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EVALUATION OF ZEARALENONE IN FLOURS

Master dissertation in Food Safety, orientated by Professor Doctor Celeste de Matos Lino and presented to the Faculty of Pharmacy, University of Coimbra

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Y con esto llego al final del camino. Un último paso para concluir una pequeña gran etapa, en la cual debo agradecer la presencia de muchas personas.

A mi familia, en especial a mis padres, a los cuales siempre estaré muy agradecido por haberme dejado cumplir mis sueños y darme las herramientas necesarias para conseguir una autonomía personal. A mis hermanas, por centrarme en la vida y a mis sobrinos por alegrarme cada día y hacer que fuese más fácil. A mis tías, por hacer también todo lo posible para que alcanzase la meta.

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ABSTRACT

An analytical methodology based on extraction with acetonitrile:water (90:10), clean-up with immunoaffinity columns (IACs), and detection and quantification by liquid chromatography with fluorescence detection (LC-FD) was validated in order to evaluate zearalenone (ZEA) in different types of flours (wheat, maize, mixed cereals) used for human consumption with different purposes, originated from Coimbra (Portugal), Utrecht (The Netherlands) and Valencia (Spain).

Linearity, in the working standards solutions, between 12.5 ng/mL and 200ng/mL, was good (r^2=0.998). Linearity in the matrix-matched assay, prepared between 20 and 250µg/Kg, was r^2=0.997. Matrix-effect was 92.5%. Recovery values ranged between 97.6 and 105.3%, and precision between 2 and 13.6%. The accuracy and precision results comply with the requirements established by the EC directive 401/2006. LOD and LOQ were 3.75 and 12.5µg/Kg, respectively.

The application of the procedure to 50 samples from the three cities showed that 36% of the samples were contaminated. One sample with baby flour purpose exceeded the maximum limit established by EC legislation of 2007, and another one was close to the limit. A maize flour sample exceeded the ML established by EC with a concentration of 111.7µg/kg. Thus, two of the tested samples from Coimbra were contaminated above the established maximum limits for processed maize-based food for infants and maize intended for direct human consumption.

The estimated daily intake (EDI) ranged between 0.013 and 0.14 µg/kg b.w./day, which represents $52x10^2$% and $560x10^2$% of the TDI established by EFSA in 2011, 0.25 ng/kg b.w./day. Therefore, all the studied populations are at risk, being this risk higher for babies than for adults, both in Portuguese and Dutch population.

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Index of abbreviations

AFB1: aflatoxin B1

AFB2: aflatoxin B2

AFG1: aflatoxin G1

AFG2: aflatoxin G2

AUC: area under time-concentration

b.w.: body weight

CDC: centers for disease control and prevention

CYP: cytochrome p450

DON: deoxynivalenol

EC: European Commission

EDI: estimated daily intake

EFSA: European Food Safety Authority

ELISA: enzyme-linked immunosorbent assay

F.: *Fusarium*

FAO: Food Agriculture Organization

FB1: fumonisin B1

FB2: fumonisin B2

HPLC: high performance liquid chromatography

HPLC-FD: high performance liquid chromatography – fluorescence detection

HSD: dehydrogenase

IAC: immunoaffinity columns

IARC: International Agency of Research on Cancer

INE: Instituto Nacional de Estitística

Inh.: inhabitant

JECFA: Joint Expert Committee on Food Additives

Kg: kilogram

λ exc: excitation wavelength

λ em: emission wavelength

LC: liquid chromatography

LC-MS: liquid chromatography – mass spectrometry

LD: lethal dose

LOD: limit of detection

LOQ: limit of quantification

ME: matrix effect

μg: micrograms

ML: maximum limit

mL: milliliters

NaCl: sodium chloride

ng: nanograms

nm: nanometers

NOEL: non-observed effect level

PMTDI: provisional maximum tolerable daily intake

RIVM: Rijksinstituut voor volksgezondheid en milieu

SCF: Scientific Committee on Food

SD: standard deviation

SPE: solid phase extraction

SULT: sulphotransferase

TDI: tolerable daily intake

t-TDI: temporary tolerable daily intake

UGT: uridinediphosphate-glucuronosyltransferase

Vol.: volume

WHO: world health organization

ZEA: zearalenone

I. LITERATURE REVIEW

I.1 Mycotoxins

General considerations

Currently, more than 400 mycotoxins are identified in the world, but the most important groups of mycotoxins that are of major health concern for humans and animals, and occur quite often in food are aflatoxins, trichothecenes, fumonisins, ochratoxin A and zearalenone (Salem and Ahmad, 2010).

Aflatoxins are a group of difurocoumarolactones (difurocoumarin derivatives) produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus* and are recognised as human carcinogens by the International Agency of Research on Cancer (IARC, 2002). Only four compounds are naturally produced by aflatoxigenic fungi: aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2), which are significant in maize, cereals grains, and nuts (Diaz et al. 2001).

The deoxynivalenol (DON) is a mycotoxin which belongs to trichothecenes type B. This mycotoxin predominates in grains as wheat, corn, sorghum, rice, barley and oat. The pathogenic agents which produce this mycotoxic are *Fusarium graminearum* and *F. culmorum* (Beyer et al., 2006). T-2 toxin belongs, as deoxynivalenol, to trichothecenes type B and it is produced by the following species of the fungus *Fusarium*, *F. sporotrichioides*, *F. poae*, *F. equiseti* y *F. acuminatum*, affecting to the grains of wheat, corn, sorghum, rice, barley and oat.

Fumonisins are produced mostly by the fungus *Fusarium verticillioides* but can be produced by other *Fusarium* spp. and also by *Alternaria* spp. There exist different types of fumonisins, which fumonisin B1 (FB1) and fumonisin B2 (FB2) are the most predominant metabolites produced by the fungus. The fumonisins occur primarily in maize, and their toxicological relevance is limited to maize and maize-based animal feeds and human foods (CAST, 2003).

Ochratoxin A is an immunosuppressant fungal compound, produced by toxigenic species of *Aspergillus* and *Penicillium* fungi in a wide variety of climates and geographical regions. The contamination of food by this mycotoxin takes place primarily during preharvest periods. Almost all types of food can be contaminated (Al-Anati and Petzinger, 2006). The IARC (2002) classified this mycotoxin as a possible carcinogen for humans (Group 2B).

Zearalenone (ZEA) is produced by many species of the fungus Fusarium, and affects a wide variety of cereals. It was classified in the group 3 (IARC) as not carcinogenic for humans.

The aim of this study was evaluate the degree of exposure of different populations to zearalenone and subsequent risk assessment through the consumption of different flours. In order to obtain a good analytical performance, different experimental conditions, such as the mobile phase composition, were primarily optimized using high performance liquid chromatography (HPLC) with fluorescence detection (FD). Afterwards, the occurrence and levels of ZEA were determined, in 50 samples originated from three cities of three European countries, in order to verify the compliance with European legislation regarding maximum permitted levels.

I.2 Zearalenone

Zearalenone, "6-(10-hydroxy-6-oxo-trans-1-undecenyl) β-resorcylic-acid-lactone", is a secondary metabolite, an estrogenic mycotoxin (Briones-Reyes et al., 2007) mainly produced by *Fusarium* spp., particularly, *F. graminearum*, but also *F. culmorum, F. equiseti* and *F. verticillioides*.

Corn is the most contaminated cereal by ZEA and also its derivate foodstuffs. ZEA can also contaminate wheat, oat, barley, sorghum and rye crops (Martos, 2010). ZEA is a field contaminant of crops, because the toxins production takes place before the harvest and to a lesser extent during the storage of the crops (EFSA, 2011).

I.2.1 Physical-chemicals characteristics

ZEA is biosynthsesized via the polyketide pathway, via acetate-malonil-CoA. ZEA is stable and it is not degraded by high temperatures. The empirical formula of this mycotoxin is $C_{18}H_{22}O_5$, corresponding to (3,4,5,6,9,10-hexahydroxy-14,16-dihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1,7-(8*H*)-diona. It is a macrocyclic β-resorcylic-acid-lactone. Its molecular weight is 318.147 g/mol. ZEA and its metabolites structure is represented in Figure I.1.

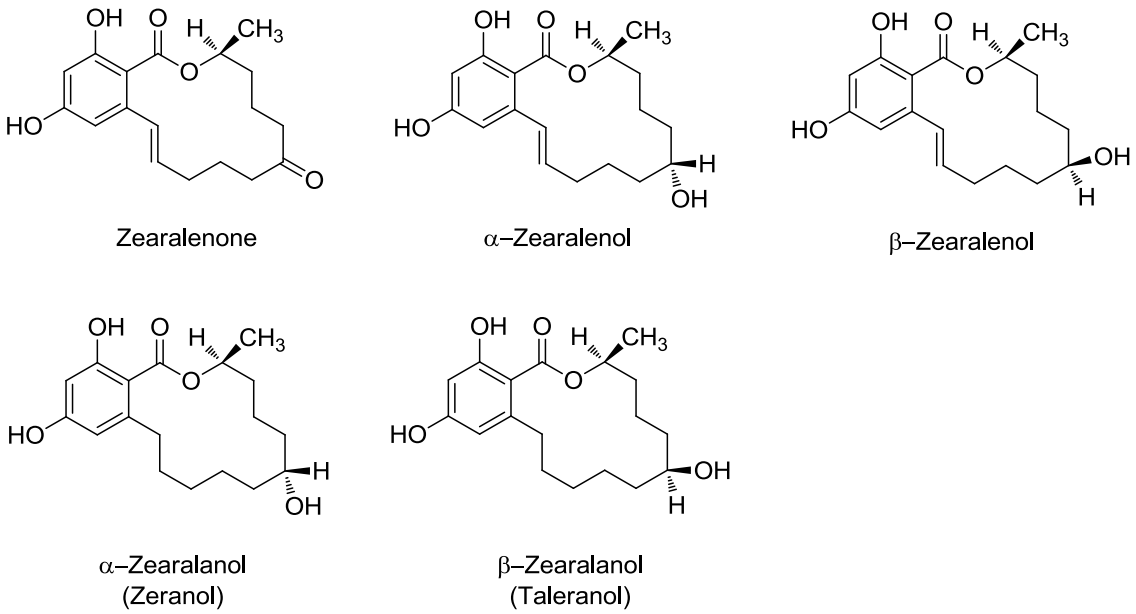

Figure I.1- ZEA and its metabolites chemical structure (adaptation of EFSA, 2011).

ZEA is a white and crystallising substance, soluble in methanol, dietilic eter, benzene, acetonitrile, etile acetate and alcohols, being insoluble in water. Its melting point is 165°C and under ultraviolet light (366 nm) emits a blue fluorescence (Marques, 2007).

Zearalenone is generally stable during cooking, except under alkaline conditions or during extrusion cooking (heating under a high degree of pressure) (EFSA, 2011).

I.2.2 Toxicokinetics

As for any other toxin, the kinetic parameters including absorption, distribution in the body, metabolism and excretion determine the internal dose and the toxin concentration at target sites.

I.2.2.1 Absorption

ZEA is rapidly absorbed after oral administration. Although the degree of absorption is difficult to measure owing to extensive biliary excretion, it appears to be extensively

absorbed in rats, rabbits, pigs, and humans (Kuiper-Goodman et al., 1987) with the formation of α and β-zearalenol and α and β-zearalanol (Figure I.2) which are subsequently conjugated with glucuronic acid (EC, 2000). The uptake in a pig after a single oral dose of 10 mg/kg b.w. was estimated to be 80-85% (Biehl et al., 1993). The absolute bioavailability of zearalenone in rats, which is defined as the ratio of parent compound area under the time-concentration curve (AUC) following oral versus intravenous administration, was low (2.7%) and linearly related to dose in the range of 1-8 mg/kg b.w. (EFSA, 2011).

I.2.2.2 Distribution

Many studies demonstrate that ZEA is widely distributed and slowly eliminated from tissues, likely resulting from enterohepatic recycling of ZEA and its metabolites. In male rats, zearalenone is distributed to tissues other than the gastrointestinal tract, including kidney, liver, adipose, lung, heart, spleen, muscle, brain, and testes (Shin et al., 2009).

Placental transfer of zearalenone and α-zearalenol has been demonstrated in rats following intravenous administration. The zearalenone and α-zearalenol levels in the whole fetus were 5 to 38% and 2 to 6%, respectively, of the maternal liver levels, and the placental levels were approximately twice those of the fetus (Bernhoft et al., 2001).

The carry-over rate into dairy milk remained low and confirms, together with studies on tissue disposition, that human exposure via foods from animal origin is much lower than direct exposure via contaminated grains and cereals used in the human diet (Fink-Gremmels et al., 2007).

I.2.2.3 Metabolism

Three important biotransformation pathways for zearalenone in animals have been reported (Figure I.2).

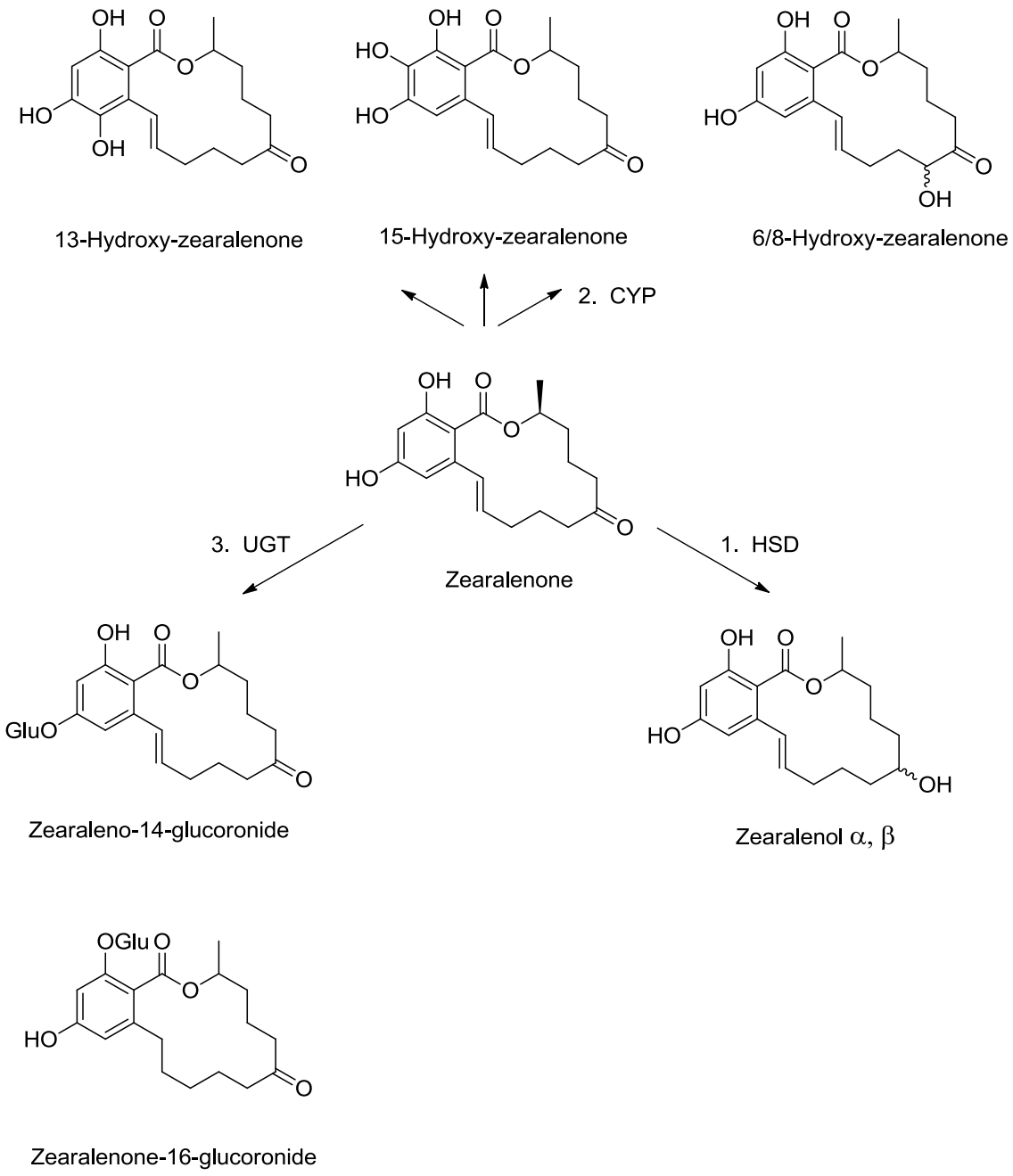

Figure I.2 - Major metabolic pathways for ZEA (adaptation of EFSA, 2011).

1. Enzymatic reduction of ZEA catalyzed by 3α- and 3β-hydroxysteroid dehydrogenases (HSDs) produces α- and β-zearalenol, respectively, and smaller amounts of the corresponding zearalanols. The primary reduced form α-zearalenol has more oestrogenic activity than the parent compound (Zinedine, 2007). Malekinejad et al. (2006) reported differences between mammalian species in hepatic transformation of zearalenone to its reduced and glucuronide metabolites.

2. ZEA is also monohydroxylated by recombinant human cytochromes P450 (CYPs) and human liver microsomes in vitro (Bravin et al., 2009). Hydroxylation occurs at the 6/8-position (aliphatic) and 13/15-position (aromatic). Studies with zearalenone oxidation by recombinant human CYP isoforms suggest that CYP 1A2 is the major isoform with a lesser contribution from CYP 3A4 (Pfeiffer et al., 2009). The major oxidative metabolites appear to arise through aromatic hydroxylation and are catechols. These metabolites undergo oxidation to quinines, which can redox cycle and covalently modify biological macromolecules (Pfeiffer et al., 2009). While the oestrogenic properties of zearalenone catechols are unknown, the aliphatic C6/8 hydroxy-zearalenone appeas to be approximately an order of magnitude less active than the parent compound (Bravin et al., 2009).

3. Phase II conjugation of ZEA and its reduced metabolites with glucuronic acid and sulphate, is catalyzed by uridinediphosphate-glucuronosyltransferases (UGTs) and sulphotransferases (SULTs), respectively. Zearalenone, α- and β-zearalenol, and the further reduced metabolites (α- and β-zearalanol) are readily glucuronidated both in the liver and intestine as well as in other extrahepatic organs of human and various animal species (Pfeiffer et al., 2010).

1.2.2.4 Excretion

Rats excreted approximately 55% of the administrated dose (1 or 100 mg/kg b.w.) in the faeces with 15-20% excreted in urine (Fitzpatrick et al., 1988). Faecal (97-98%) and urinary zearalenone (86-88%) was primarily in the unconjugated form. Approximately 10% of the administrated dose was excreted as α-zearalenol. The respective zearalenone, α-zearalenol and β-zearalenol concentrations found in human male urine exclusively as glucuronide conjugates after oral dose of 100 mg zearalenone were: 3.7 μg/mL, 3 μg/mL, and <LOD after 6 hours; 6.9, 6 and 2.7 μg/mL after 12 hours; and 2.7, 4 and μg/mL after 24 hours (Mirocha et al., 1981). The presence of C6/8-hydroxy-zearalenone in rat liver and urine has been reported (Bravin et al., 2009).

I.2.3 Physiological effects and toxicity

Studies on the physical and chemical properties of zearalenone revealed traits ideal for an easy diffusion into the tissues. Zearalenone is low toxic, and there is no evidence on its carcinogenetic potential in all studies conducted on animals. On the other hand, this mycotoxin has strong estrogenic and anabolic properties because of the agonistic effect on the estrogenic receptors, many animal species displaying severe disturbances of the reproductive system (Duca et al., 2009).

I.2.3.1 Acute

It is acknowledged that ZEA is of a relatively low acute toxicity (oral LD values of >2000-20000 mg/kg b.w.) after oral administration in mice, rats and guinea pigs. It is more toxic by intraperitoneal injection (Zinedine et al., 2007).

I.2.3.2 Subacute and subchronic

In oral toxicity studies of up to 90 days, the effects seen in experimental as well as in domestic animal appeared to be dependant on interaction of ZEA or its metabolites with the estrogen receptors. Pig and sheep appear to be more sensitive than rodents; in controlled studies with well-defined exposure to multiple does, the NOEL in pigs was 40 μg/kg b.w./day compared with the NOEL of 100 μg/kg b.w./day in rats (Kuiper-Goodman et al., 1987).

I.2.3.3 Chronic and carcinogenesis

Chronic toxicity and carcinogenicity of ZEA confirmed effect in rodents exposed to long-term administration of this mycotoxin. From long-term toxicity studies in rats a NOEL of 0.1 mg/kg b.w./day can be derived, based on the absence of increase in weight of uterus at this dose level. These studies provided limited evidence of carcinogenic activity of ZEA in experimental animals (hepatocellular adenomas in female mice and pituitary adenomas in

both male and female mice but no effects in rats). This conclusion is in agreement with the evaluation of ZEA by IARC (EFSA, 2011).

1.2.3.4 Effects in humans

There is little substantive information on the effects of ZEA in humans. However, observations of high concentrations of ZEA in foodstuffs and the occurrence of oestogenic-related pathologies in humans, such as precocious puberty and breast cancer has resulted in speculation that ZEA may contribute to such effects (EFSA, 2011).

1.2.4 Incidence

The weather conditions, especially at the growing and flowering time, greatly influence *Fusarium* infection of the plant and the mycotoxin production, and therefore ZEA concentrations vary from year to year (EFSA, 2011).

Many studies have been carried out showing the occurrence and levels of ZEA in cereals and derivatives (Table I.1) in the last years.

In the studies, different samples of cereals were analysed (barley, oat, wheat) but the most studied cereal, due to its normal higher contamination of *Fusarium*, is corn and its derivatives, such as popcorn, snacks, oil or flakes.

ZEA concentrations were investigated in a total of 99 cereal samples (41 samples of wheat, 17 of oat and 41 of corn) in Germany (Schollenberger et al., 2006). In wheat, oat and corn the incidences of ZEA were 63.5, 23.5 and 85.4%, respectively, and the mean concentrations 15, 21 and 48 μg/kg, respectively. In a later study of Reinhold and Reinhardt (2011), 58 samples were analysed (12 of popcorn, 18 of maize snacks, 8 of maize flour and 20 of maize germ oil) with incidences of 50, 88.9, 75 and 100%, respectively, and mean concentrations of 16.2, 8.0, 31.7 and 63.9 μg/kg, respectively.

A total of 91 grain samples (54 wheat, 18 barley and 19 maize samples) were collected in Bulgaria during 2007 and tested for ZEA (Manova and Mladenova, 2009). The incidence of positive samples was higher in maize (21%) than in barley (11%) and wheat samples (2%). The mean levels in the wheat, barley and maize samples were 10.0, 29.0 and 80.6 μg/kg, respectively. The highest level was observed in maize (148.0 μg/kg). In Serbia, Skrbic et al. (2011) tested 15 samples of wheat flour. The incidence was 33.3% with a mean contamination of 4.6 μg/kg and a maximum level of 21.1 μg/kg. In Croatia, 40 maize samples were analysed, which 87.5% were positive to ZEA and the highest level was 5.11 mg/kg (Pleadin et al., 2012).

In Spain, Vidal et al. (2013) analysed 67 cereal samples (37 of wheat and 30 of oat) with an occurrence of 13.5 and 16.7%, respectively, and a ML of 25 μg/kg was observed in an oat sample. Cano-Sancho et al. (2012) analysed 486 samples (70 of pasta, 71 of corn flakes, 29 of wheat flakes, 72 of sweet corn, 71 of sliced bread, 71 of beer and 30 of baby food) with frequencies of occurrence of 14.3, 0, 13.8, 23.6, 18.1, 43.7, 11.3, 23.3%, respectively. The mean concentrations were 3.8, 0, 6.3, 5.9, 4.9, 3.7, 3.1 and 4.1 μg/kg, respectively. The highest level (22.8 μg/kg) was observed in a corn snack sample.

In Indonesia, Nuryono et al. (2005) tested 32 samples (4 of industrially-produced food, 5 of home-made food, 2 of maize for food, 3 of maize for feed and 18 of poultry feed) and found frequencies of contamination of 75, 100, 50, 100 and 72.2%, respectively. The mean levels of contamination were 12.4, 219, 6.9, 31.0 and 32.2, respectively. The highest level observed was 589 μg/kg of ZEA in a home-made food sample. In Malaysia, Rahmani et al. (2010) analysed 60 cereal samples (11 of barley, 6 of wheat, 8 of maize meal, 4 of oat and 31 of rice) with frequencies of contamination of 36.4, 0, 25, 25 and 13%, respectively. The highest level was observed in a rice sample (73.11 μg/kg).

In Iran, Reza Oveisi et al. (2005) tested corn flour and cheese snack samples (19 and 19) with a 100% of frequency of contamination of ZEA in both type of samples. In Mexico, Briones-Reyes et al. (2007) analysed 24 corn samples, observing a frequency of contamination of 70.8% and levels of contamination ranging between 3 and 83.63 μg/kg. In Brazil, Silva and Vargas (2001) analysed 380 samples of corn, observing a frequency of contamination of 7.9%, a mean contamination level of 232 μg/kg and a highest level of contamination of 719.4 μg/kg. Vargas et al. (2001) tested 214 corn samples, 30.4% of them

were positive to ZEA, with a mean contamination of 155 μg/kg, and a highest level of contamination of 719 μg/kg.

Table I.1 - Occurrence (%) and levels (μg/kg) of ZEA in cereals and derivatives.

Country	Sample	No. Samples	Frequency (%)	Range (μg/kg)	Mean±SD (μg/kg)	References
Malaysia	Barley	11	4 (36.4)	2.38-24.43	-	Rahmani et al. (2010)
Malaysia	Wheat	6	n.d. (0)	n.d.	-	Rahmani et al. (2010)
Malaysia	Maize meal	8	2 (25)	2.5-2.9	-	Rahmani et al. (2010)
Malaysia	Oat	4	1 (25)	2.8	-	Rahmani et al. (2010)
Malaysia	Rice	31	4 (12.9)	2.8-73.11	-	Rahmani et al. (2010)
Serbia	Wheat flour	15	5 (33.3)	1.9-21.1	4.6	Skrbic et al. (2011)
Bulgaria	Barley	18	2 (11)	n.d.-36.6	29.0	Manova and Mladenova (2009)
Bulgaria	Maize	19	4 (21)	n.d.-148.0	80.6	Manova and Mladenova (2009)
Bulgaria	Wheat	54	1 (2)	n.d.-10.0	10.0	Manova and Mladenova (2009)
Croatia	Maize	40	35 (87.5)	n.d.-5110	-	Pleadin et al. (2012)
Germany	Popcorn maize	12	6 (50)	n.d.-22.0	16.2	Reinhold and Reinhardt (2011)
Germany	Maize snacks	18	16 (88.9)	n.d.-19.8	8.0	Reinhold and Reinhardt (2011)
Germany	Maize flour	8	6 (75)	n.d.-71.8	31.7	Reinhold and Reinhardt (2011)
Germany	Maize germ oil	20	20 (100)	-97.7	63.9	Reinhold and Reinhardt (2011)
Indonesia	Industrially-produced food	4	3 (75)	11.1- 13.7	12.4	Nuryono et al. (2005)
Indonesia	Home-made food	5	5 (100)	19.1-589	219	Nuryono et al. (2005)
Indonesia	Maize for food	2	1 (50)	6.9	6.9	Nuryono et al. (2005)
Indonesia	Maize for feed	3	3 (100)	8.1-86.6	31.0	Nuryono et al. (2005)
Indonesia	Poultry	18	13 (72.2)	10.1-122	32.2	Nuryono et al.

Table I.1 - Occurrence (%) and levels (μg/kg) of ZEA in cereals and derivatives.

Country	Sample	No. Samples	Frequency (%)	Range (μg/kg)	Mean±SD (μg/kg)	References
	feed					(2005)
Spain	Wheat	37	5 (13.5)	n.d.-21	-	Vidal et al. (2013)
Spain	Oat	30	5 (16.7)	n.d.-25	-	Vidal et al. (2013)
Spain	Pasta	70	10 (14.3)	n.d.-5.9	3.8 ± 1.8	Cano-Sancho et al. (2012)
Spain	Corn flakes	71	0 (0)	-	-	Cano-Sancho et al. (2012)
Spain	Wheat flakes	29	4 (13.8)	n.d.-12.1	6.3 ± 5.4	Cano-Sancho et al. (2012)
Spain	Corn snacks	72	17 (23.6)	n.d.-22.8	5.9 ± 6.8	Cano-Sancho et al. (2012)
Spain	Sweet corn	72	13 (18.1)	n.d.-5.9	4.9 ± 0.7	Cano-Sancho et al. (2012)
Spain	Sliced bread	71	31 (43.7)	n.d.-20.9	3.7 ± 4.5	Cano-Sancho et al. (2012)
Spain	Beer	71	8 (11.3)	n.d.-5.1	3.1 ± 1.4	Cano-Sancho et al. (2012)
Spain	Baby food	30	7 (23.3)	n.d.-5.4	4.1 ± 0.6	Cano-Sancho et al. (2012)
Mexico	Corn	24	17 (70.8)	3-83.63	-	Briones-Reyes et al. (2007)
Iran	Corn flour	19	19 (100)	0.036-0.889	-	Reza Oveisi et al. (2005)
Iran	Cheese snack	19	19 (100)	0.371-1.471	-	Reza Oveisi et al. (2005)
Brazil	Corn	380	30 (7.9)	46.7-719.4	232	Silva and Vargas (2001)
Brazil	Corn	214	65 (30.4)	36.8-719	155	Vargas et al. (2001)
Germany	Wheat	41	26 (63.5)	-	15	Schollenberger et al. (2006)
Germany	Oat	17	4 (23.5)	-	21	Schollenberger et al. (2006)
Germany	Corn	41	35 (85.4)	-	48	Schollenberger et al. (2006)

I.2.5 Methods of determination

The methods of sampling and analysis for concentrations of zearalenone in foodstuffs are stipulated in the Commission Regulation (EC) No 401/2006 of 23 February 2006 (EC, 2006), which lays down the methods of sampling and analysis for the official control of the

levels of mycotoxins in foodstuffs. The foodstuffs for which the regulation stipulates the sampling and analytical methods include cereals and cereal products, baby foods and processed cereal based foods for infants and young children and vegetable oils.

The methods require appropriate extraction and clean-up procedures, such as the use of immunoaffinity columns. Analysis mostly uses high performance liquid chromatography coupled to fluorescence detection (HPLC-FD) or triple quadrupole mass spectrometers (LC-MS). Quantification can be achieved via matrix calibration or by using stable isotope labelled standards (EFSA, 2011).

Different analytical methodologies have been studied in several studies reported by different researchers (Table I.2).

I.2.5.1 Extraction

For the extraction procedure different organic solvents, mixed in different percentages, were used. For example, methanol:water was used in the percentage (80:20) by Rahmani et al. (2010). Other percentages, used by other authors, were (75:25) and (70:30) by Nuryono et al. (2005). Reinhold and Reinhardt (2011) also used this mixture.

Other solvent mixtures used were acetonitrile: water: acid acetic (79:20:1) by Vendl et at. (2010), methanol: acetonitrile: water (25:25:50) by (Manova and Mladenova, 2009), and (5:80:15) by Llorens et al. (2002).

The most common mixture used, and the one used in our study, was acetonitrile: water, also in different percentages. Skrbic et al. (2012) and Llorens et al. (2002) used acetonitrile: water (84:16). In Josephs et al. (2001) study this mixture was also used.

I.2.5.2 Clean-up

Most of the authors used, for the clean-up procedure, immunoaffinity columns (Visconti and Pascale, 2010; Rahmani et al., 2010; Manova and Mladenova, 2009; Reinhold and Reinhardt, 2011; Nuryono et al., 2005; Josephs et al., 2001 and Llorens et al. 2002).

Other authors, for this procedure, opted for other solid-phase extraction, such as C-18 columns (Vendl et al., 2010; Briones-Reyes et al., 2007; Llorens et al., 2002), and Florisil (Llorens et al., 2002).

Gel permeation chromatography was used by Reinhold and Reinhardt (2011).

I.2.5.3 Detection and Quantification

The method more employed for the detection and quantification of ZEA contamination was High Performance Liquid Chromatography (HPLC) with different detectors. HPLC with fluorescence detection (HPLC-FD) was used by Rahmani et al. (2010), Manova and Mladenova (2009), Josephs et al. (2001), Llorens et al. (2002), and Urraca et al. (2004). HPLC with mass spectrometry was employed by Vendl et al. (2010), Skrbic et al. (2012), Reinhold and Reinhardt (2011), and Visconti and Pascale (2010).

Other less used methods were Liquid Chromatogray (LC) with an UV diode array detector (Briones-Reyes et al., 2007).

Nuryono et al. (2004) used an Enzyme-Linked ImmunoSorbent Assay (ELISA) kit.

Table I.2 - Methods of extraction, clean-up, and determination of ZEA in cereals and derivatives.

Sample	Extraction	Clean-up	Detection	Mobile phase	Analytical performance	References
Wheat and derivatives		Immunoaffinity columns	LC- tandem mass spectrometry			Visconti and Pascale (2010)
Rice, oat, barley, maize, wheat	Methanol: water (80:20)	Multifunctional immnunoaffinity column	HPLC-FD λ exc: 276 nm λ em: 460 nm	Methanol: water (50:50 v/v)	LOQ: 5 ng/g Fortification: 20-400 ng/g Recovery: 86-93%	Rahmani et al. (2010)
Flakes, wheat flakes, raw pasta, maize	Acetonitrile: water: acetic acid (79:20:1)	C- 18 columns	HPLC-tandem mass spectrometry			Vendl et al. (2010)
Wheat flour	Acetonitrile: water (84:16)		HPLC-tandem mass spectrometry Injection vol: 10 µL	95%A:5%B A: water: acetic acid (99:1) B: methanol: acetic acid (99:1)	LOD: 0.4 µg/kg LOQ: 1.3 µg/kg Fortification: 35 µg/kg Recovery: 82%	Skrbic et al. (2012)
Wheat, barley, maize	Methanol: acetonitrile: water (25:25:50)	Immunoaffinity columns	HPLC-FD λ exc: 274 nm λ em: 440 nm Injection vol: 0.1 mL	Acetonitrile: water: metanol (46:46:8)	LOQ: 12 µg/kg LOD: 4 µg/kg Recovery: 84-102%	Manova and Mladenova (2009)
Maize products (except oil)	Methanol: water	Immunoaffinity columns	HPLC-FD	Methanol: water (70:30 v/v)	LOD: 1 µg/kg LOQ: 4 µg/kg	Reinhold and Reinhardt (2011)
Maize oil		Gel permeation chromatography	HPLC-tandem mass spectrometry		LOD: 5 µg/kg LOQ: 10 µg/kg	
Corn kernels		C-18 reversed-phase column	LC-UV (diode array detector) λ exc: 236 nm λ em: 316 nm	Acetonitrile: water: methanol (50:42:8 v/v)	Fortification: 20-80 µg/g	Briones-Reyes (2007)

Table I.2 - Methods of extraction, clean-up, and determination of ZEA in cereals and derivatives.

Sample	Extraction	Clean-up	Detection	Mobile phase	Analytical performance	References
			injection vol: 50 µL			
Indonesian maize	Methanol: water (70:30)	Immunoaffinity columns	ELISA test	Methanol: water (70:30 v/v)	Fortification: 200-750 µg/kg Recovery: 97.2-101.5%	Nuryono et al. (2005)
	Methanol: water (75:25)	Immunoaffinity columns	HPLC		Fortification: 200-750 µg/kg Recovery 85.5-88.7% LOD: 3 µg/kg	
Wheat and corn	Acetonitrile or methanol and water or buffer	Immunoaffinity columns	HPLC-FD or triple quadrupole mass spectrometer			EFSA (2011)
Maize and wheat	Acetonitrile: water	Immunoaffinity columns	HPLC-FLD		Fortification: 102 µg/kg Recovery: 60-104%	Josephs et al. (2001)
Corn, rice, wheat	Acetonitrile: water (84:16) Acetonitrile: methanol: water (80:5:15)	Immunoaffinity columns reversed-phase	HPLC photodiode array or fluorescence detection λ exc: 236 nm λ em: 440 nm Vol: 20 µL	Methanol:water (80:20 v/v)	Fortification: 0.005-25 µg/g Recovery: 91.5-116%	Llorens et al. (2002)
Wheat, corn, rye, barley, rice, swine food	Acetonitrile, methanol and acetonitrile/methanol mixtures (25:75), (50:50)		LC-fluorescence detection λ exc: 271 nm λ em: 452 nm Vol: 8 µL	Acetonitrile: metanol: water (10:55:35 v/v)	Fortification: 50-200 µg/g Recovery: 76-96%	Urraca et al. (2004)

I.2.6 Legislation framework

Previously, the Commission Regulation (EC) No 1881/2006 of 19 December 2006 laid down MLs for certain contaminants foodstuff, including MLs for ZEA. In 2007, this regulation was substituted by the Commission Regulation (EC) No 1126/2007 of 28 September 2007 amending the previous one and setting maximum levels for certain contaminant foodstuffs as regards *Fusarium* toxins in maize and maize products (Table I.3). The MLs apply to the edible part of the foodstuff unless it is otherwise specified. The MLs set for first-stage processing. The Regulation specifies that "First-stage processing" shall mean any physical or thermal treatment, other than drying, of or on the grain. Cleaning, sorting and drying procedures are not considered to be "first-stage processing" insofar no physical action is exerted on the grain kernel itself and the whole grain remains intact after cleaning and sorting. In integrated production and processing systems, the MLs applies to the unprocessed cereals in case they are intended for "first-stage processing". Because of the low concentration levels of *Fusarium* toxins found in rice, no MLs are set for rice or rice products. Therefore for the application of MLs for ZEA, rice is not included in 'cereals' and rice products are not included in 'cereal products'.

ZEA was previously evaluated by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee of Food Additives (JECFA) which established a provisional maximum tolerable daily intake (PMTDI) of 0.5 μg/kg b.w. in 2000, based on the oestrogenic activity of ZEA and its metabolites in the most sensitive animal species, the pig. Also, in 2000, the Scientific Committee on Food (SCF) established a temporary TDI (t-TDI) of 0.2 μg/kg b.w. This TDI was designated as temporary and included an additional uncertainly factor because of some deficiencies in the data base. The SCF recommended that additional studies were needed to determinate the no-hormonal-effect level in pre-pubertal pigs, on the potential genotoxicity of ZEA, on species differences in metabolism, and o blood levels of ZEA in humans in order to help clarify the toxicokinetic behaviour.

The European Commission (EC), in considering if changes were needed to the legal provisions for the presence of ZEA in bran and breakfast cereal, asked the European Food Safety Authority (EFSA) to provide a scientific opinion on the effects on consumer health risk.

Toxicodynamic information indicates that it is likely that the human female would not be more sensitive to ZEA and its metabolites than the female pig. For derivation of a TDI, it was therefore not necessary to include an uncertain factor 2.5 for toxicodynamic difference between pigs and humans. Using the NOEL of 10 μg/kg b.w. per day and an uncertain factor of 40 (4 for interspecies differences in toxicokinetics and 10 for interhuman variability), a TDI of 0.25 μg/kg b.w. could be derived (EFSA, 2011).

Table I.3 - Regulation No. 1126/2007 establishing maximum levels (μg/kg) for ZEA in foodstuff in EU.

I	**Foodstuff**	**μg/kg**
1.1	Unprocessed cereals other than maize	100
1.2	Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	350
1.3	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuff listed in 1.6, 1.7, 1.8, 1.9 and 1.10	75
1.4	Refined maize oil	400
1.5	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereals	50
1.6	Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals	100
1.7	Processed cereal-based foods (excluding processed maize-based foods) and baby food for infants and young children	20
1.8	Processed maize-based foods for infants and young children	20
1.9	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	200
1.10	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	300

II. EXPERIMENTAL PART

II.1 MATERIALS AND METHODS

II.1.1 Sampling

The present study was carried out in Coimbra, a city located in the interior centre of Portugal, at the Group of Health Surveillance of the Center of Pharmaceutical Studies, in the Faculty of Pharmacy.

A total of 50 samples of flours (19 wheat flours, 12 corn flours, 13 mixed-flours with mainly wheat flour and 6 baby foods) were analysed. The samples were purchased in different supermarkets of Coimbra (Portugal) (n= 42), Utrecht (The Netherlands) (n= 6), and Valencia (Spain) (n= 2), during the winter season of 2013, between December 2012 and March 2013.

After purchase, the samples were brought to the laboratory under ambient condition. All the information concerning the samples was obtained from the labels. Afterwards they were kept in the same conditions until their analysis. After the analysis, the positive samples were frozen.

II.1.2 Reagents and materials

The reagents of HPLC grade used were acetonitrile and methanol (Carlos Erba, Milan, Italy). Acetic acid glacial 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 μm, 50 mm, Whatman GmbH, Dassel, Germany) were used. Immunoaffinity columns (IAC) ZearalaTest™ were from VICAM (Watertown, USA).

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

II.1.3 Solutions

The mobile phase was a vacuum-filtered solution of acetonitrile:water (60:40) with an adjusted pH at 3.2 with acid acetic glacial. All liquid chromatographic reagents were degassed for 15 minutes in an ultrasonic bath.

The ZEA standard stock solution was prepared at 5 mg/mL, by diluting 10 mg of ZEA in 2 mL of acetonitrile, and stored at -20°C. The intermediate solution was prepared by diluting the standard solution at 50 μg/mL, in acetonitrile, and a working standard solution at 1 μg/mL in acetonitrile, by diluting the intermediate solution. The calibration curve standard solutions with solvent were prepared between 12.5 and 200 ng/mL (12.5, 25, 50, 100, 200) in acetonitrile. The concentrations for the matrix-matched calibration curve were prepared between 20 and 250 μg/Kg (20, 50, 75, 125, 250).

II.1.4 Apparatus

A SPE of Ashcroft (Stratford, CT, USA) connected to a vacuum manifold of Macherey-Nagel (USA), a pump of Dinko (mol. D-95, 130W, 220V), a RapidVap Vertex™ evaporator of LabConco (Kansas City, MO, USA), an analytical balance of Mettler Toledo (Greifensee, Switzerland), a pH-meter of Jenway (Staffordshire, UK), a Meditronic centrifuge of P-Selecta (Barcelona, Spain), a Retsh vortex mixer (Haan, Germany) and a Sonorez RK 510S ultrasonic bath (Berlin, Germany) were used.

The LC instrument was equipped with a pump (Model 307, Gilson Medical Electronics, Villiers-le-Bel, France), and a guard column Hichrom Ltd., HI-173, (30 x 4 mm i.d) (England) preceding a Hichrom C_{18} column (5 μm, 250 x 4.6 mm i.d.). The spectrofluorimeter was a Perkin-Elmer Model LS45 (Beaconsfield, UK). The results were recorded on a Hewlett-Packard 3390A integrator (Philadelphia, PA, USA).

II.1.5 Calculation of estimated daily intake

Estimated Daily Intake (EDI) was calculated through a deterministic method (IPCS, 2009, chap. 6) 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 adult Portuguese population was considered 69 Kg (Arezes et al., 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 Pediatrics based on CDC, USA.

II.2 EXPERIMENTAL PROCEDURE

The method used for determining the zearalenone content of the different flour samples was based on Lino et al. (2006) and the clean-up step on described by Vicam ZearalaTest manual and authors as Rahmani et al. (2010), Manova and Mladenova (2009), Reinhold and Reinhardt (2011), and Reza Oveisi et al. (2005).

II.2.1 Sample extraction and clean-up

For the extraction, 20 g of sample were weight with 2 g salt (NaCl) and mixed in a centrifuge glass. Then, 50 mL of acetonitrile:water (90:10) were added, homogenized for 2 minutes and centrifuged for 15 minutes at 2500 *g*. After this, the supernatant was extracted with a pipette to an Erlenmeyer flask and the process was repeated twice with 50 mL of the same solution. Afterwards, 10 mL were extracted and mixed with 40 mL of water Milli-Q. The mixture was filtered through micro-glass paper and collected in an Erlenmeyer flask.

Ten milliliter 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 in an evaporator at 42°C under a gentle nitrogen flow, and the dried extract was stored at -20°C. Before injection, the dried extract was redissolved in 500 μL of acetonitrile.

II.2.2 Detection and quantification

Liquid chromatography-fluorescence detection analyses were performed using a 100 μL volume, and with the mobile phase flowing at 1 mL/min. Wavelengths used were 274 nm, for excitation, and 455 nm, for emission.

II.2.3 Fortification assays

ZEA validation method was performed by spiking a ZEA-free maize flour sample at three different levels (20, 75 and 200 μg/Kg), with three replications for each level. After this, the above protocol was followed.

II.2.4 Limit of detection and limit of quantification

Limits of detection (LOD) and limit of quantification (LOQ) were determinate as three and ten times the noise of the lowest level detected and determined, respectively.

II.3 RESULTS AND DISCUSSION

II.3.1 Optimization of the analytical procedures

Several experimental conditions were tested in order to obtain adequate resolution of the ZEA peak. Different mobile phases, with different concentrations of water and acetonitrile (60:40, 50:50, 55:45 and 57:43) were evaluated. Mobile phases (50:50 and 55:45) had unclear peaks and the retention time was too long. Good analytical performance was obtained using a mobile phase acetonitrile:water (60:40) with a flow proportion of 1,0

mL/min. Figure II.1 shows the HPLC spectrofluorimeter chromatograms of the ZEA standard, one sample and one sample fortified.

The filtration process therefore required modification, since the slurry produced after extraction clogged the Whatman N°1 filter paper, with or without vacuum, 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.

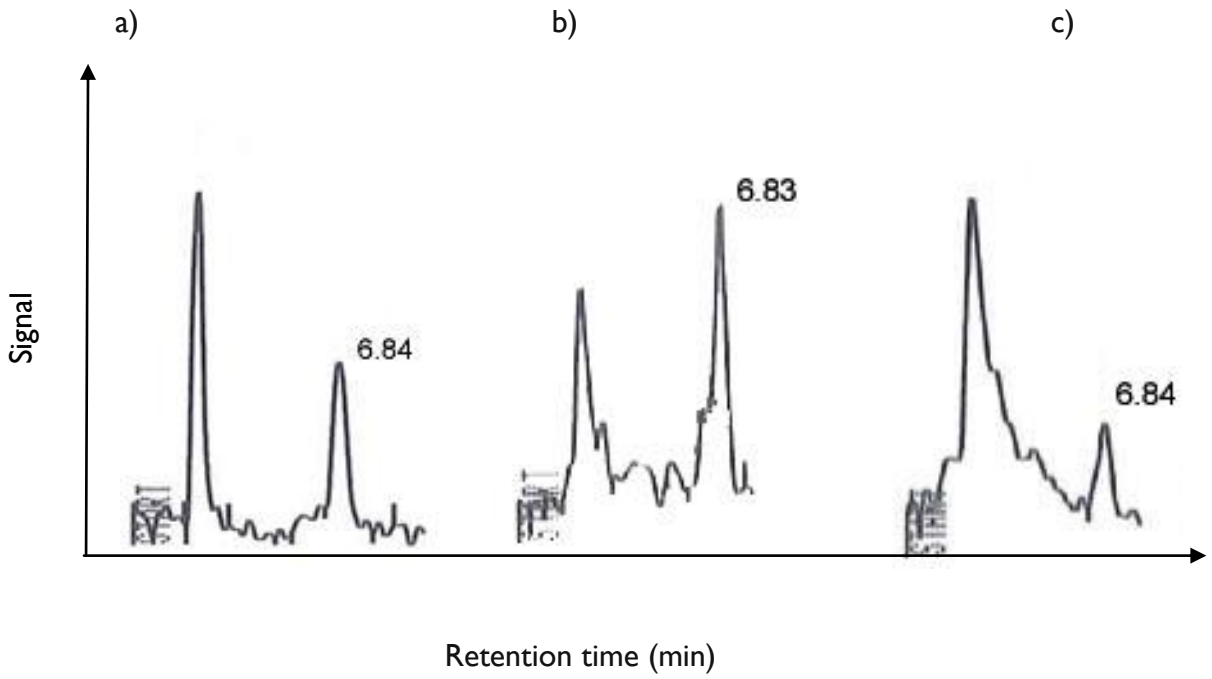

Figure II.1 - Liquid chromatography spectrofluorimetric chromatograms of ZEA standard (a) (retention time 6.84), one fortified sample at 75 μg/kg (b) and one contaminated sample (c) obtained using the optimized method.

II.3.2 Analytical performance

The calibration curve was obtained using the linear least squares regression procedure of the peak area versus the concentration. ZEA linearity, in the working

standards solutions at three determinations of five concentration levels, between 12.5 ng/mL and 200 ng/mL, was good as shown by the correlation coefficient (r^2=0.9977) (Figure II.2).

ZEA linearity obtained with the concentrations for the matrix-matched calibration curve, prepared between 20 and 250 μg/Kg, was either good as shown by the correlation coefficient (r^2=0.997) (Figure II.3). Both matrix and standard calibration curves were used to calculate the matrix effect (ME), matrix-matched calibration slope (B) is divided by the slope of the standard calibration in solvent (A). Thus, the ratio (B/A x 100) was defined as the absolute matrix effect (ME %). The obtained value was interpreted as follows: a value of 100 % denoting an absence of matrix effects, above 100 % a signal enhancement and below 100 % a signal suppression (Rupert et al., 2011).

The obtained value, 92.5%, can be considered negligible.

The accuracy of the used method was evaluated by analyzing a ZEA-free maize flour sample spiked at three different levels 20, 75 and 200 μg/Kg with three replicates for each level. The mean recoveries ranged from 97.6% and 105.3% for 200 μg/Kg and 75 μg/Kg, respectively. The intra-day repeatability varied between 2% 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 (Table II.1). The accuracy and precision results comply with the requirements established by the EC directive 401/2006 (EC, 2006).

LOD and LOQ were 3.75 and 12.5 μg/Kg, respectively. LODs and LOQs were established as the amount of analyte that produces a signal-to-noise ratio of 3:1 and 10:1 respectively. These values are satisfactory considering the maximum levels established by European Commission (Commission Directive, 2007/1126/EC) and similar with those obtained by Manova and Mladenova (2009) and Reinhold and Reinhardt (2011). These authors found LODs of 4 μg/Kg (Manova and Mladenova, 2009) and 1 μg/Kg (Reinhold and Reinhardt, 2011) and LOQs oscillating between 4 μg/Kg (Reinhold and Reinhardt, 2011) and 12 μg/Kg (Manova and Mladenova, 2009).

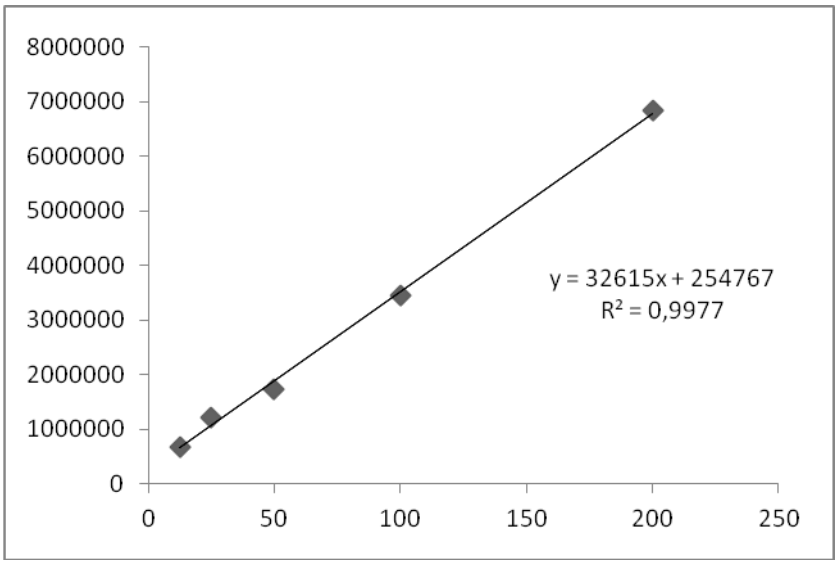

Figure II.2 - ZEA linearity in the working standards solutions.

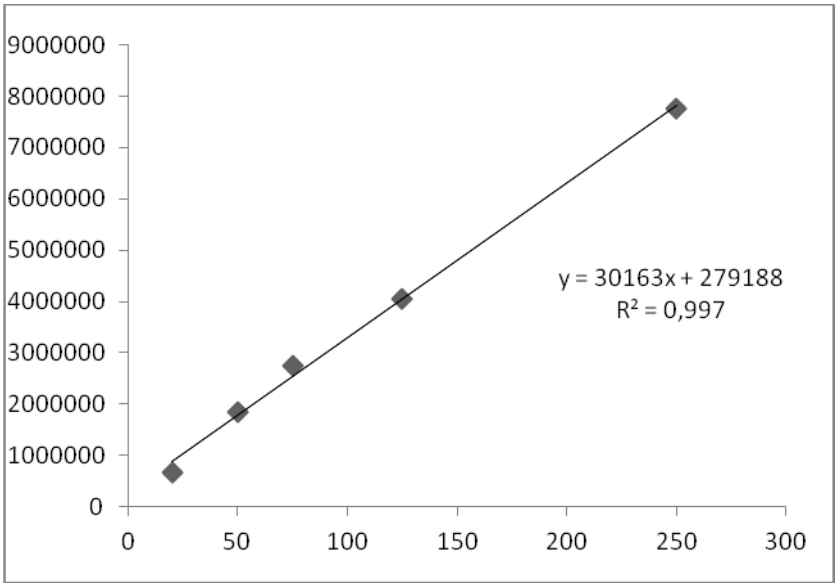

Figure II.3 - ZEA linearity in the matrix-matched solutions.

Table II.1- Validation studies of the analytical methodology.

Fortification levels (µg/Kg)	Accuracy	Intra-day repeatability	Inter-day repeatability
20	105.2	8.3	6.5
75	105.3	2.0	13.6
200	97.6	9.0	12.5

II.3.3 Occurrence of ZEA in flour

In the present study, different flour samples obtained in three different countries were compared. As shown in Table II.2, 23.5 % of wheat flour samples from Portugal were contaminated with ZEA in contrast with the wheat flour from The Netherlands, with 100%. The mean concentrations were 10.7 and 13.1 μg/Kg for Portugal and The Netherlands, respectively. Nevertheless, the maximum level (ML) for ZEA (15.3 μg/Kg) detected in one wheat flour sample was below the ML (75 μg/Kg) for cereals (including cereal flour) for direct human consumption established by EC regulation (EC, 2007). About one third (30.8%) of the Portuguese mixed cereal flours (mixed-flour) were contaminated while the Dutch samples presented frequencies of 50 %. In the Spanish mixed-flour samples ZEA was not detected. The mean concentrations for mixed-flour samples were 20.4 and 28.5 for Portuguese and Dutch samples, respectively, being these concentrations lower than the MLs established by EC regulation. In the case of maize flour, the samples were exclusively from Portugal, presenting a frequency of contamination of 50%, with a mean contamination of 28.0 μg/Kg and a maximum level of contamination of 111.7 μg/Kg. This ML is higher than the limit (75 μg/Kg) for cereals intended for direct human consumption, cereal flour, bran germs and product marketed for direct human consumption established by EC regulation (EC, 2007).

The EC regulation established different maximum limits of contamination depending on the final purpose of the flour. In Table II.3, five different uses (baby flour, culinary, for bread, for frying and semolina) of the samples are observed. The baby flour samples presented a frequency of contamination of 50%, with a mean contamination of 19.0 μg/Kg and a maximum contamination of 25.2 μg/Kg. This sample exceeded the limit of 20 μg/Kg set by EC (2007) for allowed presence of ZEA in processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children. Another baby flour sample from The Netherlands presented a value close to the limit, 19.8 μg/Kg. The flours for culinary uses and the flour for bread presented a frequency of contamination of 36 and 46.2%, respectively. Their mean contaminations were 26.6 and 13.3 μg/Kg and the maximum levels of contamination were 111.7 and 37.2 μg/Kg, respectively. ZEA was not detected in flours used for frying and semolina.

Table II.2 - Occurrence (%) and levels (μg/Kg) of ZEA in flours of different countries.

Sample	Sample size	>LOD (%)	>LOQ (%)	Range (μg/Kg)	Mean ± SD (μg/Kg)
PORTUGAL					
Wheat flour	17	4 (23.5)	1 (5.9)	7.4-15.3	10.7±3.5
Maize flour	12	6 (50)	2 (16.7)	5.9-111.7	28.0±41.4
Mixed-flour	13	4 (30.8)	2 (15.4)	5.4-39.4	20.4±15.1
SPAIN					
Mixed-flour	2	0 (0)	0 (0)	0	0
THE NETHERLANDS					
Wheat flour	2	2 (100)	1 (50)	12.4-13.7	13.1±1.0
Mixed-flour	4	2 (50)	2 (50)	19.8-37.2	28.5±12.3

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

Purpose	Sample size	>LOD (%)	>LOQ (%)	Range (μg/Kg)	Mean ± SD (μg/Kg)
Baby flour	6	3 (50)	2 (33.3)	11.8-25.2	19.0±6.7
Culinary uses	25	9 (36)	5 (20)	5.9-111.7	26.6±33.4
For bread	13	6 (46.2)	1 (7.7)	5.4-37.2	13.3±11.9
For frying	2	0 (0)	0 (0)	0	0
Semolina	4	0 (0)	0 (0)	0	0

For the totality of the analyzed samples, the prevalence of ZEA in flours of different cereals is shown in Table II.4. The analyses were successfully applied to 50 samples of different types of flours and the presence of ZEA was verified in 18 samples (36%) in which

the higher frequency (50%) was found for maize flour. The analyzed samples revealed contamination levels between 5.4 and 111.7 μg/Kg, being the maximum level of contamination and the highest mean concentration observed also in maize flour. The mayor quantity of samples with a LOQ higher than 12.5 μg/Kg was observed in mixed-cereal flour. For the totality of samples, the average level of contamination was 21.0 μg/Kg.

For wheat flour, these results are similar to those reported in The United Kingdom (<10 μg/Kg) by Vendl et al. (2010) and in Spain (8 μg/Kg) by Vidal et al. (2013), and higher than in France (3.3 μg/Kg), as referred by Sirot et al. (2013), and in Serbian market (4.3 μg/Kg) (Skrbic et al., 2012), respectively. In the Bulgarian market the results, obtained in 2009 and reported by Manova and Mladenova (2009) were also higher (29 μg/Kg) than the results obtained in our study (11.7 μg/Kg). For maize flour the results were lower (6.9 μg/Kg) in the Indonesian study carried out by Nuryono et al. in 2005.

The frequency of contamination with ZEA in wheat flours was lower in the studies carried out by Vidal et al. (2013) in the Spanish market (13%) and by Manova and Mladenova (2009) in Bulgaria (1.9%). Inversely, the study reported by Skrbic et al. (2012), in Bulgaria, showed a higher occurrence, 33.3%. For maize flour, the occurrence of ZEA was lower as reported by Nuryono et al. (2005) in Indonesia, 15.4%, and Manova and Mledanova (2009) in Bulgaria, 21.1%, and higher in Iran, 63%, as referred by Reza Oveisi et al. (2005).

In summary, wheat samples showed less concentration and frequency of ZEA than maize samples. Higher concentration of ZEA in maize samples has been also reported by Martos et al. (2010).

Table II.4 - Prevalence (%) and levels (μg/Kg) of ZEA in different types of flour samples.

Sample	Sample size	>LOD (%)	> LOQ (%)	Range (μg/Kg)	Mean ± SD (μg/Kg)
Wheat flour	19	6 (31.58)	3 (15.8)	7.4-15.3	11.7±3.1
Maize flour	12	6 (50)	2 (16.7)	5.9-111.7	28.0±41.4
Mixed-flour	19	6 (31.6)	4 (21.1)	5.4-39.4	23.1±11.7
TOTAL	50	18 (36)	9 (18)	5.4-111.7	21.0±24.7

II.3.4 Estimated daily intake of ZEA

For the calculation of the EDI the following premises were assumed: the flour daily consume for babies was 10% of the adult daily consume, in both Portugal and the Netherlands. The Dutch consumption was obtained by calculating the mean consume of the three groups of population, namely 19 to 30, 31 to 50 and 51 to 69 year-old, for male and female separately.

Despite the maize flour samples presented higher levels of contamination compared to wheat flour, the risk of excess the tolerable daily intake (TDI) is higher in wheat flour due to its higher consumption (Table II.5).

As shown in Table II.5, the EDI for both male and female Dutch population through the wheat flour consumption is higher than the Portuguese adult population, representing 348 x 10^2 - 388 x 10^2% and 196 x 10^2%, respectively, of the TDI proposed by EFSA, in 2011, of 0.25 ng/Kg b.w./day. A similar situation is observed for babies, once the TDI % obtained through this study is 396 x 10^2% and 560 x 10^2% for Portuguese and Dutch babies, respectively. This 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). The estimated daily intake (EDI) ranged between 0.013 and 0.14 μg/kg b.w./day, which represents 52x10^2% and 560x10^2% of the TDI established by EFSA. Therefore, all the studied populations are at risk, being this risk higher for babies than for adults, both in Portuguese and Dutch population.

The EDIs for babies (0.099 μg/kg b.w./day) and for adults in Portugal (0.049 μg/kg b.w./day) and in The Netherlands (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 6-9 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 bw/day, and in France the mean exposure for adults (15 years and older) was estimated as 33 ng/kg bw/day, while for children (3-14 years) was estimated as 66 ng/kg bw/day (Maragos, 2010).

Table II.5 - EDI and exposure assessment.

ZEA		**TDI**[b]	**Wheat flour**		**Maize flour**		**Baby flour**	
			EDI[a]	TDI(%)	EDI[a]	TDI(%)	EDI[a]	TDI(%)
Portugal[c, d]		0.25 ng/Kg b.w/day	0.049	196×10^2	0.013	52×10^2	0.099	396×10^2
The Netherlands	Male[e]		0.097	388×10^2	-	-	0.14	56×10^2
	Female[f]		0.087	348×10^2	-	-		

[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 (according the 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, based on CDC, USA)

[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 (according to INE, 2013)

[e]*C* is the mean annual intake estimated per Dutch male inhabitant in 2007-2010 (227.7 Kg/inh according to 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 according to RIVM, 2011) and K is the mean body weight for male adults, which was considered 70 Kg

II.4 CONCLUSIONS

Extraction with acetonitrile:water (60:40), centrifugation, and dilution with acetonitrile allows the supernatant to be applied onto an IAC column, making it possible to achieve low limits of detection. This optimized analytical methodology provides good results in terms of accuracy, repeatability, intermediate precision and sensitivity, and has been shown to be reliable for determination of ZEA in different types of flour presenting a limit of detection of 3,75 μg/Kg.

The application of the procedure to 50 samples from Coimbra (Portugal), Valencia (Spain), and Utrech (The Netherlands) showed that 36% of the samples were contaminated. One sample of baby flour exceeded the maximum limit established by EC (2007) and another one was close to the limit. A maize flour sample exceeded the ML established by EC with a concentration of 111.7 μg/Kg. Thus, two flour samples exceeded the limit, being both of them from Portugal.

Considering the percentage of TDI, ranging between 52 $x10^2$ and 560 $x10^2$%, the risk assessment linked with the exposure to ZEA was considered to be of concern for the studied populations. Additional work is needed to assess the impact of different management measures, e.g. maximal limits in main food contributors, based on the general methods defined by the Codex Alimentarius Commission (CAC, 2005).

Children are especially a vulnerable group due to their higher food consumption level per Kg body weight. Therefore, results implied that constant monitoring throughout the cereals production chain is necessary in order to minimize health risks related to the intake of ZEA present in flour.

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