by comparing obtained data to that already existing, attempt to ascertain whether those levels present an appreciable health risk to the national population, as well as if such risk is gender-dependent.

2. Materials and methods

2.1. Sampling

A total of 155 morning urine samples were collected, during the winter of 2007, from 67 male and 88 female volunteers along with signed disclaimers of their informed consent. Collection took place in several Portuguese regions, namely Alentejo, Algarve, Bragança, Coimbra, and Porto, after which the samples were frozen until extraction. The anthropometrics, detailed by region, are summarised in Table 1.

2.2. Reagents and equipment

The reagents acetonitrile (Carlo Erba, Milan, Italy), methanol (Panreac Química Sau, Barcelona, Spain), toluene (Barker , J. T. Barker, Holland) and benzene (HACH Company, USA) were all of HPLC grade.

Boron–trifluride–methanol 14% solution and acetic acid were purchased from Sigma-Aldrich (Laborchemikalien, Germany), hydrochloric acid, sodium hydroxide, potassium chloride, potassium dihydrogenphosphate and anhydrous disodium hydrogenphosphate from Merck (Darmstadt, Germany), and sodium chloride from Baker Ltd. (Pagenham, England), all of analytical grade.

Filter paper Whatman N°4 (150 mm, Whatman International Ltd. Maidstone, England) and polyamide membrane filters (0.2 µm, 50 mm, Whatman GmbH, Dassel, Germany) were used. Immunoaffinity columns (IAC) Ochratest™ were acquired from VICAM (Watertown, USA).

Water was obtained daily from a Milli-Q System (Millipore, Bedford, MA, USA).

The OTA standard was obtained from Sigma Chemical Co. (St. Louis, MO, USA) with ≥98% purity.

A vacuum manifold of Macherey-Nagel (USA), a pump of Dinko (mol. D-95, 130 W, 220 V), a magnetic stirrer (Agimatic-S, Selecta, Barcelona, Spain), a Retsh vortex mixer (Haan, Germany), and a Sonorex RK 100 ultrasonic bath (Berlin, Germany) were used.

The LC instrument was equipped with a pump (Model 307, Gilson Medical Electronics, Villiers-le-Bel, France), and a guard column Hichrom Ltd, HI-173 (30 mm × 4 mm i.d.) (England) preceding a Hichrom C_{18} column (5 µm, 250 mm × 4.6 mm i.d.). Detection and confirmation were performed on a Perkin-Elmer Model LS45 spectrofluorimeter (Beaconsfield, UK). The results were recorded on a Hewlett–Packard 3390A integrator (Philadelphia, PA, USA).

The mobile phase was a vacuum-filtered solution of acetonitrile/water/acetic acid (49.5:49.5:1.0, v/v/v).

The OTA standard stock solution was prepared by diluting 1 mg of OTA from *A. ochraceus* in 4 ml of toluene:acetic acid (99:1) at 250 µg/ml, and stored at −20 °C. The intermediate solutions were prepared at 10 µg/ml and 1 µg/ml, in toluene:acetic acid, and a working standard solution at 0.01 µg/ml, in mobile phase. The calibration curve standard solutions were prepared between 1 and 10 ng/ml in mobile phase.

Phosphate-buffered saline (PBS) solution was prepared by diluting 0.2 g potassium chloride, 0.2 g potassium dihydrogenphosphate, 1.2 g anhydrous disodium hydrogen phosphate, and 8 g sodium chloride in 1 l of distilled water, with pH adjusted to 7.4 through the use of 0.1 M HCl or 0.1 M NaOH.

All liquid chromatographic reagents were degassed for 15 min in an ultrasonic bath prior to use. For decontamination, glassware was washed with a sodium hypochlorite solution and then immersed in 4 ml/l H_2SO_4. It was returned to neutral pH through rinsing with distilled water.

2.3. Experimental procedure

The methodology used for ochratoxin A content determination was based on that described by Pena et al. (2006).

Briefly, 10 ml of the sample were mixed with 10 ml of 5% $NaHCO_3$, and then filtered. The filtrate was cleaned-up through the IAC at a flow rate of 1 drop per second. The column was then washed with 2 × 5 ml of distilled water, and afterwards OTA was eluted with 3 ml methanol. The eluate was dried in a bath at 50 °C under a gentle nitrogen flow, and the dried extract was stored at −20 °C. Before injection, the dried extract was dissolved in 125 µl of mobile phase.

LC-FD analysis was performed using a vacuum-filtered solution of acetonitrile/water/acetic acid (49.5:49.5:1.0, v/v/v) as mobile phase, flowing at a 1 ml/min rate. Wavelengths used were 333 nm for excitation and 460 nm for emission, both with a spectral bandwidth of 10 nm. An OTA working standard solution of 0.01 µg/ml was analyzed between each sample. All injections were of a volume of 20 µl.

Table 1
Anthropometric data of the populations.

Region (size)	Gender		Age (years)		Stature (cm)		Weight (kg)	
	Women	Men	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
Bragança (n = 30)	19	11	[22;65]	40.8 ± 12.7	[150;192]	168.4 ± 9.7	[48;110]	69.1 ± 14.8
Porto (n = 30)	15	15	[18;83]	49.6 ± 18	[155;183]	169.3 ± 7.2	[47;90]	67.9 ± 10
Coimbra (n = 30)	18	12	[22;80]	39 ± 15.6	[150;188]	169 ± 8.6	[51;98]	67 ± 10.4
Alentejo (n = 40)	22	18	[23;96]	47.5 ± 16.0	[150;182]	163.8 ± 8.1	[52;95]	70.5 ± 10.8
Algarve (n = 25)	14	11	[20;82]	48 ± 15.0	[148;180]	166.2 ± 8.6	[49;120]	73.3 ± 15.8
Total (n = 155)	88	67	[18;96]	45.1 ± 16	[148;192]	167.1 ± 8.6	[47;120]	69.5 ± 12.4

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