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Occurrence and risk assessment of zearalenone in flours from Portuguese and Dutch markets

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ABSTRACT

The occurrence of zearalenone (ZEA) in different flours for human consumption, from the Portuguese and Dutch markets, was evaluated. Good analytical performance was obtained through extraction with acetonitrile:water (90:10), clean-up with immunoaffinity columns, and detection and quantification by liquid chromatography-fluorescence detection. ZEA levels were determined in 48 samples to verify the compliance with the maximum permitted levels by European legislation. Two flour samples from Portugal exceeded the maximum limit established by EC. A major presence and levels in maize flours was shown. Coimbra (Portugal) and Utrecht (The Netherlands) samples showed that 37.5% of the samples were contaminated. Considering the percentage of TDI, ranging between 5.2 and 56%, the risk assessment linked with the exposure to ZEA was considered to be of concern for some studied populations, especially for babies. This is the first study on the intake assessment of ZEA present in different types of flour through their consumption.

1. Introduction

Zearalenone (ZEA), 6-(10-hydroxy-6-oxo-trans-1-undecenyl) β-resorcylic-acid-lactone, is associated mainly with cereal crops and found most commonly in maize. It is a secondary metabolite biosynthesized by a large range of *Fusarium* fungi, including *Fusarium graminearum* (*Gibberella zeae*), *Fusarium culmorum*, *Fusarium cerealis*, *Fusarium equiseti*, *Fusarium crookwellense*, and *Fusarium semitectum*. Members of the *Fusarium* genus infect cereals in the field, leading to toxin production mainly before harvesting, but also post-harvest, if the crop is not dried properly and stored in suitable conditions. Infestation of cereal grain and derivatives is especially prevalent in temperate climates, when relatively cool temperatures and high humidity coincide with flowering and early kernel filling stages of the grain (Zinedine, Soriano, Moltó, & Mañes, 2007).

Because the toxins production takes place before the harvest and to a lesser extent during the storage, ZEA is a field contaminant of crops, affecting a wide variety of cereals, being maize the most contaminated cereal, although other cereals such as wheat, oat, barley, sorghum and rye may be contaminated (Martos, Thompson, & Diaz, 2010).

Worldwide several studies have reported high ZEA contamination in a wide variety of important agricultural products, especially cereals. However, only few of them refer to a very restricted number of flour samples. Some studies for wheat flour have been reported in The United Kingdom (Vendl, Crews, MacDonald, Krska, & Berthiller, 2010), Spain (Vidal, Marín, Ramos, Cano-Sancho, & Sanchis, 2013), France (Sirot, Fremy, & Leblanc, 2013), Serbian market (Škrbić, Živančev, Đurišić-Mladenović, & Godula, 2012), and Bulgaria (Škrbić et al., 2012). For maize flour few studies were also reported in Indonesia (Nuryono, Noviandi, Böhm, & Razzazi-Fazeli, 2005), Germany (Reinhold & Reinhardt, 2011), and Iran (Reza Oveisi, Hajimahmoodi, Memarian, Sadeghi, & Shoeibi, 2005).

The European Commission, in 2007, through EC legislation N° 1126/2007 (European Commission, 2007), established regulatory limits in order to protect public health. These limits oscillate between 20 μg/kg, for processed cereal-based foods (excluding processed maize-based foods), baby foods for infants and young children, processed maize-based foods for infants and young children, and 400 μg/kg for refined maize oil, being of 75 μg/kg for cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption.

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ZEA produces estrogenic effects in humans and animals leading to hyperestrogenism. ZEA can act as an estrogen analog and in humans has been recently considered as a triggering factor for central precocious puberty at least in prepubertal girls (Vidal et al., 2013). ZEA may induce troubles of the reproduction function: lower fertility, fetal wastage, and lower hormone levels (Sirot et al., 2013). Despite being a non-steroidal estrogenic toxin, it was categorized in the group 3 (not classifiable as to its carcinogenicity to humans) by the International Agency for Research on Cancer (International Agency for Research on Cancer, 2002, p. 601).

In 2000, JECFA established a provisional maximum tolerable daily intake (PMTDI) of 0.5 μg/kg b.w./day for ZEA, based on the oestrogenic activity of zearalenone and its metabolites, in the most sensitive animal specie, the pig, but the SCF, in the same year, proposed a lower temporary TDI (t-TDI) of 0.2 μg ZEA/kg b.w./day based on a study on pig. Recently, in 2011, the EFSA proposed a new TDI of 0.25 μg/kg b.w./day based on more recent data on pig, but also taking into account comparisons between pigs and humans (EFSA, 2011).

This work was aimed to evaluate the ZEA levels in maize, wheat, and mixed-flours for human consumption, from the Portuguese and Dutch markets. In order to obtain a good analytical performance, different experimental conditions, such as the mobile phase composition, and extraction procedures were primarily optimized using high performance liquid chromatography (HPLC) with fluorescence detection (FD). Afterwards, the occurrence and levels of ZEA were determined in 48 samples in order to verify the compliance with the maximum limits of the European legislation. The estimated daily intake of ZEA was also assessed in different populations for both countries, in order to evaluate their risk assessment through the consumption of different flour types.

2. Materials and methods

2.1. Sampling

A total of 48 samples of flours (17 wheat flours, 12 corn flours, 13 mixed-flours with mainly wheat flour and 6 baby foods) were analyzed. The samples were purchased in different supermarkets of Coimbra, central zone of Portugal ($n = 42$), and Utrecht (The Netherlands) ($n = 6$), during the winter season of 2013, between December 2012 and March 2013. The samples collected in Portugal are those commercially available on the national market. Regarding the Dutch samples, a limited number was possible to achieve, nonetheless, it was considered interesting to include them in the study.

After purchase, the samples were brought to the laboratory under ambient conditions, and all the information available on the labels was assembled. Samples were kept in the same conditions until their analysis, and the positive samples were frozen.

2.2. Chemical and reagents

The reagents of HPLC grade used were acetonitrile and methanol (Carlos Erba, Milan, Italy). Glacial acetic acid was obtained from Panreac Química (Sau, Barcelona, Spain). Sodium chloride was obtained from Pronolab (Lisboa, Portugal).

Micro-glass fiber paper (150 mm, Munktell & Filtrak GmbH, Bärenstein, Germany), Whatman N°1 filter paper, and polyamide membrane filters (0.2 ìm, 50 mm, Whatman GmbH, Dassel, Germany) were used. Immunoaffinity columns (IAC) ZearalaTest™ were from VICAM (Watertown, USA).

Water was daily obtained from Milli-Q System (Millipore, Bedford, MA, USA) and the ZEA standard, a white powder, with a purity degree ≥99.0 was obtained from Sigma–Aldrich (St. Louis, MO, USA).

A mobile phase (acetonitrile:water 60:40) with an adjusted pH at 3.2 with glacial acetic acid, at 1 mL/min, was used. All liquid chromatographic reagents were degassed for 15 min in an ultrasonic bath.

ZEA standard stock solution was prepared at 5 mg/mL, diluting 10 mg of ZEA in 2 mL of acetonitrile, and stored at −20 °C. The intermediate solution was prepared by diluting the stock solution at 50 μg/mL in acetonitrile, and a working standard solution, at 1 μg/mL in acetonitrile, was prepared by diluting the intermediate solution. They were stored in darkness, at 4 °C, until the analysis.

The calibration curve standard solutions, in solvent, were prepared between 12.5 and 200 ng/mL (12.5, 25, 50, 100, 200 ng/mL) in acetonitrile. The concentrations for the matrix-matched calibration curve were prepared between 20 and 250 μg/kg (20, 50, 75, 125, 250 μg/kg).

2.3. Sample extraction and clean-up

Samples (20 g) were weight with 2 g salt (NaCl) and mixed in a centrifuge glass. Then, they were extracted twice with 50 mL of acetonitrile:water (90:10) each time, and centrifuged for 15 min at 2500 *g*. The supernatants (10 mL) were mixed with 40 mL of Milli-Q water, and the mixture filtered through micro-glass fiber paper. Ten milliliters of the resulting filtered were passed through the IAC at a vacuum-induced rate of 1 drop per second. After, the IAC was washed with 10 mL of water, before the elution with 1.5 mL of methanol. The eluate was dried at 42 °C under a gentle nitrogen flow. The dried extract was stored at −20 °C until re-dissolution in acetonitrile (500 μL), and injection in the LC-FD system.

2.4. LC conditions

The LC instrument was equipped with a pump (Model 307, Gilson Medical Electronics, Villiers-le-Bel, France), and a Hichrom Nucleosil C_{18} column (5 μm, 250 × 4.6 mm i.d.). For detection a spectrofluorimeter, Perkin–Elmer Model LS45 (Beaconsfield, UK) was used and excitation and emission wavelengths were set, respectively, at 274 nm and 455 nm. The results were recorded on a Hewlett–Packard 3390A integrator (Philadelphia, PA, USA). LC-FD analyses were performed using an injection volume of 100 μL.

2.5. Recovery studies

Recoveries were determined by spiking ZEA – free flours at three different levels, 20, 75, and 200 μg/kg, using three replicates for each level, according to the maximum limits (MLs) established by the EC legislation No 1126/2007 for processed cereal-based foods and baby foods for infants and young children, cereal flour, and milling fractions of maize with particle size > 500 micron and other maize milling products with particle size > 500 micron not used for direct human consumption, respectively.

2.6. Calculation of estimated daily intake

Estimated Daily Intake (EDI) was calculated through a deterministic method (IPCS, 2009) using the equation $EDI = (\Sigma c)(CN^{-1}\,D^{-1}\,K^{-1})$, where Σc is the sum of zearalenone in the analyzed samples (μg/kg), C is the mean annual intake estimated per person, N is the total number of analyzed samples, D is the number of days in a year, and K is the body weight. The latest assessment of the cereal consumption in Portugal corresponding to 2012 is 133.9 kg/inhabitant, being 115.5 kg for wheat and 11.8 kg for maize (INE, 2013). For Dutch population, the total cereal consumption was, for male, 227.7 kg/inhabitant, and 171.3 kg/inhabitant for females, during 2007–2010, according to RIVM (2011). Mean body weight for the