Determination of ochratoxin A content in wheat bread samples collected from the Algarve and Bragança regions, Portugal: Winter 2007

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by fungi such as Penicillium verrucosum and Aspergillus spp. and has been found to have a variety of potentially deadly toxic effects. The favoured substrate for fungal growth and OTA production appears to be cereals and flour-based products, including bread. Due to the dietary relevance of bread for the Portuguese population, it is imperative that its OTA content remains well within safe quantities. As such, bread samples collected from commercial surfaces across the Algarve region and from the city of Bragança during the winter of 2007 were tested for OTA through extraction with immunoaffinity columns and quantification by liquid chromatography coupled with fluorescence detection. Although OTA content was found to be above the limit of quantification in approximately 60% and 50% of the analysed samples from Algarve and Bragança, respectively, all samples were found to be compliant with European Commission. OTA content reached maximums of 0.49 ng/g in Algarve and 0.43 ng/g in Bragança, and was thus below the maximum limit established by European legislation for bread of 3 ng/g. The results of the present study put the estimated daily intake of OTA from bread at approximately 0.26 ng/kg bw/day in Algarve and 0.38 ng/kg bw/day in Bragança, circa 1.5% and 2.0% of the TDI established by either the EFSA or the FAO/WHO, or over 4.5% and 6.5% if we consider the FAO/WHO advised bread consumption of 250 g/day. These results seem to suggest that, in these two Portuguese regions, OTA contamination is well under control and unlikely to represent a threat to consumer health.

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1. Introduction

Despite their almost opposite geographical positioning in the country, Portugal’s Algarve—the southernmost region—and Bragança—one of the north-easternmost towns—regions (Fig. 1) are famous holiday destinations. Bragança derives much of its tourist trade from its proximity to Spain, its typical crafts and nearby natural preserve, while Algarve’s beaches fuel the tourist industry by attracting people from all over the world.

According to the Köppen–Geiger climate classification, Algarve falls under the CsA class (tropical, with warm dry summer) while Bragança is classified as a Csb (tropical, with temperate dry summer) [1].

Despite these seemingly close environments, temperature differences are quite noticeable throughout the year, with Bragança significantly cooler than Algarve. A significant part of the tourist trade, for both regions, is the typical Portuguese cuisine, which generally includes significant amounts of bread, whether as an ingredient, an entry, or a complement to the main dish. One of its possible contaminants is OTA (structure in Fig. 2).

OTA is an UV-decomposable mycotoxin produced by fungi such as Penicillium verrucosum and varied Aspergillus species, especially in cold and wet climates [2]. Production continues while the cereals are stored, especially under improper storage conditions, and processing methods are insufficient for their efficient detoxification, resulting in OTA-bearing cereal-derived products, such as contaminated bread. OTA has been proven to have a variety of toxic effects, including nephrotoxic—having been linked to the Balkan Endemic Nephropathy [3]—immunotoxic [4], carcinogenic [5] and teratogenic [6], while also being a possible genotoxic. In the last ten years some work has indicated that its mode of carcinogenicity is ‘genotoxic’ [7]. However, OTA’s genotoxicity is not certain, and recent work funded by Directorate-General (DG) XII of the EU has demonstrated that DNA binding of OTA is not detectable with sensitive analytical methods, and is unlikely to represent a mechanism for OTA-induced tumour formation [8].

The by far largest source of dietary OTA intake in Europe are cereals and their derived products, which account for around half of said intake, with wine and coffee taking a paltry second and third places, contributing about 10 and 9%, respectively [9]. To ensure the safety of the consumers, the EC has put the maximum permitted OTA level of cereals at 5 ng/g and of their derived products at 3 ng/g [10] and OTA’s Tolerable Weekly Intake (TWI) at 120 ng/kg bw/week [11].
OTA clean-up required a vacuum-manifold (Macherey–Nagel, USA) and a Dinko pump (mol. D-95, 130 W, 220 V) to drive the liquidified sample through Ochratest immunoaffinity columns (IACs) (Vicam, Watertown, MA, USA).

Dried samples were reconstituted with the help of a Retsh vortex mixer (Haan, Germany).

The LC-FD instrumentation consisted of a spectrofluorimeter (Perkin–Elmer Model LS45, Perkin–Elmer, Beaconsfield, UK) connected to a HPLC system constituted by a pump (Model 307, Gilson Medical Electronics, Villiers-le-Bel) and a 20 µL Rheodyne injector which forced the reconstituted samples through a Hichrom Ltd., HI-173 guard column (30mm×4 mm, i.d., England), and then through a Hichrom C18 column (5 µm, 250mm×4.6 mm, i.d.). Fluorescence was recorded by a Hewlett-Packard 3390A integrator (Philadelphia, PA, USA).

2.3. Solutions

Phosphate-buffered saline (PBS) solution was prepared by diluting the following amounts per litre of distilled water: 0.2 g each of potassium chloride and potassium dihydrogen-phosphate, 1.2 g anhydrous disodium hydrogen phosphate, and 8 g sodium chloride. pH was adjusted to 7.4 with recourse to 0.1 M HCl and 0.1 M NaOH.

The OTA standard stock solution was prepared by diluting OTA from A. ochraceus with toluene:acetic acid (99:1) at 250 µg/mL, and stored at −20 °C. It was then used as a starting point to prepare intermediate solutions at 10 µg/mL and 1 µg/mL, in toluene:acetic acid, and working standard solutions at 0.1 µg/mL and 0.01 µg/mL, in mobile phase. To draw the calibration curves, additional solutions were prepared at the concentration range of 1 to 10 ng/mL, also in mobile phase.

The OTB standard stock solution was acquired at 50 µg/mL and used to prepare an intermediate solution at 10 µg/mL in benzene:acetic acid (99:1), which was then evaporated and reconstituted in mobile phase in order to obtain a 100 ng/mL working solution. A second working solution was prepared from this one at 25 ng/mL.

An OTA/OTB joint standard solution was prepared, in mobile phase, at 5 ng/mL and 25 ng/mL, respectively, by mixing appropriate amounts of working solutions.

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

All LC reagents were submitted to a 15 min sonic bath for degassing. Glassware was decontamination by first washing it with a sodium hypochlorite solution, immersing it 4 ml/L H2SO4, and finally returning it to pH neutrality with distilled water.

2.4. Sampling

A total of 30 samples were purchased across Algarve and another 20 in Bragança and its outskirts in commercially available size during the Winter of 2007. Milled subsamples of 100 g each were stored in plastic bags at −20 °C until analysis.

All information on the samples was obtained from the labels. The milled samples were analysed as quickly as possible after purchase, being stored at −20 °C in the interim.

Fig. 1. Map of Portugal indicating the Algarve and Bragança regions.

Fig. 2. Chemical structure of ochratoxin A.
2.5. Sample processing and analysis

The method used was previously described by Juan et al. [13] and is as follows.

An aliquot (20 g) of each sample was extracted with 100 mL PBS/methanol (50:50, v/v) using the Braun Minipimer homogeniser for 5 min; the mixture was filtered by gravity through a Whatman filter paper. A 20 mL aliquot of the filtered was then diluted with 30 mL PBS and the resulting solution run through a IAC column for clean-up using a vacuum manifold. The column was washed with 10 mL of water before elution of OTA with 3 mL of methanol. The eluted was dried at ±50 °C under a gentle nitrogen stream and stored until needed at −20 °C.

Prior to injection, the residue was reconstituted in 250 µL of mobile phase by mixing. Injection volume in the LC system was of 20 µL, and flow was a constant 1 mL/min. Excitation wavelength was 333 nm and emission wavelength 460 nm, both with a 10 nm bandwidth.

2.6. Revalidation assays

Revalidation of the method was achieved by, during the course of three days, spiking samples in triplicate with 0.1, 0.5, and 2.0 ng/g of OTA. Before the above protocol was performed, samples were left to stand in the dark for 15 min.

The same procedure was employed to evaluate OTB recoveries, at the concentration levels of 2.0 and 5.0 ng/g.

2.7. OTA confirmation procedure

The process for OTA presence confirmation was similar to that described by Guillamont et al. [14] and Pena et al. [15], based on the conversion of OTA into its methyl ester form. The procedure used in this study differed from that used in the studies above in that a higher volume of boron trifluoride methanol solution was used, 600 µL instead of the described 150 µL.

3. Statistical analysis

All statistical analyses were carried out with using the data analysis software system STATISTICA (v.7) from StatSoft, Inc. (2004). The t test was used to evaluate the significance of the differences observed between groups. Whenever p<0.05 (two-tailed), differences were considered to be statistically significant. For statistical analysis, if the concentration was below the limit of quantification (LOQ) it was set to 50% of that limit when the mean and SD were calculated.

4. Results and discussion

4.1. Revalidation assays

Method linearity was verified through the drawing of the calibration curve and applying the linear least squares regression method to peak area versus four concentration levels, ranging from 1 to 10 ng/mL. This resulted in a correlation coefficient ($r^2$) of 0.9998, confirming linearity for that concentration range.

Recoveries ranged between 76.7 and 103.7% for fortification levels at 2.0 and 0.1 ng/g, respectively. Exactitude and intra and inter-day repeatability were found to be fully compliant with EC directives [16], as shown in Table 1.

Chromatograms of one solution of OTA and OTB standards at 5 and 25 ng/mL, respectively, a wheat bread sample contaminated with both ochratoxins, and OTA methyl ester of one bread sample contaminated with OTA are shown in Fig. 3.

Some of the tested samples presented a fluorescence peak at around 7 min, in addition to that of OTA (Fig. 3b). The injection of an OTB standard solution confirmed the suspicion that said peak was due to the presence of that substance in the samples. Attempts were made to quantify the OTB content, but recovery assays showed recoveries of around 50%, revealing this method to be inadequate for such purpose. This is probably due to the fact that the immunoaffinity columns used contained monoclonal antibodies, therefore granting them high specificity for OTA.

Nevertheless, OTB peak area was, as a rule, greater than OTA peak area. This, combined with the low recovery rates, indicates that the
samples were contaminated with OTB (Table 2). Fortunately, and even though in vitro toxicity is similar, OTB, lacking OTA’s chlorine group, is quickly metabolised and rapidly eliminated when in vivo conditions. This results in a lack of toxicity and therefore OTB seems to present no health risk [17].

4.2. Limit of quantification (LOQ)

LOQ was determined as the lowest OTA concentration that could be used to spike a blank sample and still originate repeatable and accurate results when the experimental procedure was applied to the referred sample. This value was revealed to be 0.1 ng/mL, which was considered sufficient for an accurate hazard evaluation.

4.3. OTA content in real sample

OTA quantification results are summarised in Table 3. Its analysis shows that, independently of origin, 74% of the samples were contaminated, revealing a widespread presence of ochratoxin A in the flour. This suggests that legislation concerning its storage should either be rethought or more tightly enforced.

Also, many samples featured OTA content above the LOQ determined for this study. However, since the LOQ is relatively low when compared to the legal limit, this is not particularly worrisome.

The maximum OTA levels for samples from both regions were similar, 0.49 and 0.43 ng/g for Algarve and Bragança, respectively, roughly one-seventh to one-sixth of the legal limit.

Although mean OTA content in the samples from the two areas does not present a significant statistical difference, with a p value of 0.35, those from Bragança feature, in average, a 33% higher mean contamination. This difference only becomes more pronounced if only the samples above the LOQ are taken into account, in which case the average contamination of the Bragança samples is a full 50% higher. These greater bread contamination levels may be due to the fact that Bragança is significantly colder than Algarve, which favours fungal OTA production. In all instances, the contamination levels remain far below the legal limit, and thus should present no health risk.

Standard deviations, however, are fairly high, in one instance even surpassing its corresponding mean value. This signifies that OTA content values are distributed over a large range, and even though the maximums, too, are below the legal limit, this indicates that storage conditions vary greatly within the same region, further emphasising the need for better surveillance.

As previously mentioned, one study acquired data concerning the OTA content of both wheat and maize bread in the Portuguese city of Coimbra [13]. Since maize and maize flour are much more vulnerable to fungal contamination than their wheat counterparts, comparing their results for that type of bread with those obtained here is of arguable relevance, and will therefore not be undertaken. However, comparing the results for wheat bread from all three sources, Coimbra’s bread, 0.02 ng/g, is far less contaminated than that from Algarve, 0.2 ng/g, and Bragança, 0.3 ng/g. Incidence in Coimbra is roughly one-fourth of Bragança’s and one-fifth of Algarve’s. The maximum value of OTA content for that city, 0.26 ng/g, is nearly half of that of the other two, while its mean is one-sixth of Algarve’s and one-eighth of Bragança’s.

On the other hand, studies have been performed that determined the OTA content of wheat bread in other countries [9,18,19]. The data collected from those studies is presented below, in Table 4.

<table>
<thead>
<tr>
<th>Country</th>
<th>Incidence (%)</th>
<th>Mean (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spain</td>
<td>93/93 (100%)</td>
<td>0.45</td>
</tr>
<tr>
<td>Holland</td>
<td>29/29 (100%)</td>
<td>0.39</td>
</tr>
<tr>
<td>U.S.A.</td>
<td>24/24 (100%)</td>
<td>0.41</td>
</tr>
<tr>
<td>Switzerland</td>
<td>20/20 (100%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Brazil</td>
<td>15/15 (100%)</td>
<td>0.09</td>
</tr>
<tr>
<td>France</td>
<td>14/14 (100%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Italy</td>
<td>12/12 (100%)</td>
<td>0.34</td>
</tr>
<tr>
<td>Germany</td>
<td>11/11 (100%)</td>
<td>0.35</td>
</tr>
<tr>
<td>Ireland</td>
<td>9/9 (100%)</td>
<td>0.36</td>
</tr>
<tr>
<td>Austria</td>
<td>9/9 (100%)</td>
<td>0.08</td>
</tr>
<tr>
<td>Tunisia</td>
<td>9/9 (100%)</td>
<td>0.30</td>
</tr>
<tr>
<td>Belgium</td>
<td>7/7 (100%)</td>
<td>0.23</td>
</tr>
<tr>
<td>Morocco</td>
<td>48/48 (48%)</td>
<td>13.00</td>
</tr>
<tr>
<td>Germany</td>
<td>897/986 (91%)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 3: Results of OTA quantification by LC-FD in wheat bread samples from the Algarve and Bragança regions of Portugal

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Sample size</th>
<th>Incidence</th>
<th>Mean ± RSD</th>
<th>Mean ± RSD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algarve</td>
<td>30 (80%)</td>
<td>18 (60%)</td>
<td>n.d. -0.49</td>
<td>0.12 ± 0.12</td>
<td>0.22 ± 0.1</td>
</tr>
<tr>
<td>Bragança</td>
<td>20 (65%)</td>
<td>10 (30%)</td>
<td>n.d. -0.43</td>
<td>0.36 ± 0.17</td>
<td>0.3 ± 0.14</td>
</tr>
</tbody>
</table>

Table 4: Incidence and mean values of OTA content of wheat bread from different countries [18,19,20]

*aAll samples.
*bSamples >LOQ only.
4.4. OTA confirmation procedure

The figures presented above are reproductions of the integrator print-outs for the ochratoxins’ standards (Fig. 3a), an ochratoxin-contaminated bread sample (Fig. 3b), and a sample containing OTA and subsequently esterified (Fig. 3c). Not only is the OTA peak both isolated and well-defined in both the standard and the sample, but it is also replaced by one at approximately 27 min after the sample has been subjected to methyl esterification, thus further confirming OTA presence and identity.

4.5. OTA Estimated Daily Intake (EDI)

To calculate the EDI of OTA from the obtained results, two assumptions were made: one, that the average weight of the Portuguese population is the same 65 kg as reported by Miraglia and Brera in their SCOOP report of 2002 [9], and two, that the average per capita consumption of bread by the Portuguese people has remained at the same level since 1994, that is, 32 kg/year, or 88 g/day [12].

These two assumptions, along with the data obtained in this study, result in an EDI of 0.26 ng/kg bw/day for the Algarve region and one of 0.38 ng/kg bw/day for Bragança and its outskirts, values correspondingly roughly to 1.5% and 2.0%, respectively, of the Tolerable Daily Intake (TDI) established by EC regulation [11]. However, the assumed bread consumption per capita falls far behind that advised by the WHO, 250 g/day. If the 88 g/day are replaced by the advised 250 g/day, the EDI would be 0.75 and 1.10 ng/kg bw/day, instead, which would be, for their part, 4.5% and 6.5% of TDI value established by EC.

The aforementioned SCOOP report contained data concerning the bread-derived EDI for three European countries, namely Spain (0.77 ng/kg bw/day), Germany (0.36 ng/kg bw/day), and Denmark (0.19 ng/kg bw/day). These would put the Portuguese people in line with their European counterparts in OTA EDI terms, with those from Bragança ingesting roughly as much as the Germans and those from Algarve half-way between the German and the Danish.

The SCOOP report also indicates the total Portuguese OTA EDI as being 0.81 ng/kg bw/day, and the cereals and cereal-derived products fraction of the diet as contributing half of that value. Though it is unknown how much of that fraction corresponds to bread, if we assume a not unreasonable 75%, we will end up with a total OTA EDI of 0.69 ng/kg bw/day in Algarve, and 1.01 ng/kg bw/day in Bragança. The average of these values is 0.85 ng/bw kg/day, which is very close to the national average reported by SCOOP.

5. Conclusions

Wheat bread from Bragança is noticeably more contaminated than that from Algarve. However, Bragança also features a colder climate which promotes OTA production. Most samples were contaminated, suggesting a widespread, potentially dangerous fungal growth in stored foodstuffs. On the other hand, none of those samples was found to be above the legal limit; furthermore, both mean and incidence values and the extrapolated EDI place those two regions below the European average, and in line with the expected national total intake.

Therefore, OTA contamination of bread in the two studied regions seems to be well under control and unlikely to pose a threat to consumer health. This, however, does not implicate that the current numbers can’t or shouldn’t be improved upon, nor that regular retesting should not be performed to monitor the situation.

Acknowledgments

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References