

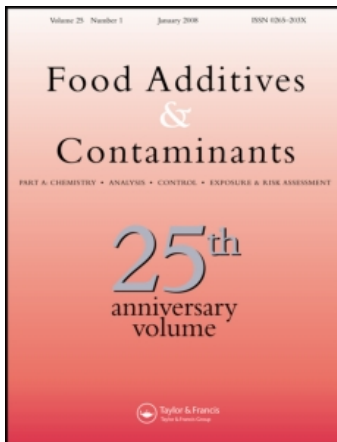
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Ochratoxin A exposure assessment of the inhabitants of Lisbon during winter 2007/2008 through bread and urine analysis

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A survey on the occurrence of ochratoxin A (OTA) in 41 bread samples was carried out in the Portuguese capital, Lisbon. Maize (5) and wheat bread (36) and 43 representative urine samples from the Lisbon region were assayed for OTA levels using immunoaffinity column cleanup (IAC) and HPLC with fluorimetric detection (LC–FD). The percentage of OTA-positive samples was slightly higher for maize bread (80%) than wheat bread (70.8%), although, due to its higher consumption, the latter contributes more to OTA exposure, featuring a higher estimated daily intake (EDI). In the urine samples analyzed, both female and male residents displayed similarly high levels of OTA frequency and average contamination. In summary, OTA is a food contaminant of concern and may constitute a hazard for public health through consumption of cereal-based products.

Keywords: exposure; HPLC; mycotoxins; ochratoxin A; bread

Introduction

The attention of public health authorities and toxicologists around the world has recently been focused on the systematic control of xenogenous substances in foodstuffs which might endanger the health of the population. This is the case of mycotoxins – toxic secondary metabolites of mycelial microscopic fungi, moulds (Malir et al. 2001) – of which ochratoxin A (OTA) is an example. Since its discovery in 1965, considerable efforts have been devoted to its study and it is currently regarded as nephrotoxic, immunotoxic and genotoxic (Schilter et al. 2005). Additionally, since 1993, it has been included in group 2B (possible human carcinogens) by the International Agency for Research on Cancer (IARC) of WHO, based on sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans. In several areas of Eastern Europe, where chronic exposure to OTA occurs, the involvement of this mycotoxin in cancer etiology of the urinary system and in kidney pathologies, typically Balkan endemic nephropathy (BEN), has long been suspected. Studies on the correlation between OTA and BEN (Vrabcheva et al. 2000; Puntarić et al. 2001) have shown higher OTA contamination level in cereals from endemic versus non-endemic areas (Palermo et al. 2002). Hence, most countries and organizations have established maximum permitted levels in foodstuffs to reduce the

exposure of its citizens to this contaminant. Additionally, tolerable intakes have been recommended, specifically of 120 ng/kg bw/week (EFSA 2006) and 100 ng/kg bw/week (JEFCA 2007). For their part, Canadian authorities proposed a TDI of 1.2–5.7 ng/kg bw/day (Kuiper-Goodman 1996). The Nordic Working Group on Food Toxicology and Risk Evaluation (1991) has suggested a maximum TDI of OTA by humans of 5 ng/kg body weight, based on carcinogenicity studies with OTA in adult rats. The Scientific Committee on Food also proposed that exposure should be below 5 ng/kg bw/day (SCF 1998).

The importance of OTA is further underlined by its global occurrence in a large variety of commodities of both plant and animal origin. Cereals and their derived products, assumed to be the major dietary source of OTA, are some of the most widely consumed foodstuffs. Of these, bread is the most popular yeast-leavened cereal product by far, providing more nourishment for humans than any other food source (FAO 2002). Even today, the importance of bread is stated by its symbolic power (solidarity), its existence in every civilization, and its major role in many religions, popular festivities and in most family meals worldwide (www.world-bread-day.com 2008).

This universal product can be made of different types of cereal grain, characterized by the many

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fermentation processes and the different ways of baking, a wide variety of sizes, shapes, textures and tastes, depending on the agricultural, cultural and eating habits of the populations (González-Osnaya et al. 2007, www.world-bread-day.com 2008). By far the most consumed type is wheat bread. In Portugal, the latest estimate of average bread consumption is from 1994, with a level of 32 kg/person/year. Maize bread, a traditional Portuguese type of bread, is estimated to represent a quarter of the total nationwide bread consumption (Instituto Português do Consumidor 2005). The famous and traditional “Mafra” bread is a regional wheat bread, sold in different shapes and sizes, but always with the same dough made of wheat flour, water, yeast and salt. Although typically from a certain region of Lisbon, most of the shops, bakeries and supermarkets in the capital sell this kind of bread.

To study the OTA exposure, different approaches have been employed. OTA levels in plasma and urine are considered typical biomarkers of exposure, and no other appropriate effect-related biomarkers have as yet been identified (Pacin et al. 2008). Although elimination in urine is considered the main route of OTA excretion from the human body, urinary monitoring is relatively unexplored due to the low concentrations involved (Pena et al. 2006) and due to the fact that, opposed to its content in plasma, it is not possible to relate concentrations or total amount of OTA excreted in urine to exposure. Recent developments in analytical methodology have resulted in more practical urinary monitoring, which has the advantage of being less invasive than blood monitoring. However, data on the frequency and concentration of OTA in human urine remain scarce (Pena et al. 2006). In a 1-month duplicate diet study in the UK, OTA in urine was found to be a better indicator of OTA intake than plasma, although levels in urine were two orders of magnitude lower; there was no significant correlation between plasma concentrations and OTA intake (Gilbert et al. 2001). It is thus suggested that urine could provide a more useful marker of OTA intake than plasma (Pascale and Visconti 2001).

Compared to food products, urine has an additional advantage as a target for OTA exposure quantification. The large variability in contamination levels and sampling difficulties means that large numbers of individual food samples need to be analyzed. Estimates of exposure based on determining OTA in food products will inevitably be vulnerable to large sources of error. Furthermore, the fact that estimated exposure levels use methods based on food intake/analysis, which are inherently inaccurate, appears to indicate that exposures close to the TDI require a better approach for assessing OTA exposure. Initial studies have used plasma levels to estimate intake, but the study of Gilbert et al. (2001) suggests

that this may not be the most suitable method. A disadvantage of urine analysis in assessing OTA exposure is the need to define further the relationship between consumption and excretion to estimate actual intake levels. Further work is needed to verify if urine concentrations are a short- or a long-term indicator of exposure to this mycotoxin (Manique et al. 2008). According to Gilbert et al. (2001), the correlation might be stronger if comparisons were made between urine excretion and previous day intake.

The present study aims to estimate the OTA contamination levels of different types of bread, a widely consumed foodstuff, in the Lisbon region during the winter of 2007/2008. Moreover, Lisbonians’ exposure to OTA was evaluated, based on bread intake and the appearance of the toxin in urine.

Materials and methods

Bread and urine sampling

The present study was carried out on samples collected in the Portuguese capital city, Lisbon, located on the country’s central western coast. The samples were collected during the winter of 2007/2008, between November 2007 and March 2008.

The bread samples, consisting of 24 units of regular wheat bread, 12 units of regional Mafra wheat bread and five units of maize bread, were commercially acquired from random bakeries and supermarkets located in the Lisbon region. After purchase, the samples were brought to the laboratory under ambient condition for milling. All information on the samples was obtained from the respective labels. Each milled sample, stored in sealed plastic bags, was frozen at -20°C , and kept in this condition until analysis.

The 43 urine samples were collected from healthy individuals residing in the same area where the bread samples were sold. From the 43 local participants involved, 48.8% ($n=21$) were female, aged 18–69 years old, and 51.2% ($n=22$) were male, aged 23–75 years old. The sampled population is characterized in Table 1.

Reagents

HPLC-grade acetonitrile (Carlo Erba, Milan, Italy), methanol (Panreac Química Sau, Barcelona, Spain), toluene (Baker Analyzed; JT Baker, Deventer, Holland) and benzene (HACH Company, Loveland, CO, USA) were used. Analytical grade boron-trifluoride-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 supplied

Table 1. Anthropometric data of the sampled population.

Gender	Size	Weight (kg)		Age (years)		Height (cm)	
		Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD
Female	48.8% (21/43)	[45;89]	65.67 \pm 11.59	[18;69]	45.95 \pm 15.63	[150;170]	157 \pm 7
Male	51.2% (22/43)	[62;108]	77.5 \pm 10.47	[23;75]	46.09 \pm 13.61	[170;180]	171 \pm 7
Total	100% i.e. 43	[45;108]	71.72 \pm 12.44	[18;75]	46.02 \pm 14.45	[150;180]	164 \pm 10

by Merck (Darmstadt, Germany), and sodium chloride was purchased from Baker Ltd. (Pagenham, UK).

The OTA standard was acquired from Sigma Chemical Co. (St. Louis, MO, USA) with $\geq 98\%$ purity. OTA standard stock solutions were prepared by diluting 1 mg OTA from *A. ochraceus* in 4 ml toluene/acetic acid (99:1, v/v) at 250 $\mu\text{g}/\text{ml}$ and stored at -20°C . The intermediate solutions were prepared at 10 and 1 $\mu\text{g}/\text{ml}$ in toluene/acetic acid, and working standard solutions at 0.1 and 0.01 $\mu\text{g}/\text{ml}$ in mobile phase. Calibration curve standard solutions were prepared in mobile phase at concentrations between 0.001 and 0.01 $\mu\text{g}/\text{ml}$.

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

Equipment

Whatman No. 4 filter paper (150 mm; Whatman International Ltd., Maidstone, UK) and polyamide membrane filters (0.2 μm , 50 mm; Whatman GmbH, Dassel, Germany) were used. Immunoaffinity columns (IAC) OchratestTM were from VICAM (Watertown, MA, USA).

A Braun MR 5000M multiquick/minipimer 500 W (220–230 V, 50–60 Hz; Esplungues del Llobregat, Spain), a Moulinex blender 700 W (230–240 V, 50–60 Hz; Barcelona, Spain), a Macherey-Nagel vacuum manifold (Düren, Germany), a Dinko pump (mol. D-95, 130 W, 220 V), a magnetic stirrer (Agimatic-S; Selecta, Barcelona, Spain), and a Retsh vortex mixer (Haan, Germany) were employed. A Sonorex RK 100 ultrasonic bath (Berlin, Germany) was used for 15-min degassing of all chromatographic solvents prior to use, and also for cleaning and decontaminating the material used in the experiments.

The LC instrument incorporated a pump (Model 307; Gilson Medical Electronics, Villiers-le-Bel, France), and a Hichrom HI-173 guard column (30 \times 4 mm I.D.; Reading, England) preceding a Hichrom C₁₈ column (5 μm , 250 \times 4.6 mm I.D.). Detection and quantification were performed on a Perkin-Elmer Model LS45 spectrofluorimeter (Beaconsfield, UK), and results were recorded on a Hewlett-Packard 3390A integrator (Philadelphia, PA, USA).

Experimental procedure

The methodologies used for OTA determination were based on those described by Juan et al. (2007) and Pena et al. (2006) for bread and urine samples, respectively.

Briefly, for OTA extraction from the bread matrix, 100 ml of PBS/methanol (50:50, v/v) were added to 20 g of milled bread and homogenized (5 min) and filtered. Then, 20 ml of the resulting filtered solution were diluted in 30 ml of PBS before passage through the IAC at a vacuum-induced rate of 1 drop per second. The IAC was then washed with 10 ml of water prior to elution with 3 ml of methanol.

For extraction from the urine matrix, 10 ml of the sample were mixed with 10 ml of 5% NaHCO₃ and then filtered. The filtrate was cleaned-up through the IAC at a flow-rate of about 1 drop per second. The column was then washed twice with 5 ml of distilled water, and the OTA eluted with 3 ml methanol.

The eluates were dried in a bath at 50°C under a gentle nitrogen flow, and dried extract stored at -20°C . Before injection, the dried extract was dissolved in 250 or 125 μl of mobile phase, for bread or urine samples, respectively.

LC–FD analysis was performed using a vacuum-filtered solution of acetonitrile/water/acetic acid (49.5:49.5:1.0, v/v) as mobile phase, at a flow-rate of 1 ml/min. 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 $\mu\text{g}/\text{ml}$ was analyzed at the beginning of each run. A 20 μl injection volume was used for both bread and urine extracts.

Analytical quality assurance

OTA method revalidation was performed by spiking an OTA-free wheat bread sample, in triplicate, over 3 days at fortification levels of 0.1, 0.5 and 2 ng/g. After fortification, the sample was left to stand in the dark for 15 min, after which the Juan et al. (2007) protocol was followed.

The fortification assays on urine, in triplicate of a blank sample and over three different days, were performed at 0.02, 0.1 and 0.5 ng/ml. After fortification,

the sample was left to stand in the dark for 15 min, after which the Pena et al. (2006) protocol was followed.

OTA presence in bread samples was confirmed as described by Guillamont et al. (2005) and Pena et al. (2005), by conversion to its methyl ester form, adding 150 μ l of boron trifluoride methanolic solution ($\text{BF}_3\text{-CH}_3\text{OH}$, 14%) to the dried sample extracts and evaporating the mixture at 60°C for 10 min, before reconstitution in 250 μ l of mobile phase. For urine samples, the method described by Castegnaro et al. (1990) was used, differing from the previous in its lower reconstitution volume of 150 μ l.

The OTA methyl esters of both samples were analyzed according to the LC conditions described above.

Results and discussion

Analytical performance

Linearity for OTA quantification was verified by plotting OTA peak areas against calibration samples (concentrations of 1, 2, 5 and 10 ng/ml). The resulting calibration curve had a coefficient of correlation of 0.9998.

Recovery values for bread ranged from 76.7 to 103.7% at fortification levels of 2.0 and 0.1 ng/g, respectively, with intra-day repeatabilities ranging from 3.6 to 14.4%, for levels of 0.5 and 0.1 ng/g, and inter-day ones from 7.2 to 12.9%, at the levels of 0.5 and 2.0 ng/g.

For urine, recovery values ranged from 90.6 to 96.0% at the 0.02 and 0.5 ng/ml fortification levels. Intra- and inter-day repeatabilities varied between 2.9 and 5.4%, and 4.3 and 8.9% for fortification levels of 0.5 and 0.02 ng/ml, respectively.

The results for accuracy and precision, for both bread and urine, were in agreement with the EC directive No. 401/2006 (EC 2006).

Determination of LOQs was performed by spiking blank samples with OTA standard solutions. Repeatable precision and trueness were achieved at minimum concentrations of 0.1 ng/ml and 0.008 ng/ml for bread and urine samples, respectively, thus setting the LOQs at those values.

As shown by the chromatograms displayed in Figure 1, there are no interfering peaks near the retention time of OTA (11.30 min) in either bread or urine samples. The different retention time of OTA and its methyl ester, and the almost complete disappearance of the OTA peak and appearance of a new peak at 27.30 min, confirmed the presence of OTA in the sample (Figure 1).

Occurrence of OTA in bread

As shown in Table 2, of the 41 bread samples analyzed from Lisbon, 68.3% contained OTA, and 51.2% showed OTA levels above the LOQ.

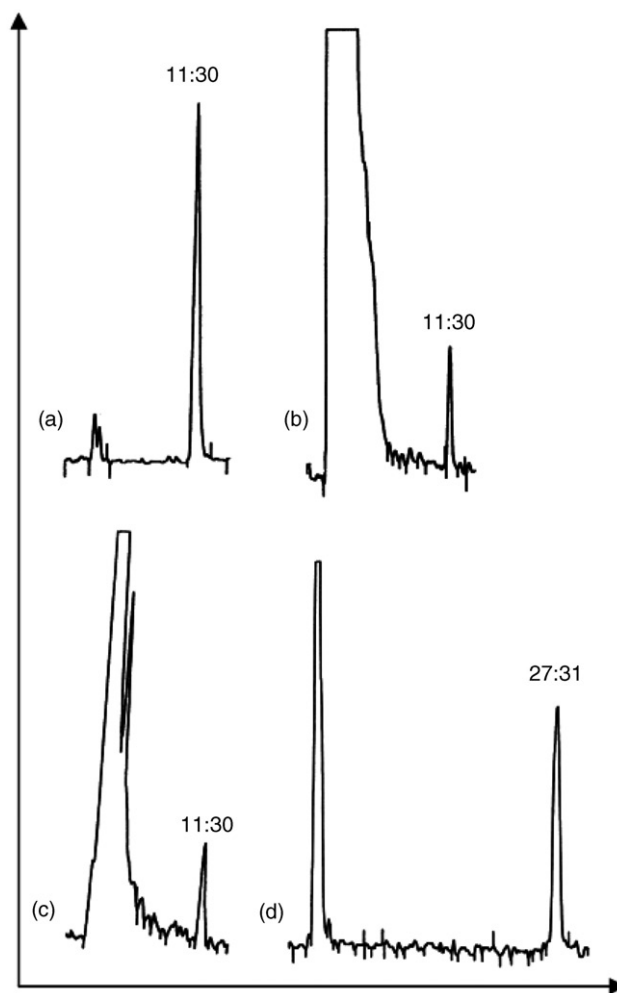


Figure 1. LC-FD chromatograms from (a) OTA standard solution (5 ng/mL; RT 11.30 min), (b) bread sample naturally positive to OTA (RT 11.30 min), (c) urine sample naturally positive to OTA (RT 11.30 min) and (d) an OTA methyl ester peak after boron fluoride-methanol derivatization of a wheat bread sample (RT 27.31 min).

The incidence was found to be high in both maize and wheat bread, and nearly all positive samples featured OTA content levels above the LOQ. In both instances, values were slightly higher, though not significantly so, in maize bread. Similarly, average contamination levels were also more elevated in maize bread than in common wheat bread (0.28 versus 0.21 ng/g). On the other hand, less than half of the Mafra bread samples contained detectable levels of OTA, and none of those featured OTA levels quantifiable through this method.

Similar results were detected in studies of other Portuguese regions (Juan et al. 2007; Bento et al. 2009). A notable exception is that of Juan et al. (2008), whose study found much lower incidence and contamination levels in wheat bread. A possible explanation for this discrepancy may lie in the fact that the samples in this

Table 2. Prevalence (%) and mean levels (ng/g) of OTA in different types of bread samples.

Sample ^a	Sample size	≥LOQ ^b (%)	≥LOD (%)	Range (ng/g)	Mean ^b ± SD	Mean ^c ± SD
Maize bread	5	4 (80)	4 (80)	n.d.–0.36	0.28 ± 0.09	0.22 ± 0.14
Wheat bread	24	17 (70.8)	19 (79.2)	n.d.–0.41	0.21 ± 0.09	0.15 ± 0.12
Mafra-type wheat bread	12	0 (0)	5 (41.7)	<0.1	n/a	0.02 ± 0.03
TOTAL	41	21 (51.2)	28 (68.3)	n.d.–0.41	0.22 ± 0.09	0.12 ± 0.12

Notes: ^aBread type.

^bSamples ≥ LOQ (≥0.1 ng/g) only.

^cAll samples.

Table 3. Compilation of this and international studies on OTA in maize and wheat grains and derivate derived breads.

Country	Sample type, number	Incidence	Mean ± SD	Range (µg/kg)	Source
Italy	Maize, 70 (1999/2000)	27.1%	1.7 ± 1.6	0.2–5.2	Palermo et al. (2002)
	Wheat, 70 (1999/2000)	8.6%	0.8 ± 0.3	0.4–1.4	
Croatia	Maize, 49	38.7%	1.47 ± 0.38	0.9–2.54	Domijan et al. (2005)
Ivory Coast	Maize, 16 (1998)	100%	44 ± 12	27–64	
	Maize, 15 (2001)	100%	266 ± 428	3–1738	
	Maize, 10 (2002)	100%	37 ± 25	9.8–86	
	Maize, 10 (2002)	100%	–	0.16–0.86	
Turkey	Corn bread, 10	–	4.94 ± 0.20	4.08–5.28	Sangare-Tigori et al. (2006b) Cengiz et al. (2007)
	Corn flour, 10	–	6.39 ± 1.10	3.86–15.67	
	White bread, 25	–	3.36 ± 0.73	n.d.–11.40	
	White flour, 22	–	6.89 ± 0.46	1.87–7.44	
	Whole meal bread, 28	–	7.84 ± 0.39	3.54–12.61	
	Whole meal flour, 14	–	9.30 ± 1.33	1.07–16.70	
Spain	Organic wheat bread, 20	20%	–	0.03–0.81	González-Osnaya et al. (2007)
	Other organic cereals bread, 6	33.3%	–	0.39–0.81	
	Conventional wheat bread, 67	19%	–	0.04–10.81	
	Other conventional cereals bread, 7	14.3%	–	2.59	
Portugal	Maize bread, 15	60%	0.43	–	Juan et al. (2007)
	Maize bread, 30	70%	0.44	–	
	Wheat bread, 31	12.9%	0.02	–	Bento et al. (2009)
	Wheat bread, 30	80%	0.2 ± 0.1	n.d.–0.49	
	Wheat bread, 20	65%	0.3 ± 0.14	n.d.–0.43	
	Maize bread, 5	80%	0.28 ± 0.09	n.d.–0.36	
	Wheat bread, 24	79.2%	0.21 ± 0.09	n.d.–0.41	
	Mafra wheat bread, 12	41.7%	n/a	<0.1	

study originated from a different area and period, and were thus subject to different conditions during processing and storage, as well as different edafo-climatic influences during crop growth.

Nonetheless, data from around the world (Table 3) categorize Portuguese bread as the least contaminated. Since some of the data in Table 3 refers to the cereal grain, it is possible that processing methods result in a similar OTA content in the bread of some of those countries, namely Italy and Croatia.

These studies concur with ours in that both maize grains and their derivatives tend to feature higher frequencies of OTA contamination than their wheat counterparts. In the specific case of bread, Cengiz et al. (2007) detected statistically significant differences between average OTA levels in white and corn bread samples, corn and whole meal bread samples, and corn and corn whole meal samples, with the latter in each case featuring higher levels. Zummo and Scott (1992) demonstrated that maize

(*Zea mays*) has an ideal nutrient composition for fungal development and is, thus, constantly exposed to the risk of fungal contamination. Moreover, tropical and subtropical countries, a few of which are among the world's principal producers of maize, have favorable environmental conditions for the development of the main types of genotoxicant fungi, *Aspergillus*, *Fusarium* and *Penicillium* (Sekiyama et al. 2005). However, OTA quantification studies offer contradictory results on the subject.

A study from Brazil (Sekiyama et al. 2005) found a single sample of corn flour (0.8%) with an OTA content of 64 µg/kg, while another (Machinski et al. 2001) found two samples (1.8%) with contamination levels of 128 and 206 µg/kg, values that exceed the maximum permitted legal limit. Yet another (Caldas et al. 2002) found no samples with contamination levels above their limit of detection (25 µg/kg).

In Argentina, however, a different study (Paolo and Tosi 1998) found a high content of OTA

(1250–2500 µg/kg) in all the analyzed maize-derived foodstuffs, though non-processed maize featured consistently higher levels of contamination. Food processing, thus, does contribute slightly to decontamination, though it does so in a clearly insufficient level.

The cited Brazilian results corroborate the low levels of OTA contamination in Brazilian maize. On this subject, it is worth noting that Argentina is significantly colder than Brazil and, though OTA-producing fungi favor warm and wet environments, mycotoxin production seems to be encouraged by cold wet climates (Eskola 2002). Yet, the Ministry of Agriculture, Fishing and Food UK found that crude maize samples imported from Argentina – as well as from a number of European countries – featured contamination levels no higher than 1.5 µg/kg, and only in 10.1% of the samples. It is, however, possible that produce destined to export is subjected to tighter controls and the most contaminated samples are discarded.

Conversely, other studies reveal higher mean levels of OTA in wheat than in maize, irrespective of their ripening season, as in BEN endemic regions (Puntarić et al. 2001). This high contamination of wheat cereal can be explained by the fact that glutamic acid is incorporated into OTA during its production, and so a high content of this amino acid in cereals, typical in wheat, could be a cofactor for the presence of OTA, as suggested by González-Osnaya et al. (2007).

Table 4. Adult estimated daily intake (EDI) and weekly intake (EWI) of OTA, according to the type of bread.

Bread type	EDI ^a (ng/kg bw/day)	% TWI ^b
Maize bread contribution	0.09	0.54
Wheat bread contribution	0.21	1.22
Mafra-type wheat bread contribution	0	n/a
Total	0.30	1.76

Notes: ^aEDI was calculated using the equation $EDI = (\sum c) / (N \cdot D \cdot K)$ (CN⁻¹ D⁻¹ K⁻¹), where $\sum c$ is the sum of OTA concentration in the analyzed samples, C is the mean annual intake estimated, N is the total number of analyzed samples, D is the number of days in a year, and K is the mean body weight, which was considered 65 kg (mean of body weight of the studied population).

^bAccording to EFSA-established TWI (120 ng/kg bw/week).

Furthermore, and according to Laca et al. (2006), OTA level differences between cereals depend on multiple factors, such as grain condition at harvest, care in grain drying and storage conditions (Gonzalez-Osnaya et al. 2007).

Taking into account the latest data, which indicates that a Portuguese citizen has an average adult body-weight of 65 kg (Miraglia and Brera 2002) and an average bread consumption of 32 kg/year (Instituto Português do Consumidor 2005), consisting of 25% maize and 75% wheat bread, the estimated contributions to OTA daily intake are 0.09 and 0.21 ng/kg bw/day for maize and wheat bread, respectively (Table 4).

It should be noted that, although maize bread tends to feature higher levels of contamination, the much higher consumption of wheat bread results in the exposure of Lisbonians to higher OTA levels through the consumption of the latter, 0.21 versus 0.09 ng/kg bw/day for maize bread, well below the values established by the SCF in 1998 (5 ng/kg bw/day) and the JECFA in 2001 (17 ng/kg bw/day). These values are lower than those recorded by Leblanc et al. (2005) for the French population, and by the SCOOP report for Spanish (0.77) and German (0.36) inhabitants (Miraglia and Brera 2002). Conversely, the values of the EDI in the present study are similar to those calculated for Danish residents (0.19) by the same SCOOP report. Furthermore, the EDI from wheat bread, 0.21 ng/kg bw/day, represents over 25% of the national average, and support the major contribution of bread, in particular, and cereals, in general, to OTA exposure.

Mafra bread, although also made of wheat, did not present any detectable levels of OTA, and so does not contribute to OTA exposure. This difference is likely due to the fact that, as previously mentioned, while Mafra bread is, as a rule, baked with only wheat flour, water, yeast and salt, the more common wheat bread also tends to include other components that generally lead to an increase in OTA, such as seeds, bran, etc. (Scudamore et al. 2003; Juan et al. 2008), and even flour made from other cereals (Czerwiecki et al. 2002).

Occurrence of OTA in urine

The present study shows an elevated incidence of OTA in the urine of Lisbonians (Table 5). Incidence

Table 5. Prevalence (%) and mean levels (ng/ml) of OTA in urine.

Gender	Size	Incidence	≥LOQ*	Mean ± SD	Range
Females	48.8% (21/43)	71.4% (15/21)	57.1% (12/21)	0.027 ± 0.015	n.d–0.055
Males	51.2% (22/43)	72.7% (16/22)	68.2% (15/22)	0.025 ± 0.020	n.d–0.071
Total	100%	72.1% (31/43)	62.8% (27/43)	0.026 ± 0.017	n.d–0.071

Note: *≥0.008 ng/ml.

Table 6. Occurrence of OTA and/or its metabolites in urine in different countries.

Country	Biomarker	Sampled population, number	Incidence	Range (mean) (ng/ml)	Reference
UK	OTA	Volunteers, 50	92%	n.d.-0.058	Gilbert et al. (2001)
Italy (South & North)	OTA	Healthy, 38	57.9%	0.012-0.046	Pascale & Visconti (2001)
Italy (Southern)	OTA	Healthy	100%	0.12-2 (0.53)	Breitholtz-Emanuelsson et al. (1994)
Hungary	OTA	Healthy, 88	61%	0.006-0.065 (0.013)	Fazekas et al. (2005)
Bulgaria (Gomo Peshitene village)	OTA	Healthy BEN area residents, 5	100%	0.010-0.33 (0.0508)	Petkova-Bocharova et al. (2003)
Bulgaria (Beli Izvor village)	OTA	Healthy BEN area residents, 11	100%	0.010-1.910 (0.168)	
Bulgaria (1984-1990)	OTA	BEN/UTT patients, 36	38.9%	0.005-0.604	Nikolov et al. (2002)
		Healthy persons from BEN families, 25	48%	0.005-0.033	
		Healthy persons from non-BEN families in BEN villages, 32	44%	0.005-0.043	
		Healthy persons from non-BEN villages in BEN area, 31	12.9	0.017-0.041	
Bulgaria	OTA	Healthy persons from non-BEN area, 3	0	n.a.	Castegnaro et al. (1991)
	4-hydroxyOTA	BEN/UTT patients and controls, 152	40%	0.005-0.03	
Sierra Leone	OTA	Children (<5 years), 54	0%	-	Jonsyn (1999)
	4-hydroxyOTA	Children (5-14 years), 434	24%	0.3-26.6	
	4-hydroxyOTA	Boys (5-14 years), 231	44.4%	0.04-21	
	OTA	Girls (5-14 years), 203	38.5%	n.d-37	Jonsyn-Ellis (2000)
Portugal (Coimbra)	OTA	Healthy, 60	23.8%	0.07-72	
		Healthy, 30	27.1%	0.08-148	
			70%	0.021-0.105 (0.038)	Pena et al. (2006)
Spain (Valencia)	OTA	Healthy, 31	M: 43.3%	M: 0.011-0.208 (0.019)	Manique et al. (2008)
			A: 46.7%	A: 0.008-0.11 (0.018)	
Portugal (Lisbon)	OTA	Healthy women, 21	M: 80.6%	M: 0.007-0.124 (0.032)	
		Healthy men, 22	A: 83.9%	A: 0.008-0.089 (0.028)	
		Total, 43	71.4%	n.d.-0.055 (0.027)	Present study
			72.7%	n.d.-0.071 (0.025)	
			72.1%	n.d.-0.071 (0.026)	

Note: M: morning samples; A: afternoon samples; BEN: Balkan Endemic Nephropathy; UTT: Urinary Tract Tumors.

was high, with 72.1% of the samples being positive for OTA, and 62.8% featuring contamination levels above the LOQ, with concentrations ranging from not detected (n.d.) to 0.071 ng/ml. The mean concentrations and standard deviations were 0.026 and 0.017 ng/ml, respectively.

Except in the cases of OTA occurrence in the Balkans, where the high incidence of positive cases and contamination level reflect endemic exposure to OTA, the results of most surveys are comparable with those reported in the present work (Table 6). Previous studies in some other European countries, where BEN has not been observed, have recorded similar results. However, a difference in analytical methods and/or eating habits can result in higher values of contamination (Baydar et al. 2005), as exemplified by the values for Sierra Leone detected by Jonsyn (1999) and Jonsyn-Ellis (2000).

Our study also reinforces previous results for samples from central Portugal and Valencia, Spain (Dinis et al. 2007; Lino et al. 2008; Manique et al. 2008), demonstrating a worrying exposure of Iberian residents to this mycotoxin, corroborated by the presence of OTA in Spanish and Portuguese foodstuffs such as cereals and cereal products, rice, dried fruits, milk and wine (Manique et al. 2008).

According to Scott (2005), the OTA level in urine is not correlated with age (Gilbert et al. 2001) or gender (Fazekas et al. 2005). Others (Manique et al. 2008; Pena et al. 2006) also reported the absence of significant differences between genders. Pena et al. (2006) observed differences between gender only in the age group between 20 and 39 years ($p=0.044$). In Sierra Leone, Jonsyn-Ellis (2000) found no correlation between nutritional status and the prevalence of OTA in urine samples of children. On the other hand, Gilbert et al. (2001) found no significant differences associated with the ethnic diet of his subjects. Vegetarians had higher OTA consumption on average, although their plasma or urine levels were not significantly higher.

The low levels of OTA observed in the study of Pascale and Visconti (2001) are clearly indicative of the much lower amount of toxin transported to the urine compared to blood. However, Gilbert et al. (2001) observed a significant correlation between consumption in controlled diets and OTA concentration in urine (ranging from <0.01 to 0.058 ng/ml), suggesting that the content of OTA in urine could be a useful marker of OTA intake.

Conclusion

In conclusion, the high percentage of bread samples featuring significant levels of OTA, corroborated by a similarly high frequency of contamination in urine

samples, suggests a real and worrying exposure of Lisbon residents to this mycotoxin. Although maize bread is more contaminated, wheat bread, due to its higher consumption, contributes more to exposure. An exception is Mafra wheat bread, which has an insignificant level of contamination.

This study contributes to the database on exposure to OTA, which is important in making decisions on the regulation of chemical contaminants and the safety of foodstuffs. Further studies are needed to expand our knowledge and pursue surveillance in cereals and derived products, as major contributors to OTA intake.

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