# EXPOSURE ASSESSMENT OF PORTUGUESE POPULATION TO OCHRATOXIN A: CONTAMINATED FOODS AND URINE LEVELS

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Contaminated Foods and Urine Levels

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# Abstract

Ochratoxin A (OTA) is a secondary metabolite mainly produced by fungi from *Aspergillus* and *Penicillium* genera. Several toxic effects have been ascribed following exposure, namely nephrotoxicity, hepatotoxicity, teratogenicity and immunotoxicity. In addition, OTA has been classified as possible human carcinogenic - group 2B according to the classification of the International Agency for Research on Cancer (IARC). In fact OTA remains in the list of Balkan endemic nephropathy (BEN) differential diagnosis and it was already established as the cause of porcine endemic nephropathy (PEN) in Denmark. Consequently, a considerable effort has been and is being expended to characterize human and animal exposure to this mycotoxin.

Once formed OTA can enter the food chain, through contamination of ingredients or foodstuffs consumed by humans or the feed chain, through contamination of feeds for animals destined for human consumption. Humans are therefore directly and indirectly exposed to this mycotoxin. OTA has been extensively reported as a worldwide contaminant of a wide variety of raw and processed stored commodities, including cereals and their derivatives, wine, coffee, beer, cocoa, dried fruits, meat and spices. However, nearly all studies point to a higher contribution of cereals and their derivatives to exposure, and a comparatively limited importance of meat and other animal-derived products.

To assess the exposure of the Portuguese population to OTA a study over a two year period, encompassing four collection periods (winter of 2007/2008, summer of 2008, winter of 2008/2009 and summer of 2009) was conducted. It involved analysis of morning urine samples from inhabitants of four Portuguese mainland regions (Porto, Coimbra, Lisboa and Alentejo), simultaneously with a survey of regionally commercialised bread and pork samples. These two analysed foodstuffs are two staple foods in the Portuguese diet, with a high and transversal consumption to the majority of the population.

OTA content the total 738 bread and 472 urine samples was evaluated through previously validated methods, involving clean-up with immunoaffinity columns (IAC)

and high performance liquid chromatography coupled to fluorescence detection (HPLC/FD). The 254 pork samples were analysed through a developed method entailing IAC clean-up and liquid chromatographic coupled to tandem mass spectrometry (LC/MS/MS). The daily intake of OTA by the Portuguese population through both bread and pork consumption was also estimated.

With this study it was possible to draw several conclusions. First the bread samples analysed in all the studied regions presented a widespread low level contamination, although the contamination range was broad, up to European Union (EU)-surpassing levels. Maize bread (*broa*), first and foremost Avintes *broa*, was the most contaminated, followed by whole grain-, rye- and wheat-based bread. However, because more consumed, the later contributes more to OTA exposure of the Portuguese consumers.

The analysis of pork revealed a relatively high average level of contamination, along with a low frequency of contamination, in contrast with previous national and foreign surveys.

The observed high within-subject variability of OTA in urine limits the use of this exposure biomarker at the individual level, but not at a population or subgroup of subjects scale. Among the studied population a widespread exposure was confirmed by the high frequency of contamination of OTA in the urine surveyed, although characterised by a low average contamination level. The urine survey revealed that the population from Alentejo was the most exposed one, and comparing the two studied staple foods, pork emerged as the major contributor. Indeed bread commercialised in this region featured the lowest contamination, as the typical Alentejano wheat bread constituted most of analysed bread samples in such region. As quite the opposite, in general, pork samples from Alentejo featured a higher contamination, probably explained by the traditional extensive feeding-system of the native swine breed, to which some of the surveyed animals belonged.

In all the studied regions' populations' the contribution of pork to the OTA intake was higher than that of bread, which was clearly in contrast with previous studies, according to which cereals and their derived products were the major contributors, while food of animal origin makes only a small contribution to the total human dietary exposure to OTA. These differing features of the exposure of the Portuguese population

in comparison to previous studies reinforce the need of a regular testing to monitor the situation and protect human health.

The rare occurrence of limit-surpassing food samples indicated that in general the exposure to OTA is unlikely to pose a threat to the health of an average Portuguese consumer, although a continuous intake at low levels can still carry risks.


## Keywords

Ochratoxin A; exposure; Portugal; population; bread; pork; urine

# Resumo


A ocratoxina A (OTA) é um metabolito secundário produzido por fungos do género Aspergillus e Penicillium. Vários efeitos tóxicos foram associados à exposição à OTA, designadamente nefrotoxicidade, hepatotoxicidade, teratogenicidade e imunotoxicidade. Adicionalmente, esta micotoxina foi classificada como agente possivelmente carcinogénico para seres humanos – grupo 2B de acordo com a classificação da Agência Internacional para a Investigação *do* Cancro (IARC). De facto a OTA permanece na lista de diagnósticos diferenciais da nefropatia endémica dos Balcãs (BEN) e foi previamente identificada como responsável pela nefropatia endémica suína (PEN) descrita na Dinamarca. Consequentemente, têm sido dedicados importantes esforços na caracterização da exposição humana e animal a esta micotoxina.

Uma vez formada, a OTA pode invadir a cadeia alimentar humana pela contaminação de ingredientes ou alimentos consumidos por humanos ou a cadeia alimentar animal, pela contaminação de alimentos destinados ao consumo de animais de produção. Os seres humanos são assim expostos a esta micotoxina de forma directa e indirecta. A OTA tem sido mundialmente descrita como um contaminante de uma grande variedade de matérias-primas e produtos alimentares processados, incluindo cereais e respectivos produtos derivados, vinho, café, cerveja, cacau, frutos secos, carne e especiarias. Contudo, praticamente todos os estudos apontam para uma contribuição superior dos cereais e seus produtos derivados para a exposição humana, a par de uma contribuição de importância comparativamente insignificante da carne e outros produtos de origem animal.

Para avaliação da exposição da população Portuguesa à OTA foi efectuado um estudo durante dois anos, abrangendo quatro períodos de recolha (Inverno de 2007/2008, Verão de 2008, Inverno de 2008/2009 e Verão de 2009). Este estudo envolveu a análise de amostras de urina de habitantes de quatro regiões do território continental (Porto, Coimbra, Lisboa e Alentejo), juntamente com uma análise de amostras de pão e de carne de suíno comercializadas nas quatro regiões. Os dois alimentos analisados são parte importante da dieta Portuguesa, com um consumo elevado e transversal à maioria da população.

O teor de OTA de um total de 738 amostras de pão e 472 amostras de urina foi avaliado através de métodos previamente validados, envolvendo purificação com colunas de imunoafinidade (IAC) e determinação por cromatografia líquida de elevada eficiência acoplada a um detector de fluorescência (HPLC/FD). As 254 amostras de carne de suíno foram analisadas através de um método desenvolvido envolvendo purificação com IAC e cromatografia líquida acoplada com espectroscopia de massa em tandem (LC/MS/MS). A ingestão diária de OTA pela população Portuguesa através do consumo de pão e carne de suíno foi igualmente estimada.

O estudo realizado possibilitou várias conclusões. As amostras de pão analisadas em todas as regiões em estudo apresentaram uma contaminação disseminada com valores médios baixos, apesar dos intervalos de contaminação ultrapassarem num dos casos o limite máximo estabelecido na União Europeia (EU). O pão de milho (broa), particularmente a broa de Avintes, foi determinado como o tipo de pão mais contaminado, seguido pelo pão integral, de centeio e trigo. Contudo, uma vez que é mais consumido, a contribuição do pão de trigo para a exposição dos consumidores Portugueses à OTA é superior.

A análise da carne suína revelou um valor médio de contaminação relativamente elevado, paralelamente a uma baixa frequência de contaminação, em contraste com estudos efectuados previamente em Portugal e no estrangeiro.

Foi observada uma elevada variabilidade intra-individual da OTA na urina, o que limita a utilização deste biomarcador de exposição ao nível individual, mas não à escala de uma população ou um subgrupo de indivíduos. Foi igualmente confirmada uma ampla exposição pela elevada frequência de contaminação da micotoxina na urina analisada, embora caracterizada por valores médios de contaminação relativamente baixos. A análise da urina revelou que a população do Alentejo apresentou uma exposição superior e numa comparação entre os dois alimentos estudados, a carne de suíno apresentou uma contribuição superior para tal exposição. Com efeito, o pão comercializado nesta região apresentou uma contaminação reduzida, uma vez que o pão de trigo Alentejano típico constituiu a maioria das amostras de pão analisadas na região em questão. De forma inversa, as amostras de carne de suíno do Alentejo apresentaram em geral uma contaminação superior, provavelmente explicada pelo sistema de

produção extensivo da raça suína autóctone, à qual parte das amostras desta região pertencia.

Em todas as populações das regiões em estudo a contribuição da carne de suíno para a ingestão de OTA foi superior à do pão, e portanto em desacordo com estudos prévios, de acordo com os quais os cereais e seus produtos derivados são os que mais contribuem para a exposição, enquanto produtos de origem animal apresentam uma contribuição insignificante para exposição alimentar humana à OTA. Estas características divergentes da exposição da população Portuguesa à OTA relativamente a estudos anteriores reforçam a necessidade da análise regular para monitorização da situação e protecção da saúde dos consumidores.

A rara ocorrência de amostras de alimentos com valores de OTA superiores aos limites máximos implementados indica que em geral a exposição a esta micotoxina provavelmente não ameaça a saúde de um consumidor Português médio, embora uma ingestão contínua a baixos níveis possa ainda assim acarretar riscos.


## Palavras-chave

Ocratoxina A; exposição; Portugal; população; pão; carne; suíno; urina

# List of Publications

The following original publications were prepared in the scope of the works conductive to the present dissertation.

## Articles in international peer-reviewed journals

Duarte, S.C., Alves, R., Pena, A, Lino, C.M. Determinants of ochratoxin A exposure - a one year follow-up study of urine levels. *International Journal of Hygiene and Environmental Health*.DOI: 10.1016/j.ijheh.2011.12.001.

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Duarte, S.C., Lino, C.M., Pena, A. (2011). Ochratoxin A in feed of food-producing animals: an undesirable mycotoxin with health and performance effects. *Veterinary Microbiology*. 154, 1-13.

Duarte, S.C., Pena, A., Lino, C.M. (2011). Human ochratoxin A biomarkers - from exposure to effect. *Critical Reviews in Toxicology*. 41, 187-212.

Duarte, S.C., Pena, A., Lino, C.M. (2010). Ochratoxin A in Portugal: a review to assess human exposure. *Toxins*. 2, 1225-1249.

Duarte, S.C., Bento, J., Pena, A., Lino, C.M., Delerue-Matos, C., Oliveira, M.B.P.P., Alves, M.R., Pereira, J.A. (2010). Influencing factors on bread-derived exposure to ochratoxin A: Type, origin and composition. *Food and Chemical Toxicology*. 48, 2139-2147.

Duarte, S., Bento, J., Pena, A., Lino, C.M., Delerue-Matos, C., Oliva-Teles, T., Morais, S., Correia, M., Oliveira, M.B.P.P., Alves, M.R., Pereira, J.A. (2010). Monitoring of ochratoxin A exposure of the Portuguese population through a nationwide urine survey – Winter 2007. *Science of the Total Environment*. 408, 1195-1198.

Duarte, S.C., Pena, A., Lino, C.M. (2010). A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. *Food Microbiology*. 27, 187-198

Duarte, S.C., Pena, A., Lino, C. (2010). Mycotoxin food and feed regulation and the specific case of ochratoxin A - review of the worldwide status. *Food Additives & Contaminants - Part A*. 27, 1440-1450.

Duarte, S.C., Tanello, A., Pena, A., Lino, C.M., Matos, C.D., Oliveira, M.B.P.P., Alves, M.R. (2010). Evaluation of Ochratoxin A exposure degree in two Portuguese cities through wheat and maize bread consumption during the winter 2007. *Food Control*. 21, 702-707

Duarte, S.C., Pena, A., Lino, C.M. (2009). Ochratoxin A non-conventional exposure sources - A review. *Microchemical Journal.* 93, 115-120.

Duarte, S.C., Bento, J.M.V., Pena, A., Lino, C.M. (2009). Ochratoxin A exposure assessment of the inhabitants of Lisbon during winter 2007/2008 through bread and urine analysis. *Food Additives & Contaminants - Part A*. 26, 1411-1420.

## Articles in national journals

Duarte, S.C., Pena, A., Lino, C.M., Perestrelo R. (2008). Micotoxinas em suinicultura. *Revista Técnica de Suinicultura.* 33, 26-30.

## Book Chapters

Food Analysis of Ochratoxin A and Fumonisins B1 and B2: State-of-the-Art. Duarte, S.C., Silva, L.J.G. Lino, C.M., Pena. A. (*At press*). In 'Food Quality: Control, Analysis and Consumer Concerns'. Edited by Medina, D.A. & Laine, A.M. Nova Science Publishers: New York.

Ochratoxin A in sorghum and derived products. Duarte, S.C., Pena, A., Lino, C.M. (*in press*). In 'Sorghum: Food and Energy Source' Edited by Vázquez, M. & Ramírez, J.A. Nova Science Publishers: New York.

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This dissertation takes in parts of the aforementioned authored chapters, published original and review articles.

# Dissertation scope and outline

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The present dissertation provides the theoretical background, describes the studies and corresponding results conducted in the exposure assessment of the Portuguese population to ochratoxin A. Such exposure was evaluated through the analysis of ochratoxin A in bread and pork, as well as urine of the inhabitants consuming these commodities according to different surveyed regions, seasons and years. Accordingly this dissertation was structured in four main parts as follows:

**Part I:** This part intends to assist the subsequently presented original research. Tailored in accordance, it thus includes and reviews studies that are of direct relevance for the evaluation of OTA exposure.

Specifically, this part comprises a focused review description of the history, origin and proprieties of ochratoxin A, as well as of the toxicokinetic features and toxicological data. It further addresses ochratoxin A exposure providing with an overview of the sources, extent and significance, as well as the main exposure assessment approaches and tools. The analytical methods reported for the mycotoxin determination in foods and urine are also covered. Finally the applicable legislative framework is surveyed.

**Part II**: In this part the rationale, scope and scheme of the original research conducted is presented. The global and specific objectives are set out. For each individual study the experimental procedure and results are detailed and followed by a discussion and concluding remarks.

**Part III**: This part comprises the general conclusions that can be taken from the studies conductive to the present dissertation. The independent results obtained for each of the three analysed matrices are combined to allow a contextualised and critical overview and final conclusion. The main limitations of the studies are also identified, and finally, the new perspectives fostered by obtained results are pointed out.

**Part IV**: This closing part lists all cited references in the previous parts.

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# List of abbreviations and symbols

**AAC** Aptamer affinity column
**$AFB_1$** Aflatoxin $B_1$
**$AFB_2$** Aflatoxin $B_2$
**$AFG_1$** Aflatoxin $G_1$
**$AFG_2$** Aflatoxin $G_2$
**AFs** Aflatoxins
**AOAC** Association of Analytical Communities
**APCI** Atmospheric pressure chemical ionization
**ASE** Accelerated solvent extraction
**ASEAN** Association of Southeast Asian Nations
**$a_w$** Water activity
**bw** Body weight
**BEN** Balkan endemic nephropathy
**BMI** Body mass index
**C18** Octadecyl carbon chain
**C8** Octyl carbon chain
**CA** Cyclopiazonic acid
**CAC** Codex alimentarius commission
**CCFAC** Codex Committee on Food Additives and Contaminants
**CCα** Decision limit
**CCβ** Detection capability
**CEC** Commission of the European Communities
**CEN** European Committee for Standardization
**CIN** Chronic interstitial nephropathy
**CIT** Citrinin
**CKDue** Chronic kidney disease of uncertain etiology
**CRM** Consecutive reaction monitoring
**CRM** Consecutive reaction monitoring
**CZE** Capillary zone electrophoresis
**DNA** Deoxyribonucleic acid
**DON** Deoxynivalenol
**EC** European Commission
**EDI** Estimated daily intake
**EFSA** European Food Safety Authority
**ELISA** Enzyme-linked immunosorbent assay
**EMAN** European Mycotoxins Awareness Network
**ESI** Electrospray ionization
**EU** European Union
**FAO** Food and Agricultural Organization
**FAPAS** Food analysis performance assessment scheme
**$FB_1$** Fumonisin $B_1$
**$FB_2$** Fumonisin $B_2$
**FBs** Fumonisins
**FDA** Food and Drug Administration

| | |
|---|---|
| **FP** | Fluorescence polarization |
| **GC** | Gas chromatography |
| **GC/MS** | Gas chromatography coupled to mass spectrometry |
| **GC/MS/MS** | Gas chromatography coupled to tandem mass spectrometry |
| **GEMS/FOOD** | Global Environment Monitoring System/Food Contamination Monitoring and Assessment Programme |
| **HPLC** | High performance liquid chromatography |
| **HPLC/FD** | High performance liquid chromatography coupled to fluorescence detection |
| **IAC** | Immunoaffinity columns |
| **IARC** | International Agency for Research on Cancer |
| **ID** | Internal diameter |
| **IPC** | *Instituto Português do Consumidor* |
| **IPCS** | International Programme on Chemical Safety |
| **IRMM** | Institute of Reference Materials and Measurements |
| **IS** | Internal standard |
| **IUPAC** | International Union of Pure and Applied Chemistry |
| **JECFA** | Joint FAO/WHO Expert Committee on Food Additives |
| **LIF** | Laser-induced fluorescence |
| **LC** | Liquid chromatography |
| **LC/FD** | Liquid chromatography coupled to fluorescence detection |
| **LC/MS** | Liquid chromatography coupled to mass spectrometry |
| **LC/MS/MS** | Liquid chromatography coupled to tandem mass spectrometry |
| **$LD_{50}$** | Median lethal dose |
| **LLE** | Liquid-liquid extraction |
| **LOD(s)** | Limit(s) of detection |
| **LOQ(s)** | Limit(s) of quantification |
| **LR** | Linear range |
| ***m/z*** | Mass to charge ratio |
| **MAE** | Microwave-assisted extraction |
| **MAFF** | Ministry of Agriculture, Forestry and Fisheries |
| **ME** | Matrix effect |
| **MERCOSUL** | *Mercado Comum do Sul* |
| **MIAC** | Molecular imprinted affinity column |
| **MIPs** | Molecularly imprinted polymers |
| **ML(s)** | Maximum limit(s) |
| **MS** | Mass spectrometry |
| **MSPD** | Matrix-solid phase dispersion |
| **n.a.** | Non-applicable |
| **n.c** | Non-collected |
| **n.d.** | Non-detected |
| **n.g.** | Not given |
| **n.s.** | Non-specified |
| **NIV** | Nivalenol |
| **NTP** | National Toxicology Program |
| **NWGFTRE** | Nordic Working Group on Food Toxicology and Risk Evaluation |
| **OTA** | Ochratoxin A |

| | |
|---|---|
| **OTA-Me** | Methyl ester of ochratoxin A |
| **OTB** | Ochratoxin B |
| **OTC** | Ochratoxin C |
| **OTα** | Ochratoxin α |
| **OTβ** | Ochratoxin β |
| **PA** | Penicillic acid |
| **PBS** | Phosphate-buffered saline |
| **PDA** | Photodiode array |
| **PDMS/DVB** | Polydimethylsiloxane/divinylbenzene |
| **PEN** | Porcine endemic nephropathy |
| **Phe** | Phenylalanine |
| **PLE** | Pressurized liquid extraction |
| **PS** | Particle size |
| **R** | Recovery |
| $R^2$ | Correlation coefficient |
| **RASFF** | Rapid Alert System for Food and Feed |
| **RP** | Reversed phase |
| **RSD** | Relative standard deviation |
| $RSD_b$ | Relative standard deviation between-day (inter-day) |
| $RSD_w$ | Relative standard deviation within-day (intra-day) |
| **SAX** | Strong anion exchange |
| **SCF** | Scientific Committee on Food |
| **SCOOP** | Scientific cooperation |
| **SD** | Standard deviation |
| **SELEX** | Systematic Evolution of Ligands by Exponential Enrichment |
| **SIM** | Selected ion-monitoring |
| **SPE** | Solid phase extraction |
| **SPME** | Solid phase micro-extraction |
| **SPR** | Surface plasmon resonance-based |
| **SR** | Spiking range |
| **SRM** | Selected reaction monitoring |
| $t_{1/2}$ | Half-life |
| **TDI(s)** | Tolerable daily intake(s) |
| **TDS** | Total diet study |
| **TLC** | Thin layer chromatography |
| **tRNA** | Transfer ribonucleic acid |
| **TWI** | Tolerable weekly intake |
| **UK** | United Kingdom |
| **UPLC** | Ultra-performance liquid chromatography |
| **USA** | United States of America |
| **UTT** | Urinary tract tumours |
| **UV** | Ultraviolet |
| **WAX** | Weak anion exchange |
| **WHO** | World Health Organization |
| **ZEA** | Zearalenone |
| $\lambda_{emi}$ | Wavelength of emission |
| $\lambda_{exc}$ | Wavelength of excitation |
| **4-OH-OTA** | 4-hydroxyochratoxin A |

# Part I

# Literature review

---

**SCOPE:** This part aims to assist the following original research studies. It comprises a focused review description of the history, origin and proprieties of ochratoxin A, as well as of the toxicokinetic features and toxicological data. This part further addresses ochratoxin A exposure providing with an overview of the sources, extent and significance, as well as the main exposure assessment approaches and tools. The analytical methods reported for the mycotoxin determination in foods and urine are also covered. Finally the applicable legislative framework is surveyed.

**ABRIDGED CONTENTS:** 1. Historical insight; 2. Family and Physico-Chemical properties; 3. Ochratoxigenic fungi; 4. Toxicokinetics; 5. Toxicological data; 6. Sources of OTA exposure; 7. Extent and significance of OTA exposure; 8. OTA exposure assessment; 9. OTA analytical methods; 10. Legislative framework.

# 1. Historical insight

The term mycotoxins refers to low molecular weight natural products, produced as secondary metabolites by filamentous fungi. It was coined in 1962 in the aftermath of an unusual veterinary crisis near London, England, during which approximately 100,000 turkey poults died. When this mysterious "Turkey X disease" was linked to a peanut (groundnut) meal contaminated with secondary metabolites from *Aspergillus flavus* (aflatoxins; AFs), it sensitised scientists to the possibility that other unknown mould metabolites might be equally deadly. This triggered large-scale screenings targeted at mycotoxin discovery and identification, particularly between 1960 and 1975, named the "mycotoxin gold rush" (Bennett & Klich, 2003). Ochratoxin A (hereinafter OTA) was discovered in this context, as a metabolite of *Aspergillus ochraceus* (hence its name) by van der Merwe and co-workers in 1965 (Van der Merwe *et al.*, 1965) in South Africa, who isolated the toxic metabolite from corn meal intentionally inoculated with the aforementioned fungus. Shortly thereafter, naturally occurring OTA was isolated for the first time from a commercial corn sample in the United States of America (USA) by Shotwell *et al.* (1969). In the same year van Walbeek *et al.* (1969) isolated the same mycotoxin from *Penicillium verrucosum*. Later, it was recognised as a secondary metabolite of several other *Aspergillus* and *Penicillium* spp.

Due to OTA's description as the first of a group of fungal metabolites that are toxic to animals, it is considered that this mycotoxin, together with the AFs, launched the distinctive and individualised science of mycotoxicology in the 1960s (Zinedine *et al.*, 2010). Even today, after the identification of more than 300 mycotoxins, OTA remains one of only about 20 mycotoxins known to occur in foodstuffs at sufficient levels and frequencies to cause food safety concerns (Clark & Snedeker, 2006).

In Portugal, research on OTA started with a delay of 35 years after its first description in 1965, when OTA was reported for the first time in Portugal during a survey of wines (Festas *et al.*, 2000).

# 2. Family and physico-chemical proprieties

Ochratoxins are a structurally related family of mycotoxins, of which OTA is the most important and most commonly occurring member (Monaci & Palmisano, 2004). The ochratoxins group (chemical structures given in Figure 1), with exception of OTα and OTβ, is comprised of compounds featuring a polyketide-derived

dihydroisocoumarin moiety linked via the 7-carboxy group to L-β-phenylalanine by an amide bond. Ochratoxins consist of OTA, its methyl ester, its ethyl ester also known as ochratoxin C (OTC), 4-hydroxyochratoxin A (4-OH-OTA), ochratoxin B (OTB) and its methyl and ethyl esters, and also OTβ and OTα; in the last two, phenylalanine (Phe) moiety is missing (Ringot *et al.*, 2006).

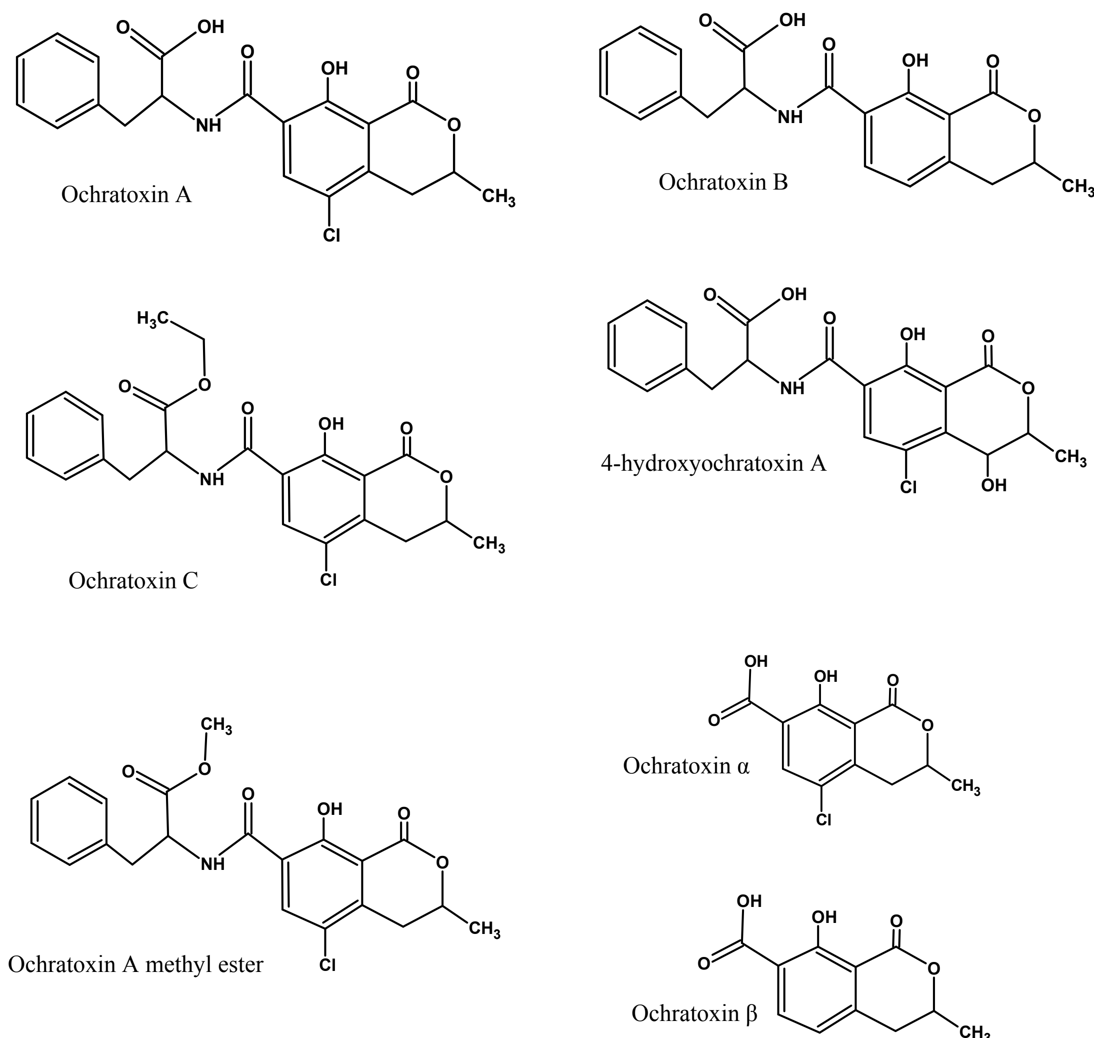


**Figure 1.** Ochratoxins basic chemical structure (modified from Almela *et al.*, 2007).

OTA is rapidly formed *in vivo* from OTC and attracted far more attention since it is distinctly (10- to 20-fold) more toxic (according to *in vivo* and *in vitro* evidences) than OTB (the second more toxic member). Both differ only by the presence of the chloride at the $C_5$ of the isocoumarin moiety, which in OTB is substituted by hydrogen (O'Brien & Dietrich, 2005; Zöllner & Mayer-Helm, 2006; Heussner *et al.*, 2007). The difference

in structure is assumed to be responsible for the differences in toxic potentials and may indicate different modes of action (Heussner *et al.*, 2007). It appears, therefore, that strict structure-activity relationships are a feature of ochratoxin toxicity (O'Brien & Dietrich, 2005).

OTA's configuration was determined using optical rotator dispersion spectroscopy. The empirical formula is $C_{20}H_{18}O_6NCl$ and the molecular weight is 403.82. The International Union of Pure and Applied Chemistry (IUPAC) developed formula of OTA is l-phenylalanine-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)carbonyl]-(*R*)-isocoumarin. It is a white, crystalline compound, highly soluble in polar organic solvents, slightly soluble in water and soluble in aqueous sodium hydrogen carbonate. OTA presents the melting points of 90 and 171 ºC, when recrystallised from benzene (containing 1 mol benzene/mol) or xylene, respectively (Ringot *et al.*, 2006).

OTA exhibits ultraviolet (UV) adsorption at $\lambda^{MeOH}$max (nm; ε) = 333 (6400). The fluorescence emission maximum is at 467 nm in 96 % ethanol and 428 nm in absolute ethanol. The infrared spectrum in chloroform includes peaks at 3380, 1723, 1678 and 1655/cm (Ringot *et al.*, 2006).

OTA has weak acidic proprieties. The pKa values are in the ranges 4.2-4.4 and 7.0-7.3, respectively, for the carboxyl group of the phenylalanine moiety and the phenolic hydroxyl group of the isocoumarin part (Ringot *et al.*, 2006). Although OTA is stable under the acidic conditions used in most extraction procedures, low pH can provoke acid hydrolysis of the OTA amide bond, yielding phenylalanine and OTα. Under alkaline conditions, the lactone ring opens, resulting in a less toxic compound. This situation is considered reversible by simply lowering the pH (Valenta, 1998; Monaci & Palmisano, 2004; Castegnaro *et al.*, 2006).

Another outcome of the effect of alkaline conditions is that all glassware used in OTA analysis must be free of alkaline soap or detergent residues to avoid loss of the toxin from neutral solvents by salt formation, precipitation and/or adsorption onto glassware, resulting in lower detection rates (Valenta, 1998; Turner *et al.*, 2009). Due to the potent desorbent character against OTA, glassware can be rinsed with methanol prior to use (Valenta, 1998). Other practices involve sodium hypochlorite solution to decontaminate glassware, followed by an acid-washed immersion in a solution of 4mL/L $H_2SO_4$ before a final rinsing with distilled water (Juan *et al.*, 2007).

For analytical procedures it is also important to refer that OTA is a substituted isocoumarin related to the fungal metabolite mellein, which is also an innately fluorescent compound (Richard, 2007; Maragos *et al.*, 2008). Hence it presents (blue) fluorescence under UV light (Monaci & Palmisano, 2004). However, OTA fluorescence cannot be measured directly in contaminated samples, without prior extraction or clean-up, because many natural compounds fluoresce at wavelengths close to that of OTA (Cruz-Aguado & Penner, 2008).

Furthermore, although OTA is a stable, heat resistant compound, it is UV-decomposable, so special care should be taken to protect it from daylight, e.g. through amber glass material (Valenta *et al.*, 1998; Yang *et al.*, 2010) or aluminium foil (Blesa *et al.*, 2004). An alternative to the storage of OTA standard solutions is to evaporate the solvent, store the toxin as a film in the glass vial and reconstitute it only when needed (Valenta, 1998).

## 3. Ochratoxigenic fungi

Two genera, *Penicillium* and *Aspergillus*, enclose all the OTA producing fungal species. Although the widespread occurrence of ochratoxigenic species has been confirmed, each shows different behaviours in respect to ecological niches, the commodities (substrates) affected and in the frequency of their occurrence in different geographical regions (Uysal *et al.*, 2009).

*P. verrucosum* grows optimally below 30 ºC and in water activity ($a_w$) values as low as 0.80, and is therefore usually found in cool temperate regions of northern Europe and Canada, though occasionally in Mediterranean sea localities, with temperate climates like Italy, Spain, France and Portugal (Logrieco et al., 2003; Cabañas et al., 2008). According to the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA, 2008) it appears to be uncommon, indeed almost unknown, in warm climates or in other kinds of foods. This species, slow-growing under any condition, has been reported almost exclusively in cereal and cereal derived products (Cabañes *et al.*, 2002). *P. verrucosum* is more frequently isolated where cooler damp harvesting conditions exist, and so inefficiently drying of grain can result in pockets of growth by this species in storage (Aldred *et al.*, 2008). Recently, *Penicillium nordicum*, formed by some strains isolated mainly from fermented meat, cheese and other dried proteinaceous foods, split from the latter species, although expectedly presenting similar physiological characteristics. At present,

these two species are the only OTA producers known and accepted in the *Penicillium* genera, although *P. nordicum* appears to be a minor source of OTA in foodstuffs in comparison with *P. verrucosum* (Cabañas *et al.*, 2008; JECFA, 2008).

*A. ochraceus* and its other mesophilic xerophile kindred species, *A. westerdijkiae* and *Aspergillus steynii*, pertain to the *Aspergillus* section *Circundati*, characterised by the golden brown coloured conidia. They present optimal growth at warm temperatures of 24-31ºC (range 8-40ºC), high $a_w$ values of 0.95-0.99 (down to 0.80) and pH between 3 and 10. Although they can sporadically affect cereals, other plant products and stored food, they are most important in stored dried fruits, nuts, coffee and cocoa beans during sun-drying (Logrieco *et al.*, 2003; Cabañas *et al.*, 2008; JECFA, 2008).

The second *Aspergillus* ochratoxigenic group - the black *Aspergillus* species, are classified under *Aspergillus* section *Nigri*, of which *A. carbonarius* is the main representative. Some authors have indicated that the very closely related species *A. carbonarius* and *A. niger* are found many times in association, and are the source of OTA in tropical and sub-tropical foods, namely in maturing fruits, especially grapes and dried fruits for the recognised high resistance to sunlight and ultraviolet light, as well as to relatively high temperatures. They are also very acid tolerant and prefer a somewhat reduced $a_w$ (Cabañes *et al.*, 2002; Logrieco *et al.*, 2003; JECFA, 2008). *A. carbonarius* produces larger spores, grows at rather lower temperatures than *A. niger*, with an optimal temperature condition at 30 ºC. The ability to grow at reduced $a_w$ is also more restricted: germination occurs down to 0.85 $a_w$ at 25 and 30 ºC. *A. niger* aggregates are often found in warm and tropical climates, because it grows optimally at the relatively high temperatures of 35-37 ºC (range 6-47 ºC). *A. niger* is a xerophile, with germination reported at 0.77 $a_w$ at 35 ºC. Besides nuts, fresh fruit, dried wine fruit and some vegetables (Magnoli *et al.*, 2007), *A. niger* in association with *A. carbonarius* is also isolated, although not as frequently, from tropical cereals. It is important to underline that only a few of the *A. niger* isolates are believed to be able to produce OTA in commercially grown crops (JECFA, 2008).

So, since each product tends to host a specific OTA-producing mould, the environmental conditions and factors that encourage the subsequent formation of OTA need to be understood (Scudamore, 2005).

Furthermore, the composition of the fungal population of a crop, and hence the potential occurring mycotoxins, will depend on the length and conditions of storage so that the species present at harvest may decline or thrive with time, while the “storage”

fungi can increase rapidly (Domijan *et al.*, 2005). Historically, fungi have been divided into two groups - the first includes the toxigenic fungi which invade and produce their toxins before harvest, and is known as the "field fungi". The second, which becomes a problem after harvest, is known as the "storage fungi" group. However, the original source of the fungi in both circumstances is the field (Miller, 1995). The ochratoxigenic species are considered "storage" fungi, because they are isolated at increased frequency as storage progresses.

## 4. Toxicokinetic features

OTA is well absorbed in the gastrointestinal tract and (approximately 99 %) binds to blood proteins, with considerable variations in serum half-lives across species that are apparently dependent on the affinity and extent of protein binding (Dietrich *et al.*, 2005; Ringot *et al.*, 2006). Also, reabsorption of OTA from the intestine, enterohepatic recirculation (Roth *et al.*, 1988), and reabsorption in every nephron segment, but mainly in the proximal and distal tubules favour its accumulation in the organism (Dahlmann *et al.*, 1998).

Among all studied models, human appears to have the longest serum half-life ($t_{1/2}$), determined at about 840 hours (35 days), due to the aforementioned unfavourable elimination kinetics (Schlatter *et al.*, 1996). Among farm animals, the highest reported serum half-life is in swine, varying between 8.8 and 150 hours (Galtier *et al.*, 1981; Hult *et al.*, 1979). Again, this long serum half-life is sustained by the high affinity for proteins, enterohepatic circulation and biliary excretion, responsible for a prolonged elimination from the body (Fuchs *et al.*, 1988; Roth *et al.*, 1988; Solti *et al.*, 1999).

Poultry species appear to eliminate OTA faster than mammals do and this leads to a lower OTA accumulation in the blood. In fact, the half-life of OTA in pig serum is up to 20–30 times longer than that in poultry serum, leading to higher OTA contamination and incidence in pigs (Galtier *et al.*, 1981; Pozzo *et al.*, 2010; Schiavone *et al.*, 2008).

Conversely to monogastric animals, ruminants are less susceptible to the adverse health effects associated with OTA exposure (Fink-Gremmels, 2008) because the protozoan fraction of rumen fluid is capable of enzymatic degradation into OTα and other less toxic metabolites (Ribelin *et al.*, 1978; Kiessling *et al.*, 1984). The degradation into OTα, a lacking phenylalanine moiety metabolite, contributes so as to

only small amounts of intact OTA are absorbed, thus explaining the high comparable tolerance of ruminants to OTA exposure (Hult *et al.*, 1976).

In swine tissue, the accumulation pattern was reported as blood>kidney>liver>muscle>fat (Curtui *et al.*, 2001; Curtui & Gareis, 2001; Chiavaro *et al.*, 2002), and also kidney>urinary bladder>liver>spleen (Ceci *et al.*, 2007). Concerning other species, bioaccumulation was reported to follow the patterns kidney>liver>muscle (Bozzo *et al.*, 2011) and liver>kidney>gizzard (Milićević *et al.*, 2011) in broilers. In the case of rabbits, the pattern was described as kidney>liver>mammary gland>muscle (Ferrufino-Guardia *et al.*, 2000). It is important to underline that the concentration of the toxin and its metabolites in tissues and plasma depends on the animal species, the dose and route of administration, the form of the administered OTA (crystalline or naturally occurring in feed), the diet composition as well as the health status of the animal. The same factors determine the relative contribution of each route of OTA excretion, which is rather slow in contrast with the rapid absorption (Ringot *et al.*, 2006). In all species, both faecal and urinary excretions play important roles in plasma clearance of the toxin. Given the high plasma protein binding potential of OTA, its glomerular filtration is limited. OTA rather undergoes tubular elimination to the urine, and subsequently is reabsorbed in all nephron segments (Dahlman *et al.*, 1998). The reabsorption of filtered and secreted OTA delays its excretion, and leads to the accumulation of the toxin in the renal tissue, thus contributing to its renal toxicity (Ringot *et al.*, 2006).

Through faeces, both OTA and its metabolite OTα are excreted, mainly due to biliary excretion (Kumagai & Aibara, 1982; Roth *et al.*, 1988; Fuch *et al.*, 1988). Additionally, mammals also excrete OTA in their milk (Ferrufino-Guardia *et al.*, 2000). Independently of the significance of the OTA biotransfer, even small amounts of this mycotoxin in milk are of importance for consumers of large quantities of milk, like youths, for whom milk can represent a proportionally important portion of OTA total intake (González- Osnaya *et al.*, 2008).

Toxicokinetics clearly determines OTA toxicity, beyond the features of biomonitoring, namely regarding the type, nature, and levels of metabolites or parent compound itself present, and the type of biological specimens where these can be searched for (Muñoz *et al.*, 2010).

# 5. Toxicological data

Toxicity of this natural contaminant entering the feed and food chains has been extensively reviewed and much of the research can be found summarised in the outputs of various international meetings and assessments (for review see International Agency for Research on Cancer - IARC, 1993; JECFA, 2001, 2008), as well as in comprehensive reviews (Petzinger & Ziegler, 2000; Ringot *et al.*, 2006; Pfohl-Leszkowicz & Manderville, 2007)

Considerable species differences in sensitivity towards OTA acute toxicity have been demonstrated (O'Brien & Dietrich, 2005). In fish median lethal dose ($LD_{50}$) varied between 0.29 for marine sea bass (oral – El-Sayed *et al.*, 2009) and 4.67 mg/kg of body weight (bw) for rainbow trout (intraperitoneal – Doster *et al.*, 1972). Among land animals oral $LD_{50}$ values were shown to be 0.2 mg/kg bw for dogs (Marquardt & Frohlich, 1992), 0.5 mg/kg bw for ducks, 1 mg/kg bw for swine (El-Sayed *et al.*, 2009), 3.3–3.9 mg/kg bw for cockerel chicks (Peckham *et al.*, 1971), 5.9 mg/kg bw for turkeys (Prior *et al.*, 1976), 10 mg/kg bw for rabbits (Mir *et al.*, 1999), 16.5 mg/kg bw for Japanese quail (Prior *et al.*, 1976), and 20-30 mg/kg for rats (Galtier *et al.*, 1981; Ballinger *et al.*, 1986).

Marked gender and species differences have also been observed, namely in nephrotoxicity studies (Bendele *et al.*, 1985; Castegnaro *et al.*, 1998), according to which male animals are more sensitive than females and rats are considerably more sensitive than mice. Age differences were also observed in nephro- and immunotoxicity studies, with male adult rats being more sensitive to OTA than young ones (Dortant *et al.*, 2001; Vettorazzi *et al.*, 2009).

Variations in kinetic parameters may play a major role in explaining these differences (Dietrich *et al.*, 2005).

## 5.1. Molecular mechanisms

Several hypotheses on the mechanism of interaction of OTA and its metabolites with endogenous molecules have been put forward to explain its toxicity. They are related to specific interactions, based on highly specific binding onto specific sites of a target molecule, and nonspecific interactions, based on the chemical reactivity of OTA and its metabolites and their vicinity to the target molecule (Ringot *et al.*, 2006)

Because it contains a Phe moiety, OTA is a structural analogue of this amino acid and as a result can act on all metabolic systems involving phenylalanine. Two major biochemical mechanisms have been proposed to explain these observations: inhibition of Phe-transfer ribonucleic acid (tRNA) synthase (Könrad & Röschenthaler, 1977; Creppy *et al.*, 1984) and inhibition of Phe-hydroxylase (Creppy *et al.*, 1990). Competition with phenylalanine in the phenylalanyl-tRNA synthase-catalysed reaction results in inhibition of protein synthesis (Bunge *et al.*, 1978). Although Creppy *et al.* (1995) also described an inhibition of OTA's nephrotoxic effects in rats by the presence of phenylalanine or OTA- and phenylalanine analogues such as aspartame, the recent study of Stoev (2010) showed the inefficiency of phenylalanine as a renal protector against the carcinogenic or toxic effects of OTA in chicks. Furthermore, since OTA disrupts protein synthesis, it indirectly impairs the activity of several cellular enzymes, and particularly the activity of cytosolic phosphoenolpyruvate carboxykinase, a key enzyme of the gluconeogenic pathway (Meisner & Meisner, 1981). Therefore, an indirect toxicological consequence of OTA is the alteration of the carbohydrate metabolic pathways (Suzuki *et al.*, 1975).

Although the inhibition of protein synthesis is considered the primary cause of OTA toxicity, it cannot individually explain the diversity of OTA toxic effects. Several studies suggest the involvement of oxidoreductive stress in the toxicity of OTA in lipid peroxidation (Rahimtula *et al.*, 1988), and disruption of calcium homeostasis (Khan *et al.*, 1989; Chong & Rahimtula 1992; Dopp *et al.*, 1999). In addition, OTA results in mitochondrial dysfunction through inhibition of mitochondrial respiration chain (Meisner & Chan, 1974; Aleo *et al.*, 1991). Oxidative stress has also been implicated in OTA-mediated cytotoxicity and apoptosis (Atroshi *et al.*, 2000; Petrik *et al.*, 2003).

Regarding the involvement of OTA in deoxyribonucleic acid (DNA) adducts formation there is no agreement and conflicting results have been reported. Some studies support the existence of OTA-DNA adducts (Obrecht-Pflumio & Dirheimer, 2000; Castegnaro *et al.*, 2006; Mantle *et al.*, 2010), and use them for instance to provide evidence of the implication of OTA in endemic human nephropathies (Pfohl-Leszkowicz *et al.*, 2007); whereas several other publications have alleged the opposite (Schlatter *et al.*, 1996; Gautier *et al.*, 2001; Gross-Steinmeyer *et al.*, 2002; Mally *et al.*, 2004) and reject its relevance to a genotoxic mode of action for OTA, in favour of oxidative stress. This last hypothesis is in harmony with the opinion of the most recent evaluation of the European Food Safety Authority (EFSA, 2006).

## 5.2. Toxic effects

Regardless of the source of exposure, the primary target organ for OTA toxicity is the kidney due to the aforementioned unfavourable elimination kinetics (Gekle & Silbernagl, 1994; Schlatter *et al.*, 1996; Dahlmann *et al.*, 1998). OTA has been found to be a potent renal toxin in all of the animal species tested with considerable differences between species, the pig and chicken being the most sensitive (Vettorazzi *et al.*, 2009). OTA nephrotoxic effects were reported in poultry (Peckham *et al.*, 1971; Huff *et al.*, 1975; Gupta *et al.*, 2008; Bozzo *et al.*, 2011), swine (Krogh *et al.*, 1973; Krogh *et al.*, 1974), rat (Boorman *et al.*, 1992; Dortant *et al.*, 2001), and rainbow trout (Doster *et al.*, 1972).

In addition the association among human nephropathies and data from dietary OTA exposure and biomarkers of exposure arises from several epidemiological studies. The results of such studies support the involvement of OTA in the aetiology of endemic human nephropathies: Balkan endemic nephropathy (BEN) - described in residents of the alluvial plains along the tributaries of the Danube river in Serbia, Bosnia, Croatia, Bulgaria, and Romania (Krogh *et al.*, 1977; Pfohl-Leszkowicz *et al.*, 2007), and Chronic Interstitial Nephropathy (CIN) - in North African countries, namely Tunisia (Grosso *et al.*, 2003) and Egypt (Wafa *et al.*, 1998). OTA was also implicated in chronic kidney disease of uncertain etiology (CKDue) in the North Central Province of Sri Lanka (Desalegn *et al.*, 2011).

BEN is characterised by progressive renal fibrosis and by urinary tract tumours (UTT), such as carcinoma of the renal pelvis, ureters and bladder (Radavanovic *et al.*, 1991; Marquardt & Frohlich, 1992; IARC, 1993; Pfohl-Leszkowicz *et al.*, 2002; Ceci *et al.*, 2007). It is also of concern that BEN resembles porcine endemic nephropathy (PEN) which was clearly related to the ingestion of OTA by pigs as reported in Denmark during the 1960s-1970s (Krogh *et al*, 1973; Krogh, 1977).

Spontaneous porcine nephropathies are also frequently observed during meat inspection in Bulgaria (Stoev *et al.*, 2010a) and South Africa (Stoev *et al.*, 2010b). However, these differ from PEN morphologically and feature a multi-mycotoxin aetiology, involving OTA, penicillic acid (PA), and fumonisin $B_1$ ($FB_1$).

Supported by sufficient evidence of carcinogenicity in animal studies, contrarily to inadequate evidence in humans, it is classified as “Possible carcinogenic to humans”

(group 2B) by IARC (1993) and as "Reasonably anticipated to be carcinogenic to humans" in the US National Toxicology Program (NTP) classification (Abnet, 2007).

Although the role of OTA in human disease is not definite, several toxic effects have been described in animal models.

Hepatic pathological changes were observed among broilers (Gentles *et al.*, 1999; Birò *et al.*, 2002; Gupta *et al.*, 2008), and pigs (Malagutti *et al.*, 2005). Neurotoxicity was observed in rats (Belmadani *et al.*, 1998), but conflicting results have been reported (Mantle & Nolan, 2010).

This mycotoxin has also proved to be teratogenic among rats (Patil *et al.*, 2006), rabbits (Wangikar *et al.*, 2004), chick embryos (Kunjamma & Nair, 1997) and quail (Dwivedi, 1984). However, OTA teratogenicity potential can be antagonised by a simultaneous exposure to aflatoxin $B_1$ ($AFB_1$) as observed in rabbits (Wangikar *et al.*, 2005). Conversely, considering embryotoxicity, additive effects were observed in chicken when OTA and citrinin (CIT) were injected sub-germinally or intra-amniotically in embryonated eggs (Veselà *et al.*, 1983).

In addition, OTA exerts an immunotoxic activity (Al-Anati & Petzinger, 2006; Ferrante *et al.*, 2008), even at OTA concentrations far below the doses used in carcinogenicity studies. Such toxicity is of concern given that immune response processes are inevitably involved in the defence against microbial invasion and tumour cell propagation (Petzinger & Weidenbach, 2002).

OTA has been reported to impair humoral immune response in broilers (Santin *et al.*, 2002; Verma *et al.*, 2004; Elaroussi *et al.*, 2006), and mice (Thuvander *et al.*, 1995). Comparably cell-mediated immunity has also been shown to be affected in turkeys (Dwivedi & Burns, 1985), broilers (Elaroussi *et al.*, 2006), pigs (Harvey *et al.*, 1994; Müller *et al.*, 1999), mice (Thuvander *et al.*, 1996), rats (Àlvarez *et al.*, 2004) and rabbits (Verma & Mathew, 1998).

The suppression in both humoral and cell-mediated immune response renders animals more susceptible (Gupta *et al.*, 2008) as recognised in several secondary infectious diseases (Stoev *et al.*, 2000) or to a heavy progression of some often encountered parasitic diseases (Stoev *et al.*, 2002a)

In poultry, OTA was recognised to increase the susceptibility and aggravate the clinicopatological picture in case of coccidiosis (*Eimeria acervulina* and *E. tenella* – Huff & Ruff, 1982; *Eimeria tenella* – Stoev *et al.*, 2002a), salmonelosis (*Salmonella typhimurium* – Elissalde *et al.*, 1994; Fukata *et al.*, 1996; *S. gallinarum* – Gupta *et al.*,

2005, 2008) and colibacillosis (*Escherichia coli* – Kumar *et al.*, 2003, 2004). Rabbits also presented a higher susceptibility to *Pasteurella multocida* as evidenced by higher mortality and severity of the lesions (Mir & Dwivedi, 2010). In swine, Stoev *et al.* (2000) demonstrated that ingestion of OTA contaminated feed increases the susceptibility of growing pigs to natural infection by *Salmonella cholerasuis*, *Serpulina hyodysenteriae* and *Campylobacter coli*. Koynarski *et al.* (2007) reported a more heavy and rapid progress of duodenal coccidiosis (by *Eimeria acervulina*) in chicks fed with OTA contaminated diets as perceived from the changes in lesions and oocyst indices, growth depression, and especially from the chick mortality.

### 5.2.1. Effects of mycotoxins combination

In controlled experiments, toxicological effects are studied for individual or a defined group of mycotoxins. In contrast, under field conditions exposure to complex mixtures of toxins frequently occur (Fink-Gremmels, 2008), resulting in various interactions, namely additivism, synergism, potentiation, and antagonism (Boermans & Leung, 2007), with unexpected repercussions. The possibility of exposure to various combinations of mycotoxins is of much concern and warrants investigation of the potential effects of possible combinations occurring naturally (Gentles *et al.*, 1999).

Nevertheless, not many studies have been performed. As reviewed by Speijers & Speijers (2004), when data on the mycotoxins involved is considered, it becomes clear that the animal species, the endpoint studied and the type of mycotoxins largely encompass the resulting toxicity and determine whether the effects of the combined mycotoxins will be antagonist, additive or synergistic. Indeed, the impacts and clinical features can be distorted by the presence of other mycotoxins.

In field animal ochratoxicosis episodes, the suspected involvement of other mycotoxins beyond OTA was suspected in both endemic (Stoev *et al.*, 2010b) and outbreak occurrence (Shlosberg *et al.*, 1997).

In rats, antagonistic interaction between OTA and OTB has been observed in the histological damage of the proximal tubules (Stormer *et al.*, 1985). The combination of OTA with CIT resulted in synergistic effects for endpoints related to the nephrotoxicity (Siray *et al.*, 1981; Mayura *et al.*, 1984). In mice, Arora *et al.* (1983) reported an antagonist effect in teratogenicity studies between OTA and zearalenone (ZEA).

In swine OTA and AFs have an additive effect on swine weight gain, with reductions by 26 %, 24 %, and 52 % for animals consuming diets containing AF, OTA, or both, respectively (Huff *et al.*, 1988). Contrary results were obtained by Tapia & Seawright (1985) reporting no significant differences in the body weights of pigs receiving both OTA and $AFB_1$ in combination compared with controls. In growing barrows for OTA and T-2 toxin co-occurrence an additive interaction was suggested regarding performance parameters (Harvey *et al.*, 1994), while OTA and PA interaction resulted in synergistic effects on pig performance (Stoev *et al.*, 2001).

In poultry the effects of exposure to several combinations of mycotoxins have generally been reported to be additive. That is the case of toxic effects and performance parameters as evaluated in the interaction OTA and cyclopiazonic acid (CA) in broilers (Gentles *et al.*, 1999), OTA and T-2 toxin in broilers (Kubena *et al.*, 1989; Garcia *et al.*, 2003; Wang *et al.*, 2009), OTA and AFs in laying hens (Verma *et al.*, 2003), OTA and diacetoxyscirpenol in broilers (Kubena *et al.*, 1994), OTA and $FB_1$ in turkey poults (Kubena *et al.*, 1997). However, also in broilers the interaction AFs and OTA produce a synergistic toxicity according to Huff *et al.* (1988) and Verma *et al.* (2004).

In rabbits, Wangikar *et al.* (2005) reported antagonistic interaction between OTA and $AFB_1$ since in foetuses exposed to both mycotoxins, most of the gross, skeletal and visceral anomalies observed in individual treatment either with OTA or $AFB_1$ were either reduced or even absent. In the corresponding dams, the clinical signs were also less severe in combination treatment as compared with those of individual toxins given alone.

## 6. Sources of ochratoxin A exposure

Described sources of human exposure to OTA are food and air. Like other mycotoxins, OTA exposure is mainly of foodborne nature, and hence the inhalation route is far less significant. Thus, considering further the scope of the present dissertation, attention is drawn mainly to ingestion of contaminated foods.

### 6.1. Air

OTA can also be found in floating or settled dust (Wang *et al.*, 2008), so components of heating, ventilation and air conditioning act as reservoirs of fungi (Tarin *et al.*, 2004). This mode of exposure has been recognised in agricultural food production

environment, in which the workers may be exposed to the dust originating from the handling and processing of contaminated foodstuffs, e.g. coffee (Tarin *et al.*, 2004), cocoa and spices (Brera *et al.*, 2002; Iavicoli *et al.*, 2002). Farm workers can also be occupationally exposed to contaminated airborne aerosols especially when tending cows (Skaug *et al.*, 2000; Skaug, 2003), or poultry houses (Wang *et al.*, 2008) by contacting with mouldy feeding and bedding materials. Mycotoxins in airborne particulates can also be found in laboratories, harbours, and warehouses (Di Paolo *et al.*, 1994), as well as in water-damaged buildings (Richard *et al.*, 1999). This exposure can be attributed to lack of prevention and protection devices, such as masks and ventilation systems (Brera *et al.*, 2002).

Although inhalation of air contaminated with OTA and fungal conidia represents an additional source of exposure to the mycotoxin (Skaug *et al.*, 2000; Iavicoli *et al.*, 2002) to both humans and animals, it is considered rare and not yet exhaustively investigated (Brera *et al.*, 2002). In fact, at present the toxicokinetics of airborne OTA in humans are still unclear (Iavicoli *et al.*, 2002). Historically, OTA airborne intoxication was related to the legendary “old-books disease” or “the curse of Tutankhamon” (Di Paolo *et al.*, 1994).

## 6.2. Food

OTA has been extensively documented as a global contaminant of a wide variety of food commodities, but certainly first and foremost in cereals (Prickett *et al.*, 2000; Pena *et al.*, 2005; Zaied *et al.*, 2009) and their derivatives such as bread (Legarda & Burdaspal, 2001; Juan *et al.*, 2007; Bento *et al.*, 2009), flour (Jørgensen & Jacobsen, 2002; Vega *et al.*, 2009), and breakfast cereals (Araguás *et al.*, 2005; Zinedine *et al.*, 2010). OTA has also been reported in wine (Pena *et al.*, 2010), coffee (Otteneder & Majerus, 2001; Gopinandhan *et al.*, 2008; Vatinno *et al.*, 2008), beer (Visconti *et al.*, 2000; Kumagai *et al.*, 2008), cocoa (Tafuri *et al.*, 2004; Sánchez-Hervás *et al.*, 2008) and derivatives (Burdaspal & Legarda, 2003), dried fruits (Ghali *et al.*, 2008; Zinedine *et al.*, 2007; Bircan, 2009), and spices (Fazekas *et al.*, 2005a; Shundo *et al.*, 2009; Iha & Trucksess, 2010; Jalili *et al.*, 2010).

An indirect exposure can also occur by ingestion of animal products tainted by virtue of contaminated feed. Such animal based products include meat (Milićević *et al.*,

2008), kidneys (Curtui *et al.*, 2001), liver (Jiménez *et al.*, 2001), milk (Boudra *et al.*, 2007), blood (Krüger *et al.*, 2010), and their derivatives.

Although representing as a group a less significant contribution to general population's exposure, other commodities have also been found contaminated with OTA, such as fruit juices (Zimmerli & Dick, 1996), namely grape-based juices (Filali *et al.*, 2001), with OTA being further reported in Turkish typical grape juice-derived product, "pekmez" (Arici *et al.*, 2004). OTA was also described in vinegar (Markaki *et al.*, 2001; Varga & Kozakiewicz, 2006), olive oil (Papachristou & Markaki, 2004), and olives (Zinedine *et al.*, 2004; El Adlouni *et al.*, 2006). OTA occurrence was further reported in liquorice and derived products intended for the food industry (liquorice extract and liquorice block), health products (liquorice extract), and confectionery (as flavouring and sweetening agents) (Ariño *et al.*, 2007). Last, but not the least, OTA was also reported in baby and infant foodstuffs (Beretta *et al.*, 2002; Lombaert *et al.*, 2003; Biffi *et al.*, 2004). The importance of the contribution of these "other foodstuffs" to OTA intake should not be discounted by the low or moderate levels found in these items. In fact, the consumption of these minor food commodities, even at low levels, can contribute to a chronic exposure (Papachristou & Markaki, 2004).

### 6.2.1. Cereals

Though human exposure to OTA can result from the ingestion of various foodstuffs, as mentioned earlier, OTA is found as a natural contaminant mainly of cereals, and therefore these and their derivate products stand as the main contributors.

Cereals have a variety of uses as foods, in an assortment that includes the usage of different technological processing methods, industrial or domestic/traditional. Bread is one of the most important, being specially made out of wheat (Indian *roti*; French baguette), rye (German pumpernickel) and maize (Portuguese *broa*). Breakfast cereals are another main food product that is increasingly consumed worldwide, just as bakery products, like cookies and cakes. Another common usage of cereals is in the preparation of alcoholic drinks such as whiskey and beer (barley; sorghum), vodka (wheat), American bourbon (rye), Japanese sake (rice), etc. A variety of unique, indigenous fermented foods (Turkish *boza*; Ethiopian *injera*; Ghanaian *kenkey*), other than leavened breads and alcoholic beverages, are also produced in regions of the world that rely mainly on plant sources for protein and calories.

Due to the global importance of cereals in the diet, their susceptibility to be invaded by moulds and, in certain climatic conditions, act as substrates for the production of mycotoxins, that can furthermore persist from the crops to the final products in spite of many years of research and the introduction of good agricultural practices in the food production, storage and distribution chain, are worrying facts (Molinié *et al.*, 2005).

### 6.2.1.1. Ochratoxin A occurrence and determinant factors

The differences in OTA contamination between cereals are multifactorial. Hence, it is especially delicate to establish a direct relationship between an individual factor and OTA content, and that is why it is very difficult to anticipate with all certainty the OTA content of each type of cereal (González-Osnaya *et al.*, 2007). That is one of the drawbacks, besides the ones reviewed by Garcia *et al.* (2009), associated with the incipient development of predictive mycotoxicology, which would be of paramount importance in the prevention of food spoilage.

Despite the fact that OTA does not normally occur before harvest (Magnoli *et al.*, 2006) attention should be paid to that period. In a Danish survey, Elmholt & Rasmussen (2005) found that most of the harvested grain samples contained *P. verrucosum* prior to drying, suggesting that much grain is contaminated prior to storage. Once established, this competitive fungal species is able to dominate under conductive environmental conditions in stored grain (Cabañas *et al.*, 2008).

As Bhattacharya & Raha (2002) demonstrated, during storage seeds were easily invaded by storage fungi, resulting in loss of germinability and degradation, decreasing their value for sowing and for food and feed, thus posing a serious safety, quality and economical problem to producers. So, beside the risk of producing OTA, fungi growing on stored grains can reduce the germination rate along with loss in the quantum of carbohydrate, protein and total oil content, and induce increased moisture and free fatty acid content, enhancing other biochemical changes.

Nevertheless, the presence of the mould is not always indicative that OTA occurs. The production of secondary metabolites is not essential to the synthesizing organism but it is regulated by several often interwoven environmental signals (Mühlencoert *et al.*, 2004). Furthermore, it is known that optimal conditions of $a_w$ and temperature for mycotoxin production are more restrictive than those for fungal growth (Magnoli *et al.*, 2007). As a general phenomenon, stress is frequently mentioned as a cause for

mycotoxin synthesis (Birzele *et al.*, 2000). The contrary situation is also true: since OTA is generally stable, it might be detected long after the producing fungi have died out or have been outgrown by other species (Ayalew *et al.*, 2006). The presence of the ochratoxigenic fungi may however be regarded as an indicator of OTA formation. For example, according to Lund & Frisvad (2003), an infestation of *P. verrucosum* greater than 7 % indicates OTA contamination.

The composition of the fungal population is furthermore important regarding the potential interaction and competition between mycotoxigenic species and other spoilage fungi in cereal grain substrates. For example, the xerophilic spoilage fungus *A. ochraceus* is an important coloniser of maize and inevitably interacts and competes with other contaminant *Fusarium*, *Aspergillus* and *Penicillium* species for the maize grain niche. It has been recently demonstrated that niche overlap and dominance by *A. ochraceus* is influenced by water availability and temperature, and that *in vitro* interaction and competition markedly influenced the production of OTA by this species. Nevertheless, the same authors (Lee & Magan, 2000) suggest that, to a large extent, *A. ochraceus* is not as competitive as some other spoilage fungi in primary resource capture on maize grain at $a_w$ of 0.95 or above, although it may modify resource quality and influence secondary colonization by other species under the appropriate conditions.

The production and occurrence of OTA in cereal grains is considered to depend first and foremost on the condition of the grain at harvest, how carefully the grain is dried and the quality of the storage facilities (Eskola, 2002a). In the specific cases of Northern and Western Europe, Canada and other temperate areas, cereals are at greater risk of OTA formation because grain is often harvested at high moisture content, sometimes above 20 % (Scudamore *et al.*, 2003). The occurrence of OTA in such grains is attributed to the insufficient drying or over-long storage before drying (Uysal *et al.*, 2009). Given favourable conditions, e.g. cereals harvested with a high content of water, inefficient drying or storage under humid conditions, high levels of OTA in cereals can occur (Jørgensen & Jacobsen, 2002).

Although it is impossible to completely eliminate all sources of mould infection, it is possible to lessen or even avoid some particular conditions conductive to mould growth. In simple terms, the cereal must be dried to below 15 % moisture ($a_w$ of about 0.8) and kept at this level throughout storage. Delays in drying then put the grain at risk, which can lead to subsequent problems during storage (Scudamore, 2005).

So, since the weather conditions vary between different harvest years, the year in which the sampling and analysis is made is also influential. That was observed by Czerwiecki *et al.* (2002a, b) when analysing two different consecutive harvest years. In average, regardless of the type of cultivation and the kind of cereal grain, the frequency and contamination levels of OTA in cereal from 1998 were substantially higher than those from 1997. The authors justified the results with a higher mean level of precipitation in Poland in the year of 1998. Similar relation was described by Jørgensen *et al.* (1996) in Denmark, when studying wheat and rye for OTA contamination. The study reported that harvests from the years of 1986-1987, characterised by normal to wet climate, featured higher average contamination levels than the following years, considered dry or very dry. It was also perceived that the contamination level was not affected during the cereals' storage period, but instead during the period immediate following the harvest, before drying would diminish water activity. If storage conditions are not correct, after a long period OTA production can occur. In the same study, no differences in OTA levels between bran and internal grain were found. Additionally, only 10-50 % of OTA could be extracted and removed from the surface of the grain, indicating that the mould is not limited to the surface, because of a deeper penetration of the hyphae of the mould. Nevertheless, other studies (Juan *et al.*, 2008a) report higher OTA frequency for the whole grain cereal samples when compared with the non-whole grain cereal samples analysed (33 % *versus* 14 %). In this study, the maximum value was detected in an organic sample of whole grain rye (27 µg/kg). The fact of the analysed samples being only kernels or conversely whole grain is important since most fungi are located on the surface of the grain, so it is where the higher contamination is expected. So, whole grain is likely to present higher levels, which is why the study of the effect of technological processing is so important and when elevated levels are detected in processed cereals, it could indicate the existence of much higher levels in whole grain. This was observed by Park *et al.* (2005), in the Korean polished rice samples analysed that presented levels above the EU maximum limit. The degree of technological processing of the cereal grain is also a determining aspect, as pointed out below.

The differences in nutritional composition of the grain, that vary according to the type of cereal grain analysed is another factor that might influence the contamination levels. Some authors observed higher incidence of contamination on maize, such as Araguás *et al.* (2003) that reported that one of the contaminated corn sample exceeded

the EU maximum limit. Nevertheless, several authors have suggested that glutamic acid plays an important factor in the incidence, because it is indirectly involved in the production of OTA in culture and that proline could be substituted. A study of the metabolism of glutamic acid during the production of OTA indicated that portions of it were incorporated into the mycotoxin. A high content of this amino acid in cereals, such as wheat, could be a cofactor for the presence of OTA (González-Osnaya *et al.*, 2007). Other studies reveal a higher OTA concentration in rye grains (Czerwiecki *et al.*, 2002a; Jørgensen & Jacobsen, 2002; Čonková *et al.*, 2006).

The chemical nature of the seeds influences their moisture. Seeds with high oil content possess lower moisture than those with high protein or starch. It is well known that water availability, i.e. $a_w$ plays an important role in the deterioration of stored seeds. The $a_w$ may increase as a result of absorption of water from the internal seed atmosphere in order to reach an equilibrium with the prevailing high relative humidity of the storage atmosphere, particularly during rainy months (Bhattacharya & Raha, 2002).

The type of agricultural practices involved in crop production is also believed to be determinant. In the study of Juan *et al.* (2008a), the organic cereal samples showed the highest incidence of contamination. Likewise, the 400 polish cereal grain samples studied by Czerwiecki *et al.* (2002a, b) from the 1997 and 1998 harvests, obtained from conventional and ecological farms were investigated for the presence of OTA. In the 1997 crop, the frequency of OTA in the samples of all types of cereal grain originating from ecological farms was substantially higher than that from conventional farms. Specifically, OTA contamination of ecological rye was over six times more frequent than that from conventional cultivation. An analogous relation was observed for the rye samples analysed by Jørgensen & Jacobsen (2002), presenting a multiyear mean concentration of OTA higher for organic production than for the conventional production, both for the raw grain and flour. This was not observed for the wheat samples. In the case of rice samples the higher contamination from products proceeding from organic practice was also observed by Gonzalez *et al.* (2006). According to the later authors, this fact can be attributed to the cultivation practices and limited use of chemical products like fertilizers and fungicides in organic crop growing.

Another factor that might influence the reported contamination of cereal products is the testing and quality of the measurements, all the way through the sampling, sample preparation, detection and interpretation of results (Bao *et al.*, 2006). In the absence of

harmonised guidelines or directives, contamination reports constantly reveal variability that is not always a reflection of the real contamination status. When evaluating assorted cereal samples in Finland, Eskola (2002a) did not detect OTA (*n*=115), and in Spain, Blesa *et al.* (2004) reported an almost absence of contamination (*n*=43). The authors suggested a low sensitivity of the method employed as an explanation. Furthermore, the lack of regulation and/or control programs on OTA occurrence, at national or regional level, is also affecting the conditions allowed to exist in grain harvest, storage and commercialization and that pose a risk to human and animal population. This situation is particularly concerning in the developing countries, which justifies the need of surveys and exposure assessments. So, although considered a natural contaminant of grain primarily in northern temperate areas, recent cereal surveys in Africa have encountered high levels of incidence and average concentration. According to Riba *et al.* (2008), in North African countries the foods most susceptible to OTA contamination are precisely cereals, locally produced or imported, of which Durum wheat is of paramount importance in the dry Mediterranean regions of North Africa, as demonstrated by the cultural tradition of use in the production of couscous, pasta, traditional bread and *frik*.

The worldwide occurrence of OTA in unprocessed cereal grains is shown in Table 1. Ayalew *et al.* (2006) reported maximum levels that surpassed the ones reported in most European surveys. The authors suggested low quality of the storage facilities (practice of grain storage in underground pits) and the weather conditions (mild sub-tropical climate in most of the sampling areas) to justify the values encountered. From all the mycotoxins studied, i.e. $AFB_1$, OTA, deoxynivalenol (DON), nivalenol (NIV), ZEA, fumonisins (FBs), OTA was the most widespread mycotoxin in the barley, sorghum, wheat and teff (a common and staple cereal in Ethiopia) grains surveyed. Most of the remaining African surveys refer to Mediterranean basin countries, leaving the epidemiology of the rest of the continent not quite known. In Turkey, Terken *et al.* (2005) detected 96 % of cereal-based flour samples with co-occurrence of AFs/OTA. In the same country, the seed-, pulses-, and cereal-flours (wheat, barley, potato, oat, rye, vetch, corn, rice, lentil, soy) samples randomly collected from Ankara supermarkets and traditional bazaars by Baydar *et al.* (2005) showed a 100 % contamination frequency. In Morocco, Zinedine *et al.* (2006) registered 5 % of OTA/ZEA contamination.

**Table 1**.
Occurrence of OTA in unprocessed cereals.

| Cereal sample | Country (Year of survey) | Incidence rate (%) | Contamination range (mean) (µg/kg) | Reference |
|---|---|---|---|---|
| **Barley** | Spain (2008-2010) | 21/105 (20 %) | n.d.-1.6 (0.10) | Mateo *et al.* (2011) |
| **Wheat** | China | 8/22 (36.4 %) | n.d.-9.10 (4.248) | Zhang *et al.* (2011) |
| **Corn** | | 6/23 (26.1 %) | n.d.-23.02 (7.360) | |
| **Rice** | | 3/20 (15 %) | n.d.-3.67 (3.382) | |
| **Rice** | Chile (2006) | 13/31 (42 %) | n.d.-12.5 | Vega *et al.* (2009) |
| **Wheat** | Tunisia | 42/110 (38 %) | n.d.-250 (55) | Zaied *et al.* (2009) |
| **Barley** | | 41/103 (40 %) | n.d.-940 (96) | |
| **Sorghum** | | 43/113 (38 %) | 8-950 (117) | |
| **Rice** | | 27/96 (28 %) | n.d.-150 (44) | |
| **Rice** | Tunisia (2004-2005) | 4/16 (25 %) | n.d.-2.3 (1.4) | Ghali *et al.* (2008) |
| **Organic cereals** | Spain/Portugal (2005) | 13/41 (31.7 %) | n.d.-27.10 (1.64) | Juan *et al.* (2008a) |
| **Non-organic cereals** | | 5/42 (11.9 %) | n.d.-0.90 (0.05) | |
| **Rye** | Japan (2003-2004) | 9/10 (90 %) | n.d.-1.59 (1.05) | Kumagai *et al.* (2008) |
| **Rice** | Vietnam | 35/100 (35 %) | n.d.-2.78 (0.75) | Nguyen *et al.* (2007) |
| **Rice** | Morocco (2005) | 18/20 (90 %) | n.d.-32.4 (4.15) | Zinedine *et al.* (2007) |
| **Barley** | Ethiopia (1999) | 27/103 (26.2 %) | n.d.-164 (17.2) | Ayalew *et al.* (2006) |
| **Sorghum** | | 17/78 (21.8 %) | n.d.-2106 (174.8) | |
| **Teff** | | 9/33 (27.3 %) | n.d.-80 (32.7) | |
| **Wheat** | | 25/107 (23.4 %) | n.d.-66 (19.6) | |
| **Wheat** | Poland | 1/22 (4.6 %) | n.d.-0.015 (0.0007) | Čonková *et al.* (2006) |
| **Rye** | | 4/23 (17.4 %) | n.d.-0.055 (0.038) | |
| **Wheat** | Slovakia | 6/48 (12.5 %) | n.d.-2.940 (0.0063) | |
| **Non-organic rice and rice products** | Spain | 5/63 (7.9 %) | n.d.-27.3 (13.44) | González *et al.* (2006) |
| **Organic rice and rice products** | | 6/20 (30 %) | n.d.-7.1 (3.6) | |
| **Maize (1998)** | Ivory Coast | 16/16 (100 %) | 27-64 (44) | Sangare-Tigori *et al.* (2006) |
| **Maize (2001)** | | 15/15 (100 %) | 3-1738 (266) | |
| **Corn** | Morocco | 40 % | n.d.-7.22 (1.08) | Zinedine *et al.* (2006) |
| **Wheat** | | 40 % | n.d.-1.73 (0.42) | |
| **Barley** | | 55 % | 0.04-0.80 (0.17) | |

(n.d.: non-detected)

**Table 1. (Continued)**
Occurrence of OTA in unprocessed cereals.

| Cereal sample | Country (Year of survey) | Incidence rate (%) | Contamination range (mean) (µg/kg) | Reference |
|---|---|---|---|---|
| **Maize** | Croatia | 19/49 (38.7 %) | n.d.-2.54 (1.47) | Domijan *et al.* (2005) |
| **Barley** | Korea (2003) | 5/22 (22.7 %) | n.d.-0.9 (0.8) | Park *et al.* (2005) |
| **Polished rice** | | 5/60 (8.3 %) | n.d.-6.0 (1.0) | |
| **Rice** | Portugal | 6/42 (14.3 %) | n.d.-3.52 | Pena *et al.* (2005) |
| **Cereal grain** (Wheat, Barley and Corn) | Spain | 58/115 (50.4 %) | 0.219 | Araguás *et al.* (2003) |
| **Conventional production grain:** | Poland (1997) | **4/110 (3.6 %):** | **0.30-2.50 (1.11):** | Czerwiecki *et al.* (2002a) |
| - Rye | | - 3/52 (5.8 %) | - 0.82-2.5 (1.38) | |
| - Wheat | | - n.d. | - n.d. | |
| - Barley | | - 1/26 (3.9 %) | - 0.30 | |
| **Ecological production grain:** | | **24/127 (18.9 %):** | **0.21-57.0 (5.70):** | |
| - Rye | | - 18/48 (37.5 %) | - 0.21-10.0 (3.17) | |
| - Wheat | | - 3/39 (7.7 %) | - 0.48-1.20 (0.83) | |
| - Barley | | - 3/40 (19.9 %) | - 6.7-57.0 (25.73) | |
| **Conventional production grain:** | Poland (1998) | **23/110 (20.9 %):** | **0.60-1024 (202):** | Czerwiecki *et al.* (2002b) |
| - Rye | | - 4/37 (10.8 %) | - 4.73-8.80 (6.75) | |
| - Wheat | | - 18/37 (48.6 %) | - 0.60-1024 (267) | |
| - Barley | | - 2/36 (5.5 %) | - 1.20-9.70 (5.45) | |
| **Ecological production grain:** | | **15/97 (15.5 %):** | **0.80-35.3 (7.92):** | |
| - Rye | | - 5/46 (10.9 %) | - 2.0-35.3 (14.5) | |
| - Wheat | | - 8/34 (23.5 %) | - 0.8-1.60 (1.17) | |
| - Barley | | - 2/17 (11.8 %) | - 1.43-35.3 (18.4) | |
| **Conventional wheat kernels** | Denmark (1993-1999) | 217/405 (53.6 %) | n.d.-32 (0.3) | Jørgensen & Jacobsen (2002) |
| **Organic wheat kernels** | | 6/14 (42.9 %) | n.d.-1.6 (0.3) | |
| **Conventional rye kernels** | | 257/405 (63.5 %) | n.d.-63 (0.9) | |
| **Organic rye kernels** | | 14/17 (82.4 %) | n.d.-45 (3.9) | |
| **Maize** | Italy (1999-2000) | 19/70 (27.1 %) | n.d.-5.2 (1.7) | Palermo *et al.* (2002) |
| **Wheat** | | 6/70 (8.6 %) | n.d.-1.4 (1.47) | |
| **Rice** | Vietnam | 2/25 (8 %) | n.d.-26.2 (23.75) | Trung *et al.* (2001) |
| **Cereal grain:** | UK (2000) | **52/320 (16.25 %):** | **0.3-231**: | Prickett *et al.* (2000) |
| - Wheat | | - 32/201 (15.9 %) | - 0.3-231 | |
| - Barley | | - 20/106 (18.9 %) | - 0.3-117 | |
| - Oats | | - 0/13 (0 %) | - n.d. | |

Furthermore, Zinedine *et al.* (2006) detected between 40 % and 55 % of contamination in wheat, corn and barley, in line with the neighbouring survey results (40 % of contamination) on Algerian wheat (Riba *et al.*, 2008). All the positive cereal samples investigated by Zaied *et al.* (2009) in Tunisia surpassed the level of European regulation (5 µg/kg). The determined high incidence of contamination agrees with the previous national results of Maaroufi *et al.* (1995).

Also in Tunisia, but more recently, the average levels of OTA found by Ghali *et al.* (2008), were 1.9, 3.0 and 3.5 µg/kg for barley, wheat, corn and their derivatives, respectively. Twenty six percent of the cereal and cereal based samples were about the European maximum permitted level (3 µg/kg). The higher incidences were registered for wheat (60.7 %), sorghum (52.9 %) and barley (52 %). These last two showed the highest incidence of AF and OTA co-occurrence, with 47 and 28 %, respectively. Sorghum also registered the higher co-occurrence of AF, OTA and ZEA (23.5 %). An equally higher co-contamination percentage was determined in OTA positive rice samples with AFs (21.6 %) and CIT (61.5 %) in Vietnam (Nguyen *et al.*, 2007). This high occurrence alone or with other mycotoxins should be a concern, especially in developing countries, like those mentioned above, in which the best quality cereals are exported while the produce with poorer quality is consumed in the homeland. Hence the citizens in developing countries, mainly living in rural areas, are especially sensitive to adverse health effects of mycotoxins due to the malnutrition and low standards of living (Eskola *et al.*, 2001). It is generally assumed that the mycotoxin problem is more serious in developing countries where the climatic conditions and the agricultural and storage practices are considered conductive to fungal growth and toxin production (Magnoli *et al.*, 2007).

On the contrary, in South American countries, several studies report the scarce incidence of OTA in foodstuffs for human consumption, conversely to the incidence in feedstuffs. For example, in Brazil, Caldas *et al.* (2002) analysed, from July 1998 to December 2001, the presence of OTA in several food samples, including maize and maize-derived products (popcorn and kernels). None of the samples were found positive.

#### 6.2.1.2. Effects of cereal processing in ochratoxin A content

Dietary exposure to OTA varies considerably depending on different factors, among which food-processing systems must be considered. These systems are often traditional and characteristic of the different geographical regions and, to this day, their influence on the mycotoxin content of the food finished products ready for consumption has been scarcely studied (Valle-Algarra *et al.*, 2009).

Mycotoxins, in general, are stable compounds, and OTA, in particular, is a moderately heat stable molecule that can survive most food processing operations and, therefore, it appears in final and derived products (Bullerman & Bianchini, 2007).

The main foods for which some studies have endeavoured to quantify the conditions and the extent under which OTA is degraded are cereals and coffee, although some work has been carried out on other foods as well (JECFA, 2001; Araguás *et al.*, 2005). In technological food processing, and as far as grains are concerned, a more accurate relationship between the amounts of OTA and the microbiological status of the cereals have to take the entire mycobiota into account, as many complex interactions may occur and influence the amount of OTA produced (Riba *et al.*, 2008). In a general way, it can be stated that OTA is relatively stable once formed, but under certain conditions of high temperature, acidic or alkaline conditions or even in the presence of enzymes breakdown can occur (Scudamore, 2005; Valle-Algarra *et al.*, 2009).

OTA levels can be reduced initially by cleaning to remove dust and broken grains. However, the reduction is small and probably depends on the condition of the grain when received (González- Osnaya *et al.*, 2007). In the study of Scudamore *et al.* (2003), only a 2-3 % reduction of OTA content in barley was achieved by cleaning. Removal of the surface layers by abrasive scouring or polishing and milling to remove outer layers for white flour production lowers OTA levels, since the mycotoxin tends to be concentrated in the outer bran layers of cereals. Because in this process of milling there is no stage or operation that destroys mycotoxins, which may only be redistributed or concentrated in certain mill fractions, this raises the question of redistribution during milling so that both reduction and increase in concentration can occur depending on the milled fraction examined (González- Osnaya *et al.*, 2007). It also supports the concern about the use of these by-products in feed (because of the carry-over effect) and in food (like in bran-based breakfast cereals, or whole cereal bread). Several authors described a higher contamination level of OTA in whole wheat (Scudamore *et al.*, 2003; Juan *et al.*,

2008a) and whole spelt (Biffi *et al.*, 2004). Conversely, other authors reported an absence of significant differences in mould counts between whole wheat and white wheat flour (Weidenbörner *et al.*, 2000) or a even higher incidence of OTA in white wheat bread than in whole wheat bread samples (González-Osnaya *et al.*, 2007). These results are paradoxical given that the physical processes of scouring and milling, where the inner bran coats are removed, decrease the levels of this mycotoxin in white flour as demonstrated by Osborne *et al.* (1996) and Subirade (1996). Therefore, in flour manufacture, given that some parts of the wheat grain are removed, there is a reduction of OTA concentrations in flour and subsequent products (Osborne *et al.*, 1996). As a result, in theory white flour for baking contains much lower concentrations than whole meal flour because the bran and offal containing high levels of OTA have been removed (González-Osnaya *et al.*, 2007). In the study of Osborne *et al.* (1996) whole meal flour and bread showed much smaller reductions in the concentration of OTA during processing, as might be expected, because less of the grain is discarded.

Furthermore, separate studies cited by González-Osnaya *et al.* (2007) showed that OTA was redistributed by milling into the bran, where the highest OTA content is found, and into the white flour fraction with the lowest concentration. Moreover during mixing of food ingredients, flour can serve as a source of fungal contamination to the atmosphere of the processing establishment, leading to a recontamination and a possible mycotoxin contamination of the products after baking.

Although OTA is relatively heat stable, the thermal processes can exert different effects, according to the temperature reached. For example, OTA is stable during bread baking, with no loss or reduction of its concentration (Scudamore *et al.*, 2003). However, baking of biscuits resulted in about two-thirds of the toxin being destroyed or immobilised (Bullerman & Bianchini, 2007). The higher diminution of OTA content in biscuits can be explained by the elevated temperature reached when compared to bread and for the lower water content. The same happens in breakfast cereal production (Subirade, 1996; JECFA, 2001).

In the specific case of the effects of the bakery processing on OTA, the studies are scarce. Furthermore, because baking is done in so many different ways, according to the geographic regions and the cultural tradition, and hence includes different cereal grains and ingredients, different levels of mycotoxins according to the origin and storage of the grain and flour, different temperatures, sizes, etc, the research is further complicated. In a study carried by Valle-Algarra *et al.* (2009), the different bread making conditions

customary in Spain were assessed in relation to the levels of several mycotoxins present in the flour used to bake bread, including OTA. The results indicated that during dough fermentation a significant reduction, ranging from 29.8 to 33.5 %, of the OTA level takes place, depending on the OTA amount added to the wheat flour. The variation in the toxin reduction compared to previous studies (Scudamore *et al.*, 2003) may be related to the dependence on the particular strain involved in fermentation, which in turn might be regarded as a possible mean to control OTA in food and beverages frequently OTA contaminated (González-Osnaya *et al.*, 2007). In respect to the factor temperature/time combination no significant differences were found, with OTA featuring the lesser reduction (32.9 %) in comparison with the also studied type B trichothecenes.

The same study determined a higher and statistically significant reduction in OTA stability in the bread crust (ranging from 20.4 to 51.3 %) than in the centre (7.3-38.2 %). The size of the bread can therefore, according to the same authors, have a relevant effect and explain the conflicting results reported by different authors. Valle-Algarra *et al.* (2009) underscore that the heating and boiling can themselves produce new toxic compounds, and so this reduction might not be innocuous. Furthermore, they remind of other little studied aspects of processed foods, such as the binding of toxins to matrix compounds such as proteins or carbohydrates, may be possible and bear the risk that during digestion the toxin is released as reported for FBs.

A schematic outline of the main operations and conditions that along the cereal processing chain (from the grain harvest to the finished product) can influence the concentrations of OTA in the final cereal-based product is given in Figure 2. It should however be remembered that, as stated above, a number of cultivation practices, preceding the harvest or even the storage phase are evenly crucial, as the strategic choice of the cereal variety to cultivate in a given geographic region and the type of agriculture employed, namely integrated, organic or conventional.

The knowledge of the processing effects is not only important to recognise their effects on OTA distribution and/or chemical modification, but can also be employed in the decontamination or reduction of contamination efforts, such as segregation of contaminated from non-contaminated kernels, milling, cleaning or washing, sieving, dehulling, and in a more judicious selection on the yeast strains used, for example, for bread fermentation.

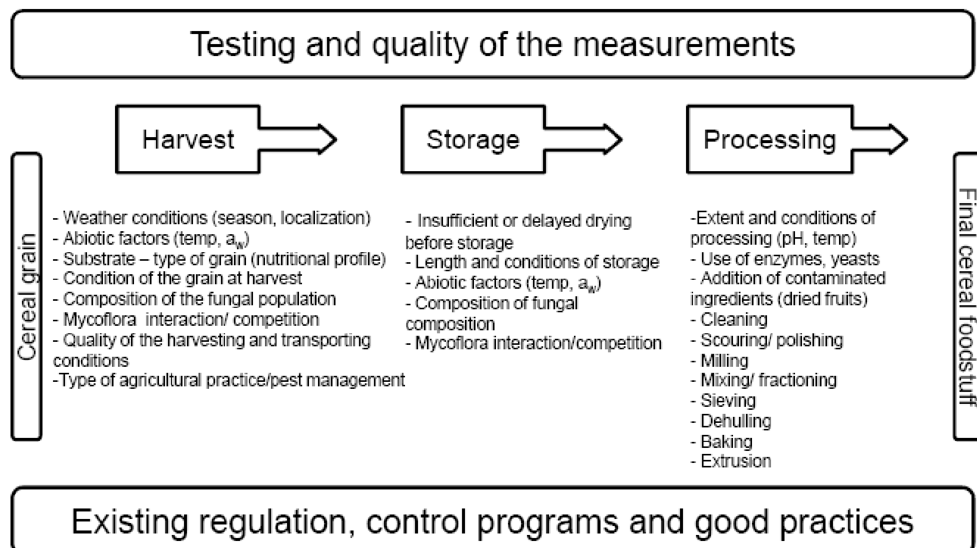


**Figure 2.** Practices and conditions that can influence OTA concentrations in cereals along the production and processing chain.

Finally, as recommended by Scudamore (2005), despite the valuable data gathered so far on the topic, considerable care must be observed when comparing experimental laboratory results with those obtained in commercial practice. The exact recipes and processing conditions used by industry are often of a highly sensitive nature and are rarely made public.

### 6.2.2. Edible animal tissues

In the context of mycotoxins, the health status of food animals that are destined to enter the human food supply chain is often overlooked. Moreover, low quality commodities, with visible mould growth or with levels of OTA unsuitable to enter the food chain are many times diverted into the feed chain. Such actions have considerable potential consequences, from the animal production and sanitary perspective to a public health perspective. The latter arises as a consequence of an indirect exposure through the contaminated animal derived foods consumed (Manning *et al.*, 2003; Gupta *et al.*, 2008).

Farm animal feeds and diets are largely based on cereals and cereal by-products, precisely the preferred substrate for *Penicillium* and *Aspergillus* growth (Petzinger & Weindenbach, 2002). Pigs and poultry are fed with complete diets, which are manufactured commercially or prepared at the farm level. Roughly, the diets consist of 40 - 80 % cereal grains including by-products, 0 - 50 % protein concentrates, 0 - 10 % oil and 0 - 10 % minerals, vitamins and other supplements. In general, the proportion of

protein concentrates is higher and the cereal proportion is lower in diets for growing animals, and for poultry as compared to pigs. The great variability in diet composition for the major farm animal species and the heterogeneity in distribution of OTA in animal feeds preclude a calculation of actual exposure levels based on the occurrence of OTA in individual feed materials (EFSA, 2004). Moreover, animal-derived products and tissues for human consumption may well present OTA contamination even if the animal has been nourished with feeds contaminated with low levels of OTA. Carry-over of OTA from feed to animal products has been demonstrated in swine and poultry (Micco *et al.*, 1987), as well as in other species.

Despite the indirect exposure pathway that it may represent, OTA occurrence in animal products is generally not thought of as a major public health concern perhaps due to the insufficiency of studies to assess the significance of the products originating from mycotoxin-exposed food production animals as an exposure pathway for OTA among humans. EFSA considers negligible the risk associated with the consumption of food derived from animals fed with OTA-contaminated feeds (EFSA, 2006). Based on the data provided by the SCOOP (scientific cooperation) report (Miraglia & Brera, 2002), for the EU population, the overall contribution of products of animal origin to human exposure has been estimated to be not more than 3% of the total ingested OTA. However, this contribution may reach 10 % in certain regions with distinct preferences for traditional meat products, like blood puddings. However, this report does not include results from the new European Union member states where there are some indications from the literature that incidence of occurrence of OTA in cereals and levels might be significantly higher than elsewhere. This might be related to differences in practices in drying and storing grains, and in climatic conditions, which make cereals more vulnerable to OTA contamination (EFSA, 2004).

Furthermore, a number of studies show an evident association between OTA contamination assessed through exposure biomarkers and dietary intake of food originating from animals. Even if not always statistically significant, high consumption of pork (Turconi *et al.*, 2004; Biasucci *et al.*, 2010), cured pork (Galvano *et al.*, 2008), liver paste/pâté (Skaug *et al.*, 2001), bovine milk (Turconi *et al.*, 2004), and cheese (Skaug *et al.*, 2001; Turconi *et al.*, 2004) was related to higher OTA levels in human milk. In the case of blood exposure biomarkers, consumption of pork and chicken (Muñoz *et al.*, 2006), or beef, turkey and cold meat (Medina *et al.*, 2010) were also related to higher plasma levels.

#### 6.2.2.1. Ochratoxin A occurrence and determinant factors

Although meat is defined as "the muscle tissue of slaughter animals" (FAO, 2007), other edible parts of the slaughtered animal are often used in further processing, namely fat, internal organs (e.g. liver, kidneys) and other slaughter byproducts (e.g. blood). As depicted in Table 2, several studies have reported the occurrence of OTA in some of these tissues detailing a pattern of tissue bioaccumulation echoing the toxicokinetic features of the mycotoxin (*vide* section 4. Toxicokinetic features). In agreement, OTA content of some specific traditional meat products can be increased by adding pig tissues, like blood (blood sausages), liver (pâtés, sausages) or even foreign contaminants, like spices (Shundo *et al.*, 2009).

In a study of the presence of OTA throughout the chain production of the pig meat, through artificial feed contamination (up to 327 µg/kg dry weight), several organs of the slaughtered animals, specifically kidney, urinary bladder, intestine, spleen, liver, lymph nodes and muscles were tested. OTA was detected only in kidneys, urinary bladder and spleen. The highest values were found in kidneys, while the lowest were found in the spleen. The levels found in the urinary bladder and in the liver were two- and five-fold lower, respectively, than in kidneys (Ceci *et al.*, 2007).

Considering the reported pattern of distribution of OTA in tissues serum>kidney>liver>muscle, and taking serum as 100% reference, Curtui *et al.* (2001) described this distribution as 22.6 %: 8.5 %: 2.57 % which resulted in a dilution factor from the serum concentration equal to 4.42: 11.69: 38.9. Other studies also confirmed a low carry-over into muscle (Dall'Asta *et al.*, 2010).

Regarding the correlation between serum and edible tissues, the strongest was reported between serum and kidney (Curtui *et al.*, 2001; Milićević *et al.*, 2008). This correlation allows the usage of kidney instead of blood as alternative sample for OTA control in slaughtered pigs or pig herds. The ratio between pig kidneys and pig meat is more variable, ranging between 10 and 90 % according to some reported studies (Curtui *et al.* 2001; Jørgensen & Petersen, 2002; Matrella *et al.*, 2006). At low levels of OTA in kidney the ratios' variability was higher. This ratio depends on many factors, e.g. the content of OTA in feed, feeding period, natural or synthetic contaminated feed, feeding in relation to time of slaughtering (Jørgensen & Petersen, 2002; Matrella *et al.*, 2006).

**Table 2.**
OTA occurrence (%) and contamination in edible tissues (µg/kg) and blood (µg/L) from healthy and nephropathic animals reported in different countries.

| Animal species and health status | Sample | Country (year of survey) | LOD (LOQ) | Positives (%) | Mean±SD | Contamination range | Reference |
|---|---|---|---|---|---|---|---|
| Broiler (Healthy) | **Central region:** | Serbia | 0.2 | | | | Milićević *et al.* (2011) |
| | - Liver | | (0.3) | - 0/30 (0%) | - | - | |
| | - Kidney | | | - 0/30 (0%) | | | |
| | - Gizzard | | | - 0/30 (0%) | | | |
| | **North region:** | | | | | | |
| | - Liver | | | - 23/60 (38.3%) | - 0.58±1.04 | - n.d.-3.9 | |
| | - Kidney | | | - 17/60 (28.3%) | - 0.51±1.38 | - n.d.-7.02 | |
| | - Gizzard | | | - 16/60 (26.6%) | - 0.51±1.75 | - n.d.-9.94 | |
| Swine (Healthy) | **Ham:** | Italy | 0.1 | - 32/110 (29.1%) | - 0.24 | - n.d.-4.66 | Dall'Asta *et al.* (2010) |
| | - Inner part | | (0.3) | - 84/110 (76.4%) | - 0.98 | - n.d.-12.51 | |
| | - Outer part | | | | | | |
| Swine (Healthy) | **Serum - Collection region:** | Brazil | - | | - | | Krüger *et al.* (2010) |
| | - Santa Catarina | | | -60/100 (60%) | | -n.d.-75400 | |
| | - Mato Grosso | | | -75/100 (75%) | | -n.d.-46790 | |
| | - Bahia | | | -36/100 (36%) | | -n.d.-4130 | |
| | - Rio de Janeiro | | | - 68/100 (68%) | | -n.d.-115000 | |
| Swine (Healthy) | **Serum:** | Italy | - | | - | | Pozzo *et al.* (2010) |
| | -Conventional breeding | (2006-2009) | | - 205/205 (100%) | | -0.03-0.87 | |
| | -Organic breeding | | | - 80/80 (100%) | | -0.15-6.24 | |
| Swine (Nephropathic) | **Serum - Year of collection:** | Bulgaria | - | - 8/10 (80%) | - 28.8 | - | Stoev *et al.* (2010a) |
| | -2006 | | | - 9/10 (90%) | - 6.3 | | |
| | -2007 | | | | | | |
| Swine (Healthy) | **Paired samples per animal:** | Serbia | | | | | Milićević *et al.* (2008) |
| | - Kidney | (2006-2007) | - 0.01 | - 30/90 (33.3%) | - 1.26 | - n.d.-52.5 | |
| | - Liver | | - 0.01 | - 24/90 (26.6%) | - 0.63 | - n.d.-14.5 | |
| | - Serum | | - 0.1 | - 28/90 (31.1%) | - 3.70 | - n.d.-221 | |

(LOD: Limit of detection; LOQ: Limit of quantification; n.d.: not detected; SD: Standard deviation)

**Table 2. (Continued)**
OTA occurrence (%) and contamination in edible tissues (µg/kg) and blood (µg/L) from healthy and nephropathic animals reported in different countries.

| Animal species and health status | Sample | Country (year of survey) | LOD (LOQ) | Positives (%) | Mean±SD | Contamination range | Reference |
|---|---|---|---|---|---|---|---|
| Poultry (Healthy) | **Serum - Conventional breeding:** | Italy (2006) | 0.001 | **26/55 (47%):** | **0.013:** | **n.d.-0.131:** | Schiavone *et al.* (2008) |
| | -Laying hens | | | - 13/25 (52%) | - 0.015 | - n.d.-0.082 | |
| | -Broilers | | | - 13/30 (43%) | - 0.012 | - n.d.-0.131 | |
| | **Serum - Organic breeding:** | | | **24/39 (61%):** | **0.022:** | **n.d.-0.165:** | |
| | -Laying hens | | | -19/26 (73%) | - 0.030 | - n.d.-0.165 | |
| | -Broilers | | | - 5/13 (38%) | - 0.004 | - n.d.-0.014 | |
| Swine (Healthy) | -Kidneys | Italy | (0.3) | - | - 25.6 | - 23.9-27.5 | Ceci *et al.* (2007) |
| | -Urinary bladder | | | | - 10.5 | - 9.8-11.5 | |
| | -Liver | | | | - 4.4 | - 3.2-5.3 | |
| | -Spleen | | | | - 0.4 | - 0.3-0.5 | |
| Swine (Healthy) | **Serum - Collection region:** | Serbia | 2 | | | | Milićević *et al.* (2007) |
| | -Baćka Topola | | | -10/15 (66.6%) | -2.17 | -n.d.-5.2 | |
| | -Kovilj | | | -11/15 (73.3%) | -5.26 | -n.d.-33.3 | |
| | -Šabac | | | -6/15 (40%) | -1.41 | -n.d.-5.0 | |
| | -Senta | | | -7/15 (46.6%) | -2.66 | -n.d.-16.0 | |
| Swine (Healthy) | **Dry-cured ham, paired:** | Italy | 0.02 | - 2/10 (20%) | - 0.9 | - n.d.-1.52 | Toscani *et al.* (2007) |
| | -Inner samples | | (0.06) | - 5/10 (50%) | - 3.88 | - n.d.-7.28 | |
| | -Outer samples | | | | | | |
| Swine (Healthy) | Serum | Brazil | 0.073 | 4/87 (4.59%) | 0.6 | n.d.-1.5 | Krüger (2006) |
| Swine (Healthy) | Kidney | Southern Italy | 0.01 | 54/54 (100%) | 0.29 | 0.01-0.9 | Matrella *et al.* (2006) |
| Swine (Healthy) | Salami | Southern Italy | 0.06 (0.22) | 14/30 (46.7%) | - | n.d.-0.4 | Monaci *et al.* (2005) |
| Swine (Healthy) | Kidney | Italy | 0.14 (0.52) | 52/54 (96.3%) | - | n.d.-3.05 | Monaci *et al.* (2004) |

(LOD: Limit of detection; LOQ: Limit of quantification; n.d.: not detected; SD: Standard deviation)

**Table 2. (Continued)**
OTA occurrence (%) and contamination in edible tissues (µg/kg) and blood (µg/L) from healthy and nephropathic animals reported in different countries.

| Animal species and health status | Sample | Country (year of survey) | LOD (LOQ) | Positives (%) | Mean±SD | Contamination range | Reference |
|---|---|---|---|---|---|---|---|
| Swine (Healthy) | **Ham:**<br>- Middle of ripening (6months)<br>- End of maturation (12 months) | Italy | 0.04 | - 17/21 (80.9%)<br>- 18/21 (85.7%) | - | - n.d.-2.2<br>- n.d.-2.3 | Chiavaro *et al.* (2002) |
| Swine (Healthy) | Kidney | Denmark (1999) | 0.02 (0.06) | 284/300 (94.7%) | 0.50 | n.d.-15 | Jørgensen & Petersen (2002) |
| Swine (Healthy) | **Matching samples per animal:**<br>- Kidney<br>- Liver<br>- Serum | Romania (1998) | - 0.01<br>- 0.01<br>- 0.1 | - 41/52 (79%)<br>-39/52 (75%)<br>-51/52 (98%) | - 0.54<br>- 0.16<br>- 2.43 | - n.d.-3.18<br>- n.d.-0.61<br>- n.d.-13.4 | Curtui *et al.* (2001) |
| Swine (Healthy) | Liver-derived pâtés | Spain | 0.56 (0.84) | 3/38 (7.9%) | - | n.d.-1.77 | Jiménez *et al.* (2001) |
| Swine (Healthy) | Serum | Poland (1999) | 2 | 26/45 (57.8%) | - | n.d.-69.5 | Kotowski *et al.* (2000) |
| Swine | Kidney | France<br>-1997<br>-1998 | - 0.17-0.20 (0.34-0.50)<br>- 0.11-0.20 | - 40/300 (13.3%)<br>- 238/710 (33.5%) | - | - n.d.-1.4<br>- n.d.-6.1 | Dragacci *et al.* (1999) |
| Swine | Kidney | France 1997 | 0.05 (0.16) | 26/100 (26%) | - | n.d.-0.48 | |
| Duck | Liver | Denmark (1993-1994) | 0.03 | 4/7 (57.1%) | 0.06 | n.d.-0.16 | Jørgensen (1998) |
| Goose | Liver | | 0.03 | 4/12 (33.3%) | 0.02 | n.d.-0.06 | |
| Turkey | Liver | | 0.03 | 3/17 (17.7%) | 0.04 | n.d.-0.28 | |
| Swine | Serum | Canada (1989-1990) | 0.3 | 572/1588 (36%) | 14.1±0.3 | n.d.-211 | Ominski *et al.* (1996) |

(LOD: Limit of detection; LOQ: Limit of quantification; n.d.: not detected; SD: Standard deviation)

Despite the nephrotoxic effects of OTA, kidneys from animals with visible nephropathies are not always more contaminated than those from healthy animals. Such conclusion is drawn from a French monitoring programme for determining OTA occurrence in pig kidneys (Dragacci *et al.*, 1999), where almost comparable incidences were determined.

Regional differences were noticed in studies of OTA occurrence in edible porcine tissues, particularly higher levels of contamination in regions neighbouring Yugoslavia (Curtui *et al.*, 2001; Milićević *et al.*, 2008). Studies of OTA in swine blood also detail significant effects regarding the region from which the herds originated (Ominski *et al.*, 1996).

A significant effect on OTA contamination of swine blood was observed according to the season of sample collection. In Canada (Manitoba region) the incidence was higher in July, as compared with April, October, or January (Ominski *et al.*, 1996). Similarly, Milićević *et al.* (2007) reported a higher incidence in the collection month of June (80 %) as compared with May (20 %). In Poland, the incidence in the winter (February) collection period was considerably higher than in the samples from the spring season (May), in a consistent pattern with simultaneously collected feed samples (Kotowski *et al.*, 2000). This strong seasonal variation could be due to particular climatic conditions that could have been responsible for a higher contamination of animal feed in the period immediately preceding collection.

Already previous surveys conducted elsewhere in the world have indicated that the incidence and concentration of OTA in swine sera and tissues are also influenced by geographic location, moisture content of the feed at harvest, origin of the feed, length of storage, and the drying procedure used (Golinski *et al.* 1984, 1985, Hult *et al.* 1984; Holmberg *et al.* 1990).

Although swine is the most susceptible animal species to OTA a small number of studies describe its occurrence in the species considered as more resistant, like poultry. Schiavone *et al.* (2008) conducted a survey on Italian conventional and organic poultry farms. Contrarily to the 100 % incidence found in feed samples, only 53 % of the sera samples were positive. In addition, no statistically significant differences in OTA contamination of feed or sera were observed either between the organic *versus* conventional group or between the laying hens *versus* broiler group. The highest value

(0.165 μg/L) found during this survey was greatly inferior to the highest values described for pig sera in the above reported studies.

Furthermore, in contrast to swine (Curtui *et al.*, 2001; Milićević *et al.* 2011), higher OTA contamination was found in the poultry's hepatic tissue, as compared to kidney (Milićević *et al.*, 2008).

#### 6.2.2.2. Effects of meat processing in ochratoxin A content

The effects of meat processing in OTA content have been scarcely studied. From the available data processing procedures such as heating and ripening as well as storage have no obvious effects on the reduction of the OTA levels in meat products (Gareis, 1996; Monaci *et al.*, 2005).

In general, OTA occurrence in swine derived products can be attributed to either direct fungal contamination, due to toxigenic mould growth in the outer layers of meat products, or indirect transmission, via the ingestion of OTA-contaminated feed, i.e. carry-over effects (Clark & Snedeker, 2006; Matrella *et al.*, 2006; Toscani *et al.*, 2007). Direct fungal growth intentionally occurs in traditional foods, such as dry-cured meat products. During the ripening steps the surface growth of moulds takes place, some of them ochratoxigenic, like some strains of *Penicillium* spp. and *Aspergillus* spp.

The presence of the moulds does not necessarily imply the production of mycotoxins, because the production of the toxin is more demanding in terms of suitable environment and substrate conditions. This fungal growth is appreciated for its enzymatic activities that contribute to the development of the characteristic flavour of these products (Toscani *et al.*, 2007). Despite the technological interest, the usage of mould and mould starters in the manufacture of sausages and other foodstuffs should be carefully considered. That is why, from a safety point of view and prior to technological evaluation, isolates must be tested for potential mycotoxigenicity (López-Díaz *et al.*, 2001).

In the specific case of ham, the duration of the ripening period does not seem to affect the OTA contamination level (Chiavaro *et al.*, 2002), while the incorporation of flavouring materials such as spices or other sources of OTA can be a risk factor (Toscani *et al.*, 2007). In addition, some authors explain OTA occurrence in the analysed ham samples by an indirect transmission from animals exposed to naturally contaminated feed (Chiavaro *et al.*, 2002), and others to a direct fungal contamination

based on a significantly higher OTA levels is the outer parts of ham (Toscani *et al.*, 2007; Dall'Asta *et al.*, 2010). In the latter case, given that OTA is rarely encountered in the interior, removal of moulds from the casing may limit or eliminate this risk entirely (Iacumin *et al.*, 2009). Several microbiological surveys performed on the environmental microflora, showed the presence of OTA-producing mould strains in the air of the ripening plant rooms (Battilani *et al.*, 2007) or on the products' surface (Pietri *et al.*, 2006; Iacumin *et al.*, 2009).

## 7. Extent and significance of ochratoxin A exposure

The FAO has estimated that up to 25 % of the world's food crops are significantly contaminated with mycotoxins (FAO, 2004), featuring an unavoidable hazard under current agricultural practices, given that no way of efficiently predicting and avoiding mycotoxins exists (Prandini *et al.*, 2009). Moreover, the many interacting factors in the pathogenesis of a mycotoxicosis, along with their general insidious nature make diagnosis difficult, as is confirming mycotoxin exposure (Bryden, 2007).

The hazard and impact that OTA can embody for both human and animals is readily obvious once the range of toxic effects, commodities affected, and widespread incidence are considered.

In the past, the concern about OTA toxicity addressed its carcinogenic and nephrotoxic potential. In fact, OTA is considered a possible human carcinogen (IARC, 1993) and, even if not yet definitely demonstrated, OTA was involved in the aetiology of endemic nephropathies, in both humans (such as BEN and CIN), and animals (such as PEN). Although sub-chronic and chronic effects of OTA are considered of greatest concern, natural field outbreaks in livestock have occurred in different countries. These ochratoxicosis episodes, characterised by nephropathy, were reported in poultry (Elling *et al.*, 1975; Hamilton *et al.*, 1982; Visconti & Bottalico, 1983; Shlosberg *et al.*, 1997; Bozzo *et al.*, 2008), swine (Rutqvist *et al.*, 1978; Cruz *et al.*, 1984), rabbits (Visconti & Bottalico, 1983), and dairy and beef cattle (Abramson *et al.*, 1983).

In addition, as documented in recent studies, OTA-induced immunomodulation is also of significant concern. Amongst farm animals, from a public health perspective, increased infections may well result in increased animal-to-human transmission of pathogens and/or increased antibiotic concentrations in meat or milk, as a consequence of animal treatment. Second, from an agricultural standpoint, it is conceivable that

altered immune function may contribute mechanistically to the symptoms of some animal mycotoxicoses and influence the clinicomorphological picture, and also predispose livestock to infectious diseases, eventually reactivate chronic infection, reduce vaccine and therapeutic efficacy and reduce productivity (Stoev *et al.*, 2000, 2002b; Oswald *et al.*, 2005).

The economic impact of rearing animals with OTA contaminated feed, just like with other mycotoxins, results from increased veterinary care costs, reduced livestock production, and disposal of contaminated feeds (Hussein & Brasel, 2001).

Of no less importance, OTA exposure in livestock animals through contaminated feed is above all problematic for the carry-over phenomena by which animal derived products can become contributors to OTA human exposure (Micco *et al.*, 1987).

Such human exposure, proven to occur globally, is nevertheless overlooked in the poorer and developing countries, due to public ignorance about mycotoxin existence, lack of regulatory mechanisms, dumping of food products, and the introduction of contaminated commodities into the human food chain during chronic food shortage due to drought, wars, political and economic instability (Wagacha & Muthomi, 2008). In contrast, increasing attention is devoted to mycotoxins in more developed countries. The current situation of mycotoxins in Europe can be followed weekly in the web site of the Rapid Alert System for Food and Feed - RASFF. Mycotoxins are consistently the hazard category with the highest number of notifications, and OTA the second most frequently notified, after AFs. According to latest available RASFF annual report (RASFF, 2009) the notifications were mainly related with paprika, dried figs, and raisins, followed by cereals and bakery products.

The current, although incomplete, knowledge of its toxicology and incidence of contamination renders it important to raise awareness among the public and health authorities, and take action to both assess and control exposure. However, both result in a further burden, in this case of economic nature. The inherent requirements of analysis, monitoring and surveillance programs, as well as the inspection actions entail a rise of costs. Further, the lack of harmonization in the regulation of maximum limits among countries that are commercial partners leads to trade disruption and economic conflicts, with greater cost to the final consumer (Dohlman, 2004; FAO, 2004; Kendra & Dyer 2007).

## 8. Ochratoxin A exposure assessment

Exposure assessment, an important tool in public health, is the third stage of risk assessment, and has been defined by Codex Alimentarius Comission - CAC (2008a) as the qualitative and/or quantitative evaluation of the likely intake of biological, chemical or physical agents via food, as well as exposures from other sources if relevant. Although the definition is broad, when applying the principles of exposure assessment to mycotoxins a number of factors need to be recognised, particularly the unpredictability and definite unavoidability of occurrence (Shephard, 2008a).

For exposure assessment the total concentration of the mycotoxin with which the individual contacts through all exposure pathways can be measured in the various media, e.g. food, air. Such approach evaluates the so called external dose. The internal dose measurement for exposure assessment can be carried out by means of analysis of biomarkers of exposure in various biological specimens, e.g. urine, blood, milk (Ritter & Arbuckle, 2007). However, each strategy presents advantages as well as disadvantages as systematised in Table 3 for OTA exposure assessment.

**Table 3**.
Advantages and disadvantages of different monitoring methods in OTA exposure assessment.

| Exposure assessment | Matrix | Advantages | Disadvantages |
|---|---|---|---|
| Internal dose | Blood | - Relatively high OTA levels: less sensitive methods required; | - Invasive collection, involving medical personnel;<br>- Estimation of exposure: based on assumptions (bioavailability and plasma clearance);<br>- Short-term biomarker;<br>- High within-subject and seasonal variability: limited use at the individual level; |
| | Urine | - Ease, fast and non invasive collection; | - Lower levels than serum: sensitive and accurate methods mandatory;<br>- Incomplete knowledge about indication of time; |
| | General | - Total OTA exposure: reflects exposure by all routes, from all sources and bioavailability of the mycotoxin;<br>- Only one type of sample needs to be analysed, while through estimated daily intake (EDI), several samples would have to be considered;<br>- Enhanced individual and group risk assessments; | - Lack of specific and validated markers, compliance difficulties and/or missing information on the toxicokinetics of OTA in humans; |

**Table 3. (Continued)**
Advantages and disadvantages of different monitoring methods in OTA exposure assessment.

| Exposure assessment | Matrix | Advantages | Disadvantages |
|---|---|---|---|
| External dose | Food | - Determining the potential risk associated with exposure of a particular foodstuff or type of diet; | - Assessment of exposure through analysis of foods particularly problematic: wide range of food types, the sporadic occurrence, and the low levels at which OTA is found;<br>- Problem with obtaining a representative sampling;<br>- Several types of foods need to be analysed to retrieve a proportional contribution to the total estimated exposure (in relation to one for bio-monitoring);<br>- Only population-based estimated exposure given the heterogeneous contamination of food it is not always indicative of potential individual exposures;<br>- Changing nature of food consumption patterns within individuals over time, that is from day to day, season to season and also between different life periods (age, marital/family status, illness, income);<br>- Up-to-date, correct and available food consumption, of the general population or risk groups data mandatory;<br>- Measurement of exposure through one or a group of foodstuffs cannot always provide a full picture of human exposure. |
| | Air | - Possible significant source of exposure, in domiciliary and occupationally settings; | - Neglects the main source of exposure: ingestion; |
| | General | - Necessary to: quantify the increment of exposure derived from particular sources or media and identify the strategies necessary for exposure reduction and source control; | - Considers only one source of exposure, and neglects multiple sources of exposures;<br>- Measures the external dose, which according to the individual susceptibility or type of exposure may vary; |

Given the several approaches and tools for OTA exposure assessment, and bearing in mind the scope of the present dissertation, attention was focused on the ones involving analysis of foods, regarding external dose assessment, and exposure biomarkers, for internal dose assessment. The foremost of such approaches and tools

were referenced and discussed in relation to their appropriated use, along with their central advantages and drawbacks.

## 8.1. Food

The amount of OTA ingested via food is referred to as dietary exposure or dietary intake. The general equation for both acute and chronic dietary exposure according to the Principles and Methods for the Risk Assessment of Chemicals in Food established by the International Programme on Chemical Safety (IPCS, 2009) is depicted in Figure 3.

Dietary exposure = [Σ (Contamination level in food × Food consumption)]/Body weight (kg)

**Figure 3.** General equation for calculation of dietary exposure (IPCS, 2009).

Reported studies use two basic approaches to obtain OTA contamination levels in food. Most studies use their own determined OTA contamination levels through monitoring works (Zimmerli & Dick, 1996; Zinedine *et al.*, 2007; Juan *et al.*, 2008b; Bento *et al.*, 2009), while others use the results of available literature data (Thuvander *et al.*, 2001; Coronel *et al.*, 2009). In monitoring studies food samples are obtained at or closer to the point of consumption in the chain of commerce. This data generally provides a better characterization of contaminants in foods as purchased by consumers (IPCS, 2009). Nevertheless, and as a general rule, estimating exposure to a contaminant from analysis of individual foods invariably involves significant uncertainty. In the case of OTA, the wide range of food sources, the sporadic occurrence and the sub-μg/kg levels at which the mycotoxin is found make assessment of exposure through analysis of foods particularly challenging (Gilbert *et al.*, 2001). Part of such problems can be circumvented by the second basic approach reported, the total diet study (TDS) that, in theory, provides the most accurate measure of the average contamination level actually ingested in foods, after they have been prepared for normal consumption, by the whole population or, if possible, population subgroups. However, the accuracy of some TDSs is lowered by using limited sample sizes and survey durations. Owing to resource considerations, TDSs usually have a small number of mean concentration data (usually *n*=1–8) for each individual food or food group, in contrast to data usually generated

through monitoring or surveillance of individual food commodities (where *n*=30–50 or more) (IPCS, 2009). For OTA, the only recent TDSs studies published are in the form of 30-days diet study (Gilbert *et al.*, 2001), 24-hour diet study (Sizoo & Van Egmond, 2005), and nation-wide study (Leblanc *et al.*, 2005).

Given that mycotoxin contamination of foods and feeds highly depends on environmental conditions that lead to mould growth and toxin production, it is thus anticipated that the observed variability in contamination incidence and levels over space and time leads to dietary exposure levels that differ in terms of central tendency, higher percentiles as well as dispersion parameters as a function of region, country, season and year. Of course, another component of the variability in dietary exposure to OTA is the changing nature of food consumption patterns within individuals over time, that is from day to day, season to season and also between different life periods (Counil *et al.*, 2005).

In order to get a clear picture of what the distribution of exposures in a given population might look like over time, it is important to include the potential variability of input data, and particularly that of contamination levels, in the exposure modelling process. Single point or deterministic modelling involves using a single (*best guess*) estimate of each variable within the model to determine the model's outcome(s). In the case of contamination data, the *best-guess* typically consists of the average of all observed values (assuming this value represents the long-term average of truly encountered contamination levels) or a high percentile or even the maximum limit (ML) proposed by national or international food authorities. In the meanwhile, probabilistic modelling accounts for every possible value that each variable could take and weighs each of them by its probability of occurrence. The structure of a probabilistic model may be similar to a deterministic model with all the operators that link the variables together, except that each variable is represented by a distribution function instead of a single value, thus providing far more realistic results than those produced by simple deterministic scenarios (Counil *et al.*, 2005). In the case of OTA dietary exposure, the majority of reported studies use a deterministic approach, rather than a probabilistic distributional analysis. Although the latter provides a more complete description of the range of exposures that occur in the exposed population and also helps increase the accuracy of combining exposure levels across different pathways, it can introduce a significant uncertainty into an exposure assessment if derived from scarce or deficient detailed data on contamination levels and consumption patterns, which is unfortunately

frequent (Counil *et al.*, 2005; Shephard, 2008a). Nevertheless, a small number of probabilistic modelling approaches, through Monte-Carlo simulation, were reported for OTA dietary exposure of French population (Counil *et al.*, 2005), to assess OTA exposure through milk consumption (Coffey *et al.*, 2009), to evaluate the impact of the existing standards (Tressou *et al.*, 2004), or even for OTA risk assessment in a market economy (Kuiper-Goodman *et al.*, 2010).

Regarding food consumption, most OTA exposure assessments use available dietary data derived from food consumption databases such as Global Environment Monitoring System/Food Contamination Monitoring and Assessment Programme regional diets (GEMS/FOOD, 2003). Additionally, several works obtained the food consumption data through individual dietary surveys, that comprise recall methods, food records, and food frequency questionnaires with different reference time frames (Thuvander *et al.*, 2001; Akdemir *et al.*, 2010; Coronel *et al.*, 2011a,b; Soubra *et al.*, 2009). Although individual dietary surveys more closely reflect actual consumption (Kroes *et al.*, 2002), it is more difficult to perform as not only the frequency, but also the amount of consumption of foodstuffs, and the weight of the food portions have to be evaluated and in some cases daily registered requiring an active involvement of the participants (Coronel *et al.*, 2011b).

## 8.2. Biomarkers of exposure

OTA human exposure can be measured directly through biological monitoring (or biomonitoring) rather than calculated based on assumed exposure parameters. A suitable biomarker for OTA exposure has been sought over a number of years and various putative biomarkers have been investigated, with promising results to a correct evaluation of the risk associated with the exposure to these natural toxins. Indeed, in exposure assessments the monitoring of biomarkers is preferable over the evaluation of food contamination, due to variations in food preparation methods, food consumption, contamination level, intestinal absorption, toxin distribution, and excretion leading to individual variations in toxin exposure that are more readily measured with a biomarker (Shephard *et al.*, 2007; IPCS, 2009). It further circumvents the heterogeneous nature of mycotoxin food contamination (Shephard, 2008a). This primacy is also maintained over the air contamination. Owing to the limitations in sampling, collection, and analytical

methods, authors have gradually combined the air measurements with the level in body fluids, namely blood, to evaluate the exposure (Iavicoli *et al.*, 2002).

The approach involving biomarkers does not represent a new, replacing alternative to the classic evaluation of dietary intake and risk assessment, but it has to be considered as a means of confirming dietary intake data obtained in the traditional way and, furthermore, of supplying the most relevant information on the real impairment to human health attributable to the toxic substances (Miraglia *et al.*, 1996). Although some heterogeneity of the OTA levels found in biological fluids may be explained by differences in the detection limit of the employed methods, they can also result from variations in dietary habits, which fluctuate between individuals and render additional importance to the use of biomarkers (Skaug *et al.*, 2001). If properly validated, data on biomonitoring provides the ultimate evidence that exposure has taken place (Thuvander, 2001).

Biomarkers of exposure are measured, in body fluids or tissues, as the molecule itself, a metabolite(s), or a product of a reaction with a biological molecule. Biomarkers of exposure can be divided into a) markers of internal dose and b) markers of effective dose. The former is an indication of the occurrence and extent of exposure of the organism, whereas the latter is an indication of the extent of exposure of what is believed to be the target molecule, structure, or cell (Timbrell, 1998). Because of the many possible interindividual differences in the rate and route of metabolism of compounds, the effective dose at the target site is a preferred measurement over the internal dose (Timbrell, 1998). This happens because the former reflects not only individual differences in absorption and distribution but also differences in the metabolism (activation *versus* detoxification) and the extent of repair of DNA damage (Pfohl-Leszkowicz *et al.*, 2007). The effective dose is often determined by measuring specific adducts in tissues or body fluids. The weight of evidence supports the notion that exposure to most chemical carcinogens results in damage to the structural integrity of DNA, which occurs primarily as covalent carcinogen binding and is referred to as carcinogen-DNA adduct formation (Poirier *et al.*, 2000). But in the specific case of OTA, as previously referred, there is no agreement on the formation of these OTA-related DNA adducts and conflicting results have been reported (*vide* section 5.1. Molecular mechanisms).

Although considered of less value, mainly for the lack of reliable information on the toxicokinetics of OTA in humans (Larsen, 1995; Ministry of Agriculture, Forestry and

Fisheries of the United Kingdom - MAFF/UK, 1999; Palli *et al.*, 1999; JECFA, 2008), biomarkers of internal dose are consistently used to indicate that exposure has taken place by measuring OTA in blood, urine, and milk. OTA in blood is by far the most studied approach. It is considered a short time biomarker and despite its usefulness to characterise and assess the relative exposure of a population or subgroups of subjects, it features a high within-subject variability, which limits its use at the individual level as shown by Palli *et al.* (1999). Furthermore, as blood samples are collected by an invasive procedure, requiring medical personnel and inherent difficulties to the sampling from the study population, some studies (Breitholtz *et al.*, 1991; Peraica *et al.*, 2001; Thuvander *et al.*, 2001; Muñoz *et al.*, 2006; Pacin *et al.*, 2008; Coronel *et al.*, 2009; Coronel *et al.*, 2011b) make use of regular blood donation campaigns to assemble the samples. The results of this type of studies should be, however, extrapolated to the general population from which the blood donors emerge only with great care, since this type of study population is not representative of the whole population, as children and elder people do not donate blood, for instance.

From OTA occurrence in blood it is possible to estimate human exposure. The advantage of performing exposure estimation through OTA levels in blood is the fact that it is not necessary to be aware of the source of contamination involved - ingestion of contaminated food or inhalation of contaminated air. Additionally it requires a single determination per person, and saves all the problems associated with the food sampling methods and consumption data collection. It can, however, confer an underestimation of the intake, given the approximations in respect to toxicokinetic properties and the assumptions from which it stands (Miraglia *et al.*, 1996; Coronel *et al.*, 2009). Further studies on this underprovided knowledge of OTA kinetics are needed.

Additionally, recent comparative studies to correlate OTA blood levels and the dietary intake of the toxin raised doubts about the reliability of this biomarker. Indeed, the analysis of blood levels for comparison with the analysis of food consumed by the volunteers (Gilbert *et al.*, 2001; Coronel *et al.*, 2011b), or even with the analysis of food consumption data retrieved from questionnaires combined with use of analytical data from the literature (Thuvander *et al.*, 2001; Muñoz *et al.*, 2006; Coronel *et al.*, 2009), failed to confirm an unequivocal association. Therefore, additional studies on the kinetics and bioavailability of OTA in humans are needed to prove the viability of the plasma level of OTA as a biomarker of dietary exposure (Thuvander *et al.*, 2001).

More recently, attention has been drawn to the urinary biomarkers that present several advantages as opposed to blood testing. These advantages begin to appear as early as the collection process, with urine collection being a rapid and easy non invasive procedure, thus enabling a comparatively more keen involvement of participants as compared to blood. Urine can be obtained in large quantities by non-invasive sampling and repeat sampling is not a problem (Ryan *et al.*, 2011).

Although OTA levels in urine are very low in comparison to those in blood, they are regarded as a promising biomonitoring approach, more than ever with the advent of new methodologies that are more sensitive and accurate (JECFA, 2008). Although currently no prospect exists that are designed for individual exposure calculation from OTA urinary levels, recent data (Gilbert *et al.*, 2001; Coronel *et al.*, 2011a) sustain a stronger correlation between OTA urinary concentration and the level of consumption, in comparison with plasma concentration. This is justified by the fact that OTA in human blood samples is compromised by the long half-life of the toxin, for that a frequent dietary exposure will result in a steady-state concentration (EFSA, 2006). This stronger correlation with the level of consumption is perhaps one of the strongest advantages in using urine, given the major source of human exposure: contaminated food intake. Further studies, but conducted in a BEN-affected area, tried to draw a correlation not only between OTA food intake and the contamination of urine and blood, but also with the amount of excreted OTA in the whole day urine. By applying the same methodology as Gilbert *et al.* (2001), 16 human participants were studied and the results reported by Petkova-Bocharova *et al.* (2003) and Castegnaro *et al.* (2006). The authors describe an apparent correlation between average values of OTA intake, serum OTA, and urinary OTA excretion. They observed that an increase of OTA intake does not result in an immediate increase of OTA elimination, but only the week after high contamination. In contrast, when the OTA intake is low, OTA elimination is modulated by OTA blood concentration. Altogether, the results indicate that OTA blood level is relatively stable over a period of one month for a given individual and is regulated by urinary excretion. As long as the OTA food intake is relatively low, a variation of OTA food intake is not directly reflected by a variation of OTA blood concentration. The fact that high OTA intake is not always reflected by high serum OTA concentration and the highest serum concentration is not related to the highest OTA consumption renders blood of less use as a very recent exposure biomarker. In this aspect, although it lacks a correlation with

serum levels, daytime OTA urine excretion is a more valuable tool than the urinary concentration (Petkova- Bocharova *et al.*, 2003; Castegnaro *et al.*, 2006).

It is also important to underline the low levels at which the OTA occurs in urine, comparatively to blood, which may compromise results in the absence of a reasonably sensitive and accurate analytical technique. This happens because, as Studer-Rohr *et al.* (2000) demonstrated on a human volunteer, only 42–54 % of intact OTA (parent compound) was detected in urine, which might add importance to the alternative determination of OTA metabolites and/or conjugates in urine as exposure biomarkers, such as OTα (Muñoz *et al.*, 2010; Coronel *et al.*, 2011a), 4-OH-OTA (Jonsyn-Ellis, 2001; Castegnaro *et al.*, 2006), and OTB (Jonsyn Ellis, 2001). The occurrence of OTA conjugates with glucuronic acid have been also claimed (Pena *et al.*, 2006), although discordant results have been also reported (Vatinno *et al.*, 2007).

In urine, OTα occur in higher levels when compared to OTA as reported by Muñoz *et al.* (2010) and Coronel *et al.* (2011a). Such difference is expected given that the major pathway of detoxification of OTA is the hydrolysis of its amide bound, which results in OTα, facilitating its excretion. Moreover, due to enterohepatic circulation, remnant OTA can be converted in the large intestine to OTα and still recirculated until its excretion. Because OTA occurs at quite low levels in urine, the analysis of OTα may therefore increase sensitivity and facilitate biomonitoring. However some drawbacks that might greatly compromise the use of OTα as an alternative or simply additional biomarker have been recognised. Muñoz *et al.* (2010) reported a considerable interindividual variation in the urine levels of total OTα, perhaps due to a substantial difference on human individual ability for OTA detoxification related to interindividual differences in OTA degradation by gastrointestinal microbial or mammalian hydrolytic enzymes, known to convert OTA into OTα. Furthermore, Coronel *et al.* (2011a) found no significant correlations between OTA and OTα levels in urine, neither in the total samples nor in the positive ones.

The measurement of OTA in milk permits the estimation of the degree of dietary exposure of infants to the toxin through milk (EI-Sayed *et al.*, 2002) and may in addition be regarded as an indicator of exposure of a subgroup of the overall population, the lactating women (Jonsyn *et al.*, 1995; Micco *et al.*, 1995). The disadvantages of OTA milk biomarker include the low levels as opposed to the ones detected in blood (Galtier *et al.*, 1977; Breitholtz-Emanuelsson *et al.*, 1993), in addition to that lactating

women are not representative of the general population regarding eating and drinking habits, age, or smoking (Skaug *et al.*, 2001). Furthermore, in the need of a whole-population monitoring survey, this matrix is not useful, since nonlactating individuals - men, children, elderly, and nonlactating women - are not represented.

# 9. Ochratoxin A analytical methods

The analytical methods for mycotoxins have developed and expanded along with the general advances in analytical science. The basic requirements of extraction, clean-up and separation for mycotoxin determination remain the same in current methods. Advances have come in the areas of sample purification techniques and in separation science with the development of liquid chromatography (LC) and associated detectors (Shephard, 2008b). However, despite all the advances in instrumental techniques and detection systems, the complexity of matrices requires, in most cases, an extensive, time-consuming, sample-preparation step, which is often still the bottleneck of the whole analytical procedure (Dorne *et al.*, 2009).

This section covers the main strategies and methods of choice regarding sample preparation, extraction and purification of OTA from food and biological matrices, according to the available equipment, economic accessibility and analytical requirements, such as sensitivity and time of analysis.

Given the scope of the dissertation, in this section in the case of foodstuffs attention was devoted to determination of OTA in cereals and their derived products, in addition to animal edible tissues; in the case of biological samples, attention was devoted to determination of OTA in human urine samples.

## 9.1. Foodstuffs: cereals and animal edible tissues

### 9.1.1. Sampling

Nearly 90 % of the error associated with mycotoxin assays could be attributed to how the original sample was collected (Turner *et al.*, 2009), i.e. the probability of errors due to sampling is higher than those of analytical procedure. The sampling error is the greatest source of variance in the analytical procedure primarily due to the fungal non-homogeneous distribution in stored agricultural commodities and products intended for human and animal consumption. Given the fungal general tendency to develop in

isolated pockets, highly contaminated so called "hot-spots" can occur (Reiter *et al.*, 2009). The uneven distribution of the mycotoxins in food products is especially reported in the case of those produced by *Aspergillus* spp., e.g. AFs and OTA (Krska *et al.*, 2008; Songsermsakul & Razzazi-Fazeli, 2008). Whereas it is considered to exist an uneven distribution of the mycotoxin contaminated kernels within a lot of grain or feed or other food products with large particle size, it can be assumed that mycotoxins in liquid samples (well-mixed liquids, such as milk) are homogeneously distributed (European Mycotoxins Awareness Network - EMAN, 2010; Krska *et al.*, 2008). In addition, although it is not a constant, processing of the agricultural commodity can improve the homogeneity of the material (Shephard, 2008b; Tittlemier *et al.*, 2011).

Furthermore, the presence of a recognised toxin-producing fungus does not, in fact, necessarily mean that the associated toxin will also be present, as many factors are involved in its formation. Equally, the absence of any visible mould will not guarantee freedom from toxins as the mould may have already died out while leaving the toxin intact (Turner *et al.*, 2009).

Several published papers review and compare different sampling procedures and their statistical in different foods, e.g. Spanjer *et al.* (2006), Vargas *et al.* (2006), Tittlemier *et al.* (2011). For OTA, in the EU, following the establishment of maximum levels for certain foodstuffs, the EC has laid down the general criteria which the sampling method for the official control should comply with through the European Commission (EC) Regulation No. 401/2006 (Commission of the European Communities - CEC, 2006a) and No. 178/2010 (CEC, 2010a).

### 9.1.2. Extraction

The type of matrix that is analysed can strongly influence the efficiency of the extraction process. Indeed, low recovery rates are still a critical issue, since OTA binds very effectively to proteins and DNA affecting negatively the extraction/purification process in tissues (Zöllner & Mayer-Helm, 2006). This implies the use of very low pH during extraction from these matrices in order to break the bond OTA-proteins, which is not always compatible with the following purification step, for example immunoaffinity columns (IAC), on which the low pH can denature and disrupt the interaction between the antibodies and the mycotoxin, resulting in very low recoveries. The need to reduce the pH of OTA extraction step was also demonstrated in cereal matrices (Castegnaro *et*

*al.*, 2006). In fact, the authors observed that for positive samples, the amount of OTA detected decreased by increasing pH of extraction, setting the ideal pH at 1.5.

Usually, OTA is extracted with ethyl acetate (Monaci *et al.*, 2004), chloroform (Guillamont *et al.*, 2005), acetonitrile/water (Ali *et al.*, 2010), hexane (Jimenez *et al.*, 2001) and dichloromethane/ethanol mixtures (Jørgensen & Vahl, 1999), containing additives like sodium hydrogen carbonate (Barna-Vetró *et al.*, 1996), phosphoric acid (Solfrizzo *et al.*, 1998) or magnesium chloride, to enhance solubility and extraction efficiency (Zaied *et al.*, 2009). The addition of modifiers (e.g. acids, bases, etc.) can also be considered (Krska *et al.*, 2008).

In the final choice of the solvent(s), further consideration must also be given to the next step of the analytical process, namely sample clean-up. It is desirable that the extractant mixture should be compatible with the extract purification process. Pure organic extractants (ethyl acetate, n-hexane) are suitable for clean-up on silica columns, whereas aqueous mixtures are suitable for reversed phase (RP) or ion exchange clean-up. In the case of OTA, for which IACs exist, aqueous methanol extractants allow, after suitable dilution, larger volumes to be used on the antibody column than would be advisable for acetonitrile or acetone (Shephard, 2008b). In fact, IACs are not compatible with organic solvents given that these can damage the antibody and can interfere with the antibody–antigen interaction (Şenyuva & Gilbert, 2010). So, aqueous-based mixtures have to be employed, such as methanol/water (Papachristou & Markaki, 2004), acetonitrile/water (Corneli & Maragos, 1998), phosphate-buffered saline (PBS)/methanol (Pena *et al.*, 2005; Juan *et al.,* 2007) with differing amounts of water (Şenyuva & Gilbert, 2010).

Of no lesser importance in the choice of the solvent, are the environmental concerns. Due to the cost and environmental implications of chlorinated solvents, proven to be ecological hazards, the reduction on the consumption of organic solvents in general and the use of aqueous mixtures of methanol, acetonitrile or acetone has come to the fore (Shephard, 2008b). Still, different procedures employing chlorinated solvents were recently developed and published for OTA extraction (Jørgensen & Vahl, 1999; Guillamont *et al.*, 2005; Toscani *et al.*, 2007).

The physical process of extraction is generally achieved by individual or combined use of methods of mincing, shaking, shredding, grinding, pulverizing and/or pressurizing of the sample to disrupt sample architecture. This step is usually followed by the addition of the extractant - solvents and/or buffers that can be likewise added

during the physical process of extraction, *e.g*. blending a sample of meat in the solvent mixture (Guillamont *et al.*, 2005), in an effort to more completely disrupt cellular and architectural composition and initiate the extraction and fractionation of various sample components from the analyte of choice (Barker *et al.*, 2007).

An additional system of extraction is the pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction) methodology, which uses solvents at relatively high pressure and temperature, at or above the boiling point, without their critical point being reached, to accelerate extraction of compounds from solid and semi-solid samples (Osnaya *et al.*, 2006). Pressure increases the contact between the extracting fluid and sample, and maintains the solvent in liquid state given that the technique works at temperatures higher than the boiling point of the fluid. Temperature breaks the analyte–matrix bonds and modifies the relative permittivity of the extracting fluid, thus increasing selectivity (Liazid *et al.*, 2007). However, the major attracting features of this sample preparation technique are shortened extraction times, reduced organic solvent consumption, high extraction yields, complete absence of air (preventing oxidation) and providing a high level of automation, are still hindered by the costs of the instruments (Osnaya *et al.*, 2006; Liazid *et al.*, 2007; Shephard, 2008b).

Application of PLE-based extraction techniques to OTA were reported in rice (Juan *et al.*, 2005), barley grains (Mateo *et al.*, 2011), and bread (Osnaya *et al.*, 2006). Similarly to PLE, microwave-assisted extraction (MAE) helps to speed-up, automate the extraction process, and offer a robust and time-saving alternative to classical solvent extraction techniques (Krska *et al.*, 2008). In both extraction techniques, PLE and MAE, the effect of extraction temperature on OTA stability was evaluated by Liazid *et al.* (2007). It was reported that OTA can be extracted using MAE procedures for the duration of 20 min at temperatures up to 150 ºC without degradation. PLE can be used at temperatures up to 100 ºC, for extraction times of less than 30 min.

In liquid-liquid extraction (LLE), solvents such as chloroform, toluene, hexane and cyclohexane are used to remove non-polar contaminants, e.g. lipids and cholesterol, to precipitate proteins and impurities (Turner *et al.*, 2009). Some representative examples of LLE operating conditions for OTA analysis in different matrices are given in Table 4. Typical procedures include extraction with acidic chloroform (Curtui *et al.*, 2001; Polisenska *et al.,* 2010), acidic ethyl acetate containing sodium chloride (Jørgensen & Petersen, 2002) or methanol/1 % sodium carbonate (70:30) (Chiavaro *et al.*, 2002).

**Table 4.**
Examples of LLE methods used in OTA extraction from different cereal and animal edible tissues.

| Matrix | LLE method outline | Clean-up | Reference |
|---|---|---|---|
| Dry-cured pork meat | $CH_3CN$ /85 % orthophosphoric acid homogenization (Ultra-Turrax)<br>Filtration<br>Partition (twice) with 0.2M Tris–HCl pH 8.5<br>Aqueous phases collection<br>$CH_3CN$ addition to achieve a 0.2M Tris–HCl/$CH_3CN$ (90:10, v/v) | - | Toscani *et al.* (2007) |
| Muscle | $CH_3CN$ /85 % orthophosphoric acid homogenization<br>Filtration<br>Partition of chloroformic extract with 1M $NaHCO_3$ solution<br>Dilution of aliquot of aqueous phase in water | IAC | Guillamont *et al.* (2005) |
| Pig kidney and muscle | 1 mol/L phosphoric acid<br>Ultra Turrax homogenization (pH ±2.5)<br>Extraction with ethyl acetate<br>Back-extraction with 0.5 M $NaHCO_3$, (pH 8.4)<br>Acidification to pH 2.5 with 7 M $H_3PO_4$<br>Back-extraction into ethyl acetate<br>Evaporation ($N_2$) and reconstitution in mobile phase | - | Monaci *et al.* (2004) |
| Pig liver-derived pâtés | 60 % acetonitrile homogenization<br>Filtration<br>Partition of the filtrate with hexane (by centrifugation)<br>Lower phase diluted in water | SPE (C8) | Jiménez *et al.* (2001) |

(C8: Octyl carbon chain)

**Table 4. (Continued)**
Examples of LLE methods used in OTA extraction from different cereal and animal edible tissues.

| Matrix | LLE method outline | Clean-up | Reference |
|---|---|---|---|
| Pig kidney and rye flour | Dichloromethane/ethanol (4:1, v/v) and 0.1M phosphoric acid<br>Filtration through dried sodium sulphate<br>Extraction (3 times) with $NaHCO_3$ solution, in water/methanol (5:2, v/v).<br>Washing collected water phases with dichloromethane.<br>2M phosphoric acid addition to water phase (pH< 3)<br>Extraction (twice) with dichloromethane<br>Filtration through dried sodium hydrogen carbonate,<br>Dichloromethane evaporated<br>Dissolution in dichloromethane/acetic acid (99:1, v/v)<br>Solvent evaporation ($N_2$)<br>Residue derivatization | - | Jørgensen & Vahl (1999) |
| Wheat and oats | $CH_3CN$ and 0.1 M phosphoric acid<br>Shaking and filtration of the extract<br>Evaporation<br>Reconstitution in acetonitrile/water/acetic acid (41:58:1, v/v)<br>Extract deffated with *n*-hexane<br>Centrifugation<br>Lower phase collection | - | Solfrizzo *et al.* (1998) |
| Maize | $CH_3CN$ /phosphoric acid 0.1M homogenization<br>Lower layer evaporation to dryness<br>Dissolution in hexane and methanol/water (1+1, v/v)<br>Extraction (twice) with methanol/water (1:1, v/v) | SAX | Pelegri *et al.* (1997) |
| Cereals | Dichloromethane and 1M citric acid<br>Centrifugation<br>Dichloromethane phase added to 1% sodium bicarbonate<br>Centrifugation<br>Upper buffer solution added to 1N HCl | - | Barna-Vetró *et al.* (1996) |

(SAX: strong anion exchange)

The procedure is compatible with different subsequent clean-up procedures, such as solid phase extraction (SPE; Becker *et al.*, 1998; Degelmann *et al.*, 1999; Lindenmeier *et al.*, 2004) and IAC (Guillamont *et al.*, 2005).

LLE procedure can be considered effective for OTA and works well in small-scale preparations (Shephard, 2008b; Turner *et al.*, 2009). However, it is dependent on which matrix is being used, is time-consuming (Turner *et al.*, 2009), error-prone and not feasible for automation (Zöllner & Mayer-Helm, 2006). Other disadvantages lie with a possible loss of sample by adsorption onto the glassware (Turner *et al.*, 2009) and the high amount of applied organic and chlorinated solvents (Reiter *et al.*, 2009), which are not suitable for a modern analytical method from an ecological point of view (Buttinger *et al.*, 2004).

### 9.1.3. Clean-up

SPE has found widespread use and is an integral part of many OTA clean-up protocols. The most frequently used packing is RP-octadecyl carbon chain (C18). Conversely, ion exchange materials are rarely used. Weak anion exchange (WAX) columns were employed in cereals and coffee beans matrices (Akiyama *et al.*, 1997) and strong anion exchange (SAX) in cereals and feed (Biancardi & Riberzani, 1996) and pig kidneys (de Saeger *et al.*, 2004). Pelegri *et al.* (1997) offered a sensitive protocol for OTA detection and quantification in maize using a LLE procedure followed by clean-up through SAX columns and HPLC/FD analysis. Matrix-solid phase dispersion (MSPD) was first applied to OTA detection in cereals and their derivatives (Blesa *et al.,* 2004; Juan *et al.*, 2008a). Cereals and their derivatives were dispersed on C8, and OTA eluted with a mixture of methanol/formic acid (99:1, v/v) and analysed by LC/FD. Latter, Rubert *et al.* (2010) combined MSPD-based extraction and LC/MS/MS, for the simultaneous extraction OTA and AFs in malt, coffee, and instant-based cereal-breakfast beverage. The dispersant used was C18 and the mycotoxins were eluted with acetonitrile. The lower recoveries and higher detection limits, in comparison to the previous studies might be related to the need to compromise conditions for different mycotoxins and the type of detector used. This study also demonstrated that matrix effects vary with samples and analytes, and it can considerably affect quantification accuracy, requiring a matrix-matched calibration.

In general SPE procedures rely on relatively unselective interactions and the resulting clean-up levels might therefore be insufficient for some challenging matrices (Monaci & Palmisano, 2004), like food. As a result, many affinity-based sorbent materials have been developed to selectively extract and purify the target mycotoxin, such as antibodies (immobilised in IAC), molecularly imprinted polymers (MIPs) immobilised in MIAC (molecular imprinted affinity columns), and aptamers immobilised in aptamer affinity columns (AAC) (*vide* Table 5). Each of these affinity-based sorbent materials present advantages as well as disadvantages as summarised in Table 6. Furthermore, among these affinity techniques, both IACs and MIPs can be used as single analyte- or group-selective sorbents (Yu & Lai, 2010).

OTA purification from food samples by IAC have been reported in many different matrices, with a high degree of success. However to be compatible with IAC, the solvent extraction mixture needs to be aqueous-based and with a pH close to neutrality. This is important in the case of OTA analysis in protein-rich matrices, where acidic solvents must be used to break the link, but that can damage the antibodies. Additionally, some debate exists as to whether alkaline conditions can adversely affect recovery (Castegnaro *et al.*, 2006).

A possible strategy to reduce the costs associated with IAC, perhaps its biggest drawback, would be the reuse of the column. However, the elution step mostly based on methanol or methanol/acid acetic is highly damaging to the antibodies limiting its regeneration. Pena *et al.* (2005) regenerated the columns a single time by passing 10 mL of methanol, followed by 25 mL of PBS, and stored them filled with PBS at 2-8 ºC. Previously, Zimmerli & Dick (1996) had reported that an IAC filled with PBS (containing sodium azide) had to be stored for at least 20 hours in the refrigerator to allow renaturing of the antibody.

The use of the IACs is not, however, devoid of problems (*vide* Table 6). Moreover, despite the specificity of the antibody it is also possible that non-specific binding occurs with some matrix compounds. Indeed, OTA cross-reactivity can be observed in regard to OTB and OTC. In view of that, attempts have been made to clearly differentiate OTA from OTB through the production of monoclonal antibodies against OTB (Heussner *et al.*, 2007).

**Table 5.**
Examples of affinity-based clean-up methods reported in the OTA analysis of cereals and animal edible tissues.

| Prior steps | Clean-up | Matrix | Reference |
|---|---|---|---|
| Extraction with acetonitrile/water (60:40, v/v)<br>Blender homogenization<br>Dilution in 0.1M HCl solution | **MIAC** (Commercial)<br>Elution: Methanol with 2% acetic acid | Wheat | Ali *et al.* (2010) |
| Extraction with PBS/methanol (50:50, v/v)<br>And blender homogenization<br>Dilution in PBS | **IAC**<br>Extraction solvent: PBS<br>Elution: Methanol | Bread | Juan *et al.* (2007) |
| Extraction with PBS/methanol (50:50, v/v)<br>and Ultra-Turrax homogenization | **IAC**<br>Extract solvent: PBS<br>Elution: methanol | Rice | Pena *et al.* (2005) |
| LLE ( 0.1 M $H_3PO_4$ and chloroform)<br>Chem Elut column<br>(elution with chloroform and formic ac.)<br>Evaporation<br>Reconstitution<br>(Methanol/water, 1:1, v/v) | **MIAC**<br>Template: OTA<br>Polymer: *N*-phenylacrylamide<br>Pulsed Elution: methanol/triethylamine (99:1, v/v) | Wheat | Zhou *et al.* (2004) |
| Extraction with<br>acetonitrile/water (60:40, v/v)<br>Filtration<br>PBS-Dilution | **Automated IAC**<br>Extract solvent: PBS<br>Elution: methanol/acetic acid (98:2, v/v) | Cereals | Eskola *et al.* (2002b) |
| Extraction with<br>Methanol/1% sodium bicarbonate (70:30, v/v)<br>Stirring<br>Filtration | **IAC**<br>Extract solvent: PBS-2%Tween-20<br>Elution: 0.1 N sodium hydroxide | Ham | Chiavaro *et al.* (2002) |
| Extraction with<br>methylene chloride and phosphoric acid (0.1M)<br>Filtration | **SPE** (Silica) followed by<br>**IAC**<br>Extract solvent: Water/acetonitrile (9:1, v/v)<br>Elution: Methanol | Corn<br>Sorghum | Corneli & Maragos (1998) |

**Table 6.**
Comparison of the major claimed advantages and disadvantages between classical and affinity-based SPE systems.

| SPE system | Strengths | Weakness |
|---|---|---|
| **Classical** | • Very fast and inexpensive<br>• Different sorbent materials<br>• Moderate use of solvents | • Each type can operate in certain conditions and its performance can be affected by pH, solvent and ion concentration of the sample<br>• Lack of selectivity<br>• There is no single universal type of cartridge useful for extraction of all toxins |
| **IAC** | • High specificity gained by using monoclonal antibodies<br>• Offer excellent recovery of analyte<br>• Greater degree of sample purification.<br>• High selectivity gained by effective exclusion of matrix interferences (less impurity peaks and flat baselines)<br>• Quite repeatable results<br>• Simple and rapid clean-up procedure<br>• Less skills or experience required | • More expensive than conventional clean up methodologies<br>• Difficult to reuse (due to denaturation of antibodies), so one column can only be used once<br>• Short shelf-life<br>• Limited resistance to harsh conditions (pH, temperature, organic solvents, ultrasounds)<br>• Composition of the matrices may interfere with toxin structure, making them not extractable and/or not recognizable by antibodies |
| **MIAC** | • Long shelf life at room temperature and humidity.<br>• High resistance to harsh conditions of pH and ultrasounds<br>• Thermal stability and chemical inertness and<br>• Stability not affected by organic solvents<br>• Promising tool to replace IAC whenever compatibility between sample extract and antibody is a major limiting factor.<br>• Reusable<br>• There is no need of a solid support: MIP itself is the support | • Polymer swelling in unfavourable solvents<br>• Slow binding kinetics of analytes<br>• Potential sample contamination by template bleeding<br>• Mycotoxins are costly for the large-scale preparation of MIPs<br>• Inconsistent molecular recognition characteristics (variable non-specific binding)<br>• Binding mechanisms known, but some aspects under debate<br>• Some health risks exist because grinding produces sub-micrometric particles, dangerous if inhaled. Some monomers (acrylamide, styrene) are toxic |
| **AAC** | • Unlimited shelf-life<br>• More stable than antibodies under a broad array of conditions and can resist physical and chemical denaturation with little or no loss of activity<br>• Can be generated against any target<br>• Produced by chemical synthesis:<br>  • can be purified to a high degree, and modifications to the structure can be introduced enhancing stability, affinity and specificity<br>  • do not involve use of animals for their production,<br>  • can be produced *in vitro* in a few minutes | • Small number of literature available<br>• Still need to prove applicability in routine analysis and in complex food samples |

The approach of using an IAC as a clean-up method is very attractive for determining mycotoxins in complex food or feed extracts containing potential interferences. Despite the self-evident attractions of IAC clean-up there have been a number of publications comparing IAC with alternative clean-up methods.

When an IAC method was compared with a multi-functional column for the analysis of OTA in cereals, raisins and green coffee beans (Sugita-Konishi *et al.*, 2006), the HPLC chromatograms using the multi-functional column contained multiple peaks eluting on the tail of a large co-extracted peak. In comparison the IAC chromatograms were very clean showing only the OTA peak, enabling measurement at levels of a few µg/kg in all three matrices (Şenyuva & Gilbert, 2010).

IACs for simultaneous determination of AFs and OTA have been the most widely tested (Şenyuva & Gilbert, 2010) in different matrices including maize and derived products (Chan *et al.*, 2004), featuring recoveries above 70 %. Lattanzio *et al.* (2007) reported a method for the simultaneous extraction of 11 mycotoxins (AFs, OTA and *Fusarium* toxins) that involved a clean-up with a multi-toxin-IAC containing antibodies for all these mycotoxins. However, double extraction approach was essential, as well as a LC separation with a linear gradient flow, and the highly selective tandem MS detectors, with an electrospray ionization (ESI) interface. Only after that were satisfactory recoveries (>79 %) achieved.

Automation of IAC clean-up is also possible, as reported for OTA in wheat, rye, barley and oat samples followed by HPLC/FD analysis (Eskola *et al.*, 2002b).

Attempts have been made to replace the bio-recognition element of IAC by a less expensive and more stable bio-mimetic counterpart (Monaci & Palmisano, 2004), more robust and ideally reusable (Şenyuva & Gilbert, 2010).

MIPs are highly promising in the food contaminant analysis, and also in the mycotoxicology (Şenyuva & Gilbert, 2010) assisted by the fact that mycotoxins possess a variety of structures, which allows a selective analysis and detection via molecular imprinting (Yu & Lai, 2010). However, mycotoxins can be considered very difficult analytes for MIAC. Difficulties arise from the elevated toxicity of this class of food contaminants and the high costs to purchase quantities of template suitable to prepare imprinted polymers (Baggiani *et al.,* 2007).

The first mycotoxin for which a successful molecular imprinting has been reported was OTA by Baggiani *et al.* (2001) and Jodlbauer *et al.* (2002), with the two groups employing an almost similar synthetic approach (Baggiani *et al.,* 2008). However, the

preparation of MIPs for OTA recognition is challenging. Direct imprinting of OTA was found to pose some problems in terms of cost and safety when using OTA as template (Visconti & Girolamo, 2005; Ali *et al.*, 2010) and additionally be hindered by the slow release of OTA template molecules. The undesirable template leakage during the analyte elution step, resulting in sample contamination, can be avoided using the mimic template method (Baggiani *et al.*, 2007). Therefore, mimic templates, with analogue structures, as reviewed by Yu & Lai (2010) have been used as a surrogate in the preparation of MIPs for OTA recognition. Still, the structural analogue should be different from the analyte in such a way that the analytical separation performed after the MIAC step discriminates clearly between the analyte and the residual template molecules released by the imprinted material (Baggiani *et al.*, 2007). In the study of Baggiani *et al.* (2001) it was found that only the simultaneous presence of the carboxyl, the phenolic hydroxyl, and some peculiar substructures such as the chlorine atom ensures full recognition of the template (Monaci & Palmisano, 2004).

Beyond MIPs, alternatives to the use of antibodies to manufacture high specificity clean-up columns being explored also include aptamers, which are single stranded DNA or RNA ligands which can be selected for different targets starting from a huge number of molecules containing randomly created sequences. These nucleic acids are able to fold into well-defined three-dimensional structures, which can show high affinity and specificity for target molecules (Şenyuva & Gilbert, 2010). From this very large number of molecules, the ones of interest are selected, for instance by the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) technique as in the study of Cruz-Aguado & Penner (2008). Once this process is complete, the aptamer sequence can be established and consequently through chemical synthesis unlimited amounts of the aptamer can be prepared (Şenyuva & Gilbert, 2010).

The first application of aptamers in the analysis of food contaminants was for detection of OTA (Cruz-Aguado & Penner, 2008). In that study, with the use of an AAC, OTA was concentrated and separated from other grain components, followed by fluorimetry detection. In comparison to OTA, OTB presented one hundred-fold less affinity to one of the tested aptamer, while the difference in reactivity between OTA and OTB with a monoclonal antibody developed for OTA was only 3-fold, and there is less than 20% difference between these toxins in the case of polyclonal antibodies.

### 9.1.4. Detection and quantification

Common analytical techniques, chromatography-based, are by far, the instrumental methods more often used for OTA analysis, offering good performances in terms of accuracy, precision, sensitivity, and reproducibility (Sforza *et al.*, 2006).

In this section focus is given to the most frequently described chromatographic techniques, i.e. LC, and thin layer- (TLC) chromatography. Furthermore, since good chromatographic separation of analytes is mandatory in combination with all detectors except, to some extent, in Mass spectrometry (MS), where some peak overlapping is allowed, the chromatographic conditions described in OTA analysis of foods will be approached during the description of the liquid chromatography assisted with fluorescence detectors.

#### 9.1.4.1. Liquid chromatography

The most widely used method for the determination of OTA in food is HPLC/FD, taking advantage of its innate fluorescence.

Most methods use isocratic elution of the mobile phase, although gradient has been used (Monaci *et al.*, 2004). The mobile phase generally comprises an acidic aqueous phase to prevent ionization of carboxylic groups (Wilkes & Sutherland, 1998).

Practically all studies apply external calibration or sometimes standard addition method. Conversely internal standards (IS), such as diflunisal (Aboul-Enein *et al.*, 2002), are rarely used. The use of OTB as an IS should be approached with caution, given the possible co-occurrence with OTA in foods (Monaci *et al.,* 2004).

LC/FD using RP shows to be fully adequate for OTA analysis, with lower limits of detection (LODs) than LC/MS/MS methods, being chromatograms usually clean enough to ensure a clear identification of the peak. For confirmation of a positive result obtained in the LC/FD, the available approaches include the subsequent use of MS (Blesa *et al.*, 2004; Milićević *et al.*, 2010), post-column pH shift confirmation by addition of 1.1 M ammonium hydroxide solution (Eskola *et al.*, 2002b), or derivatization of the sample. The latter commonly entails addition of boron trifluoride (Monaci *et al.*, 2004; Pena *et al.*, 2005), boron trifluoride methanolic solution (Guillamont *et al.*, 2005), or methanol and concentrated hydrochloric acid mixture (Blesa *et al.*, 2004; Juan *et al.*, 2005). The conversion of OTA to its methyl ester and re-injection allows confirmation through the disappearance of the chromatographic peak

due to the toxin and the appearance of one corresponding to the methyl ester (OTA-Me). The fluorescing OTA-Me obtained possesses longer retentions times. Toscani *et al.* (2007) used carboxypeptidase to cleave OTA into OTα as a confirmation procedure in beverages dry-cured pork meat.

For OTA detection and quantification, FD seems to be more satisfying than MS in terms of sensitivity, not to mention the easiness of operation and the lower cost of the instrument (Sforza *et al.*, 2006). Some representative methods of LC/FD for cereals and animal edible tissues are summarised in Table 7.

Nonetheless, the introduction of mass spectrometry (*vide* Table 8) as a viable detector system can be considered one of the greatest advances in mycotoxin analysis during the previous decade (Shephard, 2008b). In fact, the selectivity of the mass spectrometer detector is unchallenged if compared to common LC (and also GC) detection methods, and accuracy and precision are generally high (Sforza *et al.*, 2006). Furthermore, it provides a detection method independent of the formation of chemical derivatives or of the UV absorption or fluorescence properties of the molecule (Shephard, 2008b). However, compared to FD detectors, MS-based techniques, single (-MS) or tandem (-MS/MS) possess less exact quantitative determination capability (Sforza *et al.*, 2006).

The accurate choice in the calibration mode is one possible approach to overcome the matrix effects that can decrease ionization efficiency (Zöllner & Mayer-Helm, 2006). Some authors report good results with external calibration. Others, select IS for calibration purposes, namely ZEA, OTB (de Saeger *et al.*, 2004; Losito *et al.*, 2004; Chung & Kwong, 2007), and isotope-labelled standards (Lindenmeier *et al.*, 2004). Regarding OTB, higher concentrations are needed because of significantly lower instrument sensitivity and additionally the use of OTB as an IS in food matrices can be compromised, since it might be present as contaminant (Sforza *et al.*, 2006).

Conversely, stable isotope-labelled standards are distinctly less problematic and more reliable since they offer almost identical physical and chemical properties to OTA and so compensate any kind of matrix effects arising from different sample matrices. In contrast to other IS the labelled ones can be used in combination with IAC, since they are retained analogously to the non-labelled analyte. Jørgensen & Vahl (1999) used ($D_3$)-OTA methyl ester as IS, in an analysis of pig kidney and rye flour with derivatization to the methyl ester before MS/MS detection.

**Table 7.**
Main recent representative LC/FD methods for OTA determination in cereals and animal edible tissues.

| Matrix | LC separation | | | | | FD conditions | Analytical Performance | LOD (LOQ) (µg/kg) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| | Column | Mobile phase | Injection volume | Retention time | Standard | | | | |
| Cereals and Derivatives | Packing:<br>RP-C18<br>Dimension:<br>PS: 5 µm,<br>ID: 150x4.6 mm | **Flow-rate:** 0.7 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Methanol/0.1 M formic acid (70:30, v/v) | 20 µL | 15.8 min | External | $\lambda_{exc}$=333nm<br>$\lambda_{emi}$=460nm | SR: 1-10 µg/kg<br>R: 69-89 %<br>RSD: 3.66-4.3 % | 0.05 (0.19) | Juan *et al.* (2008a) |
| Dry-cured pork meat | Packing:<br>RP-C18<br>Dimension:<br>PS:3 µm,<br>ID:250x2.1 mm | **Flow-rate:** 0.2 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>$NH_3/NH_4Cl$ (20 mM, pH 9.8):$CH_3CN$ (85:15, v/v) | 20 µL | 20 min | External | $\lambda_{exc}$=380nm<br>$\lambda_{emi}$=440nm | LR: 0.5-4 µg/kg<br>SR: 0.5-1.5 µg/kg<br>R: 67.2-90.5 %<br>$RSD_w$: 6.12-18.6 %<br>$RSD_b$: 7.29-23.7 % | 0.02 (0.06) | Toscani *et al.* (2007) |
| Rice | Packing:<br>RP-C18<br>Dimension:<br>PS: 5 µm,<br>ID: 250x4.6 mm | **Flow-rate:** 1 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Acetonitrile/water/ acetic acid (49.5:49.5:1.0, v/v/v) | 50 µL | 12.6 min | External | $\lambda_{exc}$=333nm<br>$\lambda_{emi}$=460nm | SR: 0.05-0.4 µg/kg<br>R: 92-99 % | (0.05) | Pena *et al.* (2005) |
| Rice | Packing:<br>RP-C18<br>Dimension:<br>PS: 5 µm,<br>ID: 150x4.6 mm | **Flow-rate:** 0.7 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Methanol/0.1 M formic acid (70:30, v/v) | 20 µL | 8.53 min | External | $\lambda_{exc}$=334nm<br>$\lambda_{emi}$=464nm | SR: 5-15 µg/kg<br>R: 94-90.7 %<br>RSD: 2.5-2.8 % | 0.01 (0.03) | Juan *et al.* (2005) |
| Cereals | Packing:<br>RP-C18<br>Dimension:<br>PS: 5 µm,<br>ID: 150x3.9 mm | **Flow-rate:** 0.4 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Methanol/0.1 M formic acid (70:30, v/v) | 20µL | 8.5 min | External | $\lambda_{exc}$=334nm<br>$\lambda_{emi}$=464nm | SR: 10 µg/kg<br>R: 86 %<br>RSD: 5 % | 0.25 (0.75) | Blesa *et al.* (2004) |

(LOQ: Limit of quantification; ID: Internal diameter; LR: Linear range; PS: Particle size; R: Recovery; RSD: Relative standard deviation; $RSD_b$: Relative standard deviation between-day (inter-day); $RSD_w$: Relative standard deviation within-day (intra-day); SR: Spiking range; $\lambda_{exc}$: wavelength of excitation; $\lambda_{emi}$: wavelength of emission)

**Table 8.**
Main representative LC/MS and LC/MS/MS methods for OTA determination.

| Matrix | LC separation | MS detection | Monitored precursor and fragment ions (*m/z*) | Standard | LOD (LOQ) (µg/kg) | Reference |
|---|---|---|---|---|---|---|
| Cereals | **Column**: RP-C18<br>**Mobile phase**: (gradient; 0.25 mL/min)<br>1th gradient: 35% of 5mM ammonium acetate in water<br>2nd gradient: 65% of 5mM ammonium acetate in acetonitrile | LC/MS/MS<br>ESI Positive ion mode (triple quad.) | 404, 358, 239 | External (OTA) | 0.8 (2) | Rubert *et al.* (2010) |
| Bread | **Column**: RP-C18<br>PS: 3µm; ID: 150 x 2.1 mm<br>**Mobile phase**: (Isocratic; 0.25 mL/min)<br>Methanol/water/acetic acid (65:34.3:0.7) | LC/MS/MS<br>ESI Positive ion mode<br>MRM ((triple quad.) | 404<br>358<br>239 | External (OTA) | 0.03 (0.09) | Juan *et al.* (2008a) |
| Pig tissues (kidney, liver, muscle) | **Column**: RP-C18<br>(PS: 5µm; ID: 250 x 2.1 mm)<br>**Mobile phase**: (Gradient; 0.2 mL/min)<br>Water (1% $CH_3COOH$)/acetonitrile<br>1th gradient: 50:50%<br>2nd gradient: 40:60% | LC/MS/MS<br>ESI Positive ion mode<br>SRM→CRM (Ion trap) | **For OTA**:<br>404, 358, 341<br>**For IS**:<br>370, 324, 307 | Internal (OTB) | 0.6 (1.5) | Losito *et al.* (2004) |
| Various foods | **Column**: RP-C18<br>(PS: 5 µm; ID: 250 x 2.1 mm)<br>**Mobile phase**: (Gradient; 0.8 mL/min)<br>1th gradient: 0.05% trifluoroacetic acid<br>2nd gradient: trifluoroacetic acid in methanol 0.05% | LC/MS/MS<br>ESI Positive ion mode<br>SRM (triple quad.) | **For OTA**:<br>404, 358<br>**For IS**:<br>409, 363 | Internal ($^2H_5$-OTA) | 0.5 (1.4) | Lindenmeier *et al.* (2004) |
| Pig kidney & rye flour | **Column**: RP-C18<br>(PS: 5µm; ID: 250 x 2 mm)<br>**Mobile phase**: (Isocratic; 0.25 mL/min)<br>Acetonitrile/water/methanol/acetic acid (50:30:20:0.5) | LC/MS/MS<br>ESI Positive ion mode<br>MRM (triple quad.) | **For OTA methyl ester**:<br>418, 239, 221, 193<br>**For IS**:<br>421, 239 | Internal (($D_3$)-OTA methyl ester) | 0.02 | Jørgensen & Vahl (1999) |

(CRM: consecutive reaction monitoring; LOQ: Limit of quantification; ID: Internal diameter; LR: Linear range; PS: Particle size; SRM: Selected reaction monitoring)

Furthermore, to limit matrix interferences and attempt to remove the ion-suppression phenomena and thus guarantee good accuracy and precision, in addition to the use of isotope labelled or IS an intensive clean-up may be required (Cigić & Prosen, 2009). It has been shown that, in many cases, the quality of the analytical result does not suffer when conventional SPE approaches are used. Of course, this also depends on the selectivity of the MS equipment itself. It is worth mentioning, that, given the lower specificity of the single MS separation in LC/MS, and GC/MS methods, the efficiency of the sample preparation steps may determine much more decisively the quality of the analytical result than in LC/MS/MS, and GC/MS/MS methods (Tsikas, 2010). For instance, single-stage MS in selected ion-monitoring (SIM) mode might need selective clean-up to remove matrix interferences, while those interferences might not be visible with multi-stage MS in selected reaction-monitoring mode (Zöllner & Mayer-Helm 2006; Krska *et al.*, 2008). So, when avoiding the clean-up step, care must be used so that impurities in the injected sample co-eluting with the mycotoxins do not suppress the ionization of the compounds of interest (Shephard, 2008b).Thus the high selectivity of MS/MS offers the additional advantage that it can be used successfully and very effectively with less selective but cheaper clean-up sorbent materials, as e.g. SPE RP-C18. The first methods published for OTA analysis utilised ESI. The same interface has featured in most methods up to date, used either in positive or negative mode, the former offering a more specific fragmentation pattern and thus more frequently employed (Cigić & Prosen, 2009). Conversely, compared to the ESI (in the positive mode), the use of atmospheric pressure chemical ionization (APCI) interface results in lower sensitivity due to extensive fragmentation of OTA. Furthermore, Sforza *et al.* (2006) underline that the APCI interface requires high flows and, therefore, the propensity of the source can be easily contaminated, affecting both sensitivity and reproducibility.

In the first determination of OTA by LC-ESI-MS/MS (Becker *et al.*, 1998), in beer, coffee and wheat samples, good recoveries were retrieved and an almost comparable LOD (0.01 μg/kg) was obtained, to that with fluorescence detectors. A good LOD (0.020 μg/kg) was also reported in an interesting LC-ESI-MS/MS method (Jørgensen & Vahl, 1999) applied in pig kidney and rye flour that involved LLE extraction, concentration and derivatization to the methyl ester.

Tandem MS was also coupled to ultra-performance liquid chromatography (UPLC) for OTA analysis. The main advantage of UPLC is the tremendous time and efficiency by using an external standard method. Both mycotoxins could be analysed within 3.2 min (Songsermsakul & Razzazi-Fazeli, 2008). The fast resolution of UPLC-ESI-MS/MS method was also explored in different commodities by Béltran *et al.* (2009) for the simultaneous determination of eleven mycotoxins (4 min runs). Continuous switching of positive-negative ionization mode and the absence of SPE clean-up contributed to the multi-residuality of the method, and the matrix-matched standards method contributed to overcome the matrix effects.

LC/MS methods aren't as frequently encountered for OTA given that sensitive LC/FD methods exist exploiting its' natural fluorescence (Cigić & Prosen, 2009). LC/MS has been comparatively more often used for the elucidation of OTA metabolites (Zöllner & Mayer-Helm, 2006). In general, MS hyphenated techniques are now commonly established as highly reliable analyte confirmation tools in residue analysis and so used in agreement. The number of published multi-analyte LC/MS methods for mycotoxins from different chemical groups and their metabolites, in most of which OTA is included, is increasing and ranges from "small-size" (Rubert *et al.*, 2010; Sørensen *et al.*, 2010) and "medium-size" multi-analyte methods - above 10 different compounds (Kokkonen *et al.*, 2005; Lattanzio *et al.*, 2007; Ren *et al.*, 2007) to "large-size" - above 30 compounds in the same chromatographic run (Berthiller *et al.*, 2007; Sulyok *et al.*, 2007; Spanjer *et al.*, 2008).

Compared to the mass spectrometric and fluorescence detectors, all other detectors available in HPLC are seldom used in mycotoxin analysis. The reasons might be the higher LODs unsuitable for trace amounts of the determined substances and lack of specificity for some of detectors (Cigić & Prosen, 2009). Photodiode array (PDA) has been reported for OTA detection in culture media, such as bread analogues, in a multi-toxin procedure by Kokkonen *et al.* (2005).

### 9.1.4.2. Thin layer chromatography

Despite the great development of the chromatographic techniques, TLC still has a place in some analytical laboratories, especially in developing countries. TLC offers the advantage of testing a number of samples simultaneously and can also be used as a screening test prior to more sophisticated instrumental methods. Mycotoxins are polar

compounds and have mostly been separated on normal phase silica TLC plates using a range of organic solvent mixtures as mobile phase. Given the low levels at which mycotoxins occur, confirmatory analyses are frequently conducted to provide confidence in the analytical result (Shephard, 2008b).

Examples of TLC methods published for OTA analysis include the one reported by Nesheim *et al.* (1973) for barley, collaboratively evaluated. In the method of Dawlatana *et al.* (1996), a series of solvent steps was used to separate OTA from rice, and quantify it by fluorescence. This method required large quantities of solvent, intensive laboratory procedures and was characterised by a lack of automation. However, it was possible to use it to screen rapidly a variety of samples.

For OTA, sodium bicarbonate, $AlCl_3$ and $NH_3$ vapour can all be used to distinguish the mycotoxin from interferences. OTA itself fluoresces greenish blue and under alkaline conditions of the confirmatory spray, changes to blue with an increase in intensity. In another confirmatory method OTA, which contains a carboxylic acid, can be derivatised by esterification with ethanol to the resultant ethyl ester. Confirmation is achieved by TLC separation of the derivatised solution and observing the disappearance of the OTA spot and the appearance of a spot corresponding to the ester (Shephard, 2008b).

### 9.1.4.3. Immunoassays

A range of analytical methods has been developed for mycotoxins (with hapten nature) that rely on immunological principles, i.e. the interaction between an antigen (the analyte of interest) and an isolated (polyclonal or monoclonal) antibody raised against the antigen. Since the first description of antibodies against OTA (Chu *et al.*, 1976) many papers have been published describing their use to analyse food sample materials. When applied to these food samples, two critical aspects need to be considered and controlled in immunoassays: sample matrix effects and antibody cross-reactivity (Schneider *et al.*, 2004).

Available immunoassays for OTA detection in food matrices include instrumental and non-instrumental test formats. The latter generally are commercially available or intended to, and are being constantly improved, to become less expensive, more rapid, easier to use, accurate, and sensitive. Of note is the fact that, some authors (Fremy &

Usleber, 2003) suggest the need of policy establishment for standardized description of specificity and other performance characteristics of commercial immunoassays.

Several papers have been published about the development of enzyme-linked immunosorbent assay (ELISA) methods for OTA analysis, in direct and competitive assays, using polyclonal and/or monoclonal antibodies, and providing quantitative or semi-quantitative results (*vide* Table 9). Given that OTA, similarly to other mycotoxins, is a small molecule only competitive assays are often considered (Bonel *et al.*, 2010). For the use in rapid monitoring, most methods eliminate two important stages: clean-up or analyte enrichment step, which leads to a significant influence of the sample material (matrix effects) and also extraction step (for solid samples) which influences detection limits and sensitivity (Krska *et al.*, 2008).

Although some ELISA methods show detection limits comparable to those of LC methods, the former often suffer from false positive results, due to the antibody cross-reaction with matrix components or other ochratoxins (OTB, OTC, OTα, OTA methyl ester), and, more importantly, from false negative results due to inadequate sensitivity, especially when tissue extracts are analysed (Monaci & Palmisano, 2004; Visconti & Girolamo, 2005).

The fluorescence polarization (FP) immunoassay format for mycotoxin analysis has been also developed in recent years. A FP competitive immunoassay method was developed for the determination of OTA in standard solution, and then applied to spiked barley samples. When compared to indirect competitive ELISA the analysis of naturally contaminated barley samples showed some disagreements, due to a stronger matrix effect observed with ELISA (Shim *et al.* 2004). Immunosensors are emerging as a cost-effective alternative for screening and quantitative determination of OTA, developed to minimise the matrix effects of real samples, owing to the selectivity of the appropriately selected primary antibody (Krska *et al.*, 2008; Bonel *et al.*, 2010). Surface plasmon resonance-based (SPR) competitive immunoassay without prior clean-up was developed for OTA detection in cereals (LOD 0.1 μg/kg; van der Gaag *et al.*, 2003). Since no signal enhancement is normally included in SPR, the test sensitivity is relatively low compared with enzyme immunoassay (Schneider *et al.*, 2004).

**Table 9.**
Main representative immunoassays for OTA determination in cereal matrices.

| Matrix | Immunoassay | Extraction and clean-up | LOD (LOQ) (µg/kg) | % of recovery (Spiking range, µg/kg) | Reference |
|---|---|---|---|---|---|
| Wheat<br>Corn<br>Rice | Competitive indirect ELISA | Extraction:<br>Methanol/PBS (1:1, v/v)<br>Centrifugation<br>Filtration | 0.15 | 96.4-101.4<br>(2.5-10) | Zhang *et al.* (2011) |
| Wheat | Nanostructured immunosensor<br>(Competitive indirect) | Extraction:<br>Acetonitrile/water (6:4, v/v) in orbital shaker<br>Centrifugation | 0.21 | 104-107 %<br>(0.4-7.2) | Bonel *et al.* (2010) |
| Cereals | Time-resolved fluoroimmunoassay<br>(Competitive indirect) | Extraction:<br>Mixing with methanol/water (1:1, v/v)<br>Filtration | 0.02 | 95.8 %<br>(1-200) | Huang *et al.* (2006) |
| Wheat | Electrochemical immunosensor<br>(Competitive direct) | Extraction:<br>Acetonitrile<br>Clean-up:<br>none | 0.4 | - | Alarcón *et al.* (2006) |
| Milo<br>Barley<br>Wheat<br>Soybeans<br>Corn | Competitive direct ELISA | Extraction:<br>70 % methanol<br>In orbital shaker | 3.8<br>2.8<br>3.5<br>2.5<br>1.9 | 92-99.7 %<br>(20) | Zheng *et al.* (2005) |
| Wheat pasta<br>Corn meal<br>Corn flakes<br>Pasta | Competitive array biosensor<br>(Evanescent field-induced fluorescence) | Extraction:<br>Methanol<br>Clean-up:<br>none | 14<br>3.8<br>25<br>100 | - | Ngundi *et al.* (2005) |
| Cereals | Competitive direct ELISA | LLE:<br>Dichloromethane/citric acid mixture<br>Back extraction:<br>1 % sodium bicarbonate | 0.5 | 90-130 %<br>(5-100) | Barna-Vetró *et al.* (1996) |

(LOQ: Limit of quantification)

A rapid and selective electrochemical immunosensor, combining the high selectivity of a competitive enzyme-linked immunosorbent assay with the sensitivity of electrochemical screen-printed carbon electrodes, was developed for OTA determination in wheat samples. A good correlation coefficient ($R^2$) was found ($R^2$=0.9992) by comparative analysis of naturally contaminated wheat samples with an IAC-LC/FD (Alarcón *et al.*, 2006).

In recent years, interest in rapid membrane-based immunoassay methods has strongly increased due to the need for fast "on-site" (pre)-screening, in which the use of expensive and heavy electronic equipment is prohibitive. Therefore, several attempts have been made to prepare self-contained tests for mycotoxins, which can be used without specific laboratory equipment necessary, given that results are visually evaluated (Schneider *et al.*, 2004; Krska *et al.*, 2008). The techniques involved are membrane-based assays in the format of dipstick, enzyme-linked immunofiltration or flow-through devices, and lateral flow or capillary migration, as reviewed by Schneider *et al.* (2004).

The most advanced immunochemical technique in aspects of simplification and rapid on-site testing is visual immunoassays using dry reagent lateral flow technology. For small molecules, like mycotoxins, the competitive test principle determines the test design of lateral flow tests (Schneider *et al.*, 2004). In combination with IAC clean-up and a quantitative reader, lateral flow devices showed a potential application for detection of OTA in grains and other matrices at European legislative levels (Danks *et al.,* 2003). However, the most frequently reported immunochemical techniques are flow-through set-ups. Membrane based flow-through enzyme portable immunoassays were developed to screen OTA in cereals (de Saeger & Peteghem, 1999).

### 9.1.4.4. Other analytical methods

Apart from the range of chromatographic and immunological analytical methods, described in the previous sections, a number of other techniques have been investigated as potential methods for mycotoxin determination. However, these have found little application outside the research environment (Shephard, 2008b).

Capillary zone electrophoresis (CZE) is such an example for which the introduction of suitable lasers for detection has lowered the detection limits to levels suitable for analysis of contaminated food samples (Shephard, 2008b). A CZE-laser-induced

fluorescence (LIF) method has been described for quantification of OTA in roasted coffee, corn, and sorghum (Corneli & Maragos, 1998). The method had sensitivity comparable to LC (0.2 μg/kg) and proved excellent separation of OTA from interferences. The use of small volumes of samples and less expensive and versatile capillaries, together with the absence of organic solvents during the determinative step, make this method an adequate alternative to LC/FD (Monaci & Palmisano, 2004; Visconti & Girolamo, 2005).

## 9.2. Biological samples: human urine

Many biological specimens can be used for OTA exposure assessment; nevertheless a detailed discussion of the different reported strategies for each is beyond the scope of this dissertation. Thus this section will entail a focused review on the strategies for OTA analysis in human urine.

Even though some researchers recognised that measurement of OTA in urine can be definitely a good marker of human exposure to this mycotoxin, literature about OTA analysis is urine is relatively scarce. In fact, the purpose of most of the studies measuring OTA in urine is to gather pharmacokinetic and epidemiologic information. As the levels of OTA consumed by humans are much lower than those administered to experimental animals, the expected levels in human urine are, therefore, at trace levels (Castegnaro *et al.*, 1990). Development of analytical methodologies for OTA determination in urine can be challenging given the low concentration of OTA in urine which requires accurate and sensitive methods for its qualitative and quantitative determination (Vatinno *et al.*, 2007; 2008).

The use of urine as an analytical tool has a number of advantages over other biofluids, as detailed previously. Furthermore, analytical advantages claimed for urine relative to serum, for instance, include the need for less complex sample pre-treatment due to lower protein content, the relatively small size and higher thermodynamic stability of urinary peptides/proteins, and the lower sample complexity including less intermolecular interaction (Ryan *et al.*, 2011).

### 9.2.1. Sampling

Most reported works make use of a point sample collection to evaluate OTA urinary concentration (Jonsyn-Ellis 2001; Domijan *et al.*, 2003; Pena *et al.*, 2006; Manique *et*

*al.*, 2008; Muñoz *et al.*, 2010; Coronel *et al.*, 2011a; Rubert *et al.*, 2011). To guarantee a higher concentration of OTA in urine, in the one point collection most of these studies use the morning samples.

A 24-hour sampling to evaluate daytime OTA urine excretion (Gilbert *et al.*, 2001; Pascale & Visconti, 2001; Petkova- Bocharova *et al.*, 2003; Fazekas *et al.*, 2005b; Castegnaro *et al.*, 2006) has also been used.

A different way of presenting results was used by Akdemir *et al.* (2010) and Desalegn *et al.* (2011), by making a point sample collection and normalising the OTA levels in urine to 24-hour urine with creatinine concentration, suggesting that in this way their point result adjusted by creatinine represents the mean daily OTA excretion. However, this correction is useless whenever the biomarker being monitored is variable in its time of excretion (Ryan *et al.*, 2011).

### 9.2.2. Extraction

The majority of the reported strategies for OTA extraction from urine samples encompass a LLE method (Table 10), particularly with a partition against chloroform in order to reduce matrix interferences (Vatinno *et al.*, 2007).

A LLE procedure on commercially available columns has been reported for urine, and when compared with common SPE technique, the results showed better analytical performance, but failed to achieve a reasonable sensitivity (Domijan *et al.*, 2003).

### 9.2.3. Clean-up

The application of IAC in human urine by Pascale & Visconti (2001) was of note, circumventing time consuming procedures and the need of large amounts of often-hazardous solvents. After this first reported application in urine, single- (Pena *et al.*, 2006; Ahn *et al.*, 2010; Desalegn *et al.*, 2011) and multi-toxin (Rubert *et al.*, 2011) IAC has been increasingly used as a clean-up step for the quantitative determination of OTA in urine (*vide* Table 11). The selective pre-concentration provided by IAC results in good sensitivity, as observed by the LODs and limits of quantification (LOQ) reported.

The attempt of Domijan *et al.* (2003) to avoid the use of IAC's was practically ineffective due to inadequate LOQ and the poor extent of sample purification achieved.

**Table 10.**
Examples of LLE methods used in OTA extraction from urine samples.

| LLE method | Clean-up | Reference |
|---|---|---|
| Dilution in $NaHCO_3$<br>pH 3-4 adjustment with 1M phosphoric acid<br>Partitioning with $CHCl_3$/isopropanol (97:3, v/v)<br>Centrifugation<br>Organic phase evaporation to dryness | - | Muñoz *et al.* (2010) |
| Centrifugation<br>Filtration<br>pH ≥ 8 (NaOH 1M)<br>Partitioning with $CHCl_3$<br>Centrifugation<br>Aqueous phase acidification with 37 % HCl<br>Dilution 1:5 in phosphate buffer (10 mM; pH 3) | SPME | Vatinno *et al.*, 2007 |
| Urine acidification (pH 2.5) with HCl<br>Solvent extraction with $CHCl_3$<br>Centrifugation<br>Organic phase ($CHCl_3$) evaporation to dryness | IAC | Petkova-Bocharova *et al.* (2003) |
| Urine transferred into Chem Elut columns<br>Double extraction with $CHCl_3$/formic acid mixture (9+1, v/v)<br>Collected eluate evaporated to dryness under a nitrogen stream in water bath at 60 ºC | - | Domijan *et al.* (2003) |
| Urine dilution in methanol acidified with 1 M HCl<br>Extraction with $CHCl_3$<br>Centrifugation<br>$CHCl_3$ washing with redistilled water | SPE | |
| Solvent extraction ($CHCl_3$; 0.05 M HCl; 0.1 M $MgCl_2$)<br>Centrifugation<br>Organic phase ($CHCl_3$) evaporation to dryness | SPE | Jonsyn-Ellis (2001) |
| Addition of 0.2 M $MgCl_2$ and 0.1 M HCl<br>Adjustment to pH 2.5 with 1 N HCl<br>Extraction with $CHCl_3$<br>Organic phase washing with 0.1 M sodium bicarbonate<br>Aqueous alkaline phase adjusted to pH 2.5 with 1 N HCl<br>Extraction with $CHCl_3$<br>$CHCl_3$ layer evaporated to dryness | - | Wafa *et al.* (1998) |
| Urine acidification (pH 2.4-2.5) with HCl<br>Extraction with $CHCl_3$/methanol (2+1)<br>Double extraction with $CHCl_3$<br>Evaporation to dryness of the pooled organic extracts | SPE | Castegnaro *et al.* (1990) |

(SPME: solid phase micro-extraction)

**Table 11.**
Examples of SPE immunological- and non-immunological-based methods for OTA extraction and clean-up from human urine.

| Prior steps | SPE method and operative conditions | Reference |
|---|---|---|
| Sample filtration<br>Dilution in PBS (1:1) | **IAC**<br>Extraction solvent: PBS<br>Elution: Methanol/acetonitrile (50:50, v/v) | Rubert *et al.* (2011) |
| Sample centrifugation<br>Supernatant dilution in PBS | **IAC**<br>Extraction solvent: PBS<br>Elution: Methanol | Desalegn *et al.* (2011) |
| Sample dilution (1:1) in 3% $NaHCO_3$<br>Centrifugation<br>Supernatant loaded into IAC | **IAC**<br>Extraction solvent: $NaHCO_3$<br>Elution: 2% acetic acid in methanol | Ahn *et al.* (2010) |
| Sample dilution (1:1) in PBS (0.5 M) for pH 3.0 | **SPME**<br>Extraction phase: Carbon tape fibres<br>Desorption solvent: Methanol | Vatinno *et al.* (2008) |
| Sample partitioning against $CHCl_3$ | **SPME**<br>Extraction phase: Silica fibre coated with a PDMS/DVB film<br>Desorption solvent: Mobile phase | Vatinno *et al.* (2007) |
| Sample dilution (1:1) in 5% $NaHCO_3$<br>Filtration<br>Total volume loaded into IAC | **IAC**<br>Extraction solvent: $NaHCO_3$<br>Elution: Methanol | Pena *et al.* (2006) |
| Reconstitution of urine extract in methanol<br>Dilution with TRIS/HCl pH 7.5<br>Total volume loaded into IAC | **IAC**<br>Extraction solvent: Methanol+TRIS/HCl<br>Elution: Methanol/acetic acid (98:2, v/v) | Petkova-Bocharova *et al.* (2003) |
| Sample partition against a $CHCl_3$:methanol acidified mixture | **SPE**<br>Stationary phase: Silica gel<br>Elution: $CHCl_3$/formic acid (95:5, v/v). | Domijan *et al.* (2003) |
| Sample dilution (1:1) in 5% $NaHCO_3$<br>Filtration<br>Half volume loaded into IAC | **IAC**<br>Extraction solvent: $NaHCO_3$<br>Elution: Methanol | Pascale & Visconi (2001) |
| Reconstitution of urine extract in $CHCl_3$ | **SPE**<br>Stationary phase: Silica gel column<br>Elution: $CHCl_3$/glacial acetic acid (99:1) | Jonsyn-Ellis (2001) |
| Sample partition against a $CHCl_3$:methanol and $CHCl_3$ | **SPE**<br>Stationary phase: Silica gel column<br>Elution: $CHCl_3$/glacial acetic acid (100:0.2) | Castegnaro *et al.* (1990) |

(PDMS/DVB: polydimethylsiloxane/divinylbenzene; SPME: solid phase micro-extraction)

Solid phase micro-extraction (SPME), introduced in early 1990s (Arthur & Pawliszyn, 1990) has become very popular in recent years as a sample extraction "solvent-free technique" that can be coupled to GC, HPLC or capillary electrophoresis.

It satisfies most of the requirements of a good sample preparation technique, including simplicity of use, automation, and low consumption of materials. The SPME device itself is portable, and the technique is relatively fast. However, the coated fibres may be considered to be expensive. For some applications the fibres have limited lifetimes, and careful analyte desorption is mandatory to avoid carry-over of analyte from extraction to extraction (Lee *et al.*, 2008). SPME has been employed in detection of OTA in human urine, with detection by fluorescence (Vatinno *et al.*, 2007) and mass spectrometry (Vatinno *et al.*, 2008) coupled with LC. This solventless extraction technique resulted in LOD and LOQ closer to those obtained by IAC when coupled to LC/FD, which provided enhanced sensitivity.

### 9.2.4. Detection and quantification

When determining OTA in urine samples most studies exploit the native fluorescence of the mycotoxin through reversed phase LC with flourescence detection (*vide* Table 12), after suitable extraction/clean-up step.

Reported methods for OTA confirmation in urine after LC/FD analysis involve methyl ester formation, through derivatization (Castegnaro *et al.*, 1990; Pena *et al.*, 2006; Muñoz *et al.*, 2010), OTα formation through hydrolysis (Wafa *et al.*, 1998), and standard addition method (Muñoz *et al.*, 2010).

As opposed to fluorescence detection, the studies that make use of mass spectrometric detection of OTA in human urine are unquestionably scarcer (*vide* Table 13). Moreover, some of the reported ones (Ahn *et al.*, 2010; Rubert *et al.*, 2011) aimed for multi-mycotoxin monitoring, thus inevitably compromising sensibility. Rubert *et al.* (2011) reported a method for determining 11 mycotoxins , specifically DON, T-2 toxin, HT-2 toxin, ZEA, OTA, $AFB_1$, aflatoxin $B_2$ ($AFB_2$), aflatoxin $G_1$ ($AFG_1$), aflatoxin $G_2$ ($AFG_2$), $FB_1$ and fumonisin $B_2$ ($FB_2$), through a multi-mycotoxin IAC clean-up.

In MS detection of OTA in urine, the mass to charge ratio (*m/z*) of the reported transitions of precursor and product ions were *m/z* 402.1 → 357.9 (Vatinno *et al.* 2008; Muñoz *et al.*, 2010) and *m/z* 404.0 → 239.0 (Ahn *et al.*, 2010; Rubert *et al.*, 2011), in negative and positive ion mode, respectively.

**Table 12.**
Main representative LC/FD methods for OTA determination in human urine.

| LC separation | | | | FD conditions | Analytical Performance | LOD (LOQ) (µg/L) | Reference |
|---|---|---|---|---|---|---|---|
| **Column** | **Mobile phase** | **Injection volume (µl)** | **Retention time (min)** | | | | |
| Packing: RP-C18<br>Dimension: PS: 5 µm, ID:250x3 mm | **Flow-rate:** 1 mL/min<br>**Elution:** Gradient<br>**Composition:**<br>A: acetic acid 2%/methanol (66:34, v/v)<br>B: methanol/isopropanol (90:10, v/v). | 80 | 22 | $\lambda_{exc}$=333nm<br>$\lambda_{emi}$=450nm | SR: 0.05-2 µg/L<br>R: 74.4-104 %<br>RSD: 4.2-13 % | 0.02 (0.05) | Muñoz *et al.* (2010) |
| Packing: RP-C18<br>Dimension: PS: 5 µm, ID:150x4.6 mm | **Flow-rate:** 1 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Water/acetonitrile/acetic acid (111:87:2, v/v/v) | 20 | 13.61 | $\lambda_{exc}$=332nm<br>$\lambda_{emi}$=460nm | SR: 0.01-1 µg/L<br>R: 76.0 %<br>RSD: 3.5 % | 0.01 (0.05) | Vatinno *et al.* (2007) |
| Packing: RP-C18<br>Dimension: PS: 5 µm, ID:250x4.6 mm | **Flow-rate:** 1 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Acetonitrile/water/acetic acid (49.5:49.5:1, v/v/v) | 50 | 12:35 | $\lambda_{exc}$=333nm<br>$\lambda_{emi}$=477nm | SR: 0.02-0.5 µg/L<br>R: 90.6-96.0 %<br>$RSD_b$: 4.3-8.9 %<br>$RSD_W$: 2.9-5.4 % | (0.02) | Pena *et al.* (2006) |
| Packing: RP-C18<br>Dimension: PS: 5 µm, ID:250x4.6 mm | **Flow-rate:** 1 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Methanol/acetonitrile/0.005 M sodium acetate solution /acetic acid (300:300:400:14, v/v/v/v) | 50 | 7-8 | $\lambda_{exc}$=335nm<br>$\lambda_{emi}$=465nm | SR: 0.4 µg/L<br>R: 70 % | (4) | Petkova-Bocharova *et al.* (2003) |

(ID: Internal diameter; LR: Linear range; PS: Particle size; R: Recovery; RSD: Relative standard deviation; $RSD_b$: Relative standard deviation between-day (inter-day); $RSD_w$: Relative standard deviation within-day (intra-day); SR: Spiking range; $\lambda_{exc}$: wavelength of excitation; $\lambda_{emi}$:,wavelength of emission.)

**Table 12. (Continued)**
Main representative LC/FD methods for OTA determination in human urine.

| LC separation | | | | FD conditions | Analytical Performance | LOD (LOQ) (µg/L) | Reference |
|---|---|---|---|---|---|---|---|
| **Column** | **Mobile phase** | **Injection volume (µl)** | **Retention time (min)** | | | | |
| Packing:<br>RP-C18<br>Dimension:<br>PS: 5 µm,<br>ID:125x4 mm | **Flow-rate:** 0.5 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Acetonitrile/water/acetic acid (49.5:49.5:1, v/v/v) | 50 | 8 | $\lambda_{exc}$=336nm<br>$\lambda_{emi}$=464nm | Chem Elut method:<br>SR: 1-10 µg/L<br>R: 92-99 %<br>RSD: 1-4 %<br>SPE method:<br>SR: 1-10 µg/L<br>R: 67-98 %<br>RSD: 3-8.4% | Chem Elut method:<br>0.3 (0.9)<br>SPE method:<br>0.5 (1.5) | Domijan *et al.* (2003) |
| Packing:<br>RP-C18<br>Dimension:<br>PS: 5 µm,<br>ID:150x4.6 mm | **Flow-rate:** 1 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Acetonitrile/water/acetic acid (49.5:49.5:1, v/v/v) | 100 | - | $\lambda_{exc}$=333nm<br>$\lambda_{emi}$=460nm | SR: 0.05-0.1 µg/L<br>R: 88.4-93.2 %<br>RSD: 1.1-8.2 % | 0.005 | Pascale &Visconti (2001) |
| Packing:<br>RP-C18<br>Dimension:<br>PS: 5 µm,<br>ID:250x5 mm | **Flow-rate:** 1 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Methanol/water /acetic acid (65:35:1, v/v/v) | - | - | $\lambda_{exc}$=365nm<br>$\lambda_{emi}$=418nm | R: 93 % | 0.2 | Jonsyn-Ellis (2001) |
| Packing:<br>RP-C18<br>Dimension:<br>PS: 10 µm,<br>ID:250x4 mm | **Flow-rate:** 1.5 mL/min<br>**Elution:** Isocratic<br>**Composition:**<br>Methanol/acetonitrile/sodium acetate 5 mM/acetic acid (300:300:400:14, v/v/v/v) | 50 | | $\lambda_{exc}$=340nm<br>$\lambda_{emi}$=465nm | - | - | Wafa *et al.* (1998) |

(ID: Internal diameter; LR: Linear range; PS: Particle size; R: Recovery; RSD: Relative standard deviation; $RSD_b$: Relative standard deviation between-day (inter-day); $RSD_w$: Relative standard deviation within-day (intra-day); SR: Spiking range; $\lambda_{exc}$: wavelength of excitation; $\lambda_{emi}$:,wavelength of emission.)

**Table 13.**
Main recent representative LC/MS and LC/MS/MS methods for OTA determination in human urine.

| LC separation | MS detection | Standard | Analytical Performance | LOD (LOQ) (µg/L) | Reference |
|---|---|---|---|---|---|
| **Column**:<br>RP-C18<br>PD: 3µm<br>ID: 150 x 2 mm<br>**Mobile phase**:<br>Gradient<br>0.2 mL/min<br>A: Water acidified with 0.1% formic acid and 5 mM ammonium formate<br>B: Methanol with 5 mM ammonium formate | LC/MS/MS<br><br>ESI<br>Positive ion mode<br>Triple quadupole | OTA (External) | SR: 1.5-150 µg/L<br>R: 83-94 %<br>$RSD_w$: 3-4 %<br>$RSD_b$: 5-9 % | 0.5 (1.5) | Rubert *et al.* (2011) |
| **Column**:<br>RP-C18<br>PD: 5µm<br>ID: 150 x 4.6 mm<br>**Mobile phase**:<br>Gradient<br>0.9 mL/min<br>A: 0.2 % formic acid in water<br>B: 0.2 % formic acid in acetonitrile | LC/MS/MS<br><br>ESI<br>Positive ion mode<br>Triple quadrupole | $^{13}C$-labelled OTA (Internal) | SR: 0.01-0.05 µg/L<br>R: 83.7-91.0 %<br>RSD: 3-15.6 % | 0.001 (0.004) | Ahn *et al.* (2010) |
| **Column**:<br>RP-C18<br>PD: 5µm<br>ID: 50 x 2.1 mm<br>**Mobile phase**:<br>Gradient<br>0.5 mL/min<br>A: Water/acetonitrile/acetic acid (90:10:0.1)<br>B: Acetonitrile/acetic acid (100:0.1) | LC/MS/MS<br><br>ESI<br>Negative ion mode<br>Triple quadrupole | OTB (internal) | SR: 1-50 µg/L<br>R: 44-57 %<br>$RSD_w$: 2.1-11.5 % | 0.3 (0.7) | Vatinno *et al.* (2008) |

(ID: Internal diameter; LR: Linear range; PD: Particle diameter; R: Recovery; RSD: Relative standard deviation; $RSD_b$: Relative standard deviation between-day (inter-day); $RSD_w$: Relative standard deviation within-day (intra-day); SR: Spiking range)

A method for high throughput determination of OTA in human urine (>150 samples per day) based on an automated multi-fibre SPME-LC/MS/MS procedure has been presented by Vatinno *et al.* (2008). Although hindered by a lower sensitivity, the use of

LC/MS/MS guarantees specificity and eliminates the need for additional confirmation experiments. The best application for MS detectors is thus the clear-cut confirmation of OTA in samples already turned out to be positive by LC/FD analysis given the unambiguous analyte identification (Sforza *et al.*, 2006).

The use of immunoassays for OTA determination in human urine has only been reported in a survey in Sri Lanka (Desalegn *et al.*, 2011). The determination through an competitive ELISA commercial kit was preceded by a clean-up and toxin concentration through an IAC which allowed good detection limit, set at 0.005 µg/L. Average recovery was calculated as 105 % with a relative standard deviation (RSD) of 13 %.

## 10. Legislative framework

As with many public food-safety regulations, domestic and trade regimes governing mycotoxins in most countries focus on the product, rather than on the production or marketing process standards. That is, tolerance levels for the amount of mycotoxin in a product are established, rather than regulating in a mandatory manner the production or treatment of the commodity along the marketing chain (Dohlman, 2004). Therefore, the mycotoxins regulations are reactive rather than proactive (Kendra & Dyer, 2007).

This is also true for OTA, although in the EU some raw materials or ingredients are regulated, such as malt (used in beer production), and cereals (used for a variety of cereal-derived products), as established by Commission Regulation No. 1881/2006 (CEC, 2006b).

### 10.1. Ochratoxin A maximum limits in food

Historically, the EU has put limits on the permissible maximum limits of OTA in several foodstuffs in order to minimise exposure of the public justified by the characterization of this bio-contaminant as a serious hazard (FAO, 2004). At a central level, for harmonization the European Commission set regulatory limits for some commodities, which are amended or updated regularly whenever new scientific information justifies it. The regulatory levels, as established by Commission Regulation No. 1881/2006 (CEC, 2006b), were based on the updated scientific opinion of the EFSA (2006), and food incidence information collected in the last SCOOP report (Miraglia & Brera, 2002). Recently, these MLs were amended by Commission Regulation No. 105/2010 (CEC, 2010b) purposely for spices and liquorice. In the light

of this latest information, EU levels were only established for the Table 14 specified commodities, for their significant contribution to general human exposure to OTA or to the exposure of vulnerable groups of consumers such as children.

In the case of staple commodities such as grain, a maximum value reduces the average exposure of the public to the toxin. With foodstuffs such as coffee, where few people consume enough to affect their average exposure, the intention is to protect the public from any instances of high exposure (FAO, 2004). The European Commission is currently considering the appropriateness of establishing a maximum level for OTA in food commodities other than those for which already a ML exists at the EU level (CAC, 2008b).

**Table 14.**
Maximum levels (µg/kg) for OTA in foodstuffs in EU, according to Regulations No. 1881/2006 (CEC, 2006b) and 105/2010 (CEC, 2010b).

| Commodity | µg/kg |
|---|---|
| 1. Unprocessed cereals | **5** |
| 2. All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption with the exception of foodstuffs listed in 9 and 10 | **3** |
| 3. Dried vine fruit (currants, raisins and sultanas) | **10** |
| 4. Roasted coffee beans and ground roasted coffee, excluding soluble coffee | **5** |
| 5. Soluble coffee (instant coffee) | **10** |
| 6. Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15 % vol) and fruit wine | **2** |
| 7. Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails | **2** |
| 8. Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption | **2** |
| 9. Processed cereal-based foods and baby foods for infants and young children | **0.5** |
| 10. Dietary foods for special medical purposes intended specifically for infants | **0.5** |
| 11. Spices (chillies, chilli powder, paprika, pepper, ginger, nutmeg, turmeric alone or in mixtures) | **30**[a] |
| 12. Liquorice: | |
| Root (for herbal infusion) | **20** |
| Extract (for beverages and confectionary) | **80** |
| 13. Green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products | **-** |

( [a] as from 1.7.2010 until 30.6.2012. Replaced by 15µg/kg as from 1.7.2012)

The majority of EU member states only follow the EU harmonised regulations, but in some cases, in view of some individual situations that deserve tighter ruling, the

countries themselves narrow the limits or set limits in commodities not specified by the EU harmonised guidelines. For example, although there are no regulations in the EU for OTA in meat, Denmark has enforced limits between 10 µg/kg in pig kidneys for condemnation of liver and kidney and an entire carcass condemnation level at 25 µg/kg in pig kidney. Italy launched in 1999 a guideline value of 1 µg/kg in swine meat and derived products (Monaci *et al.* 2004). In Romania, the maximum permissible limit for meat is 5 µg/kg (Curtui *et al.* 2001).

OTA regulatory limits obtained by the FAO international inquiry, carried out in 2002 and 2003, regarding cereals and animal edible tissues are specified in Table 15. According to the same international inquiry, the EU harmonised regulations are followed by some European extra-EU countries (e.g. Iceland and Norway), EU candidate member states (e.g. Turkey), and small countries or principalities (e.g. Liechtenstein) in some commodities. In contrast to the EU, the South American common market (MERCOSUL, Portuguese acronym of *Mercado Comum do Sul*), whose member states are Argentina, Brazil, Paraguay and Uruguay, do not have harmonised regulations for OTA. Only Uruguay has regulations in the case of OTA for rice, barley, beans, coffee and corn. Likewise, the Association of Southeast Asian Nations (ASEAN), comprising Brunei Darussalam, Cambodia, Indonesia, the Lao People's Democratic Republic, Malaysia, Myanmar, the Philippines, Singapore, Thailand and Vietnam, does not regulate the MLs for OTA (FAO, 2004). The Food and Drug Administration (FDA) from the USA has not set any action, advisory or guidance levels for OTA. This official body is currently evaluating data on OTA levels in domestic and imported commodities, according to Clark & Snedeker (2006).

At a global scale, it is the FAO- and WHO-supported CAC, through the Codex Committee on Food Additives and Contaminants (CCFAC) that regulates the maximum limits (standards). The committee established a maximum level of 5 µg/kg for OTA in the cereals wheat, barley, and rye and derived products. The member states (117) with no specific legislation relating to maximum residue limits for OTA, like, for example, Peru, Thailand or United Arab Emirates, follow the CAC proposed limit (CAC, 2008b).

**Table 15.**
Maximum tolerated levels regulations of OTA in cereals and animal edible tissues as of December 2003 (modified from FAO, 2004).

| Country | Foodstuffs | Maximum level (µg/kg) |
|---|---|---|
| Bulgaria | Cereals and processed products thereof intended for direct human consumption or as an ingredient in foodstuffs | 3 |
| | Cereals to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs | 5 |
| Cuba | Cereals | 5 |
| Czech Republic | Child and baby nourishment | 1 |
| | Flours and cereal products | 3 |
| Denmark | Pig kidney[a] | 10 |
| | Pig kidney[b] | 25 |
| Estonia | Pig liver | 10 |
| | Cereals, cereal flours, cereal groats and flakes, pasta products, ordinary baker's wares, fine baker's wares; isolates, Concentrates and hydrolysates of cereals protein | 5 |
| Hungary | Cereals (including the rice and *Fagopyrum sp.*) | 5 |
| | Every cereal product including milled products and those cereal products used for direct human consumption | 3 |
| Islamic Republic Of Iran | Baby food based on cereals without milk | 1 |
| | Baby instant food (ready to use) | 1 |
| | Barley, maize | 50 |
| | Rice, wheat | 5 |
| Israel | Cereals, cereal products and other foods | 50 |
| Italy | Pig meat and derived products | 1 |
| | Baby food | 0.5 |
| Latvia | Cereals | 5 |
| Morocco | Cereals | 30 |
| Romania | Alimentary products | 20 |
| | Alimentary products for babies 3 years old | 5 |
| Serbia And Montenegro | All foodstuffs | 10 |
| Singapore | Cereal | 2.5 |
| Slovakia | Meat, poultry, flour and its products, rice | 5 |
| | Infant formulae and food for children | 1 |
| | Other foodstuffs | 10 |
| Sudan | Wheat | 15 |
| Switzerland | Processed cereal-based foods and baby foods for infants and young children | 0,5 |
| | All foodstuffs | 5 |
| Turkey | Raw grain | 5 |
| | Foodstuffs produced from grain | 3 |
| Uruguay | Rice, barley, corn | 50 |

(Viscera condemned; visible damaged kidneys are analysed chemically; [b] whole carcass condemned; visible damaged kidneys are analysed chemically)

## 10.2. Ochratoxin A tolerable intake

A number of tolerable daily intakes (TDIs) have been proposed by official organizations or simply by experts in the subject. Kuiper-Goodman (1990) suggested a provisional TDI of 1.2–5.7 ng/kg bw/day for a risk level of $10^{-5}$. The Nordic Working Group on Food Toxicology and Risk Evaluation (NWGFTRE, 1991) supported their calculations (5 ng OTA/kg bw/day) with the carcinogenicity studies in adult rats with a safety factor of 5000. The same value for TDI was proposed by the Scientific Committee on Food (SCF, 1998) and the Upper Council for French Public Health (*Conseil Supérieur d'Hygiène Publique de France*, 1999).

More recently, EFSA (2006) established a tolerable weekly intake (TWI) of 120 ng/kg bw/week (about 17.14 ng/kg bw/day) and JECFA (2007) reconfirmed the already previously established value of 100 ng/kg bw/week (about 14.28 ng/kg bw/day) based on pig nephrotoxicity. More recently, Kuiper-Goodman *et al.* (2010) reevaluated the TDI as 4 ng/kg bw/day.

Unfortunately, risk evaluation does not differentiate between risk groups, like children *versus* adults, despite the critical dose/body weight ratio of the former (Skaug 1999). Moreover, the fact that the majority of the calculated intakes are inferior to the tolerable dose should not be neglected. First, because between countries and even between regions/cities there may exist wide variations in consumption of the more risky foods, and so if calculation is based on an average that means that some individuals may exceed this value, and therefore be at risk. Second, individuals may differ in their sensitivity to OTA. Finally, this mycotoxin may be additive to, or synergistic with, other chemicals in food and the environment (Mantle, 2002).

## 10.3. Ochratoxin A maximum levels in feedstuffs

Although there is supporting evidence of OTA accumulating in the organs and blood of several livestock species after consuming contaminated feed, the only mycotoxin with EU-harmonised specific maximum permitted levels in feed remains $AFB_1$ (FAO, 2004). Therefore, there are no enforceable values, merely guidance values for products intended for animal feeds, for orientation purposes only. These non-compulsory values laid down by Commission recommendation 2006/576/EC (CEC, 2006c) are 0.25 mg/kg in the case of cereals and cereal-derived products and for complementary and complete feedstuffs 0.05 and 0.1 mg/kg for pigs and poultry,

respectively. In this legal framework, although recognizing that OTA can be transferred from feed into food of animal origin, since exposure assessments indicate that food of animal origin makes only a small contribution to the total human dietary exposure to OTA, no maximum levels were established. Nevertheless, in the same document, the Commission advises member states to increase their monitoring for the presence of OTA in cereals and cereal products intended for animal feeding and compound feedstuffs. This recommendation was based on the opinion of the Scientific Panel on Contaminants in the Food Chain of the EFSA, on a request from the Commission related to OTA as an undesirable substance in animal feed, adopted on 22 September 2004 (EFSA, 2004). This European authority body highlights the need for all member states to conduct surveys to study the occurrence of OTA in animal blood and tissues, not only to assess the efficacy of feed control programmes, but also to assess the significance of residue levels in animal tissues, both with respect to animal health and to human exposure.

Although EU-harmonised specific limits for OTA in animal feedstuffs have not yet been proposed, limits have been established at the national level in eight member states. According to FAO (2004), these countries include Estonia, Lithuania, Slovenia and Sweden, reporting the existence of limits for OTA in various feedstuffs, including feeds for cattle, pigs, poultry and other farm animals (Table 16).

In the rest of the world, the FAO (2004) documented few other individual national regulations for OTA occurrence in animal feed in the form of guideline limits (Canada) or enforceable maximum limits (Israel, Mozambique), as presented in Table 16. The CAC, through the Codex Committee on Food Additives and Contaminants (CCFAC), developed codes of practice for the reduction of mycotoxin contamination in cereals, which includes an annex on OTA (CAC, 2003).

### 10.4. Analytical performance criteria and quality assurance

Performance criteria are important in the analysis of contaminants in food and feed for obtaining reliable results. In the EU, the minimum performance criteria for official control of foodstuffs are established by EC regulation No. 401/2006 (CEC, 2006a) and Commission Decision No. 2002/657/EC (CEC, 2002), assisted by Council Directive 96/23/EC (CEC, 1996).

**Table 16.**
Regulation on OTA in various animal feedstuffs as of December 2003 (modified from FAO, 2004).

| Country | Feed product | Limit (µg/kg) | Remarks |
|---|---|---|---|
| Bangladesh | Maize and mixed feed for poultry | Not given | In preparation |
| Canada | Feed for swine and poultry | 2000 | Guideline limit |
| Estonia | Feedingstuffs of vegetable origin | 100 | |
| | Complete feedingstuffs for cattle, pigs and other farm animals | 100 | |
| | Complete feedingstuffs for young cattle, young pigs and other young farm animals | 50 | |
| | Complementary feedingstuffs for cattle, pigs and other farm animals | 200 | |
| | Complementary feedingstuffs for young cattle, young pigs and other farm animals | 50 | |
| Israel | All grains for feed | 300 | |
| Lithuania | Feeds for pigs and poultry | 50 | |
| | Feeds for young pigs and young poultry | 20 | |
| Mozambique | Corn for feed | Unknown | |
| Slovenia | Feedstuffs for pigs | 200 | |
| | Feedstuffs for poultry | 1000 | |
| Sweden | Complete feedstuffs for pigs | 100 | |
| | Complete feedstuffs for poultry | 200 | |
| Yugoslavia (Serbia/Montenegro) | Feed for pigs | 100 | |
| | Feed for swine | 200 | |
| | Feed for poultry | 1000 | |
| | Feed for egg laying hen | 250 | |

The European Committee for Standardization (CEN) also establishes minimum performance criteria (CR 13505:1999) that are used for official control and surveillance and in cases of dispute (CEN, 1999). Hence, for regulatory purposes where analytical methods are required for food control or as referee methods for disputes, there has been a move away from prescribed “official methods” to a criteria-based approach, more flexible (Şenyuva & Gilbert, 2010).

Nevertheless, there are various methods for mycotoxin analysis available that are validated and have been accepted as standards by official authorities such as CEN, and the Association of Analytical Communities (AOAC). The official or validated methods

for OTA analysis in different food matrices have been compiled in several reviews (Gilbert & Anklam, 2002; Monaci & Palmisano, 2004; Visconti & Girolamo, 2005).

Other important tools during both the validation process of a method and the routine analysis, to assure the quality of analytical data during routine analysis, are the certified reference materials. Unfortunately there is a lack of certified reference materials and calibrators for mycotoxin analysis designed for the different sample matrices and concentration ranges (Krska *et al.* 2008). In the September 2011 catalogue of the Institute of Reference Materials and Measurements (IRMM) only one reference material was available for OTA analysis, a wheat blank sample (BCR-471) (IRMM, 2011).

Inter-comparison studies, in the form of collaborative studies or proficiency testing schemes, also play an important role in the validation of analytical methods and the production of RMs, as well as acting as quality assurance tools for laboratories. These inter-comparison studies are mostly conducted under the auspices of official bodies such as IRMM, AOAC, CEN, and Food Analysis Performance Assessment Scheme (FAPAS). The performance criteria of the CEN methods are usually derived from collaborative studies (Krska *et al.*, 2008).

Finally, and despite the several protocols and guidelines available for method validation, e.g. the guide “The fitness for purpose of analytical methods” (Eurachem, 1998), not all of the published methods for the determination of mycotoxins, give information about the major validation parameters, hindering the production of reliable results in terms of comparability and traceability (Krska *et al.*, 2008). That is an important aspect to improve in the future.

Contrarily, for biological matrices, no performance criteria are currently implemented for OTA analysis. However, some authors follow FDA’s Guidance for Industry: Bioanalytical Method Validation (FDA, 2001; Vatinno *et al.*, 2008).

# Part II

# Original Research

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**SCOPE:** In this part the rationale, scope and scheme of the original research conducted is presented. The global and specific objectives are set out. For each individual study the experimental procedure and results are detailed, followed by a thorough discussion and concluding remarks.

**ABRIDGED CONTENTS:** 1. Rationale and scope; 2. Objectives; Study A - OTA evaluation in bread; Study B - OTA evaluation in pork; Study C - OTA evaluation in human urine.

## 1. Rationale and scope

It is imperative to have knowledge on the current situation and trends with regard to the occurrence of mycotoxins in the food production chain; furthermore, this data needs to be updated continuously so that appropriate responses can be prepared, i.e. through surveillance work. The continual monitoring of OTA is also important because of the recognized variation of the incidence and level of OTA contamination from year to year, ensuing from the characteristic unpredictability of occurrence. Multiyear surveillance studies are thus valuable to better characterize the exposure levels of humans (Ates *et al.*, 2011).

In Portugal there was a lack of investigations related to OTA exposure. This stands out as a large fault, for several reasons; the Portuguese diet, in line with the traditional Mediterranean diet, presents a high consumption of cereals, with a second place in the EU ranking (Alea, 2010). Since cereals are considered the main contributors to OTA exposure in Europe, the OTA exposure should merit more vigilance. Furthermore, the worldwide occurrence of the mycotoxin and the wide range of foods susceptible of being contaminated are of significance; even more so when considering that not all the foodstuffs consumed in Portugal are produced nationally, so the origin of OTA in Portugal can also be traced back to the countries from which the importation takes place. Indeed some of the extra-EU imports come from countries with no statutory or action levels, nor awareness of OTA contamination. Even though a legislative framework at an EU-scale exists not all of the imported commodities are verified and analysed. Finally, the recognized toxic effects should also contribute to a higher concern about this mycotoxin exposure.

Apart from mycological studies, OTA research in Portugal was limited to small sized surveys to determine the mycotoxin occurrence in individual food commodities, specifically bread (Juan *et al.*, 2007; Juan *et al.*, 2008b), raw cereals (Juan *et al.*, 2008a), rice (Pena *et al.*, 2005), wine (Festas *et al.*, 2000; Ratola *et al.*, 2004; Pena *et al.*, 2010), grapes (Serra *et al.*, 2006), cereal- and milk-based baby and infant food (Alvito *et al.*, 2010), and meat (Guillamont *et al.*, 2005). Moreover only four biomonitoring small-scale surveys have been carried out, in both urine (Pena *et al.*, 2006; Manique *et al.*, 2008), and blood (Dinis *et al.*, 2007; Lino *et al.*, 2008)

These biomonitoring surveys showed a widespread prevalence of OTA contamination of the national population. Furthermore, the absence of persistent significant differences between most of the anthropometric parameters strongly suggested that the source of OTA is transversal to the population, which means that, considering that the major source of exposure is the ingestion of contaminated food, some common dietary foodstuff is probably implicated (Ueno *et al.*, 1998).

The aforementioned food surveys conducted in Portugal sustained a higher contribution of cereals and their derived products to OTA exposure, particularly bread. In fact, the reported levels for other commodities are several times lower, and simply reach significance because of a high consumption, as in the case of pork and wine. Already previously, in a total diet study in France (Leblanc *et al.*, 2005) and Canada (Tam *et al.*, 2011) bread was identified as a major contributing food to OTA exposure. Cereals and their derived products were established as major contributors for the EU citizens in general (Miraglia & Brera, 2002), and some populations in particular, e.g. France (Verger *et al.*, 1999). Moreover, the contaminated cereals can represent a direct source of human exposure, by its direct consumption, or an indirect source through the consumption of products derived from animals fed with contaminated feed.

Bread and pork, two highly consumed foodstuffs in the Portuguese staple diet, with an intake transversal to the entire population, thus emerge as potentially significant contributors to OTA human exposure, encouraging additional studies to glean information concerning the features of their contribution. Indeed, such characterization regarding the year and season, local of commercialization, composition and production nature is lacking.

Given the importance of these staple foods for OTA exposure, the factors that determine the incidence and contamination level, could theoretically be echoed in greater or lesser extent in the OTA exposure biomarker determined in urine samples collected in the same seasons and regions where the analysed foodstuffs were marketed. OTA levels in urine samples present a stronger correlation with the level of consumption, allowing a rapid and easy non invasive procedure of collection, thus promoting a more keen involvement of participants, as compared to blood.

Exposure of the Portuguese population has never been assessed through a large-scale survey, the simultaneous use of more than one food matrix, nor combined with an exposure biomarker. Furthermore, whether considering food or biological samples, the published studies conducted so far never used more than one sample collection period. The absence of this type of studies applied to the nation's residents prompted and supported the idea to perform the studies herein presented.

It is important to underline that the studies of OTA evaluation in bread and urine presented in this dissertation are part of a broader project supported by *Fundação para a Ciência e Tecnologia* (referenced PTDC/AGR-ALI/65528/2006) that surveyed a total of six mainland regions, Bragança, Porto, Coimbra, Lisboa, Alentejo and Algarve. Of all such regions, only four, specifically Porto, Coimbra, Lisboa and Alentejo, are scoped and addressed by the present dissertation.

## 2. Objectives

Taken the abovementioned justification of the experimental work carried out, the stirring general objective was to evaluate the exposure degree of the Portuguese population to OTA, through determination of the mycotoxin levels in bread, pork, as well as through its occurrence in the urine of healthy individuals.

To achieve the general objective a two-year surveillance plan, at a nationwide scale, was carried out. Winter and summer seasons were selected for simultaneous sample collection of the commercialized bread and pork, as well as urine of the inhabitants of the Porto, Coimbra, Lisboa, and Alentejo regions.

In order to accomplish the previously stated general objective, three main studies were designed, each addressing specific objectives, as follows:

**Study A - OTA evaluation in bread** aimed to:

- optimize a previously validated method to determine OTA occurrence and levels of contamination;
- determine the effect of the type, composition, origin, season and year of commercialization on OTA contamination of bread;

- assess human dietary exposure to OTA through bread consumption, according to the region, season or year considered;
- analyse and compare the obtained results with the ones previously reported, in Portugal and in foreign countries.

**Study B - OTA evaluation in pork** aimed to:

- develop and validate an analytical methodology to determine OTA levels in pork;
- determine the effect of the origin and year of commercialization;
- assess human dietary exposure to OTA through pork consumption, according to the region, season or year considered;
- analyse and compare the obtained results with the ones previously reported, in Portugal and in foreign countries.

**Study C - OTA evaluation in human urine** aimed to:

- optimize a previously validated method to determine OTA occurrence and levels of contamination;
- determine the occurrence of OTA in the urine of healthy individuals;
- correlate OTA urine levels with anthropometric characteristics;
- correlate the ingestion of contaminated foods and OTA urine levels, according to the region, season and year surveyed;
- analyse and compare the obtained results with the ones previously reported, in Portugal and in foreign countries.

# Part II

# Original Research

## STUDY A

**Ochratoxin A evaluation in bread**

# 1. Foreword and definitions

Bread is one of the most popular yeast leavened cereal product by far, providing more nourishment for humans than any other food source (FAO, 2002). In Portugal, according to the Portuguese official statistics institution (*Instituto Nacional de Estatística* - INE), bread is one of the most consumed foodstuffs. In 2005, 323 194 tons of bread were produced, of which 278 161 tons were wheat bread (INE, 2007; INE, 2008).

In fact, despite its small size, the country has different types of bread, being the wheat (white) bread the most consumed. It is characterised by the many different fermentation processes and the different ways of baking, a wide variety of sizes, shapes, textures and tastes, depending on the agricultural, cultural and eating habits of the populations. This white bread can also include additional ingredients, in lesser amounts, such as rice, sesame seed, poppy seed, sunflower seed, linseed, milk, malt extract, fat, sugar, among others. According to their main ingredient, it can also be distinguished rye bread, fibre-enriched or whole grain bread, oat-enriched and corn-enriched bread.

Alentejano bread is a particularly famous and typical type, characterised by a constant (large) size and a traditional bread-making process, in which only wheat flour, water, salt and yeast are used. It features a dense dough and an extended time of fermentation (Aldeia do pão, 2011). Another typical type of wheat bread is the Mafra bread, consumed specially in the capital region, where it is sold in different shapes and sizes. Mafra bread is baked only with wheat-based dough, water, salt and yeast. It features a high percentage of incorporated water and a reduced time of fermentation (Café Portugal, 2010)

The most traditional Portuguese maize bread, commonly known as *broa*, is typical of the central and northern regions and is made of white or yellow milled maize mixed with wheat. However, in some regions, other cereals such as rye are added, in addition to hot water, yeast and leavened dough from the preceding *broa*. After mixing, working up and leavening, the dough is cooked in a wood-fired oven or, in more urban areas, in a common electric oven (Lino *et al.*, 2007). The most famous, typical, and highly consumed variety of *broa* from Porto region is the Avintes *broa*, made of equal parts of corn and rye. It is distinguished from the conventional one not only by the flavour and dark colour, provided by the added rye, but also by the soft and smooth texture and moist consistency.

Plain wheat (white) bread along with rye, fibre-, oat-, corn-, soy-, and raisins-enriched bread will be henceforth identified as common bread, to distinguish them from *broa* (maize bread).

# 2. Materials and methods

## 2.1. Bread sampling

The bread samples were commercially purchased in bakeries, supermarkets, and other commercial surfaces. Collection occurred during the seasons of two consecutive years, specifically: winter of 2007/2008, summer of 2008, winter of 2008/2009, and summer of 2009. As in the following studies (B and C) collection of winter samples was carried out in the period between December and March of the subsequent year, whereas that of summer samples occurred in the period between June and September. The collection of bread samples took place in four mainland regions, Porto, Coimbra, Lisboa and Alentejo, which were also simultaneously surveyed in the following studies (B and C). In Alentejo, *broa* was not collected because of the uncommon commercialization and consumption.

All information concerning the samples was obtained from their respective labels. The sample size, regions and types of bread considered are summed up in Table A.1.

**Table A.1.**
Distribution of the analysed samples by region, season and type of bread.

| Region | Winter 2007/2008 | | Summer 2008 | | Winter 2008/2009 | | Summer 2009 | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | **Common** | ***Broa*** | **Common** | ***Broa*** | **Common** | ***Broa*** | **Common** | ***Broa*** | |
| Porto | 47 | 46 | 48 | 22 | 25 | 34 | 30 | 40 | **292** |
| Coimbra | 30 | 15 | 31 | 14 | 29 | 14 | 27 | 13 | **173** |
| Lisboa | 36 | 5 | 43 | 8 | 42 | 10 | 34 | 5 | **183** |
| Alentejo | 15 | n.c. | 33 | n.c. | 22 | n.c. | 20 | n.c. | **90** |
| **Total** | **128** | **66** | **155** | **44** | **118** | **58** | **111** | **58** | **738** |

(n.c.: non-collected)

Immediately after collection, each sample was finely grinded and mixed thoroughly to assure complete homogenization, and stored in plastic bags at -20 ºC until extraction.

## 2.2. Bread consumption data

From the literature consulted, the three available individual consumption patterns were given by the outcome of a national survey carried out in 1994 - 87.7 g/day (*Instituto Português do Consumidor* - IPC, 2005), the definition of European diet that established a 117.2 g/day intake of white bread and 58.6 g/day of whole grain bread (GEMS/Food, 2003), and by a local dietary survey on adult residents in Porto - 113.6 and 136 g/day for women and men, respectively (Lopes *et al.*, 2006).

In face of some most needed missing data some assumptions were made (Kroes *et al.*, 2002). First in the absence of recent data regarding the differential consumption of the different types of bread, the consumption of maize bread was considered as representing a quarter of the total consumption of bread, in the regions of Porto, Coimbra and Lisboa according to the data provided by IPC (2005). Second, no critical groups of the population were taken into account. The only study with sufficient data to support this differentiation was available only for Porto region, and only for gender and adult age (Lopes *et al.*, 2006).

## 2.3. Estimation of daily intake

The exposure of OTA through bread consumption was calculated using the information about body weight (65 kg, according to Miraglia & Brera, 2002) and average annual bread consumption (according to the three mentioned sources) as goes after: $EDI = (\sum c)(CN^{-1} D^{-1} K^{-1})$, where EDI is the estimated daily intake, $\sum c$ is the sum of OTA concentration in the analysed samples, C is the mean annual intake estimated, N is the number of analysed samples, D is the number of days in a year, and K is the mean body weight.

The percentage of tolerable daily intake (TDI) from the consumption of bread was calculated as follows: %TDI = EDI/TDI x 100, where TDI was derived from the tolerable intake values established by EFSA, at 120 ng/kg bw/week (EFSA, 2006), JECFA, at 100 ng/kg bw/week (JECFA, 2007), and NWGFTRE, at 5 ng/kg bw/day (NWGFTRE, 1991).

## 2.4. Reagents and materials

### 2.4.1. Materials and equipments

Whatman No. 4 filter paper (150 mm; Whatman International Ltd., Maidstone, UK) and polyamide membrane filters (0.2 µm, 50 mm; Whatman GmbH, Dassel, Germany) were used. IAC Ochratest$^{TM}$ were from VICAM (Watertown, MA, USA).

A Moulinex blender 700W (230–240 V, 50–60 Hz, Barcelona, Spain), a Braun MR 5000 M multiquick/minipimer 500W (220–230 V, 50–60 Hz, Esplungues del Llobregat, Spain), a vacuum manifold of Macherey–Nagel (Düren, Germany), a Dinko pump (mol. D-95, 130 W, 220 V), a magnetic stirrer (Agimatic-S, Selecta, Barcelona, Spain), a Retsh vortex mixer (Haan, Germany), and a Sonorex RK 100 ultrasonic bath (Berlin, Germany) were employed.

LC/FD assay was performed on a Perkin-Elmer Model LS45 spectrofluorimeter (Beaconsfield, UK), and results were recorded on a Hewlett-Packard 3390A integrator (Philadelphia, PA, USA). The LC instrument was equipped with a pump (Model 307; Gilson Medical Electronics, Villiers-le-Bel, France), and a Hichrom HI-173 guard column (30x4 mm internal diameter, ID); Hichrom Ltd., Reading, England) preceding a Hichrom C18 column (5 µm, 250x4.6 mm ID).

LC/MS/MS assay was carried out on a LCQ Advantage MAX mass spectrometer coupled to a Thermo Finnigan Surveyor MS Pump plus and a Thermo Finnigan Surveyor autosampler (Thermo Finnigan, San Jose, California, USA). The analytical column (Waters Spherisorb ODS2; 3 µm, 150x2.1 mm ID) was preceded by a guard cartridge (Waters Spherisorb ODS2; 5 µm, 10x4.6 mm ID; Waters Corporation, Milford, Massachusetts, USA).

As OTA is UV-decomposable, special care was taken to protecting it from daylight, through the use of amber glass material (Valenta *et al.*, 1998; Yang *et al.*, 2010).

### 2.4.2. Reagents, standards and solutions

HPLC-grade acetonitrile and methanol (Panreac Química Sau, Barcelona, Spain), toluene (Baker Analyzed, J.T. Baker, Holland) and benzene (HACH Company, Loveland, USA) were used. Analytical grade reagents were comprised of boron trifluoride-methanol 14 % solution, acetic acid (Sigma-Aldrich, Laborchemikalien,

Germany), chloric acid, sodium hydroxide, potassium chloride, potassium dihydrogenphosphate and anhydrous disodium hydrogen phosphate (Merck, Darmstadt, Germany) and sodium chloride (Baker Ltd., Pagenham, UK).

The OTA standard from *A. ochraceus* was obtained from Sigma Chemical Co. (St. Louis, MO, USA) with ≥ 98% purity, and OTB standard solution, at 50 µg/mL, from Supelco (Bellefont, PA, USA).

The OTA standard stock solution was prepared by diluting 1 mg of OTA from *A. ochraceus* in 4 mL of toluene:acetic acid (99:1) at 250 µg/mL, and stored at -20 °C. The intermediate solutions were prepared at 10 µg/mL and 1 µg/mL, in toluene:acetic acid. The working standard solutions were prepared at 0.1, 0.01 and 0.005 µg/mL in mobile phase. Calibration curve standard solutions were prepared in mobile phase at concentrations of 0.001, 0.002, 0.005, and 0.01 µg/mL for LC/FD analysis and 0.016, 0.024, 0.032, 0.048, and 0.064 µg/mL for LC/MS/MS analysis.

An OTB intermediate standard solution at 10 µg/mL was prepared by diluting the stock solution at 50 µg/mL in benzene:acetic acid (99:1). One working solution was prepared at 0.1 µg/mL by evaporation and subsequent reconstitution in mobile phase, which was then used to prepare another solution at 0.025 µg/mL.

Furthermore, a combined standard solution containing 0.005 µg/mL of OTA and 0.025 µg/mL of OTB was prepared.

PBS solution was prepared by diluting 0.2 g potassium chloride, 0.2 g potassium dihydrogenphosphate, 1.2 g anhydrous disodium hydrogen phosphate, and 8 g sodium chloride in 1 L of distilled water, with pH adjusted to 7.4 through the use of 0.1 M HCl and 0.1 M NaOH.

The mobile phase, common to the LC/FD and LC/MS/MS assays, was a vacuum-filtered solution of acetonitrile/water/acetic acid (49.5:49.5:1.0, v/v/v).

All liquid chromatographic reagents and solutions were vacuum-filtered in polyamide membrane filters and degassed for 15 min in an ultrasonic bath prior to use.

Water was obtained daily from a Milli-Q system (Millipore, Bedford, MA, USA).

## 2.5. Safety measures

The safety precautions applicable to OTA handling, through personal protective equipment and waste disposal were observed throughout all the experimental work.

Hazardous wastes were treated with sodium hypochlorite and safely stored in accurate labelled residue containers regularly collected and disposed through an employed certified company.

Routine implemented decontamination procedures of the glassware and materials used in OTA analysis included immersion in sodium hypochlorite solution, before detergent washing followed by an abundant rinsing with water, and a final rinsing with distilled water (Valenta *et al.*, 1998; Juan *et al.*, 2007).

## 2.6. Experimental procedure

The method used for determining the OTA content of the bread samples was based on that described by Juan *et al.* (2007), entailing an IAC clean-up step and LC/FD determination.

### 2.6.1. Sample extraction and clean-up

For extraction 100 mL of the PBS:methanol solution (50:50, v/v) were added to 20 g of milled bread and then homogenised for 5 min and filtered by gravity through a filter paper. A 20 mL aliquot of the filtrate was diluted with 30 mL of PBS and then 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 3 mL of methanol.

The eluates were dried in a bath at 50 ºC under a gentle nitrogen flow, and the dried extracts were stored at -20 ºC.

### 2.6.2. Immunoaffinity columns regeneration

Immediately after their first use, IACs were regenerated according to the procedure of Scott & Trucksess (1997), modified by Pena *et al.* (2006). They were cleaned-up by passage with 5 mL of methanol followed by 20 mL of PBS solution. The regenerated IACs were stored filled with PBS at 4 ºC. This regeneration was never made more than once.

### 2.6.3. Detection and quantification

For LC/FD analysis the dried extracts were dissolved in 250 µL of mobile phase. A 20 µL (full loop) injection volume was used with the mobile phase flowing at 1 mL/min

(isocratic elution). Wavelengths used were 333 nm for excitation and 460 nm for emission, both with a spectral bandwidth of 10 nm.

The use of an OTA external standard, at a concentration of 0.01 µg/mL allowed the identification and quantification of the OTA detected in the bread samples.

### 2.6.4. Analytical quality assurance

Performance parameters were determined under repeatability conditions, namely with same bread sample, same operator, same apparatus, same laboratory, and during the course of three days. OTA-devoid bread samples were spiked in triplicate at 0.1, 0.5 and 2.0 µg/kg, and were left in the dark for 15 min before applying the aforementioned protocol of extraction, clean-up and analysis.

Recovery was calculated as the percentage mean recovery from three results at each spike level. The RSD was calculated from results generated within-day/intra-day ($RSD_w$) and between-day/inter-day ($RSD_b$) (CEC, 2006a).

The LOQ was determined as the lowest OTA concentration at which a blank sample could be spiked with OTA standard solutions and still originate accurate and repeatable results up to bylaw standard (CEC, 2006a), through the application of the experimental procedure (signal-to-noise ratio of approximately 10:1).

Linear regression of the peak area *versus* the OTA concentration was performed by least squares method, using three determinations of working standard solutions at four concentration levels, namely 0.001, 0.002, 0.005, and 0.01 µg/mL.

For OTB, fortification levels at 2 and 5 µg/kg were evaluated.

## 2.7. Confirmation procedures

For confirmation, methyl ester derivatization and LC/MS/MS analysis were both applied to the most highly contaminated samples, specifically the ones above 1.4 µg/kg. The former procedure performed as a qualitative confirmation only, and the latter as a quantitative confirmation procedure.

### 2.7.1. Methyl ester derivatization

OTA presence was confirmed as described by Guillamont *et al.* (2005) and Pena *et al.* (2005), by conversion into its methyl ester form, adding 150 µL of boron trifluoride-methanolic 14 % solution ($BF_3$-$CH_3OH$) to the dried extracts, obtained as described in

section 2.6.1. The mixture was evaporated at 60 ºC for 10 min, before reconstitution in 250 µL of mobile phase.

The OTA methyl esters were analysed through LC/FD according to the abovementioned conditions (*vide* section 2.6.3).

### 2.7.2. LC/MS/MS

For the LC/MS/MS assay the dried extracts, obtained after the described extraction and clean-up procedures (*vide* section 2.6.1) were dissolved in 250 µL mobile phase. A 20 µL (partial loop) injection volume was used with the mobile phase isocratic flow maintained at 200 µL/min.

Mass spectrometer was operated in the positive ESI mode using SRM scanning mode. Source and capillary temperatures were set at 0 and 200 ºC and voltages at 5 and 32 V, respectively. Nitrogen was used as nebulising gas, with a sheath gas flow of 80 (arbitrary unit) and the auxiliary sweep gas flow of 20 (arbitrary unit). Collision gas was Helium with a normalised collision energy of 25 %.

Precursor-to-fragment transitions used were the following: $[M+H]^+ \rightarrow [M+H-HCOOH]^+$ (*m/z* 404 → *m/z* 358) and $[M+H]^+ \rightarrow [M+H-H_2O]^+$ (*m/z* 404 → *m/z* 386).

For interpretation of results, the calibration curve of fortified bread samples was used to quantify OTA concentrations. This curve was constructed by plotting OTA peak area against fortified bread samples at different concentrations. The relative abundance of the selected product ions, expressed as a percentage of the abundance of the most abundant ion ($[M+H-HCOOH]^+$; *m/z* 358), was 60 % ($[M+H-H_2O]^+$; *m/z* 386) and 20 % ($[M+H]^+$; *m/z* 404).

Recovery experiments were performed during the course of three days, spiking OTA-blank bread samples in triplicate at addition levels of 1.0, 1.5, 2.0, 3.0, and 4.0 µg/kg, and leaving them to stand in the dark for 15 min before applying the aforementioned protocol of extraction, clean-up and analysis. These levels of fortification were also used to construct a calibration curve.

Linearity was evaluated using a calibration curve constructed by plotting OTA peak area *versus* OTA concentration of standard solutions determined in triplicate at five levels of 0.016, 0.024, 0.032, 0.048, and 0.064 µg/mL.

Both matrix and standard calibration curves were used to calculate the matrix effect (ME) for OTA in bread, through the signal suppression-enhancement as described by Rubert *et al.* (2011). The percentage of the matrix-matched calibration slope (B) 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.

The LOD and LOQ were calculated through the matrix-matched calibration curve as $|3.3S_{y/x}|/b$ and $|10.S_{y/x}|/b$, respectively, where *b* is the slope and $S_{y/x}$ the residual standard deviation of linear function. The latter was calculated as follows:

$$S_{y/x} = \sqrt{\frac{SS}{N-2}}$$

where SS is the sum of the square of the vertical distances of the points from the curve and N is the number of fortification levels of the standard solutions.

## 2.8. Statistical analysis

For statistical analysis, when the concentration was below the LOQ it was set to 50 % of that limit.

All data produced during this work was analysed with the support of appropriate statistical tools.

Whenever two independent samples had to be compared in relation to OTA levels, the parametric test used was the t test for independent samples. If the parametric method was not recommended, the non-parametric methods used were the Kolmogorov-Smirnov two-sample test and the Mann-Whitney U test (also known as the Wilcoxon test). In situations where doubts persisted after the utilization of the latter, confirmations were carried out using the Kolmogorov-Smirnov two-sample test. If more than two samples had to be compared, the parametric statistic was the one-way ANOVA, and the non-parametric equivalents were the Kruskal-Wallis ANOVA by ranks and the Median test.

Significative ANOVAs where followed by student's t tests and/or Tukey HSD post hoc test for multiple comparisons. Significative Kruskal-Wallis ANOVA by ranks were followed by Kruskal multiple comparison tests.

In order to compare two dependent samples in relation to OTA levels, the statistical methodologies applied were the t test for dependent samples (a parametric technique), the Sign test and the Wilcoxon's matched pairs test (both non-parametric techniques).

For the purpose of comparing multiple dependent samples, repeated measures ANOVA was used for the parametric situations and the Friedman's two-way analysis of variance was used in the non-parametric cases.

Correlations between variables were studied mainly in the search for possible trends between OTA levels and one or more categorical determinants under study, such as season, region or year. Due to the non-parametric nature of nearly of the data, the Spearman's R and Kendall's Tau were used to assess those trends, instead of the common Person's coefficient of correlation.

In many situations, and in contrast to what happens with parametric techniques, for a given problem, two or more non-parametric techniques are available. Therefore, all non-parametric methods available for any given type of problem were usually applied, together with scatter plots or box and whisker plots, in order to be able to fully understand the data characteristics.

All these statistical techniques were applied as implemented in the Statistica$^{TM}$ for Windows statistical package (2004). The theoretical support for the non-parametric methods can be found in Conover (1999) and the strategies for the utilization of analysis of variance (ANOVA) followed Roberts & Russo (1999).

# 3. Results and discussion

## 3.1. Analytical quality assurance

Recovery for spiked bread samples ranged from 76.7 to 103.7 %, whereas the within-day RSD between 3.6 and 14.4 % and the between-day RSD between 7.2 and 12.9 % (*vide* Table A.2). These results of accuracy and precision were found to lie within the acceptable range for all test samples, and so the method was shown to perform in line with current EU regulation (CEC, 2006a).

Repeatable precision and trueness were achieved at minimum concentration of 0.1 μg/kg, thus laying down the LOQ.

With the employed method peak area responded to OTA concentration in the 0.001-0.01 μg/mL range with clear linearity ($R^2$=0.9998).

Absence of any interfering peak at the retention time of OTA (about 11:30 min) was constant, as clearly evident in Figure A.1, depicting typical chromatograms obtained from an OTA standard solution and a naturally contaminated bread sample.

**Table A.2.**
Performance data obtained for triplicate OTA spiked bread in within- and between-day variability assays.

| **Spiking level (µg/kg)** | **0.1** | **0.5** | **2.0** |
|---|---|---|---|
| Mean recovery (%) | 103.7 | 98.6 | 76.7 |
| RSD $_{within-day}$ (%) | 14.4 | 3.6 | 4.5 |
| RSD $_{between-day}$ (%) | 10.8 | 7.2 | 12.9 |

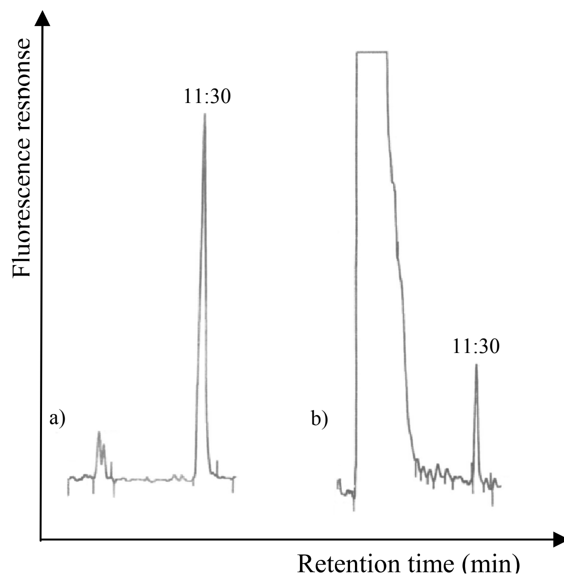


**Figure A.1.** HPLC/FD chromatograms obtained after the described experimental procedure, from a) OTA standard solution (0.005 µg/mL), and b) bread sample naturally positive to OTA (Retention time 11:30 min).

## 3.2. Bread contamination

Main descriptive statistics regarding the contamination of bread are summarised in Table A.3. Frequency of contamination ranged between 60 % and 100 %, but it was for the most part above 80 %. Maximum levels varied from 0.104 to 3.848 µg/kg, the latter surpassing current EU maximum levels permitted in bread (3 µg/kg).

Standard deviations were fairly high, exceeding in some instances its corresponding mean value. This signifies that OTA content values were distributed over a large range, and even though the maximum values, were in general below the legal limit, this indicates a marked variability in contamination levels between analysed bread samples.

A discrepancy between mean and median values was also noticeable in nearly all cases. As most of the results were asymmetrical, parametric statistics could not be used in those cases. In such circumstances, mean levels and standard deviations lose value, with ensuing errors. For that reason, median and inter-quartile range or in alternative, median, quartiles, minimum and maximum values were used.

This survey provided results that can be analysed from a seasonal and regional point of view, according to the type of bread considered or even in respect to the main cereal included in the bread making.

Starting with a seasonal analysis, it was discernible that whether taking into account frequency of contamination or median values, there was a general increase from winter to summer, although not always noticeable (Figure A.2).

Despite the test of Kruskal-Wallis ANOVA by Ranks verified statistically significant differences between seasons, these were random, whether considering common bread ($p>0.0003$), or *broa* ($p<0.0000$). Thus, in presence of favourable conditions, production of mycotoxins can occur, regardless the season in question. Exposure to this mycotoxin through bread consumption cannot therefore be truthfully predicted.

Analysing the results from a regional point of view, marked lower levels of contamination were observed in common bread commercialised in Alentejo. However, statistically significant differences were only found between Alentejo and Coimbra (Kruskal-Wallis test; $p=0.0067$) and Alentejo and Porto (Kruskal-Wallis test; $p=0.0012$). Differences were also observed between bread from Porto and Lisboa (Kruskal-Wallis test; $p=0.054$). Regarding OTA contamination in maize bread, there was no statistically significant difference between the three different surveyed regions (Kruskal-Wallis test; $p=0.0895$).

**Table A.3.**
Descriptive statistics regarding OTA occurrence and contamination levels in common and maize bread according to the region and season of collection.

| Collection season | Region | Bread type | Sample size | Positive (%) | Maximum (µg/kg) | ≥ LOQ (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Number (%) | Mean±SD (µg/kg) | Median (µg/kg) |
| **Winter 2007/2008** | Porto | *Broa* | 46 | 38 (82.6 %) | 3.848 | 32 (69.6 %) | 0.393±0.646 | 0.250 |
| | | Common | 47 | 42 (89.4 %) | 0.889 | 22 (46.8 %) | 0.187±0.164 | 0.158 |
| | Coimbra | *Broa* | 15 | 13 (86.7 %) | 0.945 | 9 (60.0 %) | 0.481±0.286 | 0.569 |
| | | Common | 30 | 24 (80.0 %) | 1.509 | 16 (53.3 %) | 0.338±0.337 | 0.220 |
| | Lisboa | *Broa* | 5 | 4 (80.0 %) | 0.356 | 4 (80.0 %) | 0.276±0.087 | 0.296 |
| | | Common | 36 | 24 (66.7 %) | 0.406 | 17 (47.2 %) | 0.210±0.090 | 0.166 |
| | Alentejo | Common | 15 | 9 (60.0 %) | < LOQ | 0 | n.a. | n.a. |
| **Summer 2008** | Porto | *Broa* | 22 | 19 (86.4 %) | 1.423 | 15 (68.2 %) | 0.580±0.453 | 0.423 |
| | | Common | 48 | 48 (100 %) | 0.479 | 34 (70.8 %) | 0.206±0.095 | 0.187 |
| | Coimbra | *Broa* | 14 | 14 (100 %) | 0.436 | 8 (57.1 %) | 0.273±0.107 | 0.304 |
| | | Common | 31 | 31 (100 %) | 0.708 | 16 (51.6 %) | 0.232±0.141 | 0.194 |
| | Lisboa | *Broa* | 8 | 7 (87.5 %) | 0.304 | 2 (25 %) | 0.266±0.053 | 0.266 |
| | | Common | 43 | 31 (72.1 %) | 0.813 | 17 (39.5 %) | 0.218±0.170 | 0.154 |
| | Alentejo | Common | 33 | 32 (97.0 %) | 0.204 | 10 (30.3 %) | 0.158±0.028 | 0.159 |
| **Winter 2008/2009** | Porto | *Broa* | 34 | 28 (82.4 %) | 0.201 | 4 (11.8 %) | 0.149±0.037 | 0.142 |
| | | Common | 25 | 24 (96.0 %) | 0.142 | 2 (8.0 %) | 0.121±0.029 | 0.121 |
| | Coimbra | *Broa* | 14 | 14 (100 %) | 0.104 | 1 (7.1 %) | 0.104 | 0.104 |
| | | Common | 29 | 27 (93.1 %) | 0.316 | 11 (37.9 %) | 0.166±0.068 | 0.136 |
| | Lisboa | *Broa* | 10 | 7 (70.0 %) | 0.382 | 1 (10.0 %) | 0.382 | 0.382 |
| | | Common | 42 | 42 (100 %) | 1.638 | 17 (40.5 %) | 0.250±0.362 | 0.148 |
| | Alentejo | Common | 22 | 21 (95 %) | 0.223 | 5 (22.7 %) | 0.152±0.049 | 0.150 |
| **Summer 2009** | Porto | *Broa* | 40 | 38 (95 %) | 1.480 | 30 (75.0 %) | 0.495±0.492 | 0.182 |
| | | Common | 30 | 30 (100 %) | 0.287 | 20 (66.7 %) | 0.170±0.042 | 0.165 |
| | Coimbra | *Broa* | 13 | 12 (92.3 %) | 0.238 | 3 (23.1 %) | 0.180±0.061 | 0.185 |
| | | Common | 27 | 26 (96.3 %) | 0.390 | 15 (55.6 %) | 0.196±0.076 | 0.183 |
| | Lisboa | *Broa* | 5 | 4 (80 %) | 0.673 | 4 (80.0 %) | 0.397±0.184 | 0.313 |
| | | Common | 34 | 33 (97.1 %) | 0.247 | 8 (23.5 %) | 0.170±0.049 | 0.157 |
| | Alentejo | Common | 20 | 20 (100 %) | 0.307 | 10 (50.0 %) | 0.180±0.063 | 0.179 |

(n.a.: non-applicable; SD: standard deviation)

**Table A.3. (Continued)**
Descriptive statistics regarding OTA occurrence and contamination levels in common and maize bread according to the region and season of collection.

| **Collection season** | **Region** | **Bread type** | **Sample size** | **Positive (%)** | **Maximum (µg/kg)** | **≥ LOQ (%)** | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | **Number (%)** | **Mean±SD (µg/kg)** | **Median (µg/kg)** |
| **Four collection seasons** | Porto | *Broa* | 142 | 123 (86.6 %) | 3.848 | 81 (57.0 %) | 0.454±0.543 | 0.223 |
| | | Common | 150 | 144 (96.0 %) | 0.889 | 78 (52.0 %) | 0.189±0.109 | 0.166 |
| | Coimbra | *Broa* | 56 | 53 (94.6 %) | 0.945 | 21 (37.5 %) | 0.341±0.234 | 0.286 |
| | | Common | 117 | 108 (92.3 %) | 1.509 | 58 (49.6 %) | 0.239±0.204 | 0.191 |
| | Lisboa | *Broa* | 28 | 22 (78.6 %) | 0.673 | 11 (39.3 %) | 0.328±0.130 | 0.307 |
| | | Common | 155 | 130 (83.9 %) | 1.638 | 59 (38.1 %) | 0.218±0.218 | 0.160 |
| | Alentejo | Common | 90 | 90 (100 %) | 0.307 | 25 (27.8 %) | 0.166±0.048 | 0.159 |
| | **Four regions** | **Total** | **738** | **662 (89.7 %)** | **3.848** | **333 (45.1 %)** | **0.280±0.324** | **0.181** |

(n.a.: non-applicable; SD: standard deviation)

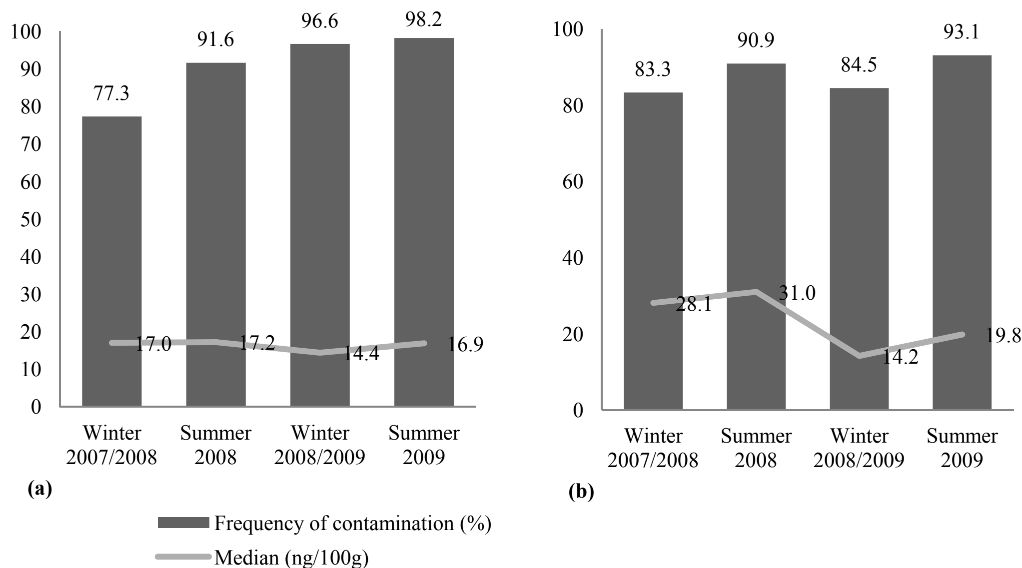


**Figure A.2.** Contamination of common (a) and maize (b) bread, with frequency of contamination (%) depicted in bars and the median OTA level (ng/100 g) with points united in a continuous line to assist in comparison.

The importance of studying the regional differences between bread products, whether regionally typical or simply regionally produced is because each type of bread presents different $a_w$ and pH levels, according to the type of grains or ingredients included, the yeast strain employed or the different practices of making the dough, baking (time/temperature) and the size of the bread. So, different conditions result in different rates of fungal growth and production of OTA (Arroyo *et al.*, 2005; Valle-Algarra *et al.*, 2009).

Common bread showed less than half of the average level (0.208 *versus* 0.420 µg/kg) and maximum value (1.638 *versus* 3.848 µg/kg) of *broa*. However, both shared similar incidences of contamination. When individually analysed, each region presented average values of contamination lower for common bread in comparison to *broa*. Mann-Whitney U test confirmed highly significant differences between common bread and *broa* in all seasons (winter of 2007/2008; p=0.000009, summer of 2008; p=0.0381, winter of 2008/2009; p=0.001148, summer of 2009 p=0.005468).

A possible explanation for the differences in contamination may lie in the nutritional composition of the various grains. For instance, Zummo & Scott (1992) identified maize as a grain that is highly vulnerable to fungal contamination, as it has an ideal

nutrient composition to serve as substrate for fungal growth. The chemical composition of each cereal grain used might explain the different susceptibilities of fungal growth. However these differences in chemical composition cannot be ascertained since considerable differences in nutrient contents exist for the same food commodity, between different ecological zones and even within countries. Reasons can be variation in temperature, rainfall and access to water, use of fertilizer, nutrient content of the soil, as well as different species (Barikmo *et al.*, 2007).

For common and maize bread, different types were studied, given that, as mentioned before, different bread products might present different features such as $a_w$ and pH levels and grains included.

When the samples of common bread are divided according to their main composing cereal (*vide* Table A.4), and considering the four collection regions as one, it was observed that the bread samples assembled in the category “others”, featured an incidence of 100 % and the highest average levels. Nevertheless, such category was composed by a small number of samples, in a miscellaneous assembly, given that it included oat-, corn-, soy- and raisins-enriched bread. Thus, by excluding this category, whole grain bread appeared as the most frequently contaminated, and featured the highest maximum and average levels. On the other end of the scale, wheat bread, composed exclusively – or almost so – of wheat, featured the lowest mean and median value. Comparing with wheat bread, rye bread presented comparable frequency of contamination (about 90 %), and a slightly higher average contamination level. Nevertheless, it presented the highest median level.

Considering each season individually, differences between cereals included were not robust determinants. In fact, significant differences were only found in winter of 2007/2008 between wheat and whole grain (Kruskal-Wallis ANOVA by ranks: $p=0.0313$; median multiple comparison tests: $p=0.0633$). The same analysis in the following seasons did not reveal any difference between each main type of composing cereal.

The source of bread contamination can be a direct one, since OTA present in contaminated flour is not sufficiently eliminated during baking and yeast fermentation (Valle-Algarra *et al.*, 2009), or an indirect one, since in the place where flour is manipulated, simultaneously with baked bread, an indirect contamination can occur through aerosol.

**Table A.4.**
Occurrence and levels (µg/kg) of OTA in common bread according to the prevailing cereal grain included or added ingredient in the four sampled seasons.

| Collection season | Bread by main cereal | Sample size | Positive (%) | Maximum (µg/kg) | ≥ LOQ (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Number (%) | Mean±SD (µg/kg) | Median (µg/kg) |
| **Winter 2007/2008** | Wheat | 86 | 64 (74.4 %) | 0.478 | 31 (36.0 %) | 0.189±0.086 | 0.169 |
| | Rye | 32 | 25 (78.1 %) | 0.423 | 16 (50.0 %) | 0.219±0.101 | 0.204 |
| | Whole grain [a] | 7 | 7 (100 %) | 0.476 | 6 (85.7 %) | 0.223±0.159 | 0.136 |
| | Others [b] | 3 | 3 (100 %) | 1.509 | 2 (66.7 %) | 1.199±0.438 | 1.199 |
| | **Total (Winter 2007/2008)** | **128** | **99 (77.3 %)** | **1.509** | **55 (43.0 %)** | **0.238±0.220** | **0.170** |
| **Summer 2008** | Wheat | 124 | 112 (90.3 %) | 0.813 | 56 (45.2 %) | 0.207±0.118 | 0.169 |
| | Rye | 20 | 20 (100 %) | 0.708 | 14 (70.0 %) | 0.233±0.154 | 0.189 |
| | Whole grain [a] | 9 | 8 (88.9 %) | 0.218 | 6 (66.7 %) | 0.165±0.039 | 0.164 |
| | Others [c] | 2 | 2 (100 %) | 0.149 | 1 (50.0 %) | 0.149 | 0.149 |
| | **Total (Summer 2008)** | **155** | **142 (91.6 %)** | **0.813** | **77 (49.7 %)** | **0.208±0.120** | **0.172** |
| **Winter 2008/2009** | Wheat | 95 | 91 (95.8 %) | 0.316 | 28 (29.5 %) | 0.160±0.061 | 0.139 |
| | Rye | 11 | 11 (100 %) | 0.224 | 4 (36.4 %) | 0.151±0.059 | 0.140 |
| | Whole grain [a] | 12 | 12 (100 %) | 1.638 | 3 (25.0 %) | 0.670±0.840 | 0.223 |
| | **Total (Winter 2008/2009)** | **118** | **114 (96.6 %)** | **1.638** | **35 (29.7 %)** | **0.202±0.256** | **0.144** |
| **Summer 2009** | Wheat | 94 | 93 (98.9 %) | 0.307 | 46 (48.9 %) | 0.174±0.050 | 0.164 |
| | Rye | 5 | 5 (100 %) | 0.257 | 4 (80.0 %) | 0.181±0.073 | 0.182 |
| | Whole grain [a] | 11 | 10 (90.9 %) | 0.191 | 2 (18.2 %) | 0.187±0.005 | 0.187 |
| | Others [d] | 1 | 1 (100 %) | 0.390 | 1 (100 %) | 0.390 | 0.390 |
| | **Total (Summer 2009)** | **111** | **109 (98.2 %)** | **0.390** | **53 (47.7 %)** | **0.179±0.058** | **0.169** |
| **Four collection seasons** | Wheat | 399 | 360 (90.2 %) | 0.813 | 161 (40.4 %) | 0.186±0.088 | 0.161 |
| | Rye | 68 | 61 (89.7 %) | 0.708 | 38 (55.9 %) | 0.213±0.118 | 0.184 |
| | Whole grain [a] | 39 | 37 (94.9 %) | 1.638 | 17 (43.6 %) | 0.277±0.364 | 0.172 |
| | Others [e] | 6 | 6 (100 %) | 1.509 | 4 (66.7 %) | 0.734±0.601 | 0.639 |
| | **Total** | **512** | **464 (90.6 %)** | **1.638** | **220 (43.0 %)** | **0.208±0.169** | **0.165** |

(SD: standard deviation; [a]: includes fibre-enriched bread; [b]: consists of oat- and corn-enriched bread; [c]: consists of soy enriched bread; [d]: consists of raisins-enriched bread; [e]: consists of oat-, corn, soy-, raisins-enriched bread)

Considering the direct source of contamination of bread, the observed difference between wheat and whole grain bread can be explained by the fact that OTA accumulates on and directly beneath the epidermis of grain seeds (Osborne *et al.*, 1996; Rafai *et al.*, 2000). Removal of the surface layers by abrasive scouring or polishing and milling to remove outer layers for white flour production lowers OTA levels as demonstrated by several studies (Osborne *et al.*, 1996; Subirade *et al.*, 1996; Scudamore *et al.*, 2003). Thus white flour for baking contains lower concentrations of OTA than whole meal flour because the bran and offal containing high levels of this mycotoxin have been removed. Further loss during the baking stage is small and so the presence of OTA in bread mainly comes from the wheat flour used for its manufacture (Arroyo *et al.*, 2005; Cabañas *et al.*, 2008). Bran incorporated in the baking mixture can thus be considered a risk factor, as previously confirmed not only in bread (Cengiz *et al.*, 2007; Tam *et al.*, 2011), but also among breakfast cereals (Araguás *et al.*, 2005; Molinié *et al.*, 2005; Kabak, 2009; Villa & Markaki, 2009).

Such data supports the concern about the use of bran in foods, allied to the belief of FAO (2002) that bread consumption particularly that of breads prepared with whole grain flours and with multi-grain flours tends to increase in developed countries. This is mainly due to an increase in a nutritionally conscious population that wants to reduce the consumption of simple carbohydrates, fat and cholesterol while increasing the consumption of complex carbohydrates, dietary fibre and plant proteins (FAO, 2002).

In view of the overall results from all four seasons, common bread made out of rye showed a tendency of higher OTA levels than wheat bread (Kruskal-Wallis test; $p=0.033$). A possible explanation for the differences in contamination may lie in the nutritional composition of the various grains. Previous studies also revealed higher OTA concentration in rye bread (Tam *et al.*, 2011) and raw rye grains (Czerwiecki *et al.*, 2002a; Jørgensen & Jacobsen, 2002) than in their wheat counterparts.

Besides wheat, rye and whole grain, common bread also included bread made out of other cereals, unfortunately with insufficient sample size to reach significance. Such breads revealed high contamination, particularly the oat-based sample (1.509 μg/kg) and one of the corn-based samples (0.889 μg/kg). The latter cereal is usually related to high contamination levels, as the results of the present study showed. Regarding the oat-based bread contamination level, due to small sample size it was unclear whether this value is typical of products derived from that cereal or if the sample was a true outlier,

but merits further investigation. Previous studies reported higher contamination among oat-based cereal derived products, such as in breakfast cereals (Roscoe *et al.*, 2008; Kabak, 2009). Raisins-based bread also presented a high value of contamination. It is noteworthy that several earlier studies pointed out dried grapes as risk factors when incorporated in cereal derived products, namely breakfast cereals (Molinié *et al.*, 2005; Kabak, 2009; Villa & Markaki, 2009), given their highly susceptibility to be contaminated with OTA.

Analysing typical wheat breads, i.e. Mafra and Alentejano bread, the low levels of contamination when comparing with the remaining common wheat breads were noteworthy. In fact, the maximum levels featured by these typical wheat breads are close to the average levels of the remaining common wheat breads. In winter of 2007/2008 none of the typical wheat bread presented a contamination above the LOQ, a fact not observed in the following winter. Furthermore, in the same season, frequency of contamination was very low (around 40 %), particularly for Mafra bread, a situation repeated in the following summer (Table A.5). It was also of note the low variability of contamination levels, as mean and median values were more in agreement. This was particularly visible in Alentejano bread.

Although each type of typical wheat bread was significantly less contaminated than common bread, this difference was far more noticeable with Alentejano bread (Kruskal-Wallis test; $p=0.000345$), than with Mafra bread (Kruskal-Wallis test; $p=0.0313$), as displayed in figure A.3.

These results may also serve to explain some of the differences observed in the regional analysis. Alentejano bread is the type of bread more consumed in Alentejo region, as Mafra bread is vastly consumed in Lisboa region. Given that these typical bread products present lower contamination levels, presumably for their composition limited to wheat (white) flour they may also contribute to a lower exposure in their region of origin, that is to say among the local populations who consume it regularly.

Analysing the contamination of maize bread according to the prevailing cereal grain that is to say maize (conventional), multigrain cereals or rye (Avintes), it was observed that considering all four seasons collectively, rye bread presented the highest average and median levels of contamination (Table A.6).

**Table A.5.**
Occurrence and levels (µg/kg) of OTA in different Portuguese typical wheat breads.

| Collection season | Typical wheat bread | Sample size | Positive (%) | Maximum (µg/kg) | ≥ LOQ (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Number (%) | Mean±SD (µg/kg) | Median (µg/kg) |
| **Winter 2007/2008** | Mafra | 12 | 5 (41.7 %) | < LOQ | 0 | n.a. | n.a. |
| | Alentejano | 13 | 8 (61.5 %) | < LOQ | 0 | n.a. | n.a. |
| **Summer 2008** | Mafra | 11 | 5 (45.5 %) | 0.143 | 2 (18.2 %) | 0.128±0.022 | 0.128 |
| | Alentejano | 29 | 28 (96.6 %) | 0.177 | 7 (24.1 %) | 0.150±0.021 | 0.159 |
| **Winter 2008/2009** | Mafra | 12 | 12 (100 %) | 0.300 | 8 (66.7 %) | 0.190±0.075 | 0.177 |
| | Alentejano | 10 | 9 (90 %) | 0.109 | 1 (10.0 %) | 0.109 | 0.109 |
| **Summer 2009** | Mafra | 7 | 6 (85.7 %) | 0.247 | 1 (14.3 %) | 0.247 | 0.247 |
| | Alentejano | 9 | 9 (100 %) | 0.252 | 3 (33.3 %) | 0.188±0.058 | 0.173 |
| **Four collection seasons** | Mafra | 42 | 28 (66.7 %) | 0.300 | 11 (26.2 %) | 0.184±0.071 | 0.162 |
| | Alentejano | 61 | 54 (88.5 %) | 0.252 | 11 (18.0 %) | 0.157±0.039 | 0.159 |
| | **Total** | **103** | **82 (79.6 %)** | **0.300** | **22 (21.4 %)** | **0.170±0.057** | **0.159** |

(n.a.: non-applicable; SD: standard deviation)

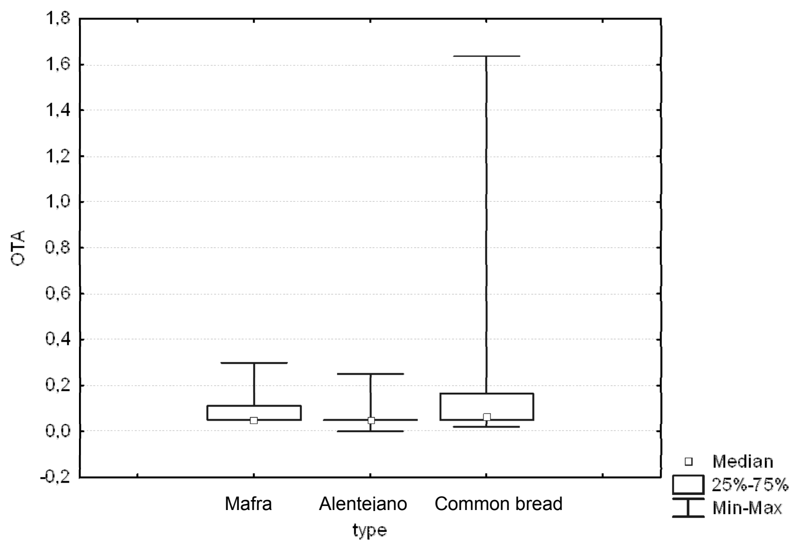


**Figure A.3.** Variation in OTA levels in Mafra, Alentejano and the remaining common bread, displayed as box and whisker plots based on medians, quartiles and extreme values.

**Table A.6.**
Occurrence and levels (µg/kg) of OTA in *broa* according to the prevailing cereal grain included or added ingredient, in the four sampled seasons.

| Collection season | *Broa* by main cereal | Sample size | Positive (%) | Maximum (µg/kg) | ≥ LOQ (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Number (%) | Mean±SD (µg/kg) | Median (µg/kg) |
| **Winter 2007/2008** | Conventional | 36 | 27 (75.0 %) | 0.717 | 21 (58.3 %) | 0.288±0.172 | 0.220 |
| | Multigrain cereals | 7 | 6 (85.7 %) | 0.945 | 5 (71.4 %) | 0.517±0.310 | 0.569 |
| | Avintes | 23 | 22 (95.7 %) | 3.848 | 19 (82.6 %) | 0.494±0.826 | 0.282 |
| | **Total (Winter 2007/2008)** | **66** | **55 (83.3 %)** | **3.848** | **45 (68.2 %)** | **0.401±0.559** | **0.281** |
| **Summer 2008** | Conventional | 31 | 28 (90.3 %) | 0.608 | 15 (48.4 %) | 0.304±0.140 | 0.306 |
| | Multigrain cereals | 5 | 5 (100 %) | 0.436 | 3 (60.0 %) | 0.258±0.154 | 0.170 |
| | Avintes | 8 | 7 (87.5 %) | 1.423 | 7 (87.5 %) | 0.869±0.508 | 0.930 |
| | **Total (Summer 2008)** | **44** | **40 (90.9 %)** | **1.423** | **25 (56.8 %)** | **0.457±0.383** | **0.310** |
| **Winter 2008/2009** | Conventional | 40 | 33 (82.5 %) | 0.382 | 3 (7.5 %) | 0.229±0.141 | 0.201 |
| | Multigrain cereals | 4 | 4 (100 %) | < LOQ | 0 | n.a. | n.a. |
| | Avintes | 14 | 12 (85.7 %) | 0.145 | 3 (21.4 %) | 0.132±0.017 | 0.139 |
| | **Total (Winter 2008/2009)** | **58** | **49 (84.5 %)** | **0.382** | **6 (10.3 %)** | **0.181±0.105** | **0.142** |
| **Summer 2009** | Conventional | 37 | 33 (89.2 %) | 1.086 | 19 (51.4 %) | 0.243±0.225 | 0.154 |
| | Multigrain cereals | 6 | 6 (100 %) | 0.673 | 3 (50.0 %) | 0.382±0.262 | 0.307 |
| | Avintes | 15 | 15 (100 %) | 1.480 | 15 (100 %) | 0.748±0.549 | 0.848 |
| | **Total (Summer 2009)** | **58** | **54 (93.1 %)** | **1.480** | **37 (63.8 %)** | **0.459±0.454** | **0.198** |
| **Four collection seasons** | Conventional | 144 | 121 (84.0 %) | 1.086 | 58 (40.3 %) | 0.274±0.181 | 0.214 |
| | Multigrain cereals | 22 | 21 (95.5 %) | 0.945 | 11 (50.0 %) | 0.410±0.264 | 0.307 |
| | Avintes | 60 | 56 (93.3 %) | 3.848 | 44 (73.3 %) | 0.616±0.677 | 0.344 |
| | **Total** | **226** | **198 (87.6 %)** | **3.848** | **113 (50.0%)** | **0.420±0.474** | **0.269** |

( n.a.: non-applicable; SD: standard deviation)

However, statistically significant differences only arose between conventional and Avintes *broa* (Kruskal-Wallis test; p=0.000021). This difference is even higher when considering this Porto's typical type of *broa* with both regular types (maize and multigrain cereals) taken together (Mann-Whitney U Test; p=0.000004).

As mentioned before, already previous studies reported high contamination in rye grains (Czerwiecki *et al.*, 2002a; Jørgensen & Jacobsen, 2002) and flour (Jørgensen & Jacobsen, 2002).

From all 738 bread samples analysed, it was precisely an Avintes *broa* that surpassed the EU maximum limit (3 µg/kg; CEC, 2006b), with 3.848 µg/kg. Of the remaining ones, 195 (58.6 %) featured an OTA content below 0.20 µg/kg and 13 (3.9 %) values above 1 µg/kg as depicted in figure A.4.

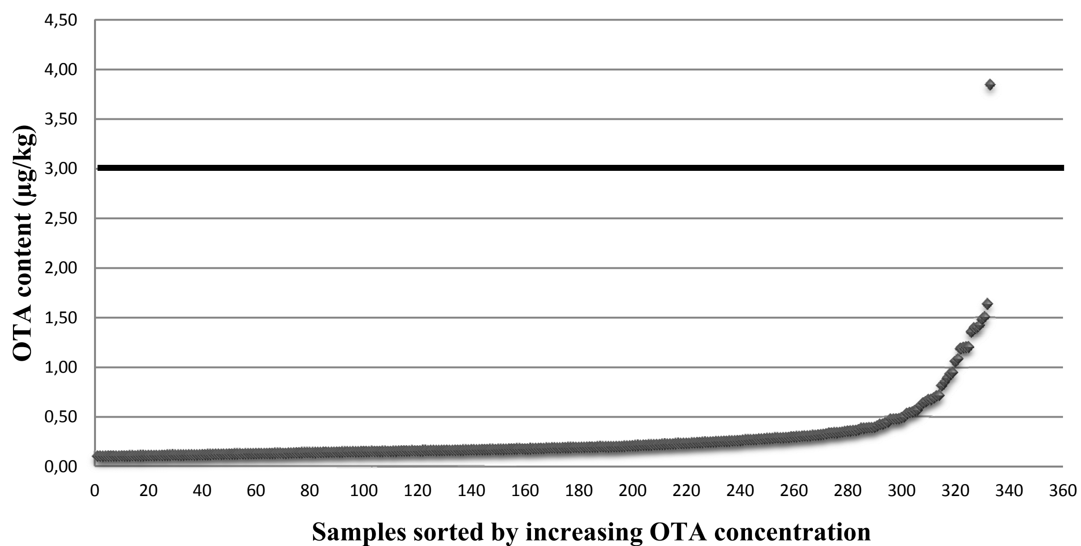


**Figure A.4**. Distribution of OTA concentration values of the total analysed bread samples. Only samples with OTA content above the LOQ (0.1 µg/kg) were depicted. EU maximum level (3 µg/kg) established for bread was represented with a horizontal line.

A comparison between results in this study and from other countries is limited in view of the scarce data (*vide* Table A.7).

**Table A.7.**
Compilation of some international surveys regarding OTA occurrence in bread.

| Country | Bread sample | Positive (%) | LOD (LOQ) | Maximum (µg/kg) | Mean±SD (µg/kg) | Reference |
|---|---|---|---|---|---|---|
| China | n.s. | 1/10 (10 %) | 0.01 (0.03) | 0.01 | 0.01 | Wu *et al.* (2012) |
| Portugal | Wheat bread<br>Wheat bread | 24/30 (80 %)<br>13/20 (65 %) | (0.1) | 0.49<br>0.43 | 0.2±0.1<br>0.3±0.14 | Bento *et al.* (2009) |
| | Maize bread | 21/30 (70 %) | 0.015 (0.03) | - | 0.44 | Juan *et al.* (2008b) |
| | Wheat bread | 4/31 (12.9 %) | | - | 0.02 | |
| | Maize bread | 9/15 (60 %) | (0.033) | - | 0.43 | Juan *et al.* (2007) |
| Spain | Organic wheat bread | 4/20 (20 %) | 0.02 (0.06) | 1.11 | - | González-Osnaya *et al.* (2007) |
| | Other organic cereals bread | 2/6 (33.3 %) | | 0.81 | - | |
| | Conventional wheat bread | 14/67 (20.9 %) | | 19.61 | - | |
| | Other conventional cereals bread | 1/7 (14.3 %) | | 2.59 | - | |
| Turkey | Corn bread | 10 | - | 5.28 | 4.94±0.20 | Cengiz *et al.* (2007) |
| | White bread | 11 | - | 9.75 | 6.38±0.48 | |
| | Whole meal bread | 28 | - | 12.61 | 7.84±0.39 | |
| Morocco | Wheat bread | 48/100 (48 %) | 0.017 (0.051) | 149 | 13.0±1.5 | Zinedine *et al.* (2007) |
| Spain | Bread | 2/20 (10 %) | 0.02 (0.06) | 2.19 | 2.55 | Osnaya *et al.* (2006) |
| Spain | Wheat bread | 0/4 (0 %) | 0.25 (0.75) | n.a. | n.a. | Blesa *et al.* (2004) |
| Germany | Bread | 897 /986 (91 %) | 0.01 | 5.54 | 0.17 | Miraglia & Brera (2002) |
| Spain | Wheat bread | 93/93 (100 %) | 0.005 | 7.37 | 0.45 | Legarda & Burdaspal (2001) |
| Holland | Bread | 29/29 (100 %) | | - | 0.39 | |
| U.S.A. | | 24/24 (100 %) | | - | 0.41 | |
| Switzerland | | 20/20 (100 %) | | - | 0.07 | |
| Brazil | | 15/15 (100 %) | | - | 0.09 | |
| France | | 14/14 (100 %) | | - | 0.25 | |
| Italy | | 12/12 (100 %) | | - | 0.34 | |
| Germany | | 11/11(100 %) | | - | 0.35 | |
| Ireland | | 9/9 (100 %) | | - | 0.36 | |
| Austria | | 9/9 (100 %) | | - | 0.08 | |
| Tunes | | 9/9 (100 %) | | - | 0.30 | |
| Belgium | | 7/7 (100 %) | | - | 0.23 | |

(n.a.: non-applicable; n.s.: non-specified; SD: Standard deviation)

Comparison with national data can only be done with three previous studies. In both studies of Juan *et al.* (2007, 2008b) the sampling area was Coimbra region. Both reported higher average values of contamination, although lower frequencies of contamination in comparison to Coimbra's maize bread as determined in the present study (0.341 µg/kg; 94.6 %). Regarding wheat bread, the study of Juan *et al.* (2008b) determined seven times lower frequency of contamination and average value of contamination when compared to the wheat bread sampled in Coimbra in the present study (92.3 %; 0.152 µg/kg, respectively). Finally, the third previous survey, in Bragança and Algarve regions, reported resembling average levels of contamination but lower frequency of contamination as compared with the overall results of the four regions in the present study, as 0.208 µg/kg and 90.6 %, respectively.

Comparing with foreign data it is noticeable that Portuguese wheat bread is more widely contaminated than that from Spain (González-Osnaya *et al.*, 2007), and Morocco (Zinedine *et al.*, 2007), as shown by a higher OTA frequency of contamination. However, samples from those countries feature a much higher average value than the Portuguese, and thus can present a higher health risk to the consumer. Though the data from other countries such as Germany, USA, Switzerland and Brazil (Legarda & Burdaspal, 2001), was drawn from a sample size that can be considered insufficient, that which does exist gives higher values for both frequency and degree of bread contamination by OTA. The highest levels of contamination were reported in Morocco (Zinedine *et al.*, 2007) and Turkey (Cengiz *et al.*, 2007). On the other end of the scale, the lowest levels of contamination were reported in China (Wu *et al.*, 2012).

In the study of Cengiz *et al.* (2007) statistically significant differences between average OTA levels in white and corn bread samples, and corn and corn whole meal samples were detected, with the latter in each case featuring higher levels. Although in the present study such elevated average levels of contamination of whole grain bread were also noticeable, that was not the case of wheat (white) bread. In fact, in the present study maize bread featured the highest average levels of contamination, exactly the opposite of the results of Cengiz *et al.* (2007), and in line with the one of Juan *et al.* (2007; 2008b).

Although acute human intoxication by OTA is unlikely to occur at most of the OTA levels found in this study, the long term effects of continuous low dose exposure to OTA on human health could be significant (Skaug *et al.*, 1998).

Finally, it is noteworthy that OTA content of bread is believed to be a risk for human health either directly, as a result of people eating contaminated bread, or indirectly, as a result of consumption of products of animals fed with contaminated bread. For instance, in the Italian outbreak responsible for the death of two dogs, several rabbits and chickens in a farm an acute gastroenteritis was the most evident clinical sign. The mouldy bread responsible for the toxicosis was part of restaurant-refused bread, and at that time used in this farm as feed. Only OTA (80 mg/kg) and OTB (9.6 mg/kg) were detected, while AF, patulin, CIT, and PA were not (Visconti & Bottalico, 1983).

## 3.3. Confirmation procedures

As an essential quality assurance requirement, results were confirmed both by methyl ester derivatization and by LC/MS/MS analysis.

### 3.3.1. Methyl ester derivatization

The conversion of OTA to its methyl ester and re-injection allowed confirmation by observation of the almost complete disappearance of the chromatographic peak due to the mycotoxin (11:30 min) and the appearance of a new one corresponding to the methyl ester (OTA-Me). The fluorescing OTA-Me obtained present longer retention time (27:31 min.). Although in each case confirmation of presence and identity was achieved, derivatization was not always complete as shown by the presence of un-reacted OTA (Zimmerli & Dick, 1995), which hindered its use for quantitative purposes (Juan *et al.*, 2007).

### 3.3.2. LC/MS/MS

With the previously described analytical conditions, the LOD and LOQ values obtained through this method were 0.23 and 0.70 µg/kg, respectively.

The RSD values were below 6.67 and 6.97 %, for within- and between-day assays, and the mean recovery values varied between 96.54 and 102.93 %, in the five fortification levels, as summed up in Table A.8. The method was thus shown to fulfil current EU regulation (CEC, 2006b).

**Table A.8.**
Performance data obtained for triplicate OTA spiked bread.

| Spiking level (µg/kg) | 1.0 | 1.5 | 2.0 | 3.0 | 4.0 |
|---|---|---|---|---|---|
| Mean recovery (%) | 96.54 | 101.20 | 102.93 | 98.85 | 99.93 |
| RSD $_{\text{within-day}}$ (%) | 6.64 | 2.22 | 5.13 | 6.57 | 6.67 |
| RSD $_{\text{between-day}}$ (%) | 5.55 | 5.87 | 4.37 | 2.93 | 6.97 |

The calibration curve (y=45848x+41793) of the standard solutions confirmed method linearity in the studied range ($R^2$=0.9975). The matrix-matched calibration curve (y=42806x + 39849) also featured a linear trend with a coefficient of correlation of 0.9991. The matrix effect calculated as described in section 2.7.2 was set at 93.4 %, and so the ensuing signal enhancement was considered not significant (Rubert *et al.*, 2011).

LC/MS/MS chromatograms and corresponding spectra of a bread sample fortified with OTA at 1 µg/kg are depicted in Figure A.5. The LC/MS/MS developed method applied for confirmation of the most contaminated bread samples, as determined by the LC/FD analysis confirmed OTA contamination.

In figure A.6 the LC/MS/MS chromatograms and mass spectrum of a naturally contaminated bread sample are depicted. In general, highest OTA content were found in the LC/FD analysis, as compared with the LC/MS/MS. For instance, the bread sample that presented the highest OTA contamination in the LC/FD analysis (3.848 µg/kg), showed a slightly lower content in the LC/MS/MS (3.57 µg/kg), although still above the EU maximum limit.

As the previously described confirmation method of derivatization, LC/MS/MS method was developed without changes in the pre-analytical step (i.e. in the extraction and clean-up procedures of the bread samples), as compared with the LC/FD method used. Thus the only optimization was performed in the analytical step, which also contributed to the higher LOD and LOQ. However, given that the LC/MS/MS was employed as a confirmatory method of OTA in samples already turned out to be positive by LC/FD analysis these limits obtained were found to be suitable for the purpose. Already previous studies reported better sensitivity for LC/FD, including in bread (Juan *et al.*, 2008b).

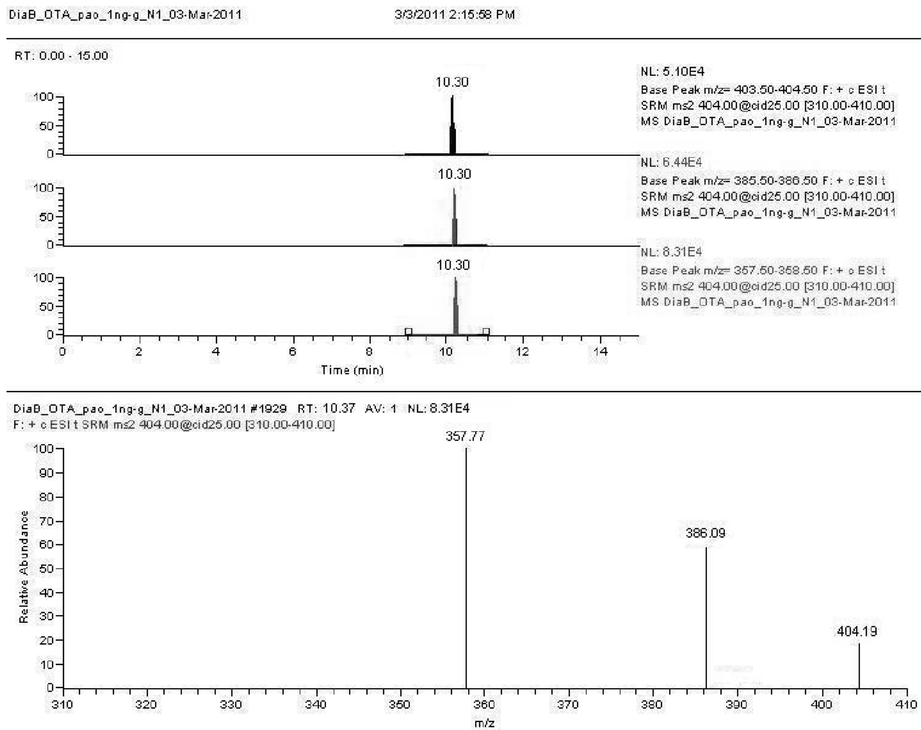


**Figure A.5.** ESI-MS/MS chromatograms and mass spectrum of a blank bread sample fortified at 1µg/kg.

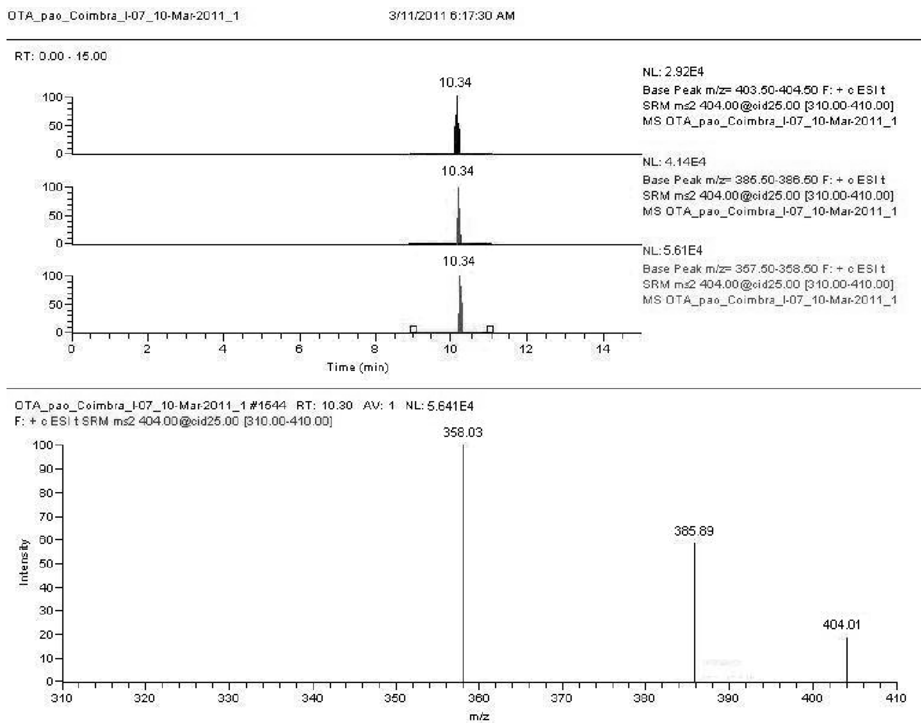


**Figure A.6.** ESI-MS/MS chromatograms and mass spectrum of a naturally contaminated bread sample with 1.14 µg/kg of OTA.

## 3.4. Estimated daily intake

The quantitative evaluation of the likely intake of this contaminant via bread products is summed up in Table A.9. It is clear that bread consumption contributes to higher OTA exposure through common bread than through maize bread that although more contaminated is less consumed. Differences between regions and seasons did not retrieve any significant difference, given that the levels of contaminations were highly variable in the bread samples analysed. Indeed, EDI derived from wheat and maize bread consumption represented 1.23 and 0.83 % of EFSA- (EFSA, 2006) and 1.47 and 0.99 % of JECFA-established TWI (JECFA, 2007), respectively.

**Table A.9.**
OTA estimated daily intake from bread in each studied region based on IPC (2005) reported data.

| **Collection season** | **Region** | **EDI (ng/kg bw/day)** | |
|---|---|---|---|
| | | ***Broa*** | **Common bread** |
| **Winter 2007/2008** | Porto | 0.133 | 0.190 |
| | Coimbra | 0.162 | 0.342 |
| | Lisboa | 0.093 | 0.213 |
| | Alentejo | - | 0 |
| | **Total (Winter 2007/2008)** | 0.135 | 0.241 |
| **Summer 2008** | Porto | 0.196 | 0.208 |
| | Coimbra | 0.092 | 0.234 |
| | Lisboa | 0.090 | 0.220 |
| | Alentejo | - | 0.160 |
| | **Total (Summer 2008)** | 0.154 | 0.210 |
| **Winter 2008/2009** | Porto | 0.050 | 0.123 |
| | Coimbra | 0.035 | 0.168 |
| | Lisboa | 0.129 | 0.253 |
| | Alentejo | - | 0.154 |
| | **Total (Winter 2008/2009)** | 0.061 | 0.205 |
| **Summer 2009** | Porto | 0.167 | 0.172 |
| | Coimbra | 0.061 | 0.199 |
| | Lisboa | 0.134 | 0.172 |
| | Alentejo | - | 0.182 |
| | **Total (Summer 2009)** | 0.155 | 0.181 |
| **Four collection seasons** | **Four regions** | 0.142 | 0.210 |

It is noteworthy that daily bread intake *per capita* falls far behind that recommended by a number of European countries, as 250 g/day (EUFIC, 2011). If the 88 g/day were

replaced by the advised 250 g/day, the estimated intake of wheat and maize bread would be, instead, 0.599 and 0.404 ng/kg bw/day.

Even considering the maximum value (3.85 μg/kg), the EDI (1.30 ng/kg bw/day) would still be bellow the recommended value and at 7.57 % of EFSA- (EFSA, 2006) and at 9.09 % of JECFA-established TWI (JECFA, 2007). On the other hand it would represent roughly 26.0 % of the TDI established at 5 ng/kg bw/day by NWGFTRE (1991).

Previous national studies, all with winter-collected samples, reported estimates of OTA daily intake within the range of the herein reported ones. Wheat bread-based EDI was estimated at 0.20 for Coimbra (Juan *et al.*, 2008b), 0.26 for Algarve and 0.38 ng/kg bw/day for Bragança (Bento *et al.*, 2009), and maize bread-based EDI of 0.16 ng/kg bw/day for Coimbra (Juan *et al.*, 2008b).

Given that the WHO agency GEMS/Food (2003) (Table A.10) settled on a higher intake of bread, a higher contribution to OTA exposure as compared with data provided by IPC (2005) ensued. Indeed, considering that in the present study common bread includes wheat (white) and whole grain such disparity is more evident.

According to the GEMS/Food bread intake in the ‘‘European diet” (175.8 g/person/day on average) as established by this agency is only surpassed by the ‘‘Middle Eastern” one (323 g/person/day on average). So in theory, with the same contamination levels, the people with this last type of diet would be more exposed to OTA through bread consumption.

**Table A.10.**
OTA estimated daily intake from white and whole grain bread based on GEMS/FOOD (2003) reported data.

| Collection season | EDI (ng/kg bw/day) | |
|---|---|---|
| | White bread | Whole grain bread |
| **Winter 2007/2008** | 0.342 | 0.201 |
| **Summer 2008** | 0.374 | 0.148 |
| **Winter 2008/2009** | 0.288 | 0.604 |
| **Summer 2009** | 0.314 | 0.169 |
| **Four collection seasons** | 0.336 | 0.250 |

Based on more recent data provided by a study conducted in the Porto region (Lopes *et al.*, 2006), the EDI was similar to the one calculated from IPC (2005) data taking into account that the earliest includes all types of bread, from wheat to maize bread. Moreover, this study in Porto population found that total consumption of bread is different between genders. Thus ensuing EDI calculations featured a higher value for men (0.396 ng/kg bw/day) than for women (0.331 ng/kg bw/day) (Table A.11).

**Table A.11.**
OTA estimated daily intake from bread in the Porto region according to gender and age based on data reported by Lopes *et al.* (2006).

| Collection season | Age (years) | EDI (ng/kg bw/day) | |
|---|---|---|---|
| | | **Women** | **Men** |
| **Winter 2007/2008** | [18; 39] | 0.261 | 0.364 |
| | [40; 49] | 0.317 | 0.381 |
| | [50; 64] | 0.356 | 0.405 |
| | >65 | 0.354 | 0.406 |
| | **Mean (Winter 2007/2008)** | 0.328 | 0.392 |
| **Summer 2008** | [18; 39] | 0.287 | 0.401 |
| | [40; 49] | 0.349 | 0.419 |
| | [50; 64] | 0.392 | 0.445 |
| | >65 | 0.390 | 0.447 |
| | **Mean (Summer 2008)** | 0.360 | 0.431 |
| **Winter 2008/2009** | [18; 39] | 0.169 | 0.236 |
| | [40; 49] | 0.206 | 0.247 |
| | [50; 64] | 0.231 | 0.262 |
| | >65 | 0.230 | 0.263 |
| | **Mean (Winter 2008/2009)** | 0.212 | 0.254 |
| **Summer 2009** | [18; 39] | 0.237 | 0.331 |
| | [40; 49] | 0.288 | 0.346 |
| | [50; 64] | 0.323 | 0.367 |
| | >65 | 0.321 | 0.368 |
| | **Mean (Summer 2009)** | 0.297 | 0.356 |
| **Four collection seasons** | **Mean (Four collection seasons)** | 0.331 | 0.396 |

Lopes *et al.* (2006) also reported a difference on the consumption of bread between the different studied adult age groups. Whether in women or men, as age increased so did bread consumption, and so did the exposure to this potent nephrotoxic fungal contaminant, that might therefore present an additional risk to the elderly, already fragile by aging-dependent reduced renal function, that functionally results in a decline

in the glomerular filtration rate, because of the decrease in the number of functional nephrons (Chattopadhyay, 2003). It is also worth mentioning that although bread contamination did not show any seasonal pattern, this study showed a bread-derived EDI always lower in winter seasons, whether women or men were considered.

When compared to internationally reported ones, the bread-derived EDI among the Portuguese residents was inferior to equal to the contribution by wheat bread in Spain (0.77 ng/kg bw/day), bread and rolls in Germany (0.36 ng/kg bw/day for adults over 14 years, 0.58 ng/kg bw/day for children under 14 years, and 0.90 ng/kg bw/day for 4-6 years old girls) and rye bread in Denmark (0.50 ng/kg bw/day). This data, drawn together in SCOOP report task 2.3.7 (Miraglia & Brera, 2002) corresponded to the main contributing food commodity to total dietary intake in each of the cited countries. They were however much different from the one reported in Morocco by Zinedine *et al.* (2007), as 126 ng/kg bw/day, which was inflated not only by the high contamination levels encountered but also by the high dietary intake of bread (assumed as 577 g/day/person, i.e. seven times higher than the Portuguese mean bread consumption).

It is acknowledged that for every exposure food source there will be differences between different individuals in the level of exposure at a specific location due to differences in intake rates, body weights, exposure frequencies, and exposure durations. Regrettably no up to date information exists about the individual consumption that permits an alimentary characterization according to gender, age, socio-economical position or urban or rural residency, a much needed tool when evaluating external chemical exposure.

For instance, in the total diet study in France (Leblanc *et al.*, 2005) children (3-14 years) presented higher exposure due to a higher bread consumption than adults (15 years and over) allied to lower weight, as revealed by the estimated daily intake (EDI; ng/kg bw/day): 0.77 *versus* 0.71 for ‘‘Bread, Rusk” and 0.21 *versus* 0.07 for ‘‘Viennese bread and buns”, for children and adults, respectively. The same study showed an additional group, the vegetarians that present different consumption levels of bread not only comparing to the rest of the population, but also among their groups being the ovolactovegetarian the higher consumers of bread products followed by the lactovegetarian and finally the vegan/macrobiotics.

Based on the results of the present study it can be suggested, regardless of the source of data about bread intake, that the OTA maximum tolerable intakes as established by EFSA (2006), JECFA (2007) or NWGFTRE (1991) were not exceeded

for the seasons and areas of the survey. However, it is important to bear in mind that bread is not the only consumed food contaminated with OTA, which signifies that contribution of all remaining food sources should be taken into account and incorporated in final OTA intake estimations.

## 3.5. Co-occurrence with ochratoxin B

A non-specific peak was detected in some of the tested samples with a retention time around 6.40 min, in addition to that of OTA. The injection of an external standard of OTB permitted to verify concordant retention times (Figure A.7).

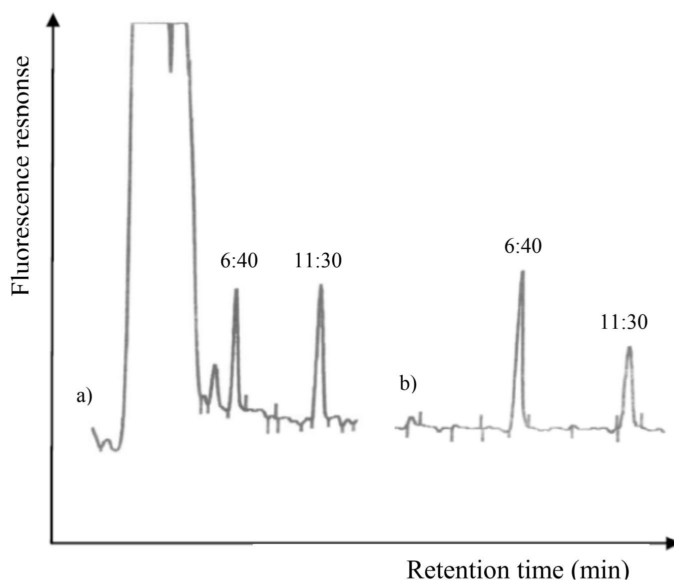


**Figure A.7.** HPLC/FD chromatograms obtained after the described experimental procedure, from a) wheat bread sample naturally positive to both OTB (Retention time 6:40 min) and OTA (Retention time 11:30 min), and b) OTB (0.025 µg/mL; Retention time 6:40 min) and OTA (0.005 µg/mL; Retention time 11:30 min) standard solution.

OTB was detected in different samples, especially in wheat bread. This implies that the choice of OTB as an internal standard should be approached with caution because of the stated possibility of simultaneous occurrence with OTA in real-life samples. However, the inadequate recoveries, below 50 %, did not permit to evaluate, with accuracy, the OTB levels, using the method for OTA determination. These low accuracy values may result from the immunoaffinity columns used for the clean-up contained monoclonal antibodies, with high specificity and affinity to OTA.

## 4. Conclusions

The two-year survey carried out in the four Portuguese mainland regions, encompassing four collection periods, as herein described contributed for the database of bread-derived human exposure in Portugal.

A widespread low level contamination was observed in all regions and types of bread products analysed. However, OTA content values were distributed over a large range, with the highest detected level surpassing the EU maximum level. High variability was also verified by the fairly high standard deviations of the average levels, which in turn were divergent to the corresponding median levels, thus hindering the use of parametric analysis.

No seasonal pattern was confirmed through statistical analysis, although a trend toward higher values in summer was observed among the frequency of contamination and median levels.

The bread commercialised in Alentejo region featured the lowest contamination, as the typical Alentejano bread constituted most of analysed bread samples in such region.

In general, common bread presented lower average OTA levels than maize bread. Among the first, wheat bread was the least contaminated, as opposed to whole grain and rye-based bread in line with previous surveys. Rye was also among the maize bread the cereal that contributed to highest mycotoxin detected levels, as observed in Avintes *broa*.

It was estimated a higher OTA intake through common bread than through maize bread that although more contaminated is less consumed. Bread-derived EDI among the Portuguese residents was inferior to equal to the contribution by wheat bread in foreign populations as reported previously.

Nevertheless, given that OTA content values were distributed over a large range, and even though the maximum values, were in general below the legal limit, this indicates a marked variability in contamination levels between analysed bread samples. Such variability could pose a risk, given that in case of the maximum value determined in bread would result in more than a quarter of the TDI set at 5 ng/kg bw/day. Considering than other food sources can also be contaminated with OTA, and consumption patterns may vary between populations or seasons, such TDI could be reached in certain individuals.

Finally, concurrently with the widespread contamination of bread with OTA, the co-occurrence of OTB in several bread samples was observed, thus hindering the use of the latter as an internal standard.

# Part II

# Original Research

## STUDY B

**Ochratoxin A evaluation in pork**

# 1. Foreword and definitions

As in the rest of the world, pork is the most consumed meat in Portugal with an annual consumption in 2009 estimated as of 47.8 kg *per capita*, thus surpassing that of poultry, which has a reported consumption of 34.8 kg *per capita* (INE, 2011a). The rising demand was a result of the Bovine Spongiform Encephalopathy crisis affecting bovine meat consumption, as well as of its lower cholesterol levels. Given the gastronomic versatility of pork and increasing consumption of derived products – ham, smoked ham, sausages, etc., it is anticipated a trend towards an even higher demand for swine meat in the following years. In 2009 a total of 50 084 swine farms existed in Portugal, rearing 1 913 161 pigs. In the same year, swine farms and allied industry featured a total production value of 318 168 000 Euros (INE, 2011b).

In response to the rising consumption of pork, it was observed a gradual introduction of foreign breeds and their crossbreds, with faster growth and greater muscle proportion in the carcass, explored in intensive farming which currently constitutes the almost totality of the national herds. For the number of farmed animals and excellent productive indicators Landrace and Large White are the most important breeds, followed by Duroc and Pietrain (MADRP, 2006).

Contrarily, the Alentejano native swine breed, typically produced in an extensive farming system in Alentejo, is perfectly adapted in a well defined agro-pasture system, allowing a sustainable farming, the preservation of *montado* (anthropurgic ecosystem of *Quercus ilex* and *Quercus suber*) and resulting in meat and derived products with high quality and growing interest and demand from consumers (Fernandes *et al.*, 2008). The strategic element of such production system is the *montanheira* phase that takes place at the end of the production cycle (finishing phase), during three or four months as a rule between late October and late February. During this intensive fattening phase the free-ranged animals are reared in the *montado* eating what the field provides like acorn, grass, stubble of cereals, stubble of pulses, which the farmers can complement with cereals like maize, barley and triticale, mostly produced in-farm (Fernandes *et al.*, 2008). Nowadays, this type of production has been changing towards a semi-intensive system to respond to the increasing demand.

For disambiguation, it is stated that muscle samples collected from pigs will be henceforth indiscriminately identified as pork or meat, equally meaning muscle tissue of pig.

# 2. Materials and methods

## 2.1. Pork sampling

All muscle samples were collected in the major slaughterhouses that supplied the Porto, Coimbra, Lisboa and Alentejo regions. With the exception of Coimbra, the sampling occurred during two consecutive years, specifically: winter of 2007/2008, summer of 2008, winter of 2008/2009, and summer of 2009. In Coimbra, samples were collected only during one year, in two collection periods: winter of 2008/2009 and summer of 2009 (Table B.1).

Samples were collected from the thick muscular dorsal attachment (pillar) of the diaphragm of healthy slaughtered pigs, randomly selected in the slaughterhouse after meat inspection, and before being transported to the local butchers, supermarkets and transforming industries.

Immediately after collection, samples were brought to the laboratory. To each sample the macroscopically visible fat and ligaments were separated and removed. Then they were minced to assure homogenization and stored at -20 °C until extraction.

**Table B.1.**
Distribution of the analysed samples by region and season.

| | Winter 2007/2008 | Summer 2008 | Winter 2008/2009 | Summer 2009 | Total |
|---|---|---|---|---|---|
| **Porto** | 15 | 19 | 20 | 14 | **68** |
| **Coimbra** | n.c. | n.c. | 20 | 20 | **40** |
| **Lisboa** | 20 | 20 | 10 | 20 | **70** |
| **Alentejo** | 20 | 20 | 20 | 16 | **76** |
| **Total** | **55** | **59** | **70** | **70** | **254** |

(n.c.: non-collected)

## 2.2. Pork consumption data

According to the INE, the annual consumption of pork in 2009 was estimated as of 47.8 kg *per capita* (INE, 2011a).

## 2.3. Estimation of daily intake

The exposure of OTA through pork consumption was calculated as described previously (*vide* Study A, section 2.3).

## 2.4. Reagents and materials

### 2.4.1. Materials and equipments

A 3-16k Sigma centrifuge (Reagente 5, Porto, Portugal), a Braun MR 5000 M multiquick/minipimer 500W (220–230 V, 50–60 Hz, Esplungues del Llobregat, Spain), a vacuum manifold of Macherey–Nagel (Düren, Germany), a Dinko pump (mol. D-95, 130 W, 220 V), a magnetic stirrer (Agimatic-S, Selecta, Barcelona, Spain), a Retsh vortex mixer (Haan, Germany), and a Sonorex RK 100 ultrasonic bath (Berlin, Germany) were employed. Polyamide membrane filters (0.2 µm, 50 mm; Whatman GmbH, Dassel, Germany) were used. Ochratest IACs were from VICAM (Watertown, MA, USA).

LC/MS/MS analysis was carried out on a Thermo Finnigan Surveyor Autosampler coupled to a Thermo Finnigan Surveyor MS Pump plus and a LCQ Advantage MAX mass spectrometer (Thermo Finnigan, San Jose, California, USA). The analytical column (Waters Spherisorb ODS2; 3 µm, 150x2.1 mm ID) was preceded by a guard cartridge (Waters Spherisorb ODS2; 5 µm, 10x4.6 mm ID; Waters Corporation, Milford, Massachusetts, USA).

### 2.4.2. Reagents, standards and solutions

HPLC-grade ≥99.8 % acetic acid (BDH Ltd., England, UK), acetonitrile (LiChrosolv, Merck, Darmstadt, Germany), toluene (Baker Analyzed, J.T. Baker, Holland) and benzene (HACH Company, Loveland, USA) were used. Analytical grade reagents were comprised of 85 % orthophosphoric acid, chloric acid, sodium hydroxide, potassium chloride, potassium dihydrogenphosphate and anhydrous disodium hydrogen phosphate (Merck, Darmstadt, Germany), and sodium chloride (Baker Ltd., Pagenham, UK).

The OTA standard from *A. ochraceus* was obtained from Sigma Chemical Co. (St. Louis, MO, USA) with ≥98 % purity.

PBS and OTA stock and intermediate standard solutions were prepared as described previously (*vide* Study A, section 2.4.2). OTA working standard solutions were prepared at 0.1 µg/mL in mobile phase. Calibration curve standard solutions were prepared in mobile phase at five concentrations, specifically 0.004, 0.006, 0.01, 0.016, 0.020 µg/mL.

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

## 2.5. Safety measures

The safety precautions applicable to OTA handling, waste disposal and decontamination of glassware and materials used in OTA analysis was described previously (*vide* Study A, section 2.5).

## 2.6. Experimental procedure

For evaluation of OTA content in pork samples a new analytical methodology was developed, entailing an IAC clean-up and a LC-ESI-MS/MS assay. The extraction and clean-up steps were adapted from a method of determination of OTA in olive oil described by Papachristou & Markaki (2004).

### 2.6.1. Sample extraction and clean-up

After defrosting and reaching room temperature, a 10 g sample of minced and homogenate muscle was taken and placed in a 25 mL centrifuge tube, and mixed up with 4 mL of methanol and 40 µL of 85 % orthophosphoric acid, to reach final pH lower than 2. The sealed and light-protected tube was then submitted to a 10 min cycle of ultrasound waves, in ice. After that 16 mL of methanol were added and followed by a vigorous vortex agitation.

A 30 min centrifugation at 5440 *g* at 5 ºC resulted in phase separation. Six millilitres of the supernatant were diluted in 40 mL PBS in an amber flask, before the clean-up step. The mixture was then slowly passed (vacuum free) through the IAC, which was washed with 10 mL bi-distilled water before the elution with 3 mL of methanol. Finally, the eluate was evaporated to dryness under a gentle stream of nitrogen at 40 ºC, and the dried extracts were stored at -20 ºC until analysis.

### 2.6.2. Immunoaffinity columns regeneration

Immediately after their first use, IACs were regenerated, only once, as previously described (*vide* Study A, section 2.6.2).

### 2.6.3. Detection and quantification

For LC/MS/MS analysis the dried eluate was taken in 150 µL of mobile phase. A 25 µL (full loop) injection volume was used with the mobile phase isocratic flow maintained at 200 µL/min.

Mass spectrometer was operated in the positive ESI mode using SRM acquisition. Source and capillary temperatures were set at 0 and 200 ºC and voltages at 5 and 32 V, respectively. Nitrogen was used as nebulising gas, with a sheath gas flow of 80 (arbitrary unit) and the auxiliary sweep gas flow of 20 (arbitrary unit). Collision gas was helium with a normalised collision energy of 25 %.

Precursor-to-fragment transitions used were the following: $[M+H]^+ \rightarrow [M+H-HCOOH]^+$ (*m/z* 404 → 358) and $[M+H]^+ \rightarrow [M+H-H_2O]^+$ (*m/z* 404 → 386). The precursor and two daughter ions corresponded to four identification points as established by Commission Decision 2002/657/EC (CEC, 2002).

For interpretation of results, the calibration curve of fortified pork samples was used to quantify OTA concentrations. This curve was constructed by plotting OTA peak area against fortified pork samples at five different concentrations, specifically 0.2, 0.3, 0.5, 0.8, 1 µg/kg. The relative abundance of the selected product ions, expressed as a percentage of the abundance of the most abundant ion ($[M+H-HCOOH]^+$; *m/z* 358), was 60 % ($[M+H-H_2O]^+$; *m/z* 386) and 20 % ($[M+H]^+$; *m/z* 404).

### 2.6.4. Validation assays

To verify that the analytical method complies with performance criteria for the performance characteristics as established by Commission Decision 2002/657/EC and Commission Regulation (EC) No. 401/2006, the following described validation procedures were carried out.

Specificity was determined by analysis of 20 different blank samples, checking for possible interferences in the region of interest where the target product ion was expected to elute.

During the course of four days, OTA-blank pork samples were spiked in triplicate at addition levels of 0.2, 0.3, 0.5, 0.8, and 1.0 µg/kg, and were left to stand in the dark for 15 min before applying the aforementioned protocol of extraction, clean-up and analysis. In each day, a blank sample was left to analyse without any added OTA. These fortification assays were used to determine recovery, within- and between-day precision, ME, decision limit (CCα) and detection capability (CCβ).

The accuracy was evaluated through spiking and recovery testing. Recovery was determined by experiments using fortified blank matrix as above-mentioned given that currently no CRM was available, which absence also hampered the establishment of trueness.

Precision was evaluated though the RSD (%) of the fortified samples, in within- and between-day assays.

The limit at and above which it can be concluded with an error probability of α that a sample was non-compliant, i.e. CCα, was calculated as follows (Commission Decision 2002/657/EC; Antignac *et al.*, 2003):

$$CC\alpha = \frac{2.33\sigma_N}{a}$$

where $\sigma_N$ was the standard deviation of the inter-day precision and $a$ was the mean slope of the calibration curve.

The lowest concentration at which the method was able to detect truly contaminated samples with a statistical certainty of 1 – β, i.e. CCβ, was calculated as follows:

$$CC\beta = CC\alpha + 1.64\sigma_N$$

Although not included in the validation procedures as established by Commission Decision 2002/657/EC, linearity was evaluated through the coefficient of correlation of a calibration curve constructed by plotting OTA peak area *versus* OTA concentration of standard solutions determined in triplicate at five levels of 0.004, 0.006, 0.01, 0.016, 0.020 µg/mL. This calibration curve was also used to calculate ME for OTA in pork through the signal suppression–enhancement, as previously described by Rubert *et al.* (2011). ME equalled to the percentage of the matrix-matched calibration slope (B) 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 % denoted an absence of matrix effects, above 100 % a signal enhancement and below 100 % a signal suppression.

At last, LOD and LOQ were calculated through the matrix-matched calibration curve as $|3.3S_{y/x}|/b$ and $|10.S_{y/x}|/b$, respectively, where *b* is the slope and $S_{y/x}$ the residual standard deviation of linear function. The latter was calculated as follows:

$$S_{y/x} = \sqrt{\frac{SS}{N-2}}$$

where SS was the sum of the square of the vertical distances of the points from the curve and N was the number of fortification levels of the standard solutions.

### 2.7. Statistical analysis

For statistical analysis, when the concentration was below the LOQ it was set to 50 % of that limit.

SAS/STAT$^{TM}$ Software was used for statistical analysis, through test of independence, Chi-square, Kruskal-Wallis, and Median tests.

## 3. Results and Discussion

### 3.1. Method optimization

Meat is a particularly challenging matrix, posing difficult problems in analysis. These problems are compounded by the strong binding between proteins and OTA in addition to the need to remove fat and other interferences that could be co-extracted (Sørensen *et al.*, 2010). Thus, different attempts were made in the conducted study from which the following discussed set of data was retrieved.

Available methods for OTA determination in pig meat and tissues employ high amount of chlorinated solvents like chloroform, dichloromethane and hexane in partition techniques as reviewed earlier (*vide* Part 1, section 9.1.2). However, these techniques present low extraction efficiency, and do not fit the green chemistry basic principles of waste reduction and use of safer products. Moreover, even if mass spectrometry is used for detection, previous studies indicate that LLE (Losito *et al.*, 2004) or SPE (Lindenmeier *et al.*, 2004) do not eliminate the problem of co-elution of interference compounds (Chung & Kwong, 2007).

In the devised approach, methanol was chosen as extraction solvent because it is less toxic and less expensive than acetonitrile (Domijan *et al.*, 2003), yet likewise miscible in water to ensure good distribution throughout the sample (Valenta, 1998). Moreover, contrary to OTA, fat is not soluble in methanol, thus preventing its co-extraction. In each sample, a total volume of 20 mL of methanol was used, far less than the volume of solvents used in previous studies.

The introduction of the IAC aimed to limit matrix interferences as well as to remove the ion-suppression phenomena due to matrix effects (Shephard, 2008b; Cigić & Prosen, 2009). Even though IAC provides high specificity and selectivity, which is critical in complex matrices, its use could entail several problems. Indeed, IAC has a known limited resistance to harsh conditions, such as pH, temperature, organic solvents, and ultrasounds. However, as already reported (Valenta, 1998), OTA presents a strong binding to proteins at neutral or alkaline pH that appears weakened with increasing ionic strength of the extraction solution and/or adjusting the pH to less than 2. Thus to extract OTA linked to proteins in this complex matrix, a decrease of the pH (to less than 2) as well as the use of methanol, coupled to a brief ultrasound cycle were used. Indeed, it was observed that recoveries were improved by decreasing pH value in the extraction step, provided that the pH of the solution to be loaded into the IAC was close to a value of 7.

Thus, to ensure good recovery of OTA from muscle, the acidification of the extraction solvent down to pH<2 and the ultrasound cycle were both performed in the extraction step. Afterward the prolonged strong centrifugation at 5 ºC ensured a good separation of the two phases, facilitating an easy withdrawal of the methanol supernatant, as orthophosphoric acid and solid particles (i.e. muscle tissue and fat) were forcedly deposited in the bottom of the tube. With dilution of 6 mL of the supernatant in PBS (to raise the pH close to 7) the IAC could be safely loaded without risk of damaging the antibodies. It is of note that PBS provided better results in comparison to the remaining tested buffers, namely (2.5 and 5 %) $NaHCO_3$ and phosphate buffer (1.5 and 1 M).

In the LC/MS/MS analysis, ESI interface operated in positive ion mode was used given that several studies reported a higher sensitivity of ESI when compared with APCI (Lau *et al.*, 2000; Losito *et al.*, 2004). Possible interference effects (e.g. suppression of ESI efficiency) due to co-elution of other matrix components with OTA

were also minimised by the use of a matrix-matched calibration curve (Losito *et al.*, 2004).

Until today, there have been few attempts to use mass spectrometric detection of OTA in animal edible tissues, in general, and only one in muscle samples, in particular. With mass spectrometry as a detection system, no further time-consuming confirmation step is necessary, as so often required for unambiguous analyte identification with fluorescent detectors. In effect, a second chromatographic run for confirmation of OTA by methyl ester formation was required in the two published FD-based protocols developed for OTA determination in muscle (Monaci *et al.*, 2004; Guillamont *et al.*, 2005)

The sole application of mass spectrometry to OTA determination in pig muscle presented ten-times higher LOD and LOQ in comparison to the herein proposed method (Losito *et al.*, 2004). In addition, it relied on a LLE procedure, allowing the use of an internal standard, but concurrently presenting all the drawbacks already mentioned, namely more time-consuming and error-prone.

In sum, the designed method provided for a quicker and simpler pre-analytical set-up, with particular use where speed and simplicity are at a premium, for instance for routine analysis. Combined with the power of ESI and sequential mass spectrometry ($MS^2$), the designed approach provided MS/MS identification and quantification of the mycotoxin in muscle at a lower range than the previously published method (Losito *et al.*, 2004).

## 3.2. Method validation

Specificity assays showed no interfering peaks of eligible size near the retention times (about 10.30 min) of the product ions analysed, as displayed in Figures B.1 and B.2.

Recovery for spiked pork was found to range from 98.46 to 100.60 % for fortification levels of 0.5 and 1 µg/kg, respectively. Within-day RSD was at all times below 1.52 % and the between-day RSD was below 2.16 %, as summed-up in Table B.2. Good extraction efficiency was thus attained.

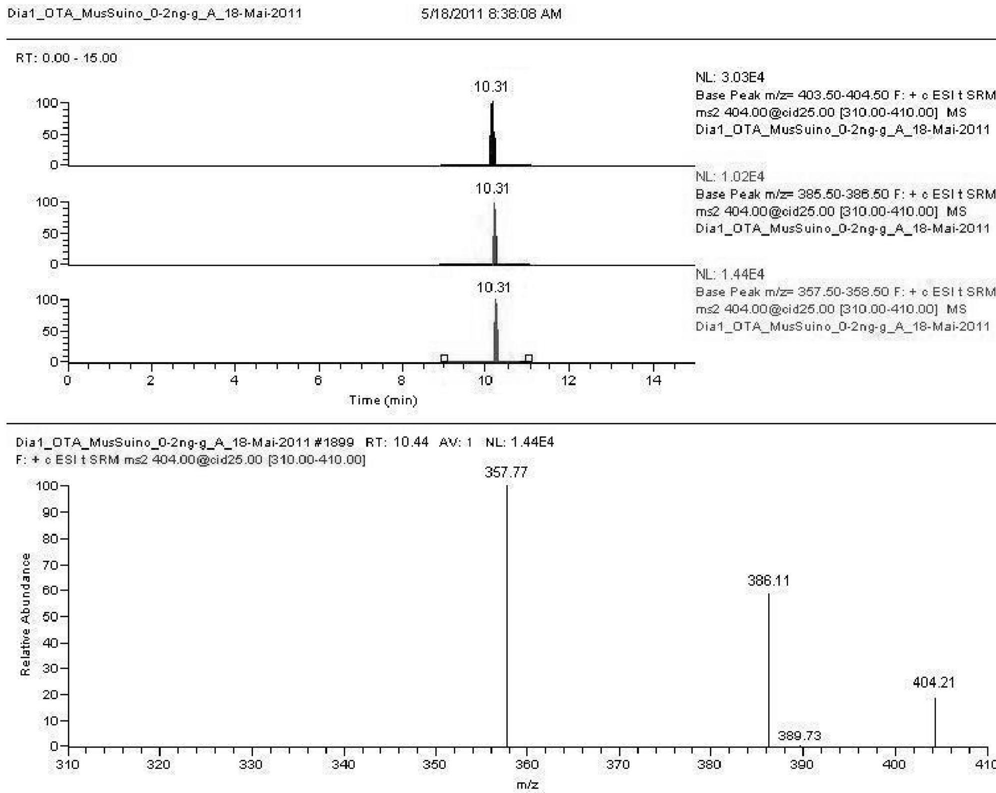


**Figure B.1.** ESI-MS/MS chromatograms and mass spectrum of a pork sample fortified with OTA at 0.2 µg/kg.

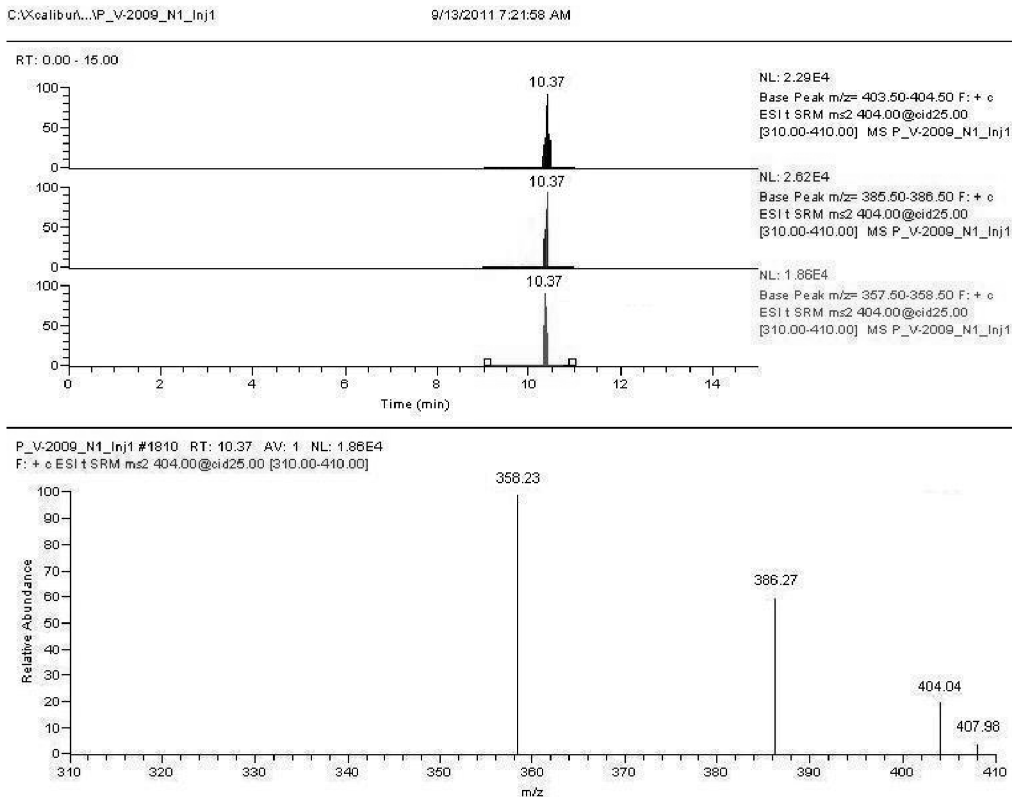


**Figure B.2.** ESI-MS/MS chromatograms and mass spectrum of a naturally contaminated pork sample with 0.44 µg/kg of OTA.

**Table B.2.**
Performance data obtained for triplicate OTA spiked pork during four days.

| Spiking level (µg/kg) | Mean recovery (%) | RSD $_{within-day}$ (%; *n*=3) | RSD $_{between-day}$ (%; *n*=4) |
|---|---|---|---|
| 0.2 | 100.10 | 0.21 | 0.34 |
| 0.3 | 100.38 | 1.10 | 0.70 |
| 0.5 | 98.46 | 1.52 | 1.46 |
| 0.8 | 98.77 | 0.70 | 1.61 |
| 1 | 100.60 | 0.60 | 2.16 |

The values determined for CCα and CCβ were 0.01 and 0.50 µg/kg, whereas for LOD and LOQ were calculated as 0.06 and 0.19 µg/kg, respectively.

With the employed method peak area responded to concentration in the 0.004-0.020 µg/mL range for OTA with clear linearity, featuring a correlation coefficient of 0.9961 (y=12347x+10865). Matrix-matched calibration curve (y=13756x+12593) featured a good linearity ($R^2$=0.9976) between 0.2 and 1 µg/kg. Matrix effect was calculated as 111 %, and so considered minor.

The only previously described LC/$MS^n$ method for OTA determination in muscle involved LLE (Losito *et al.*, 2004). When compared with the present method, it featured lower sensitivity (LOQ=1.5 µg/kg), accuracy (mean recoveries of 86 %), and precision (RSD=9 %).

Through LC/FD analysis, the two published methods describing OTA determination in muscle also entailed LLE, which carries the already mentioned disadvantages. When compared with the herein described method, Monaci *et al.* (2004) reported lower recoveries (74 %) and sensitivity (LOQ=0.67 µg/kg). In turn, Guillamont *et al.* (2005) reported comparable recoveries (90.3-103.2 %) and sensitivity (LOQ=0.04 µg/kg), although lower precision (RSD up to 20.4 %).

The developed method thus proved to be specific, as well as more sensitive and accurate than reported conventional LC/FD methods of OTA determination in muscle samples. By permitting a large sample throughput and requiring a small amount of sample and reagents, it can be considered suitable for routine analysis and surveys. On the other hand, because validation procedures were performed in accordance to the European regulations, namely Commission Decision 2002/657/EC and Commission Regulation (EC) No. 401/2006, it can also be considered for official testing purposes.

## 3.3. Pork contamination

Descriptive statistics of the analysis of 254 muscle samples from slaughter pigs in the four surveyed regions were summed-up in Table B.3. Frequency of contamination varied from 10 to 40 %. Maximum OTA level was detected in a pork sample from Alentejo, at 1.161 µg/kg. Average levels, which ranged from 0.243 to 0.698 µg/kg, presented in general high standard deviations, in addition to discrepancies with the corresponding median levels.

Although no statistical significance was confirmed between seasons, it was observed a trend towards higher levels in winter seasons, regarding the median levels. In the contamination frequency such trend was only noticeable in the second year (*vide* Figure B.3). Winter climatic conditions may influence the OTA content in muscle, for instance because of inappropriate storage conditions of the feed with which the animals were being reared before slaughter (Jørgensen & Petersen, 2002).

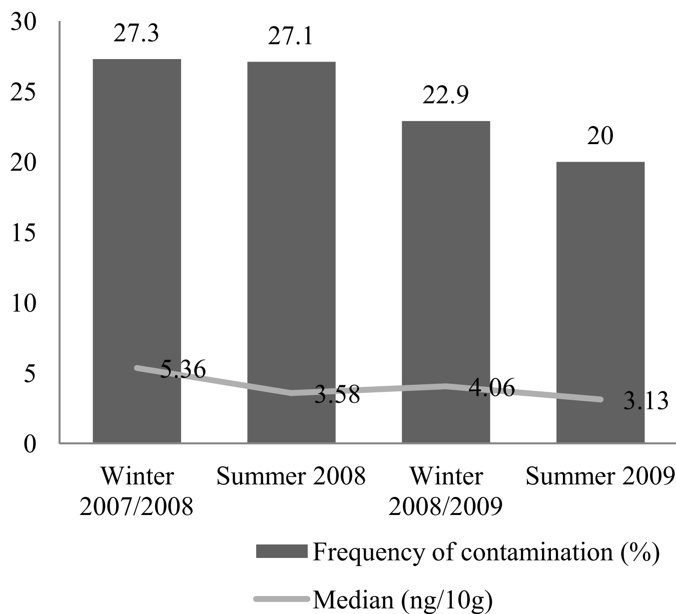


**Figure B.3.** Contamination of pork, with frequency of contamination (%) depicted in bars and the median OTA level (ng/10 g) with points united in a continuous line to assist in comparison.

**Table B.3.**
Descriptive statistics regarding OTA occurrence and contamination levels in pork according to the region and season of collection.

| Collection season | Region | Sample size | Positive (%) | Maximum (µg/kg) | ≥ LOQ (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Number (%) | Mean±SD (µg/kg) | Median (µg/kg) |
| **Winter 2007/2008** | Porto | 15 | 4 (26.7 %) | 0.485 | 3 (20 %) | 0.414±0.093 | 0.448 |
| | Coimbra | n.c. | n.c. | n.c. | n.c. | n.c. | n.c. |
| | Lisboa | 20 | 3 (15.0 %) | 0.621 | 3 (15.0 %) | 0.495±0.189 | 0.587 |
| | Alentejo | 20 | 8 (40.0 %) | 1.161 | 8 (40.0 %) | 0.698±0.343 | 0.735 |
| | **Total (Winter 2007/2008)** | **55** | **15 (27.3 %)** | **1.161** | **14 (25.5 %)** | **0.593±0.294** | **0.536** |
| **Summer 2008** | Porto | 19 | 5 (26.3 %) | 0.589 | 4 (21.1 %) | 0.364±0.158 | 0.323 |
| | Coimbra | n.c. | n.c. | n.c. | n.c. | n.c. | n.c. |
| | Lisboa | 20 | 5 (25.0 %) | 0.649 | 5 (25.0 %) | 0.446±0.172 | 0.441 |
| | Alentejo | 20 | 6 (30.0 %) | 0.796 | 5 (25.0 %) | 0.442±0.215 | 0.356 |
| | **Total (Summer 2008)** | **59** | **16 (27.1 %)** | **0.796** | **14 (23.7 %)** | **0.421±0.174** | **0.358** |
| **Winter 2008/2009** | Porto | 20 | 5 (25.0 %) | 0.578 | 4 (20.0 %) | 0.405±0.128 | 0.385 |
| | Coimbra | 20 | 3 (15.0 %) | 0.840 | 3 (15.0 %) | 0.599±0.332 | 0.736 |
| | Lisboa | 10 | 1 (10.0 %) | 0.476 | 1 (10.0 %) | 0.476 | 0.476 |
| | Alentejo | 20 | 7 (35.0 %) | 0.948 | 7 (35.0 %) | 0.444±0.279 | 0.261 |
| | **Total (Winter 2008/2009)** | **70** | **16 (22.9 %)** | **0.948** | **15 (21.4 %)** | **0.467±0.240** | **0.406** |
| **Summer 2009** | Porto | 14 | 5 (35.7 %) | 0.437 | 3 (21.4 %) | 0.332±0.120 | 0.358 |
| | Coimbra | 20 | 2 (10.0 %) | 0.255 | 2 (10.0 %) | 0.243±0.017 | 0.243 |
| | Lisboa | 20 | 4 (20.0 %) | 1.029 | 4 (20.0 %) | 0.560±0.376 | 0.485 |
| | Alentejo | 16 | 3 (18.8 %) | 0.583 | 3 (18.8 %) | 0.405±0.165 | 0.374 |
| | **Total (Summer 2009)** | **70** | **14 (20.0 %)** | **1.029** | **12 (17.1 %)** | **0.411±0.247** | **0.313** |
| **Four collection seasons** | Porto | 68 | 19 (27.9 %) | 0.589 | 14 (20.6 %) | 0.380±0.119 | 0.361 |
| | Coimbra | 40 | 5 (12.5 %) | 0.840 | 5 (12.5 %) | 0.456±0.305 | 0.255 |
| | Lisboa | 70 | 13 (18.6 %) | 1.029 | 13 (18.6 %) | 0.495±0.232 | 0.476 |
| | Alentejo | 76 | 24 (31.6 %) | 1.161 | 23 (30.3 %) | 0.526±0.293 | 0.427 |
| | **Total (Four regions)** | **254** | **61 (24.0 %)** | **1.161** | **55 (21.7 %)** | **0.475±0.247** | **0.406** |

(n.c.: non-collected; SD: standard deviation)

In a regional analysis, considering the four collection seasons jointly, pork from Alentejo featured the highest contamination, in terms of frequency of contamination and average levels. However, such differences only reached significance when comparing the frequency of contamination with the one of Coimbra (Chi-square; p=0.034).

Given the main source of the animals' OTA exposure, i.e. contaminated feed, the obtained results point toward a safer feed in the regions of Coimbra in what refers to OTA content. Contrarily, the highest contamination values in pork from Alentejo indicate a higher exposure to the mycotoxin. Given that some of the analysed samples from Alentejo corresponded to the native swine breed, this higher exposure of the animals may lie in the type of feeding system, probably during the final intensive fatting (*montado* phase), when different cereals and stubbles mostly produced in-farm are provided (Fernandes *et al.*, 2008). Such results thus merit further investigation given the increasing demand of products derived from the Alentejano native breed.

Other additional influencing factors of OTA levels found in pigs can be climate conditions during harvest as well as practices of grain/feed storage (Jørgensen & Petersen, 2002). However, as no particular information on the origin of feed and their storage conditions was available, it is difficult to assert if the development of OTA-producing moulds, and mycotoxin production in this regions was due to climatic conditions or not.

As depicted in Figure B.4, of the fifty five samples above the LOQ, 63.6 % featured an OTA content below 0.5 µg/kg, and 30.9 % between 0.5 and 1 µg/kg. Only three samples (5.5 %) surpassed the Italian maximum level in meat, i.e. 1 µg/kg. The maximum level determined in a pork sample was 1.161 µg/kg and therefore none of the analysed samples reached the Romanian established maximum level in meat (5 µg/kg).

Pork samples analysed in the present study featured a frequency of contamination only higher than that found in Italy (Monaci *et al.*, 2004) and Romania (Curtui *et al.*, 2001). Nevertheless, the average levels of contamination determined were the highest reported in swine muscle, as shown in Table B.4. Possible explanations include differences in the analytical performance of the method employed. It is worth noting that such studies employed determinations based on LC/FD technique, which retrieves better sensitivity as broadly accepted.

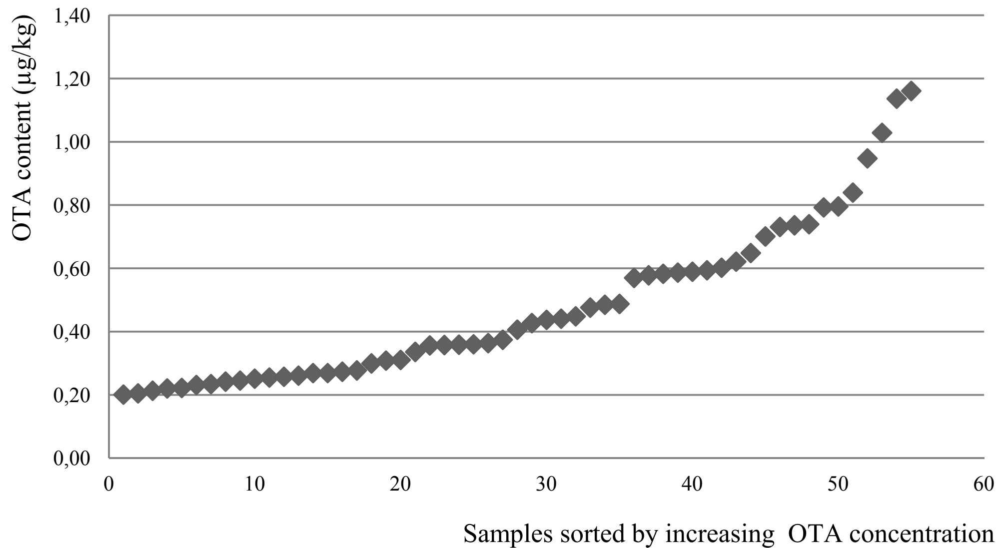


**Figure B.4**. Distribution of OTA concentration values of the total analysed pork samples. Only samples with OTA content above the LOQ (0.19 µg/kg) were depicted.

Based on the results of previous studies with matching samples per animal, concentration of OTA in muscle is lower than in kidney (Monaci *et al.*, 2004; Matrella *et al.*, 2006; Jørgensen & Petersen, 2002; Curtui *et al.*, 2001; Ceci *et al.*, 2007) and liver (Jørgensen, 1998; Curtui *et al.*, 2001) because of a low carry-over into muscle (Dall'Asta *et al.*, 2010). Is thus reasonably to assume that, based on the muscle average value found in the present study, the matching kidneys and liver would present even higher OTA concentrations.

Furthermore, taking into account the OTA pattern of distribution as reported by Curtui *et al.* (2001), the average OTA level in muscle would correspond to OTA concentrations of 18.48 µg/L in serum, 4.18 µg/kg in kidney and 1.57 µg/kg in liver. Such OTA (back-calculated) value in serum was higher than the one reported in healthy slaughtered pigs in Canada (Omisnki *et al.*, 1996), Romania (Curtui *et al.*, 2001), Brazil (Krüger, 2006), Serbia (Milićević *et al.*, 2007, 2008). It was however lower than the value reported in Bulgaria in nephropathic slaughtered swine by Stoev *et al.* (2010a). In kidney and liver, the back-calculated value was also above the ones previously reported in healthy slaughtered swine in Romania (Curtui *et al.*, 2001), Denmark (Jørgensen & Petersen, 2002), and Serbia (Milićević *et al.*, 2008). In view of these estimates, OTA contamination of swine tissues in Portugal should thus merit additional attention, given that some of cited foreign studies were performed in PEN- as well as BEN-endemic countries, to which OTA exposure has been associated.

**Table B.4.**
Compilation of the most recent international studies regarding OTA occurrence in muscle from healthy farm animals.

| Species | Country (year of collection) | LOD (LOQ) | Positive (%) | Mean (µg/kg) | Maximum (µg/kg) | Reference |
|---|---|---|---|---|---|---|
| Pig | Southern Italy | 0.01 | 42/54 (77.8 %) | 0.024 | n.s. | Matrella *et al.* (2006) |
| Pig | Portugal | 0.01 (0.04) | 7/13 (53.8 %) | 0.01 | n.d.-0.12 | Guillamont *et al.* (2005) |
| Turkey | | | 9/13 (69.2 %) | 0.02 | n.d.-0.04 | |
| Chicken | | | 9/13 (69.2 %) | <LOQ | <LOQ | |
| Pig | Italy | 0.15(0.67) | 0/12 (0 %) | n.a. | n.a. | Monaci *et al.* (2004) |
| Pig | Denmark (1999) | 0.03(0.09) | 228/300 (76 %) | 0.12 | n.d.-2.9 | Jørgensen & Petersen (2002) |
| Pig | Romania (1998) | 0.01 | 9/52 (17 %) | 0.15 | n.d.-0.53 | Curtui *et al.* (2001) |
| Pig | Denmark | 0.02 | | | | Jørgensen (1998) |
| Conventional | (1993-1994) | | 64/76 (84.2 %) | 0.11 | n.d.-1.3 | |
| Organic | | | 4/7 (57.1 %) | 0.05 | n.d.-0.12 | |
| Duck | | | 11/19 (57.8 %) | 0.02 | n.d.-0.09 | |
| Goose | | | 5/12 (41.7 %) | 0.03 | n.d.-0.10 | |
| Turkey | | | 10/17 (58.8 %) | 0.02 | n.d.-0.11 | |
| Chicken | | | 36/65 (55.4%) | 0.03 | n.d.-0.18 | |

(n.a.: non-applicable; n.d.: non-detected; n.s.: non-specified)

### 3.4. Estimated daily intake

The likely intake of OTA through pork consumption in the Portuguese population was summed up in Table B.5.

The results showed that the importance of the surveyed meat as source of OTA to the human exposure cannot be neglected. Indeed, the total contribution of pork to the total intake of OTA of a common Portuguese reached 0.958 ng/kg bw/day for the average consumers from the four regions. This value represents 5.59 and 6.71 % of the tolerable intake as established by EFSA (2006; about 17.14 ng/kg bw/day) and JECFA (2007; about 14.29 ng/kg bw/day), respectively.

**Table B.5.**
Estimated daily intake from pork consumption according to data from INE (2011a).

| Season of collection | Region | EDI (ng/kg bw/day) |
|---|---|---|
| **Winter 2007/2008** | Porto | 0.834 |
| | Coimbra | n.c. |
| | Lisboa | 0.997 |
| | Alentejo | 1.406 |
| | **TOTAL (Winter 2007/2008)** | **1.196** |
| **Summer 2008** | Porto | 0.734 |
| | Coimbra | n.c. |
| | Lisboa | 0.899 |
| | Alentejo | 0.890 |
| | **TOTAL (Summer 2008)** | **0.849** |
| **Winter 2008/2009** | Porto | 0.816 |
| | Coimbra | 1.206 |
| | Lisboa | 0.959 |
| | Alentejo | 0.894 |
| | **TOTAL (Winter 2008/2009)** | **0.940** |
| **Summer 2009** | Porto | 0.669 |
| | Coimbra | 0.490 |
| | Lisboa | 1.129 |
| | Alentejo | 0.816 |
| | **TOTAL (Summer 2009)** | **0.829** |
| **Four collections seasons** | Porto | 0.765 |
| | Coimbra | 0.920 |
| | Lisboa | 0.997 |
| | Alentejo | 1.061 |
| | **TOTAL (Four regions)** | **0.958** |

(n.c.: non-collected)

It is worth mentioning that a higher consumption of meat contaminated at the average level, just as a regular consumption of meat contaminated above the determined average, may result in an even higher EDI. In effect, if assumed that an individual's exposure to OTA was limited to pork consumption, then at the herein determined average contamination level (0.475 µg/kg), a person would need to consume up to 684.2 g of pork per day to reach the TDI established at 5 ng/kg bw/day by the Nordic Working Group on Food Toxicology and Risk Evaluation (NWGFTRE, 1991). On the other hand, if the pork consumed was contaminated at the maximum value as detected in the present study (1.161 µg/kg) then the consumption of roughly two pork steaks (totalizing 279.9 g together) in a daytime would be enough to reach the same TDI (5 ng/kg bw/day).

Hence, concern over the potential OTA contamination of pork is justified, especially in some regions where EDI values surpassing 1 ng/kg bw/day were estimated. Moreover, as a common trend among the surveyed regions, the EDI was higher in winter, as compared to summer, thus mirroring the similar pattern in the mean OTA concentration in the analysed meat.

When comparing with the previous survey in Portugal (Guillamont *et al.*, 2005; EDI=0.023 ng/kg bw/day), the likely intake of OTA through pork consumption was notoriously increased (over 40 times), as expected given the higher contamination levels encountered during the present survey. Nevertheless, if Guillamont *et al.* (2005) calculated the average levels of their analysed samples in the same way as in the present study, such difference would be reduced to 3 times. Indeed, if Guillamont *et al.* (2005) calculated the EDI considering the single pork sample above the LOQ (0.04) with an OTA content of 0.12 µg/kg, then the corresponding EDI would be estimated at 0.298 ng/kg bw/day, with the same average consumption (149 g/day) and adult body weight (60 kg).

According to the total diet study of the French population (Leblanc *et al.*, 2005) the consumption of meat (without specifying the animal species) contributed with 0.06 ng/kg bw/day to OTA exposure among the adult population (15 years and over), considering a contamination level of 0.25 µg/kg and a daily consumption of 14.9 g/day. Such low EDI, as compared with the one herein estimated for the Portuguese population resulted from the very low meat consumption (almost 10 times lower) and contamination level considered.

## 4. Conclusions

A sensitive method for the determination of OTA in swine muscle was developed. Extraction involved the use of acidified methanol, ultrasound treatment and centrifugation, followed by an IAC clean-up step before mass spectrometric detection.

The developed IAC-LC-ESI-MS/MS was shown to be a valuable alternative to the already established and reliable, whether FD- or MS-based, protocols. More sensitive and accurate than previously reported methods it further avoids a prior LLE step and therefore the use of toxic chlorinated solvents. Furthermore, the uncomplicated and quick pre-analytical step permits its use in routine analysis, whereas the fulfilment of the validation criteria as established by current European Commission regulations permits it to be considered for official testing purposes.

Although LC/MS systems cannot be considered standard equipment, the combination of sample preparation with LC/MS/MS detection technique has several advantages in terms of easy automation, well-suited for high throughput of large amounts of samples and unambiguous analyte identification without any further time-consuming and error-prone confirmation steps. This becomes especially important if maximum levels for OTA in meat are established at an EU level in the near future.

The developed method was applied to pork samples destined to commercialization, in the four Portuguese mainland regions (Porto, Coimbra, Lisboa and Alentejo). The two-year survey performed, encompassed four collection periods, to estimate pork-derived human exposure in Portugal.

Among the analysed pork samples, a relatively high average level of contamination along with a low frequency of contamination was observed, contrasting with previous national and foreign surveys. In effect, some of the analysed samples surpassed the maximum levels established by some EU-member states.

Although statistical analysis failed to establish major determinants of the OTA content in meat, several trends were found. In fact, winter-collected samples constantly featured higher contamination, as well as the samples from Alentejo region. The first potential factor may be explained by the practices and conditions of feed storage, while the second one may be explained by the traditional extensive feeding-system of the native swine breed, to which some of the surveyed animals belonged.

The resulting EDI of the Portuguese consumers mirrored such seasonal and regional factors, with an exposure on occasion surpassing 1 ng/kg bw/day.

# Part II

# Original Research

## STUDY C

**Ochratoxin C evaluation in human urine**

# 1. Foreword and ethical statement

The participant subjects were all healthy volunteers, with no history of hepatic or nephropathic conditions. No ingestion or administration was given to the subjects, and, therefore, this study did not result in any risk for the participants. Nonetheless, the collection of human urine was performed according to Declaration of Helsinki and it was based only in the evaluation of OTA concentration in the urine of different groups of populations. For this reason, all participants signed a written informed consent form before sampling.

Populations were identified and described with respect to those parameters under study, specifically basic anthropometric measures and region, searching for possible correlations with OTA levels in urine.

# 2. Materials and methods

## 2.1. Urine sampling

Collection occurred at the same time as in the previously described studies (*vide* study A and B), specifically in four collection periods (winter of 2007/2008, summer of 2008, winter of 2008/2009, summer of 2009), and across the same regions (Porto, Coimbra, Lisboa and Alentejo).

A total of 472 morning urine samples were collected from 252 female and 220 male volunteers (female-male ratio 1.1:1) along with a signed statement of their informed consent.

The enrolled subjects were selected by a simple random process. All respected the criteria of being healthy, with no history of hepatic or nephropatic conditions.

The volunteers were given clean 60 mL non-sterile plastic vessels with screw cap and were requested to fill it up with first morning midstream urine the day they were instructed. The filled plastic vessels with the fasting urine sample were returned to the laboratory in cold chain by icebox, and kept at -20 ºC until extraction.

## 2.2. Anthropometric and individual data

A small questionnaire provided information regarding anthropometrics and individual characteristics, as well as the home region of each participant (Table C.1).

**Table C.1.**
Anthropometric and individual characteristics of the sampled populations.

| Region (n) | Season | Gender | | Age (years) | | Stature (m) | | Weight (kg) | | BMI (kg/m$^2$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Female** | **Male** | **Mean±SD** | **Range** | **Mean±SD** | **Range** | **Mean±SD** | **Range** | **Mean±SD** | **Range** |
| **Porto (111)** | Winter 2007/2008 | 14 | 16 | 49.6±18.1 | 18-83 | 1.69±0.07 | 1.55-1.83 | 67.9±10.0 | 47-90 | 23.7±3.3 | 17.6-30.8 |
| | Summer 2008 | 10 | 9 | 53.9±18.9 | 24-83 | 1.69±0.08 | 1.55-1.83 | 66.8±11.8 | 44-90 | 23.3±3.9 | 17.2-30.8 |
| | Winter 2008/2009 | 22 | 21 | 47.2±18.5 | 23-84 | 1.68±0.08 | 1.52-1.84 | 66.3±11.1 | 45-100 | 23.6±3.3 | 17.6-30.8 |
| | Summer 2009 | 10 | 9 | 41.6±18.0 | 19-82 | 1.71±0.09 | 1.56-1.87 | 70.2±12.1 | 50-100 | 23.9±3.1 | 19.0-29.5 |
| | **Total - Porto** | 56 | 55 | 48.1±18.5 | 18-84 | 1.69±0.08 | 1.52-1.87 | 67.5±11.0 | 44-100 | 23.6±3.3 | 17.2-30.8 |
| **Coimbra (94)** | Winter 2007/2008 | 18 | 12 | 39.4±15.6 | 22-80 | 1.69±0.09 | 1.50-1.88 | 66.8±10.4 | 51-98 | 23.5±2.6 | 17.6-23.5 |
| | Summer 2008 | 6 | 7 | 42.4±13.6 | 24-61 | 1.73±0.11 | 1.50-1.90 | 72.9±14.3 | 51-100 | 24.2±3.7 | 17.6-30.9 |
| | Winter 2008/2009 | 13 | 8 | 32.6±13.5 | 21-59 | 1.72±0.11 | 1.50-1.97 | 70.6±17.1 | 49-115 | 23.8±4.3 | 17.6-34.3 |
| | Summer 2009 | 18 | 12 | 39.2±17.4 | 9-82 | 1.67±0.13 | 1.31-1.96 | 64.9±14.4 | 29-90 | 23.1±4.3 | 16.9-35.3 |
| | **Total - Coimbra** | 55 | 39 | 38.2±15.6 | 9-82 | 1.70±0.11 | 1.31-197 | 67.9±14.0 | 29-115 | 23.5±3.7 | 16.9-35.3 |
| **Lisboa (150)** | Winter 2007/2008 | 22 | 21 | 46.0±14.5 | 18-75 | 1.67±0.09 | 1.49-1.85 | 71.7±12.4 | 45-108 | 25.8±3.7 | 18.7-33.3 |
| | Summer 2008 | 19 | 21 | 46.7±13.5 | 24-70 | 1.68±0.09 | 1.50-1.85 | 72.1±11.6 | 54-110 | 25.7±3.6 | 20.6-36.8 |
| | Winter 2008/2009 | 18 | 16 | 48.1±13.8 | 24-70 | 1.68±0.09 | 1.50-1.85 | 72.9±12.0 | 50-105 | 25.9±3.9 | 20.2-37.3 |
| | Summer 2009 | 18 | 15 | 47.9±14.4 | 25-71 | 1.67±0.09 | 1.50-1.85 | 72.3±12.2 | 55-104 | 25.8±3.6 | 20.9-34.4 |
| | **Total - Lisboa** | 77 | 73 | 47.1±13.9 | 18-75 | 1.67±0.09 | 1.49-1.85 | 72.2±12.0 | 45-110 | 25.8±3.7 | 18.7-37.3 |
| **Alentejo (117)** | Winter 2007/2008 | 22 | 18 | 46.0±14.5 | 18-75 | 1.67±0.09 | 1.49-1.85 | 71.7±12.4 | 45-108 | 25.8±3.7 | 18.7-33.3 |
| | Summer 2008 | 14 | 16 | 51.5±22.0 | 9-83 | 1.65±0.11 | 1.47-1.88 | 72.1±12.2 | 53-113 | 26.6±4.9 | 20.9-42.5 |
| | Winter 2008/2009 | 14 | 8 | 45.7±21.3 | 6-82 | 1.63±0.13 | 1.22-1.82 | 73.2±17.1 | 21-114 | 27.4±6.1 | 14.1-45.7 |
| | Summer 2009 | 14 | 11 | 58.9±14.7 | 25-79 | 1.65±0.10 | 1.50-1.82 | 75.8±12.5 | 50-110 | 28.1±5.1 | 19.3-43.0 |
| | **Total - Alentejo** | 64 | 53 | 50.6±18.9 | 6-96 | 1.64±0.10 | 1.22-1.88 | 72.6±12.9 | 21-114 | 26.9±4.8 | 14.1-45.7 |
| **Four regions (472)** | **Total (Four seasons)** | **252** | **220** | **46.4±17.2** | **6-96** | **1.67±0.10** | **1.22-1.97** | **70.3±12.6** | **21-115** | **25.1±4.2** | **14.1-45.7** |

Body mass index (BMI) was calculated as defined by the WHO (1995), i.e. the weight in kilograms divided by the square of the height in metres (kg/m$^2$).

The individuals were divided in four categories regarding region (Porto, Coimbra, Lisboa, Alentejo,) and gender (female, male). Age-categories considered were defined as follows: [0;9], [10;19], [20; 29], [30;39], [40; 49], [50; 59], [60; 69], [70; 79], [80; 89], [90; 99] years.

Cut-off points regarding height (<160, [160;169], [170;179], [180;189], >190 cm), weight (<50, [50;59], [60;69], [70;79], [80;89], >90 kg) and BMI (<18,5, [18.5;24.9], [25;29.9], [30;34.9], [35;39.9], >40 kg/m$^2$) were also established.

## 2.3. Reagents and materials

### 2.3.1. Materials and equipments

Filter paper Whatman No. 4 (150 mm, Whatman International Ltd. Maidstone, England) and polyamide membrane filters (0.2 µm, 50 mm, Whatman GmbH, Dassel, Germany) were used. Ochratest™ IACs were acquired from VICAM (Watertown, USA).

A vacuum manifold of Macherey-Nagel (USA), a pump of Dinko (mol. D-95, 130 W, 220 V), a magnetic stirrer (Agimatic-S, Selecta, Barcelona, Spain), a Retsh vortex mixer (Haan, Germany), and a Sonorex RK 100 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 (30x4 mm ID) (England) preceding a Hichrom C18 column (5 µm, 250x4.6 mm ID). Detection and confirmation were performed on a Perkin-Elmer Model LS45 spectrofluorimeter (Beaconsfield, UK). The results were recorded on a Hewlett–Packard 3390A integrator (Philadelphia, PA, USA).

As OTA is UV-decomposable, special care was taken to protecting it from daylight, through the use of amber glass material (Valenta *et al.*, 1998; Yang *et al.*, 2010).

### 2.3.2. Reagents, standards and solutions

The reagents acetonitrile (Carlo Erba, Milan, Italy), methanol (Panreac Química Sau, Barcelona, Spain), toluene (Barker, J. T. Barker, Holland) and benzene (HACH Company, USA) were all of HPLC grade.

Boron trifluoride–methanol 14 % solution and acetic acid were purchased from Sigma-Aldrich (Laborchemikalien, Germany), hydrochloric acid, sodium hydroxide, sodium hydrogen carbonate, potassium chloride, potassium dihydrogenphosphate and anhydrous disodium hydrogen phosphate from Merck (Darmstadt, Germany), and sodium chloride from Baker Ltd. (Pagenham, England), all of analytical grade.

The OTA standard was obtained from Sigma Chemical Co. (St. Louis, MO, USA) with ≥98 % purity. OTA stock and intermediate standard solutions were prepared as described previously (*vide* Study A, section 2.4.2). The working standard solution was prepared at 10 µg/L, in mobile phase. The calibration curve standard solutions were prepared in mobile phase at concentrations of 1.0, 2.0, 5.0 and 10 µg/L.

Water was purified by distillation and passage through Milli-Q system (Millipore, Bedford, MA, USA).

PBS was prepared as described previously (*vide* Study A, section 2.4.2), and the solution of 5 % $NaHCO_3$ was prepared in distilled water.

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

All liquid chromatographic reagents and solutions were vacuum-filtered in polyamide membrane filters and degassed for 15 min in an ultrasonic bath prior to use.

## 2.4. Safety measures

The safety precautions applicable to OTA handling, waste disposal and decontamination of glassware and materials used in OTA analysis was described previously (*vide* Study A, section 2.5).

## 2.5. Experimental procedure

The methodology used for OTA determination in urine samples was based on that described by Pena *et al.* (2006).

### 2.5.1. Sample extraction and clean-up

Briefly, 10 mL of the sample were mixed with 10 mL of 5 % $NaHCO_3$, and then passed through a filter paper. The filtrate was cleaned-up through the IAC at a flow rate of 1 drop per second. The column was then washed twice with 5 mL of distilled water, and afterwards OTA was eluted with 3 mL of methanol.

The eluate was dried in a bath at 50 °C under a gentle nitrogen flow, and the dried extract was stored at -20 °C.

### 2.5.2. Immunoaffinity columns regeneration

Immediately after their first use, IACs were regenerated, only once, as previously described (*vide* Study A, section 2.6.2).

### 2.5.3. Detection and quantification

Before injection, the dried extract was dissolved in 125 µL of mobile phase. LC/FD analysis were performed using a 20 µL injection volume, and with an isocratic flow of the mobile phase set at 1 mL/min. Wavelengths used were 333 nm for excitation and 460 nm for emission, both with a spectral bandwidth of 10 nm.

The use of an OTA external standard, at a concentration of 0.01 µg/mL allowed the identification and quantification of the OTA detected in the urine samples.

### 2.5.4. Analytical quality assurance

Performance parameters were determined under repeatability conditions, namely with same urine sample, same operator, same apparatus, same laboratory, and during the course of three days. OTA method revalidation was carried out using a blank urine sample with no detectable mycotoxin.

Recovery assays were performed by spiking in triplicate an OTA-free urine sample, during three days at fortification levels of 0.02, 0.05 and 0.1 µg/L. After fortification, the sample was left to stand in the dark for 15 min, after which the Pena *et al.* (2006) protocol was followed.

Linearity for OTA quantification was verified using the linear least-squares regression procedure by plotting OTA peak areas against calibration samples at concentrations of 1.0, 2.0, 5.0 and 10.0 µg/L.

LOQ was determined as the lowest OTA concentration at which a sample could be spiked and still be clearly quantified, i.e. originate accurate and repeatable results with the application of the experimental procedure (signal-to-noise ratio of approximately 10:1).

OTA peak identity was confirmed by converting OTA into its methyl ester form, adding 150 μL of boron trifluoride methanolic 14 % solution ($BF_3$-$CH_3OH$ 14 %) to the dried sample extracts, and evaporating the mixture at 60 °C for 10 min. This was similar to the method described by Castegnaro *et al.* (1990), differing from it in its lower reconstitution volume of 150 μL. The OTA methyl esters were analysed under the LC conditions previously described.

### 2.6. Statistical analysis

For statistical analysis, when the concentration was below the LOQ it was set to 50 % of that limit. All data produced during this work was analysed with the support of appropriate statistical tools, as described in Study A (section 2.8).

## 3. Results and Discussion

### 3.1. Analytical quality assurance

Recoveries ranged from 96.43 to 100.53 % for fortification levels of 0.02 and 0.05 μg/L, respectively. Detailed data on exactitude and within- and between-day repeatability are shown in Table C.2.

**Table C.2.**
Performance data obtained for triplicate OTA spiked urine in within- and between-day variability assays.

| **Spiking level (μg/L)** | **0.02** | **0.05** | **0.1** |
|---|---|---|---|
| Mean recovery (%) | 96.43 | 100.53 | 96.53 |
| RSD $_{within-day}$ (%) | 9.30 | 7.39 | 7.41 |
| RSD $_{between-day}$ (%) | 9.70 | 12.93 | 4.12 |

The calibration curve in the linearity assay showed a coefficient of correlation of 0.9998. LOQ was calculated as 0.008 μg/L.

Figure C.1 shows the chromatograms for the 10 µg/L standard solution, and an OTA-contaminated urine sample. The conversion of OTA to its methyl ester, with a longer retention time, confirmed the identity of the toxin.

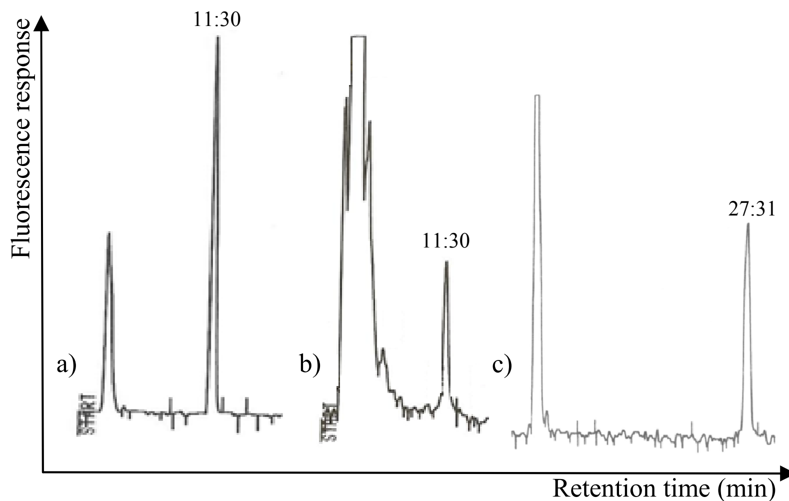


**Figure C.1.** Representative LC/FD chromatograms obtained with: a) an OTA standard (10 µg/L; Retention time 11:30min); b) an OTA naturally contaminated urine sample (Retention time 11:30 min); c) OTA methyl ester after boron fluoride-methanol derivatization of a urine sample (Retention time 27:31 min).

## 3.2. Urine contamination

Among a subgroup of individuals (n=65) that provided samples in more than one collection season, specifically in winter of 2007/2008 and summer of 2008, a high within-subject variation of OTA urine content was observed (Figure C.2). This observation was supported by a Kolmogorov-Smirnov test and a Shapiro-Wilk’s test. As a result, it is reasonable to consider that OTA urine levels, like OTA serum levels (Palli *et al.*, 1999), have a limited use at the individual level but can be used to characterise populations or subgroups of subjects. The high within-subject variability is also highly suggestive and consistent with OTA in urine being a short-time biomarker. It is possible in this way to identify the strongest determinants of OTA urine levels, considering the group of enrolled populations.

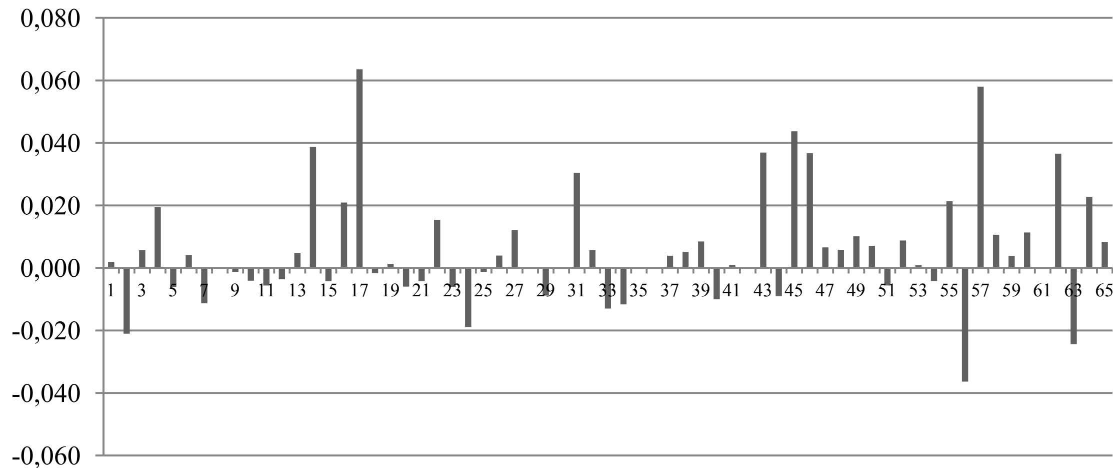


**Figure C.2.** Distribution of differences in OTA levels between winter of 2007/2008 and summer of 2008 in each of the 65 subjects.

The main descriptive statistics of the testing results with reference to the total enrolled participants (n=472) are summarised in Table C.3. The urine survey showed an OTA incidence of 86.4 %, with an average contamination level of 0.019 µg/L. However, there was a great variability, whether considering the incidence (ranging from 57.9 % to 100 %), maximum levels (ranging from 0.025 to 0.122 µg/L) or mean levels (ranging from 0.013 to 0.029 µg/L).

The distribution of the results called for non-parametric statistical analysis. In general, and in contrast to what occurs with parametric techniques, for a given problem, two or more non-parametric techniques are available. Therefore, all non-parametric methods available for any given type of problem were usually applied, together with scatter plots or box and whisker plots, in order to be able to fully understand the data characteristics.

Going into regional detail, it was found that all regions presented a high frequency of contamination (higher than 80 %), with Alentejo population featuring the highest (close to 97 %). Such difference is maintained when comparing the percentage of samples above the LOQ, in which all the regions presented nearly 60 % and Alentejo nearly 85 %. Moreover average contamination levels were comparable across all regions except for Alentejo population, which surpassed all the remaining ones with 0.023±0.016 µg/L. Alentejo populations also exhibited the greatest variation in the range, varying from non-detected to 0.122 µg/L.

**Table C.3.**
Descriptive statistics regarding OTA occurrence and contamination levels in urine according to the region and season of collection.

| Collection season | Region | Sample size | Positive (%) | Maximum (µg/L) | ≥ LOQ (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Number (%) | Mean±SD (µg/L) | Median (µg/L) |
| **Winter 2007/2008** | Porto | 30 | 29 (96.7 %) | 0.062 | 22 (73.3 %) | 0.021±0.014 | 0.016 |
| | Coimbra | 30 | 22 (73.3 %) | 0.034 | 16 (53.3 %) | 0.014±0.007 | 0.011 |
| | Lisboa | 43 | 31 (72.1 %) | 0.071 | 27 (62.8 %) | 0.026±0.017 | 0.019 |
| | Alentejo | 40 | 39 (97.5 %) | 0.064 | 36 (90.0 %) | 0.023±0.012 | 0.018 |
| | **Total** | **143** | **121 (84.6 %)** | **0.071** | **101 (70.6 %)** | **0.022±0.014** | **0.017** |
| **Summer 2008** | Porto | 19 | 11 (57.9 %) | 0.040 | 9 (47.4 %) | 0.017±0.011 | 0.015 |
| | Coimbra | 13 | 13 (100 %) | 0.069 | 10 (76.9 %) | 0.021±0.021 | 0.011 |
| | Lisboa | 40 | 36 (90.0 %) | 0.041 | 24 (60.0 %) | 0.014±0.008 | 0.013 |
| | Alentejo | 30 | 30 (100 %) | 0.063 | 27 (90.0 %) | 0.024±0.013 | 0.020 |
| | **Total** | **102** | **90 (88.2 %)** | **0.069** | **70 (68.6 %)** | **0.019±0.013** | **0.016** |
| **Winter 2008/2009** | Porto | 43 | 33 (76.7 %) | 0.036 | 20 (46.5 %) | 0.013±0.006 | 0.012 |
| | Coimbra | 21 | 19 (90.5 %) | 0.043 | 14 (66.7 %) | 0.015±0.009 | 0.012 |
| | Lisboa | 34 | 29 (85.3 %) | 0.094 | 18 (52.9 %) | 0.020±0.019 | 0.016 |
| | Alentejo | 22 | 21 (95.5 %) | 0.122 | 19 (86.4 %) | 0.029±0.026 | 0.020 |
| | **Total** | **120** | **102 (85.0 %)** | **0.122** | **71 (59.2 %)** | **0.019±0.018** | **0.015** |
| **Summer 2009** | Porto | 19 | 17 (89.5 %) | 0.033 | 17 (89.5 %) | 0.018±0.007 | 0.016 |
| | Coimbra | 30 | 23 (76.7 %) | 0.025 | 17 (56.7 %) | 0.014±0.005 | 0.012 |
| | Lisboa | 33 | 32 (97.0 %) | 0.053 | 26 (78.8 %) | 0.016±0.009 | 0.014 |
| | Alentejo | 25 | 23 (92.0 %) | 0.042 | 17 (68.0 %) | 0.014±0.008 | 0.012 |
| | **Total** | **107** | **95 (88.8 %)** | **0.053** | **77 (72.0%)** | **0.016±0.008** | **0.013** |
| **Four collection seasons** | Porto | 111 | 90 (81.1 %) | 0.062 | 68 (61.3 %) | 0.017±0.010 | 0.014 |
| | Coimbra | 94 | 77 (81.9 %) | 0.069 | 57 (60.6 %) | 0.016±0.011 | 0.012 |
| | Lisboa | 150 | 128 (85.3 %) | 0.094 | 95 (63.3 %) | 0.019±0.014 | 0.015 |
| | Alentejo | 117 | 113 (96.6 %) | 0.122 | 99 (84.6 %) | 0.023±0.016 | 0.018 |
| | **Total (Four regions)** | **472** | **408 (86.4 %)** | **0.122** | **319 (67.6 %)** | **0.019±0.014** | **0.015** |

These assessments were corroborated by the statistical analysis. Kruskal-Wallis test (p=0.0000) revealed differences between Alentejo and each of the three remaining regions, Porto (p=0.000002), Coimbra (p=0.000000), and Lisboa (p=0.000009). Box and whisker plots for each region are shown in Figure C.3.

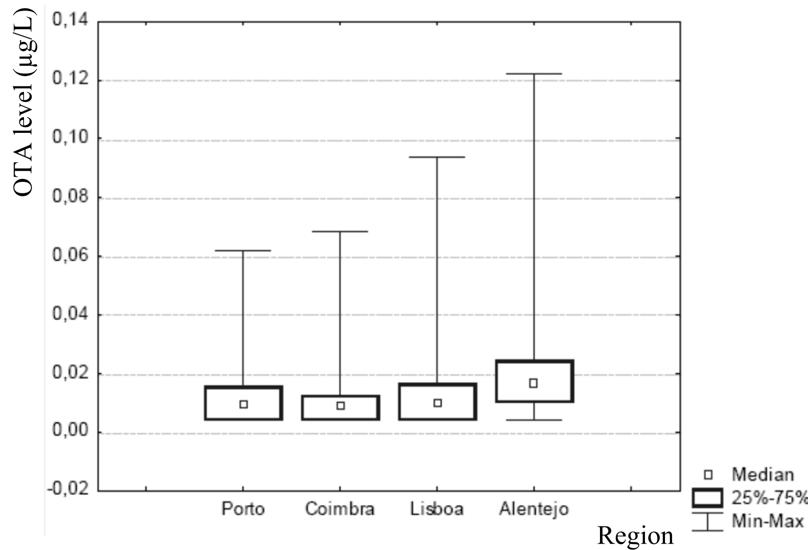


**Figure C.3.** Variation in OTA levels per region, displayed as box and whisker plots based on medians, quartiles and extreme values.

Without data from food intake (types of foods, consumption and corresponding contamination level) one can only speculate on the rationales of such difference. The Portuguese mainland presents a typical Mediterranean climate, although the influence of factors such as the Atlantic Ocean and the landscape results in obvious contrasts, by provoking a degradation of the typically Mediterranean characteristics. So the Mediterranean climate loses its characteristics on the mainland from South to North and from the coast to the interior. A Mediterranean climate is characterised by having a long, hot, and dry summer period, and a moderate winter, with a relatively low total atmospheric precipitation, while the Atlantic influence increases the overall humidity (IGP, 2010; Minerva, 2010). Climatic conditions conductive to mould spoilage, especially during storage, can thus be echoed.

Though significant in view of the specificity of the ecological niches of each ochratoxigenic species, regional climate conditions are not the only influential factor. Between regions different dietary habits persist, explained by different food

consumption patterns or socio-economical power. For instance, Alentejo is the region that presents lower human, social and economical development rates from all the four regions considered (DPP, 2002). Geographical variations observed can also derive from differences in the ingestion of contaminated foodstuffs, namely in respect to dietary habits or origin of the food consumed. Innermost peripheral regions are more dependent on locally grown or stored foodstuffs that can influence mould growth and mycotoxin production in a different way than in the central or littoral regions for the existing variations of climate and humidity levels. Bigger population centres are more dependent on imported supplies.

Regional differences have also been observed in OTA exposure evaluation using blood biomarkers in several countries, like Sweden (Breitholtz *et al.*, 1991), Lebanon (Assaf *et al.*, 2004), Argentina (Pacin *et al.*, 2008), and Tunisia (Grosso *et al.*, 2003; Maaroufi *et al.*, 1995).

Taking into account Table C.3, it was noticeable a seasonal pattern of increased OTA incidence from winter (nearly 85 %) to summer (nearly 88 %), in each of the studied years. Concurrently a decrease in OTA mean levels was observed, from winter of 2007/2008 (0.022±0.014 μg/L) to summer of 2008 (0.019±0.013 μg/L), and from winter of 2008/2009 (0.019±0.018 μg/L) to summer of 2009 (0.016±0.008 μg/L). Even so, between the summer of 2008 and the winter of 2008/2009 the average values were similar.

Statistical analysis confirmed the lack of differences between years and seasons, although multiple comparison through Kruskal-Wallis test showed a difference at $p=0.0623$ level, relatively close to significance level ($\alpha=0.05$) According to such analysis, average OTA values of the winter of 2007/2008 were significantly higher than winter of 2008/2009 ones ($p=0.0489$).

No study is available for comparison and interpretation of the seasonal variation of the average OTA values in urine. However, most studies of OTA in blood do show opposite results, i.e. increase of summer OTA levels. For instance, higher values were reported in blood samples collected during the summer in several Mediterranean countries, like Italy (Palli *et al.*, 1999), Turkey (Erkekoğlu *et al.*, 2010), and Croatia (Domijan *et al.*, 1999; Peraica *et al.*, 2001).

In urine of children (<5 years old) from Sierra Leone, Jonsyn-Ellis (2001) observed a higher incidence of OTA contamination during the collection period of dry season

comparatively to the rainy season (correspondingly 14.5 % and 10.8 %). This result is in agreement with the higher incidence of contamination observed in the present study.

Particular climatic conditions could have been responsible for a higher contamination of the ingested food in the period preceding collection, as well as a possible seasonal variation of the dietary and drinking habits that could lead to a different intake during different periods of the year. Evaluation of average and specific short-term individual dietary information would be necessary to clarify this issue (Palli *et al.*, 1999).

Considering gender, no significant differences were detected between female and male-provided samples, whether on incidence (85.71 *versus* 87.27 %) or mean level (0.020 *versus* 0.018 µg/L) as detailed in Table C.4.

**Table C.4.**
Descriptive statistics regarding OTA occurrence and contamination levels in urine according to the gender and season of collection.

| Collection season | Gender | Sample size | Positive (%) | Maximum (µg/L) | ≥ LOQ (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Number (%) | Mean±SD (µg/L) | Median (µg/L) |
| **Winter 2007/2008** | Female | 76 | 64 (84.2 %) | 0.064 | 52 (68.4 %) | 0.022±0.015 | 0.016 |
| | Male | 67 | 57 (85.1 %) | 0.071 | 49 (73.1 %) | 0.022±0.013 | 0.017 |
| **Summer 2008** | Female | 49 | 44 (89.8 %) | 0.069 | 35 (71.4 %) | 0.019±0.013 | 0.015 |
| | Male | 53 | 46 (86.8 %) | 0.063 | 35 (66.0 %) | 0.020±0.013 | 0.016 |
| **Winter 2008/2009** | Female | 67 | 56 (83.6 %) | 0.122 | 41 (61.2 %) | 0.023±0.022 | 0.016 |
| | Male | 53 | 46 (86.8 %) | 0.036 | 30 (56.6 %) | 0.014±0.006 | 0.013 |
| **Summer 2009** | Female | 60 | 52 (86.7 %) | 0.053 | 40 (66.7 %) | 0.016±0.009 | 0.013 |
| | Male | 47 | 43 (91.5 %) | 0.042 | 37 (78.7 %) | 0.015±0.006 | 0.013 |
| **Four collection seasons** | **Female** | **252** | **216 (85.7 %)** | **0.122** | **168 (66.7 %)** | **0.020±0.016** | **0.015** |
| | **Male** | **220** | **192 (87.3 %)** | **0.071** | **151 (68.6 %)** | **0.018±0.011** | **0.015** |

Both Mann-Whitney U ($p=0.999$) and Kolmogorov-Smirnov ($p>0.10$) statistical tests supported the lack of significant differences between genders, as furthermore readily obvious in the displayed graphic (*vide* Figure C.4).

Already in previous studies, during urine biomonitoring surveys of Coimbra (Pena *et al.*, 2006; Manique *et al.*, 2008) and Valencia (Manique *et al.*, 2008) inhabitants during winter, no significant differences were found between male- and female-provided samples. The only exception was the 20-39 years group of the study of Pena *et*

*al.* (2006), in which male-provided samples presented higher values of incidence and average level.

In view of these earlier results, allied with the absence of significant differences reported by surveys in foreign populations (Fazekas *et al.*, 2005b; Coronel *et al.*, 2011a), little to no difference between genders was expected. But on the other hand, one could anticipate gender effect as a key determinant with a role in OTA metabolism or simply reflecting different exposure levels between genders, ultimately attributable to different dietary patterns or quantity of ingested food.

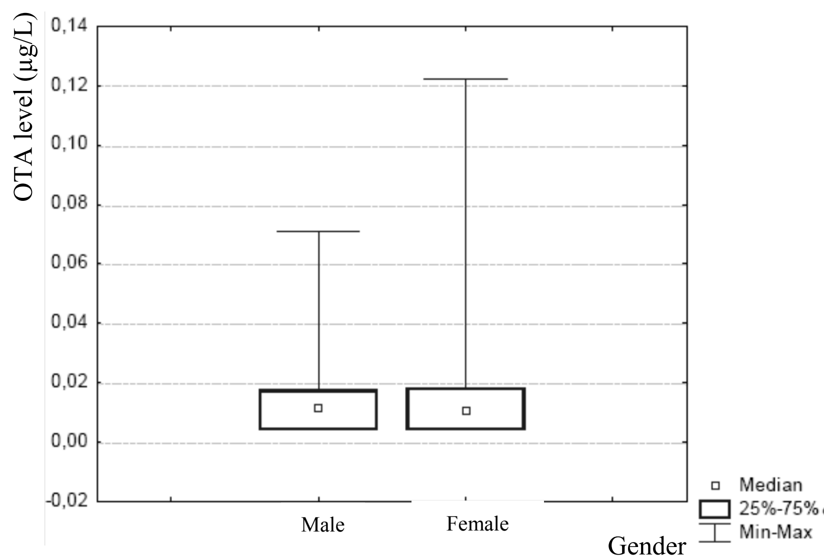


**Figure C.4.** Variation in OTA levels according to gender, displayed as box and whisker plots based on medians, quartiles and extreme values.

According to clinical observations in humans and studies in experimental animals *in vivo* and in *in vitro* models, renal structure and functions under various physiological, pharmacological and toxicological conditions are different between genders, which may reflect gender-hormone-regulated expression and action of transporters in the apical and basolateral membranes of nephron epithelial cells; specifically, renoprotective effects of estrogens were demonstrated in nephrotoxicity induced by OTA. The renoprotective effects of estrogens were further demonstrated in various pathophysiological conditions and diseases in humans and experimental animals, including nephrotoxicity induced by the mycotoxin OTA (Sabolić *et al.*, 2007).

Nevertheless, it is important to underline that such weight of evidence does not explain potential differences in OTA urine concentrations obtained between genders; it merely suggests that further work is missing, for instance regarding the susceptibility of each gender in nephropathic condition presumably induced by or in which OTA might be involved, like BEN and/or CIN.

Additionally, exposure levels could also reflect different consumption patterns or simply different amount of ingested food potentially contaminated. In fact, as demonstrated for the European population (Miraglia & Brera, 2002), wine, beer and coffee are, right after cereals and their derivatives, the major contributors to OTA exposure. Their consumption is definitely higher for the male gender which could also induce a potential gender-related difference. The recognised low level contamination of a wide range of different consumed foods may however attenuate such contribution, and thus dilute potential gender-related differences of consumption.

Like the gender factor, age does not appear to be a robust determinant of OTA exposure. No differences of OTA urine contamination were found between the different age-categories considered (*vide* Table C.5). These results were supported by statistical analysis, involving both Kruskal-Wallis ANOVA by Ranks and Median Tests.

**Table C.5.**
Descriptive statistics regarding OTA occurrence (%) and contamination levels (µg/L) in urine according to the age in the four seasons of collection.

| **Age (Years)** | **Sample size** | **Positive (%)** | **Maximum (µg/L)** | **≥ LOQ (%)** | | |
|---|---|---|---|---|---|---|
| | | | | **Number (%)** | **Mean±SD (µg/L)** | **Median (µg/L)** |
| [0;9] | 2 | 2 (100 %) | 0.027 | 2 (100 %) | 0.019±0.011 | 0.019 |
| [10;19] | 4 | 4 (100 %) | 0.024 | 4 (100 %) | 0.016±0.006 | 0.015 |
| [20;29] | 88 | 75 (85.2 %) | 0.063 | 62 (70.5 %) | 0.017±0.010 | 0.014 |
| [30;39] | 91 | 75 (82.4 %) | 0.044 | 53 (58.2 %) | 0.017±0.009 | 0.015 |
| [40;49] | 88 | 77 (87.5 %) | 0.062 | 62 (70.5 %) | 0.019±0.011 | 0.016 |
| [50;59] | 82 | 75 (91.5 %) | 0.122 | 55 (67.1 %) | 0.026±0.023 | 0.016 |
| [60;69] | 64 | 53 (82.8 %) | 0.071 | 42 (65.6 %) | 0.019±0.014 | 0.012 |
| [70;79] | 36 | 32 (88.9 %) | 0.062 | 26 (72.2 %) | 0.020±0.013 | 0.015 |
| [80;89] | 16 | 14 (87.5 %) | 0.027 | 12 (75.0 %) | 0.015±0.007 | 0.014 |
| [90;99] | 1 | 1 (100 %) | 0.015 | 1 (100 %) | 0.015 | 0.015 |

On the basis of non-parametric descriptive statistics (quartiles and extreme values as summarised in Figure C.5) it appears that younger individuals tend to feature higher

OTA levels, which can be masked by extreme values in the remaining age groups. However the number of individuals in the two younger age-categories was so small, that it is more prudent to conclude that no significant differences exist.

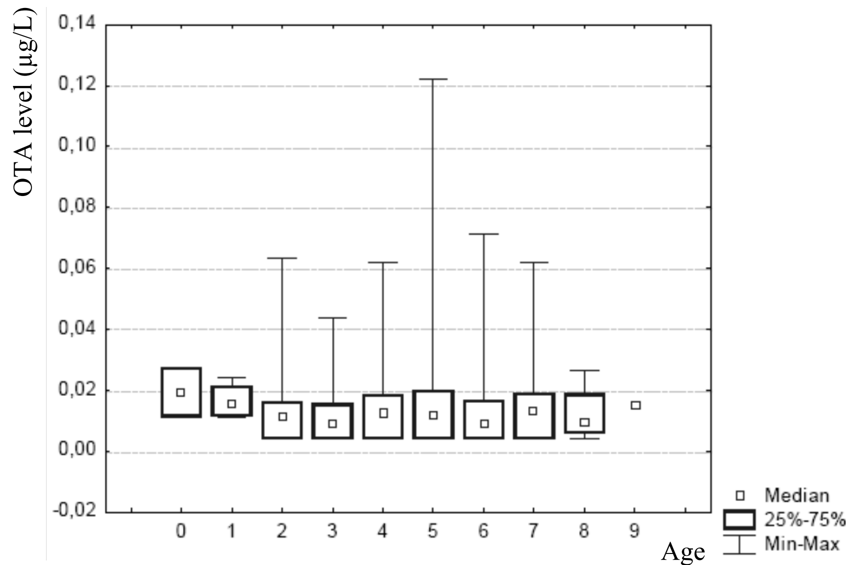


**Figure C.5.** Variation in OTA levels according to age-categories, displayed as box and whisker plots based on medians, quartiles and extreme values. Age-categories considered were as follows: 0 [0;9]; 1 [10;19]; 2 [20; 29]; 3 [30;39]; 4 [40; 49]; 5 [50; 59]; 6 [60; 69]; 7 [70; 79]; 8 [80; 89]; 9 [90; 99].

Among published studies, Gilbert *et al.* (2001) observed a lower mean level on the >45-year-old participants. However, significant correlations between OTA urine contamination levels and age were absent in more recent studies, whether considering average daily OTA excretion (Akdemir *et al.*, 2010), or conventional point observations with morning urine (Coronel *et al.*, 2011a)

Likewise, no correlation was found between OTA levels and the remaining selected individual characteristics (height, weight, BMI) as analysed by the Spearman rank order correlation and the Kendall's Tau correlation coefficient, as well as the scatter plots.

Ultimately, these findings suggest that OTA contamination is widespread in foods consumed by the Portuguese population. In other words, the number of different contaminated foods contributing to this exposure is probably big, or alternatively the major contributors are transversal food to the entire population, regardless the age, gender, region or economic power. However additional analyses are needed to explore

the dietary determinants of OTA levels in this population (Ueno *et al.*, 1998; Palli *et al.*, 1999).

Comparison of the present results with previous Portuguese surveys is restricted to two prior surveys conducted in Coimbra region, both in winter season. In the first survey (Pena *et al.*, 2006) populations featured higher OTA urine mean level (0.038 μg/L), although an almost comparable frequency of contamination (70 %). As for the second survey (Manique *et al.*, 2008) a quite similar mean level was observed (0.019 μg/L), although the frequency of contamination was set at about half the value (43.3 %) determined in the present study. Although weather conditions are not the sole determinants, it is perhaps worthy of notice that the collection period of the latter study (Manique *et al.*, 2008) was preceded by the hottest summer of the previous 75 years, with an air temperature 2.38 ºC above the average value (IM, 2009). As mentioned above, such climate condition could have been influential in the fungal development or OTA production throughout the food chain of the food commodities consumed by the surveyed populations.

Analysing international values, it can be seen that, despite the recognised role of OTA, alone or with additive or synergistic effects from two or more mycotoxins (e.g. CIT, PA), in the chronic renal diseases described in the Balkans (BEN) and North African countries (CIN), for the high contamination levels reported (Bacha *et al.*, 1999; Nikolov *et al.*, 2002; Sangare-Tigori *et al.*, 2006: Pfohl-Leszkowicz *et al.*, 2007), it is found in urine of study populations worldwide. In fact, and as summed up in Table C.6, the highest incidences of contamination, of 100 %, were observed mutually in BEN-endemic (Petkova-Bocharova *et al.*, 2003) and non-endemic regions, such as Italy (Breitholtz- Emanuelsson *et al.*, 1994) and Germany (Muñoz *et al.*, 2010). Equally higher incidences were found in Sri Lanka, among healthy individuals and patients with chronic kidney disease of uncertain aetiology (Desalegn *et al.*, 2011). In the present study, OTA incidence in the urine of the surveyed Portuguese populations is among the highest, along with the similar value for Valencia populations (Manique *et al.*, 2008), and close to the incidence reported in UK (Gilbert *et al.*, 2001) and in eastern Croatia (Domijan *et al.*, 2003). However, the OTA mean value in Portuguese regions is one of the lowest reported. In reality, the only lower values were those found for Hungarian (Fazekas *et al.*, 2005b) and Croatian populations, in both BEN-endemic and non-endemic areas (Domijan *et al.*, 2009).

**Table C.6.**
Occurrence of OTA in urine in different countries.

| Country (Region) [Year] | Urine sampling | Sampled population | LOD (LOQ) | Incidence (%) | Maximum (µg/L) | Mean±SD (µg/L) | Reference |
|---|---|---|---|---|---|---|---|
| Spain (Valencia) [2010] | Point sampling | Adult volunteers | 0.5 (1.5) | 3/27 (11.1 %) | <LOQ | n.a. | Rubert *et al.* (2011) |
| Sri Lanka (North Central Province) [2009] | Point sampling | CKDue patients (early stage)<br>CKDue patients (late stage)<br>Relatives of CKDue patients | 0.005 | 14/14 (100 %)<br>15/17 (88.2 %)<br>6/6 (100 %) | 0.360<br>0.058<br>0.223 | 0.037<br>0.012<br>0.085 | Desalegn *et al.* (2011) |
| Spain (Lleida) [2009] | Point sampling | Adult volunteers | 0.034 (0.112) | 9/72 (12.5 %) | 0.562 | 0.237±0.181 | Coronel *et al.* (2011a) |
| Germany (Dortmund) [2008] | Point sampling | Adult volunteers | 0.02 (0.05) | 13/13 (100 %) | 0.14 | 0.07±0.05 | Muñoz *et al.* (2010) |
| Croatia [2000] | Point sampling | Healthy non-BEN-area<br>Healthy BEN-area | 0.005 | 5/18 (28 %)<br>19/45 (43 %) | 0.02<br>0.086 | 0.003±0.005<br>0.007±0.014 | Domijan *et al.* (2009) |
| Croatia [2005] | Point sampling | Healthy non-BEN-area<br>Healthy BEN-area | 0.005 | 1/18 (6 %)<br>8/45 (18 %) | 0.01<br>0.015 | 0.005±0.024<br>0.001±0.003 | |
| Portugal (Coimbra) [2005;2006] | Point sampling | Healthy | (0.007) | M: 13/30 (43.3 %)<br>A: 14/30 (46.7 %) | M: 0.208<br>A: 0.110 | M: 0.019±0.041<br>A: 0.018±0.027 | Manique *et al.* (2008) |
| Spain (Valencia) [2005] | Point sampling | Healthy | (0.007) | M: 25/31 (80.6 %)<br>A: 26/31 (83.9 %) | M: 0.124<br>A: 0.089 | M: 0.032±0.031<br>A: 0.028±0.019 | |
| Portugal (Coimbra) [2004] | Point sampling | Healthy adults | (0.02) | 42/60 (70 %) | 0.105 | 0.038 | Pena *et al.* (2006) |

(A: afternoon samples; BEN: Balkan Endemic Nephropathy; CKDue: chronic kidney disease of uncertain etiology ; M: morning samples; n.a.: non-applicable; n.g.: not given; UTT: Urinary Tract Tumors)

**Table C.6. (Continued)**
Occurrence of OTA in urine in different countries.

| Country (Region) [Year] | Urine sampling | Sampled population | LOD (LOQ) | Incidence (%) | Maximum (µg/L) | Mean±SD (µg/L) | Reference |
|---|---|---|---|---|---|---|---|
| Hungary [2003] | 24h sampling | Healthy | 0.004 (0.006) | 54/88 (61.4 %) | 0.065 | 0.013 | Fazekas *et al.* (2005b) |
| Bulgaria (Gorno Peshtene) (Beli Izvor) | 24h sampling | Healthy BEN area residents | (0.004) | 5/5 (100 %)<br>11/11 (100 %) | 0.330<br>1.910 | 0.051±0.044<br>0.168±0.111 | Petkova-Bocharova *et al.* (2003) |
| Croatia (Eastern) [2000] | Point sampling | Healthy | 0.3 (0.9) | 33/35 (94.3 %) | 5.22 | 2.39±1.29 | Domijan *et al.* (2003) |
| Bulgaria [1984-1990] | Point sampling | BEN/UTT patients | n.g. | 14/36 (38.9 %) | 0.604 | n.g. | Nikolov *et al.* (2002) |
| | | Healthy persons from BEN families | | 12/25 (48.0 %) | 0.033 | | |
| | | Healthy persons from non-BEN families in BEN villages | | 14/32 (43.8 %) | 0.043 | | |
| | | Healthy persons from non-BEN villages in BEN area | | 4/31 (12.9 %) | 0.041 | | |
| | | Healthy persons from non-BEN area | | 0/3 (0 %) | n.a. | | |
| Italy (South & North) | 24h sampling | Healthy | 0.005 | 22/38 (57.9 %) | 0.046 | n.g. | Pascale & Visconti (2001) |
| U.K. | 24h sampling | Volunteers | 0.01 | 46/50 (92.0 %) | 0.058 | n.g. | Gilbert *et al.* (2001) |
| Sierra Leone [1992-1993] | Point sampling | Children in school (5-14years) | 0.2 | 110/434 (25.3 %) | 148 | n.g. | Jonsyn-Ellis (2001) |
| Sierra Leone | Point sampling | Children in hospital (<5years) | n.g. | 13/54 (24.1 %) | 26.6 | n.g. | Jonsyn (1999) |
| Bulgaria | Point sampling | BEN/UTT patients and controls | 0.005 | 61/152 (40.1 %) | 0.03 | n.g. | Castegnaro *et al.* (1990) |

(A: afternoon samples; BEN: Balkan Endemic Nephropathy; CKDue: chronic kidney disease of uncertain etiology ; M: morning samples; n.a.: non-applicable; n.g.: not given; UTT: Urinary Tract Tumors)

Nevertheless comparison of the incidence and mean levels between different studies is difficult as different sampling groups and different years were surveyed. Furthermore the limits of detection or limits of quantification of the analytical methods are different in each case, given namely the different extraction and detection methods employed (Rubert *et al.*, 2011).

## 4. Conclusions

The urine biomonitoring conducted in the four Portuguese mainland regions in a two-year surveillance design, encompassing four collection periods, as herein described contributed for the database of human exposure in Portugal.

With reference to the obtained results, a high within-subject variation of OTA urine content was observed, suggesting and consistent with OTA in urine being a short-time biomarker, with a limited use at the individual level, but useful at a population or subgroup of subjects scale.

The urine survey showed a widespread and high OTA incidence, although at a relatively low average contamination level. Comparing with foreign studies, incidence was among the highest reported, while average levels were among the lowest.

The observed distribution free of the results determined an analysis through non-parametric statistical testing.

Among the surveyed regions, Alentejo population was identified as the most exposed, featuring the highest average level and frequency of OTA urine contamination, which might be related to different food consumption patterns or socio-economical power.

Winter of 2007/2008 was the collection season during which populations presented significantly higher OTA urine content, possibly explained by particular weather conditions conductive to OTA production affecting the food supply.

Concerning the remaining seasonal, gender and individual characteristics no other strong association was observed. Thus, a large number of foodstuffs were OTA contaminated and could therefore contribute to human exposure, or otherwise, major contributors to OTA exposure were probably transversal foods to the whole population.

These observations reinforce the need for OTA exposure evaluation, possibly specifically targeting the staple foods and dietary habits that sustain potential determinants of exposure.

# Part III

# Integrated overview

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**SCOPE:** This part comprises the general conclusions that can be taken from the studies conductive to the present dissertation. The independent results obtained for each of the three analysed matrices are combined to allow a contextualized and critical overview and final conclusion. The main limitations of the studies are also identified, and finally, the new perspectives fostered by obtained results are pointed out.

**ABRIDGED CONTENTS:** 1.Integrated overview of the obtained results; 2.General conclusions.

# 1. Integrated overview of the obtained results

The risks associated with mycotoxins depend on both hazard and exposure. Whilst the hazard of mycotoxins to individuals is probably more or less the same all over the world, the exposure is not the same, because of different levels of contamination and dietary habits in the various parts of the world (van Egmond *et al.* 2007). *Per se*, this last fact makes evident the importance of regular surveys and testing to evaluate exposure in each population.

Despite the fact that human exposure to OTA can proceed from the ingestion of various foodstuffs, OTA is found as a natural contaminant mainly of cereals. The contaminated cereals can represent a direct source of human exposure, by its direct consumption, or an indirect source through the consumption of products derived from animals fed with contaminated feed. Among these food-producing animals, pig is considered the most sensitive species, with the highest OTA half-life in serum, leading to higher OTA contamination and incidence in this species (Galtier *et al.*, 1981; Pozzo *et al.*, 2010; Schiavone *et al.*, 2008).

Bread and pork, two highly consumed foodstuffs in the Portuguese staple diet, with an intake transversal to the entire population, thus emerge as potentially significant contributors to OTA human exposure. Inevitably, the choice and analysis of two single foodstuffs limited the assessment of the total dietary exposure given that other important dietary intake sources could have been left out. On the other hand, the fact that only two food matrices were studied allowed the analysis of a higher number of samples in each of the two as opposed to the total diet studies (*vide* Part I, section 8.1).

Previous foreign studies (Verger *et al.*, 1999; Miraglia & Brera, 2002; Leblanc *et al.*, 2005; Kuiper-Goodman *et al.*, 2010; Tam *et al.*, 2011) demonstrated that cereals and cereal derivatives are the food groups contributing the most to OTA exposure. For instance, in the EU, cereals and their derivatives accounted for 50 % (Miraglia & Brera, 2002) while in France, the contribution of cereals and cereal products for OTA exposure was calculated as 70 %, with bread alone corresponding to almost 33 % (Leblanc *et al.*, 2005).

Despite the indirect exposure pathway that it may represent, OTA occurrence in animal products is generally not thought of as a major public health concern. Perhaps this is due to the insufficiency of studies to assess the significance of the products originating from mycotoxin-exposed food-producing animals as an exposure pathway for OTA among humans. EFSA considers negligible the risk associated with the consumption of food derived from animals fed with OTA-contaminated feeds (EFSA, 2006). In the EU, the overall contribution of products of animal origin to human exposure has been estimated to be not more than 3 % of the total ingested OTA (Miraglia & Brera, 2002). In France, meat, without differentiating its species, contributed with merely 2.6 % (Leblanc *et al.*, 2005). Even so, some studies have showed an evident association between OTA human exposure, assessed through exposure biomarkers, and dietary intake of pork (Turconi *et al.*, 2004; Muñoz *et al.*, 2006; Galvano *et al.*, 2008; Biasucci *et al.*, 2010).

Previous studies in Portugal reported higher contribution from bread to the human exposure to OTA (0.16-0.38 ng/kg bw/day: Juan *et al.*, 2008b; Bento *et al.*, 2009), as compared with pork (0.023 ng/kg bw/day: Guillamont *et al.*, 2005). Thus it was expected that bread, as a major cereal derivative, with a consumption transversal to the entire population, would contribute more to OTA exposure in the Portuguese population. However, that was not observed in the results described in the present dissertation, that portrayed a higher contribution of pork (0.958 ng/kg bw/day), as compared with bread (0.352 ng/kg bw/day) to the Portuguese dietary exposure to OTA. The sum of the individual contribution of these two surveyed foodstuffs to the OTA dietary intake of the Portuguese population was thus calculated as 1.31 ng/kg bw/day.

In the French total diet study, Leblanc *et al.* (2005) reported that bread and rusks were the main contributors to OTA exposure which alone contributed with 32.6 % (0.71 ng/kg bw/day), while meat, without differentiating its species, contributed with merely 2.6% (0.06 ng/kg bw/day) to the total OTA exposure (estimated at 2.16 ng/kg bw/day). For comparison purposes, the dietary exposure to OTA in the Portuguese population through consumption of bread and pork only (1.31 ng/kg bw/day) was more than half of the one estimated in the French population, the latter of which was calculated considering 29 different food groups (Leblanc *et al.*, 2005).

Through bread and pork consumption only, the total OTA dietary intake of an average Portuguese regular consumer (1.31 ng/kg bw/day) was inferior to the tolerable daily intake for OTA laid down at 4 ng/kg bw/day (Kuiper-Goodman *et al.*, 2010) or else at 5 ng/kg bw/day (NWGFTRE, 1991; SCF, 1998; *Conseil Supérieur d'Hygiène Publique de France*, 1999). Moreover it was far below the ones established by JECFA (2007) and EFSA (2006), at roughly 14.28 and 17.14 ng/kg bw/day, respectively. However, it was very close to the virtual safety dose of 1.5 ng/kg bw/day calculated by Kuiper-Goodman & Scoot (1989).

The fact that the calculated intake of the Portuguese population was inferior to the tolerable dose should not be neglected because it was compounded by merely two food items. Furthermore between regions/cities there may exist wide variations in consumption of the more analysed foods, and so if calculation is based on an average that means that some individuals may exceed this value, and so be at risk. Second, individuals may differ in their sensitivity to OTA. Finally, this mycotoxin may be additive to, or synergistic with, other chemicals in food and the environment (Mantle 2002).

It is worth mentioning that differences in the analytical performance of the method employed for OTA determination in pork, namely extraction efficiency, sensitivity and specificity, could to some extent justify the high contribution of pork, for OTA exposure among the Portuguese population.

In addition when comparing data from different surveys, it is important to notice that factors such as climate conditions during harvest, conditions and practices for grain/feed storage, kinds of feed, the content of OTA in feed, feeding systems, and feeding in relation to time of slaughtering can have a major influence on the degree of contamination of feedstuffs, and thus can also influence the OTA levels found in pork (Jørgensen & Petersen, 2002; Bryden, 2012).

Likewise, the microeconomic scenario could also, although in part and temporarily justify such high contribution. In effect, the three simultaneously conducted surveys (urine, bread, and pork) occurred during and after a troublesome cereal crisis (i.e. 2007-2008), characterised by a shortage of cereals and a consequent worsening of the financial speculation, which provoked a rise in the price of these commodities. Shortages in crop resources may have to be compensated with different crops or the same crop of a lower quality grade, potentially containing mycotoxins of a different

nature or with higher prevalence, depending on the weather conditions and the modifications of the biogeographical scenarios of crop cultivation. Farmers are often tempted to incorporate mouldy grain into animal diets to reduce feed costs. Low quality commodities, with visible mould growth or with levels of OTA unsuitable to enter the food chain are thus many times diverted into the feed chain. However, this practice does carry a risk not only from mycotoxin contamination but in some circumstances, altered nutrient content of the grain. If moulded grains are used in animal diets possible changes in composition must be accounted for in least-cost feed formulations. Moreover, changes in nutrient supply to the animal may alter its response to mycotoxin exposure and complicate diagnosis.

In the context of mycotoxins, the health status of food animals that are destined to enter the human food supply chain is often overlooked. Such actions have considerable potential consequences. Firstly, from the animal production and sanitary standpoint the concerns range from impairment of animal health and increased susceptibility to secondary infections, to decrease in the performance of food producing animals. Ochratoxicosis outbreaks can also ensue, although only seldom is it possible to relate the episodes with contaminated feed, as already reported in swine (Rutqvist *et al.*, 1978; Cruz *et al.*, 1984). Secondly, but not of less importance, another major setback is the potential carry-over effect linking feed and food chain, whenever humans consume derived products. A public health concern thereby arises, consequential to an indirect exposure through the contaminated animal derived foods consumed (Manning *et al.*, 2003; Gupta *et al.*, 2008).

Although recognizing that OTA can be transferred from feed into food of animal origin, through carry-over effect, since exposure assessments indicate that food of animal origin makes only a small contribution to the total human dietary exposure to OTA, no maximum levels were established for animal-derived products at an EU-scale. Furthermore, there are no enforceable values, merely guidance values for products intended for animal feeds, for orientation purposes only. Nevertheless, in the same document (CEC, 2006c) the Commission advises that member states to increase their monitoring for the presence of OTA in cereals and cereal products intended for animal feeding and compound feedingstuffs to assess the significance of residue levels in animal tissues, both with respect to animal health and to human exposure. The observed

high OTA contamination of pork, thus greatly contributing to Portuguese dietary exposure to OTA validated this recommendation.

However, one of the greatest difficulties in detecting mycotoxins in animal feed is their heterogeneous distribution in the raw and finished products. The case of OTA is particularly challenging since, contrary to other mycotoxins ($AFB_1$, DON, FBs), there is a lack of information on the distribution of contaminated units, which precludes the correct analysis and estimation of levels along the feed production supply chain. In contrast, their direct detection and quantification in the animals themselves, whether in blood, liver, kidneys or muscles, confirms the existence of the problem and minimizes the errors involved in feed sampling and analysis (EFSA, 2004; Vilar *et al.*, 2008).

The presence of OTA in the urine of healthy individuals as determined in the herein described study (*vide* Study C), in agreement with previous national surveys (Pena *et al.*, 2006; Manique *et al.*, 2008; Bento *et al.*, 2009) confirmed a constant and widespread exposure. The high within-subject variation of OTA urine content observed limited its use at the individual level, although it remained useful at a population or subgroup of subjects scale. Devoid of all the problems associated with the food sampling methods and consumption data collection, OTA determination in urine requires a single sample per person. However assessment of human exposure through determination of OTA in urine does not point out the food source of contamination involved. In any case, the observed absence of persistent significant differences between most of the anthropometric parameters, seasonal or regional factors strongly suggested that the source of OTA was transversal to the population, which means that, considering that the major source of exposure is the ingestion of contaminated food, some common dietary foodstuff was probably implicated (Ueno *et al.*, 1998).

Future projects are thus required to continue surveillance of OTA exposure, if possible entailing a higher number of foodstuffs. Particular attention needs to be paid to the exposure of specific population groups who could be exposed to OTA in quantities that exceed the tolerable intake levels. That was not possible in this dissertation given the absence of data of food consumption according to age, gender, socio-economical or other categories. Equally, the co-occurrence of OTA with other mycotoxins should also be ascertained in the foodstuffs surveyed.

Given the contribution of pork to OTA exposure among the Portuguese population, it is important in the future to ascertain if other animal products, like kidneys, liver, blood, and their derivatives embody a similar input. In addition, more scientific data are needed to ascertain the importance of animal products in general and pork in particular to substantiate the appropriateness of the current nonexistence of OTA maximum limits in meat and feeddingstuffs destined to food-producing animals.

## 2. General conclusions

i. The urine biomonitoring conducted simultaneously with a survey of the commercialised bread and pork in four Portuguese mainland regions (Porto, Coimbra, Lisboa and Alentejo) in a two-year surveillance design, encompassing four collection periods (winter of 2007/2008, summer of 2008, winter of 2008/2009 and summer of 2009), as herein described contributed for the database of human exposure to OTA in Portugal.

ii. Concurrently an input to improved analytical methodologies was presented, through the development and validation of a sensitive method for the determination of OTA in swine muscle. Extraction entailed the use of acidified methanol, ultrasound treatment and centrifugation, followed by an IAC clean-up step before mass spectrometric detection. The developed IAC-LC-ESI-MS/MS was shown to be a valuable alternative to the already established and reliable, whether FD- or MS-based, protocols. More sensitive and accurate than previously reported methods it further avoids a prior LLE step and therefore the use of toxic chlorinated solvents. Furthermore, the uncomplicated and quick pre-analytical step permits its use in routine analysis, whereas the fulfilment of the validation criteria as established by current European Commission regulations permits it to be considered for official testing purposes.

iii. The bread products analysed in all the studied regions presented a widespread low level contamination. However, OTA content values were distributed over a large range, with the highest detected level surpassing the EU maximum level. In general, common bread presented lower average OTA levels than maize bread. Among the first, wheat bread was the least contaminated, as opposed to whole

grain and rye-based bread in line with previous surveys. Rye was also among the maize bread the cereal that contributed to highest mycotoxin detected levels, as observed in Avintes *broa*. Co-occurrence of OTB in several bread samples was observed, thus limiting its use as an internal standard.

iv. The survey of the pork revealed a relatively high average level of contamination along with a low frequency of contamination, contrasting with previous national and foreign surveys. In effect, some of the analysed samples surpassed the maximum levels individually established by some EU-member states.

v. The urine biomonitoring study showed a widespread and high OTA incidence, although at a relatively low average contamination level. Comparing with foreign studies, incidence was among the highest reported, while average levels were among the lowest. Furthermore, a high within-subject variation of OTA urine content was observed, suggesting and consistent with OTA in urine being a short-time biomarker, with a limited use at the individual level, but useful at a population or subgroup of subjects scale.

vi. Among the studied regions, Alentejo population was identified as the most exposed one, according to the urine survey, featuring the highest average level and frequency of OTA urine contamination, which might be related to different food consumption patterns or socio-economical power. Comparing the two studied staple foods, pork emerged as the major contributor. Indeed bread commercialised in this region featured the lowest contamination, as the typical Alentejano bread constituted most of analysed bread samples in such region. On the contrary, as rule pork samples from Alentejo featured higher contamination, probably explained by the traditional extensive feeding-system of the native swine breed, to which some of the surveyed animals belonged.

vii. Though statistical analysis failed to establish major determinants of the OTA content in both bread and pork, contrary trends were observed. Indeed, while a trend toward higher values in summer was observed among the frequency of contamination and median levels in bread, winter collected pork samples constantly featured higher contamination. Analysed urine did not confirm any

seasonal trend. The fact that no other strong association was observed among the season, gender and individual characteristics studied, suggests two possible justifications: a large number of foodstuffs were OTA contaminated and could therefore contribute to human exposure, or otherwise, major contributors to OTA exposure were probably transversal foods to the whole population.

viii. Considering the two only surveyed foods, bread and pork, the sum of the individual contribution to the total OTA dietary intake of the Portuguese population was estimated at 1.31 ng/kg bw/day. Bread-derived EDI (0.352 ng/kg bw/day) among the Portuguese residents was inferior to equal to the contribution by wheat bread in foreign populations as reported previously. About 60 % of the bread-derived OTA intake proceeded from common bread consumption, against the 40% contribution of maize bread that although more contaminated is less consumed. EDI of OTA through pork consumption (0.958 ng/kg bw/day) was higher than in previously reported national and foreign studies. In fact, the contribution of pork to the OTA intake of the studied population was higher than that of bread, which was clearly divergent with previous studies, according to which cereals and their derived products were the major contributors, while food of animal origin makes only a small contribution to the total human dietary exposure to OTA. These results underline the importance of the quality of the feed with which food-producing animals are reared given the carry-over effect.

ix. Although the calculated intake of the Portuguese population through consumption of bread and pork only, was inferior to the recommended OTA tolerable intakes, OTA content values were distributed over a large range, which allied to the fact that other food sources can also be contaminated with OTA as well as potential variations in dietary habits according to season, region or socio-economic features, it is reasonable to assume that inevitably some individuals may exceed this value, although the average contamination levels are unlikely to pose a threat to consumer health. Nevertheless, a continuous intake at low levels can still carry risks.

x. These observations reinforce the need for more detailed and up-to date OTA exposure evaluation, possibly specifically targeting the staple foods and dietary habits that sustain potential determinants of exposure.

# Part IV

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