Ochratoxin A Detection by HPLC-FL in Processed Baby Foods

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Received Date: August 27, 2017; Accepted Date: August 30, 2017; Published Date: August 31, 2017

Abstract

270 samples of different types of pasta and cereal products, distributed in a nursery school canteen in Milan (Italy), were analyzed to estimate the occurrence of ochratoxin A.

OTA was found in all analyzed samples (100%), 39 samples exceeded the maximum limit established by European Commission Regulation for OTA in the foodstuffs listed in the relevant category.

The mean concentration of ochratoxin A in samples of pasta, intended for children (over 5 months) consumption was 0.56 µg/kg; the mean concentration in organic pasta was 0.91 µg/kg and the mean concentration detected in cereal products was 3.12 µg/kg.

Considering that in European Countries wheat products are the first solid food eaten by babies from the earliest stages of weaning and in the light of the multiple and repeated international food alert notification on OTA occurrence in cereals and cereal products, surveillance should be continuous for an effective risk assessment, especially in these vulnerable groups of consumers.

Keywords: Ochratoxin A; HPLC-FL; Baby food

Introduction

Ochratoxins are fungal secondary metabolites produced in particular climatic conditions by several fungal species in the Penicillium and Aspergillus genera [1,2] detected in several food commodities, especially during storage of contaminated wheat, oat and barley [3,4].

The most prominent of these toxins is ochratoxin A (OTA), followed by ochratoxin B (OTB) the dechloro analog of OTA, and ochratoxin C (OTC) its ethyl ester.

OTA has several adverse effects; depending on its nephrotoxicity in the IARC (International Agency for Research on Cancer) has classified this mycotoxin as a possible human carcinogen (Group 2B) [5]. However, human epidemiology is still inconclusive and expert group, as Joint FAO/WHO Expert Committee on Food Additives (JECFA), continually reviews epidemiological data.

In humans, exposure to OTA has been linked with Balkan endemic nephropathy (BEN), a chronic tubule-interstitial disease associated with progressive renal fibrosis and tumours of the renal pelvis and urethra [6-10].

Furthermore, the Scientific Committee on Food (SCF) in 1998 underlined that OTA possesses carcinogenic, nephrotoxic, teratogenic, immunotoxic and possibly neurotoxic properties [11].

OTA is commonly found in raw and processed food commodities such as cereals, oleaginous seeds, coffee, cocoa, beer, wine and spices [12], also due to its stability to food processing [13]. In addition, OTA was detected in meat products (including cured meats, sausages, pork, and chicken meat) as a result of carryover from contaminated animal feed [14-16].

As a consequence of the possible health hazards, the Regulation (EC) No 1881/2006 [17], and subsequent amendments thereto, set the maximum level for OTA in human food in order to perform preventive-based controls across the food supply chain.

The Panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority (EFSA), taking into account all data currently available, has established a Tolerable Weekly Intake (TWI) of 120 ng per kg body weight for OTA [18].

The CONTAM specified that infants and children, as well as distinct segments of the population, representing high consumers of cereals and processed cereal products, may have high rates of exposure to OTA.

Human exposure seems to be associated predominantly with the consumption of contaminated plant-derived products. Thus pasta, frequently made from durum wheat, is an important contributor to the human diet [19].

In recent years, there have been different reports about the presence of OTA in durum wheat products above the limit imposed by the legislation in UE and non-EU countries.

The most famous case to hit the headlines was the presence of OTA at three times the permissible European Union limits in Canadian-grown durum wheat that was imported into Italy in 2006 [20]. In 2008, the Rapid Alert System of Food and Feed of the European Union notified 20 alerts concerning OTA in different cereal products [21]. In
October 2014 (Notification details - 2014.1391) an elevated level of the OTA (14 µg/kg) was detected in whole emmer wheat pasta from Italy, distributed also to Austria, Belgium, Czech Republic, France, Germany, Ireland, Portugal, Romania, Spain, Switzerland and United Kingdom [22].

There are many studies on OTA levels in infant formulas and baby foods [23-29], but there are few data on the occurrence of OTA in pasta and bread consumed by children in Italy.

In Italy Vallone et al. [30], during a pilot program, showed OTA presence in samples of processed cereal baby food.

The aim of this study was to estimate the OTA concentration in: (i) pasta, (ii) organic pasta and (iii) cereals food samples administered to children in a nursery school in Milan (Italy) over a monitoring period of 10 months.

Materials and Methods

Samples

The study was carried out on 3 different groups of products: (i) group 1 consisted of four types of dedicated pasta named A, B, C and D, made from durum wheat and intended exclusively for children over 5 months; (ii) group 2 consisted of two types of organic pasta named E and F; (iii) group 3 consisted of three types of processed cereal products used for bread or crumbing foods: wheat flour, semolina and bread crumbs, named G, H, I, respectively.

Samples were collected from unopened packs, every ten days, for a period of 10 months (September - June). 30 samples for product, for a total of 270 samples analyzed for OTA detection.

Analytical reagents

Supelco OTA (product no. 469912), packaged in sealed ampoules at a concentration of about 50 ng/µl in benzene/acetic acid (99:1 v/v) was used as a standard. This standard was prepared according to official AOAC methods. The standards for the OchraTest by HPLC were prepared as follows:

Ochratoxin-working solution 1: Diluting Supelco ochratoxin A standard (50 ng/µl) 50 times, 20 µl of Ochratoxin A (50 ng/µl) + 980 µl ethyl alcohol ≥ 1 ng/µl.

Ochratoxin-working solution 2: Diluting Ochratoxin-working solution 1 (1 ng/µl) 10 times, 100 µl of Ochratoxin-working solution 1 (1 ng/µl) + 900 µl ethyl alcohol ≥ 0.1 ng/µl.

The calibration curve was obtained by diluting Ochratoxin-working solution 2 with methanol to obtain the following concentrations: 1.0, 10.0, 20.0 and 50.0 (ng/g).

We added 1.5 ml water to all our standards and samples before injecting onto the HPLC to make the solvent for the standards and samples similar to the mobile phase.

HPLC grade ethanol, methanol, acetonitrile, acetic acid, PBS buffer (Phosphate Buffered Saline 10X concentrate) and water were purchased from Sigma Aldrich (St. Louis, USA).

Blank (Wheat-Ochratoxin A Reference Number BCR-471) was purchased from LGC Limited (Middlesex, UK).

Boron trifluoride methanol complex, 0.5 mL 20% solution in methanol, supplied by Merck.

Mycoxtoxin Reference Material (Wheat, Article Number TR-O100, batch number O-W-825, OTA concentration 7.0 ppb, Expiry date 08/01/2016) was purchased from Trilogy Analytical Laboratory, INC. (Washington, USA): was used this reference material because “Pasta certified material” was not available.

Apparatus and chromatographic conditions

For liquid chromatography analysis, an Agilent 1100 Series instrument equipped with pumps, a Rheodyne Model 7125 injector (100 µl loop) and a fluorescence detector was used. A LC Restek column C18 (5 µm) (250 mm x 4.6 mm internal diameter) was used with a mobile phase consisting of a mixture of water:acetonitrile: acetic acid (49.5:49.5:1 v/v/v), degassed at a flow rate of 0.9 ml/min. Detection of OTA was carried out using 333 and 477 nm as wavelengths for excitation and emission, respectively. The Retention time (RT) was 10.47 (± 0.10) min.

Immunooaffinity clean-up

To measure ochratoxin levels, samples were prepared by mixing with an extraction solution (see below), followed by blending and filtering. The extract was then applied to the OchraTest WB column (VICAM, Milford, USA), which contained specific antibodies for OTA. Using monoclonal affinity chromatography, OchraTest is the only ochratoxin test that produces precise numerical results. At this stage, the ochratoxin binds to the antibody on the column. The column is then washed to rid the immunoaffinity column of impurities. By passing methanol through the column, the ochratoxin is removed from the antibody: The methanol can then be injected into an HPLC system.

Sample extraction and clean-up of samples

For the extraction and clean-up phases of Blank, Reference Material and samples was used the method already described by Rahmani et al. [31] with some modifications.

A 50 g aliquot of blank/Reference Material/sample was extracted with 100 ml of acetonitrile/water (60:40 v/v) for 5 minutes. A 10 ml aliquot of the homogenate was filtered and diluted with 40 ml of PBS buffer (pH 7.0).

The diluted extract was loaded onto an OchraTest WB column. After washing with 10 ml PBS Buffer and 10 ml of water, the mycotoxin was eluted with 1.5 ml of methanol. 1.5 ml water was added to all samples before injecting onto the HPLC to make the solvent for the standards and samples similar to the mobile phase.

Method validation

The linearity of the method was determined by performing five concentration levels of standard solutions (0.1, 1.0, 2.5, 5.0, 10.0 ng/g). All samples, including blank and reference material, were analysed in triplicates.

The LODs and LOQs were calculated on the basis of signal-to-noise ratio (S/N) of three (3:1) and ten times (10:1) of the background chromatographic noise, respectively. Accuracy and precision of the method were evaluated via intra- and inter-day analyses, which were performed in the same laboratory by the same instrument. Intra-day precision (repeatability) was determined at three concentration levels (0.1, 0.5, 5 ng/g) by using Ochratoxin A fortified blank sample (wheat). Inter-day precision (reproducibility) was conducted at the same three
concentration levels of OTA and determined on three different days. Accuracy was expressed as percentage of recovery, and intra-day and inter-day precision for peak area were expressed as RSD (Table 1).

<table>
<thead>
<tr>
<th>Spiked concentration (ng/g)</th>
<th>Accuracy (mean % ± SD)</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day (n=9)</td>
<td>Inter-day (n=27)</td>
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<tr>
<td>0.1</td>
<td>103.1 ± 7.6</td>
<td>7.4</td>
</tr>
<tr>
<td>0.5</td>
<td>98.6 ± 3.3</td>
<td>3.9</td>
</tr>
<tr>
<td>5</td>
<td>91.9 ± 2.7</td>
<td>3.3</td>
</tr>
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</table>

Table 1: Intra- and inter-day accuracy and precision for OTA detection.

The OTA presence confirmation was performed by the methyl ester derivative of OTA (MET-OTA) identification method according to Lerch and Müller [32] 1 ml of immunoaffinity eluate, after drying, was dissolved in boron trifluoride methanolic solution and heated to 60°C for 15 min. Under the HPLC conditions previously described the confirmation of the presence of OTA was showed by the OTA peak disappearance [Retention time (RT) at 10.47 (± 0.10) min] and by the METOTA peak appearance of [(RT) at 15.4 (± 0.10) min].

Statistical analysis

The different concentrations of OTA found in the collected samples were analyzed using two-way analysis of variance with replication (ANOVA) considering the time and the type of food. The OTA concentrations reported for each month in the food samples were subsequently shown by means of flow diagrams of OTA concentration in the 3 groups of food: (i) pasta used exclusively for children over 5 months; (ii) organic pasta and (iii) processed cereal products.

Results

Method performance

The OTA curve calibration of standard solution performing at five concentration levels was \( y=7.2389x+1.9874 \) with \( R^2=0.998 \).

The limit of detection (LOD) was 0.036 ng/g, while the limit of quantification (LOQ) was 0.12 ng/g.

The OTA-LOD results to be lower when compared with LODs of OTA in cereal based products reported by other authors [33-35,37].

Accuracy, expressed as percentage of recovery (mean recoveries with standard deviation, ranged 91.9 ± 2.7-103.1 ± 7.6% (intra-day) and 91.4 ± 5.8-103 ± 8.1 (inter-day). These recoveries according to other authors [36], are higher than those obtained with other methods (IAC, pressurized liquid extraction, multifunctional column, matrix solid phase dispersion) by other authors [33-35,37].

Precision, expressed as RSDs, was between 3.3% and 7.4% (Intra-day) and 6.2% and 7.9% (Inter-day).

To check reliability of the applied method, certified reference materials consisted of wheat flour, which was certified to contain 7 ppb of OTA was analyzed. The quantified OTA (three replicates mean) content was of 6.8 ± 0.3 ng/g (Figure 1).

Occurrence of OTA

OTA was detected in all collected samples: the lowest and highest values found were 0.45 and 4.85 ng/g, respectively.

The mean OTA concentration evaluated in Group 1 was 0.56 ng/g (0.57; 0.70; 0.53 and 0.46 ng/g for A, B, C and D, respectively). The mean OTA concentration, evaluated in Group 2 was 0.91 ng/g (0.89 and 0.94 ng/g for E and F, respectively). Finally the mean OTA concentration measured in Group 3 was 3.12 ng/g (3.92; 3.33 and 2.11 ng/g for G, H and I respectively) (Table 2 and Figure 2).
The chromogram of a positive wheat flour sample (OTA level equal to 3.71 ng/g) was reported as Figure 3.

Discussion

Although many authors consider that climatic and environmental conditions during growth, harvest and storage have great influence on mycotoxic levels, two-way analysis of variance with replication (ANOVA), showed that OTA concentration was not influenced by time of sampling (p>0.05), but was only related to type of food (p<0.001).

Mean OTA value in Group 1 was slightly higher than allowed by Regulation (EC) No 1881/2006, i.e., 0.5 µg/kg. In the Group 2 samples mean OTA concentration was lower than the limit set by Regulation (EC) No 1881/2006, i.e., 3 µg/kg. Finally, in the Group 3 samples, mean OTA concentration was slightly higher than allowed by Regulation (EC) No 1881/2006, i.e., 3 µg/kg; with the exception of the breadcrumbs (I) that showed a mean value of 2.11 µg/kg (Table 1 and Figure 2).

The OTA concentration evaluated in Group 2 (organic pasta) represents a contradiction if compared to the mean value found in Group 1. Indeed these are products on which consumers pay more on the market and buy because they are considered safer due to the presumed absence of chemical contaminants. Antifungal agents are not employed in organic farming products: it is likely that fungi develop at the risk of producing secondary metabolite like OTA.

In addition, the type of organic pasta administered in this kindergarten was not specifically for children consumption as well as wheat flour, semolina and breadcrumbs. Moreover, the EU legal limit above reported (3 µg/kg) refers to the limit set for all products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption that is for adults of 70 kg of body weight.
The detection of OTA in 100% of the tested samples can probably be explained by analysing the sampling, that occurred in a single canteen of Milan that will surely has a single source purchasing for the products considered in this study.

This analytical evidence is not comparable with others data in the literature concerning the occurrence of OTA in pasta and semolina [25,38-40], because the reported cases concerning exclusively food proposed for adults. However, OTA concentrations detected in these studies were usually below 2 μg/kg, except for only one pasta sample analysed in Germany with a very high level of OTA (29.8 μg/kg) [41].

To the best of our knowledge, the occurrence and the relative intake of OTA from wheat flour, semolina and bread crumbs used for breading or crumbing foods was not properly investigated, however, even for this type of cereal products should be chosen dedicated products for children, considering the OTA high concentration found.

**Conclusion**

As set out in Regulation (EC) No 1881/2006 [17], this widespread contamination by OTA is a parameter that can be used to judge the quality of raw food used in nursery school.

To ensure food quality and safety, it would be necessary to adopt an integrated approach to the production line that can monitor the safety of the pasta and cereals used for children’s regular menu, in order to meet the limits set by Regulation (EC) No 1881/2006 [17].

Exposure to OTA may result in accumulation and chronic intoxication with renal damage. Other mycotoxins, as citrinin (CIT) and fumonisin B1 (FB1), have the same target organ (kidney) and their effects can be exerted on the renal tissues [27,42].

As regards the risk of accumulation of specific substances such as mycotoxins, it would be necessary to consider that baby foods and infant formulae can be contaminated simultaneously by different mycotoxins producing additive or synergistic toxic effects [43-45]. Since it is difficult to remove mycotoxins once formed, the best control measure is the prevention.

Traditionally, in European countries, wheat products are the first solid food eaten by babies from the earliest stages of weaning. Therefore, child consumers have higher cereal consumption than adults in relation to body weight. Furthermore in Italy pasta, bread and cereal products are considered staple foods.

The quality of the processed baby foods necessarily relies on precise controlling of every steps of production.

**Conflict of Interest**

The authors declare no conflict of interest.

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J Nephrol Ther, an open access journal
ISSN: 2161-0959
Volume 7 • Issue 4 • 1000301
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