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Estimation of OTA exposure of Lisbon inhabitants through analysis of bread and urine in winter 2007

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3 Estimation of ochratoxin A exposure of Lisbon inhabitants through
4 analysis of bread and urine in winter 2007/2008
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19 **Abstract**
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23 A survey on the occurrence of ochratoxin A (OTA) in 41 bread samples was carried out in the
24 Portuguese capital. Maize (5) and wheat bread (36) and 43 representative urine samples from
25 the Lisbon region were assayed for OTA content using immunoaffinity column cleanup (IAC)
26 and HPLC with fluorimetric detection (LC-FD). The percentage of OTA-positive samples
27 was slightly higher for maize bread (80%) than for wheat bread (70.8%), although, due to its
28 higher consumption, the latter contributes more to OTA exposure, featuring a higher
29 estimated daily intake (EDI). In the urine samples analyzed, both female and male residents
30 displayed similar high levels of OTA frequency and average contamination. Therefore, OTA
31 is a food contaminant of concern and may constitute a hazard for public health through
32 consumption of cereal-based products.
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42 **Keywords :-** Ochratoxin A; bread; urine; LC-FD; Portugal; Lisbon
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Introduction

The attention of public health authorities and toxicologists in the world has recently been focused on systematic control of the presence 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). The mycotoxin OTA is an example. Since its discovery in 1965, several authors and projects were devoted to its study. It is currently regarded as nephrotoxic, immunotoxic, and genotoxic (Schilter *et al.*, 2005). Additionally, since 1993, it is included in the 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, involvement of this mycotoxin in the cancer etiology of the urinary system, and in kidney pathologies typical of Balkan endemic nephropathy (BEN) has 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 areas with respect to cereals from 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 were recommended, specifically of 120ng/kg bw/week (EFSA, 2006), and 100ng/kg bw/week (JEFCA, 2007). For their part, Canadian authorities proposed a TDI of 1.2–5.7ng/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 5ng/kg body weight, based on carcinogenicity studies with OTA in adult rats. The Scientific Committee on Food also proposed that exposure should be below 5ng/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 consumed foodstuffs in the world. Of these, bread is the most popular yeast-leavened cereal product by far provides 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, in many popular festivities and in most family meals worldwide (www.world-bread-day.com, 2008).

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3 This universal product can be made of different types of cereal grain, characterized by
4 the many fermentation processes and the different ways of baking, a wide variety of sizes,
5 shapes, textures and tastes dependent on the agricultural, cultural and feeding habits of the
6 populations (González-Osnaya *et al.*, 2007, www.world-bread-day.com, 2008). By far the
7 most consumed type is wheat bread. In Portugal, the latest estimate of the average bread
8 consumption is from 1994, at a level of 32 kg/person/year. Maize bread, a traditional
9 Portuguese type of bread, is estimated to represent a quarter of the total nationwide bread
10 consumption (Instituto Português do Consumidor, 2005). The famous and traditional 'Mafra'
11 bread is a regional wheat bread type, commercialized in different shapes and sizes, but always
12 with the same dough, which is made of wheat flour, water, yeast and salt. Although typical
13 from a certain region of Lisbon, most of the shops, bakeries and supermarkets of the capital
14 feature this kind of bread.
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24 To study the OTA exposure, different approaches have been attempted. OTA levels in
25 plasma and urine are considered as typical biomarkers of exposure, and no other appropriate
26 effect-related biomarkers have as yet been identified (Pacin *et al.*, 2008). Although
27 elimination in urine has been considered the main route of OTA excretion from the human
28 body, the urinary monitoring is relatively unexplored due to the lower concentrations
29 involved (Pena *et al.*, 2006) and due to the fact that, conversely to its content in plasma, it is
30 not possible to relate concentrations or total amount of OTA excreted in urine to exposure.
31 Recent developments in analytical methodology resulted in a more practical urinary
32 monitoring, which has the advantage of being less invasive than blood monitoring. However,
33 data on the frequency and concentration of OTA in human urine remain scarce (Pena *et al.*,
34 2006). In a one-month duplicate diet study in the UK OTA in urine was found to be a better
35 indicator of OTA consumption than OTA in plasma, although levels in urine were two orders
36 of magnitude lower; there was no good correlation between plasma concentrations and
37 consumption of OTA (Gilbert *et al.*, 2001). It is thus suggested that urine could provide a
38 more useful marker of OTA intake than plasma (Pascale & Visconti, 2001).
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51 When compared to food products, urine has an additional advantage as a target for
52 OTA exposure quantification. The large variability in contamination levels and sampling
53 difficulties means that large numbers of individual food samples need to be analyzed.
54 Estimates of exposure based on determining OTA in food products will thus inevitably be
55 vulnerable to large sources of error. Further, the fact that estimated exposure levels use
56 methods based on food intake/analysis, which are inherently inaccurate, appears to indicate
57 that exposures close to the TDI require a better approach for assessing OTA exposure. Initial
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3 studies have used plasma levels to estimate intake, but the study of Gilbert *et al.* (2001)
4 suggests that this may not be the most suitable method. A disadvantage of urine analysis to
5 assess OTA exposure is the need to define further the relationship between consumption and
6 excretion to estimate actual intake levels. So, further work needs to be done to verify if urine
7 concentrations are a short- or a long-term indicator of exposure to this mycotoxin (Manique *et*
8 *al.*, 2008). According to Gilbert *et al.* (2001), the correlation might be stronger if comparisons
9 were made between urine excretion and previous day intake.

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12 The present study aims to estimate the OTA contamination levels of different types of
13 bread, a widely consumed foodstuff, in the Lisbon region during the Winter of 2007/2008.
14 Moreover, Lisbonians' exposure to OTA was evaluated, based on bread intake and the toxin's
15 presence in urine.

24 **Materials and Methods**

28 **Bread and urine sampling**

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30 The present study was carried out with samples collected in the Portuguese capital
31 city, Lisbon, located in the country's central western coast. The samples were collected during
32 the Winter of 2007/2008, between November 2007 and March 2008.

33
34 The bread samples, consisting of 24 units of regular wheat bread, 12 units of regional
35 Mafra wheat bread, and 5 units of maize bread, were commercially acquired from random
36 bakeries and supermarkets located in the Lisbon region. After purchase, the samples were
37 brought to the laboratory under ambient condition for milling. All of the information on the
38 samples was obtained from the respective labels. Each milled sample, stored in sealed plastic
39 bags, was frozen at -20°C, and kept in this condition until analysis.

40
41 The 43 urine samples were collected from healthy individuals residing in the same
42 area where the bread samples were commercialized. From the 43 local participants involved,
43 48.8% (n=21) were female, aged 18-69 years old, and 51.2% (n=22) were male, aged 23-75
44 years old. The sampled population is characterized in Table I.

54 **Reagents**

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56 HPLC grade acetonitrile (Carlo Erba, Milan, Italy), methanol (Panreac Química Sau,
57 Barcelona, Spain), toluene (Barker Analyzed, J. T. Barker, Holland) and benzene (HACH
58 Company, USA) were used. Of analytical grade were used boron-trifluoride-methanol 14%

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3 solution and acetic acid purchased from Sigma-Aldrich (Laborchemikalien, Germany),
4 hydrochloric acid, sodium hydroxide, potassium chloride, potassium dihydrogenphosphate
5 and anhydrous disodium hydrogenphosphate supplied by Merck (Darmstadt, Germany), and
6 sodium chloride purchased from Baker Ltd., Pagenham, England.
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10 The OTA standard was acquired from Sigma Chemical Co. (St. Louis, MO, USA)
11 with $\geq 98\%$ purity. The OTA standard stock solution was prepared by diluting 1mg of OTA
12 from *A. ochraceus* in 4mL of toluene:acetic acid (99:1) at 250 $\mu\text{g}/\text{mL}$, and stored at -20°C . The
13 intermediate solutions were prepared at 10 $\mu\text{g}/\text{mL}$ and 1 $\mu\text{g}/\text{mL}$, in toluene:acetic acid, and
14 working standard solutions at 0.1 $\mu\text{g}/\text{mL}$ and 0.01 $\mu\text{g}/\text{mL}$, in mobile phase. The calibration
15 curve standard solutions were prepared in mobile phase, at concentrations between 0.001 and
16 0.01 $\mu\text{g}/\text{mL}$.
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22 Water was obtained daily from a Milli-Q System (Millipore, Bedford, MA, USA).
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26 **Equipment**

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28 Filter paper Whatman N°4 (150mm, Whatman International Ltd. Maidstone, England)
29 and polyamide membrane filters (0.2 μm , 50mm, Whatman GmbH, Dassel, Germany) were
30 used. Immunoaffinity columns (IAC) OchratestTM were from VICAM (Watertown, USA).
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33 A Braun MR 5000M multiquick/minipimer 500W (220-230V, 50-60Hz, Esplungues
34 del Llobregat, Spain), a Moulinex blender 700W (230-240V, 50-60Hz, Barcelona, Spain), a
35 vacuum manifold of Macherey-Nagel (USA), a pump of Dinko (mol. D-95, 130W, 220V), a
36 magnetic stirrer (Agimatic-S, Selecta, Barcelona, Spain), and a Retsh vortex mixer (Haan,
37 Germany) were employed. A Sonorex RK 100 ultrasonic bath (Berlin, Germany) was used for
38 15min degassing of all chromatographic solvents prior to use, and also for cleaning and
39 decontaminating of the material used in the experiments.
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46 The LC instrument used possessed a pump (Model 307, Gilson Medical Electronics,
47 Villiers-le-Bel, France), and a guard column Hichrom Ltd, HI-173 (30mmx4mm i.d.)
48 (England) preceding a Hichrom C₁₈ column (5 μm , 250 mm x 4.6mm i.d.). Detection and
49 quantification were performed with a Perkin-Elmer Model LS45 (Beaconsfield, UK). A
50 Hewlett-Packard 3390A integrator (Philadelphia, PA, USA) recorded the results.
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56 **Experimental Procedure**

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

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3 Briefly, for OTA extraction from the bread matrix, 100mL of PBS:methanol (50:50)
4 were added to 20g of milled bread and then homogenized (5min) and filtered. Twenty
5 milliliters of the resulting filtered solution were diluted in 30mL of PBS before the passage
6 through the IAC at a vacuum-induced rate of 1 drop per second. The IAC was then washed
7 with 10mL of water prior to the elution with 3mL of methanol.
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11 For extraction from the urine matrix, 10mL of the sample were mixed with 10mL of
12 NaHCO₃, 5%, and then filtered. The filtrate was cleaned-up through the IAC at a flow rate of
13 about 1 drop per second. The column was then washed twice with 5mL of distilled water, and
14 afterwards OTA was eluted with 3mL methanol.
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19 The eluates were dried in a bath at 50°C under a gentle nitrogen flow, and the dried
20 extract was stored at -20°C. Before injection, the dried extract was dissolved in 250µL or
21 125µL of mobile phase, in the case of bread or urine samples, respectively.
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24 LC-FD analysis was performed using a vacuum-filtered solution of
25 acetonitrile/water/acetic acid (49.5:49.5:1.0, v/v/v) as mobile phase, flowing at a 1mL/min
26 rate. Wavelengths used were 333nm for excitation and 460nm for emission, both with a
27 spectral bandwidth of 10nm. An OTA working standard solution of 0.01µg/mL was analyzed
28 at the beginning of each run. A 20µL injection volume was used for both bread and urine
29 extracts.
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37 Analytical quality assurance

38 OTA method revalidation was performed by spiking an OTA-free wheat bread sample,
39 in triplicate, during three days at fortification levels of 0.1, 0.5 and 2ng/g. After fortification,
40 the sample was left to stand in the dark for 15min, after which the Juan *et al.* (2007) protocol
41 was followed.
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46 The fortification assays performed on urine, in a triplicate of a blank sample and
47 during three different days, were made at 0.02, 0.1 and 0.5ng/mL. After fortification the
48 sample was left to stand in the dark for 15min, after which the Pena *et al.* (2006) protocol was
49 followed.
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53 OTA presence in bread samples was confirmed as described by Guillamont *et al.*
54 (2005) and Pena *et al.* (2005), by conversion into its methyl ester form, adding 150µL of
55 boron trifluoride methanolic solution (BF₃-CH₃OH 14%) to the dried sample extracts, and
56 evaporating the mixture at 60°C for 10min, before reconstitution in 250µL of mobile phase.
57 For urine samples, a similar method was used as described by Castegnaro *et al.* (1990),
58 differing from the previous in its lower reconstitution volume of 150µL.
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3 The OTA methyl esters of both samples were analyzed according to the LC method
4 described above.
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8 9 **RESULTS AND DISCUSSION**

10 11 **Analytical performance**

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14 Linearity for OTA quantification was verified by plotting OTA peak areas against
15 calibration samples (concentrations of 1, 2, 5 and 10ng/mL). The resulting calibration curve
16 featured a coefficient of correlation of 0.9998.
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20 Recovery values for bread ranged from 76.7 to 103.7%, at fortification levels of 2.0
21 and 0.1ng/g, respectively, with intra-day repeatabilities ranging from 3.6 to 14.4%, for levels
22 of 0.5 and 0.1ng/g, and inter-day ones from 7.2 to 12.9%, at the levels of 0.5 and 2.0ng/g.
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26 For urine, recovery values were from 90.6 to 96.0% at the 0.02 and 0.5ng/mL
27 fortification levels. Intra- and inter-day repeatabilities varied between 2.9 and 5.4%, and 4.3
28 and 8.9% for fortification levels of 0.5 and 0.02ng/mL, respectively.
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31 The above results for accuracy and precision, for both bread and urine, were in
32 agreement with the EC directive N° 401/2006 (EC, 2006).
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35 Determination of LOQs was performed by spiking blank samples with OTA standard
36 solutions. Repeatable precision and trueness were achieved at minimum concentrations of
37 0.1ng/g and 0.008ng/mL for bread and urine samples, respectively, thus setting the LOQs at
38 those values.
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41 As shown by the chromatograms displayed in figure 1, there are no interfering peaks
42 near the retention time of OTA (ca.11.30) in neither bread nor urine samples. The different
43 retention time of OTA and its methyl ester and the almost complete disappearance of the
44 OTA peak and the appearance of a new one, at 27.30min, permitted the confirmation of OTA
45 presence in the sample (Figure 1).
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50 51 **Occurrence of OTA in bread**

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53 As shown in Table II, of the total 41 bread samples analyzed from Lisbon, 68.3%
54 contained OTA, and 51.2% showed OTA levels above the LOQ.
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57 Incidence was found to be high in both maize and wheat bread, and nearly all positive
58 samples featured OTA content levels above the LOQ. In both instances, values were slightly
59 higher, though not significantly so, in the maize bread. Similarly, average contamination
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3 levels were also somewhat more elevated in maize bread than in common wheat bread (0.28
4 vs. 0.21ng/g). On the other hand, less than half of the Mafra bread samples contained
5 detectable levels of OTA, and none of those featured OTA content quantifiable through this
6 method.
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10 Similar results were detected in studies concerning other Portuguese regions (Juan *et*
11 *al.*, 2007; Bento *et al.*, 2009). A notable exception is that of Juan *et al.* (2008), whose study
12 found much lower incidence and contamination levels in wheat bread. A possible explanation
13 for this discrepancy may lie in the fact that the samples for this study originated in a different
14 area and period, and were thus subject to different conditions during processing and storage,
15 as well as different edafo-climatic influences during crop growth.
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21 Independently of study, however, data from across the world (Table III) puts
22 Portuguese bread as the least contaminated. Since some of the data in that table refers to the
23 cereal grain, it is possible that processing methods result in a similar OTA content in the
24 bread of some of those countries – namely, Italy and Croatia.
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28 Those same studies concur with the present one in that they have found that both
29 maize grains and their derivates tend to feature higher frequencies of OTA contamination than
30 their wheat counterparts. In the specific case of bread, Cengiz *et al.* (2007) have detected
31 statistically significant differences between OTA content average levels in white and corn
32 bread samples, corn and whole meal bread samples, and corn and corn whole meal samples,
33 with the latter of each case featuring higher levels. Earlier, Zummo & Scott (1992)
34 demonstrated that maize (*Zea mays*) has an ideal nutrient composition for fungi development
35 and is thus constantly exposed to the risk of fungal contamination. Moreover, tropical and
36 subtropical climate countries, a few of which are among the worldwide top producers of
37 maize, have favorable environmental conditions to the development of the main types of
38 genotoxicant fungi, *Aspergillus*, *Fusarium* and *Penicillium* (Sekiyama *et al.*, 2005). However,
39 studies of OTA quantification offer contradictory results on the subject.
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49 A study from Brazil (Sekiyama *et al.*, 2005) found a single sample of corn flour
50 (0.8%) with an OTA content of 64µg/kg, while another (Machinski *et al.*, 2001) found two
51 samples (1.8%) with contamination levels of 128 and 206µg/kg, values that exceed the
52 maximum permitted legal limit. Yet another (Caldas *et al.*, 2002) found no samples with
53 contamination levels above their limit of detection (25µg/kg).
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58 In Argentina, however, a different study (Paolo & Tosi, 1998) found a high content of
59 OTA (1250-2500µg/kg) in all the analyzed maize-derived foodstuffs, though non-processed
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3 maize featured consistently higher levels of contamination. Food processing, thus, does
4 contribute slightly to decontamination, though it does so in blatantly insufficient levels.
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7 The cited Brazilian papers results corroborate the low levels of OTA contamination in
8 Brazilian maize. On this subject, it is worth noting that Argentina is significantly colder than
9 Brazil, and though OTA-producing fungi favor warm and wet environments, mycotoxin
10 production seems to be spurred by cold and wet climates (Eskola, 2002). Yet, the Ministry of
11 Agriculture, Fishing and Food of the United Kingdom found that crude maize samples
12 imported from Argentina – as well as from a number of European countries – featured
13 contamination levels no higher than 1.5µg/kg, and only in 10.1% of the samples. It is,
14 however, possible that produce destined to exportation is subjected to tighter control and the
15 most contaminated samples are discarded.
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23 Conversely, other studies reveal higher mean levels of OTA in wheat, than in maize,
24 irrespective of their ripening season, like in BEN endemic regions (Puntarić *et al.*, 2001). This
25 high contamination of wheat cereal can be explained by the fact that glutamic acid is
26 incorporated into OTA during its production, and so a high content of this aminoacid in
27 cereals, as typical in wheat, could be a cofactor for the presence of OTA, as suggested by
28 (González-Osnaya *et al.*, 2007). Furthermore, and according to Laca *et al.*, 2006, OTA
29 content differences between cereals depend on multiple factors, such as the grain condition at
30 harvest, care in grain drying, and storage conditions (Gonzalez-Osnaya *et al.*, 2007).
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37 Taking into account the latest data, which claims that a Portuguese citizen has an
38 average adult bodyweight of 65kg (Miraglia & Brera, 2002) and an average bread
39 consumption of 32kg/year (Instituto Português do Consumidor, 2005), consisting one quarter
40 maize bread and three quarters wheat bread, the estimated contributions to OTA daily intake
41 are 0.09 and 0.21ng/kg bw/day for maize and wheat bread, respectively (Table IV).
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46 It should be noted that, though maize bread tends to feature higher levels of
47 contamination, the much higher consumption of wheat bread results in that the exposure of
48 the Lisbonian residents to OTA is higher through the consumption of the latter, 0.21ng/kg
49 bw/day vs. the 0.09ng/kg bw/day for maize bread, although far from the values established by
50 the SCF in 1998 (5ng/kg bw/day) and the JECFA in 2001 (17ng/kg bw/day). These values are
51 lower than the ones demonstrated by Leblanc *et al.* (2005) for the French population, and by
52 the SCOOP report for the Spanish (0.77) and German (0.36) inhabitants (Miraglia & Brera,
53 2002). Conversely, the values of the EDI in the present study are similar to the ones
54 calculated for the Danish residents (0.19) by the same SCOOP report. Further, the EDI from
55 wheat bread, 0.21ng/kg bw/day, represents over a quarter of the national average, and thus
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3 support the major contribution of bread in particular, and cereals in general, as the main
4 contributors to OTA exposure.
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7 Mafra bread, although also made of wheat, did not present any detectable level of
8 OTA, and so does not contribute to the exposure to OTA. This difference is likely due to the
9 fact that, as previously mentioned, while Mafra bread is, as a rule, baked with only wheat
10 flour, water, yeast, and salt, the more common wheat bread also tends to include other
11 components that generally lead to an increase of the OTA content in bread, such as seeds,
12 bran (Scudamore *et al.*, 2003; Juan *et al.*, 2008), and even flour made of other cereals
13 (Czerwiecki *et al.*, 2002).
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21 **Occurrence of OTA in urine**

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23 The present study shows an elevated incidence of OTA occurrence in the urine of the
24 Lisbonian residents (Table V). Incidence was high, with nearly three-quarters (72.1%) of the
25 samples being positive for OTA, and 62.8% featuring contamination levels above the LOQ,
26 with concentrations ranging from n.d. to 0.071ng/mL. The mean concentration and standard
27 deviation were 0.026 and 0.017ng/mL, respectively.
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32 Except in the cases of OTA occurrence in Balkan region where the high incidence of
33 positive cases and contamination level reflect the endemic condition of exposure to OTA, the
34 results of most surveys are comparable with those reported by the present work (Table VI).
35 Previously, other studies showed similar results in some other European countries where BEN
36 has not been observed. However, a difference in analytical methods and/or eating habits can
37 result in far higher values of contamination (Baydar *et al.*, 2005), as exemplified by the values
38 for Sierra Leone detected by Jonsyn (1999) and Jonsyn-Ellis (2000).
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44 This study also reinforces the previous results obtained for samples of central Portugal
45 and Valencia, Spain (Dinis *et al.*, 2007; Lino *et al.*, 2008; Manique *et al.*, 2008),
46 demonstrating a concerning exposure of Iberian residents to this mycotoxin, corroborated by
47 the presence of OTA in Spanish and Portuguese foodstuffs such as cereals and cereal
48 products, rice, dried fruits, milk, and wine (Manique *et al.*, 2008).
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53 According to Scott (2005), the OTA level in urine does not present any correlation
54 with age (Gilbert *et al.*, 2001), nor gender (Fazekas *et al.*, 2005). Other authors (Manique *et*
55 *al.*, 2008, Pena *et al.*, 2006) also reported the absence of significant differences between
56 genders. Pena *et al.* (2006) observed differences between gender only in the group aged
57 between 20 and 39 years ($p=0.044$). In Sierra Leone, Jonsyn-Ellis (2000) discarded the
58 correlation with the nutritional status and the prevalence of OTA in the urine samples of the
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3 children analyzed. On the other hand, Gilbert *et al.* (2001) found no significant differences
4 associated with the ethnic diet of the subjects. Vegetarians had higher OTA consumption on
5 average, although their plasma or urine levels were not significantly higher.
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9 The low levels of OTA observed in the study of Pascale & Visconti (2001) are clearly
10 indicative of the much lower amount of the toxin which is transmitted to the urine as
11 compared to the blood. However, Gilbert *et al.* (2001) observed a significant correlation
12 between OTA human consumption through controlled diets and OTA concentration in urine
13 (ranging from <0.01 to 0.058ng/ml) suggesting that the content of OTA in 94 urine samples
14 could be a useful marker of OTA intake.
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21 **Conclusion**

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23 In conclusion, the high percentage of bread samples featuring significant levels of
24 OTA, corroborated by a similarly high frequency of contamination in urine samples, suggests
25 a real and concerning exposure of Lisbon residents to this mycotoxin. Although maize bread
26 is more contaminated, the wheat bread, due to its higher consumption, contributes more to the
27 exposure to OTA. An exception is the Mafra wheat bread type, which has an insignificant
28 level of contamination.
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34 This study contributes thus to the gathering of information about exposure to OTA,
35 which is also important when making decisions on the regulation of chemical contaminants
36 and the safety of foodstuffs. Further studies need to be conducted to expand this data and
37 pursue the surveillance in cereals and derived products, since they are the major contributors
38 to OTA intake.
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47 the grant SFRH/BD/37409/2007.
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Table I – Anthropometric data of the sampled population.

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

Table II - 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	Deleted: Frequency ^c
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	

^a Bread type, ^b Samples ≥LOQ (≥0.1ng/mL) only, ^c All samples

Table III – Compilation of some international studies and the present on OTA occurrence in maize and wheat grains and derivate bread.

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 <i>et al.</i> (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 <i>et al.</i> (2005)
Ivory Coast	Maize, 16 (1998)	100%	44±12	27-64	Sangare-Tigori <i>et al.</i> (2006a)
	Maize, 15 (2001)	100%	266±428	3-1738	
	Maize, 10 (2002)	100%	37±25	9.8-86	Sangare-Tigori <i>et al.</i> (2006b)
	Maize, 10 (2002)	100%	-	0.16-0.86	
Turkey	Corn bread, 10	-	4.94±0.20	4.08-5.28	Cengiz <i>et al.</i> (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	
Spain	Whole meal flour, 14	-	9.30±1.33	1.07-16.70	González-Osnaya <i>et al.</i> , 2007
	Organic wheat bread, 20	20%	-	0.03-0.81	
	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	
	Maize bread, 15	60%	0.43	-	Juan <i>et al.</i> (2007)
	Maize bread, 30	70%	0.44	-	Juan <i>et al.</i> (2008)
	Wheat bread, 31	12.9%	0.02	-	
Portugal	Wheat bread, 30	80%	0.2±0.1	n.d.-0.49	Bento <i>et al.</i> (2009)
	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	Present study
	Mafra wheat bread, 12	41.7%	n/a	<0.1	

Table IV – 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

^aEDI was calculated using the equation $EDI = (\sum c) (CN^{-1} D^{-1} K^{-1})$, 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 65Kg (mean of body weight of the studied population)

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

Table V - Prevalence (%) and mean levels (µg/L) of OTA in urine

Gender	Size	Incidence	≥LOQ*	Mean±SD	Range
Females	48,8%	71.4%	57.1%	0.027±0.015	n.d.-0.055
	(21/43)	(15/21)	(12/21)		
Males	51.2%	72.7%	68.2%	0.025±0.020	n.d.-0.071
	(22/43)	(16/22)	(15/22)		
Total	100%	72.1%	62.8%	0.026±0.017	n.d.-0.071

* ≥0.008ng/mL

Table VI – Occurrence of OTA and/or its metabolites in urine in different countries

Country	Biomarker	Sampled population, number	Incidence	Range (Mean) (ng/mL)	Reference
U.K.	OTA	Volunteers, 50	92%	n.d.-0.058	Gilbert <i>et al.</i> (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 <i>et al.</i> (1994)
Hungary	OTA	Healthy, 88	61%	0.006-0.065 (0.013)	Fazekas <i>et al.</i> (2005)
Bulgaria (Gorno Peshtene village)	OTA	Healthy BEN area residents , 5	100%	0.010-0.33 (0.0508)	Petkova-Bocharova <i>et al.</i> (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 <i>et al.</i> (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	
		Healthy persons from non-BEN area, 3	0	n.a.	
Bulgaria	OTA	BEN/UTT patients and controls, 152	40%	0.005-0.03	Castegnaro <i>et al.</i> (1991)
	4-hydroxyOTA		0%	-	
Sierra Leone	OTA	Children (<5years), 54	24%	0.3-26.6	Jonsyn (1999)
	4-hydroxyOTA	Children (<5years), 54	44.4%	0.04-21	
	4-hydroxyOTA	Children (5-14years), 434	38.5 %	n.d.-37	Jonsyn-Ellis (2000)
	OTA	Boys (5-14years), 231	23.8 %	0.07-72	
Portugal (Coimbra)	OTA	Girls (5-14years), 203	27.1%	0.08-148	Pena <i>et al.</i> (2006)
		Healthy, 60	70%	0.021-0.105 (0.038)	
		Healthy, 30	M: 43.3% A: 46.7%	M: 0.011-0.208 (0.019) A: 0.008-0.11 (0.018)	Manique <i>et al.</i> (2008)
Spain (Valencia)	OTA	Healthy, 31	M: 80.6% A: 83.9%	M: 0.007-0.124 (0.032) A: 0.008-0.089 (0.028)	
Portugal (Lisbon)	OTA	Healthy women, 21	71.4%	n.d.-0.055 (0.027)	Present study
		Healthy men, 22	72.7%	n.d.-0.071 (0.025)	
		Total, 43	72.1%	n.d.-0.071 (0.026)	

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

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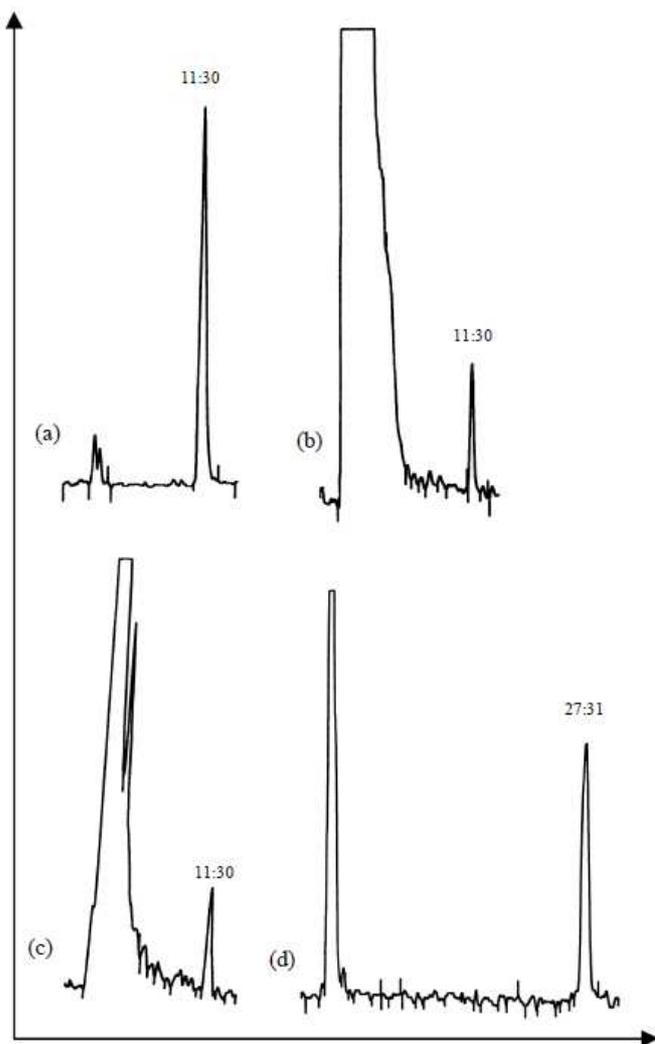


Figure 1: LC-FD chromatograms obtained after the described experimental procedure, from (a) OTA standard solution (5ng/mL; RT 11.30), (b) bread sample naturally positive to OTA (RT 11.30), (c) urine sample naturally positive to OTA (RT 11.30) and (d) an OTA methyl ester peak after boron fluoride-methanol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