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

Juan Ramos Aldana [a,b], Liliana J.G. Silva [a], Angelina Pena [a], Jordi Mañes V. [b], Celeste M. Lino [a,*]

[a] Group of Health Surveillance, CEF, Faculty of Pharmacy, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal
[b] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

ARTICLE INFO

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 postharvest, 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.

* Corresponding author. Tel.: +351 239488477; fax: +351 239488503.
E-mail addresses: clino@ff.uc.pt, cmlino@ci.uc.pt (C.M. Lino).

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 = (Σc) $(CN^{-1} D^{-1} K^{-1})$, 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

Portuguese adult population was considered 69 kg (Arezes, Barroso, Cordeiro, Costa, & Miguel, 2006), and for Dutch population was 84 kg for male adults and 70 kg for female adults (RIVM, 2011). For babies, the considered body weight was 7.5 kg, according to Portuguese Society of Paediatrics (Sociedade Portuguesa de Pediatria, 2013).

3. Results and discussion

3.1. Analytical performance

Several experimental conditions were tested in order to obtain adequate resolution of the ZEA peak. Different mobile phases, with different concentrations of acetonitrile and water (50:50, 55:45, and 60:40) were evaluated. Mobile phases at 50:50 and 55:45 had unclear peaks and the retention time was too long. Good analytical performance was obtained using a mobile phase consisting of acetonitrile:water (60:40) with a flow rate of 1.0 mL/min.

The mixture acetonitrile:water showed high efficiency, as previously described for fumonisins B1 and B2 extraction in maize and maize-based samples (Lino, Silva, Pena, & Silveira, 2006). Various extraction mixtures of acetonitrile/water and methanol/water have been used to extract ZEA from cereals (Juan, Ritieni, & Mañes, 2012). However, some authors found low recoveries when the methanol/water mixture was used (Sulyok, Berthiller, Krska, & Schuhmacher, 2006).

Initially, an extraction procedure consisting of sample blending with the extraction solvent, following filtration through a Whatman N°1 filter paper, was attempted. Nonetheless, the slurry produced after extraction clogged the filter paper leading to losses. Due to the characteristics of the sample, an efficient process for separating the matrix residue from the solvent extract was essential. Centrifugation was crucial to improve this step. Moreover, the time expended when the method with centrifugation step was applied was much lower. The centrifugation step allowed good separation between sample residue and extraction solution.

Linearity, in standard solutions (12.5–200 ng/mL) and in matrix-matched assays (20–250 μg/kg), was adequate, $r^2 = 0.998$ and $r^2 = 0.997$, respectively. Both matrix and standard calibration curves were used to calculate the matrix effect (ME) (Rubert, Soriano, Mañes, & Soler, 2011). The obtained value, 92.5%, can be considered negligible.

Recovery values, for fortification levels at 20, 75 and 200 μg/kg, ranged between 97.6 and 105.3% for 200 μg/kg and 75 μg/kg, respectively. The intra-day repeatability varied between 2.0% and 9.0% for the level at 75 and 200 μg/kg, respectively. The inter-day repeatability oscillated between 6.5% and 13.6% for 20 and 75 μg/kg, respectively. The validation results comply with the requirements established by the EC directive 401/2006 (European Commission, 2006).

LODs and LOQs were established as the amount of analyte that produces a signal-to-noise ratio of 3:1 and 10:1 respectively. LOD and LOQ were 3.75 and 12.5 μg/kg, respectively. These values are satisfactory considering the maximum levels established by the Commission Directive, 2007/1126/EC of the European Commission (European Commission, 2007) and similar with those obtained by other authors (Manova & Mladenova, 2009; Reinhold & Reinhardt, 2011). These authors found LODs of 4 μg/kg (Manova & Mladenova,

Table 1
Frequency (%) and levels (μg/kg) of ZEA in different flours.

Sample	Sample size	Frequency (%)	Range (μg/kg)	Mean ± SD (μg/kg)
Wheat flour	19	6 (31.6)	7.4–15.3	11.7 ± 3.1
Maize flour	12	6 (50)	5.9–111.7	28.0 ± 41.4
Mixed-flour	17	6 (35.2)	5.4–39.4	23.1 ± 11.7
TOTAL	48	18 (37.5)	5.4–111.7	21.0 ± 24.7

Table 2
Frequency (%) and levels (μg/kg) of ZEA in flours of different countries.

Sample	Sample size	Frequency (%)	Range (μg/kg)	Mean ± SD (μg/kg)
Portugal				
Wheat flour	17	4 (23.5)	7.4–15.3	10.7 ± 3.5
Maize flour	12	6 (50)	5.9–111.7	28.0 ± 41.4
Mixed-flour	13	4 (30.8)	5.4–39.4	20.4 ± 15.1
The Netherlands				
Wheat flour	2	2 (100)	12.4–13.7	13.1 ± 1.0
Mixed-flour	4	2 (50)	19.8–37.2	28.5 ± 12.3

2009) and 1 μg/kg (Reinhold & Reinhardt, 2011) and LOQs oscillating between 4 μg/kg (Reinhold & Reinhardt, 2011) and 12 μg/kg (Manova & Mladenova, 2009).

3.2. Surveillance results

ZEA content was evaluated in the totality of maize, wheat, and mixed-flour samples (Table 1). Fifty per cent of maize flour samples were contaminated with ZEA in contrast with 35.2% of mixed-flours and 31.6% of wheat flours. Maize flours also showed the highest mean levels, 28.0 μg/kg, followed by mixed and wheat flours, with 23.1 and 11.7 μg/kg, respectively. One maize flour, with 111.7 μg/kg, exceeded the ML of 75 μg/kg proposed by EC legislation No 1126/2007 (European Commission, 2007) for cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption. One mixed-flour for babies, with 25.2 μg/kg, also surpassed the ML of 20 μg/kg for processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children, proposed by the same EC legislation (European Commission, 2007), and another one was close to the limit, with 19.8 μg/kg.

Wheat flours from The Netherlands presented higher mean levels than those from Portugal, 13.1 and 10.7 μg/kg, respectively (Table 2). One similar situation was observed for mixed-flours with 28.5 and 20.4 μg/kg, respectively. The two flour samples that exceeded the ML were marketed in Portugal.

With regard to the purpose of the samples, as shown in Table 3, the most contaminated samples where those intended for culinary uses, 26.6 μg/kg, followed by baby flours, 19.0 μg/kg, and for bread making, 13.3 μg/kg. ZEA was not detected in flours for frying or in semolina.

For wheat flour, the results obtained in the present study are higher than those reported for The United Kingdom (<10 μg/kg) (Vendl et al., 2010), for Spain (8 μg/kg) (Vidal et al., 2013), in the Serbian market (4.3 μg/kg) (Škrbić et al., 2012), and in France (3.3 μg/kg) (Sirot et al., 2013). In a previous study, performed by GC–MS, in Portugal, ZEA was found in one of the seven analyzed samples, with 27.0 μg/kg (Cunha & Fernandes, 2010). The frequency of contamination in wheat flours was lower in a study carried out in the Spanish market (13%) (Vidal et al., 2013). Inversely, a study from Bulgaria (Škrbić et al., 2012) showed a higher occurrence, 33.3%. However, in some studies carried out in Spain, ZEA was not detected in 8 flour samples (Serrano, Font, Ruiz, & Ferrer, 2012)

Table 3
Frequency (%) and levels (μg/kg) of ZEA in flours according to the purpose.

Purpose	Sample size	Frequency (%)	Range (μg/kg)	Mean ± SD (μg/kg)
Baby flour	6	3 (50)	11.8–25.2	19.0 ± 6.7
Culinary uses	24	9 (36)	5.9–111.7	26.6 ± 33.4
For bread	13	6 (46.2)	5.4–37.2	13.3 ± 11.9
For frying	1	0 (0)	n.d.	n.d.
Semolina	4	0 (0)	n.d.	n.d.

n. d. – not detected.

Table 4
Estimated Daily Intake (EDI) by different populations and the respective comparison with tolerable daily intake (TDI) proposed by EFSA in 2011.

ZEA		TDI[b]	Wheat flour		Maize flour		Baby flour	
			EDI[a]	TDI(%)	EDI[a]	TDI(%)	EDI[a]	TDI(%)
Portugal[c,d]		0.25 μg/kg b.w./day	0.049	19.6	0.013	5.2	0.099	39.6
The Netherlands	Male[e]		0.097	38.8	–	–	0.14	56.0
	Female[f]		0.087	34.8	–	–		

[a] Calculated in μg/kg b.w./day.
[b] TDI proposed by EFSA (2011).
[c] EDI was calculated using the equation EDI = $(\sum c)\,(CN^{-1}\,D^{-1}\,K^{-1})$, where $\sum c$ is the sum of zearalenone in the analyzed samples (μg/kg), C is the mean annual intake estimated per Portuguese inhabitant in 2012 (INE, 2013), N is the total number of analyzed samples, D is the number of days in a year, and K is the mean body weight for adults, which was considered 69 kg and 7.5 kg for babies (mean of body weight of the Portuguese population from data retrieved from Arezes et al. (2006) and the Portuguese Society of Paediatrics (Sociedade Portuguesa de Pediatria, 2013), respectively.
[d] C in the EDI equation is 115.5 kg/inh of wheat flour, 11.8 kg/inh of maize flour and 14.6 kg/inh of baby flour (INE, 2013).
[e] C is the mean annual intake estimated per Dutch male inhabitant in 2007–2010 (227.7 kg/inh) (RIVM, 2011) and K is the mean body weight for male adults, which was considered 84 kg and for babies (male and female) 7.5 kg.
[f] C is the mean annual intake estimated per Dutch female inhabitant in 2007–2010 (171.3 kg/inh) (RIVM, 2011) and K is the mean body weight for male adults, which was considered 70 kg.

neither in 119 samples of wheat-based cereals (Rodríguez-Carrasco, Moltó, Berrada, & Mañes, 2014).

As regards maize flours, few data are disposable on scientific literature. Some authors (Marques, Martins, Costa, & Bernardo, 2008) detected 2 samples contaminated at levels between 0.1 and 1.0 mg/kg, but ZEA was not detected in the five analyzed samples, in Porto, Portugal (Cunha & Fernandes, 2010). Rodríguez-Carrasco et al. (2014) detected ZEA in one of 17 maize-based cereals sampling in Spain, in 2012, at level <LOQ. In Germany, Reinhold and Reinhardt (2011) detected two samples contaminated, among the eight analyzed, with mean levels of 31.7 μg/kg, containing one of them 71.8 μg/kg. The obtained mean levels in the Indonesian study carried out by Nuryono et al. (2005), in 2005, 6.9 μg/kg, were lower than those found in this study, 28 μg/kg. In Iran, Reza Oveisi et al. (2005) found ZEA in the nineteen maize flours ($n = 19$), whose levels oscillated between 36 and 889 μg/kg. The occurrence of ZEA was also lower in Indonesia, 15.4%, as reported by Nuryono et al. (2005), and in Bulgaria, 25%, Reinhold and Reinhardt (2011). However, in Iran, the frequency was higher 63%, as referred by Reza Oveisi et al. (2005).

Wheat flour samples showed less concentration and frequency of ZEA than maize samples. Higher concentrations of ZEA, in maize samples, have been also reported by Martos et al. (2010).

3.3. Estimated daily intake and risk assessment

As far as we know, this is the first study on the intake assessment of ZEA present in different types of flour through their consumption. Due to the lack of data about the risk assessment resulting from the flour consumption, a comparison between the results of this study with other countries is impossible.

Despite the maize flour samples present higher levels of contamination compared to wheat flour, the risk of exceeding the tolerable daily intake (TDI) is higher in wheat flour due to its higher consumption (Table 4).

As shown in Table 4, the EDI for male and female Dutch populations through the wheat flour consumption is higher than the Portuguese adult population, representing 34.8–38.8% and 19.6%, respectively, of the TDI proposed by EFSA, in 2011, of 0.25 μg/kg b.w./day. This situation is explained by the highest consumption by the Dutch inhabitants (227.7 kg/inhabitant for male and 171.3 kg for females) in comparison with Portuguese population (115.5 kg/inh). A similar situation was observed for babies, once the TDI % obtained through this study is 39.6% and 56% for Portuguese and Dutch babies, respectively. The risk assessment resulting of maize flour consumption is the lowest for the Portuguese population, 5.2%.

The estimated daily intake (EDI) ranged between 0.013 and 0.14 μg/kg b.w./day, which represents 5.2% and 56% of the TDI established by EFSA.

According to the review of Maragos (2010), the EDIs for babies (0.099 μg/kg b.w./day) and for adults (0.049 μg/kg b.w./day), in Portugal, and in The Netherlands (0.14 μg/kg b.w./day for babies) (0.097 μg/kg b.w./day for males/0.087 μg/kg b.w./day for females) are higher than that for infants aged between 6 and 9 months (<0.06 μg/kg b.w./day) and for adults (<0.016 μg/kg b.w./day), in Canada. In Germany, for infants, and in the UK, for ages 4–6, the mean intake were 6.5 ng/kg b.w./day and 54.8 ng/kg b.w./day, respectively. The mean intake for the Swiss population was estimated to be <0.02 μg/kg b.w./day, and in France the mean exposure for adults (15 years and older) was estimated at 33 ng/kg b.w./day, while for children (3–14 years) was estimated at 66 ng/kg b.w./day. Škrbić et al. (2012) estimated an intake of 0.02 μg/kg b.w./day through consumption of the wheat flour and wheat-based products in Novi Sad, Serbia. Among Catalonian populations, Cano-Sancho, Marin, Ramos, and Sanchis (2012) found, for infants and toddler, the highest mean estimated intake of ZEA, 12.2–17.9 ng/kg b.w./day, and the lowest for elders, 0.3–0.5 ng/kg b.w./day.

For the studied populations, the risk is higher for babies than for adults, both in Portuguese and Dutch populations, due to their higher food consumption level per kg body weight, which makes them an especially vulnerable group. Therefore, results imply that constant monitoring throughout the cereals production chain is required in order to minimize health risks related to the intake of ZEA present in flours.

4. Conclusions

The performed analytical methodology fulfilled the requirements established by the EC directive 401/2006.

ZEA contamination was found less frequently in wheat flours, followed by mixed-flours, whereas the occurrence and incidence were higher in maize flours. For the studied populations, the risk is higher for babies than for adults both in Portuguese and Dutch populations.

These results show that systematic control is required and indicate the need of preventative research to ensure the safety of food products. Continuous surveillance is necessary to avoid overlap the statutory limits in order to protect the human health.

References

Arezes, P. M., Barroso, M. P., Cordeiro, P., Costa, L. G., & Miguel, A. S. (2006). *Estudo Antropométrico da População Portuguesa* (1st ed.). Lisboa: Instituto para a Segurança, Higiene e Saúde no Trabalho.

Cano-Sancho, G., Marin, S., Ramos, A. J., & Sanchis, V. (2012). Occurrence of zearalenone, an oestrogenic mycotoxin, in Catalonia (Spain) and exposure assessment. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 50*(3–4), 835–839. http://dx.doi.org/10.1016/j.fct.2011.11.049.

Cunha, S. C., & Fernandes, J. O. (2010). Development and validation of a method based on a QuEChERS procedure and heart-cutting GC–MS for determination of five mycotoxins in cereal products. *Journal of Separation Science, 33*(4–5), 600–609. http://dx.doi.org/10.1002/jssc.200900695.

EFSA, P. on C. in the F. C. (2011). Scientific Opinion on the risks for public health related to the presence of zearalenone in food. *EFSA Journal, 9*(6). http://dx.doi.org/10.2903/j.efsa.2011.2197, 2197, 1–124.

European Commission. (2006). COMMISSION REGULATION (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Union, L70*, 12–34.

European Commission. (2007). COMMISSION REGULATION (EC) No 1126/2007 of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products. *Official Journal of the European Union, L255*, 14–17.

INE. (2013). *Consumo humano de cereais per capita (kg/hab.) por espécie de cereais.* Instituto Nacional de Estatística. Retrieved September 13, 2013, from http://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_indicadores&indOcorrCod=0000181&contexto=bd&selTab=tab2.

International Agency for Research on Cancer. (2002). *Some traditional herbal medicines, some mycotoxins, naphthalene and styrene.* In *Monograph on the evaluation of carcinogenic risk to humans* (Vol. 82). Lyon: IARCPress.

IPCS. (2009). Dietary exposure assessment of chemicals in food. In W. H. Organization (Ed.), *Principles and methods for the risk assessment of chemicals in food* (p. 98). Geneva, Switzerland: International Programme on Chemical Safety.

Juan, C., Ritieni, A., & Mañes, J. (2012). Determination of trichothecenes and zearalenones in grain cereal, flour and bread by liquid chromatography tandem mass spectrometry. *Food Chemistry, 134*(4), 2389–2397. http://dx.doi.org/10.1016/j.foodchem.2012.04.051.

Lino, C. M., Silva, L. J. G., Pena, A. L. S., & Silveira, M. I. (2006). Determination of fumonisins B1 and B2 in Portuguese maize and maize-based samples by HPLC with fluorescence detection. *Analytical and Bioanalytical Chemistry, 384*, 1214–1220. http://dx.doi.org/10.1007/s00216-005-0295-z.

Manova, R., & Mladenova, R. (2009). Incidence of zearalenone and fumonisins in Bulgarian cereal production. *Food Control, 20*(4), 362–365. http://dx.doi.org/10.1016/j.foodcont.2008.06.001.

Maragos, C. M. (2010). Zearalenone occurrence and human exposure. *World Mycotoxin Journal, 3*(4), 369–383. http://dx.doi.org/10.3920/WMJ2010.1240.

Marques, M. F., Martins, H. M., Costa, J. M., & Bernardo, F. (2008). Co-occurrence of deoxynivalenol and zearalenone in crops marketed in Portugal. *Food Additives and Contaminants: Part B, 1*(2), 130–133. http://dx.doi.org/10.1080/02652030802253983.

Martos, P. A., Thompson, W., & Diaz, G. J. (2010). Multiresidue mycotoxin analysis in wheat, barley, oats, rye and maize grain by high-performance liquid chromatography-tandem mass spectrometry. *World Mycotoxin Journal, 3*(3), 205–223. http://dx.doi.org/10.3920/WMJ2010.1212.

Nuryono, N., Noviandi, C. T., Böhm, J., & Razzazi-Fazeli, E. (2005). A limited survey of zearalenone in Indonesian maize-based food and feed by ELISA and high performance liquid chromatography. *Food Control, 16*(1), 65–71. http://dx.doi.org/10.1016/j.foodcont.2003.11.009.

Reinhold, L., & Reinhardt, K. (2011). Mycotoxins in foods in Lower Saxony (Germany): results of official control analyses performed in 2009. *Mycotoxin Research, 27*(2), 137–143. http://dx.doi.org/10.1007/s12550-011-0086-7.

Reza Oveisi, M., Hajimahmoodi, M., Memarian, S., Sadeghi, N., & Shoeibi, S. (2005). Determination of zearalenone in corn flour and a cheese snack product using high-performance liquid chromatography with fluorescence detection. *Food Additives and Contaminants, 22*(5), 443–448. http://dx.doi.org/10.1080/02652030500073709.

RIVM. (2011). *Dutch National Food Consumption Survey.* RIVM – National Institute for Public Health and the Environment. Retrieved September 13, 2013, from http://www.rivm.nl/en/Topics/Topics/D/Dutch_National_Food_Consumption_Survey.

Rodríguez-Carrasco, Y., Moltó, J. C., Berrada, H., & Mañes, J. (2014). A survey of trichothecenes, zearalenone and patulin in milled grain-based products using GC–MS/MS. *Food Chemistry, 146*, 212–219. http://dx.doi.org/10.1016/j.foodchem.2013.09.053.

Rubert, J., Soriano, J. M., Mañes, J., & Soler, C. (2011). Rapid mycotoxin analysis in human urine: a pilot study. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 49*(9), 2299–2304. http://dx.doi.org/10.1016/j.fct.2011.06.030.

Serrano, A. B., Font, G., Ruiz, M. J., & Ferrer, E. (2012). Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area. *Food Chemistry, 135*(2), 423–429. http://dx.doi.org/10.1016/j.foodchem.2012.03.064.

Sirot, V., Fremy, J.-M., & Leblanc, J.-C. (2013). Dietary exposure to mycotoxins and health risk assessment in the second French total diet study. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 52*, 1–11. http://dx.doi.org/10.1016/j.fct.2012.10.036.

Škrbić, B., Živančev, J., Đurišić-Mladenović, N., & Godula, M. (2012). Principal mycotoxins in wheat flour from the Serbian market: levels and assessment of the exposure by wheat-based products. *Food Control, 25*(1), 389–396. http://dx.doi.org/10.1016/j.foodcont.2011.10.059.

Sociedade Portuguesa de Pediatria. (2013). Retrieved September 13, 2013, from http://www.spp.pt/.

Sulyok, M., Berthiller, F., Krska, R., & Schuhmacher, R. (2006). Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Communications in Mass Spectrometry, 20*, 2649–2659. http://dx.doi.org/10.1002/rcm.

Vendl, O., Crews, C., MacDonald, S., Krska, R., & Berthiller, F. (2010). Occurrence of free and conjugated Fusarium mycotoxins in cereal-based food. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 27*(8), 1148–1152. http://dx.doi.org/10.1080/19440041003801166.

Vidal, A., Marín, S., Ramos, A. J., Cano-Sancho, G., & Sanchis, V. (2013). Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 53*, 133–138. http://dx.doi.org/10.1016/j.fct.2012.11.020.

Zinedine, A., Soriano, J. M., Moltó, J. C., & Mañes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 45*(1), 1–18. http://dx.doi.org/10.1016/j.fct.2006.07.030.